Supplementary Materials for

RNA-guided DNA insertion with CRISPR-associated transposases

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Materials and Methods

Cyanobacteria RNA Sequencing

Scytonema hofmanni (UTEX B 2349) and *Anabaena cylindrica* (PCC 7122) were cultured in BG-11 media (ThermoFisher) at 25°C with light periodicity of 14 hours on, 10 hours off. RNA was isolated using the miRNeasy Mini Kit (Qiagen) and treated with DNase I (NEB). rRNA was removed using RiboMinus (ThermoFisher). RNA libraries were prepared from rRNA-depleted RNA using NEBNext Small RNA Library Prep Set for Illumina (NEB).

RNA-Sequencing Analysis

RNA libraries were sequenced using a NextSeq 500/550 High Output Kit v2, 75 cycles (Illumina). Paired-end reads were aligned to their respective reference genomes using BWA (*35*) and entire transcripts were extracted using BEDTools. Resulting transcript sequences were analyzed using Geneious Prime 2019.0.4.

Generation of Heterologous Plasmids

Purified gDNA from *Scytonema hofmanni* and *Anabaena cylindrica* were prepped using the DNeasy Blood and Tissue Kit (Qiagen). Subsequently, CAST loci, excluding cargo genes, were amplified from the purified gDNA using KAPA HiFi HotStart ReadyMix (Kapa Biosystems) and cloned into pUC19. A *lac* promoter was placed in front of the CAST transposase genes and Cas12k gene, and a J23119 promoter was added in front of a shortened CRISPR array with two direct repeats. The first endogenous spacer in the array was replaced with the FnCpf1 protospacer 1 (PSP1) sequence (5'-GAGAAGTCATTTAATAAGGCCACTGTTAAAA-3'). The CAST open reading frames (ORFs) and downstream tracr regions were unchanged. Sequences of all bacterial expression plasmids can be found in Table S1.

PAM and Motif Screens

A randomized target PAM and insertion motif library was generated using synthesized ssDNA oligonucleotides (IDT) with 6 randomized bases upstream of PSP1 and 8 randomized bases starting 55 bp downstream of the spacer. Oligonucleotides were used to generate a PCR product for subsequent Gibson assembly (NEB) into pACYC184 vectors. Gibson products were

electroporated into Endura ElectroCompetent cells (Lucigen), recovered for 1 hour, and plated on chloramphenicol plates. Cells were harvested 16 hours after plating and plasmid DNA was harvested using a Maxi-prep kit (Macherey-Nagel). 100 ng of library target DNA was co-electroporated with 100 ng of both pHelper and pDonor into TransforMax EC100D pir+ *E. coli*. Cells were recovered for 1 hour and plated on ampicillin, kanamycin, and chloramphenicol-containing plates. Insertion products containing the randomized PAM sequence or motif sequence were amplified and sequenced using a MiSeq Reagent Kit v2, 300-cycle (Illumina). In addition, the PAM and motif sequences in the library targets were amplified and sequenced alongside insertion samples.

PAM and Motif Discovery Pipeline

For sequence verified insertion events, the randomized PAM region and motif regions were extracted, counted, and normalized to the total number of reads from the corresponding sample. The enrichment of a given randomized sequence was determined by its ratio in the insertion sample to its abundance in the library target. These ratios were used to create PAM wheels using Kronos Plot (https://github.com/marbl/Krona/wiki) (*36*). PAMs and motifs above a $\log_2$ enrichment threshold of 4 and 1, respectively, were collected and used to generate sequence logos.

Droplet digital PCR (ddPCR)

ddPCR Supermix for Probes (BioRad), primers, product specific probes, and sample were combined into 20 μL reactions and droplets were generated using the QX200 Droplet Generator (BioRad). Insertion events were quantified using insertion PCR specific primers and a donor specific probe (Table S4). Targets were quantified using target specific PCR primers and a corresponding probe (Table S4). Thermal cycling conditions for ddPCR reactions were as follows: 1 cycle, 95°C, 10 min; 40 cycles, 94°C, 30 sec, 60°C, 1 min; 1 cycle, 98°C, 10 mins; 4°C hold; 2°C/sec ramp for every step. ddPCR plates were sealed with a foil heat seal (BioRad) and read with a QX200 Droplet Reader. Absolute concentrations of inserts and targets were determined using QuantaSoft (v1.6.6.0320) and insertion frequency calculated by inserts/(inserts+targets).

E. coli Plasmid-Targeting Assays

Targeted transposition into target plasmids was performed by transformation of 5 ng each of pHelper, pInsert, and pTarget into One Shot Pir1 Chemically Competent *E. coli* (Invitrogen). Cells were recovered for 1 hour and plated on ampicillin, kanamycin, and chloramphenicol-containing plates. Cells were harvested 16 hours after plating and grown for 8 hours in LB media containing ampicillin, kanamycin, and chloramphenicol. Plasmid DNA was isolated using a Qiaprep Miniprep Kit (Qiagen), diluted approximately 500-fold, and quantified using ddPCR as described above.

Purification of shCAST Proteins

ShCAST genes were cloned into bacterial expression plasmids (T7-TwinStrep-SUMO-NLS-Cas12b-NLS-3xHA) and expressed in BL21(DE3) cells (NEB #C2527H) containing a pLysS-tRNA plasmid (from Novagen #70956). Cells were grown in Terrific Broth to mid-log phase and the temperature lowered to 20°C. Expression was induced at 0.6 OD with 0.25 mM IPTG for 16-20 h before harvesting and freezing cells at -80°C. Cell paste was resuspended in lysis buffer (50 mM TRIS pH 7.4, 500 mM NaCl, 5% glycerol, 1 mM DTT) supplemented with EDTA-free cOmplete protease inhibitor (Roche). Cells were lysed using a LM20 microfluidizer device (Microfluidics) and cleared lysate was bound to Strep-Tactin Superflow Plus resin (Qiagen). Resin was washed using lysis buffer and protein was eluted with lysis buffer supplemented with 5 mM desthiobiotin, with the exception of tniQ. The TwinStrep-SUMO tag was removed by overnight digest at 4°C with homemade SUMO protease Ulp1 at a 1:100 weight ratio of protease to target. tniB, tniC, and Cas12k protein was diluted with 50 mM TRIS pH 7.4, 50 mM NaCl to a final concentration of 200 mM NaCl and purified using a HiTrap Heparin HP column on an AKTA Pure 25 L (GE Healthcare Life Sciences) with a 200 mM-1M NaCl gradient. Fractions containing protein were pooled and concentrated and loaded onto a Superdex 200 Increase column (GE Healthcare Life Sciences) with a final storage buffer of 25 mM TRIS pH 7.4, 500 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM DTT. tniQ was cleaved from Strep-Tactin Superflow Plus resin with SUMO protease Ulp1 overnight at 4°C and loaded onto a Superdex 200 Increase column with a final storage buffer of 25 mM TRIS pH 7.4, 500 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM DTT. All proteins were concentrated to 1 mg/mL stocks and flash-frozen in liquid nitrogen before storage at -80°C.

In vitro Transposition Assays

Purified proteins were diluted to 2 uM in 25 mM Tris pH 8, 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 25% glycerol. All RNA was generated by annealing a DNA oligonucleotide containing the reverse complement of the desired RNA with a short T7 oligonucleotide or by adding the T7 promoter through PCR. In vitro transcription was performed using the HiScribe T7 High Yield RNA synthesis kit (NEB) at 37°C for 8-12 hours and RNA was purified using Agencourt AMPure RNA Clean beads (Beckman Coulter).

In vitro transposition reactions were carried out with 50 nM of each protein where indicated, 20 ng of pTarget plasmid, 100 ng of pDonor, 600 nM final RNA concentration in a final reaction buffer of 26 mM HEPES pH 7.5, 4.2 mM TRIS pH 8, 50 ug/mL BSA, 2 mM ATP, 2.1 mM DTT, 0.05 mM EDTA, 0.2 mM $MgCl_2$, 28 mM NaCl, 21 mM KCl, 1.35% glycerol, (final pH 7.5) supplemented with 15 mM $MgOAc_2$ as previously described for Tn7 (*37*). Total reaction volumes were 20 uL and reactions were incubated for 2 hours at the indicated temperature and purified using Qiagen PCR Purification columns before bacterial transformation or PCR readout.

E. coli Genome-Targeting Assays

48 guides with NGTN PAMs were randomly chosen in non-coding regions of the *E. coli* genome (Table S3) and cloned into pHelper with the sgRNA configuration. 5 ng of pHelper constructs targeting the genome were transformed into Pir1 cells harboring pDonor, recovered for 15 minutes, and plated on ampicillin and kanamycin-containing plates. Successful insertion was identified by performing nested colony PCR using KAPA HiFi HotStart ReadyMix (Kapa Biosystems). The remainder of cells were harvested 16 hours after plating and gDNA was purified using DNeasy Blood and Tissue Kit (Qiagen) for further analysis.

Genome insertions were sequence verified by insertion-specific amplification and sequenced using a MiSeq Reagent Kit v2, 150-cycle (Illumina). Paired end reads were trimmed of donor sequence and mapped to the genome using BWA (*35*). Resulting sequences were used to determine insertion position relative to the guide sequence. Frequency of genome insertions was determined with ddPCR as described above with a guide specific forward primer (Table S3).

Target abundance was determined by ddPCR amplification of the target sequence using guide specific primers (Table S3) and QX200 ddPCR EvaGreen Supermix (Bio-Rad).

E. coli Specificity Analysis

100 ng of pHelper with sgRNA targeting PSP15, PSP42, or PSP49 was electroporated alongside 100 ng of a modified pDonor harboring a temperature sensitive pSC101 origin into Endura ElectroCompetent cells. After 1 hour of recovery, cells were grown for 6 hours in LB media containing ampicillin and kanamycin at 30°C. Recovered cells were plated on media containing ampicillin and grown for 12 hours at 43°C. gDNA was purified using DNeasy Blood and Tissue Kit. Unbiased detection of transposition events was performed as previously described (*7*). Purified gDNA was tagmented with Tn5, followed by QIAquick PCR purification (Qiagen). Tagmented DNA samples were amplified using two rounds of PCR with KOD Hot Start DNA Polymerase (Millipore) using a Tn5 adapter-specific primer and nested primers within the DNA donor. The resulting libraries were sequenced using a NextSeq v2 kit, 75 cycle. Paired end reads were filtered to remove sequences not matching donor sequence, either due to low quality or amplification artefacts. Remaining reads were trimmed of donor sequence and mapped to the genome using BWA (*35*) to determine insertion position. Insertion positions with more than two unique reads were called as genome insertions for subsequent analysis. On-target rate was defined as the number of reads mapping to the region 55-75 bp downstream of the targeting protospacer compared to all reads mapping to genome insertions.

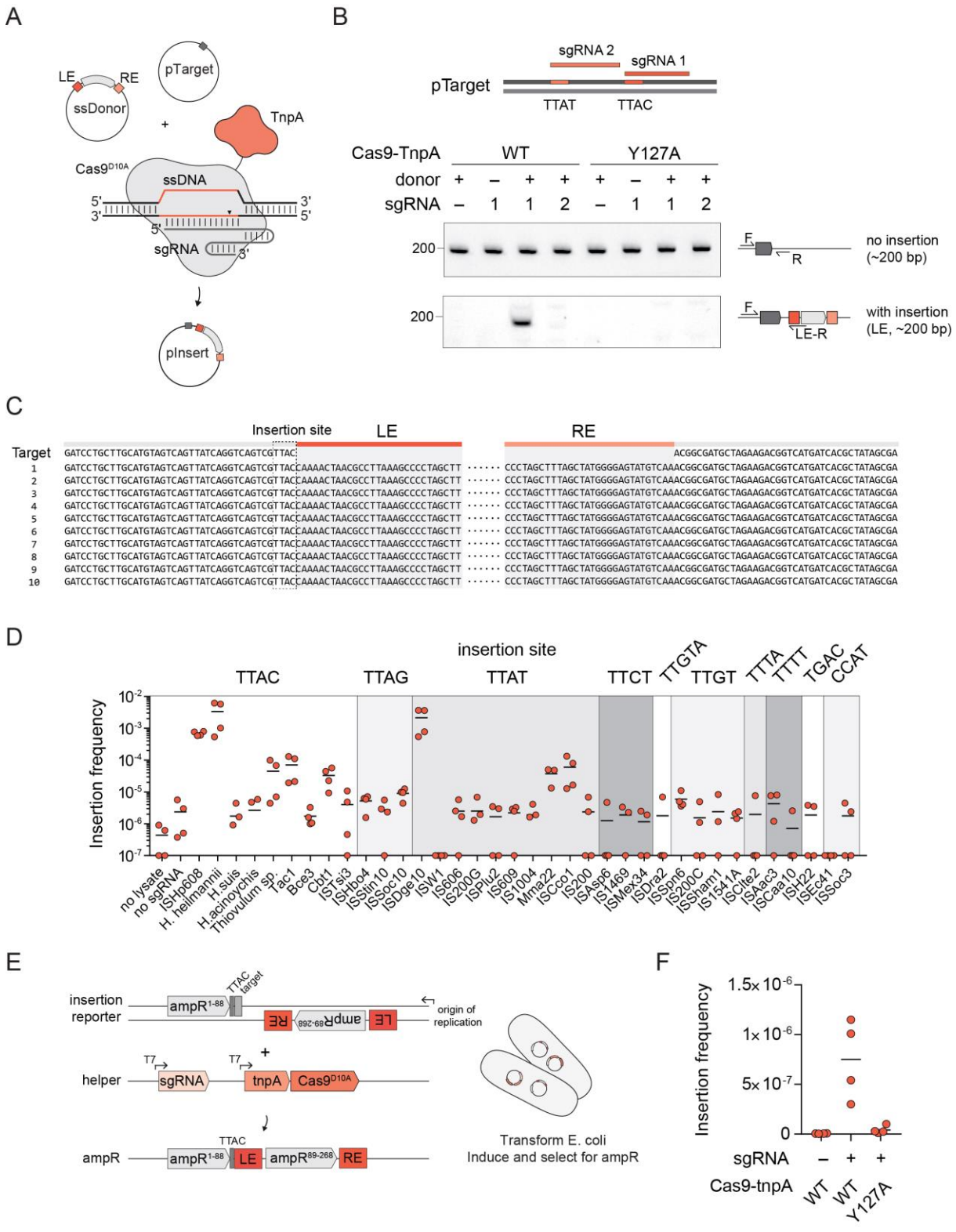

Fig. S1. Engineering Cas9-TnpA fusions for targeted DNA transposition

A. Schematic of in vitro insertion reactions using TnpA fused to Cas9^{D10A}. Cas9 binding creates an R-loop and exposes a window of ssDNA that is accessible to the ssDNA-

specific transposase TnpA (*17*, *38*). We fused tnpA from *Heliocobacter pylori* to $Cas9^{D10A}$ which nicks the target strand with the hypothesis that host-repair machinery would fill-in the opposite strand of the inserted ssDNA donor. Reactions were performed with HEK293T cell lysate and plasmid targets with circular ssDNA RE-LE joint donor intermediates.

B. In vitro insertions with Cas9-TnpA into a plasmid target. Insertions were detected by PCR and are dependent on donor DNA, an active transposase, and an sgRNA which exposes the TTAC insertion motif in the R-loop. Mutation of TnpA-Y127 has previously been shown to abolish transposase activity (*18*).
C. Deep sequencing of in vitro reaction products with flanking primers reveals precise insertions downstream of the TTAC insertion site. LE and RE elements are annotated.
D. In vitro testing of TnpA family proteins from across a variety of insertion site substrates. All TnpA proteins were fused to $Cas9^{D10A}$ and expressed in HEK293T cells. Insertion frequency was determined using ddPCR, n = 4 replicates.
E. Schematic of a reporter plasmid in *E. coli* with a split beta-lactamase gene. The DNA donor was placed adjacent to the plasmid origin to be on the lagging DNA strand during replication to promote donor excision. Insertion of LE-$ampR^{89\text{-}268}$-RE into the target site generates a functional resistance gene and insertion frequency was determined by counting the number of resistant colonies. Resistant colonies were Sanger sequenced which revealed correct insertion into the target site (8 tested).
F. Insertion frequency of TnpA-Cas9 in *E. coli* as measured by ampicillin resistant colonies. n = 4 replicates.

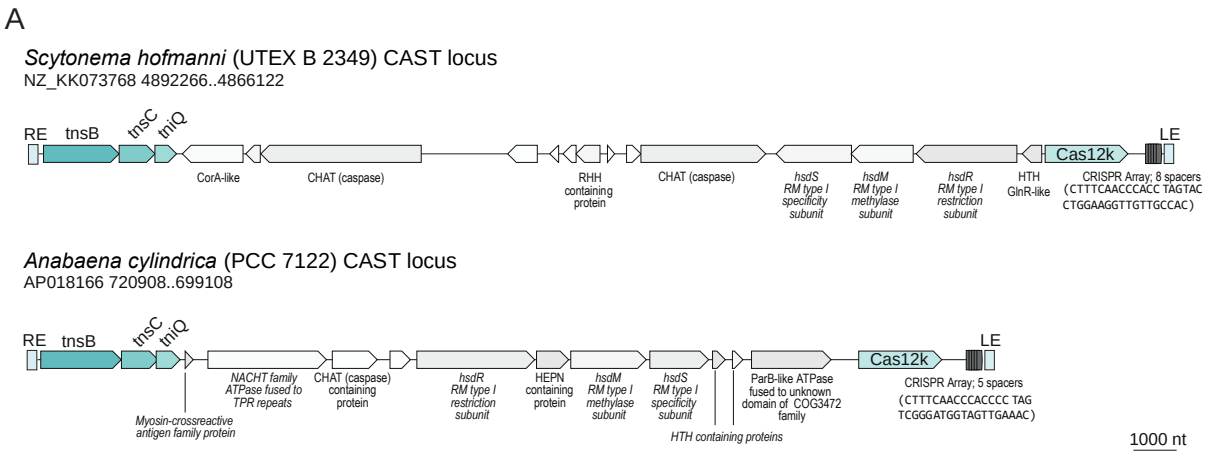

Fig. S2. CRISPR-associated transposase (CAST) systems and sequence features of TnsB, TnsC and TniQ proteins

A. Annotated genome maps for the two Tn7-like elements analyzed in this work. Species name, genome accession number and nucleotide coordinates are indicated. The genes are shown by block arrows indicating the direction of transcription and drawn roughly to

scale. The CAST-related genes are colored. Annotated cargo genes are shown in light gray and a short description is provided according to statistically significant hits (probability>90%) from the respective HHpred searches. The number of spacers in the CRISPR arrays and the sequence of the CRISPR repeats are indicated at the right end of the schemes.

B. Sequence features and domain organizations of three core proteins of the CAST transposase. Proteins are shown as rectangles drawn roughly to scale. Domains are shown inside the rectangles as gray boxes based on the statistically significant hits (probability>90%) from the respective HHpred searches. The most relevant hits from the PFAM database are mapped and are shown above the respective rectangles. ShTniQ protein is compared with selected homologs from different Tn7-like elements. The catalytic motifs are indicated for ShTnsB and ShTnsC. Abbreviations: CHAT, caspase family protease; HEPN, predicted RNase of HEPN family; HTH- helix-turn-helix DNA binding domain; RHH, ribbon-helix-helix DNA binding domain; RM, restriction-modification; TPR, Tetratricopeptide repeats containing protein.

C. Small RNA-seq reveals active expression of AcCAST CRISPR array and predicted tracrRNA.

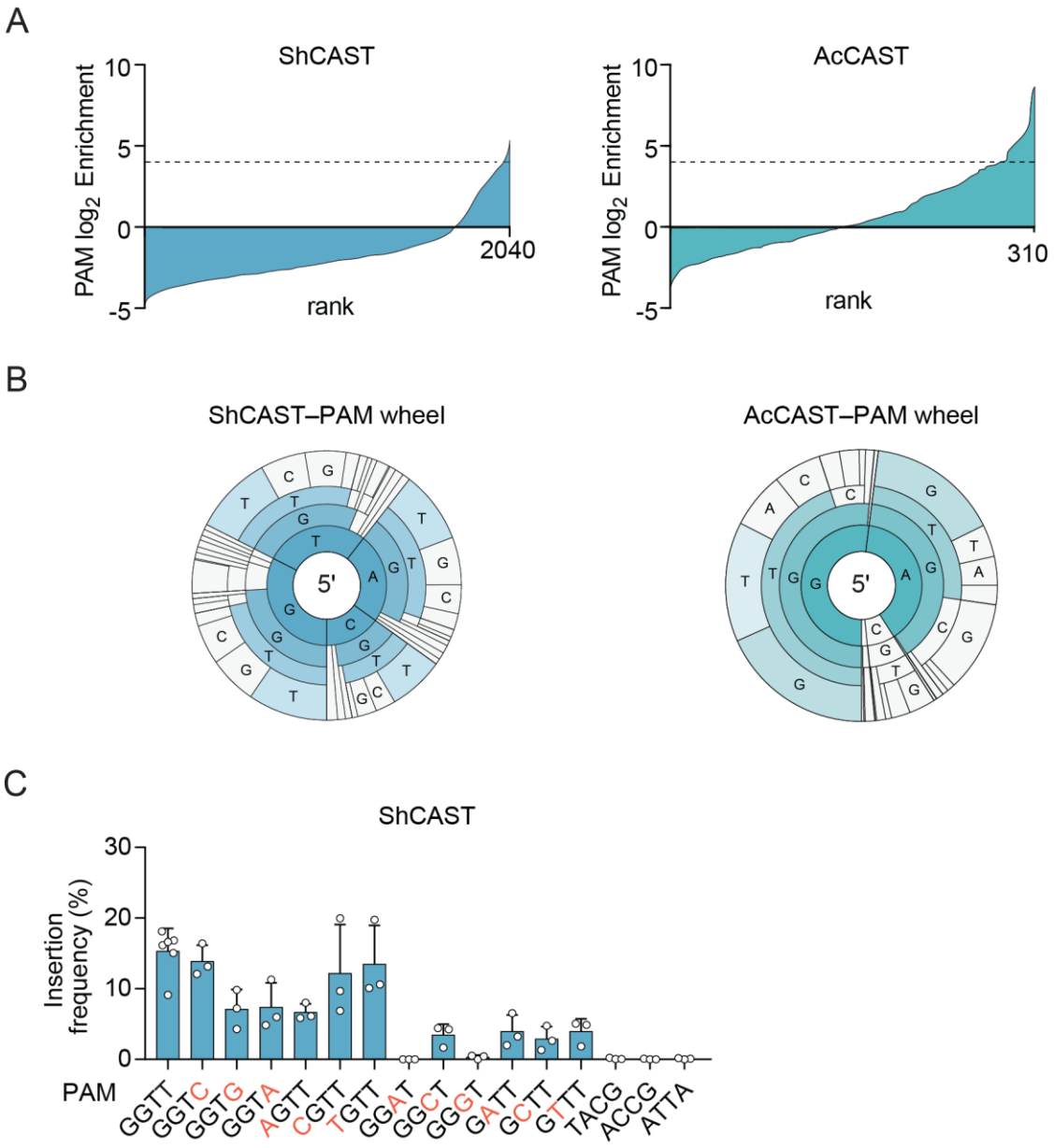

Fig. S3. Targeting requirements for RNA-guided insertions

A. Transformation of a library of PAMs, pDonor, and ShCAST pHelper or AcCAST pHelper into *E. coli* was used to discover PAM targeting requirements. Insertion products were selectively amplified and PAMs with detectable insertions were ranked and scored based on their $\log_2$ enrichment score. A $\log_2$ enrichment cutoff of 4 was used for subsequent analysis of preferred PAMs.

B. PAM wheel interpretation of preferred PAM sequences for ShCAST and AcCAST.

C. Validation of individual PAMs in ShCAST was performed by transformation of pHelper, pDonor, and pTarget with a defined PAM. Insertion frequency was determined by ddPCR.

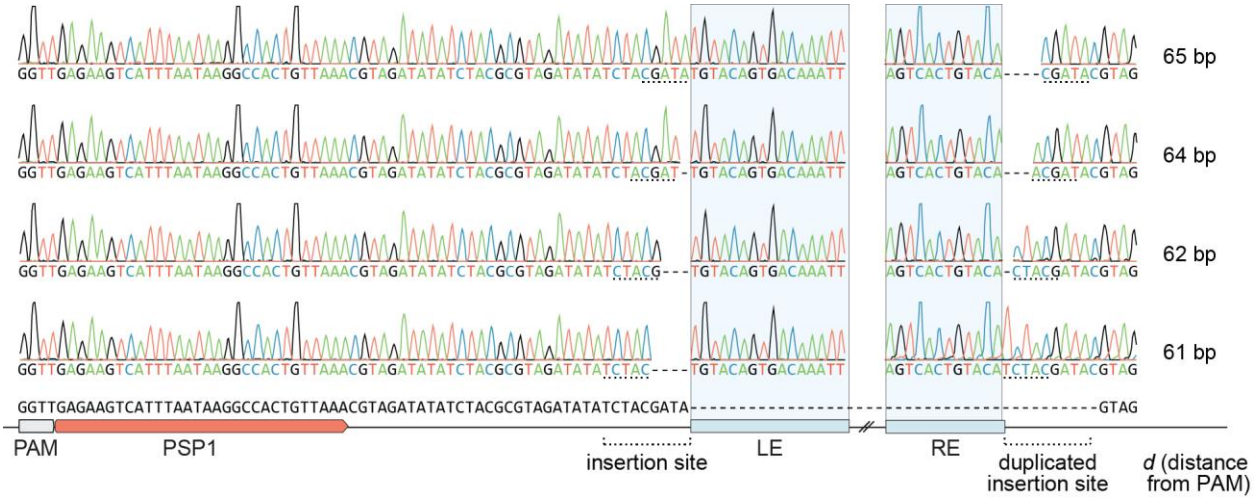

Fig. S4. Sanger sequencing of targeted insertion products in *E. coli*

Plasmid DNA from *E. coli* transformed with pHelper, pDonor, and pTarget$_{GGTT}$ was re-transformed into *E. coli* and Sanger sequenced verified. The duplicated insertion site is underlined in each trace.

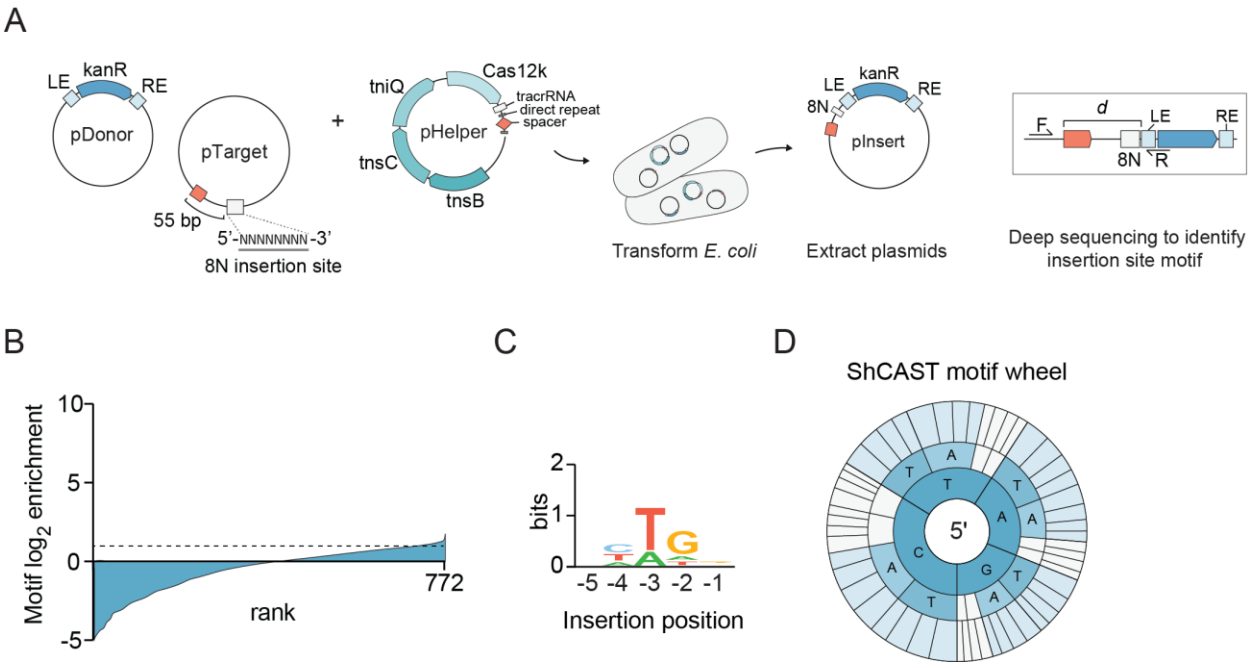

Fig. S5. Insertion site requirements for RNA-guided insertions

A. Schematic of insertion motif library screen. pDonor, pTarget, and pHelper are transformed into *E. coli* and insertions are enriched by PCR for subsequent sequencing analysis.

B. 5N motifs upstream of the insertion site were ranked and scored based on their $\log_2$ enrichment relative to the input library. The 5 bp upstream of the most abundant insertion position (62 bp) were used for analysis. A $\log_2$ enrichment cut-off of 1 was used for subsequent analysis of preferred motifs, showing a very weak motif preference.

C. Sequence logo of 5N preferred motifs shows minor preference for T/A nucleotides 3 bp upstream of the insertion site.

D. Motif wheel interpretation of identified preferred motif sequences.

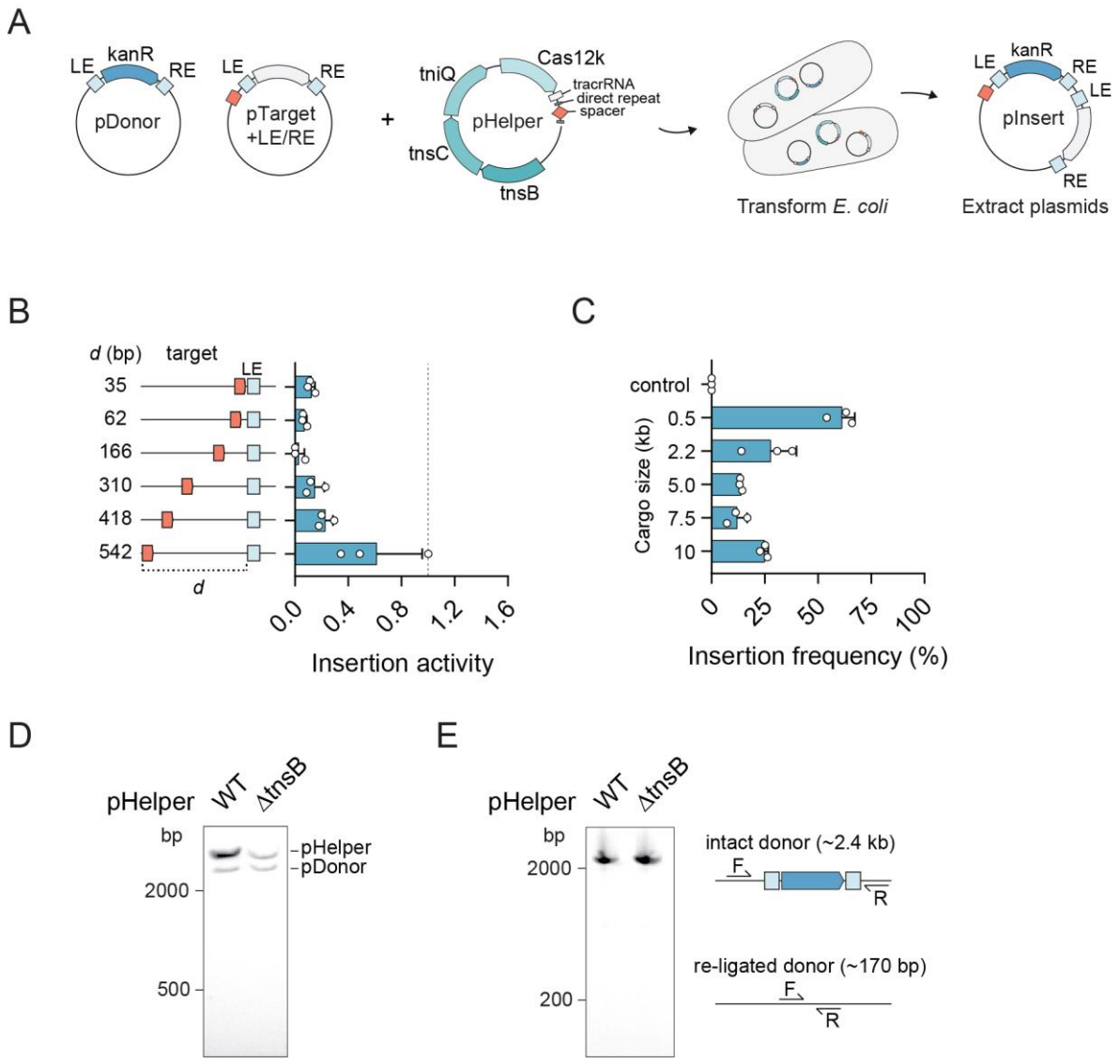

Fig. S6. Transposition properties of ShCAST

A. Schematic of plasmid insertion assay targeting a plasmid containing ShCAST transposon ends.
B. Insertion activity into pTarget containing ShCAST transposon LE. Insertion activity for each target is defined as the ratio of insertion frequency into pTarget containing ShCAST transposon LE to frequency into pTarget with no transposon ends.
C. Insertion frequency of ShCAST into pTarget with different donor cargo sizes. Cargo size includes transposon ends.
D. Re-ligation of pDonor after transposition cannot be detected in harvested plasmids from *E. coli* targeting PSP49 (Table S3) with and without tnsB.
E. Re-ligated donor is undetectable by PCR in harvested plasmids from *E. coli* targeting PSP49.

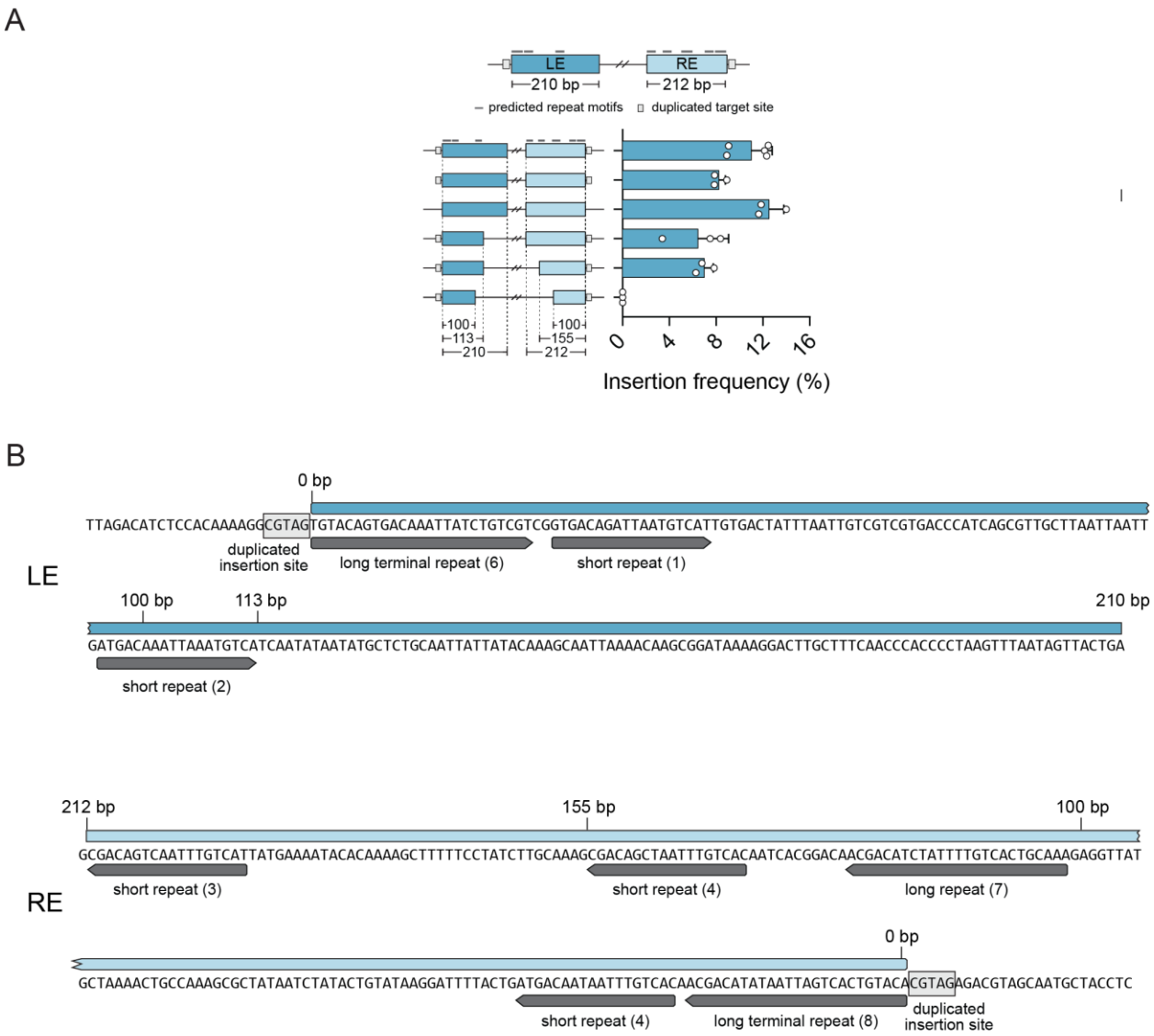

Fig. S7. ShCAST transposon ends sequence analysis

A. Insertion activity with donor truncations of LE and RE. Predicted transposase binding sites are indicated with grey lines. For all panels, experiments were carried out in E. coli

and insertion frequency was determined by ddPCR on extracted plasmid DNA. Error bars represent s.d. from n=3 replicates.

B. Sequence of ShCAST transposon ends highlighting short and long repeat motifs.
C. Alignment of ShCAST repeat motifs and the canonical Tn7 TnsB binding sequence.

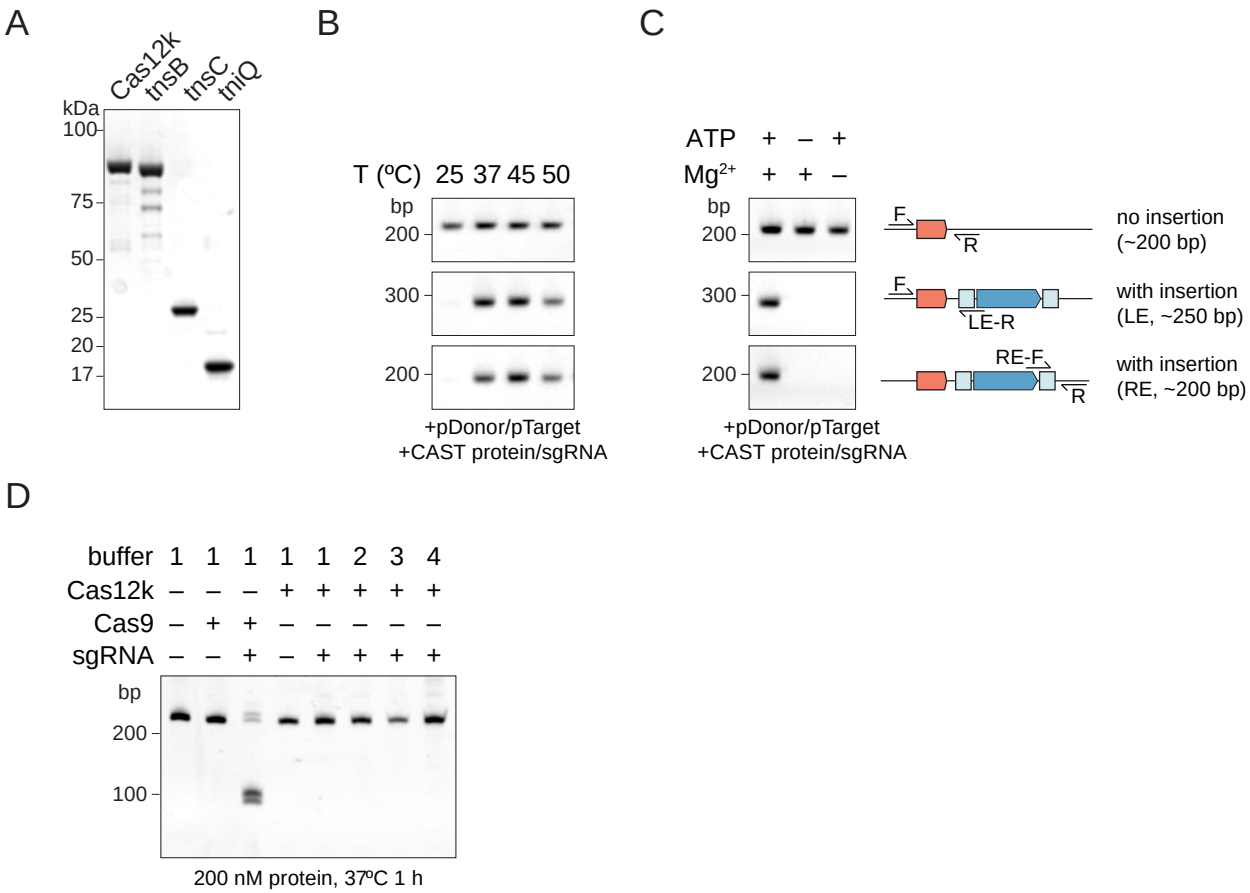

Fig. S8. In vitro reconstitution of an RNA-guided transposase

A. Coomassie stained SDS-PAGE gel of purified ShCAST proteins.
B. Temperature dependence of in vitro transposition activity of ShCAST.
C. In vitro reactions in the absence of ATP and $MgCl_2$.
D. In vitro cleavage reactions with Cas9 and Cas12k on $pTarget_{GGTT}$. Buffer 1: NEB CutSmart, buffer 2: NEB 1, buffer 3: NEB 2, buffer 4: Tn7 reaction buffer.

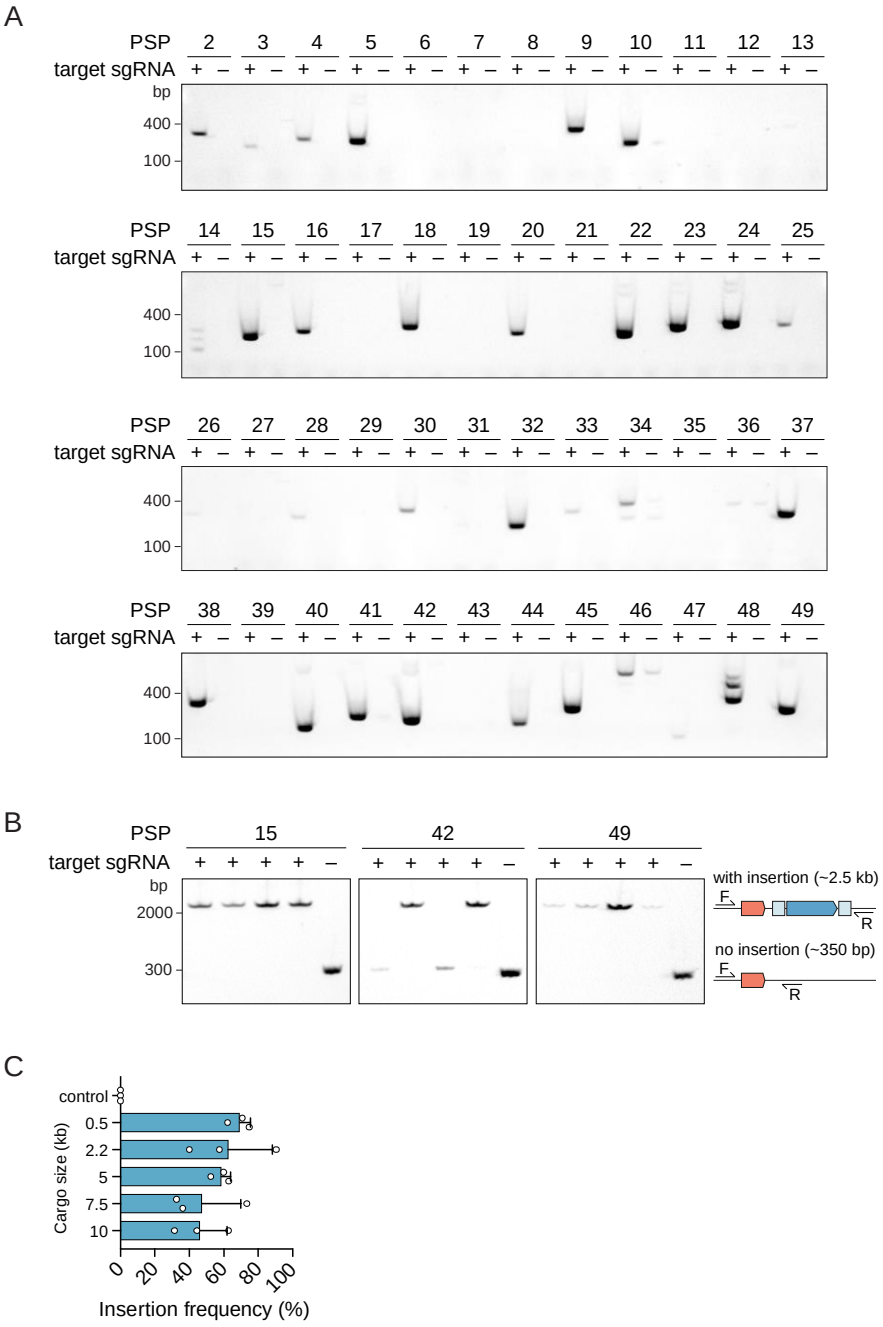

Fig. S9. ShCAST mediates genome insertions in *E. coli*

A. Screening for insertions at 48 target sites in the *E. coli* genome by nested PCR for LE junctions.

B. Re-streaking *E. coli* that were transformed with pHelpers with genome-targeting sgRNA and pDonor demonstrates the ability to recover clonal populations of bacteria with the insertion product of interest.

C. Genome insertion frequency of pDonor containing multiple cargo sizes using pHelper with sgRNA targeting PSP42.

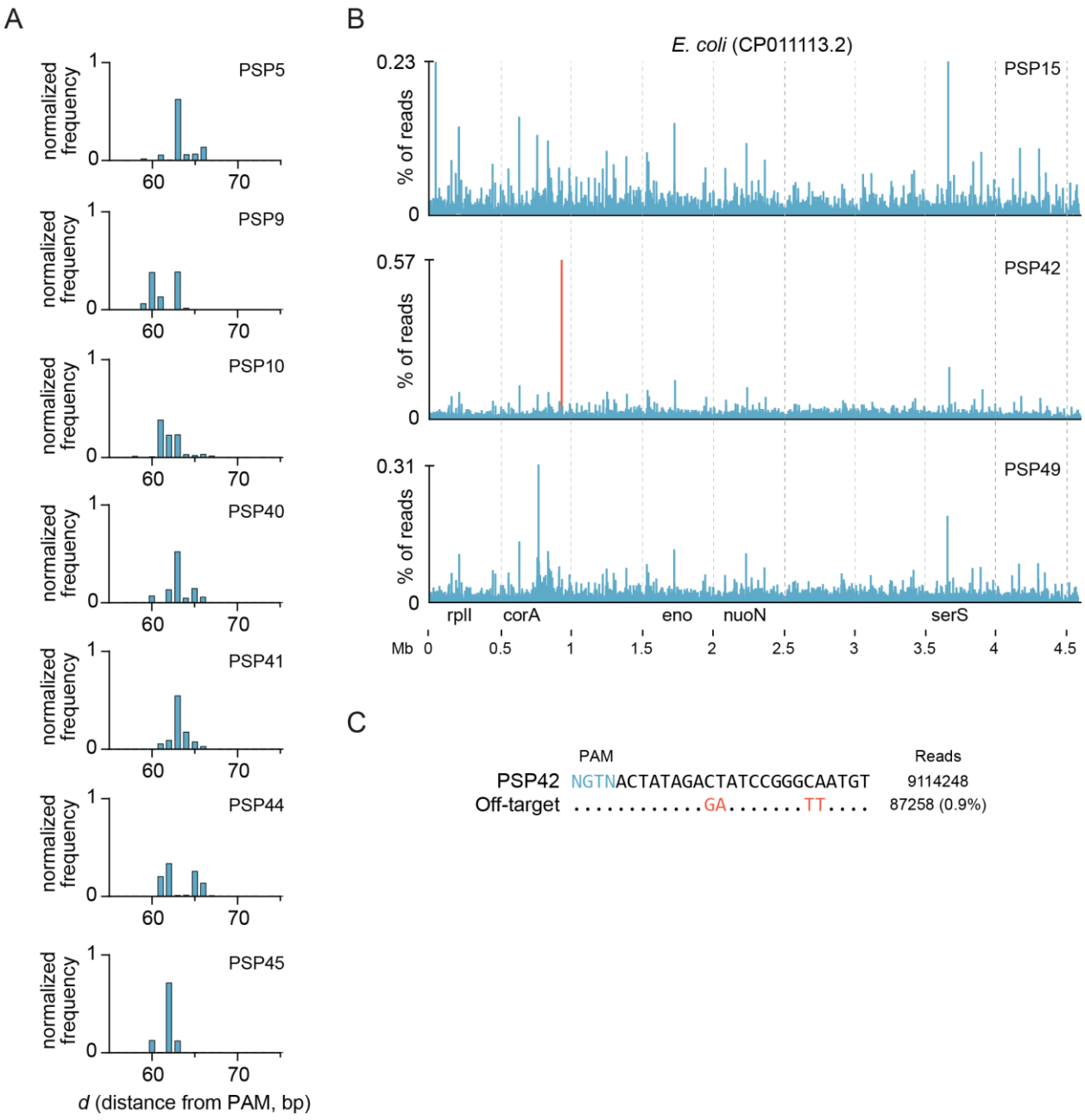

Fig. S10. Sequence analysis of *E. coli* genome insertions

A. Targeted amplification of genomic insertions and deep sequencing to identify position of insertions.
B. Off-target insertion reads for pHelper targeting the genome. Proximal genes for the most abundant guide-independent off targets are labelled. An identified guide-dependent off-target is highlighted in red.
C. Alignment of PSP42 and identified guide dependent off-target spacer.

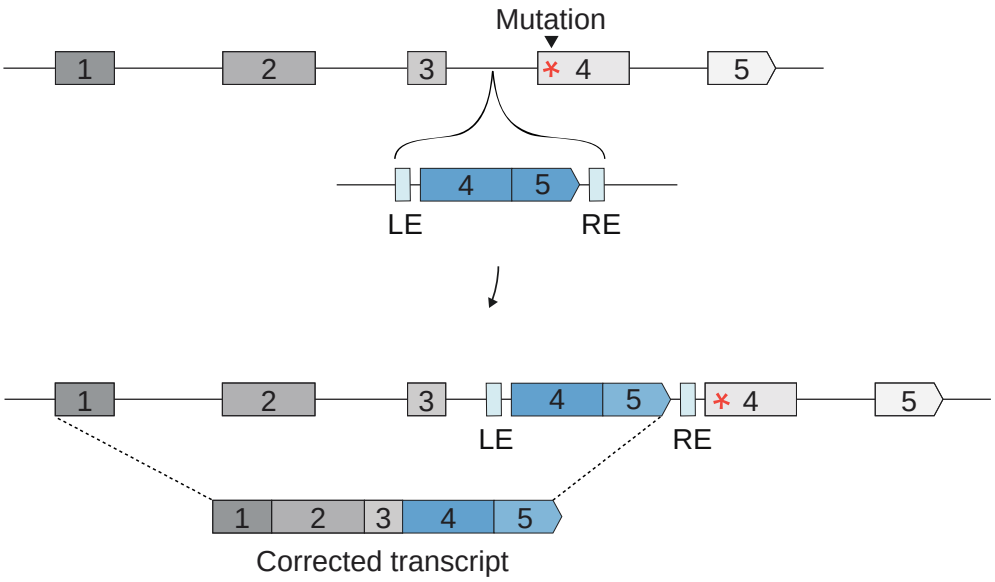

Fig. S11. Potential strategy for CAST-mediated gene correction.

Replacement of a mutation-containing exon by targeted DNA insertion.

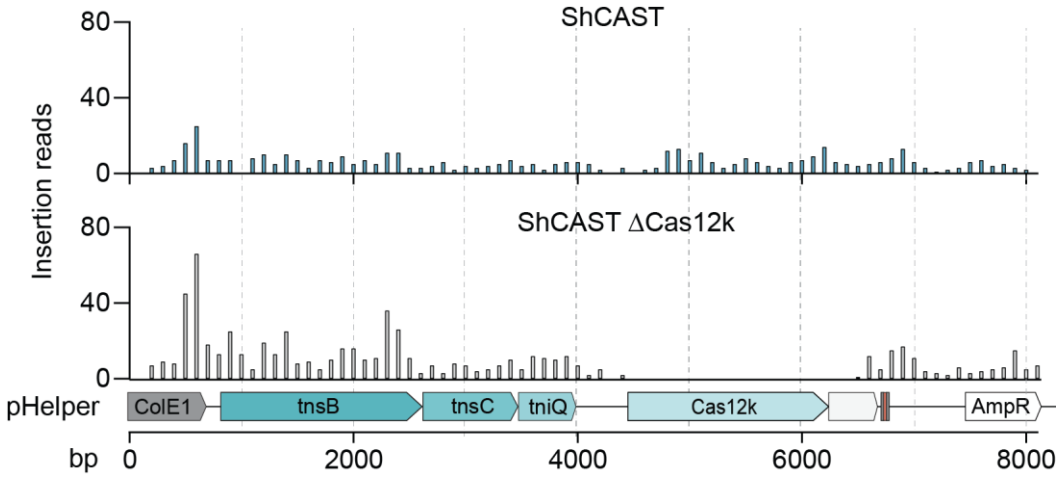

Fig. S12. ShCAST insertions into plasmids are independent of Cas12k

Sequence analysis of insertions into pHelper with wildtype ShCAST and a non-targeting sgRNA and ShCAST with Cas12k deleted.

Table S1. DNA Sequences

Protein	Accession	DNA Sequence
ShTnsB	WP_084763316.1	atgaacagtcagcaaaatcctgatttagctgttcatcccttggcaattcctatggaaggct tactaggagaaagtgctacaactcttgagaagaatgtaattgccacacaactctcagagga agcccaagtaaagctagaggtaatccaaagtttactggaaccctgcgatcgcacaacttat gggcaaaagttgcgggaagcagcagagaaactaaatgtatcgttgcgaacggtacaaaggt tggtgaaaaactgggaacaagatggcttagtcggactcactcaaacaagtagggctgataa aggaaaacaccgcattggtgagttttgggaaaacttcattaccaaaacctacaaggagggt aacaagggaagtaaacgtatgacccctaaacaagttgctctcagagtcgaggctaaagccc gtgaattaaaagactctaagccgcccaattacaaaaccgtgttacgggtattagcacccat tttggaaaagcaacaaaaagccaagagtatccgcagtcctggttggagaggaactacgctt tcggttaaaacccgtgaaggaaaagatttatcggttgattacagtaaccatgtttggcaat gtgaccatacccgcgtggatgtgttgctggtagatcaacatggtgaaattttaagtcgtcc ctggctaacaacagtaattgatacttactctcgttgcattatgggtatcaacttgggcttt gatgcacccagttctggggtagtagcattagcgttacgccatgcaattctaccaaagcgtt acggttccgagtacaaactgcattgtgagtggggaacctatggaaaaccagaacatttta tactgatggcggtaaagactttcgctctaaccacttgagtcagattggggcgcaattggga tttgtctgtcatttacgcgatcgcccttctgaaggtggagtagtagaacgtcccttcaaaa cattaaatgaccaactattttcaacgcttcctgggtacaccggatctaatgtgcaggaacg cccagaagatgcagagaaggacgcaagacttactttgcgagaactagaacagttacttgtg cgttacatcgtagatcgttacaaccaaagtattgatgcgcggatgggcgaccaaacgcgct ttgagcgttgggaagcaggattgcctacagtgccagtaccaataccagaacgagatttgga tatttgtttaatgaagcagtcacggcgcactgtgcaaagaggtggttgtttgcagtttcag aatttaatgtatcgggggaatatttggcaggttatgccggagaaactgtcaacttaaggt ttgaccccagagacattacaacaattttggtttatcgccaggaaaacaatcaggaagtatt tctgactcgcgctcacgctcaaggtttggagacagagcaactggcattagatgaggctgag gcagcaagtcgcagactccgtaccgcagggaaaactatcagtaaccaatcattattgcaag aagttgttgaccgcgatgctcttgtcgctaccaagaaaagccgtaaggagcgtcaaaaatt ggaacagactgttttgcgatctgctgctgttgatgaaagtaatagagaatccttgccttct caaatagttgaaccagatgaagtggaatctacagaaacggttcactctcaatacgaagaca ttgaggtgtgggactatgaacaacttcgtgaagaatatgggttttaa
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		aattcaaaggcttaccgaaaagttaataagtcggatgcctaaaggtcgagatttgaccaat
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		ccaaacgctggcaggatattctgttaactcgatcaagttctctcccattccccttgtttt
		tgaaaccaacgaggatatggtttggtcaaagaatcaaaagggtaggctgtgtgttcacttc
		aatggcttaagcgatttaatttttgaggtgtactgcggcaatcgtcaacttcactggtttc
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		cgaataaacaattcctttgagcgtcctagccaacccctttatcaaggtcaatcacacattt
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Table S2. RNA Sequences

RNA	Sequence (5' to 3')
ShCas12k tracrRNA1	AGGUGCGCUCCCAGCAAUAAGGGCGCGGAUGUACUGCUGUAGUGGCUACUGAAUCACCCCCGAUCAAGG GGGAACCCUCCC
ShCas12k tracrRNA2	AGACAGGAUAGGUGCGCUCCCAGCAAUAAGGGCGCGGAUGUACUGCUGUAGUGGCUACUGAAUCACCCC CGAUCAAGGGGGAACCCUCC
ShCas12k tracrRNA3	AAAUACAGUCUUGCUUUCUGACCCUGGUAGCUGCUCACCCUGAUGCUGCUGUCAAUAGACAGGAUAGGU GCGCUCCCAGCAAUAAGGGCGCGGAUGUACUGCUGUAGUGGCUACU
ShCas12k tracrRNA4	AAAUACAGUCUUGCUUUCUGACCCUGGUAGCUGCUCACCCUGAUGCUGCUGUCAAUAGACAGGAUAGGU GCGCUCCCAGCAAUAAGGGCGCGGAUGUACUGCUGUAGUGGCUACUGAAUCACCCCCGAUCAAGGGGGA ACCCUC
ShCas12k tracrRNA5	UUAAAUGAGGGUUAGUUUGACUGUAUAAAUACAGUCUUGCUUUCUGACCCUGGUAGCUGCUCACCCUGA UGCUGCUGUCAAUAGACAGGAUAGGUGCGCUCCCAGCAAUAAGGGCGCGGAUGUACUGCUGUAGUGGCU ACUGAAUCACCCCCGAUCAAGGGGGAACCCU
ShCas12k tracrRNA6	AUAUUAAUAGCGCCGCAAUUCAUGCUGCUUGCAGCCUCUGAAUUUUGUUAAAUGAGGGUUAGUUUGACU GUAUAAAUACAGUCUUGCUUUCUGACCCUGGUAGCUGCUCACCCUGAUGCUGCUGUCAAUAGACAGGAU AGGUGCGCUCCCAGCAAUAAGGGCGCGGAUGUACUGCUGUAGUGGCUACUGAAUCACCCCCGAUCAAGG GGGAACCC
ShCas12k sgRNA6.1*	AUAUUAAUAGCGCCGCAAUUCAUGCUGCUUGCAGCCUCUGAAUUUUGUUAAAUGAGGGUUAGUUUGACU GUAUAAAUACAGUCUUGCUUUCUGACCCUGGUAGCUGCUCACCCUGAUGCUGCUGUCAAUAGACAGGAU AGGUGCGCUCCCAGCAAUAAGGGCGCGGAUGUACUGCUGUAGUGGCUACUGAAUCACCCCCGAUCAAGG GGGAACCCUCCAAAAGGUGGGUUGAAAGnnnnnnnnnnnnnnnnnnnnnnn
ShCas12k sgRNA6.2*	AUAUUAAUAGCGCCGCAAUUCAUGCUGCUUGCAGCCUCUGAAUUUUGUUAAAUGAGGGUUAGUUUGACU GUAUAAAUACAGUCUUGCUUUCUGACCCUGGUAGCUGCUCACCCUGAUGCUGCUGUCAAUAGACAGGAU AGGUGCGCUCCCAGCAAUAAGGGCGCGGAUGUACUGCUGUAGUGGCUACUGAAUCACCCCCGAUCAAGG GGGAACCCUAAAUGGGUUGAAAGnnnnnnnnnnnnnnnnnnnnnnn
ShCas12k E. coli crRNA*	GUGGCAACAACCUUCCAGGUACUAGGUGGGUUGAAAGnnnnnnnnnnnnnnnnnnnnnn
ShCas12k in vitro crRNA*	UAGGUGGGUUGAAAGnnnnnnnnnnnnnnnnnnnnnn

* 23 nt guide sequences added to the 3' end of sgRNA and crRNA

Table S3. Genomic targets and primers

Protospacer	PAM	Guide sequence (24 nt)	Forward primer	Reverse primer	Position
2	TGTG	TCAGAAGGTTAGCATCAAATGAT	AGATAACCGGGCACGTTTTT	TTCCTCCACATCCACTGTCT	37315
3	GGTA	TGTGAAGTAATACCCTAACCACC	GAGCCGGTGTGGAATGGTAA	ATTCTGGCGCTTGCTACCTT	4455464
4	CGTT	TTACATGTCCTGTACCCGGCAGA	ACGAAAGGCAGGTGAGAAGG	ACCATTCTCACCCGGCAATT	61356
5	CGTT	ATAGTGAATCCGCTTATTCTCAG	ACGTTCGAAAGGCGTACCAA	TGAGTGCCATTGTAGTGCGA	1445845
6	AGTG	GGATTCACACAACGAAACAATTA	CAGGATCCAGGATTCACGGG	AACCGGGTATTCCACACACC	208056
7	CGTT	TATTGCGAAGGGAGGGTGACGAA	TTGGTAGACGCGCTAGCTTC	TCGGTTTCGCCATCACATGA	647688
8	CGTT	CTGTGCCAAAAGCGGAAGTTGGA	AGCCAGAAATATGCCGAGCC	GGCAGACCAGAAAGCGTTTG	1855018
9	AGTT	CTGTAACGTAATCATTAACATGC	GCGGCCGCCAATTTTAGTTT	GCTCGGCATATTTGTCTGCG	853988
10	AGTC	TCAGACCTATTTGGCCGGTAATC	CAGATTGCCGCGGCTTTAAT	GTCCCAGTCCGATCTCTTGC	2762104
11	GGTA	ATGCCGGTCATTCCGGGGTTTTG	CGTTTCGTTTTCCGGTGCTT	AAGAAGCCTCACCACAACCC	164858
12	CGTT	TTAGGATAATTGGAATGAATATC	CGCGTCTTATCAGGCCTACA	ACCCAAAAACATTTCGGGCG	393437
13	TGTA	TTCAAAAGAGTATAAATGCCTGA	TGGGTTGAACATAACGCCGA	GCAAGTAAGCCCGCAATAGC	1343329
14	TGTG	TTTGCGGCATTAACGCTCACCAG	GAACGGCCTCAGTAGTCTCG	TGTTTAGAGTGTTCCCCGCG	1726131
15	TGTT	ACCCTCTTAAACTATCCCACTAA	AAGGCTGGGAAATCAGACGG	TATCTGCAAAGTCGCTGGGG	3058735
16	TGTC	AACCTCACTACTATCGAAGACTC	CGATTGGCATTAACCCGCTT	AAACGGCACATTCAACTGGC	2167400
17	AGTA	TAAAAAACGAACGATAACCGTGA	GCACAACACTGCCTGAAACA	GATGAACACGCGGACGAGTA	2665227
18	GGTT	GAGACTGTTGATAAAACGTAAAA	CATCAGCATTCCTGGCCGTA	ACGCCCGTGACAGTAAACAT	999636
19	AGTC	AGATGTTATTTTTTACTCACAAC	CGGGTGAATAGAGGGCGTTT	TCAGGCACGCACTTATAGCA	4541043
20	AGTC	GTTCTGTACACTTTGTTTTGTCA	GCTCGACGCATCTTCCTCAT	GGACAGAGCCGACAGAACAA	87136
21	GGTA	AAGTTTGGTAGATTTTAGTTTGT	ACACAGGTTTATCCCCGCTG	CGCCTCTGAAAACTCCTCCA	1725870
22	TGTG	TTAATGAAACCTTCTTGACGCTG	CTGGCGCTCATCAACAATCG	CAATTTTGCCTTCCCCGAGC	1435660
23	CGTT	TAGCTTATATTGTGGTCATTAGC	CGACCGACGATTATCCCCTG	AGCACGAGGGTCAGCAATAC	259290
24	CGTT	ACCACCTCAAGCTATGCCGCCAG	TTGGTAGGCCTGATAAGCGC	GTAGCAGATGACCTCGCCTC	550349
25	TGTC	TATTCATCGTGTTGATAAGATAT	TGTGATGTTCTACGGGCAGG	CTCAGCGATCACCCGAAACT	964477
26	GGTC	TTTACTTGCTCATCGTTATAATT	TTAAACCGTGGGAAGGAGGC	TTTTGCGAGGCGTTTTCCAG	551608
27	AGTA	AAAACTGCTTCATAGCGCGGATT	GCAGTATAAAAAGCGCGCCA	GCTGTTGATTGACGCCAGTG	1707979
28	GGTT	TTTTATACCTGTAGATCATCATA	CAGGTGTCAGGTCGGAAACA	GCCGATAGTGTTCCTTGCCT	1647991
29	AGTT	CTCTTCGGACTTCGCGGGACAAA	TTTGTTAGTGGCGTGTCCGT	TTTGGCGGCTTTGATTTCCG	1874378
30	AGTC	TGAGTTATTTTTGTAGGGCTATA	AGTTTGCGGGGTGATGAGAG	AATGACACACAAGACCAGCT	4077502
31	GGTA	ACGCCGTGAAAAGACGGGCTTAC	GGTCAGCCGATTTTGCATCC	GAAATGTCTTCAGGCGTGGC	160388
32	GGTA	ACCAGTTCAGAAGCTGCTATCAG	TCATGGCATTGCTGACGACT	CTGTCTGTGCGCTATGCCTA	4587210
33	CGTT	TTGTTAAAAAATGTGAATCACTT	GTTCTTCAGCAGGCGGGATA	GATGACGAGTGGTACTCCGC	2556691
34	TGTA	GTCTGCGATCCTGCCAGCAAATA	GGAGAGGCTTTCCCGTTTCA	TAGACTGCTTGCATGGCGAA	2470836
35	CGTA	ATTTTGGTGAGACCCAAAATCGA	GCTCCACTTTTCCACGACCA	GTGGTCTGATCCAGCGTTGA	2991491
36	TGTG	GATATTGTGATACACATTGAGGT	TGTGGCGAAGTTGAGATACCA	ACTATCTGAACTCTTCGTGGCT	4562646
37	TGTC	AGAAGGTTAGCATCAAATGATAA	GCATTCTGCGGGAAGGGATA	TTTTGCAGCATCCTGGCAAC	37317

38	GGTG	GATGGAAAGGTGATTGAAAACTC	AACCAGCGTTGACCATTTGC	ATAACTTCCAGTGGGCGTGG	994483
39	GGTT	TTACCCCTGTTACACGGGAAGTG	AGTAGTTCTGACAACGGGCG	CAACTCCGCTGGCAGAAAAG	41015
40	TGTC	AATGGGTGGTTTTTGTTGTGTAA	CAAATTATACGGTGCGCCCC	TCGGCGCTAAGAACCATCAT	707736
41	AGTT	TTGTCAGATATTACGCCTGTGTG	TATCCACCCGTGCGATTACG	CCAGAATGACCTCGGCAACT	2687062
42	AGTG	ACTATAGACTATCCGGGCAATGT	TGAGTGCCAGAATCTTGCGT	ACGTACTTCGCCACCTGAAG	188387
43	GGTG	ATTTTGTGATCTGTTTAAATGTT	GAGCGAAAACAGCAGCCATC	GTCATGATTGGCCTGCGTTC	1138064
44	TGTG	TCTGTAAATCACGACAATGGGTG	AGTCGGTGAATGAGCCACTG	GCAGTTGGGGTAAGTCGTCA	1938877
45	AGTC	ACTGCCCGTTTCGAGAGTTTCTC	GCAGGCTCGGTTAGGGTAAG	GGCTAACGTGGCAGGAATCT	470870
46	TGTA	GGCCGGACAAGACGTTTATCGCA	TGTAGGCCTGATAAGACGCG	TGAAGGGGTACGAGTCGACA	4225144
47	AGTG	GTGCTGATAGGCAGATTCGAACT	AGGTAGCCGAGTTCCAGGAT	TACGGTAGTGATTGCAGCGG	453402
48	AGTT	GGTGGCTCTGGCTGGAGTGAGAG	CCTCCGCCAGCTGAAGAAAT	CCAGACGGGTTTCATCAGCA	982236
49	AGTT	ATAGCGATCCCTTGCTGAAAATA	GTCAGGTAGCCAGAACACCC	GCCGGGATACGTTCCTTCTT	762880

Table S4. ddPCR primers and probes

Insert Probe	CTGTCGTCGGTGACAGATTAATGTCATTGTGAC
Target Probe	TGGGCAGCGCCCACATACGCAGCGATTTC
pTarget Forward Primer	AAAACGCCTAACCCTAAGCAGATTC
pTarget Reverse Primer	GGTGCCGAGGATGACGATGAG
T14 LE Reverse Primer	AACGCTGATGGGTCACGACG

Table S5. Off-target insertions

	Ratio to on-target			
Genome Position (bp)	**PSP15**	**PSP42**	**PSP49**	**Nearby Genes**
37000-37999	4.30E-03	1.40E-04	1.90E-04	tRNA-Leu
147000-147999	1.60E-03	1.30E-03	1.10E-03	valS
200000-200999	2.50E-03	1.60E-03	1.90E-03	rplI, rpsR, priC, rpsF
439000-439999	1.40E-03	9.70E-04	1.20E-03	rpoC, rpoB, rplL, rplJ
627000-627999	2.80E-03	1.90E-03	2.40E-03	corA
755000-755999	2.20E-03	1.40E-03	1.70E-03	gyrB, recF, dnaN
763000-763999	2.20E-04	1.60E-04	5.50E-03	lbpA, lbpB
764000-764999	5.70E-04	3.20E-04	1.60E-03	lbpA, lbpB
765000-765999	2.10E-04	1.10E-04	1.40E-03	lbpA, lbpB
766000-766999	6.20E-04	3.50E-04	1.50E-03	lbpA, lbpB
831000-831999	2.10E-03	1.50E-03	2.00E-03	waaU, rfaJ, rfaY, rfaI, rfaS
832000-832999	1.50E-03	1.00E-03	1.60E-03	waaU, rfaJ, rfaY, rfaI, rfaS
909000-909999	1.30E-03	9.60E-04	1.40E-03	glyS
924000-924999	2.60E-05	9.70E-03	2.90E-05	tRNA-Pro
1245000-1245999	1.80E-03	1.50E-03	1.30E-03	ArgS
1385000-1385999	1.60E-03	1.40E-03	1.30E-03	uxaC, uxaA, ygjV, SstT
1531000-1531999	1.80E-03	1.70E-03	1.50E-03	TrmB, C4J69-19770, yggN
1542000-1542999	1.50E-03	1.30E-03	1.10E-03	metK, galP
1724000-1724999	2.60E-03	2.30E-03	2.10E-03	eno, pyrG, A610-3350
1944000-1944999	1.30E-03	1.20E-03	1.00E-03	glyA, hmp
2234000-2234999	2.00E-03	1.80E-03	1.90E-03	NuoN, NuoM
2364000-2364999	1.50E-03	1.20E-03	1.30E-03	fruA, fruK, fruB
3420000-3420999	1.30E-03	1.10E-03	1.20E-03	nagK, NudJ, lolD, lolE
3661000-3661999	4.30E-03	3.10E-03	3.40E-03	serS
3838000-3838999	1.50E-03	1.10E-03	1.30E-03	sucB, sucA, sdhB, sdhA
3895000-3895999	1.80E-03	1.70E-03	1.30E-03	glnS
4168000-4168999	1.90E-03	1.30E-03	1.50E-03	secF, secD, YajC, tgt, QueA
4304000-4304999	1.90E-03	1.20E-03	1.50E-03	tRNA-thr

Table S6. NGS primers

pTarget Primer LE	CTTTCCCTACACGACGCTCTTCCGATCTCGCAGACCAAAACGATCTCAAG
pTarget Primer RE	CTTTCCCTACACGACGCTCTTCCGATCTGGTGCCGAGGATGACGATGAG
T14 LE Primer	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTAATGACATTAATCTGTCACCGACG
T14 RE Primer	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTATGCTAAAACTGCCAAAGCGC
T1 LE Primer	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTCTGAAAAACAACCACCACGAC
T1 RE Primer	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGCTTTTCGCAAATTAGTGTCG

References and Notes

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