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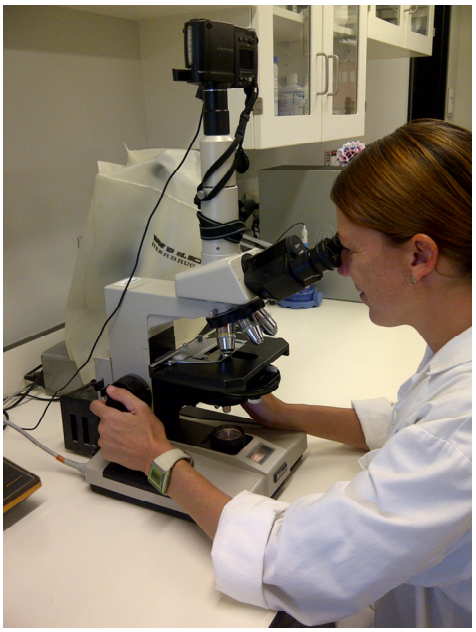
Great Lakes Forestry Centre
Insect Production Services

STANDARD OPERATING PROCEDURE

Number: IPS/008/002

Quantifying Microbial Organisms



Effective Date: 18 September 2014

Canada

TITLE: Quantifying Microbial Organisms

APPROVING OFFICIAL:

DD / MM / YY

Manager, Insect Production Services _____ ___/___/___

SIGNIFICANT CHANGES FROM PREVIOUS VERSION:

-the title of this SOP has been revised
-new definitions have been added; others have been revised.
-this SOP has been formatted to meet current IPS requirements.

1.0 INTRODUCTION

1.1 Purpose

This Standard Operating Procedure (SOP) has been established to ensure that all quantifications of aqueous suspensions of microbial organisms (e.g., NPV occlusion bodies, fungal spores, microsporidia, etc.) are performed following a consistent and statistically valid format.

1.2 Scope

This SOP shall be followed by all Quality Control Unit (QCU) personnel for the quantification of suspensions of microbial organisms.

1.3 Definitions

Biological Safety Cabinet (BSC) – A class 2 containment cabinet designed for both worker and sample protection; room air is drawn into the front of the unit; the unit is designed in such a way that room air is HEPA filtered before blowing over the work area; air-borne hazardous particles coming off samples in the work area are pulled away from the worker and the air is vented back into the room after HEPA filtration; this type of unit is not suitable for worker protection from chemical fumes.

Chemical Fume Hood – safety cabinet designed for worker protection but not sample protection; room air is drawn into the front of the unit, chemical fumes or air-borne hazardous particles pulled away from the worker and are vented to the outside of the building.

Controlled Copy – A copy of an SOP distributed to select GLFC personnel having a unique copy number and dated signature of the IPS manager. Controlled copies are intended to ensure that GLFC personnel follow the most recent version of the SOP.

Effective Date – The date from which the procedures given in an SOP are to be implemented.

Great Lakes Forestry Centre (GLFC) – One of five Canadian Forest Service (CFS) research facilities in Canada.

Insect Production Services (IPS) – A GLFC work team consisting of the Insect Production Unit (IPU), the Quality Control Unit (QCU) and Insect Quarantine (IQ) personnel who perform insect rearing, quality control and quarantine activities in support of forest pest research activities internal and external to the CFS.

Insect Production Services Manager – The individual who has overall responsibility for activities of the IPS team.

Insect Production Unit (IPU) – A work unit of IPS consisting of personnel who perform insect rearing, diet making and methods development activities at GLFC.

Quality Control (QC) Lab – An analytical laboratory under the control of IPS used by the QCU for monitoring production, process and product control for all IPU insect colonies, and for developing new QC methods and procedures.

Quality Control Unit (QCU) – A work unit of IPS consisting of personnel who conduct routine production, process and product control testing and develop new QC methodology in support of IPU activities.

Standard Operating Procedures (SOPs) – Directives describing routine administrative or technical procedures conducted by IPS personnel or users of the IQ facility.

1.4 Safety

1.4.1 Lab coats and disposable gloves shall be worn when working with microbial organisms.

1.4.2 Disposable lab supplies (e.g., gloves, microtubes, pipet tips, microscope slides, etc.) which have come in contact with microbes or insects infected with microbial organisms shall be autoclaved before being discarded.

1.4.3 Re-usable lab supplies (e.g., glassware, plastic-ware, etc.) which have come in contact with microbial organisms must be soaked in a 6% bleach solution for at least 10 minutes, washed with soap and water, then autoclaved if appropriate.

1.4.4 Where feasible, a chemical fume hood or biological safety cabinet shall be used to minimize exposure of the worker to microbial organisms.

1.4.5 After working with microbial organisms, work surfaces must be cleaned using a 6% bleach solution and a 10 minute contact time.

1.5 Materials

1.5.1 Egg Albumin.
1.5.2 Napthalene Black 10B or 12B Stain.
1.5.3 Slide warmer set at 40-45°C.
1.5.4 Nikon Optiphot microscope fitted with a squared eyepiece graticule ($0.01mm^2$), 10X occulars and a 100X oil immersion objective.
1.5.5 IPS Form Number 0055/001, *1X Count of Suspension* (refer to Appendix 6).
1.5.6 IPS Form Number 0056/001, *4X Count of Suspension* (refer to Appendix 7).
1.5.7 6% bleach solution.

2.0 PROCEDURES

2.1 General Procedure

Enumeration of microbes in purified or crude suspensions is essential to investigations involving the ecology, detection or elimination of pathogens in insect cultures or in the rearing facility . Although microbes can be enumerated using a counting chamber, accurate quantification of aqueous samples containing host tissue or impurities is unreliable since bi-refringent particles may be mistaken as the occlusion that is being counted. Accurate, reproducible counts are achieved using this stained film method, whereby a sample of defined volume is spread evenly over a known area, dried, stained, and counted under an oil immersion objective using a squared eyepiece graticule. Ten counts are made along the radii of four replicate circular films at predetermined intervals measured using a stage micrometer. The method described in this SOP is an adaptation of a stained film method first described by Wigley, 1980 (refer to section 8.0). Microbe concentrations significantly lower than 10^6 per ml cannot be counted accurately using this procedure. Microbe concentrations significantly greater than 10^8 per ml require dilution with water for accurate quantification. Alternatively, high concentrations of microbes can be accurately enumerated by removing a defined volume subsample, diluting with a defined volume of water and adjusting the dilution factor (refer to 2.6.4) accordingly. To determine whether or not the test suspension concentration is suitable for counting, it is recommended that a preliminary count be done using only one stained film.

2.2 Making the Films

2.2.1 Mix the test suspension for 30 seconds then combine 20µl of the suspension with 20µl thawed counting medium (0.25g egg albumin, 25ml distilled H_2O, stir, filter through Kleenex®, store at -20ºC; Fisher Scientific A-388). Samples are diluted in egg albumin to promote adhesion to a glass microscope slide.
2.2.2 Place a 14mm diameter white disk on a black background (refer to Appendix 1) under a stereoscopic dissecting microscope and place an alcohol-cleaned glass microscope slide over the disk.

2.2.3 Mix the suspension to be counted by gently tapping the side of the tube several times with your finger.

2.2.4 Remove a 5µl sample of the counting suspension and dispense onto the glass slide in the center of the area defined by the underlying disk. If air bubbles in the sample on the slide do not break immediately, discard the slide and prepare another. (Bubbles cause an uneven distribution of microbial organisms on the slide).

2.2.5 Using the convex side of a mounted needle with a bent tip (pre-wetted by dipping into the counting suspension and wiping against the inside of the container), carefully spread the suspension in a continuous outward spiral motion to the edge of the disk (refer to Appendix 2, Fig. A). (Pre-wetting the tip prevents the removal of microbial organisms from the suspension on the slide).

2.2.6 Without lifting the needle from the surface of the slide, rotate the needle so that the tip is in contact with the slide and draw the tip across the film with a slight zig-zag motion while lifting it from the surface (refer to Appendix 2, Fig B). (This method of removing the needle allows for an even distribution of microbial organisms across the slide).

2.2.7 Make three more films on the glass slide following the same procedure.

2.2.8 Air dry the slide flat at room temperature.

2.2.9 When an extremely precise count is required, repeat steps *2.2.1* through *2.2.8* three more times, making a total of four slides (i.e., make 16 films).

2.3 Staining

2.3.1 Place the slides in staining tray and immerse in Naphthalene Black 10B or 12B solution (2.4g Naphthalene Black 10B or 12B, 70ml Acetic Acid, 130ml distilled water, store at room temperature, replace monthly) at 40-45°C.

2.3.2 After 10 minutes, remove the slide tray and rinse the slides by dipping gently 2 times in a reservoir of tap water. (Gentle dipping prevents microbial organisms from being washed off the slide).

2.3.3 Dry the under-side of the slides using paper towel then lay face up to dry either at room temperature or on a slide warmer at 40-45°C.

2.4 Counting Procedure

Use the 100X oil immersion objective of the Nikon Optiphot microscope and the 10X occular fitted with an eyepiece grid (0.01mm^2). Count microbes only if they lie wholly within the grid or they touch the left hand or upper edge (refer to Appendix 3). Microbes stain deep blue-black with a light blue background when examined using bright field optics.

2.5 Sampling Pattern

Count along four radii on each slide, one from each of the four replicate films, with each radius being counted in a different direction (refer to Appendix 4). (Counts along four different radii will average-out any slight irregularities in distribution of microbial organisms across the slide). The location of the first count is determined by placing the edge of the microscope field-of-view on the outer edge of the film and using the stage micrometer to move the field 0.25mm toward the center of the film. Use the stage micrometer to make an additional seven 0.5 mm movements of the slide followed by two 1.0mm movements (refer to Appendix 5). Record counts on IPS Form Number 0055/001 (refer to Appendix 6). When conducting a more precise count (i.e., when four slides are being examined), record counts on IPS Form Number 0056/001 (refer to Appendix 7).

2.6 Calculation of Microbe Concentration

Calculations for steps 2.6.1 through 2.6.6 can best be performed using the Excel spread sheet that is available on the IPS network drive.

2.6.1 Add together the four counts of each stratum and divide by four to obtain mean counts for the strata (when performing the counting procedure using four slides, the mean count will be determined using 16 counts).

2.6.2 Multiply the mean counts by the corresponding weight factors to obtain corrected means. (Counts at each of the 10 strata have to be given a weight proportional to the area of the stratum; Appendix 8).

2.6.3 Sum the corrected means to obtain an overall corrected mean.

2.6.4 Calculate the microbe concentration per ml of the undiluted stock suspension by:

microbes/ml = Y x M x V x D

where Y = overall corrected mean
M = microscope factor (15394*)
V = sample volume factor (1000÷5)
D = dilution factor (2**)

*film area divided by eyepiece grid area (153.94mm^2 ÷ 0.01mm^2)

**the dilution factor shall be revised when enumerating a diluted subsample as per section 2.1 (e.g., a subsample diluted 10-fold with water and mixed 1:1 with the egg albumin counting medium would have a resulting dilution factor of 20).

2.6.5 Calculate the Standard Error of the mean count per grid by:

$$S.E. = \sqrt{\sum W_i^2 \frac{X_s}{N_r}}$$

Where: W_i = individual weight factors

X_s = mean count at each stratum
N_r = number of radii counted

2.6.6 Calculate 95% confidence limits for the microbe concentration by:
95% confidence limits = dilution factor x S.E. x 200 x microscope factor

2.7 Calculations

2.7.1 The bleach working solution for general cleaning shall have a final sodium hypochlorite concentration of 0.3%. Bleach stock material with a 5.25% sodium hypochlorite concentration (e.g., Javex®) shall be diluted by combining 60ml bleach and 940ml water (i.e., 6% dilution). Bleach stock material with a 6.0% sodium hypochlorite concentration (e.g., Ultra Javex®) shall be diluted by adding 53ml bleach and 947ml water (i.e., 5.25% dilution). If another brand of bleach is used, volumes may need to be adjusted to provide a 0.3% sodium hypochlorite working solution.
[Note: minimum contact time of 10 minutes is required for effective sanitation]

2.8 Documentation and Reporting

2.8.1 Counting data sheets (IPS Form Number 0055/001 or 0056/001) shall completed for each suspension being enumerated.

3.0 DISTRIBUTION AND ARCHIVING

3.1 Distribution

This SOP shall be distributed by the IPS manager to all QCU personnel.

3.2 Archiving

3.2.1 The IPS manager shall maintain a historical file of this SOP when it is replaced by a new version.
3.2.2 QCU personnel shall ensure that completed counting data sheets (IPS Form Numbers 0055/001 or 0056/001) are maintained with records for the investigation being conducted.

3.3 Destruction of Outdated SOPs

When a new version of this SOP is available for distribution, all persons in possession of a *Controlled Copy* shall ensure that the retired version is returned to the IPS manager upon request.

4.0 ASSURING SOP VALIDATION AND COMPLIANCE

4.1 Responsible Individual

4.1.1 The head QC technician is responsible for assuring that this SOP is valid.
4.1.2 The head QC technician is responsible for assuring that this SOP is followed by QC personnel and that these persons have been appropriately trained in its use.
4.1.3 QCU personnel are responsible for complying with procedures specified on a *Controlled Copy* of this SOP and shall never use non-controlled copies which could be outdated.

5.0 REVISION OF THE SOP

5.1 Responsible Individual

The head QC technician is responsible for assuring that this SOP is current. If necessary, the head QC technician shall initiate the revision process.

5.2 Revision Schedule

This SOP shall be revised when its provisions no longer agree with current practices or GLFC policies, and shall be approved by the IPS Manager.

6.0 CONTINGENCIES

When QCU personnel find circumstances that do not permit compliance with this SOP, the head QC technician shall be consulted.

7.0 CONFIDENTIALITY

IPS SOPs are not considered to be confidential documents and may be distributed to outside parties. *Controlled Copies* shall not be reproduced.

8.0 REFERENCES

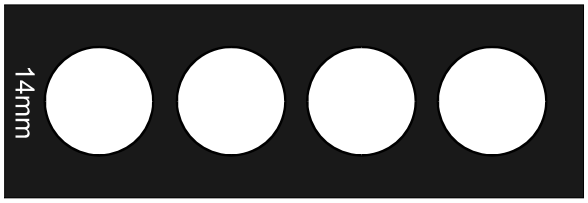
Wigley PJ, Counting micro-organisms, in *Microbial control of insect pests*, ed by Kalmakoff J and Longworth JF, NZ Dep Sci Ind Res Bull 228, Wellington, NZ, pp 29-35 (1980).

9.0 APPENDICES

Appendix 1: Template for Film Preparation.
Appendix 2: Technique for Film Preparation
Appendix 3: Microbial organisms to be omitted or counted at each counting position along the radius of the stained film.
Appendix 4: Sampling pattern for counts along the radius of four replicate films.
Appendix 5: Location of the ten counting positions along the radius of the stained film.
Appendix 6: IPS Form Number 0055/001, *1X Count of Suspension.*
Appendix 7: IPS Form Number 0056/001, *4X Count of Suspension.*
Appendix 8: Counting positions and sample weight factors for each of 10 counts along the 7.0 mm radius of a circular film.

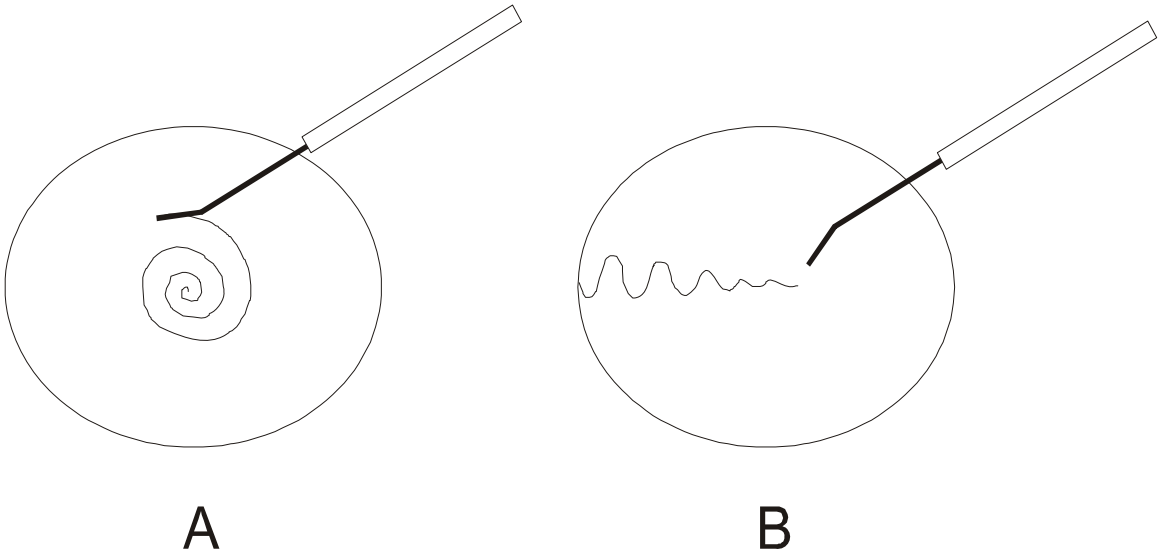
Appendix 1

Template for Film Preparation



Appendix 2

Technique for Film Preparation

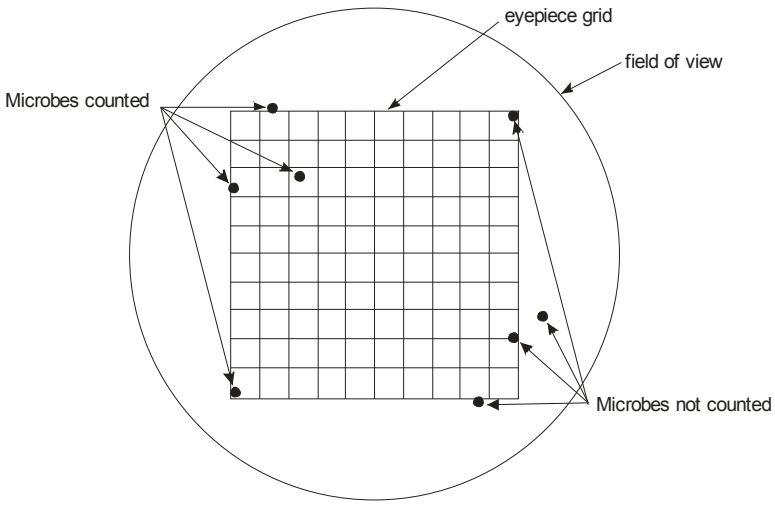


A B

A. Spreading of the suspension in a continuous outward spiral motion to the edge of the disk.
B. Drawing the tip across the film with a slight zig-zag motion while lifting it from the surface of the slide.

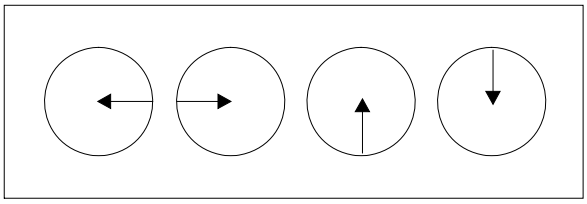
Appendix 3

Microbial organisms to be omitted or counted at each counting position along the radius of the stained film



Appendix 4

Sampling pattern for counts along the radius of four replicate films



Appendix 5

Location of the ten counting positions along the radius of the stained film

Appendix 6

1X Count of Suspension

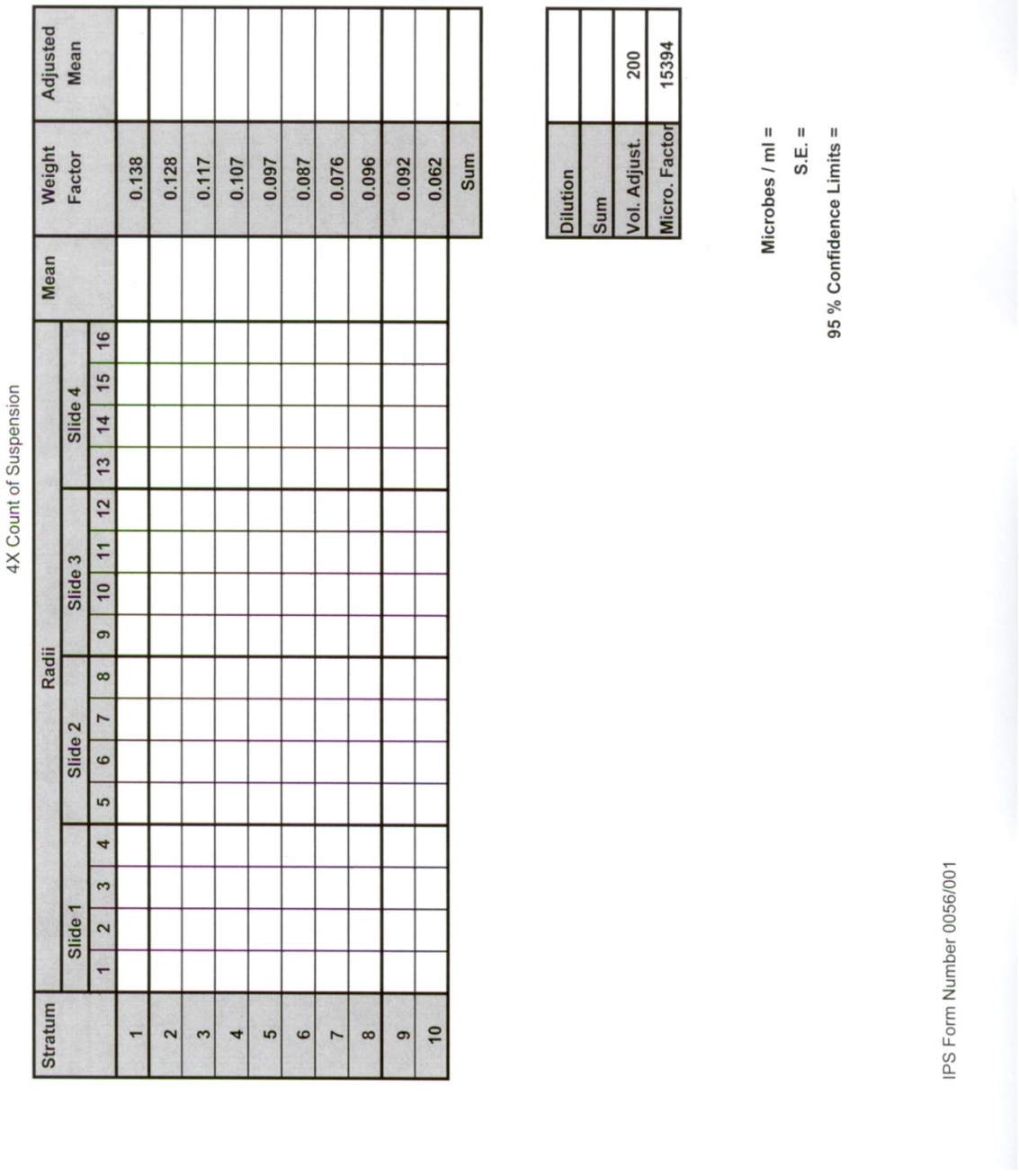
Stratum	Radii 1	2	3	4	Mean	Weight Factor	Adjusted Mean
1						0.138	
2						0.128	
3						0.117	
4						0.107	
5						0.097	
6						0.087	
7						0.076	
8						0.096	
9						0.092	
10						0.062	
						Sum	

Dilution	
Sum	
Vol. Adjust.	200
Micro. Factor	15394

Microbes / ml =
S.E. =
95 % Confidence Limits =

IPS Form Number 0055/001

Appendix 7



4X Count of Suspension

Stratum	Radii																Mean	Weight Factor	Adjusted Mean
	Slide 1				Slide 2				Slide 3				Slide 4						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16			
1																		0.138	
2																		0.128	
3																		0.117	
4																		0.107	
5																		0.097	
6																		0.087	
7																		0.076	
8																		0.096	
9																		0.092	
10																		0.062	
																		Sum	

Dilution	
Sum	
Vol. Adjust.	200
Micro. Factor	15394

Microbes / ml =

S.E. =

95 % Confidence Limits =

IPS Form Number 0056/001

Appendix 8

Counting positions and sample weight factors for each of 10 counts along the 7.0 mm radius of a circular film

Count no.	Distance moved (mm)	Count position (mm from film center)	Outer and inner radii (mm)	Weight factor*
1	0.25	6.75	7.0 - 6.5	0.138
2	0.5	6.25	6.5 - 6.0	0.128
3	0.5	5.75	6.0 - 5.5	0.117
4	0.5	5.25	5.5 - 5.0	0.107
5	0.5	4.75	5.0 - 4.5	0.097
6	0.5	4.25	4.5 - 4.0	0.087
7	0.5	3.75	4.0 - 3.5	0.076
8	0.5	3.25	3.5 - 2.75	0.096
9	1.0	2.25	2.75 - 1.75	0.092
10	1.0	1.25	1.75 - 0.0	0.062

*weight factor = $(r_1^2-r_2^2) \div R^2$ where: r_1 = outer stratum radius
r_2 = inner stratum radius
R = film radius

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