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

Title: sigB PCR Protocol			
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***sigB PCR* Protocol**

FILE NAME: *sigB* PCR Protocol_11252020.doc

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to set forth standard guidelines for performing a PCR to amplify a 780 bp fragment of *sigB* to be used for sequence determination and subsequent phylogenetic analysis.

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

Hot start PCR: Enzymatic hot start PCR using AmpliTaq Gold reduces non-specific amplification of DNA by inactivating the Taq polymerase at lower temperatures. It significantly reduces nonspecific priming, the formation of primer dimers, and often increases product yields. *Hot start PCR is required for sigB PCR, in contrast to most other PCR procedures.*
Master Mix: a mix of all the components required for PCR (taq polymerase, buffer, and $MgCl_2$), except the DNA template.
$MgCl_2$: a cofactor for the polymerase
AmpliTaq gold: a taq polymerase that activates at a higher temperature to prevent unspecific binding and annealing.
dNTPs: deoxyribonucleotide monomers, which are the building blocks of DNA. There are four dNTPs, cytosine, adenine, guanine and thymine

1.4 Safety

Be aware when handling BSL-2 pathogen. Appropriate protective measures need to be taken. 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
- LM sigB15 (forward) Primer, 12.5µM (5' - AAT ATA TTA ATG AAA AGC AGG TGG AG -3') - Room 352C, chest freezer
- LM sigB16 (reverse) Primer, 12.5µM (5' - ATA AAT TAT TTG ATT CAA CTG CCT T -3') - Room 352C, chest freezer
- AmpliTaq gold DNA Polymerase - Room 352, "Dumb" freezer
- AmpliTaq 10X PCR buffer - Room 352, "Dumb" freezer
- AmpliTaq $MgCl_2$ (25mM) - 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
- 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
- LM sigB15 (forward) Primer, 12.5µM (5' - AAT ATA TTA ATG AAA AGC AGG TGG AG -3') - Room 352C, chest freezer
- LM sigB16 (reverse) Primer, 12.5µM (5' - ATA AAT TAT TTG ATT CAA CTG CCT T -3') - Room 352C, chest freezer
- AmpliTaq gold DNA Polymerase - Room 352, "Dumb" freezer
- AmpliTaq 10X PCR buffer - Room 352, "Dumb" freezer
- AmpliTaq $MgCl_2$ (25mM) - 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
- 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 3.3: Heat Lysis" when making Listeria cell lysates.

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 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 Taq polymerase solution or mastermix after the Taq 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 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: sigB PCR Master Mix

Master Mix:	Volume per reaction	Final reaction concentration
• dH_20	29.75 µL	
• AmpliTaq 10X PCR buffer	5.0 µL	
• AmpliTaq $MgCl_2$ (25mM)	4.0 µL	2mM
• dNTPs (1mM each)	5.0 µL	100uM
• LM sigB15 primer (12.5µM)	2.0 µL	0.5uM
• LM sigB16 primer (12.5µM)	2.0 µL	0.5uM
• AmpliTaq gold polymerase	0.25 µL	
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	48 µL per tube	

3.2.4 Enzymatic Hot Start (AmpliTaq gold polymerase)

3.2.4.1 Dispense 48 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.

3.2.4.3 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.

3.2.4.4 Briefly centrifuge the PCR plate or tubes to concentrate reagents at the bottom of the tube.

3.2.4.5 Perform the reaction using the following set of cycling conditions:

Touchdown PCR Conditions:

94°C	10 min	
94°C	30 sec	
AT*	30 sec	20 cycles at 55-45°, then
72°C	1 min	20 cycles at 45°C
72°C	7 min	
4°C	Hold	

* the first 20 cycles have an AT that decreases by 0.5°C for each cycle (touch down PCR). Then, 20 cycles with an AT at 45°C

3.2.4.6 Place the rack in the PCR machine, with a compression pad, and begin the cycle.

3.2.4.7 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.

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 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	11/01/2012	N/A	Original SOP
Version 2-5	06/24/2015	N/A	N/A
Version 6	09/28/2017	Genevieve Sullivan, Jordan Skeens	Updated and changed to SOP format
Version 7	10/11/2019	N/A	Updated and reformatted
Version 8	11/14/2019	Sherry Roof	Updates
Version 9	01/30/2020	Jordan Skeens	Added instructions to make dNTPs and primers, and final reaction concentrations. Removed the Go-Taq manual hot start protocol
Version 10	11/25/2020	Jordan Skeens	-Introduction: Removed references to manual hot start. -Section 3.1: Reference to another SOP erroneously stated “Section 3.2: Heat Lysis” and was fixed to say “Section 3.3: Heat Lysis” -Section 6: Method Versions added -MQIP Logo added to page headers