Ministry of Health

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Acronyms:

BA	Blood agar
CA	Chocolate agar
MAC	MaConkey
ATCC	American Type Culture Collection
H&S	Health and Safety
ID	Identification
IQC	Internal Quality Control
MDRO	Multidrug Resistant Organism
MRSA	Methicillin Resistant Staph. Aureus
SOP	Standard operating procedure

1. **Purpose**

   This document provides instructions on how to subculture and maintain reference bacterial strains in the laboratory.

2. **Scope**

   This document is applicable for all medical laboratories under MOH and other collaborative governmental and non-governmental health institutions.

3. **Definitions**

   3.1 **Reference Culture:** is a microorganism preparation that is acquired from a culture type collection.

   3.2 **Stock Culture**: is a microorganism preparation that is derived from a reference culture.

   3.3 **Working Cultures**: is growth derived from a stock culture.

   3.4 **Subcultures (Passages):** is simply the transfer of organisms from a viable culture to fresh medium with growth of the microorganisms or transfer of established microorganism growth on media to fresh media. The subsequent growth on the fresh media constitutes a subculture or passage. Resuscitating frozen cultures by thawing is not by itself considered a passage. Subculturing the reference strain from ATCC's vial to the stock culture is the first passage. Subculturing stock cultures to working cultures is the second passage from the original reference strain. Any subsequent subculture is another cumulative passage.

4. **Procedure**

   4.1. Clinical background:

   Reference bacterial cultures are used as controls for various tests including antibiotic susceptibility testing, identification system, stains, media, reagent, kits, biochemical testing and incubation environment . Reference cultures are obtained from a recognised National Collections in order to demonstrate traceability: e.g., National Collection of Type Cultures (NCTC), American Type Culture Collection (ATCC). Proper maintenance of quality control strains is essential for ensuring acceptable performance and to preserve the viability, purity, and genotypic and phenotypic characteristics.

   4.2. Principle:

   Improper storage and repeated subculturing of Reference bacterial cultures can produce alterations in characteristic and antimicrobial susceptibility test results of the reference

strain, therefore it is important to follow the recommended guideline for subculturing and maintaining Reference bacterial cultures.

4.3. Pre – analytical stage:

4.3.1. Sample: Lyophilized Quality control microorganism pellet coming in different forms (e.g disc, stick) obtained from a recognised National Collections.

4.3.2. Material:

Reagents	**Consumables/Supplies**	**Equipment**
Media Agar (BA , nutrient agar)	Disposable loop	incubator
lyophilised cultures ATCC strains	Sterile Swab	-20 freezer
Skimmed milk		-50 -70 freezer
Preservative cryo-bank		<-70 freezer
BHI		2-8 fridge

4.3.3. Safety precaution:

- All specimens need to be treated as potentially infectious. Standard procedures for handling of biohazard material must be followed at all times. Universal Precautions must be practiced at all stages of these procedures.
- Standard procedures for handling of biohazard material must be always followed.
- Process ampoules in a Class 1 safety cabinet and minimise the formation of aerosols.
- Refer to the manufacture Safety Data Sheet (SDS) for more details on safety of hydrating fluid chemical or any other chemical.

4.3.4. Quality control:

- Check the expiry dates of all media, reagents and stains before use.
- All media, reagents, kits, and stains **MUST** be quality controlled before use.
- Identification tests should be run with appropriate controls.
- Record the quality control results in the appropriate QC sheet.

- A logbook should be kept to record the following:
  - Lot number
  - Date of receipt
  - Strains received
  - Quantity received
  - Expiration date
  - Label all strain's information of all sub-cultured strains in the plate.

4.4. Analytical stage:

4.4.1 **Procedure for Preparation and Maintenance of quality control strain Cultures (stick) (figure 1) :**

4.4.1.1 Identify the culture by checking the paper inside the ampoule, to ensure that the correct organism has been received. Check the certificates of the passage number. Keep for documentation.

4.4.1.2 For susceptibility testing, maximum excepted stick passage is between passage 1 or 2. Check for certificate.

4.4.1.3 For identification and other biochemical tests, up to passage 5 is acceptable.

4.4.1.4 Follow manufacturer's instruction to subculture lyophilized Stock cultures.

4.4.1.5 Reference Culture should be subculture on a non-selective Agar such as Blood agar or Nutrient agar. Broth is not recommended because contaminants can easily be introduced.

4.4.1.6 This growth is called the stock Culture "pellet plate "or monthly working stock culture.

4.4.1.7 Label as stock culture with the name of the organism and date of inoculation. Incubate under recommended conditions favourable for growth.

4.4.1.8 Reference cultures sub-cultured only once to provide a stock culture.

4.4.1.9 Do not test using the stock Culture (pellet plate). This is the plate on which the lyophilized pellet was started. The organisms growing on this plate are not fully revived.

4.4.1.10 Store the stock control at 2 to 8 C for less than four weeks. Seal tightly if plates are used.

4.4.1.11 Prepare a fresh stock culture at least once per month from the refrigerated reference culture (stick).

4.4.1.12 Subculture stock culture weekly into non-selective agar (blood, nutrient agar) and then daily from the weekly plate.

4.4.1.13 Always use fresh microorganisms for testing. Many tests require that the microorganisms are not more than 24 hours old.

4.4.1.14 Avoid multiple serial subcultures of quality control organisms over extended periods of time.

4.4.1.15 Select isolated colonies for the test. Do not test colonies from a contaminated plate

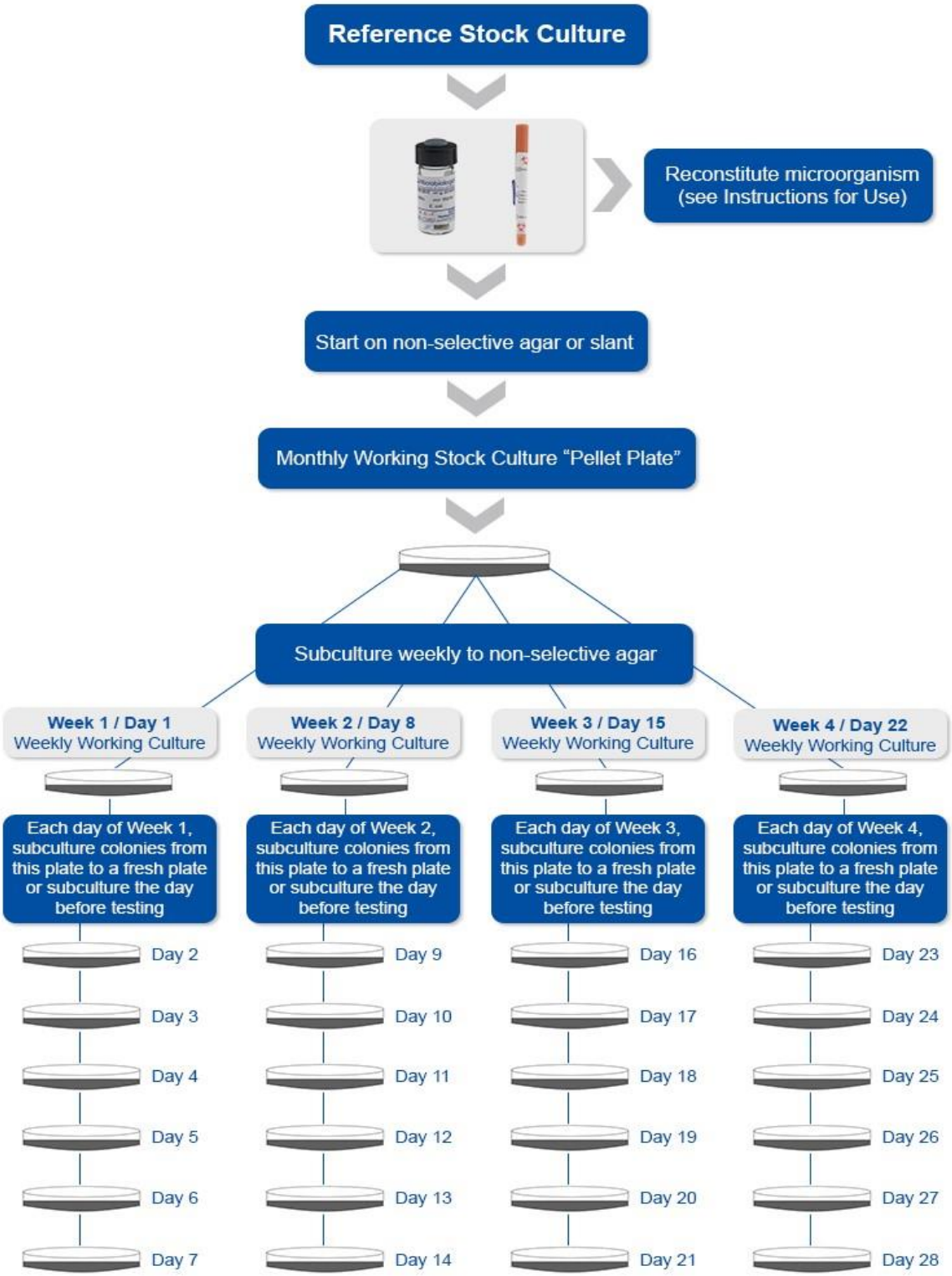

Figure 1: Procedure for Preparation and Maintenance of quality control strain Cultures

4.4.2 Seed lot system.

Seed lot Technique is recommended for storage and maintenance of reference strains in laboratories. In this system, the ATCC reference strain is sub-cultured to several replicates at one time (stock cultures), all of which are within one passage. These replicates of the stock culture are the seed vials for the laboratory. The seed stock is sub-cultured—the second passage—to make replicates of working cultures (slants). Maximum of 5 passages are allowed from the ATCC reference strains

4.4.3 Procedure for seed lot system. (Figure 2).

4.4.3.1 ATCC reference strains are sub-cultured into appropriate culture media plates and from them into cryo-vials with beads (stock cultures). This considered as the first passage. These cryo-vials are stored in -80°C indefinitely, in -50 to -70°C for one year or in -20°C for 3 months.

4.4.3.2 Cryo-vials should be labeled with ATCC strain details, expiry date and LOT number.

4.4.3.3 The strains can be sub-cultured from the beads into growth plates (non – selective) and then Subculture weekly into non-selective agar or slant (second passage) and then daily from the weekly plate(third passage).

4.4.3.4 Weekly working culture plates stored at 2-8°C for up to 1 week and used whenever needed.

4.4.3.5 Stocks from cryovials, must only be thawed once and should not be refrozen and reuse.These cultures are replaced monthly.

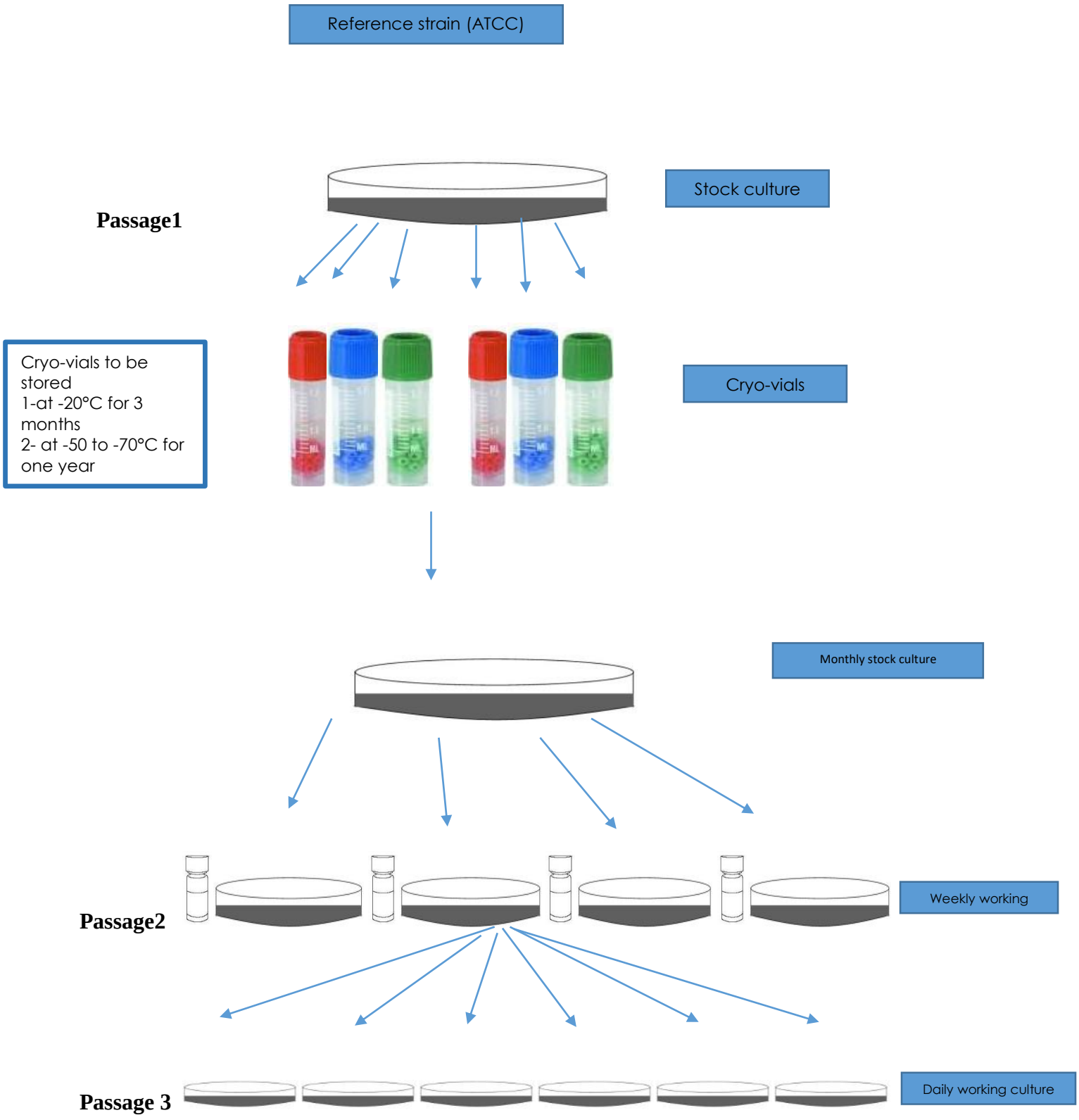

Figure 2: Seed lot system

4.4.4 The number of passages

4.4.4.1 It is t important to minimize the number of passages to reduce the chance of contamination, reduce/prevent genetic drift and mutations and to minimize phenotypic variations

4.4.4.2 The viable microorganisms used in the test must not be more than five passages removed from the original ATCC culture. (according to USP <51> Antimicrobial Effectiveness Testing guideline and CLSI guide.

4.4.4.3 Another term used by commercial sources other than ATCC is "ATCC derived." A culture is derived from the ATCC reference strain by subculturing in other words, one or more passages. However, lab must consider that these cultures are at least one or two passages from the ATCC reference strains. Therefore, always should request the ATCC certificate from the supplier to verify the passages.

4.4.5 Quality Control Organisms

- Storage temperature of stock and working cultures can affect growth characteristics and viability.
- Aerobic non-Fastidious organisms should be stored as below

Quality Control Organisms	**Type of Stock Culture**	**Storage**	**Media or storage**	**Length of Storage**
All ATCC	Lyophilized	Per manufacturer	Per manufacturer	Until expiration date
Aerobic Bacteria non-fastidious	Stock QC Cultures	2-8 oC	a non-selective agar or slants	4 weeks
		-20 oC	Suspension in Cryo-vials	3month
		−50 to −70°C	Suspension in	1 year

			Cryo-vials	
		≤-70 oC	Suspension in Cryo-vials	Indefinitely
	Working culture	2-8°C	a non-selective agar or slants	One week

Fastidious organisms have shorter survival periods than aerobic bacteria. They will. For example: *Streptococcus pneumonia, Neisseria gonorrhoea, Haemophilus influenza*, and *Campylobacter* need to be sub-cultured every three days.

4.4.6 Favourable conditions for QC organism maintenance:

Aerobic	Store at 2-8°C
CO2 dependent species	Store at room temperature in a jar with CO2 packet
Anaerobic Bacteria	Store in anaerobic conditions at room temperature
Campylobacter	Store on *Campylobacter* agar at 37°C in microaerophilic conditions

4.4.7 QC Reference strains for and Frequency of testing

Tests/methods	*ATCC QC strains	Expected result		Frequency of testing
Oxidase Test	*P. aeruginosa* ATCC 27853	Positive control	Purple color	Daily and when new lot no. and shipment is received
	Escherichia coli ATCC 25922	Negative control	No color change	
Catalase Test	*Staph. aureus*	Positive	Bubble formation	Daily and

	ATCC 25923	control		when new lot no. and shipment is received
	Strep. pyogenes ATCC 19615	Negative control	No bubble formation	
Staph Latex Rapid Test (Plasmatec)	*Staph. aureus ATCC 25923*	Positive control	Visible agglutination	Daily and when new lot no. and shipment is received
	Staph. saprophyticus ATCC BAA-750	Negative control	No visible agglutination	
Coagulase test (Tube method)	*Staph. aureus ATCC 25923*	Positive control	Complete or partial coagulation in 1 to 4 hours.	Weekly and when new lot no. and shipment is received
	Staph. saprophyticus ATCC BAA-750	Negative control	No coagulation	
DNase Test for Staph. aureus	*Staph. aureus ATCC 25923*	Positive control	Shows a clear zone area in the growth medium surrounding the bacterial growth	Daily
	Staph. saprophyticus ATCC BAA-750	Negative control	Shows no clear zone area in the growth medium surrounding the bacterial growth	
Latex agglutination Test for Streptococcus	*Strep. pyogenes ATCC 19615*	Positive for group A	visible agglutination of the latex particles	Each day of patient testing and when new
		Positive for	indicated by a milky	

species	*Enterococcus faecalis ATCC 29212*	group D	appearance without any visible agglutination of the latex particles	lot no. and shipment is received
Optochin Test	*Streptococcus pneumoniae* ATCC 49619	Positive control	Zone ≥ 14mm surrounding a 6mm-diameter disk	Weekly and when new lot no. and shipment is received
	Streptococcus pyogenes ATCC 12384 Or Streptococcus mitis NCTC 10712	Negative control	Zone <14mm surrounding a 6mm-diameter disk	
Gram Staining	*Staph. aureus ATCC 25923*	Gram Positive control	Purple cells	Daily and when new lot no. and shipment is received
	E. coli ATCC 25922	Gram Negative control	Pink to red cells	
"X" and "V" FACTORS (MAST group) For Differentiation of Haemophilus Species	*Haemophilus influenzae ATCC 49766*	XV	(+) growth	Each day of patient testing and when new lot no. and shipment is received
		X	(-) no growth	
		V	(-) no growth	

*The recommended ATCC strains might change according to manufacturer recommendation

Note: for AST and media, refer to their SOP

4.5. **Post – analytical stage:**

4.5.1 Reference strains maintenance record: Recoded in the following sheet

Preparation of **stock** culture ATCC Name …… ATCC number ……..			Preparation of **working** culture							
ATCC lot number	Date / sign subculture	Date expires	Week 1		Week2		Week3		Week4	
			Prepared Date/sign	Expired	Prepared Date/sign	expired	Prepared Date/sign	expired	Prepared Date/sign	expired

4.5.2 Procedures for abnormal results or Failed QC Results.

4.5.2.1 When strain does not pass quality control (e.g due to contamination of growth or discordance with expected disc diffusion zone / MIC results),

- Check the expiration date on the QC vial, test kit, test strips or any reagent being used. DO NOT use QC or reagents that are expired. it should be discarded, and correction action request form should be filled with corrective action taken
- Repeat the QC test. Follow the procedure regarding proper handling of QC materials and technique.
- If repeat QC passes, patient testing can be performed. Record QC results and document all action taken in the QC Logbook.
- If repeat QC is still out of range, DO NOT perform patient testing. Repeat the QC test using a fresh vial of test strips, QC or test kit.
- If second repeat QC passes, patient testing can be performed. Record QC results and the documentation of all action taken in the QC form. If second and third repeat QC fails: DO NOT perform patient testing.
- Document all action taken in QC form and call instrument technical support, inform the supervisor
- The problem should be investigated by the Q.C. focal point

5. Responsibilities

5.1. Responsible (incharge, supervisor,..etc) staff:

- To ensure the adherence to reference strains maintenance procedure
- Responsible to give their utmost ability to ascertain the reliability of the QC Test results consistently.

5.2. Quality manager /officer

- To follow up the implementation of the procedure
- To monitor regularly QC results and raise non-conformance with corrective action once needed.

5.3. All lab staff:

- Follow manufacturer’s recommendation in performing all QC and test procedures

- To adhere to the laboratory procedure.
- To document record and release results as recommended
- To report test failures or incident, report failure or incidents.

6. Document History and Version Control

Version	Description	Review Date
1	Initial Release	May 2026

7. References

Title of book/ journal/ articles/ Website	Author	Year of publication	**Page**
department of microbiology Standard Operating Procedures. Bacteriology Procedures. Quality Control Manual	University health network/mount sinai hospital,	(2021).	-
Department of Medical Microbiology, Division of Clinical Microbiology, Galway University Hospitals Maintenance of Reference Cultures Ref: NSRLSOP012	UK Standards for Microbiology Investigations	2019	B 57 Issue 3.5.
The Central Public Health Laboratory Technical Procedural Manual ATCC Reference strains	CPHL LAB	6/5/2015	
Quality Control for Commercially Prepared Microbiological Culture Media; Approved Standard—Third Edition	CLSI	2004	
Reference strains: how many passages are too many? ATCC TECHNICAL DOCUMENTS, at www.atcc.org, Credible	at www.atcc.org,	2022	
Subculture and Maintenance of QC strains G00-06-001/23.06.2017	WHO Collaborating Centre External Quality Assurance System (EQAS	2017	
Quality Assurance in Bacteriology and Immunology	World Health	2012	

	Organization		
Maintenance of Quality Control Strains	MICROBIOLGICS		
Lot seed system	http://pharmaceuticalmicrobiologi.blogspot.com/		
ATCC Microbiology –Best Practices for Stock Maintenance	Carol Horton ATCC		