

Supplementary Materials:

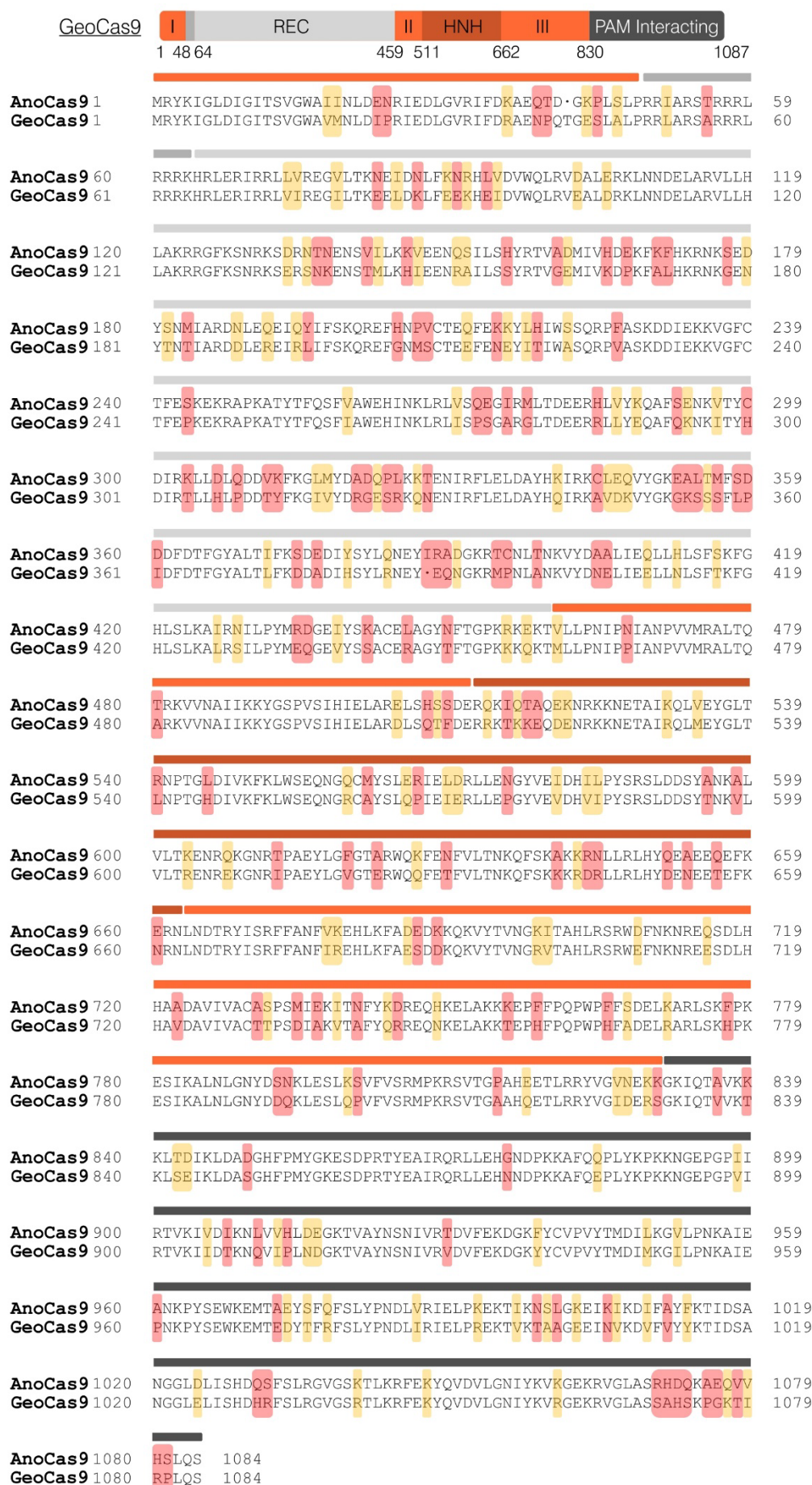


Figure S1. Alignment of AnoCas9 and GeoCas9 protein sequences. Mismatches are highlighted in red and conservative substitutions are highlighted in yellow. GeoCas9 domain structure is presented with domains marked with varying colors.

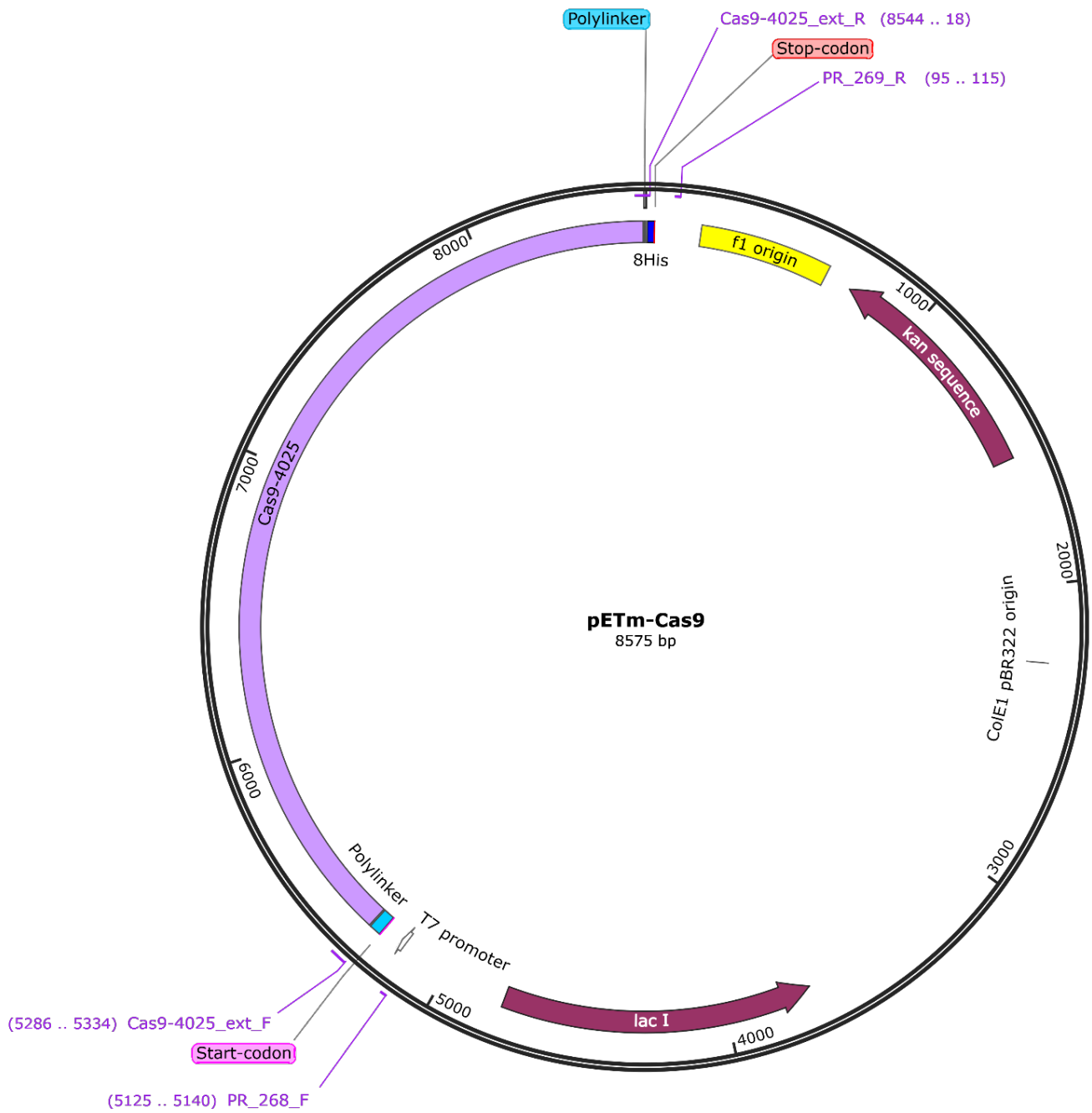


Figure S2. pETm-AnoCas9 map.

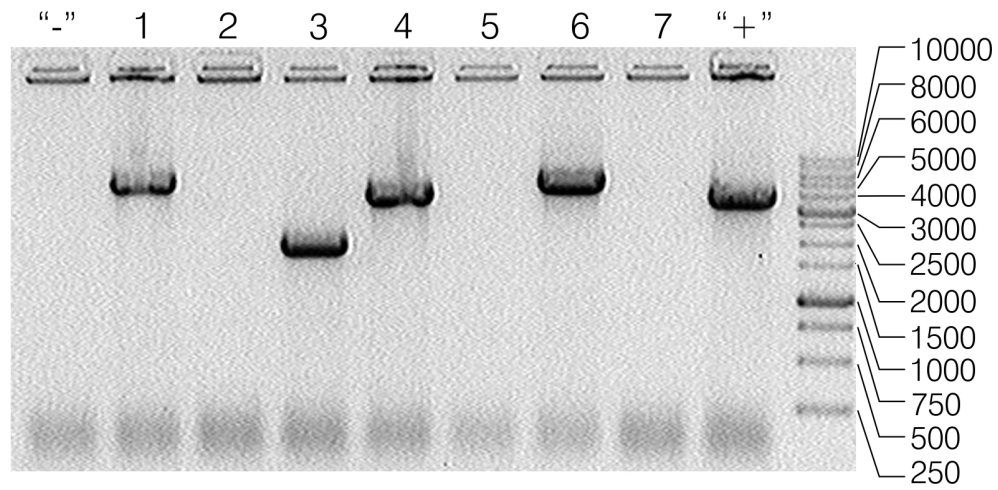


Figure S3. Analysis of PCR products after electrophoresis in 1% agarose gel. The expected product length was 3,580 bp in the case of the *anoCas9* insertion. Lane "-" – negative control, lane "+" – positive control (ligation mixture was used as a template). Lanes 1-7 refer to the colonies 1-7, respectively.

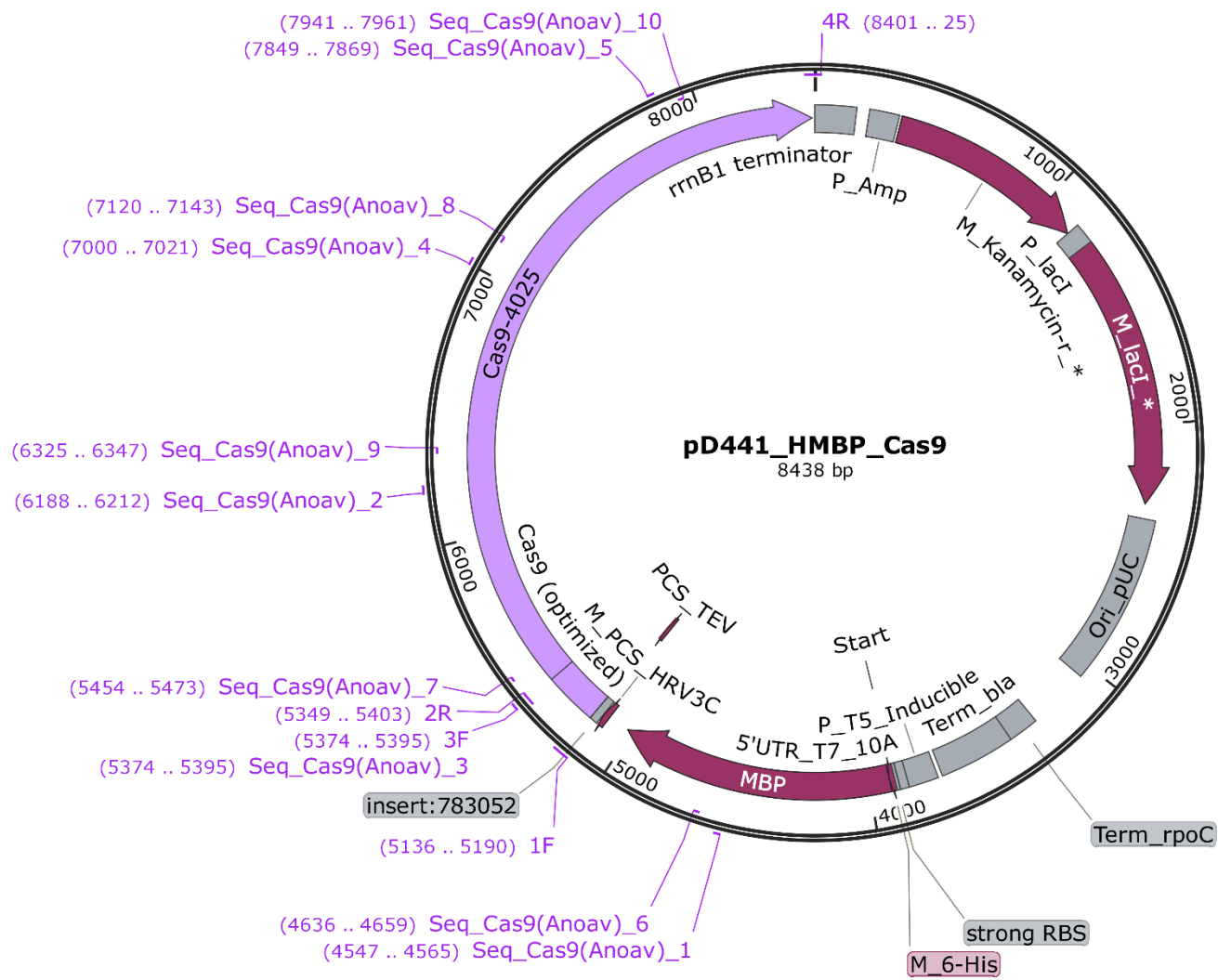


Figure S4. pD441-HMBP-AnoCas9 map.

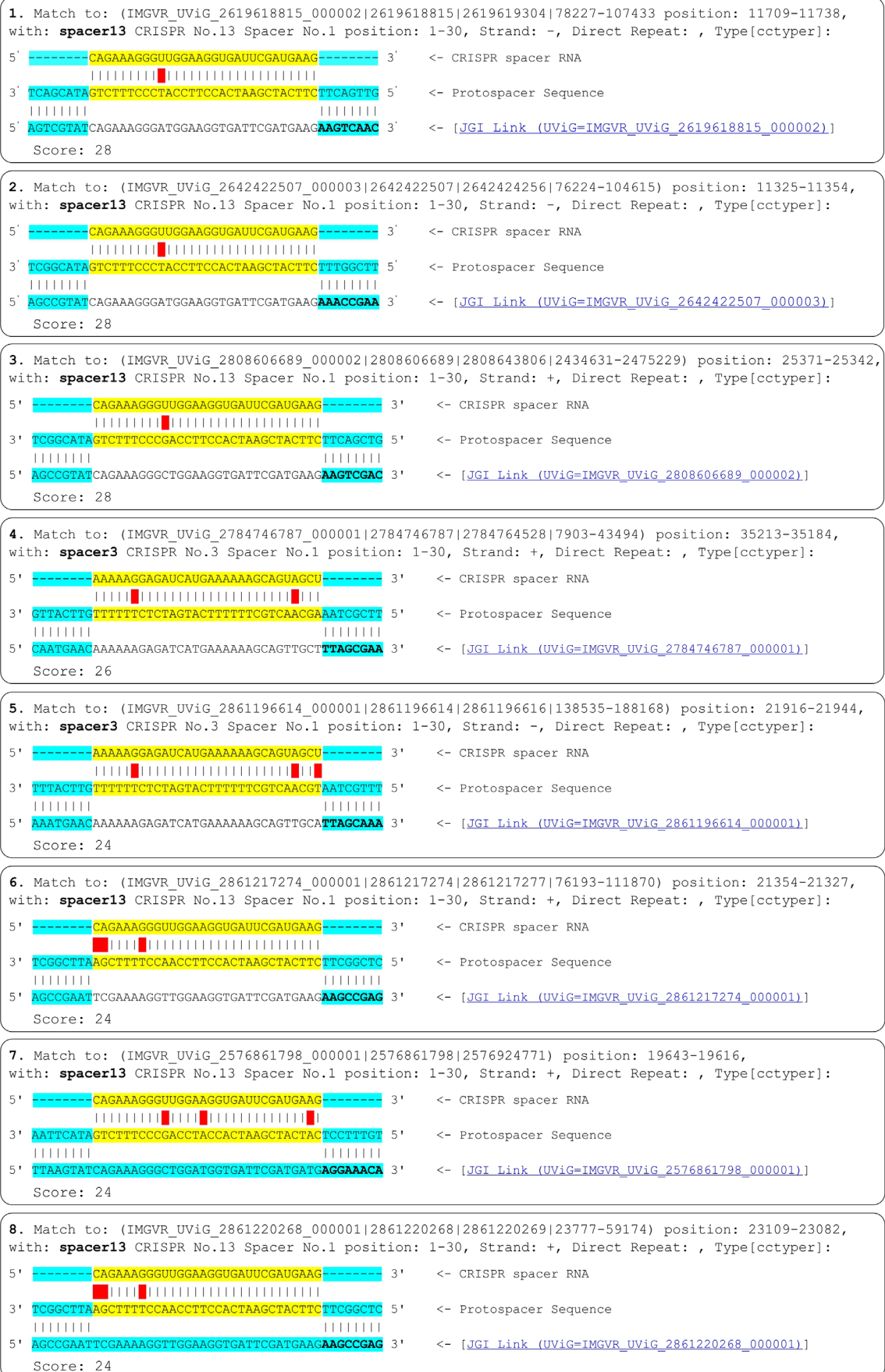
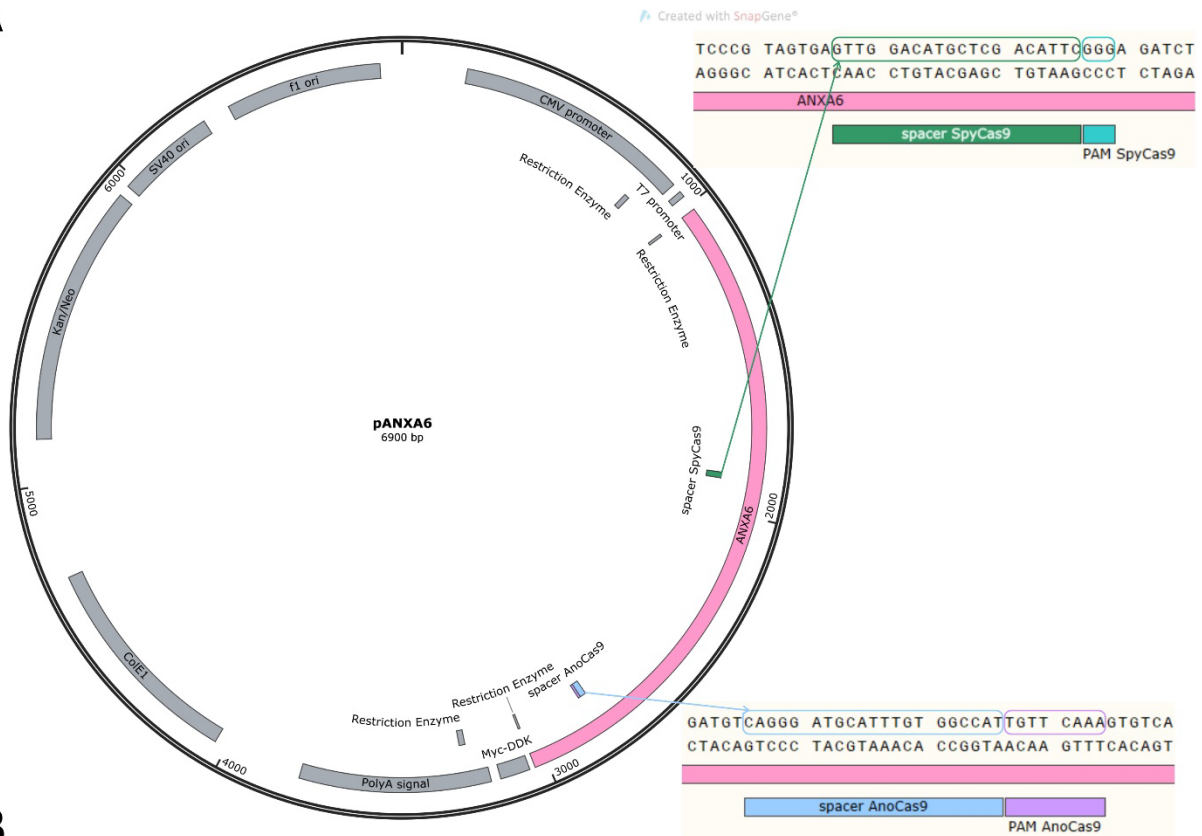


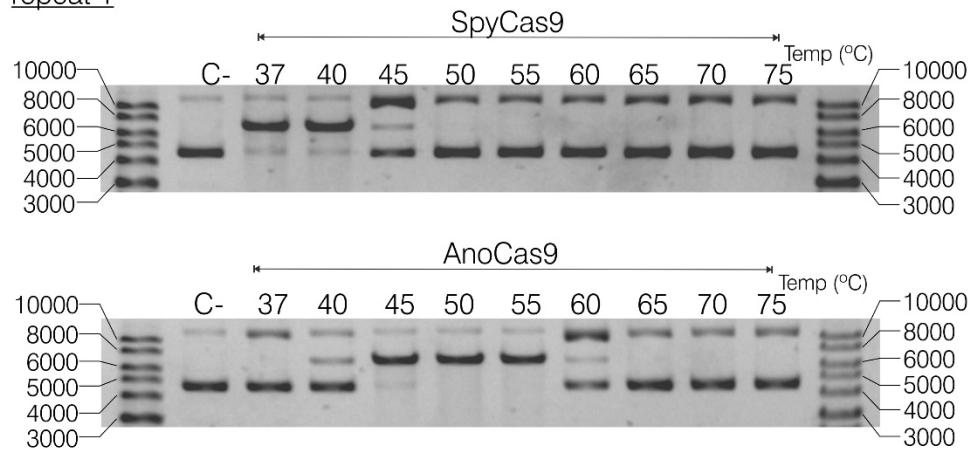
Figure S5. CRISPRTarget spacer analysis. First 8 targets are presented that were used to predict and create the PAM sequence logo.

A



B

repeat 1



repeat 2

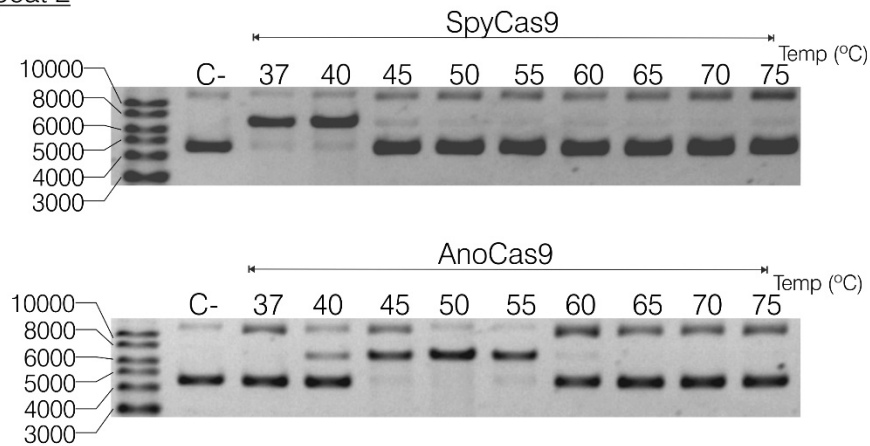


Figure S6. Experimental design for the Cas9 cleavage assay. The map of pANXA6 is presented with the spacer and PAM sequences for SpyCas9 and AnoCas9 highlighted (A). Technical replicates of the temperature gradient cleavage assay are presented (B). Reaction mixtures were resolved in the 1% agarose gel after incubation at the indicated temperature and quenching. Plasmid DNA without Cas9 treatment (C-) was used as a negative control.

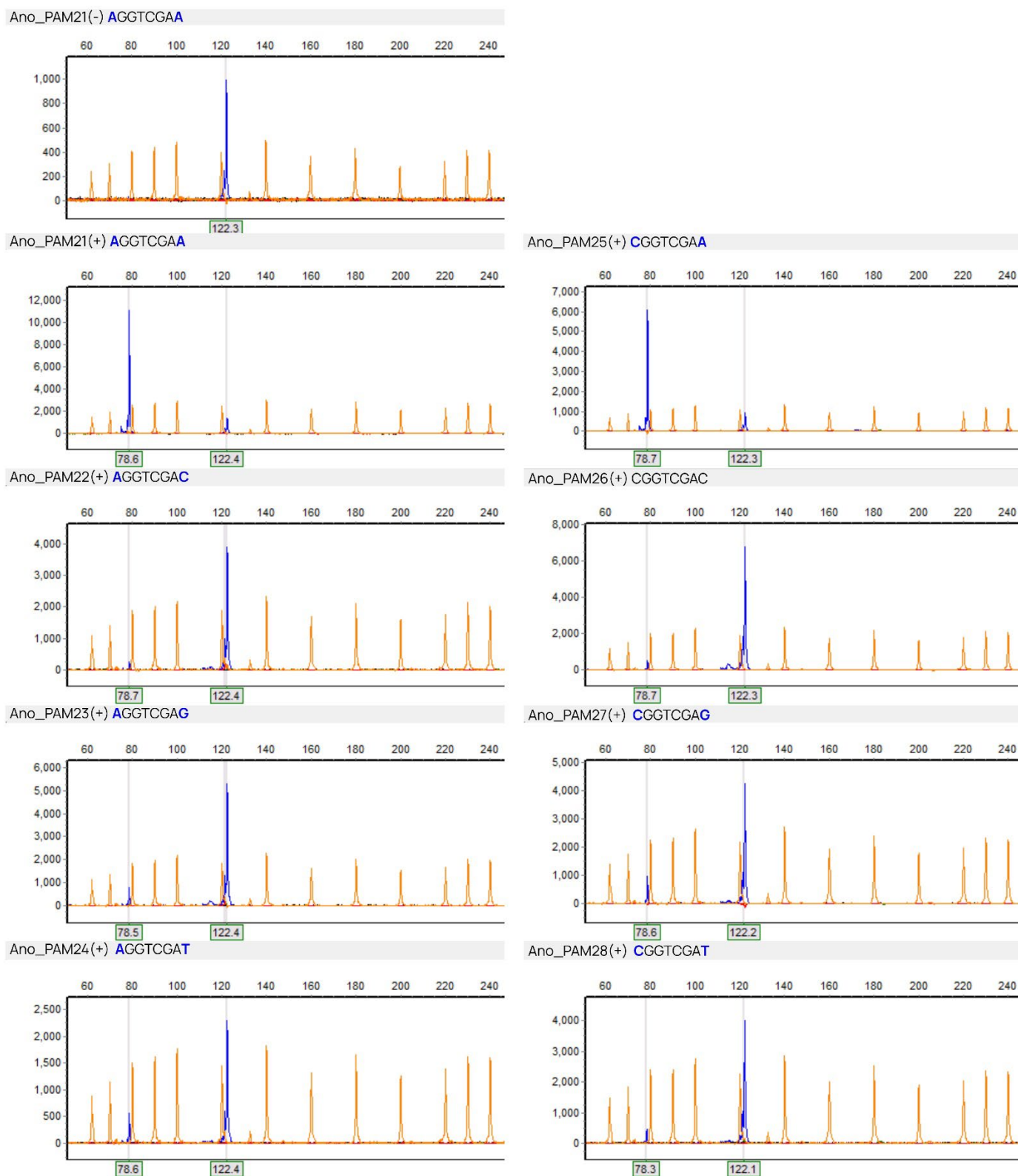


Figure S7. A few examples of electropherograms of the AnCas9 digestion for the FAM-labeled PAM-variable DNA substrates. PAM (-) control refers to the sample with no Cas9 nuclease added. Variable PAM nucleotide positions for the displayed subset of substrates are marked in blue. The observed difference in the lengths of uncut and cut fragments compared to the expected ones from the experiment design is related to the overall short length of the substrates tested and seemingly lowered resolution of the gel analyzer in the selected area.

Table S1. Amplification primers used for the generation of FAM-labeled dsDNA PAM-templates.

Name	Structure (5'-3')
Uni-Spacer-F	caagaaggagatgtctggggatgtcagggatgcatttgtggccat
FAM-Uni-R	ttctcatctgtgccagcaccttcatggatttgaagttgtcggcaaagaagaga
Spacer-PAM-R_1	ttgtcggcaaagaagagaggcttgttcttgacacttgaacaatggccacaaatgcatc
Spacer-PAM-R_2	ttgtcggcaaagaagagaggcttgttcttgacacttgaacaatggccacaaatgcatc
Spacer-PAM-R_3	ttgtcggcaaagaagagaggcttgttcttgacacttgaacaatggccacaaatgcatc
Spacer-PAM-R_4	ttgtcggcaaagaagagaggcttgttcttgacacttagaacaatggccacaaatgcatc
Spacer-PAM-R_5	ttgtcggcaaagaagagaggcttgttcttgacactttgatcaatggccacaaatgcatc
Spacer-PAM-R_6	ttgtcggcaaagaagagaggcttgttcttgacacttggatcaatggccacaaatgcatc
Spacer-PAM-R_7	ttgtcggcaaagaagagaggcttgttcttgacacttcgatcaatggccacaaatgcatc
Spacer-PAM-R_8	ttgtcggcaaagaagagaggcttgttcttgacacttagatcaatggccacaaatgcatc
Spacer-PAM-R_9	ttgtcggcaaagaagagaggcttgttcttgacactttgagcaatggccacaaatgcatc
Spacer-PAM-R_10	ttgtcggcaaagaagagaggcttgttcttgacacttggagcaatggccacaaatgcatc
Spacer-PAM-R_11	ttgtcggcaaagaagagaggcttgttcttgacacttcgagcaatggccacaaatgcatc
Spacer-PAM-R_12	ttgtcggcaaagaagagaggcttgttcttgacacttagagcaatggccacaaatgcatc
Spacer-PAM-R_13	ttgtcggcaaagaagagaggcttgttcttgacactttgaccaatggccacaaatgcatc
Spacer-PAM-R_14	ttgtcggcaaagaagagaggcttgttcttgacacttggaccaatggccacaaatgcatc
Spacer-PAM-R_15	ttgtcggcaaagaagagaggcttgttcttgacacttcgaccaatggccacaaatgcatc
Spacer-PAM-R_16	ttgtcggcaaagaagagaggcttgttcttgacacttagaccaatggccacaaatgcatc
Spacer-PAM-R_18	ttgtcggcaaagaagagaggcttgttcttgacacgtcgaccaatggccacaaatgcatc
Spacer-PAM-R_19	ttgtcggcaaagaagagaggcttgttcttgacacctcgaccaatggccacaaatgcatc
Spacer-PAM-R_20	ttgtcggcaaagaagagaggcttgttcttgacacatcgaccaatggccacaaatgcatc
Spacer-PAM-R_21	ttgtcggcaaagaagagaggcttgttcttgacacttcgacctatggccacaaatgcatc
Spacer-PAM-R_22	ttgtcggcaaagaagagaggcttgttcttgacacgtcgacctatggccacaaatgcatc
Spacer-PAM-R_23	ttgtcggcaaagaagagaggcttgttcttgacacctcgacctatggccacaaatgcatc
Spacer-PAM-R_24	ttgtcggcaaagaagagaggcttgttcttgacacatcgacctatggccacaaatgcatc
Spacer-PAM-R_25	ttgtcggcaaagaagagaggcttgttcttgacacttcgaccgatggccacaaatgcatc
Spacer-PAM-R_26	ttgtcggcaaagaagagaggcttgttcttgacacgtcgaccgatggccacaaatgcatc
Spacer-PAM-R_27	ttgtcggcaaagaagagaggcttgttcttgacacctcgaccgatggccacaaatgcatc
Spacer-PAM-R_28	ttgtcggcaaagaagagaggcttgttcttgacacatcgaccgatggccacaaatgcatc
Spacer-PAM-R_29	ttgtcggcaaagaagagaggcttgttcttgacacttcgacctatggccacaaatgcatc
Spacer-PAM-R_30	ttgtcggcaaagaagagaggcttgttcttgacacgtcgacctatggccacaaatgcatc
Spacer-PAM-R_31	ttgtcggcaaagaagagaggcttgttcttgacacctcgacctatggccacaaatgcatc
Spacer-PAM-R_32	ttgtcggcaaagaagagaggcttgttcttgacacatcgacctatggccacaaatgcatc
Spacer-PAM-R_33	ttgtcggcaaagaagagaggcttgttcttgacacttcgtcttatggccacaaatgcatc
Spacer-PAM-R_34	ttgtcggcaaagaagagaggcttgttcttgacacttcgtcttatggccacaaatgcatc
Spacer-PAM-R_35	ttgtcggcaaagaagagaggcttgttcttgacacttcgtcttatggccacaaatgcatc
Spacer-PAM-R_36	ttgtcggcaaagaagagaggcttgttcttgacacttcgtcttatggccacaaatgcatc
Spacer-PAM-R_37	ttgtcggcaaagaagagaggcttgttcttgacacttcggcttatggccacaaatgcatc
Spacer-PAM-R_38	ttgtcggcaaagaagagaggcttgttcttgacacttcggcttatggccacaaatgcatc
Spacer-PAM-R_39	ttgtcggcaaagaagagaggcttgttcttgacacttcggcttatggccacaaatgcatc
Spacer-PAM-R_40	ttgtcggcaaagaagagaggcttgttcttgacacttcggcttatggccacaaatgcatc
Spacer-PAM-R_41	ttgtcggcaaagaagagaggcttgttcttgacacttcgccttatggccacaaatgcatc
Spacer-PAM-R_42	ttgtcggcaaagaagagaggcttgttcttgacacttcgccttatggccacaaatgcatc
Spacer-PAM-R_43	ttgtcggcaaagaagagaggcttgttcttgacacttcgccttatggccacaaatgcatc
Spacer-PAM-R_44	ttgtcggcaaagaagagaggcttgttcttgacacttcgccttatggccacaaatgcatc
Spacer-PAM-R_45	ttgtcggcaaagaagagaggcttgttcttgacacttcgacttatggccacaaatgcatc
Spacer-PAM-R_46	ttgtcggcaaagaagagaggcttgttcttgacacttcgacttatggccacaaatgcatc
Spacer-PAM-R_48	ttgtcggcaaagaagagaggcttgttcttgacacttcgacatatggccacaaatgcatc

Table S2. Sequences of CRISPR array elements derived from the *Anoxybacillus flavithermus* genome.

Name	Structure (5'-3')
CRISPR repeat	gtaatagttcccctgaggtattgctgtgttatgat
CRISPR spacer 1	aaaatgacacacttatttgataaaaagaaa
CRISPR spacer 2	ccgaaagtttagactcactataaaatcctc
CRISPR spacer 3	aaaaaggagatcatgaaaaagcagtagct
CRISPR spacer 4	cgtgggtcaattcggtaaatggaagtttt
CRISPR spacer 5	tgctacacttttgaccgaaacggagcgga
CRISPR spacer 6	agaccagtccaactagaaattaattttaga
CRISPR spacer 7	tgaagaaaaagaaaactgtagagaacaac
CRISPR spacer 8	tcatcagcaaaaatgtatagcgagaacggc
CRISPR spacer 9	tttctgtctagcagaacaactatttctgaa
CRISPR spacer 10	tttgctactatgatagaagaacattagtg
CRISPR spacer 11	tttctgtctagcagaacaactatttccgaa
CRISPR spacer 12	tttctgtctagcagaacaactatttcggcg
CRISPR spacer 13	cagaaagggttggaaggtgattcgatgaag
CRISPR spacer 14	ccaaattcatgtttaagaacccgctgata
CRISPR spacer 15	atcatttaacgtcttactgatccgcgtatt
CRISPR spacer 16	tgggtggtgatatgcctaataacgaattaaa

Table S3. Structure of dsDNA templates used as a library to determine AnCas9 PAM specificity. The PAM motif is highlighted in green.

Name	Structure (5'-3')
Spacer-PAM_1	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGTTCAAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_2	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGTTCCAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_3	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGTTCGAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_4	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGTTCTAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_5	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGATCAAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_6	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGATCCAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_7	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGATCGAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_8	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGATCTAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_9	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGCTCAAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_10	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGCTCCAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_11	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGCTCGAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_12	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGCTCTAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_13	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGGTCAAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_14	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGGTCCAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_15	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGGTCGAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_16	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGGTCTAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_18	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGGTCGACGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA

Spacer-PAM_19	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGGTCGAGGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_20	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT TGGTCGATGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_21	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT AGGTCGAAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_22	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT AGGTCGACGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_23	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT AGGTCGAGGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_24	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT AGGTCGATGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_25	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT CGGTCTGAAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_26	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT CGGTCTGACGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_27	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT CGGTCTGAGGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_28	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT CGGTCTGATGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_29	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT GGGTCTGAAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_30	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT GGGTCTGACGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_31	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT GGGTCTGAGGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM_32	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT GGGTCTGATGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM-R_33	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT AAGACGAAAGT GTCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGC TGGCACAGATGAGAA
Spacer-PAM-R_34	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT ACGACGAAAGT GTCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGC TGGCACAGATGAGAA
Spacer-PAM-R_35	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT AGGACGAAAGT GTCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGC TGGCACAGATGAGAA
Spacer-PAM-R_36	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT ATGACGAAAGTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA

Spacer-PAM-R_37	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT AAGCCGAA GT GTCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT TGGCACAGATGAGAA
Spacer-PAM-R_38	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT ACGCCGAA GTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM-R_39	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT AGGCCGAA GT GTCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT TGGCACAGATGAGAA
Spacer-PAM-R_40	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT ATGCCGAA GTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM-R_41	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT AAGGCCAA GT GTCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT TGGCACAGATGAGAA
Spacer-PAM-R_42	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT ACGGCGAA GT GTCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT TGGCACAGATGAGAA
Spacer-PAM-R_43	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT AGGGCGAA GT GTCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT TGGCACAGATGAGAA
Spacer-PAM-R_44	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT ATGGCGAA GTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM-R_45	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT AAGTCGAA GTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM-R_46	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT ACGTCGAA GTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
Spacer-PAM-R_48	CAAGAAGGAGATGTCTGGGGATGTCAGGGATGCATTTGTGGCCAT ATGTCGAA GTG TCAAGAACAAGCCTCTCTTCTTTGCCGACAACTTTACAAATCCATGAAGGGTGCT GGCACAGATGAGAA
