SAS Short Library

	A	C	G	T	Sum
A	-	558	190	610	1358
C	731	-	204	774	1709
G	312	539	-	587	1438
T	472	524	518	-	1514
Sum	1515	1621	912	1971	6019

SAS Long Library

	A	C	G	T	Sum
A	-	542	310	500	1352
C	649	-	231	571	1451
G	394	241	-	497	1132
T	353	492	235	-	1080
Sum	1396	1275	776	1568	5015

Error-Prone PCR

	A	C	G	T	Sum
A	-	73	347	22	442
C	52	-	12	146	210
G	143	19	-	83	245
T	20	324	21	-	365
Sum	215	416	380	251	1262

Supplementary Table 1

Mutation distribution for SAS mutation libraries and error-prone PCR

Mutant singleton reads per 10,000 for each nucleotide (rows) to each alternative nucleotide (columns). Transitions are highlighted in red. SAS short library was 217bp (including primer sequence) and was fully sequenced in both directions on an Illumina MiSeq. The SAS long fragment was a >1188bp linear product amplified from a plasmid and the distal 3' 607bp were sequenced in three sequence-barcoded segments each of ~200bp. The mutation rate along the length of the sequenced section of this SAS product is in Supplementary Figure 5. Error prone PCR was performed using Taq polymerase on the same 217bp sequence as the SAS short library. The expected transition:transversion ratio is observed for the error-prone PCR.

	Mutagenized Region Length	Sequenced Region length	# Clones	# of Mutant Clones (%)	# of Singleton Mutant Clones (%)	% of mutations present within 10x of mean % of reads
SAS Long Fragment	1188bp+	607bp	1106756	634248 (57%)	555115 (50%)	98%
SAS Short Fragment	202bp+	202bp	4007170	2724875 (68%)	2411915 (60%)	100%
High Fidelity PCR	202bp+	202bp	3747273	40245 (1%)	40060 (0.9%)	100%
Error Prone PCR	202bp+	202bp	3077664	419177 (13%)	388401 (12%)	100%

Supplementary Table 2

Characteristics of SAS libraries versus PCR

Characteristics of SAS libraries compared to either error-prone PCR or high fidelity PCR of the same regions. For the SAS long fragment, the distal end of the mutagenized region was sequenced in three overlapping, sequence tagged sections and aligned *in silico* by assuming reads harboring the same tag originated from the same DNA molecule.

Supplementary Note

Protocol for SAS Mutagenesis using Reversibly-Terminated Inosine (Patent Pending)

References

LABELED NUCLEOSIDE TRIPHOSPHATES WITH REVERSIBLY TERMINATING AMINOALKOXYL GROUPS *Nucleosides, Nucleotides and Nucleic Acids*, 29:879–895, 2010

Creation of 3'-O-NH2, 2'-deoxyinsoine triphosphate

Starting with 1ul of 100mM 3'-O-NH2, 2'-deoxy adenosine triphosphate (rtATP) (of which we purchased 5umoles total, i.e. 50 ul from Firebird Biomolecular Sciences, LLC), rSAP (NEB) treat the rtATP for 1 hr at 37 deg. The reaction is 1ul rtATP, 1ul cutsmart buffer and 8ul water (for a total of 10ul) then add 1 ul rSAP. Inactivate the rSAP by denaturing at 95 deg for 20 min. Add 5ul of adenosine deaminase (in ammonium sulfate) to a microcentrifuge tube and spin. Add denatured rSAPed rtATP to this protein precipitate after removing supernatant. Check for deamination using spectrophotometer watching for shift from 260nm to 250nm (ATP to ITP). This product can then be used as the substrate for the next kinase reaction. Add 2ul of 10x T4 ligase buffer and 2ul of T4 PNK buffer (NEB), 1ul T4 PNK (NEB), 5ul of pyruvate kinase (diluted in water and stored at -20deg) (Sigma) and 5ul of myokinase (adenylate kinase) (Sigma) (diluted in water and stored at -20). Both enzymes are ~5u/ul. The total reaction is now 25ul and the concentration of the rtITP should be 4mM.

Linear amplification of targets with rtITP

Using a biotinylated primer, perform a PCR with 50% rtITP.

16 ul	H2O
5 ul	5x GoTaq Flexi Buffer (colorless)
1 ul	4 mM dNTPs (~1ug/ul)
1 ul	4 mM rtITP (~1ug/ul)
1 ul	10uM biotinylated F primer
0.4 ul	Firebird Taq 475
1ul (200ng/ul)	Template
25 ul	TOTAL

Perform default PCR conditions on ABI thermocycler (25 cycles).
Note: This will produce 25x more product than template (assuming the primers and dNTPs are in excess), which means that the product is 1x dsDNA and 25x ssDNA that has been randomly terminated. **It is important to store beads in 1x TE for the stability of the ssDNA.**

**Before bead binding, linear products can be extracted from an agarose gel. The desired section of the gel can be melted using gel extraction buffer (yellow) from qiagen and streptavidin beads added directly to the melted gel in the gel extraction buffer and incubated for 30 min rocking at room temperature.

This process was used for shorter products to increase the proportion of reads harboring inosine nucleotides after linear amplification. We extracted a band consisting of sizes larger than primer and smaller than full length or where the reverse primer was to hybridize.

Bind biotinylated DNA to DYNA beads (streptavidin coated)

Take 10 ul of streptavidin coated beads and remove supernatant using a magnet in a lo-bind tube. Wash beads in 200ul Binding buffer (see solutions at end of protocol) in a Lo-Bind tube. Add 150 ul Binding Buffer linear amplification product and transfer to washed beads.
Incubate 30 min at room temp, rocking
(Pre-warm wash buffer 2 at 65C)
Wash 1x with 200ul Wash buffer 1, 15 min room temp rocking
Pre-warm Wash Buffer 2 to 65C. Perform next 3 washes one at a time to keep solutions at 65C
Wash 3x with 200ul Wash buffer 2, resuspend by pipetting, incubate 15 min at 65C
Resuspend in 1x TE. Beads are stable at this step if you choose to pause here.

Reverse Termination of rtlTP and extension

*****Note: 5 min of exposure of rtATP to 700mM sodium nitrite, pH 5.5 will enable PCR amplification in the absence of dATP, thus proving that it reverses the termination sufficiently enough to allow use in PCR.
Take beads resuspended in 1x TE and remove TE. Add 20ul of 700mM sodium nitrite, pH 5.5. Allow to sit for 5 min (not longer). Prolonged exposure may damage DNA. Remove sodium nitrite, wash 3x with TE and add PCR reaction containing:

All	Streptavidin beads
18 ul	H2O
5 ul	5x GoTaq Flexi Buffer (colorless)
1 ul	10 mM dNTPs
1 ul	Firebird Taq 475
25 ul	TOTAL

Perform default PCR conditions on ABI thermocycler (8 cycles).
Note: Each additional cycle allows a different set of unextended products to find a template and extend. If you choose to perform only 1 cycle or fewer cycles, I suggest adding sufficient template such that all of the unexpended products have a template. Theoretically, after a few cycles all of the unextended product will have had a chance to sit down on a full length template and extend.

Removal of Template DNA

In order to make sure you aren't just amplifying the template, plasmids can be degraded with DPN1 enzyme if obtained directly from bacteria and DNA amplified with deoxyuricil can be degraded with uracil-DNA-glycosylase and exonuclease VIII. If these are not true of your template, having used a biotinylated primer is the only way to get rid of the template.
To do this, ***add 1ul of the R primer only and amplify for 2 additional cycles.***
Wash 1x with 200ul Wash buffer 1, 15 min room temp rocking
Pre-warm Wash Buffer 2 to 65C. Perform next 3 washes one at a time to keep solutions at 65C
Wash 3x with 200ul Wash buffer 2, resuspend by pipetting, incubate 15 min at 65C
Resuspend in 1x TE. Beads are stable at this step if you choose to pause here.

Amplify from beads

All	Streptavidin beads
17 ul	H2O
20 ul	2x NEB Next HF Mastermix
1 ul	10 mM dNTPs
1ul	F primer (not biotinylated)
1ul	R primer
40 ul	TOTAL

Perform default PCR conditions on ABI thermocycler (25 cycles).
Purify either using a Qiagen PCR cleanup kit or AmPure beads with ½ 50% 3350 glycerol and ½ 5M NaCl.

Reagents

Streptavidin Bead Binding Buffer: 10 mM Tris-HCL (pH 7.5), 1 mM EDTA (pH 8) and 1 M NaCl
Wash Buffer 1: 1X SSC with 0.1% SDS
Wash Buffer 2: 0.1X SSC with 0.1% SDS
Elution Buffer: 0.1 M NaOH (use only if you want to ensure there is no template)