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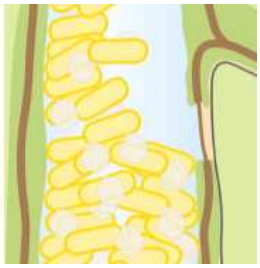
Unmarked_Gene_Deletion_Ralstonia_Gibson_Assembly_&_Transformation



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We use this protocol and it's working

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Abstract

This protocol describes the linearization of the *pUFR80* plasmid and Gibson Assembly of upstream and downstream flanking regions for unmarked gene deletion in *Ralstonia*. It includes the subsequent chemical transformation of *E. coli* with the assembled plasmid.

The expected outcome is the recovery of *E. coli* colonies that have successfully taken up the plasmid and can grow in the presence of kanamycin. These colonies will be screened by colony PCR to confirm plasmid presence, followed by plasmid extraction and sequencing for validation.

Guidelines

This protocol is intended for generating an unmarked gene deletion in *Ralstonia* using the *pUFR80* vector backbone. It assumes the upstream and downstream flanking regions were amplified according to the approach outlined in the *Unmarked_Gene_Deletion_Ralstonia_Upstream&Downstream_PCR_Amplification* protocol, with overhangs compatible for the Gibson Assembly method described in this protocol. If the flanking regions were amplified using a different strategy, such as primer design based on alternative restriction sites for vector linearization, modifications to this protocol may be necessary.

Materials

Reagents

- Upstream PCR product from Unmarked_Gene_Deletion_Ralstonia_Upstream&Downstream_PCR_Amplification
- Downstream PCR product from Unmarked_Gene_Deletion_Ralstonia_Upstream&Downstream_PCR_Amplification
- Chemically competent *E. coli*
- Nuclease-free water
- pUFR80 plasmid DNA (undigested stock)
- HindIII-HF (NEB #R3104)
- EcoRI-HF (NEB #R3101)
- 10X rCutSmart Buffer (NEB #B6004)
- Zymo DNA Clean & Concentrator Kit
- Gibson Assembly Master Mix (NEB #E2611)
- SOC medium
- LB agar plates with kanamycin
- 1 kb Plus DNA Ladder (NEB #N3200)
- Loading dye (6X)
- Agarose powder
- Ethidium bromide
- Lithium Borate Buffer

Equipment

- Nitrile gloves
- PCR tubes
- Microcentrifuge tubes (1.7 mL)
- Ice bucket and ice
- Thermocycler
- Microcentrifuge (for quick spin-downs and pelleting)
- Pipettes (P2, P20, P200, P1000) 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
- Benchtop shaker incubator (37ºC, 250 rpm)
- Waste container for Ethidium bromide gels and contaminated materials
- Parafilm
- Sterile glass beads or sterile hockey stick (for spreading cells)

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.
- **Band Interpretation:**
  Undigested pUFR80 may appear as a single, diffuse or smeared band rather than two distinct bands representing supercoiled and nicked/open circular forms. This is because the different conformations can migrate similarly and overlap, especially in lower-percentage agarose gels. In contrast, the linearized plasmid, produced by restriction digest, should appear as a single, sharper band that migrates more slowly and thus runs higher on the gel than the undigested form.

Before start

Follow upstream and downstream amplification steps in Unmarked_Gene_Deletion_Ralstonia_Upstream&Downstream_PCR_Amplification to obtain flanking regions with overhangs for Gibson Assembly.

Restriction Digest

1 **Restriction Digest Calculations**

Calculate the volume of pUFR80 plasmid needed to obtain 1000 ng in a 50 µL reaction.

Example: If pUFR80 is at 250 ng/µL, add 4 µL.

2 **Restriction Digest Setup**

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

	Item	Volume
	Autoclaved Milli-Q water (fill to 50 µl)	43 - X µl
	10X rCutSmart Buffer (NEB #B6004)	5 µl
	pUFR80 at initial concetration	X µl
	HindIII-HF (NEB #R3104)	1 µl
	EcoRI-HF (NEB #R3101)	1 µl

3 **Restriction Digest Incubation**

Briefly spin down the PCR tube and load it into the thermocycler.

Run the following program (total reaction volume: 50 µL, lid temperature: 105ºC):
- Incubation: 37ºC for 3 hours

Start the program. Proceed to the next step during the run, or pause after completion and store product 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: 4 µL of non-digested *pUFR80* plasmid + 1 µL loading dye
- Well 3: 4 µL of *pUFR80* plasmid digested with HindIII-HF and EcoRI-HF + 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.

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.
- Expected bands for undigested *pUFR80* is two bands both smaller then the linearized product
- Expected band for linearized *pUFR80* product is a single band ~7.8 kb.

6 **Plasmid Purification**

Use the Zymo Clean & Concentrator Kit to purify the digested *pUFR80* plasmid according to the manufacturer's instructions. Elute in nuclease-free water.

Gibson Assembly

7 **Gibson Assembly Calculations**

Perform calculations based on the concentration of *pUFR80* plasmid digested with HindIII-HF and EcoRI-HF obtained after plasmid purification.

A reaction with a final volume of 10 µl should contain 40 ng of *pUFR80* plasmid digested with HindIII-HF and EcoRI-HF.

8 **Gibson Assembly Setup**

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

	Item	Volume
	Autoclaved Milli-Q water (fill to 10 µl)	8.5 - X µl
	Upstream PCR product	0.25 µl
	Downstream PCR product	0.25 µl
	Digest pUFR80 (40 ng total)	X µl
	2X Gibson Assembly Mix (NEB #E2611)	1 µl

9 **Gibson Assembly Program**

Briefly spin down the PCR tube and load it into the thermocycler.

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

- Incubation: 50ºC for 1.5 hours

Start the program. Proceed to the next step after the run has completed, or pause after completion and store product at –20ºC.

E. coli Transformation

10 **Transformation Setup**

Retrieve a chemically competent *E. coli* stock in a 1.7 micro centrifuge tube from -80ºC and place it on ice.

Immediately after it has thawed, add 2 µl of Gibson Assembly product and allow the cells to sit for 15 minutes, still on ice.

Recovery

After the 15 minutes, add 1000 µl of SOC to the *E. coli* tube and place the entire tube in a 37ºC shaker incubator (250 rpm) for an outgrowth of 1 hour

11 **Dilution and Plating**

Take the recovered *E. coli* cells from the outgrowth and prepare a 10 fold and 100 fold dilution of the cells.

Label 4 LB + kanamycin plates. Plate as follows:

- Plate 1: 100 µL undiluted
- Plate 2: 100 µL of 10^{-1} dilution
- Plate 3: 100 µL of 10^{-2} dilution
- Plate 4: Pellet remaining cells, discard supernatant, resuspend pellet, and plate residual volume

12 **Plate Handling**

Spread evenly with sterile beads or hockey stick.

Allow plates to dry.

Parafilm seal, invert, and incubate overnight at 37°C.

13 **Colony Picking & Streaking**

Check plates for colonies and streak out 10 isolated colonies onto new LB plates with kanamycin.

Incubate plates overnight at 37ºC.

Store original plates at 4ºC in case restreaks fail.

14 **Plate Storage**

Check the streak outs and store the plates at 4ºC with Parafilm until ready to proceed with colony PCR.