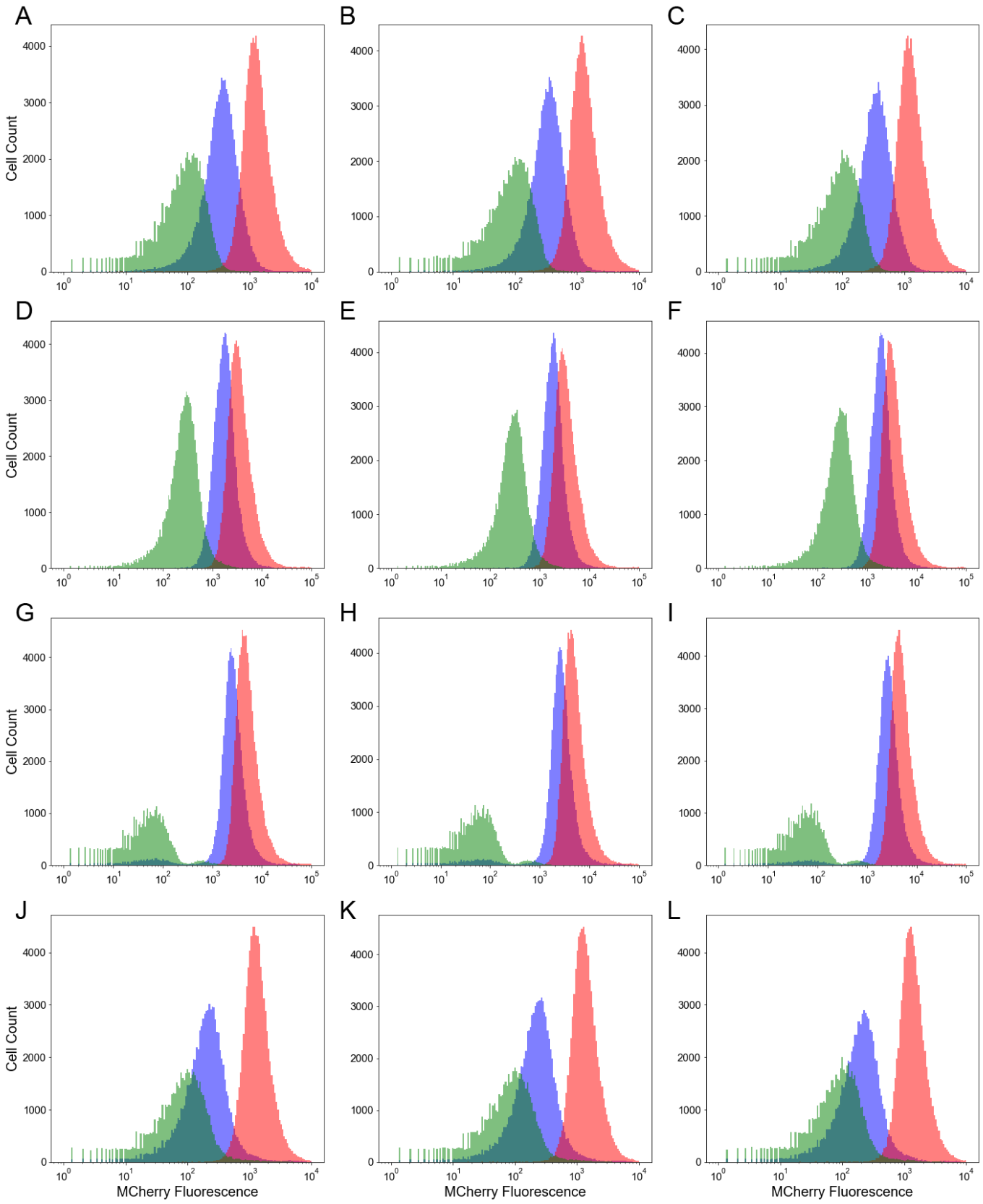

Figure S1 Fluorescence intensity distributions of EXP (blue), NC (red) and PC groups (green). Three independent biological replicates were conducted for **(A-C)** R1, **(D-F)** R2, **(G-I)** R3, and **(J-L)** R4.

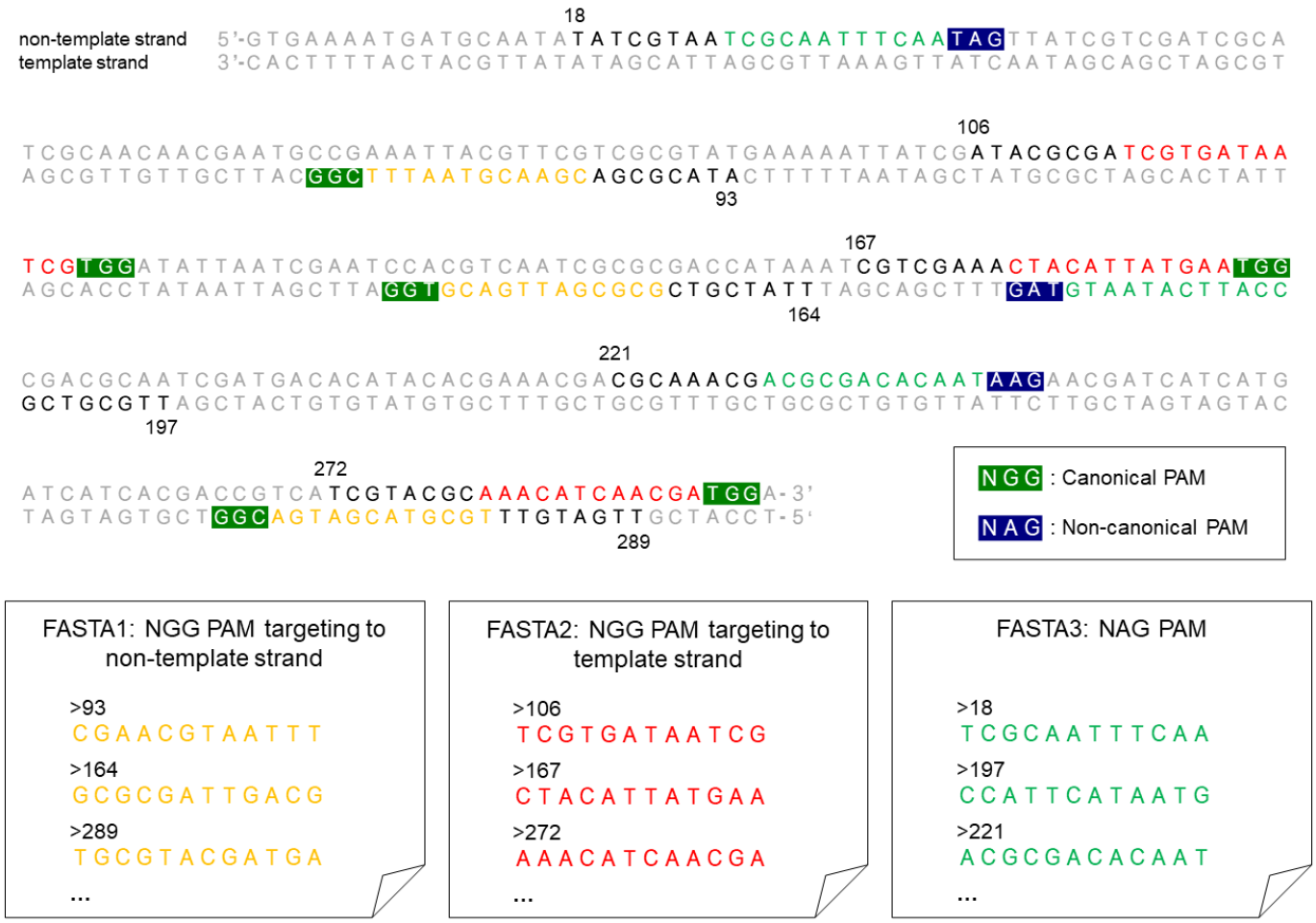

Figure S2. A toy example to illustrate the identification and classification of candidate sgRNAs. sgRNAs are searched by regular expressions and classified into three groups based on the PAM and targeted strands. The start position and PAM-proximal 12-bp of each sgRNA are saved in three separate FASTA files.

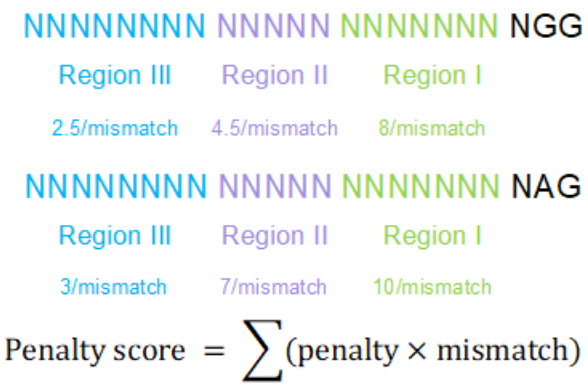

Figure S3. Schematic diagram of the calculation of penalty scores

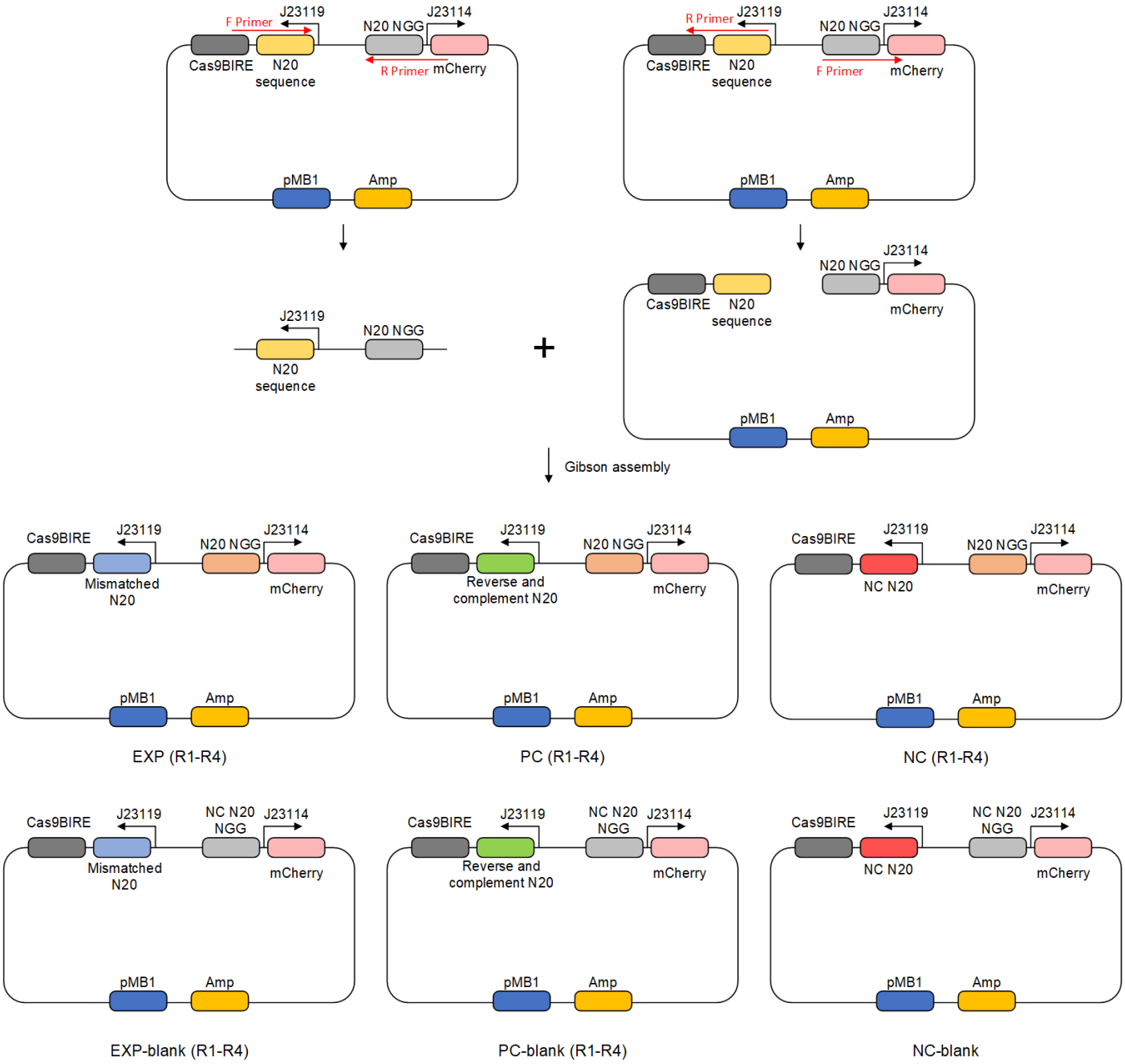

Figure S4. Workflow of the plasmid construction. The sgRNA and N20 sequences were introduced via Gibson assembly.

Table S1: Clusters of all multi-copy coding genes of *E. coli* MG1655 identified by GLiDe

Gene name	Copy number
insL	3
insB	7
insA	7
insI	3
insH	11
insE	5
insF	5
insC	6
insD	6
rzpD, rzoR	2
ylcI, ynfO	2
nohD, nohA	2
rhsC, rhsB, rhsA	3
ybfD, ydcC, yhhI	3
ldrA, ldrB, ldrC	3
tpr, ychS	2
tfaR, tfaQ	2
pinR, pinQ	2
ynaE, ydfK	2
ynaM, ynfT	2
hokB, mokB	2
gadB, gadA	2
yqgG, yqgC	2
tufA, tufB	2
yriA, yriB	2
cyaY, yzcX	2

Table S2: PCR Primers used in this study

Name	Sequence
R1_F	CCGTGGAACCATTCGAATTAACTAGTATTATACCTAGGACTGAGC
R1_R	CTAGTTAATTCGAATGGTTCCACGGGTTTTAGAGCTAGAAATAGCAAGTT
N20_R1_F	TCCTTCCTCTGGTTCCACGGCGGTTTATGGCTAGCTCAGTCCTAG
N20_R1_R	AACCGCCGTGGAACCAGAGGAAGGAGTTGGATGTACTGCGGCTCCGTCTA
R1_PC_F	CCGTGGAACCAGAGGAAGGAACTAGTATTATACCTAGGACTGAGC
R1_PC_R	CTAGTTCCTTCCTCTGGTTCCACGGGTTTTAGAGCTAGAAATAGCAAGTT
R2_F	CGGTGTGGTGGGCATCGTGCACTAGTATTATACCTAGGACTGAGC
R2_R	CTAGTGCACGATGCCCACCACACCGGTTTTAGAGCTAGAAATAGCAAGTT
N20_R2_F	CGGATGGGCCCACCACACCGCGGTTTATGGCTAGCTCAGTCCTAG
N20_R2_R	AACCGCGGTGTGGTGGGCCCATCCGGTTGGATGTACTGCGGCTCCGTCTA
R2_PC_F	CGGTGTGGTGGGCCCATCCGACTAGTATTATACCTAGGACTGAGC
R2_PC_R	CTAGTCGGATGGGCCCACCACACCGGTTTTAGAGCTAGAAATAGCAAGTT
R3_F	CGCCGAAGCCATGCGCAGAAACTAGTATTATACCTAGGACTGAGC
R3_R	CTAGTTTCTGCGCATGGCTTCGGCGGTTTTAGAGCTAGAAATAGCAAGTT
N20_R3_F	GCGCGGCGATGGCTTCGGCGCGGTTTATGGCTAGCTCAGTCCTAG
N20_R3_R	AACCGCGCCGAAGCCATCGCCGCGCGTTGGATGTACTGCGGCTCCGTCTA
R3_PC_F	CGCCGAAGCCATCGCCGCGCACTAGTATTATACCTAGGACTGAGC
R3_PC_R	CTAGTGCGCGGCGATGGCTTCGGCGGTTTTAGAGCTAGAAATAGCAAGTT
R4_F	ATGTTGAGCGATCTGAGCTCACTAGTATTATACCTAGGACTGAGC
R4_R	CTAGTGAGCTCAGATCGCTCAACATGTTTTAGAGCTAGAAATAGCAAGTT
N20_R4_F	TCTCTTGAATCGCTCAACATCGGTTTATGGCTAGCTCAGTCCTAG
N20_R4_R	AACCGATGTTGAGCGATTCAAGAGAGTTGGATGTACTGCGGCTCCGTCTA
R4_PC_F	ATGTTGAGCGATTCAAGAGAACTAGTATTATACCTAGGACTGAGC
R4_PC_R	CTAGTTCTCTTGAATCGCTCAACATGTTTTAGAGCTAGAAATAGCAAGTT
NC_F	ATGTCCGGGCTCCGATAATAACTAGTATTATACCTAGGACTGAGC
NC_R	CTAGTTATTATCGGAGCCCGGACATGTTTTAGAGCTAGAAATAGCAAGTT
N20_NC_F	CCAACCATGTATTATACACGAAGTTCGGTTTATGGCTAGCTCAGTCCTAG
N20_NC_R	AACTTCGTGTATAATACATGGTTGGATGTACTGCGGCTCCGTCTA

Table S3: Strains and plasmids used in this study

Strains/plasmids	Sequence	Sources
Strains		
E. coli K12 MG1655	Wild type	ATCC 700936
E. coli MCm	Carries chloromycetin expression cassette integrated into *smf* locus of *E. coli* K12 MG1655	Ref (1)
Plasmids		
pdCas9-J23111	Expresses dCas9, p15A, Km^R	Ref (2)
pN20test-J23114-MCherry-R1-EXP pN20test-J23114-MCherry-R1-PC pN20test-J23114-MCherry-R1-NC pN20test-J23114-MCherry-R2-EXP pN20test-J23114-MCherry-R2-PC pN20test-J23114-MCherry-R2-NC pN20test-J23114-MCherry-R3-EXP pN20test-J23114-MCherry-R3-PC pN20test-J23114-MCherry-R3-NC pN20test-J23114-MCherry-R4-EXP pN20test-J23114-MCherry-R4-PC pN20test-J23114-MCherry-R4-NC	Express MCherry with different exp/NC/PC sgRNA and N20 sequence, pMB1, Amp^R	This study
pN20test-J23114-MCherry-R1-EXP-blank pN20test-J23114-MCherry-R1-PC-blank pN20test-J23114-MCherry-R2-EXP-blank pN20test-J23114-MCherry-R2-PC-blank pN20test-J23114-MCherry-R3-EXP-blank pN20test-J23114-MCherry-R3-PC-blank pN20test-J23114-MCherry-R4-EXP-blank pN20test-J23114-MCherry-R4-PC-blank pN20test-J23114-MCherry-NC-blank	Express MCherry with negative control N20 sequence and different exp/NC/PC sgRNA, pMB1, Amp^R	This study

References

1. Wang,T., Guan,C., Guo,J., Liu,B., Wu,Y., Xie,Z., Zhang,C. and Xing,X.-H. (2018) Pooled CRISPR interference screening enables genome-scale functional genomics study in bacteria with superior performance. *Nat Commun*, **9**, 2475.
2. Feng,H., Guo,J., Wang,T., Zhang,C. and Xing,X. (2021) Guide-target mismatch effects on dCas9–sgRNA binding activity in living bacterial cells. *Nucleic Acids Res*, **49**, 1263–1277.