**SOP First Isolation-RCM**

**Document Number: XX**

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**Purpose**

This SOP describes the procedure used to isolate individual anaerobic bacteria from human fecal material and make frozen stocks of isolates.

**Scope:**

**Regulatory References:** NA

**Responsibility of experimentalist:** understanding and performing this procedure as described; reporting any deviations or problems to area supervisor; adequately documenting the procedures and results.

**Responsibility of area manager or supervisor:** ensuring that the analyst performing this procedure is qualified; ensuring that the procedure is followed, and updating the procedure as necessary.

**Definitions/Abbreviations**

Reinforced Clostridial Media: RCM

Brain Heart Infusion: BHI

Phosphate-buffered saline buffer: PBS

pre-reduced: refers to liquid that has either been sparged with Nitrogen gas for 30 minutes in a capped serum bottle or a liquid that contains a reducing agent (L-cysteine) that has been stored in the anaerobic chamber for 24+ hours. All media and buffers should be pre-reduced before use. Ones reduced liquid can be stored in the anaerobic chamber and used within 1 month

uL – microliter

**Related Documents**

Laminar flow hood operation manual SOP

Anaerobic chamber operation manual SOP

Sample collection SOP

Liquid Sparging Protocol SOP

Reinforced Clostridial Media SOP

Brain Heart Infusion Media SOP

**Required Equipment and Materials / Reagents (in order of use)**

* 150mm petri plates (VWR: Cat# 25384-326; or equivalent)
* RCM media (see Reinforced Clostridial Media SOP)
* 2x Agar (Sigma: Cat A5431; or equivalent)
* PBS (Sigma Aldrich: C7352; or equivalent)
* L-cysteine (Sigma Aldrich; Cat# C7532)
* Disposable hockey sticks/cell spreaders (VWR: Cat# 89042-018; or equivalent)
* 100mm petri plates (VWR: Cat# 25384-094; or equivalent)
* BHI media (HiVegMedia: Cat# MV210)
* Disposable sterile inoculation loops (VWR, Cat# 12000-808)
* 96 Well Clear Flat Bottom TC-Treated Culture Microplate, with Lid, Individually Wrapped, Sterile (Falcon® : Cat# 353072)
* 200uL multichannel pipette
* 200uL filter pipette tips
* 10uL pipette
* 10uL filter pipette tips
* Glycerol (BDH, Cat# 11724LP)
* Aluminum foil plate covers (VWR: Cat# 60941-076)

**Precautions**

Personal protection equipment including gloves and lab coat must be worn when executing this procedure.

**Procedure**

Note 1: Prepare all medium, buffer and solution before starting this procedure (see SOPs)

Note 2: PBS and 50% Glycerol solution have to be autoclaved and pre-reduced adding 0.1% L-cysteine and stocked in the anaerobic chamber for 24h before using it

**Day 1: Prepare “150mm RCM plates” (one day before inoculation, should be carried out in the Laminar Flow Hood)**

1. Autoclave 2x Agar and let it cool at room temperature to 50°C
2. Mix with filter-sterilized 2x Reinforced Clostridial Media (pre-warmed to 50°C in water-bath)
3. Pour plates and let them solidify and dry in Laminar Flow Hood to ensure their sterility. (Plates may require drying open to eliminate condensation)
4. Move plates in anaerobic chamber. Plates have to be lid-side down and in the anaerobic chamber for 24h for reducing before used

**Day 2: Inoculate sample (should be carried out in the anaerobic chamber)**

1. Since start concentration are different, dilute samples in serial dilution in pre-reduced PBS to 1:50.000; 1:100.000; 1:500.000 and 1:1.000.000 (and choose the better to obtain about 5.000 colonies per plate)
2. Plate 200uLs of diluted sample onto “150mm RCM plates” and spread with disposable hockey sticks (Include medium control plate and PBS control plate)
3. Let plates incubate for 6 days. Plates have to be lid-side down, stored in sterile bag and placed in the 37°C incubator into the anaerobic chamber

Note 3: This should result in 5,000 colonies per plate

**Day 7: Prepare “100mm BHI plates” (one day before inoculation, should be carried out in the Laminar Flow Hood)**

1. Mark quadrants on bottom of each plates
2. Autoclave 2x Agar and let it cool at room temperature to 50°C
3. Mix with filter-sterilized 2x BHI Media (pre-warmed to 50°C in water-bath)
4. Pour plates and let them solidify and dry in Laminar Flow Hood to ensure their sterility. (Plates may require drying open to eliminate condensation)
5. Move plates in anaerobic chamber. Plates have to be lid-side down and in the anaerobic chamber for 24h for reducing before used

**Day 8: First isolation from “150mm RCM plates” to “100mm BHI plates” (should be carried out in the anaerobic chamber)**

Note 4: Plates have to be lifted but facing down and with the lid opened just enough to pick from the “150mm RCM plates” and to streak on the “100mm BHI plates” to limit exposure to contamination for all this procedure

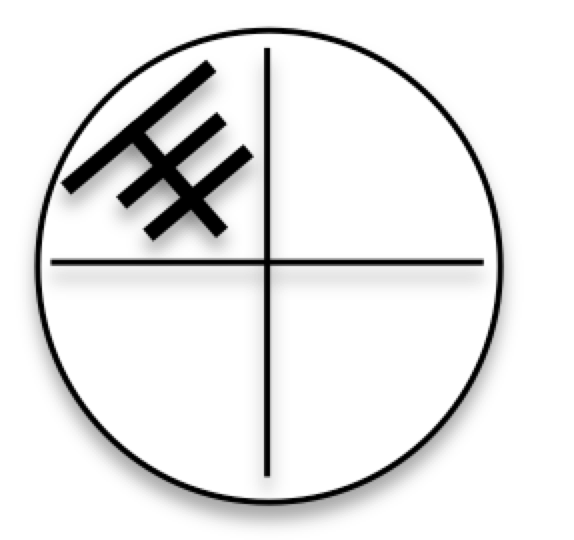
Note 5: Use two separate, sterile inoculating loops for each colony pick,

1. Pick one colony with an inoculation loop on “150mm RCM plates”
2. Use the same initial loop to streak a “T” shape onto a quadrant of one of the “100mm BHI plates”

Note 6: Flip the loop in between the top horizontal line and the vertical lines of the “T shape”

1. Use the second loop to make two more additional lines perpendicular to the vertical line of the “T shape”

Note 7: Flip the loop in between the two additional lines

Note 8: The streak should likes to: 

Note 9: Pick and streak colonies as many as you have quadrants on all your “100mm BHI plates”

1. Store all “100mm BHI plates” lid-side down, in sterile bag and placed in the 37°C incubator into the anaerobic chamber
2. Let streak grow until single colonies can be picked from the majority of them (48h usually)

**Day 10: Second isolation from “100mm BHI plates” to liquid BHI media (should be carried out in the anaerobic chamber)**

Note 10: Prepare liquid BHI media earlier (see Brain Heart Infusion Media SOP and Liquid Sparging Protocol SOP)

1. When single colonies can be picked, aliquot 200uLs of liquid BHI media into a 96 well cell culture plate
2. Use a pipette with a 10uL tip to pick a single colony from each individual streak and inoculate a well in the 96 well plate. Pipette 10uL up and down a few times to mix the colony into the well.

Note 11: Do this with the “100mm BHI plates” facing down to limit contamination

1. Let 96 well plate incubate in sterile bag and placed in the 37°C incubator into the anaerobic chamber until the majority of the wells appear turbid to the eye (48h usually)

**Day 12: Make Glycerol stocks (should be carried out in the anaerobic chamber)**

Note 12: Start when wells of the 96-well culture plate look turbid by visual inspection

1. Use a multichannel pipette to fill a new 96 well cell culture plate with 100uLs of pre-reduced 50% glycerol
2. Transfer 100uLs from the initial 96-well culture plate (mixing by pipetting before transfer) to the plate with 50% glycerol
3. Add and additional 100uLs of 50% glycerol to the original 96-well culture plate, which should now contain 200uLs per well, and mix by pipetting
4. Cover both culture plates with aluminum foil plate covers and their plastic plate lids and store in the -80C freezer.

Note 12: One of these plates will be used for Sanger sequencing and the other will serve as a stock plate for reviving the isolates.

Note 13: each plate is labeled as follows: NumberOfPlate – MediaUsed – Date - Experimentor

**Version History**

NA

**Worksheets**

Laboratory notebook pages 5 and 6 (Mathilde Poyet)

Each user record results and/or observations in their own notebook.

**Appendix**

NA