

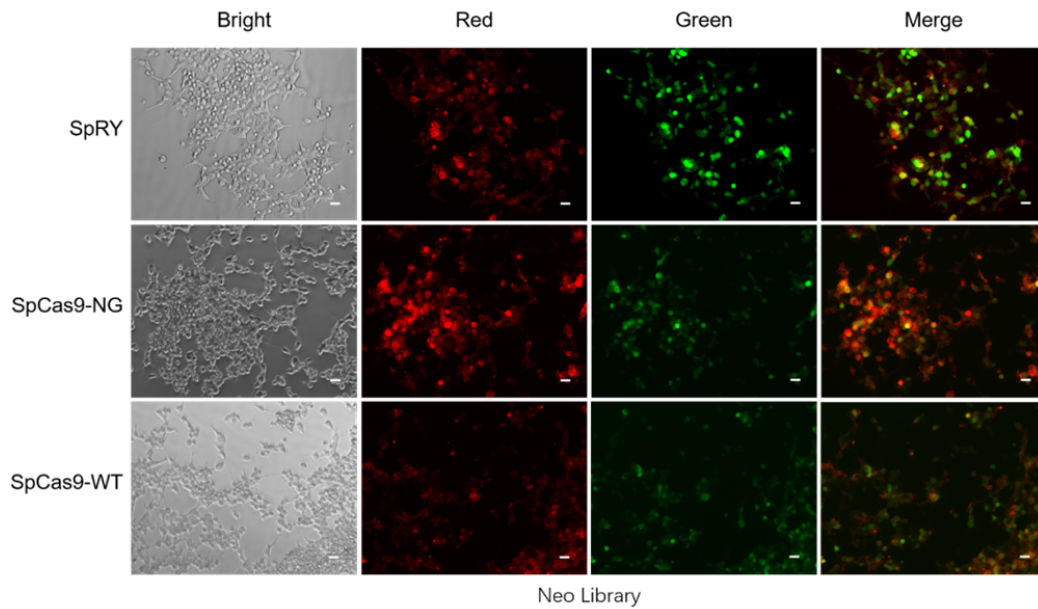
## Supplementary information

### Can SpRY recognize any PAM in human cells?

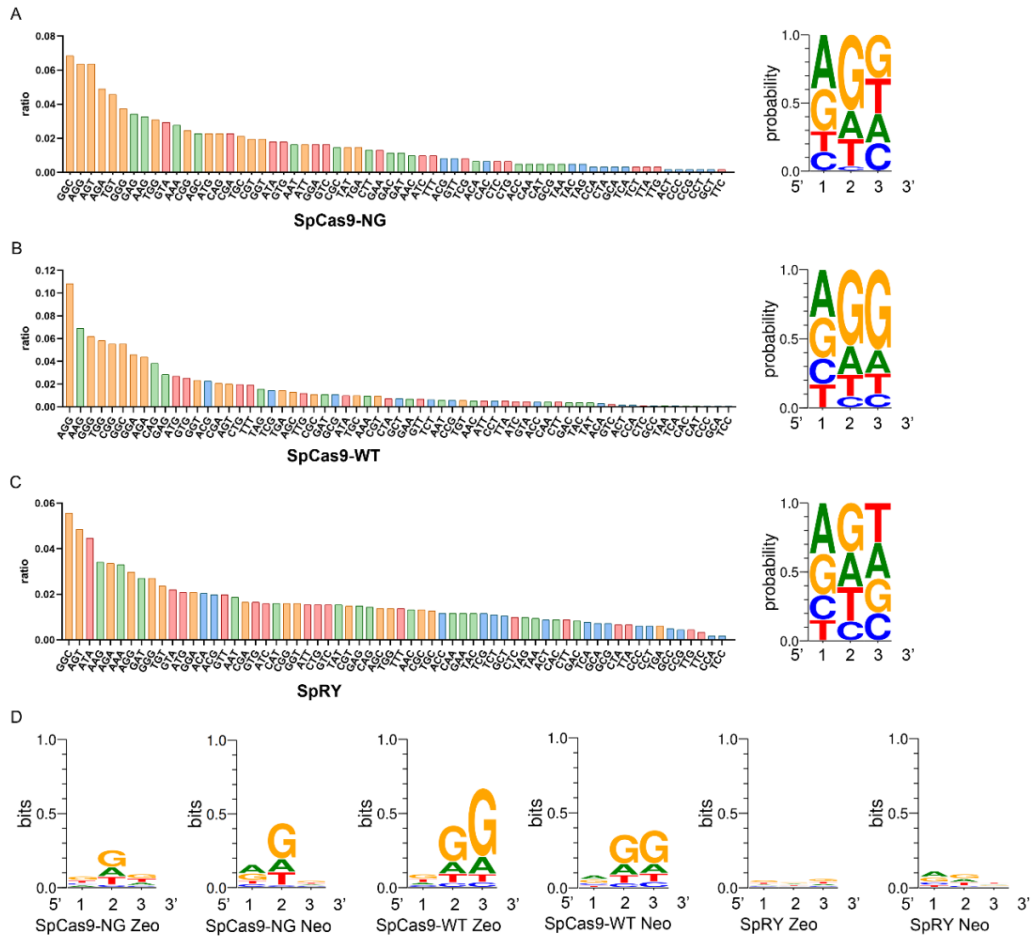
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**Fig. S1** The results of PAM-DOSE via fluorescent microscopy at Neo site. HEK-293 cells were co-transfected with a pmTmG-N<sub>4</sub>-Neo PAM library, and the plasmids expressing Cas and sgRNA for the cleavage and images were obtained 48 h after transfection. Scale bar is 100  $\mu$ m.



**Fig. S2** The NGS data of SpCas9-NG, SpCas9-WT, and SpRY using PAM-DOSE at the Neo site. (A, B, C) Ratio of recognized PAMs calculated and sequence logo based on NGS results at the Neo site of SpCas9-NG, SpCas9-WT and SpRY. (D) The sequence logo of SpCas9-NG, SpCas9-WT and SpRY at the Zeo and Neo site (Y axis was bits).

**Table S1 Oligonucleotide sequences for plasmids construction of Cas9 variants**

Primer name	Primer sequence
SpRY-F1	TACTCCCACAGGTGAGCGG
SpRY-R1	GTTGGTGCCGATGTCCAGG
SpRY-F2	CCTGGACATCGGCACCAACTCTGTGG
SpRY-R2	CGAGCTCTAGGAATTCTTAGACTTTCCTCTTCTTCTTGGGCTCG

**Table S2 Target sequence of three mTmG PAM libraries with random PAMs**

Name	Sequence
Neo	CGCGTAGTGGCGGGCGACTTGACAAGCGTGCT <u>NNNN</u> CCTCGTACCCGCGGACTAGTACG
Zeo	CGCGTAGTGGCGGGAGGACGACTTCGCCGGTG <u>NNNN</u> CCTCGTACCCGCGGACTAGTACG
sloxP	TAATAACTTCGTATAGCATACTAG <u>GGT</u>

**Table S3 Oligonucleotide sequences for sgRNA construction targeting Neo, Zeo, and sloxP**

Name	SgRNA
SaCas9 sloxP F	CACCGTAATAACTTCGTATAGCATAC
SaCas9 sloxP R	AAACGTATGCTATACGAAGTTATTAC
SpCas9 Neo F	CACCGGGCGACTTGACAAGCGTGCT
SpCas9 Neo R	AAACAGCACGCTTGTC AAGTCGCCC
SpCas9 Zeo F	CACCGGGAGGACGACTTCGCCGGTG
SpCas9 Zeo R	AAACCACCGGCGAAGTCGTCCTCCC

**Table S4 *EGFP* target sequences**

<b>Name</b>	<b>EGFP target sequence</b>	<b>PAM</b>
sgRNA-AAAC	GTCGAGCTGGACGGCGACGT	AAAC
sgRNA-AAAG	GCACCCAGTCCGCCCTGAGC	AAAG
sgRNA-AACA	TCGACTTCAAGGAGGACGGC	AACA
sgRNA-GACG	TGCCCATCCTGGTCGAGCTG	GACG
sgRNA-AAGA	TCAAGGACGACGGCAACTAC	AAGA
sgRNA-GAGA	CCCTGAGCAAAGACCCCAAC	GAGA
sgRNA-TAGG	ACTTCAGGGTCAGTTGCCG	TAGG
sgRNA-CATC	CTGTTACCCGGGTGGTGCC	CATC
sgRNA-GATG	TGTCCGGCGAGGGCGAGGGC	GATG
sgRNA-ACAA	GGACGGCGACGTAAACGGCC	ACAA
sgRNA-GCAC	GCTGACCCTGAAGTTCATCT	GCAC
sgRNA-ACCA	AAGGCTACGTCCAGGAGCGC	ACCA
sgRNA-ACCG	GCAAGGGCGAGGAGCTGTT	ACCG
sgRNA-CCCA	AGCTGTTACCCGGGTGGTG	CCCA
sgRNA-GCCC	GAGCTGTTACCCGGGTGGT	GCCC
sgRNA-TCCA	CGCCATGCCCCGAAGGCTACG	TCCA
sgRNA-TCCG	ACGGCCACAAGTTCAGCGTG	TCCG
sgRNA-ACGG	CGAGCTGGACGGCGACGTAA	ACGG
sgRNA-TCGG	CTACCAGCAGAACACCCCA	TCGG
sgRNA-TCTG	CAAGCTGACCCTGAAGTTCA	TCTG
sgRNA-ACTT	CGACCACATGAAGCAGCACG	ACTT
sgRNA-TGAA	CACCTACGGCAAGCTGACCC	TGAA
sgRNA-AGAC	CAAGGACGACGGCAACTACA	AGAC
sgRNA-GGCA	TGAAGTTCATCTGCACCACC	GGCA
sgRNA-TGCA	AGCTGACCCTGAAGTTCATC	TGCA
sgRNA-CGCC	CAGCACGACTTCTTCAAGTC	CGCC
sgRNA-TGCC	GGAGCTGTTACCCGGGTGG	TGCC
sgRNA-GGCG	CCATCCTGGTCGAGCTGGAC	GGCG
sgRNA-AGCT	GTTTATCTGCACCACCGCA	AGCT
sgRNA-CGCT	ACGGCGTGCAGTGCTTCAGC	CGCT
sgRNA-CGGA	GGCATCGCCCTCGCCCTCGC	CGGA
sgRNA-GGGA	TGGAGTTCGTGACCGCCGCC	GGGA
sgRNA-TGGC	CCGGCAAGCTGCCCGTGCC	TGGC
sgRNA-GGGT	GGGCGAGGAGCTGTTACCG	GGGT
sgRNA-TGTC	AAACGGCCACAAGTTCAGCG	TGTC
sgRNA-AGTT	CGGCGACGTAAACGGCCACA	AGTT
sgRNA-CGTT	GGACACGCTGAACTTGTGGC	CGTT
sgRNA-GGTT	GATGCCCTTCAGCTCGATGC	GGTT
sgRNA-GTAC	ATCCTGGGGCACAAGCTGGA	GTAC
sgRNA-ATAT	CTACAACAGCCACAACGTCT	ATAT
sgRNA-GTCC	AACGGCCACAAGTTCAGCGT	GTCC
sgRNA-GTCT	ACAACACTACAACAGCCACAAC	GTCT
sgRNA-TTGA	AGCCTTCGGGCATGGCGGAC	TTGA
sgRNA-GTGT	TAAACGGCCACAAGTTCAGC	GTGT
sgRNA-GTTG	ACCTCGGCGCGGTCTTGTA	GTTG
sgRNA-GTTT	GACACGCTGAACTTGTGGCC	GTTT

**Table S5 Oligonucleotide sequences for sgRNA construction targeting *EGFP* gene**

<b>Primer name</b>	<b>Primer sequence</b>
sgRNA-AACA-F	CACCGTCGACTTCAAGGAGGACGGC
sgRNA-AACA-R	AAACGCCGTCCTCCTTGAAGTCGAC
sgRNA-AAGA-F	CACCGTCAAGGACGACGGCAACTAC
sgRNA-AAGA-R	AAACGTAGTTGCCGTCGTCCTTGAC
sgRNA-ACAA-F	CACCGGGACGCGCAGCTAAACGGCC
sgRNA-ACAA-R	AAACGGCCGTTTACGTCGCCGTC
sgRNA-GACG-R	CACCGTGCCATCCTGGTCGAGCTG
sgRNA-GACG-R	AAACCAGCTCGACCAGGATGGGCAC
sgRNA-CGGA-F	CACCGGGCATCGCCCTCGCCCTCGC
sgRNA-CGGA-R	AAACGCGAGGGCGAGGGCGATGCC
sgRNA-GAGA-F	CACCGCCCTGAGCAAAGACCCCAAC
sgRNA-GAGA-R	AAACGTTGGGGTCTTTGCTCAGGGC
sgRNA-GGCA-F	CACCGTGAAGTTCATCTGCACCACC
sgRNA-GGCA-R	AAACGGTGGTGCAGATGAACTTCAC
sgRNA-GGGA-F	CACCGTGGAGTTCGTGACCGCCGCC
sgRNA-GGGA-R	AAACGGCGGGGTCACGAACTCCAC
sgRNA-ACCA-F	CACCGAAGGCTACGTCCAGGAGCGC
sgRNA-ACCA-R	AAACGCGCTCCTGGACGTAGCCTTC
sgRNA-ACCG-F	CACCGGCAAGGGCGAGGAGCTGTTC
sgRNA-ACCG-R	AAACGAACAGCTCCTCGCCCTTGCC
sgRNA-CCCA-F	CACCGAGCTGTTACCGGGGTGGTG
sgRNA-CCCA-R	AAACCACCACCCCGGTGAACAGCTC
sgRNA-GCCC-F	CACCGGAGCTGTTACCGGGGTGGT
sgRNA-GCCC-R	AAACACCACCCCGGTGAACAGCTCC
sgRNA-TCCA-F	CACCGCGCCATGCCCCAAGGCTACG
sgRNA-TCCA-R	AAACCGTAGCCTTCGGGCATGGCGC
sgRNA-TCCG-F	CACCGACGGCCACAAGTTCAGCGTG
sgRNA-TCCG-R	AAACCACGCTGAACTTGTGGCCGTC
sgRNA-TGAA-F	CACCGCACCTACGGCAAGCTGACCC
sgRNA-TGAA-R	AAACGGGTCAGCTTGCCGTAGGTGC
sgRNA-TGCA-F	CACCGAGCTGACCCTGAAGTTCATC
sgRNA-TGCA-R	AAACGATGAACTTCAGGGTCAGCTC
sgRNA-TTGA-F	CACCGAGCCTTCGGGCATGGCGGAC
sgRNA-TTGA-R	AAACGTCCGCCATGCCCCAAGGCTC
sgRNA-AAAC-F	CACCGGTCGAGCTGGACGGCGACGT
sgRNA-AAAC-R	AAACACGTCGCCGTCCAGCTCGACC
sgRNA-AGAC-F	CACCGCAAGGACGACGGCAACTACA
sgRNA-AGAC-R	AAACTGTAGTTGCCGTCGTCCTTGC
sgRNA-CATC-F	CACCGCTGTTACCGGGGTGGTGCC
sgRNA-CATC-R	AAACGACAAGTGCCCCACCACGGC
sgRNA-CGCC-F	CACCGCAGCACGACTTCTCAAGTC
sgRNA-CGCC-R	AAACGACTTGAAGAAGTCGTGCTGC
sgRNA-GCAC-F	CACCGGCTGACCCTGAAGTTCATCT
sgRNA-GCAC-R	AAACAGATGAACTTCAGGGTCAGCC
sgRNA-GTAC-F	CACCGATCCTGGGGCACAAGCTGGA
sgRNA-GTAC-R	AAACTCCAGCTTGTGCCCCAGGATC
sgRNA-GTCC-F	CACCGAACGGCCACAAGTTCAGCGT
sgRNA-GTCC-R	AAACACGCTGAACTTGTGGCCGTTC

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sgRNA-TGCC-F	CACCGGGAGCTGTTACCGGGGTGG
sgRNA-TGCC-R	AAACCCACCCCGGTGAACAGCTCCC
sgRNA-TGGC-F	CACCGCCGGCAAGCTGCCCGTGCCC
sgRNA-TGGC-R	AAACGGGCACGGCAGCTTGCCGGC
sgRNA-TGTC-F	CACCGAAACGGCCACAAGTTCAGCG
sgRNA-TGTC-R	AAACCGCTGAACTTGTGGCCGTTTC
sgRNA-AAAG-F	CACCGGCACCCAGTCCGCCCTGAGC
sgRNA-AAAG-R	AAACGCTCAGGGCGGACTGGGTGCC
sgRNA-ACGG-F	CACCGCGAGCTGGACGGCGACGTAA
sgRNA-ACGG-R	AAACTTACGTCGCCGTCCAGCTCGC
sgRNA-GATG-F	CACCGTGTCCGGCGAGGGCGAGGGC
sgRNA-GATG-R	AAACGCCCTCGCCCTCGCCGGACAC
sgRNA-GGCG-F	CACCGCCATCCTGGTCGAGCTGGAC
sgRNA-GGCG-R	AAACGTCCAGCTCGACCAGGATGGC
sgRNA-GTTG-F	CACCGACCTCGGGCGGGTCTTGTA
sgRNA-GTTG-R	AAACTACAAGACCCGCGCCGAGGTC
sgRNA-TAGG-F	CACCGACTTCAGGGTCAGCTTGCCG
sgRNA-TAGG-R	AAACCGGCAAGCTGACCCTGAAGTC
sgRNA-TCGG-F	CACCGCTACCAGCAGAACACCCCCA
sgRNA-TCGG-R	AAACTGGGGGTGTTCTGCTGGTAGC
sgRNA-TCTG-F	CACCGCAAGCTGACCCTGAAGTTCA
sgRNA-TCTG-R	AAACTGAACTTCAGGGTCAGCTTGC
sgRNA-ACTT-F	CACCGCGACCACATGAAGCAGCACG
sgRNA-ACTT-R	AAACCGTGCTGCTTCATGTGGTCGC
sgRNA-AGCT-F	CACCGGTTTCATCTGCACCACCGGCA
sgRNA-AGCT-R	AAACTGCCGGTGGTGCAGATGAACC
sgRNA-AGTT-F	CACCGCGGCGACGTAAACGGCCACA
sgRNA-AGTT-R	AAACTGTGGCCGTTTACGTCGCCGC
sgRNA-ATAT-F	CACCGCTACAACAGCCACAACGTCT
sgRNA-ATAT-R	AAACAGACGTTGTGGCTGTTGTAGC
sgRNA-CGCT-F	CACCGACGGCGTGCAGTGCTTCAGC
sgRNA-CGCT-R	AAACGCTGAAGCACTGCACGCCGTC
sgRNA-CGTT-F	CACCGGGACACGCTGAACTTGTGGC
sgRNA-CGTT-R	AAACGCCACAAGTTCAGCGTGTC
sgRNA-GGGT-F	CACCGGGGCGAGGAGCTGTTACCG
sgRNA-GGGT-R	AAACCGGTGAACAGCTCCTCGCCCC
sgRNA-GGTT-F	CACCGGATGCCCTTCAGCTCGATGC
sgRNA-GGTT-R	AAACGCATCGAGCTGAAGGGCATCC
sgRNA-GTCT-F	CACCGACAACACTACAACAGCCACAAC
sgRNA-GTCT-R	AAACGTTGTGGCTGTTGTAGTTGTC
sgRNA-GTGT-F	CACCGTAAACGGCCACAAGTTCAGC
sgRNA-GTGT-R	AAACGCTGAACTTGTGGCCGTTTAC
sgRNA-GTTT-F	CACCGGACACGCTGAACTTGTGGCC
sgRNA-GTTT-R	AAACGGCCACAAGTTCAGCGTGTC

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**Table S6 PCR Primers for Sanger sequencing and NGS**

Name	Primer
Sanger F	AATTCGTACTAGTCCGCGGGTACGAGG
Sanger R	GGGACTTCCTTTGTCCCAAATC
NGS F1	CCCTACACGACGCTCTCCGATCTAATTCGTACTAGTCCGCGGGTACGAGG
NGS R1	GACTGGAGTTCAGACGTGTGCTCTCCGATCTGGGCTCGACACTAGTAGATCTCCTA
NGS F2	AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTCCGATCTA
NGS R2	CAAGCAGAAGACGGCATAACGAGATCGCTGATCGTGACTGGAGTTCAGACGTGTGCTCTT

**Table S7 The raw read counts of NGS for each PAM via PAM-DOSE**

	PAM	3	A	A	A	A	C	C	C	C	G	G	G	G	T	T	T	T
	1	2	4	A	C	G	A	C	G	T	A	C	G	T	A	C	G	T
SpRY Zeo	NA		4	19	12	9	13	5	12	4	17	22	6	12	8	8	20	3
	NC		6	7	4	7	10	4	4	9	4	11	10	14	1	18	11	6
	NG		14	6	14	12	7	4	8	7	13	15	17	15	13	12	14	31
	NT		9	13	7	10	10	6	3	4	12	6	16	19	8	5	16	9
SpRY Neo	NA		53	24	23	19	23	20	19	14	46	25	46	16	40	39	30	31
	NC		26	9	29	3	11	11	14	8	16	25	18	19	8	17	19	22
	NG		34	34	40	32	23	14	116	20	55	28	31	43	30	35	33	89
	NT		39	50	36	20	17	28	20	16	34	20	25	25	15	20	36	34
SpCas9-NG Zeo	NA		23	48	28	28	35	9	47	7	54	71	22	79	44	60	38	33
	NC		3	17	3	12	5	5	5	15	7	12	21	24	5	7	31	7
	NG		78	48	107	65	27	39	57	42	64	64	76	86	67	80	85	100
	NT		28	35	29	14	21	13	10	13	52	35	75	81	18	15	49	28
SpCas9-NG Neo	NA		16	3	8	4	8	3	7	2	19	12	23	4	5	2	17	5
	NC		3	0	5	2	2	0	1	1	4	4	5	1	0	2	0	3
	NG		11	20	19	13	10	9	50	9	27	18	23	28	16	16	29	30
	NT		7	10	15	1	4	2	12	3	6	3	12	10	7	6	11	5
SpCas9-WT Zeo	NA		4	25	16	13	8	1	9	1	121	171	50	142	10	17	17	8
	NC		1	14	12	7	7	13	0	27	17	15	84	35	3	7	18	6
	NG		147	100	330	62	19	41	53	54	344	145	250	237	40	187	77	81
	NT		4	59	5	14	6	9	2	5	52	69		125	4	13	17	11
SpCas9-WT Neo	NA		13	10	13	5	9	3	4	9	80	98	70	45	16	8	1	15
	NC		4	1	7	0	4	0	8	0	8	36	37	23	16	11	3	9
	NG		58	74	72	38	27	40	93	12	142	128	119	161	23	18	28	44
	NT		22	13	6	11	5	3	4	3	30	38	53	41	40	2	12	14