Supplementary Material

CRISPR-based self-cleaving mechanism for controllable gene delivery in human cells

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Supplementary Figures

Supplementary Figure 1: Translated open reading frames; cloning steps available in Supplementary Cloning Information.

Cas9-2A-mKate2-PEST
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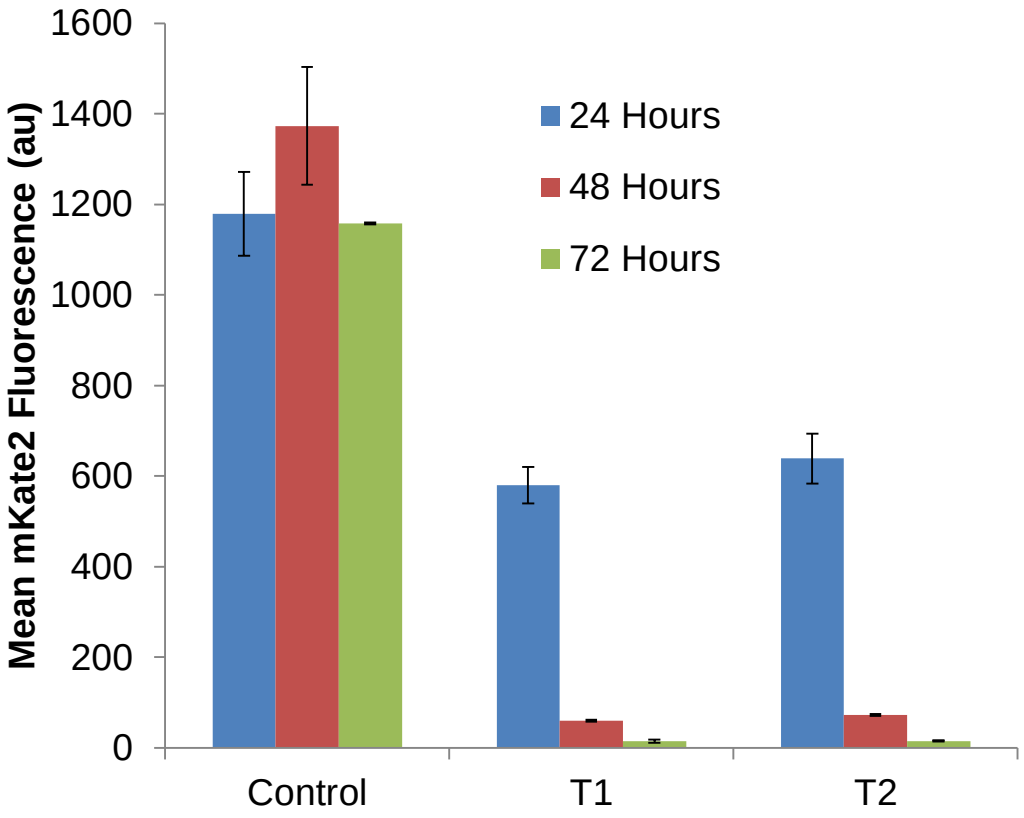
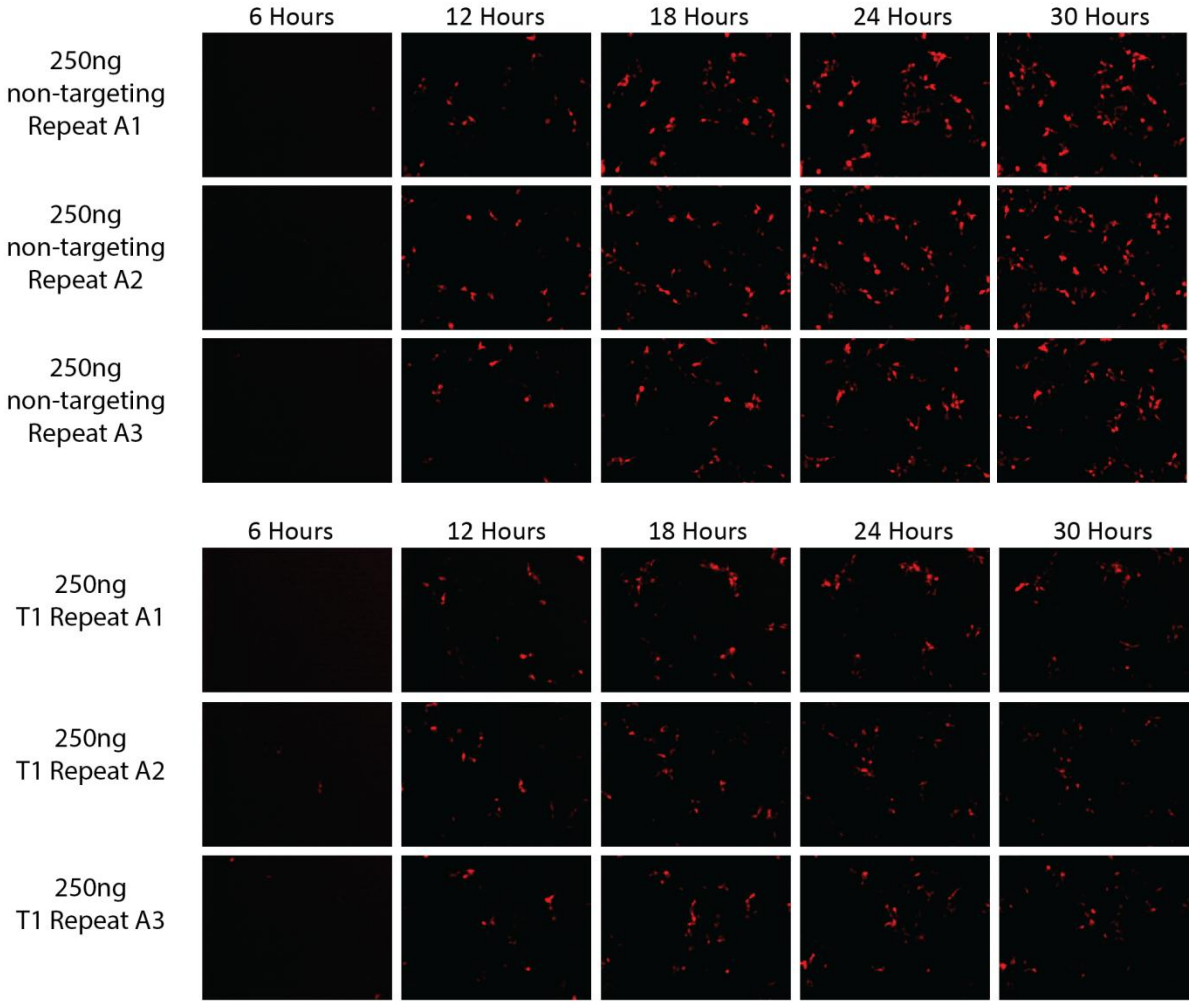
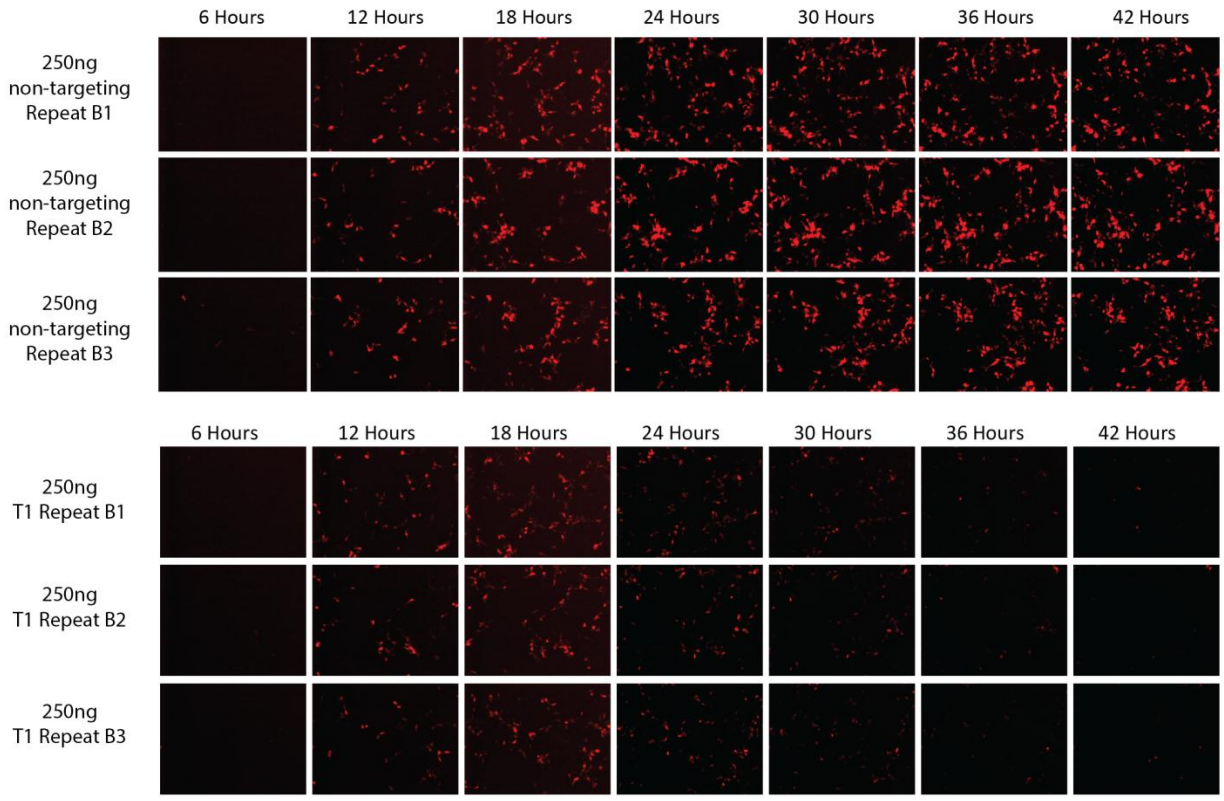


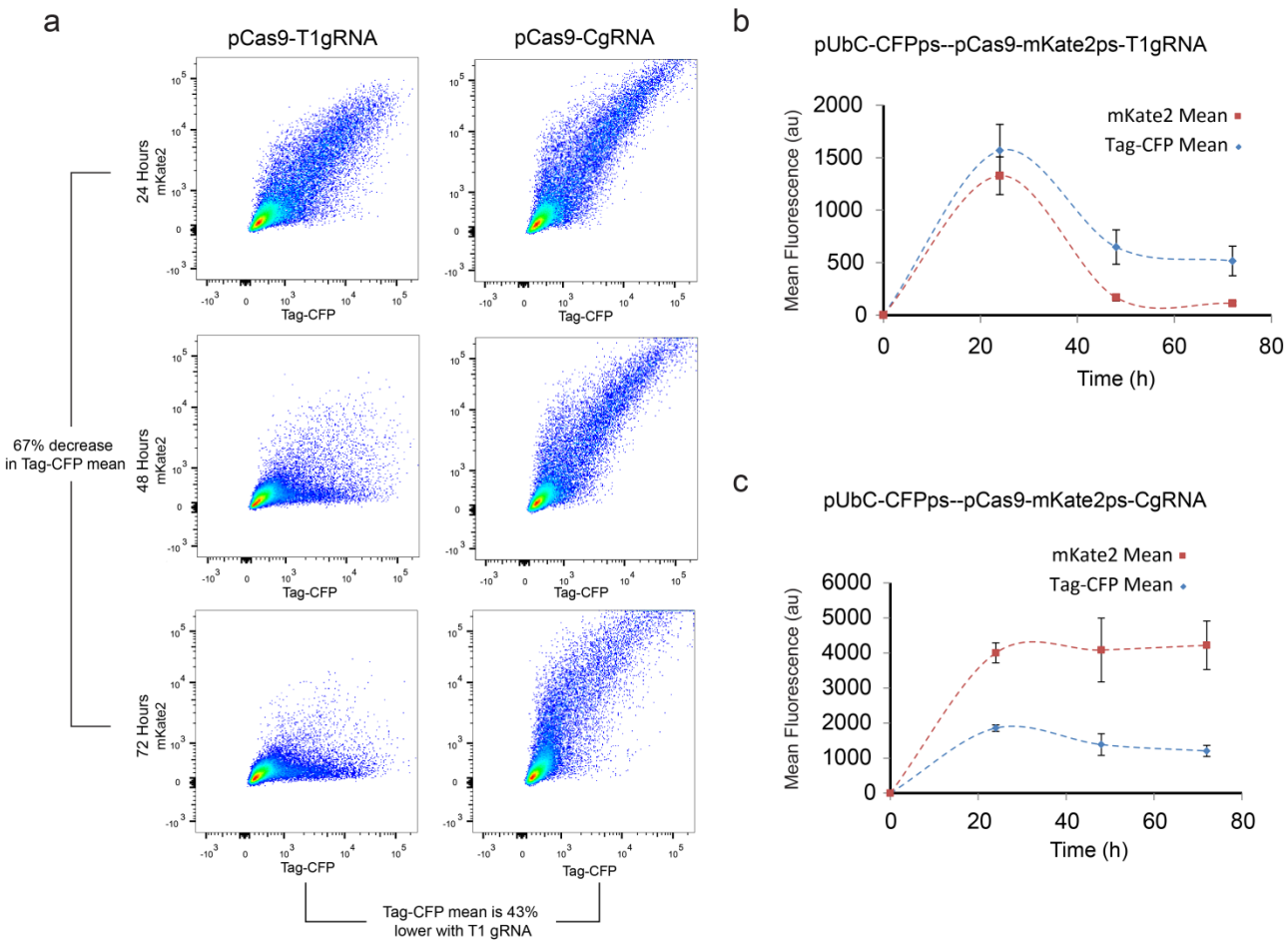
Supplementary Figure 2: Triplicate flow cytometry time lapse of 100ng of each plasmid transiently transfected in HEK-293 cells. Control contains the non-targeting gRNA (pCas9-mKate2ps-CgRNA) while T1 (pCas9-mKate2ps-T1gRNA) and T2 (pCas9-mKate2ps-T2gRNA) contain the gRNAs targeting the mKate2 ORF. Error bars show standard deviation of the calculated means from three biological replicates.



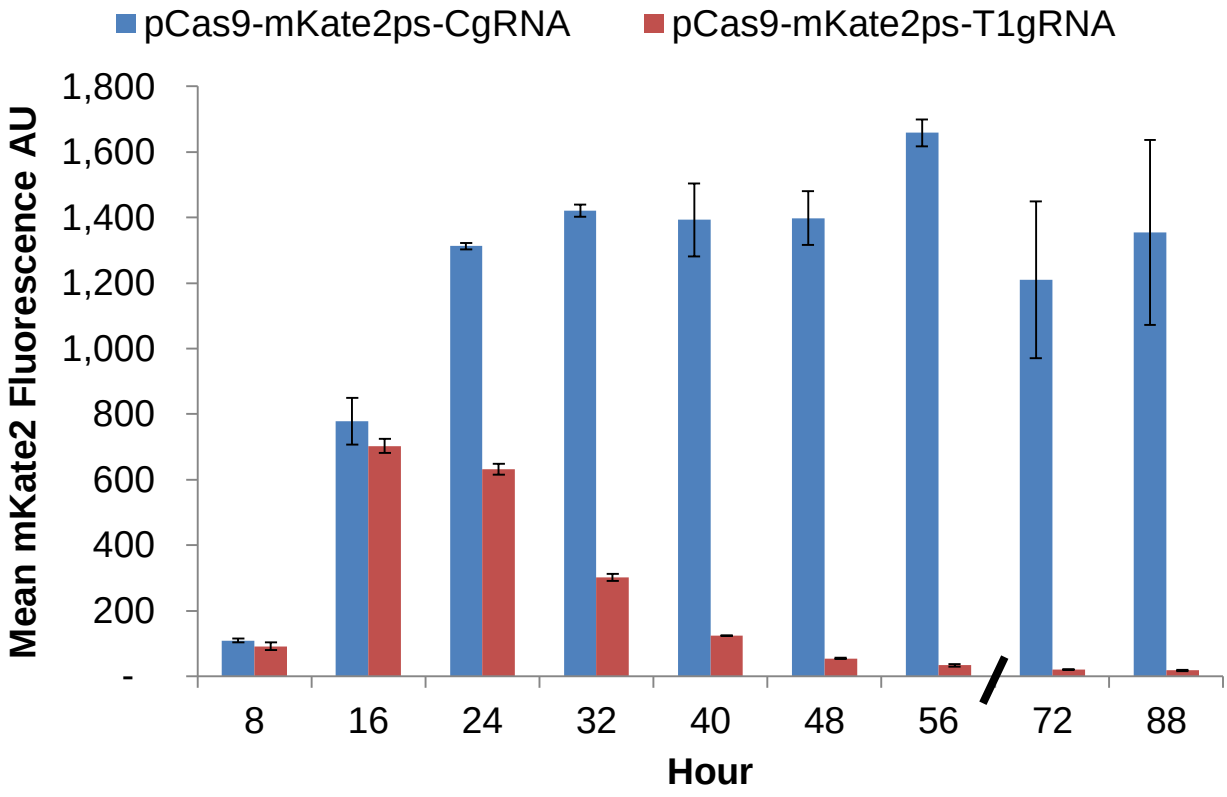
Supplementary Figure 3: 6-30 hour time-lapse microscopy measurements of mKate2 reporter expression for pCas9-mKate2ps-T1gRNA and the non-targeting pCas9-mKate2ps-CgRNA. The repeats correspond to different positions in the same well (technical replicates T1gRNA: A1, A2, A3 and non-targeting CgRNA: A1, A2, A3) from two wells transiently transfected with 250ng of the plasmids. The time-lapse protocol is available in Microscopy Methods.



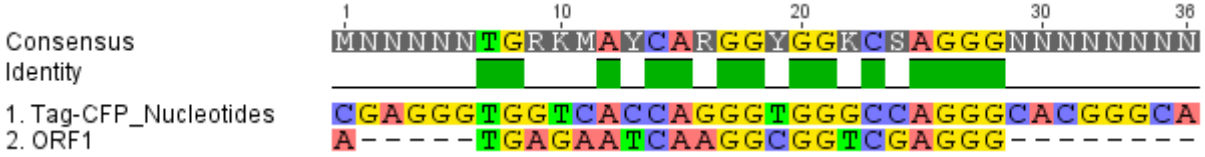
Supplementary Figure 4: 6-42 hour microscopy time-lapse measurements of mKate2 reporter expression for pCas9-mKate2ps-T1gRNA and the non-targeting pCas9-mKate2ps-CgRNA. The time-lapse protocol is available in Microscopy Methods. The repeats correspond to different positions in the same well (technical replicates T1gRNA: B1, B2, B3 and non-targeting CgRNA: B1, B2, B3) from two wells transiently transfected with 250ng of the plasmids.



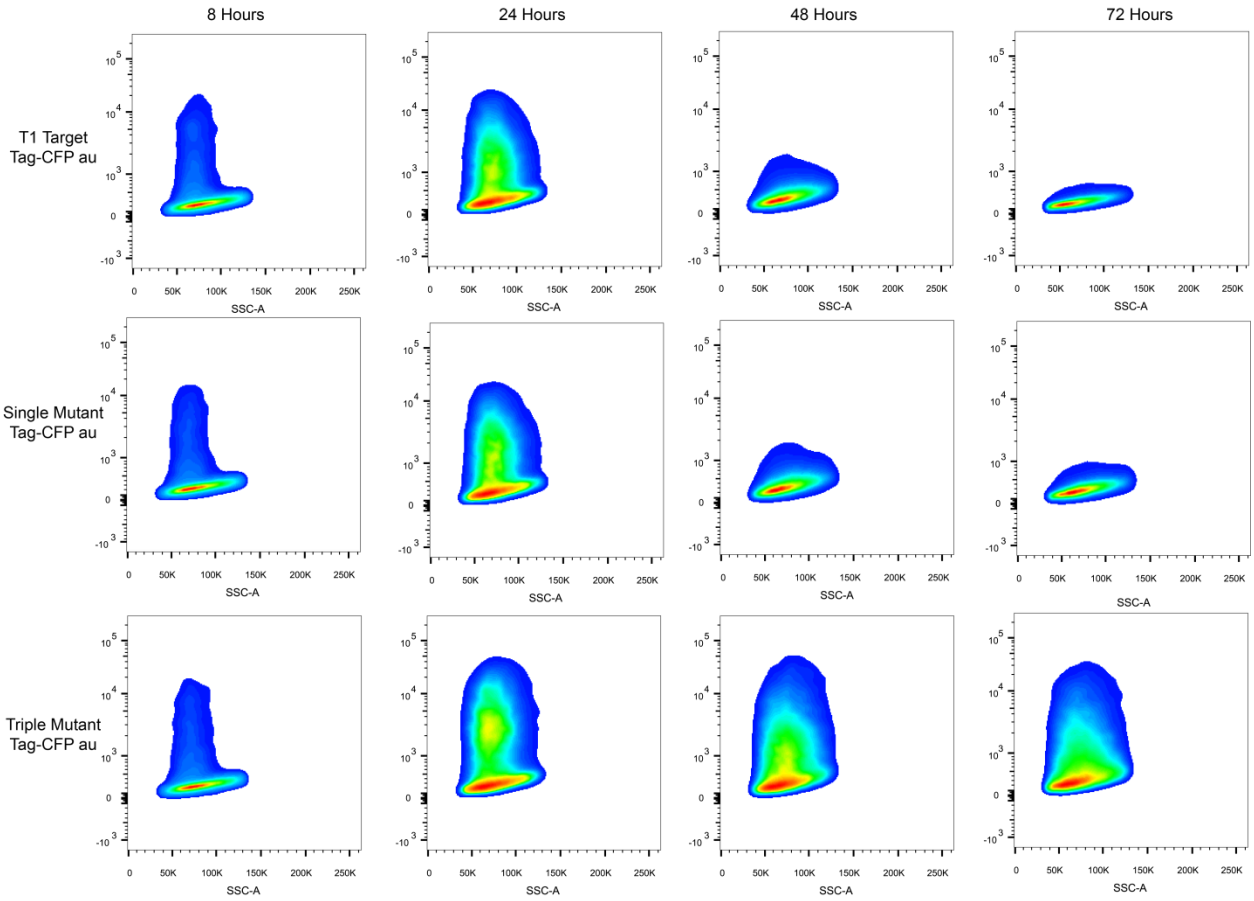
Supplementary Figure 5: (a) Flow cytometry scatter plot data of the T1gRNA two reporter system (pUbC-CFPps--pCas9-mKate2ps-T1gRNA) transiently transfected at 100ng versus the non-targeting two reporter system (pUbC-CFPps--pCas9-mKate2ps-CgRNA). Mean Tag-CFP with the T1gRNA decreases by 67% from 24 to 72 hours as calculated form triplicate biological replicates (1567 AU to 516 AU, SD +/- 250 and 141 respectively). (b) The T1gRNA two reporter system (pUbC-CFPps--pCas9-mKate2ps-T1gRNA). Error bars show standard deviation of the calculated means from three biological replicates. (c) Non-targeting two reporter system (pUbC-CFPps--pCas9-mKate2ps-CgRNA). Error bars show standard deviation of the calculated means from three biological replicates.



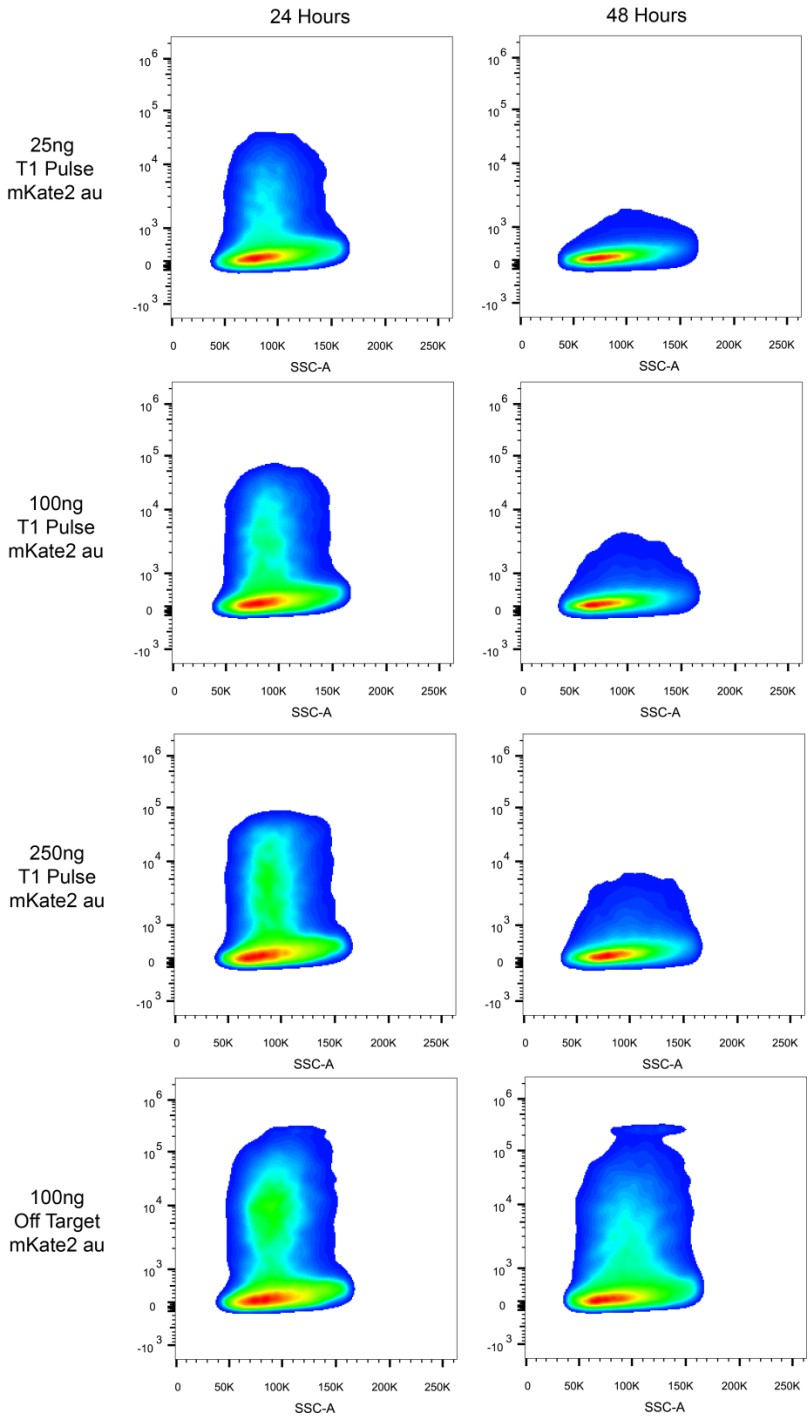
Supplementary Figure 6: Triplicate flow cytometry time lapse of 100ng of each plasmid transiently transfected. The plasmid pCas9-mKate2ps-CgRNA contains the non-targeting gRNA while pCas9-mKate2ps-T1gRNA has a gRNA targeting the mKate2 ORF. Error bars represent standard deviation of the means.



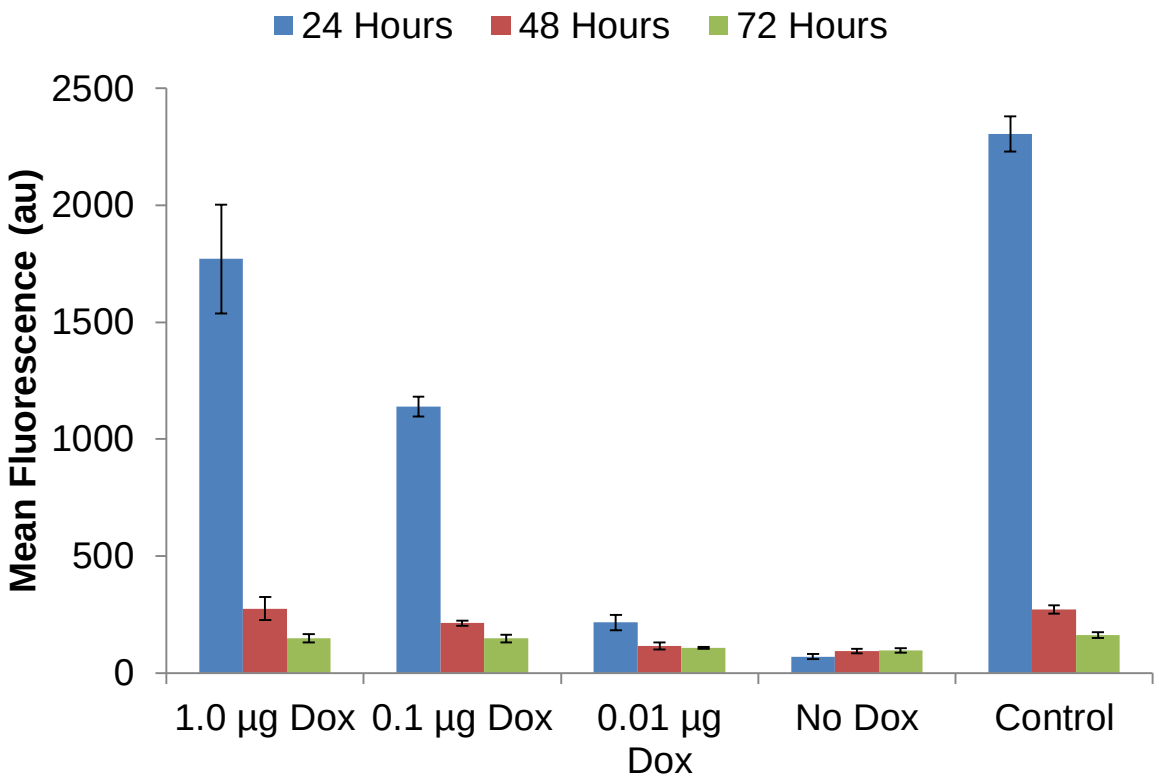
Supplementary Figure 7: Nucleotides 166 to 182 of the ORF of Tag-CFP are shown. Alignment with the T1 gRNA indicates a protospacer with 9 mismatches.



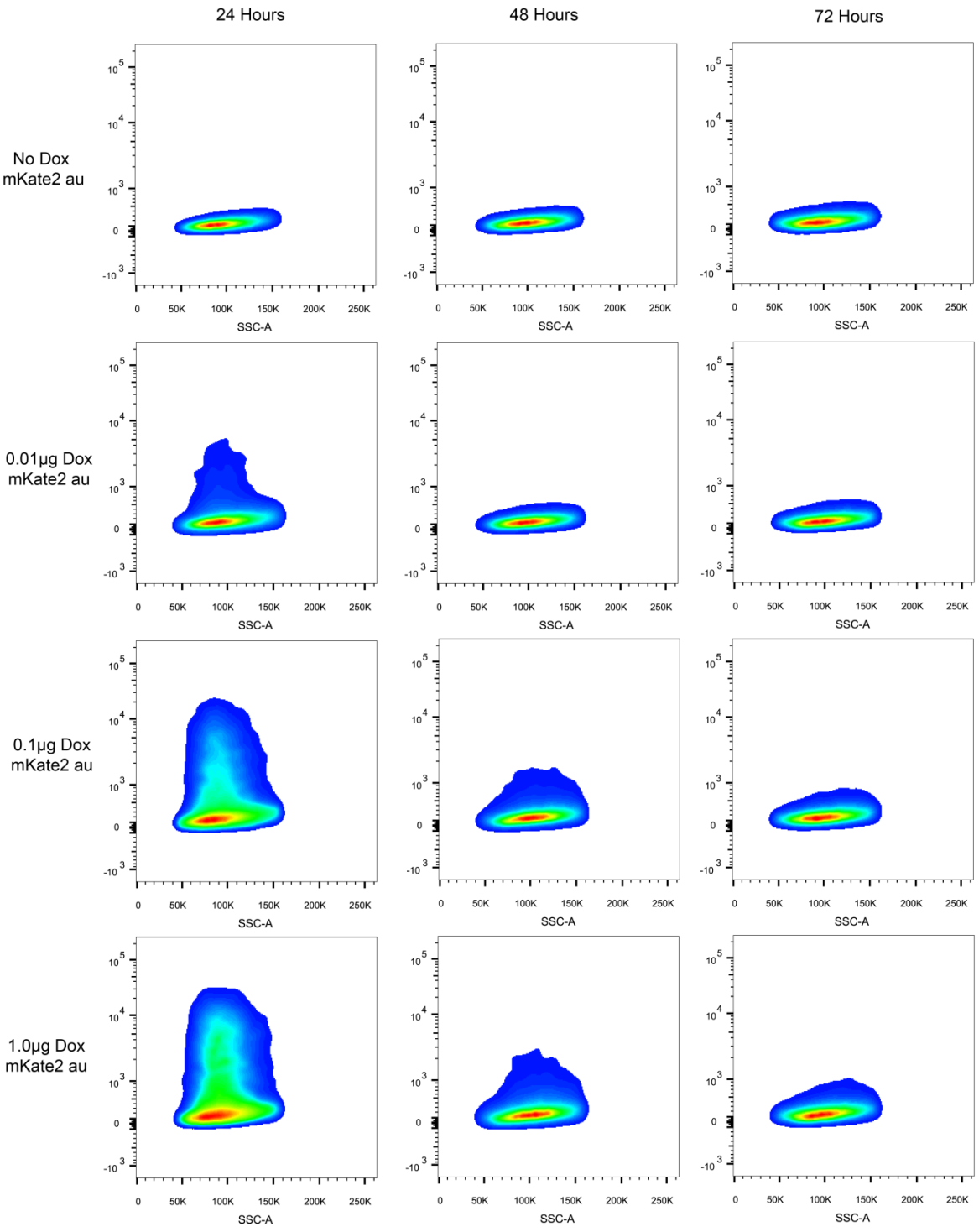
Supplementary Figure 8: Representative flow cytometry analysis showing Tag-CFP au intensity versus SSC for the original T1 target (pCas9-t1-CFPps-T1gRNA), the single mutant (pCas9-mut1-CFPps-T1gRNA) and triple mutant (pCas9-mut3-CFPps-T1gRNA).



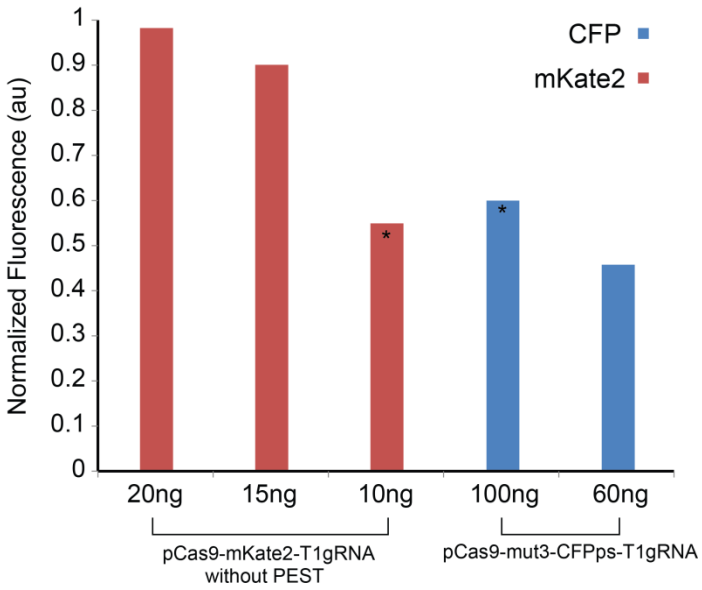
Supplementary Figure 9: Representative flow cytometry analysis showing mKate2 versus SSC for the titrations of 25ng, 100ng and 250ng of pCas9-mKate2ps-T1gRNA. Positive control is 100ng of the single plasmid with the non-targeting CgRNA (pCas9-mKate2ps-CgRNA).



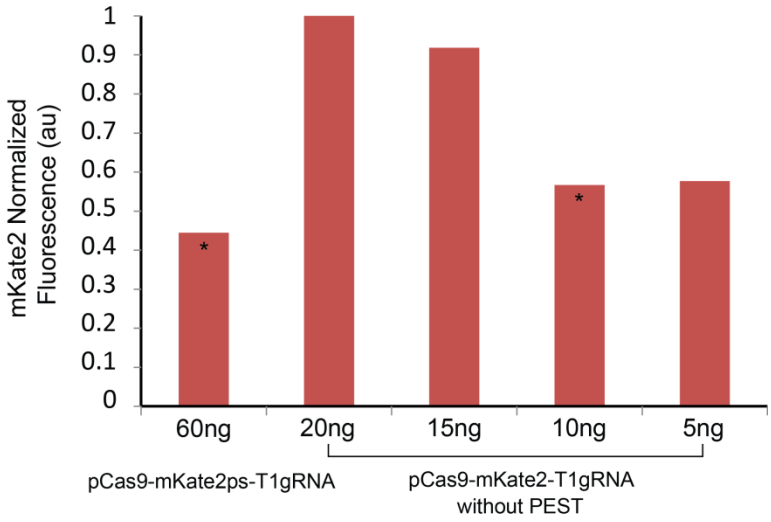
Supplementary Figure 10: Triplicates of 100ng of each plasmid transiently transfected. Control is pCas9-mKate2ps-T1gRNA plasmid with Cas9 under control of CMV. For the Dox experiments the Cas9 is under TRE promoter control (ptreCas9-mKate2-T1gRNA). Error bars show standard deviation of the calculated means from three replicates.



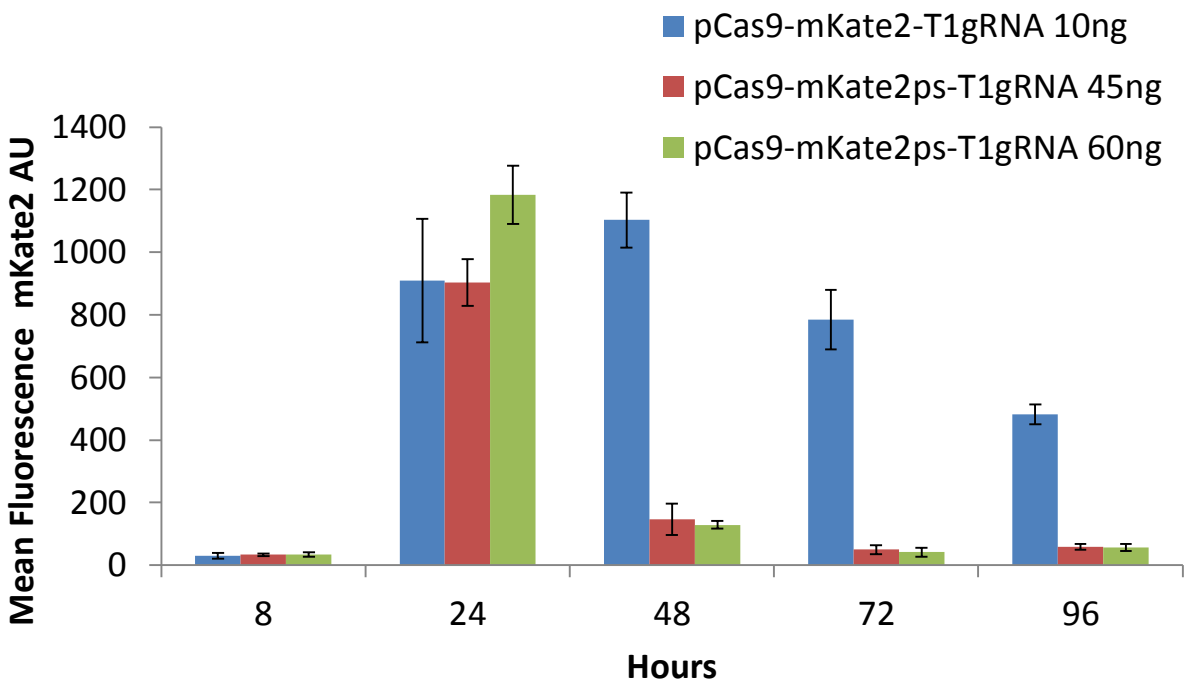
Supplementary Figure 11: Representative FACS analysis showing mKate2 au intensity versus SSC for the Dox titrations of no Dox, 0.01µg, 0.01µg, and 1.0µg; construct is ptreCas9-mKate2-T1gRNA.



Supplementary Figure 12: Normalized reporter expression for the triple mutant (pCas9-mut3-CFPps-T1gRNA, Tag-CFP output) and pCas9-mKate2-T1gRNA (without PEST domain, mKate2 output) measured at 24 hours. We selected the 100ng triple mutant versus 10ng of pCas9-mKate2-T1gRNA , which yield approximately the same fluorescence (indicated with the star).



Supplementary Figure 13: Normalized reporter expression for pCas9-mKate2ps-T1gRNA plasmid with PEST and pCas9-mKate2-T1gRNA without the PEST domain. We selected the ratio of approximately 6:1.



Supplementary Figure 14: Mean reporter expression for pCas9-mKate2ps-T1gRNA (45ng and 60ng) and pCas9-mKate2-T1gRNA (10ng) without the PEST domain on mKate2 (biological triplicates shown). Transient transfection masses are shown in legend. Error bars represent standard deviation of the means.

Supplementary Video 1: Pulse behavior on a population level for our self-cleaving plasmid (pCas9-mKate2ps-T1gRNA). The first frame represents 5 hours post-transfection and each frame represents a 20 minute interval. Final frame shows 45 hours post-transfection.

Supplementary Video 2: mKate2 expression on a population level for our non-targeting plasmid (pCas9-mKate2ps-CgRNA); this control has no targets for the gRNA. The first frame represents 5 hours post-transfection and each frame represents a 20 minute interval. Final frame shows 45 hours post-transfection."

Supplementary Tables

Supplementary Table 1: List of plasmids

Identifier	Description	Plasmid
pCas9-mKate2ps-T1gRNA	Pulse system with T1 gRNA (targeting ORF of mKate2)	CMV-Cas9-2A-mKate2-PEST--U6-T1gRNA
pCas9-mKate2ps-T2gRNA	Pulse system with T2 gRNA (targeting ORF of mKate2)	CMV-Cas9-2A-mKate2-PEST--U6-T2gRNA
pCas9-mKate2ps-CgRNA	Control with gRNA which does not target the plasmid	CMV-Cas9-2A-mKate2-PEST--U6-CgRNA
pUbC-CFPps--pCas9-mKate2ps-T1gRNA	Control with additional Tag-CFP reporter (with T1 gRNA)	UbC-TagCFP-PEST--CMV-Cas9-2A-mKate2-PEST--U6-T1gRNA
pUbC-CFPps--pCas9-mKate2ps-CgRNA	Control with additional Tag-CFP reporter (Non-targeting C gRNA)	UbC-TagCFP-PEST--CMV-Cas9-2A-mKate2-PEST--U6-CgRNA
pCas9-mKate2-T1gRNA	Pulse system with T1 gRNA and mKate2 lacking PEST	CMV-Cas9-2A-mKate2--U6-T1gRNA
ptreCas9-mKate2-T1gRNA	Pulse system driven by the inducible TRE3G (with T1 gRNA)	TRE3G-Cas9-2A-mKate2-PEST--U6-T1gRNA
pCas9-t1-CFPps-T1gRNA	Pulse system with CFP reporter preceded by T1 target	CMV-Cas9-2A-T1tgt(orig)-TagCFP-PEST--U6-T1gRNA
pCas9-mut1-CFPps-T1gRNA	Pulse system with CFP reporter preceded by single mutant T1 target	CMV-Cas9-2A-T1tgt(1x mut)-TagCFP-PEST--U6-T1gRNA
pCas9-mut3-CFPps-T1gRNA	Pulse system with CFP reporter preceded by triple mutant T1 target	CMV-Cas9-2A-T1tgt(3x mut)-TagCFP-PEST--U6-T1gRNA

Supplementary Table 2: Primers

Identifier	Description	Oligo
P1	Cas9 Forward with NheI	GCCACCGCTAGCCTTGCCACCATGGACAAGAAGTACTCC
P2	Cas9 Reverse 2A Overlap	AAGACTTCCTCTGCCCTCAGCCACCTTCCTCTTCTTCTTGGGGTCAGC
P4	2A Peptide FWD (for mKate2) no Target	GCTGAGGGCAGAGGAAGTCTTC

P6	PEST Reverse with TAA (for mKate2) with NotI (preserves Poly A tail of CFP backbone)	gccaccGCGGCCGCTTAGACGTTGATCCTGGCGCTG
P7	mKate2 Forward	ACCGAGACCCTGTACCCCGC
P8	mKate2 Reverse	CAGGGCCATGTCGGCTCTGC
P9	U6 gRNA seq Forward	GGAACCAATTCAGTCGACTGGATC
P10	Non-targeting gRNA Forward	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGAG GCAAGAGTGCCTTGACG
P11	Non-targeting gRNA Reverse	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCGTCA AGGCACTCTTGCCTC
P12	Screened Target 1 mKate2 CDS Forward	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGTGA GAATCAAGGCGGTCGA
P13	Screened Target 1 mKate2 CDS Reverse	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCGAC CGCCTTGATTCTCAC
P14	Screened Target 2 mKate2 CDS Forward	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGATG AGAATCAAGGCGGTCG
P15	Screened Target 2 mKate2 CDS Reverse	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCGACC GCCTTGATTCTCATC
P16	gRNA Extract Forward BsaI (BsaI native to Evrogen CFP)	GCCACCccaccgagaccTCAGTCGACTGGATCCGGTA
P17	gRNA Extract Reverse BsaI	GCCACCggtctcggtggGATCCACTAGTAACGGCCG
P23	ApaI mKate2 FWD	gccaccGGGCCCATGGTGAGCGAGCTGATTAAGGAGAA
P24	FseI mKate2 REV with STOP	gccaccGGCCGGCCTTATCTGTGCCCCAGTTTGCTAGG
P25	CFP PEST No Mutation F (open reading frame #1 from mKate2)	gccaccGGGCCCATGAGAATCAAGGCGGTCGAGGGgATGAGCG GGGGCGAGGAGCTGTTC
P26	CFP PEST REV	gccaccGGCCGGCCTTAGACGTTGATCCTGGCGCTGGC
P27	CFP PEST 1 Mutation F (G-T,A-C,C-A,T-G)	gccaccGGGCCCATGAGAATCAAGGCGGTCGATGGgATGAGCG GGGGCGAGGAGCTGTTC

P28	CFP PEST 3 Mutation F (G-T,A-C,C-A,T-G)	gccaccGGGCCCATGAGAATCAAGGCTGTAGATGGgATGAGCGGGGGCGAGGAGCTGTTC
P32	TRE-3G-FWD (truncate left cmv min) with rep origin overlap	TGTGGATAACCGTATTACCGCCCCGTACACGCCACCTCGACATA
P33	TRE-3G-REV with NheI	gccaccGCTAGCCGGAAAGTTGGTATAAGACAAAAGTGTTGTGG
P34	Plasmid Rep Origin FWD with SpeI	CGGCCGTTACTAGTGGATCCGC
P35	Plasmid Rep Origin REV	GGCGGTAATACGGTTATCCACAGAATCAGG
P36	CPCR 3G FWD - Anneal 52	TGGAAAGGACGAAACACCGT
P37	CPCR 3G REV - Product is 473	TTTTCTACGGGGTCTGACGC
P38	Cas9 REV (near beginning)	TTTGCTCGGCACCTTGTACT
P39	UbC Forward	gccaccACTAGTGGCCTCCGCGCCGGGTTTTG
P40	UbC Reverse without SpeI	CTAACACTGAAAATTACATATTGACCC
P41	UbC Reverse	TCGTCTAACAAAAAAGCCAAAAACGGCCAGAATTTAGCGGACAATTTAGTACTCTAACACTGAAAATTACATATTG
P42	TagCFP-PEST FWD	CGTTTTTGGCTTTTTTGTTAGACGAgccaccATGAGCGGGGGCGAGGAGCT
P43	TagCFP-PEST REV	gccaccACTAGTGATGAGTTTGGACAAACCACAAC

Supplementary Table 3: gRNA targets

gRNA Name	gRNA Sequence
T1: mKate2 Open Reading Frame	ATGAGAATCAAGGCGGTCGA
T2: mKate2 Open Reading Frame	CATGAGAATCAAGGCGGTCG
C: Non-targeting; no match in our complete plasmids	TAGGCAAGAGTGCCTTGACG

Supplementary Table 4: Screened gRNA targets for the mKate2 open reading frame. Only the targets with quality score above 80% are shown.

#	gRNA	PAM	Score %
1 (T1)	ATGAGAATCAAGGCGGTCGA	GGG	91
2 (T2)	CATGAGAATCAAGGCGGTCG	AGG	90
3	GGCGAAGGCAAGCCCTACGA	GGG	88
4	AAGTGCACATCCGAGGGCGA	AGG	88
5	CACTTCAAGTGCACATCCGA	GGG	83
6	AGAATCAAGGCGGTCGAGGG	CGG	83
7	ATGGTCTGGGTGCCCTCGTA	GGG	82
8	GGGCGAAGGCAAGCCCTACG	AGG	82
9	GTAGGGCTTGCCTTCGCCCT	CGG	82
10	CCACTTCAAGTGCACATCCG	AGG	80

Supplementary Table 5: Model ODEs

[Pulse Plasmid] -> mRNACas9_2A_mKate2 + [Pulse Plasmid]
mRNACas9_2A_mKate2 -> null
mRNACas9_2A_mKate2 -> mRNACas9_2A_mKate2 + Cas9
Cas9->null
mRNACas9_2A_mKate2 -> mRNACas9_2A_mKate2 + mKate2
mKate2->null
[Pulse Plasmid] -> gRNA + [Pulse Plasmid]
gRNA ->null
Cas9+gRNA->Cas9-gRNA
Cas9-gRNA->null
[Pulse Plasmid] + Cas9-gRNA -> [Pulse Plasmid-Cas9-gRNA]
[Pulse Plasmid-Cas9-gRNA] ->null

Supplementary Table 6: Simulation Parameters

Parameter (1)	**Value (1/s)**
k_mRNA_Cas9_2A_mKate2	0.08227
k_mRNA_Cas9_mKate2_deg	0.004214
k_gRNA	0.00004214
k_gRNA_deg	0.42×10^{-5}
k_pr_Cas9	7.5916×10^{-6}
k_pr_Cas9_deg	9.20×10^{-7}
k_pr_mKate2	7.30×10^{-5}
k_pr_mKate2_deg	1.82×10^{-5}
k_Cas9_gRNA_Complex	1.00×10^{-6}
k_Cas9_gRNA_Complex_deg	0.42×10^{-3}
k_Cas9_gRNA_Plasmid_cutting	1.00×10^{-3}

Supplementary Cloning Information

Plasmid: pCas9-mKate2ps-T1gRNA (CMV-Cas9-2A-mKate2-PEST--U6-T1gRNA)

Guide RNA were constructed according to option B of the gRNA synthesis protocol from Mali et al(2). T1 gRNA used oligos P12 and P13, T2 gRNA used oligos P14 and P15 and the non-targeting gRNA used oligos P10 and P11. The gRNAs were sequenced using primer P9. Each gRNA construct was amplified along with its U6 promoter using primers P16 and P17 and cloned into Tag-CFP-N (Evrogen) using BsaI. To accomplish this BsaI cloning, each U6-gRNA amplicon was PCR purified and then digested with BsaI at 37°C; the digested products were gel purified. Tag-CFP-N was digested with BsaI at 37°C, CIP treated and then gel purified. Each gRNA was ligated with the Tag-CFP-N backbone at a 1:3 ratio using T4 ligase at 4°C overnight and then transformed into NEB-5-alpha high efficiency cells. The transformations were plated on Kanamycin plates and colonies were screened by test digestion with BsaI. These three vectors containing each U6-gRNA were subsequently used as a backbone for Cas9-2A-mKate2-PEST described in the following steps. To create Cas9-2A-mKate2-PEST, Cas9 was amplified from hCas9 (Addgene) using primers P1 and P2 and gel purified. Next, 2A-mKate2-PEST was amplified using primers P4 and P6 and gel purified. Both Cas9 and 2A-mKate2-PEST amplicons were combined using overlap extension PCR with primers P1 and P6. The completed amplicon from the overlap extension was then digested with NheI and NotI at 37°C and gel purified. Each U6-gRNA-CMV-Tag-CFP backbone (T1, T2 and C non-targeting) was digested with NheI and NotI at 37°C gel purified. Next, the digested U6-gRNA backbones were ligated with the digested Cas9-2A-mKate2-PEST at a 1:1 ratio with T4 ligase overnight at 4°C along with a negative reaction containing only the backbone. The

following day, the ligations were transformed into XL10-Ultracompetent cells and spread on Kanamycin plates. Colonies were inoculated and screened by transfection. Sequencing was performed with primers P7 and P8.

Plasmid: pCas9-mKate2-T1gRNA (pCas9-mKate2-T1gRNA)
U6-gRNAT1—CMV-Cas9-2A-mKate2-PEST was digested with ApaI at 25°C for one hour and subsequently with FseI at 37°C for one hour to remove mKate2-PEST and then gel purified. Next, mKate2 was amplified without PEST using primers P23 and P24 and digested with ApaI at 25°C for one hour subsequently with FseI at 37°C for one hour; the digestion was followed by gel purification. The two gel purified products were ligated 1:3 with T4 ligase overnight at 4°C and transformed the following day in XL10-Ultracompetent cells. Sequencing was performed with primer P19 and verified by transfection.

Plasmid: pCas9-t1-CFPps-T1gRNA (CMV-Cas9-2A-T1tgt(orig)-TagCFP-PEST--U6-T1gRNA)
U6-gRNAT1_CMV-Cas9-2A-mKate2-PEST was digested with ApaI at 25°C for one hour and subsequently with FseI at 37°C for one hour to remove mKate2-PEST and then gel purified. Target T1 was added to the Tag-CFP-PEST sequence using primers P25 and P26. Single mutant target T1 was added to the Tag-CFP-PEST sequence using primers P27 and P26. Triple mutant target T1 was added to the Tag-CFP-PEST sequence using primers P28 and P26. Each of the three amplicons of Tag-CFP-PEST preceded by targets was digested with ApaI at 25°C for one hour and subsequently with FseI at 37°C for one hour; all three digestions were gel purified. Each Target-Tag-CFP-PEST was ligated with gel purified U6-gRNA-T1-CMV-Cas9-2A with T4 ligase overnight at 4°C and transformed the following day in XL10-Ultracompetent cells.

Plasmid: ptreCas9-mKate2-T1gRNA (TRE3G-Cas9-2A-mKate2-PEST--U6-T1gRNA)
The replication origin from U6-gRNAT1—CMV-Cas9-2A-mKate2-PEST was amplified using 34 and 35 while the TRE3G promoter was amplified using primers P32and P33. The two amplicons were gel purified and then an overlap PCR was performed with primers P34 and P33. This product was digested with SpeI and NheI and then gel purified. U6-gRNAT1—CMV-Cas9-2A-mKate2-PEST was digested with SpeI and NheI, CIP treated, and then gel purified. The two gel purified digestions were joined with T4 ligase and transformed in XL10-Ultracompetent cells. The clones were screened for the correct orientation using primers P36 and P37 and then sequenced with primer P38. Subsequently, the construct was tested by transfection.

Plasmid: pUbC-CFPps--pCas9-mKate2ps-T1gRNA (UbC-TagCFP-PEST--CMV-Cas9-2A-mKate2-PEST--U6-T1gRNA)

The UbC promoter was amplified from UbC-mKate2-PEST (unpublished work) using primers P39 and P40. This amplicon was re-amplified with P39 and P41. Next, we amplified TagCFP-PEST from U6-gRNAT1_CMV-TargetT1-Cas9-2A-TagCFP-PEST using primers P42 and P43. Next, we performed overlap PCR of UbC and TagCFP-PEST using oligos P39 and P43 to generate the amplicon UbC-TagCFP-PEST. We digested this overlap PCR product with SpeI and digested the backbone U6-gRNAT1_CMV-Cas9-2A-mKate2-PEST with SpeI. After CIP treating the backbone, we ligated the digested products and screen for appropriate orientation using test digestion to yield UbC-TagCFP-PEST--U6-gRNAT1_CMV-Cas9-2A-mKate2-PEST.

References

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