SUPPLEMENTARY INFORMATION FOR

Plasmid-based single-pot saturation mutagenesis

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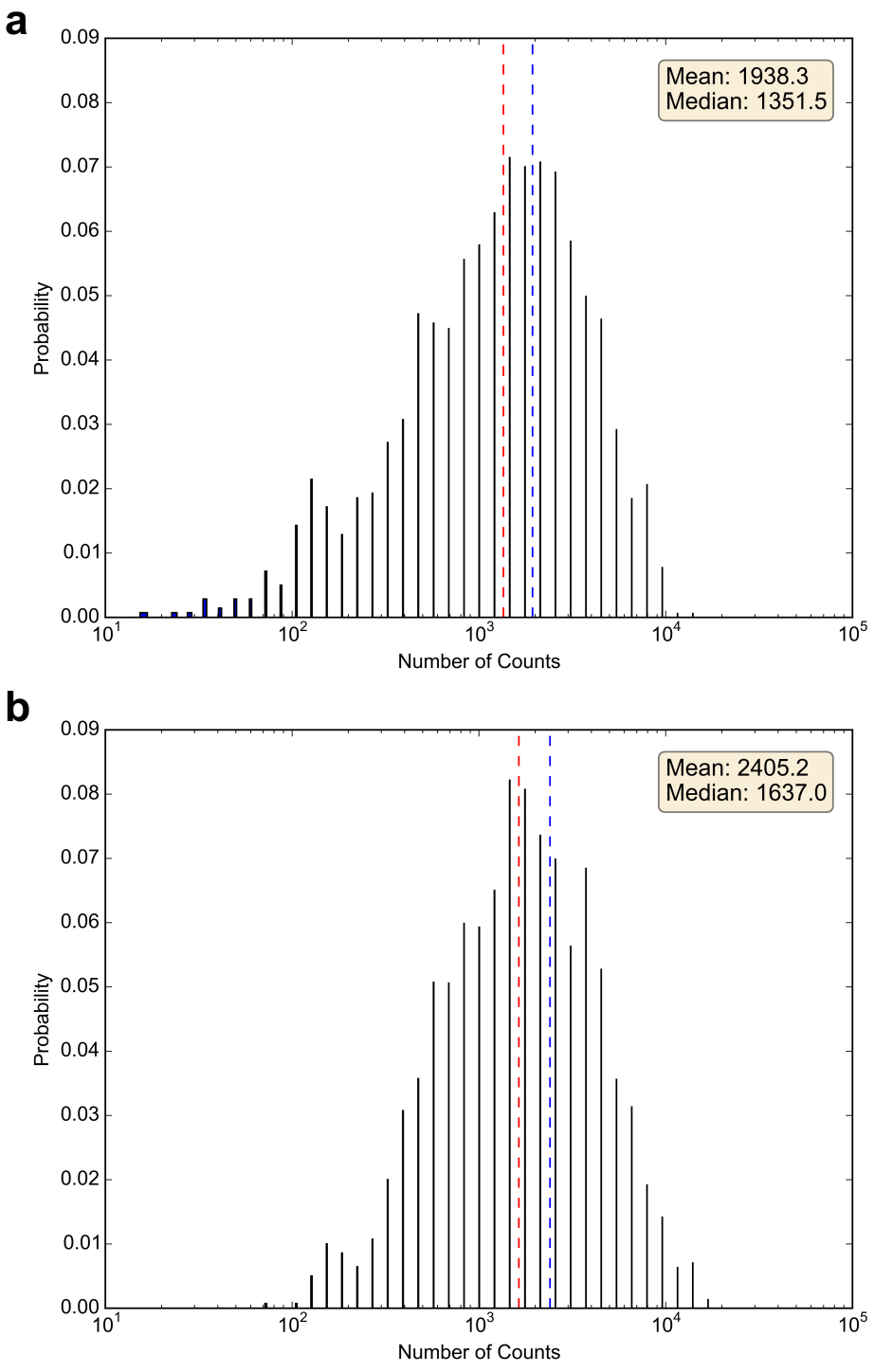
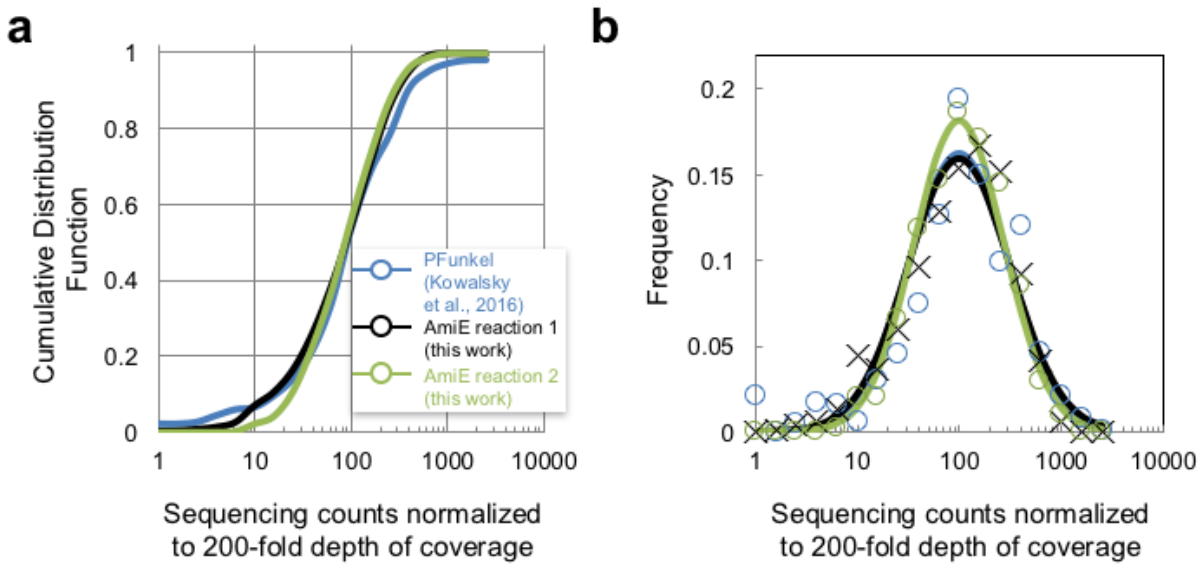
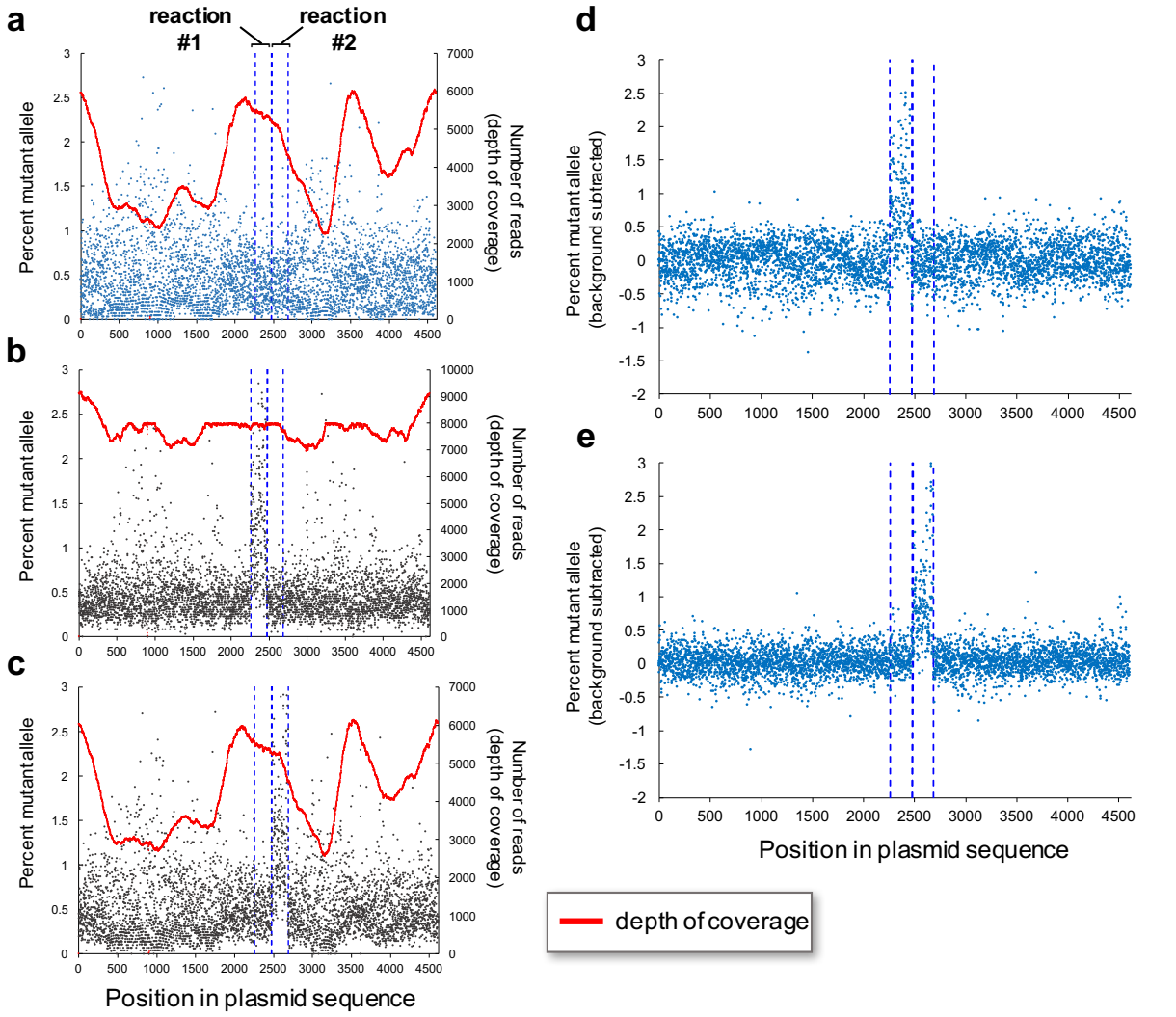


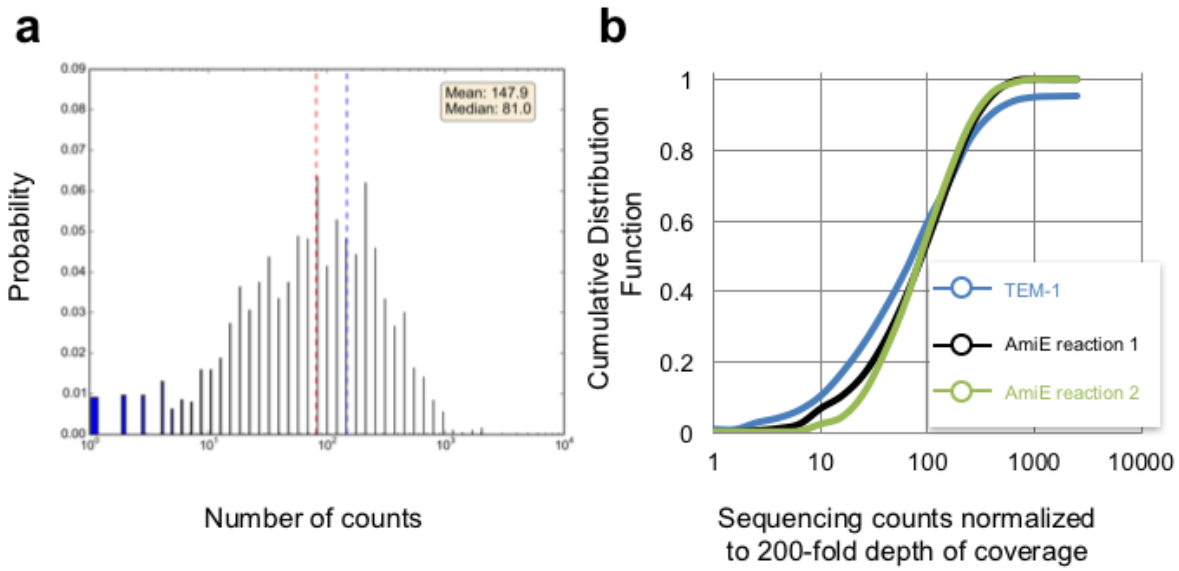
Supplementary Figure 1 | Probability distribution of mutation counts in AmiE comprehensive nicking mutagenesis libraries. Dashed vertical lines represent median (red) and mean (blue) library member read coverage. Panel **a** shows distribution for Tile 1 and panel **b** shows the distribution for Tile 2.



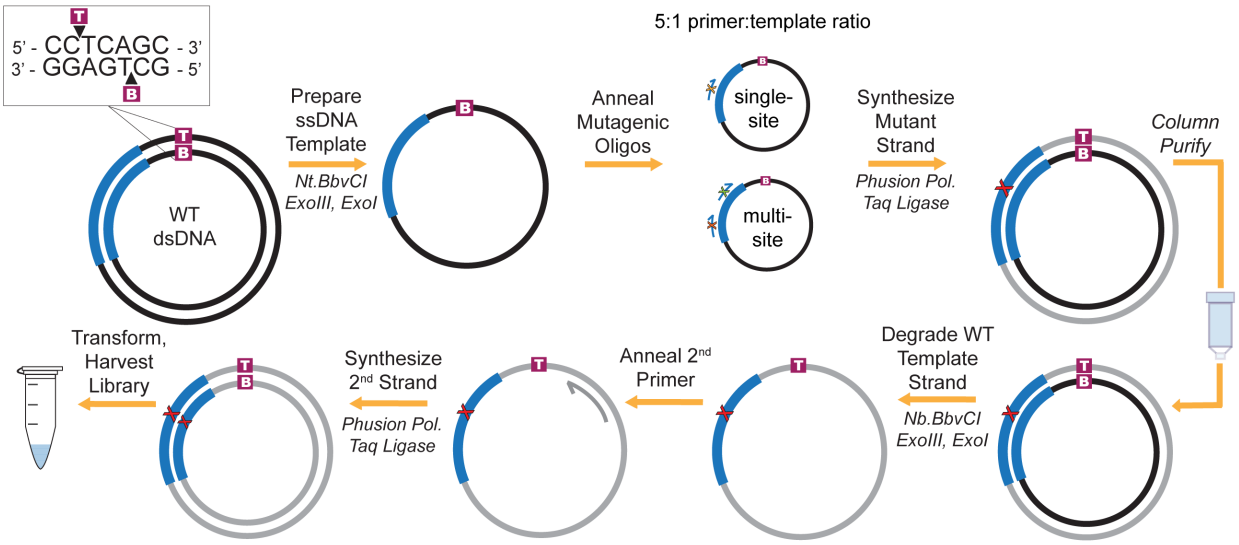
Supplementary Figure 2 | Comparison of the probability distributions of site-saturation mutagenesis libraries resulting from nicking mutagenesis or PFunkel mutagenesis[1,2]. Because the depth of sequencing coverage varied between the three methods, all samples were normalized to a 200-fold depth of coverage of possible single non-synonymous mutations. The expected library diversity is 820 for Kowalsky et al.[2] and 1420 for AmiE T1 & T2 (this work). **a.** Cumulative distribution function for the three libraries as a function of normalized sequencing counts. 91.7%, 93.2%, and 97.8% of the library is represented above a threshold of 10 sequencing counts for PFunkel library, AmiE T1, and the AmiE T2 libraries, respectively. **b.** Frequency is plotted as a function of sequencing counts for the same three libraries. The experimental data are plotted as symbols, with lines representing a best fit of the data using a log-normal distribution (PFunkel: μ=2, σ=0.49, AmiE T1: μ=2, σ=0.50. AmiE T2: μ=2, σ=0.44).



Supplementary Figure 3 | Off-target mutational analysis of AmiE input plasmid and mutational libraries by Shotgun Sequencing. **a-c**. Percent mutant allele at each position in the plasmid sequence for the input plasmid (**a**) AmiE reaction 1 library (**b**) and AmiE reaction 2 library (**c**). Shotgun sequencing reads were aligned to the pEDA3_amiE plasmid using BWA aligner[3,4] and the frequency of each base at each position was counted using bam-readcount (www.github.com). Percent mutant allele was calculated for each position by summing all non-wildtype allele counts and diving by total reads at that position. Overlain red curves indicate depth of sequencing coverage at each position. **d-e**. Background subtracted percent mutant allele for each position in plasmid sequence of AmiE reaction 1 library (**d**) and AmiE reaction 2 library (**e**).



Supplementary Figure 4 | TEM-1 library coverage distributions. **a**. Probability distribution of mutation counts in TEM-1 lactamase comprehensive nicking mutagenesis libraries. Dashed vertical lines represent median (red) and mean (blue) library member read coverage. **b.** Cumulative distribution function for the three libraries as a function of normalized sequencing counts.



Supplementary Figure 5 | Schematic overview of single- or multi-site nicking mutagenesis. After the preparation of an ssDNA template, an annealing reaction is set up with a single or mixed set of mutagenic oligos at a 5:1 primer:template ratio (for each oligo). Next, reagents and enzymes necessary to synthesize the mutant strands are added. The remainder of the protocol is identical to comprehensive nicking mutagenesis.

Supplementary Table 1 | Performance metrics of published comprehensive mutagenesis methods[5–9]. **Bolded** text indicates metrics that are comparatively inefficient to nicking mutagenesis and PFunkel mutagenesis. NS = nonsynonymous.

Mutagenesis method Publication data gathered from Gene (# codons mutated)	**Library type**	**Library coverage**	**Percent of mutants with NS mutations**			**Scalability** *mutatable codons/ reaction*
			Single	**Zero**	**Multiple**	
Casette Mutagenesis Hieptas et al. Hsp90 (9)	user-defined	100%	nd	nd	nd	**20**
Error-Prone PCR Doolan et al. mouse PrP (211)	random	nd	**28.2%**	**60.6%**	**11.08%**	all
Chemical Synthesis Fowler et al. hYAP65 WW domain (25)	random	**83.2%**	nd	20*	nd	**30**
PALS Mutagenesis Kitzman et al. Gal4 DBD and p53 (457 total)	user-defined	**94.3%**	**35%**	**29.2%**	**33%**	all
PFunkel Mutagenesis Kowalsky et al. Ct Cohesin (162)	user-defined	97.1%	73.6%	20.5%	5.9%	all
NSM This work AmiE (142)	user-defined	100.0%	64%	26.8%	9.3%	all

*estimated from Supplementary Figure 3 of original publication

Supplementary Table 2 | Estimated time required for comprehensive library construction using nicking mutagenesis.

Step number		Hands-on time (min)	On-thermal cycler time (min)
1a*	Phosporylate oligos	30	60*
1b*	ssDNA template strand preparation	5	80*
2a	Comprehensive codon mutagenesis strand 1	10	146
2b	Column purification I	5	
3	Degrade template strand	5	80
4a	Synthesize complimentary mutagenic strand	10	32
4b	DpnI DNA cleanup	2	60
4c	Column purification II	5	
	Subtotal (hr):	1.2	6.6
	Total (hr):	**7.8**	

*Steps can be performed concurrently

Supplementary Table 3 | Primer sequences

Plasmid construction primers	
pED_BbvCI	gcggccccacgggtcctcagcgcgcatgat
pED_kRBS3	gacgagctaatatcgccatgtctcatatgtataaaaacttcttaaagttaaacaaaattatttctagaaagttaaa
GFP_Y66H	gcaaagcattgaacaccatgaccgaaagtagtgacaagt
Green/white screening mutagenic oligos	
GFP_H66Y	gcaaagcattgaacaccataaccgaaagtagtgacaagt
GFP_H66Y_RC	acttgtcactactttcggttatggtgttcaatgctttgc
Green/white screening secondary primer	
pED_2ND	ggtgattcattctgctaa
AmiE and TEM-1 secondary primers	
pED_2ND (AmiE)	ggtgattcattctgctaa
pSALECT/pETconNK_2ND (TEM-1)	ggtttcccgactggaaag
Gene amplification: inner primers	
amiE_NMT1_FWD	gttcagagttctacagtccgacgatcgcaaatgtttggggtgtg
amiE_T2_FWD	gttcagagttctacagtccgacgatcctgcgatgacggtaat
amiE_T1_REV	ccttggcacccgagaattccactctccaaatttccggata
amiE_NMT2_REV	ccttggcacccgagaattccattcgccgcattcacccagagt
TEM1_T3_FWD	gttcagagttctacagtccgacgatcattaactggcgaactacttact
pETconNK_REV	ccttggcacccgagaattccaaagcttttgttcggatc
blue = Illumina sequencing primer; black = gene overlap	
Gene amplification: outer primers	
Illumina_FWD	aatgatacggcgaccaccgagatctacacgttcagagttctacagtccga
RPI30	caagcagaagacggcatacgagat**CCGGTG**gtgactggagttccttggcacccgagaattcca
RPI31	caagcagaagacggcatacgagat**ATCGTG**gtgactggagttccttggcacccgagaattcca
RPI21	caagcagaagacggcatacgagat**CGAAAC**gtgactggagttccttggcacccgagaattcca
red = Illumina adapter sequence; **BOLD** = barcode; blue = Illumina sequencing primer	

Supplementary Table 4 | Cost analysis of nicking mutagenesis compared with PFunkel mutagenesis[1]. Library preparation cost was calculated by totaling cost of enzymes (price information gathered from New England Biolabs) and reagents (price information gathered from Sigma-Aldrich, Qiagen, and Zymo Research) on a per reaction basis. Price of chemically synthesized degenerate NNN oligos based on IDT pricing for a 40bp primer[10] at the 500 pmole scale: $0.10/base*40bp = $4/codon. Prices obtained February 2016.

	PFunkel	**NSM**
Library preparation cost per reaction	$53	$55
NNN oligo cost per codon (source)	$4 (IDT)	$4 (IDT)
Total cost per 100 scanned codons	**$453**	**$455**

Supplementary Note 1 | Comprehensive site-saturation Nicking Mutagenesis protocol.
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: 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'. 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 efficiencies.

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)

Reagents:
Nuclease-Free H_2O (NFH_2O, Integrated DNA Technologies)
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):
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)
*Diluent for all enzymes is 1X NEB CutSmart Buffer

PROTOCOL

1.) Phosphorylate Oligos

1. Make a mixture of NNN/NNK mutagenic oligos at 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.
5. Store phosphorylated oligos at -20°C. The day of mutagenesis, dilute phosphorylated mutagenic oligos 1:1000 and secondary primer 1:20 in NFH_2O.

2.) ssDNA Template Strand Preparation

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	

PCR Program:

37°C	60 minutes
80°C	20 minutes
4-10°C	Hold

3.) Comprehensive Codon Mutagenesis Strand 1

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)

PCR Program:

98°C	2 minutes	
98°C	30 seconds	→ x15 cycles; add additional 4.3 µL oligo at beginning of cycles 6 and 11
55°C	45 seconds	
72°C	7 minutes	
45°C	20 minutes	
4-10°C	Hold	

4.) Column Purification Using a Zymo Clean and Concentrate Kit

Following the manufacturer's instructions:

1. Add 5 volumes of DNA binding buffer to each reaction and mix
2. Transfer to a Zymo-Spin Column in a collection tube
3. Centrifuge at maximum speed for 30 seconds and discard flow through
4. Add 200 µL of DNA wash buffer to the column
5. Centrifuge at maximum speed for 30 seconds and discard flow through
6. Repeat steps 4 and 5
7. Add **15 µL** of NFH_2O directly to the column in a new clean 1.5mL microfuge tube and incubate at room temperature for 5 minutes
8. Centrifuge at maximum speed for one minute

5.) Degrade Template Strand

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)

PCR Program:

37°C	60 minutes
80°C	20 minutes
4-10°C	Hold

6.) Synthesize 2nd (Complementary) Mutagenic Strand

To above PCR tube, 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)

PCR Program:

98°C	30 seconds
55°C	45 seconds
72°C	10 minutes
45°C	20 minutes
4-10°C	Hold

7.) DNA cleanup

Add into each reaction:

2 µL DpnI (20 U/µL)

PCR Program:

37°C	60 minutes

8.) Zymo Clean and Concentrate Kit

Follow instructions in step 4 but elute in **6 µL** of NFH_2O.

9.) DNA Transformation

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.

Green/White Screening Primer Sequences:

GFP_H66Y: gcaaagcattgaacaccataaccgaaagtagtgacaagt

pED_2ND: ggtgattcattctgctaa

Supplementary Note 2 | Single- or multi-site Nicking Mutagenesis protocol. See Notes, Troubleshooting, Materials, Reagents, and Enzymes sections from **Supplementary Note 1**.

PROTOCOL:

1.) Phosphorylate Oligos

Phosphorylate each oligo separately and then mix to obtain a final dilute oligo mixture.

1. To phosphorylate each NNN/NNK oligo, in PCR tubes add:

18 µL	NFH_2O
3 µL	T4 Polynucleotide Kinase Buffer
7 µL	100 µM mutagenic oligo
1 µL	10 mM ATP
1 µL	T4 Polynucleotide Kinase (10 U/µL)

2. To phosphorylate the secondary primer, 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)

3. Incubate at 37°C for 1 hour.
4. Dilute phosphorylated oligos 1:20. If performing multi-site nicking mutagenesis, add 2 µL of each oligo into a single tube, then add NFH_2O to 40 µL final volume. Dilute secondary primer 1:20.

2.) ssDNA Template Strand Preparation

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	

PCR Program:

37°C	60 minutes
80°C	20 minutes
4-10°C	Hold

3.) Anneal Oligos
Add the following to the appropriate tube (50 µL final volume):

16.7 µL	NFH_2O
3.3 µL	1:20 diluted mutagenic oligos (single or mixed)
10 µL	5X Phusion HF Buffer

PCR Program:

98°C	2 minutes
gradually decrease to 55°C over 15 minutes	
55°C	5 minutes
55°C	Hold

4.) Single- or Multi-Site Mutagenesis Strand 1
Keeping the tubes on the thermocyler, add the following into each tube (100 µL final volume):

11 µL	NFH_2O
10 µL	5X Phusion HF Buffer
20 µL	50 mM DTT
1 µL	50 mM NAD^+
2 µL	10 mM dNTPs
5 µL	Taq DNA Ligase (40 U/µL)
1 µL	Phusion HF Polymerase (2 U/µL)

PCR Program:

72°C	10 minutes
45°C	20 minutes
4-10°C	Hold

5.) Column Purification Using a Zymo Clean and Concentrate Kit
Following the manufacturer's instructions:

1. Add 5 volumes of DNA binding buffer to each reaction and mix
2. Transfer to a Zymo-Spin Column in a collection tube
3. Centrifuge at maximum speed for 30 seconds and discard flow through
4. Add 200 µL of DNA wash buffer to the column
5. Centrifuge at maximum speed for 30 seconds and discard flow through
6. Repeat steps 4 and 5
7. 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
8. Centrifuge at maximum speed for one minute

6.) Degrade Template Strand
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 diluted Nb.BbvCI (final concentration of 1 U/µL)
1 µL	Exonuclease I (20 U/µL)

PCR Program:

37°C	60 minutes
80°C	20 minutes
4-10°C	Hold

7.) Synthesize 2nd (Complementary) Mutagenic Strand
To each tube, add (100 µL final volume):

27.7 µL	NFH_2O
20 µL	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)

PCR Program:

98°C	30 seconds
55°C	45 seconds
72°C	10 minutes
45°C	20 minutes
4-10°C	Hold

8.) DNA cleanup
Add into each reaction:

2 µL	DpnI (20 U/µL)

PCR Program:

37°C	60 minutes

9.) Zymo Clean and Concentrate Kit
Follow instructions in step 5 but elute in **6 µL** NFH_2O.

10.) DNA Transformations
Transform entire 6 µL reaction product as described in **Supplementary Note 1**.

Supplementary Note 3 | Orientation of BbvCI site and design of primers.

In oligonucleotide-programmed mutagenesis, mutagenic oligos are designed to be complementary to the wild-type template sequence on either side of the programmed mutation such that they can anneal to the template. For Kunkel mutagenesis[11], the ssDNA template strand is made by replication and packaging within a phage host. The directionality of the ssDNA template strand (sense or anti-sense) is dependent upon the directionality of the F1-origin of replication. If the F1-origin is such that the template strand made is sense, then mutagenic oligos are designed anti-sense.

For nicking mutagenesis, the directionality of the template strand is dependent upon the orientation of the BbvCI site. The set of enzymes, Nt.BbvCI (Nick-top BbvCI) and Nb.BbvCI (Nick-bottom BbvCI) will create nicks on the strands containing their respective recognition sequence. If the Nt.BbvCI nicking enzyme is used for template preparation and its recognition sequence is encoded on the anti-sense strand, the ssDNA template formed will be sense. Thus, mutagenic oligos should be designed anti-sense. The opposite is true if Nb.BbvCI was used to create the template strand.

Another consideration is that a target gene of interest may contain a BbvCI nicking site. In such a case, confirm that the orientation of the BbvCI nicking site is the same on the gene as on the backbone.

References:

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