ANALYTICAL METHOD

VALIDATION PROTOCOL

FOR

PRE & PROBIOTIC CAPSULES

SUPERSEDE PROTOCOL No.	
DATE OF VALIDATION	
VALIDATION BATCH No.	
VALIDATION BATCH SIZE	

ANALYTICAL METHOD VALIDATION PROTOCOL FOR PRE & PROBIOTIC CAPSULES

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ANALYTICAL METHOD VALIDATION PROTOCOL FOR PRE & PROBIOTIC CAPSULES

1.0 PROTOCOL APPROVAL:

INITIATED BY:

DESIGNATION	NAME	SIGNATURE	DATE
OFFICER/EXECUTIVE (QUALITY CONTROL)			

REVIEWED BY:

DESIGNATION	NAME	SIGNATURE	DATE
HEAD (QUALITY CONTROL)			
MANAGER (QUALITY ASSURANCE)			

APPROVED BY:

DESIGNATION	NAME	SIGNATURE	DATE
HEAD (QUALITY ASSURANCE)			

ANALYTICAL METHOD VALIDATION PROTOCOL FOR PRE & PROBIOTIC CAPSULES

2.0 OBJECTIVE:

To provide documented evidence that the analytical method for the **Assay test of Pre & Probiotic capsules** is validated and suitable for testing samples of commercial batches.

3.0 SCOPE:

This Protocol is applicable for **Assay Test of Pre & Probiotic capsules** for Accuracy, Precision, Specificity, Linearity & Range, Robustness in the Quality Control Department and covers the Analytical procedure, Responsibilities, Training, Methodology of Validation and Acceptance criteria.

4.0 RESPONSIBILITY:

The validation group, comprising of a representative from each of following departments, shall be responsible for the overall compliance of this report and protocol:

DEPARTMENTS	RESPONSIBILITIES
Quality Control	• To Prepare and Review of Protocol. • To conduct Analytical Method Validation activity as per the approved Protocol. • To Compile the Validation Data. • Protocol Training.
Quality Assurance	• To Review and Approval of Protocol. • To monitor all Analytical Method Validation Activities and ensuring the Validation are carried out as per the Protocol. • To monitor Protocol completeness and Technical Accuracy.

5.0 TRAINING DETAILS:

- The Validation Team shall be authorized by Head-QA or his/her designee.
- All the personnel involved in the Analytical Method Validation shall be appropriately trained both in their job related activities and on the Analytical Method Validation Protocol
- Verify the Training Records of the persons involved in the Analytical Method Validation.

6.0 INSTRUMENT CALIBRATION VERIFICATION:

Ensure that all instruments to be used in Analytical Method Validation are calibrated. The standard Calibration Documents shall be verified

7.0 PROCEDURE:

7.1 SELECTION OF ANALYTICAL PERFORMANCE PARAMETERS:

As per the cGMP guidelines the test methods, which are used for assessing the quality of pharmaceutical products with established specifications, must be validated for analytical performance, parameters viz., Accuracy, Precision, Specificity, Linearity, Range & Ruggedness. However method validation performance parameters shall be decided as per the requirement of submission authority. Unless otherwise justified and authorized all above mentioned performance parameters shall be carried out.

7.1 DESCRIPTION OF ANALYTICAL METHOD:

The detailed test method shall be described including limits given in the specification.

7.2 DESCRIPTION OF ANALYTICAL PARAMETERS :

Analytical method validation for **Assay of Pre & Probiotic** shall be carried out for specificity, Accuracy, Precision, Linearity, Range & Ruggedness parameters.

7.3 REVALIDATION CRITERIA:

Method shall be revalidated for following changes:

- Method
- Drug substance (including route of synthesis)
- Change in the Composition of Drug product
- FDA mandated Up-date
- Technology

8.0 MATERIALS AND INSTRUMENTS USED:

- List of all Media, chemical/reagents with B. No./Lot No.
- Details of the Instrument(s) used (Make/Model/No.)
- Sample details (B. No./Batch Size)

9.0 STUDY DESIGN:

Following parameter are covered in method validation:

- Specificity
- Precision
- Linearity and Range
- Accuracy/Recovery
- Ruggedness

10.0 METHODOLOGY

Instrument Requirement:

- LAF
- Autoclave
- Micropipette
- Colony Counter
- pH Meter
- Analytical Balance
- Butter paper
- Spatula
- Ultra Sonicator
- Vortex Mixture
- Mortar & Pestle
- Anaerobic Gas Jars

Material & Glassware Requirement:

- Conical (100 ml, 250 ml and 500 ml)
- Measuring cylinder
- Test tubes

Media & Chemical Requirement:

- Mitis Salviris Agar
- PNY Agar
- Nutrient Agar

- Columbia Agar
- NaCl (AR grade)
- 1 % Potassium tellurite solution
- Gentamicin
- Millipore Water or equivalent
- Buffer Peptone Water

REQUIREMENT & PREPARATION OF MEDIAS:

Preparation Mitis Salviris Agar:

- Weigh accurately about 90.07 gm of Mitis Salviris Agar into dried conical flask. Add 100 ml of water. Disperse in water and heat to about 60°C to get uniform suspension.
- Sterilize in an autoclave at 121°C, under the pressure of 15 lbs for 20 minutes. Cool to 50°C to 55°C and add 1 ml of sterile 1% potassium tellurite solution.

Preparation of Nutrient Agar:

- Weigh accurately about 28.0 g of Nutrient Agar into dried conical flask. Add 1000 ml of water. Disperse and heat to about 60°C to get uniform suspension.
- Sterilize in an autoclave at 121°c, under the pressure of 15lbs for 20 minutes.

Preparation of Glucose yeast Extract Agar:

- Weigh accurately about 28.32 g of Nutrient Agar into dried conical flask.
- Add 1000 ml of water. Disperse and heat to about 60°C to get uniform suspension.
- Sterilize in an autoclave at 121°C, under the pressure of 15lbs for 20 minutes.

Preparation of 0.9% Sodium Chloride Solution:

- Weigh accurately about 0.9 g of Sodium Chloride into dried conical flask. Add 1000ml of water.
- Disperse and heat to about 60°C to get uniform solution. Plug with cotton wool, Cover with aluminum foil.
- Sterilize the solution in an autoclave at 121°C, under the pressure of 15 lbs for 20 minutes.

Preparation of PNY Medium:

- Weigh accurately about 31.28 gm of PNY Medium Agar into dried conical flask. Add 1000 ml of water.

- Disperse in water and heat to about 60°C to get uniform suspension.
- Sterilize in an autoclave at 121°C, under the pressure of 15 lbs for 20 minutes.

Preparation of Columbia Agar:

- Weigh accurately about 44.0 g of Columbia Agar into dried conical flask. Add 1000 ml of water. Disperse in water and heat to about 60°C to get uniform suspension.
- Sterilize in an autoclave at 121°c, under the pressure of 15 lbs for 20 minutes. Cool to 50°C to 55°C and add 1 vial of sterile Gentamicin selective supplement solution.

10.1 Determination of Content of *Streptococcus faecalis*:

Sample preparation:

- Weigh accurately about 1.0 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-7} dilution series is obtained.
- Transfer 1.0 ml of 10^{-7} dilution to 3 nos. of labeled sterile Petri plates by using 1.0 ml sterile pipettes.
- Pour approximately 20 ml of the **Mitis Salviris Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under aerobic condition for 72 hours.

Reporting of results: (*Streptococcus faecalis*)

Calculation:

$$\frac{\text{Number of colonies observed in 3 plates}}{\text{No. of plates}} = \text{Average Cfu}$$

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

10.2 Determination of content of *Clostridium butyricum*:

Sample preparation:

- Weigh accurately about 1.0 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-5} dilution series is obtained.
- Transfer 1.0 ml of 10^{-5} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **Columbia Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under anaerobic conditions (CO_2) gas pack in Anaerobic jar for 72 hours.

Reporting of results: *(Clostridium butyricum*) (An aerobically)

Calculation:

$$\frac{\text{Number of colonies observed in 3 plates}}{\text{No. of plates}} = \text{Average Cfu}$$

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

10.3 Determination of content of *Bacillus mesentricus*:

Sample preparation:

- Weigh accurately about 1.0 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.

- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-5} dilution series is obtained.
- Transfer 1.0 ml of 10^{-5} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **Nutrient Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under aerobic condition for 72 hours.

Reporting of results: (*Bacillus mesentricus*)

Calculation:

$$\frac{\text{Number of colonies observed in 3 plates}}{\text{No. of plates}} = \text{Average Cfu}$$

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

10.4 Determination of content of Lactic Acid Bacillus *(Lactobacillus sporogenes)*:

Sample preparation:

- Weigh accurately about 1.0 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-7} dilution series is obtained.
- Transfer 1.0 ml of 10^{-7} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **PNY Agar or Glucose Yeast Extract Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under aerobic condition for 72 hours.

Reporting of results: (*Lactic Acid Bacillus*)

Calculation: Number of colonies observed in 3 plates
---------- = Average Cfu
No. of plates

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

11.0 PROCEDURE FOR METHOD VALIDATION:

11.1 SPECIFICITY:

The specificity of an analytical method is it stability to measure unequivocally the analyte in the presence of components that may be expected to be present in the sample matrix**.**

Note:

For detailed procedure, refer. Methodology as described under section 10.0

Definition: Specificity of method the assay placebo (i.e. formulation without active under condition) and ensuring that there is no response i.e. there should not be any growth.

Determination: Specificity of method is demonstrated from the results obtained the assay placebo (i.e. formulation without active under condition) and ensuring that there should not be any growth.

Demonstration: Specificity of the method is demonstrated from the results obtained by placebo. There is no growth obtained.

11.2 PRECISION:

The measure of how close the data values are to each other for a number of determinations under the same analytical condition.

Definition: The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samples of homogeneous sample.

Determination: The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogeneous sample, precision is determined in terms of Method precision

Acceptance criteria: System precision: The relative standard deviation of replicate analysis shall not be more than 10.0 %.

Method Precision:

Duplicate measurement of area for six sample preparations.

Intermediate Precision:

Second analyst shall repeat the method precision on different instrument on different day.

11.2.1 METHOD PRECISION:

Note: For detailed procedure, refer methodology as described under section 10.0

Acceptance Criteria:

The % RSD of Assay results obtained for six different preparations should not be more than 10.0 %.

11.2.2 INTERMEDIATE PRECISION:

The second analyst repeats method precision on different day.

Note: For detailed procedure, refer methodology as described under section 10.0

Acceptance criteria:

The % RSD of Assay results obtained for six solutions by second analyst should not be more than 10.0 %.

The relative difference between the average results obtained by both analysts should not be more than 10.0 %.

11.3 LINEARITY AND RANGE:

Ability to obtain results that are directly proportional to analyte concentration in the sample (linearity). Interval between the upper and lower concentration of analyte for which a suitable level of precision, accuracy and linearity has been demonstrated.

Note: For detailed procedure, refer methodology as described under section 10.0

Definition: The linearity of an analytical method is its ability to elicit test results that are directly or by a well defined mathematical transformation, proportional to the concentration of analyze in sample within a given range.

Determination: The linearity is determine from the plot of Concentration vs No. of colony forming units and the expressed in terms of the coefficient of variation Tabulate No. of colony forming unit against Corresponding concentration in Plot a graph for Concentration vs No. of colony forming units and observe for linearity.

Demonstration: The linearity is demonstrated from the aliquots of five different concentration 60%, 80%, 100%, 120% and 140% of working assay concentration.

Procedure: Separately prepared minimum five different concentration levels of sample in the range 60%, 80%, 100%, 120% and 140%.Report the Range based on the data of precision, accuracy and linearity.

Acceptance criteria:
Plot of concentration vs No. of colony forming units shall be linear coefficient of variance shall be close to 1.0

11.4 ACCURACY/RECOVERY:

To demonstrate that the analytical method is capable to yield data values close to true value, which is accepted as a conventional true value (Accuracy).

An accepted standard value and the value found (Recovery)

Note: For detailed procedure, refer methodology as described under section 10.0.

11.4.1 Test preparation: I (80% Concentration)

11.4.1.1 Determination of content of *Streptococcus faecalis*:

Sample preparation:

- Weigh accurately about 0.8 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-7} dilution series is obtained.
- Transfer 1.0 ml of 10^{-7} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **Mitis Salviris Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under aerobic condition for 72 hours.

Reporting of results: (*Streptococcus faecalis*)

Calculation:

$$\frac{\text{Number of colonies observed in 3 plates}}{\text{No. of plates}} = \text{Average Cfu}$$

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

11.4.1.2 Determination of content of *Clostridium butyricum*:

Sample preparation:

- Weigh accurately about 0.8 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-5} dilution series is obtained.
- Transfer 1.0 ml of 10^{-5} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **Columbia Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under anaerobic conditions (CO_2) gas pack in Anaerobic jar for 72 hours.

Reporting of results: (*Clostridium butyricum*) (An aerobically)

Calculation:

$$\frac{\text{Number of colonies observed in 3 plates}}{\text{No. of plates}} = \text{Average Cfu}$$

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu} \times \text{Average Wt.}$$

11.4.1.3 Determination of content of *Bacillus mesentricus*:

Sample preparation:

- Weigh accurately about 0.8 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-5} dilution series is obtained.
- Transfer 1.0 ml of 10^{-5} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **Nutrient Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under aerobic condition for 72 hours.

Reporting of results: (*Bacillus mesentricus*)

Calculation:

$$\frac{\text{Number of colonies observed in 3 plates}}{\text{No. of plates}} = \text{Average Cfu}$$

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-7} dilution series is obtained.
- Transfer 1.0 ml of 10^{-7} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **PNY Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under aerobic condition for 72 hours.

Reporting of results: (*Lactic Acid Bacillus*)

Calculation: $\dfrac{\text{Number of colonies observed in 3 plates}}{\text{No. of plates}} = \text{Average Cfu}$

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

11.4.2 Test preparation: II (100% Concentration)

11.4.2.1 Determination of content of *Streptococcus faecalis*:

Sample preparation:

- Weigh accurately about 1.0 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-7} dilution series is obtained.
- Transfer 1.0 ml of 10^{-7} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **Mitis Salviris Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under aerobic condition for 72 hours.

Reporting of results: (*Streptococcus faecalis*)

Calculation: Number of colonies observed in 3 plates
---------- = Average Cfu
No. of plates

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

11.4.2.2 Determination of content of *Clostridium butyricum*:

Sample preparation:

- Weigh accurately about 1.0 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-5} dilution series is obtained.
- Transfer 1.0 ml of 10^{-5} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **Columbia Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under anaerobic conditions (CO_2) gas pack in anaerobic jar for 72 hours.

Reporting of results: (*Clostridium butyricum*) (An aerobically)

Calculation: Number of colonies observed in 3 plates
---------- = Average Cfu
No. of plates

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

11.4.2.3 Determination of content of *Bacillus mesentricus:*

Sample preparation:

- Weigh accurately about 1.0 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-5} dilution series is obtained.
- Transfer 1.0 ml of 10^{-5} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **Nutrient Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under aerobic condition for 72 hours.

Reporting of results: (*Bacillus mesentricus*)

Calculation:

$$\frac{\text{Number of colonies observed in 3 plates}}{\text{No. of plates}} = \text{Average Cfu}$$

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-7} dilution series is obtained.
- Transfer 1.0 ml of 10^{-7} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **PNY Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under aerobic condition for 72 hours.

Reporting of results: *(Lactic Acid Bacillus*)

Calculation: $\dfrac{\text{Number of colonies observed in 3 plates}}{\text{No. of plates}} = \text{Average Cfu}$

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

11.4.3 Test preparation: III (120% Concentration)

11.4.3.1 Determination of content of S*treptococcus faecalis*:

Sample preparation:

- Weigh accurately about 1.2 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-7} dilution series is obtained.
- Transfer 1.0 ml of 10^{-7} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.

- Pour approximately 20 ml of the **Mitis Salviris Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under aerobic condition for 72 hours.

Reporting of results: (*Streptococcus faecalis*)

Calculation: $\frac{\text{Number of colonies observed in 3 plates}}{\text{No. of plates}}$ = Average Cfu

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

11.4.3.2 Determination of content of *Clostridium butyricum*:

Sample preparation:

- Weigh accurately about 1.2 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-5} dilution series is obtained.
- Transfer 1.0 ml of 10^{-5} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **Columbia Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under anaerobic conditions (CO_2) gas pack in Anaerobic jar for 72 hours.

Reporting of results: (*Clostridium butyricum*) (An aerobically)

Calculation: Number of colonies observed in 3 plates
---------- = Average Cfu
No. of plates

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

11.4.3.3 Determination of content of *Bacillus mesentricus*:

Sample preparation:

- Weigh accurately about 1.2 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-5} dilution series is obtained.
- Transfer 1.0 ml of 10^{-5} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **Nutrient Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under aerobic condition for 72 hours.

Reporting of results*: (Bacillus mesentricus*)

Calculation: Number of colonies observed in 3 plates
---------- = Average Cfu
No. of plates

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

11.4.3.4 Determination of content of Lactic Acid Bacillus *(Lactobacillus sporogenes):*

Sample preparation:

- Weigh accurately about 1.2 g of sample into a sterile conical flask.
- Aseptically add 99 ml of sterile 0.9% Sodium Chloride Solution to the above sterile conical flask. (This is 10^{-2} primary dilution)
- Vortex for 2 minutes and hold the sample at room temperature for 30 minutes to rehydrate the powder with occasional stirring.
- Mix for an additional 30 seconds and dilute by adding 1.0 ml of the primary 10^{-2} dilution to 9 ml of 0.9% Sodium Chloride Solution with 1 ml pipette so as to obtain a 10^{-3} dilution. Repeat this operation until the 10^{-7} dilution series is obtained.
- Transfer 1.0 ml of 10^{-7} dilution to 3 nos. of labeled sterile Petri plates by using 1.0ml sterile pipettes.
- Pour approximately 20 ml of the **PNY Agar** Swirl the plates to mix, and let solidify at room temperature on a cool level surface.
- Incubate the plates at 37°C under aerobic condition for 72 hours.

Reporting of results: (*Lactic Acid Bacillus*)

Calculation:

$$\frac{\text{Number of colonies observed in 3 plates}}{\text{No. of plates}} = \text{Average Cfu}$$

$$A = \frac{100}{\text{Sample wt.}} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \frac{10}{1} \times \text{Avg. No cfu x Average Wt.}$$

$$\% \text{ Recovery} = \frac{\text{Amount recovered in count in Cfu}}{\text{Amount added approx in Cfu (according to label claim)}} \times 100$$

Acceptance criteria:

The amount shall be recovered 90.0% to 110.0 % of the theoretical value for all concentration levels.

11.5 RUGGEDNESS:

Definition: The Ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different Incubation, different temperature, and different assay medium.

Determination: The Ruggedness of an analytical method is determined as degree of reproducibility of test results by analysis of aliquots from homogeneous lots in different Instruments by different analysts using operational and environmental conditions that may differ but are still within specified parameters of the assay.

Demonstration: The Ruggedness of the method is demonstrated from the results obtained by four different assay medium is used in this experiment at four different incubation parameters.

Acceptance criteria:

The microorganisms show growth only in specific conditions such as specific medium and specific incubation conditions.

12.0 VALIDATION ACCEPTANCE CRITERIA:

S.No.	Validation Parameters	Acceptance criteria
1.	**Specificity**	There should be no growth observed in Placebo
2.	**Precision**	
	Method Precision	RSD: Not more than 10.0%
	Intermediate precision **Analyst I** **Analysis II** **Relative difference**	RSD: Not more than 10.0%
3.	**Linearity**	Plot of concentration vs No. of colony forming units shall be linear coefficient of variance shall be close to 1.0
4.	**Recovery**	Recovery: Between 90.0% and 110.0%
5.	**Ruggedness**	The microorganisms show growth only in specific conditions such as specific medium and specific incubation conditions

13.0 REFERENCE:

United States Pharmacopoeia

ICH Guidelines (Q2: Validation of analytical procedures: Methodology)

14.0 SUMMARY REPORT:

Summary of the results of completed Analytical Method Validation of analytical method for Pre & Probiotic shall be tabulated in analytical Method Validation Report.

15.0 DEVIATIONS:

Any protocol deviation, non conformances and out of specification results obtained shall be investigated in accordance with corresponding SOPs and documented in the Analytical Method Validation Report.

16.0 REVIEW:

Validation data to be reviewed as per acceptance criteria & inference given in an individual result record.

17.0 CONCLUSION:

Validation data shall be written on Analytical Method Validation Report, clearly stating the achievement or non-compliance of the acceptance criteria, effect of the deviations made during the validation and in case of failure, investigation carried out and their findings.

18.0 RECOMMENDATION:

Recommendation shall be written on the Analytical Method Validation Report, clearly stating that the Analytical Method is recommended for the regular testing of commercial batches.

19.0 ABBREVIATIONS:

Sr.	:	Senior
Pvt.	:	Private
Ltd.	:	Limited
QA	:	Quality Assurance
cGMP	:	Current Good Manufacturing Practices
FDA	:	Food & Drug Administration
LAF	:	Laminar Air Flow
pH	:	Potential of Hydrogen
ml	:	Milliliter
NaCl	:	Sodium Chloride
AR	:	Analytical Grade
Gm	:	Gram
°C	:	Degree Centigrade
lbs	:	Pound
cfu	:	Colony Forming Unit
Wt.	:	Weight
CO_2	:	Carbon Dioxide
RSD	:	Relative Standard Deviation
ICH	:	International Conference on Harmonization