Nicking Mutagenesis: comprehensive single-site saturation mutagenesis

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Method Article

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Abstract

The purpose of this method is to generate comprehensive single-site saturation mutagenesis libraries. The required input for Nicking Mutagenesis is double-stranded plasmid DNA, and any plasmid dsDNA can be used provided that it contains a 7-base pair BbvCI recognition site. The method works by first creating an ssDNA template using a site and strand specific nicking endonuclease \(Nt.BbvCI) followed by exonuclease digestion of the nicked strand. After creation of a heteroduplex by thermal cycling template with mutagenic oligos, the parental template ssDNA is destroyed by employing the opposite strand nicking endonuclease \(Nb.BbvCI) followed by exonuclease digestion. A schematic of the method is outlined in Figure 1. The protocol can be completed in a single day \(with transformation) and libraries can be harvested the following day.

Introduction

Notes: Plasmid dsDNA should be prepared fresh \(<1 month old, avoid freeze/thaw) from a dam+ E. coli strain \(i.e. XL1-Blue), and should be at a concentration sufficient to add 0.76 pmol dsDNA in ≤15 µL. Quality of the input dsDNA substrate is important. Mutagenic oligos are designed using the Agilent QuikChange Primer Design Program \(www.agilent.com). **Troubleshooting/learning Nicking Mutagenesis:** Green/white fluorescent screening can be used to troubleshoot or learn the method. Plasmid pEDA5_GFPmut3_Y66H contains a constitutively expressed non-fluorescent GFPmut3 variant with a mutated chromophore \(Gly65-Tyr66-Gly67 to Gly65-His66-Gly67). A single mutagenic oligo, GFP_H66Y, encodes the restore-to-function mutation resulting in fluorescent ‘mutants’. This plasmid can be obtained freely from Addgene \(www.addgene.com, #80085). The protocol can be followed as below with the following adjustments: 1. 20 µL of 10 µM GFP_H66Y primer is added to the phosphorylation reaction \(single primer as opposed to a primer mix). 2. The secondary primer used is pED_2ND \(primer sequences listed at end of protocol). 3. Prepare serial dilution plates of the transformation to calculate transformation and mutational efficiency. **Green/White Screening Primer Sequences:** GFP_H66Y: gcaaagcattgaacaccataaccgaaagtagtgacaagt pED_2ND: ggtgattcattctgctaa

Reagents

Reagents: Nuclease-Free H_2O \(NFH_2O, IDT) Plasmid dsDNA \(see notes above on preparation) Mutagenic and secondary primers T4 Polynucleotide Kinase Buffer \(NEB) 10 mM ATP 10X CutSmart Buffer \(NEB) 5X Phusion HF Buffer \(NEB) 10 mM ATP 50 mM DTT 50 mM NAD^+ 10 mM dNTPs **Enzymes \(all purchased from NEB; diluent for all enzymes is 1X NEB CutSmart Buffer):** T4 Polynucleotide Kinase \(10 U/µL) Nt.BbvCI \(10 U/µL) Nb.BbvCI \(10 U/µL) Exonuclease III \(100 U/µL) Exonuclease I \(20 U/µL) Phusion High-Fidelity DNA Polymerase \(2 U/µL) Taq DNA Ligase \(40 U/µL) DpnI \(20 U/µL) **Materials:** Zymo Clean & Concentrator-5 kit \(Zymo Research) Corning square bioassay dishes, 245 mm x 245 mm x 25 mm \(Sigma-Aldrich) High-efficiency electrocompetent cells \(e.g. Agilent XL1-Blue Electroporation Competent cells, #200228)

Equipment

Thermal cycler

Procedure

Kinase mutagenic oligos and secondary primer 1. Make a mixture of NNN/NNK mutagenic oligos at a final concentration of 10 µM. 2. Into a PCR tube, add: 20 µL 10 µM mutagenic oligo mixture 2.4 µL T4 Polynucleotide Kinase Buffer 1 µL 10 mM ATP 1 µL T4 Polynucleotide Kinase \(10 U/µL) 3. In a separate PCR tube add: 18 µL NFH_2O 3 µL T4 Polynucleotide Kinase Buffer 7 µL 100 µM secondary primer 1 µL 10 mM ATP 1 µL T4 Polynucleotide Kinase \(10 U/µL) 4. Incubate at 37°C for 1 hour. Store phosphorylated oligos at -20°C. The day of mutagenesis, dilute phosphorylated mutagenic oligos 1:1000 and secondary primer 1:20 in NFH_2O. **Prepare ssDNA template** 5. Add the following into PCR tube\(s): 0.76 pmol plasmid dsDNA 2 µL 10X CutSmart Buffer 1 µL 1:10 diluted Exonuclease III \(final concentration of 10 U/µL) 1 µL Nt.BbvCI \(10 U/µL) 1 µL Exonuclease I \(20 U/µL) NFH_2O to 20 µL final volume 6. Run the following PCR program: 37°C 60 minutes 80°C 80 minutes 4-10°C Hold **Single-site saturation mutagenesis strand 1** 7. Add the following into each tube \(100 µL final volume): 26.7 µL NFH_2O 20 µL 5X Phusion HF Buffer 4.3 µL 1:1000 diluted phosphorylated mutagenic oligos 20 µL 50 mM DTT 1 µL 50 mM NAD+ 2 µL 10 mM dNTPs 1 µL Phusion High Fidelity Polymerase \(2 U/µL) 5 µL Taq DNA Ligase \(40 U/µL) 8. Run the following PCR program: 98°C 2 minutes ———————— 15 cycles of: 98°C 30 seconds 55°C 45 seconds 72°C 7 minutes ******add additional 4.3 µL oligo at beginning of cycles 6 and 11****** ———————— 45°C 20 minutes 4-10°C Hold **Column purification I** 9. Following the manufacturers' instructions, perform a column purification using a Zymo Clean and Concentrate Kit: a) Add 5 volumes of DNA binding buffer to each reaction and mix b) Transfer to a Zymo-Spin Column in a collection tube c) Centrifuge at maximum speed for 30 seconds and discard flow through d) Add 200 µL of DNA wash buffer to the column e) Centrifuge at maximum speed for 30 seconds and discard flow through f) Repeat steps 4 and 5 g) Add 15 µL of NFH_2O directly to the column in a new clean 1.5 mL microfuge tube and incubate at room temperature for 5 minutes h) Centrifuge at maximum speed for one minute **Degrade template strand** 10. Transfer 14 µL of the purified DNA product to a PCR tube, then add \(20 µL final volume): 2 µL 10X CutSmart Buffer 2 µL 1:50 diluted Exonuclease III \(final concentration of 2 U/µL) 1 µL 1:10 Nb.BbvCI \(final concentration of 1 U/µL) 1 µL Exonuclease I \(20 U/µL) 11. Run the following PCR program: 37°C 60 minutes 80°C 20 minutes 4-10°C Hold **Synthesize 2^{nd} \(complementary) mutagenic strand** 12. To the above PCR tubes, add \(100 µL final volume): 27.7 µL NFH_2O 20 µL 5X Phusion HF Buffer 3.3 µL 1:20 diluted phosphorylated secondary primer 20 µL 50 mM DTT 1 µL 50 mM NAD+ 2 µL 10 mM dNTPs 1 µL Phusion High Fidelity Polymerase \(2 U/µL) 5 µL Taq DNA Ligase \(40 U/µL) 13. Run the following PCR program: 98°C 30 seconds 55°C 45 seconds 72°C 10 minutes 45°C 20 minutes 4-10°C Hold **DNA clean up** 14. Add into each reaction 2 µL of DpnI \(20 U/µL) and run the following PCR program: 60°C 60 minutes **Column purification II** 15. Follow the instructions in step 9 but elute in **6 µL** of NFH_2O. **DNA Transformation** 16. Transform the entire 6 µL reaction product into a high-efficiency cloning strain following standard transformation protocols. After recovery, bring the final

volume of the transformation to 2-2.5 mL with additional sterile media. Spread on to a prepared large BioAssay dish \(245 mm x 245 mm x 25 mm, Sigma-Aldrich). Additionally, serial dilution plates should be prepared to calculate transformation efficiencies. Incubate overnight at 37°C. The next day, scrape the plate using 5-10 mL of LB or TB. Vortex the cell suspension and extract the library plasmid dsDNA using a mini-prep kit \(Qiagen) of a 1 mL aliquot of the cell suspension. Additional mini-preps \(or a midi-prep) can be done if large amounts of library DNA are required.

Timing

Please refer to Table 1.

Anticipated Results

One can expect 10^5 and 10^8 total transformants and roughly 70% mutational efficiency. The efficacy of this protocol has been demonstrated on multiple systems, and deep sequencing data to assess library composition of three different reactions can be found in Table 2.

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Figures

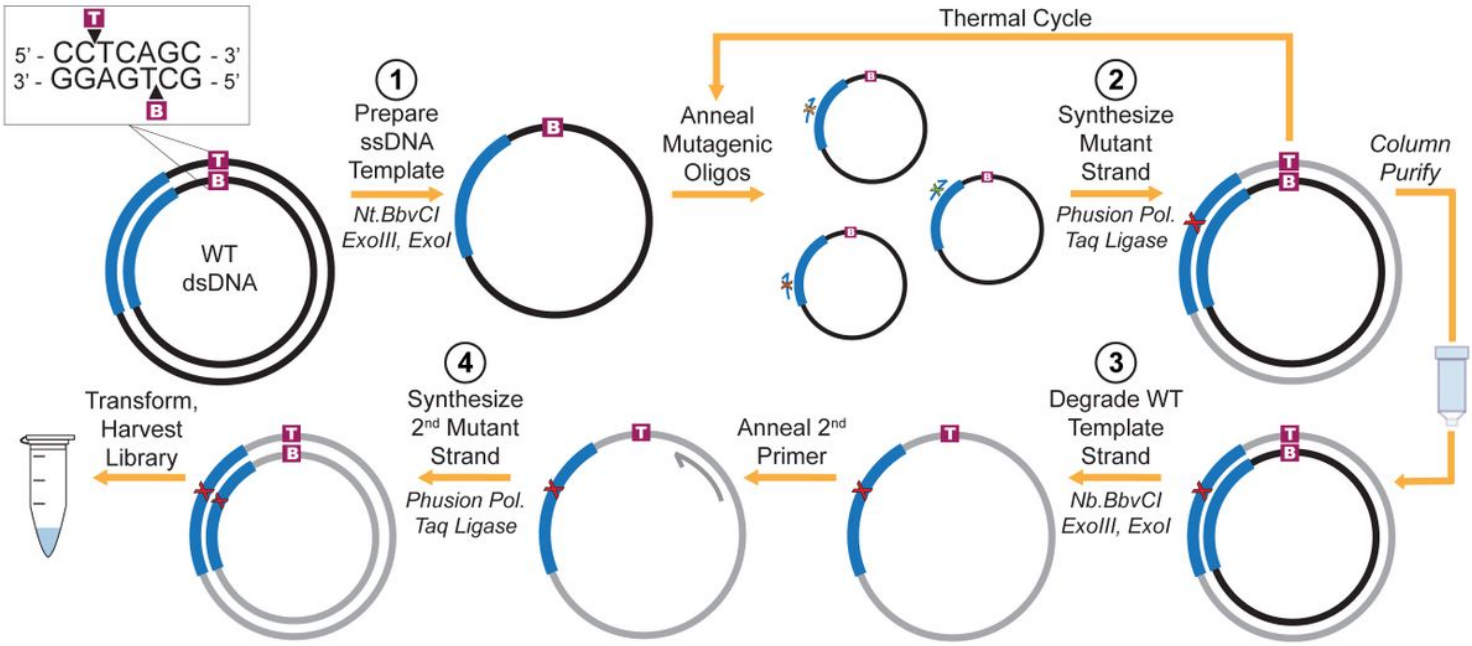

Figure 1

Method Schematic *Figure 1:* Comprehensive single-site Nicking Mutagenesis. Plasmid dsDNA containing a 7-bp BbvCI recognition site is nicked by Nt.BbvCI. Exonuclease III degrades the nicked strand to generate an ssDNA template (step 1). Mutagenic oligos are then added at a 1:20 ratio with template, and Phusion Polymerase synthesizes mutant strands and Taq DNA Ligase seals nicks (step 2). The reaction is column purified, and then the wild-type template strand is nicked by Nb.BbvCI and digested by Exonuclease III digestion (step 3). A second primer is added and the complementary mutant strand is synthesized to yield mutagenized dsDNA (step 4).

Supplementary Files

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- supplement0.xlsx
- supplement0.xlsx