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**HANDBOOK FOR THE MICROBIAL
EXAMINATION OF SPACE HARDWARE**

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FOREWORD

This Handbook is published by the National Aeronautics and Space Administration (NASA) as a guidance document to provide engineering information; lessons learned; possible options to address technical issues; classification of similar items, materials, or processes; interpretative direction and techniques; and any other type of guidance information that may help the Government or its contractors in the design, construction, selection, management, support, or operation of systems, products, processes, or services.

This Handbook is approved for use by NASA Headquarters and NASA Centers, including Component Facilities and Technical and Service Support Centers.

This Handbook establishes a compilation of procedures for the microbiological examination of space hardware and associated environments developed to meet the requirements of NASA's Planetary Protection and Space and Life Sciences Programs.

Requests for information, corrections, or additions to this Handbook should be submitted via "Feedback" in the NASA Standards and Technical Assistance Resource Tool at <http://standards.nasa.gov>.

Edward Weiler
Associate Administrator
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Approval Date

TABLE OF CONTENTS

<u>SECTION</u>	<u>PAGE</u>
DOCUMENT HISTORY LOG	2
FOREWORD	3
TABLE OF CONTENTS.....	4
LIST OF FIGURES	7
1. SCOPE	8
1.1 Purpose	8
1.2 Applicability	8
1.3 Deviations	9
2. APPLICABLE DOCUMENTS	9
2.1 General.....	9
2.2 Government Documents	9
2.3 Non-Government Documents.....	9
2.4 Order of Precedence	10
3. ACRONYMS AND DEFINITIONS	10
3.1 Acronyms and Abbreviations	10
3.2 Definitions	11
4. ASSAY CONSIDERATIONS	11
4.1 General Notes	11
4.2 Considerations for Assay Performance	12
4.2.1 Number of Assays to be Performed.....	13
4.2.2 Accuracy and Precision	13
4.2.3 Substitutions	13
4.3 Preparation of Sterile Test Supplies	13
4.3.1 Swabs, Cotton or Synthetic	14
4.3.2 Wipes	15
4.3.3 Forceps.....	15
4.3.4 Petri Plates	15
4.3.5 Glassware.....	16
4.3.6 Erlenmeyer Flask Stoppers	16
4.3.7 Stainless Steel Strips.....	17
4.3.8 Teflon [®] Ribbons	18
4.4 Preparation of Media and Reagents.....	18
4.4.1 Buffered Distilled Water	18

TABLE OF CONTENTS (Continued)

<u>SECTION</u>	<u>PAGE</u>
----------------	-------------

NASA-HDBK-6022

4.4.2	Rinse Solution.....	18
4.4.3	Trypticase Soy Agar (TSA) Culture Medium	19
4.4.4	Concentrated TSA	19
4.4.5	TSA with Neutralizers	19
4.4.6	TSB.....	19
4.4.7	TSB with Neutralizers	20
4.4.8	BSN Spores.....	20
4.5	Standard Protocols Used in Multiple Assays	20
4.5.1	Sonication	20
4.5.2	Performance Requirements for Modified Sonication Procedures	21
4.5.3	Pilot Tubes	23
4.5.4	Membrane Filter Field Monitors	23
4.5.5	Maintenance of Anaerobic Conditions for Incubation	23
4.5.6	Incubation	24
4.5.7	Calculations	24
5.	APPROVED PLANETARY PROTECTION METHODS FOR CULTURE-BASED ASSAYS.....	25
5.1	Surface Contamination Assays.....	25
5.1.1	Microbiological Sampling of Spacecraft and Environmental Surfaces Using the Swab-Rinse Method	25
5.1.2	Microbiological Sampling of Spacecraft and Environmental Surfaces Using the Wipe-Rinse Method.....	28
5.2	Hardware Contamination Assays	31
5.2.1	Assessment of Microbial Contamination on Spacecraft Hardware Parts.....	31
5.2.2	Assessment of Microbial Contamination on Spacecraft Hardware Using Detachable Strips.....	35
5.3	Airborne Contamination Assays.....	35
5.3.1	Assessment of Airborne Microbial Contamination Accumulating on Surfaces Using Stainless Steel Strips	35
5.3.2	Assessment of Airborne Microbial Contamination Accumulating on Surfaces Using Teflon Fallout Ribbons	39
5.3.3	Microbiological Sampling of Intramural Air Using Membrane Filter Field Monitors.....	41
5.3.4	Microbiological Sampling of Intramural Air Using Slit Sampler Agar Impaction Devices	43

TABLE OF CONTENTS (Continued)

<u>SECTION</u>	<u>PAGE</u>
6. PLANETARY PROTECTION METHODS FOR SUPPLEMENTAL ASSAYS	44
6.1 Assessment of Microbial Contamination on Spacecraft Surfaces Using the T-ATP Assay	44
6.1.1 Scope and Application.....	44
6.1.2 Summary of Method.....	44
6.1.3 Apparatus and Equipment	44
6.1.4 Reagents and Consumable Materials.....	45
6.1.5 Sample Collection, Preservation, and Storage.....	45
6.1.6 Extraction.....	45
6.1.7 Calibration and Standardization	46
6.1.8 Procedure for T-ATP Assay	46
6.1.9 Data Collection and Analysis	47
6.2 Assessment of Microbial Contamination Accumulating on Surfaces Using the LAL Assay.....	48
6.2.1 Scope and Application.....	48
6.2.2 Summary of Method.....	48
6.2.3 Apparatus and Equipment	48
6.2.4 Reagents and Consumable Materials.....	49
6.2.5 Sample Collection, Preservation, and Storage.....	49
6.2.6 Extraction.....	49
6.2.7 Calibration and Standardization	49
6.2.8 Procedure for LAL Assay.....	50
 APPENDICES 	
A Bibliography	52

LIST OF FIGURES

<u>FIGURE</u>		<u>PAGE</u>
1	Calculation of Total CFUs.....	22

HANDBOOK FOR THE MICROBIAL EXAMINATION OF SPACE HARDWARE

1. SCOPE

1.1 Purpose

The purpose of this Handbook is to provide a compilation of procedures for the microbiological examination of space hardware and associated environments developed to meet the requirements of NASA's Planetary Protection and Space and Life Sciences Programs.

This document supersedes the earlier NHB 5340.1B, NASA Handbook for the Microbial Examination of Space Hardware (1980), incorporates improvements, reflects current practices in the microbiological examination of space hardware and associated environments, and introduces two supplemental assay procedures. Additional revisions of this document will be issued as needed.

1.2 Applicability

This Handbook is applicable to the assay procedures that apply to all spacecraft hardware and pertinent assembly, test, and launch facilities required to meet planetary protection standards and/or requirements established by the NASA Planetary Protection Officer (PPO). This Handbook is written for use by microbiologists and other personnel who carry out sampling procedures to determine the microbial load of spacecraft and their associated environments.

This Handbook describes uniform microbiological assay procedures that are to be used as follows:

- a. Assess the degree of microbiological contamination of intramural environments where spacecraft hardware is assembled, tested, and launched.
- b. Assess the level of microbial contamination on spacecraft hardware.

This Handbook is approved for use by NASA Headquarters and NASA Centers, including Component Facilities and Technical and Service Support Centers. This Handbook may also apply to the Jet Propulsion Laboratory (JPL) or to other contractors, grant recipients, or parties to agreements only to the extent specified or referenced in their contracts, grants, or agreements.

This Handbook, or portions thereof, may be referenced in contract, program, and other Agency documents for guidance. When this Handbook contains procedural or process requirements, they may be cited in contract, program, and other Agency documents for guidance.

1.3 Deviations

NASA-HDBK-6022

It is emphasized that standardization of procedures is one of the primary objectives of this Handbook. However, portions of this Handbook have been written to include a degree of flexibility in that alternate techniques are described. Consequently, observe the following:

a. Any change in these procedures, other than those specifically mentioned in alternate sections, shall be considered a deviation.

b. Requests to approve a deviation shall be submitted to the NASA Planetary Protection Officer (PPO), Science Mission Directorate, National Aeronautics and Space Administration, Washington, DC 20546.

c. Deviation from a procedure shall not be permitted unless written approval is granted by the PPO.

2. APPLICABLE DOCUMENTS

2.1 General

The documents listed in this section are applicable to the guidance in this Handbook.

2.1.1 The latest issuances of cited documents shall apply unless specific versions are designated.

2.1.2 Non-use of specific versions as designated shall be approved by the responsible Technical Authority.

The applicable documents are accessible via the NASA Technical Standards and Technical Assistance Resource Tool at <http://standards.nasa.gov> or may be obtained directly from the Standards Developing Organizations or from other document distributors.

2.2 Government Documents

NPR 8020.12	Planetary Protection Provisions for Robotic Extraterrestrial Missions
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2.3 Non-Government Documents

ISO 14644-1	Cleanrooms and Associated Controlled Environments – Part 1: Classification of Air Cleanliness; and Part 2: Specifications for Testing and Monitoring to Prove Continued Compliance with ISO 14644-1
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2.4 Order of Precedence

This Handbook provides guidance for the microbiological examination of space hardware and associated environments developed to meet the requirements of NASA's Planetary Protection

NASA-HDBK-6022

and Space and Life Sciences Programs with a compilation of procedures but does not supersede nor waive established Agency requirements/guidance found in other documentation.

3. ACRONYMS AND DEFINITIONS

3.1 Acronyms and Abbreviations

°C	degrees Celsius
ACS	American Chemical Society
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BSN	<i>Bacillus subtilis</i> var. <i>niger</i> (renamed <i>Bacillus atrophaeus</i>)
CFU	Colony Forming Unit
cm	centimeter
cm ²	square centimeter(s)
CSE	Control Standard Endotoxin
EU	endotoxin unit
FEP	fluorinated ethylene propylene
g	gram
HCl	hydrochloric acid
Hg	mercury
H ₂ S	hydrogen sulfide
JPL	Jet Propulsion Laboratory
kHz	1,000 Hertz
LAL	Limulus Amebocyte Lysate
LPS	Lipopolysaccharide
LRW	LAL Reagent Water
M	M molar
m ²	square meter(s)
µm	micrometer
µL	microliter
µmol	micromole(s)
mg	milligram(s)
ml	milliliter(s)
mm	millimeter(s)
NaOH	sodium hydroxide
NASA	National Aeronautics and Space Administration
NHB	NASA Handbook
nm	nanometers
pH	negative log of the hydrogen ion concentration
PPO	Planetary Protection Officer
psi	pounds per square inch
PTS	Portable Test System for LAL assay
RLU	relative luminescence unit
T-ATP	Total Adenosine Triphosphate
TSA	Trypticase soy agar

NASA-HDBK-6022

TSB	Trypticase soy broth
Tween	Molecular Biology Grade
USP	United States Pharmacopeia
w/cm ²	Watts per square centimeter

3.2 Definitions

None.

4. ASSAY CONSIDERATIONS

This section presents general comments on the performance of these assays, as well as detailed subsidiary protocols relevant to multiple assays; detailed descriptions of equipment and glassware to be used in the specific assays; and detailed instructions for the preparation of reagents used in the assay protocols. The selection of assay procedures to be performed by a flight project in compliance with Planetary Protection requirements shall be determined as per NPR 8020.12, Planetary Protection Provisions for Robotic Extraterrestrial Missions, and at the discretion of the NASA PPO, and documented in the Project Planetary Protection Plan.

4.1 General Notes

Unless otherwise stated in an individual method, all reagents both chemical or biological should be graded to meet American Chemical Society (ACS) (Reagent Grade) or United States Pharmacopeia (USP) purity requirements as appropriate.

The following sections provide general notes, which apply to all operations described in these assay procedures.

4.1.1 Any deviation from the operations or equipment specified in these procedures shall require written approval from the NASA PPO (see section 1.3).

4.1.2 All operations involving the manipulation of sterile items and sample processing shall be performed in laminar flow environments meeting at least Class 100 air cleanliness requirements of ISO 14644-1, Cleanrooms and Associated Controlled Environments – Part 1: Classification of Air Cleanliness; and Part 2: Specifications for Testing and Monitoring to Prove Continued Compliance with ISO 14644-1.

4.1.3 These procedures are designed primarily for the detection and enumeration of heterotrophic, mesophilic microorganisms with aerobic and/or anaerobic growth requirements. Procedures for the detection of other microorganisms, e.g., psychrophiles and thermophiles, are included to meet the needs of specific missions. Procedures are not included for the detection of specific microorganisms that may be resistant to sterilization methods other than dry heat, and may survive space and planetary environments (e.g., halophiles, certain *Bacillus* species and extremophiles). Such procedures will be provided in subsequent revisions of this document, as warranted. Section 4 contains general comments on the assay procedures, as well as detailed information on materials and protocols that are used in more than one assay. Culture-based assay methods for planetary protection purposes are included in section 5. Section 6 includes two new assay procedures that may be used to supplement the culture-based methods or to serve as a quick

indicator of cleanliness prior to conducting the culture-based assays. These new procedures may be used to augment the required assay procedures to meet specific mission needs.

4.1.4 Each assay method described in this document makes reference to standard procedures provided in section 4.5. Modification of these methods will be made available through revisions of this document. The consistent use of terminology is an important feature of the methods described herein. When available, the name(s) of the author(s) for each method is/are provided to assist users in obtaining direct support when required.

4.2 Considerations for Assay Performance

For each of these assays and as a general approach, samples are collected from locations with potential contamination; and the collection devices are extracted into an extraction fluid that is subsequently divided into aliquots for use in each required or planned assay procedure. As required, one aliquot of a sample may be plated directly to measure all culturable organisms. A second aliquot may be heat shocked prior to plating, killing susceptible organisms and leaving only those that are heat-resistant, including spores as well as other heat-resistant microbes. As required, plates may be cultured aerobically (with oxygen) or anaerobically (without oxygen). Colonies are counted during 72 hours of growth at 32 °C, to assess the numbers of total and heat-resistant organisms that grow in the various conditions tested. This is equivalent to dividing the original extraction fluid into four aliquots and processing two of the aliquots aerobically and two of the aliquots anaerobically.

During these procedures, significant contamination can result from poor sample handling. Cleanroom requirements should be followed and good aseptic techniques should be employed at all times. Powder-free sterile gloves and other protective equipment as required or necessary to minimize contamination should be worn while performing sample collection procedures.

Sections of these protocols may be adapted for specific applications, as described within.

4.2.1 Number of Assays to be Performed

a. For all analyses, the assay procedures to be performed, the number of assays, the duration of exposure, and the number of sites analyzed shall be subject to approval by NASA management (the PPO or designee) or its authorized contractor(s). The number of samples to be taken for an assay depends on specifications in NPR 8020.12, the assay procedures, and the burden values to be demonstrated for the specific types of microorganisms evaluated.

- b. Sterility and contamination controls shall be performed as part of each assay.
- (1) For surface contamination assays, one field blank shall be taken with every ten or fewer samples collected.
 - (2) Laboratory blanks and solution blanks shall also be tested to evaluate contamination of the prepared materials.

c. The quality of prepared media shall be assessed by plating out appropriate controls using *Bacillus atrophaeus* (BSN) spores.

4.2.2 Accuracy and Precision

The accuracy and reproducibility of all methods should be tested within the laboratory prior to field use.

a. The accuracy and reproducibility of the procedures shall be internally validated at reasonable and regular intervals.

b. Records documenting the accuracy and reproducibility of the methods used shall be maintained by any laboratory carrying out Planetary Protection procedures.

c. Records documenting the accuracy and reproducibility of the methods used shall be made available to NASA management upon request.

4.2.3 Substitutions

On a case-by-case basis, the use of witness plates may be permitted as an alternative to direct sampling, subject to approval by NASA management or its authorized contractor(s).

4.3 Preparation of Sterile Test Supplies

The following paragraphs provide important information on steam sterilization:

Autoclave time, at the desired autoclave temperature, is the most critical parameter for adequate sterilization. The time required to sterilize a given dry or liquid load varies with the following:

- The volume of liquid per container.
- The number of containers/pouches per load.

NASA-HDBK-6022

- The thickness of the container walls.
- The thermal conductivity of the container.

The amount of space between the containers/pouches is also of importance because a tightly packed load may increase sterilization time by interfering with free circulation of steam. The influence of variations in sterilizers and ancillary equipment, such as steam supply, pipe size, and pressure-regulating systems also affects sterilization time. It is recommended that autoclave manufacturer's instructions and parameters be followed. In general, minimum conditions for autoclaving a dry or liquid materials is 121 °C, at 15 psi for 15 minutes.

In practice, the performance of each autoclave should be validated with indicator organisms under various conditions of loading and with various materials to establish a profile of sterilization cycle times, temperatures, and pressures for that autoclave. In most cases, the manufacturer's instructions provide adequate detail.

If the autoclave is equipped with a liquid temperature probe, place the probe in a surrogate vessel with ideally the same dimensions and containing at least as much liquid as the largest volume to be autoclaved in that load.

Each load should use a paper autoclave indicator or autoclave indicator tape, where a color change is evident, if acceptable autoclaving conditions have been met. Autoclave integrator strips providing a broader range of time and temperature conditions may also be used. It is frequently useful to include indicator strips in a variety of containers such as wipe pouches, one dry test tube in each rack, etc. In the case of team efforts, with several people working in the lab, it is necessary to mark and distinguish clearly which items have or have not been sterilized.

Inspection of the autoclave paper printouts, as each run is removed from the autoclave, is good laboratory practice. If adequate sterilization conditions are not met, correct the problem, and autoclave dry loads again. Liquid media and water blanks should be discarded and started with new tubes and media, due to liquid volume changes and changes in the chemical composition of media.

Commercial mediaclaves are another acceptable option, if demonstrated that the above criteria are met.

4.3.1 Swabs, Cotton or Synthetic

Cotton swabs are suitable for approved standard swab-test assays but should not be used for the supplemental T-ATP (Total Adenosine Triphosphate) or LAL (Limulus Amebocyte Lysate) assays. Synthetic (e.g., polyester) swabs are required for those assays, as detailed in the specific requirements for those procedures.

Use commercially available absorbent swabs firmly twisted to a suggested size of 5 by 19 mm long over one end of an applicator stick. The swabs shall be packed individually in a protective container, such as sterile test tubes with lids. Swab heads should be located toward the bottom of

NASA-HDBK-6022

the container. Sterilize swabs by autoclaving according to the manufacturer's instructions (see section 4.3) and allow them to dry prior to use, or purchase pre-sterilized.

The wooden shafts of cotton swabs may be pre-scored with a razor blade between 1-2 cm above the cotton to allow the swab head to be broken cleanly into the sample tube after collecting the sample.

Synthetic swab material may not retain the same numbers of organisms as cotton when used in a standard swab assay.

4.3.2 Wipes

Use commercially available 100 percent polyester-bonded cleanroom wipes, 23 x 23 cm. Folded wipes shall be placed in individual surgical sterilization bags (or equivalent, e.g., steam permeable autoclave pouches). Sterilize wipes by autoclaving according to the manufacturer's instructions (see section 4.3) and allow them to dry prior to use.

As an alternative to the above procedure, dry wipes may be inserted individually into clean jars or test tubes, distilled water added, and the entire jar with wipe covered with aluminum foil and sterilized by autoclaving according to the manufacturer's instructions (see section 4.3).

4.3.3 Forceps

Clean stainless steel forceps (at least 10 cm long) by washing in hot tap water (80 ± 10 °C) containing a non-ionic detergent. Rinse three times with distilled water. Rinse with reagent-grade absolute isopropyl alcohol. Air dry and package them individually in loosely wrapped aluminum foil, capped test tubes, or steam permeable autoclave bags. Sterilize by autoclaving according to the manufacturer's instructions (see section 4.3 or with dry heat at 175 ± 2 °C for 2 hours).

4.3.4 Petri Plates

Commercially available, sterile disposable plastic 100 mm diameter and 150 mm diameter Petri plates or reusable glass Petri plates of similar diameter are equally acceptable.

Glass Petri plates should be washed according to the procedure for glassware stated in section 4.3.5 below. Place clean, dry, glass Petri plates in metal Petri plate containers, and sterilize by autoclaving by autoclaving according to the manufacturer's instructions (see section 4.3).

4.3.5 Glassware

There are no requirements for the use of specific items of glassware, as long as the items used are appropriate for the purpose. Glassware commonly used in these assays include test tubes, 20 or 25 x 150 mm and other sizes; 300-500 ml jars, such as media jars; autoclavable media bottles 100, 250, 500, and 1000 ml; 125 and 250 Erlenmeyer flasks; and other glassware commonly used in biological laboratories.

a. Cleaning: Wash glassware and lids in hot tap water (at least 80 °C) containing a non-ionic detergent. Rinse five times with tap water to remove the detergent and one time with distilled water to remove contaminants from the tap water, and air dry. Alternatively, use a commercial laboratory dishwasher with a distilled water supply.

b. Sterilization: Cover the openings of the glassware with pieces of clean aluminum foil, and autoclave according to the manufacturer's instructions (see section 4.3).

Clean caps may be sterilized by autoclaving according to the manufacturer's instructions (see section 4.3) in a large beaker containing many caps and covered with aluminum foil. Caps may be placed on glassware aseptically immediately prior to use.

Glassware may be sterilized with caps in place; however, care must be taken to ensure the caps are loose to prevent vacuum lock.

An alternative sterilization method is to use dry heat at 175 ± 2 °C for a minimum of 2 hours. The sterilization time begins when the tray temperature reaches 175 ± 2 °C as determined by thermocouples.

4.3.6 Erlenmeyer Flask Stoppers

a. White or black stoppers, No. 6-1/2 or suitable sizes shall be used.

Cleaning: Prior to each use, wash stoppers in hot tap water (at least 80 °C) containing a non-ionic detergent. Rinse five times with tap water and one time with distilled water. Air dry.

b. When black or white rubber stoppers are used for the first time, the following procedures shall be used to remove toxic substances:

(1) White stoppers.

A. Prepare 0.5 normal NaOH (sodium hydroxide) in distilled water (20 grams (g)/liter).

B. Boil rubber stoppers for 30 minutes in this solution.

C. Rinse 15 minutes in hot (80 ± 10 °C) tap water.

D. Place stoppers in 0.5 normal hydrochloric acid (HCl) (18 ml of concentrated HCl/liter).

E. Boil 30 minutes in this solution.

F. Rinse 15 minutes in hot tap water (80 ± 10 °C).

- G. Rinse 5 times with distilled water.
- (2) Black stoppers.
 - A. Soak rubber stoppers in a non-ionic detergent for approximately 12 hours.
 - B. Rinse in tap water for 10 minutes.
 - C. Boil 30 minutes in fresh non-ionic detergent.
 - D. Rinse with hot tap water for 30 minutes.
 - E. Rinse 3 times with distilled water.

Sterilization: Cover each flask with a layer of aluminum foil 5-6 cm down the neck of the flask. Sterilize the aluminum foil-covered flask with dry heat at 175 ± 2 °C for a minimum of 2 hours or in an autoclave per manufacturer's instructions (see section 4.3). Sterilize the stoppers in an aluminum foil-covered beaker or equivalent (e.g., loosely wrapped in foil) in an autoclave per manufacturer's instructions (see section 4.3). Aseptically place the sterile stoppers on the sterile flasks. Replace the aluminum foil over the stoppered flasks. From each lot of 50 processed flasks, randomly select six flasks and to each aseptically add 50 ml of sterile Trypticase soy broth (TSB) (described below). Incubate these sterility controls at 32 °C for 72 hours.

4.3.7 Stainless Steel Strips

- a. Stainless steel strips shall conform to the following specifications: cold roll; type 302 or 304; No. 4 finish, 2.5 x 5.1 cm; 22-26 gauge (0.08-0.05 cm).

Cleaning: Wash in hot tap water (80 ± 10 °C) containing a non-ionic detergent. Rinse 3 times with distilled water. Rinse with 99.5 percent minimum purity isopropyl alcohol. Air dry.

- b. Marking: Each strip shall be marked to distinguish both the sample number (if appropriate) and the exposed side, using a diamond-tipped scriber or equivalent.

4.3.7.1 Preparation for Sterilization

Using aseptic techniques, place clean, dry, stainless steel strips (marked side up) on flat, non-corrosive metal trays (approximately 28 x 38 cm).

4.3.7.2 Sterilization

Arrange the strips in a single layer on each tray. Wrap each tray with degreased aluminum foil. (To degrease aluminum foil, wipe foil surfaces with a lint-free cloth wipe wetted with 99.5 percent minimum purity methyl alcohol.) Make certain that the entire tray is covered and the foil is not punctured in the process. Sterilize the wrapped trays with dry heat at 175 ± 2 °C for a minimum of 2 hours.

- a. The sterilization time shall begin when the tray temperature reaches 175 ± 2 °C as determined by thermocouples.
- b. If more than one tray is sterilized at a time, the thermocouples shall be attached to the tray nearest the center of the oven.

4.3.8 Teflon[®] Ribbons

Teflon ribbons shall conform to the following specifications: Teflon fluorinated ethylene propylene (FEP) Type 500A Transparent, Roll 7.6 by 1646 cm.

Cleaning: Wash in hot tap water (80 ± 10 °C) containing a non-ionic detergent. Rinse three times with distilled water. Air dry.

4.3.8.1 Preparation for Sterilization

Using aseptic techniques, roll up clean, dry Teflon ribbon, and place in clean 250 ml glass beaker. Cover each beaker with degreased aluminum foil.

4.3.8.2 Sterilization

Sterilize the Teflon ribbons in glass beakers with dry heat at 175 ± 2 °C for a minimum of 3 hours. New ribbons are preconditioned by two exposures to dry heat for 3 hours each at 175 ± 2 °C to allow for degassing of Teflon prior to a third heating interval for sterilization. The sterilization shall begin when the ribbon temperature reaches 175 ± 2 °C as determined by a thermocouple attached to the Teflon ribbon inside of a glass beaker located nearest the center of the oven.

4.4 Preparation of Media and Reagents

4.4.1 Buffered Distilled Water

To prepare a stock buffer solution, dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 ml of distilled water, adjust to pH 7.2 ± 0.1 with one normal NaOH, and dilute to one liter with distilled water. To prepare a working solution of buffered distilled water, add 1.25 ml of the stock buffer solution to one liter of distilled water.

4.4.2 Rinse Solution

Prepare a 0.02 percent volume-to-volume solution of certified (i.e., certified non-toxic to microorganisms) Tween 80 in buffered distilled water. The final pH shall be 7.2 ± 0.1 . Sterilize by autoclaving according to the manufacturer's instructions (see section 4.3).

NASA-HDBK-6022

4.4.3 Trypticase Soy Agar (TSA) Culture Medium

To prepare TSA, dissolve commercially available certified TSA. Mix in water per the manufacturer's instructions (usually 40 g per liter). Final product should be adjusted to pH 7.3. Sterilize by autoclaving according to the manufacturer's instructions (see section 4.3).

If desired for immediate use, after autoclaving, place the containers of sterile TSA in a 48-50 °C water bath, or allow to cool to 48-50 °C before placing in a 48-50 °C water bath or holding oven. Warm media should be used within 6 hours.

Alternatively, media may be removed from the autoclave and allowed to cool and solidify.

a. Flasks containing media shall be stored at ambient room temperature for 72 hours and observed for evidence of contamination. Discard any flask containing contaminated agar. Store remaining flasks containing agar at 4 °C.

b. Media not used within 14 days from date of sterilization shall be discarded.

Prior to the assay operation, place flasks containing solidified agar in the autoclave. Allow to remain at temperature and pressure (see section 4.3) for 10 minutes. Immediately after autoclaving, place the flasks of sterile agar in a water bath at 48-50 °C or allow cooling to 50-55 °C before placing in a 48-50 °C holding oven. Warm media should be used within 6 hours.

4.4.4 Concentrated TSA

Prepare concentrated TSA in the same manner described above using 80 g of TSA Mix per liter volume of distilled water. Use a water bath or holding oven set at 48-50 °C.

4.4.5 TSA with Neutralizers

To prepare TSA with Neutralizers, dissolve certified TSA Mix according to the manufacturers' instructions, in distilled water containing 0.5 percent volume-to-volume Tween 80 and 0.07 percent volume-to-volume lecithin. Sterilize by autoclaving per manufacture instructions (see section 4.3), mix thoroughly, cool, and use within 30 minutes.

4.4.6 TSB

To prepare TSB, dissolve commercially available certified TSB Mix in water per the manufacturer's instructions. Mix thoroughly and sterilize by autoclaving according to the manufacturer's instructions (see section 4.3). Final product should be adjusted to pH 7.3.

4.4.7 TSB with Neutralizers

To prepare TSB with Neutralizers, dissolve commercially available certified TSB with Neutralizers containing Tween 80 and lecithin to make concentrations of 0.5 percent and 0.07 percent volume to volume, respectively. Mix in water per the manufacturer's instructions and sterilize by autoclaving according to the manufacturer's instructions (see section 4.3). Use within 30 minutes.

4.4.8 BSN Spores

Bacillus subtilis var. *niger* (BSN) has been renamed *Bacillus atrophaeus*. The reference strain ATCC 9372 is the control strain of choice. *B. atrophaeus* (BSN) may be purchased from a commercial vendor, or can be prepared according to standard protocols. Spore suspensions are stable for prolonged periods in deionized water, at 4 °C. Colonies appear as an orange color on TSA.

4.5 Standard Protocols Used in Multiple Assays

The following sections provide standard protocol methods and approaches to be used in the various assay procedures.

4.5.1 Sonication

Suspend glassware containing the sampling devices and rinse solution in an ultrasonic bath filled to its normal capacity so that the bottom of the container is parallel to the bottom of the bath and the bath fluid solution (water) is above the level of the solution in the container. Racks, baskets, or similar devices designed to hold sample containers should not be used unless they have been tested as described below, as they can interfere with the process of sonication.

All sonication procedures shall conform to the following specifications:

- a. The frequency shall be 19-27 kHz.
- b. The rated power output in relation to the bottom surface area of the bath shall be at least 0.35 w/cm².
- c. If the ultrasonic bath is not automatically tuned, tuning shall be performed according to the manufacturer's directions.
- d. The inside surfaces of the bath shall be stainless steel.
- e. Each flask containing a strip or piece part to be assayed shall be suspended individually in the middle of a full bath and sonicated for 2 minutes \pm 5 seconds.
- f. The bath fluid shall be an aqueous solution of 0.02 percent volume-to-volume Tween 80.

NASA-HDBK-6022

- g. This aqueous solution shall be made up fresh each day.
- h. The temperature of the bath fluid shall be at least 20 °C and shall not exceed 32 °C.
- i. Prior to sonicating samples to be assayed, the bath shall be operated for at least 5 minutes to de-gas the bath fluid.
- j. The bath fluid shall be above the level of the liquid in the flasks being sonicated.

It is suggested that ultrasonic baths approximately 23 cm wide, 25 cm long, and 25 cm deep be used. The use of larger baths is discouraged but not prohibited.

Do not place heavy objects on the bottom center of the bath, as this may interfere with the energy transfer between the transducers and the liquid. Baskets that come with the bath have supporting rods that sit along the outside length of the bath.

4.5.2 Performance Requirements for Modified Sonication Procedures

4.5.2.1 Purpose

The sonication of more than one flask in an ultrasonic bath at one time and/or the use of containers other than flasks for the sonication procedure shall be permitted, if the modified procedure meets the standard performance requirements specified herein.

4.5.2.2 Methods

Prepare standard 2.5 x 5.1 cm stainless steel strips inoculated with BSN spores on the marked surface. Using sterile forceps, place one strip in each container to be used in the modified procedure. The strip should be placed so that the contaminated surface faces the transducers during sonication. Aseptically add the quantity of sterile rinse solution used in the modified procedure to each container. Place the container or container(s) in the ultrasonic bath according to the modified procedure and sonicate for 2 minutes \pm 5 seconds.

Pour or pipet all of the rinse solution from each container into separate 150 x 25 mm Petri plates, reserving the stainless steel strip. No more than 25 ml of liquid should be poured into any one plate. Gently swirl the solution in each plate; quickly add 75 ml of sterile, molten (50-55 °C) concentrated TSA; and *immediately* resume rapid swirling until the contents are thoroughly mixed. Allow the mixture to solidify at room temperature.

Also assay the stainless steel strip. Aseptically remove the strip from the container using sterile forceps; rinse it completely in a gentle stream of sterile distilled water for 3 to 5 seconds; and place each strip with the marked surface facing upward in a separate sterile, dry, 100 mm diameter Petri plate. Add enough sterile, molten (48-50 °C) TSA to completely cover each stainless steel strip.

NASA-HDBK-6022

Aerobically incubate all samples at 32 °C. After 24, 48, and 72 hours of incubation, count and record any colony-forming units (CFUs). Do not remove the plate covers until the final count is made at 72 hours.

4.5.2.3 Calculations

For the calculations, it is important to use the most accurate estimation of total CFUs. Typically, this means counts taken at 24, 48, and 72 hours will be aggregated to reflect the true number of colonies that were present at any time during the incubation period. This will account for merging colonies and late developing colonies. In the example below, the actual count used should be 12 colonies.

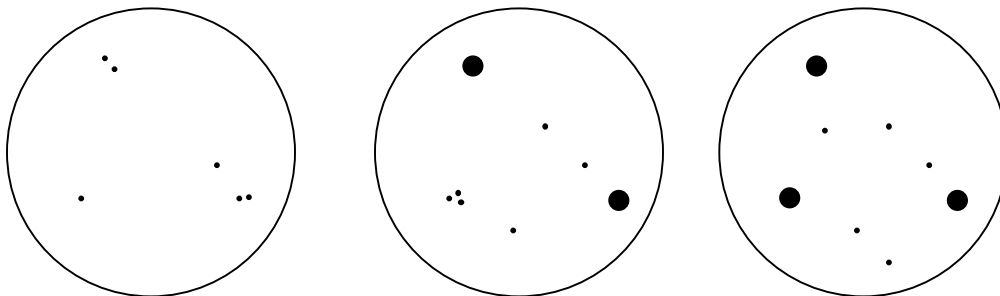


Figure 1—Calculation of Total CFUs

Divide the total colony count from all the rinse fluid in a container by the sum of the total colony count from all the rinse fluid in that container plus the colony count from the plated stainless steel strip taken from that container. Multiply this quotient by 100 to obtain the percent of heat resistant microbes, including spores, removed from the strip in that container.

4.5.2.4 Requirements

The number of standard contaminated stainless steel strips assayed by the modified procedure is to be determined by the following:

- a. If five or more containers are sonicated in one ultrasonic bath at one time, at least three sets of standard strips shall be assayed.
- b. Each set shall contain a number of strips equal to the maximum number of containers sonicated at one time with the modified procedure.

NASA-HDBK-6022

c. If less than five containers are sonicated in one ultrasonic bath at one time, a total of at least 12 standard strips shall be assayed.

d. The strips shall be sonicated singly or in sets according to the modified procedure.

The modified procedure meets performance requirements if in a above, the mean percent of heat resistant microbes including spores removed is 95 percent or greater for each of the three sets; in c above, the mean percent of spores removed from the 12 standard strips is 95 percent or greater.

4.5.3 Pilot Tubes

Using a container identical to those used for the assay samples, fill one pilot container with the same volume of rinse solution used for the test samples. A thermometer shall be placed in the pilot, which is used to monitor when the temperature of the test tubes reaches the required temperature.

4.5.4 Membrane Filter Field Monitors

a. Commercially available, sterile membrane filter monitors shall be used for air or other gas sampling (e.g., http://www.pall.com/laboratory_20841.asp).

Calibration and Preparation: Insert a 10-liter/minute flow-limiting orifice to the outlet end of an aerosol adapter. With a clinical field monitor attached to the inlet of the adapter and the outlet connected via vacuum hose to a minimum vacuum source of 500 mm Hg, a constant flow rate will be obtained. To ensure accuracy, the orifice should be calibrated with a flowmeter at the actual working vacuum level.

b. When a manifold is used, it shall be equipped with a calibrated in-line flowmeter.

A minimum vacuum source of 75-liter/minute free air is required for each manifold. Before initial operation and at monthly intervals thereafter, calibrate all manifolds to draw air at a velocity of 10 liters/minute per field monitor up to a maximum of 60 ± 0.02 liters/minute per manifold.

4.5.5 Maintenance of Anaerobic Conditions for Incubation

When performing anaerobic assays, establish and maintain anaerobic conditions using Brewer jars, either the electric or cold catalyst system, or other appropriate equipment designed for anaerobic microbiological cultures. If the electric Brewer jar is used, gaseous hydrogen and a catalyst shall be used to remove residual oxygen. Alternatively, a commercially available anaerobic chamber may be used.

Place a reducing indicator (such as methylene blue) in each incubator. In addition, the anaerobic indicator organism *Clostridium novyi* type B and optionally the aerobic indicator organism *Alcaligenes faecalis* may be included in each incubator. If the *A. faecalis* shows visible growth after 72 hours at 32 °C or the indicator remains blue after 12 hours at 32 °C, the environment

NASA-HDBK-6022

cannot be considered anaerobic. If *C. novyi* type B shows visible growth and the indicator is white, anaerobic conditions are considered to exist.

If *Clostridium sporogenes*, or any other organism that produces H₂S, is grown, the catalyst pellets should be replaced before another run.

When using a cold catalyst system below room temperature for the incubation of psychrophiles, the anaerobic chamber should be set up and allowed to set at room temperature for 30 minutes before placing into a low temperature incubator; otherwise, the non-heated catalyst will not be active.

4.5.6 Incubation

Plates should be inverted and incubated at 32 °C. Examine the aerobically incubated sample plates at 24 and 48 hours. If colonies are observed, count and record data. Examine and record final colony counts from all plates at 72 hours. Do not remove the Petri plate covers until the final 72-hour count is made.

In accordance with the assay group's needs or as required by NASA management or its authorized contractor(s), detection of anaerobic, psychrophilic, or thermophilic organisms may be attempted. To facilitate the detection and enumeration of psychrophilic organisms, plates should be incubated at 3 °C for 14 days. To facilitate the detection and enumeration of thermophilic organisms, incubation should take place at 55 °C for 24 to 48 hours.

4.5.7 Calculations

The dilution factors and conversion calculations may not be consistent throughout these assay procedures; thus, considerable care is to be taken to ensure that the correct calculations are performed when determining counts per unit.

- a. Results shall be expressed as number of microorganisms per piece part, strip, or ribbon, in the case of part assays or environmental assays.
- b. When a surface is being assayed, results shall be expressed as total number of microorganisms, or heat resistant microbes including spores, per unit area.
- c. The general categories of microorganisms shall be reported as individual groups (i.e., aerobes, anaerobes, aerobic spores, or anaerobic spores) and not be added in any combination.
- d. It is suggested that the mean and range be calculated for each series of assays. However, all extrapolations of data shall be done in accordance with the assay group's needs or, if applicable, as required by NASA management or its authorized contractor(s).

5. APPROVED PLANETARY PROTECTION METHODS FOR CULTURE-BASED ASSAYS

5.1 Surface Contamination Assays

Throughout all these assays, aseptic techniques shall be used. This includes but is not limited to the use of sterile gloves, sterile forceps, sterile wipes, sterile swabs, etc.

5.1.1 Microbiological Sampling of Spacecraft and Environmental Surfaces Using the Swab-Rinse Method

5.1.1.1 Scope and Application

This assay is used to sample microbial contamination on spacecraft surfaces, parts, cleanroom and assembly areas, or ground support equipment. This assay is often referred to as the “Standard Assay Method” and is appropriate for materials that can tolerate sample collection using a damp swab. This method is limited to sample collection from surfaces no greater than 25 cm² per swab. Multiple swabs may be used to sample a larger surface area.

5.1.1.2 Apparatus and Equipment

Sterile Test Tubes – 20 or 25 x 150 mm, capped.
Pipettes – assorted sterile pipettes (10, 25, 50 ml).
Sterile 100 x 15 mm Petri Plates.
Sterile Cotton Swabs.

Vortex Mixer.
Ultrasonic Bath.
80 °C Waterbath.
Incubators.

5.1.1.3 Reagents and Consumable Materials

Sterile Rinse Solution.
Sterile Buffered Distilled or Deionized Water.
Trypticase Soy Agar.

5.1.1.4 Sample Collection, Preservation, and Storage

Prepare a sufficient number of sterile swabs and sterile test tubes containing 10.0 ml each of sterile distilled water to accommodate all swab samples to be collected, plus controls. Aseptically remove a sterile cotton swab from its container and moisten the head of the swab in a test tube. Express excess moisture from the swab against the interior wall of the tube. Hold the swab so that the handle makes about a 30-degree 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 degrees and again swab the surface thoroughly. Complete a third coverage of the surface by again changing the direction of the swabbing motion by 90 degrees.

NASA-HDBK-6022

Return the head of the swab to the original tube containing sterile distilled water, breaking off the head of the swab against the side of the tube, below any portion of the handle that was touched by the sampler. Allow the swab head to drop into the liquid and replace the screw cap.

For each ten or fewer samples collected, also collect a field blank negative control. Remove the sterile swab from its wrapper; moisten as above with 10 ml sterile, distilled water; wave the swab through the air; and break off the head of the swab into the tube.

Transport samples to the laboratory. If processing does not commence within 1 hour, the samples can be stored at 4 °C for up to 24 hours.

5.1.1.5 Extraction

In the lab, create at least one negative control swab blank by moistening the head of a sterile swab in 10.0 ml of sterile water, as above, and breaking off the head of the swab into the tube without exposing it to air.

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

Suspend the tubes containing swab heads in an ultrasonic bath filled to normal capacity. Not more than 12 tubes should be placed into the bath at one time. Make sure that the bath liquid is above the fluid level in the tubes. Sonicate for 2 minutes \pm 5 seconds.

5.1.1.6 Swab Assay Procedures

Methods 1 and 2 are the standard swab assay procedures, commonly referred to as the NASA standard assay. (See sections 5.1.1.6.1 and 5.1.1.6.2.)

These procedures may be modified to create duplicate plates by removing several equal aliquots from the same tube for plating, when this is desirable or required by NASA management or its authorized contractor(s). Multiple plates may be desired when the assay is directed toward one specific group of microorganisms (i.e., aerobes, anaerobes, aerobic spores, anaerobic spores, psychrophiles, etc., or several groups of microorganisms).

5.1.1.6.1 Swab Assay Method 1 (in which the same tube is used for both treatments)

If necessary, make appropriate dilutions of the distilled water extraction fluid in sterile rinse solution before proceeding with plating.

a. Unheated Portions: Aseptically pipette 2.0 or 4.0 ml portions of the extraction fluid into Petri plates, using 8.0 ml total.

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

NASA-HDBK-6022

b. Heated Portions (Heat Shock Treatment): After removing samples for the unheated assay, place the tube containing the vortexed solution and the swab head in a water bath at 80 ± 2 °C for 15 minutes, as determined by a pilot tube containing a thermometer in 10 ml of water. 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.

Aseptically pipette 2.0 or 4.0 ml portions of the extraction fluid into Petri plates, using 8.0 ml total.

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

- c. Plate out at least one 4.0 ml water-only blank as a negative control.
- d. Incubate plates and perform colony counts as detailed in section 4.

5.1.1.6.2 Swab Assay Method 2 (in which different tubes are used for each treatment)

This method may be performed when the level of microbial contamination is anticipated to be low, or duplicate plates are required.

a. Unheated Portions: Aseptically pipette as much of the 10 ml extraction fluid as required from a single tube, in 2.0 ml or 4.0 ml aliquots, into separate Petri plates.

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

b. Heated Portions (Heat Shock Treatment): Place the tubes containing the swab heads and the water/swab solution in a water bath at 80 ± 2 °C. Hold at temperature for 15 minutes, as determined by a pilot tube containing a thermometer.

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.

Aseptically pipette as much of the 10 ml water/swab solution as required from a single tube, in 2.0 or 4.0 ml aliquots, into separate Petri plates.

Add 20 ml sterile, molten (48-50 °C) TSA; mix the contents by gentle swirling; and allow the mixture to solidify at room temperature.

- c. Plate out at least one 4.0 ml water-only blank as a negative control.

- d. Incubate plates and perform colony counts as detailed in section 4.

5.1.2 Microbiological Sampling of Spacecraft and Environmental Surfaces Using the Wipe-Rinse Method

5.1.2.1 Scope and Application

This assay is used to sample microbial contamination on spacecraft surfaces, parts, cleanroom and assembly areas, or ground support equipment. This assay is often referred to as the “Standard Assay Wipe Method” and is appropriate for materials that can tolerate sample collection using a damp wipe. This method collects a sample over a greater surface area (up to 1 m²) than the swab method. Multiple wipes may be used to sample even larger surface areas.

5.1.2.2 Apparatus and Equipment

Sterile Test Tubes 25 x 150 mm.

Sterile Glass Jars.

Pipettes – assorted sterile pipettes (10, 25, 50 ml).

Sterile Petri Plates 100 x 15 mm or 150 x 25 mm.

Sterile Forceps.

Sterile Wipes.

Sterile Gloves.

Vortex Mixer.

Ultrasonic Bath.

80 °C Waterbath.

Incubators.

5.1.2.3 Reagents and Consumable Materials

Sterile Rinse Solution.

Sterile Buffered Distilled Water.

Trypticase Soy Agar.

5.1.2.4 Sample Collection, Preservation, and Storage

Prepare a sufficient number of sterile wipes and dry, sterile, clean, glass transport jars (~300 ml capacity) to accommodate all samples to be collected.

Either pre-wetted sterile wipes may be used, or wipes may be wetted with sterile distilled water immediately prior to use.

If pre-wetted sterilized wipes have not been prepared, dry sterilized wipes shall be wetted. In a clean laminar-flow hood, wearing sterile gloves, aseptically remove a sterile wipe from its wrapper and place the individual wipe into a clean transport jar or sterile Petri dish. Moisten the wipe with 15-20 ml sterile distilled water.

NASA-HDBK-6022

Rinse gloved hands with filtered sterilized 70 percent isopropyl alcohol between each sample, and change gloves at least once every 24 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 after each complete sampling of the area. Transfer the wipe into a transport jar.

For each six or fewer samples collected, also collect a “field blank” negative control. Remove the sterile wipe from its wrapper; moisten with 15-20 ml sterile distilled water as above; wave the wipe through the air; and insert into a dry, sterile, glass jar.

Transport samples to the laboratory. If processing does not commence within 1 hour, the samples can be stored at 4 °C for up to 24 hours.

5.1.2.5 Extraction

In the lab, create a negative control wipe blank by moistening a wipe in 15-20 ml of sterile water as above, and aseptically insert the wipe into a dry, sterile, glass jar without exposing it to air.

Add 200 ml of sterile rinse solution to each sample and reseal the jar.

Vortex at maximum speed for 5-10 seconds, even if not very efficient for bottles and 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 in an ultrasonic bath, making sure the liquid level in the bath is above the fluid level in the sample jars and that the number of jars does not exceed the performance rating of the sonicator. Sonicate 2 minutes \pm 5 seconds.

5.1.2.6 Wipe Assay Procedures

When it is desirable or required that the assay be directed toward one specific group of microorganisms (i.e., aerobes, anaerobes, aerobic spores, or anaerobic spores) and/or when the level of microbial contamination is anticipated to be extremely low, the second assay procedure, section 5.1.2.6.2 may be used to evaluate the number of heat-shock resistant spores.

Standard membrane filter methods may be used to test both unheated and heated extraction fluid, as an alternative to these procedures.

5.1.2.6.1 Wipe Assay Method 1 (in which extraction fluid from the same wipe is used for both treatments)

If necessary, make appropriate dilutions of the extraction fluid in sterile rinse solution.

NASA-HDBK-6022

a. Unheated Portions: Using a sterile pipette, agitate the wipe in the sample jar for approximately 10 seconds.

Aseptically pipette 2.0 or 4.0 ml portions of extraction fluid into individual sterile 100 x 15 mm Petri plates. A total of 48-50 ml of solution should be plated.

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

b. Heated Portions (Heat Shock Treatment): Using a sterile pipette, agitate the wipe approximately 10 seconds, and aseptically pipette two 25.0 ml portions of extraction fluid from each sample into each of two 25 x 150 mm screw cap tubes.

Place in a water bath at 80 ± 2 °C, and hold at temperature for 15 minutes as determined by a pilot tube containing a thermometer in 25 ml of water.

After heat shock, cool the tubes rapidly so that the temperature of the fluid contents is between 30 and 35 °C.

Aseptically pipette 2.0 or 4.0 ml portions of the rinse solution into sterile Petri plates. A total of 48-50 ml from each tube should be plated.

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

c. Plate out 4.0 ml of sterile rinse solution with 20 ml TSA as a negative control.

d. Incubate plates as described in section 4.

5.1.2.6.2 Wipe Assay Method 2 (in which a large volume of extraction fluid is tested by heat shock only)

a. Heated Portions (Heat Shock Treatment): After sonication, use a sterile pipette to agitate the wipe for approximately 10 seconds.

Aseptically pipette 25.0 ml portions of the extraction fluid into each of four large sterile test tubes capable of holding 150 ml.

Place tubes in a 80 ± 2 °C water bath and allow to remain at 80 °C for 15 minutes after the tube contents have reached temperature, as determined by a pilot tube containing a thermometer in 25 ml of water.

Transfer tubes to a cold-water bath and rapidly (within 2 minutes) cool to 55 °C, as determined from the pilot tube.

NASA-HDBK-6022

Add approximately 75 ml sterile, molten (48-50 °C) double strength TSA to each tube and mix the contents by swirling. Pour plates by adding the contents of one tube to each large Petri plate (150 x 25 mm), and allow to cool at room temperature.

- b. Plate out 4.0 ml of sterile rinse solution with 20 ml TSA as a negative control.
- c. Incubate plates and perform colony counts as detailed in section 4.

5.2 Hardware Contamination Assays

5.2.1 Assessment of Microbial Contamination on Spacecraft Hardware Parts

5.2.1.1 Scope and Application

This method is used to assess the microbial contamination of representative small parts or other hardware (piece parts) without the use of swab or wipe sampling.

- a. Each piece part shall be identified.
- b. The total exposed surface area shall be determined and recorded.
- c. Results shall be expressed initially as number of microorganisms of each type assayed per piece part.
- d. It is suggested that the mean and range be calculated for each type of piece part. However, all extrapolations of data shall be done in accordance with the assay group's needs or, if applicable, as required by NASA management or its authorized contractor(s).

5.2.1.2 Apparatus and Equipment

Sterile Erlenmeyer flasks, jars, or bottles.
Sterile Petri plates.
Sterile Forceps.

Ultrasonic Bath.
80 °C Waterbath.
Incubators.

5.2.1.3 Reagents and Consumable Materials

Sterile Rinse Solution.
Sterile Buffered Distilled or Deionized Water.
Trypticase Soy Broth.
Trypticase Soy Agar.
BSN spores.

5.2.1.4 Sample Collection, Preservation, and Storage

Aseptically place the piece part to be assayed in a flask or jar containing 50 ml of sterile rinse solution (or a known volume sufficient to cover the part) at 25 ± 5 °C. Each flask should have its stopper or cap covered with aluminum foil, and the foil should extend 5-6 cm down the neck of the flask.

For large piece parts that will not fit in standard flasks, a suitable container may be used that conforms to the performance requirements for removing microorganisms from surfaces, as specified in section 4.5.2.

5.2.1.5 Extraction

- a. Suspend each flask containing the piece part and rinse solution in an ultrasonic bath containing enough water that the water is slightly above the level of the rinse solution in the flask. Flasks and jars containing sample solution should be free standing. Racks, baskets, or similar devices designed to hold sample containers should not be used.
- b. Sonicate for 2.0 minutes ± 5 seconds.
- c. At the discretion of the assay group or upon direction by NASA management or its authorized contractor(s), irregularly shaped piece parts may be sonicated for 2 minutes in each of several positions to ensure adequate exposure of all surfaces to ultrasonic energy.

5.2.1.6 Piece Part Assay Procedures

When it is desirable or required that duplicate plates be produced, the assay be directed toward one group of microorganisms (i.e., aerobes, aerobic spores, or anaerobic spores), or when microbial contamination is anticipated to be extremely low, assay procedure two (section 5.2.1.6.2) may be used.

Standard membrane filter methods (section 5.3.3) may be used to test both unheated and heated extraction fluid, as an alternative to these procedures.

5.2.1.6.1 Part Assay Method 1 (in which the same extraction fluid is tested using both treatments, and the piece part is cultured directly)

- a. Unheated Portions: Pipette 2.0 or 4.0 ml portions of the extraction fluid in each flask into Petri plates, using 8-20 ml total.

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

- b. Heated Portions (Heat Shock Treatment): Aseptically pipette 25 ml of the extraction fluid into the bottom of individual sterile test tubes (approximately 25 x 150 mm) taking care not to contaminate the upper inside surface of each tube. The tubes should be held on ice if the pipetting procedure will require more than 10 minutes, but for no longer than 45 minutes.

NASA-HDBK-6022

Place a rack containing the sample tubes and a pilot tube in a water bath at 80 ± 2 °C. Make certain the water bath level is above the level of the liquid content of each test tube. Monitor the temperature in the pilot tube, and incubate the sample tubes in the water bath for 15 minutes after the temperature in the pilot tube reaches 80 ± 2 °C.

Cool the sample tubes rapidly by immersion in cold water (10-15 °C) or by any other suitable means that affords rapid (within 2 minutes) cooling of the extraction fluid to 30-35 °C, as measured in the pilot tube.

Aseptically pipette 2.0 or 4.0 ml portions of the heat-shocked extraction fluid from each tube into Petri plates, using 8-20 ml total.

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

c. Piece Part (if possible): Using sterile forceps, aseptically remove each part from its container; rinse it completely in a gentle stream of sterile water for 3-5 seconds; and place it in a sterile, dry Petri plate.

d. Add enough sterile, molten (48-50 °C) TSA to completely cover the part, if possible, and allow the medium to solidify at room temperature.

e. Pour at least one plate containing 4.0 ml of sterile rinse solution and 20 ml TSA as a negative control.

f. Incubate plates and perform colony counts as detailed in section 4.

5.2.1.6.2 Part Assay Method 2 (in which a large volume of extraction fluid is tested by heat shock only)

a. Heated Portions (Heat Shock Treatment): After sonication, heat-shock the flask containing the extraction fluid directly in a water bath at 80 ± 2 °C for 15 minutes as determined by a pilot flask containing a thermometer. Ensure that the water bath level is above the liquid contents of the flask.

After heat shock, immediately cool the contents of the flask to approximately 50 °C.

Aseptically transfer 25 ml portions of extraction fluid into two large, sterile test tubes capable of holding 150 ml; add 75 ml of molten (48-50 °C) concentrated TSA; and mix by swirling. Pour each tube into a Petri plate (150 x 25 mm), and allow the mixture to solidify at room temperature.

b. Pour at least one plate containing 4.0 ml of sterile rinse solution and 20 ml TSA as a negative control.

- c. Incubate plates and perform colony counts as detailed in section 4.

5.2.1.7 Control Procedures (to be performed with each piece part assay)

a. Negative Control: Process a minimum of 10 sterile piece parts as sterility control check items (negative controls) on the entire assay procedure. Processing shall occur during one of the daily assays or more often as prescribed by NASA management or its authorized contractor(s).

b. Positive Control: After sterilization, perform an inhibitory test on each type of piece part assayed. Aseptically place the sterile piece part in a flask containing 50 ml of sterile TSB. Add 80-120 spores of BSN. Sonicate the flask containing the part for 2 minutes ± 5 seconds and allow it to stand for 30 minutes at room temperature (22-27 °C). Aseptically remove the piece part and incubate the flask at 32 °C for 72 hours. Growth is confirmed if the TSB becomes turbid, demonstrating that the piece part does not inhibit spore growth.

c. Perform a viability check on the spore preparation by inoculating a tube of sterile TSB and incubating it at 32 °C for 72 hours.

5.2.2 Assessment of Microbial Contamination on Spacecraft Hardware Using Detachable Strips

Hardware design should provide for the inclusion of detachable strips on the surface of each flight item.

a. The time of attachment, location, and the number of strips shall be determined by the assay group's needs or by NASA management or its authorized contractor(s).

b. The method of attachment shall be such that it does not compromise either the physical integrity of the space hardware or the validity of the microbiological assay. Use the method described next, section 5.3.1, to assess the contamination accumulating on strips.

5.3 Airborne Contamination Assays

5.3.1 Assessment of Airborne Microbial Contamination Accumulating on Surfaces Using Stainless Steel Strips

5.3.1.1 Scope and Application

This method involves the collection of airborne microbial contaminants on stainless steel strips. It may be used to monitor facilities associated with hardware or any surface that cannot be subjected to water-based assay methods.

5.3.1.2 Apparatus and Equipment

Sterile Stainless Steel Strips.
Sterile Erlenmeyer flask or other suitable glass container.
Sterile Test Tubes, 25 x 150 mm.
Sterile Pipettes – assorted volumes (10, 25, 50 ml).
Sterile Petri Plates, 100 mm.
Sterile Forceps.

Ultrasonic Bath.
80 °C Waterbath.
Incubators.

5.3.1.3 Reagents and Consumable Materials

Trypticase Soy Broth.
Sterile Rinse Solution.
Sterile Buffered Distilled Water.
Trypticase Soy Agar.
Concentrated Trypticase Soy Agar.

5.3.1.4 Sample Collection, Preservation, and Storage

5.3.1.4.1 Standard Collection Procedure

Prepare trays of sterile stainless steel strips according to section 4, containing at least six strips per sample to be collected. Place trays at each sampling site and carefully remove the aluminum foil cover of each tray. Using sterile forceps, arrange the sterile strips to form a single layer in the tray, with marked surfaces upwards.

Wearing sterile gloves, collect at least six strips per tray at each sampling interval. Using separate sterile forceps for each strip, aseptically place each strip to be assayed in a separate, dry, sterile, Erlenmeyer flask or other glass container. Ensure that the marked, contaminated surface of each strip is facing downwards toward the bottom of the flask.

As a field-blank negative control, collect at least two strips per sample immediately after the tray has been unwrapped. Hold control strips under sterile conditions and assay with the rest of the samples.

Hold the strips contained in the flasks at 4 °C and begin assay procedures within 24 hours after collection. If assayed within 2 hours, the strips may be held at 25-35 °C.

5.3.1.4.2 Alternative Collection Procedure

When an assay laboratory is adjacent or close to the test area, the following is permitted: Place each strip directly in a flask containing 50 ml of cold (0-6 °C), sterile rinse solution. Handle the flask in such a manner as to maintain the temperature of the rinse solution between

NASA-HDBK-6022

0 and 6 °C, e.g., by transport in an ice bucket. Begin sonication and subsequent procedures within 20 minutes from the time strips are collected.

5.3.1.5 Extraction

Create a “laboratory blank” by placing at least one sterile strip into a collection jar and processing with the test samples.

Aseptically add 50 ml of sterile rinse solution maintained at 20-25 °C to each flask containing a strip but no liquid. Make sure that the contaminated (marked) surface of the strip (marked “X”) is facing the bottom of the flask. Replace the foil covering.

Immediately sonicate the sample, as detailed in section 4, for 2.0 minutes \pm 5 seconds.

5.3.1.6 Stainless Steel Strip Assay Procedures

When it is desirable or required that duplicate plates be produced, that the assay be directed toward one group of microorganisms (i.e., aerobes, aerobic spores, or anaerobic spores), or when the level of microbial contamination is considered to be extremely low, assay procedure 2 (section 5.3.1.6.2) may be used.

Standard membrane filter methods may be used as an alternative to these procedures.

5.3.1.6.1 Strip Assay Method 1 (in which one strip is assayed using both treatments)

a. Unheated Portions: Pipette 2.0 or 4.0 ml portions of the extraction fluid from each flask into 100 mm Petri plates, using 20 ml total.

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

b. Heated Portions (Heat Shock Treatment): Aseptically pipette 25 ml of the extraction fluid from each flask into separate, sterile test tubes (approximately 25 x 150 mm), taking care not to contaminate the upper inside surface of the tube. The tubes should be held on ice if the pipetting procedure will require more than 10 minutes, but for no longer than 60 minutes. Place a rack containing the sample tubes and a pilot tube (see section 4) in the water bath at 80 ± 2 °C. Make certain the water bath level is above the level of the liquid content of each test tube. Monitor the temperature in the pilot tube, and incubate the sample tubes in the water bath for 15 minutes after the temperature in the pilot tube reaches 80 ± 2 °C.

Cool the sample tubes rapidly by immersion in cold water (10-15 °C) or by any other suitable means that affords rapid (within 2 minutes) cooling of the extraction fluid to 30-35 °C, as measured in the pilot tube.

Aseptically pipette 2.0 or 4.0 ml of the heat-shocked extraction fluid into Petri plates, using 20 ml total.

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NASA-HDBK-6022

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

c. Strip: Using sterile forceps, aseptically remove each strip from its flask; rinse it thoroughly in a gentle stream of sterile distilled room temperature water for 3-5 seconds; and place it, contaminated (marked) side facing up, in a sterile, dry Petri plate large enough to contain the strip.

Add enough sterile, molten (48-50 °C) TSA to completely cover the strip and allow the medium to solidify at room temperature.

d. As a negative control, also pour at least one plate containing 4.0 ml of sterile rinse solution.

e. Incubate plates and perform colony counts as detailed in section 4.

5.3.1.6.2 Strip Assay Method 2 (in which each strip is assayed using only one treatment)

a. Unheated Portions: After sonication, aseptically transfer 20 ml portions of the extraction fluid from one strip into two large, sterile Petri plates (150 x 25 mm).

Gently swirl the solution in the plate and quickly add 75 of sterile, molten (48-50 °C) concentrated TSA. *Immediately* resume rapid swirling until the contents are thoroughly mixed, and allow the mixture to solidify at room temperature.

b. Strip: Using sterile forceps, aseptically remove each strip from its flask; rinse it thoroughly in a gentle stream of sterile (room temperature) distilled water for 3-5 seconds; and place it, contaminated (marked) side facing up, in a sterile, dry Petri plate.

Add enough sterile, molten (48-50 °C) TSA to completely cover the strip and allow the medium to solidify at room temperature.

c. Heated Portions (Heat Shock Treatment): Place the flask containing extraction fluid directly in a water bath at 80₊₂ °C for 15 minutes as determined by a pilot flask containing a thermometer. Make sure the water bath level is above the liquid contents of the flask.

After heat shock, immediately cool the contents of the flask to approximately 45-50 °C.

Aseptically transfer 20 ml portions of extraction fluid into two large, sterile Petri plates (150 x 25 mm).

Gently swirl the solution in the plate and quickly add 75 ml of sterile, molten (48-50 °C) concentrated TSA. *Immediately* resume rapid swirling until the contents are thoroughly mixed, and allow the mixture to solidify at room temperature.

d. As a negative control, also pour at least one plate containing 25 ml of sterile rinse solution.

e. Incubate plates and perform colony counts as detailed in section 4.

5.3.2 Assessment of Airborne Microbial Contamination Accumulating on Surfaces Using Teflon Fallout Ribbons

5.3.2.1 Scope and Application

This method involves the collection of airborne microbial contaminants on Teflon ribbons. It may be used to monitor facilities associated with hardware or any surface that cannot be subjected to water-based assay methods.

5.3.2.2 Apparatus and Equipment

Sterile Teflon Ribbons.

Sterile Test Tubes 25 x 150 mm.

Pipettes – assorted sterile pipettes (10, 25, 50 ml).

Sterile Petri Plates.

Sterile Forceps.

Ultrasonic Bath.

80 °C Waterbath.

Incubators.

5.3.2.3 Reagents and Consumable Material

Trypticase Soy Broth.

Sterile Rinse Solution.

Sterile Buffered Distilled Water.

Trypticase Soy Agar.

5.3.2.4 Sample Collection, Preservation, and Storage

Prepare sterile, Teflon ribbons as described in section 4, allowing two ribbons per site per sample. At each sampling site, wearing sterile gloves, carefully remove a sufficient number of Teflon ribbons from sterile glass beakers and unroll ribbons on a pre-cleaned (sterilized) support surface. The duration of Teflon ribbon exposure and the sampling intervals shall be subject to approval by NASA management or its authorized contractor(s).

Wearing sterile gloves, collect two Teflon ribbons per sampling site at each sampling interval. Aseptically roll up each exposed Teflon ribbon and place in separate, dry, sterile glass jars. It is recommended that the cap of each jar be covered with aluminum foil to minimize the possibility of contaminating the lip of the jar. The foil should extend 5-6 cm down the side of the jar.

NASA-HDBK-6022

As a field blank, unroll at least one Teflon ribbon; expose it to the air in the environment for several seconds; roll it; and place it in a separate glass jar.

Hold Teflon ribbons contained in jars at 4 °C and begin assay procedures within 24 hours after collection. If assayed within 2 hours, the strips may be held at 25-35 °C.

5.3.2.5 Extraction

Create a “laboratory blank” by aseptically unrolling and rerolling a Teflon ribbon and placing it into a clean, sterile collection jar.

Aseptically add 400 ml of sterile rinse solution maintained at room temperature to each jar containing a Teflon ribbon. Immediately sonicate the samples as detailed in section 4 for 6 minutes \pm 5 seconds. Sonication time is longer for ribbons because they are coiled in the jar.

5.3.2.6 Teflon Fallout Ribbon Assay Procedures

When the level of microbial contamination is anticipated to be extremely low or the assay is directed toward one specific group of microorganisms, assay procedure two (section 5.3.2.6.2) may be used.

5.3.2.6.1 Ribbon Assay Method 1 (in which one ribbon is assayed using both treatments)

a. Unheated Portions: Aseptically pipette 2.0 or 4.0 ml portions of the extraction fluid into Petri plates, using 100 ml total.

Add approximately 20 ml sterile, molten (48-50 °C) TSA to each plate; mix by gentle swirling; and allow to solidify at room temperature.

b. Heated Portions (Heat Shock Treatment): Aseptically pipette 30 ml portions of the extraction fluid into four separate sterile test tubes (approximately 25 x 150 mm) taking care not to contaminate the upper inside surface of the tubes. The tubes should be held on ice if the pipetting procedure will require more than 10 minutes, but for no longer than 60 minutes.

Place a rack containing the sample tubes and a pilot tube in a water bath at 80 ± 2 °C. Make certain the water bath level is above the level of the liquid content of each test tube. Monitor the temperature in the pilot tube, and incubate the sample tubes in the water bath for 15 minutes after the temperature in the pilot tube reaches 80 ± 2 °C.

Cool the sample tubes rapidly by immersion in cold water (10-15 °C) or by any other suitable means that affords rapid (within 2 minutes) cooling of the extraction fluid to 50-55 °C, as measured in the pilot tube.

Aseptically pipette 25.0 ml portions of the heat-shocked extraction fluid from each tube into four large Petri plates (150 x 25 mm).

NASA-HDBK-6022

Add 75 ml of sterile, molten (48-50 °C), concentrated TSA to each plate. Mix by rapid swirling and allow to solidify at room temperature.

- c. As a negative control, also pour at least one plate containing 4.0 ml of sterile rinse solution.
- d. Incubate plates and perform colony counts as detailed in section 4.

5.3.2.6.2 Ribbon Assay Method 2 (in which each ribbon is assayed by only one treatment)

- a. Unheated Portions: After sonication, aseptically transfer the entire 400 ml portion of extraction fluid from one jar through a sterile membrane filter using a membrane filter monitor as described in section 4.

Press the filter from each jar onto the surface of a fresh, newly prepared Petri plate containing TSA.

- b. Heated Portions (Heat Shock Treatment): After sonication, place one jar containing a ribbon and 400 ml of extraction fluid in a water bath at 80 ± 2 °C and allow to remain for 15 minutes after the extraction fluid has reached a temperature of 80 ± 2 °C. A pilot jar containing 400 ml of rinse solution and a thermometer should be used to determine when the solution reaches temperature.

After heating, immediately cool the extraction fluid to 30-35 °C and filter the contents according to standard sterile membrane filter procedures.

Press the filter from each jar onto the surface of a fresh, newly prepared Petri plate containing TSA.

- c. Incubate plates and perform colony counts as detailed in section 4.

5.3.3 Microbiological Sampling of Intramural Air Using Membrane Filter Field Monitors

5.3.3.1 Scope and Application

This method is used to collect air samples using membrane filters, for plating and growth onto media in order to determine the microbial contamination present in cleanrooms and assembly areas.

5.3.3.2 Apparatus and Equipment

Membrane Filter Field Monitors.
Sterile membranes.
Sterile forceps.

Aerobic and anaerobic incubators.

5.3.3.3 Reagents and Consumable Materials

Freshly prepared TSA Petri plates.

5.3.3.4 Sample Collection, Preservation, and Storage

When required, membrane filter field monitors may be used to sample intramural air, especially in areas where larger slit sampler agar impaction devices are restricted.

- a. Each field monitor shall use a 0.45 or 0.8 micron membrane filter attached to a vacuum source via an aerosol adapter with a 10-liter/minute flow-limiting orifice.
- b. The volume of air sampled with each field monitor shall not exceed 300 liters.

When sampling larger volumes of intramural air or when isolation of different types of microorganisms by the use of selective isolation media is desired, a manifold capable of holding up to six membrane filter field monitors may be used.

- c. Each manifold shall be equipped with a flow meter calibrated to collect air at the rate of 10 liters/minute per field monitor up to a maximum of 60 ± 0.02 liters/minute per manifold.
- d. The volume of air sampled with each manifold shall not exceed 300 liters per field monitor, or 1800 liters per manifold.

5.3.3.5 Assay Procedure

For every ten or fewer membrane samples collected, process one additional sterile membrane as a negative control.

Process samples through the membrane filter monitor or manifold according to the manufacturer's instructions. Remove each membrane filter aseptically from the field monitor and place it onto the surface of a freshly prepared TSA Petri plate.

Aerobically incubate all plates at 32 °C for 72 hours in an inverted position. Scan plates at 24 and 48 hours; count and record number of detectable colonies. Perform and record final colony counts at 72 hours. Do not remove the Petri plate covers until the final count is made at 72 hours of incubation.

NASA-HDBK-6022

The number of colonies growing on the surface of the membrane filter represents the number of airborne viable particles per 300 liters of ambient air. This value divided by 300 would represent the average colony count for one liter of air. Results shall be reported as the number of CFUs per liter of air or as prescribed by NASA management or its authorized contractor(s).

5.3.4 Microbiological Sampling of Intramural Air Using Slit Sampler Agar Impaction Devices

5.3.4.1 Scope and Application

This method is used to collect air samples directly onto growth media in order to determine the level of microbial contamination present in cleanrooms and assembly areas.

5.3.4.2 Apparatus and Equipment

Slit Sampler.

Aerobic and anaerobic incubators.

Calibration and Preparation: Each sample shall be equipped with a calibrated in-line flowmeter. A vacuum source 50 percent greater than the designated sampling rate is required for each sampler. Before initial operation and semi-annually thereafter, calibrate all samplers to draw the designated volume ± 5 percent.

5.3.4.3 Reagents and Consumable Material

Volumetric Air Sampler Plates: Use sterile, 150 x 25 mm Petri plates. Prepare each plate to contain 85.0 ± 2.0 ml of sterile TSA. Wrap prepared batches of plates securely to prevent dehydration. Incubate prepared and wrapped plates at 32 °C for 72 hours. Unwrap and discard contaminated plates. Rewrap and refrigerate plates at 4-6 °C until 12-18 hours prior to use. Again unwrap plates and leave at room temperature until used. Use sterile plates within 18 hours after unwrapping and within 30 days of initial preparation.

5.3.4.4 Sample Collection, Preservation, and Storage

Perform sampling according to the slit sampler manufacturer's instructions.

For sequential sampling of intramural air in clean assembly environments, a slit sampler capable of collecting at least 850 liters of air on a 150 x 25 mm agar plate in a single revolution should be used. The volume sampled with each plate should not exceed 3500 liters. Other devices suitable for cleanroom sampling include oilless pumps and manual, spring-wound clock motors.

5.3.4.5 Assay Procedure

Aerobically incubate all plates at 32 °C for 72 hours in an inverted position. Scan plates at 24 and 48 hours. Count and record the number of colonies detected. Similarly record colony counts at 72 hours. Do not remove the Petri plate covers until the final count is made at 72 hours.

6. PLANETARY PROTECTION METHODS FOR SUPPLEMENTAL ASSAYS

The information in the following sections describes the use of specific commercial equipment and reagents. However, it may be permissible to use alternate vendors provided that the detection limits described are met.

6.1 Assessment of Microbial Contamination on Spacecraft Surfaces Using the T-ATP Assay

6.1.1 Scope and Application

This assay is used to pre-screen hardware for the presence of microbial contamination prior to conducting final assays using the methods described in sections 4 and 5. This method is useful because the results are obtained rapidly (without a 72-hour incubation period) and can be used to assess quickly if cleaning or other processing is required prior to carrying out and reporting final assays of spacecraft surfaces.

6.1.2 Summary of Method

This method describes the analysis, identification, and quantification of microbial contamination on spacecraft surfaces based upon the measurement of T-ATP. Samples are obtained from the field using procedures described below. The T-ATP detection assay is widely used in the food industry to determine gross bacterial contamination. The T-ATP assay uses a bioluminescent reagent containing luciferin and the firefly enzyme luciferase. If present, ATP transfers chemical energy to the luciferin molecule that is then acted upon by the enzyme luciferase (in the presence of magnesium ions and molecular oxygen) to form oxyluciferin, carbon dioxide, and light (bioluminescence). The bioluminescence is then measured with a luminometer using a photomultiplier tube.

6.1.3 Apparatus and Equipment

Kikkoman Lumitester™ K-120, or instrument of equal or superior sensitivity.
Kikkoman tubes – sterile, or equivalent appropriate for the instrument used.
Kikkoman test tube racks, or equivalent appropriate for the instrument used.

Sterile wire cutters.

Oxford® pipettors, or equivalent.

Vortexer.

Laminar Clean Air Flowbench.

6.1.4 Reagents and Consumable Materials

Kikkoman CheckLite™ – HS Plus Kit containing luciferin-luciferase reagent, Reconstitution Buffer, and ATP Releasing Reagent.

NASA-HDBK-6022

Sterile, Molecular Biology Grade Water, SIGMA W4502, or equivalent.

Kikkoman tubes – sterile, or equivalent appropriate for the instrument used.

Sterile Pipette Tips (100 microliter sterile – ATP free).

Sterile Polyester Texwipe swabs – (TX761 Alpha Swab with Long Handle), or equivalent.

6.1.5 Sample Collection, Preservation, and Storage

Aseptically remove a sterile polyester swab from its container and moisten the head of the swab in a known volume that may range from 3-10 ml sterile distilled 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-degree angle with the surface to be sampled. Rotate the head of the swab slowly and thoroughly over a measured surface area of no more than 25 cm². Change the direction of the swabbing motion 90 degrees and again swab the surface. Complete a third coverage of the surface by again changing the direction of the swabbing motion by 90 degrees. Return the head of the swab to the original tube of solution, using sterile cutters to cut the swab below any portion of the handle that was touched by the sampler. Allow the swab head to drop into the liquid and replace the screw cap. Transport samples to the laboratory on ice and process within 24 hours. Samples may be kept frozen at –20 °C until processed for a period of up to 1 week.

6.1.6 Extraction

Prior to sonication, place each tube containing the water and the swab on a vortex mixer and agitate at maximum power for 5-6 seconds.

Suspend the tubes containing swab heads in the middle of an ultrasonic bath filled to normal capacity. Not more than 12 tubes should be placed into the bath at one time. Make sure that the bath fluid is above the liquid level in the tubes and then sonicate for 2 minutes \pm 5 seconds. After sonication, assay the vortexed solution as described in section 6.1.8.

6.1.7 Calibration and Standardization

Calibration standards are required to determine the correlation between the bioluminescence readings from the Lumitester™ in relative luminescence units (RLU) and the concentration of total ATP in each sample. A dilute stock standard of ATP at 10^{-9} M may be prepared and frozen in aliquots to be thawed just before use. The stock standard is used to prepare working (calibration) standards prepared in the linear response range of the instrument ($\sim 10^{-9}$ - 10^{-13} M) in Sigma water. Working standards are prepared fresh prior to use (minimum daily) and are analyzed by the same method as the samples.

- a. Prior to sample analysis, instrument calibration is performed using a minimum of three standard concentrations falling within the linear response range of the instrument.
- b. In addition to preanalysis instrument calibration (above), method blanks (negative controls) are also analyzed daily to verify that the sample analysis does not reflect instrument or method contamination. The instrument/method blank is created at the time of analysis using the same matrix as the prepared samples and carried out using the same procedures as test samples. These quality control procedures ensure that results do not reflect procedural contamination or contamination introduced by reagents.
- c. Field blanks are samples that are taken in the field and subjected to all field environments. These blanks are not used for sampling, but may contain sterile water and/or a sterile swab that would be used for sampling. Field blanks are analyzed in the same manner as test samples. This quality control procedure determines if contamination occurs during any phase of the field sampling process.

6.1.8 Procedure for T-ATP Assay

Complete the full assay on one sample before starting with the next. Aseptically set-up and label the tubes by labeling the rack in front of each tube position rather than the tube itself. Labeling the tubes directly may interfere with the bioluminescence readings during analysis.

Prepare the Sample Source tube by aseptically pipetting 0.5 ml of sample into the labeled first tube position.

Aseptically pipette 0.1 ml into each of four sample tubes behind the corresponding Sample Source tube.

6.1.8.1 Complete the following assay for each tube, one at a time:

- a. Using a new pipette tip, add 0.1 ml (use volume equivalent to sample volume) of ATP Releasing Reagent to each sample tube. Discard pipette tip.
- b. Vortex the sample for 3-5 seconds. Allow the sample to sit for a minimum of 60 seconds to complete the ATP extraction before the next step.

NASA-HDBK-6022

- c. Using a new pipette tip, add 0.1 ml (or one sample volume) of Luciferin-Luciferase Reagent to the sample tube.
- d. Vortex the sample for 3-5 seconds.
- e. Immediately insert the sample tube into the Lumitester™, close the lid, and measure the bioluminescence. Each tube is read automatically ten times by the instrument; the average reading is displayed on the screen in RLUs.
- f. Repeat section 6.1.8.1 for each remaining sample tube.
- g. Record each sample tube reading on the Sample Log sheet.
- h. To process another sample, use a new pipette tip and repeat section 6.1.8.

6.1.9 Data Collection and Analysis

Sample bioluminescence readings should be performed in triplicate for each sample. Total ATP values for each sample will be taken from a least squares linear regression formulated from the calibration curve using the method blank as zero. The average bioluminescence reading for each sample will be used to determine the total ATP via the least squares fit.

The final concentration for T-ATP for the area sampled is calculated as follows:

$$\text{T-ATP} = \frac{(\text{TATPM})(\text{TSV})}{(\text{ASV})}$$

Where:

T-ATP = Total ATP in moles

T-ATPM = (Total ATP measured for the sample in moles)

TSV = Total sample volume used in the field

ASV = Assay sample volume

Details of the method and detection limits are presented in JPL document D-30970 (Kern and others, 2005) and Venkateswaran and others, 2003. For the Kikkoman Lumitester system as described above, the method detection limit was determined to be 7.0×10^{-14} $\mu\text{mol/ml}$. Instrument sensitivities may vary. Therefore, it is recommended that a demonstrated method detection limit of less than 10^{-13} $\mu\text{mol/ml}$ be required of the ATP instrument-reagent system overall.

6.2 Assessment of Microbial Contamination Accumulating on Surfaces Using the LAL Assay

6.2.1 Scope and Application

This assay is used to pre-screen hardware for the presence of microbial contamination prior to conducting final assays using the methods described in sections 4 and 5. This method is useful because the results are obtained rapidly (without a 72-hour incubation period) and can be used to assess quickly, if cleaning or other processing is required prior to carrying out and reporting final assays of spacecraft surfaces.

6.2.2 Summary of Method

LAL is a highly sensitive test developed to detect the presence of pyrogenic endotoxins or lipopolysaccharide (LPS) which may be present on medical devices. As LPS is present only in Gram negative bacteria, the method cannot be easily related to the current pour plate standard assay that analyzes spores from microorganisms which are primarily Gram positive. However, because endotoxins are prevalent in nature, the method can be used as a good indicator of the overall cleanliness of spaceflight hardware. Samples are obtained from the field using procedures contained in this Handbook. The analysis includes an extraction in LAL Reagent Water (LRW) followed by a kinetic chromogenic assay.

Extremes in pH, high concentrations of salts, and the presence of detergents or other organics can interfere with the LAL assay. Each sample is tested “unspiked” or plain and with an LPS “spike” of known concentration added. The measured Endotoxin Units per ml (EU/ml) of the spiked sample when compared to that of the unspiked sample indicates the presence of interference with the LAL assay by the sample.

6.2.3 Apparatus and Equipment

Heat depyrogenated glass dilution tubes.
Adjustable micropipetter.
Repeating pipetter with tips.

Vortex mixer.

Sterile Wire Cutters.

PTS (Portable Test System) Reader, Charles River Endosafe®.

96-well incubating plate reader with LAL capable software, Molecular Devices.

Laminar Clean Air Flowbench ThermoMAX™, or equivalent, or Charles River Endosafe®. PTS.

6.2.4 Reagents and Consumable Materials

Pyrogen-free pipettes and pipette tips.

Sterile, pyrogen-free, flat bottomed, lidded 96-well microtiter plates (Falcon #3072, or equivalent), or Charles River Endosafe® PTS cuvettes 5.0.

LAL, Charles River Endosafe® Endochrome-K or PTS-LAL cartridges.

LRW, Charles River Endosafe® LRW, or equivalent (Sigma Water for Molecular Biology, W-4502).

Control Standard Endotoxin (CSE), Charles River Endosafe® CSE,
Sterile Polyester Texwipe swabs – (TX761 Alpha Swab with Long Handle), or equivalent.

6.2.5 Sample Collection, Preservation, and Storage

Aseptically remove a sterile ultra-clean polyester swab from its container and moisten the head of the swab in 2-10 ml of LRW. Express excess moisture from the swab against the interior wall of the tube. Hold the swab so that the handle makes about a 30-degree angle with the surface to be sampled. Rotate the head of the swab slowly and thoroughly over a measured surface area of no more than 25 cm². Change the direction of the swabbing motion 90 degrees and again swab the surface. Complete a third coverage of the surface by again changing the direction of the swabbing motion by 90 degrees. Return swab to tube containing LRW by cutting the handle with sterile wire cutters and transport closed tubes using sterile procedures back to the laboratory. Samples may be used immediately or refrigerated up to 24 hours prior to testing.

6.2.6 Extraction

To begin processing, vortex each sample at maximum speed for 30 seconds. At this point, sub-samples may be aliquoted for other tests by dispensing a volume of the extract (vortexed LRW from sample tube) into smaller tubes (VWR - #60818-496 polystyrene or polypropylene or equivalent). Samples may be analyzed immediately or frozen for later analysis.

6.2.7 Calibration and Standardization

Calibration Standards are required to determine the correlation between the optical density values from the plate reader and the concentration of total endotoxin in each sample. CSE is prepared according to the manufacturer's instructions and lysate lot-specific certificate of analysis (note: different lots of CSE or LAL will require different reconstitution volumes for the CSE). Working Stock is obtained by diluting, if necessary, the CSE to a concentration of 50 EU/ml using LRW. Working Standards are prepared by diluting the working stock in 10-fold increments to prepare the following standard concentrations: 5.0, 0.5, 0.05, and 0.005 EU/ml. All dilutions should be done with LRW in heat-depyrogenated glass tubes or other pyrogen-free tube.

NASA-HDBK-6022

Calibration is established for each assay using the five endotoxin standard concentrations, 50 EU/ml to 0.005 EU/ml. Method blanks are analyzed with each assay to ensure no method contamination. This is accomplished by testing LRW negative controls with each assay. Field blanks are samples that are taken to the field and subjected to all field environments. These blanks are not used for sampling, but may contain water and/or a sterile swab that would be used for sampling. Field blanks are analyzed in the same manner as samples. This measurement determines if there is contamination occurring during any phase of the field sampling process. If sample assays are rejected due to quality control anomalies, resampling or retesting may be necessary.

6.2.8 Procedure for LAL Assay

6.2.8.1 Kinetic Chromogenic Assay Using 96-well Plate with Endochrome-K

- a. Add 25 μ L of LRW to all sample wells that will contain the aliquot of unspiked sample.
- b. Add 25 μ L of 1.0 EU/ml endotoxin spike solution to all wells that will contain spiked samples. This will give a 0.5 EU/ml spike in all samples.
- c. Add 25 μ L of the unknown sample to the appropriate LRW and endotoxin-spiked wells.
- d. Add 50 μ L of the LPS standards to the appropriate wells in the plate.
- e. Rapidly add 50 μ L of LAL to each well using a repeating pipetter.
- f. Mix the plate for 10 seconds in the plate reader, or by gently shaking side to side by hand.
- g. Take kinetic readings of the incubating plate every 30 seconds over a 60-minute period at a wave-length of 405 nm and a temperature of 37 °C.
- h. Using the appropriate software procedure, determine the onset time for each sample at an onset OD of 0.05.

The final concentration of EU/ml in the sample is determined using a log—log regression calculation of the endotoxin standard concentration and mean onset time data to determine the endotoxin concentrations in the unknown samples. The onset time for the negative control should be significantly longer than for the lowest standard.

The spike recovery for each sample should be within a two-fold dilution of the 0.5 EU/ml positive control. A value of less than 50 percent indicates inhibition of the assay by the sample. A value of greater than 200 percent indicates enhancement. If inhibition is found, the sample should be diluted further and then retested.

6.2.8.2 PTS LAL Assay

Remove PTS LAL Assay Cartridge from packaging and insert into the preheated PTS unit when prompted. When prompted by the PTS reader, enter the PTS LAL cartridge lot number followed by the lot-specific calibration code (refer to the manufacturer's certificate of analysis). For the PTS LAL assay, the use of calibration standards and spikes are not required. The PTS LAL assay utilizes an archived standard curve with an endotoxin standard curve range of 5.0-0.05 EU/ml.

When instrument prompts: "Add sample and press enter," add 25 μ L of the unknown to each sample well and then press the enter key.

PTS instrument will display calculated results when the assay is finished. Record or print test results.

Instrument sensitivities may vary. Therefore, it is recommended that a demonstrated method detection limit of less than 0.05 EU/ml be required of an LAL instrument-reagent system.

Method References

Charles River Endosafe® Limulus Amebocyte Lysate, Endochrome-K product insert.

Charles River Endosafe® Certificate of Analysis for Control Standard Endotoxin.

Charles River Laboratories PTS User's Guide.

APPENDIX A

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