**FOOD SAFETY LABORATORY/MQIP**
**CORNELL UNIVERSITY**

# ***rpoB*** PCR and Sequencing

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**Authored by: Rachel Miller and Kanika Chauhan**
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**Approved by: Martin Wiedmann**

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APPROVED BY:

_____ _____
Dr. Martin Wiedmann (date)

AUTHORED BY:

_____Rachel Miller and Kanika Chauhan_____ _____
(Name) (date)

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## SECTION 1 INTRODUCTION

### 1.1 Purpose

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

***For rpoB PCR to be performed in 96-well plates (high through-put), refer to the "High Through put PCR and Sequencing Reactions" Protocol.***

### 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

### 1.4 Safety

## SECTION 2 MATERIALS

### Lysate Preparation

- Brain Heart Infusion (BHI) agar plates
- Sterile water
- Sterile toothpicks
- Sterile 0.2 mL tubes

### PCR Amplification

- Sterile 0.2 mL tubes
- Sterile water
- Primers (see table 1.1 for primers)
- GoTaq Green 2X Master Mix (ProMega Item M7122)

### Gel Electrophoresis

- 1% agarose gel
- 0.5X TAE buffer

### ExoSAP Purification of PCR Products

- Exonuclease 1 (10 units/μL) (USB Products Item 70073X)
- Shrimp Alkaline Phosphatase (1 unit/μL) (USB Product Item 70092X)

## SECTION 3 PROCEDURES

### 1.1 Lysate Preparation

(1) Plate isolates on BHI agarose plates, and incubate plates at either 32°C or 55°C until visible growth is observed (usually overnight).
(2) Inoculate 100 µL aliquots of sterile water in 0.2 mL Eppendorf tubes with bacterial cells using sterile toothpicks. Note that the inoculated water should appear slightly cloudy, but should not be completely turbid.
(3) Heat tubes containing lysates in a thermocycler (*Note: use program "lysis" stored in the thermocyclers currently in the lab*) at 95°C for 15 minutes, then cool to 4°C. Lysates can be stored at 6°C for up to 2 weeks, or can be stored at -20°C for 6 months. Note, that if storing at -20°C, freeze-thaw cycles should be limited.

### 1.2 PCR amplification of *rpoB* gene segment

(1) Prepare a master mix containing the following reagents, and aliquot 24 µL of the prepared master mix into each 0.2 mL Eppendorf tube or well of a 96 well plate.

Table 1.1 Primers for *rpoB* PCR amplification

| Primer name | Primer Sequence (5' to 3') | Application (Genera)[1] |
|---|---|---|
| RZrpoBFV1<br>RZrpoBRV2 | AARYTIGGMCCTGAAGAAAT<br>TGIARTTTRTCATCAACCATGTG | *Bacillus, Paenibacillus* |
| RZrpoBFV2<br>RZrpoBRV2 | AARYTNGGHCCTGAAGAAAT<br>TGNARYTTRTCATCAACCATGTC | *Bacillus, Paenibacillus* |
| RZrpoBFV3<br>RZrpoBRV3 | AARYTNGGHCCDGARGAAAT<br>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 | 9 µL |
| Primer Forward (10 µM) | 1.25 µL |
| Primer Reverse (10 µM) | 1.25 µL |
| 2X GoTaq Green MasterMix | 12.5 µL |

(2) Add 1 µL of lysate to each reaction.

(3) Perform the reaction using the following set of cycling conditions:

| | | |
|---|---|---|
| 94°C | 3 min | |
| 94°C | 30 sec | 20 cycles at 55-45°, then 20 cycles at 45°C |
| AT* | 30 sec | |
| 72°C | 1 min | |
| 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 with an AT at 45°C

**1.3 Gel Electrophoresis of PCR Products**

(1) Prepare a 1% agarose gel with 0.5X TAE buffer.
*Note: Other percentages of agarose may be used.*
(2) Load 3µL of PCR product per lane.
(3) Electrophorese PCR products at 115 – 120 Volts for 25 – 30 minutes, or until loading dye reaches the edge of the gel.
(4) Image the gel, and ensure that PCR amplification resulted in PCR products of approximately 740 bp.
*Note: PCR products that show unspecific bands should not be used for sequencing. If unspecific bands are detected when the PCR is repeated, perform gel purification before sequencing (see "Troubleshooting"). If gel shows "primer dimers", do not perform ExoSAP purification, but perform column purification instead (see "Troubleshooting").*

**1.4 ExoSAP Purification of PCR Products**

(1) Prepare a master mix containing 1 µL of Exonuclease I (10 units/µL) and 1 µL of Shrimp Alkaline Phosphatase (1 unit/µL), per 20-22 µL of PCR product.
(2) Pipette 2 µL of ExoSAP master mix into each tube containing PCR products which will undergo the sequencing reaction.
(3) Heat PCR products, using a thermocycler (*Note: use program "ExoSAP" stored in current thermocyclers*) with ExoSAP master mix, at 37°C for 45 minutes, and then at 80°C for 15 minutes.

**1.5 Sequencing**

Consult your sequencing facility's requirements for sequencing submissions.

(1) For submission to Cornell BRC, prepare samples by adding 8 µL of water, 8 µL of primer (1 µM) and 2 µL of PCR product. A separate reaction must be prepared for the other primer. Reactions can be prepared in a 96 well plate, or in single tubes.

## SECTION 4 TROUBLESHOOTING

Problems previously encountered using this protocol include:

### 4.1 Lysate preparation

If lysates appear to be turbid following inoculation, lysates may be diluted prior to use as template in the PCR. The template added to each reaction should appear clear, and should not have obvious evidence of bacterial debris.

### 4.2. Failed PCR due to high template concentration

Addition of too much template will inhibit the reaction by exhausting the dNTPs and primers added, before full length products can be generated. If the lysate appears very turbid, dilute the template 1:10 or 1:100 and then use 1 μL of the diluted template for the PCR.

### 4.3 Primer dimers present in gel electrophoresed PCR product

If primer dimers (strong bands of approximately 20-30 bp) appear, use the Qiagen PCR Product Purification kit to purify the PCR product before submitting the product for sequencing.

### 4.4 Unspecific products

If the gel electrophoresis yields multiple PCR products, try running the PCR using a single annealing temperature (i.e. do not run the PCR as a touchdown reaction). Use an annealing temperature of 50°C, for 35 cycles. Alternatively, the reaction can be run performed using a hot-start enzyme.

### 4.3. Failed sequencing reactions

Sequencing reaction may be returned with comments of "Failed" or "Drop Off". If this occurs, try diluting the purified PCR product (dilute 1:10 first) and resubmit the PCR product for sequencing.

## SECTION 5 REFERENCES

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