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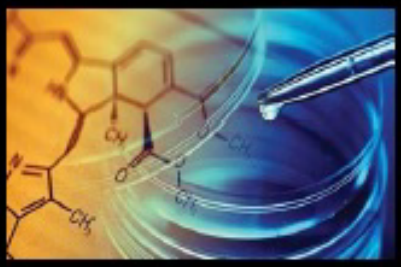
Microbiological Examination of Non-Sterile Pharmaceutical Products and Raw Materials



Ibrahim Hashim

Microbiological Examination of Non-Sterile Pharmaceutical Products and Raw Materials Analysis & Validation

Author: Ibrahim Mahmoud Hashim

ISBN: 978-1-63278-043-0

DOI: http://dx.doi.org/10.4172/978-1-63278-043-0-044

Published Date: February, 2015

Printed Version: February, 2015

Published by **OMICS Group eBooks**

731 Gull Ave, Foster City, CA 94404, USA

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Preface

Day after Day, the field of pharmaceutical microbiology has experienced numerous advances, accompanied by the publication of new and harmonized compendial methods.

It is therefore imperative for all microbiologists who are responsible for monitoring the microbial quality of pharmaceutical products to keep abreast of the latest changes

Microbiological Examination of non-sterile pharmaceutical products and Raw materials is a reference book for managers, supervisors, and scientists in the Pharmaceutical industry engaged in monitoring the microbiological Quality of non-sterile pharmaceutical products.

This book is also intended for pharmaceutical quality assurance managers, and other interested individuals.

In this book, the reader is guided through a typical, explained microbiological method, with a culmination of months of discussions with technical experts, as well as different Pharmacopeias and articles.

This book presents the simple guidance of microbiological examination of non-sterile pharmaceutical products, testing and validation methods to be introduced to all microbiologists as a standard operating procedure and validation protocol, ready to be used in the pharmaceutical laboratories.

This Book Contains a case study on microbiological analytical method validation with an imaginary product called Sabotac 600 mg/Tablets.

Ibrahim Mahmoud Hashim

About Author

Dr. Ibrahim Mahmoud Hashim is an Egyptian and internationally recognized microbiologist, subject matter expert and consultant in pharmaceutical microbiology, rapid microbiological methods, ophthalmic, contact lens care microbiology and food microbiology.

Dr. Ibrahim Hashim has held numerous positions in Quality Control Laboratories, Manufacturing and Quality Assurance at Borg Pharmaceutical Company, Medizen Pharmaceutical Company, Sabaa Pharmaceutical Company and Global Beverage Company. Also has consulting and Business Development Leadership roles in a lot of Pharmaceutical Companies and Food Manufacturing Companies with more than 13 years of experience.

Dr. Ibrahim Hashim provides practical, comprehensive and hands-on solutions for the Pharmaceutical, Biopharmaceutical, Food, Beverages and Medical Device Industries.

Acknowledgement

I would like to thank my Parents, my son Hamza, my Daughter Joudy and to thank my Wife, Mona, for her words of encouragement while this book was being written. Her companionship has enriched my life and her loving support has given me the strength and determination to always follow my dreams.

I would like to acknowledge and thank OMICS for the Scientific Publication and review Services for producing the document in the best image.

Ibrahim Mahmoud Hashim

ISBN: 978-1-63278-041-6
DOI: http://dx.doi.org/10.4172/978-1-63278-041-6-042

Microbiological Examination of Non-Sterile Pharmaceutical Products and Raw Materials Analysis & Validation

Ibrahim Hashim*

QA Manager, Global Beverage Company, KSA

***Corresponding author:** Ibrahim Hashim, QA Manager, Global Beverage Company, KSA, E-mail: ibrahim.hashim@gbc.sa; ibrahim.hashim71@yahoo.com

Purpose

To ascertain whether a given Finished product, process intermediate Product or raw material meets microbiological quality specifications by (The Quantitative Enumeration of mesophilic bacteria and fungi) that may grow under aerobic condition, Using either Pour Plate Method or Membrane Filtration Method.

To ascertain whether a given Finished product, process intermediate Product or raw material meets microbiological quality specifications by (The Qualitative Absence/Presence Tests of Some Specified Microorganisms) Using Direct Inoculation Method.

Scope

This Microbiological Reference Method is used in Pharmaceutical Microbiology Lab and is applicable for the non-sterile pharmaceutical dosage forms, also for non-sterile substances for pharmaceutical use (raw material).

Abbreviations

BTGNB: Bile Tolerant Gram Negative Bacteria; gm: Gram; IPA: Isopropyl Alcohol; ml: Milliliter; MLT: Microbial Limit Test; MEEB: Mossel Enterobacteriaceae Enrichment Broth; MPN: Most Probable Number; NLT: Not Lower Than; RVSEB: Rappaport Vassiliadis Salmonella Enrichment Broth; SDA: Sabouraud Dextrose Agar; SDB-Sabouraud Dextrose Broth; TSA: Tryptone Soy Agar; TSB: Tryptone Soy Broth; TNTC: Too Numerous To Count; TAMC: Total Aerobic Microbial Count; TYMC: Total Combined Yeast and Mold Count; USP: United States Pharmacopeia; VRBG: Violet Red Bile Glucose Agar

Responsibilities

S. No	Position	Roles
1	Microbiologists	Sterilize Sample Containers
2	QA Personnel	Collect Samples

3	Microbiologists	Follow this procedure
4	Microbiology Head	Ensure compliance with the procedure and to train microbiologists to perform this procedure

Materials/Tools

(All items must be sterile prior to use)

- Inoculating Loops
- Disposable Latex or Nitrile Gloves
- Disinfectant (70% IPA)
- Sharpie or Permanent Markers
- Sterile Glass Petri Dishes, 100x15 mm
- Sterile Syringes (10 ml)
- Scissors
- Aluminum Racks
- Bunsen Burner
- Glass Slide
- Immersion Oil
- Forceps
- Filtration Cups
- Filter Membrane 0.45
- Screw Capped test tubes
- Screw Capped Bottles
- Wipes

Sterile Liquefied Agar media/Media broth

(All Media must be Sterilized and tested prior to use)

- Liquefied Tryptone soy agar
- Liquefied Sabouraud dextrose agar
- Tryptone soy broth
- Sabouraud dextrose broth
- MacConkey broth
- MacConkey agar Selective Plates
- Cetrimide agar Selective Plates
- Mannitol salt agar Selective Plates
- Rappaport vassiliadis salmonella enrichment broth
- Xylose-Lysine Deoxychecolate agar Selective Plates

- Bismuth sulphite agar Selective Plates
- MRVP Broth
- Triple sugar Iron agar stabs and slopes
- Urease agar
- Mossel Enterobacteriaceae enrichment broth
- Violet-red bile glucose agar
- Indole Medium
- Simmons citrate agar
- EMB Agar Selective Plates

Reagents/Solutions

- Sterile Phosphate Buffer solution pH = 7.2
- Sterile Phosphate Buffer solution pH = 7.2 + Tween 80
- Sterile Tetrazolium chloride 0.15%
- Sterile saline solution
- Kovac's Indole reagent
- 0.04% w/v aqueous solution of methyl red
- Alcoholic -Naphthol
- 40% KOH
- Chloroform
- Coagulase Reagents
- Oxidase Reagents
- Crystal violet
- Gram's iodine Solution
- Ethyl alcohol (95%)
- Safranin
- Cedar Oil

Equipment

(All piece of equipment must be calibrated and/or validated prior to use as possible)

- Calibrated Balance (0.00 g)
- Colony Counter
- Incubator at 30-35°C
- Incubator at 20-25°C
- Incubator at 40-45°C

- Vortex Mixer
- Light Microscope
- Unidirectional Air Flow Hood (UDAF)
- Filtration manifold with vacuum pump

General Notes

- Proper aseptic techniques must be followed while performing these procedures.
- Plate in duplicate from a 1:10 typical sample Dilution or any other suitable sample dilution.
- The exterior of product containers should be disinfected with an ethanol soaked paper towel prior to testing.
- Sample manipulation and plating should be done in a laminar flow hood, in a separate plating room to avoid accidental contamination.
- After receiving of samples, the samples details should be recorded in the sample receipt log book and in the Results log book.
- Before carrying out the test, it is essential to be sure that all types of microbiological media used in the test are not expired and are tested for the sterility check and growth promotion fertility studies.
- Before carrying out the test, it is essential to be sure that all types of Tools/Reagents and Solutions used in the test are not expired and are Sterile whenever needed.

Procedures

Sampling

Under aseptic conditions, a representative sample shall be taken using sterile tools and sterile, containers, label the sample container with details and send it to the lab immediately.

Sample Registration & Storage

Record Samples in the Samples Receipt Record and Analysis Records.

Sample Pretreatment

Notes

- Unless otherwise specified, weigh10 g/ml of the sample by using a calibrated balance.
- Transfer by means of a sterile spatula, sterile Pipette or other sterile sampling device 10 g/ml in 90ml of phosphate buffer solution pH 7.2 to make 100ml prepared sample of (1: 10) dilution.
- Dilute further if needed, to yield not more than 250 cfu/plate in case of TAMC, 50 cfu/plate in case of TYMC.

Water soluble products

Dissolve or dilute (usually a 1 in 10 dilution is prepared) the product to be examined in Phosphate Buffer Solution pH 7.2, If necessary, adjust to a pH of 6 to 8. Further dilutions, where necessary, are prepared with the same diluent to yield not more than 250 cfu/plate in case of TAMC, 50 cfu/plate in case of TYMC.

For products or raw materials that don't dissolve completely, grind them in a sterile mortar, pestle, in an aseptic environment, to a fine powder.

Non-fatty Products Insoluble in Water

Suspend the product to be examined (usually a 1 in 10 dilution is prepared) in Phosphate Buffer Solution pH 7.2. A surface-active agent such as 1 g per L of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If necessary, adjust to a pH of 6 to 8. Further dilutions, where necessary, are prepared with the same diluent to yield not more than 250 cfu/plate in case of TAMC, 50 cfu/plate in case of TYMC.

Fatty Products

Dissolve in isopropyl myristate sterilized by filtration, or mix the product to be examined with the minimum necessary quantity of sterile polysorbate 80. Heated, if necessary, to not more than 40°C or in exceptional cases, to not more than 45°C. Mix carefully and if necessary maintain the temperature in a water bath.

Add a sufficient quantity of the prewarmed chosen diluent to make a 1 in 10 dilution of the original product.

Mix carefully, while maintaining the temperature for the shortest time necessary for the formation of an emulsion. Further serial 10-fold dilutions may be prepared using the chosen diluent containing a suitable concentration of sterile polysorbate 80 or another non inhibitory sterile surface-active reagent.

Microbial Enumeration Tests

1. Test for Total Aerobic Microbial Count Using Pour Plate Method
2. Test for Total Yeast & Mould Count Using Pour Plate Method
3. Test for Total Aerobic Microbial Count Using Membrane Filtration Method
4. Test for Total Yeast & Mould Count Using Membrane Filtration Method

Microbial Enumeration Tests Using Pour Plate Method (TAMC&TYMC)

TAMC Testing Method

- Set up the sanitized laminar air flow hood and put on sterile gloves.
- Unless otherwise specified Pipette 1 ml (aliquot) of the final dilution of the prepared sample in to each of 2 sterile Petri dishes 9 cm in diameter.
- Label the plates with sample details, Analyst, date…etc.
- Add 1.5 ml of 0.15% Tetrazolium chloride solution to 100 ml of molten TSA agar when its temperature is not more than 45°C, mix gently.
- Pour about 15:20 ml of the SCD agar in each plate of the 2 Petri dishes or increase the volume in case of larger Petri dishes.

$$DF = \frac{1}{\frac{Qt\ of\ sample\ Dissolved\ in\ Buffer}{Total Volume of\ sample\ prepared} \times Volume of\ Aliquot\ Plated}$$

- Mix the plates well by swirling, allow solidifying, incubating the plates inverted at 30:35°C for 3-5 days.
- Count the plates by the aid of colony counter; take the average count of the 2 plates.
- Calculate the dilution factor DF from the following equation:

DF equation

- Calculate the number of (cfu/gm. or ml) of product or raw material tested as follow:

$$No.of\,(cfu/gm\,or\,ml) = Average\,Count\,of\,the\,2\,Plates \times DF$$

- Record the results in the log book of microbial examination of non-sterile pharmaceutical product and raw material.

TYMC Testing Method

- Unless otherwise specified Pipette 1 ml (aliquot) of the final dilution of the prepared sample.
- Into each of 2 sterile Petri dishes 9 cm in diameter.
- Label the plates with sample details, Analyst, date…etc.
- Pour about 15:20 ml of the Sabouraud dextrose agar into each plate of the 2 Petri dishes or increase the volume in case of larger Petri dishes.
- Mix the plates well by swirling, allow solidifying, incubating the plates inverted at 20:25°C for 5-7 days.
- Count the plates by the aid of colony counter; take the average count of the 2 plates.
- Calculate the dilution factor DF from the following equation

DF equation

$$DF = \frac{1}{\frac{Qt\ of\ sample\ Dissolved\ in\ Buffer}{Total Volume\,of\ sample\ prepared} \times Volume\,of\ Aliquot\ Plated}$$

- Calculate the number of (cfu/gm or ml) of product or raw material tested as follow:

$$No.of\,(cfu/gm\,or\,ml) = Average\,Count\,of\,the\,2\,Plates \times DF$$

- Record the results in the Laboratory Records of Samples Receipt and Analysis.

Test Controls of Pour Plate Method

At the time of testing, settle plates should be exposed in the Laminar Flow Hood for evaluation of suitability of the environment for aseptic work.

The test diluent "phosphate buffer solution pH 7.2 must be evaluated for sterility check as test negative control, this is done by plating 1 ml of the diluent without product, with the same lot of media used for testing the product or raw material, be sure to use the same lot of sterile pipette, syringes used to test the product or raw material. Incubate the negative

control alongside the sample test plats at 30:35°C for 3-5 days. The test negative control must not show any growth of colonies.

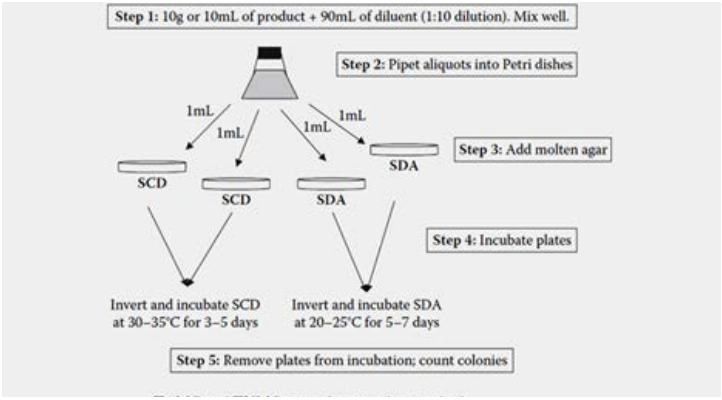


Figure 1: Microbial Enumeration Tests (TAMC & TYMC) Using Pour Plate Method.

Microbial Enumeration Tests Using Membrane Filtration Method (TAMC&TYMC)

TAMC Testing Method

- Unless otherwise specified, using syringe, pipette 10ml of the final dilution of the prepared sample into a sterile filtration unit.
- Filter the (10 ml) of the solution by using a sterile membrane filter (Pore size 0.45 µm).
- Wash the filters with 100 ml buffer diluent (phosphate buffer solution pH 7.2) for 3 times (3X100 ml).
- Remove the filter aseptically, by using a sterile forceps.
- Place the first filter onto the surface of solidified TSA agar, Incubate the plate at 30-35°C for 3-5 days.
- After incubation, count the number of cfu on filter expressed as (cfu/g or ml) of the product or raw material.
- Record the results on SCD agar as TAMC in the log book of microbial examination of non-sterile pharmaceutical product, raw material.

TYMC Testing Method

- Unless otherwise specified, using syringe, pipette 10ml of the final dilution of the prepared sample into a sterile filtration unit.
- Filter the (10 ml) of the solution by using a sterile membrane filter (Pore size 0.45 µm).
- Wash the filters with 100ml buffer diluent (phosphate buffer solution pH 7.2) for 3 times (3X100 ml).
- Remove the filter aseptically, by using a sterile forceps.
- Place the filter onto the surface of Sabouraud dextrose agar (SDA), Incubate the plate

at 20-25°C for 5-7 days.

- After incubation, count the number of cfu on filter expressed as (cfu/g or) ml) of the product or Raw material.
- Record the results on SDA as TYMC in the Laboratory Records of Samples Receipt and Analysis.

Test Controls of Membrane Filtration Method

- At the time of testing, settle plates should be exposed in the Laminar Flow Hood for evaluation of suitability of the environment for aseptic work.
- The test diluents' "phosphate buffer solution pH 7.2" must be evaluated for sterility check as test negative control; this is done by filtering the test diluent and plating the membrane filter with the same lot of media used for testing the product or raw material, be sure to use the same lot of filters, filtration units, sterile forceps, syringes used to test the product or raw material.
- Perform forceps negative control (dip the forceps in TSB).
- Incubate the test negative control, forceps negative control alongside the sample test plates at 30-35°C for 3-5 days.
- The test negative control must not show any growth of colonies.
- Forceps negative control must not show any growth (turbidity).

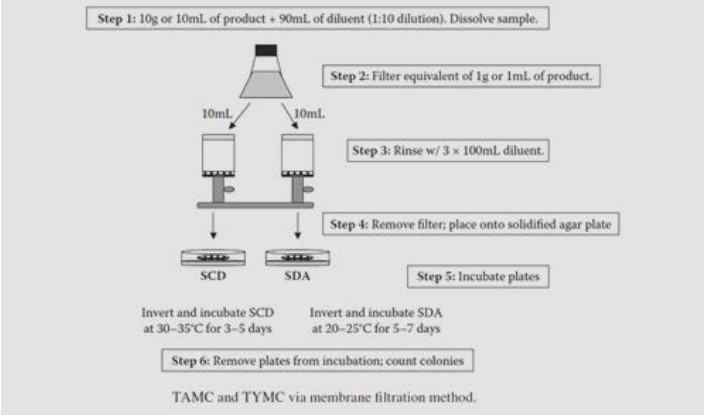


Figure 2: Microbial Enumeration Tests (TAMC&TYMC) Using Membrane Filtration Method.

Tests for Specified Microorganisms Using Direct Inoculation Method

1. Test for absence/presence of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella spp.* per 10 g/ml.
2. Test for absence/presence of *Candida albicans* per1 g/ml.
3. Test For bile tolerant Gram negative bacteria per 1 g/ml.

Test for absence/presence of Escherichia coli, Pseudomonas aeruginosa,

Staphylococcus aureus and Salmonella spp. Per 10 g/ml

Sample preparation

Directly dissolve 10 g/ml of the product or raw material into 100ml TSB as enrichment step, Mix Well.

Test for absence/presence of Escherichia coli

Inoculate 1 ml from the prepared sample in the previous enrichment TSB bottle into 100 ml MacConkey Broth, incubate at 42:44°C for 24:48 h, then subculture on MacConkey agar, incubate at 30:35°C for 18:72 h, observe the growth, the suspected growth is brick red, generally mucoid colonies, may be surrounded with a reddish bile precipitation zone.

If no suspected growth appears, pass the test.

If suspected growth appears, confirm the results as follow:

-Subculture on EMB agar.

-Subculture on Tryptone soy agar to do the following tests.

Indole: Inoculate 0.1% peptone (Indole medium) and incubate at 30-35°C for 48 h. Add 0.5 ml of Kovac's Indole reagent, shake and allow settling.

Methyl red: Inoculate glucose phosphate broth (MRVP medium) and incubate at 30-35°C for 48 h. Add 0.1 ml of a 0.04 % w/v aqueous solution of methyl red and shake gently.

Voges-Proskauer: Add 0.6 ml of alcoholic α-Naphthol and 0.2 ml of 40% KOH to 1 ml of the broth retained from the methyl red test described above. Shake well after the addition of each reagent.

Positive reaction occurs at once or within 5 minutes (the development of a copper color should be disregarded).

Citrate: Lightly streak Simmons citrate agar and incubate at 30-35°C for 48 h. If positive, repeat to confirm.

Gram's stain: Refer to Annex # 1.

Interpretation of Results

Test	Expected result if E-coli is present
EMB	Metallic sheen colonies
Indole	Red layer on medium **(+)**
Methyl red	Red color formed **(+)**
Voges-Proskauer	No pink color formed **(-)**
Citrate	No color change from green to blue **(-)**
Gram's stain	Gram negative rods, non-spore former

- Record the results in the Laboratory Records of Samples Receipt and Analysis.

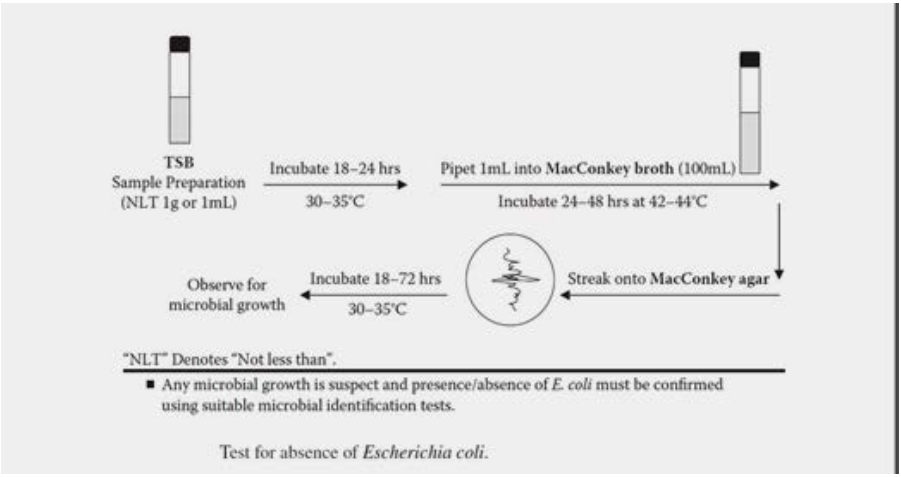


Figure 3: Diagram for test of absence/presence of E coli

Test for absence/presence of *Pseudomonas aeruginosa*

From the prepared sample in the previous enrichment TSB Bottle, subculture on Cetrimide agar, incubate at 30:35°C for 18:72 h, observe the growth, the suspected growth have Diffusible pigment or no pigment.

If no suspected growth appears, pass the test.

If suspected growth appears, confirm the results as follow.

Subculture on Tryptone soya agar or nutrient agar for the following tests:

Gram's stain: Refer to Annex # 1

Oxidase test kit

- Remove the reagent container from refrigerator and allow it to stand for 5 minutes at room temperature.
- Choose a well separated representative colony on the primary isolation medium.
- Remove one disk from the container and put it on the colony.
- Examine the disk after 30 seconds. If no color change has occurred examine again after 3 minutes.

Chloroform extraction: Inoculate Tryptone soya broth and incubate at 30-35°C for 48 h. Add 1 ml of chloroform, shake for 1 minute.

Interpretation of Results

Test	Expected result Pseudomonas aeruginosa is present
Oxidase	Changes to a blue color within 15 seconds
Chloroform extraction	Greenish pigmentation appears in the chloroform layer
Gram's stain	Gram negative rods, non-spore-former

- Record the results in the Laboratory Records of Samples Receipt and Analysis.

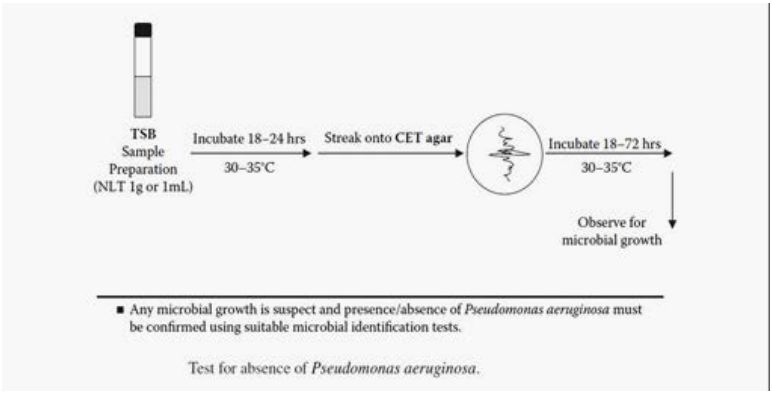


Figure 4: Diagram for test of absence/presence of Pseudomonas aeruginosa.

Test for absence/presence of Staphylococcus aureus

From the prepared sample in the previous enrichment TSB Bottle, subculture on the surface of Mannitol salt agar, incubate at 30:35°C for 18:72 h, observe the growth, the suspected growth is yellow to white colonies surrounded by a yellow zone.

If no suspected growth appears, pass the test.

If suspected growth appears, confirm the results as follow.

Subculture on Tryptone soya agar or nutrient agar for the following tests:

Coagulase test: using Coagulase kit test.

Gram's stain: Refer to Annex # 1

Interpretation of Results:

Test	Expected result
Coagulase (Staphylase)	Coagulated
Culture on TSA	Golden yellow pigmentation
Gram's stain	Gram positive cocci in clusters

- Record the results in the Laboratory Records of Samples Receipt and Analysis.

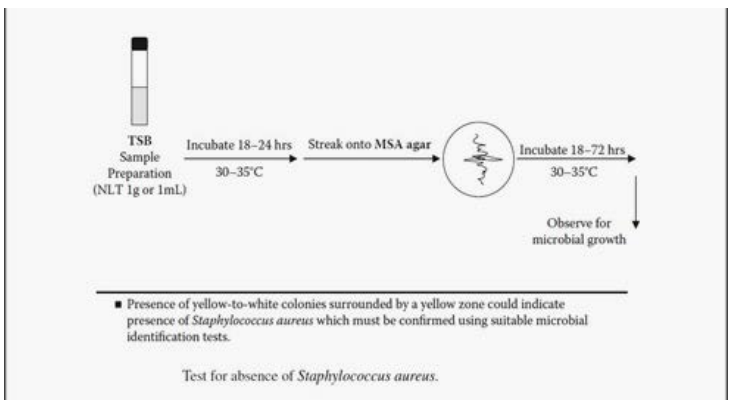


Figure 5: Diagram for test of absence/presence of Staphylococcus aureus.

Test for Absence/Presence of Salmonella spp.

Inoculate 0.1 ml from the prepared sample in the previous enrichment TSB bottle into 10 ml of Rappaport Vassiliadis Salmonella Enrichment Broth (RVSEB), incubate at 30:35°C for 18:24 h then subculture onto the surface of XLD agar, Incubate for 18-48 h at 30-35°C. The suspected growth is well developed red colonies with or without black centers.

If no suspected growth appears, pass the test.

If suspected growth appears, confirm the results as follow.

Subculture on Tryptone soya agar or nutrient agar for the following tests:

Triple sugar Iron agar (TSI): Inoculate the slope and stab the bottom of the T.S.I slope and incubate at 30-35°C for 48 h. Examine at 24 and 48 h.

Urease: Inoculate the surface or Urease agar and incubate at 30-35°C for 24 h.

Oxidase test:

- Remove the reagent container from refrigerator and allow it to stand for 5 minutes at room temperature.
- Choose a well separated representative colony on the primary isolation medium.
- Remove one disk from the container and put it on the colony.
- Examine the disk after 30 seconds. If no color change has occurred examine again after 3 minutes.

Gram's stain: Refer to Annex # 1.

Interpretation of Results:

Test	Expected results
T.S.I	H_2S: Hydrogen sulphide (black)
Urease	No color change in the medium
Oxidase	Negative
Gram's stain	Gram negative rods non-sporing cells

- Record the results in the Laboratory Records of Samples Receipt and Analysis.

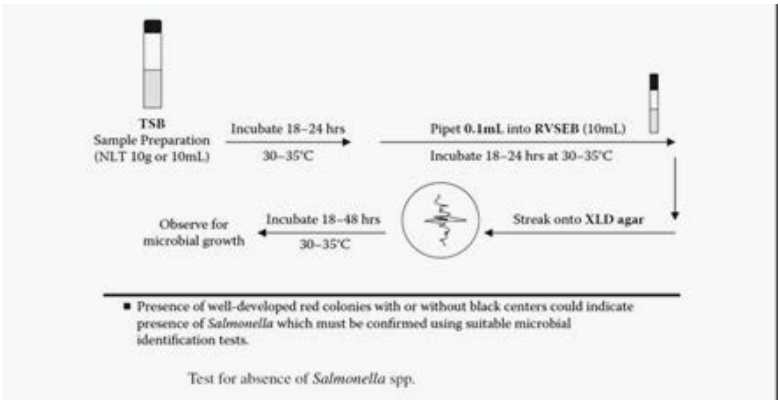


Figure 6: Diagram for test of absence/presence of Salmonella spp.

Test for absence/presence of Candida albicans per 1g/ml

Sample Preparation

Directly dissolve 1 g of the product or raw material into 10 ml SDB as enrichment step in a sterile test tube, Mix Well.

Test Method

Incubate the prepared sample in the previous enrichment SDB Test Tube at 30:35°C for 3-5 days, then subculture on the surface of solidified SDA plate, incubate at 30:35°C for 24:48 h, observe the growth, the suspected growth is white colonies.

If no suspected growth appears, pass the test.

If suspected growth appears, confirm the results as follow.

Subculture on Sabouraud dextrose agar for the following tests:

Gram's stain: Refer to Annex # 1

Interpretation of Results:

Test	Expected results
Gram's stain	Gram positive, globose, ovoid or elongated, multipolar budding yeast.

- Record the results in the Laboratory Records of Samples Receipt and Analysis.

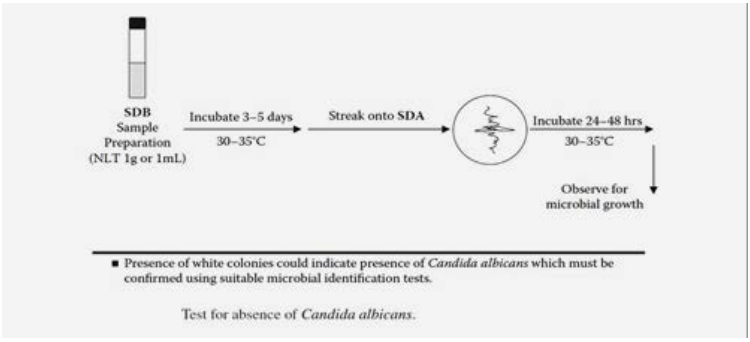


Figure 7: Diagram for test of absence/presence of Candida albicans.

Test for Bile Tolerant Gram Negative Bacteria BTGNB

Test for absence/Presence of BTGNB

- Directly inoculate 9 ml TSB with (1g or ml) of the product or raw material and incubate at 20:25°C for 2-5 h to resuscitate any bacteria that might be present.
- Transfer amount equivalent to 1 g/1 ml of the product/raw material into 100 ml MEEB, incubate at 30:35°C for 24-48 h, then streak on to the surface of VRBG Plate and incubate at 30:35°C for 18-24 h, observe the growth.

- Presence of red colonies surrounded by a reddish precipitate indicates presence of Bile-tolerant Gram-negative bacteria.
- If no growth appears, pass the test, record Results.
- If growth appears, follow the quantitative test to count BTGNVB Using MPN Method.

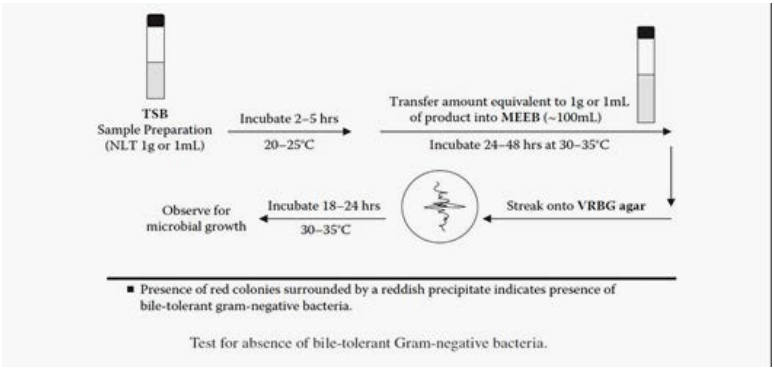


Figure 8: Diagram for test of absence/presence of BTGNB.

MPN Quantitative Test of BTGNB

- Directly inoculate 9 ml TSB with (1 g or ml) of the product or raw material and incubate at 20:25°C for 2-5 h.
- Transfer 1 ml Aliquot from TSB test tube equivalent to (0.1 g or ml of the product or raw material) to 9 ml MEEB in tube (1).
- From tube (1) transfer 1ml equivalent to (0.01 g or ml of the product or raw material) to 9 ml MEEB in tube (2).
- From tube (2) transfer 1ml equivalent to (0.01 g or ml of the product or raw material) to 9 ml MEEB in tube (3).
- Incubate the three tubes 1, 2, 3 at 30:35°C for 24-48 h then streak on to the surface of VRBG Plate and incubate at 30:35°C for 18-24 h, observe the growth.
- Presence of red colonies surrounded by a reddish precipitate indicates presence of Bile-tolerant Gram-negative bacteria.
- Use the following Table 1 to evaluate results and to determine most probable number of bile-tolerant Gram-negative bacteria.

MPN of Bacteria per g or ml	Test Result		
	0.1 g or 0.1 ml	0.01 g or 0.01 ml	0.001 g or 0.001 ml
More than 10^3	+	+	+
Less than 10^3, more than 10^2	+	+	-

Less than 10^2, more than 10	+	-	-
Less than 10	-	-	-

Table 1: Determination of most probable number of bile-tolerant Gram-negative bacteria.

- Record the results in the Laboratory Records of Samples Receipt and Analysis.

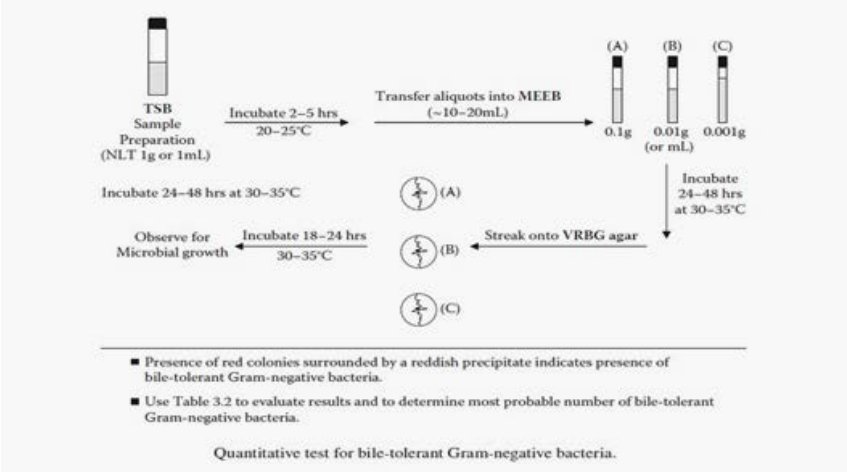


Figure 9: Diagram for MPN Quantitative Method of BTGNB.

Specification Limits (Harmonized Method)

Route of Administration	TAMC	TYMC	Specified M.Os
Non-aqueous preparations for oral use	10^3	10^2	*Escherichia coli* absent in 1 g or 1 ml
Aqueous preparations for oral use	10^2	10^1	*Escherichia coli* absent in 1 g or 1 ml
Rectal use	10^3	10^2	If Required
Oromucosal use Gingival use Cutaneous use Nasal use Auricular use	10^2	10^1	*Staphylococcus aureus* absent in 1 g or 1 ml *Pseudomonas aeruginosa* absent in 1g or 1 ml
Vaginal use	10^2	10^1	*Staphylococcus aureus* absent in 1 g or 1 ml *Pseudomonas aeruginosa* absent in 1 g or 1 ml *Candida albicans* absent in 1 g or 1 ml

Inhalation use (special requirements apply to Liquid preparations for nebulization)	10^2	10^1	*Staphylococcus aureus* absent in 1 g or 1 ml *Pseudomonas aeruginosa* absent in 1 g or 1 ml Bile-tolerant gram-negative bacteria absent in 1 g or 1 ml
Transdermal patches (limits for one patch including adhesive layers and backing)	10^2	10^1	*Staphylococcus aureus* absent in 1 g or 1 ml *Pseudomonas aeruginosa* absent in 1 g or 1 ml

Table 2: Acceptance criteria for Microbiological Quality of non- sterile dosage forms and Raw Material.

Material	TAMC	TYMC	Specified M.Os
Substances for Pharmaceutical Use	10^3	10^2	If Required

Table 3: Acceptance criteria for Microbiological Quality of Raw Material for Non Sterile Manufacturing.

Note:

USP32 chapter <61> allows variability in test results equal to a factor of 2. If the specified microbial limit is 10,the maximum accepted microbial count is 20 cfu and still meets the product specification, if the specified microbial limit is 100 (10^2),the maximum accepted microbial count is 200 cfu and still meets the product specification and so on. i.e.,

- 10^1 microorganisms: maximum acceptable count = 20.
- 10^2 microorganisms: maximum acceptable count = 200.
- 10^3 microorganisms: maximum acceptable count = 2000, and so forth.

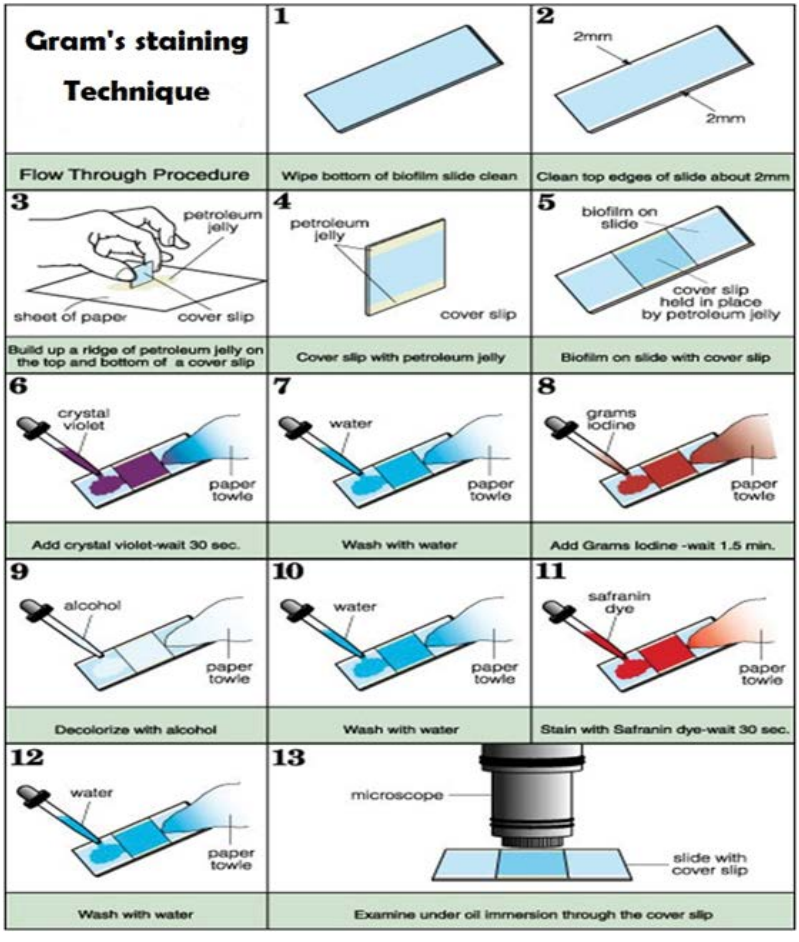
Annex # 1

Gram's Staining

Procedure

- Label a glass microscope slide with the laboratory accession number and isolate number.
- Flame the slide over the Bunsen burner.
- Place one drop of sterile saline solution on the surface of the slide.
- Mix one separate colony of the microbial culture examined with the saline drop by means of sterile loop.
- Air dry smears at room temperature or fix the smear by passing the slide through a Bunsen burner flame three or four times do not overheat.
- Allow slide to cool before staining.

- Flood the slide with crystal violet for 1 minute.
- Rinse off the crystal violet with water.
- Flood the slide with Gram's iodine for 1 minute.
- Wash off the iodine with water.
- Add drops of alcohol (95%) to the smear until the rinsed alcohol contains no stain.
- Wash off the slide with water.
- Flood the slide with Safranin for 1 minute.
- Wash off the Safranin with water; leave the slide for 10 minutes to dry.
- Examine the slide under a microscope using an oil immersion lens with a magnification power 100.
- Gram positive microorganisms appear with a purple color stain.
- Gram negative microorganisms appear with a red color stain.
- See the Following Pictures of Gram Staining Steps.



Microbiological Method Validation Protocol

Product/material Name

Example: Sabotac 600 mg/Tab

Approved by			
Printed name	**Title**	**Signature**	**Date**

Validation team			
Printed name	**Title**	**Signature**	**Date**

Study contents

Revision control

Revision	Date	Cause of revision	Author

P 01 Objective: To establish documented evidence which provide high degree of assurance that the analytical microbiological methods used for the analysis of Non-Sterile Pharmaceutical Product (Sabotac 600 mg/Tab), will be suitable for its intended use in (Company Name).

P 02 Scope: This study will be focused on The Microbiological method used for the analysis of the non- sterile pharmaceutical product named (Product Name).

The validation study will include 3 consecutive Batches # (xxx1, xxx2 and xxx3.)

The validation study will include microbial enumeration tests (TAMC-TYMC) and tests for presence/absence of specified MO.s.

Serial	Batch no.	Manufacturing date	Microbial enumeration test		Test for absence/presence of specified mo.s					
			TAMC	TYMC	*E.coli*	*P.aeru*	*S.aur*	*S.typh*	*C.alb*	*Costr*
1	Batch 1	xx/xx/xxxx	√	√	√	X	X	X	X	X
2	Batch 2	xx/xx/xxxx	√	√	√	X	X	X	X	X
3	Batch 3	xx/xx/xxxx	√	√	√	X	X	X	X	X

P 03 Responsibility matrix

Validation Team Members	Roles
Name	Develop Protocol
Name	Execute Testing
Name	Supervise Testing , Approve Protocol, Final Report

P 04 References

- Method validation
- USP 32–NF 27, <61>
- USP 32–NF 27, <62>
- European Pharmacopoeia 2009, 2.6.12
- European Pharmacopoeia 2009, 2.6.13
- Microbial Limit and Bioburden Approach, Second Edition – Lucia Clontz

P 05 Abbreviations

The Following Abbreviations will be included in this study			
Abbreviation	**Description**	**Abbreviation**	**Description**
M.Os	Microorganisms	**TSA**	Tryptone soy agar
TAMC	Total aerobic microbial count	**SDA**	Sabouraud dextrose agar
TYMC	Total combined yeast and mold count	**CFU**	Colony forming unit
E coli	*Escherichia coli*	**CG**	Control group
ATCC	American type culture collection	**TG**	Test group
SOP	Standard operating procedure	**VG**	Viability group
gm	Gram unit of weight	**ml**	Milliliter unit of volume
L	Liter unit of volume	**USP**	United State Pharmacopoeia

NA	Not applicable	***A. bras***	*Aspergillusbarsiliensis*
S. aur	*S. aureus*	***C.alb***	*C. albicans*
P. aer	*P. aeruginosa*	**µL**	Micro liter
B. sub	*Bacillus subtilis*	**NMT**	Not More Than
cm	Centimeter	**Clostr**	Clostridia

P 06 Instruments and Tools

Equipment

All instruments used during the validation study should be verified in the description section of the following table with:

Function, model, serial number and calibration status if Possible.

Copies of certificates, if possible should be enclosed to the test protocols.

Equipment	Description
Autoclave	**Function:** **Model:** **SN:** **Status:**
Oven	**Function:** **Model:** **SN:** **Status:**
Refrigerator	**Function:** **Model:** **Status:**
pH Meter	**Function:** **Model:** **SN:** **Status:**
Incubator at 30-35˚C	**Function:** **Model:** **SN:** **Status:**
Incubator at 20-25˚C	**Function:** **Model:** **SN :** **Status:**
Incubator at 40-45˚C	**Function:** **Model:** **SN:** **Status:**
Vortex Mixer	**Function:** **Model:**
Colony Counter	**Function:** **Model:** **SN :**
Light Microscope	**Function:** **Model:** **SN :**
Laminar air flow Hood	**Function:** **Model:** **Status:**
Precision Balance	**Function:** **Model:** **SN:** **Status**:

Tools

- All Tools used during the validation study should be sterile prior to use.

Tools	Description
Inoculating Loops	Aluminum loop handle insulated with a sterilizable Nickel-Chrome wire loops from (1 µL) to r (10 µL)
Gloves	Disposable Latex or Nitrile Gloves
Petri Dishes	Sterile autoclavable glass, or disposable plastic 9 cm in diameter
Syringes/Pipettes	Disposable sterile 10ml syringe or autoclavable sterile 10ml glass pipettes.
Test Tubes	Sterile glass screw capped test tubes
Bottles	100 ml, Sterile Glass Screw Capped Bottles

Reagents and Kits

All Reagents used during the validation study should not be expired and should be sterile as required.

Tools	Description
Tetrazolium chloride 0.15%	Sterilized by filtration, Dissolve 0.3 g of 2, 3, 5-triphenyl Tetrazolium chloride in 200 ml of distilled water. Filter the solution through a sterile 0.45 µm membrane filter into sterile screw capped container.
Sterile saline solution	Dissolve 9.0 g of sodium chloride in 100 ml of distilled water , sterilize by autoclaving
Kovac's Indole reagent	Amyl or isoamyl 150 ml ρ-Dimethylaminobenzaldehyde 10 g Hydrochloric acid, concentrated 50 ml Dissolve the aldehyde in alcohol, and then add slowly the acid **Notes:** The dry aldehyde should be light in color. Prepare small quantities and store in a refrigerator. Discard the solution when it becomes deep brown in color
0.04% w/v aqueous solution of methyl red	Methyl red 0.1 g Ethyl alcohol 95% 300 ml Dissolve the dye in alcohol, and add sufficient distilled water to make 500ml.
Alcoholic α-Naphthol	α-Naphthol 5 g Ethyl alcohol, absolute 100 ml
40% KOH	Potassium hydroxide 40 g Distilled water 10 ml
Crystal violet	Solution A:(Expiration period is not more than 6 months). Crystal Violet 2.0g Ethyl alcohol 95% 20.0 g Solution B: (Expiration period is not more than 6 months). Ammonium oxalate 0.8 g Distilled water 80.0 g Mix solutions A and B. Store for 24 h before use. Filter through filter paper into staining bottle
Gram's iodine Solution	Iodine 1.0 g Potassium iodide 2.0 g Distilled water 300 ml Grind the dry iodine and potassium iodide in a mortar. Add water gradually, and grind thoroughly after each addition until solution is achieved. Rinse the solution into an amber, glass bottle with the remainder of the distilled water. 6 months expiration.
Ethyl alcohol (95%)	Pure Reagent
Safranin	Stock solution: Safranine 2.5 g Ethyl alcohol 95% 100 ml Working solution: Stock solution 10.0 g Distilled water 90.0 g
Cedar Oil	Pure Reagent

Media/Diluents

All Microbiological Media and Diluents used during the validation study should be properly prepared, Sterilized and tested for growth promotion, Sterility check and Physical Check prior to use.

Tools	Description
Sterile Phosphate Buffer solution pH 7.2	**Stock solution:** Dissolve 34 g of monobasic potassium phosphate in about 500 ml of water in 1 L volumetric flask. Adjust to pH 7.2 ± 0.1 by addition of 1M sodium hydroxide, add water to volume, and mix. Dispense and sterilize at 121°C for 15 minutes. Store in refrigerator. **Working Solution:** Dilute the stock solution with water in the ratio of 1 to 800. Add 2% Tween 80 if required. Heat to aid dispersion. Dispense 90 ml into 100 ml bottles, sterilize at 121°C for 15 minutes.
Peptone water	Peptone 5 g/L Sodium chloride 5 g/L Complete till one liter distilled water. Sterilize at 121°C for 15 minutes.
Liquefied Tryptone soy agar	Prepared, Sterilized and tested prior to use.
Liquefied Sabouraud dextrose agar	Prepared, Sterilized and tested prior to use.
Tryptone soy broth	Prepared, Sterilized and tested prior to use.
MacConkey broth	Prepared, Sterilized and tested prior to use.
MacConkey agar Selective Plates	Prepared, Sterilized and tested prior to use.
MRVP Broth	Prepared, Sterilized and tested prior to use.
Indole Medium	Tryptone10.0 g/L Sodium chloride 5 g/L pH 7.5 0.2 Sterilize at 121°C for 15 minutes.
EMB Agar Selective Plates	Prepared, Sterilized and tested prior to use.
Simmons citrate agar	Prepared, Sterilized and tested prior to use.

Reference Microbial Strains

- All Challenge Reference microbial Strains used during the validation study should be purchased from a national culture collection like ATCC, properly rehydrated, transferred and diluted to yield a low level Inoculum of 10-100cfu By means of serial Dilution.
- The compendial recommendation is limiting the number of transfers to not more than five passages in order to avoid mutations.

Microbial strain	Description
S. aureus	ATCC 6538
P. aeruginosa	ATCC 9027

Bacillus subtilis	ATCC 6633
Aspergillusbarsiliensis	ATCC 16404
C. albicans	ATCC 10231
E coli	ATCC 8739

General Tools

Some general tools should be provided prior to the validation study.

Tools	Description
Disinfectant	(Isopropyl Alcohol 70%) or (Ethyl Alcohol 70%)
Markers	Sharpie or Permanent
Racks	Aluminum or stainless steel
Flame	Bunsen burner
Wipes	Napkin Wipe Rolls
Microscopic Slides	Glass Slides for microscopic examinations and Gram Staining

P 07 Change Protocol

Changes that might affect the validated status shall be formally documented, evaluated and the need for action is decided to ensure that the validated status is maintained.

Revalidation is required in case of major changes like:

- Change in formula
- Change in material origin
- Change in analysis method/new method

P 08 Deviations

Document any discrepancies found during the Microbiological Method validation.

P 09 Safety Measures

Follow the general safety rules of microbiology lab.

P 10 Documents Required

S.No	Document
1	Microbiological Method validation SOP
2	Microbial limit test SOP
3	Log book of Microbiological Method validation
4	Microbiology Lab Equipment SOP
5	Calibration/validation Certificates of Equipment
6	Safety Rules in Microbiology Lab SOP
7	Gram Staining SOP
8	Microbiological Media SOP
9	Microbial Strains SOP

P 11 Validation methodology

- Suitability testing of the counting method by Pour plate technique
- Suitability testing for screening of *E. coli* by direct inoculation technique

Suitability testing of the counting method by Pour plate technique

Test area: Suitability testing of the Counting Method by Pour Plate Technique

Product name: Sabotac 600 mg Tab

Test point: TAMC and TYMC

Batch numbers: 1-2-3

Test objective: To ensure that the Counting method (TAMC and TYMC) used for the analysis of Sabotac 600 mg Tab will be suitable for its intended use in the laboratory.

Sampling plan:

Initial validation: 3 Consecutive Batches

Revalidation: NA

Sampling container: Sterile glass screw capped bottle

Sampling Quantity: 10 gm

Sampling stage: After Blistering

Test control group:

- Dilute, homogenize10 gm of Sabotac 600 mg tab in 90 ml Phosphate buffer Solution pH 7.2 to make 1:10 product dilution.
- Shake, mix the sample preparation.
- Dispense equal volume of aliquots (10 ml) of the sample preparation into 5 separated sterile test tubes (T1, T2, T3, T4, and T5) separately.
- Inoculate each tube with 0.1 ml 100:1000 CFU pure cultures of:

(T1) - *S. aureus*

(T2) - *P. aeruginosa*

(T3) - *Bacillus subtilis*

(T4) - *Aspergillus barsiliensis*

(T5) - *C. albicans*

- Hold the inoculated preparation prior to plating for a time not exceed 1 h (15-20 minutes is recommended).
- For tube T1 (*S. aureus*) plate 1 ml aliquots in duplicates with the appropriate media, TSA medium for TAMC suitability Testing.
- For tube T2 (*P. aeruginosa*) plate 1 ml aliquots in duplicates with the appropriate media, TSA medium for TAMC suitability testing.
- For tube T3 (*Bacillus subtilis*) plate 1ml aliquots in duplicates with the appropriate media, TSA medium for TAMC suitability Testing.
- For tube T4 (*Aspergillus barsiliensis*) Plate 1 ml aliquots in duplicates with the appropriate media, TSA medium for TAMC suitability Testing. Also Plate 1 ml aliquots in duplicates with the appropriate media SDA medium for TYMC suitability testing.

- For tube T5 (*C. albicans*) Plate 1 ml aliquots in duplicates with the appropriate media, TSA medium for TAMC suitability Testing. Also Plate 1 ml aliquots in duplicates with the appropriate media SDA medium for TYMC suitability testing.
- Incubate TSA plates at 30:35°C for not more than 3 days
- Incubate SDA plates at 20:25°C for not more than 5 days.

Viability Group: For each microorganism, separately prepare a viability group by plating the inoculum (10-100 CFU) as used to challenge the test group, peptone control group, using the same dilution scheme using a suitable buffer as diluent on TSA medium for bacterial suspensions and SDA medium for fungal suspensions, incubate TSA plates for 24-48 h @ 30-35°C Incubate fungi on SDA plates for 3-5 days @ 20-25°C.

Test Negative Control: Prepare test negative control (buffer and media separately) by setting aside an unopened containers of the same lot of diluent, plate the same volume used to test the sample preparation, use the same lot of recovery medium TSA, incubate TSA plates for 24-48 h @ 30-35°C, observe for absence of turbidity.

Product Negative Control: Prepare product negative control (like Routine product testing) to evaluate any inherent product bioburden that could interfere with enumeration and evaluation of the recovered challenge organisms. Incubate TSA plates for 24-48 h @ 30-35°C - incubate fungi on SDA plates for 3–5 days @ 20-25°C.

Microbial Recovery Calculation: At the end of incubation, enumerate the recovered colonies from test group, peptone control group and viability group and calculate the arithmetic mean for replicate plates.

1. If the mean count of (Test Group) is not less than a factor 2 of the mean count of (Peptone Control Group).

*the method shows adequate neutralizer effectiveness

2- If the mean count of (Peptone Control Group) is not less than a factor 2 of the mean count of (Viability group).

*the method shows lock of neutralizer toxicity.

Acceptance Criteria:

1. For all test organisms average count from Test Group must not be less than a factor of 2 when compared to average count from Peptone Control Group.
2. For all test organisms average count from Peptone Control Group must not be less than a factor of 2 when compared to average count from Viability Group.
3. The plates for test negative control should be free microbial contamination.
4. The plates for the product negative control should be free of microbial contamination or have a very low bioburden.

Study Results:

Comment

According to the above Results , it was found that the counting method for both (TAMC-TYMC) for The Product named Sabotac 600 mg Tab, B No's 1, 2 and 3 is suitable to be held in the laboratory in accordance with a the above study.

Conclusion

PASS	FAIL
✓	-

Test committee

	Name	Signature	Date
Tester			
Verifier			

Suitability testing for screening of E. coli by direct inoculation technique

Test area: Suitability testing for screening of *E. coli* by direct inoculation technique

Product Name: Sabotac 600 mg Tab

Test Point: Absence or presence of *E. coli*

Batch Numbers: 1-2-3

Test objective: To ensure that the absence/presence of test method used to detect *E. coli* in the Product (Sabotac 600 mg Tab) by direct inoculation technique will be suitable for its intended use in the laboratory.

Sampling plan:

Initial validation: 3 Consecutive Batches

Revalidation: NA

Sampling container: Sterile glass screw capped bottle

Sampling Quantity: 1 gm

Sampling stage: After blistering

Test control group:

- Dilute, homogenize 1 gm of Sabotac 600 mg tab in 9 ml Tryptone soy Broth to make 1:10 product dilution,
- Shake, mix the sample preparation (Tube T) and inoculate the tube T with 0.1 ml 10:100 CFU pure cultures of (T) - *E. coli.*
- Hold the inoculated preparation prior to plating for a time not exceed 1 h (15-20 min recommended).
- For tube T (*E. coli*) incubate 18 h, at 30:35°C then pipette 1ml from the incubated broth into MacConkey broth (100 mL), incubate 24 h at 40-44°C then streak on to MacConkey agar, incubate 18h 30:35°C then observe the growth.

Peptone control group:

- Dilute 10 ml of Peptone water in 90 ml Tryptone soy Broth to make 1:10 dilution.

- Shake, mix the sample preparation (Tube C), inoculate the Tube C with 1 ml 10:100 CFU pure cultures of (C) - *E. coli.*
- Hold the inoculated preparation prior to plating for a time not exceed 1h (15-20 minutes recommended).
- For tube C (*E. coli*) incubate 18 h, at 30:35°C then pipette 1 ml from the incubated broth into MacConkey broth (100 mL), incubate 24 h at 40-44°C then streak on to MacConkey agar, incubate 18 h 30:35°C then observe the growth.

Viability group: Prepare a Viability group by plating the inoculum 0.1 ml (10-100 CFU) of *E. coli* which is used to challenge the test group, peptone control group, in duplicates of TSA medium, incubate plates for 24-48 h @ 30-35°C. Take the average of the 2 plates and verify the low level inoculum count.

Test negative control: Prepare test negative control using 10 ml TSB (the same lot of TSB used in case of TG and PCG), incubate the broth tube for 18 h @ 30-35°C, observe for absence of turbidity.

Microbial Recovery Calculation:

- At the end of incubation, observe the turbidity, physical appearance, colour change and biochemical reaction if needed from test group, peptone control group.
- Observe the viability group and calculate the arithmetic mean for replicate plates.
- Microbial growth recovered from the test group must be comparable to the microbial growth obtained from the peptone group, check the comparability in appearance.

Acceptance criteria:

1. The *E. coli* challenge organism must be recovered from the peptone control group.
2. The viability group confirms a low level count (10-100 CFU).
3. The test negative control shows absence of microbial contamination.
4. Microbial growth recovered from the test group is comparable to the microbial growth obtained from the peptone group, check the comparability in appearance.

Study Results

Sabotac 600 mg/Tab			
Sample Preparation	**First batch**	**Second batch**	**Third batch**
	12033	**12034**	**12035**
Special requirements for sample preparation	N.A	N.A	N.A
Tryptone soy broth Lot No.	TSB 120612	TSB 120612	TSB 120612
Tryptone soy broth Volume	90 ml	90 ml	90 ml
Sample Weight	10 g	10 g	10 g
Dilution Factor	1:10	1:10	1:10
Neutralizer/volume	N.A	N.A	N.A

Sample hold time	5 Minutes	5 Minutes	5 Minutes
Inoculum/test Ratio	1:100	1:100	1:100
MacConkey Broth B.No….	MCB 120612	MCB 120612	MCB 120612
MacConkey Broth Volume	100 ml	100 ml	100 ml
MacConkey agar B.No….	MCA 11 0612	MCA 11 0612	MCA 11 0612
Test Negative Control	**First batch**	**Second batch**	**Third batch**
	12033	**12034**	**12035**
Tryptone soy broth Lot No.	TSB 120612	TSB 120612	TSB 120612
Tryptone soy broth volume	10 ml	10 ml	10 ml
Specification	Absence of Turbidity		
Result	**Comply with the predetermined specification**		

Test Result Calculation For Suitability of Absence or Presence of *E. coli* by direct inoculation Technique (First Batch #1)

Mo.	Test group TG			Peptone Control Group CG			Viability Group VG			TG/CG Comparability	Pass/ Fail
	TSB	Mac Broth	Mac agar	TSB	Mac Broth	Mac agar	Plate 1	Plate 2	Mean		
E. coli	Turbid	Turbid with Yellow color	Typical Growth	Turbid	Turbid with Yellow color	Typical Growth	78 Cfu	86 Cfu	82 Cfu	**Comparable**	**Pass**
		color			Yellow color						

Test Result Calculation For Suitability of Absence or Presence of *E. coli*by direct inoculation Technique (Second Batch #2)

Mo.	Test group TG			Peptone Control Group CG			Viability Group VG			TG/CG Comparability	Pass/ Fail
	TSB	Mac Broth	Mac agar	TSB	Mac Broth	Mac agar	Plate 1	Plate 2	Mean		
E. coli	Turbid	Turbid with Yellow color	Typical Growth	Turbid	Turbid with Yellow color	Typical Growth	83 Cfu	74 Cfu	79 Cfu	**Comparable**	**Pass**

Test Result Calculation For Suitability of Absence or Presence of *E. coli* by direct inoculation Technique (Third Batch #3)

Mo.	Test group TG			Peptone Control Group CG			Viability Group VG			TG/CG Comparability	Pass/ Fail
	TSB	Mac Broth	Mac agar	TSB	Mac Broth	Mac agar	Plate 1	Plate 2	Mean		
E. coli	Turbid	Turbid with Yellow color	Typical Growth	Turbid	Turbid with Yellow color	Typical Growth	69 Cfu	78 Cfu	71 Cfu	**Comparable**	**Pass**

Comment

According to the above results, it was found that the method for screening of *E. coli* in the product named Sabotac 600 mg Tab, B Nos. 1, 2 and 3 is suitable to be held in the laboratory in accordance with the above study

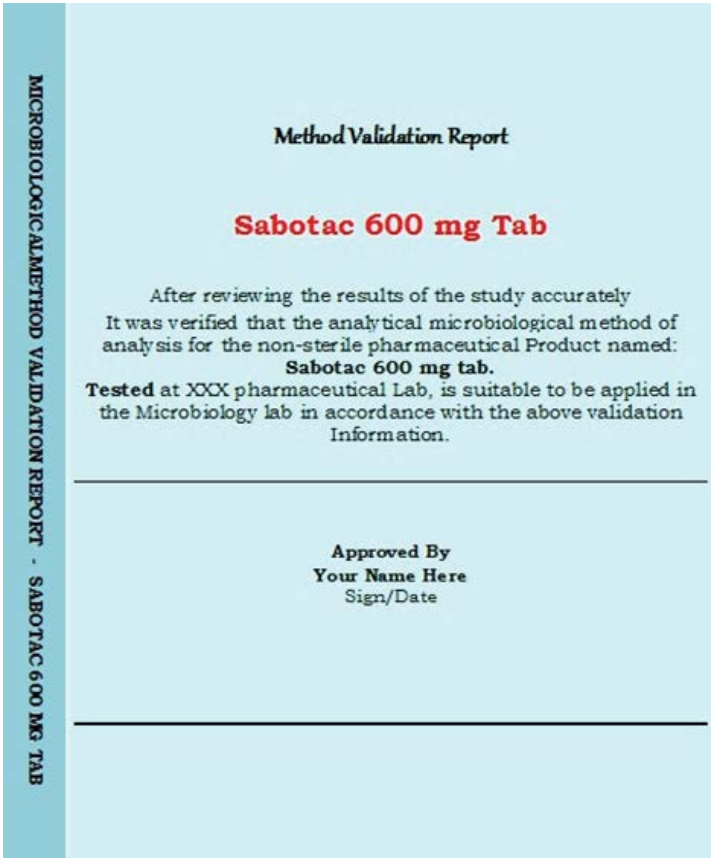
Conclusion

PASS	FAIL
✓	-

Test Committee

	Name	Signature	Date
Tester			
Verifier			

P 12 Final report



References

1. USP 32-NF 27, <61> Harmonized Method.
2. USP 32-NF 27, <62> Harmonized Method.
3. European Pharmacopoeia (2009) 2.6.12 Harmonized Method.
4. European Pharmacopoeia (2009) 2.6.13 Harmonized Method.
5. Lucia Clontz (2008) Microbial Limit and Bioburden Tests, Validation Approaches and Global Requirements (2nd Edition), CRC Press.