

USER GUIDE

life

GeneArt® CRISPR Nuclease Vector Kit

Reporter vector system for expression of Guide RNA and Cas9
in mammalian cells

Catalog Numbers A21174, A21175, A21177, A21178

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Revision C.0

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Contents and Storage

Types of Kits

This manual is supplied with the products listed below.

Product	Catalog no.
GeneArt® CRISPR Nuclease (OFP Reporter) Vector Kit	A21174
GeneArt® CRISPR Nuclease (CD4 Enrichment) Vector Kit	A21175
GeneArt® CRISPR Nuclease (CD4 Enrichment) Vector Kit with Competent Cells	A21177
GeneArt® CRISPR Nuclease (OFP Reporter) Vector Kit with Competent Cells	A21178

Contents

The following reagents are included with GeneArt® CRISPR Nuclease Vector Kits.

All GeneArt® CRISPR Nuclease Vectors are shipped at room temperature.

Store at -20°C, upon receipt.

Reagent	Buffer Composition	Amount
CRISPR nuclease vector, linearized (15 ng/μL)	10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0	1 × 20 μL
10X Oligonucleotide Annealing Buffer	100 mM Tris-HCl, pH 8.0 10 mM EDTA, pH 8.0 1 M NaCl	250 μL
DNase/RNase-Free Water	—	2 × 1.5 mL
5X Ligation Buffer	250 mM Tris-HCl, pH 7.6 50 mM MgCl ₂ 5 mM ATP 5 mM DTT 25% (w/v) polyethylene glycol-8000	80 μL
T4 DNA Ligase (1 (Weiss) U/μL)	10 mM Tris-HCl, pH 7.5 50 mM KCl 1 mM DTT 50% (v/v) glycerol	20 μL
U6 Forward Sequencing Primer (0.1 μg/μL)	TE Buffer, pH 8.0	20 μL
ds Cloning Control Oligo (50 μM)	1X Oligonucleotide Annealing Buffer	10 μL

Primer Sequences

The table below provides the sequence and the amount supplied of the primers included in the kit.

Primer	Sequence
U6 Forward	5'- GGACTATCATATGCTTACCG -3'

continued on next page

Contents and Storage, continued

Double stranded (ds) Control Oligo Sequences

The sequences of the two strands for the ds Cloning Control Oligo are listed below. The ds Cloning Control Oligo comes annealed, and is supplied in the kit as a 50 μM double-stranded oligonucleotide. The ds Cloning Control Oligo needs to be re-annealed and diluted before use in the ligation reaction (see page 7).

ds Cloning Control Oligo	Sequence
Top strand	5'- CATTCTCAGTGCTATAGAGTTTT -3'
Bottom strand	5'- TCTATAGCACTGAGAAATGCGGTG -3'

Competent cells

GeneArt® CRISPR Nuclease Vectors Kits A21177 and A21178 include One Shot® TOP10 Chemically Competent *E. coli* sufficient for 10 reactions.

Transformation efficiency is $\geq 1 \times 10^9$ cfu/ μg plasmid DNA.

One Shot® TOP10 Chemically Competent *E. coli* are shipped on dry ice.

Store Box 2 at -80°C , upon receipt.

Reagent	Composition	Amount
S.O.C. Medium (may be stored at $+4^\circ\text{C}$ or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM MgCl_2 10 mM MgSO_4 20 mM glucose	6 mL
TOP10 cells	—	11 \times 50 μL
pUC19 Control DNA	10 $\mu\text{g}/\mu\text{L}$ in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 μL

Genotype of TOP10 Cells

F⁻ *mcrA* $\Delta(mrr\text{-}hsdRMS\text{-}mcrBC)$ $\phi 80lacZ\Delta M15$ $\Delta lacX74$ *recA1* *araD139* $\Delta(ara\text{-}leu)7697$ *galU galK rpsL (Str^R) endA1 nupG*

Introduction

Product Information

Introduction

GeneArt® CRISPR Nuclease Vector Kits facilitate the generation of constructs to express non-coding guide RNA including CRISPR RNA and tracrRNA as well as Cas9 nuclease for use in CRISPR mediated target gene cleavage or gene editing in mammalian cells.

The Cas9 nuclease is based on the type II CRISPR/Cas system from the bacterium *Streptococcus pyogenes* and has been engineered for genome editing in mammalian systems (Jinek et al., 2012; Mali¹ et al., 2013; Cong et al., 2013).

GeneArt® CRISPR Nuclease Vectors with OFF allow for FACS based sorting of Cas9 and CRISPR RNA expressing cell populations, while the GeneArt® CRISPR Nuclease Vectors with CD4 enables bead based enrichment of Cas9 and CRISPR RNA expressing cells.

The linearized GeneArt® CRISPR Nuclease Vectors provide a rapid and efficient way to clone double-stranded oligonucleotides encoding a desired CRISPR RNA target into an expression cassette that allows targeting of the Cas9 nuclease in a sequence specific manner.

Although the kit has been designed to express Cas9 and guide RNA representing a particular target sequence in the simplest, most direct fashion, use of the kit for genome editing and target loci cleavage analysis assumes that users are familiar with the principles of CRISPR system, vector-based production of CRISPR RNA, and transfection in mammalian systems. We highly recommend that users possess a working knowledge of the CRISPR system.

The CRISPR system

The CRISPR system is a prokaryotic adaptive immune system that uses a RNA guided DNA nuclease to silence viral nucleic acids (Jinek et al., 2012). In bacteria CRISPR loci are composed of a series of repeats separated by segments of exogenous DNA (of ~30bp in length), called spacers. The repeat-spacer array is transcribed as a long precursor and processed within repeat sequences to generate small crRNAs that specify the target sequences (also known as protospacers) cleaved by the CRISPR nuclease. CRISPR spacers are then used to recognize and silence exogenous genetic elements at the RNA or DNA level. Essential for cleavage is a sequence motif immediately downstream on the 3' end of the target region, known as the protospacer-adjacent motif (PAM). The PAM is present in the target DNA, but not the crRNA that targets it.

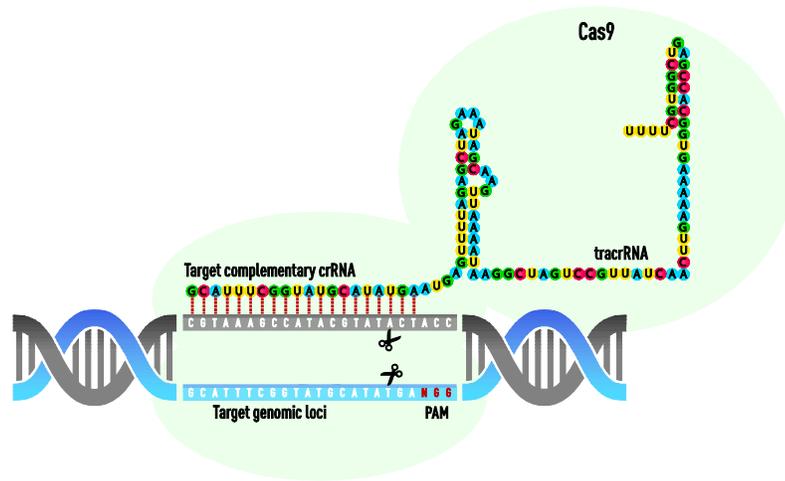
Product Information, continued

Genome editing

Genome editing involves the use of engineered nucleases in conjunction with endogenous repair mechanisms to insert, delete, or replace DNA sequences from a specific location in genomic DNA. Engineered nucleases induce a double stranded break (DSB) at a specific location in the genome, after which endogenous repair mechanisms repair the break via non-homologous end joining (NHEJ) or homology directed repair. The type II CRISPR system has been shown to function as a gene editing tool in various organisms including mammalian cells. (Mali¹ et al., 2013; Cong et al., 2013).

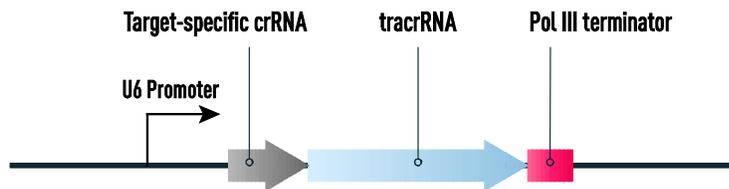
It consists of three components: the CRISPR-associated Cas9 nuclease (a double-stranded DNA endonuclease), a target complementary CRISPR RNA (crRNA), and an auxiliary trans-activating crRNA (tracrRNA). The crRNA and tracrRNA act as a short guide RNA to target the Cas9 nuclease to specific genomic loci (Figure 1).

Figure 1 Schematic representation of CRISPR/Cas9 mediated target DNA cleavage.



The crRNA and tracrRNA of the GeneArt[®] CRISPR Nuclease Vector are expressed together as a guide RNA that mimics the natural crRNA-tracrRNA hybrid in bacterial systems. The guide RNA expression is driven by a U6 polIII type promoter (Figure 2).

Figure 2 Guide RNA expression cassette in GeneArt[®] CRISPR Nuclease Vector.



The system is versatile, and simple to use, and changing target specificity only requires a change in the design of the CRISPR RNA.

Methods

Experimental Outline

Experimental outline

The following table and figure outlines the steps required to create your GeneArt® CRISPR Nuclease Vector and express it in cells.

Step	Action	Page
1	Design single-stranded DNA oligonucleotides.	4
2	Anneal single-stranded oligonucleotides to generate a double-stranded oligonucleotide.	6
3	Dilute double-stranded oligonucleotide to working concentration	7
4	Clone double-stranded oligonucleotide into CRISPR Nuclease Vector.	8
5	Transform One Shot® Chemically Competent TOP10 <i>E. coli</i> cells and select for expression clones.	9
6	Analyze transformants for the presence of insert by sequencing.	10
7	Prepare purified plasmid DNA and transfect the cell line of choice.	11

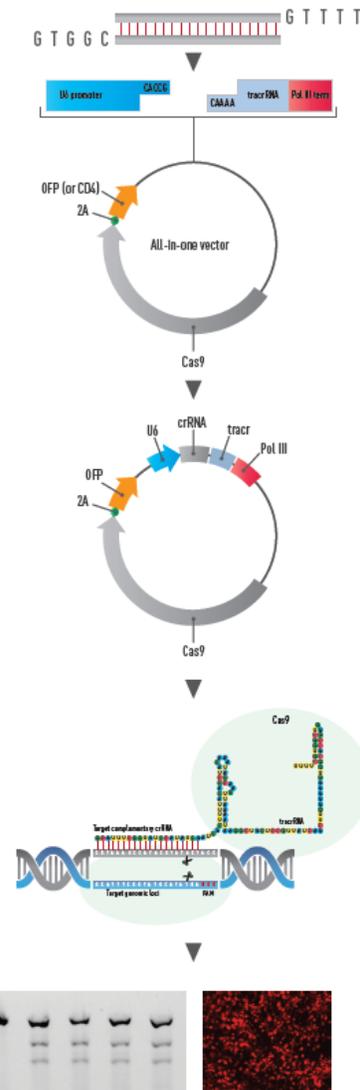
Figure 3 Cloning and analysis of target specific ds oligonucleotide.

Anneal DNA oligos that code for target-specific crRNA

Clone annealed oligos into linearized Cas9 nuclease reporter vector using T4 DNA ligase

Transform into *E. coli* competent cells and screen for desired CRISPR clone

Transfect, **enrich**, and screen for gene editing



Design Single-Stranded DNA Oligonucleotides

Introduction

To use the GeneArt® CRISPR Nuclease Vector Kit, you will first need to design two single-stranded DNA oligonucleotides with suitable overhangs to complement the linearized vector; one encoding the target CRISPR RNA (Forward strand oligonucleotide) and the other its complement (Reverse strand oligonucleotide). You will then anneal the top and bottom strand oligonucleotides to generate a double-stranded oligonucleotide (ds oligonucleotide) suitable for cloning into the linearized vector provided in the kit.

The design of the single-stranded (ss) oligonucleotides is critical to the success of both the cloning procedure. General guidelines are provided in this section to help you choose the target sequence and to design the ss oligonucleotides. Note that for a given target gene, you may need to generate and screen multiple crRNA sequences to identify one that is active in efficiently cleaving your target genomic loci.

Choose the target sequence

When performing CRISPR/Cas9 induced DNA double stranded break on a particular gene or genomic loci of interest, your choice of target sequence can significantly affect the degree of cleavage observed. We recommend following the guidelines below when choosing your target sequence. These are general recommendations only; exceptions may occur.

Length: Choose a target sequence ranging from 19 to 20 nucleotides in length that is adjacent to a NGG proto-spacer adjacent motif (PAM) sequence on the 3' end of the target sequence. **Note:** the 5' G required for transcription initiation from the U6 PolIII promoter is already included in the 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 guide gRNA-Cas9 complexes can potentially tolerate up to 1–3 mismatches. Refer to published articles for more insights into choosing target sequence. (Fu et al., 2013; Mali² et al., 2013).

Orientation: You may choose a target sequence encoding the **sense** sequence of the target Loci or the **antisense** sequence. Thus, you can generate CRISPR RNA in two possible orientations provided that it meets the PAM requirements on the 3' ends.



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Design Single-Stranded DNA Oligonucleotides, continued

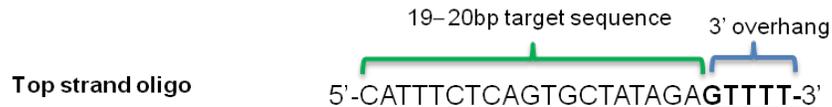
Sequences required for directional cloning

After choosing a 19–20 base pair target sequence, proceed to designing the crRNA specific oligonucleotide primers.

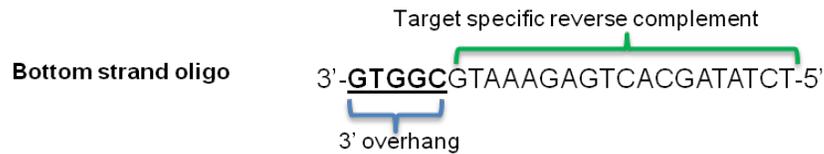
Important! Do not include the PAM sequence in the oligonucleotide primers.

To enable directional cloning of the ds oligonucleotide into the GeneArt® CRISPR Nuclease Vector, you **must** add the following 5 nucleotides to the 3' end of the corresponding ss oligonucleotides:

- **Top strand oligonucleotide:** Add GTTTT to the 3' end of the oligonucleotide. The GTTTT is complementary to the overhang sequence, CAAAA, in the linearized CRISPR Nuclease Vector (see Figure 3) and constitutes the first 5 bases of the tracrRNA.



- **Bottom strand oligonucleotide:** The bottom strand oligonucleotide should be the reverse complement of the target sequence. Add CGGTG to the 3' end of the oligonucleotide. This sequence is complementary to the overhang sequence, CACCG, in the linearized GeneArt® CRISPR Nuclease Vector (see Figure 3) and constitutes the last 4 bases of U6 promoter and the first base required for PolIII transcription start site.



Annealing the two single-stranded oligonucleotides results in a double-stranded oligonucleotide with compatible ends for cloning into the GeneArt® CRISPR Nuclease Vector.



Generate Double-Stranded Oligonucleotide

Introduction

Anneal equal amounts of each single-stranded oligonucleotide to generate a double-stranded (ds) oligonucleotide.

After annealing, dilute an aliquot of the ds oligonucleotide from 50 μM to a working concentration of 5 nM.

Materials needed

- Forward strand oligonucleotide (200 μM in water or TE Buffer)
 - Reverse strand oligonucleotide (200 μM in water or TE Buffer)
 - 50 μM stock of ds control oligonucleotide (thaw on ice)
 - 10X Oligonucleotide Annealing Buffer
 - DNase/RNase-Free Water (supplied with kit)
 - 1.5 mL sterile microcentrifuge tubes
 - 95°C heat block
-

Annealing procedure

1. Add the following reagents to a clean microcentrifuge tube at room temperature.

Forward strand oligonucleotide (200 μM)	5 μL
Reverse strand oligonucleotide (200 μM)	5 μL
10X Oligonucleotide Annealing Buffer	2 μL
DNase/RNase-Free Water	8 μL
<hr/>	
Total volume	20 μL

2. Re-anneal the ds Cloning Control Oligo. Centrifuge the tube briefly (~5 s), then transfer 5 μL to a clean microcentrifuge tube, and proceed to the next step.
Note: This procedure is also applicable when re-annealing other 50 μM ds oligonucleotides.
3. Incubate the tube at 95°C for 4 minutes in a heat block.
4. Remove the tube from the heat block, and allow the reaction mixture to cool to 25°C for 5–10 minutes.
5. Centrifuge the tube briefly (~5 seconds). Mix gently.
6. Proceed to **Dilute double-stranded oligonucleotide** (page 7)

For long-term storage, keep the 50 μM ds oligonucleotide stock solution at -20°C.

continued on next page

Generate Double-Stranded Oligonucleotide, continued

Prepare diluted double-stranded oligonucleotides

After the single-stranded oligonucleotides and Cloning Control Oligos are annealed, perform two 100-fold serial dilutions of the 50 μM ds oligonucleotide stock to prepare a 500 nM ds oligonucleotide stock solution (100-fold dilution), and a 5 nM ds oligonucleotide working solution (10,000-fold dilution).

Prepare 500 nM stock solution

Prepare a 500 nM ds oligonucleotide stock solution by diluting the 50 μM ds oligonucleotide stock 100-fold.

1. Mix the following reagents in a clean microcentrifuge tube:

50 μM ds oligonucleotide stock	1 μL
DNase/RNase-Free Water	99 μL
<hr/>	
Total volume	100 μL

2. Vortex to mix thoroughly.

For long-term storage, keep the 500 nM ds oligonucleotide stock solution at -20°C .

Prepare 5 nM working solution

Prepare a 5 nM ds oligonucleotide working solution by diluting the 500 nM ds oligonucleotide stock solution 100-fold.

Note: 5 nM ds oligonucleotide working solution is not suited for long-term storage, and should be prepared fresh each time.

1. Mix the following reagents in a clean microcentrifuge tube:

500 nM ds oligonucleotide solution	1 μL
10X Oligonucleotide Annealing Buffer	10 μL
DNase/RNase-Free Water	89 μL
<hr/>	
Total volume	100 μL

2. Vortex to mix thoroughly.
-

Handling double-stranded oligonucleotide solutions

- Thaw frozen ds oligonucleotide solutions on ice.
 - **Do not** heat or allow the temperature of ds oligonucleotide solutions to rise above room temperature. Heating of ds oligonucleotide solutions results in partial denaturation, and a reduction in cloning efficiency.
 - If the 500 nM stock solution, or 5 nM working solution become heated, prepare new diluted solutions.
 - If the 50 μM ds oligonucleotide stock becomes heated, re-anneal the oligonucleotides (page 6).
-

continued on next page

Ligation Reaction

Introduction

Once you have generated your ds oligonucleotide, and prepared the appropriate stock solutions, clone the ds oligonucleotide into the GeneArt® CRISPR Nuclease Vector.

Materials needed

- Double-stranded oligonucleotide (5 nM in 1X Oligonucleotide Annealing Buffer; thaw on ice before use)
 - Double-stranded control oligonucleotide (5 nM in 1X Oligonucleotide Annealing Buffer; thaw on ice before use)
 - Linearized GeneArt® CRISPR Nuclease Vector (thaw on ice before use)
 - 5X Ligation Buffer (supplied with kit)
 - DNase/RNase-Free Water (supplied with kit)
 - T4 DNA Ligase (supplied with kit)
-

Controls

We recommend including the ds control oligonucleotide supplied with the kit as a positive control in your ligation experiment. The ds control oligonucleotide is supplied as a 50 µM stock in 1X Oligonucleotide Annealing Buffer, and needs to be re-annealed and diluted 10,000-fold before use in a ligation reaction (see page 6).

If you wish to include a negative control, set up a separate ligation reaction but omit the ds oligonucleotide.

Ligation procedure

Set up a 20 µL ligation reaction at room temperature for each ds oligonucleotide to be cloned.

1. Add the following reagents to a clean microcentrifuge tube **in the order** shown:

5X Ligation Buffer	4 µL
Linearized GeneArt® CRISPR Nuclease Vector	2 µL
ds oligonucleotide (5 nM)	2 µL
DNase/RNase-Free Water	11 µL
T4 DNA Ligase	1 µL
<hr/>	
Total volume	20 µL

2. Mix reaction well by pipetting up and down.
Note: The presence of PEG and glycerol (supplied by the Ligation Buffer and the T4 DNA Ligase) will make the reaction mixture viscous. Be sure to mix the reaction thoroughly by pipetting up and down. **Do not vortex.**
 3. Incubate for 10 minutes at room temperature (25–27°C).
Note: The incubation time may be extended up to 2 hours and may result in a higher yield of colonies.
 4. Place the reaction on ice and proceed to **Transform One Shot® TOP10 Competent *E. coli***, page 9.
Note: You may store the remaining ligation reaction at –20°C overnight.
-

Transform Competent *E. coli* Cells

Introduction

Once you have completed the ligation reaction, transform One Shot® TOP10 chemically competent *E. coli* with the resulting CRISPR nuclease construct.

Cloning of the GeneArt® CRISPR Nuclease Vectors was optimized using One Shot® TOP10 chemically competent *E. coli*. These cells are ideal for high-efficiency cloning and plasmid propagation, and allow stable replication of high-copy number plasmids. The genotype of TOP10 cells is similar to that of the DH10B™ strain.

Note: using competent cells of different genotype may lower cloning efficiency and can also result in a higher proportion of vectors without insert.

One tube of One Shot® TOP10 *E. coli* is required for each ligation reaction.

Materials needed

- Ligation reaction (from step 4, page 8)
 - (Optional) pUC19 control (supplied with kit)
 - One Shot® TOP10 chemically competent *E. coli* cells (supplied with kit or available separately, see page 15)
 - S.O.C. Medium (warm to room temperature before use)
 - LB plates containing 100 µg/mL ampicillin (two for each transformation; warm at 37°C for 30 minutes)
 - 42°C water bath
 - 37°C shaking and non-shaking incubator
-

One Shot® TOP10 transformation procedure

1. Thaw One Shot® TOP10 chemically competent *E. coli* on ice, and proceed to the next step immediately after the cells are thawed.
 2. Add 3 µL of the ligation reaction (from step 4, page 8) into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently by swirling or tapping the tube gently. **Do not mix by pipetting up and down.**

Note: Transform 1 µL of the pUC19 plasmid if performing a positive control for transformation efficiency.
 3. Place the tube immediately on ice, and incubate for 10–30 minutes.

Note: Longer incubations seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
 4. Heat-shock the cells for 30 seconds at 42°C without shaking.
 5. Immediately transfer the tubes to ice.
 6. Add 250 µL of room temperature S.O.C. Medium.
 7. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
 8. Spread 50 µL from the transformation reaction on a pre-warmed LB agar plate containing 100 µg/mL ampicillin. Plate the remainder of the reaction on a second pre-warmed LB agar plate. Incubate the plates overnight at 37°C.

Note: Plate two different volumes to ensure that at least one plate has well-spaced colonies. When transforming the pUC19 control, plate 20–100 µL of the transformation on pre-warmed LB plates containing 100 µg/mL ampicillin.
 9. An efficient ligation reaction may produce over a hundred colonies in total. Pick 5–10 colonies for analysis (see **Analyze Transformants**, page 10).
-

Analyze Transformants

Confirm positive clones

Confirm the identity of the ds oligonucleotide insert in positive transformants by sequencing. Analyze each CRISPR nuclease construct to verify:

- That the ds oligonucleotide insert is present, and in the correct orientation
- That the ds oligonucleotide insert has the correct sequence

Note: Restriction analysis is **not** recommended due to the small size of the ds oligonucleotide insert.

Analyze transformants

1. Pick 5–10 ampicillin-resistant colonies and culture them overnight in LB medium containing 100 µg/mL ampicillin at 37°C.
 2. Isolate plasmid DNA using your method of choice. We recommend using the PureLink® HQ Mini Plasmid Purification Kit.
 3. Perform sequencing of the CRISPR nuclease construct using the U6 Forward Primer (supplied with kit).
 4. Make glycerol stocks of desired CRISPR nuclease expression plasmids (see **Long-term storage**).
 5. Proceed to **Transfection** (page 11).
-

Sequencing guidelines

If a particular CRISPR nuclease construct is difficult to sequence, follow these recommendations to improve results:

- Use high-quality, purified plasmid DNA for sequencing. We recommend preparing DNA using the PureLink® HQ Mini Plasmid Purification Kit (Cat. no. K2100-01).
 - Add DMSO to the sequencing reaction to a final concentration of 5%.
 - Increase the amount of template used in the sequencing reaction (up to twice the normal concentration).
 - Use a 7:1 molar ratio of dITP:dGTP in your sequencing reaction.
-

Long-term storage

Once the correct CRISPR nuclease construct is identified, make a glycerol stock for long-term storage.

1. Streak the original colony out on an LB plate containing 100 µg/mL ampicillin, and incubate overnight at 37°C.
 2. Isolate a single colony and inoculate in 1–2 mL of LB medium containing 100 µg/mL ampicillin.
 3. Incubate at 37°C until the culture reaches stationary phase.
 4. Mix 0.85 mL of the culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
 5. Store the glycerol stock at –80°C
-

Transfection of Mammalian Cell Lines

Methods of transfection

Methods of transfecting plasmids into the mammalian cell lines include calcium phosphate (Chen and Okayama, 1987; Wigler et al., 1977), lipid-mediated techniques (Felgner et al., 1989; Felgner and Ringold, 1989), and electroporation (Chu et al., 1987; Shigekawa and Dower, 1988).

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) (Ciccarone et al., 1999).

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.

Plasmid preparation

Plasmid DNA for transfection in eukaryotic cells must be pure, and free from contamination with phenol and sodium chloride. We recommend using high quality maxi prep DNA for transfection.

Store plasmid DNA stocks at -20°C.

Transfection guidelines

The following general guidelines are recommended for performing transfection in a standard 6 well plate:

- Perform transfection with Lipofectamine[®] 2000 with most cell lines.
- Seed cells so that they are 70% confluent on the day of transfection.
Note: Seeding density varies with cell type.
- Perform transfection with 3 µg of CRISPR/Cas9 expression vector.
Note: Results will vary depending upon cell type and passage number, and optimization of lipid:DNA concentrations may be required for best results.

For details on enriching for populations transfected with GeneArt[®] CRISPR Nuclease Vectors using either GFP or CD4 reporters, refer to Appendix B (page 18).

Cleavage efficiency

The GeneART[®] Genomic Cleavage Detection Kit is recommended for performing cleavage efficiency analysis (see page 21).

Controls

We recommend that you include a positive control and a negative control (mock transfection) in your experiment to evaluate your results.

Troubleshooting

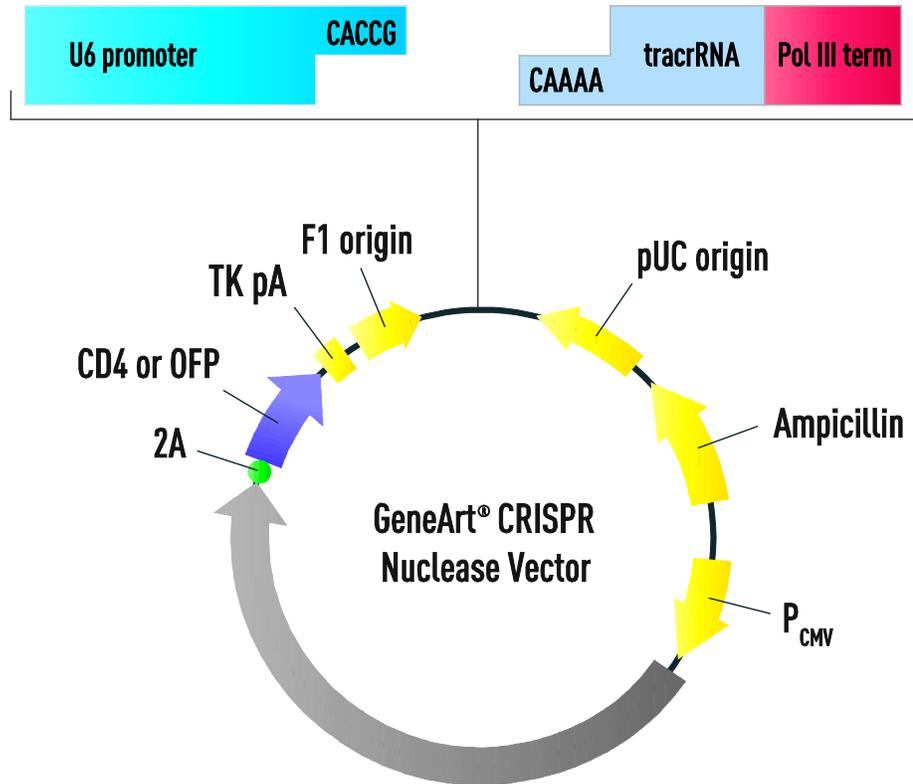
Observation	Reason	Solution
Few ampicillin-resistant colonies obtained on the selective plate	Single-stranded oligonucleotides designed incorrectly	Make sure that each single-stranded oligonucleotide contains the 5 nucleotides on the 3' end required for cloning into the GeneArt® CRISPR Nuclease Vector: <ul style="list-style-type: none">• Top strand oligonucleotide: include GTTTT on the 3' end.• Bottom strand oligonucleotide: include CGGTG on the 3' end.
	ds oligonucleotides were degraded	<ul style="list-style-type: none">• Store the 5 nM ds oligonucleotide stock in 1X Oligonucleotide Annealing Buffer.• Avoid repeated freeze/thaw cycles. Aliquot the 5 nM ds oligonucleotide stock and store at -20°C.
	Oligonucleotide annealing reaction inefficient	<ul style="list-style-type: none">• Ensure that the annealing reaction was performed as directed (page 6).• If ambient temperature is >25°C to 27°C, incubate the annealing reaction in a 25°C incubator.

Appendix A

Map and Features of GeneArt® CRISPR Nuclease Vector

GeneArt® CRISPR Nuclease Vector

The figure below shows the features of the GeneArt® CRISPR Nuclease Vector. The vector is supplied linearized at nucleotides 7335 and 7356 (CD4) or 6732 and 6752 (OFP) with 5 base pair 3' overhangs on each strand as indicated. The complete sequence of the vector is available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 16).



Continued on next page

Map and Features of GeneArt® CRISPR Nuclease Vector, continued

Features of GeneArt® CRISPR Nuclease Vector

The GeneArt® CRISPR Nuclease Vector (9822 bp CD4 and 9219 bp OFF) contains the following elements. All features have been functionally tested and the vector fully sequenced.

Feature	Benefit
tracrRNA	Auxiliary trans-activating crRNA allows loading of Cas9 nuclease onto the gRNA
F1 origin of replication	Origin of replication.
TK pA	Polyadenylation signal.
CD4	Reporter gene for bead based enrichment. Can be used for monitoring transfection efficiency when stained with a fluorescently labeled anti-CD4 antibody (Schlossman et. al., 1995).
OFFP	Reporter gene for FACS based sorting. The fluorescent protein can also be used for monitoring transfection efficiency.
2A peptide linker	A self-cleaving peptide linker connecting CD4 or OFFP reporter genes to the C-terminal end of Cas9 nuclease. Following translation, the two proteins flanking the 2A peptide are separated from each other.
CMV promoter	Allows expression of Cas9 nuclease and CD4 or OFFP reporter genes.
Human U6 promoter	Allows RNA Polymerase III-dependent expression of the guide RNA (gRNA) (Kunkel et al., 1986; Kunkel and Pederson, 1988).
U6 forward priming site	Allows sequencing of the insert.
3' overhangs	Allows ligase-mediated directional cloning of the double-stranded oligonucleotide of interest.
Pol III terminator	Allows efficient termination of RNA Polymerase III-dependent transcription.
Ampicillin resistance gene	Allows selection of the plasmid in <i>E. coli</i> .
pUC origin of replication (<i>ori</i>)	Permits high-copy replication and maintenance in <i>E. coli</i> .

Accessory Products

Introduction

The products listed in this section may be used with the GeneArt® CRISPR Nuclease Vectors. For more information, refer to our web site (www.lifetechnologies.com) or contact Technical Support (see page 16).

Ordering oligonucleotides

Custom oligonucleotides for use with the GeneArt® CRISPR Nuclease Vectors can be ordered from Life Technologies. For additional details, visit our web site at www.lifetechnologies.com/oligos or contact Technical Support (see page 16).

Additional products

Many of the reagents suitable for use with the vectors are available separately from Life Technologies. Ordering information for these reagents is provided below.

Item	Quantity	Catalog no.
T4 DNA Ligase	100 units	15224-017
One Shot® TOP10 Chemically Competent <i>E. coli</i>	20 reactions	C4040-03
PureLink® HiPure Plasmid MiniPrep Kit	25 preps	K2100-02
PureLink® HiPure Plasmid MidiPrep Kit	25 preps	K2100-04
PureLink® HiPure Plasmid MaxiPrep Kit	25 preps	K2100-07
Lipofectamine® 2000	0.75 mL 1.5 mL	11668-027 11668-019
Dynabeads® CD4 Positive Isolation Kit	5 mL	11331D
HulaMixer® Sample Mixer	1 unit	15920D
GeneART® Genomic Cleavage Detection Kit	20 reactions	A24372

Technical Support

Obtaining support For the latest services and support information for all locations, go to www.lifetechnologies.com

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
 - Search through frequently asked questions (FAQs)
 - Submit a question directly to Technical Support (techsupport@lifetech.com)
 - Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
 - Obtain information about customer training
 - Download software updates and patches
-

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at www.lifetechnologies.com/support

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.lifetechnologies.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Limited product warranty

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Appendix B

Enrichment of GeneArt® CRISPR Nuclease Expressing Cells

Introduction

Populations of cells transfected with GeneArt® CRISPR Nuclease Vectors (with OFP or CD4 reporters) can be enriched using fluorescence activated cell sorting (FACS), or in the case of vectors with the CD4 reporter, using Dynabeads® CD4 magnetic beads.

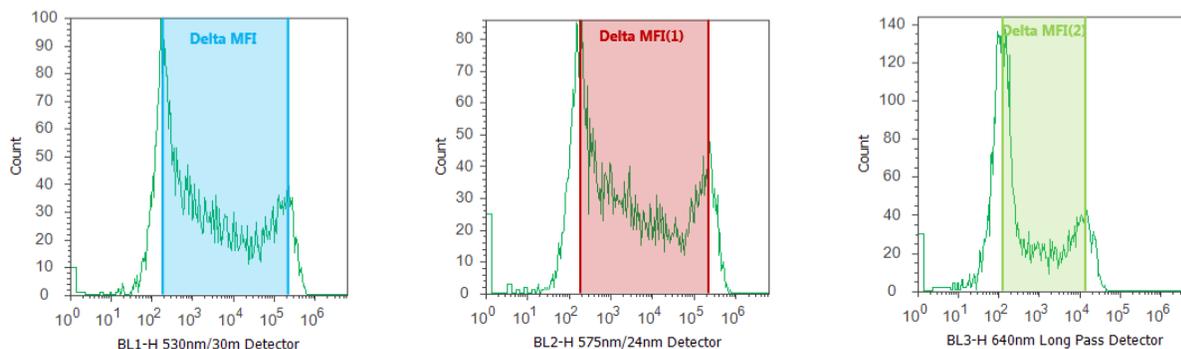
Enrichment by FACS

Use the following guidelines to enrich for cells transfected with the GeneArt® CRISPR Nuclease (OFP Reporter) Vector:

- Harvest live transfected cells and resuspend in a FACS buffer for the cell line being used. For example, a 0.2 micron sterile-filtered FACS buffer (1X PBS containing 1 mM EDTA, 25 mM HEPES, 1% FBS) works well for adherent cell line such as HEK 293.
- OFP has a peak excitation of 548 nm, and emission of 560 nm.
 - A 488 nm laser is recommended for efficient excitation.
 - Standard 530/30, 574/26 and 603/48 emission filters are recommended for detection.

The optimal collection buffer will depend on the downstream application of choice, but RPMI media with 2% FBS, and FACS buffer (above) are all viable options.

Figure 1 The Δ MFI (median Fluorescence intensity) of cells transfected with GeneArt® CRISPR Nuclease OFP vector when excited by a 488 laser and measured by different detectors.



Use the following guidelines to enrich for cells transfected with the GeneArt® CRISPR Nuclease (CD4 Reporter) Vector:

- Harvest live transfected cells and resuspend in a FACS buffer for the cell line being used.
- Stain cells with a CD4 antibody conjugated to your fluorophore of choice (according to manufacturer instructions).
- Once stained, cells transfected with the GeneArt® CRISPR Nuclease (CD4 Reporter) Vector can be enriched by FACS (see above).

continued on next page

Enrichment of GeneArt[®] CRISPR Nuclease Expressing Cells, continued

Enrichment Using CD4 Dynabeads

Cells transfected with the GeneArt[®] CRISPR Nuclease (CD4 Reporter) Vector can be enriched using Dynabeads[®] CD4 magnetic beads (Cat. no. 11331D). We recommend the following protocol when using this product:

1. Harvest live transfected cells and centrifuge at $400 \times g$ for 5 minutes.
 2. Decant supernatant and resuspend in 2 mL of Buffer I (0.2 micron sterile filtered PBS with 0.1% BSA, 2 mM EDTA).
 3. Centrifuge at $400 \times g$ for 5 minutes.
 4. Wash twice with 2 mL of Buffer I.
 5. Resuspend cells in appropriate volume of Buffer I.
 6. Proceed to "Prepare Dynabeads[®] CD4 magnetic beads".
-

Prepare Dynabeads[®] CD4 magnetic beads

1. Resuspend the vial of Dynabeads[®] CD4 magnetic beads for 3 minutes using a mixer allowing tilting and rotation of tubes (e.g. HulaMixer[®] Sample Mixer).
 2. Transfer 25 μ L of beads to a sterile 1.7-mL microcentrifuge tube and place on a magnetic separator for 1 minute.
 3. With the tube still on the magnet, decant the supernatant.
 4. Resuspend beads in 100 μ L of Buffer I.
 5. Place the tube on a magnetic separator for 1 minute and with the vial still on the magnet, decant the supernatant.
 6. Resuspend beads in 25 μ L of Buffer I.
 7. Proceed to "Incubate cells with Dynabeads[®] CD4 magnetic beads" (page 20).
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Enrichment of GeneArt[®] CRISPR Nuclease Expressing Cells, continued

Incubate cells with Dynabeads[®] CD4 magnetic beads

A 1:1 bead to cell ratio is recommended (25 μ L of beads $\sim 10^7$ beads), though this number may need to be optimized based on application.

1. Add harvested cells to resuspended beads, and bring to a final volume of 1 mL with Buffer I.
 2. Incubate at 4°C for 30 minutes on a mixer allowing tilting and rotation of tubes.
 3. Place the tube on a magnetic separator for 1 minute.
 4. With the tube still on the magnet, decant the supernatant.
 5. Resuspend the cells and beads with 500 μ L of Buffer I.
 6. Incubate on a mixer for 2 minutes.
 7. Repeat steps 3–6 a total of 5 times.
 8. After the final wash, resuspend the cells and beads in 100 μ L of Buffer II (0.2 micron sterile filtered RPMI with 2% FBS).
 9. Add 10 μ L of DETACHaBEAD[®] CD4 (provided with 11331D kit).
 10. Incubate on a mixer at room temperature for 45 minutes.
 11. Place the tube on a magnetic separator for 1 minute.
 12. Transfer the supernatant (containing CD4+ cells) to a fresh tube.
 13. Resuspend the beads in 500 μ L of Buffer II.
 14. Place the tube on a magnetic separator and add the supernatant to the tube containing the recovered CD4+ cells.
 15. Repeat steps 13–14 a total of 3 times to obtain the maximum yield of recovered CD4+ cells.
 16. Bring the tube containing recovered CD4+ cells to a final volume of 4 mL.
 17. Centrifuge at 400 $\times g$ for 6 minutes.
 18. Resuspend enriched cells in a solution appropriate for your downstream application (e.g. staining solution, flow cytometry buffer, media).
-

Determining CRISPR/Cas9 Cleavage Efficiency

Introduction

CRISPR/Cas9 mediated double stranded cleavage efficiency can be detected using a mismatch cleavage assay. This technique leverages mismatch detection endonucleases (e.g. T7E1) to detect genomic insertions or deletions (indels) incorporated during cellular NHEJ repair mechanisms.

GeneART® Genomic Cleavage Detection Kit

The GeneART® Genomic Cleavage Detection Kit (see page 15 for ordering details) offers a complete workflow that helps you analyze the cleavage efficiency of your CRISPR targets. A brief description of the assay follows:

- Extract Genomic DNA from cells transfected with your CRISPR nuclease construct.
 - Amplify loci where the gene-specific double-strand breaks occur by PCR.
 - Denature and reanneal the PCR product so that mismatches are generated as strands with an indel re-anneal to strands with no indel or a different indel.
 - Incubate heteroduplex DNA with the Detection Enzyme to allow mismatches to be detected and cleaved.
 - Analyze resultant bands by gel electrophoresis.
 - Quantitate bands by densitometry.
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25 February 2014

