	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*

Title: PCR Amplification of 16S rDNA Internal Region, Supplement			
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Supplement: PCR Amplification of 16S rDNA Internal Region

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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_Internal SOP 2016-11-18"* authored by Sam Riechler, which was 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 protocols can be found archived 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
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

1.4 Safety

16S rDNA 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

Single Tube Reactions

- Sterile water - Room 358B and Room 350A
- Sterile 0.2 mL tubes - Room 358B and Room 350A
- 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
- 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
- Vortex Mixer - Room 358B
- Miniature Centrifuge - Room 358B
- Thermocycler - Room 356
- Micropipette and sterile filter tips
- Crushed Ice

High Throughput Reactions (96-well plate)

- Sterile reservoir basin - Room 358B
- Sterile 96-well plates - Room 358B
- Multi-channel pipette
- Sterile water - Room 358B and Room 350A
- 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
- 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
- Vortex Mixer - Room 358B
- Miniature Centrifuge - Room 358B
- Thermocycler - Room 356
- Adhesive aluminum foil lids - Room 358B
- Micropipette and sterile filter tips
- Crushed Ice

SECTION 3 PROCEDURES

3.1 Lysate Preparation

Refer to the "Preparing DNA Lysates" SOP on the Food Safety Lab wiki.

3.2 PCR Amplification

3.2.1 Working in Room 358B, prepare a master mix of the components listed in Table 1 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 errors.

Table 1: 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 µL**

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 Set up the thermocycler 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 Confirmation of Amplification

Refer to the "Gel Electrophoresis of PCR Product" SOP on the Food Safety Lab wiki.

3.4 ExoSAP Purification and Sequencing Submission

Refer to the "PCR Product Purification and Sanger Sequencing Submission" SOP on the Food Safety Lab wiki.

3.5 Sequence Analysis

Refer to the "Automated Sequence Editing" SOP on the Food Safety Lab wiki.

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 may result in primer dimerization, which may reduce amplification.

4.4 **Wrong PCR Program**

4.4.1 Check your program while it's 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. If the lysate appears very turbid, dilute the template 1:10 or 1:100 and then use 1 µL of the diluted template for the PCR.

4.5.2 Too little template and amplification may be undetectable. Make sure you correctly prepared your dirty lysate 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 due to Mg2 depletion. Between 40-200 µM is optimal. dNTPs are sensitive to repeated freeze-thaw cycles. Make small aliquots when you get a fresh batch and turn over your stock frequently.

4.6.2 Note that the dNTP solution concentration suggested by the GoTaq Flexi manufacturer Promega suggests having each dNTP at 10mM , while this protocol uses 1mM due to less Mg2 being used.

4.7 Isolates that do not amplify with this protocol may be yeasts or molds. Check for this by utilizing morphological assessments such as Gram staining with observations under a microscope

4.8 Note that the dNTP solution concentration suggested by the GoTaq Flexi manufacturer Promega suggests having each dNTP at 10mM

SECTION 5 REFERENCES

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