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Molecular Cloning

In 1 collection

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We use this protocol and it's working

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Abstract

Protocol for cloning GEM-SCOPe plasmids via Gibson reaction

Design Cloning Reaction

1 Acquire sequences for the plasmid backbone and inserts

2 Upload into NEBuilder Assembly Tool (nebuilder.neb.com) and follow the steps to get primers with 5' and 3' ends compatible for Gibson assembly

3 Order primers and necessary templates

Linearize Plasmid

4 Mix 5ug of backbone plasmid (pFUW; Addgene #14882) with 2 uL NheI-HF (NEB R3131), 2 uL BamHI-HF (NEB R3136), 5 uL 10x CutSmart Buffer.

5 Add nuclease free water to a final volume of 50uL

6 Incubate at 37°C for at least 1 hour

7 Heat inactivate at 65°C for 20 minutes 20m

8 Run the whole sample on a 1% agarose gel

9 Purify the desired gel fragment with a gel purification kit of choice

PCR Amplification of Inserts

10 Combine 1 uL 10uM Forward Primer, 1 uL 10uM Reverse Primer, and 10 uL Q5 High-Fidelity 2X Master Mix (NEB M0493)

11 Add 20ng of template DNA

12 Add nuclease free water to a final volume of 20 uL

13 Repeat steps 7-9 for each insert

14 Run the PCR in a thermocycler. The following is for inserts of about 1000bp. Adjust annealing temperature and elongation time accordingly.
1. 98°C for 30s
2. Repeat the following 12x
- 98°C for 10s
- 72°C for 20s
- 72°C for 30s
3. Repeat the following 28x
- 98°C for 15s
- 60°C for 15s
- 72°C for 30s
4. 72° for 2m
5. Hold at 4°C

15 Run the PCR reactions on a 2% agarose gel to verify band size. Purify the gel fragments with a gel purification kit.

Gibson Assembly

1h

16 Add nuclease free water to a final volume of 10 uL

17 Combine 5uL HiFi DNA Assembly Master Mix (NEB E2621), 50 ng of linearized backbone with 3-fold molar excess of each insert (**NEBioCalculator**)

18 Prepare a negative control with 5uL HiFi DNA Assembly Master Mix, 50 ng of linearized backbone, and nuclease free water to a final volume of 10 uL.

19 Incubate at 50°C for 1h

1h

Purify Plasmid

30m

20 Thaw a tube of 10-beta Competent *E. coli* (NEB C3019) on ice for 10 minutes. Transfer 10uL into a fresh tube on ice.

21 Add 2uL of Gibson reaction to the bacteria and flick to mix

22 Keep the mixture on ice for 30m 30m

23 Heat shock at 42°C for 30s 30s

24 Place on ice for 5m 5m

25 Add 250uL of room temperature NEB 10-beta/Stable Outgrowth Medium

26 Place at 37°C for 60 minutes with vigorous shaking 1h

27 Meanwhile, warm up LB Agar plates with 100ug/mL ampicillin to 37°C

28 Spread 150uL of bacterial mixture onto plates

29 Incubate overnight at 37°C

30 Pick 1-3 colonies for mini prep isolation