Supporting Information

uPIC–M: efficient and scalable preparation of clonal single mutant libraries for high-throughput protein biochemistry

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Figure S1. Timeline of uPIC–M library generation.

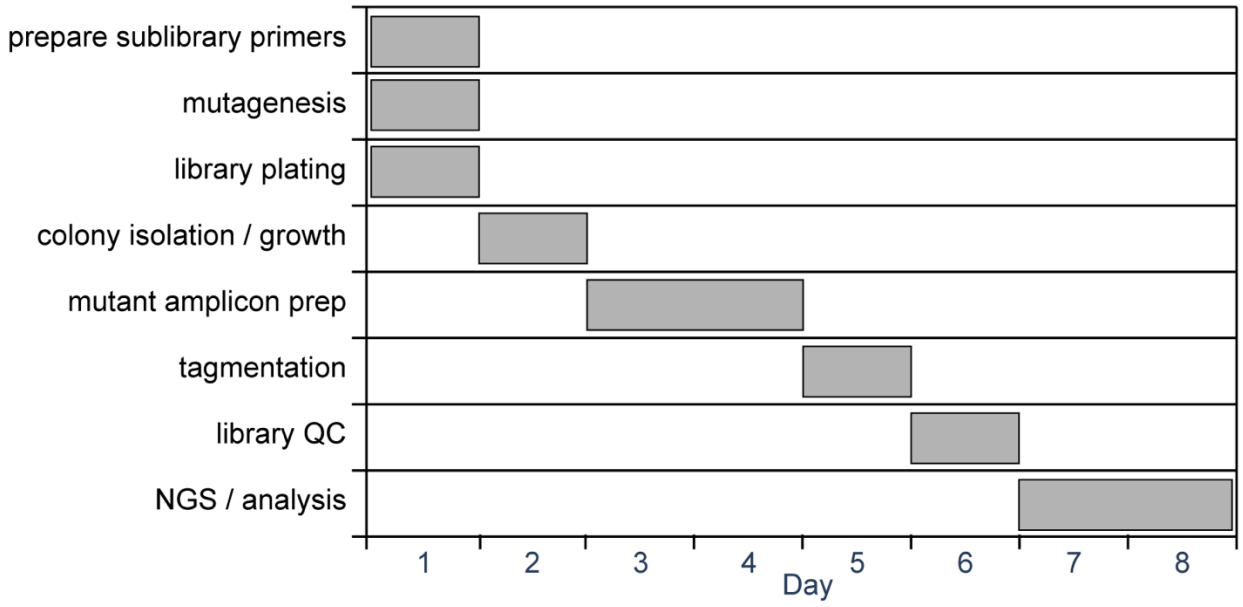


Figure S2. Amplification of window-specific sublibrary pools from an oligo array.
(**A**) Microelectrophoresis results for PCR-amplified sublibrary mutagenic primers after column purification. (**B**) Plot of predicted and observed lengths for mutagenic primer pools corresponding to sublibrary windows 1–13.

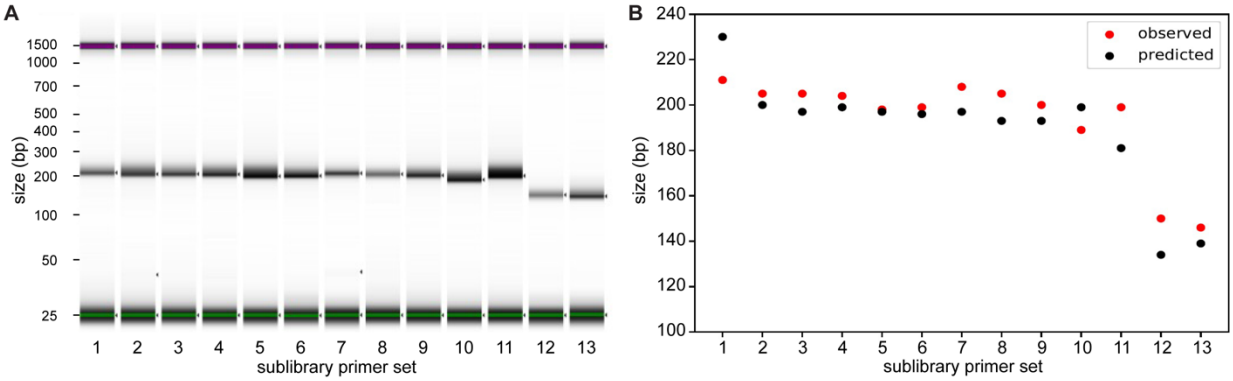
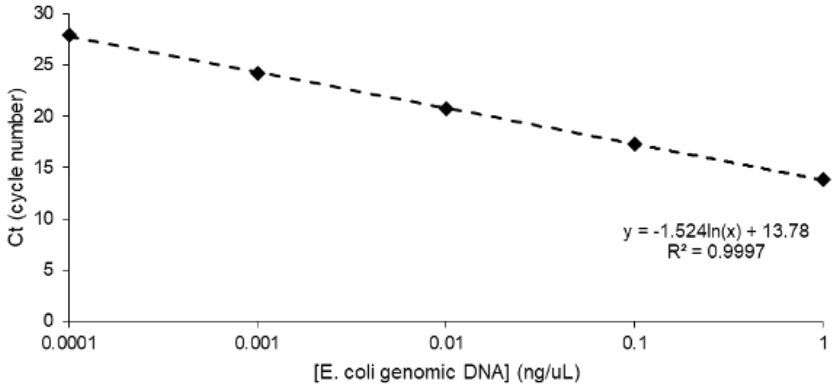


Figure S3. Quantification of *E. coli* genomic DNA in diluted mutant culture templates.
(**A**) Standard curve of *E. coli* genomic DNA concentration measured by qPCR using previously reported primers to the *rodA* gene. Each point represents the average of 4 technical replicates. (**B**) Measurement of *E. coli* genomic DNA concentrations in diluted mutant cultures by qPCR. Six saturated mutant cultures were serially diluted in H_2O and assayed alongside the standard curve in (**A**). The average of two technical replicates is plotted for each of the six biological replicates at each dilution. The black horizontal line represents the median across biological replicates.

A



B

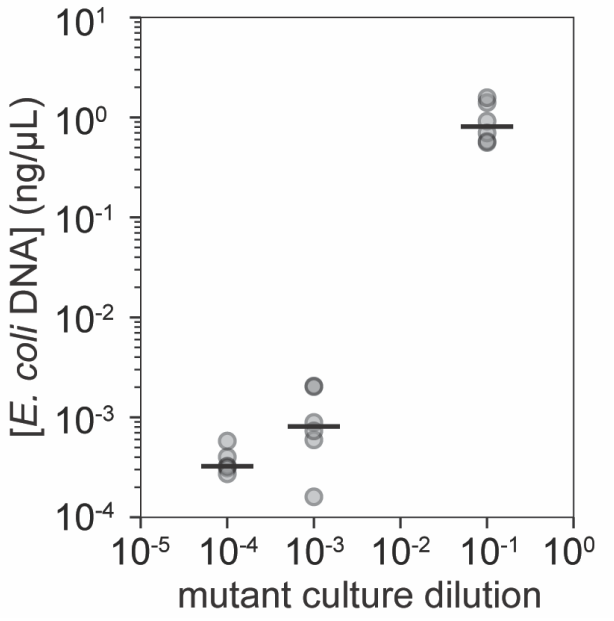


Figure S4. Selection of PCR conditions for SpAP mutant amplicons.
Twelve sample wells containing *E. coli* cultures of SpAP mutants grown to saturation were selected from sublibrary plates 1 and 5 (6 each). Cultures were then diluted from 1:10 to $1:10^4$ with H_2O and used as PCR templates for 18 or 25 cycles of PCR amplification using KAPA HiFi polymerase. Primers were selective for a 1737 bp region of the PURExpress-SpAP-eGFP plasmid (F: 5'-CCCGCGAAATTAATACGACTCACTATAGG 3'; R: 5'- CTTGCTCACCATGCCACTG -3'). Following PCR, samples were diluted with H_2O and loading buffer and run at equal volumes on a 0.8% TAE agarose gel.

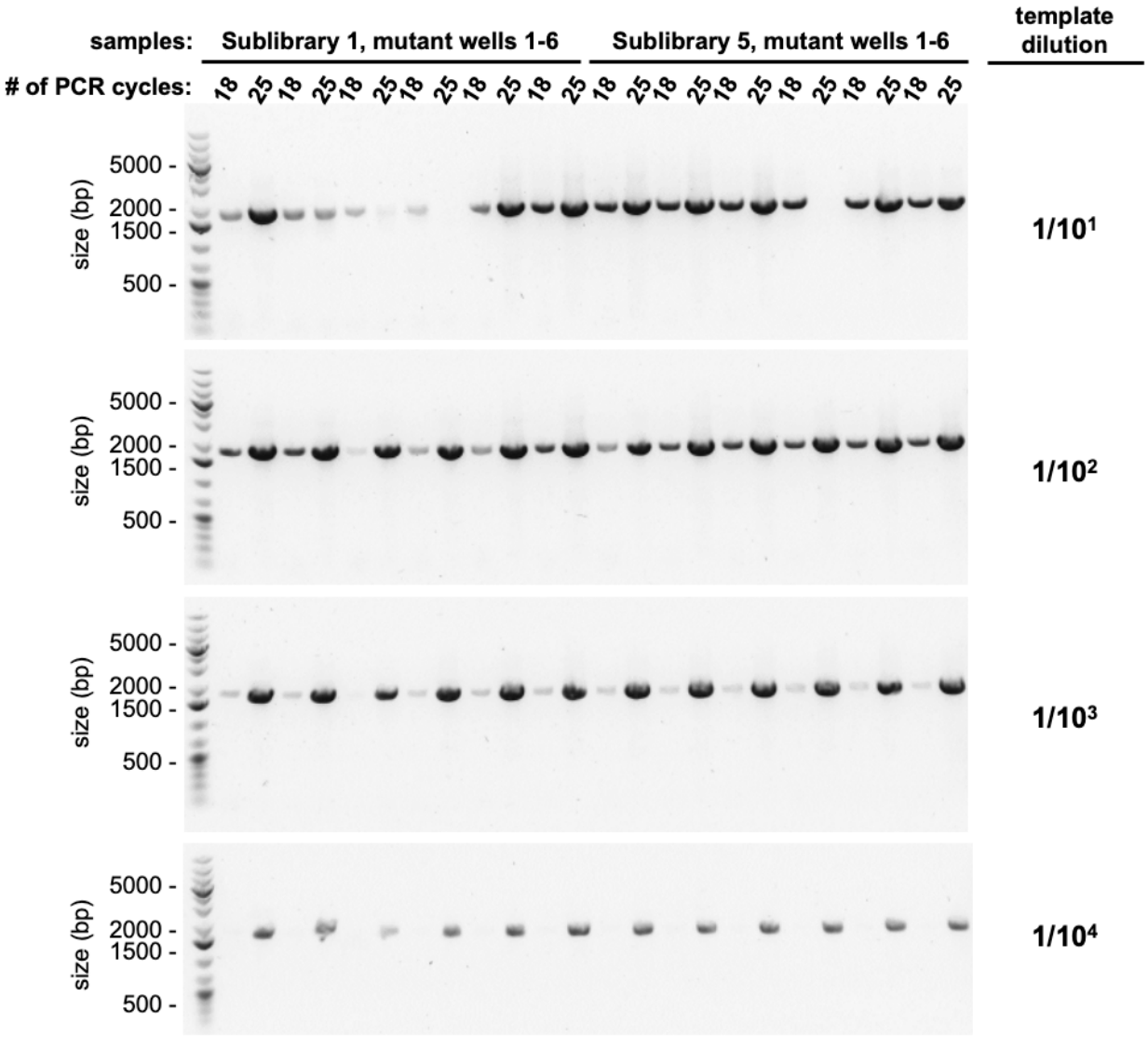


Figure S5. Quantification of amplicon DNA concentrations per sublibrary plate.
DNA concentrations were measured for each sublibrary plate (184 or 368 sample wells out of 384 possible) using the PicoGreen fluorescence assay. Amplicon samples were diluted 5-fold with H_2O and measured alongside a λ phage DNA standard curve.

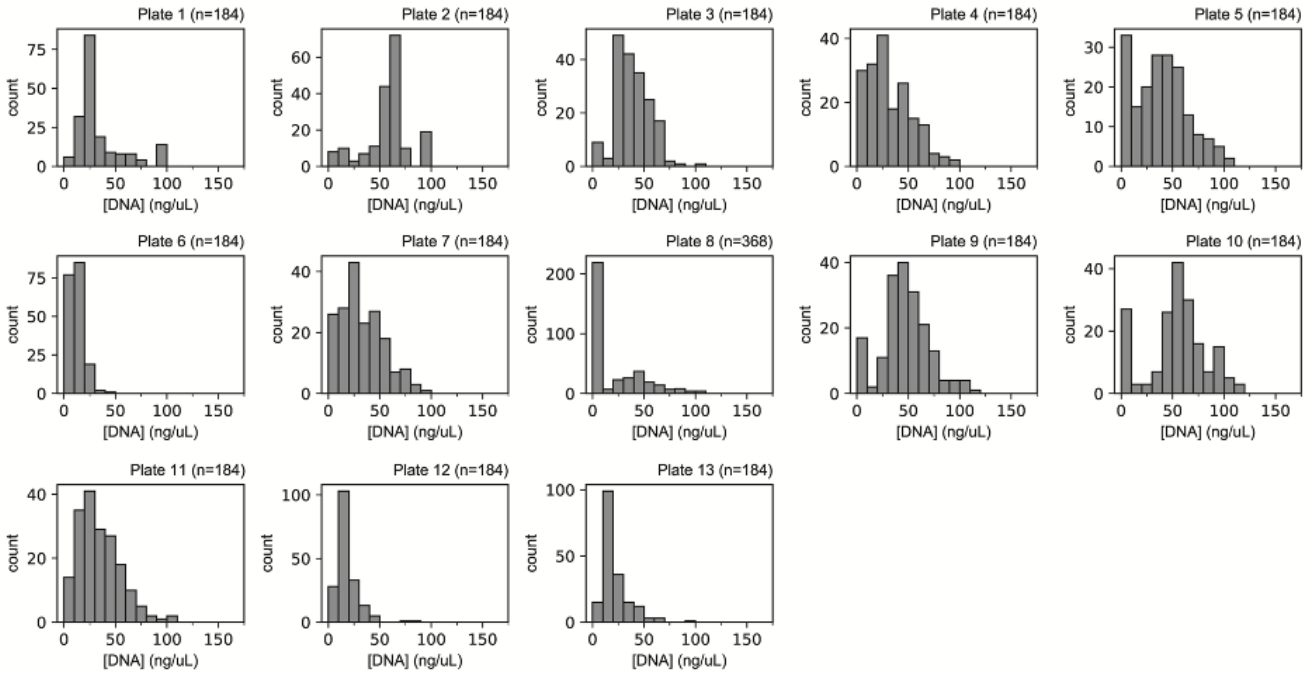


Figure S6. Testing Tn5 tagmentation reaction conditions.
(**A**) Comparison of library content and concentration with or without AmpureXP bead cleanup of Tn5 templates. Following mutant amplicon preparation, two unique scanning library sample plates were either purified by AmpureXP bead cleanup or simply diluted prior to Tn5 tagmentation. The Tn5 stock used here was in-house purified from the protocol of Picelli et. al., 2014. (**B**) Comparison of library content and concentration at template DNA concentrations of 0.1–0.5 ng/uL, Tn5 enzyme dilutions of 1/50 and 1/100, and tagmentation times of 3 and 7 minutes. The Tn5 template was purified SpAP amplicon DNA. Samples were allowed to react for specified times, quenched, and then amplified by library preparation PCR (see Materials and Methods). Amplified libraries were purified by AmpureXP bead cleanup and analyzed by microelectrophoresis. In both (**A**) and (**B)**, DNA peak concentrations represent the integrated signal of the entire library peak (~200–1000 bp).

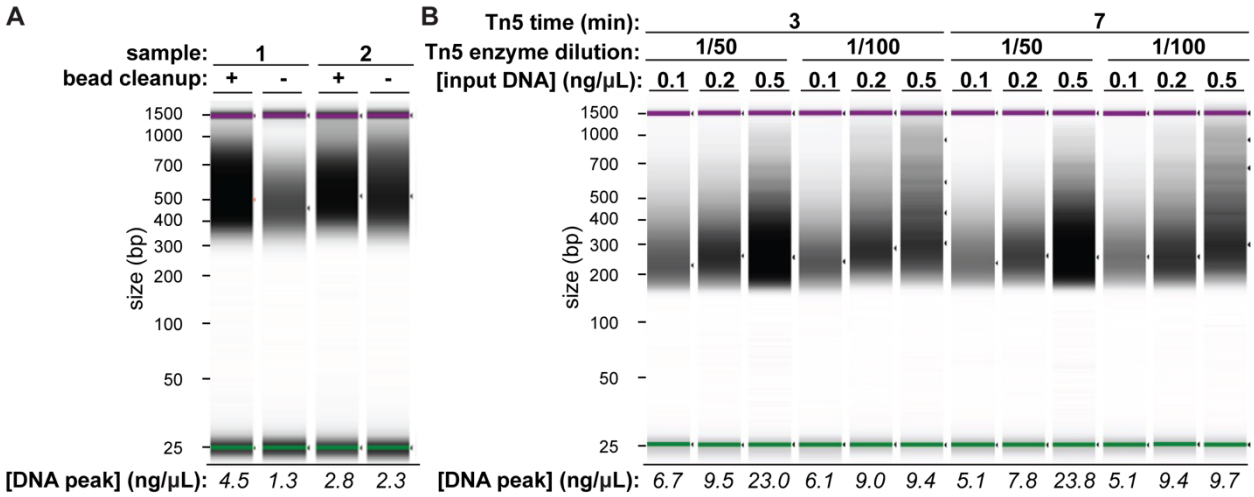


Figure S7. Electropherograms of tagmented and amplified mutant sublibraries.

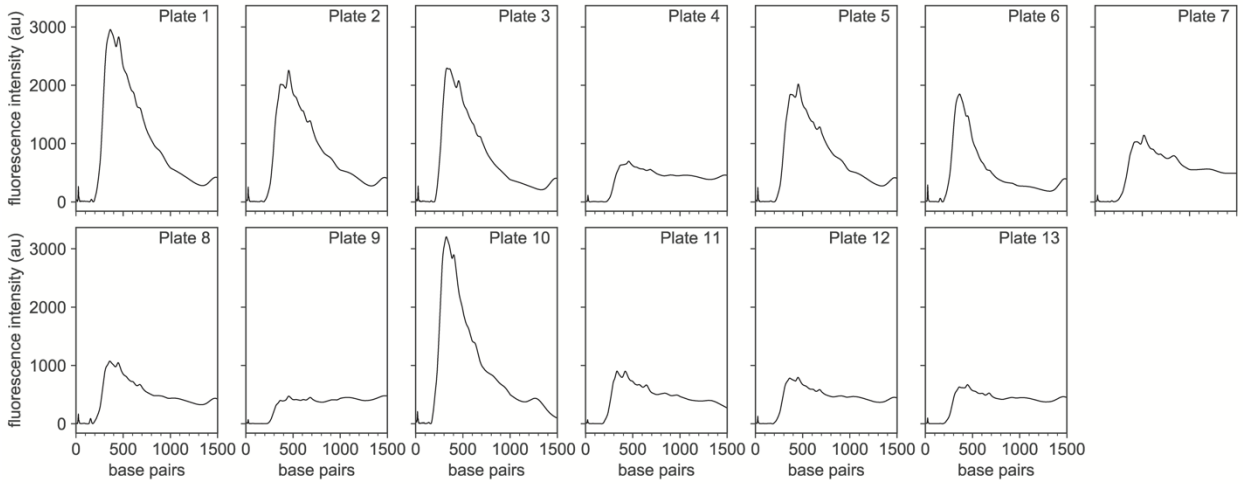


Figure S8. Histogram of variant:WT read ratios among single, double, and triple and greater mutants.

Read counts for variant and WT sequences represent the sum of forward and reverse reads of each genotype, averaged across each nucleotide for each codon substitution. If higher order mutants originated solely from the presence of co-occurring mutations on the same plasmid genome, the rate of WT reads for each substitution would be comparable for all types of mutants. However, a higher variant:WT read ratio for single mutants compared to double, and triple and greater is consistent with the model that many higher order mutants originate from well-to-well cross-contamination during plate handling steps prior to barcoding.

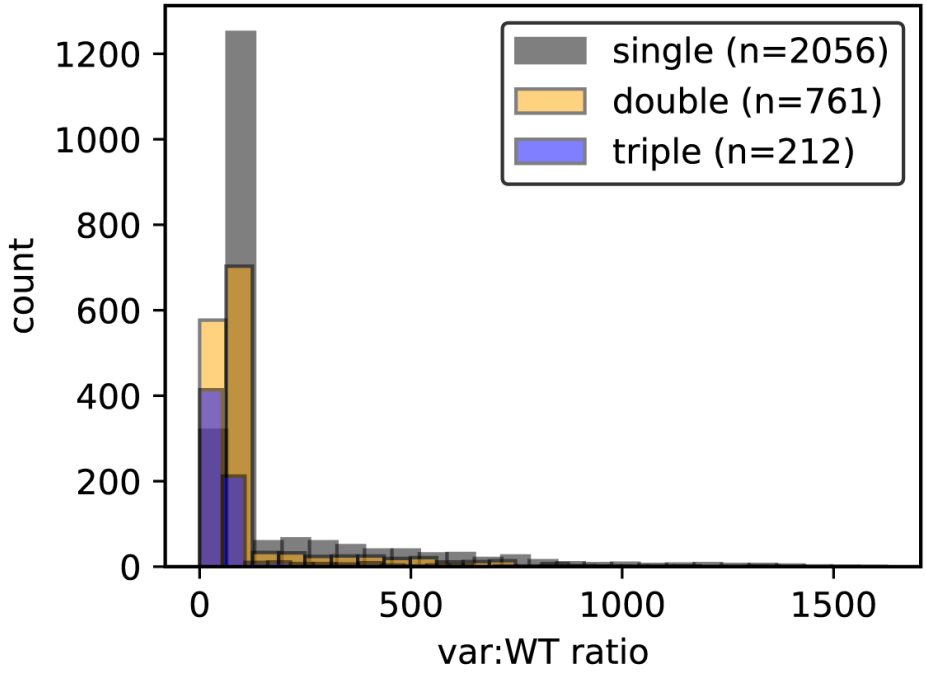


Figure S9. Comparison of observed and simulated single mutant frequency distributions.
We simulated predicted single mutant frequency distributions per sublibrary (assuming equal relative abundances among individual single mutant genotypes) and calculated the following three parameters: total number of barcodes (of 384 possible) meeting a read depth threshold, fraction of intended single mutants compared to all other library variants, and the number of desired unique single mutants. These parameters were used to simulate picking experiments as described in Results and Discussion, and repeated for 1000 replicates (orange bars). Simulated distributions were plotted alongside observed single mutant frequencies for each sublibrary (blue bars).

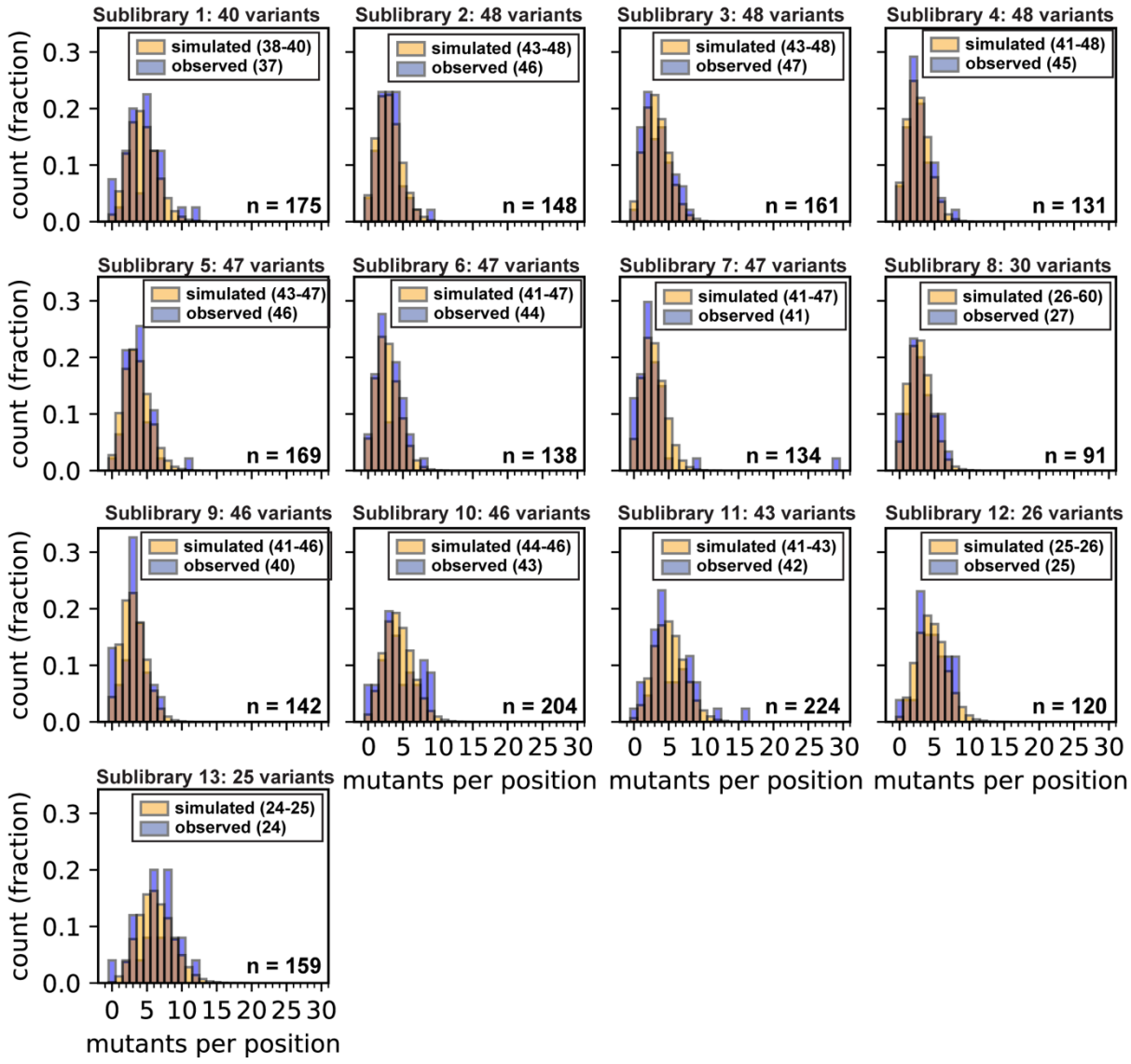


Figure S10. Plasmid map of PURExpress-SpAP-eGFP.

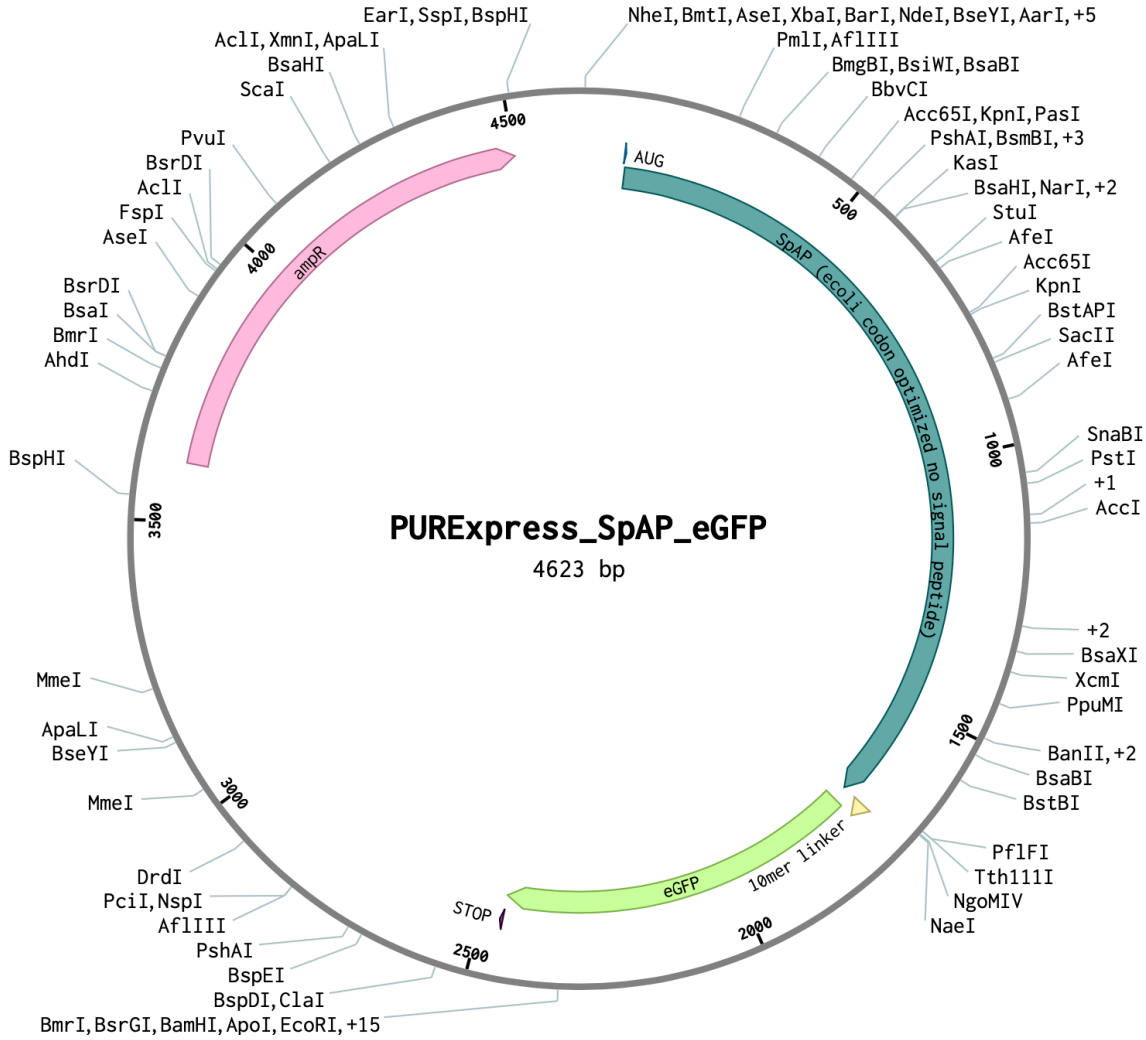


Figure S11. Complete DNA sequence of PURExpress-SpAP-eGFP plasmid.

GCTAGTGGTGCTAGCCCCGCGAAATTAATACGACTCACTATAGGGTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAC
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Figure S12. Protein sequence of SpAP-(10mer linker)-eGFP.

MQSPAPAAAPAPAARSIAATPPKLIVAISVDQFSADLFSEYRQYYTGGLKRLTSEGAVFPRGYQ
SHAATETCPGHSTILTGSRPSRTGIIANNWFDLDAKREDKNLYCAEDESQPGSSSDKYEASPLH
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LSKDPNEKRDHMVLLEFVTAAGITLGMDELYK

Table S1. Time and cost calculations for uPIC–M and conventional mutagenesis. All costs are listed in USD.

MUT-seq mutagenesis

Materials:

item	manufacturer	quantity	units	total cost (USD)	units per reaction	reactions per mutant	cost per mutant (USD)	Notes
oligos	Agilent	3750	number of oligos	2857	1	1	0.76	7500 oligo array with 2X replicates, cost from agilent
KAPA HiFi HotStart 2X Master Mix	Roche	6.25	mL	678.56	0.0013	7.68	1.04	384/50 reactions per desired mutant
pA-Tn5	Diagenode	15	uL	585.9	0.004	7.68	1.20	1:50 dilution of pA-Tn5, 0.2 uL per reaction
KAPA HiFi	Roche	250	U	303.49	0.08	7.68	0.75	
biorad hard shell	Biorad	50	each	327	0.0026	46.08	0.78	~6 replicate plates for dilutions prior to PCR and Tn5 needed for every reaction, and 384/50 reactions per desired mutant
96-head liquidator tips	Rainin	960	each	125	1	30.72	4.00	~4 plate transfers with the liquidator needed for every reaction, and 384/50 reactions per desired mutant
							8.53	

Sequencing: MUT-seq, as above | MUT-seq, liquidator tip washing

mutant library size	sequencing capacity	total sequencing cost (USD)	cost per mutant (USD)	Sequencing and materials cost per mutant (USD):	total sequencing and materials cost (USD)	Sequencing and materials cost per mutant (USD):	total sequencing and materials cost (USD)	Notes
1-50	1000000	600	12.00	20.53	1026.74	16.53	826.74	1M read miseq nano run, 2x250, for 50 mutants in 1x384 well plate of barcodes
500	25000000	3300	3.40	11.93	5967.36	7.93	3967.36	25M read miseq nano run, 2x300, for ~500 mutants in ~13x384 well plates
5000	250000000	2250	0.45	8.98	44923.58	4.98	24923.58	250M read HiSeq 2x150 for 5000 mutants
20000	-	-	-	8.98	179600.00	4.98	99694.30	Same unit cost as 5000

Time and labor costs:

task	time (days)	mutant capacity	days for 1 mutant	days for 50 mutants	days for 500 mutants	days for 5000 mutants	days for 20000 mutants	Notes
sublibrary pre-amp, purification, quikc	1	1000	-	1	1	5	20	
Colony picking and outgrowth	1	500	-	1	1	10	40	
Dilution and ORF PCR amplification	1	500	-	1	1	10	40	
DNA dilution and quantification	1	1000	-	1	1	5	20	
Tn5 tagmentation	1	1000	-	1	1	5	20	
library QC	1	1000	-	1	1	3	5	Library QC will not scale linearly, as samples are pooled at this stage, for 20000 mutants, with approximately 50 possible per 384 well plate, 400 plates and 400 pooled samples to QC and sequence
sequencing and analysis	2	1000	-	2	2	2	2	Sequencing and analysis will not scale linearly, see above
		total days:	-	8	8	40	147	
		Labor costs (at 200 USD/day):	-	1600	1600	8000	29400	Labor costs calculated at a 50k USD annual salary

Conventional mutagenesis

Materials & sequencing:

item	manufacturer	quantity	units	total cost (USD)	units per reaction	reactions per mutant	cost per mutant (USD)	Notes
oligos	IDT	2	each	5.73	2.00	1	5.73	
PFU Turbo polymerase	Agilent	1000	U	791.7	0.63	1	0.49	
Chemical competent E. coli	NEB	1200	uL	153	12.50	1	1.59	
Miniprep columns	Qiagen	1	each	1	1.00	1.5	1.50	Estimate 1.5 minipreps per desired mutant, to account for the rate of picking colonies that do not have the correct sequence
sanger sequencing	-	1	each	2	3.00	1.5	9.00	For a 1500 base pair ORF, with 1 sanger read for each 500 bp
							18.32	

Time and labor costs:

task	time (days)	mutant capacity	days for 1 mutant	days for 50 mutants	days for 500 mutants	days for 5000 mutants	days for 20000 mutants	Notes
quikchange, dpnI digestion	1	100	-	1	5	50	200	1 reaction per mutant
transformation and plating	1	100	-	1	5	50	200	1 transformation and plate per mutant
colony picking and outgrowth	1	100	-	1	5	50	200	
miniprep and sequence	1	100	-	1	5	50	200	
		total days:	-	4	20	200	800	Conventional mutagenesis, without additional automated handling, will scale approximately linearly
		Labor costs (at 200 USD/day):	-	800	4000	40000	160000	Labor costs calculated at a 50k USD annual salary

Table S2. Oligo array and window design details for SpAP scanning mutant library.

sublibrary	length (nt)	primers (forward, reverse)	SpAP positions mutated	substitution
1	200	CGACTCACTATAGGGTCTAGAAATA, CCTCCGGTGTAATATTGACG	2–41	non-val → val val → ala
2	200	GTTTAGTGCAGACTTGTTCTCGGAGT, CGTCCAAGTCGAACCAGTTATTAGCG	42–89	non-val → val val → ala
3	197	CTGACAGGATCACGTCCGTCAC, GCAGGATTGGCGGCTTTCAT	90–137	non-val → val val → ala
4	199	CTTAAAGGTACCCACCCTGGGG, CTGATTGACCTGTGTTACAAGGGGAG	138–185	non-val → val val → ala
5	197	GGGGTATGTTTCGTATAAGGGTGTAGC, CTTTGTAGTCACCAGCATCACGGGC	186–232	non-val → val val → ala
6	196	CGGGAAATCGCACAGTGGGTAC, GTGTGTCCCACGTAATCCGTAGC	233–279	non-val → val val → ala
7	197	GCCCAGACCGATATTATTAGCATTGGAC, GGCAGATCGTGTCCTCCATGATC	280–326	non-val → val val → ala
8	193	CGTTTAGACACGGAGCTTGGTG, CTTTCTCAGCGATGGTAGCATTCAG	327–356	non-ala → ala ala → ala (synonymous)
9	193	GACATGGCCCTGACACCTAAAGC, CAGTCTGTACTTGGGGATGCGC	357–402	non-ala → ala ala → ala (synonymous)
10	199	CAACGTGCCCGTGTTGAAACC, CACGAGGTTTCAATAAAAGTAACAGGTC	403–448	non-ala → ala ala → ala (synonymous)
11	181	CGTCATTTTACCCGTCGCGCTC, CCTAAGGGTTGTTCGAAATGCTGC	449–491	non-ala → ala ala → ala (synonymous)
12	134	GCCGTGTGCCTATCCTGTTTTG, GCCGTCGATCTGATCCTTAGGAAC	492–517	non-ala → ala ala → ala (synonymous)
13[a]	139	CCTTGGCTGCACTTATTAAGCTTCC, TTGCTCACCATGCCACTGCCTC	518–542	non-ala → ala ala → ala (synonymous)

[a]This sublibrary also encodes a mutation for position 542, which is the first residue within a 10 amino acid linker between the SpAP and eGFP ORFs.

Table S3. Concentration of purified sublibrary mutagenic primer pools

sublibrary	primer pool concentration (nM)[a]
1	17.5
2	28.8
3	23.8
4	31.6
5	51.2
6	37.2
7	20.4
8	14.7
9	27.6
10	40.0
11	61.2
12	19.1
13	34.0

[a]Purified dsDNA samples were quantified by UV absorbance.

Table S4. Expected mutant yields from simulations of mutant sampling.
Sequenced clones are reported for achieving unique mutant yields equivalent to 90% of total mutants.

total mutants	single mutant frequency	sequenced clones (median)[a]	sequenced clones (lower)[b]	sequenced clones (upper)[c]
50	0.10	>500	>500	>500
50	0.25	445	324	>500
50	0.50	218	162	473
50	0.75	144	105	450
50	1.00	106	85	330
500	0.10	>5000	>5000	>5000
500	0.25	4726	4290	>5000
500	0.50	2322	2081	4793
500	0.75	1538	1407	4702
500	1.00	1147	1056	4309
5000	0.10	>50000	>50000	>50000
5000	0.25	47703	46405	49957
5000	0.50	23431	22589	49832
5000	0.75	15503	15080	49446
5000	1.00	11586	11296	46054

[a]The minimum number of sequenced clones required to obtain 90% yield of unique single mutants, as determined by the median unique mutant yield of 100 simulated picking experiments.
[b]The lower bound was calculated as the minimum number of sequenced clones required to obtain a 90% yield of unique single mutants within the 95% confidence interval of unique mutant yields expected for this volume, from 100 simulated picking experiments.
[c]The upper bound was calculated as the maximum number of sequenced clones required to obtain a 90% yield of unique single mutants within the 95% confidence interval of unique mutant yields expected for this volume, from 100 simulated picking experiments.

Table S5. Variant composition of small-scale QuikChange-HT reactions.

variant type[a]	count (n=96)	fraction
WT	11	0.11
single	60	0.63
double	4	0.04
triple+	1	0.01
indels, errors[b]	20	0.21

[a]Clones were sequenced with one forward primer spanning the mutational region

[b]Includes indels, with or without the presence of intended codon substitution(s), and includes errors likely attributable to sanger sequencing

Table S6. Sublibrary transformation and colony picking results.

sublibrary	primer pool concentration (nM)	colonies[a]	repeat QuikChange[b]
1	17.5	76	Y
2	28.8	136	N
3	23.8	130	N
4	31.6	200	N
5	51.2	440	N
6	37.2	25	Y
7	20.4	26	Y
8	14.7	130	N
9	27.6	220	N
10	40.0	80	N
11	61.2	150	N
12	19.1	120	N
13	34.0	220	N

[a]Number of colonies obtained after QuikChange mutagenesis using normalized primer concentrations of 15 nM. Plating details: 1 µL of reaction volume used to transform 20 µL NEB-5α cells, followed by addition of 200 µL SOC, of which 100 µL was plated on a 150 mm LB agar plate. Sublibraries not meeting colony yield requirements (400–500 colonies) were plated and/or transformed again at higher volume.

[b]QuikChange mutagenesis was repeated for these sublibraries using the maximum possible concentrations of stock primer pools allowed by reaction volumes.

Table S7. Amplicon DNA and library concentration statistics.

sublibrary	plate	sample wells total	sample wells assayed[a]	median[b]	mean[b]	standard deviation[b]	tagmented library concentration[b, c]
1	1	384	184	25	34	24	17
2	2	384	184	61	58	22	12
3	3	384	184	37	39	17	14
4	4	384	184	28	32	22	3
5	5	384	184	39	40	26	12
6	6	384	184	11	11	7	9
7	7	384	184	29	32	21	4
8	8	384	368	1	20	27	5
9	9	384	184	47	47	23	3
10	10	384	184	57	54	29	17
11	11	384	184	31	34	21	3
12	12	384	184	16	18	11	4
13	13	384	184	17	21	14	3

[a]Number of amplicon wells measured by fluorescence assay for DNA concentration

[b]In units of ng/µL

[c]Following tagmentation, barcoding/amplification PCR, and pooling of all 384 sample wells per plate, concentration represents total upon integration of all fragmentation peaks (see Figure 5)

Table S8. Unique single mutant yields for the SpAP scanning library.
Total and fractional yields for the entire library (bold text) and within each mutational sublibrary are shown at read threshold values of 0, 1, 10, 100, and 1000. The read threshold value represents the minimum number of variant reads for each single mutant, and, the minimum ratio of var:WT reads (sum of forward and reverse reads in each case).

sublibrary	residues	total positions	yield at read thresholds: 0–1000 (fraction of total)			
			n/a	10	100	1000
all	**2–542**	**541**	**507 (0.94)**	**498 (0.92)**	**484 (0.89)**	**60 (0.11)**
1	2–41	40	37 (0.93)	36 (0.9)	35 (0.88)	0 (0)
2	42–89	48	46 (0.96)	45 (0.94)	45 (0.94)	12 (0.25)
3	90–137	48	47 (0.98)	46 (0.96)	45 (0.94)	7 (0.15)
4	138–185	48	45 (0.94)	41 (0.85)	36 (0.75)	2 (0.04)
5	186–232	47	46 (0.98)	46 (0.98)	45 (0.96)	6 (0.13)
6	233–279	47	44 (0.94)	44 (0.94)	42 (0.89)	1 (0.02)
7	280–326	47	41 (0.87)	40 (0.85)	40 (0.85)	3 (0.06)
8	327–356	30	27 (0.9)	26 (0.87)	26 (0.87)	4 (0.13)
9	357–402	46	40 (0.87)	40 (0.87)	38 (0.83)	1 (0.02)
10	403–448	46	43 (0.93)	43 (0.93)	42 (0.91)	8 (0.17)
11	449–491	43	42 (0.98)	42 (0.98)	42 (0.98)	16 (0.37)
12	492–517	26	25 (0.96)	25 (0.96)	24 (0.92)	0 (0)
13	518–542	25	24 (0.96)	24 (0.96)	24 (0.96)	0 (0)

Table S9. Comparison of uPIC–M performance with simulated picking experiments, per sublibrary.

sublibrary	total barcodes	single mutant frequency	total positions	observed positions[a]	expected positions, median (95% CI)[b]
1	318	0.55	40	**37**	40 (38–40)
2	274	0.54	48	46	46 (43–48)
3	298	0.54	48	47	46 (43–48)
4	247	0.53	48	45	45 (41–48)
5	315	0.54	47	46	46 (43–47)
6	264	0.52	47	44	45 (41–47)
7	241	0.56	47	41	45 (41–47)
8	146	0.62	30	27	29 (26–30)
9	260	0.55	46	**40**	44 (41–46)
10	352	0.58	46	**43**	46 (44–46)
11	329	0.68	43	42	43 (41–43)
12	216	0.56	26	25	26 (25–26)
13	270	0.59	25	24	25 (24–25)

[a]Number of unique mutants obtained for sublibrary. Sublibraries not meeting expected yields (based on the 95% confidence interval) are in bold red text.

[b]Predicted number of unique mutants given the observed single mutant frequency and number of clones sampled for plate, reported as the median of 1000 simulated sampling events.

Table S10. Oligo array price summary.

array size (unique oligos)	array cost (USD)	cost per oligo (USD)[a]
7.5×10^3	2,857	0.38
1.5×10^4	5,714	0.38
6.0×10^4	8,435	0.14
1.0×10^5	10,856	0.11
2.44×10^5	23,883	0.10

[a]Oligo array price summary (academic pricing, obtained 01/2021 from Agilent Technologies, personal communication). Prices are provided for arrays containing oligos of length 191–210 nt.