Supporting Information

Semenova et al. 10.1073/pnas.1104144108

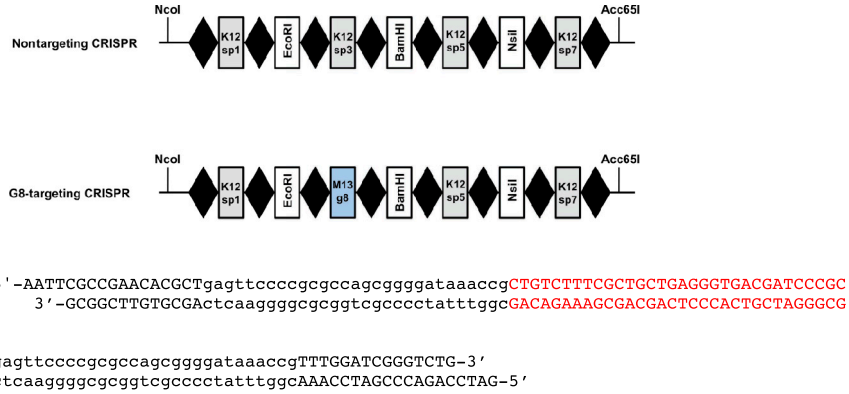


Fig. S1. Design of nontargeting CRISPR (clustered regularly interspaced short palindromic repeats) (pWUR477, *S1*) and M13 g8-targeting CRISPR cassettes; rectangles indicate spacers, rhombi—repeats. Below synthetic DNA used for generating M13 phage matching CRISPR cassette plasmid is shown. Two oligonucleotides were annealed to create double-stranded DNA with sticky EcoRI and BamHI ends. The g8 spacer is shown in red color, repeat sequences are shown in lowercase.

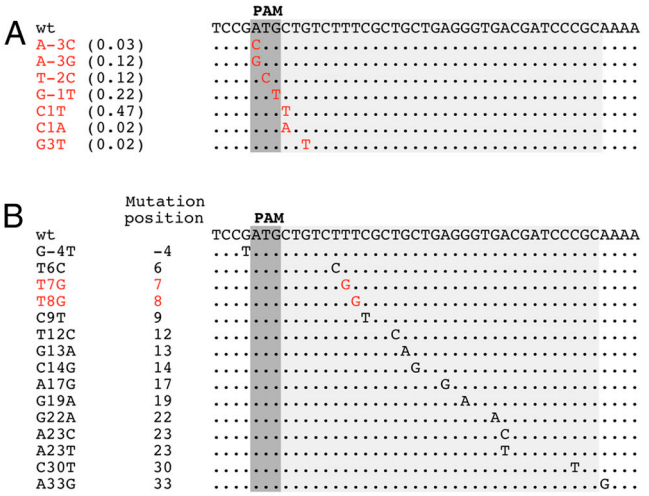


Fig. S2. (*A*) Natural M13 escape mutants obtained on lawns of cells expressing g8 crRNA. The wild-type sequence is shown (*Top*). Individual mutations are indicated by red-color font. Numbers indicate frequencies of individual mutations (a total of 50 escape mutant phages was sequenced). (*B*) Engineered point mutations in g8 protospacer, which were not included in Fig. 1*B*. Mutations indicated with red color led to escape, whereas mutations shown in black were restricted by CRISPR/Cas (the phage mutants had nonescape phenotype on lawns of cells expressing the g8 crRNA). PAM, protospacer-adjacent motif.

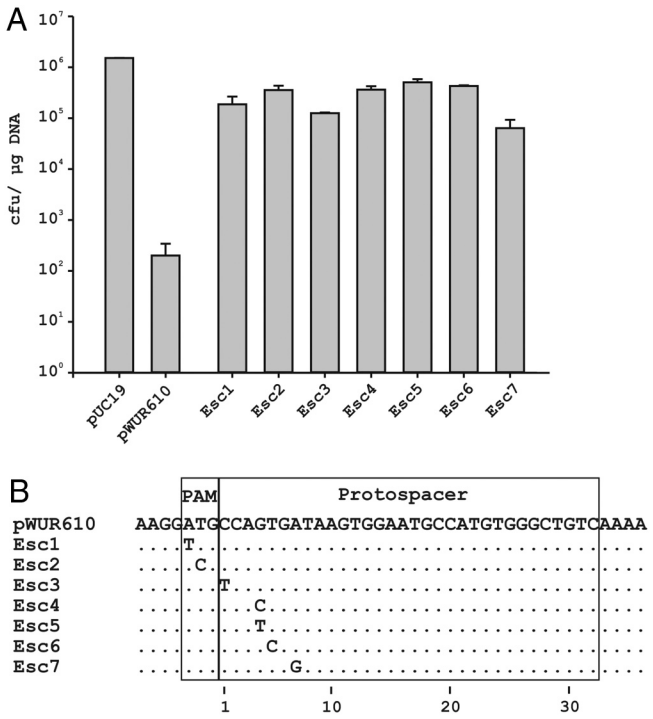


Fig. S3. Mutations in PAM and protospacer seed region abolish CRISPR/Cas mediated plasmid transformation inhibition. (*A*) Transformation efficiencies of pUC19, pWUR610, and pWUR610 escape mutant series. pWUR610 is a pUC19 plasmid containing the J3 protospacer on a 350-bp fragment of bacteriophage λ genome (Table S1). The recA⁻ strain *Escherichia coli* KRX (Promega) was used to overproduce Cascade, Cas3, and J3 crRNA. Error bars indicate the standard deviation. (*B*) Overview of the identified escape mutants. The sequence of the protospacer as well as escape point mutations are shown.

	Mutation positions	PAM
wt		TCCGATGCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCAAAA
G-4C,C1T	-4,1	...C...T.....
G-4T,C1T	-4,1	...T...T.....
T6A,G-1T	6,-1	.....T.....A.....
T6C,C1T	6,1	.....T....C.....
C9T,A-3G	9,-3	....G.........T.....
C9T,C1T	9,1	.....T.......T.....
T12A,C1T	12,1	.....T..........A.....
T12C,C1T	12,1	.....T..........C.....
T15A,T-2C	15,-2	.....C..............A.....
T15A,G-1C	15,-1	.....C..............A.....
G18A,T-2C	18,-2	.....C..................A.....
G18A,A-3C	18,-3	....C..................A.....
T21C,T-2C	21,-2	.....C.....................C.....
T21C,A-3G	21,-3	....G.....................C.....
C24T,G-1T	24,-1	.....T.....................T.....
C24T,C1T	24,1	.....T.....................T.....
T27C,A-3C	27,-3	....C.........................C.....
C30T,G3T	30,3	.........T.....................T.....
A33C,C1T	33,1	.....T.....................C...
A33G,G-1T	33,-1	.....T.....................G...

Fig. S4. Escape mutants obtained on lawns of cells expressing g8 crRNA with individual nonescape phages carrying point mutations in the g8 protospacer. Each row indicates a nonrestricted point mutant of M13, with mutational substitution indicated with black font color. In red, substitutions observed in phages that escaped the block imposed by cells expressing the g8 crRNA are shown.

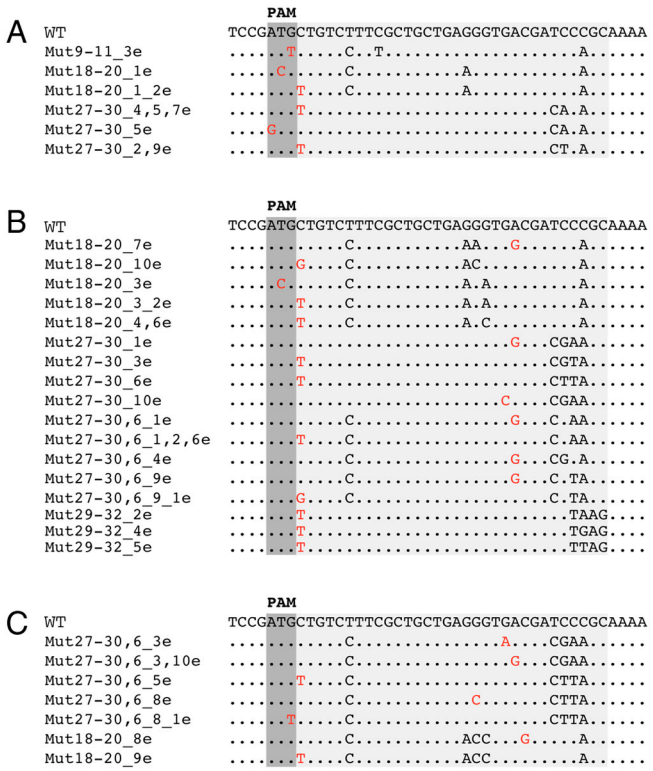


Fig. S5. Escape mutants obtained on lawns of cells expressing g8 crRNA with triple (*A*), quadruple (*B*), and quintuple (*C*) nonescape phages carrying point mutations in the g8 protospacer. See Fig. S3 legend for details.

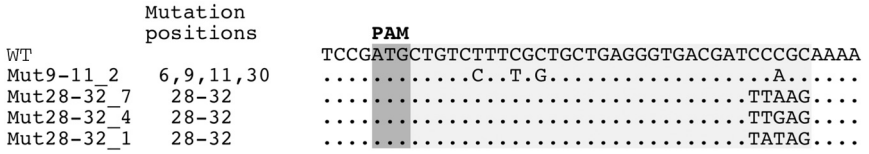


Fig. S6. Quadruple and quintuple mutants that escape CRISPR/Cas-imposed block on cells expressing g8 crRNA.

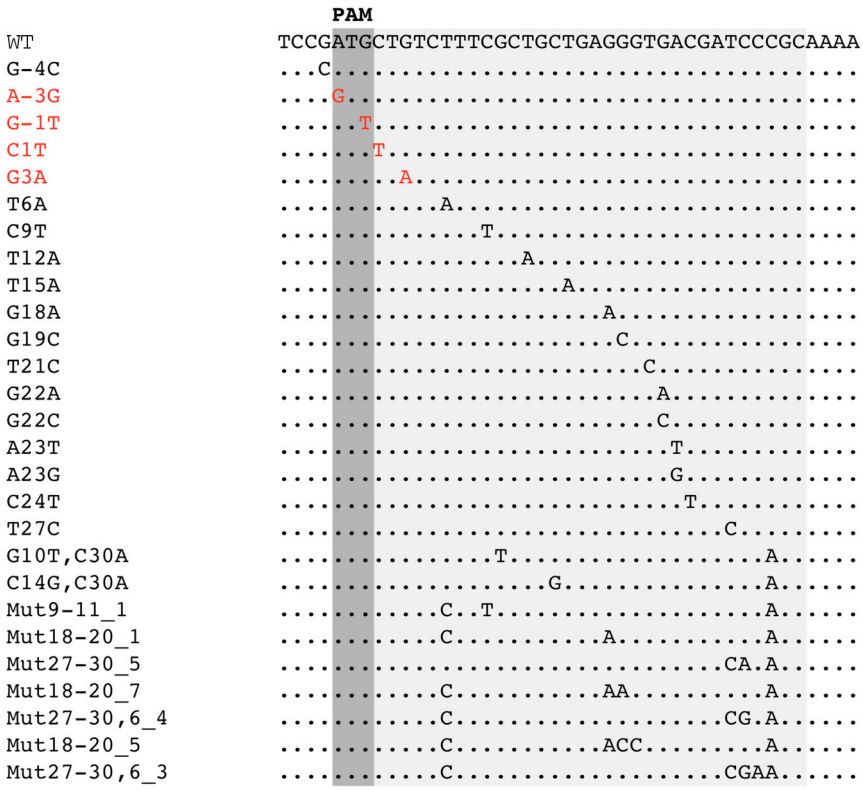
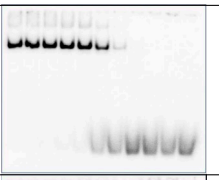
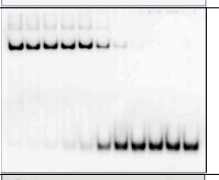
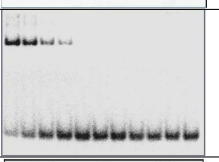
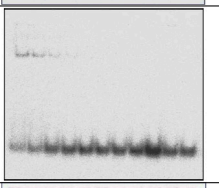
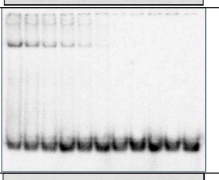
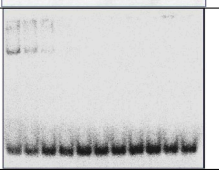
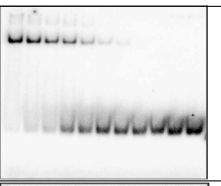
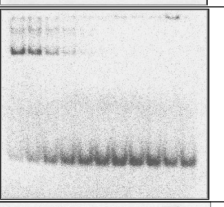
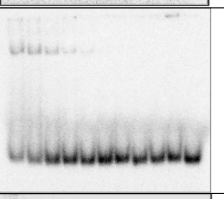
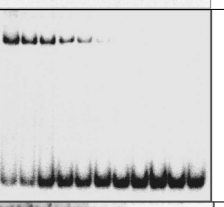
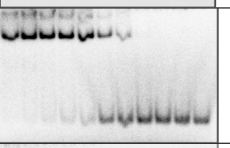
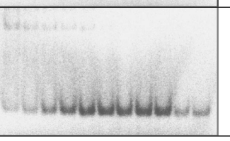


Fig. S7. Phenotypes of indicated phages on lawns of hns⁻ *E. coli* cells containing an engineered g8 CRISPR spacer in their chromosomal CRISPR cassette. See Fig. 1 legend for nomenclature.

Probe pair	Position (mutation)	Phenotype	Kd (nM)	EMSA gel
BG3233+BG3234	wild type		24 ± 9	
BG3333+BG3334	-4 (G→C)	nonescape	31 ± 7	
BG3235+BG3236	-3 (A→C)	escape	285 ± 32	
BG3237+BG3238	-2 (T→C)	escape	> 1,230	
BG3239+BG3240	-1 (G→T)	escape	> 1,230	
BG3241+BG3242	1 (C→T)	escape	> 1,230	

BG3243+BG3244	2 (T→A)	escape	270 ± 20	
BG3245+BG3246	3 (G→T)	escape	790 ± 254	
BG3247+BG3248	4 (T→G)	escape	> 1,230	
BG3249+BG3250	5 (C→G)	escape	782 ± 132	
BG3251+BG3252	6 (T→A)	nonescape	38 ± 14	
BG3253+BG3254	7 (T→C)	escape	> 1,230	

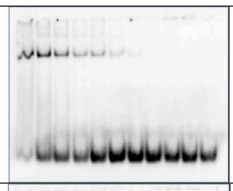
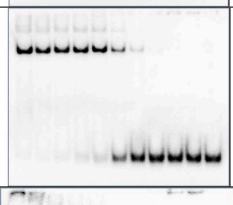
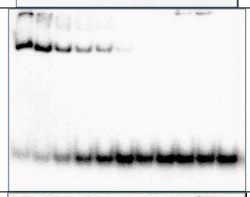
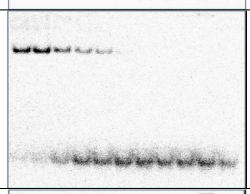
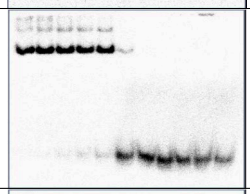
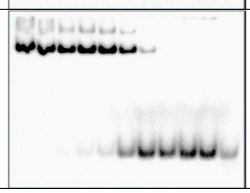
BG3255+BG3256	8 (T→C)	escape	1176 ± 112	
BG3331+BG3332	9 (C→T)	nonescape	38 ± 11	
BG3365+BG3366	10 (G→T)	nonescape	310 ± 28	
BG3367+BG3368	15 (T→A)	nonescape	439 ± 43	
BG3369+BG3370	24 (C→T)	nonescape	45 ± 20	
BG3371+BG3372	30 (C→A)	nonescape	29 ± 8	

Fig. S8. Electrophoretic mobility shift assays of Cascade complex binding various double-stranded DNA probes. The probes are listed in Table S2. Cascade concentration range (nM): 1200, 600, 300, 150, 75, 37.5, 18.8, 9.4, 4.7, 2.3, 1.2, respectively.

Table S1. Plasmids used in this study

Plasmids	Description and order of genes (5′-3′)	Restriction sites	Primers	Source
pWUR477	nontargeting CRISPR in pACYCDuet-1			Brouns et al. (1)
pWUR397	*cas3* in pRSF-1b, no tags			Brouns et al. (1)
pWUR399	*casA-casB-casC-casD-casE-cas1-cas2* in pCDF-1b, no tags			Brouns et al. (1)
pWUR400	*casA-casB-casC-casD-casE* in pCDF-1b, no tags			Brouns et al. (1)
pWUR408	*casA* in pRSF-1b, no tags			Brouns et al. (1)
pWUR514	*casB* with Strep-tag II (N-term)-*casC-casD-CasE* in pET52b			Jore et al. (2)
pWUR610	lambda fragment 17918–18250, in pUC19	BamHI/HindIII	BG3218 + BG3219	this study
pWUR615	*E. coli* CRISPR, 7 x spacer g8, in pACYCDuet-1*	EcoNI/Acc65I		Geneart, Germany
pWUR630	*E. coli* CRISPR, 4 x spacer J3, in pACYCDuet-1†	NcoI/KpnI		Geneart, Germany

*pWUR615—*E. coli* CRISPR, 7x spacer g8 (underlined), in pACYCDuet-1
CCTGCATTAGGTAATACGACTCACTATAGGATAAACCGCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCGAGTTCCCCGCGCCAGCGGGGATAAACCG
CTGTCTTTCGCTGCTGAGGGTGACGATCCCGCGAGTTCCCCGCGCCAGCGGGGATAAACCGCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCGAGTTC
CCCGCGCCAGCGGGGATAAACCGCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCGAGTTCCCCGCGCCAGCGGGGATAAACCGCTGTCTTTCGCTGCT
GAGGGTGACGATCCCGCGAGTTCCCCGCGCCAGCGGGGATAAACCGCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCGAGTTCCCCGCGCCAGCGGG
GATAAACCGCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCGAGTTCCCCGCGCCAGCGGGGATAAACCGGGTACC.
†pWUR630—*E. coli* CRISPR, 4x spacer J3 (underlined), in pACYCDuet-1
CCATGGAAACAAAGAATTAGCTGATCTTTAATAATAAGGAAATGTTACATTAAGGTTGGTGGGTTGTTTTTATGGGAAAAAATGCTTTAAGAACAAATG
TATACTTCTAGAGAGTTCCCCGCGCCAGCGGGGATAAACCGCCAGTGATAAGTGGAATGCCATGTGGGCTGTCGAGTTCCCCGCGCCAGCGGGGATAAA
CCGCCAGTGATAAGTGGAATGCCATGTGGGCTGTCGAGTTCCCCGCGCCAGCGGGGATAAACCGCCAGTGATAAGTGGAATGCCATGTGGGCTGTCGAG
TTCCCCGCGCCAGCGGGGATAAACCGCCAGTGATAAGTGGAATGCCATGTGGGCTGTCGAGTTCCCCGCGCCAGCGGGGATAAACCGCAGCTCCCATTTT
CAAACCCAGGTACC.

1. Brouns SJ, et al. (2008) Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* 321:960–964.
2. Jore MM, et al. (2011) Structural basis for CRISPR RNA-guided DNA recognition by Cascade. *Nat Struct Mol Biol* 18:529–536.

Table S2. Primers used in this study

Experiment	Primer	Sequence (5′-3′)	Description
M13 mutagenesis*	N-4	GCTACCCTCGTTCC***H***ATGCTGTCTTTCGC	Mutation at position −4
	N2	CCTCGTTCCG*ATG*C***V***GTCTTTCGCTGCTG	Mutation at position 2
	N4	TCGTTCCG*ATG*CTG***V***CTTTCGCTGCTGAG	Mutation at position 4
	N5	CGTTCCG*ATG*CTGT***D***TTTCGCTGCTGAGG	Mutation at position 5
	N6	GTTCCG*ATG*CTGTC***V***TTCGCTGCTGAGGG	Mutation at position 6
	N7	TTCCG*ATG*CTGTCT***V***TCGCTGCTGAGGGT	Mutation at position 7
	N8	TCCG*ATG*CTGTCTT***V***CGCTGCTGAGGGTG	Mutation at position 8
	N9	CCG*ATG*CTGTCTTT***T***GCTGCTGAGGGTGA	Mutation at position 9
	N10	CG*ATG*CTGTCTTTC***H***CTGCTGAGGGTGAC	Mutation at position 10
	N11	G*ATG*CTGTCTTTCG***D***TGCTGAGGGTGACG	Mutation at position 11
	N12	*ATG*CTGTCTTTCGC***V***GCTGAGGGTGACGA	Mutation at position 12
	N13	*TG*CTGTCTTTCGCT***H***CTGAGGGTGACGAT	Mutation at position 13
	N14	*G*CTGTCTTTCGCTG***D***TGAGGGTGACGATC	Mutation at position 14
	N15	CTGTCTTTCGCTGC***V***GAGGGTGACGATCC	Mutation at position 15
	N16	TGTCTTTCGCTGCT***H***AGGGTGACGATCCC	Mutation at position 16
	N17	GTCTTTCGCTGCTG***B***GGGTGACGATCCCG	Mutation at position 17
	N18	TCTTTCGCTGCTGA***A***GGTGACGATCCCGC	Mutation at position 18
	N19	CTTTCGCTGCTGAG***H***GTGACGATCCCGCA	Mutation at position 19
	N22	TCGCTGCTGAGGGT***H***ACGATCCCGCAAAA	Mutation at position 22
	N23	CGCTGCTGAGGGTG***B***CGATCCCGCAAAAG	Mutation at position 23
	N24	GCTGCTGAGGGTGA***T***GATCCCGCAAAAGC	Mutation at position 24
	N27	GCTGAGGGTGACGAC***C***CCCGCAAAAGCGGC	Mutation at position 27
	N30	GAGGGTGACGATCC***D***GCAAAAGCGGCCTT	Mutation at position 30
	N33	GGTGACGATCCCGC***B***AAAGCGGCCTTTAA	Mutation at position 32
	N9-11(T6C)	CCG*ATG*CTGTC***C***TT***TNN***TGCTGAGGGTGACG	Mutation at positions 6, 9–11
	N18-20(T6C,C30A)	TC***C***TTCGCTGCTGA***ANN***TGACGATCC***A***GCAA	Mutation at positions 6, 18–20, 30
	N27-29(C30A)	GCTGAGGGTGACGA***CNNA***GCAAAAGCGGCCT	Mutation at positions 27-30
	N29-32	CGCTGCTGAGGGTGACGATC***DDAG***TAAAGCGGCCTTTAACTC	Mutation at positions 29–32
	N28-32	CTTTCGCTGCTGAGGGTGACGAT***DDDAG***TAAAGCGGCCTTTAACTC	Mutation at positions 28–32
Mutant phage sequencing	G8_F	CTTTAGTCCTCAAAGCCTCTG	g8 protospacer forward primer
	G8_R	GCTTGCTTTCGAGGTGAATTTC	g8 protospacer reverse primer
Plasmid construction	BG3218	GGCCC**GGATCC**GTCGGGCGAGCGATGATGCG	lambda fragment + BamHI (fw)
	BG3219	CGCGC**AAGCTT**CATCGGCGTTTCATTCCCGTTT	lambda fragment + HindIII (rv)

Experiment	Primer	Sequence (5′-3′)	Description
EMSA	BG3233	GCTACCCTCGTTCCG*ATG*CTGTCTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Native target
	BG3234	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCGAAAGACAG*CAT*CGGAACGAGGGTAGC	Native target
	BG3235	GCTACCCTCGTTCCG***CTG***CTGTCTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position −3
	BG3236	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCGAAAGACAG*CA****G***CGGAACGAGGGTAGC	Mutation at position −3
	BG3237	GCTACCCTCGTTCCG***AC****G*CTGTCTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position −2
	BG3238	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCGAAAGACAG*C****G****T*CGGAACGAGGGTAGC	Mutation at position −2
	BG3239	GCTACCCTCGTTCCG*AT****T***CTGTCTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position −1
	BG3240	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCGAAAGACAG***A****AT*CGGAACGAGGGTAGC	Mutation at position −1
	BG3241	GCTACCCTCGTTCCG*ATG****T***TGTCTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position 1
	BG3242	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCGAAAGACA***A****CAT*CGGAACGAGGGTAGC	Mutation at position 1
	BG3243	GCTACCCTCGTTCCG*ATG****CA***GTCTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position 2
	BG3244	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCGAAAGAC***T****G**CAT*CGGAACGAGGGTAGC	Mutation at position 2
	BG3245	GCTACCCTCGTTCCG*ATG*CT***T****TCTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position 3
	BG3246	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCGAAAGA***A****AG*CAT*CGGAACGAGGGTAGC	Mutation at position 3
	BG3247	GCTACCCTCGTTCCG*ATG*CTG***G****CTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position 4
	BG3248	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCGAAAG***C****CAG*CAT*CGGAACGAGGGTAGC	Mutation at position 4
	BG3249	GCTACCCTCGTTCCG*ATG*CTGT***G****TTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position 5
	BG3250	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCGAAA***C****ACAG*CAT*CGGAACGAGGGTAGC	Mutation at position 5
	BG3251	GCTACCCTCGTTCCG*ATG*CTGTC***A****TTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position 6
	BG3252	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCGAA***T****GACAG*CAT*CGGAACGAGGGTAGC	Mutation at position 6
	BG3253	GCTACCCTCGTTCCG*ATG*CTGTCT***C****TCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position 7
	BG3254	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCGA***G****AGACAG*CAT*CGGAACGAGGGTAGC	Mutation at position 7
	BG3255	GCTACCCTCGTTCCG*ATG*CTGTCTT***C****CGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position 8
	BG3256	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCG***G****AAGACAG*CAT*CGGAACGAGGGTAGC	Mutation at position 8
	BG3266	GCAGAACAATGGTTACTTTTTCGATACGTGAAACATGTCCCACGGTAGCCCAAAGACTTGAGAGT	Random target
	BG3267	ACTCTCAAGTCTTTGGGCTACCGTGGGACATGTTTCACGTATCGAAAAAGTAACCATTGTTCTGC	Random target
	BG3331	GCTACCCTCGTTCCG*ATG*CTGTCTTT***T****GCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position 9
	BG3332	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGC***A****AAAGACAG*CAT*CGGAACGAGGGTAGC	Mutation at position 9
	BG3333	GCTACCCTCGTTCC***C****ATG*CTGTCTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position -4
	BG3334	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAGCGAAAGACAG*CAT****G***GAACGAGGGTAGC	Mutation at position −4
	BG3365	GCTACCCTCGTTCCG*ATG*CTGTCTTTC***T****CTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position 10
	BG3366	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTCAGCAG***A****GAAAGACAG*CAT*CGGAACGAGGGTAGC	Mutation at position 10
	BG3367	GCTACCCTCGTTCCG*ATG*CTGTCTTTCGCTGC***A****GAGGGTGACGATCCCGCAAAAGCGGCCTTTAA	Mutation at position 15
	BG3368	TTAAAGGCCGCTTTTGCGGGATCGTCACCCTC***T****GCAGCGAAAGACAG*CAT*CGGAACGAGGGTAGC	Mutation at position 15
	BG3369	GCTACCCTCGTTCCG*ATG*CTGTCTTTCGCTGCTGAGGGTGA***T****GATCCCGCAAAAGCGGCCTTTAA	Mutation at position 24
	BG3370	TTAAAGGCCGCTTTTGCGGGATC***A****TCACCCTCAGCAGCGAAAGACAG*CAT*CGGAACGAGGGTAGC	Mutation at position 24
	BG3371	GCTACCCTCGTTCCG*ATG*CTGTCTTTCGCTGCTGAGGGTGACGATCC***A****GCAAAAGCGGCCTTTAA	Mutation at position 30
	BG3372	TTAAAGGCCGCTTTTGC***T****GGATCGTCACCCTCAGCAGCGAAAGACAG*CAT*CGGAACGAGGGTAGC	Mutation at position 30
Plasmid construction	BG3218	GGCCC**GGATCC**GTCGGGCGAGCGATGATGCG	lambda fragment + BamHI (fw)
	BG3219	CGCGC**AAGCTT**CATCGGCGTTTCATTCCCGTTT	lambda fragment + HindIII (rv)
Mutant plasmid sequencing	BG2455	TTTCCCAGTCACGACGTTG	J3 protospacer forward primer
	BG2456	GGATAACAATTTCACACAGG	J3 protospacer reverse primer

Mutations are indicated in bold italics.
The protospacer region is underlined.
PAM is indicated in italics.
*Only one of two complementary primers used for mutagenesis is shown.