GeneArt® CRISPR Nuclease Vector Kit

Rapid and efficient genome editing from a single vector

Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) system is the latest addition to the genome editing toolbox, offering a simple, rapid, and efficient solution. The CRISPR/Cas system is a prokaryotic adaptive immune system that uses an RNA-guided DNA nuclease to silence viral nucleic acids [1]. The type II CRISPR/Cas system from the bacterium *Streptococcus pyogenes* has been modified to enable editing of mammalian genomes [2, 3]. As a simple two-component system composed of Cas9 protein and a non-coding guide RNA (gRNA), the engineered type II CRISPR/Cas system can be utilized to cleave genomic DNA at a predefined target sequence of interest.

The gRNA has two molecular components, a target complementary CRISPR RNA (crRNA), and an auxiliary trans-activating crRNA (tracrRNA). The gRNA unit guides the Cas9 nuclease to a specific genomic locus and the Cas9 protein induces a double-strand break (DSB) at the specific genomic target sequence (Figure 1). Following CRISPR/Cas9-induced DNA cleavage, the DSB can be repaired by the cellular repair machinery using either non-homologous end joining (NHEJ) or a homology-directed repair mechanism. This product bulletin provides detailed information about our GeneArt® CRISPR Nuclease Vector technology along with recommendations for using the kits.

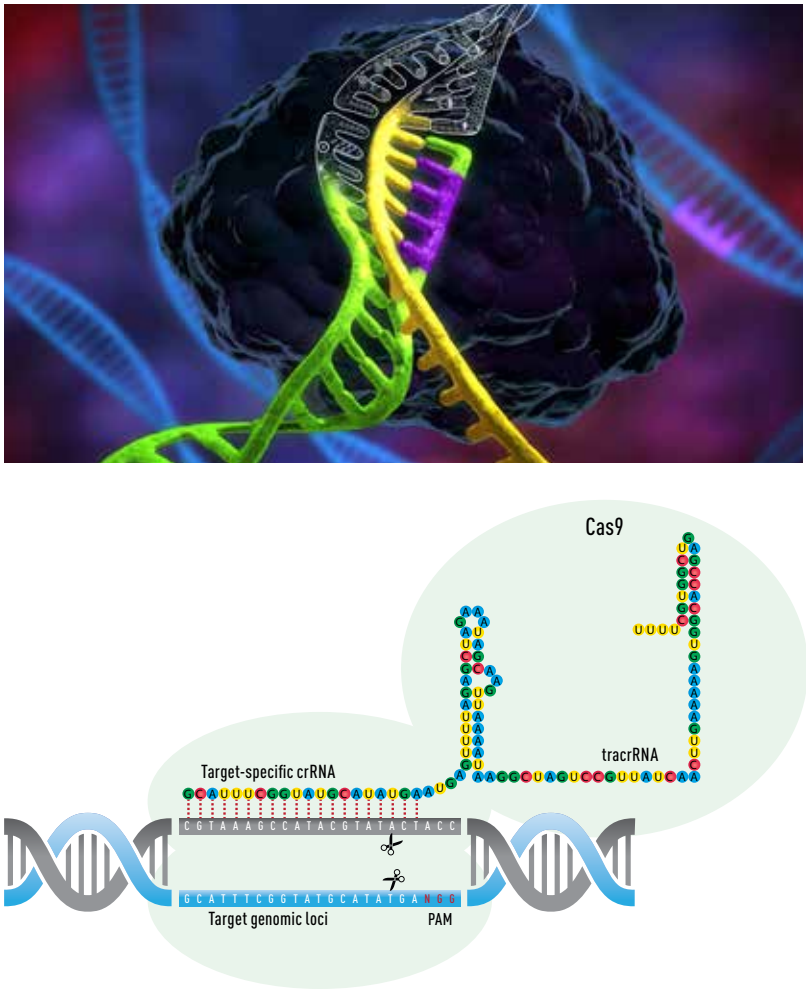

Figure 1. A CRISPR/Cas9 targeted double-strand break. Cleavage occurs on both strands, 3 bp upstream of the NGG proto-spacer adjacent motif (PAM) sequence on the 3′ end of the target sequence.

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Product details

The GeneArt® CRISPR Nuclease Vector Kit offers an all-in-one expression vector consisting of both a Cas9 nuclease expression cassette and gRNA cloning cassette (Figure 2) for simple and efficient cloning of a double-stranded DNA oligo encoding a target-specific crRNA. This system allows you to edit and engineer a genomic locus of choice in a sequence-specific manner from a single plasmid in mammalian cells. Key features of the kit are detailed below.

- A single vector is provided for expression of all the components needed for CRISPR/Cas9-mediated gene editing. Two different formats are available for enrichment of transfected cells (Figure 3):
  - **GeneArt® CRISPR Nuclease Vector with OFP:** In this vector, Cas9 is fused to orange fluorescent protein (OFP) via a self-cleaving 2A peptide linkage [4], thereby allowing the expression of Cas9 and OFP from the same mRNA. This allows for fluorescence-based tracking of transfection efficiency as well as FACS-based sorting and enrichment of cells expressing Cas9 and gRNA. Note: excitation and emission of OFP are in the same range as GFP—therefore, no new filter is needed.
  - **GeneArt® CRISPR Nuclease Vector with CD4:** In this vector, Cas9 is fused to CD4 surface antigen via a self-cleaving 2A peptide linkage, thereby allowing the expression of Cas9 and CD4 from the same mRNA. This provides an option for magnetic bead–based sorting and enrichment of cells expressing Cas9 and gRNA using Dynabeads® CD4 magnetic beads (Cat. No. 11145D). Transfection efficiency can also be tracked using an anti-CD4 fluorescent antibody.

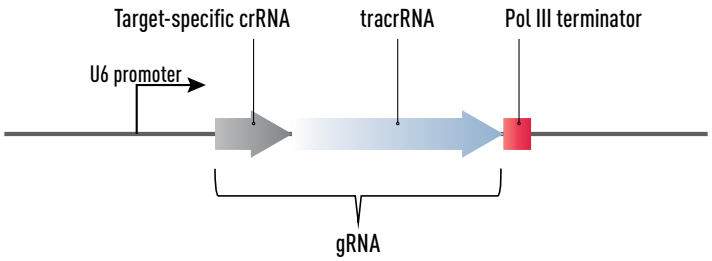


Figure 2. The gRNA expression cassette. The gRNA cassette is expressed from the U6 promoter (a pol III promoter) as a single transcript and is comprised of crRNA and tracrRNA.

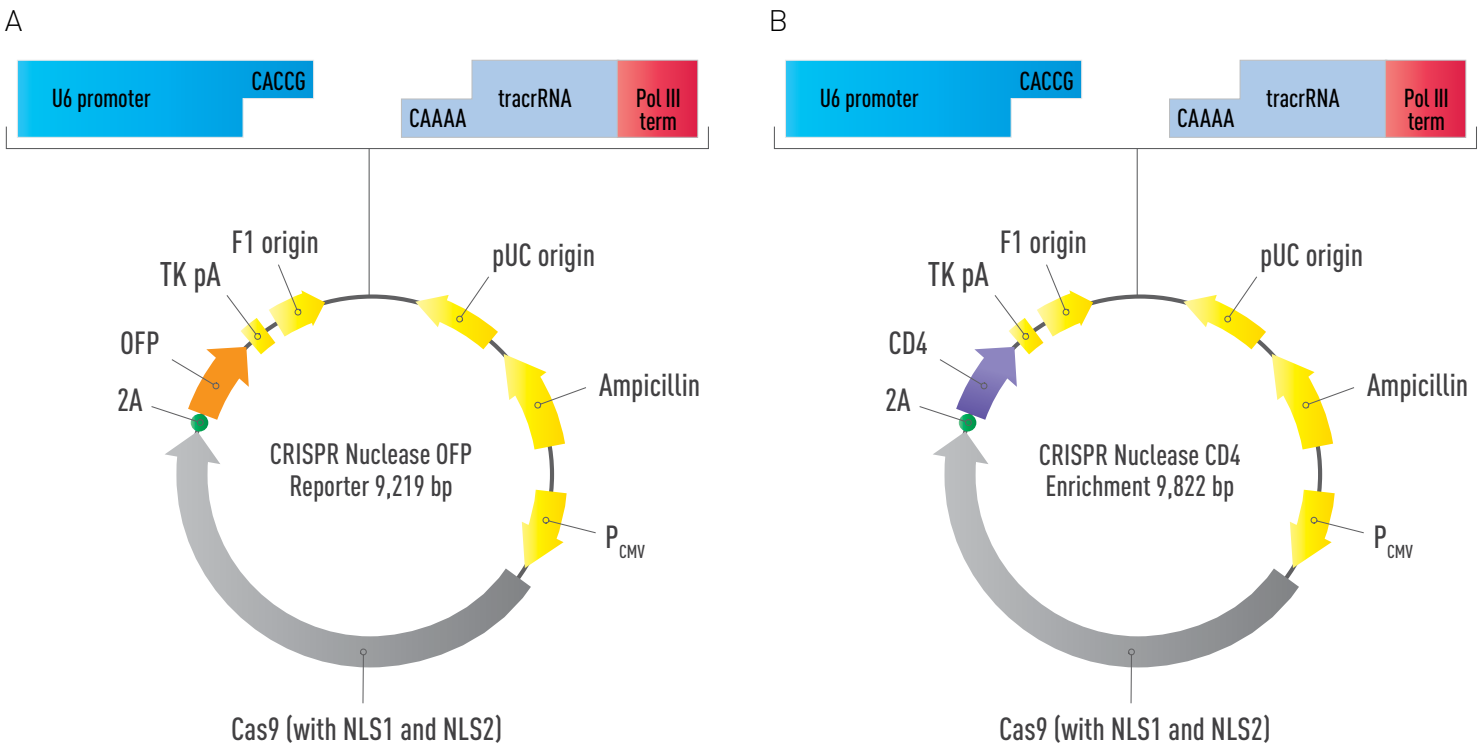


Figure 3. GeneArt® CRISPR Nuclease Vector maps. The vector is pre-linearized with 5 base pair overhangs for easy cloning of your double-stranded DNA oligo that encodes a target-specific crRNA. Maps are shown of the vectors with (**A**) OFP reporter and (**B**) CD4 reporter. The gRNA, Cas9, and reporter are expressed from the same vector. The expression of Cas9 and the reporter gene (either OFP or CD4) is driven by the CMV promoter. Cas9 is directed to the nucleus by nuclear localization signals (NLS1 and NLS2).

Cloning workflow and analysis

1. Design strategy and target specificity

To use the GeneArt® CRISPR Nuclease Vector Kit, you first design two single-stranded DNA oligonucleotides (24–25 bp), one encoding the crRNA specific to the target (forward-strand oligonucleotide) and the other its complement (reverse-strand oligonucleotide). The choice of genomic target sequence can significantly affect the degree of cleavage observed. Therefore, we recommend that you test more than one target-specific crRNA sequence per locus of interest. Guidelines are provided below for choosing your target sequence, and an example is provided in Figure 4. Note that these are general recommendations only and exceptions may occur.

- **Length:** Choose a target sequence ranging from 19 to 20 nucleotides in length that is adjacent to an NGG proto-spacer adjacent motif (PAM) sequence on the 3' end of the target sequence. The 5' G required for transcription initiation from the U6 pol III promoter is already included in the vector overhangs and does not need to be included in the target sequence.
- **Homology:** Make sure that the target sequence does **not** contain significant homology to other genes as this can increase off-target effects. Recently published work has shown that gRNA-Cas9 complexes can potentially tolerate 1–3 or more mismatches, depending on their location in the gRNA [5]. Refer to published articles for more insights into choosing a target sequence [5, 6].
- **Orientation:** You may choose a target sequence encoding the sense sequence of the target locus or the antisense sequence. Thus, you can generate CRISPR RNA in two possible orientations, provided that it meets the PAM requirements on the 3' end.

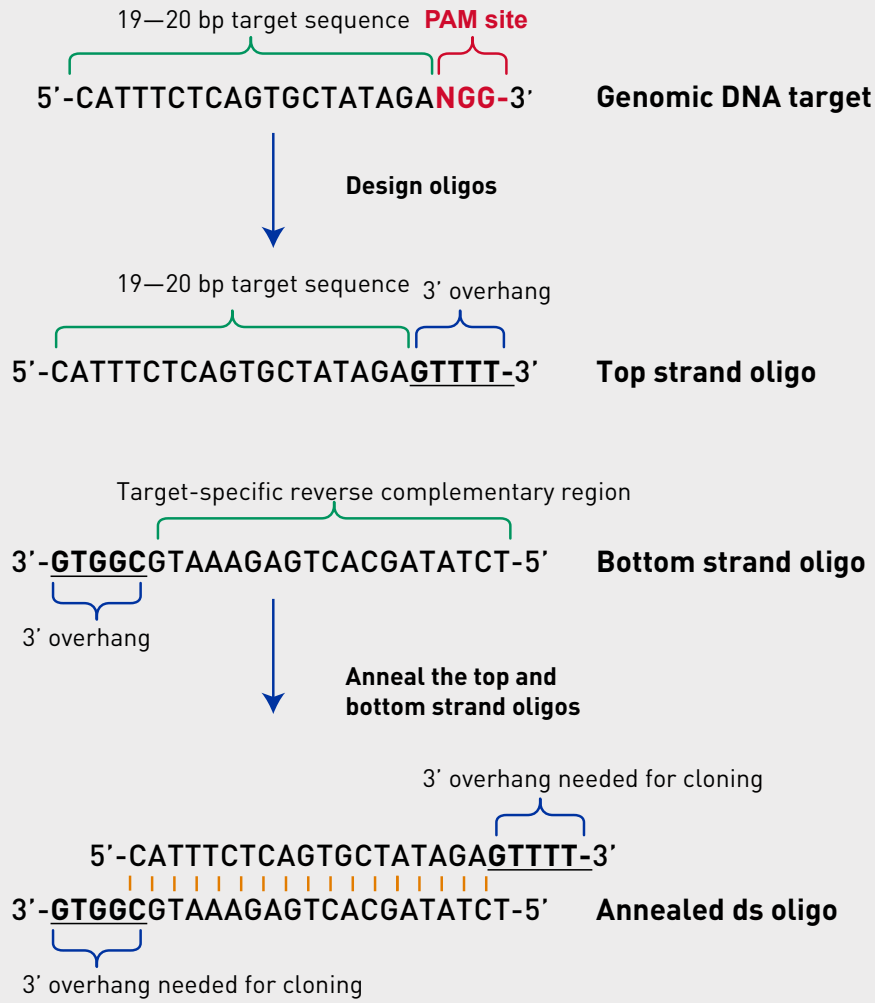
Figure 4. An overview of the oligo design workflow.

1. **Choose genomic DNA target sequence:** Choose a 19—20 bp target sequence upstream of the NGG PAM site. You can choose a target site either in the sense or antisense strand of the genomic DNA provided it meets the PAM requirements.

2. **Top strand oligo design:** Add a 5 base, GTTTT 3' overhang needed for cloning to the selected 19—20 bp target sequence to generate the top strand oligo. Please note that the PAM site is not included in the oligo.

3. **Bottom strand oligo design:** Generate reverse complementary sequence specific to the 19—20 bp target sequence and add a 5 base, CGGTG 3' overhang to generate the bottom strand oligo.

4. **Anneal oligos:** Anneal top and a bottom strand oligos to generate a double-stranded (ds) oligo with compatible ends for cloning into GeneArt® CRISPR Nuclease Vector.



2. Anneal and clone DNA oligos

For cloning, simply anneal DNA oligos that code for your target-specific crRNA and ligate into the pre-linearized vector (Figure 5). Enough reagents are provided for 10 reactions, including:

- Linearized cloning vector, 10X annealing buffer, T4 DNA ligase, 5X DNA ligase buffer, and DNase/RNase-free water.
- A control double-stranded DNA oligo for monitoring cloning efficiency.
- U6 forward sequencing primer for checking the orientation and sequence of the crRNA-specific double-stranded oligo insert.

3. Transform

Once you have completed the ligation reaction, transform One Shot® TOP10 Chemically Competent *E. coli* (Cat. No. C4040-10) with the resulting CRISPR nuclease construct. One Shot® TOP10 Chemically Competent *E. coli* are ideal for high-efficiency cloning and plasmid propagation. They allow stable replication of high-copy number plasmids. The genotype of TOP10 cells is similar to that of the DH10B™ strain. One tube of One Shot® TOP10 *E. coli* is required for each ligation reaction.

4. Transfect CRISPR plasmids for cell culture applications

a. Delivery method: Method for transfections varies based on cell type. Consult original references or the supplier of your cell line for the optimal method of transfection. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. For high–efficiency transfection in a broad range of mammalian cell lines, we recommend using the cationic lipid-based Lipofectamine 2000 Reagent (Cat. No. 11668-027).

b. DNA purity: For transfection in eukaryotic cells, DNA must be pure, and free from contamination with phenol and sodium chloride. We recommend using high-quality DNA prepared with the PureLink® HiPure Plasmid MidiPrep Kit (Cat. No. K2100-04). Store plasmid DNA stocks at –20°C.

c. Amount of DNA: Depending on the transfection reagent and cell line, the dosage of DNA that yields the best transfection efficiency will vary. It is advisable to do a dose response study to determine optimal transfection conditions. In our experience with 293FT cells in a 6-well transfection format, 3 µg of CRISPR/Cas9 expression plasmid gives optimal transfection efficiency when cells are at 70% confluency.

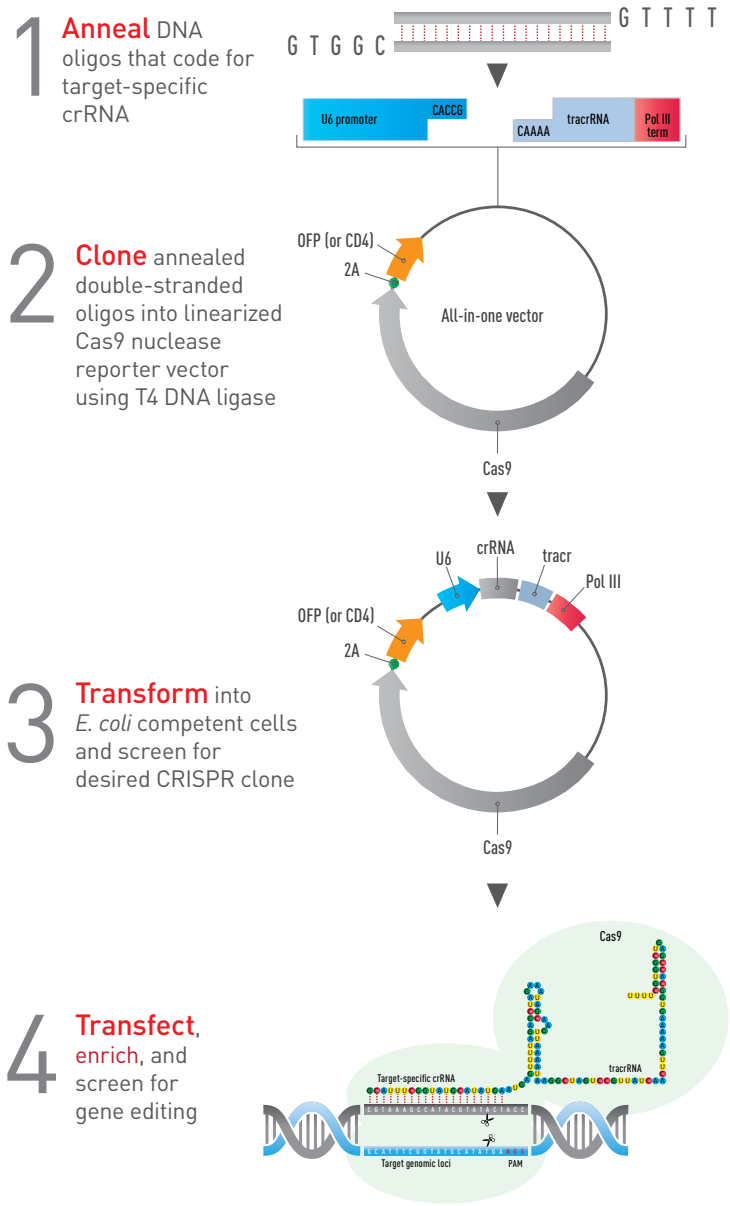


Figure 5. The cloning and analysis workflow. After transfection, the samples can be analyzed for transfection efficiency based on the choice of reporter used, cleavage efficiency using our GeneArt® Genomic Cleavage Detection Assay (Cat. No. A24372), or enriched for the CRISPR/Cas9-expressing cell population (Figures 6, 7, and 8).

5. Analysis

CRISPR/Cas9-mediated cleavage efficiency

Cleavage efficiency can be detected using the GeneArt® Genomic Cleavage Detection Assay (Cat. No. A24372), which is a technique that leverages mismatch detection endonucleases to detect insertions and deletions (indels) generated during cellular NHEJ repair (Figure 6).

Alternatively, NGS or Sanger sequencing can be used to analyze and measure the indels induced by the CRISPR/Cas9 system. In the case of low or minimal cleavage or transfection efficiency, cells expressing Cas9 and gRNA can be enriched with the following method.

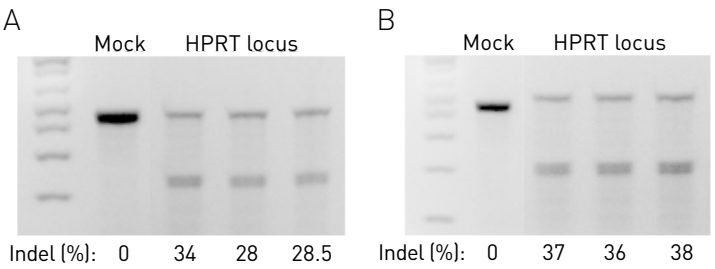


Figure 6. CRISPR/Cas9-mediated cleavage efficiency. Gel image of a cleavage assay using the GeneArt® Genomic Cleavage Detection Assay (Cat. No. A24372) for the HPRT locus. **(A)** Results using the GeneArt® CRISPR Nuclease OFP Vector expressing HPRT-specific CRISPR RNA. (**B**) Results obtained using the GeneArt® CRISPR Nuclease CD4 Vector expressing HPRT-specific CRISPR RNA. Following transfection into HeLa cells, triplicate cleavage assays were performed and the percentage of indels were calculated.

Enrichment of the Cas9- and gRNA-expressing cell population

Since Cas9 is linked to either OFP or CD4 in the expression vector, transfection efficiency can be monitored using fluorescence microscopy or FACS (Figure 7). In addition, cells that express Cas9 and gRNA can be enriched by FACS using the OFP reporter or magnetic bead–based enrichment using the CD4 reporter. Dynabeads® CD4 magnetic beads (Cat. No. 11145D) were used for enrichment (Figure 8). We have noticed that cells with the highest Cas9-OFP or Cas9-CD4 expression exhibit the best genomic DNA cleavage efficiency. Therefore, while using FACS-based enrichment, we recommend sorting (1) dim, (2) medium, and (3) bright cell populations, which correspond to low, medium, and high OFP- or CD4-expressing cells.

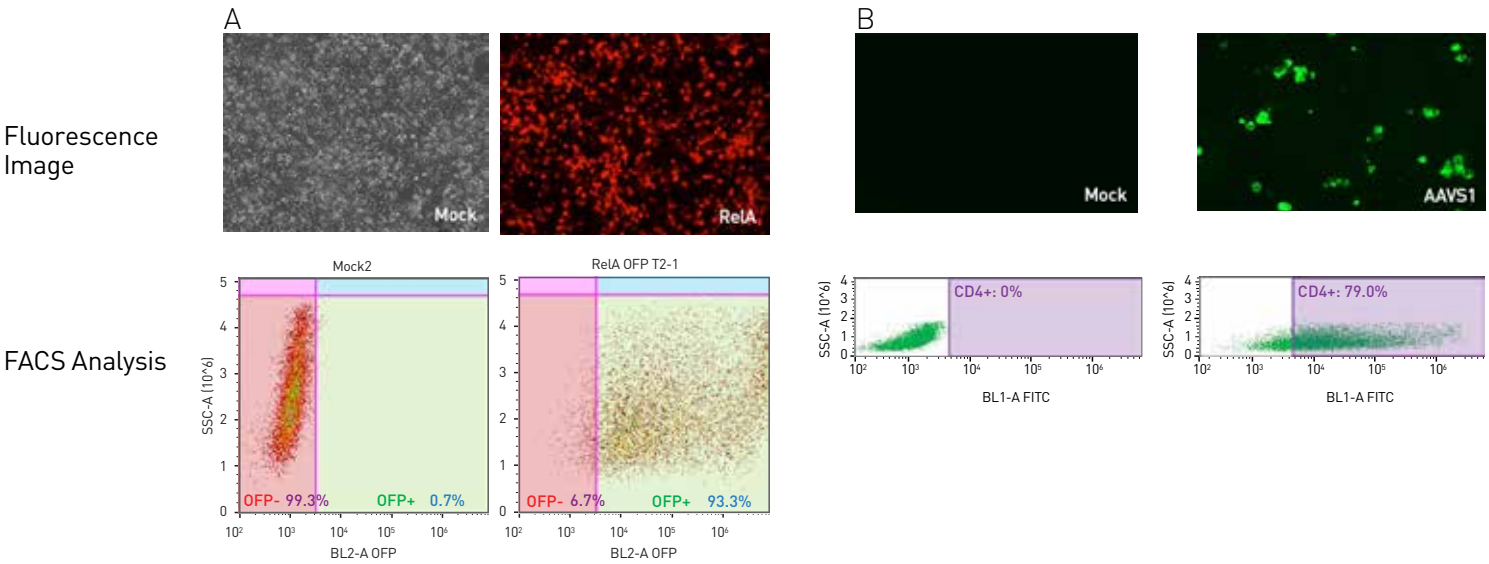


Figure 7. Enrichment of the Cas9- and gRNA-expressing cell population. (**A**) Transfection efficiency in 293T cells using the GeneArt® CRISPR Nuclease OFP Vector encoding crRNA specific for the RelA locus. Data shows >90% OFP-positive cells in transfected samples. (**B**) CD4 functionality for the GeneArt® CRISPR Nuclease CD4 Vector. 293 FT cells were transfected with AAVS1-specific GeneArt® CRISPR Nuclease CD4 Vector. Cells were harvested and stained with anti-CD4 FITC antibody and analyzed by flow cytometry for measuring transfection efficiency. A portion of the stained cells were also seeded on a plate for analysis by fluorescence microscopy.

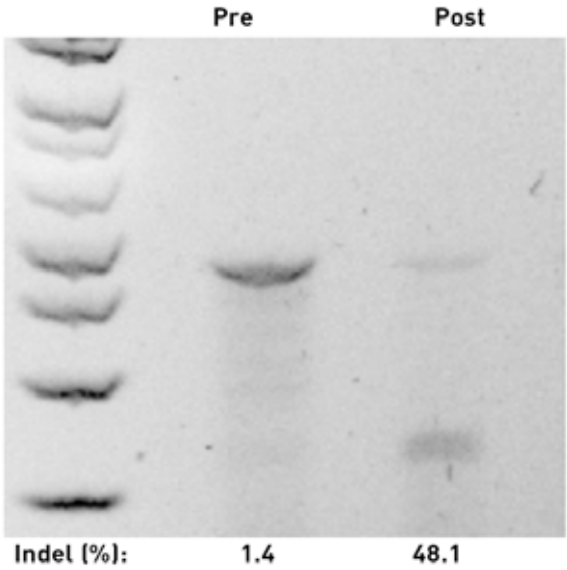


Figure 8. Bead-based enrichment using the GeneArt® CRISPR Nuclease Vector with CD4. The sample had very low CD4-expressing cell populations and hence lower cleavage efficiency (1.4%). Following transfection, CD4-expressing cells were enriched from the rest of the cell population using anti-CD4 antibody–coated magnetic beads. A GeneArt® Genomic Cleavage Detection Assay (Cat. No. A24372) was performed on both pre- and post-enriched cell pellets. Cleavage efficiency increased to 48.1% after enrichment

After relevant targets have been identified with fast and easy-to-use GeneArt® CRISPRs, the biologically relevant mutations can be precisely created with GeneArt® Precision TALs with high specificity and low off-target effect.

Ordering information

Product	Quantity	Cat. No.
GeneArt® CRISPR Nuclease: OFP Reporter Kit	10 reactions	A21174
GeneArt® CRISPR Nuclease: OFP Reporter with Competent Cells (Combo) Kit	10 reactions	A21178
GeneArt® CRISPR Nuclease: CD4 Enrichment Kit	10 reactions	A21175
GeneArt® CRISPR Nuclease: CD4 Enrichment with Competent Cells (Combo) Kit	10 reactions	A11277
Related Product Information		
GeneArt® Genomic Cleavage Detection Kit	20 reactions	A24372
T4 DNA Ligase	100 units	15224017
One Shot® TOP10 Chemically Competent *E. coli*	20 reactions	C404003
PureLink® HiPure Plasmid MiniPrep Kit	25 preps	K210002
PureLink® HiPure Plasmid MidiPrep Kit	25 preps	K210004
PureLink® HiPure Plasmid MaxiPrep Kit	25 preps	K210007
Lipofectamine® 2000	0.75 mL	11668027
Lipofectamine® 2000	1.5 mL	11668019
Dynabeads® CD4	5 mL	11145D

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References

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3. Cong L, Ran FA, Cox D et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339:819–823.
4. Kim JH, Lee SR, Li LH et al. (2011) High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. *PLoS One* 6:e18556.
5. Fu Y, Foden JA, Khayter C et al. (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature Biotechnol* 31:822–826.
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Notes:

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