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

Title: actA PCR Protocol			
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Authors: Jordan Skeens, Emily Wright		Approved by:	

actA PCR ASSAY TO DIFFERENTIATE LISTERIA MONOCYTOGENES FSL R3-0001 CONTROL STRAIN FROM WILD-TYPE STRAINS

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TABLE OF CONTENTS

1. INTRODUCTION
   Purpose
   Scope
   Definitions
   Safety

2. MATERIALS

3. PROCEDURE

4. TROUBLESHOOTING

5. REFERENCES

SECTION 1 INTRODUCTION

1.1 Purpose

A standard *actA* PCR method that allows differentiation of the *L. monocytogenes* control strain FSL R3-0001 from wild-type *L. monocytogenes* strains. FSL R3-0001 is currently used as a genetically modified control strain by Silliker laboratories and this procedure will allow the user to determine whether *L. monocytogenes* isolates from test samples represent wild-type isolates or contamination with FSL R3-0001.

This SOP is derived from an earlier protocol titled *"actA PCR ASSAY TO DIFFERENTIATE LISTERIA MONOCYTOGENES FSL R3-001 CONTROL STRAIN FROM WILDTYPE STRAINS"* authored by Emily Wright. The original protocol can be found archived on the Food Safety Wiki.

1.2 Scope

This SOP applies to the Food Safety Lab, including the Laboratory for Molecular Typing.

1.3 Definitions

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

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
- mpl-xbaF (forward) Primer, 12.5 µM (5' - GCT CTA GAT CAA CAA GCA GCG AAA GATT - 3') - Room 352C, chest freezer
- actA MassR (reverse) Primer, 12.5 µM (5' TTT ATG TGG TAA TTT GCT GTC 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
- mpl-xbaF (forward) Primer, 12.5 µM (5' - GCT CTA GAT CAA CAA GCA GCG AAA GATT - 3') - Room 352C, chest freezer
- actA MassR (reverse) Primer, 12.5 µM (5' TTT ATG TGG TAA TTT GCT GTC 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℃ after use.

3.2.1.7

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℃ after use.

3.2.3 Working in Room 358B, prepare a master mix and Taq mix of the components listed in Table 1.

3.2.3.1 **IMPORTANT**: Do not vortex the stock Taq polymerase solution or the Taq mix 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: *actA* PCR Master Mix

Master Mix:	**Volume per reaction**	**Final reaction concentration**
• dH_20	18.0 µL	
• 5X PCR buffer	8.0 µL	
• $MgCl_2$ (25mM)	3.0 µL	1.5mM
• dNTPs (1mM each)	5.0 µL	100uM
• mpl-xbaF (12.5µM)	2.0 µL	0.5uM
• actA MassR (12.5µM)	2.0 µL	0.5uM
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	38 µL per tube	

Taq Mix:	**Volume per reaction**
• 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

3.2.4 Manual Hot Start

3.2.4.1 Briefly shake the tube containing the prepared master mix, but do not vortex

3.2.4.2 Spin briefly in the mini centrifuge to concentrate all contents at the bottom of the tube.

3.2.4.3 Dispense 38 µL of prepared master mix (but not the Taq Mix) into each well (one well per lysate to be amplified) 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.

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

3.2.4.5 Briefly spin down the previously-prepared lysates in a centrifuge, then add 2µL of lysate to its corresponding PCR well or tube. Pipette up and down to mix.

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

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

Hot Start PCR Conditions:

94°C	3 min	
94°C	1 min	40 cycles
53°C	1 min	
72°C	2 min	
72°C	5 min	
4°C	Hold	

3.2.4.8 Place the rack in the PCR machine, with a compression pad, and begin the cycle. Once the sample completes its first denaturation step (1min at 94°C) and the temperature begins to decrease, pause the program when it reaches 80°C, and wait for the temperature to stabilize.

3.2.4.9 Add 10µL of the Taq Mix to each PCR tube. Ensure all the tube caps are tightly closed, then close the thermocycler lid, wait 1 min, and resume the program.

3.2.5 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

SECTION 5 REFERENCES

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