2. Correct Aseptic technique must be used when performing all Microbiological procedures.
3. All work must be carried out in the Biohazard Cabinet.

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NOTES:
The microorganisms to be used in Stasis work and Media checks are as follows:

i)	Vegetative organisms of *Candida albicans*	ATCC 10231
	Vegetative organisms of *Staphylococcus aureus*	ATCC 6538
	Vegetative organisms of *Escherichia coli*	ATCC 8739
	Vegetative organisms of *Pseudomonas aeruginosa*	ATCC 9027
	Spores of *Bacillus subtilis*	ATCC 6633
	Spores of *Clostridium sporogenes*	ATCC 19404
	Spores of *Bacillus sphaericus*	ATTC 4525
	Spores of *Aspergillus niger*	ATCC 16404

KWIK-STIK TM Plus Microorganisms are sourced from BIOMERIEUX (brand).

The microorganisms may be used after the noted expiry date providing the purity and growth is of the correct standards. The correct standards are verified by following steps 1 and 2 of this SOP.

ii) Appropriate strains of microorganisms collected from the manufacturing environment.

Procedure

Stock suspensions of the noted microorganisms are used to perform monthly dilutions, which are used for growth promotion and stasis checks (See sections 6 and 7). Stock suspensions and dilutions must be placed in the refrigerator immediately after use for preservation. See **MICLAB 035** for the requirements for Media Runs and Soup Tests. Record the bacterial counts from the dilutions.

Method for obtaining microorganisms from KWIK-STIK TM Plus Microorganisms

1.1. Remove the cylinder for the relevant strain of microorganism from the fridge.

1.2. Take one pouch from the cylinder and reseal. Return the cylinder to the fridge. Allow the pouch to equilibrate to room temperature.

1.3. Tear open the pouch at the notch and remove the KWIK-STIK™.

1.4. Tear off the pull-tab portion of the label and attach to the primary culture plate. Use Nutrient Agar (NA) for all organisms except *Cl.Sporogenes* - use Reinforced Clostridial Agar (RCA) and *A.niger* - use Saboraud Dextrose Agar (SDA).

3.6. Using a sterile pipette remove ALL the cryosolution. If all the liquid is not removed from the vial it may hinder the recovery of the microorganism later on.
NOTE: Remember to remove the liquid from the lid.

3.7. Re-cap the vials and label with the:

- Name of the microorganism {or appropriate abbreviation}
- Date of preparation and
- Number the vials for usage purposes, (i.e. Vial #1 and Vial #2).

NOTE: There is no expiry set as the organisms' viability and purity is checked with each stock set up.

3.8. Place the vial in a pre-frozen cryoblock and store in the freezer at –20°C (±1°C) {preferably towards the back for the coldest temperature}.
NOTE: The removal of individual beads will be made easier if the vial is placed horizontally in the freezer for the first few minutes.

4. Set Up of Stock Cultures

4.1. **Vegetative Micro-organisms**

Candida albicans	ATCC 10231
Staphylococcus aureus	ATCC 6538
Escherichia coli	ATCC 8739
Pseudomonas aeruginosa	ATCC 9027

4.1.1. Remove the vial for the organism required from the freezer and place in a separate pre-frozen cryoblock.

4.1.2. Transfer using a sterile disposable plastic loop, 2 beads onto 2 separate Tryptone Soy Agar (TSA) slopes.

4.1.3. Discard the loop.

4.1.4. Using a fresh sterile plastic disposable loop streak the bead onto the slope surface.

4.1.5. Remove the bead from the slope by tipping into a Hibitane-containing waste container and pulling out with the loop.

4.1.6. Repeat for the second slope using a fresh sterile plastic disposable loop.

4.1.7. Incubate slopes at 32°C (±1.5°C) for 24 – 48 hours. If sufficient growth has not occurred slopes may be incubated for up to 4 days. If suitable growth has not been obtained after 4 days incubation, re-streak the organism from the beads onto a fresh set of TSA slopes and incubate as above.
Once substantial growth has occurred, place one slope in the fridge to be kept for future use if required (spare). Label with the Organism Name, Date of Subculture, and the fact that it is a spare.

4.1.8. Wash the growth off the remaining slope with 5 – 10mL sterile Peptone Water and transfer aseptically into a sterile 20mL McCartney bottle. Store the prepared stock culture in the refrigerator at 4°C(±1°C).

NOTE:
Slopes can be kept for a period of 3 months. Upon expiry a fresh stock must be prepared. If the stock becomes contaminated prior to expiry, the spare slope can be used to produce another stock suspension.

4.2. **Spore Formers**

Bacillus subtilis	ATCC 6633
Clostridium sporogenes	ATTC 19404

5.3. If one dilution level is skipped, i.e. from 10^{-3} to 10^{-5}, then transfer 1mL from the 10^{-3} dilution to 99mL of Peptone Water. This then serves as the 10^{-5} dilution.

5.4. If two dilution levels are to be skipped, i.e. from 10^{-3} to 10^{-6}, then transfer 0.1mL from the 10^{-3} dilution bottle to 99.9mL of Peptone Water. This then serves as the 10^{-6} dilution.

Therefore in summary:

Number of Jumps	Transfer Volume	Diluent Volume
1 jump	10mL	90mL
2 jumps	1mL	99mL
3 jumps	0.1mL	99.9mL

5.5. Dilute all prepared stock suspensions to 10^{-7}, (except *E.coli*, which should be diluted to 10^{-8}).

5.6. Perform spread plates on NA using the last 3 dilutions in the series. This involves taking 100µL of the dilution and using a sterile spreader to distribute the sample over the surface of the plate. Plates are then incubated at 32°C (±1.5°C) for 48hrs.

The exceptions to this are:

- *Cl.sporogenes*: Use the pour plate technique with RCA and incubate anaerobically at 32°C (±1.5°C) for 48hrs.
- *A.niger:* Use SDA and wrap plates in foil prior to incubating at 25°C (±1.5°C) for 5 days **in a designated incubator.**

5.7. Count all plates and record the number of cfu/0.1mL in the Reference Culture File and stasis spreadsheet.

5.8. The following week, plate out 2 or 3 dilutions based on the number of cfu/0.1mL obtained from the dilutions plated from the previous week. Based on the counts from the previous week, plate out the dilutions, which will most likely give rise to 10-100 cfu/0.1mL. Incubate according to the conditions listed in 5.6. Record results as in 5.7.

NOTE: Dilutions can be stored for a period of 1 month from the preparation date. Upon expiry a fresh dilution series must be made from stock suspensions. If the dilutions become contaminated prior to expiry, the dilutions can be re-made from the stock.

6. Positive Control (Stasis) Details

All media and Peptone Waters both manufactured in the laboratory and sourced from external suppliers must be challenged for growth promotion according to the procedures outlined in the following section.

6.1. Liquid Growth Media

Four bottles from every batch of liquid media serving as positive controls must be inoculated with a fixed number of organisms to determine if the media is capable of supporting growth. Because of the nature of our injection solutions it is likely that any microbial contamination level will be low and we must therefore ensure the media is sufficiently sensitive to pick up these low levels. A suitable level of inoculum is 10-20 organisms nominally, but not more than 100 viable organisms. 2 bottles from each media type are to be inoculated as per table outlined below:

Organisms to be used for Liquid Media Stasis

Medium	**Challenge Organism**	**Incubation Temp.**
TSB x 2	*Candida albicans*	25°C (±1.5°C)
TSB x 2	*Bacillus subtilis*	25°C (±1.5°C)
FTM x 2	*Clostridium sporogenes*	32°C (±1.5°C)

6.3.4. Divide each plate into 4 quadrants and streak 2 plates with each organism according to the diagram below. Each quadrant should contain five streak lines overlapping once only at the edge of each quadrant, as well as an additional line bisecting all quadrants.

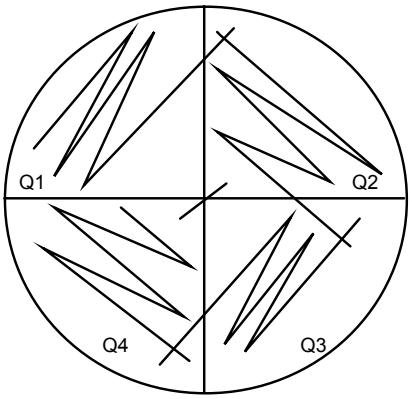

Diagram for streaking solid media

6.3.5. Incubate non-selective media for 48hr and selective media for 4 days at the appropriate temperature.

6.3.6. Score the rate of growth, awarding a score of 0.2 for each streak line with growth. The maximum score for growth in all quadrants plus the additional line bisecting all quadrants is 4.2.

NOTE: A minimum combined score of 2 must be evident for the agar to be considered acceptable for use in the Laboratory. If the media does not meet the acceptance criteria, then repeat the test using new samples of media. If again the media fails, consult the Microbiology Manager or a senior Technician. Record details in the log book.

6.3.7. The average of the scores for the replicate plates is to be determined and this information is to be recorded in log book.

6.4. Verification of pre-poured media purchased from External suppliers

Verification of growth promotion of plated media supplied from an external supplier is to take place for every new batch for each media type purchased from the external supplier. That is, verification is to be conducted on each new lot number of media for each media type delivered to the laboratory. Stasis testing and analysis of results is to be conducted as per Section 6.3 of this SOP. Growth results are recorded in the media preparation field for each lot number verified at which time the batch may be signed off for use.

6.5. Recording Stasis Results

It is the responsibility of the person on Stasis testing to enter the results from media growth promotion and verification tests into appropriate log book.

7. Media Check after Sterility Testing (Stasis Test)

7.1. Membrane Filtration and Direct Inoculation

Once every 12 months media containers from a product tested by Membrane Filtration and, if possible those from a product tested by Direct Inoculation, must be subjected to a challenge of a low number of microorganisms after the sterility test has been completed. This is to ensure that any inhibitory effects present in the products have been successfully inactivated, either by the dilution effect or the washing procedure, as appropriate to the Test Method. This challenge is to demonstrate the effectiveness of the sterility test method, ensuring that the testing procedures are allowing for the potential growth of any microorganisms within the product.

Morphological, (after 5-7 days on Sabouraud Dextrose Agar plate at 25°C (±1.5°C)).

Compact White or Yellow basal felt covered by a dense layer of Dark Brown to Black conidial heads (spores).

Microscopic

Large conidial heads (up to 3mm x 15-20μm in diameter), Dark Brown and globose.

9. Appendix 2: Kwik-Stik instruction diagram