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Unmarked_Gene_Deletion_Ralstonia_Upstream&Downstream_PCR_Amplification

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We use this protocol and it's working

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Abstract

This protocol describes the amplification and validation of upstream and downstream flanking regions for generating an unmarked gene deletion in *Ralstonia*, following primer design. It outlines the setup and execution of PCRs to obtain clean products suitable for cloning.

The expected outcome is PCR products of the correct size, with strong bands, minimal off-target amplification, and limited primer dimer formation. These products will be used in Gibson Assembly with the pUFR80 vector backbone to enable homologous recombination in later steps of the cloning workflow.

Guidelines

This protocol is intended for generating an unmarked gene deletion in *Ralstonia* using the pUFR80 vector backbone. It assumes primers were designed using the *Unmarked_Gene_Deletion_Ralstonia_Primer_Design* protocol, targeting amplification regions between 400–600 bp. If primer design deviates from that protocol, such as shorter primers or larger/smaller amplification regions, modifications to this PCR setup may be required, and a two-step PCR may not be suitable (see comment on step 3).

Materials

Reagents

- Autoclaved Milli-Q water
- Invitrogen Platinum SuperFi II PCR Master Mix (ThermoFisher #12369010)
- Template DNA (e.g., genomic DNA at 10 ng/µL)
- Upstream forward primer (10 µM)
- Upstream reverse primer (10 µM)
- Downstream forward primer (10 µM)
- Downstream reverse primer (10 µM)
- 1 kb Plus DNA Ladder (NEB #N3200)
- Loading dye (6X)
- Agarose powder
- Ethidium bromide
- Lithium Borate Buffer

Equipment

- Nitrile gloves
- PCR tubes
- Ice bucket and ice
- Thermocycler
- Microcentrifuge (for quick spin-downs)
- Pipettes (P2 and P20) and sterile tips
- Gel casting tray and comb
- Gel electrophoresis apparatus (gel box and power supply)
- UV gel imaging system (gel documentation system)
- Flash drive or other image storage device
- Computer
- Waste container for Ethidium bromide gels as well as gloves and tips that come into contact with Ethidum bromide

Safety warnings

- **Ethidium Bromide Safety:**
  Ethidium bromide is a potent mutagen. Always handle it at a dedicated EtBr work station while wearing nitrile gloves. Avoid touching anything outside the station once gloved. All tips, gloves, gels, and materials that have contacted EtBr must be disposed of in the designated hazardous waste bins at the station.
  When loading PCR products onto the gel, dispose of one glove after pipetting, and replace it with a clean glove before handling any PCR tubes or equipment outside the EtBr zone. This minimizes contamination risk to samples, surfaces, and shared equipment.
- **Master Mix Temperature Sensitivity:**
  Invitrogen Platinum SuperFi II Master Mix should be kept on ice at all times while preparing PCR reactions. Prolonged exposure to room temperature can degrade the polymerase and reduce PCR efficiency. Always return the mix to ice immediately after use.
- **PCR Tube Sealing:**
  Ensure PCR tubes are tightly sealed before placing them in the thermocycler. Improper sealing can lead to evaporation during high-temperature cycles, which may reduce yield or prevent amplification altogether.
- **Gel Ladder Visibility:**
  The smaller bands in the 1 kb Plus DNA ladder may appear faint and tightly spaced. For size estimation, prioritize the stronger reference bands at 0.5 kb, 1 kb, and 3 kb, which are more distinct and easier to identify.

Before start

Follow primer design steps in Unmarked_Gene_Deletion_Ralstonia_Primer_Design to generate your primers for this protocol and make 10 µM concentration working stocks.

PCR Amplification

1 **PCR Mix for Upstream Fragment**

To a labelled PCR tube on ice, add the following in order. Mix by pipetting:

	Item	Volume
	Autoclaved Milli-Q water	6 µl
	Invitrogen Platinum SuperFi II PCR Master Mix (ThermoFisher #12369010)	10 µl
	Template DNA (10 ng/µl)	2 µl
	Upstream forward primer (10 µM)	1 µl
	Upstream reverse primer (10 µM)	1 µl

2 **PCR Mix for Downstream Fragment**

To a separate labelled PCR tube on ice, add the following in order. Mix by pipetting:

	Item	Volume
	Autoclaved Milli-Q water	6 µl
	Invitrogen Platinum SuperFi II PCR Master Mix (ThermoFisher #12369010)	10 µl
	Template DNA (10 ng/µl)	2 µl
	Downstream forward primer (10 ng/µl)	1 µl
	Downstream reverse primer (10 ng/µl)	1 µl

3 **Thermocycler Program**

Briefly spin down both PCR tubes and load them into the thermocycler.

Run the following two-step PCR program (total reaction volume: 20 µL, lid temperature: 105ºC):

- Initial denaturation: 98ºC for 5 minutes
- **35 cycles of:**

1. Denaturation: 98ºC for 15 seconds

2. Annealing/extension: 72ºC for 15 seconds (polymerase works at 15-30 seconds per kb)
- Final extension: 72ºC for 5 minutes
- Hold: 8ºC indefinitely

Start the program. Proceed to the next step during the run, or pause after completion and store PCR products at –20ºC.

4 **Gel Electrophoresis**

Gel Preparation:
- Make a 0.8% agarose gel in Lithium Borate buffer.
- Melt the agarose completely using a microwave.
- Add 0.2–0.5 µg/mL Ethidium bromide (1 drop per 50 mL).
- Pour gel into the casting tray with a comb inserted. Let it solidify for ≥30 minutes.

Sample Loading:
1. Place the gel in the electrophoresis chamber and submerge it in Lithium Borate buffer.
2. Load the wells:
- Well 1: 2 µL of 1 kb Plus DNA ladder (NEB #N3200)
- Well 2: 5 µL of upstream PCR product mixed with 1 µL loading dye
- Well 3: 5 µL of downstream PCR product mixed with 1 µL loading dye

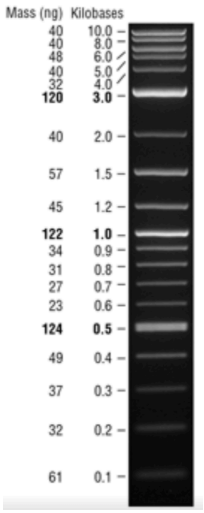
Electrophoresis Setup:
- Seal the lid of the gel box.
- Connect the negative (black) lead of the power supply to the well side of the gel box and the positive (red) lead of the power supply to the far side.
- Set the power supply to run at 90 V for 30 minutes and press start.

Store the remaining PCR products at –20°C.

5 **Product Validation**

Gel Imaging
1. Carefully remove the gel and place it in secondary containment.
2. Image the gel using a gel documentation system.
3. Save the gel image to a flash drive.
4. Clean the gel station and dispose of the Ethidium bromide gel in the appropriate waste container.

Data Analysis:
- Compare band sizes to the ladder.
- Confirm that each product is the expected size based on primer design.
- Check for off-target bands that may be faint.



1 kb Plus DNA ladder (NEB #N3200) reference ladder

If both upstream and downstream fragments are the correct size and free from off-target products, the samples are ready for Gibson Assembly.