

# Supplemental material

Table S3.1. List of bacterial strains used in this study.

Strains	Characteristics	Source
<i>E. coli</i> DH5 $\alpha$	F <sup>-</sup> <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20</i> $\phi$ 80d <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> ) U169 <i>hsdR17</i> ( <i>r<sub>K</sub>-m<sub>K</sub>+</i> ), f.	NEB
<i>E. coli</i> PAM-SCANR	<i>lacIq</i> <i>rrmB</i> <sub>T14</sub> $\Delta$ <i>lacZ</i> <sub>WJ16</sub> <i>hsdR</i> <sub>514</sub> $\Delta$ <i>araBAD</i> <sub>AH33</sub> $\Delta$ <i>rhaBAD</i> <sub>L078</sub> <i>rph-1</i> $\Delta$ <i>cas3</i> -CRISPR1 $\Delta$ P <sub>lacI</sub> - <i>lacZ</i> .	32
<i>E. coli</i> PAM-SCANR x p <i>Sp</i> ydCas9-PAmCherry2.1_Target, pTarget	PAM-SCANR strain harbouring plasmids p <i>Sp</i> ydCas9-PAmCherry2.1_Target and pTarget.	This study
<i>E. coli</i> PAM-SCANR x p <i>Sp</i> ydCas9-PAmCherry2.1_Scrambled, pTarget	PAM-SCANR strain harbouring plasmids p <i>Sp</i> ydCas9-PAmCherry2.1_Scrambled and pTarget.	This study
<i>E. coli</i> PAM-SCANR x p <i>Lbd</i> Cas12a-PAmCherry2.1_Target, pTarget	PAM-SCANR strain harbouring plasmids p <i>Lbd</i> Cas12a-PAmCherry2.1_Target and pTarget.	This study
<i>E. coli</i> PAM-SCANR x p <i>Lbd</i> Cas12a-PAmCherry2.1_Scrambled, pTarget	PAM-SCANR strain harbouring plasmids p <i>Lbd</i> Cas12a-PAmCherry2.1_Scrambled and pTarget.	This study
<i>E. coli</i> PAM-SCANR x p <i>Sp</i> RYdCas9-PAmCherry2.1_Target, pTarget	PAM-SCANR strain harbouring plasmids p <i>Sp</i> RYdCas9-PAmCherry2.1_Target and pTarget.	This study
<i>E. coli</i> PAM-SCANR x p <i>Sp</i> RYdCas9-PAmCherry2.1_Scrambled, pTarget	PAM-SCANR strain harbouring plasmids p <i>Sp</i> RYdCas9-PAmCherry2.1_Scrambled and pTarget.	This study
<i>E. coli</i> PAM-SCANR x p <i>impLbd</i> Cas12a-PAmCherry2.1_Target, pTarget	PAM-SCANR strain harbouring plasmids p <i>impLbd</i> Cas12a-PAmCherry2.1_Target and pTarget.	This study
<i>E. coli</i> PAM-SCANR x p <i>impLbd</i> Cas12a-PAmCherry2.1_Scrambled, pTarget	PAM-SCANR strain harbouring plasmids p <i>impLbd</i> Cas12a-PAmCherry2.1_Scrambled and pTarget.	This study

**Table S3.2.** List of plasmids used in this study.

Plasmid	Relevant characteristics	Source
pBeloBAC11	<i>repE, ori2, sopABC, cat.</i>	NEB
pSC101-K102E	<i>neoR, rep101(K102E).</i>	<sup>37</sup>
pd <i>Spy</i> Cas9- PAmCherry2.1_Target	pBeloBAC11, P <sub>LtetO-1</sub> -BCD- <i>Spydcas9</i> -PAmCherry2(M10L), P <sub>J23119</sub> - <i>sgRNA(targeting)</i> .	This study
pd <i>Spy</i> Cas9- PAmCherry2.1_Scrambled	pBeloBAC11, P <sub>LtetO-1</sub> -BCD- <i>Spydcas9</i> -PAmCherry2(M10L), P <sub>J23119</sub> - <i>sgRNA(scrambled)</i> .	This study
pd <i>Lb</i> Cas12a- PAmCherry2.1_Target	pBeloBAC11, P <sub>LtetO-1</sub> -BCD- <i>Lbdcas12a</i> -PAmCherry2(M10L), P <sub>J23119</sub> - <i>sgRNA(targeting)</i> .	This study
pd <i>Lb</i> Cas12a- PAmCherry2.1_Scrambled	pBeloBAC11, P <sub>LtetO-1</sub> -BCD- <i>Lbdcas12a</i> --PAmCherry2(M10L), P <sub>J23119</sub> - <i>sgRNA(scrambled)</i> .	This study
pSpRYdCas9- PAmCherry2_Target	pBeloBAC11, P <sub>LtetO-1</sub> -BCD- <i>Spydcas9</i> (A61R, L1111R, D1135L, S1136W, G1218K, E1219Q, A1322R, R1333P, R1335Q, T1337R)- PAmCherry2(M10L), P <sub>J23119</sub> - <i>sgRNA(targeting)</i> .	This study
pSpRYdCas9- PAmCherry2_Scrambled	pBeloBAC11, P <sub>LtetO-1</sub> -BCD- <i>Spydcas9</i> (A61R, L1111R, D1135L, S1136W, G1218K, E1219Q, A1322R, R1333P, R1335Q, T1337R)- PAmCherry2(M10L), P <sub>J23119</sub> - <i>sgRNA(scrambled)</i> .	This study
pimp <i>Lb</i> Cas12a- PAmCherry2_Target	pBeloBAC11, P <sub>LtetO-1</sub> -BCD- <i>Lbdcas12a</i> (D156R, G532R, K538V, Y542R, K595R)-PAmCherry2(M10L), P <sub>J23119</sub> - <i>sgRNA(targeting)</i> .	This study
pimp <i>Lb</i> Cas12a- PAmCherry2_Scrambled	pBeloBAC11, P <sub>LtetO-1</sub> -BCD- <i>Lbdcas12a</i> (D156R, G532R, K538V, Y542R, K595R)-PAmCherry2(M10L), P <sub>J23119</sub> - <i>sgRNA(scrambled)</i> .	This study
pTarget	pSC101-K102E, 5 protospacers, 5 30 bp-long distancing sequences.	This study

**Table S3.3.** List of oligonucleotides and DNA fragments used to introduce mutations. Nucleotides in bold introduced the listed mutations.

Identifier	Sequence (5'-3')	Used for
BG28281	<b>AAG</b> gttatcctcctcgcccttgctc	Introducing M10L mutation in PAmCherry2.1.
BG28278	agggcgaggaggataac <b>CTT</b> gccatcatcaaggagttcatgcg	Introducing M10L mutation in PAmCherry2.1.
BG30037	cggaggcttctccaaggaaagtatc <b>CGC</b> ccgaaaaggaaacagcgacaagctgatcgacgcaaaaagattgggacccaagaaatacggcggtatc <b>CTGTGG</b> cctacagtcgcttacagtgtactggtgtggccaaagtggagaaaggaagtctaaaaaactcaaaagcgtaagggaactgtggtgcacacaatcatggagcgatcaagcttcgaaaaaaaccccatcgactttctcgaggcgaaaggatataaagagggtcaaaaagacctcatcattaagcttccaagtactctctttgagcttgaaaacggccggaaacgaatgctcgctagtgcg <b>AAACAG</b> ctgcagaaggtaacgagctggcactgccctctaaatacgttaatttctgtatctggccagccactatgaaaagctcaaagggtctcccgaaagataatgagcagaagcagctgttcgtggaacaacacaacactaccttgatgagatcatcgagcaaa taagcgaattctccaaagagtgatcctcgccgacgctaacctcgataagggtgctttctgcttacaataagcacagggataagcccatcagggagcaggcagaaaacattatccacttgttactctgacc <b>C</b> <b>GCT</b> gggcgcgcct <b>CGC</b> gccttaagtacttcgacaccaccatagac <b>CCG</b> aag <b>CAA</b> tac <b>CGT</b> tctacaaggaggtcctggacg	Introducing L1111R, D1135L, S1136W, G1218K, E1219Q, N1317R, A1322R, R1333P, R1335Q and T1337R mutations of <i>SpRYdCas9</i> .
BG30041	gt <b>GCG</b> ttcggccgtctccccg	Introducing A61R mutation of <i>SpRYdCas9</i> .
BG30042	ggggagacggccgaa <b>CGC</b> acgcggctcaaaagaacag	Introducing A61R mutation of <i>SpRYdCas9</i> .
BG29090	tatatatgatgcaattcgtaattacgtaacccaaaagccgtacagcaagataagttcaactgta ttccagaacccgcagtttatgCGCggtgggacaaagac <b>GTT</b> gagacagac <b>CGC</b> cgcgcca ctattctgcgttacggcagcaagtactatctcgccatcatggacaaaaatatgcaaagtgtctgc agaaaatcgataaagacgacgtgaacggaaattacgaaaagattaattataagctgctgccag ggcccaacaagatgttaccgCGCgtatTTTTTccaaaaa	Introducing G532R, K538V, Y542R and K595R mutation of imp <i>LbdCas12a</i> .
BG29087	aacatattttccctgtt <b>GCG</b> gaaaaagcccgtgaaggc	Introducing D156R mutation of imp <i>LbdCas12a</i> .
BG29088	<b>CGC</b> aacaggggaaaatatgttttcagagg	Introducing D156R mutation of imp <i>LbdCas12a</i> .

## Source data

Source Data Table 3.1. Source data of non-target interaction times ( $t_{NTI}$ ), used for the bar plot of Figure 3.2C.

Nuclease	$t_{NTI}$ (ms)	Standard deviation
<i>SpydCas9</i>	16.25	0.659369446
<i>SpRYdCas9</i>	20.70376	1.195545762
<i>LbdCas12a</i>	10.66948	0.206954271
<i>impLbdCas12a</i>	12.17949	0.35222798

Source Data Table 3.2. Source data of PAM-investigating fractions, used for the bar plot of Figure 3.2D. The other fractions, totalling to 1, are also reported.

Nuclease	PAM-investigating		1D		3D	
	Prob (%)	St. dev.	Prob (%)	St. dev.	Prob (%)	St. dev.
<i>SpydCas9</i>	0.330709	0.03501	0.275591	0.030371	0.393701	0.036079
<i>SpRYdCas9</i>	0.338498	0.031611	0.300177	0.02838	0.361325	0.034974
<i>LbdCas12a</i>	0.351324	0.016209	0.198574	0.009969	0.450102	0.018814
<i>impLbdCas12a</i>	0.469588	0.030267	0.141941	0.010746	0.38847	0.02197

Source Data Table 3.3. Source data of average diffusion coefficients ( $\mu\text{m}^2/\text{s}$ ), used for the scatter plots of Figure 3.3B.

Track length	<i>SpydCas9</i>		<i>SpRYdCas9</i>		<i>LbdCas12a</i>		<i>impLbdCas12a</i>	
	Avg. D*	St. dev.	Avg. D*	St. dev.	Avg. D*	St. dev.	Avg. D*	St. dev.
3	0.57421921	0.006045	0.542011	0.00624	0.784748	0.008135	0.69277	0.007546
4	0.48265905	0.005354	0.4458916	0.005573	0.737751	0.007861	0.6554	0.00823
5	0.42657774	0.004586	0.3827837	0.004809	0.718802	0.007629	0.628498	0.008021
6	0.38228715	0.004047	0.3557168	0.004466	0.736711	0.007028	0.62063	0.007804
7	0.36854012	0.003393	0.3315628	0.004071	0.72226	0.005696	0.600112	0.007397
>8	0.39834597	0.002726	0.333857	0.005361	0.734685	0.004565	0.630357	0.00624

Source Data Table 3.4. Source data of average probability of 1 unit of each Cas nuclease to find 1 target inside the *E. coli* genome, used for scatter plot of Figure 3.4A (continuous lines).

Time (min)	<i>SpydCas9</i>		<i>SpRYdCas9</i>		<i>LbdCas12a</i>		<i>impLbdCas12a</i>	
	Avg. prob.	St. dev.	Avg. prob.	St. dev.	Avg. prob.	St. dev.	Avg. prob.	St. dev.
1	0.33	0.1006	0.2	0.0943	2.065	0.1617	0.44	0.163
5	1.645	0.1536	0.935	0.231	10.68	0.4934	2.195	0.3647
10	3.15	0.3291	1.7	0.2896	19.575	0.5181	4.075	0.5361
30	9.76	0.8171	5.54	0.395	49.93	1.3907	11.94	0.6306
60	18.525	0.8545	10.39	0.6501	74.1	0.5788	21.62	0.68
120	33.015	1.1188	19.71	0.914	93.315	0.7835	39.115	0.886
240	55.15	1.0904	35.315	1.0483	100	0	63.755	0.7286
480	79.99	0.8736	59.04	0.7923	100	0	86.57	0.7436

Source Data Table 3.5. Source data of average probability of 10 units of each Cas nuclease to find 1 target inside the *E. coli* genome, used for scatter plot of Figure 3.4A (dashed lines).

Time (min)	<i>SpydCas9</i>		<i>SpRYdCas9</i>		<i>LbdCas12a</i>		<i>impLbdCas12a</i>	
	Avg. prob.	St. dev.	Avg. prob.	St. dev.	Avg. prob.	St. dev.	Avg. prob.	St. dev.
1	3.41	0.3044	1.69	0.2923	20.735	0.6876	4.155	0.376
5	15.45	0.7785	8.665	0.5391	68.29	1.0096	18.795	1.0114
10	28.675	1.1304	16.825	1.0191	89.49	0.6096	33.66	1.0456
30	63.54	0.667	42.65	1.2383	99.89	0.0394	71.685	0.6684
60	86.995	0.8821	67.02	0.7173	100	0	91.87	0.4015
120	98.175	0.3129	88.74	0.9101	100	0	99.315	0.2274
240	99.965	0.0412	98.95	0.2646	100	0	100	0
480	100	0	99.99	0.0211	100	0	100	0

Source Data Table 3.6. Source data of  $t_{50\%}$  expressed in minutes at varying GC content percentages, used for the scatter plot in figure 3.4B (top).

GC content (%)	$t_{50\%}$ (min)			
	<i>SpydCas9</i>	<i>SpRYdCas9</i>	<i>LbdCas12a</i>	<i>impLbdCas12a</i>
30.0	98	371	66	192
40.0	152	369	46	185
50.5	205	370	30	164
60.0	249	371	17	155
70.0	270	373	8.5	153

Source Data Table 3.7. Source data of the number of PAM sequences at varying GC content percentage, used for the scatter plot in Figure 3.4B (bottom).

GC content (%)	Number of PAM sequences			
	<i>SpydCas9</i>	<i>SpRYdCas9</i>	<i>LbdCas12a</i>	<i>impLbdCas12a</i>
30.0	481,900.2	21,417,790	596,887	3,291,935
40.0	856,711.5	21,417,790	404,796.2	2,767,170
50.5	1,365,518	21,417,790	244,347	2,324,340
60.0	1,927,601	21,417,790	137,073.8	2038970
70.0	2,586,332	21,417,790	64,375.98	1,887,440

Source Data Table 3.8. Source data of target-bound fractions at different number of tracks per cell, used for the scatter plot of Figure 3.5C.

Apparent copy number	<i>SpydCas9</i>		<i>SpRYdCas9</i>		<i>LbdCas12a</i>		<i>impLbdCas12a</i>	
	Target-bound	St. dev.	Target-bound	St. dev.	Target-bound	St. dev.	Target-bound	St. dev.
7-50	15	4	5	2	35	4	29	5
51-100	13	4	3	1	30	2	26	3
101-150	10	2	3.6	1	28	4	20	3
151-200	3	2	3.2	2.5	25	3	18	3