Department	Micro Laboratory		Document no	MICLAB – METHOD 019	
Title	Validation of Microbiological Test Methods				
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1. SCOPE AND APPLICATION

This general test method applies to the determination of microbiological test method soundness. This procedure is applicable to finished products and raw materials. Test Method Validation is to be performed when any of the following occur:

- New formulation of a finished product
- Change of active raw material in a product
- Change in the site of manufacture of a product
- Use of a new brand of microbiological media

2. REAGENTS AND MATERIALS REQUIRED

2.1 Tryptone Soya Broth containing 4% Tween 80 (TSB + T), or another appropriate deactivating broth.

2.2 Tryptone Soya Agar (TSA) molten

2.3 TSA Agar plates

2.4 Sabouraud Dextrose Agar (SDA) molten

2.5 SDA Agar plates

2.6 Buffered Peptone Water (BPW)

2.7 Mannitol Selenite Cystine broth (MSC)

2.8 Rappaport - Vassiliadis broth (RV)

2.9 Xylose Lysine Desoxycholate plates (XLD)

2.10 Bismuth Sulphite Agar plates (BSA)

2.11 Pseudomonas Agar Base (PAB) plates

2.12 CFC plates

2.13 MacConkey Agar (MAC) plates

2.14 Eosin Methylene Blue (EMB) plates

2.15 Baird-Parker Agar (BP) plates

2.16 Malt Extract Agar + 35% Sucrose + 10% Glucose (MEASG)

2.17 Cooked Meat Medium (CMM)

2.18 Thioglycollate Oxoid CM173 + 0.5% Tween 80 (TH +T)

2.19 TSC Agar

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2.19 Reinforced Clostridium Agar (RCA)

2.20 Diluent (peptone) plus 0.1% Tween 80 (for rinsing)

2.21 Lauryl Tryptose Broth double strength (D/S LTB)

2.22 Violet Red Bile Glucose Agar (VRBGA)

2.23 Diluent (0.1% peptone)

2.24 Diluent (0.1% peptone) + 20% sucrose (DIL + SUC)

2.25 0.85% NaCl Solution

2.26 Soy Lecithin, 10% Sodium Thiosulphate

2.27 Tween 80

2.28 Reference cultures

2.28.1 *Aspergillus niger* A.T.C.C 16404

2.28.2 *Candida albicans* A.T.C.C 10231

2.28.3 *Escherichia coli (+ve control)* N.C.T.C 9001
OR Escherichia coli (USP) A.T.C.C 8739

2.28.4 *Pseudomonas aeruginosa (*USP, BP, +ve) A.T.C.C 9027
OR *Pseudomonas aeruginosa* N.C.T.C 6750

2.28.5 *Staphylococcus aureus* (BP + USP) A.T.C.C 6538
OR *Staphylococcus aureus* (+ve control) N.C.T.C 6571

2.28.6 *Salmonella salford* (+ve control) I.M.V.S 1710

2.28.7 *Zygosaccharomyces rouxii* N.C.Y.C 381

2.28.8 *Bacillus subtilis* (Optional) A.T.C.C 6633

2.28.9 *Corynebacterium pseudodipthериticum* (Optional) N.C.T.C 231

2.28.10 Environmental isolate (choose appropriate strain from culture collection)
2.28.11 *Clostridium perfringens* N.C.T.C 8237

2.28.12 *Salmonella choleraesuis* A.T.C.C 10708

2.29 Sterile jars

2.30 Sterile blender bags

2.31 Sterile spreaders

2.32 Sterile loops - 1µL and 10µL

2.33 Masticator

2.34 TMV and Preservative Culture log SF 020307

2.35 Membrane Filtration Technique GM062925

2.36 Results of Test Method Validation SF150108

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2.37 Manifold and collecting vessel

2.38 Anaerobic jar

2.39 Anaerobic indicators

2.40 Sterile filter holders

2.41 Hydrophobic edge membranes (0.45μm)

2.42 10mL and 1mL sterile pipettes

2.43 1M NaoH and 1M HCl

3. GENERAL TEST PROCEDURE

3.1 Preliminaries

Note: The sample under test should not contain any detectable viable microorganisms. Contaminated samples cannot be used in this study.

3.1.1 The microorganism's chosen should represent Gram positive rods, Gram negative rods, cocci, fungi and environmental isolate, eg.
Pseudomonas spp., *Corynebacterium* sp., *E. coli*
Bacillus spp. (spore suspension) *S. aureus, C. albicans, A. niger*.
Environmental isolate - *Pseudomonas* spp. would be preferable for topical products and an *Enterobacteriaceae* spp. for oral products.

3.1.2 *Z. rouxii* is to be used on products containing high levels of sugar (eg cough medicines).

3.1.3 *S. salford* is to be used for the recovery of *Salmonella* in existing products, see Section 3.7. *S.choleraesuis* is to be used only for the recovery of *Salmonella* in any new product in the category of Dental and Oral care.

3.1.4 The deactivating broth is usually Tryptone Soya broth containing 4% Tween 80, however if preliminary tests indicate lack of inactivating properties it will be substituted by another deactivating broth such as Letheen broth containing 4% Tween 80.

3.1.5 In some cases the addition of soy lecithin or filter sterilised 10% sodium thiosulphate will be necessary to combat the antimicrobial properties of some products.

3.1.6 TSA may be substituted by Letheen agar. An addition of Tween 80 and/or soy lecithin may be required in order to defy antimicrobial properties of preservatives or active substances.

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3.1.7 Test methods for Listerine Mouthwash, Listerine Coolmint, Orased Lotion/Gel and granulating solutions or any other solution with high alcohol content will be validated by membrane filtration technique.

3.2 Preparation of Inoculum

3.2.1 Streak bacterial cultures on TSA slopes and incubate for 24-48 hours at 30 ± 1°C.

3.2.2 Streak *C. albicans, A. niger* and *Z. rouxii* on SDA slopes, incubate *C. albicans* for 3 days and *A. niger* for 5 - 10 days (or until sporulation occurs) at 25 ± 1°C.

Note: Wash and dilute culture suspensions (steps 3.2.3 to 3.2.11) on day of use.

3.2.3 Wash off each bacterial culture, *C. albicans* and *Z. rouxii* with approximately 10 mL of sterile saline and transfer into a sterile jar. Label jar with date and test organism.

3.2.4 Wash off *A. niger* with 10 mL diluent containing 0.1% Tween 80 and transfer into a sterile jar. Label jar with date and test organism.

3.2.5 Prepare serial dilutions of the above cultures in sterile diluent. Aim to achieve between 10 and 100 cells in 1 mL of bacterial suspensions and *Z. rouxii*.

Note: If spread plate technique is chosen for *C. albicans* and *A. niger*, aim to have between 10-100 cells/spores in 0.1mL of suspension.

3.2.6 Pipette 1mL of bacterial cultures and *Z. rouxii* (in duplicate) from the dilution containing 10-100 cfu. Pour plates with TSA for bacteria and MEASG for *Z. rouxii*.

3.2.7 Incubate bacterial plates in an inverted position at 30 ± 1°C for 5 days and *Z. rouxii* plates in an inverted position at 25 ± 1°C for 5 days.

3.2.8 After incubation remove plates from the incubator and count colonies.

3.2.9 Counts of new suspensions are recorded in the TMV and Preservative Culture Log SF020307.

3.2.10 To enumerate *C. albicans* and *A. niger* the pour plate method OR spread plate method may be used.

Spread Plate Method

Pipette, (in duplicate) 0.1mL of the dilution containing 10-100 cells onto prepared and dried SDA plates. Spread evenly on the agar surface using a sterile spreader.

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Pour Plate Method

Pipette (in duplicate) 1mL from the dilution containing 10-100 cells and pour with SDA.

3.2.11 Incubate *C. albicans* and *A. niger* plates in an upright position at 25 ± 1°C for 5 days, checking after 3 days.

3.3 Recovery of Inoculum (Total Plate Count) by Plating Method

3.3.1 Weigh 10g of product into a sterile stomacher bag. Add 90g of deactivating broth. Mix well. This is a 1 in 10 dilution. For *Z. rouxii* add 90mL of Dil + SUC to the 10g of product.

3.3.2 Check pH - if not within 6.5 - 7.5, adjust with sterile 1N HCl or 1N NaOH.

3.3.3 Label plates for bacterial suspensions and *Z. rouxii* (and *C. albicans,* and *A. niger* if pour plate method used) as follows:

Job Number:	DEACT
Organism Name:	
Dilution of Suspension Used:	
Amount of 1 in 10 dilution i.e.:	1mL OR 0.1mL

3.3.4 Plate out 1 mL and 0.1 mL in duplicate of 1 in 10 dilution to each separate petri dish.

3.3.5 Add 1mL of one of the diluted cultures (10 - 100 cells) and pour plates with appropriate agar (TSA for bacteria, MEASG for *Z. rouxii* and SDA for *C. albicans* and *A. niger*). Mix well. Allow agar to solidify. Repeat procedure for each culture.

3.3.6 Incubate TSA plates in inverted position at 30 ± 1°C for 5 days.

3.3.7 Incubate SDA plates in an upright position for 5 days at 25 ± 1°C checking after 3 days.

3.3.8 Incubate MEASG plates in an upright position for 5 days at 25 ± 1°C.

3.3.9 If spread plate method is used for *C. albicans* and *A. niger* label plates as follows:

Job Number:	DEACT
Organism:	
Dilution of Suspension Used:	

3.3.10 To determine recovery of *C. albicans* and *A. niger* if spread plate method used, plate out 0.1 mL in duplicate of 1 in 10 dilution onto SDA plates. Add 0.1 mL of diluted culture (10-100 cells). Spread evenly on the agar surface using a sterile spreader.

3.3.11 Determine the inoculum on the day of the test by plating out 1mL in duplicate of the dilute bacterial cultures (10-100 cells) and *Z. rouxii* to determine initial count (I.C).

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If pour plate method is chosen for *C. albicans* and *A. niger* plate out 1mL in duplicate. If spread plate method used plate out 0.1mL (10-100 cells).

3.3.12 After incubation remove plates from incubator and count colonies.

3.3.13 Record counts on results of test method validation sheet SF150108.

3.4 Recovery of Inoculum (Total Plate Count) by Membrane Filtration

3.4.1 Assemble the membrane filtration apparatus as per GM062925, using one sterile filter holder per organism and one for diluent control.

3.4.2 Label plates for bacterial /yeast and mould suspensions as follows:

Job Number:
DEACT
Organism Name:

Dilution of Suspension Used:

(Include amount of product tested eg. 1mL, 0.1mL)

3.4.3 Label initial count plates with dilution of suspension used and organism name.

3.4.4 Label another plate (TSA) for diluent control with batch number of diluent used.

3.4.5 Into a sterile filter pour 100mL DIL + T. Pipette 1mL of product into filter holder. Filter through. Add 100mL DIL + T (first rinse).

3.4.6 To the first rinse, add 1mL of the diluted cultures (10 – 80 cells). Filter through and transfer membrane. Repeat procedure for each organism type.

NOTE: If the deactivation is unsuccessful with one rinse then repeat with a second and third rinse adding the organism to the third rinse.

3.4.7 For diluent control, filter 100mL and place onto plate.

3.4.8 Transfer membranes onto agar plates (TSA for bacteria, SDA for *C. albicans/A.niger*, MEASG for *Z.rouxii*).

3.4.9 Incubate TSA plates in an inverted position at 30° ± 1°C for 5 days.

3.4.10 Incubate SDA plates in an upright position at 25° ± 1°C for 5 days, check at 3 days. Incubate MEASG plates in an inverted position at 25° ± 1°C for 5 days

3.4.11 To ensure viability of cultures determine the initial count of the test by filtering 1mL of diluted suspension (10 - 80 cells) for each organism type.

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3.4.12 Transfer membranes onto agar plates appropriate for each organism type. After the required incubation period has passed remove plates from incubator and count colonies.

3.4.13 Record counts on results of test method validation sheet SF150108.

3.5 Recovery of Inoculum (presence or absence test)

3.5.1 Weigh out 10g of product into a sterile stomacher bag. Add 90g of deactivating broth. Mix well. Label bag with indicator organism name one bag per organism. Repeat for each organism under test

3.5.2 Weigh out an additional 10g of product into a sterile stomacher bag. Add 90g of deactivating broth. Label this sample as blank. This bag will be used to record pH before and after incubation.

3.5.3 Check pH using the blank sample - if not within 6.5 - 7.5, adjust all samples with sterile 1M HCl or 1M NaOH.

3.5.4 Add 1 mL of indicator organism test culture suspension containing between 10 and 100 cells. Mix well and seal the bag with masking tape.

3.5.5 Incubate the bag for 48 hours at 30 ± 1°C.

3.5.6 Repeat the above procedure for each test organism.

3.5.7 After 48 hours remove the bag from the incubator. Mix the bag's content.

3.5.8 Streak 10µL of the broth using the Presence/Absence Streaking Technique onto a selective agar plate, e.g.

Pseudomonas spp onto CFC and PAB

Coliforms onto MAC

S. aureus onto BP

E.coli onto EMB (if unable to recover per 1gram).

3.5.9 Incubate CFC and PAB plates at 30°C ± 1°C for 48 hours. Incubate BP and MAC plates at 37°C ± 1°C for 48 hours. Incubate EMB plates at 37°C ± 1°C for 24 hours.

3.5.10 Remove plates from the incubator and record results on "Results of Test Method Validation" sheet SF150108.

3.6 Recovery of Inoculum (Presence or Absence Test) by Membrane Filtration
(Use the same filter holders as per 3.4)

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3.6.1 Label deactivating broth (90mL) bottles or sterile blender bags with 90g/mL deactivating broth with organism name and job number.

3.6.2 Into a filter holder, pour 100mL DIL + T.

3.6.3 Pipette 10mL of product into the filter holder and filter through.

3.6.4 Follow step 3.4.6

3.6.5 Transfer each membrane (for each organism type) into corresponding 90mL deactivating broth bottles or blender bag.

3.6.6 Incubate samples for 48 hours at 30 ± 1°C.

3.6.7 After 48 hours remove from incubator and proceed with steps 3.5.7 – 3.5.10.

3.7 <u>Recovery of Salmonella (Presence or Absence Test)</u>

3.7.1 Weigh out 10g of product in 2 sterile stomacher bags (labelled *S. salford* or *S.choleraesuis* and Uninoculated respectively).

3.7.2 Add 90g/mL of Buffered Peptone Water (BPW).

3.7.3 Place in masticator to mix for 15 - 30 seconds.

3.7.4 Allow to stand for approximately 1 hour.

3.7.5 Check pH - if not within 6.5 - 7.5, adjust with sterile 1N HCl or 1N NaOH.

3.7.6 Add 1mL of *S. salford* (or *S.choleraesuis*) suspension from 3.2.5 into one of the bags. Mix well and seal the bag.

3.7.7 The second bag is to remain uninoculated. Seal bag. Record pH results before and after incubation from this bag.

3.7.8 Incubate primary enrichments at 37 ± 1°C for 16 - 20 hours.

3.7.9 Plate out 1mL in duplicate of the dilute (10 - 100 cells) suspension of *S. Salford* or *S.choleraesuis*.

3.7.10 Pour plates with TSA. Mix well.

3.7.11 Incubate plates in inverted position at 37 ± 1°C for 16 - 20 hours.

3.7.12 Record counts on Results of Test Method Validation sheet SF150108.

3.7.13 Remove primary enrichments from incubator after the required incubation time

3.7.14 Mix bags well and subculture primary enrichment broths

- 1mL into 10mLs of MSC (Mannitol Selenite Cystine broth) pre warmed to room temperature.
- 0.1mL into 10mL of RV (Rappaport-Vassiliadis) pre warmed to room temperature.

3.7.15 Vortex to mix.

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3.7.16 Incubate MSC at 37 ± 1°C for 18 - 24 hours.

Incubate RV at 42 ± 1°C for 18 - 24 hours.

3.7.17 Streak 10µL of each broth using the Presence/Absence Streaking Technique onto selective agar plates XLD and BSA.

3.7.18 Incubate XLD plates for 24 hours at 37 ± 1°C.

Incubate BSA plates for 48 hours at 37 ± 1°C.

3.7.19 Remove plates from the incubator and record results on "Results of Test Method Validation" sheet SF150108.

3.8 Recovery of Salmonella (Presence or Absence Test) by Membrane Filtration

3.8.1 Label 2 x BPW 90mL bottles or sterile blender bags containing 90mL BPW with organism name and job number.

3.8.2 Into a filter holder, pour 100mL DIL+ T. Pipette 10mL of product into the filter holder and filter through. Add 100mL DIL + T (first rinse).

3.8.3 To the first rinse, add 1mL of the diluted cultures (10 – 100 cells). Filter sample. Repeat for uninoculated sample using a new filter holder.

NOTE: If the deactivation is unsuccessful with one rinse then repeat with a second and third rinse adding the organism to the third rinse.

3.8.4 Transfer each membrane into the corresponding 90mL BPW bottles or blender bag.

3.8.5 Incubate bottles or blender bags for 16 – 20 hours at 37 ± 1°C.
3.8.6 Mix samples and subculture primary enrichment broths as per 3.7.15.

3.8.7 Follow steps 3.7.16 – 3.7.20.

3.9 Recovery of Cl. perfringens (Presence or Absence Test)

3.9.1 Preparation of Inoculum

3.9.1.1 Inoculate 10mLs of CMM (pre-steamed for 10 minutes) with 10µL of *Cl. perfringens* and incubate with a tight lid at 37±1°C for 24 hours.

3.9.1.2 Prepare serial dilutions of the 24hour *Cl. Perfringens* culture in sterile diluent. Aim to achieve approximately 100 cells/spore per 0.1mL.

3.9.1.3 Pipette 0.1mL onto pre-poured RCA or TSC plates (TSC plates must be overlayed prior to incubation) and incubate anaerobically at 37±1°C for 48 hours.

3.9.2 Test Procedure

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3.9.2.1 Weigh 1.0g of product into 10mLs of CMM. Perform this 3 additional times.

NOTE: If CMM has not been prepared fresh then steam the medium for 10 minutes. Cool to room temperature before use.

3.9.2.2 Add 0.1mL of inoculum from 3.9.1.2 to three of the 10mLs CMM containing 1g of sample.

3.9.2.3 Do not inoculate the 4th bottle of product + CMM

3.9.2.4 Inoculate a 5th bottle with *Cl. Perfringens* and no product

3.9.2.5 Incubate all McCartney bottles

i.e. CMM + 1g product (1 bottle)
CMM + 1g product + inoculum (3 bottles)
CMM + inoculum (control) 1 bottle

at 37 ± 1°C for 48 hours.

Note: If recovery of *Cl.perfringens* is not achieved using 10mLs of CMM then the amount of CMM may be increased. If this still proves unsuccessful then TH + T may be substituted as an alternative to CMM.

3.9.2.6 Perform a count on the inoculum (I.C.) by spreading 0.1mL of inoculum from 3.9.1.2 onto 2 dried TSC (or RCA) agar plates. Overlay plates with TSC agar without egg yolk and incubate anaerobically in an inverted position at 37±1°C for 48 hours.

3.9.2.7 After incubation, streak 10µL from each bottle of CMM onto dried TSC (or RCA) agar plates (one plate per bottle) using the Presence/Absence Streaking Technique. Overlay plates with TSC agar without egg yolk and incubate anaerobically in an inverted position at 37±1°C for 24 hours.

3.9.2.8 Remove agar plates from Step 3.9.2.5 from anaerobe jar and count the number of colonies. Record the initial counts on “Results of Test Method Validation” sheet SF150108.

3.9.2.9 Remove agar plates from Step 3.9.2.6 from the anaerobe jar and record growth or no growth on SF150108.

4. Recovery of E.coli in/per One Gram (Presence / Absence Test)

4.1 Label 3 double strength LTB tubes with job number and ‘Blank’

4.2 Label an additional 3 double strength LTB tubes with job number and ‘*E.coli’*

4.3 Prepare a suspension of E.coli test culture to contain 10-100 cfu per 1mL (Keep culture in fridge until ready to use).

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4.4 Weigh out 10grams of product into a sterile stomacher bag. Add 90grams of deactivating broth. Mix well using masticator.

4.5 Pipette 10grams (or mLs) of product and deactivating broth into each double strength LTB tube. This will give 1gram of sample per tube.

4.6 Mix each tube gently taking care not to disturb Durham tubes.

4.7 Inoculate each of 3 the double strength LTB tubes with 1mL of *E.coli* suspension prepared earlier in 4.3. Leave the remaining set of double strength LTB tubes as blank controls.

4.8 Prepare an additional double strength LTB tube containing 10mL of deactivating medium to be the broth blank.

4.9 Pipette 1mL of test culture (from 4.3) into 2 labelled petri dishes to determine the initial count for the suspension pour with TSA.

4.10 Incubate all tubes and plates @ 37°C ± 1°C for 48 hours check all tubes and plates at 24 hours.

4.11 Check initial count plates to ensure inoculum of double strength LTB is between 10-100 cfu 1g-mL.

4.12 After incubation check blank tubes. All blank tubes should produce no growth and no gas fermentation in Durham tubes.

4.13 Check all tubes labelled *'E.coli'* for growth (turbidity) and gas fermentation in Durham tubes.

4.14 Sub-culture each positive tube using 1μL – loop into Tryptone water. Incubate tubes at 44.5°C ± 1°C for 24 hours.

4.15 Record all results on test method validation sheet SF150108.

4.16 Validation is successful if 2 out of 3, or 3 out of 3 double strength LTB tubes confirm positive for E.coli and inoculum of double strength LTB tubes is between 10 – 100 cfu/ mL.

5. Recovery of Enterobacteriaceae by Plating Method

5.1 Prepare a suspension of *E.coli* to contain 10 – 100 cfu / mL. Keep the culture in the fridge until ready to use.

Label plates in duplicate as follows:

Job Number:

Deact:

E.coli:

Dilution of suspension used

Amount of dilution i.e. 1mL or 0.1mL (which will be pipetted).

Also label plates (in duplicate) for initial counts, with *E.coli* and dilution of suspension used.

5.2 Weigh out 10 grams of product into a sterile stomacher bag. Added 90grams of deactivating broth. Mix well.

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5.3 Pipette 1mL and 0.1mL in duplicate of product and deactivation broth (prepared earlier) into each petri dish.

5.4 Add 1mL of the diluted E.coli culture (10 – 100 cells) prepared earlier into each petri dish.

5.5 Pour plates with VRBGA and mix well.

5.6 For initial counts pipette 1mL (in duplicate) and 0.1mL (in duplicate) of deactivation broth and 1mL of diluted E.coli culture into each petri dish. Pour with VRBGA.

5.7 When all plates are set overlay plates with extra VRBGA.

5.8 Allow agar to solidify. Incubate plates in an inverted position for 18 – 24 hours at 37°C ± 1°C.

5.9 Record all results on test method validation sheet SF150108

6. REFERENCE DOCUMENTS

6.1 Pharmaceutical technology June 1989 "Microbes and CGMPs" - Tony Lord.

7. REVIEW HISTORY

Version #	Revision History
MICLAB – METHOD 019	New

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