Supplementary Figure 1

Gel snapshots along the optimized nicking mutagenesis method.

Plasmid dsDNA and ssDNA (prepared from bacteriophage) of pEDA5_GFPmut3 are included for size reference. NR = nicking reaction; 2 μg of pEDA5_GFPmut3_Y66H was placed in a 20 μL reaction with 10 U Nt.BbvCI in 1X CutSmart buffer. TP = template preparation; a reaction was ceased after the template preparation phase. MS = mutant strand; a reaction was ceased after the synthesis of the mutant strands, where regeneration of relaxed dsDNA can be seen. 1 kb Plus Ladder (Thermo Fischer Scientific, lane 1) included for size reference. Gel image has been cropped to size.
Supplementary Figure 2

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 reaction 1 and panel b shows the distribution for reaction 2.
Supplementary Figure 3

Comparison of the probability distributions of site-saturation mutagenesis libraries resulting from nicking mutagenesis or PFunkel mutagenesis.

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.\textsuperscript{1,2} and 1420 for \textit{amiE} reaction 1 & reaction 2 (this work). \textbf{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, \textit{amiE} reaction 1, and the \textit{amiE} reaction 2 libraries, respectively. \textbf{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: $\mu=2$, $\sigma=0.49$, \textit{amiE} reaction 1: $\mu=2$, $\sigma=0.50$, \textit{amiE} reaction 2: $\mu=2$, $\sigma=0.44$).
Supplementary Figure 4

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 5

*bla* library coverage distributions.

Probability distribution of mutation counts in *bla* 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 6

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 INFORMATION FOR

Plasmid-based single-pot saturation mutagenesis

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³ Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL 60208;
⁴ Department of Biosystems and Agricultural Engineering, Michigan State University, East Lansing, Michigan, 48824;

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**Supplementary Table 1** | Performance metrics of published comprehensive mutagenesis methods\(^{1,5-8}\). **Bolded** text indicates metrics that are comparatively inefficient to nicking mutagenesis and PFunkel mutagenesis. NS = nonsynonymous.

<table>
<thead>
<tr>
<th>Mutagenesis method</th>
<th>Library type</th>
<th>Library coverage</th>
<th>Library type</th>
<th>Library coverage</th>
<th>Library type</th>
<th>Library coverage</th>
<th>Percent of mutants with NS mutations</th>
<th>Scalability mutable codons/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cassette Mutagenesis</strong></td>
<td>user-defined</td>
<td>100%</td>
<td>Gene (# codons mutated)</td>
<td>Library type</td>
<td>Library coverage</td>
<td>Percent of mutants with NS mutations</td>
<td>Scalability mutable codons/reaction</td>
<td></td>
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<tr>
<td>Hietpas et al.(^5)</td>
<td></td>
<td></td>
<td>Hsp90 (9)</td>
<td>user-defined</td>
<td>100%</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td><strong>20</strong></td>
</tr>
<tr>
<td><strong>Error-Prone PCR</strong></td>
<td>random</td>
<td>nd</td>
<td>Library type</td>
<td>Library coverage</td>
<td>Library type</td>
<td>Library coverage</td>
<td>Percent of mutants with NS mutations</td>
<td>Scalability mutable codons/reaction</td>
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<tr>
<td>Doolan et al.(^6)</td>
<td></td>
<td></td>
<td>mouse PrP (211)</td>
<td>random</td>
<td>nd</td>
<td><strong>28.2%</strong></td>
<td><strong>60.6%</strong></td>
<td><strong>11.08%</strong></td>
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<td><strong>20</strong></td>
<td><strong>30</strong></td>
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<td><strong>Chemical Synthesis</strong></td>
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<td>Library coverage</td>
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<tr>
<td>Fowler et al.(^7)</td>
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<td>hYAP65 WW domain (25)</td>
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<td><strong>30</strong></td>
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<td><strong>all</strong></td>
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<tr>
<td><strong>PALS Mutagenesis</strong></td>
<td>user-defined</td>
<td>94.3%</td>
<td>Library type</td>
<td>Library coverage</td>
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<td>Library coverage</td>
<td>Percent of mutants with NS mutations</td>
<td>Scalability mutable codons/reaction</td>
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<tr>
<td>Kitzman et al.(^8)</td>
<td></td>
<td></td>
<td>Gal4 DBD and p53 (457 total)</td>
<td>user-defined</td>
<td>94.3%</td>
<td><strong>35%</strong></td>
<td><strong>29.2%</strong></td>
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<td></td>
<td><strong>30</strong></td>
<td><strong>all</strong></td>
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<tr>
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<td>Library coverage</td>
<td>Percent of mutants with NS mutations</td>
<td>Scalability mutable codons/reaction</td>
</tr>
<tr>
<td>Kowalsky et al.(^2)</td>
<td></td>
<td></td>
<td>Ct Cohesin (162)</td>
<td>user-defined</td>
<td>97.1%</td>
<td>73.6%</td>
<td>20.5%</td>
<td>5.9%</td>
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<td></td>
<td><strong>20</strong></td>
<td><strong>all</strong></td>
<td><strong>all</strong></td>
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<td><strong>Nicking Mutagenesis</strong></td>
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<td>Library type</td>
<td>Library coverage</td>
<td>Library type</td>
<td>Library coverage</td>
<td>Percent of mutants with NS mutations</td>
<td>Scalability mutable codons/reaction</td>
</tr>
<tr>
<td>This work</td>
<td></td>
<td></td>
<td>amiE (142)</td>
<td>user-defined</td>
<td>100.0%</td>
<td>64%</td>
<td>26.8%</td>
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<td><strong>all</strong></td>
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*estimated from Supplementary Figure 3 of original publication
**Supplementary Table 2** | Estimated time required for comprehensive library construction using nicking mutagenesis.

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<th>Step number</th>
<th>Hands-on time (min)</th>
<th>On-thermal cycler time (min)</th>
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<td>1a*</td>
<td>30</td>
<td>60*</td>
</tr>
<tr>
<td>1b*</td>
<td>5</td>
<td>80*</td>
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<tr>
<td>2a</td>
<td>10</td>
<td>146</td>
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<tr>
<td>2b</td>
<td>5</td>
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<tr>
<td>3</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>4a</td>
<td>10</td>
<td>32</td>
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<td>4b</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>4c</td>
<td>5</td>
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</table>

**Subtotal (hr):** 1.2 6.6  
**Total (hr):** 7.8

*steps can be performed simultaneously
### Supplementary Table 3 | Primer sequences

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<thead>
<tr>
<th>Plasmid construction primers</th>
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<tbody>
<tr>
<td>pED_BbvCI</td>
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<tr>
<td>pED_kRBS3</td>
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<td>GFP_Y66H</td>
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<table>
<thead>
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<th>Green/white screening mutagenic oligos</th>
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<tbody>
<tr>
<td>GFP_H66Y</td>
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<tr>
<td>GFP_H66Y_RC</td>
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<table>
<thead>
<tr>
<th>Green/white screening secondary primer</th>
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<tbody>
<tr>
<td>pED_2ND</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>amiE and TEM-1 secondary primers</th>
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</thead>
<tbody>
<tr>
<td>pED_2ND (amiE)</td>
</tr>
<tr>
<td>pSALECT/pETconNK_2ND (TEM-1)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene amplification: inner primers</th>
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</thead>
<tbody>
<tr>
<td>amiE_NMT1_FWD</td>
</tr>
<tr>
<td>amiE_T2_FWD</td>
</tr>
<tr>
<td>amiE_T1_REV</td>
</tr>
<tr>
<td>amiE_NMT2_REV</td>
</tr>
<tr>
<td>TEM1_T3_FWD</td>
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<tr>
<td>pETconNK_REV</td>
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</table>

blue = Illumina sequencing primer; black = gene overlap

<table>
<thead>
<tr>
<th>Gene amplification: outer primers</th>
</tr>
</thead>
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<td>Illumina_FWD</td>
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<tr>
<td>RPI30</td>
</tr>
<tr>
<td>RPI31</td>
</tr>
<tr>
<td>RPI21</td>
</tr>
</tbody>
</table>

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\(^9\) at the 500 pmole scale: $0.10/base*40bp = $4/codon. Prices obtained February 2016.

<table>
<thead>
<tr>
<th></th>
<th>PFunkel</th>
<th>Nicking Mutagenesis</th>
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</thead>
<tbody>
<tr>
<td>Library preparation cost per reaction</td>
<td>$53</td>
<td>$55</td>
</tr>
<tr>
<td>NNN oligo cost per codon (source)</td>
<td>$4 (IDT)</td>
<td>$4 (IDT)</td>
</tr>
<tr>
<td><strong>Total cost per 100 scanned codons</strong></td>
<td><strong>$453</strong></td>
<td><strong>$455</strong></td>
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</table>
Supplementary Note 1 | Optimization of nicking mutagenesis using green/white screening

A previously constructed GFPmut3 expression plasmid\textsuperscript{10} was modified by incorporating a BbvCI site and by changing the amino acid sequence of the GFPmut3 chromophore, Gly65-Tyr66-Gly67, to Gly65-His66-Gly67, resulting in a non-fluorescent protein. We performed nicking mutagenesis on this construct (pEDA5 GFPmut3_Y66H) with a restore-to-function mutagenic oligo (primer GFP_H66Y, see Supplementary Table 3 for sequences). Supplementary Figure 1 shows gel snapshots at different stages along the optimized process.

Initial experiments with the full nicking mutagenesis protocol showed a mutational efficiency of 23% with 3x10\textsuperscript{5} transformants. To determine the sources of high wild-type background, we performed a series of control experiments containing no mutagenic primer. Thus, any resulting transformants could be unambiguously attributed to wild-type. The number of background transformants was 10\textsuperscript{3} after the template preparation step and incubation with DpnI, but increased to 10\textsuperscript{6} if the reaction was allowed to proceed through the thermal cycling steps. We hypothesized that short stretches of incompletely degraded DNA were priming and regenerating wild-type constructs. To remedy this, Exonuclease I, which specifically degrades ssDNA, was added to both the template preparation and degradation reactions. The addition of Exonuclease I improved mutational efficiency to 56% with >5x10\textsuperscript{5} transformants. Incubation of the final reaction mixture with DpnI to remove methylated and hemi-methylated wild-type DNA increased the mutational efficiency to 68% with >3x10\textsuperscript{5} transformants.

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\textsuperscript{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.

To confirm that the order of nicking enzymes could be switched, we performed nicking mutagenesis using green/white screening in two reactions: one with Nt.BbvCI then Nb.BbvCI using the GFP_H66Y mutagenic primer (priming one strand), and the second using Nb.BbvCI first with the GFP_H66Y_RC primer (priming the opposite strand at the same location as GFP_H66Y). We observed mutational efficiencies of 46% and 44% with >8x10\textsuperscript{4} and >9x10\textsuperscript{4} total transformants, respectively, confirming that the order of nicking enzymes can be switched.

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.
**Supplementary Protocol 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 H₂O (NFH₂O, 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 NFH2O
   - 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 NFH2O.

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)

NFH2O 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 NFH2O
- 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:**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>98°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>98°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>55°C</td>
<td>45 seconds</td>
</tr>
<tr>
<td>72°C</td>
<td>7 minutes</td>
</tr>
<tr>
<td>45°C</td>
<td>20 minutes</td>
</tr>
<tr>
<td>4-10°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

→ x15 cycles; add additional 4.3 µL oligo at beginning of cycles 6 and 11

**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 NFH₂O 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.BbvCl (final concentration of 1 U/µL)
- 1 µL Exonuclease I (20 U/µL)

**PCR Program:**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>60 minutes</td>
</tr>
<tr>
<td>80°C</td>
<td>20 minutes</td>
</tr>
<tr>
<td>4-10°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

**6.) Synthesize 2nd (Complementary) Mutagenic Strand**

To above PCR tube, add (100 µL final volume):

- 27.7 µL NFH₂O
- 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)
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 NFH2O.

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: ggtgattcattgtgaagtaa

Nature Methods: doi:10.1038/nmeth.4029
**Supplementary Protocol 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:

   
<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 µL</td>
<td>NFH₂O</td>
</tr>
<tr>
<td>3 µL</td>
<td>T4 Polynucleotide Kinase Buffer</td>
</tr>
<tr>
<td>7 µL</td>
<td>100 µM mutagenic oligo</td>
</tr>
<tr>
<td>1 µL</td>
<td>10 mM ATP</td>
</tr>
<tr>
<td>1 µL</td>
<td>T4 Polynucleotide Kinase (10 U/µL)</td>
</tr>
</tbody>
</table>

2. To phosphorylate the secondary primer, in a separate PCR tube add:

   
<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 µL</td>
<td>NFH₂O</td>
</tr>
<tr>
<td>3 µL</td>
<td>T4 Polynucleotide Kinase Buffer</td>
</tr>
<tr>
<td>7 µL</td>
<td>100 µM secondary primer</td>
</tr>
<tr>
<td>1 µL</td>
<td>10 mM ATP</td>
</tr>
<tr>
<td>1 µL</td>
<td>T4 Polynucleotide Kinase (10 U/µL)</td>
</tr>
</tbody>
</table>

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 NFH₂O to 40 µL final volume. Dilute secondary primer 1:20.

2.) **ssDNA Template Strand Preparation**

Add the following into PCR tube(s):

   
<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.76 pmol</td>
<td>Plasmid dsDNA</td>
</tr>
<tr>
<td>2 µL</td>
<td>10X CutSmart Buffer</td>
</tr>
<tr>
<td>1 µL</td>
<td>1:10 diluted Exonuclease III (final concentration of 10 U/µL)</td>
</tr>
<tr>
<td>1 µL</td>
<td>Nt.BbvCl (10 U/µL)</td>
</tr>
<tr>
<td>1 µL</td>
<td>Exonuclease I (20 U/µL)</td>
</tr>
<tr>
<td></td>
<td>NFH₂O to 20 µL final volume</td>
</tr>
</tbody>
</table>

**PCR Program:**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>60 minutes</td>
</tr>
<tr>
<td>80°C</td>
<td>20 minutes</td>
</tr>
<tr>
<td>4-10°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>
3.) **Anneal Oligos**
Add the following to the appropriate tube (50 µL final volume):

- 16.7 µL NFH₂O
- 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 thermocycler, add the following into each tube (100 µL final volume):

- 11 µL NFH₂O
- 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 NFH₂O 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 NFH2O
- 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 NFH2O.

10.) DNA Transformations
Transform entire 6 µL reaction product as described in Supplementary Protocol 1.
References: