Title: Target-dependent nickase activities of CRISPR-Cas nucleases Cpf1 and Cas9

Supplemental material

Supplementary Table 1) Retention scores for individual target sequence candidates from *unc-22A* mixed target library incubated with Cas9.

Libraries and incubations were as described in methods. Individual tags with >50 counts from sequencing each of two control reactions (control = no incubation with Cas9) were chosen for further analysis. For each target sequence, time point, and condition, the total numbers of sequence reads were normalized to the count of tags for an unrelated target sequence (Protospacer 4[29]) included in the library (for sequencing depth), and to the median representation of the sequence in the unreacted libraries (for tag representation). Shown are the log2 retention scores calculated as described in methods for each tag meeting the minimum count criteria of 50 in each control. Positive retention scores are indicative of effective nicking in the reaction and tags are sorted with the criterion being the minimal retention scores for time points (from 1 to 360 minutes). Thus, the top of the list are sequences that are strongest candidates for effective nicking with positive retention at the 1-minute incubation and for which there is not substantial loss of target (cutting of the second strand) for the longest (360-minute reaction).

Target sequence design (*unc-22A*): 'NNNNNN'+'GCACCACCGTCGCCGGCATT'+'NGG'+'NNNNNN'

Reference Sequence design (PS4): 'NNNNNN'+'AAAAATTAGGTGCGCTTGGC'+'NGG'+'NNNNNN'

with N's being equimolar mixture of all four nucleotides and other bases being a mixture of 90% of the indicated base and 3.33% of each other base.

Supplementary Table 2) Table of data for each corresponding Figures.

Each file is named as follows: (miscellaneous number and letter)-(exp [+ CRISPR protein] or con [no CRISPR protein])-(target [gfp1/gfp2/rol6/unc22])-(addition of library DNA in nanograms)-(type of CRISPR protein)-……..fastq.gz

Figure	Files
2	Circular library: 1a-exp-gfp1-1-50ng-lb_S1_L001_R1_001_AF-SOL-805.fastq.gz 1b-exp-gfp1-3-50ng-lb_S2_L001_R1_001_AF-SOL-805.fastq.gz 1c-exp-gfp1-10-50ng-lb_S3_L001_R1_001_AF-SOL-805.fastq.gz 1d-exp-gfp1-30-50ng-lb_S4_L001_R1_001_AF-SOL-805.fastq.gz 1e-exp-gfp1-60-50ng-lb_S5_L001_R1_001_AF-SOL-805.fastq.gz 1f-exp-gfp1-180-50ng-lb_S6_L001_R1_001_AF-SOL-805.fastq.gz 1h-con-50ng_S7_L001_R1_001_AF-SOL-805.fastq.gz 2h-con-50ng_S14_L001_R1_001_AF-SOL-805.fastq.gz Linear library: 2a-exp-gfp1-1-50ng-lb_S7_L001_R1_001_AF_SOL_820.fastq.gz 2b-exp-gfp1-3-50ng-lb_S8_L001_R1_001_AF_SOL_820.fastq.gz 2c-exp-gfp1-10-50ng-lb_S9_L001_R1_001_AF_SOL_820.fastq.gz 2d-exp-gfp1-30-50ng-lb_S10_L001_R1_001_AF_SOL_820.fastq.gz 2e-exp-gfp1-60-50ng-lb_S11_L001_R1_001_AF_SOL_820.fastq.gz 2f-exp-gfp1-360-50ng-lb_S12_L001_R1_001_AF_SOL_820.fastq.gz 3g-con_S19_L001_R1_001_AF_SOL_820.fastq.gz 3h-con_S20_L001_R1_001_AF_SOL_820.fastq.gz
4	4a-c) Circular library: 1a-con-unc22-cas9-RVL-yeast-circ-ku_S1_L001_R1_001_AF_SOL_875.fastq.gz 1b-exp-1-unc22-cas9-RVL-yeast-circ-ku_S2_L001_R1_001_AF_SOL_875.fastq.gz 1c-exp-1-unc22-cas9-RVL-yeast-circ-ku_S3_L001_R1_001_AF_SOL_875.fastq.gz 1d-exp-2.5-unc22-cas9-RVL-yeast-circ-ku_S4_L001_R1_001_AF_SOL_875.fastq.gz 1e-exp-2.5-unc22-cas9-RVL-yeast-circ-ku_S5_L001_R1_001_AF_SOL_875.fastq.gz 2a-con-unc22-cas9-RVL-yeast-circ-by_S6_L001_R1_001_AF_SOL_875.fastq.gz 2b-exp-1-unc22-cas9-RVL-yeast-circ-by_S7_L001_R1_001_AF_SOL_875.fastq.gz 2c-exp-1-unc22-cas9-RVL-yeast-circ-by_S8_L001_R1_001_AF_SOL_875.fastq.gz 2d-exp-2.5-unc22-cas9-RVL-yeast-circ-by_S9_L001_R1_001_AF_SOL_875.fastq.gz 2e-exp-2.5-unc22-cas9-RVL-yeast-circ-by_S10_L001_R1_001_AF_SOL_875.fastq.gz Linear library: 3a-con-unc22-cas9-RVL-yeast-lin-ku_S11_L001_R1_001_AF_SOL_875.fastq.gz 3b-exp-1-unc22-cas9-RVL-yeast-lin-ku_S12_L001_R1_001_AF_SOL_875.fastq.gz 3c-exp-1-unc22-cas9-RVL-yeast-lin-ku_S13_L001_R1_001_AF_SOL_875.fastq.gz

	3d-exp-2.5-unc22-cas9-RVL-yeast-lin-ku_S14_L001_R1_001_AF_SOL_875.fastq.gz 3e-exp-2.5-unc22-cas9-RVL-yeast-lin-ku_S15_L001_R1_001_AF_SOL_875.fastq.gz 4a-con-unc22-cas9-RVL-yeast-lin-by_S16_L001_R1_001_AF_SOL_875.fastq.gz 4b-exp-1-unc22-cas9-RVL-yeast-lin-by_S17_L001_R1_001_AF_SOL_875.fastq.gz 4c-exp-1-unc22-cas9-RVL-yeast-lin-by_S18_L001_R1_001_AF_SOL_875.fastq.gz 4d-exp-2.5-unc22-cas9-RVL-yeast-lin-by_S19_L001_R1_001_AF_SOL_875.fastq.gz 4e-exp-2.5-unc22-cas9-RVL-yeast-lin-by_S20_L001_R1_001_AF_SOL_875.fastq.gz 4d) Circular library: 1a-exp-unc22-1-50ng-cas9-circ-cas9buff-RVL2_S21_L001_R1_001_AF_SOL_878.fastq.gz 1b-exp-unc22-3-50ng-cas9-circ-cas9buff-RVL2_S22_L001_R1_001_AF_SOL_878.fastq.gz 1c-exp-unc22-10-50ng-cas9-circ-cas9buff-RVL2_S23_L001_R1_001_AF_SOL_878.fastq.gz 2a-con-circ-cas9_S27_L001_R1_001_AF_SOL_878.fastq.gz 2b-con-circ-cas9_S28_L001_R1_001_AF_SOL_878.fastq.gz Linear library: 1a-exp-unc22-1-50ng-cas9-lin-cas9buff-RVL2_S7_L001_R1_001_AF_SOL_878.fastq.gz 1b-exp-unc22-3-50ng-cas9-lin-cas9buff-RVL2_S8_L001_R1_001_AF_SOL_878.fastq.gz 1c-exp-unc22-10-50ng-cas9-lin-cas9buff-RVL2_S9_L001_R1_001_AF_SOL_878.fastq.gz 2a-con-lin-cas9_S13_L001_R1_001_AF_SOL_878.fastq.gz 2b-con-lin-cas9_S14_L001_R1_001_AF_SOL_878.fastq.gz 4e) Circular library: 1a-exp-unc22-1-50ng-cas9-circ-thermo-RVL2_S15_L001_R1_001_AF_SOL_878.fastq.gz 1b-exp-unc22-3-50ng-cas9-circ--thermo-RVL2_S16_L001_R1_001_AF_SOL_878.fastq.gz 1c-exp-unc22-10-50ng-cas9-circ--thermo-RVL2_S17_L001_R1_001_AF_SOL_878.fastq.gz 2a-con-circ-cas9_S27_L001_R1_001_AF_SOL_878.fastq.gz 2b-con-circ-cas9_S28_L001_R1_001_AF_SOL_878.fastq.gz Linear library: 1a-exp-unc22-1-50ng-cas9-lin-thermo-RVL2_S1_L001_R1_001_AF_SOL_878.fastq.gz 1b-exp-unc22-3-50ng-cas9-lin-thermo-RVL2_S2_L001_R1_001_AF_SOL_878.fastq.gz 1c-exp-unc22-10-50ng-cas9-lin-thermo-RVL2_S3_L001_R1_001_AF_SOL_878.fastq.gz 2a-con-lin-cas9_S13_L001_R1_001_AF_SOL_878.fastq.gz 2b-con-lin-cas9_S14_L001_R1_001_AF_SOL_878.fastq.gz
S1-3	Circular library: 2h-con-50ng_S14_L001_R1_001_AF-SOL-805.fastq.gz 2f-exp-gfp2-180-50ng-lb_S13_L001_R1_001_AF-SOL-805.fastq.gz 2e-exp-gfp2-60-50ng-lb_S12_L001_R1_001_AF-SOL-805.fastq.gz 2d-exp-gfp2-30-50ng-lb_S11_L001_R1_001_AF-SOL-805.fastq.gz

	2c-exp-gfp2-10-50ng-lb_S10_L001_R1_001_AF-SOL-805.fastq.gz 2b-exp-gfp2-3-50ng-lb_S9_L001_R1_001_AF-SOL-805.fastq.gz 2a-exp-gfp2-1-50ng-lb_S8_L001_R1_001_AF-SOL-805.fastq.gz 1h-con-50ng_S7_L001_R1_001_AF-SOL-805.fastq.gz Linear library: 1d-exp-gfp2-1-50ng-lb_S4_L001_R1_001_AF_SOL_821.fastq.gz 1e-exp-gfp2-10-50ng-lb_S5_L001_R1_001_AF_SOL_821.fastq.gz 1f-exp-gfp2-180-50ng-lb_S6_L001_R1_001_AF_SOL_821.fastq.gz 4d-con_S22_L001_R1_001.fastq.gz 4e-con_S23_L001_R1_001.fastq.gz
S4-6	Circular library: 3a-exp-rol6-1-50ng-lb_S15_L001_R1_001.fastq_AF-SOL-805.gz 3b-exp-rol6-3-50ng-lb_S16_L001_R1_001_AF-SOL-805.fastq.gz 3c-exp-rol6-10-50ng-lb_S17_L001_R1_001_AF-SOL-805.fastq.gz 3d-exp-rol6-30-50ng-lb_S18_L001_R1_001_AF-SOL-805.fastq.gz 3e-exp-rol6-60-50ng-lb_S19_L001_R1_001_AF-SOL-805.fastq.gz 3f-exp-rol6-180-50ng-lb_S20_L001_R1_001_AF-SOL-805.fastq.gz 1h-con-50ng_S7_L001_R1_001_AF-SOL-805.fastq.gz 2h-con-50ng_S14_L001_R1_001_AF-SOL-805.fastq.gz Linear library: 3a-exp-rol6-1-50ng-lb_S13_L001_R1_001_AF_SOL_821.fastq.gz 3b-exp-rol6-10-50ng-lb_S14_L001_R1_001_AF_SOL_821.fastq.gz 3c-exp-rol6-180-50ng-lb_S15_L001_R1_001_AF_SOL_821.fastq.gz 4d-con_S22_L001_R1_001.fastq.gz 4e-con_S23_L001_R1_001.fastq.gz
S7-9	Circular library: 3a-exp-gfp1-1-50ng-as_S15_L001_R1_001_AF_SOL_809.fastq.gz 3b-exp-gfp1-3-50ng-as_S16_L001_R1_001_AF_SOL_809.fastq.gz 3c-exp-gfp1-10-50ng-as_S17_L001_R1_001_AF_SOL_809.fastq.gz 3d-exp-gfp1-30-50ng-as_S18_L001_R1_001_AF_SOL_809.fastq.gz 3e-exp-gfp1-60-50ng-as_S19_L001_R1_001_AF_SOL_809.fastq.gz 3f-exp-gfp1-180-50ng-as_S20_L001_R1_001_AF_SOL_809.fastq.gz 3g-con-50ng_S21_L001_R1_001_AF_SOL_809.fastq.gz 3h-con-50ng_S22_L001_R1_001_AF_SOL_809.fastq.gz Linear library:

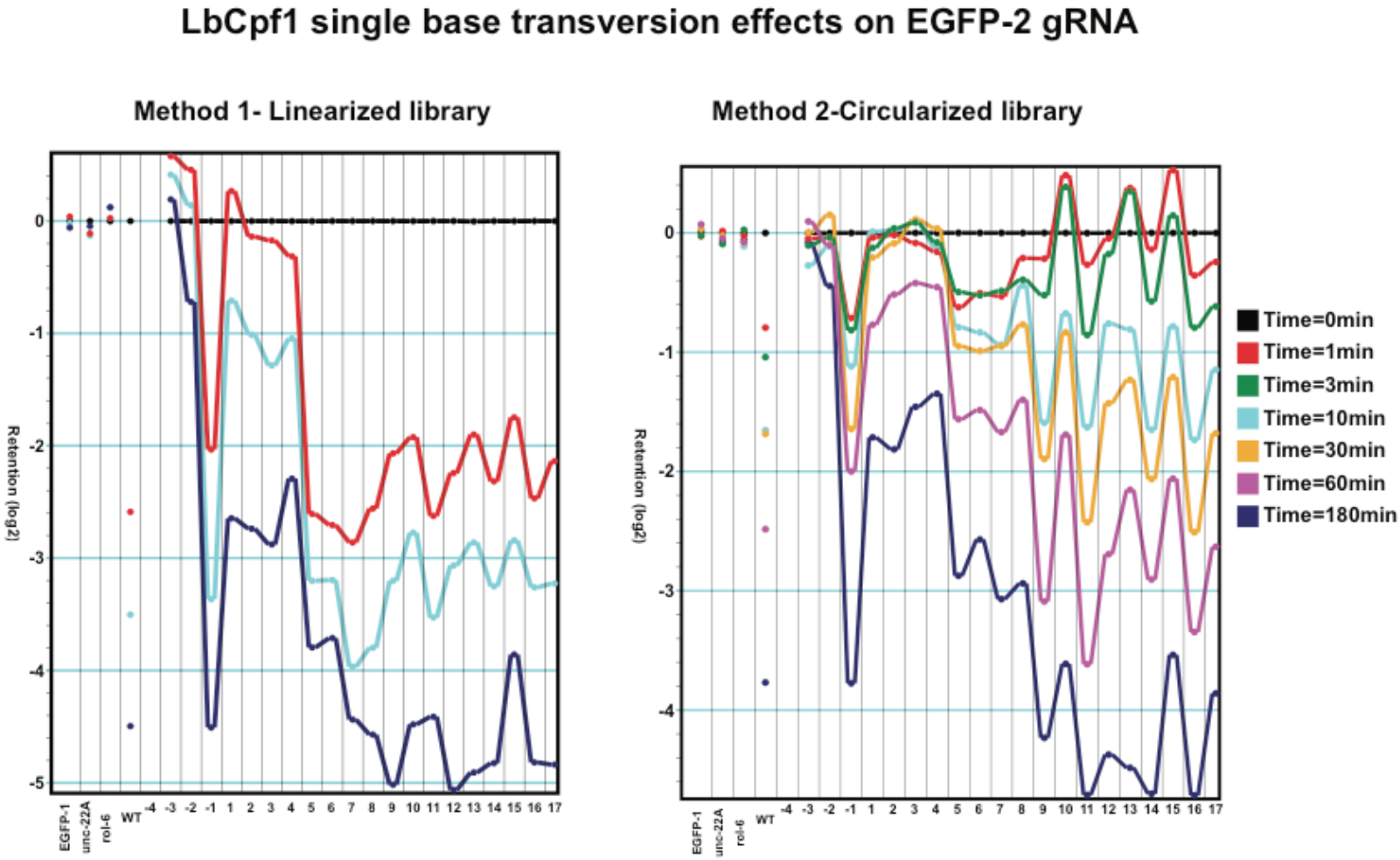
	1a-exp-gfp1-1-50ng-as_S1_L001_R1_001_AF_SOL_820.fastq.gz 1b-exp-gfp1-3-50ng-as_S2_L001_R1_001_AF_SOL_820.fastq.gz 1c-exp-gfp1-10-50ng-as_S3_L001_R1_001_AF_SOL_820.fastq.gz 1d-exp-gfp1-30-50ng-as_S4_L001_R1_001_AF_SOL_820.fastq.gz 1e-exp-gfp1-60-50ng-as_S5_L001_R1_001_AF_SOL_820.fastq.gz 1f-exp-gfp1-180-50ng-as_S6_L001_R1_001_AF_SOL_820.fastq.gz 3g-con_S19_L001_R1_001_AF_SOL_820.fastq.gz 3h-con_S20_L001_R1_001_AF_SOL_820.fastq.gz
S10-12	Circular library: 1a-exp-gfp2-1-50ng-as_S1_L001_R1_001_AF_SOL_810.fastq.gz 1b-exp-gfp2-3-50ng-as_S2_L001_R1_001_AF_SOL_810.fastq.gz 1c-exp-gfp2-10-50ng-as_S3_L001_R1_001_AF_SOL_810.fastq.gz 1d-exp-gfp2-30-50ng-as_S4_L001_R1_001_AF_SOL_810.fastq.gz 1e-exp-gfp2-60-50ng-as_S5_L001_R1_001_AF_SOL_810.fastq.gz 1f-exp-gfp2-180-50ng-as_S6_L001_R1_001_AF_SOL_810.fastq.gz 1g-con-50ng_S20_L001_R1_001_AF_SOL_810.fastq.gz 1h-con-50ng_S7_L001_R1_001_AF_SOL_810.fastq.gz Linear library: 1a-exp-gfp2-1-50ng-as_S1_L001_R1_001_AF_SOL_821.fastq.gz 1b-exp-gfp2-10-50ng-as_S2_L001_R1_001_AF_SOL_821.fastq.gz 1c-exp-gfp2-180-50ng-as_S3_L001_R1_001_AF_SOL_821.fastq.gz 4d-con_S22_L001_R1_001_AF_SOL_821.fastq.gz 4e-con_S23_L001_R1_001_AF_SOL_821.fastq.gz
S13-15	Circular library: 1a-exp-unc22-1-50ng-lb_S1_L001_R1_001_AF_SOL_809.fastq.gz 1b-exp-unc22-3-50ng-lb_S2_L001_R1_001_AF_SOL_809.fastq.gz 1c-exp-unc22-10-50ng-lb_S3_L001_R1_001_AF_SOL_809.fastq.gz 1d-exp-unc22-30-50ng-lb_S4_L001_R1_001_AF_SOL_809.fastq.gz 1e-exp-unc22-60-50ng-lb_S5_L001_R1_001_AF_SOL_809.fastq.gz 1f-exp-unc22-180-50ng-lb_S6_L001_R1_001_AF_SOL_809.fastq.gz 1h-con-50ng_S7_L001_R1_001_AF_SOL_809.fastq.gz 2h-con-50ng_S14_L001_R1_001_AF_SOL_809.fastq.gz Linear library: 1a-exp-unc22-1-50ng-lb_S1_L001_R1_001_AF_SOL_824.fastq.gz 1b-exp-unc22-10-50ng-lb_S2_L001_R1_001_AF_SOL_824.fastq.gz 1c-exp-unc22-180-50ng-lb_S3_L001_R1_001_AF_SOL_824.fastq.gz 1g-con_S20_L001_R1_001_AF_SOL_824.fastq.gz 1h-con_S19_L001_R1_001_AF_SOL_824.fastq.gz
S16-18	Circular library: 2a-exp-rol6-180-50ng-as_S8_L001_R1_001_AF_SOL_810.fastq.gz

	2b-exp-rol6-3-50ng-as_S9_L001_R1_001_AF_SOL_810.fastq.gz 2c-exp-rol6-10-50ng-as_S10_L001_R1_001_AF_SOL_810.fastq.gz 2d-exp-rol6-30-50ng-as_S11_L001_R1_001_AF_SOL_810.fastq.gz 2e-exp-rol6-60-50ng-as_S12_L001_R1_001_AF_SOL_810.fastq.gz 2f-exp-rol6-1-50ng-as_S13_L001_R1_001_AF_SOL_810.fastq.gz 1g-con-50ng_S20_L001_R1_001_AF_SOL_810.fastq.gz 1h-con-50ng_S7_L001_R1_001_AF_SOL_810.fastq.gz Linear library: 2d-exp-rol6-1-50ng-as_S10_L001_R1_001_AF_SOL_821.fastq.gz 2e-exp-rol6-10-50ng-as_S11_L001_R1_001_AF_SOL_821.fastq.gz 2f-exp-rol6-180-50ng-as_S12_L001_R1_001_AF_SOL_821.fastq.gz 4d-con_S22_L001_R1_001.fastq.gz 4e-con_S23_L001_R1_001.fastq.gz
S19-21	Circular library: 1a-exp-gfp1-1-50ng-fn_S1_L001_R1_001_AF_SOL_811.fastq.gz 1b-exp-gfp1-3-50ng-fn_S2_L001_R1_001_AF_SOL_811.fastq.gz 1c-exp-gfp1-10-50ng-fn_S3_L001_R1_001_AF_SOL_811.fastq.gz 1d-exp-gfp1-30-50ng-fn_S4_L001_R1_001_AF_SOL_811.fastq.gz 1e-exp-gfp1-60-50ng-fn_S5_L001_R1_001_AF_SOL_811.fastq.gz 1f-exp-gfp1-180-50ng-fn_S6_L001_R1_001_AF_SOL_811.fastq.gz 1g-con-50ng_S7_L001_R1_001_AF_SOL_811.fastq.gz 1h-con-50ng_S14_L001_R1_001_AF_SOL_811.fastq.gz Linear library: 3a-exp-gfp1-1-50ng-fn_S13_L001_R1_001_AF_SOL_820.fastq.gz 3b-exp-gfp1-3-50ng-fn_S14_L001_R1_001._AF_SOL_820.fastq.gz 3c-exp-gfp1-10-50ng-fn_S15_L001_R1_001_AF_SOL_820.fastq.gz 3d-exp-gfp1-30-50ng-fn_S16_L001_R1_001_AF_SOL_820.fastq.gz 3e-exp-gfp1-60-50ng-fn_S17_L001_R1_001_AF_SOL_820.fastq.gz 3f-exp-gfp1-360-50ng-fn_S18_L001_R1_001_AF_SOL_820.fastq.gz 3g-con_S19_L001_R1_001_AF_SOL_820.fastq.gz 3h-con_S20_L001_R1_001_AF_SOL_820.fastq.gz
S22-24	Circular library: 1g-con-50ng_S7_L001_R1_001_AF_SOL_811.fastq.gz 1h-con-50ng_S14_L001_R1_001_AF_SOL_811.fastq.gz 2a-exp-gfp2-1-50ng-fn_S8_L001_R1_001_AF_SOL_811.fastq.gz 2b-exp-gfp2-3-50ng-fn_S9_L001_R1_001_AF_SOL_811.fastq.gz 2c-exp-gfp2-10-50ng-fn_S10_L001_R1_001_AF_SOL_811.fastq.gz 2d-exp-gfp2-30-50ng-fn_S11_L001_R1_001_AF_SOL_811.fastq.gz 2e-exp-gfp2-60-50ng-fn_S12_L001_R1_001_AF_SOL_811.fastq.gz 2f-exp-gfp2-180-50ng-fn_S13_L001_R1_001_AF_SOL_811.fastq.gz Linear library: 2a-exp-gfp2-1-50ng-fn_S7_L001_R1_001_AF_SOL_821.fastq.gz

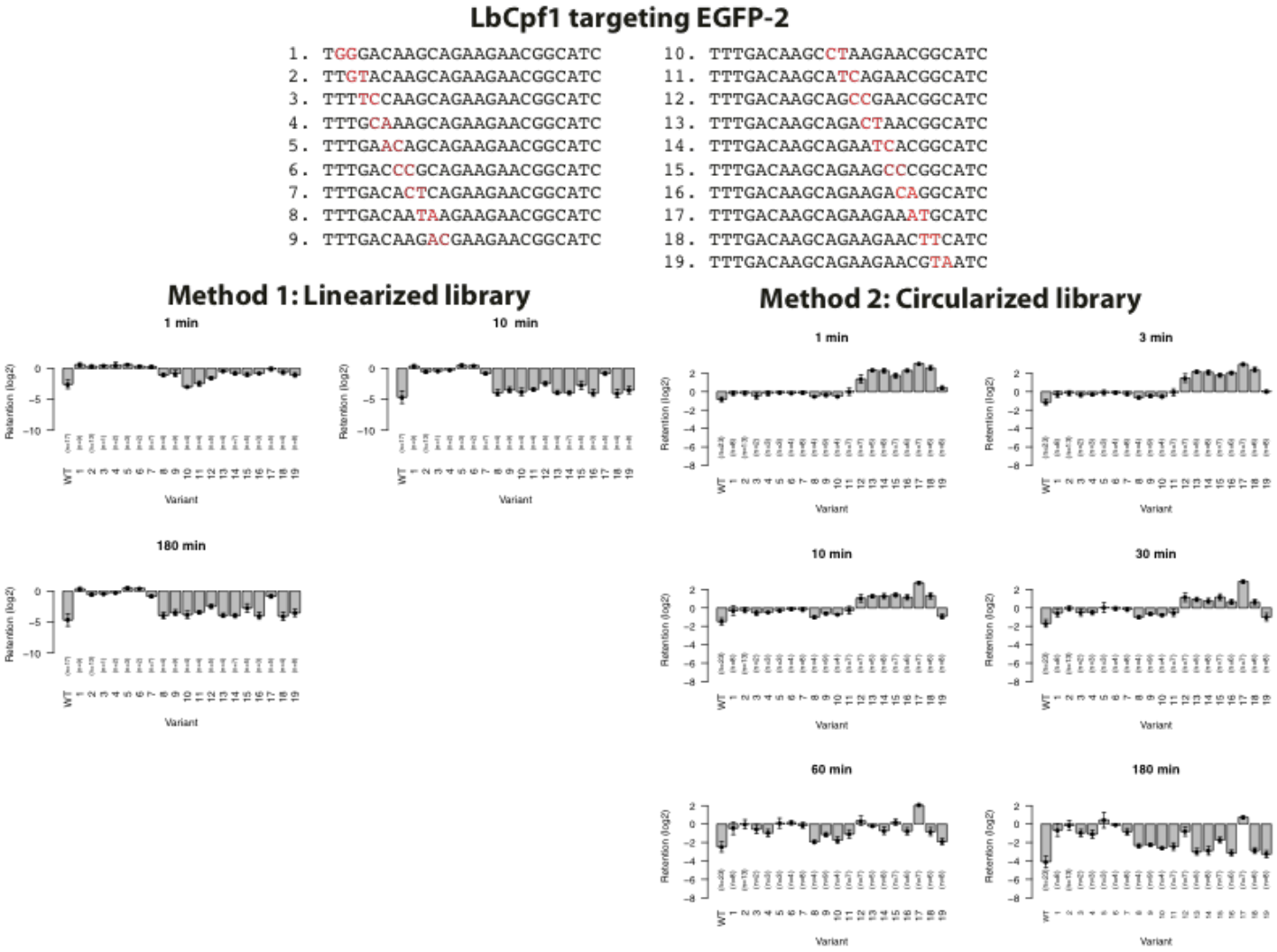
	2b-exp-gfp2-10-50ng-fn_S8_L001_R1_001_AF_SOL_821.fastq.gz 2c-exp-gfp2-180-50ng-fn_S9_L001_R1_001_AF_SOL_821.fastq.gz 4d-con_S22_L001_R1_001_AF_SOL_821.fastq.gz 4e-con_S23_L001_R1_001_AF_SOL_821.fastq.gz
S25-27	Circular library: 3a-exp-rol6-1-50ng-fn_S15_L001_R1_001_AF_SOL_811.fastq.gz 3b-exp-rol6-3-50ng-fn_S16_L001_R1_001_AF_SOL_811.fastq.gz 3c-exp-rol6-10-50ng-fn_S17_L001_R1_001_AF_SOL_811.fastq.gz 3d-exp-rol6-30-50ng-fn_S18_L001_R1_001_AF_SOL_811.fastq.gz 3e-exp-rol6-60-50ng-fn_S19_L001_R1_001_AF_SOL_811.fastq.gz 3f-exp-rol6-180-50ng-fn_S20_L001_R1_001_AF_SOL_811.fastq.gz 1g-con-50ng_S7_L001_R1_001_AF_SOL_811.fastq.gz 1h-con-50ng_S14_L001_R1_001_AF_SOL_811.fastq.gz Linear library: 3d-exp-rol6-1-50ng-fn_S16_L001_R1_001_AF_SOL_821.fastq.gz 3e-exp-rol6-10-50ng-fn_S17_L001_R1_001_AF_SOL_821.fastq.gz 3f-exp-rol6-180-50ng-fn_S18_L001_R1_001_AF_SOL_821.fastq.gz 4d-con_S22_L001_R1_001_AF_SOL_821.fastq.gz 4e-con_S23_L001_R1_001_AF_SOL_821.fastq.gz
S34-36	Circular library: 3a-exp-gfp1-3-50ng-cas9-circ-typeII_S15_L001_R1_001_AF_SOL_854.fastq.gz 3b-exp-gfp1-10-50ng-cas9-circ-typeII_S16_L001_R1_001_AF_SOL_854.fastq.gz 3c-exp-gfp1-30-50ng-cas9-circ-typeII_S17_L001_R1_001_AF_SOL_854.fastq.gz 3d-exp-gfp1-180-50ng-cas9-circ-typeII_S18_L001_R1_001_AF_SOL_854.fastq.gz 1f-con-cas9-typeII_S5_L001_R1_001_AF_SOL_854.fastq.gz 1g-con-cas9-typeII_S6_L001_R1_001_AF_SOL_854.fastq.gz
S37-39	Circular library: 2e-exp-gfp2-3-50ng-cas9-circ-typeII_S11_L001_R1_001_AF_SOL_854.fastq.gz 2f-exp-gfp2-10-50ng-cas9-circ-typeII_S12_L001_R1_001_AF_SOL_854.fastq.gz 2g-exp-gfp2-30-50ng-cas9-circ-typeII_S13_L001_R1_001_AF_SOL_854.fastq.gz 2h-exp-gfp2-180-50ng-cas9-circ-typeII_S14_L001_R1_001_AF_SOL_854.fastq.gz 1f-con-cas9-typeII_S5_L001_R1_001_AF_SOL_854.fastq.gz 1g-con-cas9-typeII_S6_L001_R1_001_AF_SOL_854.fastq.gz
S40-42	Circular library: 1f-con-cas9-typeII_S5_L001_R1_001_AF_SOL_854.fastq.gz 1g-con-cas9-typeII_S6_L001_R1_001_AF_SOL_854.fastq.gz 2a-exp-rol6-3-50ng-cas9-circ-typeII_S7_L001_R1_001_AF_SOL_854.fastq.gz 2b-exp-rol6-10-50ng-cas9-circ-typeII_S8_L001_R1_001_AF_SOL_854.fastq.gz 2c-exp-rol6-30-50ng-cas9-circ-typeII_S9_L001_R1_001_AF_SOL_854.fastq.gz 2d-exp-rol6-180-50ng-cas9-circ-typeII_S10_L001_R1_001_AF_SOL_854.fastq.gz

S43-45	Circular library: 1a-exp-unc22-3-50ng-cas9-circ-buff3-typeII_S1_L001_R1_001_AF_SOL_854.fastq.gz 1b-exp-unc22-10-50ng-cas9-circ-buff3-typeII_S2_L001_R1_001_AF_SOL_854.fastq.gz 1d-exp-unc22-30-50ng-cas9-circ-buff3-typeII_S3_L001_R1_001_AF_SOL_854.fastq.gz 1e-exp-unc22-180-50ng-cas9-circ-buff3-typeII_S4_L001_R1_001_AF_SOL_854.fastq.gz 1f-con-cas9-typeII_S5_L001_R1_001_AF_SOL_854.fastq.gz 1g-con-cas9-typeII_S6_L001_R1_001_AF_SOL_854.fastq.gz
S47	Circular library: 3a-exp-unc22-1-50ng-cas9_S1_L001_R1_001_AF_SOL_827.fastq.gz 3b-exp-unc22-3-50ng-cas9_S2_L001_R1_001_AF_SOL_827.fastq.gz 3c-exp-unc22-10-50ng-cas9_S3_L001_R1_001_AF_SOL_827.fastq.gz 3d-exp-unc22-30-50ng-cas9_S4_L001_R1_001_AF_SOL_827.fastq.gz 3e-exp-unc22-60-50ng-cas9_S5_L001_R1_001_AF_SOL_827.fastq.gz 3f-exp-unc22-180-50ng-cas9_S6_L001_R1_001_AF_SOL_827.fastq.gz 3g-con-cas9_S7_L001_R1_001_AF_SOL_827.fastq.gz 3h-con-cas9_S8_L001_R1_001_AF_SOL_827.fastq.gz
S52-54	Circular library: 1e-exp-unc22-1-50ng-cas9d10a-circ-thermobuff-typeII_S5_L001_R1_001_AF_SOL_855.fastq.gz 1f-exp-unc22-10-50ng-cas9d10a-circ-thermobuff-typeII_S6_L001_R1_001_AF_SOL_855.fastq.gz 1g-exp-unc22-30-50ng-cas9d10a-circ-thermobuff-typeII_S7_L001_R1_001_AF_SOL_855.fastq.gz 1h-exp-unc22-60-50ng-cas9d10a-circ-thermobuff-typeII_S8_L001_R1_001_AF_SOL_855.fastq.gz 2a-con-circ-cas9_S9_L001_R1_001_AF_SOL_855.fastq.gz 2b-con-circ-cas9_S10_L001_R1_001_AF_SOL_855.fastq.gz

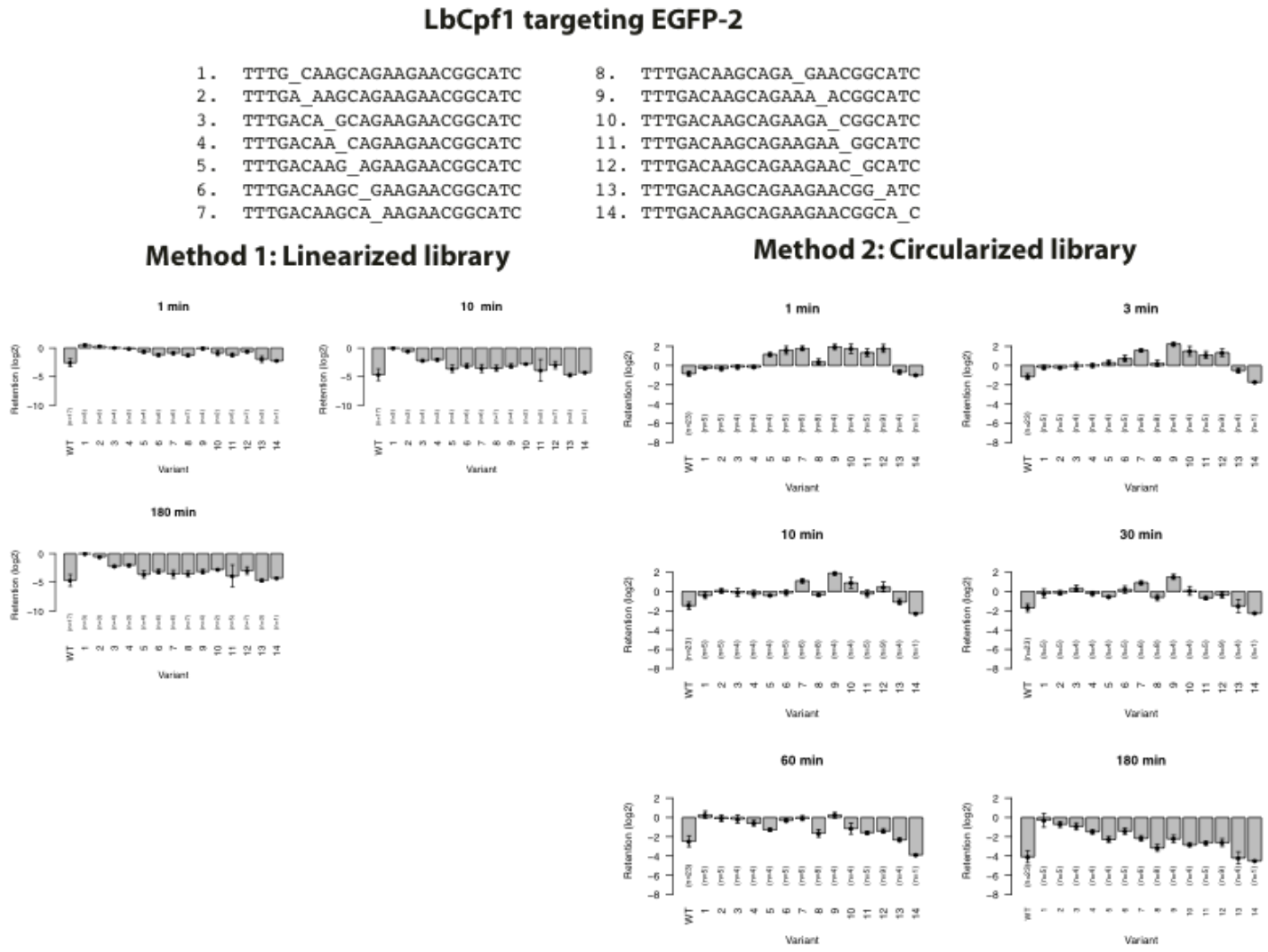
Supplementary Figure 1) Results from LbCpf1 interaction with pooled EGFP-2 targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Retention score profiles for whole-library assays with backbone cleavage step used (to avoid preferential recovery of nicked substrates; left panel) or not used (allowing preferential recovery of nicked substrates; right panel) for EGFP-2 single base transversions. X axis shows the positions, controls, and WT. Y axis shows the retention score. Refer to “Materials and Methods” for full conditions and retention calculations. (Method 1-Linearized library; AF_SOL_821, Method 2-Circular library; AF_SOL_805)



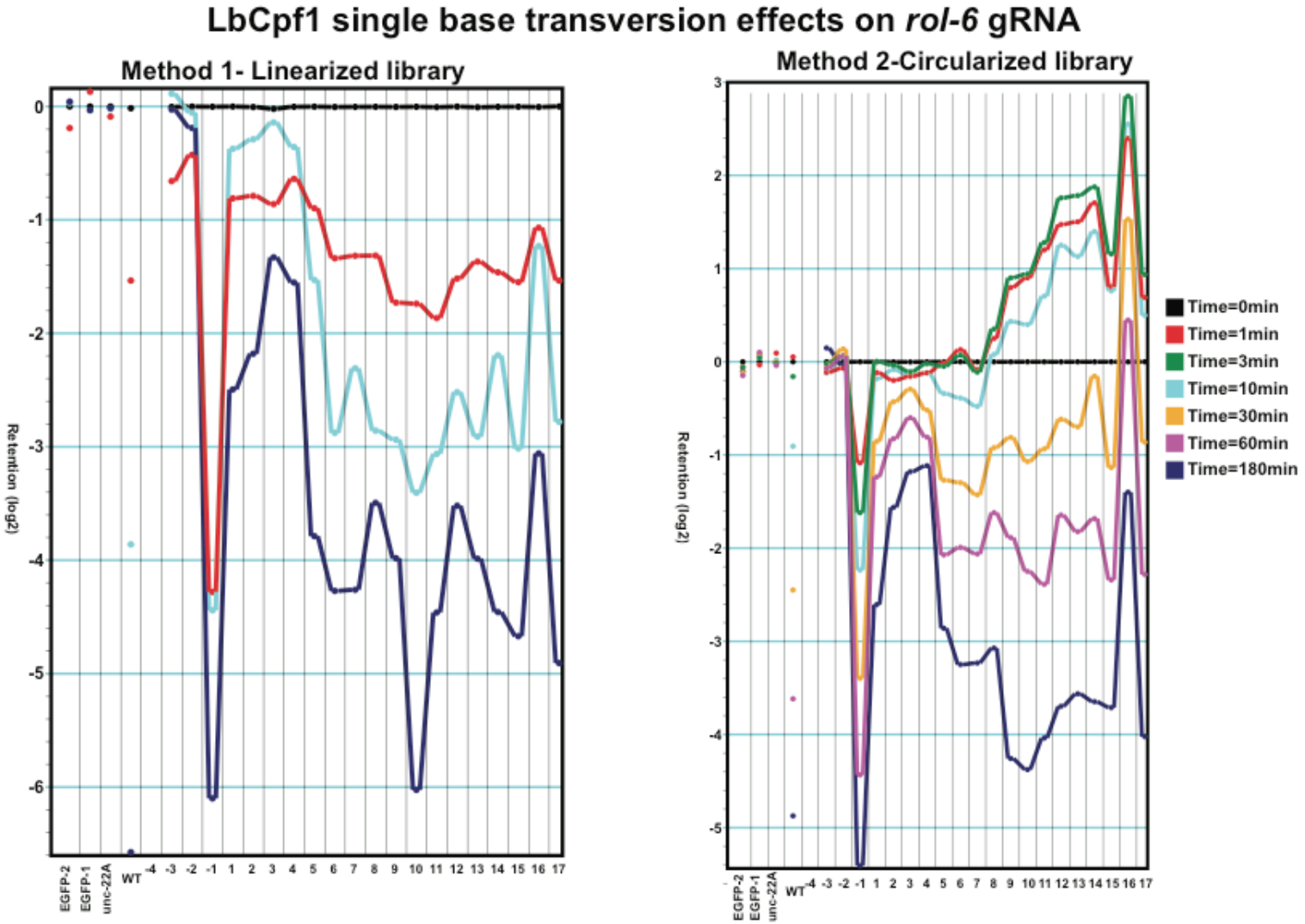
Supplementary Figure 2) Results from LbCpf1 interaction with pooled EGFP-2 targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



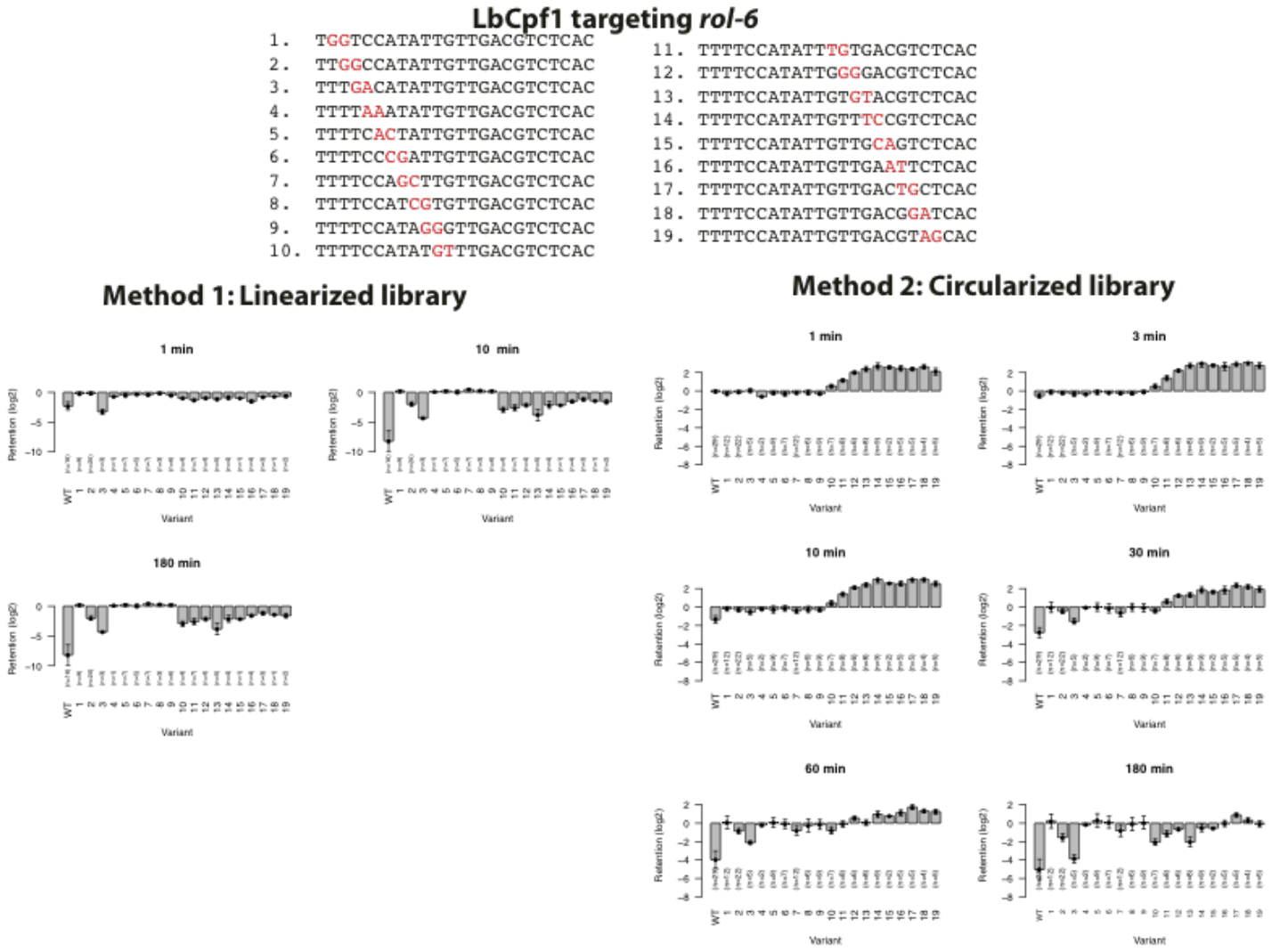
Supplementary Figure 3) Results from LbCpf1 interaction with pooled EGFP-2 targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for single-base-deletion variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



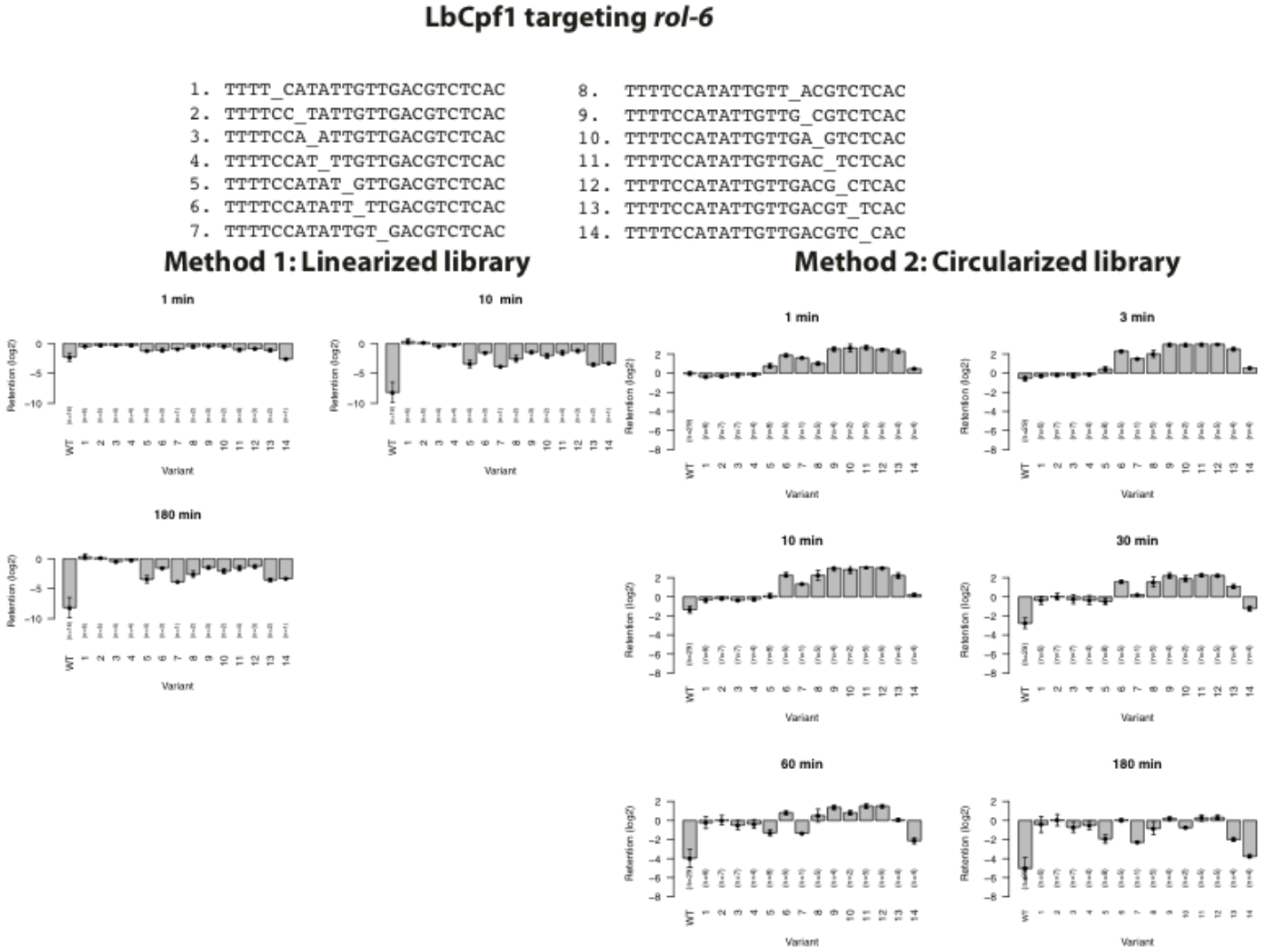
Supplementary Figure 4) Results from LbCpf1 interaction with pooled *rol-6* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Retention score profiles for whole-library assays with backbone cleavage step used (to avoid preferential recovery of nicked substrates; left panel) or not used (allowing preferential recovery of nicked substrates; right panel) for *rol-6* single base transversions. X axis shows the positions, controls, and WT. Y axis shows the retention score. (Method 1-Linearized library, Method 2-Circular library; AF_SOL_805). Refer to “Materials and Methods” for full conditions and retention calculations.



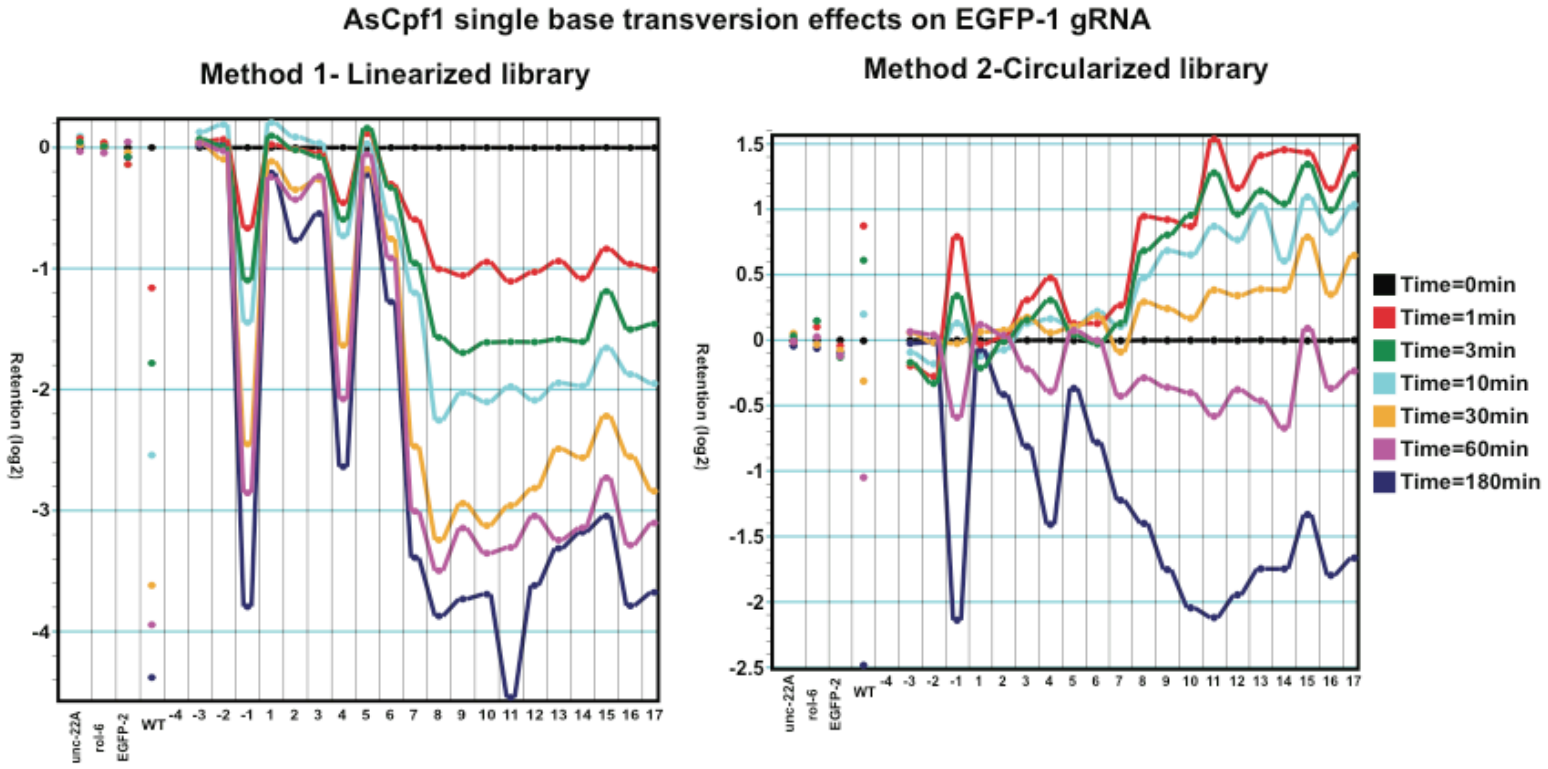
Supplementary Figure 5) Results from LbCpf1 interaction with pooled *rol-6* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



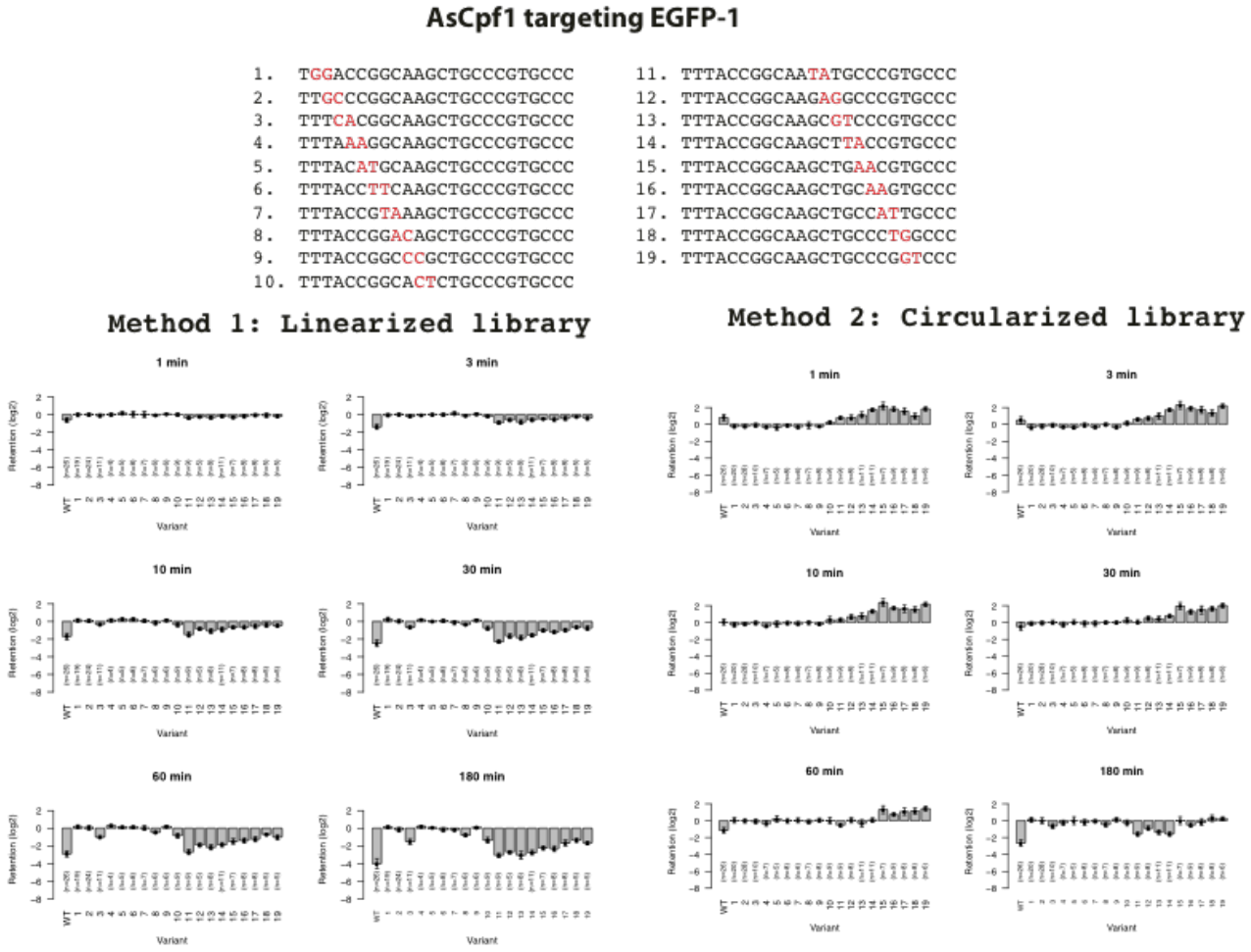
Supplementary Figure 6) Results from LbCpf1 interaction with pooled *rol-6* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for single-base-deletion variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



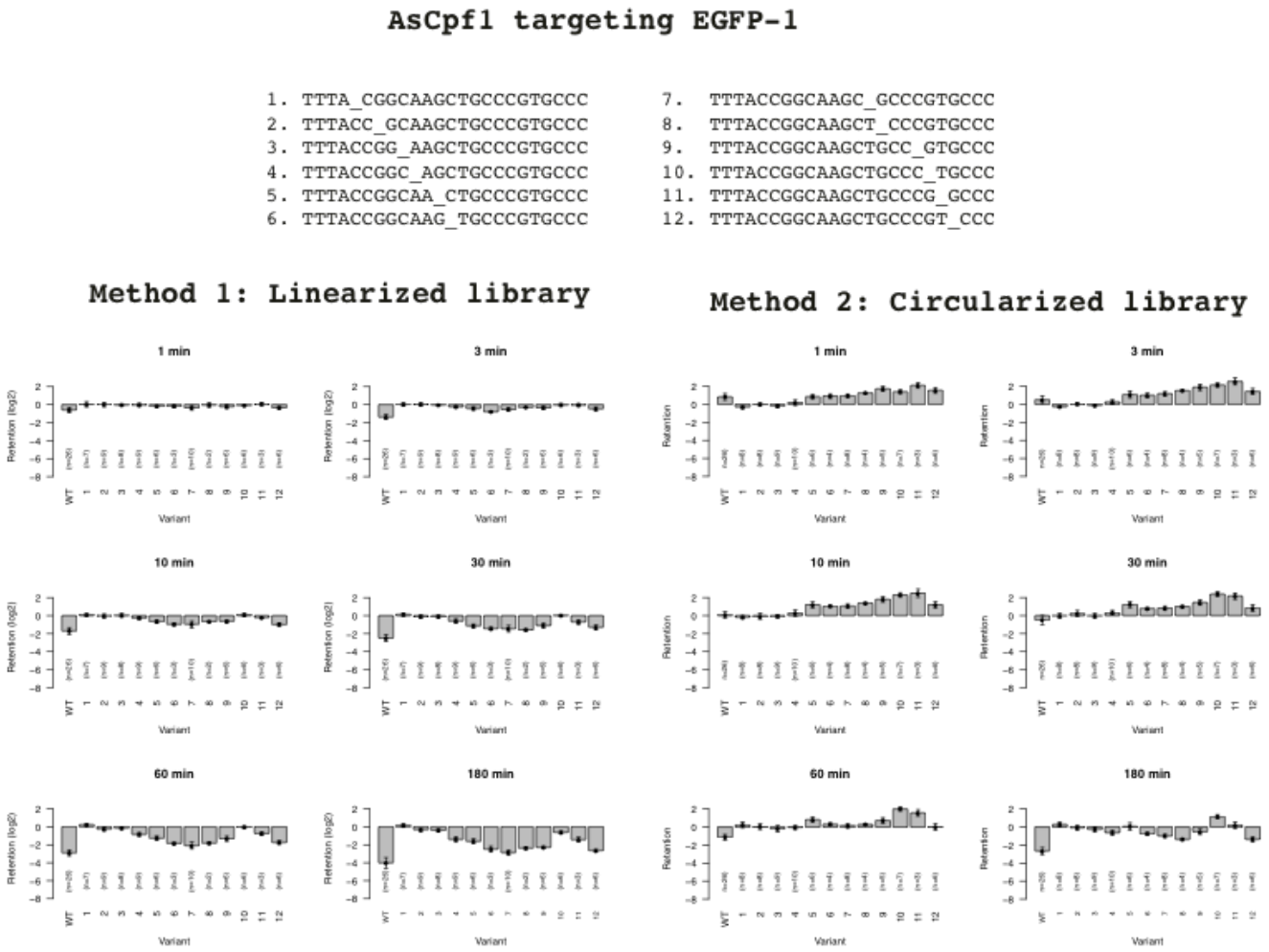
Supplementary Figure 7) Results from AsCpf1 interaction with pooled *EGFP-1* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Retention score profiles for whole-library assays with backbone cleavage step used (to avoid preferential recovery of nicked substrates; left panel) or not used (allowing preferential recovery of nicked substrates; right panel) for EGFP-1 single base transversions. X axis shows the positions, controls, and WT. Y axis shows the retention score. Refer to “Materials and Methods” for full conditions and retention calculations. (NEB buffer 3.1; Method 1-Linearized library; AF_SOL_820, Method 2-Circular library; AF_SOL_809)



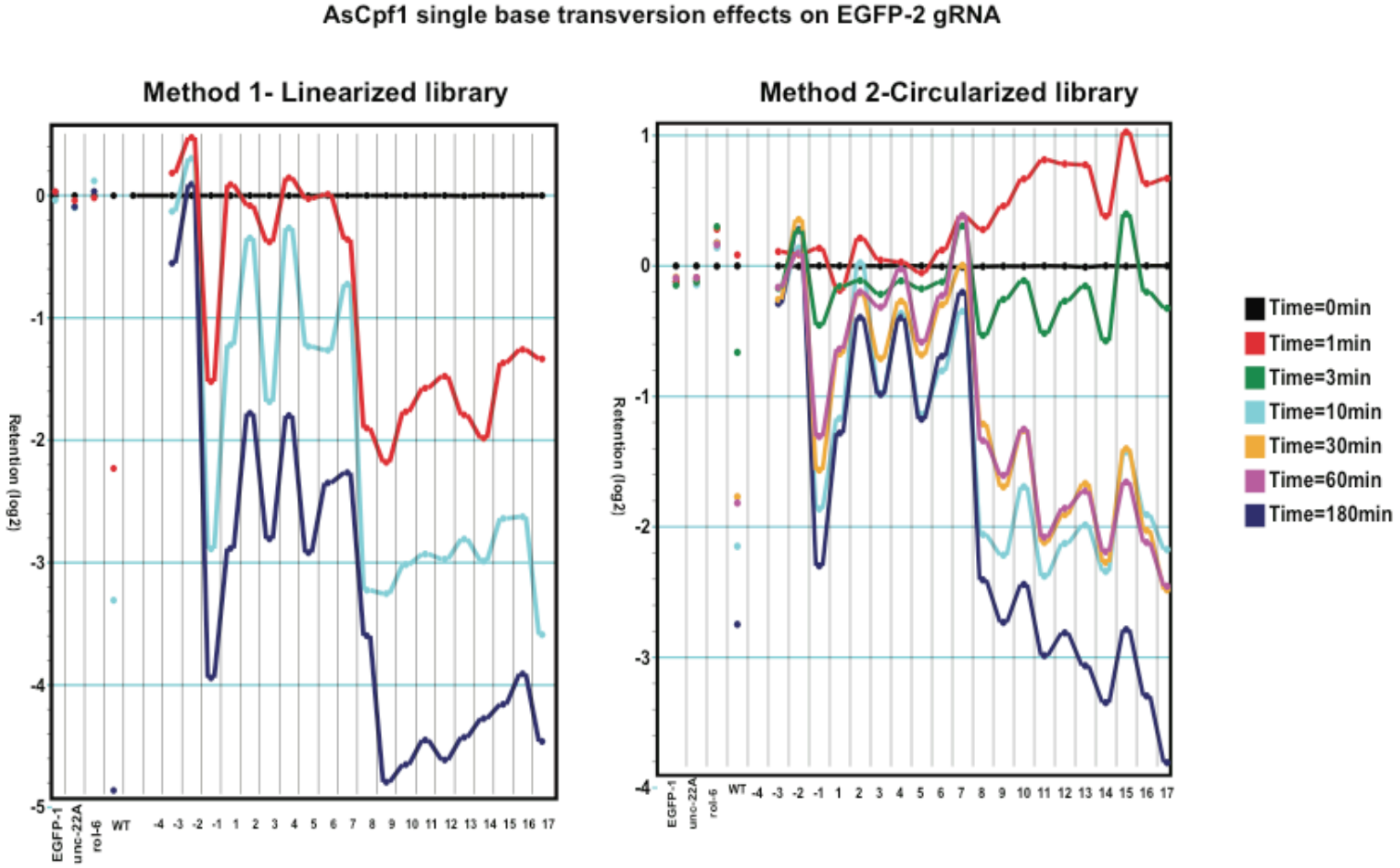
Supplementary Figure 8) Results from AsCpf1 interaction with pooled *EGFP-1* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



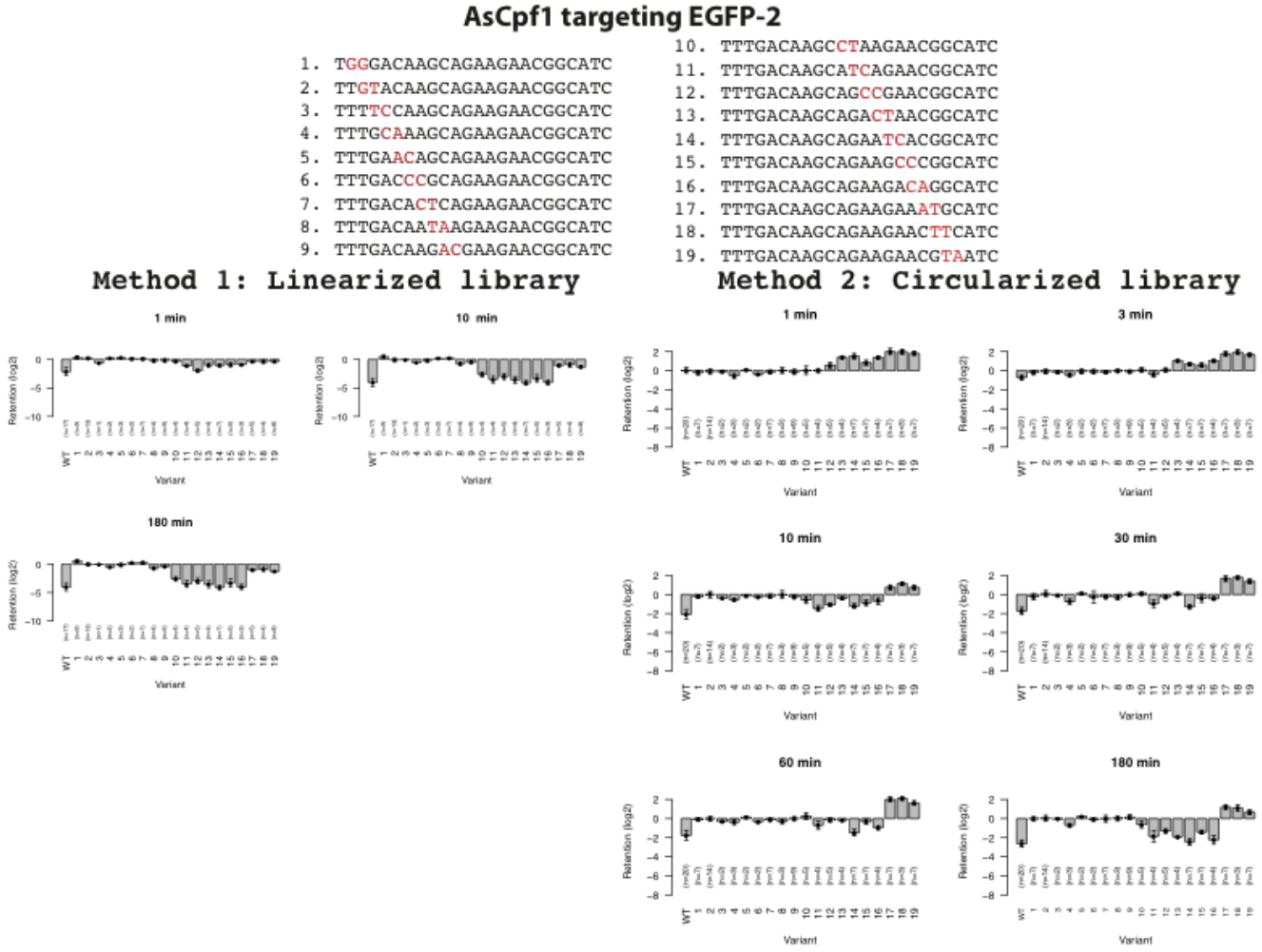
Supplementary Figure 9) Results from AsCpf1 interaction with pooled *EGFP-1* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for single-base-deletion variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



Supplementary Figure 10) Results from AsCpf1 interaction with pooled *EGFP-2 targets.* All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Retention score profiles for whole-library assays with backbone cleavage step used (to avoid preferential recovery of nicked substrates; left panel) or not used (allowing preferential recovery of nicked substrates; right panel) for EGFP-2 single base transversions. X axis shows the positions, controls, and WT. Y axis shows the retention score. Refer to “Materials and Methods” for full conditions and calculations. (Method 1-Linearized library; AF_SOL_821, Method 2-Circular library; AF_SOL_810; NEB buffer 3.1).



Supplementary Figure 11) Results from AsCpf1 interaction with pooled *EGFP-2 targets.* All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.

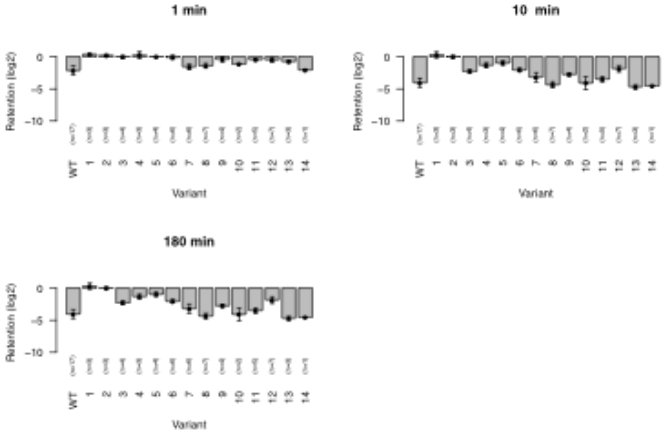


Supplementary Figure 12) Results from AsCpf1 interaction with pooled *EGFP-2 targets.* All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for single-base-deletion variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.

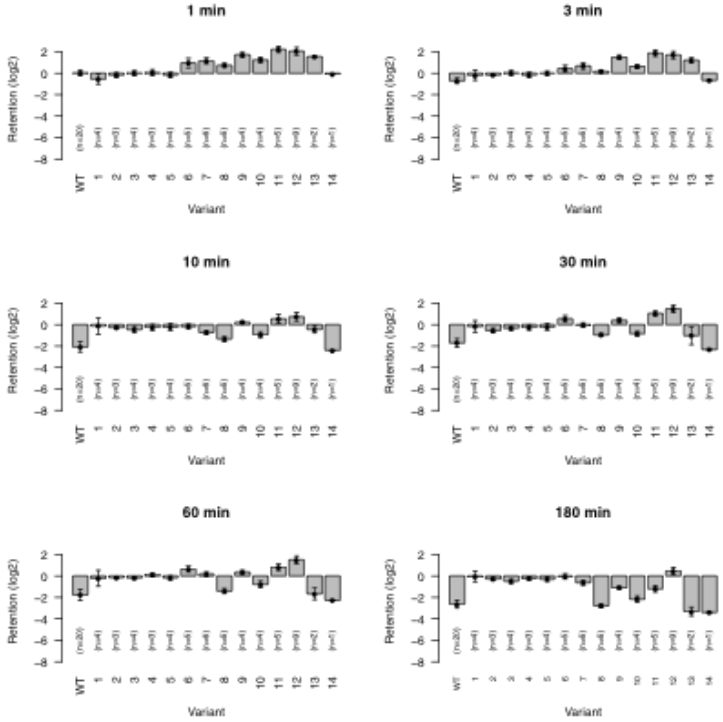
AsCpf1 targeting EGFP-2

1. TTTG_CAAGCAGAAGAACGGCATC
2. TTTGA_AAGCAGAAGAACGGCATC
3. TTTGACA_GCAGAAGAACGGCATC
4. TTTGACAA_CAGAAGAACGGCATC
5. TTTGACAAG_AGAAGAACGGCATC
6. TTTGACAAGC_GAAGAACGGCATC
7. TTTGACAAGCA_AAGAACGGCATC
8. TTTGACAAGCAGA_GAACGGCATC
9. TTTGACAAGCAGAAA_ACGGCATC
10. TTTGACAAGCAGAAGA_CGGCATC
11. TTTGACAAGCAGAAGAA_GGCATC
12. TTTGACAAGCAGAAGAAC_GCATC
13. TTTGACAAGCAGAAGAACGG_ATC
14. TTTGACAAGCAGAAGAACGGCA_C

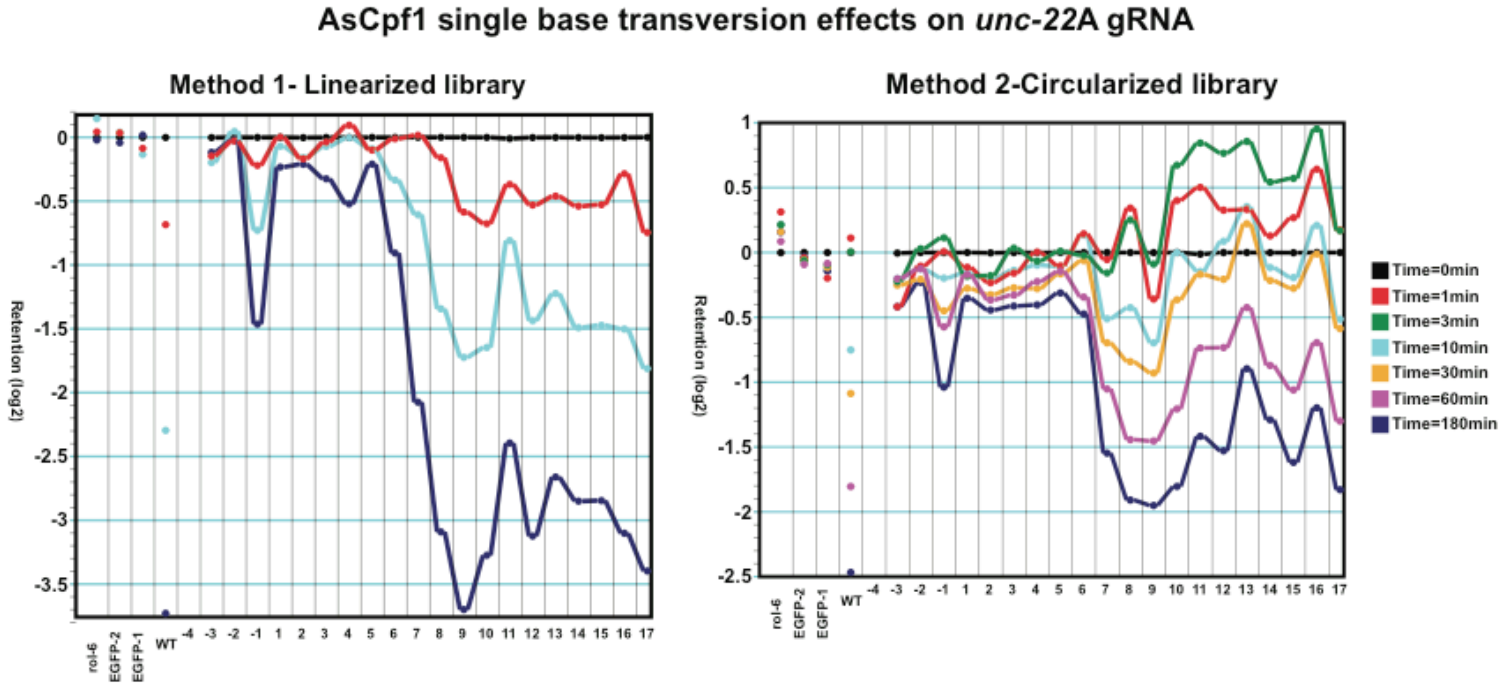
Method 1: Linearized library



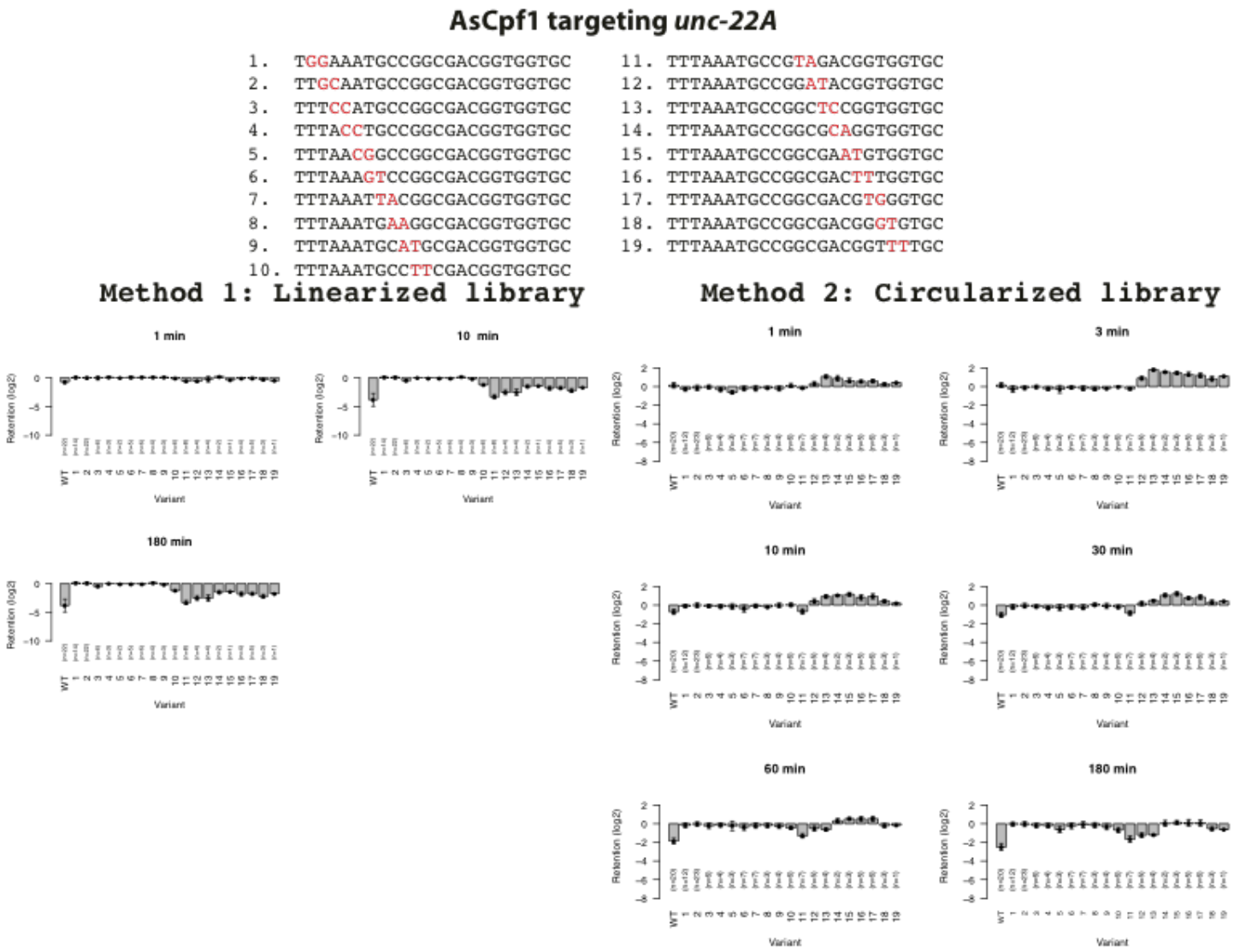
Method 2: Circularized library



Supplementary Figure 13) Results from AsCpf1 interaction with pooled *unc-22A* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Retention score profiles for whole-library assays with backbone cleavage step used (to avoid preferential recovery of nicked substrates; left panel) or not used (allowing preferential recovery of nicked substrates; right panel) for *unc-22A* single base transversions. X axis shows the positions, controls, and WT. Y axis shows the retention score. Refer to “Materials and Methods” for full conditions and retention calculations. (Method 1-Linearized library; AF_SOL_821, Method 2-Circular library; AF_SOL_810, NEB buffer 3.1)



Supplementary Figure 14) Results from AsCpf1 interaction with pooled *unc-22A* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”.Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



Supplementary Figure 15) Results from AsCpf1 interaction with pooled *unc-22A* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for single-base-deletion variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.

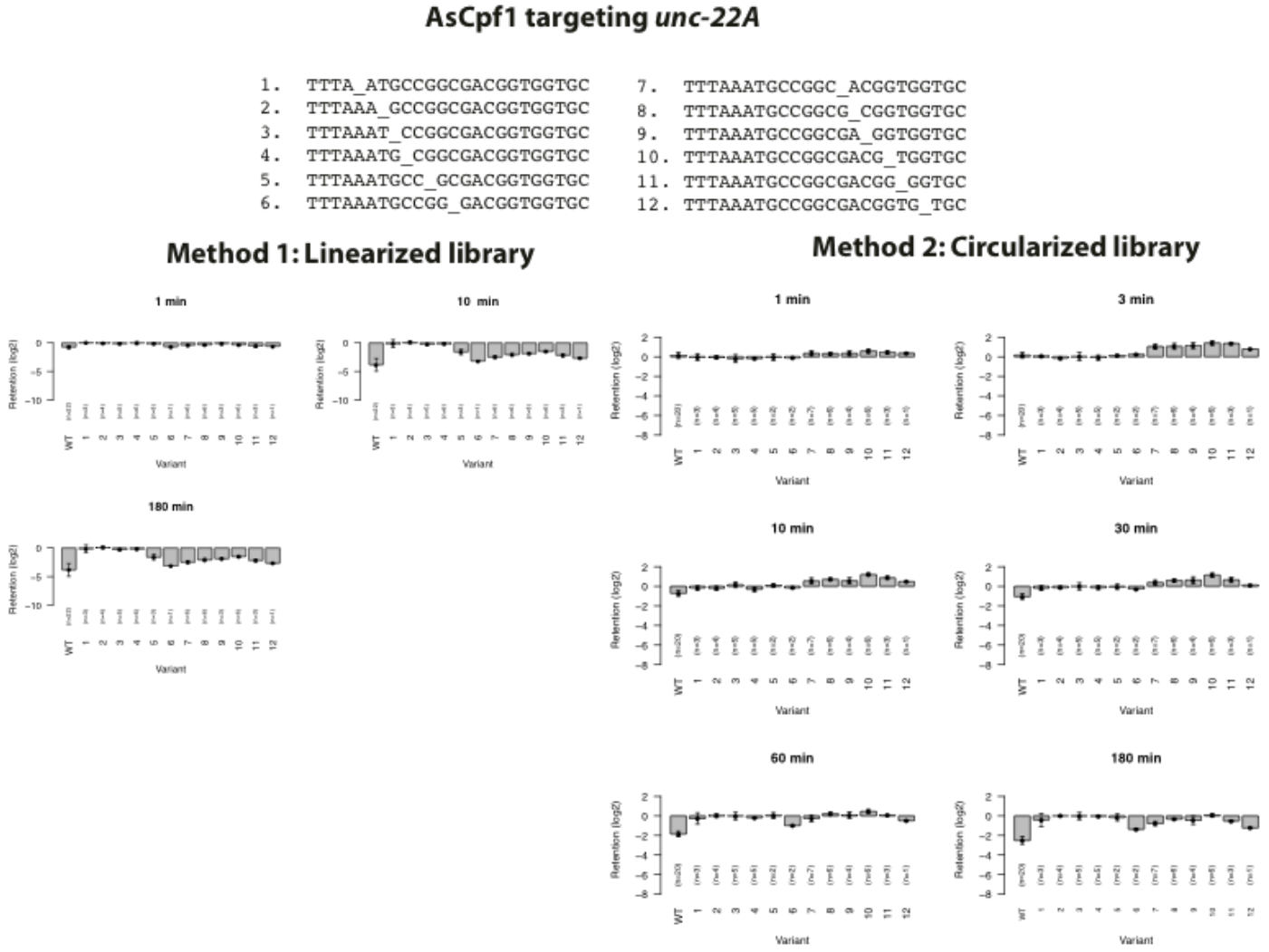
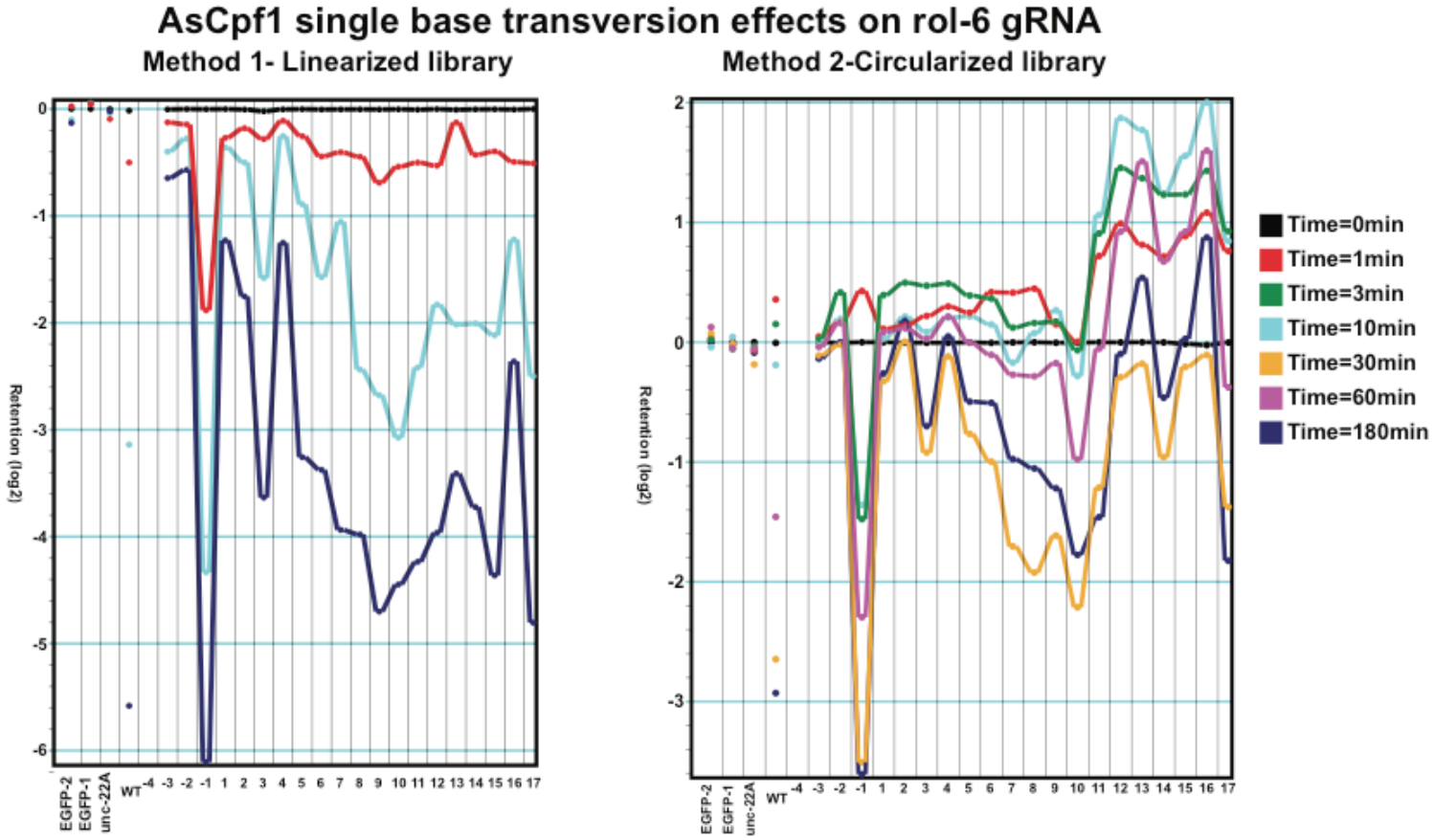
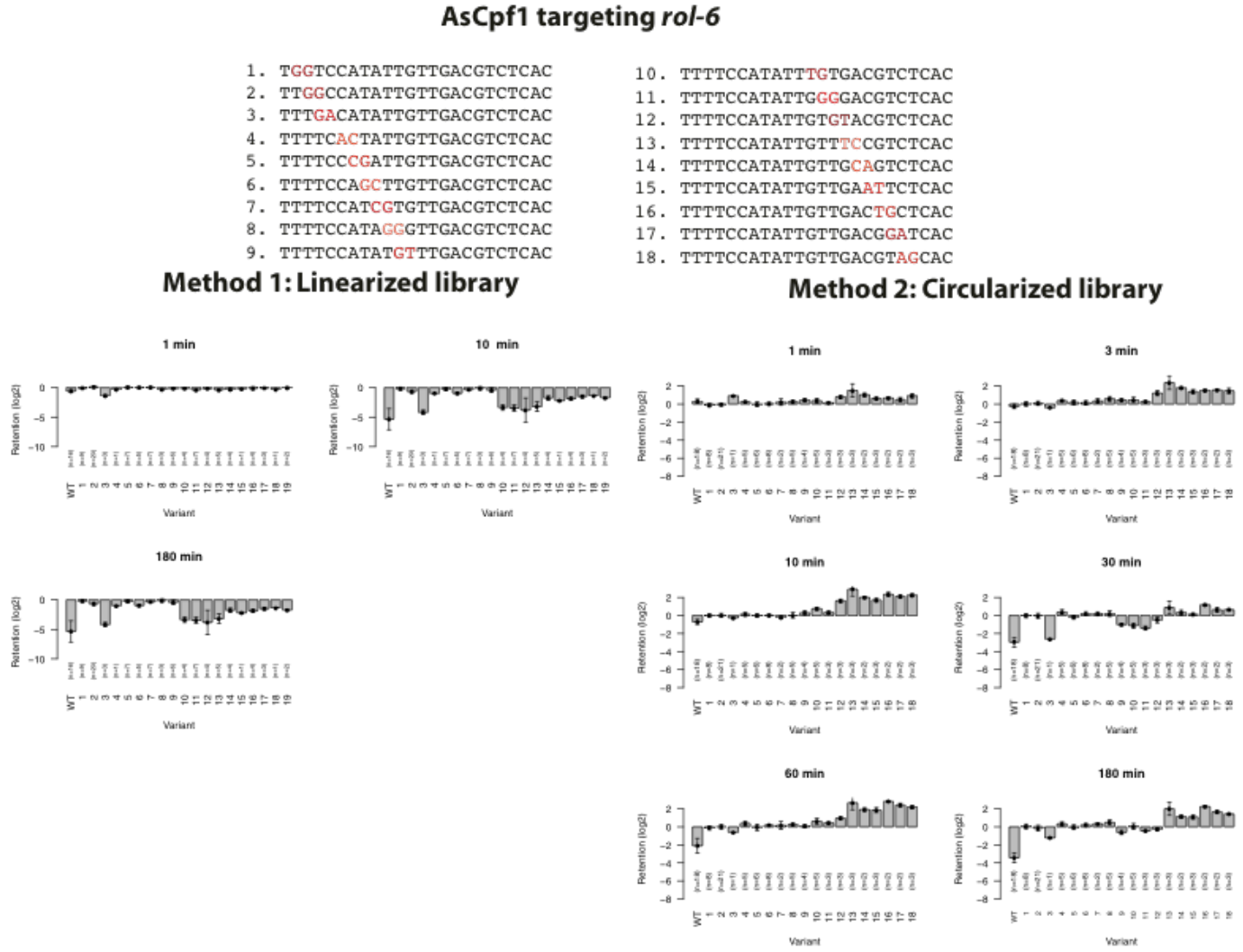


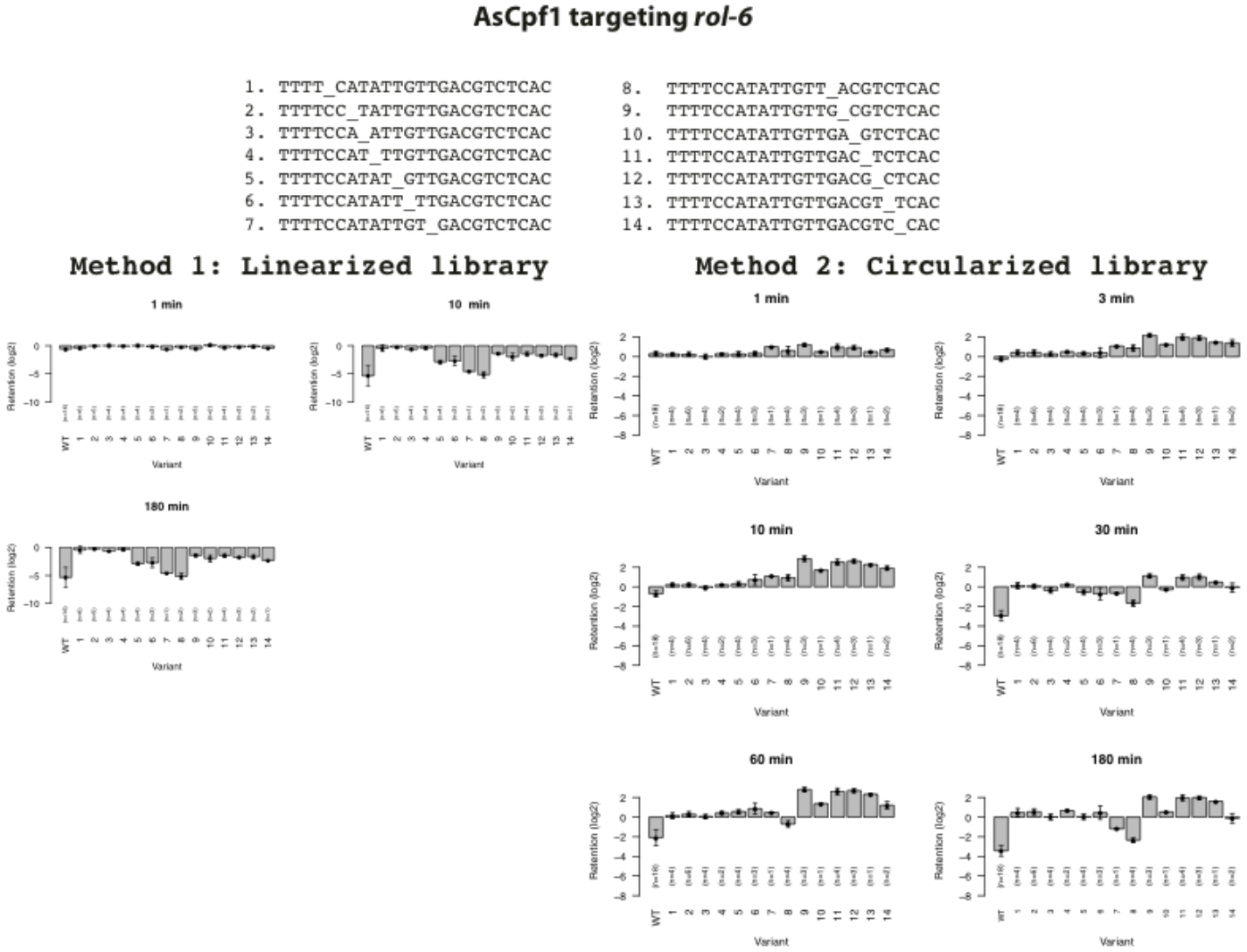
Figure 16) Results from AsCpf1 interaction with pooled *rol-6* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Retention score profiles for whole-library assays with backbone cleavage step used (to avoid preferential recovery of nicked substrates; left panel) or not used (allowing preferential recovery of nicked substrates; right panel) for *rol-6* single base transversions. X axis shows the positions, controls, and WT. Y axis shows the retention score. Refer to “Materials and Methods” for full conditions and retention calculations. (Method 1-Linearized library; AF_SOL_821, Method 2-Circular library; AF_SOL_810; NEB buffer 3.1)



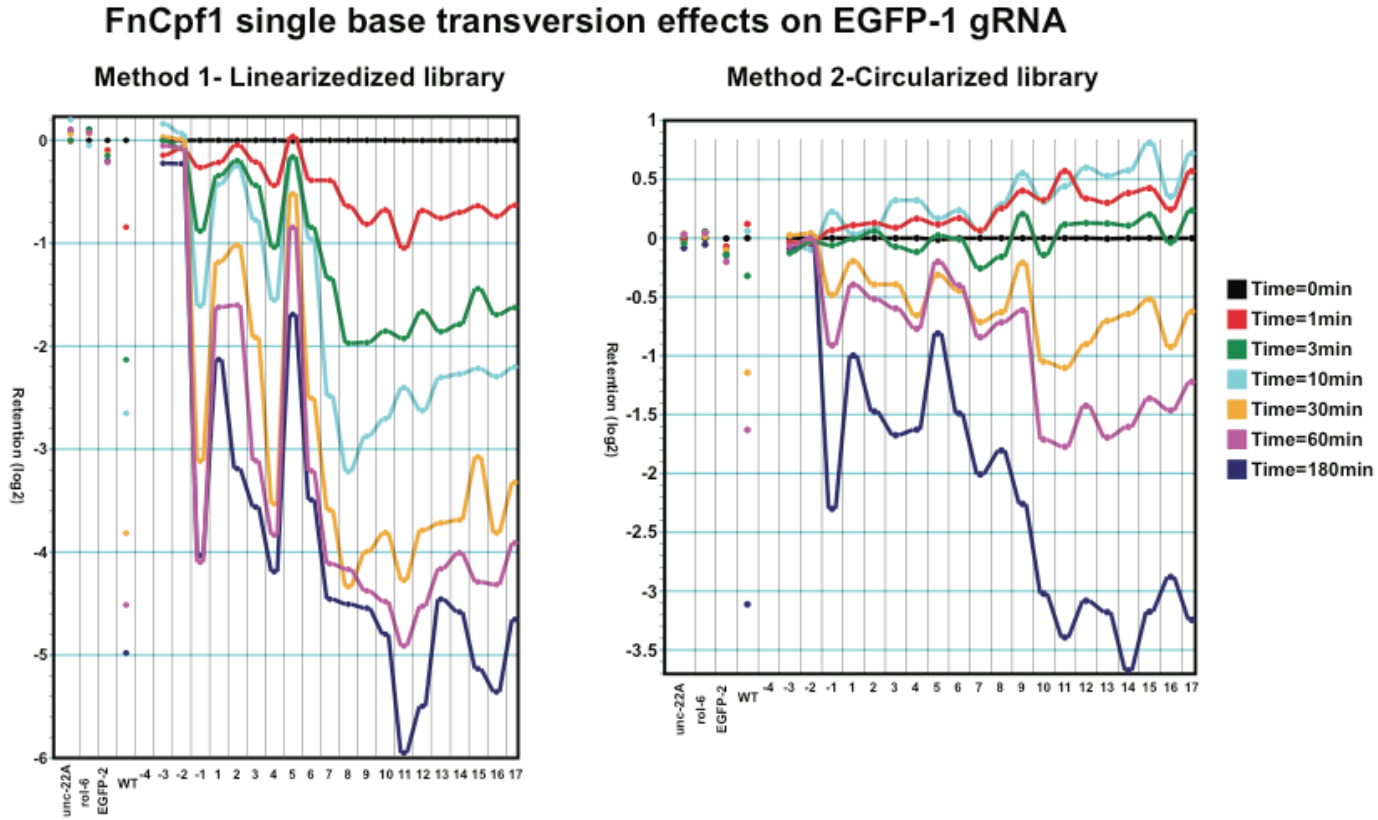
Supplementary Figure 17) Results from AsCpf1 interaction with pooled *rol-6* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



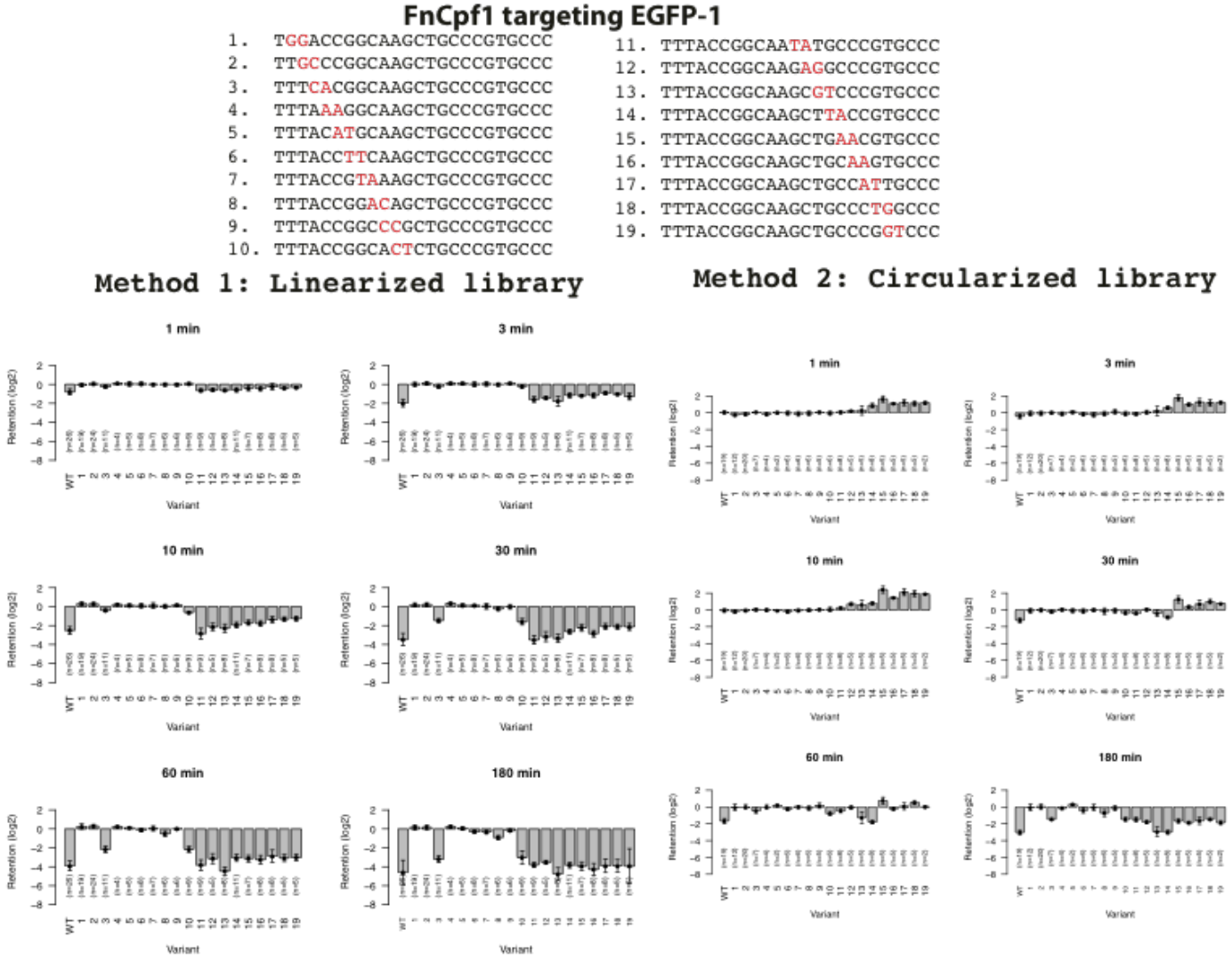
Supplementary Figure 18) Results from AsCpf1 interaction with pooled *rol-6* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”.Bar graphs of median retention scores for single-base-deletion variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



Supplementary Figure 19) Results from FnCpf1 interaction with pooled *EGFP-1* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Retention score profiles for whole-library assays with backbone cleavage step used (to avoid preferential recovery of nicked substrates; left panel) or not used (allowing preferential recovery of nicked substrates; right panel) for EGFP-1 single base transversions. X axis shows the positions, controls, and WT. Y axis shows the retention score. Refer to “Materials and Methods” for full conditions and retention calculations. (Method 1-Linearized library; AF_SOL_820, Method 2-Circular library; AF_SOL_811; NEB buffer 3.1).



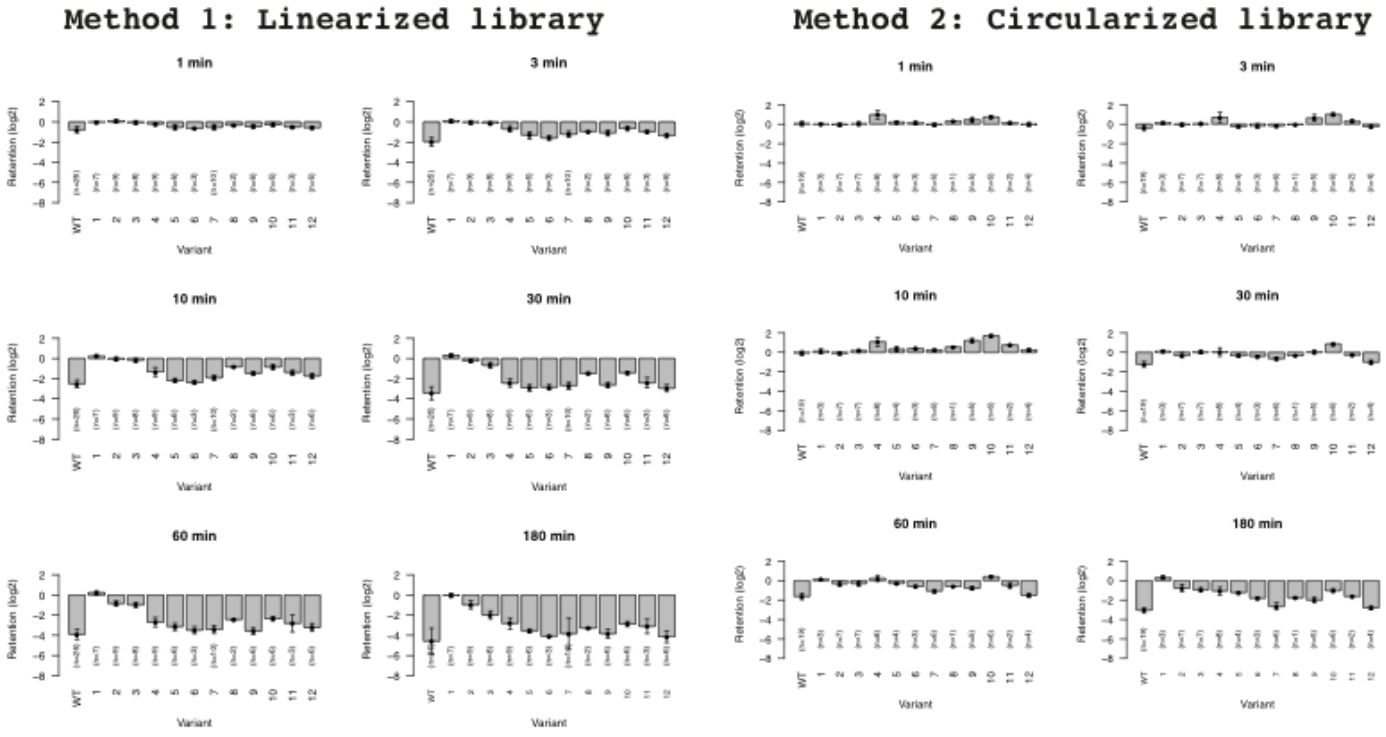
Supplementary Figure 20) Results from FnCpf1 interaction with pooled *EGFP-1* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



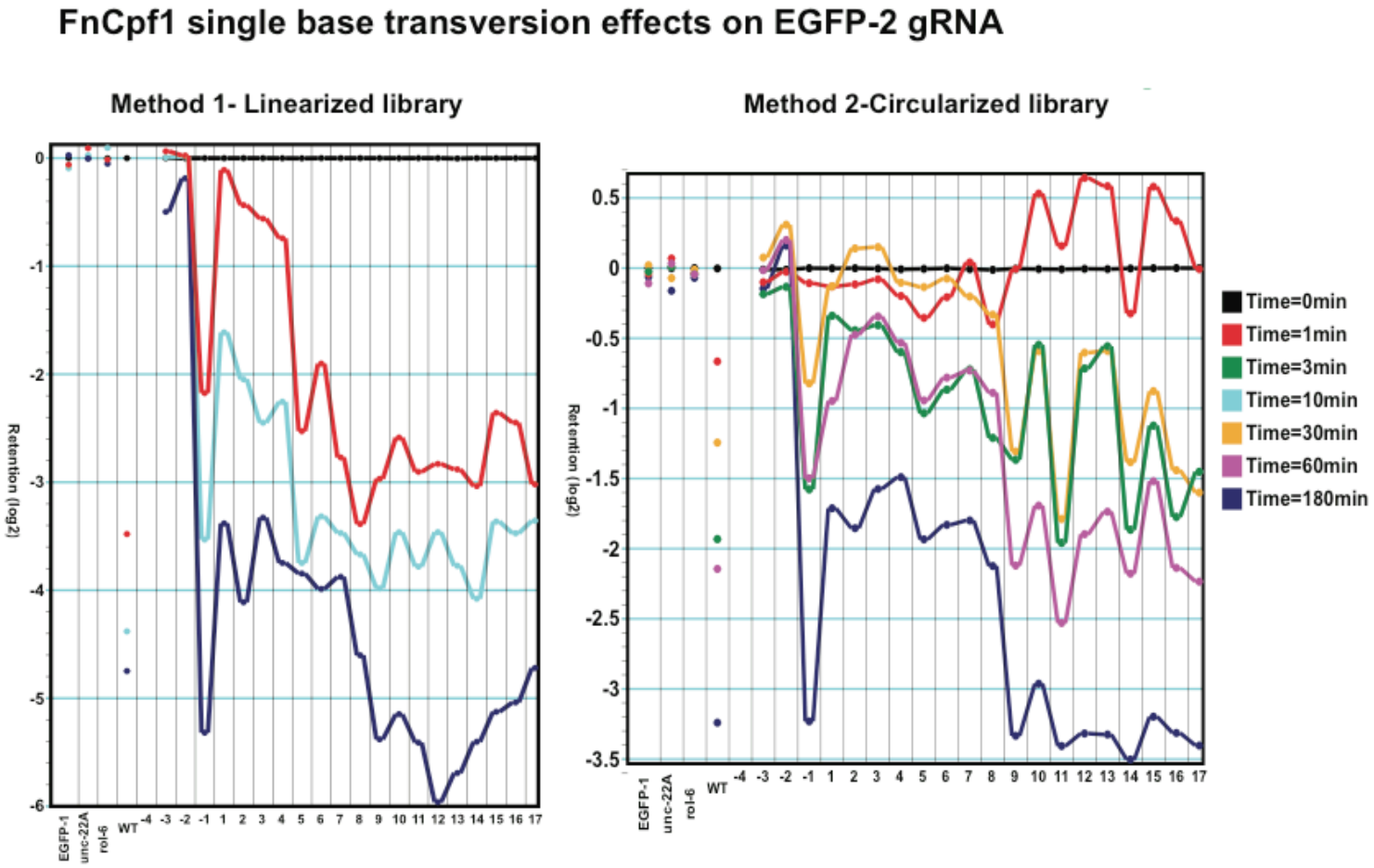
Supplementary Figure 21) Results from FnCpf1 interaction with pooled *EGFP-1* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for single-base-deletion variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.

FnCpf1 targeting EGFP-1

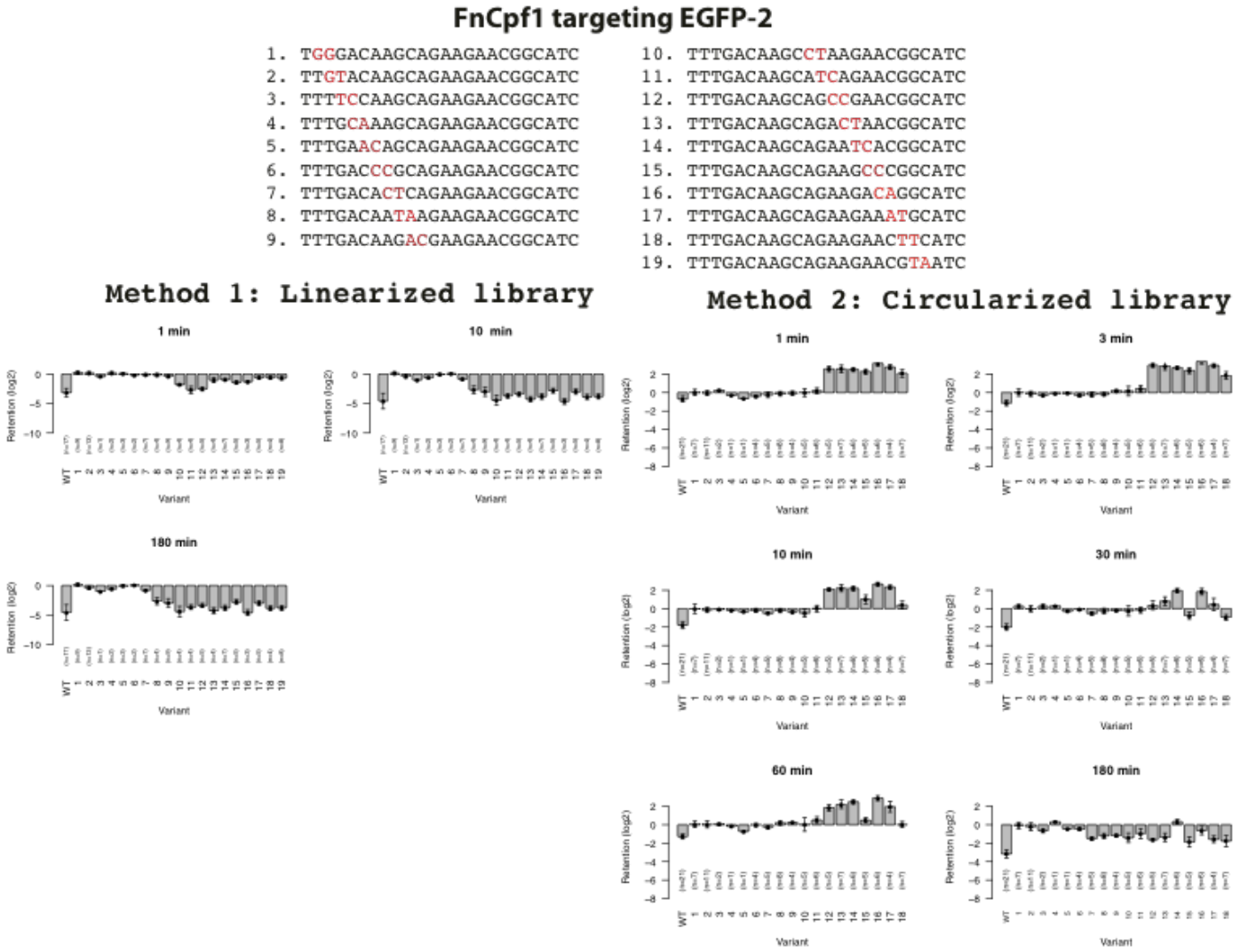
1. TTTA_CGGCAAGCTGCCCGTGCCC
2. TTTACC_GCAAGCTGCCCGTGCCC
3. TTTACCGG_AAGCTGCCCGTGCCC
4. TTTACCGGC_AGCTGCCCGTGCCC
5. TTTACCGGCAA_CTGCCCGTGCCC
6. TTTACCGGCAAG_TGCCCGTGCCC
7. TTTACCGGCAAGC_GCCCGTGCCC
8. TTTACCGGCAAGCT_CCCGTGCCC
9. TTTACCGGCAAGCTGCC_GTGCCC
10. TTTACCGGCAAGCTGCCC_TGCCC
11. TTTACCGGCAAGCTGCCCG_GCCC
12. TTTACCGGCAAGCTGCCCGT_CCC



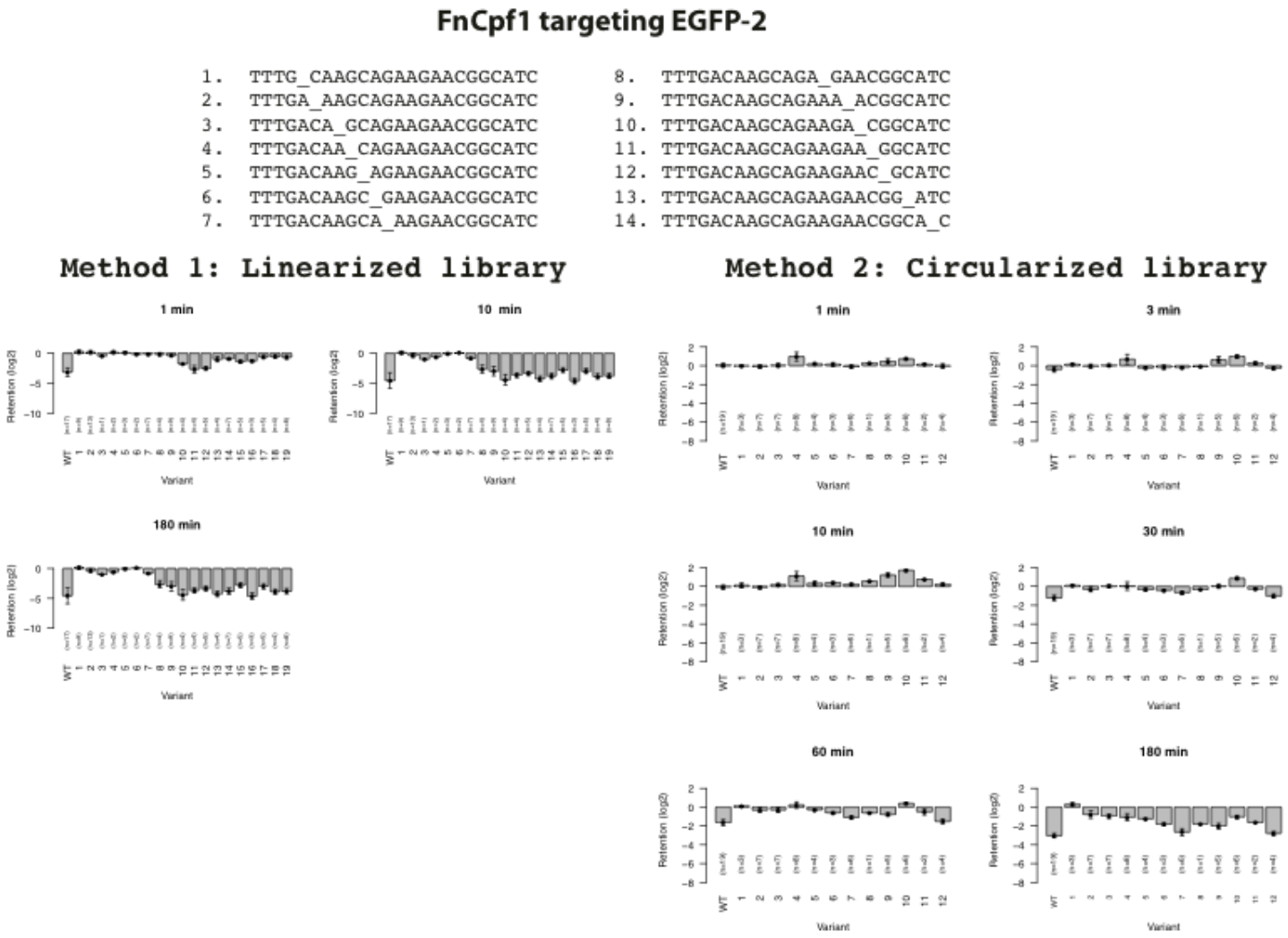
Supplementary Figure 22) Results from FnCpf1 interaction with pooled *EGFP-2* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Retention score profiles for whole-library assays with backbone cleavage step used (to avoid preferential recovery of nicked substrates; left panel) or not used (allowing preferential recovery of nicked substrates; right panel) for EGFP-2 single base transversions. X axis shows the positions, controls, and WT. Y axis shows the retention score. Refer to “Materials and Methods” for full conditions and retention calculations. (Method 1-Linearized library; AF_SOL_821, Method 2-Circular library; AF_SOL_811; NEB buffer 3.1).



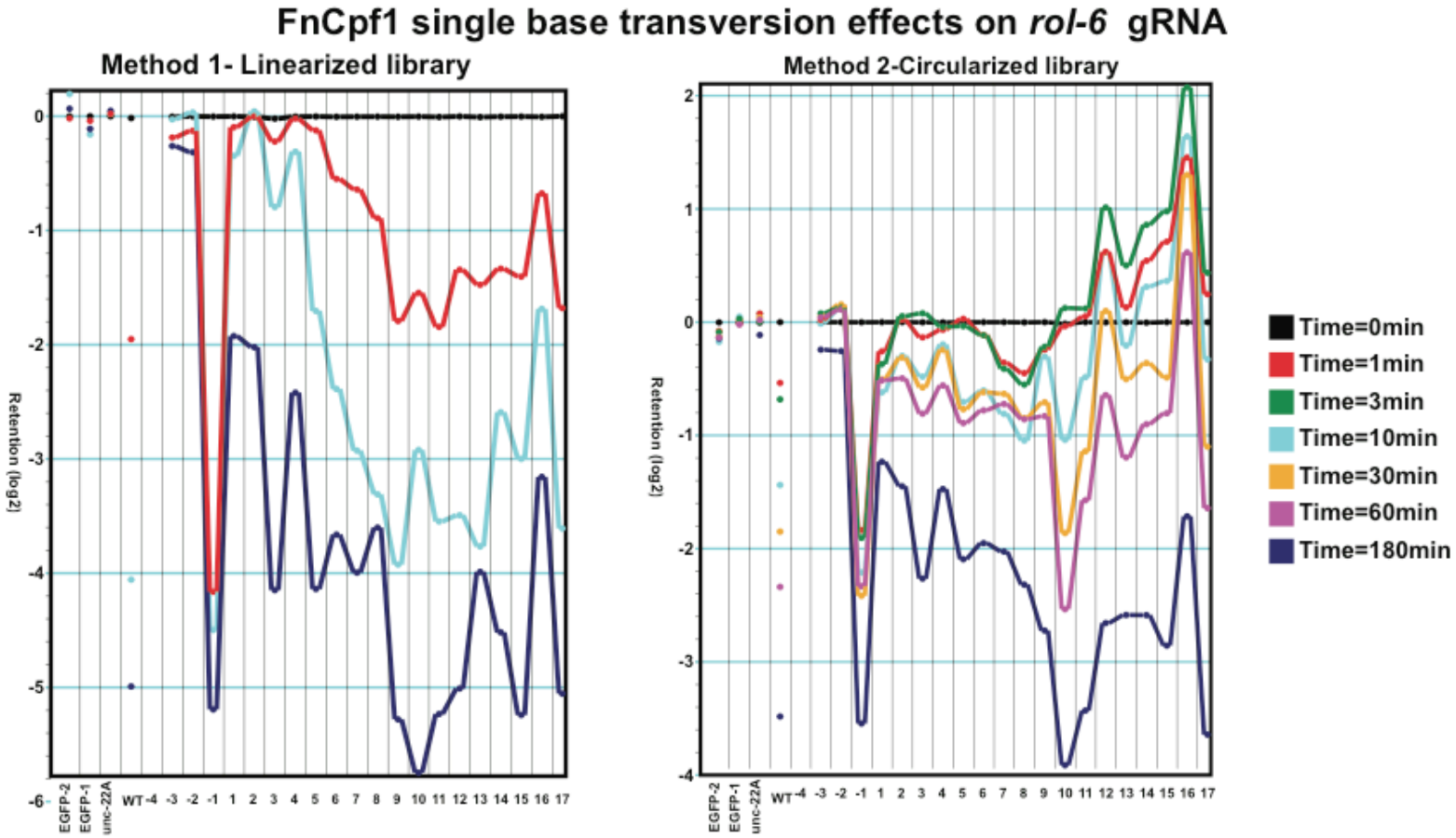
Supplementary Figure 23) Results from FnCpf1 interaction with pooled *EGFP-2* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



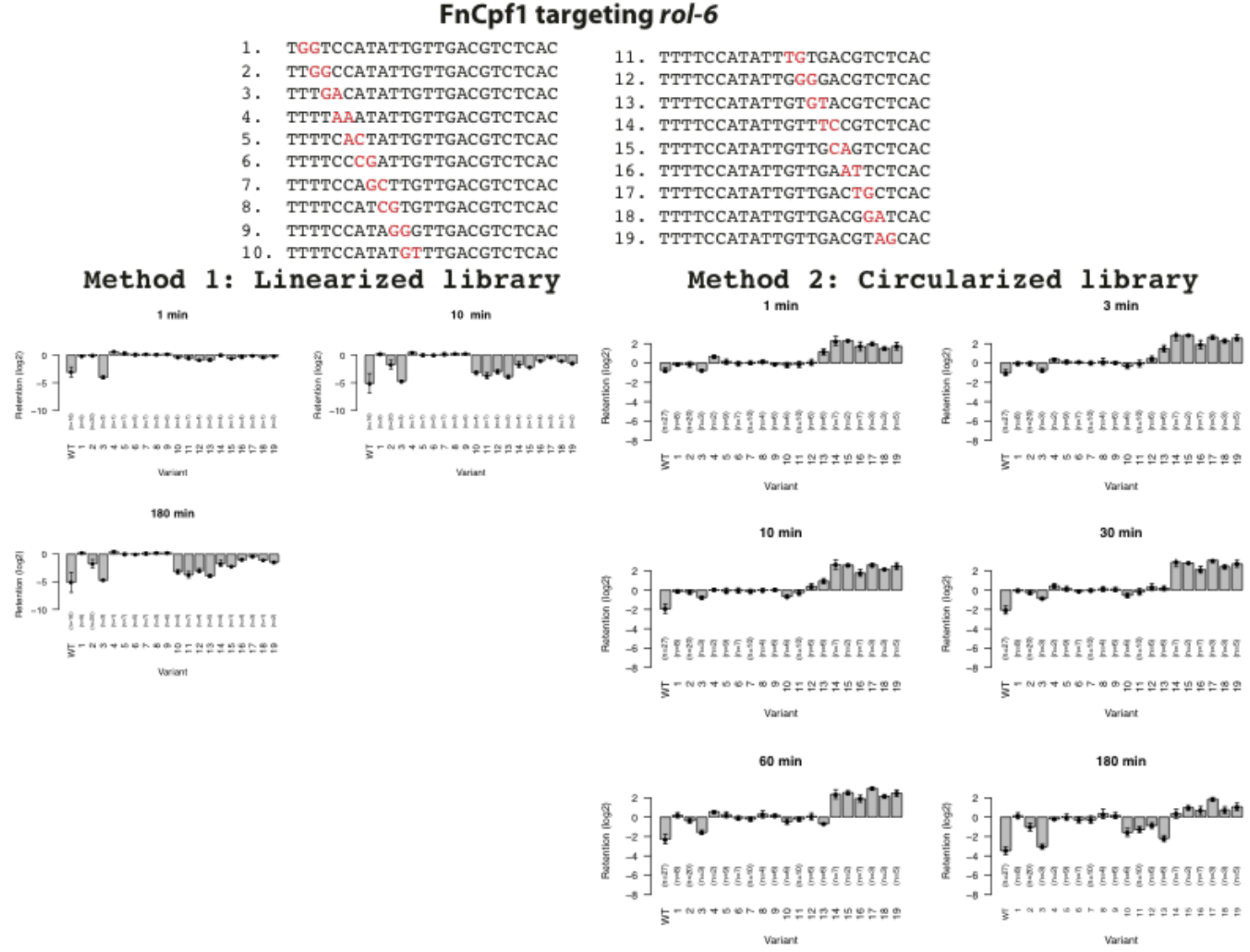
Supplementary Figure 24) Results from FnCpf1 interaction with pooled *EGFP-2* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for single-base-deletion variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



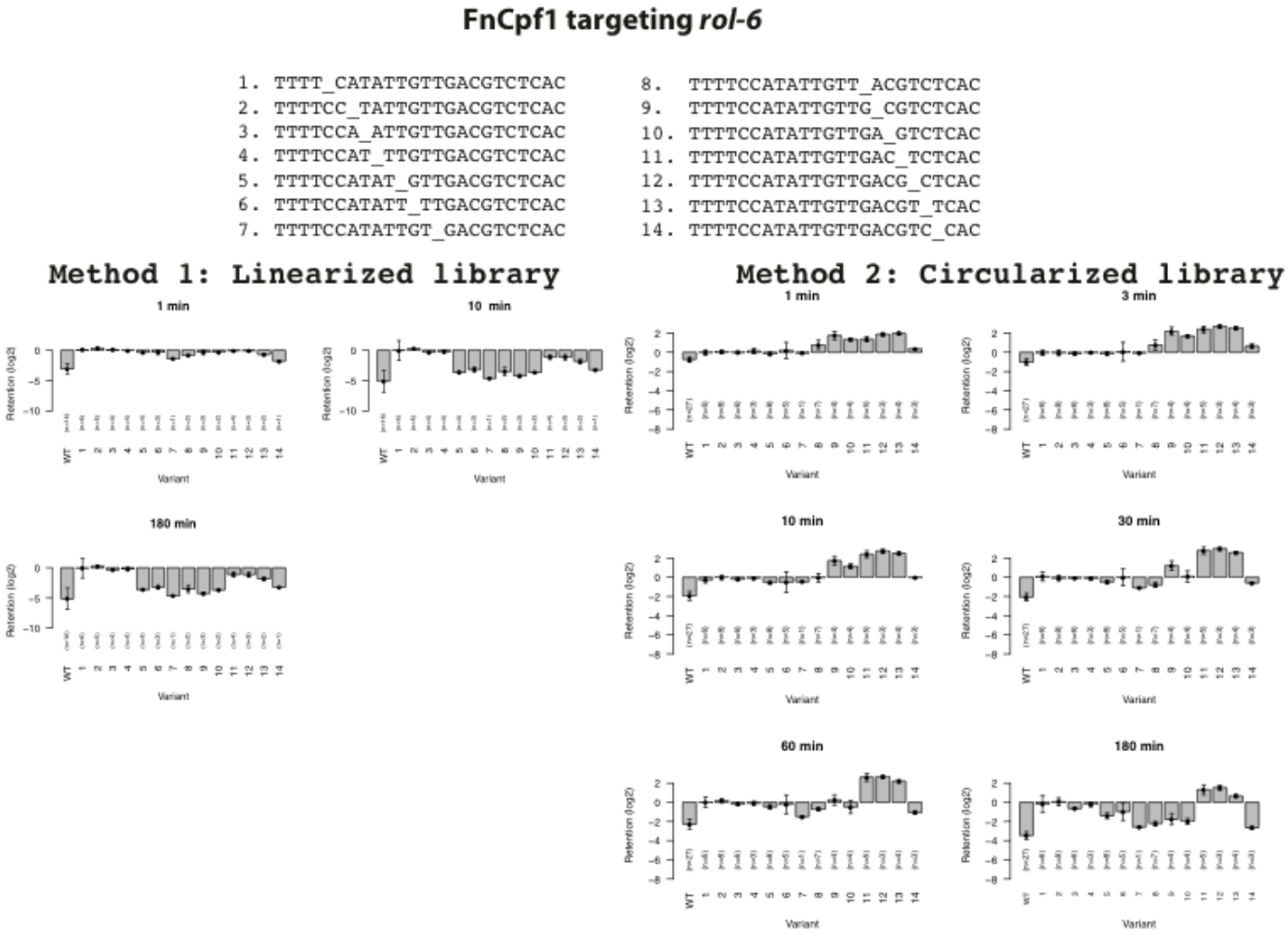
Supplementary Figure 25) Results from FnCpf1 interaction with pooled *rol-6* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Retention score profiles for whole-library assays with backbone cleavage step used (to avoid preferential recovery of nicked substrates; left panel) or not used (allowing preferential recovery of nicked substrates; right panel) for *rol-6* single base transversions. X axis shows the positions, controls, and WT. Y axis shows the retention score. Refer to “Materials and Methods” for full conditions and retention calculations.



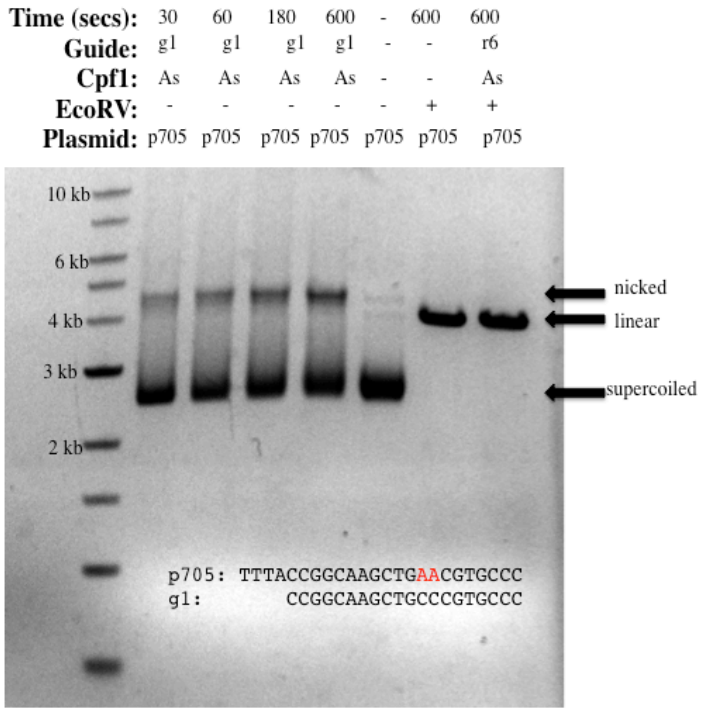
Supplementary Figure 26) Results from FnCpf1 interaction with pooled *rol-6* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.

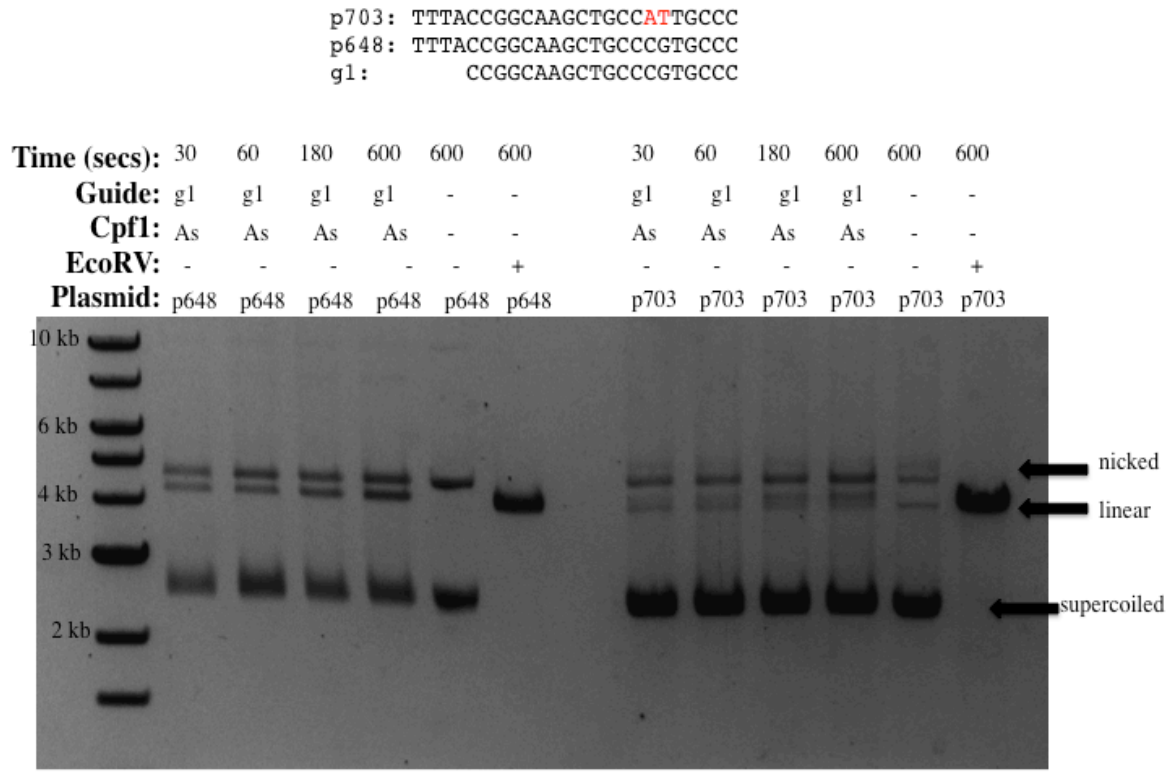


Supplementary Figure 27) Results from FnCpf1 interaction with pooled *rol-6* targets. All reactions were done with NEB buffer 3.1 and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for single-base-deletion variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.

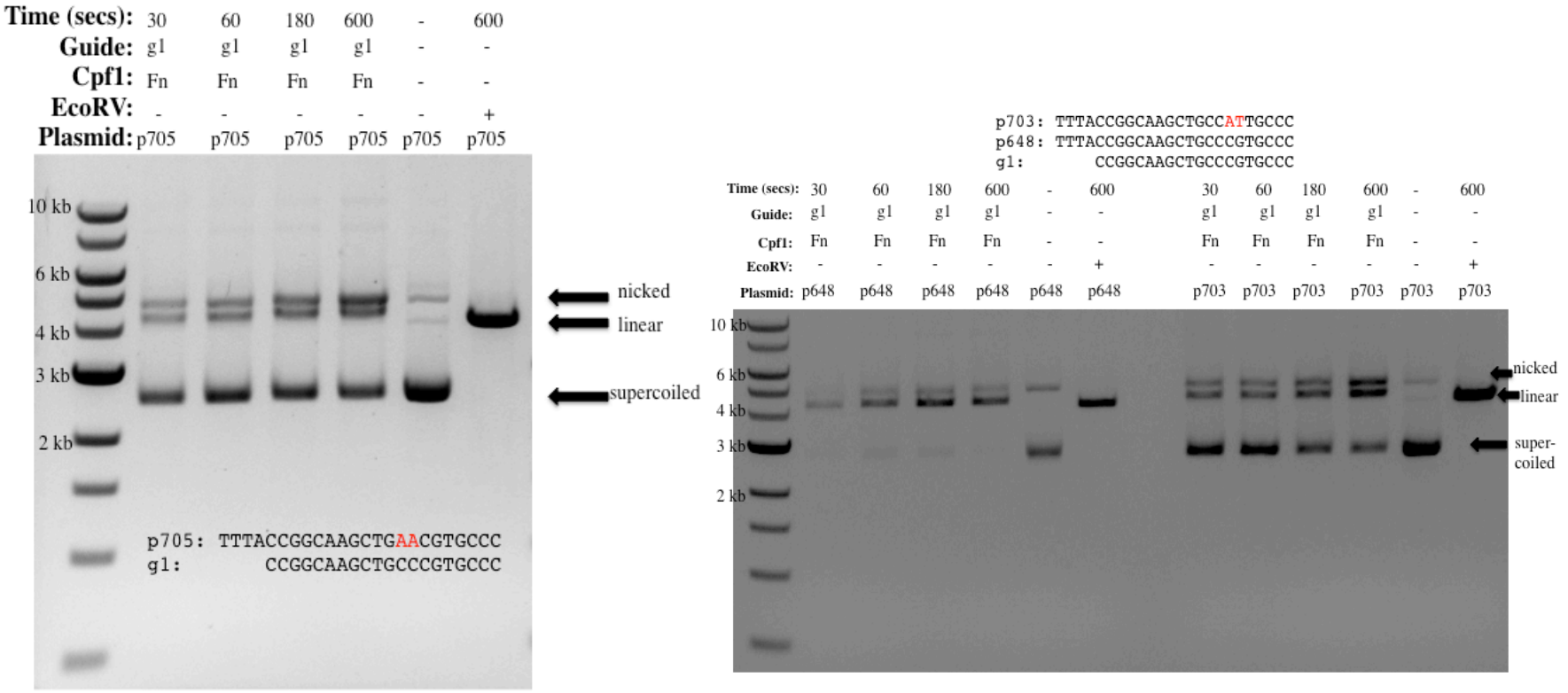


Supplementary Figure 28) AsCpf1 nicking assessments using agarose gel electrophoresis. AsCpf1 nicking assessments on individual circular plasmid targets, assessed using agarose gel electrophoresis in the presence of Ethidium Bromide intercalator. All experiments done with 50 ng of enzyme with 1:1 concentration of gRNA:protein and at least 100 ng of target plasmid DNA (supercoiled) in NEB buffer 3.1 at 37°C. Ethidium bromide staining of DNA allows visualization of electrophorectic mobility, with a 1kb ladder (left on each gel) used to determine relative mobility of linearized fragments as a function of size. Inferred mobilities of nicked, linear, and supercoiled target plasmids are indicated with arrows at the right of each gel. Sequences shown are of the target-homologous region of each plasmid and of the corresponding guide homology segment. All nicking experiments were done at least twice with similar results

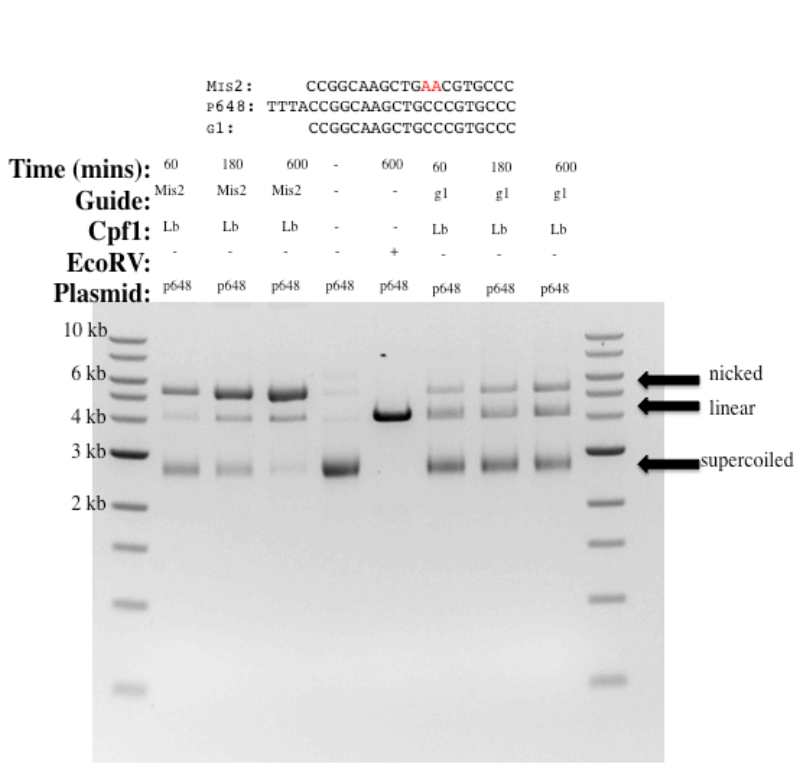


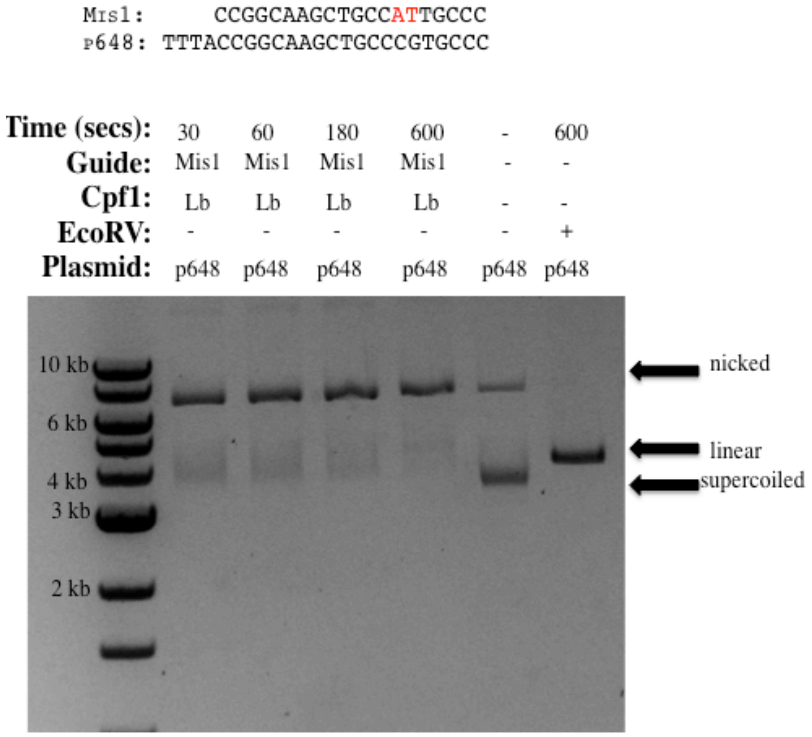


Supplementary Figure 29) FnCpf1 assessments using agarose gel electrophoresis. FnCpf1 nicking assessments on individual circular plasmid targets, assessed using agarose gel electrophoresis in the presence of Ethidium Bromide intercalator. All experiments done with 50 ng of enzyme with 1:1 concentration of gRNA:protein and at least 100 ng of target plasmid DNA (supercoiled) in NEB buffer 3.1 at 37°C. Ethidium bromide staining of DNA allows visualization of electrophorectic mobility, with a 1kb ladder (left on each gel) used to determine relative mobility of linearized fragments as a function of size. Inferred mobilities of nicked, linear, and supercoiled target plasmids are indicated with arrows at the right of each gel. Sequences shown are of the target-homologous region of each plasmid and of the corresponding guide homology segment. All nicking experiments were done at least twice with similar results



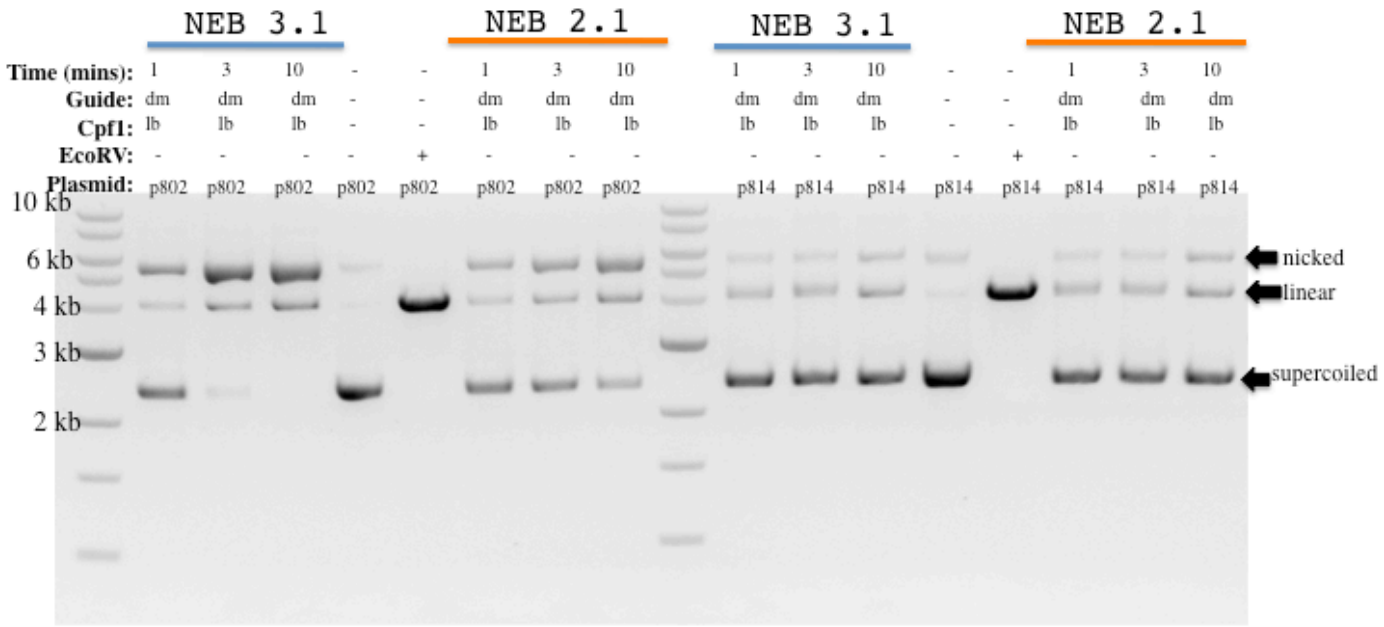
Supplementary Figure 30) LbCpf1 agarose gel nicking assessment with equivalent mismatches in EGFP-1 gRNA paired with WT EGFP-1 target. LbCpf1 nicking assessments on individual circular plasmid targets, assessed using agarose gel electrophoresis in the presence of Ethidium Bromide intercalator. All experiments done with 50 ng of enzyme with 1:1 concentration of gRNA:protein and at least 100 ng of target plasmid DNA (supercoiled) in NEB buffer 3.1 at 37°C. Ethidium bromide staining of DNA allows visualization of electrophorectic mobility, with a 1kb ladder (left on each gel) used to determine relative mobility of linearized fragments as a function of size. Inferred mobilities of nicked, linear, and supercoiled target plasmids are indicated with arrows at the right of each gel. Sequences shown are of the target-homologous region of each plasmid and of the corresponding guide homology segment. All nicking experiments were done at least twice with similar results



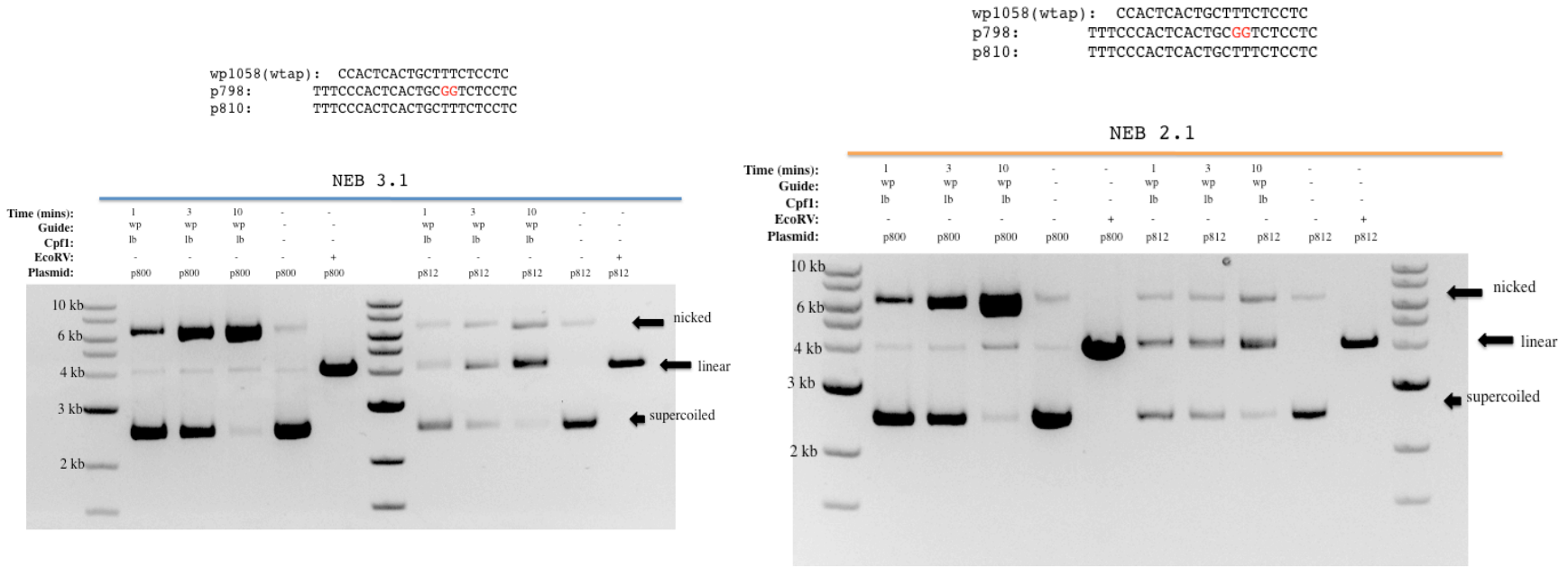


Supplementary Figure 31) Analysis of Cpf1 activities on additional target sequences. A series of additional gRNA/target combinations were tested using individual agarose gel migration assays. These were targets where no high throughput data was available. Instead, we used general rules from *unc-22A*, EGFP-1, and *rol-6* to design potential nicking guides. Sequences of guide homology regions and targets were as shown in figure. All nicking gel experiments were done at least twice with similar results. LbCpf1 agarose gel nicking assays for DNMT1 targets with NEB buffer 2.1 and 3.1 incubated at 37°C.

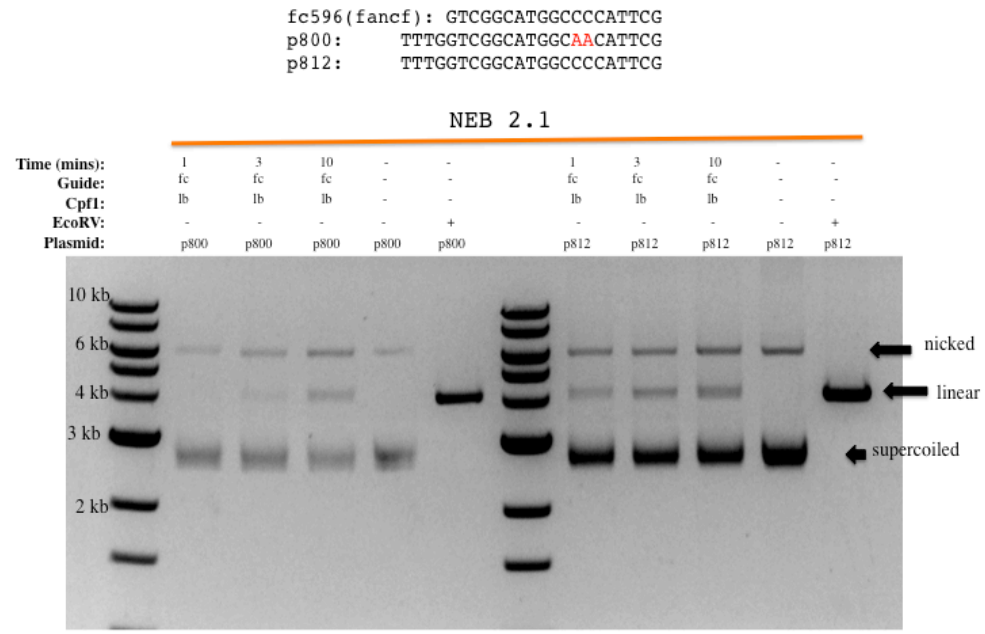
```
dm22085(dmnt1): TTTCCCTTCAGCTAAAATAA
p802:           TTTATTTCCCTTCAGAGAAAATAA
p814:           TTTATTTCCCTTCAGCTAAAATAA
```



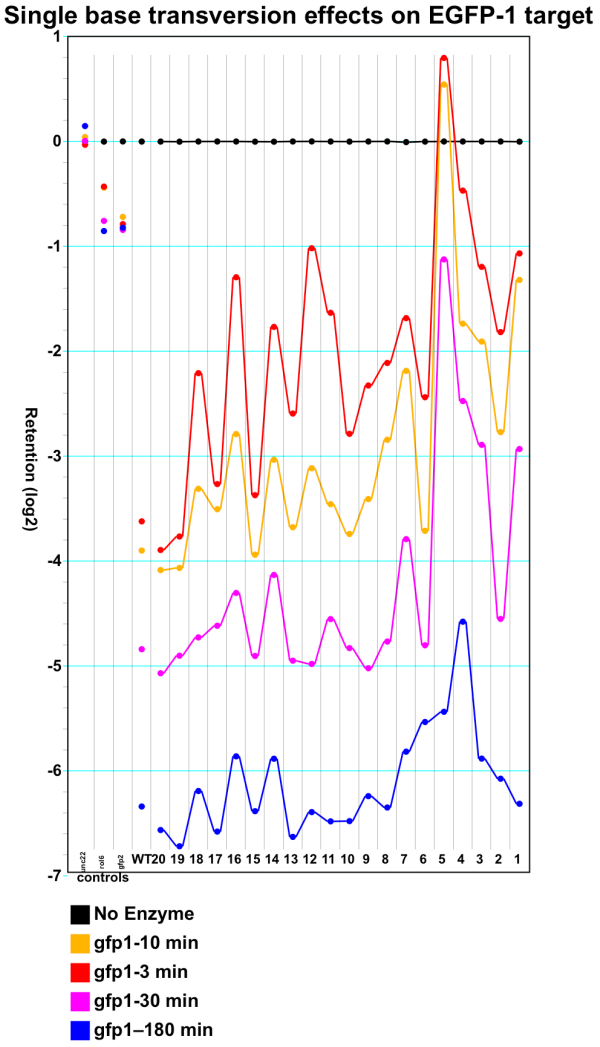
Supplementary Figure 32) LbCpf1 agarose gel nicking assays for WTAP exon8 targets with NEB buffer 2.1 and 3.1 incubated at 37°C. Analysis of Cpf1 activities on additional target sequences. A series of additional gRNA/target combinations were tested using individual agarose gel migration assays. These were targets where no high throughput data was available. Instead, we used general rules from *unc-22A*, EGFP-1, and *rol-6* to design potential nicking guides. Sequences of guide homology regions and targets were as shown in figure. All nicking gel experiments were done at least twice with similar results.



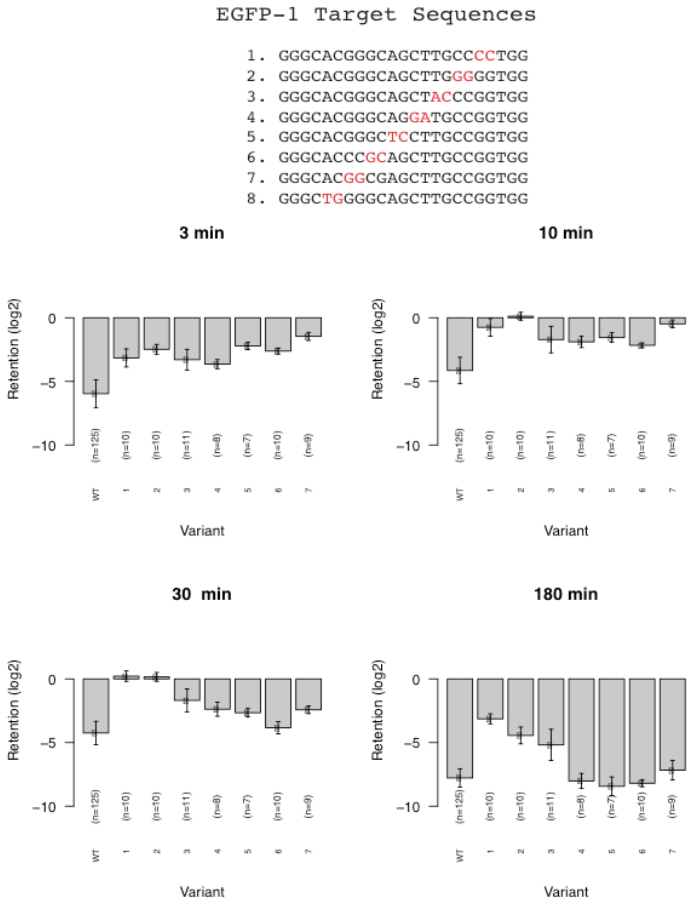
Supplementary Figure 33) LbCpf1 agarose gel nicking assays for Fancf targets with NEB buffer 2.1 incubated at 37°C. Analysis of Cpf1 activities on additional target sequences. A series of additional gRNA/target combinations were tested using individual agarose gel migration assays. These were targets where no high throughput data was available. Instead, we used general rules from *unc-22A*, EGFP-1, and *rol-6* to design potential nicking guides. Sequences of guide homology regions and targets were as shown in figure. All nicking gel experiments were done at least twice with similar results.



Supplementary Figure 34) Results from Cas9 interaction with pooled *EGFP-1* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Cas9 high throughput nicking assay with Type II libraries for EGFP-1 (Method 2-Circular Library; AF_SOL_854). Retention scores are shown for whole-library assays with backbone cleavage step used to avoid preferential recovery of nicked substrates for EGFP-1 target single base transversions. X axis shows the positions, controls, and wild type retention (WT—full target match), negative controls (no target match) and individual transversions through the full sequence. Y axis shows the retention score. Refer to “Materials and Methods” for full conditions and retention calculations.



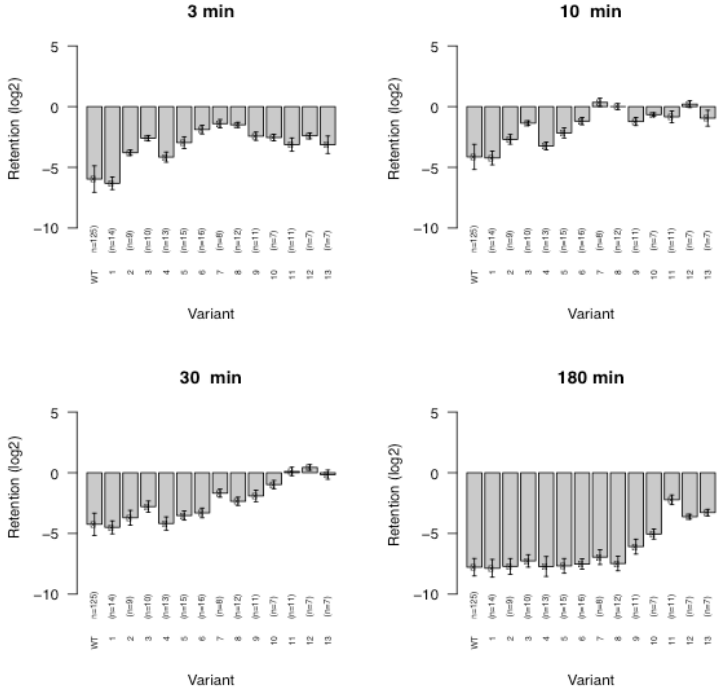
Supplementary Figure 35) Results from Cas9 interaction with pooled *EGFP-1* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Cas9 high throughput nicking assay with Type II libraries for EGFP-1 (Method 2-Circular Library; AF_SOL_854). Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



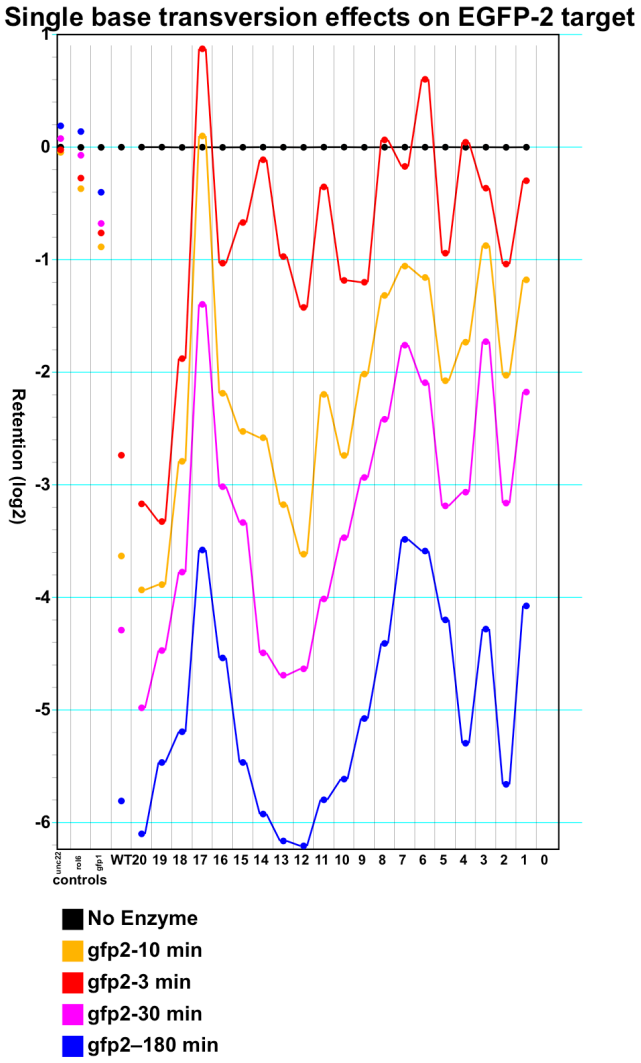
Supplementary Figure 36) Results from Cas9 interaction with pooled *EGFP-1* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Cas9 high throughput nicking assay with Type II libraries for EGFP-1 (Method 2-Circular Library; AF_SOL_854). Bar graphs of median retention scores for single deletion variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.

EGFP-1 Target Sequences

1. GG_CACGGGCAGCTTGCCGGTGG
2. GGG_ACGGGCAGCTTGCCGGTGG
3. GGGC_CGGGCAGCTTGCCGGTGG
4. GGGCA_GGGCAGCTTGCCGGTGG
5. GGGCAC_GGCAGCTTGCCGGTGG
6. GGGCACGGG_AGCTTGCCGGTGG
7. GGGCACGGGC_GCTTGCCGGTGG
8. GGGCACGGGCA_CTTGCCGGTGG
9. GGGCACGGGCAG_TTGCCGGTGG
10. GGGCACGGGCAGC_TGCCGGTGG
11. GGGCACGGGCAGCTT_CCGGTGG
12. GGGCACGGGCAGCTTG_CGGTGG
13. GGGCACGGGCAGCTTGCC_GTGG



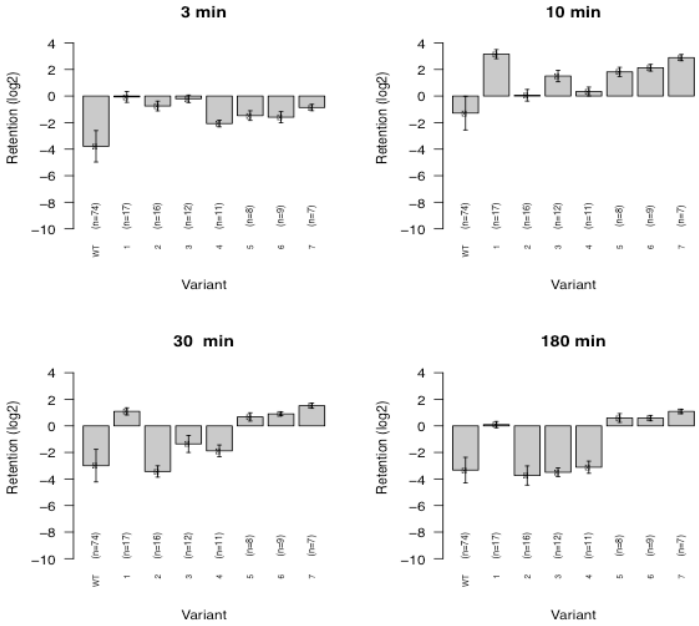
Supplementary Figure 37) Results from Cas9 interaction with pooled *EGFP-2* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”.Cas9 high throughput nicking assay with Type II libraries for EGFP-2 (Method 2-Circular Library; AF_SOL_854). Retention scores are shown for whole-library assays with backbone cleavage step used to avoid preferential recovery of nicked substrates for EGFP-2 target single base transversions. X axis shows the positions, controls, and wild type retention (WT—full target match), negative controls (no target match) and individual transversions through the full sequence. Y axis shows the retention score. Refer to “Materials and Methods” for full conditions and retention calculations.



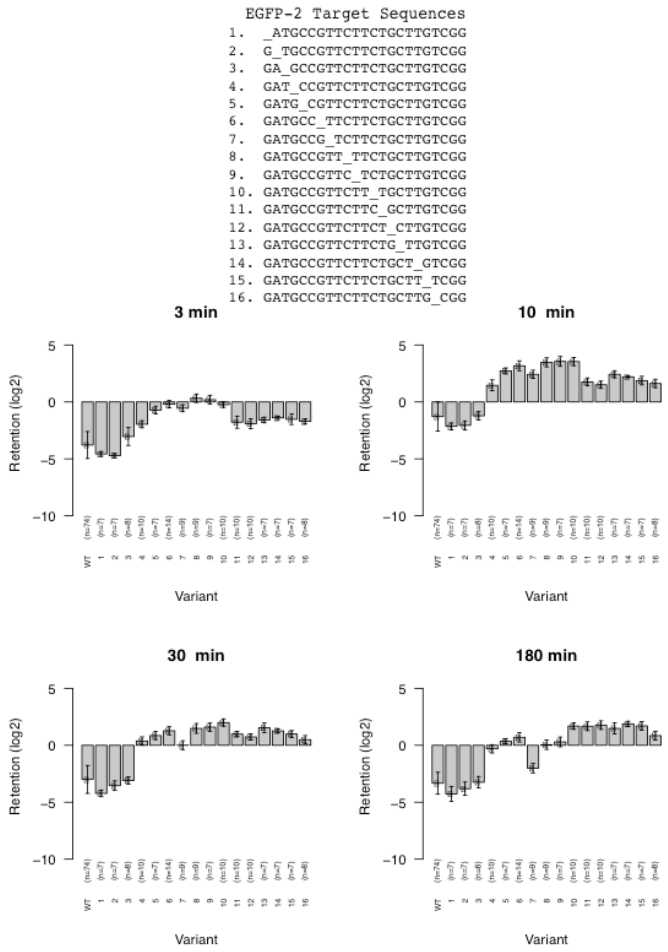
Supplementary Figure 38) Results from Cas9 interaction with pooled *EGFP-2* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”.Cas9 high throughput nicking assay with Type II libraries for EGFP-2 (Method 2-Circular Library; AF_SOL_854). Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.

EGFP-2 Target Sequences

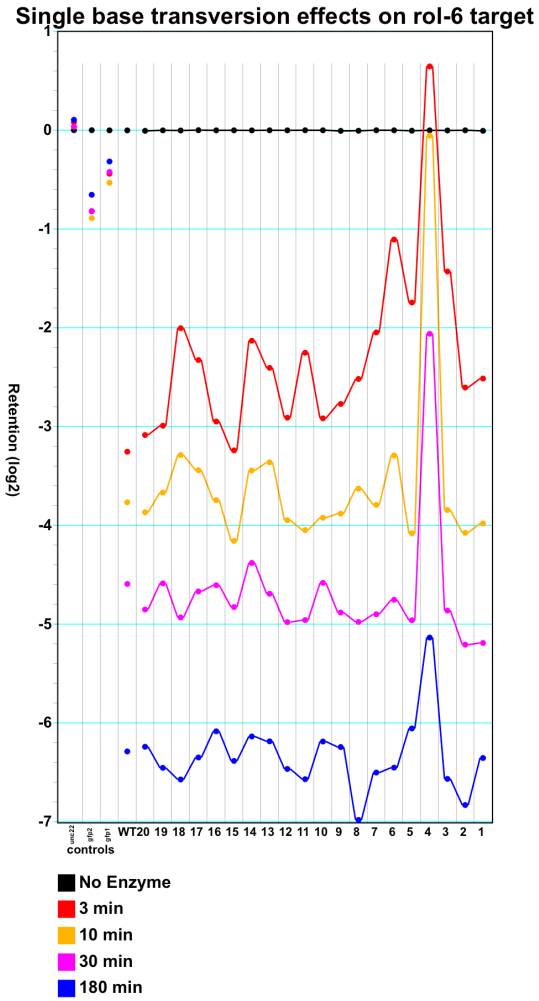
1. GATGGGTTCTTCTGCTTGTCGG
2. GATGCCCATCTTCTGCTTGTCGG
3. GATGCCGTAGTTCTGCTTGTCGG
4. GATGCCGTTCAACTGCTTGTCGG
5. GATGCCGTTCTTGAGCTTGTCGG
6. GATGCCGTTCTTCTCGTTGTCGG
7. GATGCCGTTCTTCTGCAAGTCGG
8. GATGCCGTTCTTCTGCTTCACGG



Supplementary Figure 39) Results from Cas9 interaction with pooled *EGFP-2* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”.Cas9 high throughput nicking assay with Type II libraries for EGFP-2 (Method 2-Circular Library; AF_SOL_854). Bar graphs of median retention scores for single deletion variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.



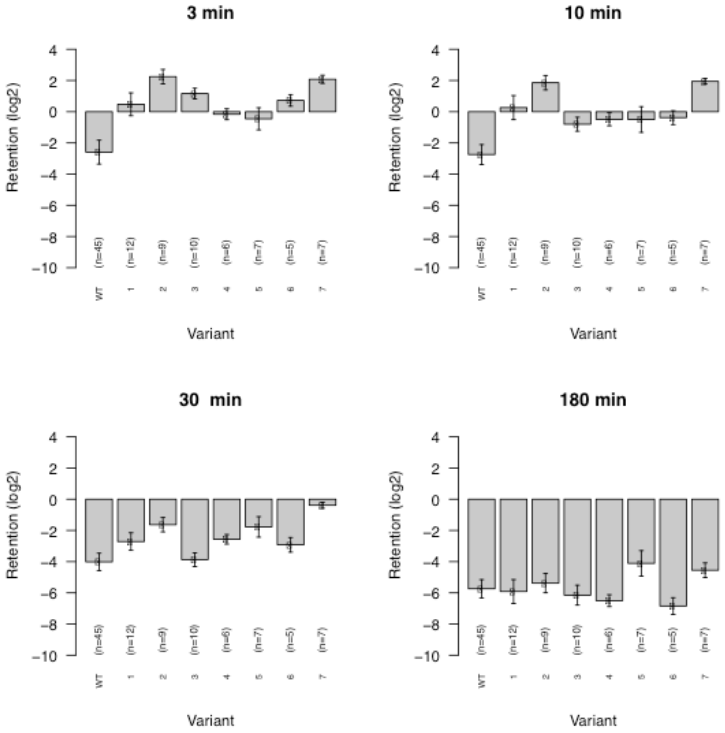
Supplementary Figure 40) Results from Cas9 interaction with pooled *rol-6* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Cas9 high throughput nicking assay with Type II libraries for *rol-6*. (Method 2-Circular Library; AF_SOL_854). Retention scores are shown for whole-library assays with backbone cleavage step used to avoid preferential recovery of nicked substrates for *rol-6* target single base transversions. X axis shows the positions, controls, and wild type retention (WT—full target match), negative controls (no target match) and individual transversions through the full sequence. Y axis shows the retention score. Refer to “Materials and Methods” for full conditions and retention calculations.



Supplementary Figure 41) Results from Cas9 interaction with pooled *rol-6* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.

rol-6 Target sequences

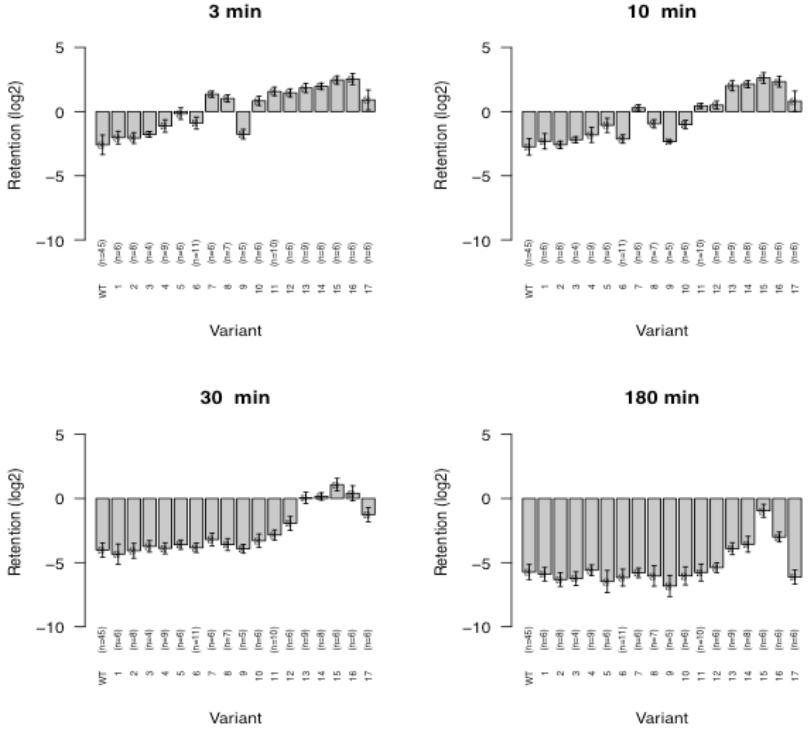
1. GTGACTCGTCAACAATATGGAGG
2. GTGAGAGCTCAACAATATGGAGG
3. GTGAGACGAGAACAATATGGAGG
4. GTGAGACGTCTTCAATATGGAGG
5. GTGAGACGTCAAGTATATGGAGG
6. GTGAGACGTCAACATAATGGAGG
7. GTGAGACGTCAACAATTAGGAGG
8. GTGAGACGTCAACAATATCCAGG



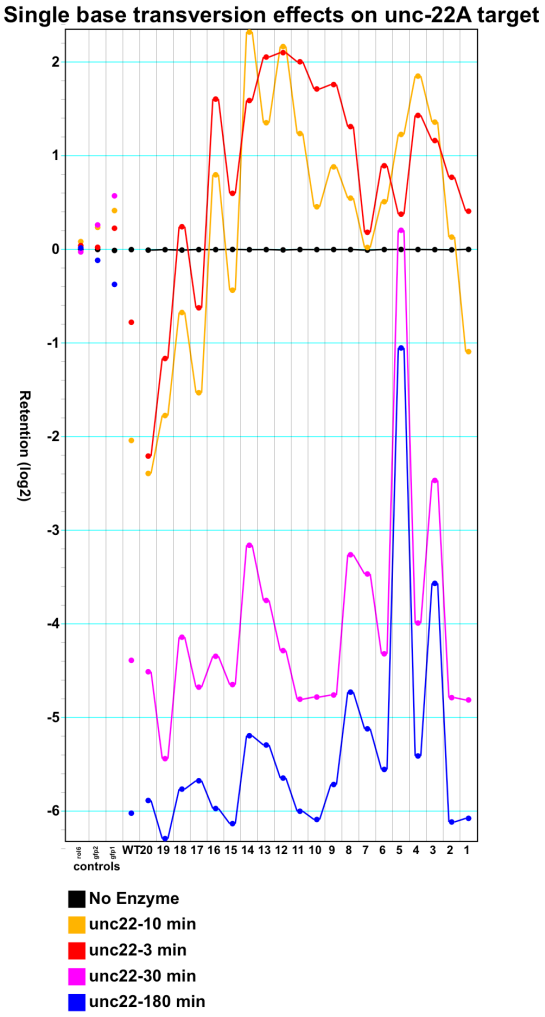
Supplementary Figure 42) Results from Cas9 interaction with pooled *rol-6* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.

rol-6 Target sequences

1. _TGAGACGTCAACAATATGGAGG
2. G_GAGACGTCAACAATATGGAGG
3. GT_AGACGTCAACAATATGGAGG
4. GTG_GACGTCAACAATATGGAGG
5. GTGA_ACGTCAACAATATGGAGG
6. GTGAG_CGTCAACAATATGGAGG
7. GTGAGA_GTCAACAATATGGAGG
8. GTGAGAC_TCAACAATATGGAGG
9. GTGAGACG_CAACAATATGGAGG
10. GTGAGACGT_AACAATATGGAGG
11. GTGAGACGTC_ACAATATGGAGG
12. GTGAGACGTCAA_AATATGGAGG
13. GTGAGACGTCAAC_ATATGGAGG
14. GTGAGACGTCAACAA_ATGGAGG
15. GTGAGACGTCAACAAT_TGGAGG
16. GTGAGACGTCAACAATA_GGAGG
17. GTGAGACGTCAACAATAT_GAGG



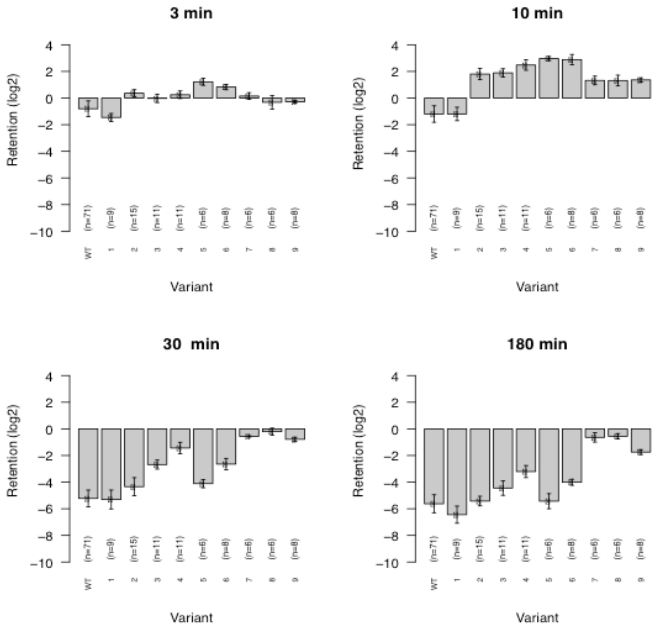
Supplementary Figure 43) Results from Cas9 interaction with pooled *unc-22A* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Cas9 high throughput nicking assay with Type II libraries for *unc-22A* (Method 2-Circular Library; AF_SOL_854). Retention scores are shown for whole-library assays with backbone cleavage step used to avoid preferential recovery of nicked substrates for *unc-22A* target single base transversions. X axis shows the positions, controls, and wild type retention (WT—full target match), negative controls (no target match) and individual transversions through the full sequence. Y axis shows the retention score. Refer to “Materials and Methods” for full conditions and retention calculations.



Supplementary Figure 44) Results from Cas9 interaction with pooled *unc-22A* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Cas9 high throughput nicking assay with Type II libraries for *unc-22A* (Method 2-Circular Library; AF_SOL_854). Bar graphs of median retention scores for double consecutive variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.

unc-22A Target Sequences

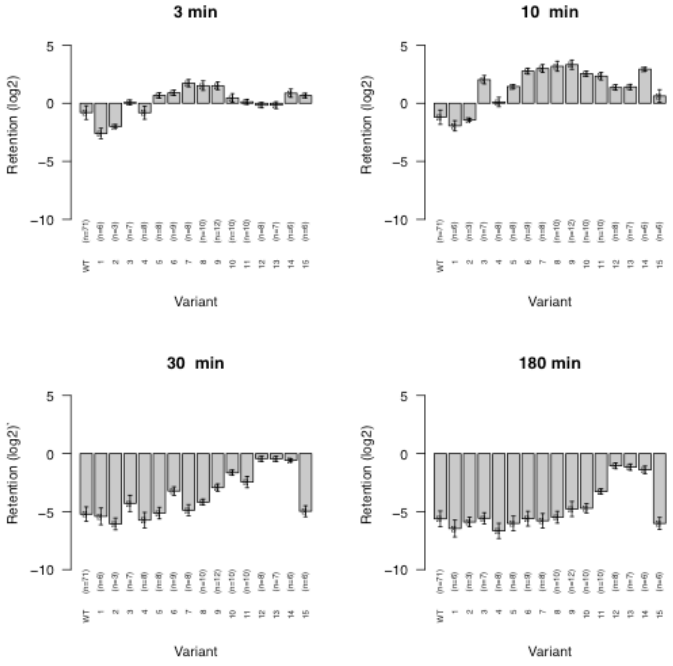
1. CGACCACCGTCGCCGGCATTTGG
2. GCTGCACCGTCGCCGGCATTTGG
3. GCACGTCCGTCGCCGGCATTTGG
4. GCACCAGGGTCGCCGGCATTTGG
5. GCACCACCCACGCCGGCATTTGG
6. GCACCACCGTGCCCGGCATTTGG
7. GCACCACCGTCGGGGGCATTTGG
8. GCACCACCGTCGCCCCCATTTGG
9. GCACCACCGTCGCCGGGTTTTGG
10. GCACCACCGTCGCCGGCAAATGG



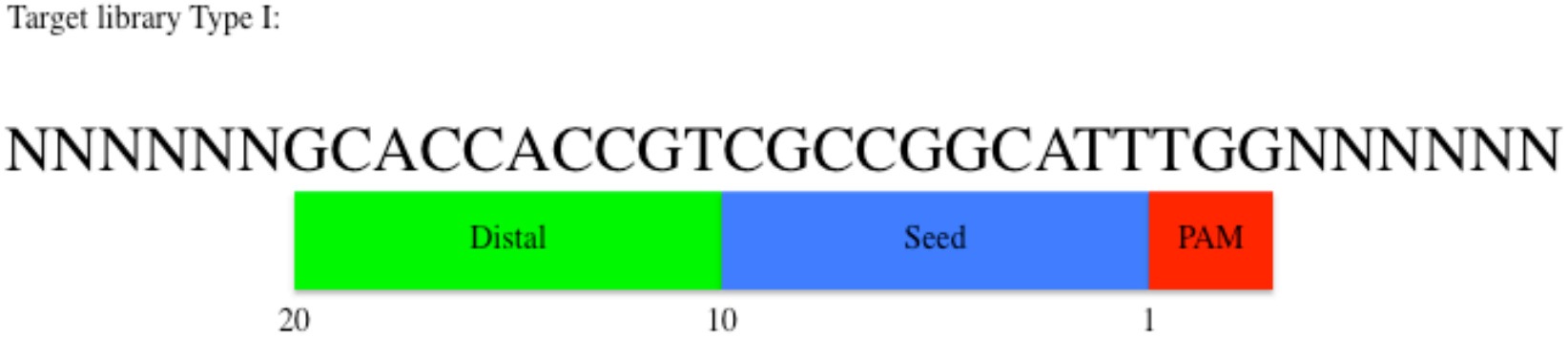
Supplementary Figure 45) Results from Cas9 interaction with pooled *unc-22A* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Cas9 high throughput nicking assay with Type II libraries for *unc-22A* (Method 2-Circular Library; AF_SOL_854). Bar graphs of median retention scores for single deletion variants. Error bars represent the standard deviation of the retentions for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention calculations.

unc-22A Target Sequences

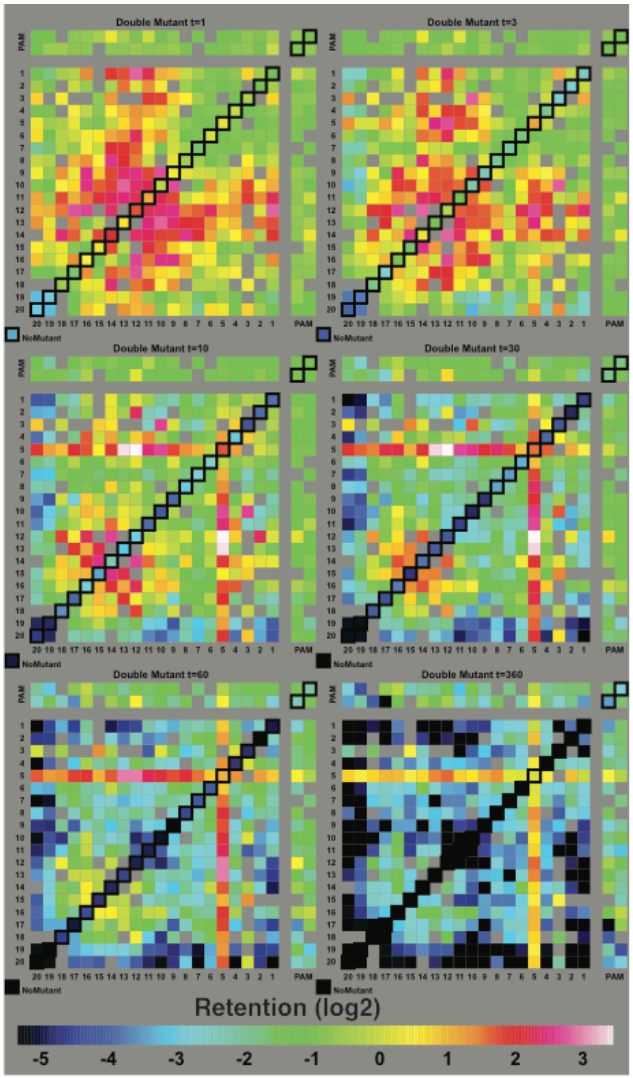
1. _CACCACCGTCGCCGGCATTTGG
2. G_ACCACCGTCGCCGGCATTTGG
3. GC_CCACCGTCGCCGGCATTTGG
4. GCA_CACCGTCGCCGGCATTTGG
5. GCACC_CCGTCGCCGGCATTTGG
6. GCACCA_CGTCGCCGGCATTTGG
7. GCACCACC_TCGCCGGCATTTGG
8. GCACCACCG_CGCCGGCATTTGG
9. GCACCACCGTG_CCGGCATTTGG
10. GCACCACCGTCCC_GGCATTTGG
11. GCACCACCGTCGCGG_CATTTGG
12. GCACCACCGTCGCCGC_ATTTGG
13. GCACCACCGTCGCCGG_ATTTGG
14. GCACCACCGTCGCCGGC_TTTGG
15. GCACCACCGTCGCCGGCATT_GG



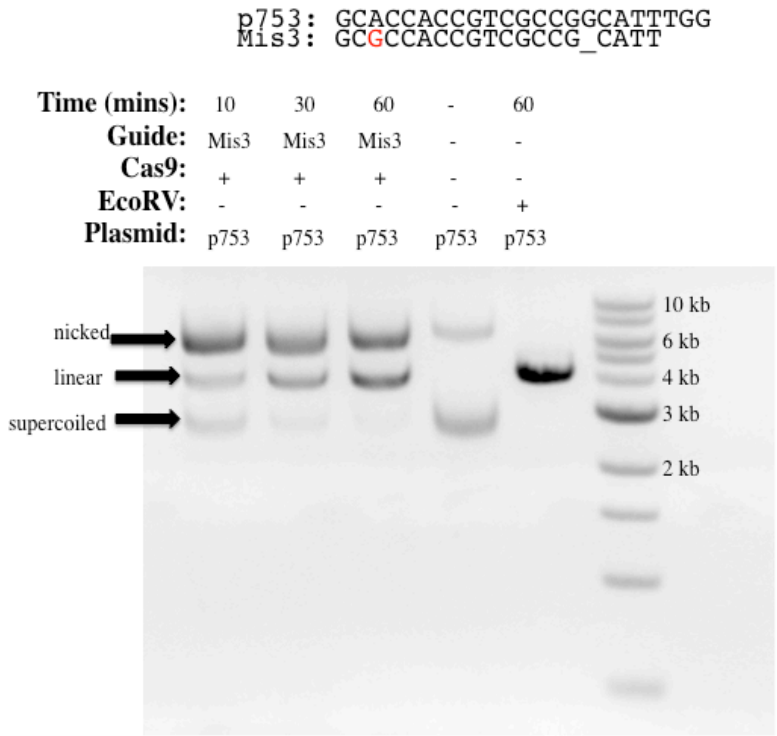
Supplementary Figure 46) Schematic of Type I library redrawn from Fu et al. (2016). Type I libraries were created using oligo pooled array synthesis. Type II libraries were made using degenerate oligo synthesis.



Supplementary Figure 47) Double mutant retention (log2) score profile with Cas9 and variant unc-22A library generated via degenerate oligonucleotide synthesis. These are non-precleaved (Method 2) libraries, so that a positive retention (log2) score indicates nicking of the indicated double mutant substrate at the indicated time point. White boxes indicate no mutant available. The single box labeled "NoMutant" at each time point indicates retention (log2) score for the wild type *unc-22A* sequence. The diagonal indicates the retention (log2) of the single mutant at that position. Refer to "Materials and Methods" for full conditions and retention (log2) calculations. (Method 2-Circular Library; AF_SOL_827)



Supplementary Figure 48) Cas9 nicking assessments on individual circular plasmid targets, assessed using agarose gel electrophoresis in the presence of Ethidium Bromide intercalator. All experiments done with 79.5 ng of enzyme with 1:1 ratio of gRNA:protein with at least 100 ng of target plasmid DNA (supercoiled) in buffer detailed in “Materials and Methods” at 37°C. Ethidium bromide staining of DNA allows visualization of electrophorectic mobility, with a 1kb ladder (left on each gel) used to determine relative mobility of linearized fragments as a function of size. Inferred mobilities of nicked, linear, and supercoiled target plasmids are indicated with arrows at the right of each gel. Sequences shown are of the target-homologous region of each plasmid and of the corresponding guide homology segment. All nicking experiments were done at least twice with similar results. *unc-22a* target with mismatched *unc-22a* gRNA.



Supplementary Figure 49) Cas9 nicking assessments on individual circular plasmid targets, assessed using agarose gel electrophoresis in the presence of Ethidium Bromide intercalator. All experiments done with 79.5 ng of enzyme with 1:1 ratio of gRNA:protein with at least 100 ng of target plasmid DNA (supercoiled) in buffer detailed in “Materials and Methods” at 37°C. Ethidium bromide staining of DNA allows visualization of electrophorectic mobility, with a 1kb ladder (left on each gel) used to determine relative mobility of linearized fragments as a function of size. Inferred mobilities of nicked, linear, and supercoiled target plasmids are indicated with arrows at the right of each gel. Sequences shown are of the target-homologous region of each plasmid and of the corresponding guide homology segment. All nicking experiments were done at least twice with similar results. EGFP-2 target with mismatched EGFP-2 gRNAs

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Mis 4: GATGCCGTTCTT_TGCTTGT
Mis 5: GATGGCG_TCTTCTGCTTTT
 p793: GATGCCGTTCTTCTGCTTGTCGG
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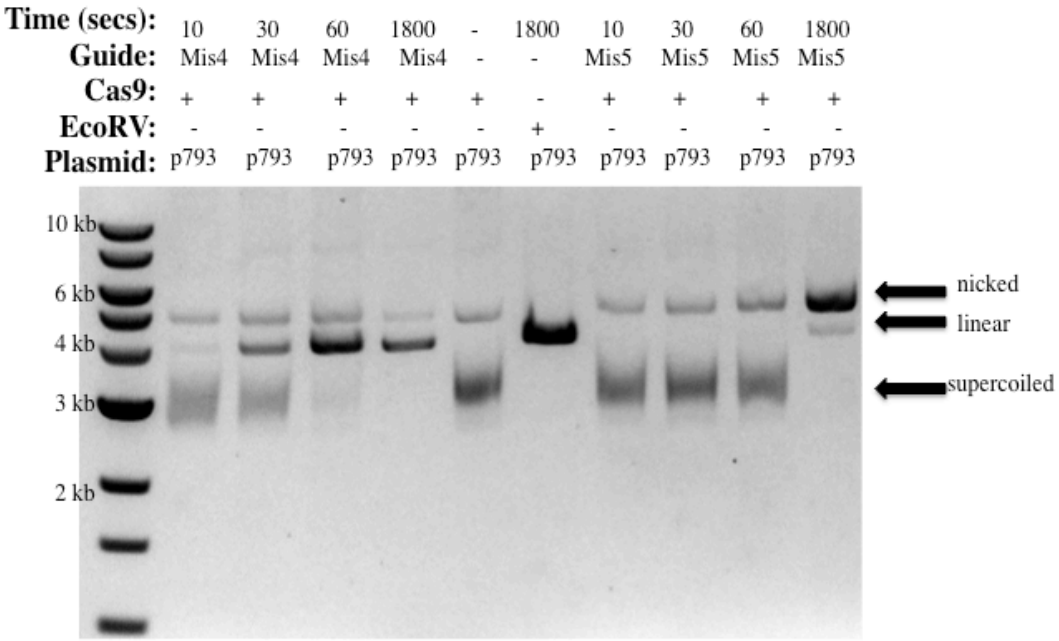
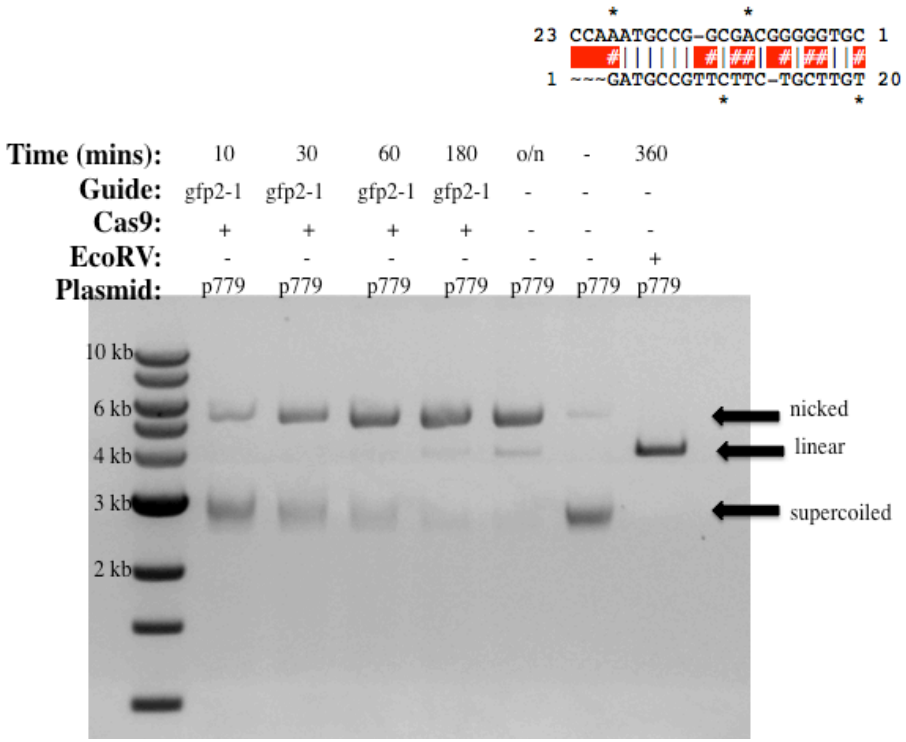
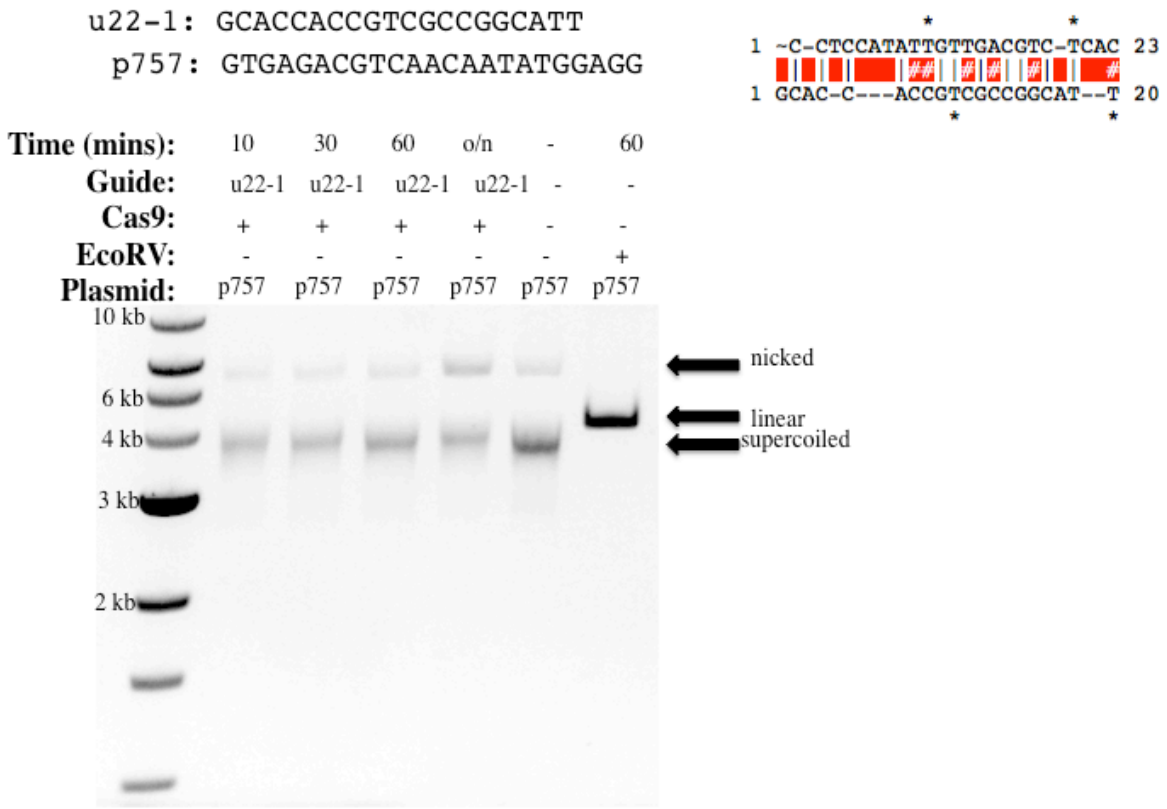


Supplementary Figure 50) Cas9 nicking assessments on individual circular plasmid targets, assessed using agarose gel electrophoresis in the presence of Ethidium Bromide intercalator. All experiments done with 79.5 ng of enzyme with 1:1 ratio of gRNA:protein with at least 100 ng of target plasmid DNA (supercoiled) in buffer detailed in “Materials and Methods” at 37°C. Ethidium bromide staining of DNA allows visualization of electrophorectic mobility, with a 1kb ladder (left on each gel) used to determine relative mobility of linearized fragments as a function of size. Inferred mobilities of nicked, linear, and supercoiled target plasmids are indicated with arrows at the right of each gel. Sequences shown are of the target-homologous region of each plasmid and of the corresponding guide homology segment. Variant *unc-22A* target with EGFP-2 gRNA.

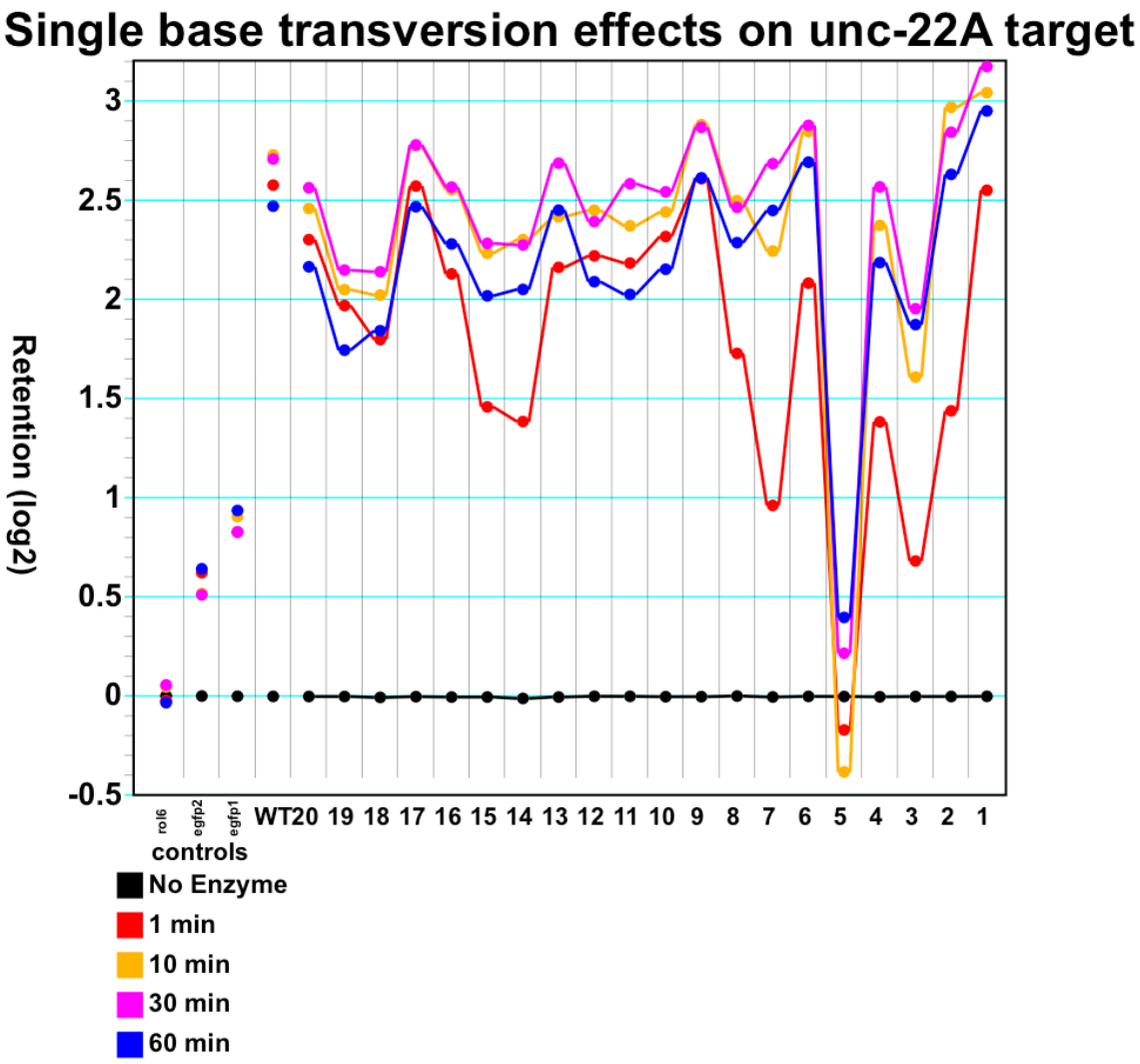


Supplementary Figure 51) Cas9 nicking assessments on individual circular plasmid targets, assessed using agarose gel electrophoresis in the presence of Ethidium Bromide intercalator. All experiments done with 79.5 ng of enzyme with 1:1 ratio of gRNA:protein with at least 100 ng of target plasmid DNA (supercoiled) in buffer detailed in “Materials and Methods” at 37°C. Ethidium bromide staining of DNA allows visualization of electrophorectic mobility, with a 1kb ladder (left on each gel) used to determine relative mobility of linearized fragments as a function of size. Inferred mobilities of nicked, linear, and supercoiled target plasmids are indicated with arrows at the right of each gel. Sequences shown are of the target-homologous region of each plasmid and of the corresponding guide homology segment. *rol-6* target with *unc-22a* gRNA.



Supplementary Figure 52) Results from Cas9D10A interaction with pooled *unc-22A* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Cas9 high throughput nicking

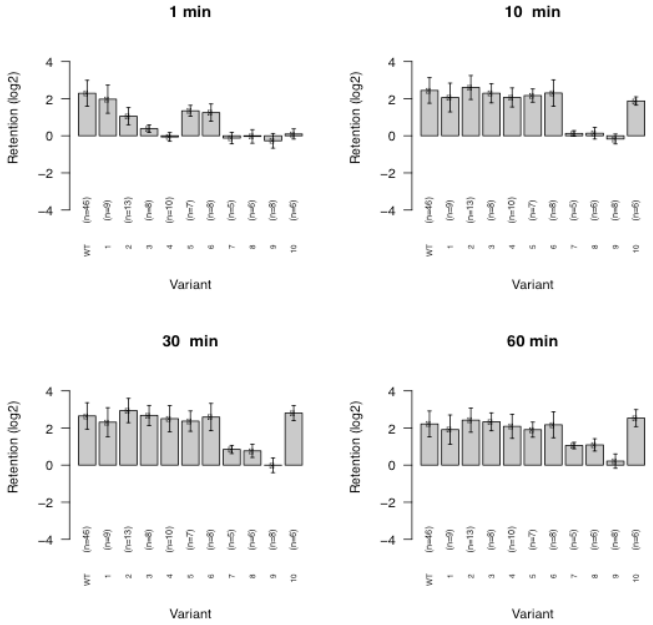
assay with Type II libraries for *unc-22A*. Retention (log2) scores are shown for whole-library assays with backbone cleavage step used to avoid preferential recovery of nicked substrates for *unc-22A* target single base transversions with Cas9D10A. X axis shows the positions, controls, and wild type retention (log2) (WT—full target match), negative controls (no target match) and individual transversions through the full sequence. Y axis shows the retention (log2) score. Refer to “Materials and Methods” for full conditions and retention (log2) calculations. (Method 2-Circular Library; AF_SOL_855)



Supplementary Figure 53) Results from Cas9D10A interaction with pooled *unc-22A* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Cas9 high throughput nicking assay with Type II libraries for *unc-22A*. Cas9D10A specificity profile results for single double consecutive transversion variants. Bar graphs of median retention (log2) scores for double consecutive variants. Error bars represent the standard deviation of the retention (log2)s for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention (log2) calculations.

unc-22A target sequences

1. CGACCACCGTCGCCGGCATTTGG
2. GCTGCACCGTCGCCGGCATTTGG
3. GCACGTCCGTCGCCGGCATTTGG
4. GCACCAGGGTCGCCGGCATTTGG
5. GCACCACCCACGCCGGCATTTGG
6. GCACCACCGTGCCCGGCATTTGG
7. GCACCACCGTCGGGGGCATTTGG
8. GCACCACCGTCGCCCCCATTTGG
9. GCACCACCGTCGCCGGGTTTTGG
10. GCACCACCGTCGCCGGCAAATGG



Supplementary Figure 54) Results from Cas9D10A interaction with pooled *unc-22A* targets. All reactions were done with thermobuffer and conditions detailed in “Materials and Methods: High throughput in vitro target specificity assays”. Cas9 high throughput nicking assay with Type II libraries for *unc-22A*. Cas9D10A specificity profile results for single deletion variants. Bar graphs of median retention (log2) scores for double consecutive variants. Error bars represent the standard deviation of the retention (log2)s for distinct sequence tags in the library matching the noted target sequence, with (n) values below each bar representing the total number of independent barcoded tags assessed for each target sequence. Refer to “Materials and Methods” for full conditions and retention (log2) calculations.

```
 unc-22A Target Sequences
1.  _CACCACCGTCGCCGGCATTTGG
2.  G_ACCACCGTCGCCGGCATTTGG
3. GC_CCACCGTCGCCGGCATTTGG
4.  GCA_CACCGTCGCCGGCATTTGG
5.  GCACC_CCGTCGCCGGCATTTGG
6.  GCACCA_CGTCGCCGGCATTTGG
7.  GCACCACC_TCGCCGGCATTTGG
8.  GCACCACCG_CGCCGGCATTTGG
9.  GCACCACCGTG_CCGGCATTTGG
10. GCACCACCGTCCC_GGCATTTGG
11. GCACCACCGTCGCGG_CATTTGG
12. GCACCACCGTCGCCGC_ATTTGG
13. GCACCACCGTCGCCGG_ATTTGG
14. GCACCACCGTCGCCGGC_TTTGG
15. GCACCACCGTCGCCGGCATT_GG
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