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# Multiplex genome engineering in human cells using all-in-one CRISPR/Cas9 vector system

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CRISPR/Cas9-mediated genome editing is a next-generation strategy for genetic modifications, not only for single gene targeting, but also for multiple targeted mutagenesis. To make the most of the multiplexity of CRISPR/Cas9, we established a system for constructing all-in-one expression vectors containing multiple guide RNA expression cassettes and a Cas9 nuclease/nickase expression cassette. We further demonstrated successful examples of multiple targeting including chromosomal deletions in human cells using the all-in-one CRISPR/Cas9 vectors constructed with our novel system. Our system provides an efficient targeting strategy for multiplex genome/epigenome editing, simultaneous activation/repression of multiple genes, and beyond.

Genome editing using clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas9 is an easy and efficient strategy for the generation of gene-modified cells and organisms[1,2]. The CRISPR/Cas9 system consists of two components: Cas9 protein and guide RNA (gRNA). The natural Cas9 protein possesses a nuclease activity and can induce a DNA double-strand break (DSB) in any genomic sequence guided by a gRNA, provided that a protospacer adjacent motif (PAM) sequence exists in the target locus[3–5]. *Streptococcus pyogenes* Cas9 (SpCas9) has been the most widely used Cas9 protein, and its PAM sequence is 5′-NGG-3′, which is the only restriction for designing CRISPR/Cas9 target sequences[6].

Since Cas9 has no DNA recognition specificity except for the PAM sequence, simple multiplication of gRNAs along with a common Cas9 protein results in multiplex genome engineering. Currently, two or more kinds of plasmids or DNA fragments are generally used for multiple targeting in cultured cells[7]. However, cotransfection of multiple plasmids can cause low targeting efficiency in cells that are inefficient in DNA transfection.

Here, we report an easy and efficient construction system for all-in-one CRISPR/Cas9 vectors expressing Cas9 protein and up to seven gRNAs. The expression cassettes of the gRNAs are tandemly ligated into a single vector using the Golden Gate cloning method. Furthermore, we demonstrate simultaneous multiple targeted mutagenesis and chromosomal deletions mediated by not only Cas9 nuclease, but also Cas9 nickase. In Cas9 nuclease-mediated genome editing, we constructed an all-in-one vector expressing Cas9 nuclease and seven gRNAs, and targeted seven genomic loci. In Cas9 nickase-mediated genome editing, we constructed an all-in-one vector expressing Cas9 nickase and six gRNAs, and targeted three genomic loci.

## Results

**Establishment of an all-in-one vector construction system for CRISPR/Cas9-mediated multiplex genome engineering.** To establish an all-in-one vector system, we modified the pX330 vector, originally developed by the Feng Zhang laboratory[4,8], containing a single gRNA expression cassette and a Cas9 nuclease expression cassette. The protocol for inserting a gRNA-targeting sequence was the same as described in a previous paper[8] (Figure 1, STEP 1). For the assembly of gRNA cassettes, we applied the Golden Gate cloning method, which has been well-established for modular assembly of DNA-binding repeats of transcription activator-like effector nucleases (TALENs) (Figure 1, STEP 2). After assembling the plasmids, we were able to easily screen for correctly-assembled clones by colony PCR, as shown in Supplementary Fig. S1. In addition, the plasmids for the second-step reaction retained the structure of a CRISPR/Cas9 expression vector harboring single gRNA and Cas9 expression cassettes, and can therefore be used separately for single gene targeting. In this study, we
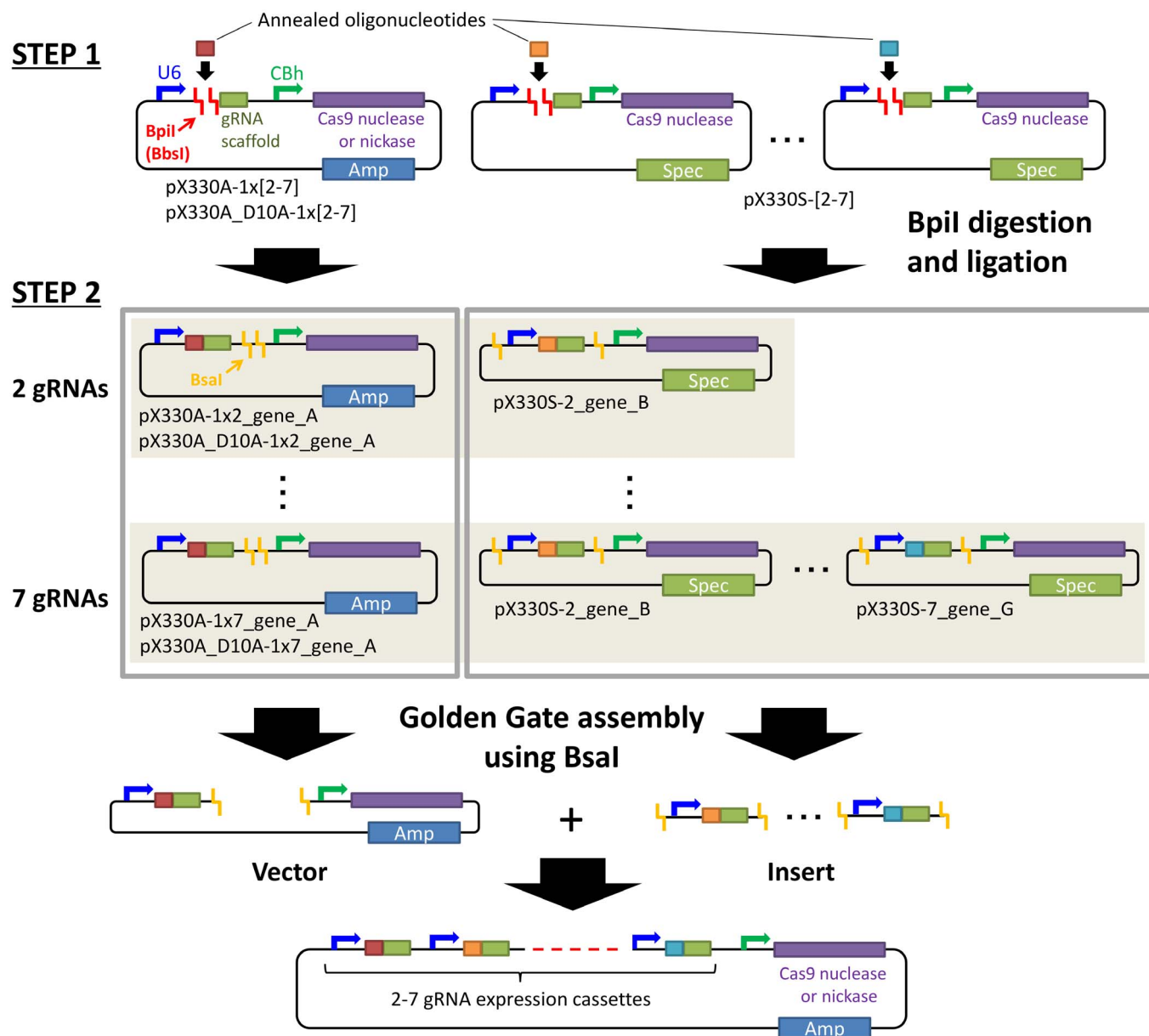
**Figure 1 | Schematic overview of the all-in-one CRISPR/Cas9 vector construction system for multiplex genome engineering.** Oligonucleotides corresponding to each target sequence are annealed and inserted into BpiI-digested pX330A or pX330S vectors (STEP 1). The constructed vectors harboring single gRNA expression cassettes are then assembled into an all-in-one vector harboring multiple gRNA cassettes using the Golden Gate assembly method (STEP 2). Amp, ampicillin; Spec, spectinomycin; U6, human U6 promoter; CBh, chicken beta-actin short promoter.

constructed plasmids to create all-in-one vectors expressing two to seven gRNAs with Cas9 nuclease or nickase (Figure 1).

**Multiplex genome engineering with a single vector expressing seven gRNAs and one Cas9 nuclease.** To prove the functionality of the all-in-one vectors created by our system, we constructed a CRISPR/Cas9 vector targeting seven genomic loci (Supplementary Fig. S2A). Oligonucleotides targeting these seven loci were synthesized and inserted separately into pX330A/S vectors. The constructed vectors were then assembled using Golden Gate cloning, resulting in an all-in-one CRISPR/Cas9 vector expressing Cas9 nuclease and seven gRNAs targeting different genomic loci (Figure 2A).

The genome editing efficiency of this multiplex CRISPR/Cas9-nuclease vector was verified using a mismatch-sensitive endonuclease assay[9]. We transfected the all-in-one vector containing seven

gRNA cassettes as well as single gRNA-expressing vectors into HEK293T cells, and performed genomic PCR followed by an endonuclease cleavage assay. The results indicated that the seven gRNA-expressing all-in-one vector was able to induce mutations at almost the same level of genome editing efficiency as the single gRNA-expressing vectors (Figure 2B). Therefore, we confirmed the usefulness of our newly-established all-in-one CRISPR/Cas9-nuclease vector system.

**Multiplex genome engineering with a single vector expressing six gRNAs and one Cas9 nickase.** Recent progress in CRISPR/Cas9-mediated genome engineering has permitted Cas9 nickase-mediated mutagenesis[10–12]. A DSB was reported to be introduced when two gRNAs induce adjacent nicks at both DNA strands. We thus applied this paired nickase strategy to our all-in-one vector system. Since two gRNAs are required for a single locus in nickase-mediated
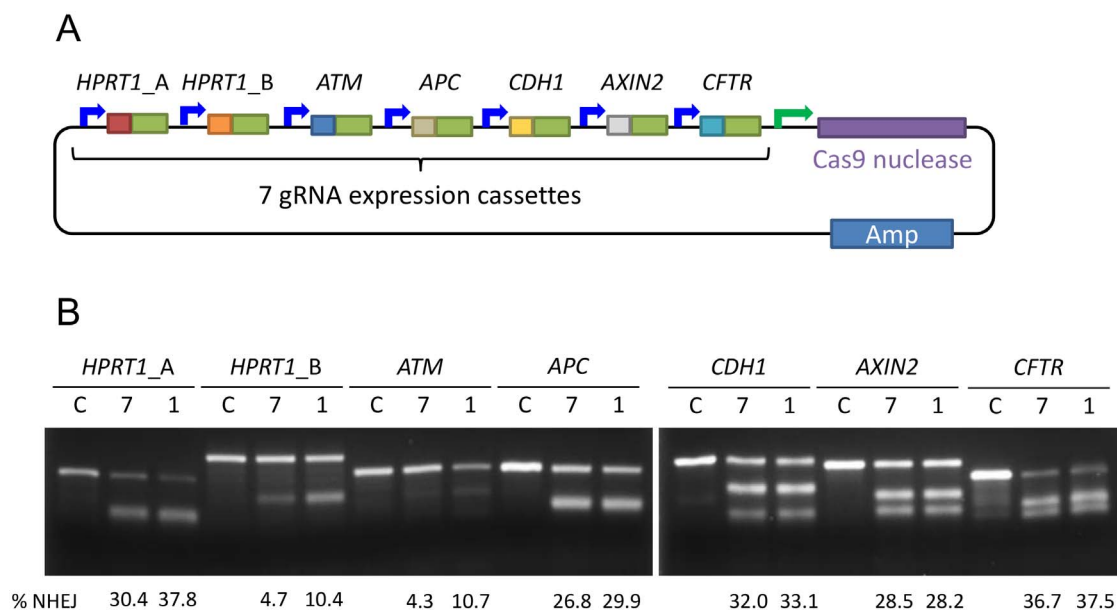
**Figure 2 | Multiplex genome editing with Cas9 nuclease and seven gRNAs.** (A) Schematic illustration of the all-in-one vector expressing seven gRNAs targeting seven different genomic loci and Cas9 nuclease. The blue and green bent arrows indicate the U6 and CBh promoters, respectively. (B) Genomic cleavage analysis of the seven genomic loci targeted with the all-in-one CRISPR/Cas9-nuclease vector. The products from untransfected control cells (C) and cells transfected with CRISPR/Cas9-nuclease vectors targeting seven (7) and single (1) loci were analyzed by agarose gel electrophoresis. The percentage of non-homologous end-joining (% NHEJ) was estimated using ImageJ software as previously described[30].

mutagenesis, up to three loci can be simultaneously targeted using our all-in-one vector containing six gRNA expression cassettes and one Cas9 nickase expression cassette.

We constructed an all-in-one CRISPR/Cas9-nickase vector expressing six gRNAs targeting three loci in the adenomatous polyposis coli (*APC*) gene (Figure 3A). Since the offset lengths of the individual loci were five, eight, and four base pairs, we named these loci off-5, off-8, and off-4, respectively (Supplementary Fig. S2B). The all-in-one CRISPR/Cas9-nickase vector targeting these three loci was transfected into HEK293T cells and an endonuclease cleavage assay was performed. The sizes of PCR products and cleaved fragments were nearly identical among the three loci (Figure 3B). As a result of the cleavage assay, cleaved products certainly appeared and the applicability of our all-in-one vector system for Cas9-nickase-mediated multiplex genome engineering was demonstrated (Figure 3C).

**Induction of chromosomal deletions using all-in-one CRISPR/ Cas9-nuclease and CRISPR/Cas9-nickase vectors.** Simultaneous introduction of DSBs on the same chromosome often causes a large deletion[4,8]. To verify the applicability of our system for such chromosomal deletions, we analyzed whether the constructed all-in-one CRISPR/Cas9-nuclease and CRISPR/Cas9-nickase vectors can generate large deletions. The CRISPR/Cas9-nuclease vector, illustrated in Figure 1A, can induce two DSBs at the *HPRT1* locus, possibly resulting in ∼1.9-kb deletion (Figure 4A). The CRISPR/ Cas9-nickase vector, illustrated in Figure 3A, can induce two DSBs at the *APC* locus, possibly resulting in ∼2.5-kb deletion (Figure 4B). Therefore, we performed genomic PCR using primers running from both outsides of DSB-inducing sites, and found that chromosomal deletions occurred at the both loci (Figure 4C). Interestingly, DNA sequencing revealed that Cas9 nuclease-mediated large deletion was likely to be repaired mainly by microhomology-mediated end-joining, resulting in the same sequence pattern, whereas Cas9 nickase-mediated large deletion resulted in a variety of sequence patterns, supposedly caused by non-homologous end-joining (Figure 4D). These differences might be due to the different DSB-inducing manner via Cas9 nuclease and Cas9 nickase. Cas9 nuclease

are known to induce blunt end, while double nicking via Cas9 nickase generates protruding end[10,11].

## Discussion

Multiplex genome engineering is one of the most attractive applications of the CRISPR/Cas9 system. Our study provides a simple and efficient strategy for single vector-mediated multiple targeting of up to seven genomic loci, which has not been reported to date. One matter for concern in CRISPR/Cas9-mediated multiplex genome editing is targeting specificity. Several reports have described high frequencies of off-target mutations using the CRISPR/Cas9-nuclease system[13–15]. However, recent improvements with a paired nickase strategy were reported to reduce off-target mutations dramatically[10–12]. Since we have shown the applicability of our all-in-one vector system with Cas9 nickase, our system should minimize the risk of off-target mutations in CRISPR/Cas9-mediated multiple gene targeting.

Thus far, we have only demonstrated the functionality of our all-in-one CRISPR/Cas9-nuclease and CRISPR/Cas9-nickase vectors in cultured cells. However, it should also be possible to apply these vectors directly, even in animal embryos, because Mashiko and colleagues reported that a pX330-based CRISPR/Cas9 plasmid could be directly injected for genome editing in mice[16,17], and CRISPR/Cas9 paired nickases were successfully applied for mouse genome editing in recent studies[18–20]. In addition, CRISPR/Cas9 system has been utilized in viral vectors such as lentiviral vectors[21]. Since our multiple-guided CRISPR/Cas9-nuclease and CRISPR/Cas9-nickase system will make it convenient to deliver multiple gRNA cassettes into viral vectors, it is possible that efficient viral vector-mediated multiplex genome engineering can be performed by using our system.

In addition to the genome editing approaches described above, our system is expected to be utilized in other CRISPR/Cas9-based technologies such as transcriptional control[22–24], epigenome editing[25], and visualization of specific genomic loci[26]. As simple replacement of Cas9 nuclease/nickase with inactivated Cas9 with or without various effector domains allows our system to be applied to such a wide range of applications, we anticipate that our report will provide
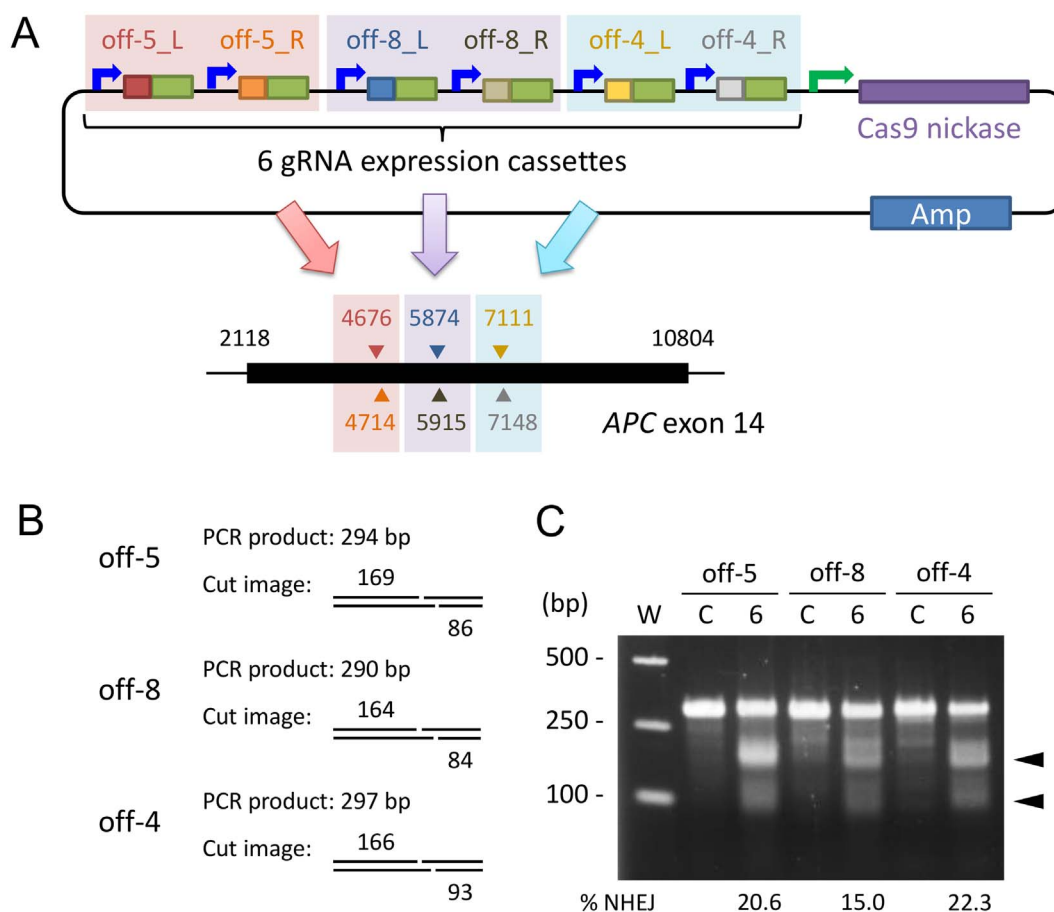
**Figure 3 | Multiplex genome editing with Cas9 nickase and six gRNAs.** (A) Schematic illustration of the all-in-one vector expressing six gRNAs targeting three different genomic loci and Cas9 nuclease. The black box indicates exon 14 of the human *APC* gene. The numbers from 2118 to 10804 represent the base positions in the *APC* gene transcript (NCBI reference sequence: NM_001127511.2). (B) The sizes of PCR products and cut images in each locus. (C) Genomic cleavage analysis of the three genomic loci targeted with the all-in-one CRISPR/Cas9-nickase vector. The products from untransfected control cells (C) and cells transfected with the CRISPR/Cas9-nickase vector expressing six gRNAs (6) were analyzed by agarose gel electrophoresis. The arrowheads indicate the approximate positions of the cleaved fragments. % NHEJ was estimated using ImageJ software as previously described[30]. W, Wide-Range DNA Ladder (100–2,000 bp) (Takara Bio, Shiga, Japan).

substantial contributions to a large number of researchers interested in innovative CRISPR/Cas9-based technologies.

## Methods

**Plasmids for the multiplex CRISPR/Cas9 vector system.** All plasmids for the multiplex CRISPR/Cas9 vector system were constructed using the In-Fusion cloning method with PCR products from pX330 (Addgene, Cambridge, MA; Plasmid 42230)[4,8] and pFUS_A30A (Addgene; Plasmid 31029)[27] as summarized in Supplementary Table S1.

**Insertion of annealed oligonucleotides into pX330A and pX330S vectors.** To construct CRISPR/Cas9 plasmids targeting human genes, sense and antisense oligonucleotides were synthesized and annealed in the following buffer: 40 mM Tris-HCl (pH 8.0), 20 mM MgCl$_2$, and 50 mM NaCl. The annealed oligonucleotides, pX330A/S vectors, BpiI enzyme (Thermo Scientific, Rockford, IL), and Quick ligase (New England Biolabs, Beverly, MA) were mixed in a single tube with T4 DNA ligase buffer (New England Biolabs), and subjected to a thermal cycling reaction as follows: 3 cycles of 37°C for 5 min and 16°C for 10 min. After the cycling reaction, additional BpiI digestion was performed at 37°C for 1 h. A list of the constructed plasmids with the vectors used and oligonucleotide sequences is shown in Supplementary Table S2.

**Golden Gate assembly and screening by colony PCR.** Golden Gate assembly was performed as described previously[27–29] with some modifications. pX330A/S plasmids, BsaI-HF enzyme (New England Biolabs), and Quick ligase were mixed in a single tube with T4 DNA ligase buffer, and subjected to a thermal cycling reaction as follows: 6–15 cycles of 37°C for 5 min and 16°C for 10 min. After the cycling reaction, additional BsaI-HF digestion was performed at 50°C for 30 min. For construction of the CRISPR/Cas9-nuclease vector targeting seven genomic loci, pX330A-1x7_HPRT1_A, pX330S-2_HPRT1_B, pX330S-3_ATM, pX330S-4_APC, pX330S-5_CDH1, pX330S-6_AXIN2, and pX330S-7_CFTR were unified into a single vector.

For construction of the CRISPR/Cas9-nickase vector targeting three genomic loci, pX330A_D10A-1x6_off-5_L, pX330S-2_off-5_R, pX330S-3_off-8_L, pX330S-4_off-8_R, pX330S-5_off-4_L, and pX330S-6_off-4_R were unified into a single vector.

Correctly-assembled clones were screened by colony PCR using CRISPR-step2-F (5′-GCCTTTTGCTGGCCTTTTGCTC-3′) and CRISPR-step2-R (5′-CGGGCCATTTACCGTAAGTTATGTAACG-3′) primers, followed by agarose gel electrophoresis and ethidium bromide staining.

**Cell culture and transfection.** HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum. Transfection of plasmids was carried out as described previously[29]. Briefly, 30,000 HEK293T cells were transfected with 400 ng of CRISPR/Cas9 plasmids using Lipofectamine LTX (Life Technologies, Carlsbad, CA) in a 96-well plate. At 48 h post-transfection, the cells were collected into PCR tubes.

**Cell lysis, genomic PCR, and cleavage assay.** Cell lysis, genomic PCR, and detection of cleavage were conducted using a GeneArt Genomic Cleavage Detection Kit (Life Technologies) according to the manufacturer's instructions except that DNA polymerase used in the experiment of Figure 3C was KOD FX Neo (Toyobo, Osaka, Japan) instead of AmpliTaq Gold 360. Briefly, 20 μl of cell lysis buffer and 0.8 μl of protein degrader were added to cell pellets prepared as described above. After an initial program in a thermal cycler (68°C for 15 min and 95°C for 10 min), genomic PCR was carried out using 1–2 μl of cell lysates and the primers listed in Supplementary Table S3. The PCR products were subjected to re-annealing and a cleavage assay according to the manufacturer's instructions. The products were then analyzed by electrophoresis in 3% agarose gels and ethidium bromide staining.

**Detection and sequencing of chromosomally deleted alleles.** Chromosomally deleted alleles were detected by genomic PCR using the primers listed in Supplementary Table S4, followed by agarose gel electrophoresis and ethidium bromide staining. The deleted PCR products were then cloned into TA cloning vector using a TArget Clone -Plus- Kit (Toyobo). Sequencing was performed using an ABI
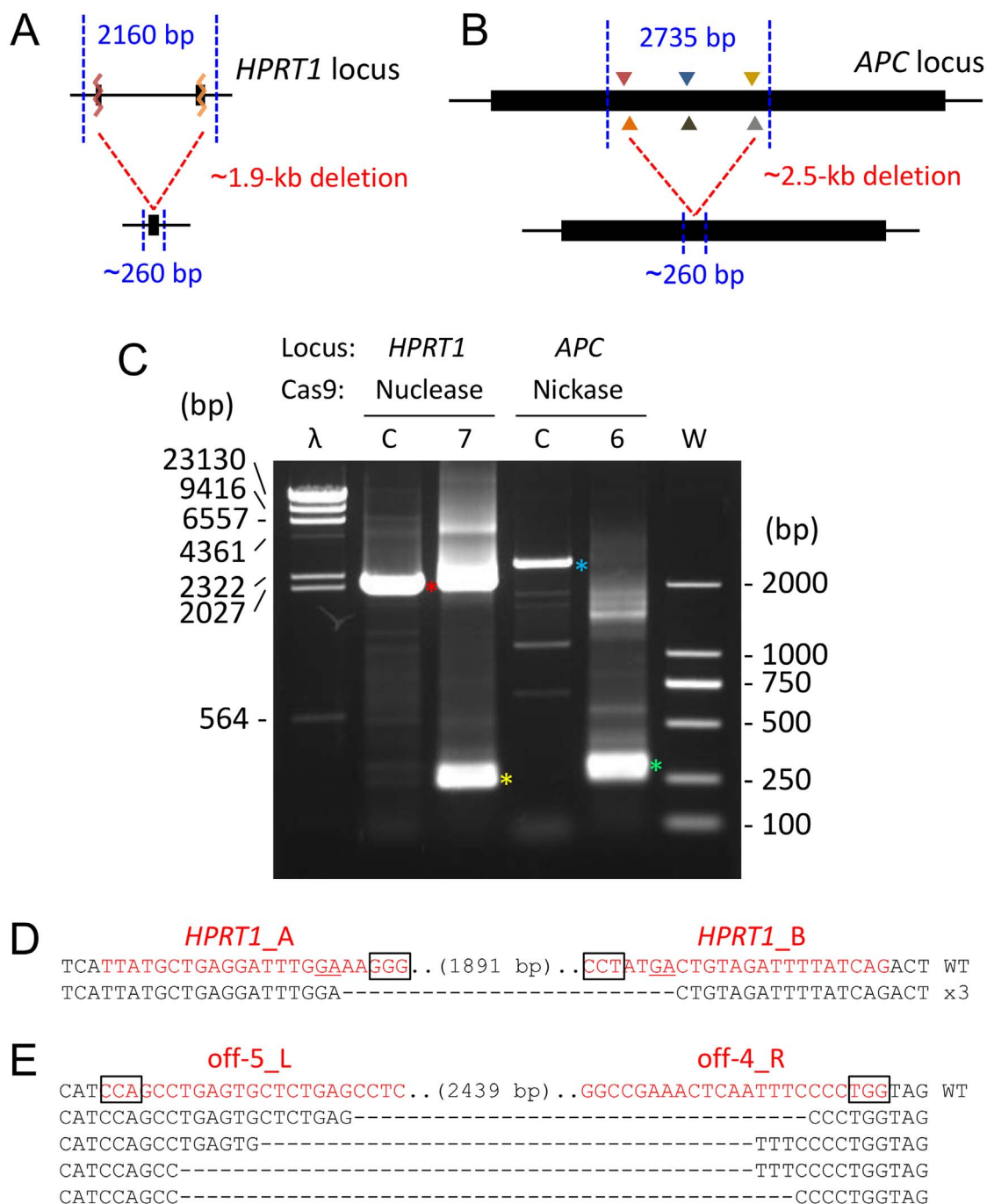
**Figure 4 | Large deletions mediated by all-in-one CRISPR/Cas9 vectors.** (A) Schematic illustration of large deletion at the *HPRT1* locus. The black boxes indicate exons. The blue lines and letters indicate PCR products. The zigzag lines indicate the *HPRT1*_A and *HPRT1*_B target sites. (B) Schematic illustration of large deletion at the *APC* locus. The black boxes indicate exons. The blue lines and letters indicate PCR products. The triangles indicate the off-5, off-8 and off-4 target sites. (C) Genomic PCR analysis of the *HPRT1* and *APC* loci. The products from untransfected control cells (C) and cells transfected with the CRISPR/Cas9-nuclease vector expressing seven gRNAs (7) or the CRISPR/Cas9-nickase vector expressing six gRNAs (6) were analyzed by agarose gel electrophoresis. Red and blue asterisks indicate PCR products from un-deleted alleles, whereas yellow and green asterisks indicate PCR products from chromosomally deleted alleles. λ, λHindIII marker. W, Wide-Range DNA Ladder (100–2,000 bp) (Takara Bio). (D) Sequences of the PCR products from deleted alleles at the *HPRT1* locus. The *HPRT1*_A and *HPRT1*_B target sites are indicated by red letters. PAM sites are indicated by black boxes. Deletions are indicated by dashes. (E) Sequences of the PCR products from deleted alleles at the *APC* locus. The off-5_L and off-4_R target sites are indicated by red letters. PAM sites are indicated by black boxes. Deletions are indicated by dashes.

3131xl Genetic analyzer (Life Technologies) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies).

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## Author contributions

T.S. designed the work, performed the experiments, and wrote the manuscript. A.N. supported the creation of CRISPR/Cas9 vectors. S.K. supported human cell experiments. K.C. provided instructions. T.Y. supervised the work. All authors reviewed the manuscript.

## Additional information