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Unmarked_Gene_Deletion_Ralstonia_Electroporation_Transformation

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Noah Guillome[1]

[1]Lowe-Power Lab, UC Davis

guillome
Lowe-Power Lab, UC Davis

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We use this protocol and it's working

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Abstract

This protocol describes the electroporation-based transformation of a modified *pUFR80* plasmid into *Ralstonia*, as part of the workflow for an unmarked gene deletion in *Ralstonia*. It includes selection on kanamycin, counter-selection on sucrose, and preparation for downstream PCR and sequencing validation.

The expected outcome is recovery of kanamycin-resistant colonies following plasmid integration via homologous recombination. A second recombination event enables sucrose resistance, yielding either wild-type revertants or the desired deletion mutants.

Guidelines

This protocol is intended for generating an unmarked gene deletion in *Ralstonia* using the *pUFR80* vector backbone. It assumes the *pUFR80* with deletion construct was assembled using the *Unmarked_Gene_Deletion_Ralstonia_Gibson_Assembly_&_Transformation* protocol, subsequently validated by the *Unmarked_Gene_Deletion_Ralstonia_Colony_PCR* protocol. If modifications were made from either of those protocols, transformation efficiency into *Ralstonia* may be reduced or fail altogether.

Materials

Reagents

- Sequence-confirmed *pUFR80* plasmid (e.g., *pUFR80* with deletion construct)
- Electrocompetent *Ralstonia* cells (same strain background used in gene deletion)
- CPG liquid medium (per liter: 1 g Casamino acids, 10 g Bacto-Peptone, 5 g Glucose (Dextrose), 1 g Yeast extract, and fill to 1000 ml total with Milli-Q water)
- CPG agar (500 µl CPG liquid medium with 7.5 g agarose)
- TZC (Triphenyltetrazolium chloride)
- Kanamycin 25 mg/ml
- Sucrose (7.5% final concentration)
- Autoclaved Milli-Q water
- Nuclease-free water
- 75% glycerol

Equipment

- Nitrile gloves
- PCR tubes
- 1 mm electroporation cuvettes
- Electroporator (settable to 900 V)
- Ice bucket and ice
- 2.0 ml microcentrifuge tubes
- Thermocycler
- Benchtop shaker incubator (28°C, 250 rpm)
- Pipettes (P2, P20, P200, P1000) and sterile tips
- Sterile glass beads or sterile hockey stick (for spreading cells)
- Parafilm

Safety warnings

- **Troubleshooting Failure (No Growth on Kanamycin)**:
  If no colonies appear after kanamycin selection, likely causes include:
  A. Delayed handling during electroporation (see *Transformation Speed* below).
  B. Low-density electrocompetent cells.
  C. Poor electroporation pulse (e.g., improper voltage or arc discharge).
  In these cases, repeat the transformation using freshly prepared competent cells and verify plasmid concentration and pulse quality.
- **False Positives (Growth on Kanamycin During Final Screen)**:
  If colonies grow on kanamycin after sucrose counter-selection, they may still carry the plasmid in a merodiploid state (first recombination only). Only isolates that fail to grow on kanamycin should move forward to colony PCR. If too few pass this screen, sucrose counter-selection may have been ineffective and it is necessary to check media preparation and sucrose concentration (should be 7.5%). If media prep is correct, return to backup kanamycin-resistant stocks, streak on non-selective plates, inoculate liquid culture in non-selective media, and continue with plating onto 7.5% sucrose. Avoid reusing backups more than once or twice and beyond that, restart the transformation process.
- **Sucrose Growth Delay**:
  Ralstonia grows slowly on 7.5% sucrose plates and may take a variable amount of time ranging from 2-3 days in the incubator to become visible and ready for the next step. Make sure to plan accordingly around this possibility.
- **Transformation Speed**:
  Work quickly once cells thaw to improve transformation efficiency.
  A. Pre-calculate plasmid volumes based on concentration.
  B. Label recovery tube in advance.
  C. Add plasmid immediately after thawing cells.
  D. Preset pipettes with required volumes.
  E. Prepare 1000 µl CPG during electroporation to add once electroporator signals the end of the pulse.

Before start

Follow validation steps outlined in *Unmarked_Gene_Deletion_Ralstonia_Colony_PCR* to confirm modified *pUFR80* contains the deletion construct.

Electroporation Transformation

1 **Transformation Setup**

Retrieve sequence-confirmed plasmid from the *Unmarked_Gene_Deletion_Ralstonia_Colony_PCR* protocol, stored at -20ºC. Thaw on ice.

Place a 1mm electroporation cuvette on ice.

Retrieve electrocompetent *Ralstonia* cells (same strain background used for gene deletion) from -80ºC. Thaw on ice.

2 **Electroporation**

Add 1000 ng of sequence-confirmed plasmid (e.g., 10 µl if 100 ng/µl) to the competent cells. Mix by gently flicking the tube.

Transfer 200 µl of the mixture into the chilled electroporation cuvette.

Wipe outside of cuvette with a Kimwipe.

Insert cuvette into the electroporator (dent toward the back).

Set electroporator to 900 volts and pulse.

Recovery

After the pulse (signaled by a beep), add 1000 µl CPG directly to the cuvette.

Resuspend by pipetting up and down twice.

Transfer full volume to a 2 ml microcentrifuge tube.

Incubate at 28ºC shaker incubator (250 rpm) for 4 hours.

3 **Dilution and Plating**

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

Label 4 CPG + 1% TZC + kanamycin plates as follows:
1. Plate 1: 100 µl undiluted

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

4 **Plate Handling**

Spread evenly with sterile beads or hockey stick.

Allow plates to dry.

Incubate for 3-4 days at 28ºC.

5 **Liquid Culture Preparation**

A. Prepare a test tube with:
- 3 mL CPG broth

B. Inoculate with an isolated colony, choosing from the colonies with kanamycin resistance
C. Incubate overnight in shaker incubator at 28ºC and 250 rpm.

SacB Counter-selection

6 **Backup Kanamycin Resistant Stock**

Use 500 µl of the overnight culture prepared the day prior to make backup glycerol stocks (500 µl culture + 500 µl 75% glycerol).

Store at -80ºC

7 **Dilution and Plating**

Prepare a 10 fold, 100 fold, and 1000 fold dilutions of the liquid culture, using autoclaved Milli-Q water to dilute.

Label 3 CPG + 1% TZC + 7.5% sucrose plates as follows:
- Plate 1: 100 µl of 10^{-1} dilution
- Plate 2: 100 µl of 10^{-2} dilution
- Plate 3: 100 µl of 10^{-3} dilution

8 **Plate Handling**

Spread evenly with sterile beads or hockey stick.

Allow plates to dry.

Incubate for 2-3 days at 28°C.

Loss of Kanamycin Resistance Screen

9 **Patch Plating**

Select 24 diverse colonies from sucrose plates.

For each isolate:

- Streak a lawn onto **CPG + 1 %TZC** (4 isolates per plate).
- Using the same stick, patch streak onto **CPG + 1% TZC + kanamycin** plate (24 isolates total).
- Use new stick per isolate to streak from lawn and fill out a quadrant of the **CPG + 1% TZC** plate.
- Incubate for 2 days at 28ºC.

10 **Liquid Culture Preparation**

For isolates that do not grow on kanamycin:
A. Prepare a test tube with:

- 3 mL CPG broth

B. Inoculate with an isolated colony from the **CPG + 1% TZC** plate.
C. Incubate overnight at 28°C, shaking at 250 rpm.

These will be used for making backup glycerol stocks as well as colony PCR for a final external PCR screen.