	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*		
Title: PCR Amplification of 16s rDNA Internal Region, Supplement			
SOP #: 8.1.1.1.16	Revision: 00	Revision Date: n/a	Effective Date: 2016-11-18
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Supplement: PCR Amplification of 16s rDNA Internal Region

FILE NAME: 16s_Internal SOP 2016-11-18.doc

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this SOP is to amplify a ~700 base pair internal region of the 16s rDNA gene by PCR for the purpose of sequencing and subsequent organism identification. Historically, this internal region has been used for bacterial identification in MQIP projects.

This SOP is derived from an earlier protocol titled *"16s PCR Protocol for GreenMaster,"* authored by Pat Wood and revised by Nicole Woodcock (Martin) and Lexi Andrus. The original protocol can be found in the archived Milk Quality Protocols on the Food Safety Wiki.

1.2 Scope

This procedure is used for amplifying 16s rDNA for use in sequencing and identification of unknown organisms by the Wiedmann-Boor Lab in the department of Food Science at Cornell University. Historically, the internal region has been used in MQIP projects.

1.3 Definitions

16s rDNA: ribosomal RNA gene that is highly conserved among bacteria and archaea
BP: base pair
dNTPs: deoxyribonucleotide monomers, which are the building blocks of DNA. There are four dNTPs, cytosine, adenine, guanine and thymine
Exonuclease I: An enzyme used for post-PCR cleanup that degrades single-stranded DNA and primers.
GelDoc: An automated gel transilluminator that is coupled with ImageLab software.
GoTaq Green: A PCR kit containing thermostable Taq polymerase, $MgCl_2$, and a PCR buffer which contains a green loading dye. This loading dye does not interfere with the PCR and saves the step of loading dye incorporation prior to running gel electrophoresis.
PCR: polymerase chain reaction, used to amplify a specific region within a DNA sequence
pGEM: acts as a reference to estimate the size of unknown DNA molecules and to approximate the mass of a band
Shrimp Alkaline Phosphatase: An enzyme used for post-PCR cleanup that degrades free dNTPs.
TBE Buffer: buffer solution containing a mixture of Tris base, boric acid, and EDTA
Thermocycler: laboratory apparatus used to amplify segments of DNA via the polymerase chain reaction (PCR) process

1.4 Safety

Molten agarose can cause severe burns. Wear all appropriate thermal PPE when preparing agarose gels.

The heated lid of the thermocycler can cause severe burns. Exercise appropriate caution to avoid contact with the thermocycler lid.

Ethidium bromide is an extremely strong mutagen. It should only be used in the designated room, Room 350C, and nitrile gloves should always be worn while there.

16srDNA PCR is used in the sequencing and identification of unknown organisms. Many of these organisms, such as L. monocytogenes, are BSL-2 pathogens. Appropriate protective measures need to be taken when working with these species.

SECTION 2 MATERIALS

Lysis

- **96-well PCR Plate, 0.2 mL PCR strip tubes, or 0.2 mL individual PCR tubes** Room 358B and Room 350A
    - **96-well PCR Plate Covers, plastic or foil** Room 358B
    - **PCR Rack** Room 358B
- **Sterile ddH_2O** Room 350A
- **Sterile Toothpicks** Room 350A
- **Plate(s) with overnight culture of organism(s)**
- **Thermocycler** Room 356
- **Micropipette and sterile filter tips**

PCR Amplification

- **96-well PCR Plate, 0.2 mL PCR strip tubes, or 0.2 mL individual PCR tubes** Room 358B and Room 350A
    - **96-well PCR Plate Covers, plastic or foil** Room 358B
    - **PCR Rack** Room 358B
- **Micropipette and sterile filter tips**
- **Sterile 1.5 mL microcentrifuge tube** Room 358B and Room 350A
- **Crushed Ice**
- **Sterile ddH_2O** Room 358B and Room 350A
- **GoTaq Green 5x PCR Buffer** Room 352, “Dumb” freezer
- **dNTP solution consisting of 1 mM each dATP, dGTP, dCTP, dTTP** dNTP stocks in Room 352, “Dumber” freezer, in covered cryoblock
- **$MgCl_2$, 25 mM** Room 352, “Dumb” freezer
- **16s-PEU7-F (forward) Primer, 10 µM (5’- GCA AAC AGG ATT AGA TAC CC -3’)** Room 352C, chest freezer

- **16s-DG74-R (reverse) Primer, 10 µM (5'- AGG AGG TGA TCC AAC CGC A -3')** Room 352C, chest freezer
- **GoTaq Flexi Polymerase** Room 352, "Dumb" freezer
- **Vortex Mixer** Room 358B
- **Miniature Centrifuge** Room 358B
- **Thermocycler** Room 356

Gel Electrophoresis

- **Micropipette and sterile filter tips**
- **250 mL Erlenmeyer flask** Room 352, glassware cabinet
- **Agarose** Room 352B
- **0.5X TBE Buffer** Room 350, carboy on gel bench
- **Kimwipes**
- **Microwave Oven** Room 350
- **Gel Trays** Room 350, shelf above gel bench
- **40-well Gel Combs** Room 350, shelf above gel bench
- **Gel Tray Seals** Room 350, shelf above gel bench
- **Lab Tape** Room 350, gel bench
- **Razor Blade** Room 350, shelf above gel bench
- **Cutting board** Room 350, pegboard above sink
- **pGEM** Room 356, "Pedro's Friend"
- **PCR Product**
- **Crushed Ice**
- **Gel Electrophoresis Chamber** Room 350, gel bench
- **Transformer** Room 350, gel bench
- **Ethidium Bromide Solution** Room 350C
- **ddH_2O**, Room 350C, carboy
- **GelDoc SR** Room 350C

Post-PCR Cleanup

- **Micropipette and sterile filter tips**
- **Sterile 1.5 mL microcentrifuge tube** Room 358B and Room 350A
- **Exonuclease I, 10 U/µL** Room 352, "Dumber" freezer
- **Shrimp Alkaline Phosphatase, 1 U/µL** Room 352, "Dumber" freezer
    - *Optional:* **10x SAP Buffer** Room 352, "Dumber" freezer
    - *Optional:* **Sterile ddH_2O** Room 350A
- **96-well PCR Plate Covers, plastic or foil** Room 358B
- **Thermocycler** Room 356

Sequencing Submission

- **Micropipette and sterile filter tips**
- **96-well PCR Plate** Room 358B **OR**

- **500 µL standalone screw top vials** Room 350, gel bench drawer
  - **Zip-top plastic bag** Room 350, gel bench drawer
- **16s-PEU7-F (forward) Primer, <u>1 µM</u> (5'- GCA AAC AGG ATT AGA TAC CC -3')** Room 352C, chest freezer
- **16s-DG74-R (reverse) Primer, <u>1 µM</u> (5'- AGG AGG TGA TCC AAC CGC A -3')** Room 352C, chest freezer
- **Purified PCR Product**

SECTION 3 PROCEDURES

3.1 Lysis and DNA Preparation

3.1.1 For each isolate to be lysed, pipet 100 µL of sterile ddH_2O into one well of the 96-well plate or one 0.2 µL PCR tube placed in a PCR rack.

3.1.2 Use a sterile toothpick to gently touch a single colony from an overnight plate of the culture to be lysed. If there is any material visible on the end of the toothpick, there is likely too much DNA.

3.1.3 Insert the toothpick into the appropriate well or tube containing 100 µL of ddH_2O and swirl the toothpick briefly to dislodge the cells.

3.1.4 Repeat steps 3.1.1-3.1.3 for each sample and for a positive and negative control.

3.1.5 Seal the PCR plate with a cover or close the lids on the PCR tubes.

3.1.6 Use a thermocycler to heat the plate or tubes containing the lysates at 95°C for 15 minutes, then cool to 4°C (most thermocyclers in the lab already have the program "Lysis").

3.1.6.1 Lysates can be stored at 4°C for up to 1 week, or can be stored at -20°C for up to 6 months.

3.1.6.1.1 If storing lysates at -20°C, freeze-thaw cycles should be limited to avoid shearing of DNA.

3.2 PCR Amplification

3.2.1 Working in Room 358B, prepare a master mix of the components listed in 3.2.1.5 by pipetting each component into a sterile 1.5 mL microcentrifuge tube.

3.2.1.1 Pipette up and down to mix after each addition and to ensure that no reagent clings to the pipette tip.

3.2.1.2 Store all master mix components and the prepared master mix on crushed ice.

3.2.1.3 Vortex and centrifuge down the contents of all reagent containers before opening.

3.2.1.4 Calculate master mix volumes required to include positive and negative controls and 1-2 additional reactions to compensate for pipetting errrors.

3.2.1.5 **16s Internal Region PCR Master Mix**

Reagent	**Volume per reaction (µL)**
Sterile ddH_2O	13.125
GoTaq Green 5x PCR Buffer	5.0
1 mM dNTPs	1.25
$MgCl_2$, 25 mM	1.0
16s-PEU7-F Primer, 10 µM	1.25
16s-DG74-R Primer, 10 µM	1.25
GoTaq Flexi Polymerase	0.125
Total	**23.0**

3.2.2 Briefly vortex the prepared master mix. Spin briefly in the mini centrifuge to concentrate all contents at the bottom of the tube.
3.2.3 Dispense 23 µL of prepared master mix into one well per lysate in a 96-well PCR plate or into individual 0.2 mL PCR tubes. Cover the plate loosely or cover the tubes and hold on ice.
3.2.4 Remove the plate or tubes containing the master mix from room 358B to a lab bench.
3.2.5 Briefly spin down the previously-prepared lysates in a centrifuge, then add 2 µL of lysate to its corresponding PCR well or tube. Pipette up and down to mix.
3.2.6 Briefly centrifuge the PCR plate or tubes to concentrate reagents at the bottom of the tube.
3.2.7 Thermocycle using the parameters described in 3.2.7.1. Most lab thermocyclers should already have this programmed as "16s" under user "MQIP."

3.2.7.1 **Thermocycling Conditions for 16s Internal Region PCR**

Time (minutes:seconds)	**Temperature (°C)**	**Number of Cycles**
2:00	94	1
1:00	94	30
1:00	50	
1:30	72	
5:00	72	1
∞	4	1

3.2.8 Promptly remove the reactions from the thermocycler when complete. PCR product can be stored at 4°C for up to 1 week or at -20°C for up to 6 months.

3.3 Gel Electrophoresis

3.3.1 Gel Preparation

3.3.1.1 Weigh 1.5 g of agarose into a 250 mL Erlenmeyer flask. Add 100 mL of 0.5x TBE buffer and swirl gently to mix.
3.3.1.2 Cover the flask by inserting two Kimwipes partway into its neck

3.3.1.3 Microwave for approximately 1 minute and 30 seconds, stopping every 15-30 seconds to swirl the contents of the flask. Wear protective gloves when handling the hot flask.

3.3.1.4 Heating is completed when all agarose particles are completely dissolved. Allow the flask containing the molten agarose to cool for 5 minutes at room temperature before pouring.

3.3.1.5 Prepare a gel tray by inserting rubber seals into the grooves on both long ends of the tray. Place a piece of lab tape over both seals as additional protection against leakage. Insert two 40-well combs into the slots on top of the gel tray. Place the gel tray into the holder.

3.3.1.6 Slowly pour the cooled molten agarose into one corner of the gel tray. If bubbles are present, use a pipette tip to burst them.

3.3.1.7 Allow the poured gel to cool at room temperature for 30-60 minutes.

3.3.1.8 Gently pull the combs from the gel, then remove the gel tray from the holder. Remove the tape and seals, and gently slide the gel off of the tray.

3.3.1.9 If not using the gel immediately, store it in a plastic container of 0.5x TBE buffer.

3.3.1.10 Using a razor blade and cutting board, cut a gel with enough wells for each PCR that will be analyzed, plus additional wells for pGEM ladder.

3.3.2 Loading and Running the Gel

3.3.2.1 Place the gel onto the tray in the center of the gel electrophoresis chamber, with the wells on the left. Ensure that the 0.5x TBE in the chamber covers the gel completely.

3.3.2.2 Place PCR product and pGEM on ice.

3.3.2.3 Add 3 µL of pGEM to the first and last wells on the gel.

3.3.2.4 Add 3 µL of PCR product to the remaining wells.

3.3.2.5 Ensure that the gel is aligned correctly within the chamber, so that the DNA will run in a straight line.

3.3.2.6 Place the cover on the gel chamber, plug the electrodes into their color-coordinated sockets on the transformer, and power on the transformer. Ensure that the voltage is set to 120-123.

3.3.2.7 If the setup is running properly, there will be a fine stream of bubbles emerging from the electrodes at both ends of the box.

3.3.2.8 Ensure that the loading dye begins to move in the direction of the cathode, and not backwards and off of the gel.

3.3.2.9 Allow the gel to run for 20-30 minutes, until the yellow loading dye nears the end of the gel. Turn off the transformer and remove the gel from the chamber.

3.3.3 Gel Staining and Imaging

3.3.3.1 Check the log on the inside of the door of room 350C to see the last time the ethidium bromide solution was prepared. Solution 1-2 weeks old will stain the gel in 30-45 seconds; older solution may take up to 5 minutes to stain the gel.

3.3.3.2 Wearing nitrile gloves and safety glasses, use the gel spatula to gently lower the gel into the ethidium bromide solution. Leave the gel immersed in the EtBr for the appropriate length of time.
3.3.3.2 Remove the gel from the EtBr solution using the spatula and immediately transfer to a plastic container with enough ddH_2O to completely cover the gel. Leave the gel in water to destain for 30-60 minutes.
3.3.3.3 Remove the gel from the destaining water and place on Kimwipes to dry slightly.
3.3.3.4 Power on the GelDoc attached to the computer labeled "Hercules." The other GelDoc is reserved for PFGE and is not to be used by anybody else.
3.3.3.5 Open the ImageLab program on the computer. Select the "New Protocol" button. Under "Application," select first "Nucleic Acid," then "Ethidium Bromide." All other parameters can be left at their default settings.
3.3.3.6 Place the gel on the center of the platform within the GelDoc. Click the "Position Gel" button in ImageLab and align the gel with the camera.
3.3.3.7 Close the door of the GelDoc, and click the "Run Protocol" button.
3.3.3.8 To save the gel image, select "Export," then "Export for Publication" in the File menu.
3.3.3.9 Close the ImageLab software and power off the GelDoc.
3.3.3.10 Remove the gel from the platform within the GelDoc, then wipe any moisture or gel fragments from the platform using Kimwipes.

3.4 Post-PCR Cleanup

3.4.1 Prepare a master mix of the components listed in 3.4.1.5 by pipetting each component into a sterile 1.5 mL microcentrifuge tube.

3.4.1.1 Pipette up and down to mix after each addition and to ensure that no reagent clings to the pipette tip.
3.4.1.2 Store all master mix components and the prepared master mix on crushed ice.
3.4.1.3 Vortex and centrifuge down the contents of all reagent containers before opening.
3.4.1.4 Calculate master mix volumes required to include positive and negative controls and 1-2 additional reactions to compensate for pipetting errors.

3.4.1.5 **ExoSAP Master Mix**

Reagent	**Volume per reaction (µL)**
Exonuclease I	1.0
Shrimp Alkaline Phosphatase	1.0
Total	**2.0**

3.4.2 Briefly vortex the prepared master mix. Spin briefly in the mini centrifuge to concentrate all contents at the bottom of the tube.

3.4.3 Briefly spin the plate or tubes containing the PCR products to concentrate the product at the bottom of the tube.
3.4.3 Dispense 2 µL of prepared ExoSAP master mix into each well or tube containing PCR product.
3.4.4 Briefly centrifuge the PCR plate or tubes to concentrate reagents at the bottom of the tube.
3.4.5 Thermocycle using the parameters described in 3.4.5.1. Most lab thermocyclers should already have this programmed as "exosap" under user "MQIP."

3.4.5.1 **Thermocycling Conditions for 16s Internal Region PCR**

Time (minutes:seconds)	Temperature (°C)	Number of Cycles
45:00	37	1
15:00	80	1
∞	4	1

3.4.6 Promptly remove the reactions from the thermocycler when complete. PCR product can be stored at 4°C for up to 1 week or at -20°C for up to 6 months, but performing ExoSAP does not reset these times.

3.5 Sequencing Submission

3.5.1 Follow all guidelines for sequencing submission from the Cornell BRC: http://www.biotech.cornell.edu/sites/default/files/uploads/Genomics/17Nov2016_SeqHandbook.pdf
3.5.1.1 Login information for the BRC sequencing portal can be obtained from lab members.
3.5.2 Each PCR product requires 2 wells/tubes for sequencing: one for forward (PEU7) and one for reverse (DG74).
3.5.3 In each well, aliquot 10 µL of purified PCR product and 8 µL of the appropriate 1 µM primer, either PEU7 or DG74.
3.5.4 When ordering sequencing, write in the "General Comments" section: *Please use KB Basecaller software for trace processing, with mixed base identification set at a 70% detection level.*

SECTION 4 TROUBLESHOOTING

4.1 Issues with $MgCl_2$
4.1.1 $MgCl_2$ forms a concentration gradient when frozen and needs to be vortexed prior to use.
4.1.2 Every PCR reaction has an optimal $MgCl_2$ concentration range, usually between 1-4 mM. Since Mg^{+2} forms complexes with dNTPs and acts as a cofactor for polymerase you may need to try several conditions to optimize your concentration.
4.2 Too Much Enzyme
4.2.1 Excess enzyme in your PCR can lead to smearing of PCR products.

4.3 Wrong Primer Concentration
- 4.3.1 If you have too little primer you won't see any product.
- 4.3.2 Too much primer and you get primer dimerization and not enough amplification. Primer concentration should be between 0.1-1.0 µM.

4.4 Wrong PCR Program
- 4.4.1 Check your program while its cycling to make sure it is the right program.

4.5 Excess or Insufficient Template
- 4.5.1 Too much template can inhibit PCR by binding all of the primers. Take a smaller sample from the culture.
- 4.5.2 Too little template and amplification may be undetectable. Make sure you correctly prepared your dirty lystate and that you took a sufficiently large sample from your culture.

4.6 Too Much dNTP or Degraded dNTP
- 4.6.1 Excess dNTP inhibits PCR. Between 40-200 µM is optimal. dNTP is sensitive to repeated freeze-thaw cycles. Make small aliquots when you get a fresh batch and turn over your stock frequently.

SECTION 5 REFERENCES

Frame, Peter. (2010). Ten Things That Can Kill Your PCR. *Bio-Synthesis*. www.biosyn.com.