1.0 Purpose

The purpose of this procedure is to determine method suitability for internal procedures D-715.0 Microbial Limits Testing using Agar plates and D-715 Microbial Limits Testing using Neogen Petrifilm System. Method suitability, as stated in USP <2021>, USP <2022>, USP <61> and USP <62>, is the ability of organism to grow in the presence of product. If organisms do not grow or show < 50% recovery the final product is considered inhibitory at the dilution tested and will be further tested by additional dilutions or neutralizing diluent. If organisms show >200% recovery the final product is considered an enhancement product at the dilution tested and will be further tested by additional dilutions or neutralizing diluents

2.0 Scope

This procedure applies to internal procedures D-715.0-Microbial Limits Testing using Agar plates, which includes enrichment and D-715-Microbial Limits Testing using Neogen Petrifilm System.

3.0 Responsibility

3.1 It is the responsibility of QC Analysts to follow this procedure.

3.2 It is the responsibility of QC Laboratory Management to implement this procedure and to ensure that the procedure is followed.

3.3 It is the responsibility of QC Laboratory Management to keep current this procedure and to oversee validations.

4.0 Definitions

4.1 **Diluent** – The sterile medium used to dissolve/suspend and dilute samples

4.2 **Inoculum** – The prepared sample that is placed on the agar dish, petrifilm or Enhancement media via pipet

4.3 **IPA** – Isopropyl Alcohol

4.4 **QC** – Quality Control

4.5 **TAPC** – Total Aerobic Plate Count

4.6 **TNTC** – Too numerous to Count

4.7 **MPN** – Most probable number

4.8 **NaOH** – Sodium Hydroxide

4.9 **HCl** – Hydrochloric Acid

4.10 **TSA** – Tryp-Soy Agar

4.11 **TSB** – Tryp-Soy Broth

4.12 **SDA** – Sabouraud Dextrose Agar

4.13 **XLD** – Xylose, Lysine, Desoxycholate Agar dishes

4.14 **MSA** – Mannitol Salt Agar Plates

4.15 **TSB w/L&T80** – Tryp-Soy Broth with Lecithin and Tween 80

4.16 **AC** – Aerobic Plate Count Plate

4.17 **EC** – E. coli / Coliform Count Plate

4.18 **EB** – Enterobacteriaceae Count Plate

4.19 **STX** – Staph Express Count Plate

4.20 **RYM** – Rapid Yeast and Mold Count Plate

5.0 References

5.1 USP <2021>Microbial Enumeration Tests-Nutritional and Dietary Supplement,

5.2 USP <2022> Microbiological Procedures for Absence of Specified Microorganisms-Nutritional and Dietary Supplements

5.3 USP <61> Microbiological Examination of Nonsterile products: Microbial Enumeration Tests.

5.4 USP <62> Microbiological Examination of Nonsterile products: Tests for Specified Microorganisms.

5.5 USP <1111>, Pharmacopeia Monograph, Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use

5.6 D-125-F1, Form, Microbial Method Suitability Petrifilm Method

5.7 D-125-F2, Form, Microbial Method Suitability Agar Method

5.8 D-125-F3, Form, Microbial Method Suitability Specified Microorganism Method

5.9 D-125-F4, Form, Finished Product Microbiology Suitability Summary for Nutritional Supplements.

5.10 D-824, SOP, Operation and Cleaning of the Tuttnauer EZ10 Autoclave

5.11 D-101, SOP, Laboratory Housekeeping

5.12 D-715, SOP, Microbial Limits Testing using Neogen Petrifilm System

5.13 D-715.0, SOP, Microbial Limits Testing using Agar Plates

5.14 D-113, SOP, Microbiological Media Validation

6.0 Required Supplies, Media and Equipment

6.1 Supplies

6.1.1 70% IPA

6.1.2 Sterile and filtered 1N HCl solution

6.1.3 Sterile and filtered 1N NaOH solution

6.1.4 10ml sterile serological pipettes

6.1.5 120mL sterile containers w/ Lid

6.1.6 200µL and 1mL variable automatic pipettes w/ sterile tips

6.1.7 250mL, 500mL and 1L wide mouth storage bottles w/ screw cap top.

6.2 Media

6.2.1 Tryp-Soy Broth

6.2.2 Tryp-Soy Agar

6.2.3 Rappaport-Versailles Media

6.2.4 MacConkey Broth

6.2.5 Mossel Enrichment Broth

6.2.6 Sabouraud Dextrose Agar

6.2.7 MacConkey Agar dishes

6.2.8 Violet Red Bile Glucose Agar dishes

6.2.9 Violet Red Bile Agar dishes

6.2.10 Cetrimide Agar dishes

6.2.11 Xylose, Lysine, Desoxycholate Agar dishes

6.2.12 Mannitol Salt Agar Plates

6.2.13 Tryp-Soy Broth with Lecithin and Tween 80

6.2.14 Butterfields Buffer

6.2.15 Peptone Salt Diluent

6.2.16 AC petrifilm

6.2.17 RYM petrifilm

6.2.18 EC Petrifilm

6.2.19 EB Petrifilm

6.2.20 STX Petrifilm

6.3 Equipment

6.3.1 Analytical Balance

6.3.2 pH Meter

6.3.3 Biological Safety Cabinet

6.3.4 Autoclave

6.3.5 25°C, 33°C, and 37°C Incubators

6.3.6 Variable Temperature Circulating Water Bath

6.3.7 Compound Microscope

6.3.8 Darkfield Colony Counter

6.4 Organism

6.4.1 *Candida albicans,* ATCC 10231

6.4.2 *Bacillus subtilis,* ATCC 6633

6.4.3 *Escherichia coli,* ATCC 8739

6.4.4 *Aspergillus brasilliensis,* ATCC 16404

6.4.5 *Pseudomonas paraeruginosa,* ATCC 9027

6.4.6 *Staphylococcus aureus,* ATCC 6538

6.4.7 *Salmonella enterica,* ATCC 14028

7.0 Procedure

7.1 Media Preparation

7.1.1 Follow the preparation instruction on-line or on container for each media type and vendor

Note: Media may be purchased ready to use.

7.1.2 pH should be confirmed and / or adjusted before using.

7.1.3 Ensure that all media has been validated prior to testing (Refer to SOP D-113, Microbiological Media Validation).

7.2 Sample Labeling and Preparation

7.2.1 Sterilize all sample preparation equipment and media prior to use as per SOP D-824 Operation and Cleaning of the Tuttnauer EZ10 Autoclave. Single use, sterile disposables may also be used.

7.2.2 Label sample containers with a minimum of sample preparation # and Sample

Control or Sample + Bacteria testing.

7.2.3 Create a parent sample by transferring no less than 10g to the containers labeled in step 7.2.2 to either TSB or BPB. Alternatively, TSB w/L&T80 or Peptone Salt diluent may be used.

7.2.4 Before inoculation, sample pH should be adjusted with 1N NaOH for acids products and 1N HCl for alkaline products. If pH indicator strips are used, sample should be adjusted to pH 7.0.

7.3 Organism Preparation

7.3.1 Commercially prepared challenge organisms are suitable for use.

7.3.2 Prepare the challenge organisms per manufacturer instruction. Once ready, use the preparation immediately. The remaining suspension can be refrigerated and subsequently used for up to 8 hours.

7.4 Organism(s) to test for each method:

Organism to Test	Agar Method	Enhancement Method	Petrifilm Method
Candida albicans	X		X
Bacillus subtilis	X		X
Escherichia coli		X	X
Aspergillus brasilliensis	X		X
Pseudomonas aeruginosa		X	
Staphylococcus aureus		X	X
Salmonella enterica		X	X

7.5 Agar Method Inoculation – TAPC and Yeast & Mold Count.

7.5.1 Sanitize the interior of the biological safety cabinet as per D-101 Laboratory Housekeeping before plating.

7.5.2 Obtain the required type of media and confirm they are not expired.

7.5.3 Prepare initial 1:10 of sample as per 7.2 section, using 90 mL TSB as the diluent.

7.5.4 Inoculate 1000 µl of diluent onto two plates and label as Negative Control.

7.5.5 Inoculate 1000 µl of initial 1:10 dilution onto two plates and label as Sample Control.

7.5.6 Inoculate two plates with the organism being tested (100ul of a 10-100 CFU/ml organism preparation) and label as Positive Control.

7.5.7 Inoculate 1000 µl of initial 1:10 sample dilution onto two plates. Label plates as Sample + Challenge Organism. Add 100ul of the 10-100 CFU/ml organism preparation to each plate on top of sample.

7.5.8 Pour TSA or SDA media into each of the plates.

Organism to Test	TSA	SDA
Candida albicans		X
Bacillus subtilis	X	
Aspergillus brasilliensis		X

7.5.9 Let cool and then incubate utilizing the table below:

Agar Media	Incubation (°C)	Incubation Time
TSA	30°C to 35°C	24 hours-48 hours
SDA	20°C to 25 °C	3 - 5 Days

7.5.10 Repeat for each organism to be tested.

7.5.11 After incubation period, visually count colonies to determine the total raw count for the plate or if the colonies are difficult to see visually, use the Darkfield Colony Counter to determine the raw count.

7.5.12 Calculate the percent recovery as follows:

$$\text{Percent Recovery} = \frac{\text{avg. of sample + organism}}{\text{avg. of organism control plate}} \text{ X } 100$$

7.5.12.1 Document results on Logbook D-125-F2 Microbial Method Suitability Agar Method.

7.5.13 The Percent recovery between the test plates and control plates must be 50-200% for the method to be considered suitable for use, refer to section 8.0.

7.5.14 If a TNTC result or a recovery greater than 200% is obtained, serial dilution and retest may be required. If organisms do not grow or show < 50% recovery, proceed to section 9.0.

7.6 Petrifilm Method

7.6.1 Sanitize the interior of the biological safety cabinet as per D-101 Laboratory Housekeeping before plating.

7.6.2 Obtain the required Petrifilm media and confirm they are not expired.

7.6.3 Prepare the initial sample dilution at 1:10 ratio with approximately 10g of sample and 90mL Buffered Phosphate Buffer or Peptone Salt as the diluent. If specific sample requires a 1g of sample, prepare 1:100 ratio using 99mL Buffered Phosphate Buffer or Peptone Salt as the diluent.

7.6.4 Before inoculation, sample pH should be adjusted to neutral level with 1N NaOH for acidic products, and 1N HCl for alkaline products. If pH indicator strips are used, sample should be adjusted to pH 7.0.

7.6.5 Inoculate petrifilm with 1000 µl of initial dilution sample in duplicate and label as Sample Control. Refer to D-715 Microbial Limits Testing using Neogen Petrifilm System for plating details.

7.6.6 Inoculate petrifilm with 1000 µl of diluent in duplicate and label as Negative Control.

7.6.7 Prepare 1000 µl of diluent containing 10-100 CFU of challenge organism (100ul of a 10-100 CFU/ml organism preparation), per film to inoculate. Inoculate in

duplicate, 1000 µl of preparation containing the organism being tested. Label as Positive Control.

7.6.8 Prepare 1000 µl of initial sample dilution containing 100ul of the 10-100 CFU/ml organism preparation, per film to inoculate. Inoculate in duplicate, 1000 µl of preparation containing the organism being tested. Label as Sample + Organism tested.

7.6.9 Organism(s) to test for each petrifilm:

Organism to Test	AC	RYM	FC	EB	STX
Candida albicans		X			
Bacillus subtilis	X				
Escherichia coli			X		
Aspergillus brasilliensis		X			
Staphylococcus aureus					X
Salmonella enterica				X	

7.6.10 Incubate petrifilm utilizing the table below:

Film	Incubation (°C)	Incubation Time
AC	30°C to 35°C	24-48 hours
RYM	20 °C to 25 °C	3-5 days
EC	30°C to 35°C	24-48 hours
EB	30°C to 35°C	24-48 hours
STX	30°C to 35°C	24-48 hours

7.6.11 Repeat for each of the organisms to be tested.

7.6.12 Count plates and then calculate Percent Recovery:

$$\text{Percent Recovery} = \frac{\text{avg. of sample + organism}}{\text{avg. of organism control plate}} \text{ X } 100$$

7.6.12.1 Document results on Logbook D-125-F1 Microbial Method Suitability Petrifilm Method.

7.6.13 The Percent recovery between the test plates and control plates must be 50-200% for the method to be considered suitable for use, refer to section 8.0.

7.6.14 If a TNTC result or a recovery greater than 200% is obtained, serial dilution and retest may be required. If organisms do not grow or show < 50% recovery, proceed to section 9.0.

7.7 Specified Microorganism Method

7.7.1 Sanitize the interior of the biological safety cabinet as per D-101 Laboratory Housekeeping before plating.

7.7.2 Obtain the required media and confirm they are not expired.

7.7.3 Inoculate plate with 1000 µl of initial 1:10 sample dilution in duplicate and label as Sample Control.

7.7.4 Inoculate plate with 1000 µl of diluent in duplicate and label as Negative Control.

7.7.5 Using TSB as the initial diluent, prepare 1:10 sample dilution as per 7.2 section.

7.7.6 Bile Tolerant Gram-Negative Bacteria/ Enterobacteriaceae Count – MPN Method

7.7.6.1 Inoculate 10 ml of (1:10) sample dilution prepared, with 100 µl of *Salmonella enterica.* Mix and incubate at 20° to 25° for 2-5 hours.

7.7.6.2 After incubation, transfer 1mL of the above dilution to 9mL of Enterobacteria Enrichment Broth Mossel, incubate at 30°C to 35°C for 24 to 48 hours.

7.7.6.3 Subculture on Violet Red Bile Glucose Agar and incubate for an additional 18 to 24 hours at 30°C to 35°C.

7.7.6.4 Growth of colonies with a purple zone around them is indicative of *Enterobacteria.*

7.7.6.5 The method is suitable if typical grow is observed at 0.1g/ml of sample solution.

7.7.7 Coliforms Count – MPN Method

7.7.7.1 Inoculate 10 ml of 1:10 sample dilution prepared, with 100 µl *Escherichia coli.* Mix and incubate at 20° to 25° for 2-5 hours.

7.7.7.2 After incubation, transfer 1mL of the above dilution to 9mL of Enterobacteria Enrichment Broth Mossel, incubate at 30°C to 35°C for 24 to 48 hours.

7.7.7.3 Subculture on Violet Red Bile Agar and incubate for an additional 18 to 24 hours at 30°C to 35°C.

7.7.7.4 Growth of lactose fermenting colonies (pink colonies) indicates the presence of coliforms.

7.7.7.5 The method is suitable if typical grow is observed at 0.1g/ml of sample solution.

7.7.8 *Escherichia coli*

7.7.8.1 Inoculate (1:10) sample dilution prepared with 100 µl of *Escherichia coli.* Mix and incubate at 30°C to 35°C for 18 to 96 hours.

7.7.8.2 After incubation, transfer 1mL of the above dilution broth to 9 mL of MacConkey Broth, incubate at 42°C to 44°C in water bath for 18 to 24 hours.

7.7.8.3 Subculture on MacConkey Agar at 30°C to 35°C for 18 to 72 hours.

7.7.8.4 Growth of lactose fermenting colonies (pink colonies) indicates the presence of *Escherichia coli.*

7.7.9 *Salmonella*

7.7.9.1 Inoculate (1:10) sample dilution prepared with 100 µl of *Salmonella enterica.* Mix and incubate at 30°C to 35°C for 18 to 24 hours.

7.7.9.2 After incubation, transfer 1mL of the above dilution broth to 9 mL of Rappaport Vassiliadis Salmonella Enrichment Broth incubate at 30°C to 35°C for 18 to 24 hours.

7.7.9.3 Subculture on XLD Agar at 30°C to 35°C for 18 to 72 hours.

7.7.9.4 Growth of well-developed, red colonies, with or without black centers is indicative of *Salmonella.*

7.7.10 *Pseudomonas aeruginosa*

7.7.10.1 Inoculate (1:10) sample dilution prepared with 100 µl of *Pseudomonas aeruginosa.* Mix and incubate at 30°C to 35°C for 18 to 24 hours.

7.7.10.2 Subculture on Cetrimide Agar at 30°C to 35°C for 18 to 72 hours.

7.7.10.3 Growth of yellow-green or blue-green colonies indicative of *Pseudomonas aeruginosa.*

7.7.11 *Staphylococcus aureus*

7.7.11.1 Inoculate (1:10) sample dilution prepared with 100 µl of *Staphylococcus aureus.* Mix and incubate at 30°C to 35°C for 18 to 24 hours.

7.7.11.2 Subculture on MSA agar at 30°C to 35°C for 18 to 72 hours.

7.7.11.3 Growth of yellow or white colonies surrounded by a yellow zone is indicative of *Staphylococcus aureus.*

7.7.12 Interpretation and reporting results

7.7.12.1 Growth of the indicated organism on both the organism control plates and the sample + organism plates is a valid result for this method. There is no percent recovery calculated as these tests are used for absence or presence of specified organism only.

7.7.12.2 Document results on Logbook D-125-F3, Microbial Method Suitability Specified Microorganism Method.

7.7.12.3 If no growth proceed to step 9.0 for neutralization methods.

8.0 Specifications

8.1 The Agar method or Petrifilm method Percent Recovery must be ≥ 50% for all organisms to be considered a valid method.

8.2 The Specified Organism method requires growth on both the sample + organism plate and the organism control plate to be considered a valid method .

8.3 If organisms do not grow or show < 50% recovery the product is considered inhibitory at the dilution tested and will be further tested by additional dilutions or neutralizing diluent. If organisms show >200% recovery the final product is considered an enhancement product at the dilution tested and will be further tested by additional dilutions or neutralizing diluents.

9.0 Neutralization / Removal of Antimicrobial Activity

9.1 Refer to USP <61>, USP <2021>Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests for common neutralizing agents/methods for interfering substances. The three most common techniques are:

9.1.1 Diluting the sample.

9.1.2 Incorporating neutralizing agents into the test specimen.

9.1.3 Performing membrane filtration of the samples to remove antimicrobial agents.

10.0 Reporting

10.1.1 Record the testing information and results on the logbook forms indicated in this protocol.

10.1.2 Upon completion of the study, prepare a summary report with the results of the microbiological suitability tests and the conclusions using form D-125-F4.

11.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	04/14/22	New procedure.	N/A	G. Shaw
1	09/30/22	Made directions more clear to actual process.	CC-22-0395	G. Shaw
2	03/24/25	Updated Procedure and Forms to reflect current practices. Addition of enumeration methods for Coliforms and Bile Tolerant Gram-Negative Bacteria. Addition of reporting section and Form D-125-F4 Finished Product Microbiology Suitability Summary.	CC-25-0018	A. Perez

HBI Ion Labs a Spectrum business	Microbial Method Suitability – Petrifilm		
	Form: D-125-F1	CCR No. CC-22-0018	Revision: 2

Logbook Number: _____ Logbook Page: _____ of _____

Finished Product Name/Formulation #							
Initial Sample Weight and Initial Dilution							
Diluent Used/Lot #/Expiration Date				**Balance #/Cal Due Date:**			
Safety Cabinet #/Cal Due Date:							
1000 ul Pipette #/Cal Due Date:				**100 ul Pipette#/Cal Due Date:**			
Negative Control Results	**P1:**	**P2:**	**Avg:**	**Sample Control Results:**	**P1:**	**P2:**	**Avg:**
AC Media Lot#/Exp date:				**EB Media Lot#/Exp date:**			
EC Media Lot#/Exp date:				**STX Media Lot#/Exp date:**			
Other:				**RYM Media Lot#/Exp date:**			

Organism	Lot Number/ Expiration Date	Organism Prep Date/ Time	Incubation Date/ Time/Tech	Positive Control Results	Sample + Organism Results	% Recovery	Pass/Fail
Candida albicans			Start: End:	P1: P2: AVG:	P1: P2: AVG:		
Aspergillus brasilliensis			Start: End:	P1: P2: AVG:	P1: P2: AVG:		
Escherichia coli			Start: End:	P1: P2: AVG:	P1: P2: AVG:		
Salmonella enterica			Start: End:	P1: P2: AVG:	P1: P2: AVG:		
Staphylococcus. aureus			Start: End:	P1: P2: AVG:	P1: P2: AVG:		
Bacillus subtilis			Start: End:	P1: P2: AVG:	P1: P2: AVG:		

Suitability for Agar Method (circle one): APPROVED (Dilution:) REJECTED (test at another dilution or use another form of diluent)

Reviewed By/Date: _____

CONFIDENTIAL: For HBI Ion Labs use only

HBI Ion Labs	Microbial Method Suitability – Agar Method		
	Form: D-125-F2	CCR No. CC-25-0018	Revision: 2

Logbook Number: _____ Logbook Page: _____ of _____

Finished Product Name/Formula#:								
Initial Sample Weight and Initial Dilution:								
Diluent Used/Lot #/Expiration Date:								
Safety Cabinet #/Cal Due Date:				**Balance #/Cal Due Date:**				
1000 ul Pipette #/Cal Due Date:				**100 ul Pipette#/Cal Due Date:**				
Negative Control Results:	**P1:**	**P2:**	**Avg:**	**Sample Control Results:**	**P1:**	**P2:**	**Avg:**	
TSA Media Lot#/Exp date:				**Other:**				

Aerobic Organism	Lot Number/ Expiration Date	Organism Prep Date/ Time	Incubation Date/ Time/Tech	Positive Control Results	Sample + Organism Results	% Recovery	Pass/Fail
Bacillus subtilis			Start : End :	P1: P2: AVG:	P1: P2: AVG:		

SDA Media Lot#/Exp Date:		

Yeast and Mold Organism	Lot Number/ Expiration Date	Organism Prep Date/ Time	Incubation Date/ Time/Tech	Positive Control Results	Sample + Organism Results	% Recovery	Pass/Fail
Candida albicans			Start : End :	P1: P2: AVG:	P1: P2: AVG:		
Aspergillus brasilliensis			Start : End :	P1: P2: AVG:	P1: P2: AVG:		

Suitability for Agar Method (circle one): APPROVED (Dilution:) or REJECTED (test at another dilution or use another form of diluent)

Comments:

Reviewed By/Date: _____

CONFIDENTIAL: For HBI Ion Labs use only

HBI Ion Labs	Microbial Method Suitability – Tests for Specified Microorganisms		
	Form: D-125-F3	CCR No. CC-25-0018	Revision: 2

Logbook Number: _____ Logbook Page: _____ of _____

Finished Product Name / Formula#:						
Initial Sample Weight and Initial Dilution:						
Diluent Used/Lot #/Expiration Date:						
Safety Cabinet #/Cal Due Date:			**Balance #/Cal Due Date:**			
1000 ul Pipette #/Cal Due Date:			**100 ul Pipette#/Cal Due Date:**			
TSB Media Lot#/Exp Date:			**RVS Broth Media Lot#/Exp Date:**			
MAC Broth Media Lot#/Exp Date:			**XLD Agar Media Lot#/Exp Date:**			
MAC Agar Media Lot#/Exp Date:			**MSA Agar Media Lot#/Exp Date:**			
Cetrimide Agar Media Lot#/Exp Date:			**EE Broth Media Lot#/Exp Date:**			
VRB Agar Media Lot#/Exp Date:			**VRBG Agar Media Lot#/Exp Date:**			

Negative Control Results:		**Sample Control Results:**	

Organism	Lot Number/ Expiration Date	Organism Prep Date/ Time	Incubation Date/ Time/Tech	Positive Control Growth/No Growth	Sample + Organism Growth/No Growth	Pass/Fail
Escherichia coli			Start: End:			
Salmonella enterica			Start: End:			
Enterobacteriacea / Bile tolerant Gram-Negative Bacteria			Start: End:			
Coliforms			Start: End:			
Staphylococcus. aureus			Start: End:			
Pseudomonas aeruginosa			Start: End:			

Suitability for Agar Method (circle one): APPROVED (Dilution:) or REJECTED (test at another dilution or use another form of diluent)

Comments:

Reviewed
By/Date:_____

CONFIDENTIAL: For HBI Ion Labs use only

Method Suitability Information

Product Name	
Formula Number	
Target Assay	

Method / SOP Number	**D-715 Petrifilm**	**D-715.0 Agar**	**D-715.0 Specified Organism**
Sample Weight			
Dilution			
Diluent			
Logbook reference			

Conclusion(s):

Role	Name	Title	Signature	Date
Initiated By:				
Approved By:				