

Supplementary Materials

Supplementary Figures

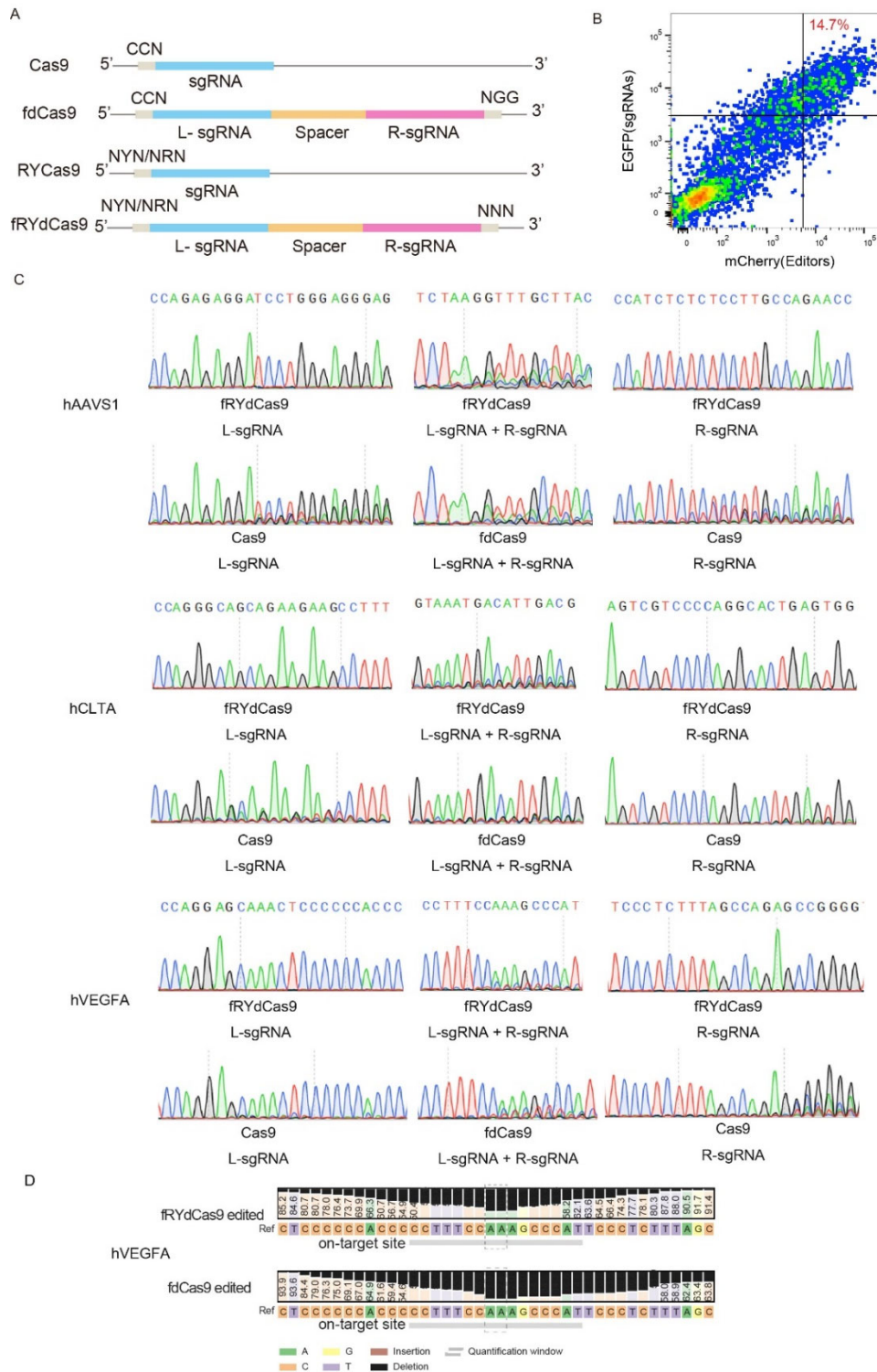


Figure S1. The construction and assessment of fRYdCas9. **(A)** Schematic diagram of sgRNA design for fdCas9, fRYdCas9, and RYCas9. **(B)** Flow cytometry gating strategy for double positive cells after being co-transfected with Editors (linked with mCherry) and sgRNA (linked with EGFP) in HEK293T cells. **(C)** Sanger sequencing chromatograms fRYCas9, fdCas9, and Cas9 at the endogenous targeting sites of hAAVS1, hCLTA1, and hVEGFA. **(D)** Schematic representation of editing windows for fRYCas9 and fdCas9 targeting the hVEGFA gene.

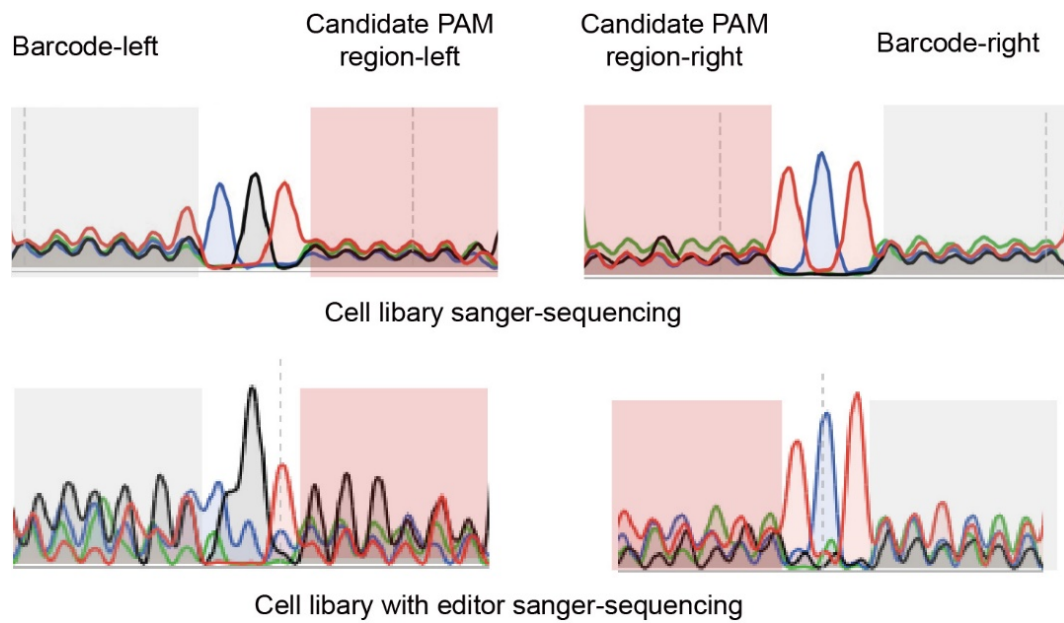


Figure S2. The optimization of spacer length for fRYdCas9. Sanger sequencing chromatograms showing the integration of the PAM library into the genome of HEK293T cells.

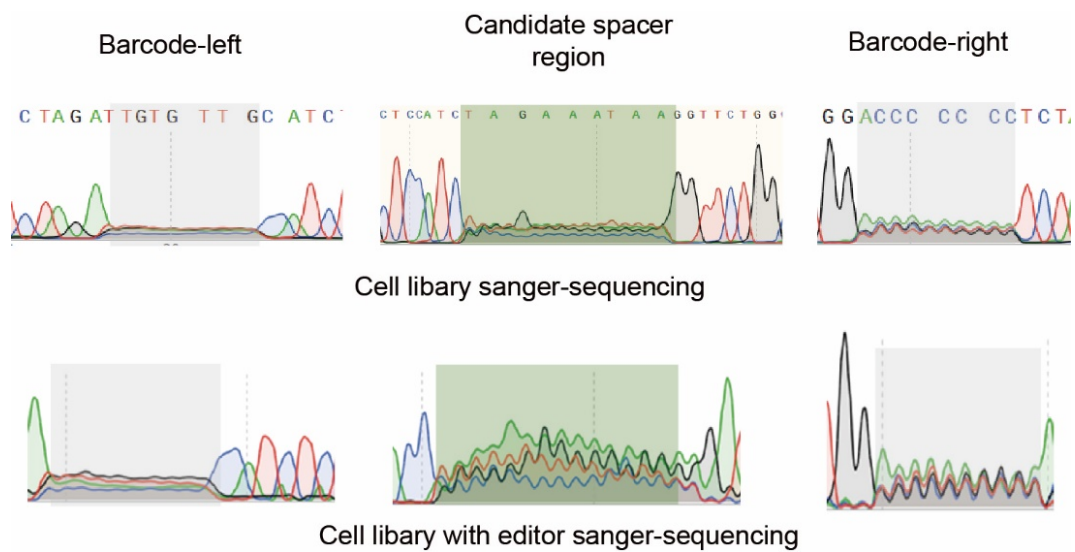
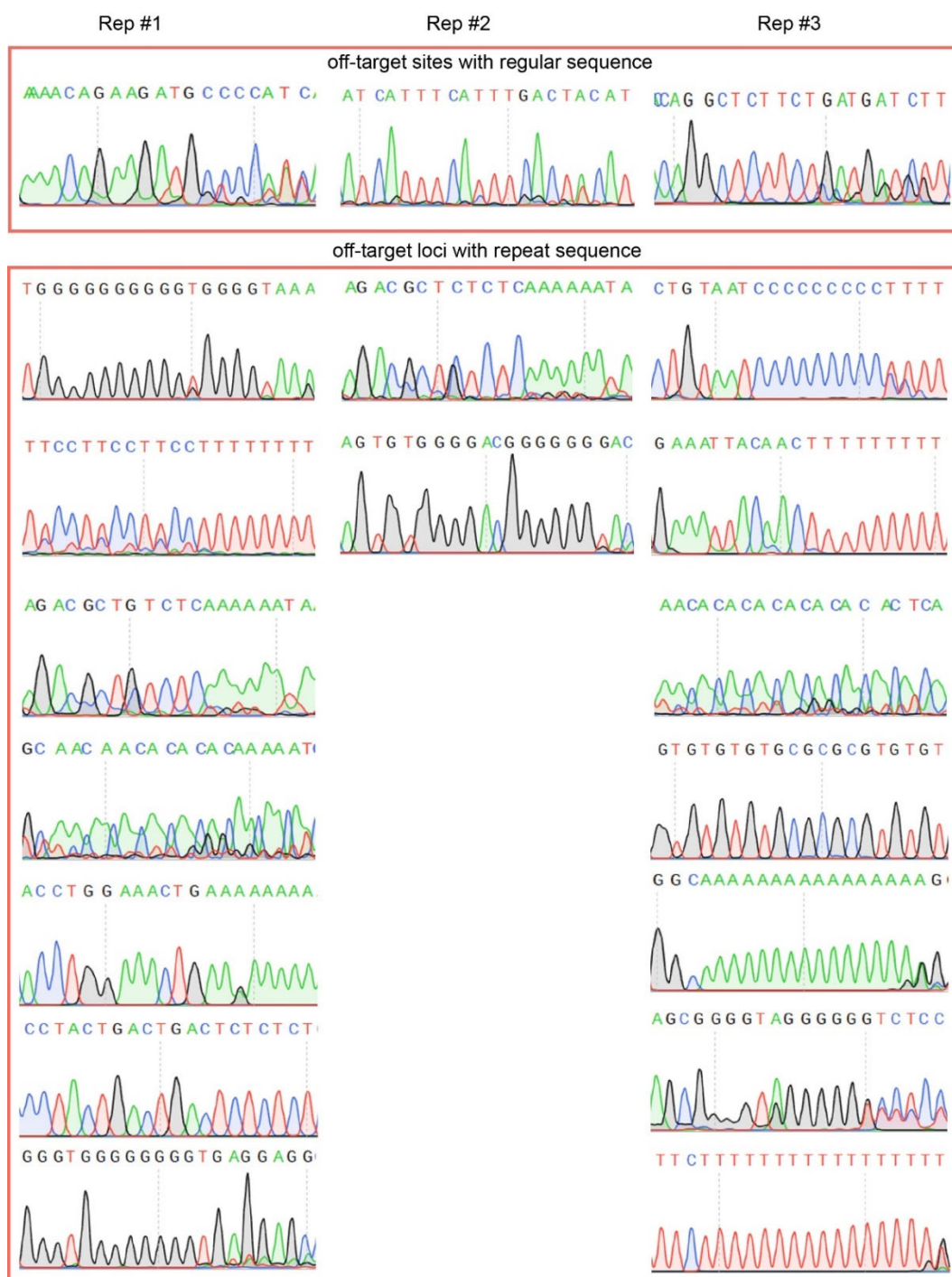


Figure S3. The evaluation of PAM restriction for fRYdCas9. Sanger sequencing chromatograms showing the integration of the editing window library into the genome of HEK293T cells.

A

Sanger validation results for off-target loci of fRYdCas9



B

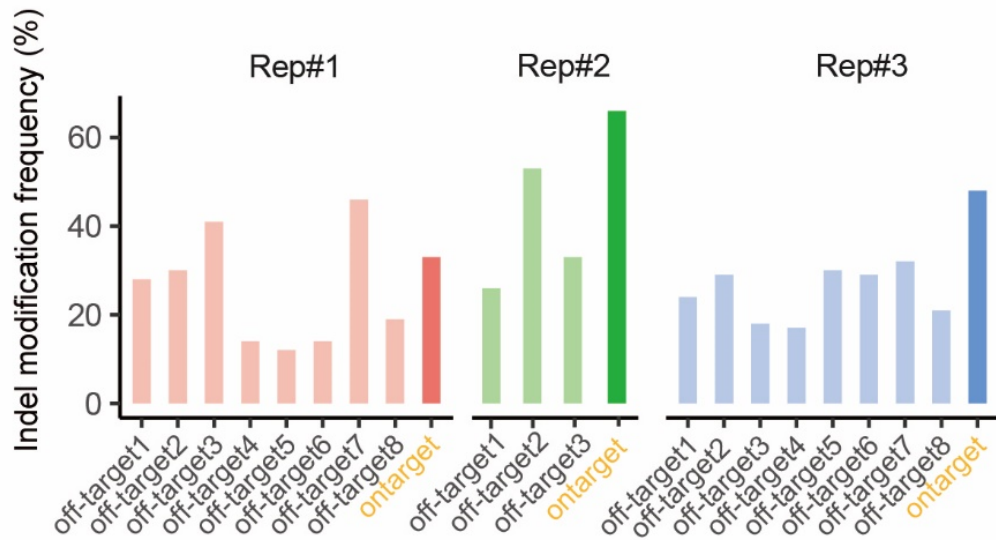


Figure S4. Validation results for off-target loci of fRYdCas9. (A) Sanger validation results for off-target loci of fRYdCas9 in three replicates. (B) The offtarget loci's indel modification frequency from Whole Genome Sequencing (WGS) data.

A

AGCACTTTCGGAGGCCACAG TGG
 AGCACTTTAGGAGGCCACAG TGA
 AGCACTTTGGGAGGCCACAG CAG
 AGCACTTTGGGAGGCCACAG CAG
 AGCACTTTGGGAGGCCACAG TGG
 AGCACTTTGGGAGGCCACAG GAG
 AGCACTTTGGGAGGCCACAG TGG
 AGCACTTTGGGAGGCCACAG CAG
 AGCACTTTGGGAGGCCACAG CAG
 AGCACTTTGGGAGGCCACAG TAG
 AGCACTTTGGGAGGCCACAG CAG
 AGCACTTTGGGAGGCCACAG CAG
 AGCACTTTGGGAGGCCACAG TAG
 AGCACTTTGGGAGGCCACAG TAG
 AGCACTTTGGGAGGCCACAG CAG
 AGCACTTTGGGAGGCCACAG GAG

B

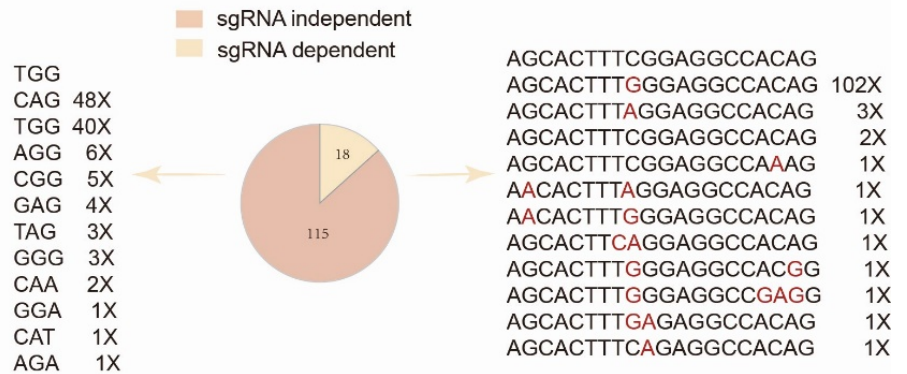


Figure S5. Off-target analysis of fRYdCas9. (A) Off-target occurrences of sgRNA observed in all three samples. (B) The off-target classification of sgRNAs-dependent and sgRNAs-independent for sgRNAs-dependent.

Supplementary Tables

Table S1. The PAM requirements and potential editing sites for fdCas9, Cas9, and fRYdCas9.

| Editors | PAM requirement | hAAVS1(146,664 bp) genebank ID:17 | hCLTA(21,186 bp) genebank ID:1211 | hVEGFA(, bp) genebank ID:7422 | GRCh38 |
|---------|-----------------|--------------------------------------|--------------------------------------|----------------------------------|------------|
| fdCas9 | CCN-56*N-NGG | 1165 | 72 | 185 | 9078468 |
| Cas9 | CCN/NGG | 26896 | 2537 | 3348 | 317350282 |
| RYCas9 | NRN/NYN | 146660 | 22203 | 16277 | 3049314921 |

Table S2. The left and right (L/R-) sgRNA for fRYdCas9 and fdCas9 at endogenous loci, including hAAVS1, hCLTA, and hVEGFA.

| L-sgRNA | | R-sgRNA |
|---------|--------|--------------------------|
| locus | hAAVS1 | gatggagccagagaggatcc tgg |
| | hCLTA | aaaggcttcttctgctgcc tgg |
| | hVEGFA | gggtggggggagtttctcc tgg |

Table S3. The 15–20 bp spacer length with 'NGG' PAM for fRYdCas9 targeting gene hAAVS1.

| Spacer length | L-sgRNA | R-sgRNA |
|---------------|--------------------------|--------------------------|
| 15 bp | tatggggcaaagtgtgggtg agg | cgggcccatagcctggctcc agg |
| 16 bp | ggtcacaggggaagggagaa tgg | caccacactttgcccata tgg |
| 17 bp | gaaagtcaagatgaggtcac agg | ccatatggttcaatgtgac cgg |
| 18 bp | aaagtcaagatgaggtcaca ggg | ccatatggttcaatgtgac cgg |
| 19 bp | aagtcaagatgaggtcacag ggg | ccatatggttcaatgtgac cgg |
| 20 bp | aagtcaagatgaggtcacag ggg | catatggttcaatgtgacc ggg |

Table S4. The increasing spacer lengths for fRYdCas9 at hAAVS1.

| Locus | hCLTA | hVEGFA |
|---------------|--------------------------|---------------------------|
| left sgRNA | R-sgRNA | L-sgRNA |
| spacer length | sgRNA-R | sgRNA-R |
| 0 bp | aagatgtctcccgcatgcgc tca | cctttccaaagccattccc tct |
| 1 bp | agatgtctcccgcatgcgc tca | ctttccaaagccattccct ctt |
| 2 bp | gatgtctcccgcatgcgc tca | tttccaaagccattccctc ttt |
| 3 bp | atgtctcccgcatgcgc tca | ttccaaagccattccctct tta |
| 4 bp | tgtctcccgcatgcgc tca | tccaaagccattccctctt tag |
| 5 bp | gtctcccgcatgcgc tca | ccaaagccattccctctt agc |
| 6 bp | tctcccgcatgcgc tca | caaagccattccctcttta gcc |
| 7 bp | ctcccgcatgcgc tca | aaagccattccctctttag cca |
| 8 bp | tcccgcatgcgc tca | aagccattccctctttagc cag |
| 9 bp | cccgcatgcgc tca | agccattccctctttagcc aga |
| 10 bp | ccgcatgcgc tca | gccattccctctttagcca gag |
| 11 bp | cgcgcatgcgc tca | cccattccctctttagccag agc |
| 12 bp | gcgcatgcgc tca | ccattccctctttagccaga gcc |
| 13 bp | catgcgcatgcgc tca | cattccctctttagccagag ccg |
| 14 bp | atgcgcatgcgc tca | attccctctttagccagagc cgg |
| 15 bp | tgcgcatgcgc tca | ttccctctttagccagagcc ggg |
| 16 bp | gcgcatgcgc tca | tcctctttagccagagccg ggg |
| 17 bp | cgcgcatgcgc tca | ccctctttagccagagccgg ggt |
| 18 bp | gctcagtcctcatctcc tca | cctctttagccagagccggg gtg |
| 19 bp | ctcagtcctcatctcc tca | ctctttagccagagccgggg tgt |
| 20 bp | tcagtcctcatctcc tca | tcctttagccagagccgggg gtg |
| 21 bp | cagtcctcatctcc tca | ctttagccagagccgggggtg tgc |

Table S5. The sgRNA of NYN PAMs for fRYdCas9.

| PAM | L-sgRNA | R-sgRNA |
|-----------|--------------------------|--------------------------|
| right-ACC | gccagaacctctaagggttg ctt | ccaggaaatgggggtgtgtc acc |
| left-TCT | tctctctcttgccagaacc tct | gggtgtgtcaccagataagg aat |
| left-CCT | catctctctcttgccagaa cct | gtgtgtcaccagataaggaa tct |
| left-GCT | tgccagaacctctaagggtt gct | caggaaatgggggtgtgtca cca |
| right-ATC | atctctctcttgccagaac ctc | ggtgtgtcaccagataagga atc |
| left-TTG | tcctggagccatctctctc ttg | cagataaggaatctgcctaa cag |
| right-CTA | ggagccatctctctcttc cag | tcaccagataaggaatctgc cta |
| left-GTT | tcctggcagaacctctaag gtt | aaatgggggtgtgtcaccag ata |

Table S6. Oligonucleotides synthesized for library construction.

| | | |
|-----------|-------------------|---|
| Library 1 | L1 forward primer | ttagtaccgggcccctacgcgtNNNNNNNcgtNNNNNNNggatcctctctggctcca |
| | L1 substrate | ggatcctctctggctccatcgtaagcaaacccttagagggtctggcaaggagagaga |
| | L1 reverse primer | tgggctatgaatacctctagaNNNNNNNagaNNNNNNNtctctctcttgcagaac |
| Library 2 | L2 forward primer | ttagtaccgggcccctacgcgtNNNNNNNNNNNNccaggatcctctctggctcca |
| | L2 substrate | ggatcctctctggctccatcNNNNNNNNNNNNNNNNNNnggttctggcaaggagagaga |
| | L2 reverse primer | tgggctatgaatacctctagaNNNNNNNNNNNNCCAtctctctcttgcagaacc |

Table S7. Primers used in the experiments.

| | | | |
|----------------------------|--------|----------------------------|-----------------------------|
| TableS2 primers | hAAVS1 | F1 gcagcaccaggatcagtg | R1 ggtaatgtggctctggttc |
| | | F2 agaaccgggcaggctacg | R2 acctctgttaggcagattc |
| | hVEGFA | F1 gaagcatccctggacacttc | R1 acgtctcactctcgaagac |
| | | F2 gtacatgaagcaactccagtccc | R2 cagcttccctgtggtggc |
| | hCLTA | F1 ggcatgtgctgtgtagcaag | R1 tctccctcctctctgaatgcca |
| | | F2 tcgtccccaggcactgag | R2 atgtagtgttccacagggtgg |
| TableS3 primers | hAAVS1 | F1 acatcatgtcttctctgccag | R1 tgcaggcccatatcacgc |
| | | F2 gctgctgggcctacgaaa | R2 ggtcagatggcagctctg |
| TableS4 primers | hCLTA | F1 cgtgtctacctgagtctgacct | R1 gcacaggtgaggctactctt |
| | | F2 ggcatgtgctgtgtagcaag | R2 tctccctcctctctgaatgcca |
| | hVEGFA | F1 gaagcatccctggacacttc | R1 cacagatctattggaatcctggag |
| | | F2 cagtcactccagcctgttg | R2 acgtctcactctcgaagac |
| TableS5 primers | hAAVS1 | F1 gcagcaccaggatcagtg | R1 ggtaatgtggctctggttc |
| | | F2 ctcccaggatcctctctg | R2 tgatattgggtctaaccgccacc |