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Title: Establishing Stable Binary Cultures of Symbiotic Saccharibacteria from the Oral Cavity

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group?

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 18
Number of Shots: 34

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Floyd Dewhirst:** Saccharibacteria are obligate parasites that require coculture with appropriate host bacteria. As human oral Saccharibacteria can be easily isolated from most individuals using known host bacterial species, this protocol will allow any investigator or laboratory to isolate their own strains in binary coculture.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested b-roll: 6.1.2*

1.2. **Floyd Dewhirst:** This technique uses simple devices and equipment present in most microbiology laboratories and has been successfully used to culture over 30 isolates representing six species of Saccharibacteria.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested b-roll: 6.2.1*

OPTIONAL:

1.3. **Floyd Dewhirst:** This protocol may be useful for isolation of other Candidate Phyla Radiation as well as Saccharibacteria from different environments and other mammalian organisms if appropriate Actinobacteria hosts can be identified and isolated.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Introduction of Demonstrator on Camera

1.4. **Floyd Dewhirst:** Demonstrating the procedure will be Andrew Collins, a former post-doctoral fellow from my laboratory.

1.4.1. INTERVIEW: Author saying the above.

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

1.5. Procedures involving human subjects have been approved by the Institutional Review Board and informed consent was obtained from all subjects

Protocol

2. Preparation – Initiating Host Cultures and Sterilizing Equipment

2.1. To begin, start cultures of host bacteria such as *Arachnia* (Pronounce: A-RACK-knee-uh) *propionica* (Pronounce: PRO-PEE-on-ick-uh) with enough time for them to grow to early stationary phase. To initiate the culture, inoculate 2 to 5 milliliters of tryptic soy broth containing 0.1% yeast extract with *Arachnia propionica* **[1]** and incubate it at 37 degrees Celsius for 24 hours **[2]**.

2.1.1. WIDE: Establishing shot of talent inoculating the tryptic soy broth.

2.1.2. Talent placing the culture in the incubator.

2.2. Next, assemble filter holders with track-etched 0.2-micrometer filter membranes **[1]**, wrap them in foil and sterilize by autoclaving, along with centrifuge tubes and cap assemblies. Sterilize extra supplies in case of accidental contact with non-sterile surfaces **[2]**.

2.2.1. Talent assembling filter holders.

2.2.2. Talent placing filter holders wrapped in foil, centrifuge tubes, and cap assemblies in the autoclave.

3. Obtaining Sample of Oral Bacteria

3.1. Scrape the plaque using a sterile paper point, Gracey curette, or a sterile toothpick or pipette tip **[1]** and transfer it a suitable buffer, such as MRD (Pronounce M-R-D) or PBS **[2-TXT]**. Keep the sample on ice until ready to proceed further **[3].**

3.1.1. Talent taking a plaque scraping.

3.1.2. Talent transferring the plaque to the buffer. TEXT: **MRD - Maximal Recovery Diluent**

3.1.3. Talent placing the sample on ice.

3.2. Resuspend the dental plaque vigorously using a combination of vortexing and pipetting with a small pipet tip **[1]**. Then, add the resuspended plaque sample to a tube containing 9 milliliters of MRD buffer **[2]**.

3.2.1. Talent resuspending the plaque.

3.2.2. Talent adding the resuspended sample to MRD buffer.

4. Preparation of Filtrate from Oral Sample

4.1. Carefully unwrap a sterile filter assembly using aseptic technique **[1]**. Untwist a quarter turn and retighten the filter holder to ensure that the threads are properly engaged and the apparatus is properly closed **[2]**. *Videographer: This step is important!*

4.1.1. Talent unwrapping a sterile filter assembly.

4.1.2. Talent untwisting and retightening the filter holder.

4.2. Wash the membrane by passing 10 milliliters of MRD buffer **[1-Txt]** through the apparatus using a syringe **[2].** *Videographer: This step is important!*

4.2.1. Talent washing the membrane. **TEXT: In case of leakage, use another sterile filter assembly**

4.2.2. Added shot**:** CU: Shot of proper flow through outlet of apparatus

4.3. To apply the sample to the washed filter, remove the plunger from a syringe and attach it to the filter apparatus **[1]**. Place a sterile centrifuge tube beneath the filter apparatus to catch the filtrate **[4.4.1]**, then pour the dispersed dental sample into the syringe and load it onto the filter **[2].** *Videographer: This step is important!*

4.3.1. Talent removing plunger and attaching syringe to the filter apparatus.

4.4.1 Talent placing the centrifuge tube beneath the filter.

4.3.2. Talent pouring sample into syringe and loading it onto the filter.

Note: Shot 4.4.1 is moved above 4.3.2 and voice over is shifted accordingly

4.4. Then apply light pressure to the plunger to push the sample through the filter **[2]**. *Videographer: This step is important!*

4.4.2 Talent applying pressure on the plunger.

4.5. Wash the membrane with another 10 milliliters of MRD buffer and collect the flow-through in the same tube as the filtered sample **[1]**. Then, cap the tube aseptically and keep on ice **[2].**

4.5.1. Talent washing the membrane.

4.5.2. Talent capping the tube and place on ice

5. Concentration of the Saccharibacteria cells by centrifugation

5.1. Make an orientation mark on the tube and cap to identify the location of the cell pellet after centrifugation **[1]**. Place the tube in a high-speed centrifuge with the mark on the upper side and centrifuge the samples at 60,000 x g for 1 hour at 4 degrees Celsius **[2]**. *Videographer: This step is important!*

5.1.1. Talent marking the tube and cap.

5.1.2. Talent placing the tube in the centrifuge with mark on the upper side clearly visible.

5.2. After centrifugation, carefully pour out the supernatant, keeping the pellet on the upper side of the tube **[1]**. Then, resuspend the pellet in 1 to 2 milliliters of MRD buffer by vigorous vortexing **[2]**. *Videographer: This step is important!*

5.2.1. Talent pouring out the liquid.

5.2.2. Talent resuspending the pellet.

6. Infection of Host Cultures with Saccharibacteria-enriched Filtrate

6.1. Prepare culture tubes by aliquoting 2 milliliters of appropriate growth media into the tubes **[1]**. Then, add 200 microliters of overnight culture of host organisms and 100 to 200 microliters of resuspended, filtered sample to each tube **[2]**.

6.1.1. Talent adding growth media to culture tubes.

6.1.2. Talent adding host organisms and filtered sample to one tube.

6.2. Incubate the combined samples under conditions appropriate for the host organism **[1-TXT]**.

6.2.1. Talent incubating the samples. **TEXT: 37 °C in an microaerobic atmosphere for *A. propionica***

6.3. Passage the cells every two to three days by transferring 200 microliters of the binary culture to 2 milliliters of fresh growth medium in a new tube **[1]**. If the passaged cultures show no growth, add 200 microliters of uninfected host culture when passaging the cells **[2-TXT]**. Return inoculated tubes to incubator [3].

6.3.1. Talent adding the binary culture to fresh media in a new tube.

6.3.2. Talent adding uninfected host culture to the culture tube. **TEXT: Perform at least 5 passages**

6.3.3. Added shot: **Talent returns inoculated tubes to incubator**

7. Confirmation of Infection by PCR and Verification of Purity

7.1. Aliquot 24 microliters of the PCR master mix into a 0.2-milliliter PCR tube **[1-TXT]**. Add 1 microliter of Saccharibacteria-infected culture **[2]** and place the tube in a thermocycler. Set the PCR program as described in the text manuscript **[3]**.

7.1.1. Talent aliquoting the master mix. **TEXT: See text for master mix preparation**

7.1.2. Talent adding the Saccharibacteria-infected culture.

7.1.3. Talent placing the tube in a thermocycler.

7.2. Load and run the PCR products on a 1% agarose gel. A band of approximately 600 bases will confirm the presence of Saccharibacteria **[1]**.

7.2.1. Talent loading the gel.

7.3. To confirm the purity of positive cultures, perform a 10-fold serial dilution of the coculture of Saccharibacteria in a sterile buffer **[1]** and spread 100 microliters on an agar plate **[2]**.

7.3.1. Talent diluting the co-culture.

7.3.2. Talent spreading the diluted culture on an agar plate.

7.4. Incubate the culture under appropriate growth conditions and observe for contaminating organisms **[1]**.

7.4.1. Talent incubating the culture.

Results

8. Results: Confirmation of Infection and Purity of Binary Cultures of Symbiotic Saccharibacteria

8.1. PCR for detection of Saccharibacteria **[1]** may appear negative in initial infection cultures due to a low number of Saccharibacteria symbionts **[2]**. However, after a few passages, a strong PCR product should appear, indicating that a stable infection has occurred **[3]**.

8.1.1. LAB MEDIA: Figure 1A.

8.1.2. LAB MEDIA: Figure 1A. *Video Editor: Emphasize absence of a band in Initial infection*

8.1.3. LAB MEDIA: Figure 1A. *Video Editor: Emphasize the strong band in passage 4.*

8.2. Testing several host species with the same Saccharibacteria filtrate will usually have a low success rate as the symbiont-host interaction is very specific **[1]**.

8.2.1. LAB MEDIA: Figure 1B

8.3. As the infected cultures grow, normal turbid growth should appear **[1]**. As the symbiosis establishes itself, infected cultures will appear less turbid **[2]** than cultures of uninfected host organisms **[3]**.

8.3.1. LAB MEDIA: Figure 2A. *Video Editor: Emphasize that curves overlap up to 20 h*

8.3.2. LAB MEDIA: Figure 2A. *Video Editor: Emphasize the decreasing OD in dotted curve after 20 h*

8.3.3. LAB MEDIA: Figure 2A. *Video Editor: Emphasize increasing OD in the solid curve after 20 h*

8.4. A contaminated culture can be purified by picking infected colonies after plating **[1]**. Infected colonies can sometimes be identified by an irregular colony shape **[2]** compared to uninfected colonies **[3]**.

8.4.1. LAB MEDIA: Figure 2B, 2C.

8.4.2. LAB MEDIA: Figure 2B, 2C. *Video Editor: Emphasize the irregular colonies indicated by the white arrows in Figure 2B and 2C.*

8.4.3. LAB MEDIA: Figure 2B, 2C. *Video Editor: Emphasize the regular colonies (circular) in Figure 2C*

8.5. The proportion of irregular colonies depends on the titer of Saccharibacteria in the binary culture **[1]**. If the titer is low, fewer colonies will appear irregular making it easy to pick the infected colonies and start a pure binary culture **[2]**.

8.5.1. LAB MEDIA: Figure 2B-2C. *Video Editor: Label 2B as High Titer and 2C as Low Titer*

8.5.2. LAB MEDIA: Figure 2B-2C. *Video Editor: Emphasize irregular colonies in Figure 2C.*

Conclusion

9. Conclusion Interview Statements

9.1. **Andrew Collins:** Cultures may not appear positive for several passages, so some patience will be key. It is also necessary to regularly check for contamination of binary cultures as this can destabilize the culture and lead to the death of the Saccharibacteria.

9.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested b-roll: 7.3.1, 7.3.2*

9.2. **Andrew Collins:** The binary culture can be used for all sorts of laboratory experiments. DNA and RNA can be extracted for genome sequencing and transcriptome analysis, respectively. Pathogenicity can also be investigated using mouse models.

9.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera