**ICEMAN Sample Processing Protocol**

**Purpose**

To culture and freeze bacterial isolates from clinical environmental and patients samples for future sequencing analysis.

**Materials & Equipment**

* Sterile PBST (0.02%) pre-aliquoted, 45mL per tube
* Sterile 50mL tubes
* Sterile 1.5mL cryovials
* Sterile 5mL cryovials
* Sterile 5mL & 50mL pipettes
* Sterile transfer pipets
* 10 µL & 1000 µL pipettors
* Sterile 10 µL & 1000 µL tips
* Sterile loops
* MALDI slides & matrix
* Stomacher bag rack
* Stomacher 400C Circulator
* Table top centrifuge
* Multi tube vortex mixer
* LabVantage Labels

**Selective Media & Incubation Times**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *Media* | *Abbreviation* | *Temperature* | *Incubation Time* | *MALDI* |
| BD MRSA Chromagar | MRSA | aerobic 35 – 37oC | 18-24 hours | No |
| Hardy Chromagar CRE | CRE | aerobic 35 – 37oC | 18-24 hours | Yes |
| *~~CHROMagar KPC\*~~* | *~~CRPA~~* | *~~aerobic 35 – 37~~~~o~~~~C~~* | *~~18-24 hours~~* | *~~Yes~~* |
| Hardy Chromagar ESBL | ESBL | aerobic 35 – 37oC | 18-24 hours | Yes |
| Anaerobe Systems CCFA-HT | CDIFF | anaerobic 35 – 37oC | 18-48 hours | Yes |
| Thermo SPECTRA VRE | VRE | aerobic 35 – 37oC | 18-24 hours | Yes |

*\*Note: as of 1/1/2021 we are no longer plating specimens on KPC media.*

**Documentation Procedure:** *See ICEMAN LabVantage Training PowerPoint for detailed instructions*

1. Upon receipt of samples, prepare a sample log for each subject and enter all swabs into LabVantage.
2. Label the patient microbiome and environmental flocked swabs that are designated to be frozen immediately.
3. Generate child samples in LabVantage from the patient E-swabs and environmental 3M swabs to make labels for all selective agars and CHOP cryovials. Pre-label the selective agars and sponge fluid cryovials prior to processing.
4. Continue documentation based on the sample log until the samples are either frozen or destroyed.
5. After plates have been subcultured, generate child subcultures in LabVantage for each subculture. Destroy samples in LabVantage that did not grow on the selective media.
6. Generate bacterial cell isolate samples for each subculture that is to be frozen. This may be after MALDI results are received for the sample.
7. Once samples have been frozen, verify that the sample log and information in LabVantage match. Frozen samples are to be filed in LabVantage to match their location in the freezer.

**Environmental Sponge Swabs Procedure:** *adapted from CDC Stomacher Processing Method*

* *Swabs must be processed within 24 hours.*
* *Plates may be split in half, however samples from different rooms/patients are to be kept separate and not put on the same plate.*
* *All plates should be pre-labeled prior to sample processing.*
* *The hood must be cleaned with 10% bleach followed by 70% ethanol before and after use.*

The following steps are to be performed in a Class II Biosafety Cabinet:

1. Place sample bags with environmental sponges in Stomacher bag rack.
2. Add a 45mL aliquot of PBST to each bag.
3. Remove the plastic backing and open the sponge so it is not doubled over itself.
4. Aseptically orient the long side of the sponge with the bottom of the Stomacher bag.
5. Squeeze the sponge until it is fully saturated in PBST.
6. Process each swab in the Stomacher 400C Circulator for 1 minute at 200 RPM.
7. Allow foam to reduce in homogenate for about 5-10 minutes.
8. Carefully hold and squeeze the sponge on one side of the bag and from the opposite side remove all of the homogenate from the stomacher bag into a labeled 50-ml centrifuge tube using a sterile 50mL pipette.
9. Concentrate sample homogenates by centrifuging at 2000 x g for 30 min (Sorvall T6000D).
10. Remove supernatant from the tube using a 50-ml pipette leaving 5ml of supernatant in the tube as indicated from the graduated markings on the tube.

*Note: There should be ~ 5 ml homogenate remaining in each tube.*

1. Place tube(s) on VWR multi-tube vortexer for a total of 2 minutes with 10 second bursts.

*Note: Additional vortexing and sonication can be performed; the specific parameters will depend upon which microorganism is being recovered.*

1. Vortex again for a few seconds then measure the final suspension volume (~ 5 ml).
2. Plate 50 µL for isolation from undiluted suspension onto each selective agar: MRSA, ESBL, CRE, CRPA, VRE and CDIFF. Each plate should be labeled with its designated LabVantage label.
3. Remove 300 µL of the remaining undiluted homogenate (from Step 13) with a sterile pipettor and place in a 1.5mL cryovial labeled “cell pellet”. This sample is for DNA extraction and sequencing at CHOP. The remaining leftover homogenate can be stored in a labeled 5mL cryovial. Both tubes are stored at -80C.
4. Incubate all media at appropriate optimum temperature for recovery of suspect organism. Document all cultures in LabVantage.

The following steps can be performed at the bench:

1. After incubation period, remove the selective media and examine for growth. CDIFF media should be left in the anaerobic chamber for 48 hours to ensure growth, do not open the anaerobic chamber after 24 hours.
2. Pick up representative isolates from each selective agar and subculture isolates by streaking for isolation on blood agar. Label each colony with a unique identifier (ex. 1D-NS1-CRE-C1) and document all subcultures in LabVantage. Incubate the subcultured blood agar plates at 35 – 37oC for 18-24 hours.

*Note: At least one isolate per species should be picked based on differentiating colors.* *If there are two colonies on the same plate of the same color but with differing morphologies or if there are two colonies on the same plate with variations in color, subculture both colonies.*

1. After the blood agar has incubated for 18-24 hours, subcultures from ESBL, CRE, VRE and CDIFF agar are to be sequestered for MALDI testing. MALDI testing is to occur every other day. See below for MALDI procedure.
2. After receiving MALDI results, freeze isolates in labeled cryovials at -80C and document in LabVantage. MRSA isolates may be frozen immediately without MALDI testing.

**Patient & Environmental E-Swabs Procedure**

* *Swabs must be processed within 24 hours\*\*. Patients may not have swabs for all locations.*

*\*\*Note: in extenuating circumstances E-swabs can be cultured up to 48 hours after collection, but this is not the standard procedure.*

* *Plates may be split in half, however patient samples are to be kept separate and not put on the same plate.*
* *All plates should be pre-labeled prior to sample processing.*
* *The hood must be cleaned with 10% bleach followed by 70% ethanol before and after use.*

The following steps are to be performed in a Class II Biosafety Cabinet:

1. Briefly vortex each E-swab.
2. Plate one drop of liquid from each E-swab using a disposable transfer pipet onto the following selective agar and streak for isolation:
   1. Nasal: MRSA
   2. Oral: MRSA, ESBL, & CRE
   3. Groin, Rectal, & Stool: MRSA, ESBL, CRE, VRE & CDIFF
3. Incubate all media at appropriate optimum temperature for recovery of suspect organism. Document all cultures in LabVantage.

The following steps can be performed at the bench:

1. After incubation period, remove the selective media and examine for growth. CDIFF media should be left in the anaerobic chamber for 48 hours to ensure growth, do not open the anaerobic chamber after 24 hours.
2. Pick up representative isolates from each selective agar and subculture isolates by streaking for isolation on blood agar. Label each colony with a unique identifier (ex. 1D-NS1-CRE-C1) and document all subcultures in LabVantage and on the Sample Log. Incubate the subcultured blood agar plates at 35 – 37oC for 18-24 hours.

*Note: At least one isolate per species should be picked based on differentiating colors.* *If there are two colonies on the same plate of the same color but with differing morphologies or if there are two colonies on the same plate with variations in color, subculture both colonies.*

1. After the blood agar has incubated for 18-24 hours, subcultures from ESBL, CRE, VRE and CDIFF agar are to be sequestered for MALDI testing. MALDI testing is to occur every other day. See below for MALDI procedure.
2. After receiving MALDI results, freeze isolates in labeled cryovials at -80C and document in LabVantage. MRSA isolates may be frozen immediately without MALDI testing.

**MALDI Procedure**

* 1. Using a sterile toothpick, select one colony from the plate of interest and carefully spread onto a new spot on the MALDI slide.
  2. Drop 1 µL of matrix solution over the spot and allow to air dry completely
  3. Record the ID of the colony and location on the MALDI slide.
  4. Repeat for all plates that require MALDI identification.
  5. Use the *E. coli* control for the center spot.
  6. After completing the slide, document the MALDI slide in BOX.
  7. Deliver the slide to HUP micro before the end of the day.
  8. Upon receipt of results from HUP micro, record results in BOX and freeze necessary isolates.

**Solution Recipes**

1L 0.02% PBST

* 200 µL Tween
* 100 mL 10X PBS
* 900 mL dH2O