<table>
<tr><td> </td><td colspan="3">FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM
Standard Operating Procedure</td><td> </td></tr>
<tr><td colspan="5">Title: rpoB PCR</td></tr>
<tr><td>SOP #: 8.1.1.4.4</td><td>Version: 02</td><td>Revision Date: 04/29/2020</td><td colspan="2">Effective Date: 05/18/2020</td></tr>
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***rpoB* PCR**

FILE NAME: 8.1.1.4.4-rpoB.docx

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SECTION 1 INTRODUCTION

1.1 Purpose

To provide a standard laboratory procedure for PCR of a 632 nt. fragment of *rpoB* (encoding the β subunit of the RNA polymerase) for *Bacillus* and related genera.

This SOP is derived from an earlier protocol titled *"rpoB PCR and Sequencing"* authored by Rachel Miller and Kanika Chauhan. The original protocol can be found in the archived section of the Food Safety Wiki.

1.2 Scope

This SOP applies to the Food Safety Lab, including the Milk Quality Improvement Program. The protocols may also be used by laboratory members from other locations.

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, MgCl2, 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

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
- Primers (see table 1.1 for primers) - Room 352C, chest freezer
- GoTaq Green 2X Master Mix (ProMega Item M7122) - 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 water - Room 358B and Room 350A
- Sterile 96-well plates - Room 358B
- Sterile reservoir basin - Room 358B
- Primers (see table 1.1 for primers) - Room 352C, chest freezer
- GoTaq Green 2X Master Mix (ProMega Item M7122) - Room 352, “Dumb” freezer
- Vortex Mixer - Room 358B
- Miniature Centrifuge - Room 358B
- Thermocycler - Room 356
- Adhesive aluminum foil lids - Room 358B
- Multi-channel pipette
- 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 of *rpoB* gene segment

3.2.1 To prepare 100 µL of the 10uM primer working solutions:
- 3.2.1.1 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.
- 3.2.1.2 Thaw the 100µM primer stock solutions on ice.
- 3.2.1.3 Fill two 1.5ml tubes with 90ul of 10mM Tris-HCL(pH8.0).
- 3.2.1.4 Add 10 µL of your forward primer to one of the 1.5ml tubes, and 10 µL of your reverse primer to the other 1.5ml tube. Label the tubes appropriately.
- 3.2.1.5 Gently vortex each tube to mix.
- 3.2.1.6 These solutions should be frozen at -20°C after use.

3.2.2 Working in Room 358B, prepare a master mix of the components listed in Table 1.1 by pipetting each component into a sterile 1.5 mL microcentrifuge tube.
- 3.2.2.1 Store all master mix components and the prepared master mix on crushed ice.
- 3.2.2.2 Gently vortex and centrifuge down the contents of all reagent containers before opening.
- 3.2.2.3 Calculate master mix volumes required to include positive and negative controls and 1-2 additional reactions to compensate for pipetting errors.
- 3.2.2.4 Pipette up and down to mix after each addition and to ensure that no reagent clings to the pipette tip.
- 3.2.2.5 Briefly vortex the prepared master mix. Spin briefly in the mini centrifuge to concentrate all contents at the bottom of the tube.

SECTION 3 PROCEDURES (CONT'D)

Table 1.1 Primers for *rpoB* PCR amplification

Primer name	Primer Sequence (5' to 3')	Application (Genera)[1]
RZrpoBFV1 RZrpoBRV2	AARYTIGGMCCTGAAGAAAT TGIARTTTRTCATCAACCATGTG	*Bacillus, Paenibacillus*
RZrpoBFV2 RZrpoBRV2	AARYTNGGHCCTGAAGAAAT TGNARYTTRTCATCAACCATGTC	*Bacillus, Paenibacillus*
RZrpoBFV3 RZrpoBRV3	AARYTNGGHCCDGARGAAAT TGNARYTTRTCRTCRACCATGTG	*Bacillus, Geobacillus, Anoxybacillus, Ureibacillus, Viridibacillus, Paenibacillus, Lysinibacillus*

[1]Confirmed amplification of *rpoB* gene segment for isolates from the genera listed

Note: Primers should be selected based on the target organism. RZrpoBV3 primers contain the highest number of ambiguous bases, and are therefore able to amplify the target rpoB sequence of isolates belonging to the broadest range of genera.

Reagent	Volume per each 25 µL reaction
Sterile water	8 µL
Primer Forward (10 µM)	1.25 µL
Primer Reverse (10 µM)	1.25 µL
2X GoTaq Green MasterMix	12.5 µL

3.2.3 Dispense 23 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 Remove the plate or tubes containing the master mix from room 358B to a lab bench.

3.2.5 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.

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

SECTION 3 PROCEDURES (CONT'D)

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

94°C	3 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 -		

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

3.2.8 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. **Wrong Primer Concentration**
- 4.1.1. If you have too little primer you won't see any product.
- 4.1.2. Too much primer may result in primer dimerization, which may reduce amplification.

4.2. **Wrong PCR Program**
- 4.2.1. Check your program while it's cycling to make sure it is the right program

4.3. **Excess or Insufficient Template**
- 4.3.1. 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.
- 4.3.2. 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.

SECTION 5 REFERENCES

Huck, J.R., B.H. Hammond, S.C. Murphy, N.H. Woodcock, and K.J. Boor. 2007. Tracking spore-forming bacterial contaminants in fluid milk-processing systems. J. Dairy Sci. 90: 4872-4883.

Ivy, R.A., M.L. Ranieri, N.H. Martin, H.C. den Bakker, B.M. Xavier, M. Wiedmann, and K.J. Boor. 2012. Identification and characterization of psychrotolerant sporeformers associated with fluid milk production and processing. Appl. Environ. Microbiol. 78:1853 1864.

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	12/20/2019		Original SOP
Version 2	4/29/2020	Ser15	Reformatted to new SOP template