	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*	MQIP Milk Quality Improvement Program

Title: **hlyα/β PCR**			
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hlyα/β PCR

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SECTION 1 INTRODUCTION

1.1 Purpose

To set forth a standard method to amplify an 858 bp fragment of the hly gene as confirmation of the presence of the hly gene for putative *Listeria monocytogenes* or hemolytic *innocua* isolates.

1.2 Scope

This SOP applies to the Food Safety and MQIP Labs

1.3 Definitions

BP: base pair
PCR: Polymerase chain reaction is a process used to amplify a specific region of a DNA sequence.
AmpliTaq Gold: A taq polymerase that activates at a higher temperature to prevent unspecific binding and annealing. This enzyme comes in a kit containing its own 10X buffer and 25mM $MgCl_2$.
$MgCl_2$: a cofactor for the polymerase
dNTPs: deoxyribonucleotide monomers, which are the building blocks of DNA. There are four dNTPs, cytosine, adenine, guanine and thymine.
Master Mix: a mix of all the components required for PCR (taq polymerase, buffer, dNTPs and $MgCl_2$), except the DNA template.

1.4 Safety

L. monocytogenes is a BSL-2 pathogen. Proper PPE (personal protective equipment) which includes safety glasses, lab coats and gloves, must be worn when performing this or any other experiment in the lab. 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
- Forward Primer ***hly* α** : CCT AAG ACG CCA ATC GAA AAG AAA
- Reverse Primer ***hly* β** : TAG TTC TAC ATC ACC TGA GAC AGA
- AmpliTaq gold DNA Polymerase -Room 352, "Dumb" freezer
- AmpliTaq 10X PCR buffer -Room 352, "Dumb" freezer
- AmpliTaq MgCl2(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
- FSL X1-0001 (10403S) for positive control

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 10mM 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 120 µL of dH2O.

3.2.1.4 Transfer 20 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 20uM 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 80.0ul of 10mM Tris-HCl (pH8.0).

3.2.2.4 Add 20.0 µL of your forward primer to one of the 1.5ml tubes, and 20.0 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 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 master 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 to ensure no reagent clings to the pipette tip.

TABLE 1: ***hly* α/ *hly* β Master Mix:**

Master Ingredients	for (1X)
d'H $_2$O	17.3 µl
10X Gold PCR Buffer	2.5 µl
Gold MgCl2 (25 mM)	1.5 µl
dNTPs (10mM)	0.5 µl
hly α (20.0 µM)	0.5 µl
hly β (20.0 µM)	0.5 µl
AmpliTaq Gold DNApolymerase	0.2 µl

Add 2 µl of template to 23 ul of master mix

3.2.4 **Enzymatic Hot Start (AmpliTaq gold polymerase)**
3.2.4.1 Dispense 23µL of prepared master mix into each tube**.**
3.2.4.2 Remove the 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 tube.
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:

10 min. at	94° C	(1 x)
10 sec.	95° C	----------(40 x)
30 sec.	60° C	
30 sec.	72° C	
10 min. at	72° C	(1 x)

∞ 4° C

3.3 Gel Electrophoresis Confirmation of Amplification

Refer to the "Gel Electrophoresis of PCR Product" SOP on the Food Safety Lab wiki.

SECTION 4 TROUBLESHOOTING

4.1 **Issues with MgCl2**

4.1.1 MgCl2forms a concentration gradient when frozen and needs to be vortexed prior to use.

4.1.2 Every PCR reaction has an optimal MgCl2Bconcentration 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 2µL 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 MgCl2depletion. Between 40-200µM 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.

4.7 **Template is not *Listeria monocytogenes***

4.7.1 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

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

Norton, Dawn M., et al. "Molecular studies on the ecology of Listeria monocytogenes in the smoked fish processing industry." Applied and environmental microbiology 67.1 (2001): 198-205.

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	8/2013	A. Andrus	Original protocol 8/2013
Version 2	04/10/2020	Ser15	Reviewed protocol, formatted to new SOP template added information to satisfy SOP requirements.