Designation: D7391 – 09

Standard Test Method for Categorization and Quantification of Airborne Fungal Structures in an Inertial Impaction Sample by Optical Microscopy[1]

This standard is issued under the fixed designation D7391; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method is a procedure that uses direct microscopy to analyze the deposit on an inertial impaction sample.

1.2 This test method describes procedures for categorizing and enumerating fungal structures by morphological type. Typically, categories may be as small as genus (for example, *Cladosporium*) or as large as phylum (for example, basidiospores).

1.3 This method contains two procedures for enumerating fungal structures: one for slit impaction samples and one for circular impaction samples. This test method is applicable for impaction air samples, for which a known volume of air (at a rate as recommended by the manufacturer) has been drawn, and is also applicable for blank impaction samples.

1.4 Enumeration results are presented in fungal structures/sample (fs/sample) and fungal structures/m^3 (fs/m^3).

1.5 The range of enumeration results that can be determined with this method depends on the size of the spores on the sample trace, the amount of particulate matter on the sample trace, the percentage of the sample trace counted, and the volume of air sampled.

1.6 This method addresses only the analysis of samples. The sampling process and interpretation of results is outside the scope of this method.

1.7 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

3. Terminology

3.1 *ASTM Definitions (see ASTM Terminology Dictionary)* :

3.1.1 *numerical aperture*

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *circular impaction sample, n*—a sample of airborne particulate matter collected via a device that draws air through a round aperture at a specified rate, impacting the particles suspended in the air onto an adhesive medium, resulting in a circular area of deposition. A circular impaction sample may be collected by means of a cassette manufactured for that purpose, or by means of a sampling device that requires slides to be pre-coated with impaction medium.

3.2.2 *debris rating, n*—a distinct value assigned to an impactor sample based on the percentage of the sample area potentially obscured by particulate matter, and ranging from 0 to 5.

3.2.3 *field blank, n*—a sample slide or cassette carried to the sampling site, exposed to sampling conditions (for example, seals opened), returned to the laboratory, treated as a sample, and carried through all steps of the analysis.

3.2.4 *hyaline, adj*—colorless.

3.2.5 *impaction medium, n*—a substance applied to a microscope slide used to collect (or capture) particulate matter during sampling.

3.2.6 *impaction sample, n*—a sample taken using impaction, for example, slit impaction sample, circular impaction sample.

3.2.7 *inertial impactor, n*—a device for collecting particles separated from an air stream by inertia to force an impact onto an adhesive surface. Inertial impactors are available in many

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

designs, including those having a slit jet, yielding a rectangular sample trace, and a circular jet, yielding a circular sample trace.

3.2.8 *magnification/resolution combination 1, n*—~150-400× total magnification and a point to point resolution of 0.7 µm or better, as checked by a resolution check slide.

3.2.9 *magnification/resolution combination 2, n*—~400× or greater total magnification and a point to point resolution of 0.5 µm or better, as checked by a resolution check slide.

3.2.10 *minimum reporting limit (fs/sample); minimum reporting limit (fs/m^3), n*—the lowest result to be reported for total spores or any spore category. Since both fs/sample and fs/m^3 are reported, there are two minimum reporting limits.

3.2.11 *morphology, n*—the form and structure of an organism or any of its parts; for fungi, the shape, form, and/or ornamentation.

3.2.12 *mounting medium, n*—a liquid, for example, lactic acid or prepared stain, used to immerse the sample particulate matter and to attach a cover slip to an impaction sample.

3.2.13 *sample trace, n*—the area of particle deposition, that is, the deposit on a slit impaction sample resembling a narrow rectangle, or the circular deposit on a circular impaction sample.

3.2.14 *septum (pl.: septa), n*—a cell wall or partition.

3.2.15 *slide adherent, n*—an adhesive or liquid used to affix an impaction sample substrate to a microscope slide.

3.2.16 *slit impaction sample, n*—a sample of airborne particulate matter collected via a device that draws air through a linear aperture at a specified rate, impacting the particles suspended in the air onto an adhesive medium, resulting in a rectangular area of deposition. A slit impaction sample may be collected via a cassette manufactured for that purpose , or via a sampling device that requires slides to be pre-coated with impaction medium.

3.2.17 *spore category, n*—a grouping used for identification and quantifation of fungal structures. A spore category may contain a specific genus (for example, *Stachybotrys*), or it may represent a combination of genera (for example, *Aspergillus*/*Penicillium* -like).

3.2.18 *traverse, n*—a portion of analysis of an impactor sample consisting of one scan under the microscope from a sample-less portion of the impaction medium across the deposit to a corresponding sample-less portion of the impaction medium on the other side.

3.3 *Symbols:*

3.3.1 *fs*—fungal structure

3.3.2 *fs/m^3*—fungal structures per cubic metre

3.3.3 *m^3*—cubic metre

3.3.4 *mm*—millimetre

3.3.5 *µm*—micrometre

4. Summary of Test Method

4.1 Samples have been previously collected utilizing an impaction device operating at the device manufacturer's recommended sample flow rate. Each sample consists of an optically clear substrate coated with an adhesive and optically transparent medium onto which particles have been deposited through inertial impaction.

4.2 A sample is mounted to a microscope slide and examined by bright field microscopy using at least two magnification/resolution combinations.

4.3 Spores are differentiated from each other, other fungal structures, and from non-fungal material by color, size, shape, presence of a septum or septa, attachment scars, surface texture, etc., by means of a taxonomic comparison with standard reference texts and/or known standard samples (see A1.1 for suggested references). The number of spores that match each spore category are then calculated in units of fungal structures per sample (fs/sample) and also fungal structures per cubic meter of air (fs/m^3).

5. Significance and Use

5.1 This test method is used to estimate and categorize the number and type of fungal structures present on an inertial impactor sample.

5.2 Fungal structures are identified and quantified regardless of whether they would or would not grow in culture.

5.3 It must be emphasized that the detector in this method is the analyst, and therefore results are subjective, depending on the experience, training, qualification, and mental and optical fatigue of the analyst.

6. Interferences

6.1 *Differentiation of Fungal Genera/Species*—Because of the similar size and morphology of some fungal spores of different genera and the absence of growth structures and mycelia in airborne samples, differentiation by microscopic examination alone is difficult and spores must be grouped into categories based strictly on morphology. In many cases, identification at the genus level is presumptive. For example, differentiation between *Aspergillus* and *Penicillium* using this method is not typical, so a combined *Aspergillus*/*Penicillium*-like category is used. When differentiation between such genera is desired, a different method must be used. Unequivocal identification of every spore in each category is not possible due to optical limitations, the atypical nature of some of the spores, and/or overlapping morphology among different spore types, and therefore, certain spores must be categorized as Miscellaneous/Unidentifiable.

6.2 *Look-alike Non-fungal Particles*—Certain types of particles of non-fungal origin may resemble fungal spores. These particles and artifacts may include air or plant resin bubbles, starch, talc, cosmetic particles, or combustion products. Standards (mounted similarly to impactor samples) should be examined by laboratory analysts to know how to identify such particles. Examination of suspect particles using optical conditions other than bright field microscopy (for example, polarized light microscopy, phase contrast microscopy, differential interference contrast) may be helpful whenever significant concentrations of look-alike particles are present. In some cases dust and debris can mimic the morphology of particles of

interest. When look-alike particles are present in high concentration, accurately counting spores with similar morphology is difficult. When these conditions exist, they should be reported in the analysis notes section of the report.

6.3 *Particle Overloading*—High levels of particulate matter on an impaction sample will bias the analysis in two ways:

(1) Particle capture efficiency decreases, and

(2) Debris obscures or covers spores.

Both of these factors produce a negative bias.

6.4 *Staining*—Staining, while optional, may help the analyst differentiate spores from debris. Without staining, clear spores (especially small ones) may exhibit negative bias because the analyst has insufficient contrast to notice them while scanning. Also, because spores of different fungal species absorb stains at different rates, under or over-staining makes identification difficult. The problem can be eliminated by careful control of stain concentrations.

6.5 *Impaction Medium Stability and Clarity*—Chemicals present in some mounting media may affect the physical stability or clarity of the impaction medium. For instance:

(1) Samples collected on silicone grease medium should first be warmed on a hot plate at approximately 40°C to "fix" the sample in place, when using lacto-phenol cotton blue stain, and

(2) Slides and cassettes using methyl cellulose ester + solvent adhesive medium, which is stable in lacto-phenol cotton blue stain, will "fog" with Calberla's stain due to the water and alcohol mixture; warming fogged slides may temporarily clear them.

The lab or analyst should develop through experimentation an impaction medium/mounting medium combination that will result in acceptable stability, clarity, and spore visibility.

6.6 *Uneven Impaction Medium Uniformity*—Uneven thickness may be present in greased slides, pre-coated slides and manufactured cassettes. The microscopist will compensate by adjusting the plane of focus. When grease is too thick, differentiating small spores from background artifacts (especially air bubbles) in the grease preparation becomes difficult. When grease is too thin, shrinkage and pooling may have occurred, causing particle loss during sampling.

7. Apparatus

7.1 *Marking pen,* for marking sample slides.

7.2 *Microscope or magnification system,* having a precision *x-y* mechanical stage. The microscope or magnification system used for analysis shall be capable of at least two magnification/resolution combinations as follows: magnification/resolution combination 1 shall be ~150-400× total magnification and a point to point resolution of 0.7 µm or better; magnification/resolution combination 2 shall be ~400× or greater total magnification and a point to point resolution of 0.5 µm or better. It is recommended that at least one microscope or magnification system in the lab be capable of magnification of ~1000× total magnification and a point to point resolution of 0.3 µm or better. That the resolution for combinations 1 and 2 is suitable is to be checked using a resolution check slide (see 13.2.3).

7.3 *Reference Slides*—a series of mounted field samples to be used as counting references. Analysts' results from these slides are expected to be within laboratory acceptance limits to prove competence.

7.4 *Reticule,* width defining, an optical device in the light path of the microscope capable of being reproducibly set to define a traverse width no larger than 0.75× the diameter of the ocular field of view, and having graduations of an appropriate dimension to allow measurement of spore size, for example, Walton-Beckett reticule (round) or 100 divisions in 10 mm (linear or square). If a non-round reticule is used, procedures must be in place to ensure that the reticule is correctly positioned for each analysis.

7.5 *Stage micrometer,* traceable to the National Institute of Standards and Technology (NIST) or equivalent international standard.

7.6 *Resolution check slide,* a microscope slide on which calibrated distances, shapes, and line widths provide reliable and simple image resolution and shape identification performance of the microscopic and analyst at magnification. Examples include: a slide onto which a variety of diatoms have been mounted, including examples of *Stauroneis phoenicenteron* and *Pleurosigma angulatum*, a brightfield resolution test slide, or equivalent.

7.7 *Syringe or dropper,* for dispensing liquid during sample preparation.

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type II of Specification D1193.

8.3 *Mounting medium (with or without stain),* for rehydrating spores and for holding the cover slip to the impaction sample, for example, lactic acid, lacto-cotton blue stain, lacto-phenol-cotton blue stain, lacto-fuchsin stain (see X2.1 for stain preparation).

8.4 *Microscope cover slips,* large enough to cover the deposit (for example, 22 mm^2); for optimum performance, choose a cover slip thickness according to the recommendations of the microscope objective lens manufacturer.

8.5 *Microscope slides*

8.6 *Slide adherent,* for affixing impaction cassette samples to microscope slides, for example, clear nail polish, immersion oil, tape.

9. Hazards

9.1 Components of re-hydrating liquids and stains, for example, lactic acid, phenol, are corrosive or hazardous. Consult the appropriate MSDS for any reagents used.

10. Preparation of Apparatus

10.1 *Microscope Alignment/Adjustments*—Follow the manufacturer's instructions.

11. Calibration and Standardization

11.1 *Diameter/Width and Graduation Spacing for Ocular Reticule*—see 13.2.2.

12. Procedure

12.1 *Sample Preparation*

12.1.1 *Preparation of a Pre-coated Slide* (the impaction medium is already on a microscope slide)

12.1.1.1 Mark each slide with a unique designation.

12.1.1.2 If necessary (for example, for grease medium), gently warm to no more than 40°C to "fix" impacted particles in place.

12.1.1.3 Place one drop of mounting medium near the deposition trace and cover with a clean cover slip. Gently lower the cover slip at a slight angle to minimize air bubble formation. If the liquid contains stain, allow the stain to fully penetrate the particles before enumeration.

12.1.2 *Preparation of a Cassette*

12.1.2.1 Cut the sealer on the cassette and dismantle into two parts.

12.1.2.2 Mark each slide with a unique designation.

12.1.2.3 If the impaction substrate is not of suitable size/thickness to be examined on the microscope, it must be mounted on a microscope slide.

(1) Place a drop of slide adherent on a clean microscope slide.

(2) Carefully pull the glass or substrate that contains the adhesive film and the sample from the cassette, and place it, sample side upwards, on the slide adherent. Gently lower the glass slip at a slight angle to minimize air bubble formation if using liquid adherent.

12.1.2.4 Place one drop of mounting medium on the sample trace or cover slip. Gently lower the cover slip onto the sample trace at a slight angle to minimize air bubble formation. If the liquid contains stain, allow the stain to fully penetrate the particles before enumeration.

12.1.2.5 (*Optional*) Mark the approximate maximum extent of scan (~3 mm larger than the visible deposit) on the underside of the slide using a marking pen. This is especially useful for lightly loaded samples, in which the area to be scanned may not be obvious when the slide is observed on the microscope.

12.2 *Preliminary Evaluation*

12.2.1 The purpose of this examination is to note possible sample problems and to assign a debris rating. Use magnification/resolution combination 1.

12.2.2 Examine the entire sample trace. Note on the worksheet non-uniform deposition or other sample problems.

12.2.3 *Debris Rating Determination*

12.2.3.1 From the amount of particulate matter present at that part of the sample trace having approximately the greatest particle load, assign the sample a debris rating on a scale from 0 to 5. (A description of the debris rating numbers is given below in Table 1). Since the amount of debris in a field of view varies with the field of view chosen and its position in the sample trace, choose a rating that is most representative of a number of fields of view taken from the middle (not the more lightly loaded edges) of the trace.

12.3 *Counting Procedure for Slit Impactor*

12.3.1 Categorize each observed spore based on color, morphology, size, etc.

12.3.2 Categorize, at a minimum, the spore categories:

(1) Alternaria,
(2) ascospores (undifferentiated),
(3) Aspergillus/Penicillium-like
(4) basidiospores (undifferentiated),
(5) Chaetomium,
(6) Cladosporium,
(7) Curvularia,
(8) Drechslera/Bipolaris-like,
(9) smuts/Myxomycetes/*Periconia*,
(10) Stachybotrys/Memnoniella,
(11) Ulocladium, and
(12) hyphal fragments

For a fungal structure having characteristics inconsistent with all reported categories, enumerate it under the general category: Miscellaneous/Unidentified.

NOTE 1—Categories other than the above minimum may be used and reported, if desired. Fungal structures in the Miscellaneous/Unidentified category may be grouped by characteristics and reported separately, if desired, for example, Miscellaneous/Unidentified 1, Miscellaneous/Unidentified 2, etc.

12.3.3 Enumerate spore categories at an appropriate magnification/resolution. Enumerate the spore categories *Aspergillus/Penicillium*-like and *Cladosporium* at magnification/resolution 2 and other spore categories at either magnification/resolution 1 or 2.

NOTE 2—Spores that are especially small or hyaline are best enumerated at magnification/resolution 2. The categories basidiospores (undifferentiated) and ascospores (undifferentiated) are so variable that some spores in these categories could be enumerated at magnification/resolution 1, but others should be enumerated at magnification/resolution 2. The spore categories *Alternaria*, *Chaetomium*, *Curvularia*, *Drechslera/Bipolaris*-like, smuts/Myxomycetes/*Periconia* -like, *Stachybotrys/Memnoniella*, *Ulocladium*, hyphal fragments, and Miscellaneous/Unidentified may be enumerated at either magnification/resolution 1 or 2.

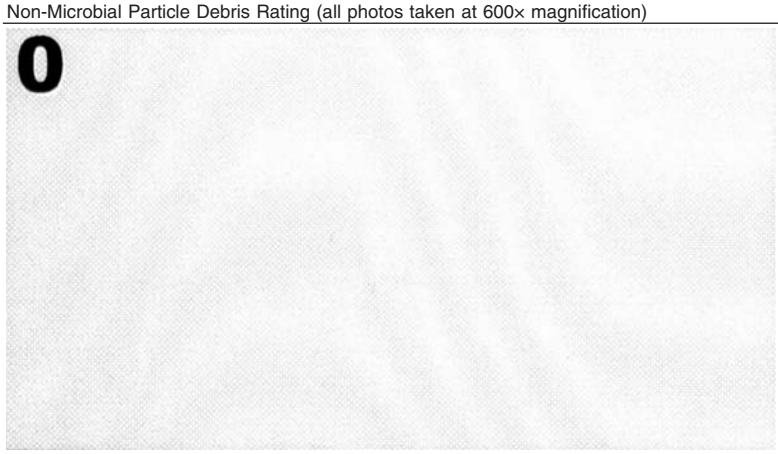
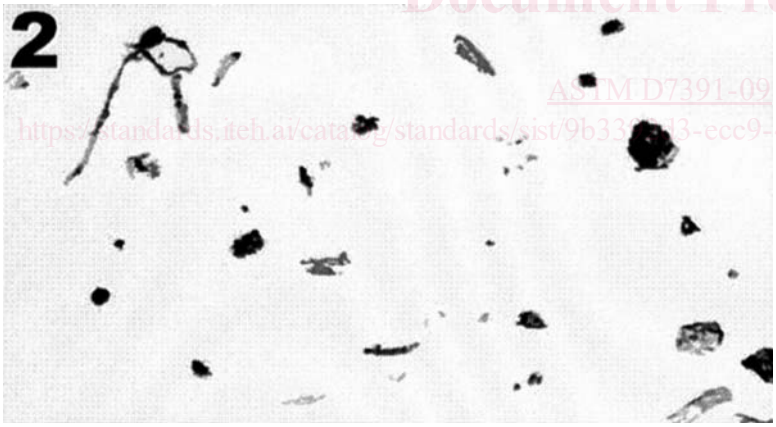
12.3.4 Enumerate a minimum of 20 % sample trace.

NOTE 3—An analyst/lab could decide to enumerate the minimum of 20 % for all spore categories, or decide to enumerate 100 % for all spore categories, or decide to enumerate for each spore category a percentage between 20 and 100 based on experience, on quality objectives or on how many spores in that category appeared to be present during the initial screening.

12.3.5 Enumerate during traverses across the sample trace. A traverse is one scan across the sample trace in a direction perpendicular to the longest dimension of the sample trace.

12.3.5.1 Start above or below the visible apparent deposit and scan across the deposit until well off the visible deposit. This process constitutes one traverse.

TABLE 1 Debris Rating Table

Non-Microbial Particle Debris Rating (all photos taken at 600× magnification)	Description	Interpretation
	No particulate matter detected in impaction area.	The absence of particulate matter in the impact area could indicate improper sampling or a blank sample, as most air samples typically contain some particles. Such absence of particulate matter should be noted on the report if the sample was not meant to be a blank.
	Minimal (>non detect to approx. 5 %) particulate matter present.	Reported values are minimally affected by particle load.
	Approx. 5 % toapprox. 25 % of the trace occluded with particulate matter.	Negative bias is expected. The degree of bias increases with the percent of the trace that is occluded.

12.3.5.2 As a traverse is made, the outer edges of the ocular reticule will be used to describe the boundaries of a rectangular counting area.

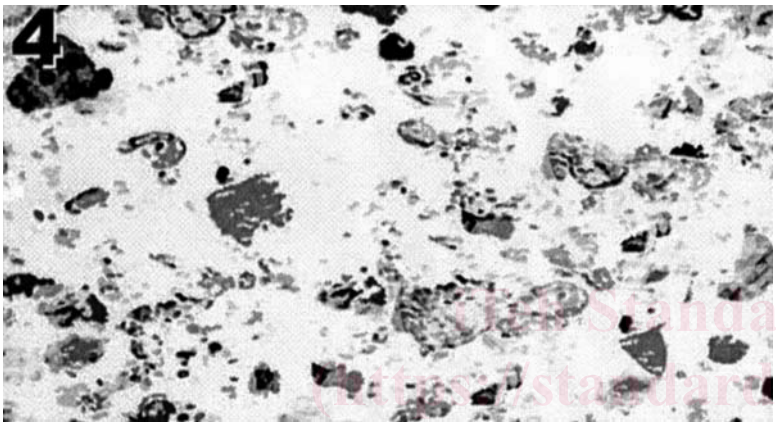
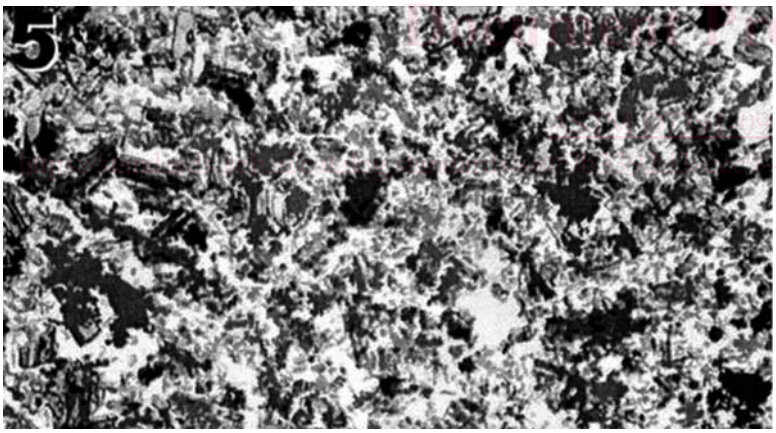
12.3.5.3 During a traverse, identify and categorize, the fungal structures that appear to fall within the outer edges of the ocular reticule If a fungal structure appears to lie on the left boundary line of the ocular reticule, count this structure. If a fungal structure appears to lie on the right boundary line of the ocular reticule, do not count it.

12.3.5.4 An analysis consists of a number of complete traverses. Do not use partial traverses.

12.3.5.5 If enumerating varying percentages for each spore category, pre-determine the % of the sample trace to be enumerated, so that traverses may be chosen to cover the sample trace more or less evenly. That is, do not start counting 100 % of the sample trace for all spore categories (using adjacent traverses), and then stop counting certain spore categories part of the way through the analysis, since such a count would be biased low due to the lightly loaded first few traverses.

12.3.5.6 If enumerating 100 % of the sample trace, start the traverses slightly outside the visible end of the sample trace, to

TABLE 1 *Continued*

	Approx. 25 % to approx. 75 % of the trace occluded with particulate matter.	Negative bias is expected. The degree of bias increases with the percent of the trace that is occluded.
	Approx. 75 % to approx. 90 % of the trace occluded with particulate matter.	Negative bias is expected. The degree of bias increases with the percent of the trace that is occluded.
	Greater than approx. 90 % of the trace occluded with particulate matter.	Quantification is not possible due to large negative bias. A new sample should be collected at shorter time interval, or other measures taken to reduce the particle load.

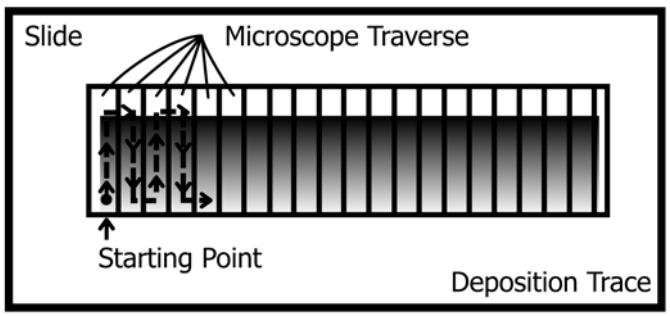


FIG. 1 Slit Impactor Location of Traverses for Counting 100 % of the Sample Trace

ensure that all spores are within the scanned area. If enumerating less than 100 % of the sample trace, start the traverses slightly inside the visible end of the sample trace, where the deposit appears to become uniform in order to obtain a representative traverse. Avoid the extreme end of the sample trace where the deposit appears to become less dense.

12.3.5.7 If enumerating 100 % of the sample trace, choose each subsequent counting area so that it abuts the previous counting area to provide full coverage, as shown in Fig. 1. If enumerating less than 100 % of the sample trace, separate traverses to prevent overlap. In this case, it is recommended