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

Title: PCR Amplification of 16S rDNA Internal Region, Supplement			
SOP #:	Revision: 00	Revision Date: n/a	Effective Date: 2020-01-30
Authors: Jordan Skeens, Sam Reichler		Approved by:	

Supplement: PCR Amplification of 16S rDNA Internal Region

FILE NAME: 16S_Internal PCR SOP_01302020.doc

TABLE OF CONTENTS

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 Reichler, 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 Cell Lysates for PCR" SOP on the Food Safety Lab wiki.

3.2 PCR Amplification

3.2.1 To prepare 200µL of the 1mM dNTP working solution:

3.2.1.1 Always make at least 200 µL to avoid pipetting 1 µL volumes, which can be inaccurate.

3.2.1.2 Thaw the individual 100mM dNTP tubes on ice.

3.2.1.3 Fill a 1.5ml tube with 192 µL of dH2O.

3.2.1.4 Transfer 2 µL of each dNTP into the 1.5mL tube and gently vortex to mix.

3.2.1.5 If only small portions of the 200µL will be used in your PCRs, aliquot this dNTP solution into 1.5ml tubes at appropriate volumes for your needs to reduce freeze thaw cycles.

3.2.1.6 This solution should be frozen at -20°C after use.

3.2.2 To prepare 100 µL of the 10uM primer working solutions:

3.2.2.1 If the primer stock has not been reconstituted, refer to the "8.1.1.1.7-Primer Ordering and Reconstitution" SOP on the Food Safety Lab wiki.

3.2.2.2 Thaw the 100µM primer stock solutions on ice.

3.2.2.3 Fill two 1.5ml tubes with 90ul of 10mM Tris-HCL(pH8.0).

3.2.2.4 Add 10 µL of your forward primer to one of the 1.5ml tubes, and 10 µL of your reverse primer to the other 1.5ml tube. Label the tubes appropriately.

3.2.2.5 Gently vortex each tube to mix.

3.2.2.6 These solutions should be frozen at -20°C after use.

3.2.3 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.

3.2.3.1 **IMPORTANT**: Do not vortex the stock Taq polymerase solution or the mastermix after Taq is added, it will be damaged by vortexing. The Taq can be the last component added to the master mix.

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

3.2.3.3 Store all master mix components and the prepared master mix on crushed ice.
3.2.3.4 Vortex and centrifuge down the contents of all reagent containers before opening (except Taq)
3.2.3.5 Pipette up and down to mix after each addition and to ensure that no reagent clings to the pipette tip.

Table 1: 16S Internal Region PCR Master Mix

Reagent	**Volume per reaction (µL)**	**Final reaction concentration**
Sterile ddH_2O	11.875	
GoTaq Green 5x PCR Buffer	5	
dNTPs, 1 mM each	2.5	100uM
$MgCl_2$, 25 mM	1	1mM
16S-PEU7-F Primer, 10 µM	1.25	0.5uM
16S-DG74-R Primer, 10 µM	1.25	0.5uM
GoTaq Flexi Polymerase	0.125	
Total	**23.0 µL**	

3.2.4 Briefly shake the tube containing the prepared master mix, but do not vortex.
3.2.5 Spin briefly in the mini centrifuge to concentrate all contents at the bottom of the tube.
3.2.6 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.
3.2.7 Remove the plate or tubes containing the master mix from room 358B to a lab bench.
3.2.8 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.
3.2.9 Briefly centrifuge the PCR plate or tubes to concentrate reagents at the bottom of the tube.
3.2.10 Set up the thermocycler using the parameters described in 3.2.10.1. Most lab thermocyclers should already have this programmed as “16S” under user “MQIP.”

3.2.10.1 **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

3.2.11 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 2 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 MgCl2 depletion. Between 40-200 µM final concentration 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 MgCl2 being used.

4.6.3 The 1mM dNTP concentration has been validated for this PCR protocol, but should not be transferred for use in other PCRs, especially if the fragment length is much longer

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

SECTION 5 REFERENCES

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