Department	Micro Laboratory		Document no	MICLAB – METHOD 013	
Title	Presence or Absence Test of Indicator Organisms				
Prepared by:		Date:		Supersedes:	
Checked by:		Date:		Date Issued:	
Approved by:		Date:		Review Date:	

1. SCOPE AND APPLICATION

This general test method shall be followed in order to determine the presence or absence of indicator organisms such as Pseudomonas spp., Coliforms (e.g. Klebsiella spp., Enterobacter spp.) and coagulase positive Staphylococcus spp. This general test method is also followed when screening for objectionable organisms in order to determine the presence or absence of any gram negative rod.

This procedure applies to products manufactured at GMP Manufacturing site as well as raw materials used to manufacture those products.

This procedure covers tests for objectionable and deleterious organisms.

2. REAGENTS AND MATERIALS REQUIRED

2.1 Tryptone Soya Broth containing 4% Tween 80 (TSB+T) or other enrichment broth as predetermined by validation study ie. Letheen Broth + 2% Lecithin + 4% Tween 80.

2.2 Stomacher bag or sterile jar

2.3 Streaking loops (10μL and 1 μL)

2.4 Pseudomonas Agar Plates (PAB)

2.5 CFC Agar Plates (CFC)

2.6 MacConkey No. 3 Agar plates (MAC)

2.7 Baird-Parker Agar plates (BP)

2.8 Lauryl Tryptose Broth (LTB)

2.9 EC Broth (EC)

2.10 Tryptone Soya Agar Plates (TSA)

2.11 Saboraud Dextrose Agar (SDA)

2.12 EMB agar plates (EMB)

2.13 Tryptone Water (TW)

2.14 Latex Gloves

3. GENERAL TEST PROCEDURE

Note: The analyst must wear latex gloves during all steps

3.1 Weigh out 10g of sample into the stomacher bag.

3.2 Make up to 100g with TSB+T (enrichment) or other enrichment broth.

3.3 Mix well.

Incubate the enrichment at 30 ± 1°C for 2 days.

NOTE: Enrichment broths of finished products and raw materials must be incubated in separate baskets to avoid cross contamination.

3.5 After the required incubation time has passed, remove broth from the incubator and streak a loopfull (10µL) onto relevant selective and non selective agar plates: CFC, MAC, BP, PAB, EMB, TSA and SDA.

PAB, CFC	-	Pseudomonas spp
MAC	-	Coliforms
BP	-	Coagulase positive Staphylococcus (S.aureus)
EMB	-	E.coli
SDA	-	Candida albicans
TSA	-	Gram negative rods

NOTE: Dispose each enrichment broth into the autoclave bag after streaking.

3.6 Incubate plates as follows:

CFC, PAB, TSA, SDA at 30 ± 1°C for 48 hours, checking after 24 hours.

BP, MAC at 37 ± 1°C for 48 hours, checking after 24 hours.

EMB at 37 ± 1°C for 24 hours.

Confirm any growth on agar plates for the presence or absence of indicator organisms as outlined below:

3.7 CONFIRMATORY TEST FOR COLIFORMS ex MAC

Coliforms that colonies are lactose fermenters that produce red-violet colonies, whilst non-lactose fermenters are colourless on MacConkey agar.

3.7.1 Confirm at least three suspect colonies. Subculture each colony into a tube of LTB.

3.7.2 Incubate tubes at 37± 1°C for up to 48 hours checking at 24 hours.

3.7.3 Any tubes producing sufficient gas at 24 hours are considered positive and need no further incubation. Tubes not producing gas at 24 hours must be re-incubated for a further 24 hours. Tubes at 48 hours showing insufficient gas production are considered negative.

A positive reaction is indicated by the production of sufficient gas to fill the concavity of the durham tube.

3.7.4. If any LTB tubes show a positive reaction then Coliforms/10g are DETECTED. Consult the Microbiology Team Leader to determine if further identification is required (eg API).

3.8 CONFIRMATORY TEST FOR E.coli ex EMB

Typical E.coli colonies appear 2-3 mm in diameter, with little tendency to confluent growth, exhibiting a greenish metallic sheen by reflected light and dark purple centres by transmitted light.

3.8.1 To determine whether E.coli is present or absent per 10g of sample proceed as follows.

3.8.2 Confirm at least three suspect colonies. Subculture each colony into a tube of EC broth.

3.8.3 Incubate tubes at 44.5°C+ 0.5°C in a waterbath for 48 hours checking at 24 hours.

3.8.4 Any tubes producing sufficient gas at 24 hours are considered positive and need no further incubation. Tubes not producing gas at 24 hours must be re-incubated for a further 24 hours. Tubes at 48 hours showing insufficient gas production are considered negative.

A positive reaction is indicated by the production of sufficient gas to fill the concavity of the durham tube.

3.8.5 Subculture each positive EC tube into a separate tube containing Tryptone Water using a 1μL loop.

3.8.6 Incubate tubes at 44.5°C+ 0.5°C in a waterbath 24 hours.

3.8.7 After incubation add 1-2 drops of Kovacs reagent to each Tryptone Water tube.

3.8.8 A positive result is indicated by the presence of a red-pink ring at the top of the tube.

3.8.9 If both EC tubes and Tryptone Water tubes show a positive reaction then E.coli/10g are DETECTED.

3.8.10 Consult the Microbiology Team Leader to determine if further identification is required (eg API).

3.9 CONFIRMATORY TEST FOR COAGULASE POSITIVE STAPHYLOCOCCUS (S. AUREUS) x BP

Typical Staphylococcus species on BP appear as black to grey shiny or non- shiny convex colonies, which may or may not produce a clear zone around the colony.

3.9.1 Perform a wet mount, if cocci proceed with agglutination test.

3.9.2 Confirm at least 3 suspect colonies

3.9.3 Check for agglutination using latex agglutination kit as per manufacturer's instruction running a control at the same time.

3.9.4 Formation of small particles is indicative of coagulase positive Staphylococcus.

3.9.5 Perform further identification tests (if necessary) “Identification of Contaminants”.

Note: This confirmatory test will be used routinely. If a full confirmatory test is required this

shall be done according to AS 1766.24 - 1986.

3.10 CONFIRMATORY TEST FOR PSEUDOMONAS SPP x PAB and CFC

The presence of blue-green or brown pigmentation or fluorescence may be taken as presumptive Pseudomonas spp, but futher testing must be carried out to confirm the identify of the organism. Confirm all typical and atypical growth on PAB and CFC agar.

3.10.1 Streak all suspect colonies onto TSA plates. Incubate at 30 ± 1°C for 18 to 24 hours check purity.

3.10.2 If a pure culture is indicated, proceed with identification “Identification of Contaminants”.

3.11 CONFIRMATORY TEST FOR CANDIDA ALBICANS x SDA

Perform a wet mount, if yeast are present (ie large round to ellipsoidal cells with or without budding) proceed with API Identification using BioMerieux API Candida identification kit as per manufacturer's instruction.

3.12 CONFIRMATORY TEST FOR GRAM NEGATIVE RODS x TSA

3.12.1 If a pure culture is indicated, perform a gram stain.
3.12.2 If gram negative rods are obtained, proceed with identification “Identification of Contaminants.”

3.13 REPORTING OF RESULTS

For specific organisms report as: Organism Not Detected per unit of sample tested Eg Coliforms ND / 10g-ml

For Objectionable or Deleterious organisms report as:

Objectionable or Deleterious organisms Not Detected per unit of sample tested. Sample is free of Gram negative rods, C.albicans, S.aureus.

Eg Objectionable or Deleterious organisms ND / 10g-ml. Sample is free of Gram negative rods, C.albicans, S.aureus.

4. REFERENCE DOCUMENTS

4.1 Edwards and Ewing's - Identification of Enterobacteriaceae, Fourth Edition, 1986.
4.2 The OXOID Manual- Ninth Edition, 2006

5. REVIEW HISTORY

Version #	Revision History
MICLAB – METHOD 013	New

