CRISPR Prime Design and Testing at the Genome Engineering and iPSC Center

Wang, Z.T., Sentmanat, M., Chen, Y.H., Cui, X.C.. Genome Engineering and iPSC Center, Washington University School of Medicine

CRISPR Prime

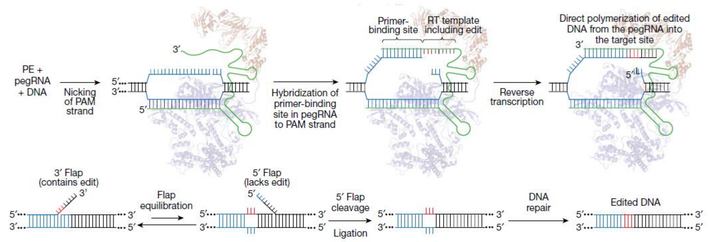
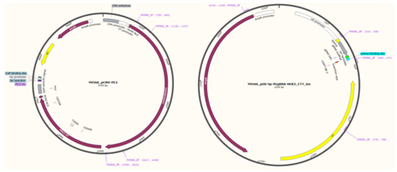


Figure 1. CRISPR Prime is a fusion of a Cas9 nickase to a reverse transcriptase which allows for guide RNA-mediated nicking of a target genomic sequence and also the simultaneous installation of a desired mutant sequence into the target site via the interaction of RT and an extended template motif on the tail end of the guide RNA (Prime Editing guide RNA, aka pegRNA).

Anzalone et. al. 2019

Validation of CRISPR Prime in human cell lines



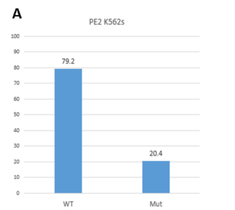
PE2 plasmid HEK3 pegRNA plasmid

	3 day PE2			5 day PE2			3 day WTCas9		
	%WT	%Mut	% NHEJ	%WT	%Mut	% NHEJ	%WT	%Mut	% NHEJ
K562	78.6	**20.2**	0.566129	77.7	**20.8**	n.d.	24.4	0	**75**
K562 GFP	99.2	0		n.d.	n.d.		99	0	
293T	82.4	**16.4**	0.619134	81	**17.7**	n.d.	38.9	0	**60.6**
293T GFP	99.3	0		n.d.	n.d.		98.8	0	
HCT116	88.8	**10.3**	0.274018	96.6	**2.5**	n.d.	32.1	0	**67.5**
HCT116 GFP	99.3	0		n.d.	n.d.		98.8	0	

Nucleofection: 250,000 cells, 800ng PE plasmid or WT Cas9 plasmid, 200ng HEK3 pegRNA plasmid

Figure 2. Both the Prime Editor 2 (PE2) plasmid construct and the pegRNA plasmid construct targeting the HEK3 locus with a CTT insertion (Anzalone et. al. 2019) were nucleofected into K562, HEK293T and HCT116 cells with Lonza nucleofection. pMax-GFP plasmid was used as a negative control. At 72hrs and 5 days post-nucleofection, NGS against the nucleofected pools showed significant on-target mutations in all three human lines, and almost all non-mutant alleles were wild-type, with no significant indels detected. Nucleofection with WT Cas9 led to high rates of NHEJ.

Profiling of Prime-edited K562 cells via single-cell sorting



Plasmid nucleofection 12/9/19
800ng PE2 plasmid, 200ng HEK3 pegRNA plasmid
K562: 250,000 cells (pass #24)
Harvested 72hr post-nucleofection

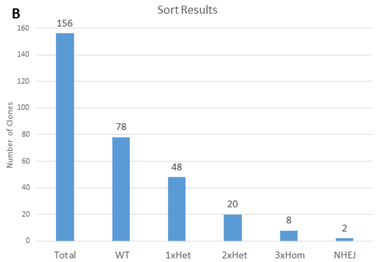
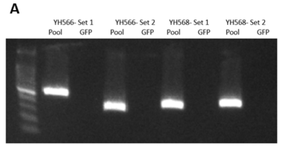


Figure 3. (**A**) K562 cells were nucleofected with PE2 plasmid and HEK3 pegRNA plasmid, and Prime editing was confirmed in the pool. K562 cells were sorted on a Sony SH-800 cytometer to isolate single-cell clones. (**B**) 156 clones in total were recovered and genotyped. 50% of clones (78) were genotypically WT, 44% (68) were heterozygous for the CTT insertion, 5% (8) were homozygous, and 1% (2) clones had other mutations at the target locus.

Detection of Random Plasmid Integration in K562 clones

A



B

Sample	Total	WT	CTT	#1-Indel	#1-Reads	#2-Indel	#2-Reads	#3-Indel	#3-Reads	%WT	%CTT
4C9	4188	2519 (60.1%)	1351 (32.3%)	0	2563 (61.2	3	1354 (32.3	-1	165 (3.9%	60.1	32.3
3D10	3702	2310 (62.4%)	1163 (31.4%)	0	2335 (63.1	3	1147 (31.0	-1	122 (3.3%	62.4	31.4
1E3	3341	3083 (92.3%)	0 (0.0%)	0	3200 (95.8	-1	123 (3.7%)	1	11 (0.3%)	92.3	0
1E5	4218	2477 (58.7%)	1428 (33.9%)	0	2495 (59.2	3	1438 (34.1	-1	170 (4.0%	58.7	33.9
1E9	2908	912 (31.4%)	1823 (62.7%)	3	1806 (62.1	0	907 (31.2%	2	107 (3.7%	31.4	62.7

Figure 4. (**A**) 2 sets of primers each were designed against the pegRNA plasmid (YH566) and the PE2 plasmid (YH568), and detection of both plasmids was confirmed in the nucleofected pool. (**B**) None of the 156 clones had detectable integration of the pegRNA plasmid, however 5 clones were found with integration of the PE2 plasmid. 3 clones were PCR-positive for both PE2 plasmid primer sets, and 2 more were positive with one primerset but not the other. Of these 5 plasmid integration clones, 1 clone is genotypically WT, 3 have one mutant allele, and 1 has two mutant alleles.

Prime Editing with synthetic pegRNAs and PE2 Protein

A

HEK3.CTT.ins.wtTrcr	ggcccagactgagcacgtgagttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctctgccatcaaagcgtgctcagtctg
HEK3.CTT.ins.mutTrcr	ggcccagactgagcacgtgagttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggGaccgagtcggtCctctgccatcaaagcgtgctcagtctg

B

pegRNA identity	species	Cell type	% mut (Plasmid)
HEK3.CTT.ins.wtTrcr	human	K562 p15	**5.3**
HEK3.CTT.ins.wtTrcr-XT	human	K562 p15	**2.4**
HEK3.CTT.ins.mutTrcr	human	K562 p15	**13.9**
HEK3.CTT.ins.mutTrcr-XT	human	K562 p15	**15.9**
GFP Ctrl	human	K562 p15	-

C

pegRNA identity	species	Cell type	% mut (Plasmid)	% mut (Protein)
HEK3.CTT.ins.wtTrcr	human	K562 p20	**5.6**	**0.3**
HEK3.CTT.ins.wtTrcr-XT	human	K562 p20	**2.4**	**2.7**
HEK3.CTT.ins.mutTrcr	human	K562 p20	**9.6**	**0.1**
HEK3.CTT.ins.mutTrcr-XT	human	K562 p20	**14.2**	**5.6**
GFP Ctrl	human	K562 p20	-	-

Figure 5. (**A**) We obtained 2 forms of synthetic pegRNAs from IDT, standard pegRNAs with the typical complement of stabilizing modifications at the 5′ and 3′ ends, and pegRNA-XT where the stabilizing modifications were extended deep into the pegRNA sequence. We also noticed two point mutations in the Anzalone HEK3 pegRNA sequence relative to the canonical TrcrRNA sequence, corresponding to a C-G > G-C inversion in a stem loop, and ordered synthetic HEK3-CTT-ins pegRNA with both the WT and mutant sequences. (**B**) The combination of PE2 plasmid with synthetic pegRNA yielded good editing activity, and the mutant TrcrRNA sequence with the inversion was >3fold more active than pegRNA with the WT trcrRNA sequence. (**C**) We obtained purified PE2 protein from UC Berkeley Macrolabs and found that overall activity level was lower with delivery of the protein directly compared to the PE2 plasmid expression construct, and the XT modifications were critical for editing activity with the protein, and the mutant TrcrRNA sequence appeared to be more active.

Design of pegRNAs for our own mutations of interest

A

pegRNA	guide	trcr type	PBS/ Ext seq	PBS length (nt)	Ext length (nt)
YH600.hTNNC1.sp4.mutant.pegRNA	cctggagttcatgaaggGtg	Mut	gcatctactcGacaTCCTTCATGAACTC	13	15
MS05.sp1.C299G	ggctcggaggcacatgaaag	WT	caaaaaaaCccacttttatgtgcctccg	13	15
MS05.sp1.G298T	ggctcggaggcacatgaaag	WT	caaaaaaaGAcacttttatgtgcctccg	13	15
MS05.sp1.C310T	ggctcggaggcacatgaaag	WT	accAgccaaaaaaaGCcacttttatgtgcctccg	13	21
MS02.sp2.G322A	ttccttctaggacgagcaat	WT	ggtgctcttccTattgctgtcctagaa	13	15
MS02.sp2.A319G	ttccttctaggacgagcaat	WT	ggtgctcttccCatCgctgtcctagaa	13	15

B

pegRNA identity	species	Cell type	% mut Plasmid
#1 YH600.hTNNC1.sp4.mutant.pegRNA	human	K562 p15	**0**
#2 YH600.hTNNC1.sp4.mutant.pegRNA-XT	human	K562 p15	**0**
#3 MS05.sp1.C299G	human	K562 p15	**0**
#4 MS05.sp1.C299G-XT	human	K562 p15	**0.1**
#5 MS05.sp1.G298T	human	K562 p15	**0.2**
#6 MS05.sp1.G298T-XT	human	K562 p15	**0.6**
#7 MS05.sp1.C310T	human	K562 p15	**0.2**
#8 MS05.sp1.C310T-XT	human	K562 p15	**0.1**
#9 MS02.sp2.G322A-XT	human	K562 p15	**0.7**
#10 MS02.sp2.G322A-XT	human	K562 p15	**0.4**
#11 MS02.sp2.A319G	human	K562 p15	**16.7**
#12 MS02.sp2.A319G-XT	human	K562 p15	**13.1**

C

pegRNA identity	species	Cell type	% mut (Plasmid)	% mut (Protein)
#11 MS02.sp2.A319G	human	K562 p20	**17.1**	**0.3**
#12 MS02.sp2.A319G-XT	human	K562 p20	**10.9**	**0.9**

D

pegRNA	guide	trcr type	PBS/ Ext seq	PBS length (nt)	Ext length (nt)
YH600.U6.sp.pegRNA.TNNC1_test-2	cctggagttcatgaaggGtg	WT	tactcGacaTCCTTCATGAACTCCA	15	9
MS05.sp1.G298T.mutTrcr	ggctcggaggcacatgaaag	Mut	accAgccaaaaaaaGCcacttttatgtgcctccg	13	15
MS02.sp2.A319G.mutTrcr	ttccttctaggacgagcaat	Mut	ggtgctcttccCatCgctgtcctagaa	13	15

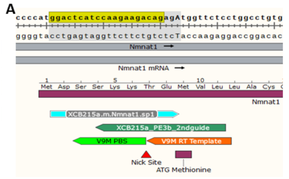
E

pegRNA identity	species	Cell type	% mut (Plasmid)
YH600.U7.sp.pegRNA.TNNC1_test_2	human	K562 p8	**0**
MS05.sp1.G298T.mutTrcr	human	K562 p8	**0.1**
MS02.sp2.A319G.mutTrcr	human	K562 p8	**15.5**
HEK3-CTT-Ins-pegRNA	human	K562 p8	**18.2**

Figure 6. (**A**) We designed six pegRNAs for six human mutations of interest and obtained synthetic standard and XT pegRNAs. (**B**) Of the six designs tested, only one pegRNA guide resulted in a significant amount of editing activity. (**C**) The active guide was re-tested with PE2 protein, and only trace amounts of editing activity was detectable despite the XT modification. (**D**) We re-designed the PBS and Ext sequences for the completely inactive guide with WT TrcrRNA, and re-synthesized one active (A319G) and one inactive (G298T) pegRNA with mutant TrcrRNA instead. (**E**) The alterations did not significantly affect activity levels.

PE3/ PE3b design and testing

A



B

C

pegRNA	guide	trcr type	PBS/ Ext seq	PBS length (nt)	Ext length (nt)
XCB215a.mNmnat1.V5M.Cprime.pegRNA.V1	ggactcatccaagaagacag	WT	ggagaaccaTctctgtctcttctggatga	13	15
XCB215a.mNmnat1.V5M.Cprime.pegRNA.V5	ggactcatccaagaagacag	WT	ggagaaccaTctctgtctcttctggatgagt	15	15
XCB215a.mNmnat1.V5M.Cprime.PE3b.g1	gagaaccaTctctgtctctct	WT	-	-	-
YH652.mKmt2c.Y4793A.Cprime.pegRNA.V1	agacattgaaaaacacacta	WT	ttccaatAGCctcgatgaccatagtgtgttttcaatgt	15	24
YH652.mKmt2c.Y4793A.Cprime.pegRNA.V2	agacattgaaaaacacacta	WT	ttccaatAGCctcgatgaccatagtgtgttttcaat	13	24
YH652.mKmt2c.Y4793A.Cprime.PE3.g1	actcataaagcttctcctttc	WT	-	-	-

D

pegRNA identity	species	Cell type	% mut (Plasmid)	% mut (Protein)
XCB215a.mNmnat1.V5M.Cprime.PEG.V1	mouse	N2A p4	**0.6**	
XCB215a.mNmnat1.V5M.Cprime.PEG.V1 + PE3b	mouse	N2A p4	**0.7**	
XCB215a.mNmnat1.V5M.Cprime.PEG.V5	mouse	N2A p4	**0.7**	
XCB215a.mNmnat1.V5M.Cprime.PEG.V5 + PE3b	mouse	N2A p4	**0.9**	
YH652.mKmt2c.Y4793A.Cprime.PEG.V1	mouse	N2A p4	**0**	
YH652.mKmt2c.Y4793A.Cprime.PEG.V1 + PE3	mouse	N2A p4	**0**	
YH652.mKmt2c.Y4793A.Cprime.PEG.V2	mouse	N2A p4	**0**	
YH652.mKmt2c.Y4793A.Cprime.PEG.V2 + PE3	mouse	N2A p4	**0**	

Figure 7. (**A**) We designed pegRNAs to create a mutation of interest in mice using a PE3b strategy, with a secondary guide RNA specific to the edited strand to induce nicking of the non-edited strand after installation of the edit. (**B**) We also designed pegRNAs for another mutation using a PE3 strategy, using a secondary guide +55 from the nick site to bias repair towards the edited strand. (**C**) Two pegRNA designs were created per target sequence. (**D**) Despite the secondary PE3/ PE3b guide, the amount of editing activity that resulted was minimal.

Conclusions

- **CRISPR Prime has the potential to allow targeted and specific mutagenesis without need for double-strand breaks and indel formation**
- **Synthetically produced pegRNAs are compatible with delivery of PE2 via both plasmid and protein formats**
- **Plasmid delivery of PE2 can sometimes lead to integration of plasmid DNA into the genome**
- **Delivery of PE2 directly as protein avoids this issue, but requires extensive modification of pegRNAs and results in reduced activity. The lack of commercially available PE2 protein is a barrier as well.**
- **Design and troubleshooting of pegRNAs for Prime Editing applications remain a difficult problem: the cost of synthesizing and testing multiple pegRNA designs quickly becomes prohibitive for large-scale applications with many targets of interest.**

References

- **Anzalone, A.V., Randolph, P.B., Davis, J.R. et al. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576, 149–157 (2019). https://doi.org/10.1038/s41586-019-1711-4**

With thanks to Ashley Jacobi and Integrated DNA Technologies (IDT) for providing us the pegRNAs and pegRNA-XTs. Thanks as well to UC Berkeley Macrolabs for providing us their preps of the PE2 protein.