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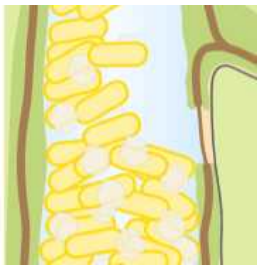
Unmarked_Gene_Deletion_Ralstonia_Primer_Design



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We use this protocol and it's working

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Abstract

This protocol describes the primer design step in generating an unmarked gene deletion in *Ralstonia* using the *pUFR80* vector backbone. It guides the design of primer pairs to amplify ~500 bp upstream and downstream homologous regions with overhangs compatible with pUFR80 linearized by HindIII-HF and EcoRI-HF.

The goal is to generate primers that minimize off-targets, have self-complementarity scores ≤ 4 (and 3′ scores ≤ 2), and avoid amplifying the target gene while preserving nearby coding and regulatory regions. These primers are then used to build fragments for later steps in plasmid construction and genome integration.

Guidelines

This protocol is intended for generating an unmarked gene deletion in *Ralstonia* using the *pUFR80* vector backbone. It assumes the use of NEBuilder for plasmid assembly and Benchling for sequence management. If working with a different organism, plasmid backbone, or assembly method, primer design parameters and workflow may need to be adjusted.

Materials

Computer with internet connection

Safety warnings

- In difficult sequence regions, it may be necessary to use primers with self-complementarity scores > 4 or 3′ scores > 2. These primers may still work, but they often have reduced efficiency and an increased risk of primer-dimer formation.
- Avoid using primer pairs with off-target products unless the unintended product is significantly larger than the expected product and can be excluded by setting a short elongation time during PCR that could not amplify the off-target product.
- For challenging regions where standard primer placement fails, refer to the troubleshooting comment in Steps 2 and 3. Ensure primer placement does not result in inclusion of coding regions from the neighboring genes in the sequence between the upstream and downstream fragments. This would result in their unintended partial deletion later during the cloning process.

Primer Design

1 **Identify the Target Gene**

- Input the gene ID of the gene targeted for deletion into **NCBI** to determine its genomic coordinates.
- Extract a **10 kb sequence** surrounding the gene of interest: **5 kb upstream** and **5 kb downstream**.
- Import the sequence into **Benchling** for annotation and future reference.
- If the gene is on the reverse strand (i.e., oriented to the left), reverse complement the sequence so that it reads left-to-right, with the upstream region on the left and downstream on the right.

2 **Upstream Primers**

- Paste the full 10 kb sequence into **NCBI's Primer-BLAST**.
- For the **reverse primer**, select the **5'→3' sequence 20 bp directly upstream** of the gene of interest on the **minus strand**. (If the upstream gene overlaps with your gene of interest, use the **last 20 bp** of the upstream gene)
- Set the desired **PCR product size** to **400–600 bp**.
- Under "Primer Pair Specificity Checking Parameters", select the **"nt" database** and set the **Organism** to match your strain. For example, if working with **GMI1000**, select:

```
Ralstonia pseudosolanacearum (taxid: 267608)
```

- Run the Primer-BLAST and select a primer pair with minimal off-targets and low complementary scores (see comments for troubleshooting information)

3 **Downstream Primers**

- Use the same 10 kb sequence and **NCBI's Primer-BLAST** tool.
- For the **forward primer**, select the **5'→3' sequence 20 bp directly downstream** of the gene of interest on the **plus strand**. (If the downstream gene overlaps with your gene of interest, use the **first 20 bp** of the downstream gene)
- Set the desired **PCR product size** to **400–600 bp**.
- Under "Primer Pair Specificity Checking Parameters", select the **"nt" database** and set the **Organism** to match your strain. For example, if working with **GMI1000**, select:

```
Ralstonia pseudosolanacearum (taxid: 267608)
```

- Run the Primer-BLAST and select a primer pair with minimal off-targets and low complementary scores (see comments for troubleshooting information)

4 **Experimental Plasmid Construction**

Open up **NEBuilder** assembly tool

Prepare the Vector Backbone

- In Benchling, open the reference plasmid ***pUFR80*** and copy its full sequence.
- In NEBuilder, select "Add New Fragment" and paste the *pUFR80* sequence and check both the Vector and Circular options.
- For producing a linearized fragment, select **Restriction Digest** method.
- Set the 5′ flank enzyme to **HindIII-HF** and the 3′ flank enzyme to **EcoRI-HF** and add the fragment.

Add the Upstream Fragment

- In Benchling, identify the full sequence amplified by the **upstream primer pair**, based on flanking regions and primer pair generated via NCBI Primer-BLAST.
- Copy this amplified upstream sequence.
- In NEBuilder, select "Add New Fragment" and paste the sequence.
- For producing a linearized fragment, select **PCR** method and add the fragment.

Add the Downstream Fragment

- In Benchling, identify the full sequence amplified by the **downstream primer pair**, based on flanking regions and primer pair generated via NCBI Primer-BLAST.
- Copy this amplified downstream sequence.
- In NEBuilder, select "Add New Fragment" and paste the sequence.
- For producing a linearized fragment, select **PCR** method and add the fragment.

Finalize and Export

- Save the NEBuilder project and export the **JSON file** for documentation.
- In the project summary, locate the primer pairs designed for each fragment. NEBuilder will color-code primer binding regions to indicate homology.
- Use this summary to **order the primers**.

Archive the Assembled Plasmid

- Copy the entire assembled plasmid sequence from NEBuilder.
- Paste the sequence into Benchling and save it as a new plasmid entry.
- Run "Auto-Annotate" using the *pUFR80* reference plasmid.
- Manually annotate the **upstream** and **downstream fragments** based on the inserted sequences. These annotations will support future alignment after plasmid sequencing.

5 **External Primers**

- Use the same 10 kb sequence and **NCBI's Primer-BLAST** tool.
- Define a search range within Primer-BLAST: forward primer at least 100 bp before the upstream region and reverse primer at least 100 bp after the downstream region.
- Set the desired **PCR product size** to **< 4500 bp**.

- Under "Primer Pair Specificity Checking Parameters", select the **"nt" database** and set the **Organism** to match your strain. For example, if working with **GMI1000**, select:

```
Ralstonia pseudosolanacearum (taxid: 267608)
```

- Run the Primer-BLAST and select a primer pair with minimal off-targets and low complementary scores