THE UNIVERSITY OF SYDNEY

NeoCol: Standard Operating Procedure (SOP) for Storage of Positive MDR, GBS & Fungal Isolates v2.0

Purpose:

The purpose of this SOP is to describe the standard procedures involved in storage of positive Group B *Streptooccus* (GBS), *Candida* species and multidrug resistant (MDR) isolates identified during participant screening.

Principal:

Swabs collected from mothers and neonates will be screened for the presence of MDR bacteria, *Candida* species and Group B *Streptococcus* (mothers only) with chromogenic agar. Each positive isolates will be transferred into a growth medium (STGG; skim milk-tryptone-glucose-glycerol broth) and stored in cryovials at -80°C for potential further analyses. Each unique colony on an ESBL and CRE chromogenic agar, Candida chromogenic agar and each GBS isolate should be stored in a separate cryotube in STGG broth.

Responsibility:

This SOP applies to any clinical and laboratory staff who are involved in handling and processing MDR, Fungal and GBS samples for NeoCOL study. It is the responsibility of those users to always follow these guidelines when handling and processing samples for the study.

Safety Requirements:

Gloves and a laboratory gown should be worn during sample handling. Standard hand hygiene practices should be followed before, and after handling of samples. Handle all specimens with care and treat them as potentially infectious material. Appropriate insulated gloves should be worn when using a -80°C Freezer.

Materials:

- Sample: positive isolate from ESBL/CRE chromogenic agar, or GBS screening (after CAMP / Maldi ID confirmation), or positive isolate from Candida chromogenic agar.
- Sterile inoculation loop (x1 per sample)
- Sterile cotton swab (x1 per sample)
- Blood agar (x1 per sample)
- Non-sterile gloves, freezer gloves, laboratory coat
- -80°C freezer with a thermometer and temperature log.
- STGG broth (1mL per isolate)
    a) Reagents:
        - Skim Milk Power (Oxoid LP0031) (2g per 100mL)
        - Tryptone Soya Broth (Oxoid CM0129) (3g per 100mL)
        - Glucose (0.5g per 110mL)
        - Glycerol (10mL per 110mL)
        - Distilled Water
    b) Equipment
        - 500mL Duran bottle
        - Cryogenic tube (1x per isolate)
        - Stirring Heat plate
        - P1000 pipette tips
        - Autoclave
        - Autoclave tape

Procedure:

1. Prepare STGG broth (if not pre-made)
    a. Mix the following ingredients into a 500mL Duran bottle. For power ingredients, carefully weight them out using disposable weighing boats

NO.	INGREDIENTS	AMOUNT	UNIT
1	Skim milk powder (Oxoid LP0031)	2	G
2	Tryptone Soya Broth (Oxoid CM0129)	3	G
3	Glucose	0.5	G
4	Glycerol	10	mL
5	Distilled water	100	mL

    b. Place the bottle on the stirring hot plate set to 100°C.
    c. Heat and stir the liquid until the power has dissolved and the solution is clear.
    d. Aliquot 1mL of the mixture into each cryotube and place in a box.
    e. Place a strip of autoclave tape on the box.
    f. Loosen the screw-cap tops and autoclave at 121°C for 10 minutes.
    g. Tighten caps after autoclaving and allow to cool.
    h. When cool:
        i. Label with the medium, date and batch number if more than one bottle is prepared.
        ii. Perform QC and sterility testing.

2. Quality Assurance of STGG broth:
    a. Every batch of agar or broth must be quality controlled for both sterility and the ability to support growth of target organisms, and suppression of non-target organisms in certain cases.
    b. To perform QC testing for STGG media select two random cryotubes from each box from the batch.
        i. Tube 1:
            1. Vortex well.
            2. Plate 100 µl onto a blood agar plate and incubate overnight at 37 °C. There should be *NO* growth on the plate.
        ii. Tube 2:
            1. Vortex well.
            2. Inoculate with Streptococcus pneumoniae ATCC 49619 (or similar known bacterial isolate eg. *S. aureus* used for CAMP testing)
            3. Freeze at -80 °C for 48 h.
            4. Thaw out at room temperature and vortex well.
            5. Subculture 100 µl onto a blood agar plate and incubate overnight at 37 °C. After this there should be *good* growth on the plate.
    c. If the batch fails QC, all tubes should be discarded and a new batch prepared: consideration should be given to the source of failure (e.g. incorrect autoclave cycle, omission of supplement).
    d. STGG batches that pass should be stored in the prepared tubes at 2-8 °C for up to six months.

3. Storage of isolates:
    a. Each unique colony on an ESBL and CRE chromogenic agar, each GBS isolate, and each *Candida spp*. isolate should be stored in a separate cryotube in STGG broth.
    b. Culture the organism on blood agar (or Sabouraud Dextrose agar for Candida spp.) and incubate overnight at 37°C. Note: Ensure that the culture is pure: if mixed, pick off a well isolated colony and re-culture, and confirm its identity before saving.
    c. Using an alcohol resistant marker (e.g. Sharpie) or a cryo-resistant label, label the side of the cryotube with the relevant unique REDCap sample ID, unique laboratory number as per laboratory labelling process and unique patient / hospital identification as per hospital process. (N.B. do not use pen to label the cryotube as this will fade in -80°C freezer. Do not use regular paper labels as these may fall off in -80°C freezer and moisture from freeze-thawing cycles may also cause label to fall off.)

d. Label the lid of the cryotube with the unique position number in the cryobox (e.g. A1).
e. Place the labelled cryotube and plate containing the isolated colony in a class II biosafety cabinet.
f. Using a sterile cotton swab / sterile loop harvest the entire growth from the young culture plate and dispense into the labelled cryotube containing 1mL STGG medium. NB: This step must be performed in the class II biosafety cabinet.
g. Place the tube vertically into a cryobox with an inbuilt grid in the next available spot (see figure 1 and 2) and store in -80ºC freezer.
h. Record Sample ID number in position number in Excel template which corresponds to box and number of positions available for isolates.

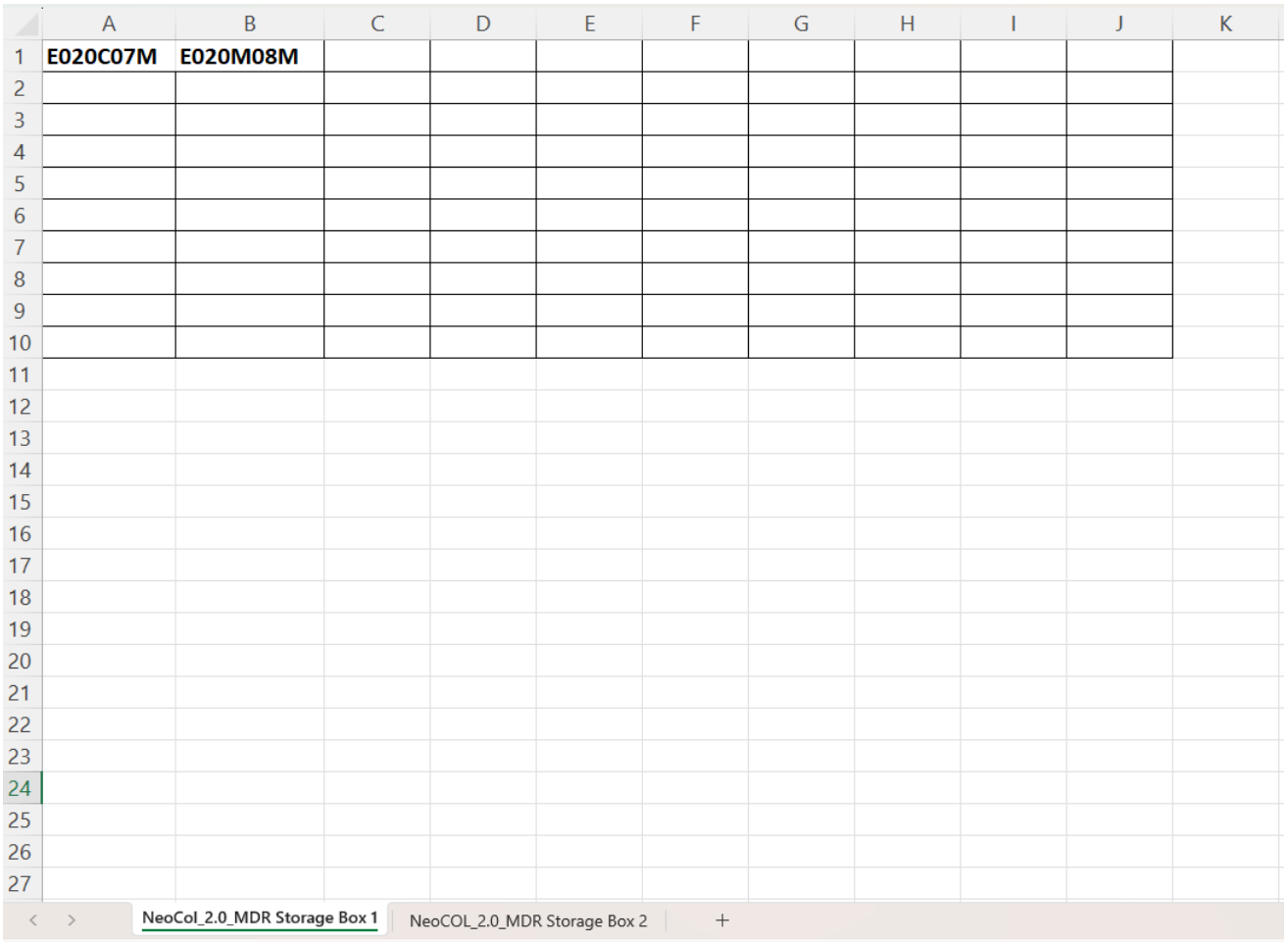
4. Complete the NeoCOL Sample Storage Log.

5. Re-Culture of frozen isolates:
   a. Remove required isolate for culture from cryobox in -80°C freezer. (Note: only thaw necessary isolate rather than whole cryobox.)
   b. Allow isolates to thaw slightly and using a disposable sterile plastic loop / sterilized wire loop inoculate 10ul onto a blood agar plate and incubate overnight at 37ºC. ***This should be performed quickly with very minimal thawing of the cryotube, as repeated freeze-thaw cycles may decrease organism viability over time.*** [1] NB: Ensure this step is carried out aseptically and, in a class II biosafety cabinet to prevent any risk of contaminating stored isolate.
   c. If stocks allow sub-culture a ***single colony*** of the isolated organism on blood agar and incubate overnight at 37ºC to ensure optimal viability of organism post freezing.

Appendix

Figure 1: Example of plastic cryobox with in-built grid system.

Figure 2: *Example of cardboard cryobox with in-built grid system.*



	A	B	C	D	E	F	G	H	I	J	K
1	**E020C07M**	**E020M08M**									
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5											
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NeoCol_2.0_MDR Storage Box 1 | NeoCOL_2.0_MDR Storage Box 2 | +

	A	B	C	D	E	F	G	H	I	J	K
1	**E050C14CF**	**E053M08F**									
2											
3											
4											
5											
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NeoCol_2.0_Fungal Storage Box 1 | NeoCOL_2.0_Fungal Storage Box 2 | +

Figure 3: Example of Excel template for identifying isolate position in cryobox.

References

- Tuttle AR, Trahan ND, Son MS. Growth and Maintenance of Escherichia coli Laboratory Strains. Curr Protoc. 2021 Jan;1(1):e20. doi: 10.1002/cpz1.20. Erratum in: Curr Protoc. 2022 Aug;2(8):e552. doi: 10.1002/cpz1.552. Erratum in: Curr Protoc. 2022 Aug;2(8):e551. doi: 10.1002/cpz1.551. PMID: 33484484; PMCID: PMC8006063.

Document History

Version	Author(s)	Approved by	Update Reason	Date	SOP No:
1.0	B. Dickson	P. Williams	New document	03AUG2023	NeoCOL_SOP07
2.0	R. Kelleghan	P. Williams	Adapted for NeoCOL 2.0	02MAR2025	NeoCOL_2.0_SOP07

Site Training Record

Trainee Name	Read/Understand SOP (Tick)	Access to SOP (Tick)	Trainee Signature	Date	Trainer Initials