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

Title: Degenerate sigB PCR			
SOP #: 8.1.1.2.2.17	Version: 02	Revision Date: 06/21/2020	Effective Date: 7/31/2020
Author: Jingqiu Liao		Approved by:	

Degenerate *sigB* PCR

FILE NAME: 8.1.1.2.2.17-Degenerate sigB PCR.docx

TABLE OF CONTENTS

SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to set forth standard guidelines for performing a PCR to amplify a 570bp fragment of the *sigB* gene to be used for sequence determination and subsequent phylogenetic analyses.

1.2 Scope

This SOP applies to the Food Safety Lab, including the Laboratory for Food Microbiology and Pathogenesis of Foodborne Diseases, and the Milk Quality Improvement Program.

1.3 Definitions

Master Mix: a mix of all the components required for PCR (taq polymerase if manual hot start, buffer, and $MgCl_2$), except the DNA template.
$MgCl_2$: a cofactor for the polymerase
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.

1.4 Safety

L. monocytogenes is a BSL-2 pathogen. Appropriate protective measures need to be taken when working with *L. monocytogenes*. All waste from these experiments needs to be treated as BSL-2 waste.

SECTION 2 MATERIALS

Single Tube Reactions

- Sterile water - Room 358B and Room 350A
- Sterile 0.2 mL tubes - Room 358B and Room 350A
- sigBdegF (forward) Primer, 12.5µM (5' – ATGAAAAGCAGGTGGAGGAGAATGC - 3') - Room 352C, chest freezer
- sigBdegR (reverse) Primer, 12.5µM (5' - CCSGTTTCTTTTTGACTRCGRTTTTC -3') - Room 352C, chest freezer
- GoTaq polymerase - Room 352C, chest freezer
- 5 x Green Buffer - Room 352C, chest freezer
- MgCl2 (25mM) - Room 352C, chest freezer
- dNTP solution consisting of 1 mM each dATP, dGTP, dCTP, dTTP dNTP stocks in Room 352, "Dumber" freezer, in covered cryoblock
- 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
- sigBdegF (forward) Primer, 12.5µM (5' – ATGAAAAGCAGGTGGAGGAGAATGC - 3') - Room 352C, chest freezer
- sigBdegR (reverse) Primer, 12.5µM (5' - CCSGTTTCTTTTTGACTRCGRTTTTC -3') - Room 352C, chest freezer
- GoTaq polymerase - Room 352C, chest freezer
- 5 x Green Buffer - Room 352C, chest freezer
- MgCl2 (25mM) - Room 352C, chest freezer
- dNTP solution consisting of 1 mM each dATP, dGTP, dCTP, dTTP dNTP stocks in Room 352, "Dumber" freezer, in covered cryoblock
- 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 section 3.1 or 3.2 of the "Preparing Cell Lysates for PCR" SOP on the Food Safety Lab wiki. Do not use "Section 3.2: Heat Lysis" when making Listeria cell lysates.

3.2 PCR Amplification

3.2.1 To prepare 200µL of the 10 mM 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 100 mM dNTP tubes on ice.

3.2.1.3 Fill a 1.5ml tube with 180 µL of dH2O.

3.2.1.4 Transfer 5 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 12.5uM 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 87.5ul of 10mM Tris-HCL(pH8.0).

3.2.2.4 Add 12.5 µL of your forward primer to one of the 1.5ml tubes, and 12.5 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 mastermix of the components listed in Table 1.

3.2.3.1 **IMPORTANT**: Do not vortex the stock GoTaq polymerase solution or mastermix after the GoTaq polymerase is added, it will be damaged by vortexing.

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 GoTaq polymerase)

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:

	1X (µL)
dH_2O	33.75
5 x Green Buffer	5
25 mM MgCl2	4
10 mM dNTPs	2
12.5 µM sigBdegF	2
12.5 µM sigBdegR	2
5 u/µL GoTaq polymerase	0.25
Total	49

3.2.4 Start PCR

3.2.4.1 Dispense 49 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.

3.2.4.2 Remove the plate or tubes containing the master mix from room 358B to a lab bench on ice.

3.2.4.3 Check the cycling conditions on the PCR machine:

Touchdown PCR Conditions:

Time	Temperature (°C)	Cycles or Holds
3 min	95	1
30 s	94	20 (-0.5°C per cycle)
30 s	60	
1 min	72	
30 s	94	20
30 s	50	
1 min	72	
7 min	72	1
∞	4	1

3.2.4.4 Begin the cycle. Once the temperature reaches 80 °C, pause the program and wait for the temperature to stabilize.

3.2.4.5 While the temperature stabilizes, add 1 µL of lysate to its corresponding PCR well or tube. Pipette up and down to mix.

3.2.4.6 Ensure all the tube caps are tightly closed, then close the thermocycler lid and resume the program.

3.2.4.7 Promptly remove the reactions from the thermocycler when complete. P CR 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 $MgCl_2$ 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 It may be wise to re-streak all cultures onto BHI plates if the organisms were isolated on LMPM and MOX plates. Make sure your positive control matches the plating medium of whatever you're testing (i.e. if you're testing cultures from LMPM plates, use a PC from an LMPM plate).

SECTION 5 REFERENCES

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	6/21/2020	Jingqiu	Original SOP
Version 2	7/31/2020	Jingqiu	Minor corrections
Version 3			