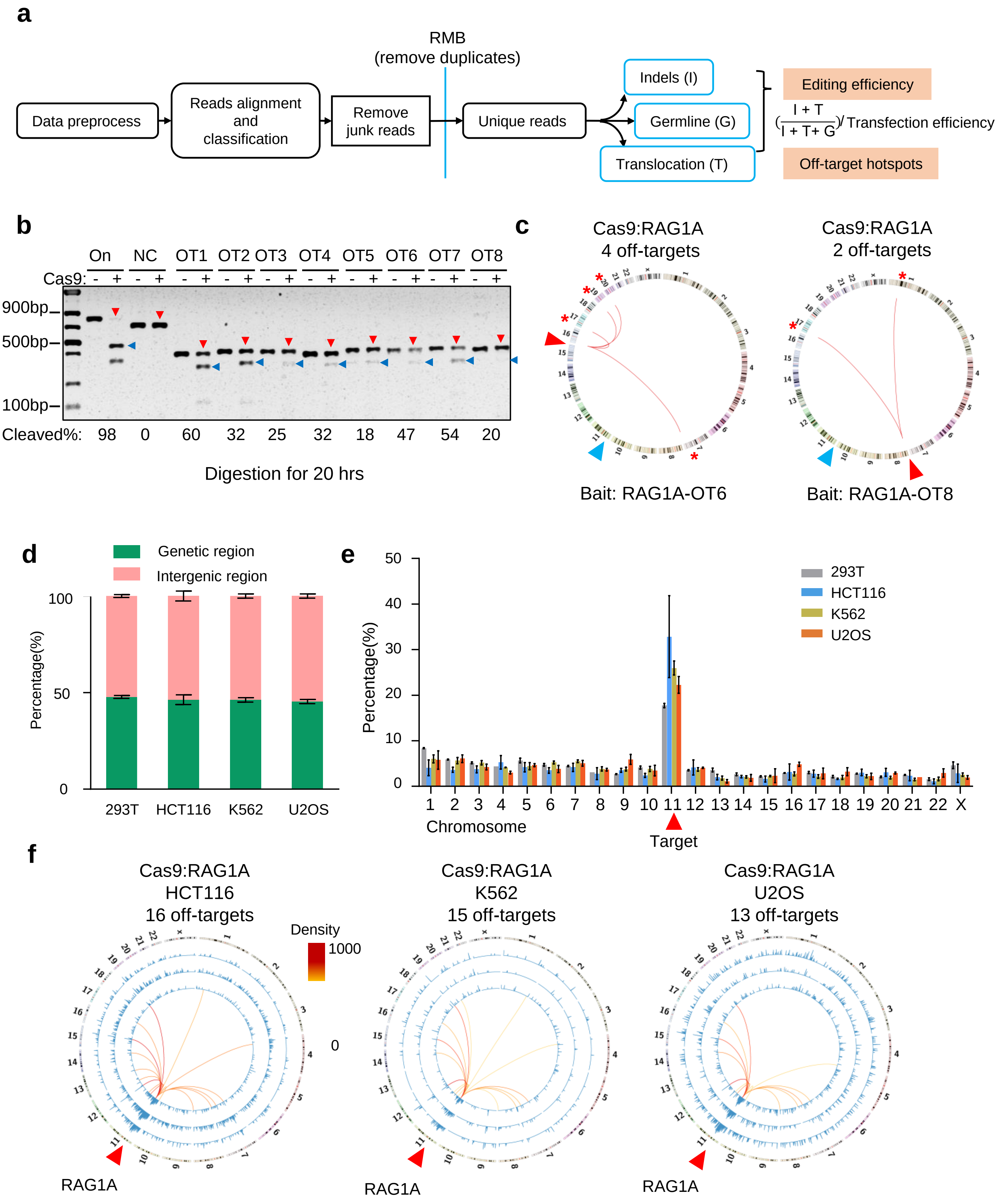
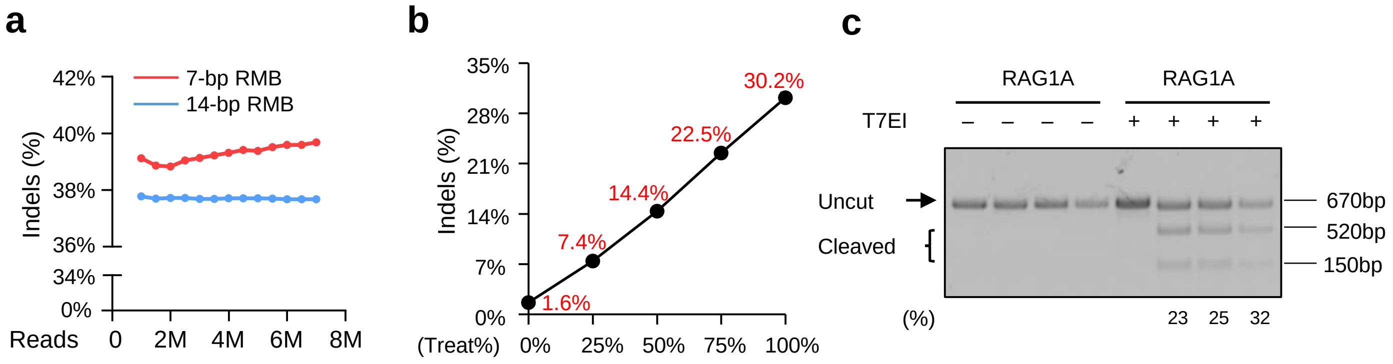
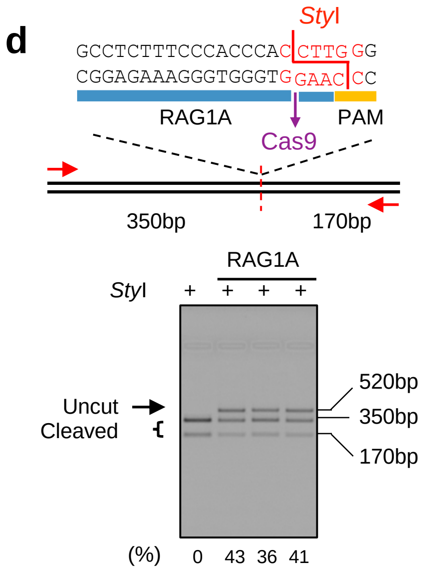
# Supplementary Fig. S1



# Supplementary Fig. S2

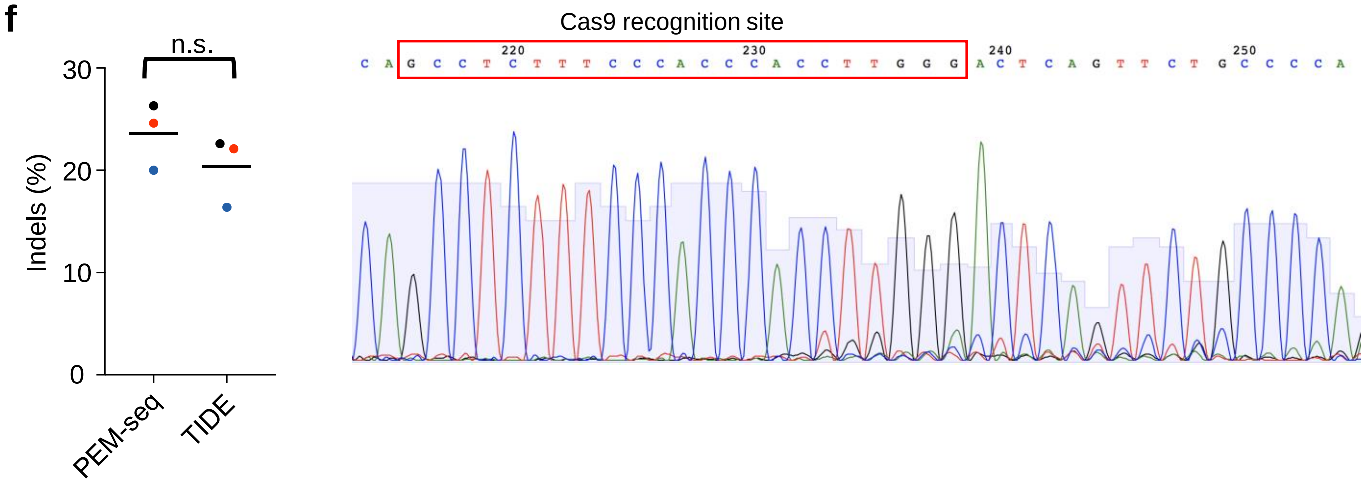




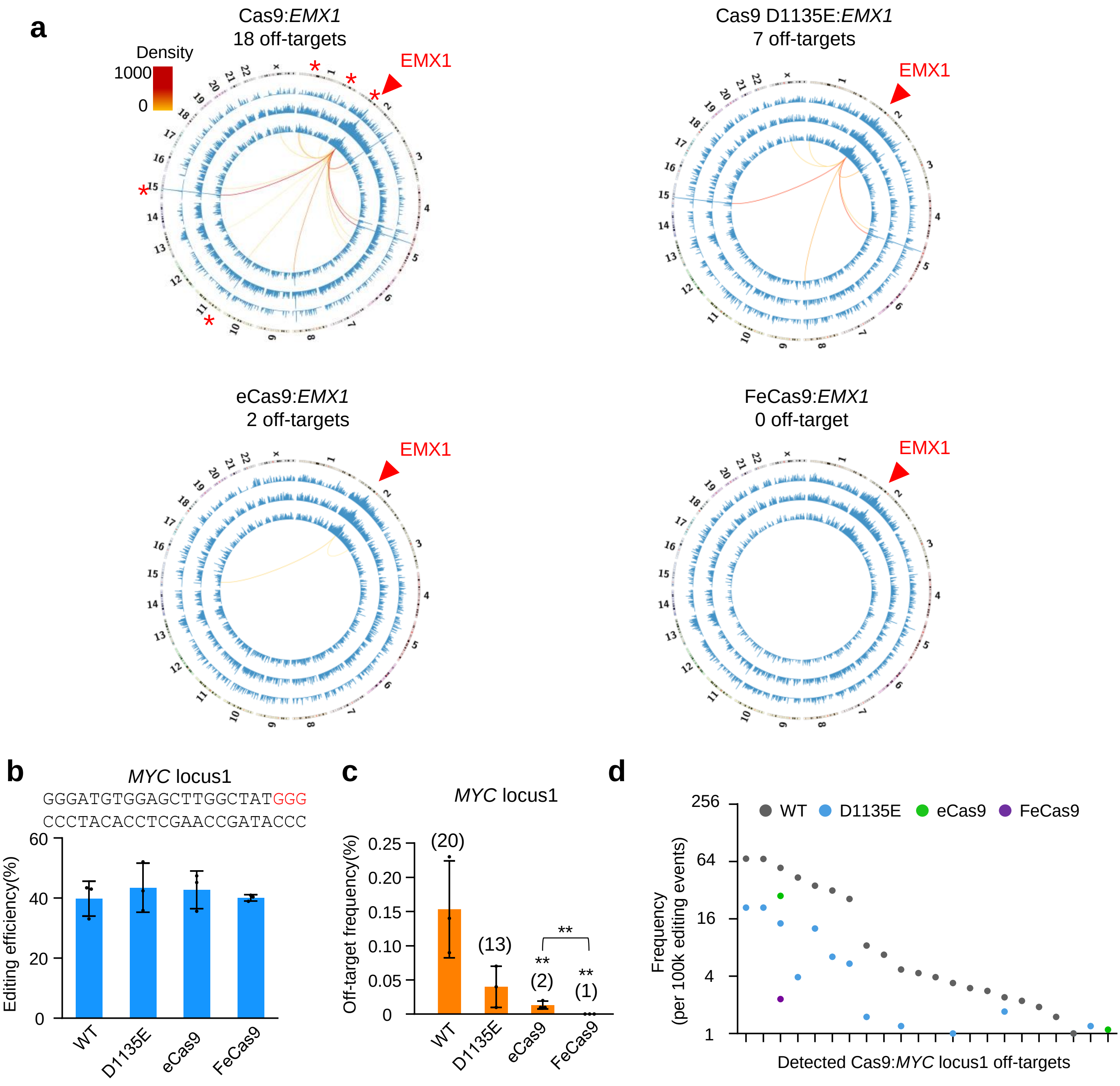
**e**

| No. | F.D. | P.D. | N.D. | Indels% |
|---|---|---|---|---|
| 1 | 34 | 87 | 46 | 46.4% |
| 2 | 23 | 28 | 76 | 29.1% |
| 3 | 16 | 27 | 64 | 27.6% |

F.D.: Fully-digested;
P.D.: Partially-digested;
U.D.: Un-digested



# Supplementary Fig. S3



**Supplementary Fig. S4**

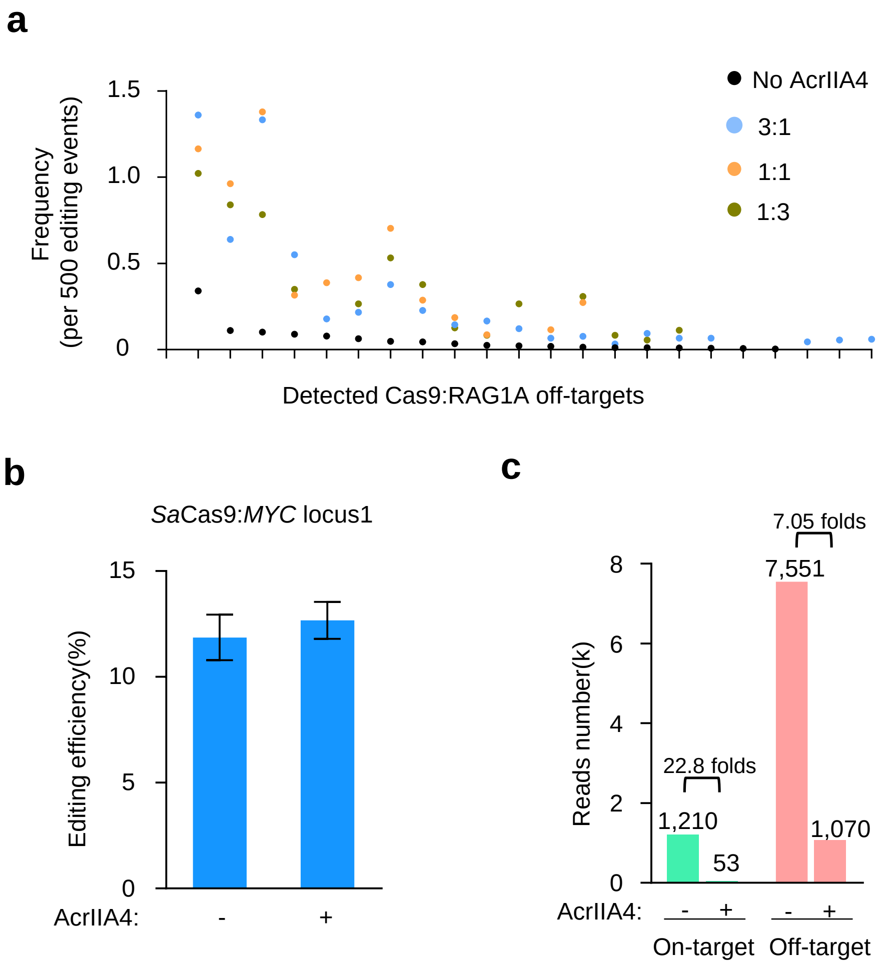


**Figure legend**

Fig. 1 PEM-seq Detects Off-target Hotspots of CRISPR/Cas9

(a) Overview of PEM-seq. To prepare PEM-seq libraries, primer extension generated a copy of template with biotinylated primer followed by bridge adapter ligation and DNA amplification. Gray bars represented bait region while orange ones for captured prey region; "N" indicated RMB in the bridge adapter. Arrows indicated positions and orientations of primers. See supplemental protocols for details.

(b) Circos plots of Cas9:RAG1A libraries. Three biological replicates were showed from outside to inside, and the displayed translocation junctions were 19,494, 16,005 and 18,078. Genome-wide translocation junctions binned into 5-Mb regions (blue lines) were plotted on a log scale. Chromosomes were showed with centromere to telomere in clockwise direction. Red arrow indicated the Cas9:RAG1A cleavage site. Colored lines connected the on-target site to the off-target hotspots.

(c) Zoomed-in view of Cas9:RAG1A translocation junctions (binned into 2Mb) on chromosome 7. Red arrows indicated identified off-target hotspots. Cen represented centromere and p/q indicated the chromosome arms. The Pearson correlation coefficient was 0.99 between replicates 1 and 2, 0.99 between replicates 1 and 3, 0.98 between replicates 2 and 3.

(d) Scatter plot of Cas9:RAG1A off-target hotspots in 293T, HCT116, K562 and U2OS cells. *y* axis showed frequency of each hotspot per 100,000 editing events (indels plus translocation). Red asterisks indicated the off-target hotspots detected by PEM-seq but not by LAM-HTGTS.

(e) Venn Diagram showing the overlap for RAG1A off-target hotspots among indicated cell lines. Legend is the same as showed in panel (d).

Fig. 2 Assessing Editing Efficiencies of CRISPR/Cas9 by PEM-seq

(a) Outcomes of Cas9-induced DSBs. Germline represents un-cut or perfect rejoining; indels is derived from errored rejoining; translocation involves a second DSB.

(b) Percentages of germline, indels and translocation showed by pie graph for Cas9:RAG1A. Mean ± SD.

(c) Frequency of indels for Cas9:RAG1A detected by PEM-seq, RFLP, T7EI assay and single cell RFLP. Averages were indicated by black lines. DNA from different batches were marked in different colors.

(d) Composition of Cas9:RAG1A translocation either joined with off-target DSBs or genome-wide low-level DSBs. Mean ± SD.

(e) Composition of indels of Cas9:RAG1A libraries within ±20 bp around cleavage-site. Note that part of deletions also involved insertion, indicated in gray. Mean ± SD.

(f) Frequency of junctions (binned into 50 bp) within ±5 kb (±20 bp excluded) around Cas9:RAG1A cleavage-site. Upper schematic showed different DNA repair products detected by PEM-seq, including inversion, deletion, and excision. Yellow box indicates the Cas9 target site. Blue and red arrows indicate the translocation orientations. Purple arrow indicates the position and orientation of primer used for PEM-seq. In the bottom panel, black dotted line indicates the Cas9 cleavage-site, while yellow dotted line indicates the primer locus. Junction numbers for each region were indicated.

(g) Frequency of junctions (binned into 1kb) for 5-50 kb downstream of Cas9:RAG1A cleavage-site. Black dotted lines indicate the boundaries of the 5-50 kb region. Purple arrow indicates the primer used for PEM-seq. Junction numbers for each region were indicated.

Fig. 3 Cas9 Nickase Shows Lower Off-target Activity with Loss of Target Editing Efficiency

Schematic of RAG1A, RAG1B, RAG1C and RAG1G target-site positions. Purple arrows indicated the biotinylated primers used for PEM-seq. Yellow boxes represented gRNA target sites and red bars indicated Cas9 cleavage site. In the lower panels, pie graph showed the compositions of germline, indels, and translocation for indicated Cas9 treatment in HEK293T cells. Mean ± SD.

Fig. 4 Editing Efficiencies and Specificity of Cas9 Variants

(a) Schematic of Cas9 domains and corresponding point mutations for Cas9 variants. Red letters indicated mutated amino acids of indicated Cas9 variants.

(b) Editing efficiencies detected by PEM-seq for Cas9 variants targeting RAG1A in HEK293T cells. Target site sequence was listed above and the red letters indicated the PAM sequence. Error bars, mean ± SD. Two-tailed t-test, *, $p < 0.05$.

(c) Frequencies of total translocation junctions in 1-kb regions around off-target hotspots for indicated variants targeting RAG1A in HEK293T cells. Total numbers of identified off-target hotspots for each Cas9 variant were showed above the bar. Error bars, mean ± SD. Two-tailed t-test, *, $p < 0.05$; ** $p < 0.01$.

(d) Scatter plot of RAG1A off-target hotspots for indicated variants. *y* axis showed frequency of each hotspot per 100,000 editing events (indels plus translocation). Note that the WT libraries presented in (b-d) were independently prepared from the ones in Fig. 1 and 2. Sequencing reads for the libraries in this figure was less (~60%) and the identified hotspots were only 38, but still more than LAM-HTGTS.

(e-g) Editing efficiencies and off-target hotspots for Cas9 variants targeting *EMX1* site in HEK293T cells, depicted as described in the legend to panels b-d.

Fig. 5 AcrIIA4 Blocks Cas9 Off-target Activity Less Effectively

(a) Editing efficiencies detected by PEM-seq for Cas9:RAG1A in HEK293T cells with indicated mass ratios of Cas9 over AcrIIA4. Error bars, mean ± SD. Two-tailed t-test, **, $p < 0.01$.

(b) Frequencies of translocation junctions in 1-kb regions around off-target hotspots for Cas9:RAG1A in HEK293T cells with indicated ratios of Cas9 over AcrIIA4. Total numbers of identified off-target hotspots for indicated samples were showed above the bars. Error bars, mean ± SD. Two-tailed t-test, *, $p < 0.05$; ** $p < 0.01$.

(c) Composition of indels and off-target junctions for Cas9:RAG1A libraries with indicated ratios of Cas9 over AcrIIA4. Total junction numbers from pooled three libraries were indicated above the bars. Note that the total bar length of each library was normalized to the same though inhibitor-treated samples always contained less junctions than un-treated ones.

(d-f) Editing efficiencies and off-target hotspots in other Cas9-targeting loci with 1:1 Cas9 over AcrIIA4, depicted as described in the legend to panels (a-c). Note that for 1:1 ratio described in panels (a-c), the amount of plasmid DNA used for transfection for Cas9:RAG1A, AcrIIA4 and blank were 2 μg, 2 μg and 4 μg respectively; while for 1:1 in panels (d-f), the amounts were 2 μg, 2 μg and 0, which led to a higher transfection efficiency of Cas9:RAG1A.

Supplementary Fig. S1 Genomic translocation features revealed by PEM-seq.

(a) Workflow of PEM-seq pipeline. Raw data was subjected to data preprocess, reads alignment and de-duplicates with RMB. Then unique reads were categorized into germline, indels and translocation according to the sequence features. See online methods for details.

(b) *In vitro* Cas9 digestion on RAG1A off-target hotspots. Indicated amplified fragments were incubated with purified Cas9 for 20 hrs. “On” was the RAG1A on-target site. “NC”, negative control with no Cas9:RAG1A target sites but can be targeted by Cas9:*MYC1*. Red triangles indicated the un-cleaved fragment, while blue triangles indicated the larger cleaved fragments. The detailed information of these off-targets were showed in Supplementary Table S2.

(c) Circos plots of Cas9:RAG1A libraries with RAG1A OT6 and OT8 loci as bait in 293T cells. Red triangles indicated the bait sites and red asterisks indicated the detected off-target, and blue triangles indicated the position of RAG1A on-target sites. The detailed information of these off-targets are showed in Supplementary Table S2.

(d) Frequencies of translocation junctions in genetic and intergenic regions for Cas9:RAG1A in indicated cell lines. Error bars, mean ± SD.

(e) Distribution profiles of off-target hotspots-excluded translocation junctions in each chromosome for Cas9:RAG1A in indicated cell lines. Red arrow indicated the target site-containing chromosome. Error bars, mean ± SD.

(f) Circos plots of Cas9:RAG1A libraries in HCT116, K562 and U2OS cell lines, depicted as described in the legend to Fig.1b.

Supplementary Fig. S2 Detecting indels generated by CRISPR/Cas9.

(a) Frequencies of indels detected by PEM-seq from titrated amounts of raw reads extracted from Cas9:RAG1A library with 14-bp or 7-bp RMB. The original read size for the Cas9:RAG1A library was 7.85 million. We randomly took 1 M to 7M (one sample per 0.5M) raw reads to go through the PEM-seq “SuperQ” pipeline individually. The 7-bp RMB was truncated from 14-bp RMB for simulation analysis. If the RMB got saturated, the frequencies of indels would increase due to the miss-assignment of germline as showed by the red line.

(b) Frequencies of indels for libraries with indicated ratios of Cas9:RAG1A-treated and un-treated DNA. The frequency of indels consistently increased about 7% per 25% more Cas9-treated DNA.

(c) Frequencies of indels detected by T7EI for Cas9:RAG1A in HEK293T cells. Cas9:RAG1A cleavge site was PCR amplified and either mock cleaved (–) or T7EI cleaved (+). Length of the uncut and cleaved DNA, and frequencies of indels for each lane was indicated. See online methods for details.

(d) Frequencies of indels detected by RFLP for Cas9:RAG1A in HEK293T cells. Cas9:RAG1A targeting sequence was underlined by blue and PAM sequence by yellow. Purple arrow indicated the Cas9 cutting site. Red sequence indicated the endonuclease *Sty*I recognition site and red line showed the cutting site. Length of the uncut and *Sty*I-cleaved DNA, and frequencies of indels for each lane were indicated. See online methods for details.

(e) Table for single cell RFLP in HEK293T cells statistics. Three biological repeats were showed. See online methods for details.

(f) Frequencies of indels for Cas9:RAG1A detected by PEM-seq and TIDE. Note that DNA used here was different batch as that used in Fig. 2. Red box indicates the Cas9:RAG1A target site. DNA from the same batch were marked in the same color. n.s. not significant. See online methods for details.

Supplementary Fig. S3 Editing efficiency and off-target hotspots detected by PEM-seq for Cas9 variants.

(a) Circos plots of Cas9 variants for *EMX1* locus libraries, depicted as described in the legend to Fig. 1b.

(b-d) Editing efficiencies and off-target hotspots for Cas9 variants targeting *MYC* locus1 in HEK293T cells, depicted as described in the legend to panels Fig.4b-d.

Supplementary Fig. S4 AcrIIA4 suppresses Cas9 activity.

(a) Scatter plot of Cas9:RAG1A off-target hotspots in HEK293T cells with indicated ratios of Cas9 over AcrIIA4. *y* axis showed frequency of each hotspot per 500 editing events (indels + translocation). There low-frequency off-target hotspots detected in 3:1 libraries but not in no-inhibitor libraries were on the list of 53 total Cas9:RAG1A off-target sites. Only 19 off-target hotspots were detected in the no-inhibitor control libraries, because blank plasmid mock added into the transfection mixture reduced the delivery efficiency of Cas9:RAG1A plasmid.

(b) Editing efficiencies detected by PEM-seq for *Sa*Cas9:*MYC* locus1 with or without AcrIIA4 inhibitor. Error bars, mean ± SD.

(c) Translocation junctions captured for Cas9:RAG1A on- and off-target sites with or without AcrIIA4 inhibitor identified by *Sa*Cas9:*MYC* locus1 bait. Total numbers of Cas9:RAG1A on- and off-target junctions for each treatment were showed above the bar. Fold changes of junctions between different treatments were indicated.

# Supplemental Tables

## Supplementary Table S1: Information of PEM-seq libraries

| RAG1A locus in different cell lines | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Nuclease** | **Cell line** | **Locus** | **GFP (%)** | **DNA** | **Reads** | **Trans-location** | **Indels** | **Germline** | **Editing efficiency** | **Pearson correlation coefficient** |
| Cas9 | 293T | RAG1A | 95% | 20ug | 7,851,886 | 19,494 | 290,742 | 460,327 | 39.7% | 1.00 |
| | 293T | RAG1A | 90% | 20ug | 6,632,310 | 16,005 | 236,954 | 459,240 | 36.5% | 1.00 |
| | 293T | RAG1A | 93% | 20ug | 6,939,348 | 18,078 | 237,726 | 406,670 | 39.0% | 1.00 |
| **Total** | | | | **60ug** | **21,423,544** | **53,577** | **765,422** | **1,326,237** | | |
| Cas9 | HCT116 | RAG1A | 84% | 20ug | 2,594,049 | 1,002 | 34,315 | 453,894 | 6.1% | 1.00 |
| | HCT116 | RAG1A | 81% | 20ug | 3,093,171 | 1,161 | 34,702 | 466,884 | 6.2% | 1.00 |
| | HCT116 | RAG1A | 79% | 20ug | 2,687,388 | 647 | 30,707 | 465,548 | 5.3% | 1.00 |
| **Total** | | | | **60ug** | **8,374,608** | **2,810** | **99,724** | **1,386,326** | | |
| Cas9 | K562 | RAG1A | 97% | 20ug | 5,714,664 | 5,564 | 177,063 | 998,723 | 13.8% | 1.00 |
| | K562 | RAG1A | 96% | 20ug | 5,171,097 | 4,977 | 129,098 | 869,775 | 11.8% | 1.00 |
| | K562 | RAG1A | 97% | 20ug | 5,088,244 | 4,586 | 139,803 | 899,627 | 12.2% | 1.00 |
| **Total** | | | | **60ug** | **15,974,005** | **15,127** | **445,964** | **2,768,125** | | |
| Cas9 | U2OS | RAG1A | 80% | 20ug | 4,069,162 | 2,496 | 60,684 | 746,677 | 7.2% | 1.00 |
| | U2OS | RAG1A | 80% | 20ug | 3,837,414 | 2,122 | 56,258 | 693,266 | 7.2% | 1.00 |
| | U2OS | RAG1A | 80% | 20ug | 3,652,282 | 2,019 | 52,057 | 657,544 | 7.0% | 1.00 |
| **Total** | | | | **60ug** | **11,558,858** | **6,637** | **168,999** | **2,097,487** | | |
| Other loci around RAG1A | | | | | | | | | | |
| Cas9 | 293T | RAG1B | 90% | 20ug | 3,080,750 | 1,654 | 90,793 | 435,451 | 18.8% | 1.00 |
| | 293T | RAG1B | 88% | 20ug | 3,212,193 | 1,295 | 79,374 | 376,688 | 19.4% | 1.00 |
| | 293T | RAG1B | 88% | 20ug | 2,126,589 | 927 | 48,035 | 200,496 | 21.7% | 1.00 |
| **Total** | | | | **60ug** | **8,419,532** | **3,876** | **218,202** | **1,012,635** | | |
| Cas9 | 293T | RAG1C | 90% | 20ug | 1,201,887 | 988 | 73,467 | 200,535 | 28.2% | 1.00 |
| | 293T | RAG1C | 88% | 20ug | 1,997,275 | 1,552 | 89,084 | 271,415 | 26.6% | 1.00 |
| | 293T | RAG1C | 82% | 20ug | 3,017,360 | 1,784 | 137,037 | 401,451 | 29.3% | 1.00 |
| **Total** | | | | **60ug** | **6,216,522** | **4,324** | **299,588** | **873,401** | | |
| Cas9n | 293T | RAG1A&G | 92% | 20ug | 5,329,428 | 2,071 | 128,249 | 360,296 | 21.6% | 1.00 |
| | 293T | RAG1A&G | 86% | 20ug | 2,612,348 | 1,785 | 89,569 | 324,397 | 17.8% | 1.00 |
| | 293T | RAG1A&G | 83% | 20ug | 2,616,692 | 1,589 | 88,624 | 318,448 | 18.4% | 1.00 |
| **Total** | | | | **60ug** | **10,558,468** | **5,445** | **306,442** | **1,003,141** | | |
| RAG1A and DNA titration libraries | | | | | | | | | | |
| 4:0 | 293T | RAG1A | - | 20ug | 2,765,758 | 10,562 | 146,721 | 328,693 | 32.4% | - |
| 3:1 | 293T | RAG1A | - | 20ug | 2,244,669 | 6,617 | 98,938 | 334,469 | 24.0% | - |

|  |  |  |  |  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|---|
| 2:2 | 293T | RAG1A | - | 20ug | 2,447,355 | 4,827 | 77,638 | 456,722 | 15.3% | - |
| 1:3 | 293T | RAG1A | - | 20ug | 1,814,991 | 2,322 | 40,017 | 497,239 | 7.8% | - |
| 0:4 | 293T | RAG1A | - | 20ug | 1,718,514 | 45 | 8,995 | 542,549 | 1.6% | - |
| Cas9:RAG1A variants |  |  |  |  |  |  |  |  |  |  |
| Cas9 | 293T | RAG1A | 87% | 20ug | 3,842,485 | 11,159 | 156,800 | 339,285 | 36.2% | 1.00 |
|  | 293T | RAG1A | 89% | 20ug | 4,726,942 | 18,801 | 227,188 | 416,858 | 40.0% | 1.00 |
|  | 293T | RAG1A | 80% | 20ug | 4,147,416 | 8,087 | 137,379 | 388,652 | 31.8% | 1.00 |
| **Total** |  |  |  | **60ug** | **12,716,843** | **38,047** | **521,367** | **1,144,795** |  |  |
| D1135E | 293T | RAG1A | 98% | 20ug | 4,752,710 | 10,281 | 196,184 | 327,216 | 36.9% | 1.00 |
|  | 293T | RAG1A | 96% | 20ug | 3,500,646 | 10,085 | 193,552 | 310,619 | 38.7% | 1.00 |
|  | 293T | RAG1A | 96% | 20ug | 4,186,260 | 10,531 | 192,087 | 281,209 | 41.2% | 1.00 |
| **Total** |  |  |  | **60ug** | **12,439,616** | **30,897** | **581,823** | **919,044** |  |  |
| eCas9 | 293T | RAG1A | 92% | 20ug | 3,225,144 | 5,381 | 137,897 | 249,179 | 37.0% | 1.00 |
|  | 293T | RAG1A | 90% | 20ug | 3,084,029 | 5,052 | 133,061 | 327,003 | 30.4% | 1.00 |
|  | 293T | RAG1A | 88% | 20ug | 1,846,304 | 3,105 | 88,385 | 211,194 | 31.6% | 1.00 |
| **Total** |  |  |  | **60ug** | **8,155,477** | **13,538** | **359,343** | **787,376** |  |  |
| FeCas9 | 293T | RAG1A | 87% | 20ug | 2,227,781 | 13,222 | 59,680 | 162,041 | 46.0% | 1.00 |
|  | 293T | RAG1A | 82% | 20ug | 2,476,621 | 17,906 | 86,764 | 188,102 | 59.8% | 1.00 |
|  | 293T | RAG1A | 81% | 20ug | 2,181,993 | 11,605 | 47,357 | 139,839 | 45.9% | 1.00 |
| **Total** |  |  |  | **60ug** | **6,886,395** | **42,733** | **193,801** | **489,982** |  |  |
| Cas9:EMX1 variants |  |  |  |  |  |  |  |  |  |  |
| *Sp*Cas9 | 293T | EMX1 | 83% | 20ug | 2,259,843 | 3,561 | 182,614 | 202,850 | 57.9% | 1.00 |
|  | 293T | EMX1 | 96% | 20ug | 1,728,790 | 13,255 | 253,009 | 138,626 | 68.9% | 1.00 |
|  | 293T | EMX1 | 97% | 20ug | 1,617,812 | 6,148 | 196,836 | 80,635 | 73.6% | 1.00 |
| **Total** |  |  |  | **60ug** | **5,606,445** | **22,964** | **632,459** | **422,111** |  |  |
| D1135E | 293T | EMX1 | 86% | 20ug | 2,245,786 | 4,408 | 281,487 | 234,844 | 63.8% | 1.00 |
|  | 293T | EMX1 | 93% | 20ug | 1,442,261 | 5,278 | 219,216 | 128,526 | 68.2% | 1.00 |
|  | 293T | EMX1 | 98% | 20ug | 1,430,186 | 4,982 | 206,844 | 84,695 | 72.9% | 1.00 |
| **Total** |  |  |  | **60ug** | **5,118,233** | **14,668** | **707,547** | **448,065** |  |  |
| eCas9 | 293T | EMX1 | 90% | 20ug | 2,825,769 | 3,978 | 252,905 | 168,454 | 67.4% | 1.00 |
|  | 293T | EMX1 | 94% | 20ug | 1,349,284 | 5,640 | 238,614 | 145,073 | 66.7% | 1.00 |
|  | 293T | EMX1 | 90% | 20ug | 1,577,042 | 4,374 | 189,306 | 93,930 | 75.2% | 1.00 |
| **Total** |  |  |  | **60ug** | **5,752,095** | **13,992** | **680,825** | **407,457** |  |  |
| FeCas9 | 293T | EMX1 | 86% | 20ug | 2,015,130 | 4,328 | 213,106 | 257,933 | 53.4% | 1.00 |
|  | 293T | EMX1 | 96% | 20ug | 1,751,609 | 5,358 | 268,147 | 156,880 | 66.0% | 1.00 |
|  | 293T | EMX1 | 96% | 20ug | 1,530,437 | 2,797 | 170,044 | 146,198 | 56.7% | 1.00 |
| **Total** |  |  |  | **60ug** | **5,297,176** | **12,483** | **651,297** | **561,011** |  |  |
| Cas9:*MYC*-locus1 variants |  |  |  |  |  |  |  |  |  |  |
| Cas9 | 293T | *MYC*-locus1 | 97% | 20ug | 3,884,455 | 9,377 | 149,327 | 207,022 | 43.0% | 1.00 |
|  | 293T | *MYC*-locus1 | 87% | 20ug | 5,007,042 | 5,956 | 134,327 | 323,853 | 33.1% | 1.00 |
|  | 293T | *MYC*- | 85% | 20ug | 3,040,133 | 8,301 | 178,424 | 297,689 | 43.4% | 1.00 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | locus1 | | | | | | | | |
| **Total** | | | | **60ug** | **11,931,630** | **23,634** | **462,078** | **828,564** | | |
| D1135E | 293T | *MYC*-locus1 | 98% | 20ug | 3,759,229 | 11,409 | 215,672 | 204,814 | 52.1% | 1.00 |
| | 293T | *MYC*-locus1 | 91% | 20ug | 1,410,295 | 4,519 | 113,828 | 175,876 | 42.4% | 1.00 |
| | 293T | *MYC*-locus1 | 73% | 20ug | 3,409,507 | 6,840 | 188,745 | 509,110 | 35.9% | 1.00 |
| **Total** | | | | **60ug** | **8,579,031** | **22,768** | **518,245** | **889,800** | | |
| eCas9 | 293T | *MYC*-locus1 | 98% | 20ug | 4,222,772 | 9,585 | 172,112 | 195,976 | 47.4% | 1.00 |
| | 293T | *MYC*-locus1 | 88% | 20ug | 3,047,053 | 6,952 | 141,436 | 302,895 | 35.6% | 1.00 |
| | 293T | *MYC*-locus1 | 98% | 20ug | 4,155,923 | 7,005 | 141,635 | 174,922 | 45.2% | 1.00 |
| **Total** | | | | **60ug** | **11,425,748** | **23,542** | **455,183** | **673,793** | | |
| FeCas9 | 293T | *MYC*-locus1 | 98% | 20ug | 3,738,130 | 5,910 | 137,411 | 199,781 | 41.0% | 1.00 |
| | 293T | *MYC*-locus1 | 89% | 20ug | 3,481,196 | 4,462 | 101,718 | 186,367 | 38.9% | 1.00 |
| | 293T | *MYC*-locus1 | 98% | 20ug | 2,374,662 | 4,426 | 114,930 | 170,927 | 40.4% | 1.00 |
| **Total** | | | | **60ug** | **9,593,988** | **14,798** | **354,059** | **557,075** | | |
| AcrIIA4 inhibitor libraries | | | | | | | | | | |
| 1:0 | 293T | RAG1A | 72% | 20ug | 1,513,624 | 2,713 | 37,828 | 207,508 | 20.3% | 1.00 |
| | 293T | RAG1A | 68% | 20ug | 1,259,867 | 2,357 | 33,008 | 199,679 | 19.6% | 1.00 |
| | 293T | RAG1A | 54% | 20ug | 1,081,871 | 825 | 17,400 | 167,655 | 15.0% | 1.00 |
| **Total** | | | | **60ug** | **3,855,362** | **5,895** | **88,236** | **574,842** | | |
| 3:1 | 293T | RAG1A | 76% | 20ug | 1,055,671 | 614 | 6,602 | 225,486 | 1.8% | 0.98 |
| | 293T | RAG1A | 76% | 20ug | 1,276,393 | 806 | 7,878 | 273,475 | 1.8% | 0.98 |
| | 293T | RAG1A | 54% | 20ug | 1,064,759 | 350 | 4,820 | 209,925 | 1.3% | 0.98 |
| **Total** | | | | **60ug** | **3,396,823** | **1,770** | **19,300** | **708,886** | | |
| 1:1 | 293T | RAG1A | 68% | 20ug | 1,253,450 | 377 | 5,776 | 278,637 | 0.7% | 0.98 |
| | 293T | RAG1A | 64% | 20ug | 1,038,947 | 293 | 5,237 | 239,284 | 0.9% | 0.97 |
| | 293T | RAG1A | 48% | 20ug | 1,214,018 | 100 | 4,703 | 252,257 | 0.4% | 0.98 |
| **Total** | | | | **60ug** | **3,506,415** | **770** | **15,716** | **770,178** | | |
| 1:3 | 293T | RAG1A | 68% | 20ug | 1,210,093 | 199 | 4,770 | 229,174 | 0.6% | 0.97 |
| | 293T | RAG1A | 69% | 20ug | 1,442,245 | 291 | 6,618 | 330,241 | 0.5% | 0.97 |
| | 293T | RAG1A | 62% | 20ug | 891,225 | 102 | 3,555 | 148,599 | 1.1% | 0.97 |
| **Total** | | | | **60ug** | **3,543,563** | **592** | **14,943** | **708,014** | | |
| 1:0 | 293T | RAG1A | 61% | 20ug | 2,958,042 | 4,174 | 69,064 | 423,828 | 22.3% | 1.00 |
| | 293T | RAG1A | 60% | 20ug | 3,545,677 | 3,962 | 72,852 | 430,571 | 23.3% | 1.00 |
| | 293T | RAG1A | 60% | 20ug | 2,538,328 | 3,129 | 56,638 | 344,469 | 22.5% | 1.00 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Total** | | | | **60ug** | **9,042,047** | **11,265** | **198,554** | **1,198,868** | | |
| 1:1 | 293T | RAG1A | 56% | 20ug | 2,638,509 | 380 | 10,624 | 515,716 | 1.6% | 0.97 |
| | 293T | RAG1A | 55% | 20ug | 2,476,639 | 364 | 7,632 | 460,536 | 0.9% | 0.96 |
| | 293T | RAG1A | 58% | 20ug | 2,620,035 | 306 | 7,815 | 433,166 | 1.1% | 0.96 |
| **Total** | | | | **60ug** | **7,735,183** | **1,050** | **26,071** | **1,409,418** | | |
| 1:0 | 293T | RAG1B | 64% | 20ug | 1,336,396 | 380 | 16,561 | 192,913 | 10.8% | 1.00 |
| | 293T | RAG1B | 65% | 20ug | 1,668,598 | 369 | 19,288 | 217,289 | 10.9% | 1.00 |
| | 293T | RAG1B | 66% | 20ug | 1,809,127 | 430 | 23,597 | 249,305 | 11.5% | 1.00 |
| **Total** | | | | **60ug** | **4,814,121** | **1,179** | **59,446** | **659,507** | | |
| 1:1 | 293T | RAG1B | 68% | 20ug | 1,603,237 | 68 | 5,932 | 252,289 | 1.7% | 1.00 |
| | 293T | RAG1B | 75% | 20ug | 1,791,627 | 58 | 7,872 | 325,862 | 1.6% | 0.99 |
| | 293T | RAG1B | 70% | 20ug | 2,005,905 | 90 | 8,364 | 336,542 | 1.8% | 0.99 |
| **Total** | | | | **60ug** | **5,400,769** | **216** | **22,168** | **914,693** | | |
| 1:0 | 293T | MYC1 | 57% | 20ug | 2,377,797 | 2,637 | 80,618 | 316,712 | 34.0% | 1.00 |
| | 293T | MYC1 | 54% | 20ug | 2,468,967 | 2,581 | 80,095 | 334,090 | 33.6% | 1.00 |
| | 293T | MYC1 | 58% | 20ug | 2,735,892 | 2,944 | 94,124 | 341,969 | 35.6% | 1.00 |
| **Total** | | | | **60ug** | **7,582,656** | **8,162** | **254,837** | **992,771** | | |
| 1:1 | 293T | MYC1 | 52% | 20ug | 3,515,540 | 296 | 14,726 | 440,508 | 3.3% | 1.00 |
| | 293T | MYC1 | 58% | 20ug | 2,374,608 | 137 | 8,351 | 251,701 | 2.9% | 1.00 |
| | 293T | MYC1 | 55% | 20ug | 2,663,791 | 300 | 15,157 | 477,481 | 2.8% | 1.00 |
| **Total** | | | | **60ug** | **8,553,939** | **733** | **38,234** | **1,169,690** | | |
| 1:0 | 293T | MYC3 | 80% | 20ug | 2,690,416 | 2,977 | 58,929 | 327,096 | 16.7% | 1.00 |
| | 293T | MYC3 | 72% | 20ug | 2,871,109 | 2,450 | 62,019 | 380,840 | 16.6% | 1.00 |
| | 293T | MYC3 | 77% | 20ug | 3,226,089 | 3,086 | 66,093 | 406,850 | 15.6% | 1.00 |
| **Total** | | | | **60ug** | **8,787,614** | **8,513** | **187,041** | **1,114,786** | | |
| 1:1 | 293T | MYC3 | 68% | 20ug | 2,983,856 | 499 | 18,096 | 517,949 | 1.4% | 0.99 |
| | 293T | MYC3 | 68% | 20ug | 2,805,560 | 278 | 15,322 | 444,385 | 1.3% | 0.99 |
| | 293T | MYC3 | 69% | 20ug | 2,964,143 | 321 | 17,917 | 510,070 | 1.4% | 1.00 |
| **Total** | | | | **60ug** | **8,753,559** | **1,098** | **51,335** | **1,472,404** | | |
| 1:0 | 293T | EMX1 | 84% | 20ug | 2,852,531 | 5,071 | 234,331 | 184757 | 63.8% | 1.00 |
| | 293T | EMX1 | 84% | 20ug | 3,267,308 | 6,209 | 246,013 | 180120 | 65.5% | 1.00 |
| | 293T | EMX1 | 84% | 20ug | 2,634,404 | 6,145 | 243,544 | 187049 | 64.1% | 1.00 |
| **Total** | | | | **60ug** | **8,754,243** | **17,425** | **723,888** | **551,926** | | |
| 1:1 | 293T | EMX1 | 74% | 20ug | 3,384,228 | 651 | 30,153 | 432677 | 6.6% | 1.00 |
| | 293T | EMX1 | 78% | 20ug | 3,537,851 | 1,121 | 39,552 | 504690 | 7.3% | 1.00 |
| | 293T | EMX1 | 78% | 20ug | 3,508,240 | 861 | 38,750 | 520959 | 6.8% | 1.00 |
| **Total** | | | | **60ug** | **10,430,319** | **2,633** | **108,455** | **1,458,326** | | |
| **Total** | | | | **60ug** | **8,551,291** | **11,523** | **518,688** | **1,176,615** | | |
| Cas9:RAG1A | 293T | RAG1A-OT32 | - | 10ug | 2,849,117 | 277 | 7,790 | 464,411 | - | - |
| | 293T | RAG1A-OT32 | - | 10ug | 1,851,189 | 225 | 5,299 | 357,882 | - | - |
| Cas9:RA | 293T | RAG1A- | - | 10ug | 4,722,803 | 80 | 21,076 | 338,448 | - | - |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| G1A | | OT29 | | | | | | | | |
| | 293T | RAG1A-OT29 | - | 10ug | 2,936,487 | 116 | 13,809 | 227,911 | - | - |

PEM-seq control libraries

| | **Cell line** | **Locus** | **DNA** | **Total reads** | **Translocation** | **Indels** | **Germline** |
|---|---|---|---|---|---|---|---|
| Cas9 variants | 293T | RAG1A | 20ug | 3,964,448 | 50 | 45,241 | 605,764 |
| Cas9 | 293T | RAG1B | 20ug | 769,966 | 14 | 4,617 | 698,247 |
| Cas9 | 293T | RAG1C | 20ug | 1,181,902 | 11 | 4,473 | 272,936 |
| Cas9 inhibitor | 293T | RAG1A | 20ug | 1,695,139 | 7 | 6,102 | 363,426 |
| Cas9 | HCT116 | RAG1A | 20ug | 3,040,041 | 13 | 11,711 | 538,051 |
| Cas9 | K562 | RAG1A | 20ug | 6,657,170 | 67 | 27,757 | 1,299,770 |
| Cas9 | U2OS | RAG1A | 20ug | 4,197,923 | 42 | 17,885 | 838,120 |
| Cas9 variants | 293T | EMX1 | 20ug | 2,185,150 | 9 | 10,682 | 477,511 |
| Cas9 variants | 293T | MYC1 | 20ug | 2,347,509 | 58 | 7,534 | 463,882 |
| 1:1 AcrIIA4 | 293T | RAG1A | 20ug | 1,239,225 | 7 | 3,273 | 279,986 |
| 1:1 AcrIIA4 | 293T | RAG1B | 20ug | 1,239,225 | 7 | 3,273 | 279,986 |
| 1:1 AcrIIA4 | 293T | EMX1 | 20ug | 3,532,528 | 6 | 10704 | 654373 |
| 1:1 AcrIIA4 | 293T | MYC1 | 20ug | 2,346,147 | 58 | 7,534 | 463,882 |
| 1:1 AcrIIA4 | 293T | MYC2 | 20ug | 1,332,715 | 25 | 7,521 | 298,450 |
| *Sa*Cas9 AcrIIA4 | 293T | MYC1 | 20ug | 2,062,054 | 23 | 5,812 | 451,326 |
| Cas9 | 293T | RAG1A-OT32 | 10ug | 2,366,218 | 26 | 5,293 | 381,173 |
| Cas9 | 293T | RAG1A-OT29 | 10ug | 1,864,579 | 8 | 12,214 | 176,109 |

(The Pearson correlation coefficient is for replicates 1 and 2, 2 and 3, 1 and 3 from top to bottom for each group.)

Supplementary Table S2: Cas9:RAG1A off-targets in different cell lines, *in vitro* digestion for Cas9:RAG1A off-targets and identification for Cas9:RAG1A off-targets with baits from Cas9: RAG1A off-targets

| Cas9:RAG1A off-targets in different cell lines | | | | | | |
|---|---|---|---|---|---|---|
| **Chromosome** | **Off-targets** | **Sequence** | **293T** | **HCT116** | **K562** | **U2OS** |
| chr11 | RAG1A | GCCTCTTTCCCACCCACCTT GGG | - | - | - | - |
| chr12 | 1 | TCCTCCTCCCCACCCACCTT CAG | 724.9 | 87.6 | 88.5 | 139.0 |
| chr15 | 2 | TCCTCTCTCCCACCCACCTC CGG | 481.7 | 104.5 | 34.3 | 58.6 |
| chr19 | 3 | ACCCCTTCCCCACCTACCTT GGG | 277.9 | 83.4 | 32.0 | 82.0 |
| chr7 | 4 | CCCTCCTTCCCACCCACTTT GGG | 277.9 | 26.8 | 6.6 | 9.4 |
| chr11 | 5 | ACGTCTTCCCCACCCACCTG GGG | 226.7 | 22.6 | 4.6 | 23.4 |
| chr14 | 6 | ACTTCCTTCCCACCCACCTT CAG | 222.5 | 25.4 | 10.7 | 12.5 |
| chr7 | 7 | GCCTCTTCCACACCCACCCT GGG | 164.5 | 17.0 | 10.2 | 12.5 |
| chr1 | 8 | TCCTCTTCTCCACCCACCTC TGG | 127.4 | 15.5 | - | - |
| chr17 | 9 | GTCTCTTTCTCACCCACTTT GGG | 76.3 | 26.8 | 4.1 | 6.3 |
| chr7 | 10 | CACCCTTTCCCATCCACCTT TGG | 69.1 | 7.1 | 20.1 | 7.8 |
| chr8 | 11 | ACCTCCTTCCCGCCCACCTG GGG | 68.1 | 2.8 | 2.3 | 7.0 |
| chr11 | 12 | TCCTCTTCTCCACCCACCAT AGG | 63.3 | 15.5 | 3.1 | 2.3 |
| chr20 | 13 | GTCTCTTTCCCATCCACCTT TGA | 56.4 | - | 3.1 | - |
| chr13 | 14 | GCCTCTTTACCACCTCACCT TGG | 47.3 | 15.5 | - | - |
| chr4 | 15 | TTCTCTTCCCCACCCACCTT TGA | 44.8 | 4.2 | 3.1 | - |
| chr11 | 16 | GCCCCTCCCCCACCCACCCT GGG | 35.1 | 11.3 | - | 2.3 |
| chrX | 17 | ACCACTTCCCCACCCACCTC AGG | 18.5 | - | - | - |
| chr17 | 18 | GACTCTTTTCCACCCACCCT AGG | 16.0 | - | - | - |
| chr12 | 19 | GCTTCTTCCCCACCCACACT TGG | 12.1 | 11.3 | - | - |
| chr3 | 20 | ACCTCTTCCCTCCCCACCTT TGG | 10.3 | - | - | - |
| chr7 | 21 | TGCCTCTCCCCACCCACCCT TGG | 9.6 | - | - | - |
| chr1 | 22 | CACCCTTCCCCACCCACCCT GGG | 9.3 | - | 1.02 | - |
| chr17 | 23 | ACCTCTTATCCACCCACCTT GGC | 9.1 | - | - | - |
| chr7 | 24 | GCCCCTGCCCCACCCACCTG TGG | 6.1 | - | - | - |
| chr11 | 25 | GCCCCTACCCCACCCACCTG GGT | 5.8 | - | 1.27 | - |
| chr5 | 26 | TTTTCTTCCCCACCCACCTC GGG | 5.8 | - | - | 3.12 |
| chr22 | 27 | TGCTCTTCCCCACCCACCCA AGG | 4.9 | - | - | - |
| chr16 | 28 | GCCCCTTCCCCACCCACCCT GTG | 3.3 | - | - | - |
| chr14 | 29 | GGCTCCTCCTCACCCACCTC TGG | 2.5 | - | - | - |
| chr11 | 30 | TACCCTTCCCCATCCACCCT GGG | 2.4 | - | - | - |
| chr11 | 31 | TCCCCTTCACCACCCACCTG AGG | 2.4 | - | - | - |
| chr3 | 32 | GTCTCTTTCCCACCCACCTT GTT | 2.4 | - | - | - |
| chr11 | 33 | TTATCTTTCCCAACCACCTT TGG | 2.2 | - | - | - |
| chr4 | 34 | ACCTCTTTCCATCCCACCTT GGG | 2.2 | - | - | - |
| chr10 | 35 | TCATCTTCCCCACCCACCGT GGG | 2.1 | - | - | - |
| chr15 | 36 | CCCTCCTTCCCACCCACCTC CGA | 1.9 | - | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| chr11 | 37 | ACCTCATCCCCACTCACCTG GGG | 1.8 | - | - | - |
| chr12 | 38 | GCCTCTTTTCCACCCACCTA ATT | 1.5 | - | - | - |
| chr6 | 39 | GTGTATTCCCCACCCACCTT GGG | 1.5 | - | - | - |
| chr8 | 40 | ACCTCGTCCCCACCCACCCT GGA | 1.5 | - | - | - |
| chr3 | 41 | GCCTCATCCACATCCACCTT CGG | 1.3 | - | - | - |
| chr11 | 42 | GCCTCTCCGCCACCCACCAG AGG | 1.2 | - | - | - |
| chr7 | 43 | GCCTCCTCCCCACCCACTCT GAG | 1.2 | - | - | - |
| chr16 | 44 | CGCTCTGTCCCACCCACCTC CGG | 1.1 | - | - | - |
| chr11 | 45 | ACATCAACCCCACCCACCGT AGG | 0.9 | - | - | - |
| chr12 | 46 | GCCTCCTTCCCCTCCACCCC AGG | 0.9 | - | - | - |
| chr2 | 47 | CTCCCCTTCCCACCCACCTC TGG | 0.8 | - | - | - |
| chr8 | 48 | GCCTCATCCCCACCCACCCA AGG | 0.8 | - | - | - |
| chr11 | 49 | GTCTCAAACCCACCCACCTT GGC | 0.5 | - | - | - |
| chr14 | 50 | CCCCCTTTCCCACCCACCTT CTG | 0.5 | - | - | - |
| chr16 | 51 | GCCTCAGTCCCACCCACCCC AGG | 0.5 | - | - | - |
| chr7 | 52 | TCCTCTTCCTCTCCCACCTC CGG | 0.5 | - | - | - |
| chr16 | 53 | TCCTCCTCTCCACCCACCTC TGG | 0.2 | - | - | - |
| *In vitro* digestion for Cas9:RAG1A off-targets | | | | | | |
| **Chromosome** | **Off-targets** | **Sequence** | | | | |
| chr12 | OT1 | TCCTCCTCCCCACCCACCTT CAG | Related to off-target #1 in Table S4 | | | |
| chr14 | OT2 | ACTTCCTTCCCACCCACCTT CAG | Related to off-target #6 in Table S4 | | | |
| chr8 | OT3 | ACCTCCTTCCCGCCCACCTG GGG | Related to off-target #12 in Table S4 | | | |
| chr17 | OT4 | GACTCTTTTCCACCCACCCT AGG | Related to off-target #18 in Table S4 | | | |
| chr10 | OT5 | AGCTCCTCCTCACCCACCTC GGG | Related to off-target #30 in Table S4 | | | |
| chr16 | OT6 | GCCTCAGTCCCACCCACCCC AGG | Related to off-target #32 in Table S4 | | | |
| chr17 | OT7 | CGCCTCTTACCATCCACCTT GGG | Related to off-target #38 in Table S4 | | | |
| chr8 | OT8 | ACCTCGTCCCCACCCACCCT GGA | Related to off-target #29 in Table S4 | | | |
| Cas9:RAG1A off-targets using baits from Cas9: RAG1A OT6 and OT8 | | | | | | |
| **Chromosome** | **Off-targets** | **Sequence** | | | | |
| chr16 | OT6 | GCCTCAGTCCCACCCACCCC AGG | Related to off-target #32 in Table S4 | | | |
| chr19 | 1 | ACCCCTTCCCCACCTACCTT GGG | Related to off-target #2 in Table S4 | | | |
| chr7 | 2 | CCCTCCTTCCCACCCACTTT GGG | Related to off-target #4 in Table S4 | | | |
| chr17 | 3 | GTCTCTTTCTCACCCACTTT GGG | Related to off-target #9 in Table S4 | | | |
| chr20 | 4 | GTCTCTTTCCCATCCACCTT TGA | Related to off-target #11 in Table S4 | | | |
| chr8 | OT8 | ACCTCGTCCCCACCCACCCT GGA | Related to off-target #29 in Table S4 | | | |
| chr1 | 1 | TCCTCTTCTCCACCCACCTC TGG | Related to off-target #7 in Table S4 | | | |
| chr17 | 2 | GTCTCTTTCTCACCCACTTT GGG | Related to off-target #9 in Table S4 | | | |

## Supplementary Table S3: Off-targets for other Cas9:RAG1 sites

| Chromosome | Off-targets | Sequence | Frequency |
|---|---|---|---|
| chr11 | RAG1B | GACTTGTTTTCATTGTTCTC AGG | - |
| chr14 | 1 | TCCTTGTTTTCATTGTTCTC TGG | 581.9 |
| chr4 | 2 | CATTTGTTTTCATTGTTCTC TGG | 69.2 |
| chr11 | RAG1A/G | GCCTCTTTCCCACCCACCTT GGG | - |
| chr12 | 1 | TCCTCCTCCCCACCCACCTT CAG | 4.1 |
| chr11 | 2 | ACGTCTTCCCCACCCACCTG GGG | 3.2 |
| chr7 | 3 | CCCTCCTTCCCACCCACTTT GGG | 1.8 |
| chr19 | 4 | ACCCCTTCCCCACCTACCTT GGG | 1.8 |
| chr13 | 5 | GCCTCTTTACCACCTCACCT TGG | 1.4 |

## Supplementary Table S4: Off-targets for Cas9:RAG1A variants

| Chromosome | Off-targets | Sequence | WT | D1135E | eCas9 | FeCas9 |
|---|---|---|---|---|---|---|
| chr11 | RAG1A | GCCTCTTTCCCACCCACCTT GGG | - | - | - | - |
| chr12 | 1 | TCCTCCTCCCCACCCACCTT CAG | 713.8 | 497.3 | 3.8 | 4.4 |
| chr19 | 2 | ACCCCTTCCCCACCTACCTT GGG | 348.1 | 179.8 | 13.0 | 6.2 |
| chr11 | 3 | ACGTCTTCCCCACCCACCTG GGG | 336.5 | 137.7 | 8.2 | - |
| chr7 | 4 | CCCTCCTTCCCACCCACTTT GGG | 221.3 | 75.5 | 1.0 | - |
| chr15 | 5 | TCCTCTCTCCCACCCACCTC CGG | 212.6 | 65.8 | 1.0 | 0.9 |
| chr14 | 6 | ACTTCCTTCCCACCCACCTT CAG | 188.0 | 99.0 | 1.4 | - |
| chr1 | 7 | TCCTCTTCTCCACCCACCTC TGG | 123.0 | 31.8 | - | - |
| chr7 | 8 | GCCTCTTCCACACCCACCCT GGG | 119.0 | 52.4 | - | - |
| chr17 | 9 | GTCTCTTTCTCACCCACTTT GGG | 77.3 | 24.9 | 0.7 | - |
| chr7 | 10 | CACCCTTTCCCATCCACCTT TGG | 65.4 | 31.8 | - | - |
| chr20 | 11 | GTCTCTTTCCCATCCACCTT TGA | 45.5 | 17.8 | - | - |
| chr8 | 12 | ACCTCCTTCCCGCCCACCTG GGG | 40.1 | 32.6 |  |  |
| chr13 | 13 | GCCTCTTTACCACCTCACCT TGG | 38.8 | 17.2 | - | - |
| chr11 | 14 | TCCTCTTCTCCACCCACCAT AGG | 36.5 | 31.6 | - | - |
| chr4 | 15 | TTCTCTTCCCCACCCACCTT TGA | 29.6 | 15.6 | - | - |
| chr11 | 16 | GCCCCTCCCCCACCCACCCT GGG | 25.8 | 4.7 | - | - |
| chr7 | 17 | GCCTCTCCCCACCCACCCTT GGC | 11.0 | 5.7 | - | - |
| chr17 | 18 | GACTCTTTTCCACCCACCCT AGG | 10.3 | 4.7 | - | - |
| chr3 | 19 | ACCTCTTCCCTCCCCACCTT TGG | 8.9 | 0.8 | - | - |
| chr12 | 20 | GCTTCTTCCCCACCCACACT TGG | 8.7 | 1.8 | - | - |
| chr1 | 21 | CACCCTTCCCCACCCACCCT GGG | 7.6 | 9.1 | - | - |
| chr11 | 22 | GCCCCTACCCCACCCACCTG GGT | 6.5 | 4.5 | - | - |
| chr16 | 23 | GCCCCTTCCCCACCCACCCT GTG | 6.1 | 2.4 | - | - |
| chr7 | 24 | GCCCCTGCCCCACCCACCTG TGG | 5.8 | 1.6 | - | - |
| chrX | 25 | ACCACTTCCCCACCCACCTC AGG | 4.9 | 2.8 | - | - |
| chr14 | 26 | GGCTCCTCCTCACCCACCTC TGG | 4.9 | - | - | - |
| chr17 | 27 | ACCTCTTATCCACCCACCTT GGC | 4.3 | 0.8 | - | - |
| chr22 | 28 | GCCCCTTACCCATCCACCCT GGG | 4.0 | - | - | - |
| chr8 | 29 | ACCTCGTCCCCACCCACCCT GGA | 2.5 | - | - | - |
| chr10 | 30 | AGCTCCTCCTCACCCACCTC GGG | 2.4 | - | - | - |
| chr11 | 31 | TACCCTTCCCCATCCACCCT GGG | 1.8 | - | - | - |
| chr16 | 32 | GCCTCAGTCCCACCCACCCC AGG | 1.6 | - | - | - |
| chr22 | 33 | TGCTCTTCCCCACCCACCCA AGG | 1.5 | 2.0 | - | - |
| chrX | 34 | CCTTCTTTCCCACCCACATG GGG | 1.5 | - | - | - |
| chr5 | 35 | TTTTCTTCCCCACCCACCTC GGG | 0.9 | 5.5 | - | - |
| chr10 | 36 | TCATCTTCCCCACCCACCGT GGG | 0.7 | - | - | - |
| chr11 | 37 | TTATCTTTCCCAACCACCTT TGG | 0.5 | - | - | - |
| chr17 | 38 | CGCCTCTTACCATCCACCTT GGG | 0.5 | - | - | - |
| chr4 | 39 | GCCTCCCTCCCAACCAGGAT GAT | - | - | 2.7 | - |

Supplementary Table S5: Off-targets for Cas9:EMX1 variants

| **Chromosome** | **Off-targets** | **Sequence** | **WT** | **D1135E** | **e*Sp*Cas9** | **Fe*Sp*Cas9** |
|---|---|---|---|---|---|---|
| chr2 | *EMX1* | GAGTCCGAGCAGAAGAAGAA GGG | - | - | - | - |
| chr15 | 1 | GAGTCTAAGCAGAAGAAGAA GAG | 129.3 | 75.1 | 0.8 | - |
| chr5 | 2 | GAGTTAGAGCAGAAGAAGAA AGG | 100.0 | 59.3 | - | - |
| chr2 | 3 | GAGGCCGAGCAGAAGAAAGA CGG | 64.9 | 8.9 | 0.5 | - |
| chr5 | 4 | AAGTCTGAGCACAAGAAGAA TGG | 25.8 | 3.3 | - | - |
| chr8 | 5 | GAGTCCTAGCAGGAGAAGAA GAG | 26.4 | 5.0 | - | - |
| chr1 | 6 | AAGTCCGAGGAGAGGAAGAA AGG | 3.8 | 0.7 | - | - |
| chr11 | 7 | AAGCCCGAGCAAAGGAAGAA AGG | 2.5 | - | - | - |
| chrX | 8 | GAGTCCGGGAAGGAGAAGAA AGG | 2.5 | 0.9 | - | - |
| chr5 | 9 | GAGCCGGAGCAGAAGAAGGA GGG | 2.5 | - | - | - |
| chr1 | 10 | GAGCCTGAGCAGAAGGAGAA GGG | 1.7 | - | - | - |
| chr15 | 11 | AAGTCCCGGCAGAGGAAGAA GGG | 1.1 | - | - | - |
| chr2 | 12 | AAGGCAGAGCAGAGGAAGAG AGG | 0.9 | - | - | - |
| chr2 | 13 | GAGTCTAAGCAGGAGAATAA AGG | 1.1 | - | - | - |
| chr6 | 14 | ACGTCTGAGCAGAAGAAGAA TGG | 0.8 | - | - | - |
| chr15 | 15 | GAGCCTGAGGAACAGGAGGA GGA | 0.5 | - | - | - |
| chr3 | 16 | GAATCCAAGCAGGAGAAGAA GGA | 0.6 | - | - | - |
| chr1 | 17 | GATTCCTACCAGAAGAAGAA TGG | 0.5 | - | - | - |
| chr13 | 18 | GAGTAGGAGCAGGAGAAGAA GGA | 0.5 | - | - | - |

## Supplementary Table S6: Off-targets for Cas9:*MYC1* variants

| Chromosome | Off-targets | Sequence | WT | D1135E | e*Sp*Cas9 | Fe*Sp*Cas9 |
|---|---|---|---|---|---|---|
| chr8 | *MYC*-1 | GGGATGTGGAGCTTGGCTAT GGG | - | - | - | - |
| chr8 | 1 | AGGAAGTGGAGCTTGGCCTT GGG | 68.2 | 21.0 | - | - |
| chr8 | 2 | AGGAAGTGGAGCTTGGCCTT GGG | 67.8 | 21.0 | - | - |
| chr9 | 3 | GGGGTGTGGAGCTTGACTAT GAG | 54.7 | 14.3 | 27.8 | 2.3 |
| chr4 | 4 | AGGATGAAGAGATTGGCTAT GGG | 43.3 | 3.9 | - | - |
| chr3 | 5 | GGGAAGTGGAACCTGGCTCT GGG | 35.7 | 12.7 | - | - |
| chr12 | 6 | TGGATGTGCAGCCTGGCTAT TGG | 31.6 | 6.4 | - | - |
| chr12 | 7 | GGTATGCAGAGCTTGGCTTT CGG | 25.8 | 5.4 | - | - |
| chr18 | 8 | GAGGTGTGGAGCTTGGTTAC TGG | 8.4 | 1.5 | - | - |
| chr1 | 9 | GGGTTGCAGAGCTTGGCTAC GGG | 6.7 | - | - | - |
| chr2 | 10 | GGGGTGTGGAGCATGGCTTT GGG | 4.7 | 1.2 | - | - |
| chr14 | 11 | GGTGTGTGGAGCATGGCTAT TGG | 4.3 | - | - | - |
| chr15 | 12 | TGAATGTGGAACTTGGCTAT TAG | 3.9 | - | - | - |
| chr20 | 13 | AATATGTGGAGCTTGGCTAT AGC | 3.4 | 0.8 | - | - |
| chr10 | 14 | GGGAAGAGGAGCCTGGCTTT GGG | 3.0 | - | - | - |
| chr2 | 15 | GGGACTTGGAGCTTGGCTTT AGG | 2.8 | - | - | - |
| chr12 | 16 | AGGGTCTGGAGCTTGGCTAA GGG | 2.4 | 1.7 | - | - |
| chr17 | 17 | AGGATGTGGAGCTTGGATAT CAG | 2.2 | - | - | - |
| chr1 | 18 | AAGATGTGGAACTTGGTTAC GGG | 1.9 | - | - | - |
| chr8 | 19 | GAGATAAGGCCCTTGGCTAT GGG | 1.5 | - | - | - |
| chr2 | 20 | GG-ATGAGGAG[G]CTTGGCTA- GGG | 0.9 | 0.6 | - | - |
| chr8 | 21 | AGGAGATGGAGCCTGGCTTA GGG | - | 1.2 | - | - |
| chr20 | 22 | GAGATGTGGAGCCTGACTAT GGA | - | - | 1.1 | - |
| chr1 | 23 | AAGACATGGAGCCTGGCTAT GGT | - | - | - | - |
| chr20 | 24 | AAGACTAGGATCTTGGATAA GGG | - | - | - | - |

Supplementary Table S7: Off-targets for Cas9:RAG1A locus with AcrIIA4 inhibitor

| Chromosome | Off-targets | Sequence | 1:0 | 3:1 | 1:1 | 1:3 |
|---|---|---|---|---|---|---|
| chr11 | RAG1A | GCCTCTTTCCCACCCACCTT GGG | - | - | - | - |
| chr12 | 1 | TCCTCCTCCCCACCCACCTT CAG | 3.4 | 13.6 | 11.6 | 10.2 |
| chr14 | 2 | ACTTCCTTCCCACCCACCTT CAG | 1.1 | 6.4 | 9.6 | 8.4 |
| chr11 | 3 | ACGTCTTCCCCACCCACCTG GGG | 1.0 | 13.3 | 13.8 | 7.8 |
| chr7 | 4 | CCCTCCTTCCCACCCACTTT GGG | 0.9 | 5.5 | 3.2 | 3.5 |
| chr19 | 5 | CCCCTTCCCC-ACCTACCTT GGG | 0.8 | 1.8 | 3.9 | - |
| chr15 | 6 | TCCTCTCTCCCACCCACCTC CGG | 0.6 | 2.2 | 4.2 | 2.7 |
| chr7 | 7 | GCCTCTTCCACACCCACCCT GGG | 0.5 | 3.8 | 7.0 | 5.3 |
| chr1 | 8 | TCCTCTTCTCCACCCACCTC TGG | 0.5 | 2.3 | 2.9 | 3.8 |
| chr17 | 9 | GTCTCTTTCTCACCCACTTT GGG | 0.4 | 1.4 | 1.9 | 1.3 |
| chr7 | 10 | CACCCTTTCCCATCCACCTT TGG | 0.3 | 1.7 | 0.9 | 0.8 |
| chr13 | 11 | GCCTCTTTACCACC[T]CACCTT GGG | 0.2 | 1.2 | - | 2.7 |
| chr11 | 12 | TCCTCTTCTCCACCCACCAT AGG | 0.2 | 0.7 | 1.2 | - |
| chr8 | 13 | ACCTCCTTCCCGCCCACCTG GGG | 0.1 | 0.8 | 2.7 | 3.1 |
| chr12 | 14 | GCTTCTTCCCCACCCACACT TGG | 0.1 | 0.3 | - | 0.8 |
| chr20 | 15 | GTCTCTTTCCCATCCACCTT TGA | 0.1 | 0.9 | - | 0.6 |
| chr11 | 16 | CGCCCCTCCCC[C]ACCCACCCT GGG | 0.1 | 0.7 | - | 1.1 |
| chr4 | 17 | TTCTCTTCCCCACCCACCTT TGA | 0.1 | 0.7 | - | - |
| chr11 | 18 | GCCCCTACCCCACCCACCTG GGT | 0.1 | - | - | - |
| chr17 | 19 | GACTCTTTTCCACCCACCCT AGG | 0.1 | - | - | - |
| chr11 | 20 | CCTCGTGATCCACCCACCTC GGG | - | 0.4 | - | - |
| chr1 | 21 | CACCCTTCCCCACCCACCCT GGG | - | 0.6 | - | - |
| chr17 | 22 | ACCTCTTATCCACCCACCTT GGC | - | 0.6 | - | - |

Supplementary Table S8: Off-targets for Cas9:RAG1A locus with AcrIIA4 inhibitor, and the bait DSBs were generated by *Sa*Cas9:*MYC1*

| **Chromosome** | **Off-targets** | **Sequence** | **1:0** | **1:1** |
|---|---|---|---|---|
| chr11 | RAG1A | GCCTCTTTCCCACCCACCTT GGG | 616.0 | 33.8 |
| chr8 | 1 | TCCTCCTCCCCACCCACCTT CAG | 1367.9 | 295.0 |
| chr12 | 2 | ACTTCCTTCCCACCCACCTT CAG | 468.8 | 55.5 |
| chr7 | 3 | ACGTCTTCCCCACCCACCTG GGG | 269.3 | 38.3 |
| chr14 | 4 | CCCTCCTTCCCACCCACTTT GGG | 255.0 | 33.8 |
| chr15 | 5 | CCCCTTCCCC-ACCTACCTT GGG | 220.9 | 33.8 |
| chr7 | 6 | TCCTCTCTCCCACCCACCTC CGG | 181.7 | 19.8 |
| chr1 | 7 | GCCTCTTCCACACCCACCCT GGG | 179.2 | 29.4 |
| chr19 | 8 | TCCTCTTCTCCACCCACCTC TGG | 172.6 | 24.9 |
| chr17 | 9 | GTCTCTTTCTCACCCACTTT GGG | 123.2 | 56.8 |
| chr7 | 10 | CACCCTTTCCCATCCACCTT TGG | 103.8 | 20.4 |
| chr13 | 11 | GCCTCTTTACCACC[T]CACCTT GGG | 75.9 | 7.7 |
| chr20 | 12 | TCCTCTTCTCCACCCACCAT AGG | 66.7 | 3.8 |
| chr11 | 13 | ACCTCCTTCCCGCCCACCTG GGG | 46.8 | 10.9 |
| chr4 | 14 | GCTTCTTCCCCACCCACACT TGG | 36.1 | 8.9 |
| chr12 | 15 | GTCTCTTTCCCATCCACCTT TGA | 34.6 | 7.0 |
| chr7 | 16 | CGCCCCTCCCC[C]ACCCACCCT GGG | 31.1 | 17.9 |
| chr11 | 17 | TTCTCTTCCCCACCCACCTT TGA | 28.5 | 7.7 |
| chr17 | 18 | GCCCCTACCCCACCCACCTG GGT | 23.9 | 3.2 |
| chr11 | 19 | GACTCTTTTCCACCCACCCT AGG | 21.4 | 3.8 |
| chr17 | 20 | CCTCGTGATCCACCCACCTC GGG | 19.9 | - |
| chr1 | 21 | CACCCTTCCCCACCCACCCT GGG | 14.3 | - |
| chr22 | 22 | ACCTCTTATCCACCCACCTT GGC | 12.2 | - |
| chrX | 23 | ACCACTTCCCCACCCACCTC AGG | 12.2 | 4.5 |
| chr2 | 24 | CTCCCCTTCCCACCCACCTC TGG | 9.7 | - |
| chr8 | 25 | GCCTCATCCCCACCCACCCA AGG | 8.1 | - |
| chr7 | 26 | GCCCCTGCCCCACCCACCTG TGG | 7.6 | - |
| chr7 | 27 | ACCTCCTAACCCCCCACCTT GGG | 6.6 | - |
| chr8 | 28 | ACCTCGTCCCCACCCACCCT GGA | 6.6 | - |
| chr16 | 29 | TCCTCCTCTCCACCCACCTC TGG | 6.1 | - |
| chr22 | 30 | GCCCCTTACCCATCCACCCT GGG | 6.1 | - |
| chr10 | 31 | TCATCTTCCCCACCCACCGT GGG | 5.6 | - |
| chr16 | 32 | GCCTCAGTCCCACCCACCCC AGG | 5.1 | - |
| chr3 | 33 | ACCTCTTCCCTCCCCACCTT TGG | 4.1 | - |
| chr1 | 34 | TCTTCTCCCCCACCCACCCT GGG | 3.6 | - |
| chr6 | 35 | ACCCCCTTCCCACCCACCCT GAG | 3.6 | - |
| chr8 | 36 | TCCTCCCTCCCACCCACCTG AGG | 3.6 | - |
| chr8 | 37 | GCCTCTTTCCTGCCCACCTG TGG | 1.5 | - |

Supplementary Table S9: Off-targets for Cas9 with 1:1 AcrIIA4

| Chromosome | Off-targets | Sequence | 1:0 | 1:1 |
|---|---|---|---|---|
| chr11 | RAG1A | GCCTCTTTCCCACCCACCTT GGG | | |
| chr12 | 1 | TCCTCCTCCCCACCCACCTT CAG | 1.9 | 5.8 |
| chr11 | 2 | ACGTCTTCCCCACCCACCTG GGG | 0.9 | 4.4 |
| chr19 | 3 | ACCCCTTCCCCACCTACCTT GGG | 0.8 | 1.4 |
| chr7 | 4 | CCCTCCTTCCCACCCACTTT GGG | 0.7 | 2.0 |
| chr14 | 5 | ACTTCCTTCCCACCCACCTT CAG | 0.6 | 3.6 |
| chr15 | 6 | TCCTCTCTCCCACCCACCTC CGG | 0.5 | 1.8 |
| chr17 | 7 | GTCTCTTTCTCACCCACTTT GGG | 0.2 | 1.4 |
| chr7 | 8 | GCCTCTTCCACACCCACCCT GGG | 0.2 | 0.9 |
| chr1 | 9 | TCCTCTTCTCCACCCACCTC TGG | 0.2 | 1.1 |
| chr11 | 10 | TCCTCTTCTCCACCCACCAT AGG | 0.2 | - |
| chr7 | 11 | CACCCTTTCCCATCCACCTT TGG | 0.2 | 1.2 |
| chr13 | 12 | GCCTCTTTACCACC[T]CACCTT GGG | 0.1 | 1.3 |
| chr20 | 13 | GTCTCTTTCCCATCCACCTT TGA | 0.1 | 0.5 |
| chr8 | 14 | ACCTCCTTCCCGCCCACCTG GGG | 0.1 | 0.9 |
| chr11 | 15 | GCCCCTCCCCCACCCACCCT GGG | 0.1 | 0.6 |
| chr4 | 16 | TTCTCTTCCCCACCCACCTT TGA | 0.1 | 0.4 |
| chr17 | 17 | ACCTCTTATCCACCCACCTT GGC | 0.1 | 0.1 |
| chrX | 18 | ACCACTTCCCCACCCACCTC AGG | 0.1 | - |
| chr11 | 19 | GCCCCTACCCCACCCACCTG GGT | 0.1 | - |
| chr17 | 20 | GACTCTTTTCCACCCACCCT AGG | 0.1 | - |
| **Chromosome** | **Locus** | **Sequence** | **1:0** | **1:1** |
| chr11 | RAG1B | GACTTGTTTTCATTGTTCTC AGG | | - |
| chr14 | 1 | TCCTTGTTTTCATTGTTCTC TGG | 2.5 | 1.1 |
| chr4 | 2 | CATTTGTTTTCATTGTTCTC TGG | 0.2 | 0.4 |
| **Chromosome** | **Off-targets** | **Sequence** | **1:0** | **1:1** |
| chr8 | *MYC*-locus1 | GGGATGTGGAGCTTGGCTAT GGG | - | - |
| chr9 | 1 | GGGGTGTGGAGCTTGACTAT GAG | 0.2 | 0.4 |
| chr8 | 2 | AGGAAGTGGAGCTTGGCCTT GGG | 0.1 | 0.6 |
| chr8 | 3 | AGGAAGTGGAGCTTGGCCTT GGG | 0.1 | 0.6 |
| chr4 | 4 | AGGATGAAGAGATTGGCTAT GGG | 0.1 | 0.2 |
| chr12 | 5 | GGTATGCAGAGCTTGGCTTT CGG | 0.1 | 0.4 |
| chr12 | 6 | TGGATGTGCAGCCTGGCTAT TGG | 0.1 | 0.4 |
| chr3 | 7 | GGGAAGTGGAACCTGGCTCT GGG | 0.1 | 0.6 |
| chr18 | 8 | GAGGTGTGGAGCTTGGTTAC TGG | 0.1 | - |
| chr20 | 9 | AAGACTAGGATCTTGGATAA GGG | 0.1 | - |
| chr2 | 10 | GGGGTGTGGAGCATGGCTTT GGG | 0.1 | - |
| chr14 | 11 | GGTGTGTGGAGCATGGCTAT TGG | 0.1 | - |
| chr15 | 12 | TGAATGTGGAACTTGGCTAT TAG | 0.1 | - |
| chr17 | 13 | AGGATGTGGAGCTTGGATAT CAG | 0.1 | - |

| Chromosome | Locus | Sequence | 1:0 | 1:1 |
|---|---|---|---|---|
| chr8 | *MYC*-locus2 | `GTACATGCAGTTCTGCATCT TGG` | - | - |
| chr6 | 1 | `ATAAATACAGTTCTGCATCT GGG` | 1.0 | 3.6 |
| chr2 | 2 | `GTAAATGCAGTTCTGCATTT AGG` | 0.1 | 0.9 |
| chr5 | 3 | `GCACATGCAGCCTTGCATCT GGG` | 0.1 | 0.4 |

## Supplementary Table S10: gRNA sequences for PEM-seq

| **Locus** | **Sequence** |
|---|---|
| RAG1A | `GCCTCTTTCCCACCCACCTT (GGG)` |
| RAG1B | `GACTTGTTTTCATTGTTCTC (AGG)` |
| RAG1C | `GCACCTAACATGATATATTA (AGG)` |
| RAG1G | `GAAAGAGGCTGCCATGCTGGCTG (AGG)` |
| *EMX1* | `GAGTCCGAGCAGAAGAAGAA (GGG)` |
| *MYC*-locus1 | `(TTTA) GGGATGTGGAGCTTGGCTAT (GGGAAT)` |
| *MYC*-locus2 | `(TTTA) GTACATGCAGTTCTGCATCT (TGGGGT)` |

Supplementary Table S11: Oligos used for PEM-seq

| Name | Sequence5'→3' | Purpose |
|---|---|---|
| **Bridge adapters oligos (PEM-seq)** | | |
| BA-up | /5phos/CCA CGC GTG CTC TAC ANN NNT NNN ANN NTN NNN AGA TCG GAA GAG CAC ACG TCT GAA CTC CAG T-NH2(C7) | link ssDNA with RMB |
| BA-lower | TGT AGA GCA CGC GTG GNN NNN N-NH2(C7) | common bridge oligo |
| **Oligos for tagging PCR** | | |
| P5-I5 | AAT GAT ACG GCG ACC ACC GAG ATC TAC ACA CAC TCT TTC CCT ACA CGA CGC | Hiseq tail |
| P7 | CAA GCA GAA GAC GGC ATA CGA GAT | Hiseq tail |
| **Oligos for nested PCR** | | |
| I7-B01 | CAG AAG ACG GCA TAC GAG ATC GTG ATG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-B02 | CAG AAG ACG GCA TAC GAG ATA CAT CGG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-B03 | CAG AAG ACG GCA TAC GAG ATG CCT AAG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-B04 | CAG AAG ACG GCA TAC GAG ATT GGT CAG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-B05 | CAG AAG ACG GCA TAC GAG ATC ACT GTG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-B06 | CAG AAG ACG GCA TAC GAG ATA TTG GCG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-B07 | CAG AAG ACG GCA TAC GAG ATG ATC TGG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-B08 | CAG AAG ACG GCA TAC GAG ATT CAA GTG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-B09 | CAG AAG ACG GCA TAC GAG ATC TGA TCG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-B10 | CAG AAG ACG GCA TAC GAG ATA AGC TAG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-B11 | CAG AAG ACG GCA TAC GAG ATG TAG CCG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-B12 | CAG AAG ACG GCA TAC GAG ATT ACA AGG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-R01 | CAG AAG ACG GCA TAC GAG ATT AGT GCG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-R02 | CAG AAG ACG GCA TAC GAG ATG CTA CAG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-R03 | CAG AAG ACG GCA TAC GAG ATA ATC CGG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |

| | | |
|---|---|---|
| I7-R04 | CAG AAG ACG GCA TAC GAG ATA CTG GTG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-R05 | CAG AAG ACG GCA TAC GAG ATT GTC ACG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-R06 | CAG AAG ACG GCA TAC GAG ATC GGT TAG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-R07 | CAG AAG ACG GCA TAC GAG ATG TCT AGG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-R08 | CAG AAG ACG GCA TAC GAG ATT GAA CTG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-R09 | CAG AAG ACG GCA TAC GAG ATC TAG TCG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-R10 | CAG AAG ACG GCA TAC GAG ATA TCG AAG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-R11 | CAG AAG ACG GCA TAC GAG ATC CGA TGG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| I7-R12 | CAG AAG ACG GCA TAC GAG ATG AAC ATG TGA CTG GAG TTC AGA CGT GTG C | Hiseq tail mutiplex |
| **oligos for RAG1A/B locus** | | |
| bioRAG1A/B | Biotin/GGA CTG CTG GAG ATT GCT CCA GAG AGG GTT TCC CCT CAA AGG AAT CCT TC | primer extension |
| I5-R1-RAG1A/B | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG CGA GGA TCT CAC CCG GAA CAG C | Hiseq tail mutiplex |
| I5-R2-RAG1A/B | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TGC CTA GGA TCT CAC CCG GAA CAG C | Hiseq tail mutiplex |
| I5-R3-RAG1A/B | ACT TTT CCC TAC ACG ACG CTC TTC CGA TCT AGG AAG GAT CTC ACC CGG AAC AGC | Hiseq tail mutiplex |
| I5-R4-RAG1A/B | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TTC AGA GGA TCT CAC CCG GAA CAG C | Hiseq tail mutiplex |
| RAG1A-RFLP-F | GGG AGG CAA AGA TGA ATC AAA G | RAG1A-RFLP |
| RAG1A-T7EI-F | CCC CCT GGA AGA CTG CTT TA | RAG1A-RFLP |
| RAG1A-R/T-R | AGG ACT GCT GGA GAT TGC TC | RAG1A-RFLP/T7EI |
| **oligos for RAG1C locus** | | |
| bioRAG1C | Biotin/CCT GAG AAC AAT GAA AAC AAG TC | primer extension |
| I5-R1-RAG1C | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG CGT ATC AAT ATC CCA CTG ATG TAT C | Hiseq tail mutiplex |
| I5-R2-RAG1C | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TGC CTT ATC AAT ATC CCA CTG ATG TAT C | Hiseq tail mutiplex |
| I5-R3-RAG1C | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG GAT ATC AAT ATC CCA CTG ATG TAT C | Hiseq tail mutiplex |
| I5-R4-RAG1C | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TTC AGT ATC AAT ATC CCA CTG ATG TAT C | Hiseq tail mutiplex |

| | | |
|---|---|---|
| **oligos for MYC locus1** | | |
| bioMYC1 | Biotin/CGG GAA GGA AGT TGG CAT CTG TCC TG | primer extension |
| I5-R1-MYC1 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG CGA GAG CCA TTC TCT GGC TCA G | Hiseq tail mutiplex |
| I5-R2-MYC1 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TGC CTA GAG CCA TTC TCT GGC TCA G | Hiseq tail mutiplex |
| I5-R3-MYC1 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG GAA GAG CCA TTC TCT GGC TCA G | Hiseq tail mutiplex |
| I5-R4-MYC1 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TTC AGA GAG CCA TTC TCT GGC TCA G | Hiseq tail mutiplex |
| **oligos for MYC locus2** | | |
| bioMYC2 | Biotin/TTG CGA CTC TCA GCT GAA TCC ACT GCT G | primer extension |
| I5-R1-MYC2 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG CGT GCT CCC ATG GCA TCA TGA C | Hiseq tail mutiplex |
| I5-R2-MYC2 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TGC CTT GCT CCC ATG GCA TCA TGA C | Hiseq tail mutiplex |
| I5-R3-MYC2 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG GAT GCT CCC ATG GCA TCA TGA C | Hiseq tail mutiplex |
| I5-R4-MYC2 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TTC AGT GCT CCC ATG GCA TCA TGA C | Hiseq tail mutiplex |
| **oligos for EMX1 locus** | | |
| biotinEMX1 | Biotin/CCC ATC AGG CTC TCA GCT CAG CCT GAG TGT TGA G | primer extension |
| I5-R1-EMX1 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG CGC CCA GGT GAA GGT GTG GTT C | Hiseq tail mutiplex |
| I5-R2-EMX1 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TGC CTC CCA GGT GAA GGT GTG GTT C | Hiseq tail mutiplex |
| I5-R3-EMX1 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG GAC CCA GGT GAA GGT GTG GTT C | Hiseq tail mutiplex |
| I5-R4-EMX1 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TTC AGC CCA GGT GAA GGT GTG GTT C | Hiseq tail mutiplex |
| **oligos for RAG1A off-targets** | | |
| bio*Sp*CAS9-RAG1A-OT6 | GGG TGT CAC GGG CCG TGG GAC AGG TC | primer extension |
| I5-R1-*Sp*CAS9-RAG1A-OT6 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG CGC ATC GGT TGC CAT GGC TAC TG | Hiseq tail mutiplex |
| I5-R2-*Sp*CAS9-RAG1A-OT6 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TGC CTC ATC GGT TGC CAT GGC TAC TG | Hiseq tail mutiplex |
| I5-R3-*Sp*CAS9-RAG1A-OT6 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG GAC ATC GGT TGC CAT GGC TAC TG | Hiseq tail mutiplex |
| bio*Sp*CAS9-RAG1A-OT8 | CCT CCC TGA CTA GTG GTT AAC GGA CTT G | primer extension |

| | | |
|---|---|---|
| I5-R1-*Sp*CAS9-RAG1A-OT8 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG CGC GCT TTC CTG GCG GTT ACA G | Hiseq tail mutiplex |
| I5-R2-*Sp*CAS9-RAG1A-OT8 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TGC CTC GCT TTC CTG GCG GTT ACA G | Hiseq tail mutiplex |
| I5-R3-*Sp*CAS9-RAG1A-OT8 | ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAG GAC GCT TTC CTG GCG GTT ACA G | Hiseq tail mutiplex |
| RAG1A-OT1-F | GGC TGT CCA TGT TGT CCT TC | *In vitro* digestion |
| RAG1A-OT1-R | ACA CCT CTG GTT AGA GAT AAA GTG | *In vitro* digestion |
| RAG1A-OT2-F | CTT GCG ATC TGT CTG TCT CC | *In vitro* digestion |
| RAG1A-OT2-R | GAT GTG GAG ACT GAT TAA GCC | *In vitro* digestion |
| RAG1A-OT3-F | AGG CCA GTG GTC ACG TGG CTC | *In vitro* digestion |
| RAG1A-OT3-R | GGC CGA GGC CTG GAG ACA GG | *In vitro* digestion |
| RAG1A-OT4-F | ACA GAC ATA CAC GTG AGG TC | *In vitro* digestion |
| RAG1A-OT4-R | AAG TAC AGT CGG ACT TGG CAG | *In vitro* digestion |
| RAG1A-OT5-F | GCT GCC TCA GCG GCC AGG GCC | *In vitro* digestion |
| RAG1A-OT5-R | TCC TCC CAT CCC AAG GTG CC | *In vitro* digestion |
| RAG1A-OT6-F | GGC CTG ATG GCC AGA GAG GG | *In vitro* digestion |
| RAG1A-OT6-R | GAA TAA TTA CAG GGG AGT CAG CC | *In vitro* digestion |
| RAG1A-OT7-F | CAT GAG CCT CTG GAA TCT GTC | *In vitro* digestion |
| RAG1A-OT7-R | GTC ACA TTC ACA GGT CCG AG | *In vitro* digestion |
| RAG1A-OT8-F | TCT GGC TAA GCC GTT AGG CGC | *In vitro* digestion |
| RAG1A-OT8-R | CCT GTG TTT TCT GGA CAC AGA AC | *In vitro* digestion |
| RAG1A-on-target-F | GGG AGG CAA AGA TGA ATC AAA G | *In vitro* digestion |
| RAG1A-on-target-R | GGA TCG AGT CAA CAT CTG CC | *In vitro* digestion |
| Negative-control-F | GCC TCT GAA AAC CTA TGG CC | *In vitro* digestion |
| Negative-control-R | GTT GCA CAA TGG CAG AGA CC | *In vitro* digestion |