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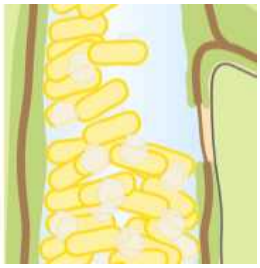
Unmarked_Gene_Deletion_Ralstonia_External_Ralstonia_Colony_PCR



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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 to validate *Ralstonia* transformants that successfully deleted the target region during unmarked gene deletion in *Ralstonia*. It outlines the setup and execution of bulk PCR to identify colonies lacking the gene of interest.

The expected outcome for a knockout is a PCR product smaller than the wildtype control, reflecting loss of the deleted region. Linear DNA from correctly sized PCR products will be purified and sequenced for verification prior to long-term storage.

Guidelines

This protocol is intended for generating an unmarked gene deletion in *Ralstonia* using the *pUFR80* vector backbone. It assumes the *Ralstonia* transformants were obtained and underwent selection and counter-selection in accordance to the *Unmarked_Gene_Deletion_Ralstonia_Electroporation_Transformation* protocol. The goal is to differentiate wildtype revertants from true gene deletion mutants using PCR amplification of the target locus followed by gel electrophoresis. PCR conditions (e.g., annealing temperature, extension time) may require optimization depending on the size and GC content of the target region.

Materials

Reagents

- Autoclaved Milli-Q water
- Nuclease-free water
- 10 µM external forward and reverse primers (from *Unmarked_Gene_Deletion_Ralstonia_Primer_Design*)
- Bio-Rad InstaGene™ Matrix (Cat# 7326030)
- 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 (0.2-0.5 µg/ml)
- Lithium Borate buffer
- 75% Glycerol
- Zymo DNA Clean & Concentrator-5 Kit (D4004)

Equipment

- PCR tubes
- Pipettes (P2, P20, P200, P1000) with sterile tips
- Ice bucket and ice
- Microcentrifuge
- Thermocycler
- Vortex mixer
- 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
- 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 DNA samples have been sequenced and a correct clone has not been obtained, it is necessary to return to the kanamycin-resistant stocks from the Unmarked_Gene_Deletion_Ralstonia_Electroporation_Transformation protocol and screen a new set of colonies. Avoid reusing backups more than once or twice and beyond that, restart the transformation process.

Before start

Prepare liquid culture of wildtype strain background to use as control in external colony PCR screen. Follow selection and counter-selection steps outlined in *Unmarked_Gene_Deletion_Ralstonia_Electroporation_Transformation* to obtain liquid cultures of transformants for external PCR screening.

Ralstonia Colony PCR

1 **Backup Glycerol Stock**

Use 500 µl of the overnight culture to make backup glycerol stocks (500 µl culture + 500 µl 75% glycerol) for each transformant that passed up to this point.

Store at -80ºC.

2 **Sample Preparation**

Label 8 PCR tubes (1 control and 7 samples).

Add 35 µl of autoclaved Milli-Q water to each tube.

Add 5 µl of homogenous Bio-Rad InstaGene™ Matrix (#7326030) to each PCR tube.

Centrifuge 1 ml of overnight culture at 16,000 rcf for 1 minute then discard the supernatant. Repeat this step for 7 total transformants and the 1 wildtype control.

Using a sterile 200 µl pipette tip, grab a small, but visible amount of cells from the pellet and mix thoroughly into the respective PCR tube to disperse cells. Store pellets at -20ºC.

Vortex for 10 seconds, then incubate at 56ºC in thermocycler for 30 minutes.

Vortex for 10 seconds, then incubate at 100ºC in thermocycler for 8 minutes.

Vortex for 10 seconds, then spin down for 2 minutes.

Use clear supernatant as the DNA template. Store at -20ºC after use.

3 **PCR Setup**

Reflect on notes from *Unmarked_Gene_Deletion_Ralstonia_Primer_Design* to identify corresponding external forward and reverse primers.

Prepare a PCR master mix on ice for 8 reactions (7 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	7.5 µl	66 µl

	Item	Volume per Reaction	Total Volume
	OneTaq® Hot Start Quick-Load® 2X Master Mix with GC Buffer (NEB #M0488)	12.5 µl	110 µl
	External forward primer (10 µM)	1 µl	8.8 µl
	External reverse primer (10 µM)	1 µl	8.8 µl

Spin down the master mix.

Prepare PCR tubes:
1. Aliquot 22 µl of the master mix into each PCR tube.
2. Add 3 µl of each colony lysate to the corresponding sample tube.

4 **Thermocycler Program**

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

Run the following PCR program (total reaction volume: 25 µl, lid temperature: 105ºC):
A. Initial denaturation: 94ºC for 5 minutes
B. **35 cycles of**:
- Denaturation: 94ºC for 30 seconds
- Annealing: 53ºC for 30 seconds
- Extension: 68ºC for X seconds (polymerase works at 1 kb per minute, set X to be long enough to amplify wildtype sequence length)

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.

5 **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 & 10: 2 µl; of 1 kb Plus DNA ladder (NEB #N3200)
- Well 2: 4 µl of wildtype control PCR product

- Well 3-9: 4 µl of sample PCR product

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 45 minutes and press start.

Store the remaining PCR products at -20°C.

6 **Product Validation**

Gel Imaging

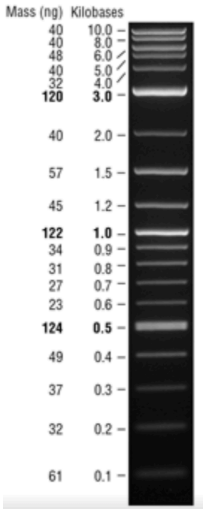
1. Carefully remove the gel and place it in secondary containment.
2. Image the gel using a gel documentation system.
3. Save the gel image to a flash drive.
4. Clean the gel station and dispose of the Ethidium bromide gel in the appropriate waste container.

Expected results:

- Wildtype reversion: bands at same height as control
- Knockout: band shifted lower (missing deleted region)

Data Analysis:

- Compare band sizes to the ladder and wildtype control.
- The difference in size between wildtype and a knockout should be equal to the length of the region targeted for deletion.
- Compare expected band sizes using Benchling files created during primer design.



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

Sequence Validation

7 **DNA Cleanup**

Use the **Zymo Scientific DNA Clean & Concentrator-5 Kit** (D4004) to purify linear DNA for PCR products with the knockout band size, following manufacturer's instructions.

Elute DNA in nuclease-free water.

8 **Sequencing**

Submit **10 µl of purified linear DNA (20–200 ng/µl)** from one PCR product to a sequencing provider such as Plasmidsaurus (Oxford Nanopore sequencing).

Store purified linear DNA samples at -20ºC.

Compare the sequence in Benchling to the original sequence from the *Unmarked_Gene_Deletion_Ralstonia_Primer_Design* step to ensure only the deletion target is missing and there are no mutations elsewhere.

If incorrect, sequence another DNA sample until a correct knockout is identified or all samples have been exhausted.