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Unmarked_Gene_Deletion_Ralstonia_Colony_PCR

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We use this protocol and it's working

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Abstract

This protocol describes the colony PCR screening step used to validate *E. coli* transformants carrying the experimental plasmid assembled during Gibson Assembly for unmarked gene deletion in *Ralstonia*. It outlines the setup and execution of bulk PCR to identify colonies that acquired the plasmid and are resistant to kanamycin.

The expected outcome is a ~1150 bp PCR product for colonies containing the pUFR80 plasmid with upstream and downstream inserts. Plasmid DNA will be extracted from correctly sized colonies and sequenced for verification prior to transformation into *Ralstonia*.

Guidelines

This protocol is intended for generating an unmarked gene deletion in *Ralstonia* using the *pUFR80* vector backbone. It assumes that Gibson Assembly and subsequent *E. coli* transformation were performed according to the *Unmarked_Gene_Deletion_Ralstonia_Gibson_Assembly_&_Transformation* protocol, with upstream and downstream inserts cloned between the M13 primer binding sites. If plasmid assembly was carried out using a different strategy, the M13 primers may not be suitable, and modifications to this protocol may be necessary.

Materials

Reagents

- Isolated colonies from transformation plates (Unmarked_Gene_Deletion_Ralstonia_Gibson_Assembly_&_Transformation)
- *pUFR80* plasmid DNA (undigested stock, for negative control)
- Autoclaved Milli-Q water
- Nuclease-free water
- M13 forward and reverse primers specific to *pUFR80* backbone
- OneTaq® Hot Start Quick-Load® 2X Master Mix with GC Buffer (NEB #M0488)
- 1 kb Plus DNA Ladder (NEB #N3200)
- Loading dye (6X)
- Agarose powder
- Ethidium bromide (EtBr)
- Lithium Borate (LB) Buffer
- LB broth
- Kanamycin (25 mg/mL stock)
- 75% Glycerol (for backup stocks)

Equipment

- PCR tubes
- Microcentrifuge tubes (1.7 mL)
- Pipettes (P2, P20, P200, P1000) with sterile filtered tips
- Ice bucket and ice
- Microcentrifuge (for quick spin-downs and pelleting)
- Thermocycler
- Gel casting tray and comb
- Gel electrophoresis apparatus (gel box and power supply)
- UV gel imagining system (gel documentation system)
- Flash drive or data storage device
- Benchtop shaker incubator (37ºC, 250 rpm)
- Parafilm
- Waste container for Ethidium bromide gels and contaminated materials

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.
- **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.
- **Troubleshooting Failed Screens:**
  If no samples amplify to the correct size or all plasmid isolates have been sequenced and a correct clone has not been obtained, it is necessary to return to the *E. coli* transformation step in the *Unmarked_Gene_Deletion_Ralstonia_Gibson_Assembly_&_Transformation* protocol and screen a new set of colonies.

Before start

Follow Gibson Assembly and *E. coli* transformation steps in *Unmarked_Gene_Deletion_Ralstonia_Gibson_Assembly_&_Transformation* to obtain the 10 sample colonies that will be used for colony PCR.

Colony PCR

1 Sample Preparation

Label 10 PCR tubes for your colony samples.

Add 9 µL of autoclaved Milli-Q water to each tube.

Using a sterile pipette tip, pick an isolated colony from each of the 10 transformation plates and mix thoroughly into the respective PCR tube to disperse cells.

Spin down the PCR tubes.

2 PCR Setup

Prepare a PCR master mix on ice for 11 reactions (10 samples and 1 control), with an extra 10% to account for pipetting error, by adding the following in order. Mix by pipetting:

	Item	Volume per Reaction	Total Volume
	Autoclaved Milli-Q water	3.6 µl	43.56 µl
	OneTaq® Hot Start Quick-Load® 2X Master Mix with GC Buffer (NEB #M0488)	5 µl	60.5 µl
	M13F primer	0.2 µl	2.42 µl
	M13R primer	0.2 µl	2.42 µl

Spin down the master mix.

Prepare PCR tubes:

1. Aliquot 9 µL of the master mix into each PCR tube.
2. Add 1 µL of each colony lysate to the corresponding sample tube.
3. For the control tube, add 1 µl of empty *pUFR80* plasmid to the PCR tube.
4. Briefly spin down the PCR tubes.

3 Thermocycler Program

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

Run the following three-step PCR program (total reaction volume: 10 µL, lid temperature: 105ºC):

A. Initial denaturation: 94ºC for 30 seconds

B. **35 cycles of**:

- Denaturation: 94ºC for 30 seconds

- Annealing: 53ºC for 30 seconds
- Extension: 68ºC for 1 minute 15 seconds (polymerase works at 15-30 seconds per kb)

C. Final extension: 68ºC for 5 minutes

D. 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 2-11: 5 µL of sample PCR product mixed with 1 µL loading dye
- Well 12: 5 µL of empty *pUFR80* 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**

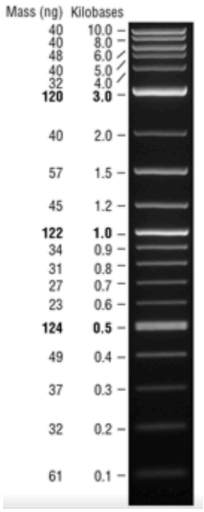
Gel Imaging

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 and no insert *pUFR80* control.

- Expected result is for bands to be ~1150 bp if the sample contains the upstream and downstream fragments inserted into the *pUFR80* plasmid.
- If samples contain empty *pUFR80* plasmid, such as the no insert control, the PCR will only amplify a 146 bp region of the plasmid.



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

6 Liquid Culture Preparation

For colonies showing correct band size:
A. Prepare test tubes with:

- 3 mL LB broth
- 3 µL of **25 mg/mL kanamycin**

B. Inoculate each with a colony from successful PCR reactions.
C. Incubate overnight at 37°C, shaking at 250 rpm.

7 Miniprep & Glycerol Stock

Use 500 µL of each overnight culture to make glycerol stocks (500 µL culture + 500 µL 75% glycerol).

Use the **IBI Scientific I-Blue Mini Plasmid Kit** (IB47170) to isolate plasmid DNA per manufacturer's instructions.

Elute DNA in nuclease-free water.

8 Sequencing

Submit **10 µL of purified plasmid DNA (20–200 ng/µL)** to a sequencing provider such as Plasmidsaurus (Oxford Nanopore sequencing).

Compare the sequence to the **NEBuilder** predicted construct in **Benchling** from the *Unmarked_Gene_Deletion_Ralstonia_Primer_Design* step.

If incorrect, sequence another plasmid isolate until a correct clone is identified or all samples have been exhausted.