	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*		
Title: SigB PCR			
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SigB PCR

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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 780bp fragment of the sigB gene 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: Hot Start PCR avoids a 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.* This can be done either **manually** (by adding the taq polymerase, such as GoTaq, after the denaturation step), or **enzymatically** (using AmpliTaq Gold).

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

Amplitaq gold: a taq polymerase that activates at a higher temperature to prevent unspecific binding and annealing.

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

Manual Hot Start:

Master Mix:	1X (50µL rxn)
• dH_20	21.5 µL
• 5X PCR buffer*	8.0 µL
• $MgCl_2$ (25mM)	3.0 µL
• dNTPs (10mM)	2.5 µL
• SigB15 (12.5µM)	2.0 µL
• SigB16 (12.5µM)	2.0 µL
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	39 µL per tube

Taq Mix:	1X (50µL rxn)
• dH_20	7.6 µL
• 5X PCR buffer*	2.0 µL
• Taq polymerase (GoTaq)	0.4 µL
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	10 µL per tube

Enzymatic Hot Start:

Master Mix:	1X (50µL rxn)
• dH_20	33.75 µL
• Amplitaq 10X PCR buffer	5.0 µL
• Amplitaq $MgCl_2$ (25mM)	4.0 µL
• dNTPs (10mM)	2.0 µL
• SigB15 (12.5µM)	2.0 µL
• SigB16 (12.5µM)	2.0 µL
• AmpliTaq gold	0.25 µL
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	49 µL per tube

*adjust volumes if buffer is 10X instead of 5X

Primer Sequences:
LM sigB15 (forward) = AAT ATA TTA ATG AAA AGC AGG TGG AG
LM sigB16 (reverse) = ATA AAT TAT TTG ATT CAA CTG CCT T

SECTION 3 PROCEDURES

3.1. Manual Hot Start (GoTaq Polymerase)

(1) Using a 10uL unfiltered pipette tip, remove a single colony from a pure plated culture. Only remove a part of the colony, as too much DNA can result in smears. Insert wooden toothpick (containing bacteria) into a 200ul PCR tube (or well in a 96-well plate) containing 100 ul of sterile water and press/swirl colony into bottom of tube. ***For Gram positive organisms, do this step without water***. Make sure to include a positive and negative control. Microwave PCR tubes or 96-well plate for 3 minutes. Let cool at bench
(2) Calculate the necessary amount of Master Mix and Taq Mix to prepare, based on the number of tubes you would like to test. In general, it is best to prepare enough for one additional tube.
(3) In the PCR room, aseptically prepare the Master Mix and the Taq Mix, however do not yet add the Taq enzyme component to the Taq Mix, leaving the Taq enzyme in the freezer until just prior to use.
(4) Vortex the Master mix thoroughly and aliquot 39 ul to each PCR tube. Keep tube rack on ice until placed in thermocycler.
(5) Check the cycling conditions on the PCR machine:

Touchdown PCR Conditions:
5 min @ 94°C
30 sec @ 94°C
30 sec @ 54-44°C *20 cycles (decreases by 0.5°C through first 20 cycles)
1 min @ 72°C
30 sec @ 94°C
30 sec @ 44°C *20 cycles
1 min @ 72°C
7 min @ 72°C
Hold @ 4°C

(6) Place the rack in the PCR machine, with a compression pad, and begin the cycle. Once the sample temperature reaches 80°C, pause the program and wait for the temperature to stabilize.
(7) While the temperature stabilizes, add the calculated volume of Taq enzyme to the Taq Mix, mix by 'flicking' the tube (do not vortex) and immediately add 10μL of the Taq Mix to each PCR tube.
(8) Ensure all the tube caps are tightly closed, then close the thermocycler lid and resume the program.

3.2. Enzymatic Hot Start (AmpliTaq gold polymerase)

(1) Using a 10uL unfiltered pipette tip, remove a single colony from a pure plated culture. Only remove a part of the colony, as too much DNA can result in smears. Insert pipette tip (containing bacteria) into a 200ul PCR tube (or well in a 96-well plate) containing 100 ul of sterile water and press/swirl colony into bottom of tube. ***For Gram positive organisms, do this step without water,*** *briefly pressing the tip to the bottom of the well*. Make sure to include a positive and negative control.
(2) Microwave PCR tubes or 96-well plate for 3 minutes. Let cool at bench
(3) Calculate the necessary amount of master mix to prepare, based on the number of tubes you would like to test. In general, it is best to prepare enough for one additional tube (e.g. if you have 14 samples, calculate and prepare enough master mix for 15 samples).
(4) In PCR room, aseptically prepare the Master Mix using Amplitaq and its accompanying 10X PCR buffer and 25 mM $MgCl_2$. Amplitaq gold should be added last to the Master Mix as shown in the set-up in the materials section. Do not vortex the Amplitaq gold tube. Everything can be vortexed except the Amplitaq gold.
(5) Mix thoroughly and aliquot 49 ul of master mix to each PCR tube. Keep tube rack on ice until placed in thermocycler.
(6) Check the cycling conditions on the PCR machine:

Touchdown PCR Conditions:
10 min @ 95°C
30 sec @ 94°C
30 sec @ 54-44°C *20 cycles (decreases by 0.5°C through first 20 cycles)
1 min @ 72°C
30 sec @ 94°C
30 sec @ 44°C *20 cycles
1 min @ 72°C
7 min @ 72°C
Hold @ 4°C

(7) Place the rack in the PCR machine, with a compression pad, and begin the cycle.

SECTION 4 TROUBLESHOOTING

(1) Keep the buffer on ice.
(2) You can spin down the mixture in thermocycler room.
(3) If your PCR smears, you may have used too much DNA. Try taking a smaller portion of the colony.
(4) If overall amplification of gene fragment is weak, increase the amount of template and decrease the amount of dH_2O in the master mix.
(5) It may be wise to re-streak all cultures onto BHI plates, as LMPM and MOX plates may not be effective in generating results. 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