USER GUIDE

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# GeneArt® Genomic Cleavage Selection Kit

## Reporter system for detecting the functionality of engineered nucleases in mammalian cells and enriching for the nuclease-modified cells

**Catalog Number** A27663

**Publication Number** MAN0013465
**Revision** A.0

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# About this guide

## Audience

This user guide is for laboratory staff using the GeneArt® Genomic Cleavage Selection Kit.

## Revision history

| Revision | Date | Description |
|---|---|---|
| A.0 | February 2015 | Establish new user guide |

# Product information

## Product description

### GeneArt® Genomic Cleavage Selection Kit

The GeneArt® Genomic Cleavage Selection Kit (Cat. no. A27663) is a reporter system for detecting the functionality of engineered nucleases in mammalian cells and enriching for nuclease-modified cell populations.

## Kit contents and storage

### Contents

The GeneArt® Genomic Cleavage Selection Kit contains the following components.

| Component | Buffer composition | Amount |
|---|---|---|
| pGCS vector, linearized (15 ng/µL) | TE Buffer, pH 8.0* | 20 µL |
| DNase/RNase-Free Water | — | 1.5 mL |
| 5× Ligation Buffer | 250 mM Tris-HCl, pH 7.6<br>50 mM $MgCl_2$<br>5 mM ATP<br>5 mM DTT<br>25% (w/v) polyethylene glycol-8000 | 80 µL |
| T4 DNA Ligase (1 Weiss U/µL) | 10 mM Tris-HCl, pH 7.5<br>50 mM KCl<br>1 mM DTT<br>50% (v/v) glycerol | 20 µL |
| pBGH-pA Sequencing Primer (0.1 µg/µL) | TE Buffer, pH 8.0 | 20 µL |
| ds Cloning Control Oligo (5 µM) | 1× ligation Buffer | 10 µL |

***TE buffer, pH 8.0:** 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

### Shipping and storage

The GeneArt® Genomic Cleavage Selection Kit is shipped on dry ice.

Upon receipt, **store at –20°C**.

# Description of the system

## Introduction

Engineered nucleases, including zinc-finger nuclease (ZFN), transcriptional activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPRs) have emerged as attractive tools for inducing genetic modifications in various organisms and cell types (Boch, 2011; Cathomen & Joung, 2008; Christian *et al.*, 2010; Cong *et al.*, 2013; Jinek *et al.*, 2012; Kim *et al.*, 1996; Mali *et al.*, 2013; Miller *et al.*, 2011).

TAL effector and zinc-finger nucleases are proteins with programmable DNA binding domains that can be engineered to bind to specific DNA sequences, thereby allowing one to target a specific genomic locus for cleavage. CRISPR/Cas9 system, on the other hand, is an RNA-guided endonuclease, using CRISPR RNA (crRNA) as a guide to locate the DNA target and directing the Cas9 protein to the DNA cleavage site.

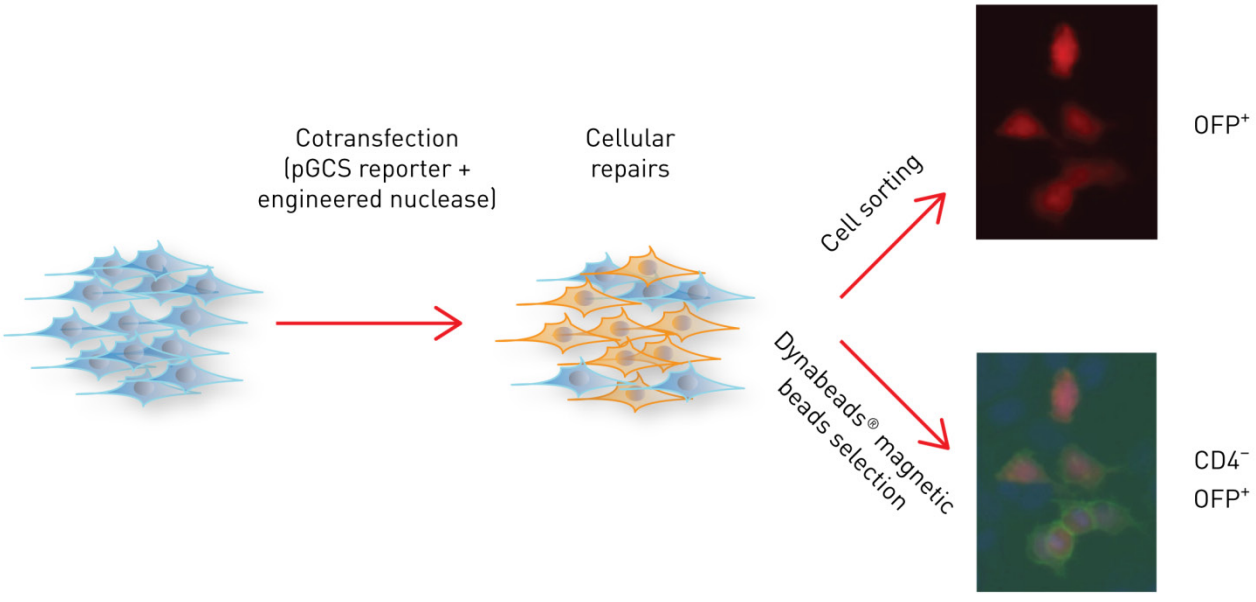
Engineered nucleases induce a double-strand break (DSB) at a specific location in the genome, enabling the creation of disease models and reporter cell lines through efficient gene knock-in via homologous recombination with a donor DNA, or gene knock-out via error-prone non-homologous end joining.

The GeneArt® Genomic Cleavage Selection Kit facilitates the generation of constructs to screen the activity of the engineered nucleases in mammalian cells, and to enrich the nuclease-modified cells.

The linearized GeneArt® Genomic Cleavage Selection vector provides a rapid and efficient way to clone the double-stranded oligonucleotides containing the target sequence into the reporter plasmid. The recombinant vector containing OFP and CD4 coding sequences, in turn, allows for FACS-based cell sorting and isolation using CD4 antibody-conjugated beads to enrich for cell populations that are likely modified by engineered nucleases (Figure 1).

Although the kit has been designed to directly detect the functionality of engineered nucleases, use of the kit for genome editing and target loci cleavage analysis assumes that users are familiar with the principles of ZFN, TALEN™, and CRISPR/Cas9 technologies, and transfection in mammalian systems.

**Figure 1** Detection and enrichment using GeneArt® Genomic Cleavage Selection Kit.



## Genomic cleavage selection

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 the 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 (HDR) pathways.

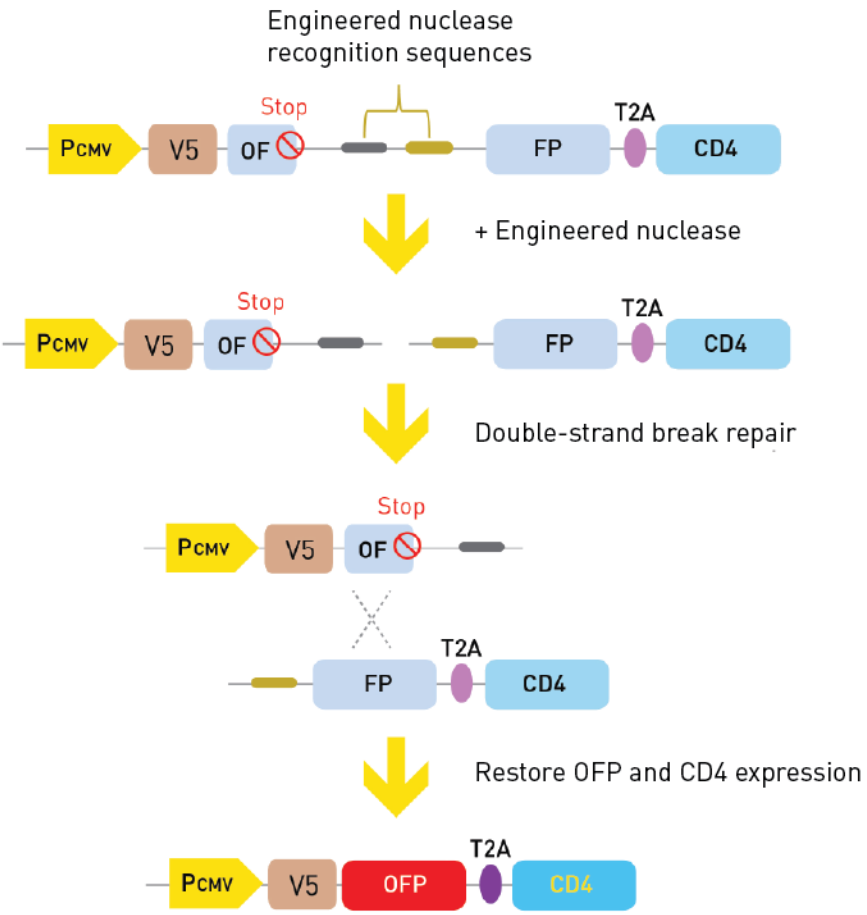
GeneArt® Genomic Cleavage Selection vector pGCS contains two tandem copies of the partial coding sequence of the Orange Fluorescence Protein (OFP) reporter (Figure 2, page 5). The upstream and downstream sequences coding for the N-terminal and C-terminal ends of the OFP gene partially overlap and are separated by a cloning site for the target sequence of the programmable nucleases. The upstream sequence contains only the N-terminal portion of OFP, followed by three stop codons to ensure no expression of the reporter gene.

When a double-stranded break is introduced into the target sequence by the nuclease, the complementary strands from each repeated sequence can recombine to yield a contiguous OFP coding sequence and restore OFP and CD4 gene expression while eliminating the extra sequence between the two complementary repeats. By using this reporter clone, it is possible to verify cleavage by the TALEN™ or CRISPR/Cas9 systems as early as 24 hours post-transfection by simply checking for OFP expression of the transfected cells under the microscope. The percentage of OFP-positive cells indicates the cleavage activity of TALEN™ or CRISPR/Cas9.

The GeneArt® Genomic Cleavage Selection vector also contains the membrane protein CD4 coding gene that is fused with OFP through the T2A self-cleavage peptide, allowing nuclease-modified cells to be enriched through cell sorting or CD4 antibody conjugated Dynabeads®. This cleavage selection vector allows simple, rapid evaluation of the functionality of the programmable nuclease, and direct enrichment of the genome-modified cells.

The design of GeneArt® Genomic Cleavage Selection vector is based on the premise that when a target sequence on a plasmid is mutated by the engineered endonucleases, the same target sequence on the chromosome in the same cell is likely to be mutated (Lee *et al.*, 2010; Perez *et al.*, 2008). However, cleavage by the engineered endonucleases on the plasmid vector is not likely to be affected by the chromatin status, such as higher order chromatin structure, methylation, and accessibility, allowing for robust functionality testing of the engineered endonucleases. For this reason, OFP and CD4 expression are considered an estimation rather than absolute quantification of genomic cleavage. We recommend using GeneArt® Genomic Cleavage Detection Kit (Cat. A24372) to verify the cleavage on endogenous genomic locus.

**Figure 2** Schematic representation of GeneArt® Genomic Cleavage Selection vector during genomic cleavage selection.




OF = N terminus of the *OFP* gene
FP = C terminus of the *OFP* gene

**Note:** For a map and a description of the features of the GeneArt® Genomic Cleavage Selection pGCS, see page 21.

# Methods

## Workflow

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| 2 | Anneal single-stranded oligonucleotides to generate a double-stranded oligonucleotide. | 11 |
| 3 | Clone double-stranded oligonucleotide into pGCS Vector. | 13 |
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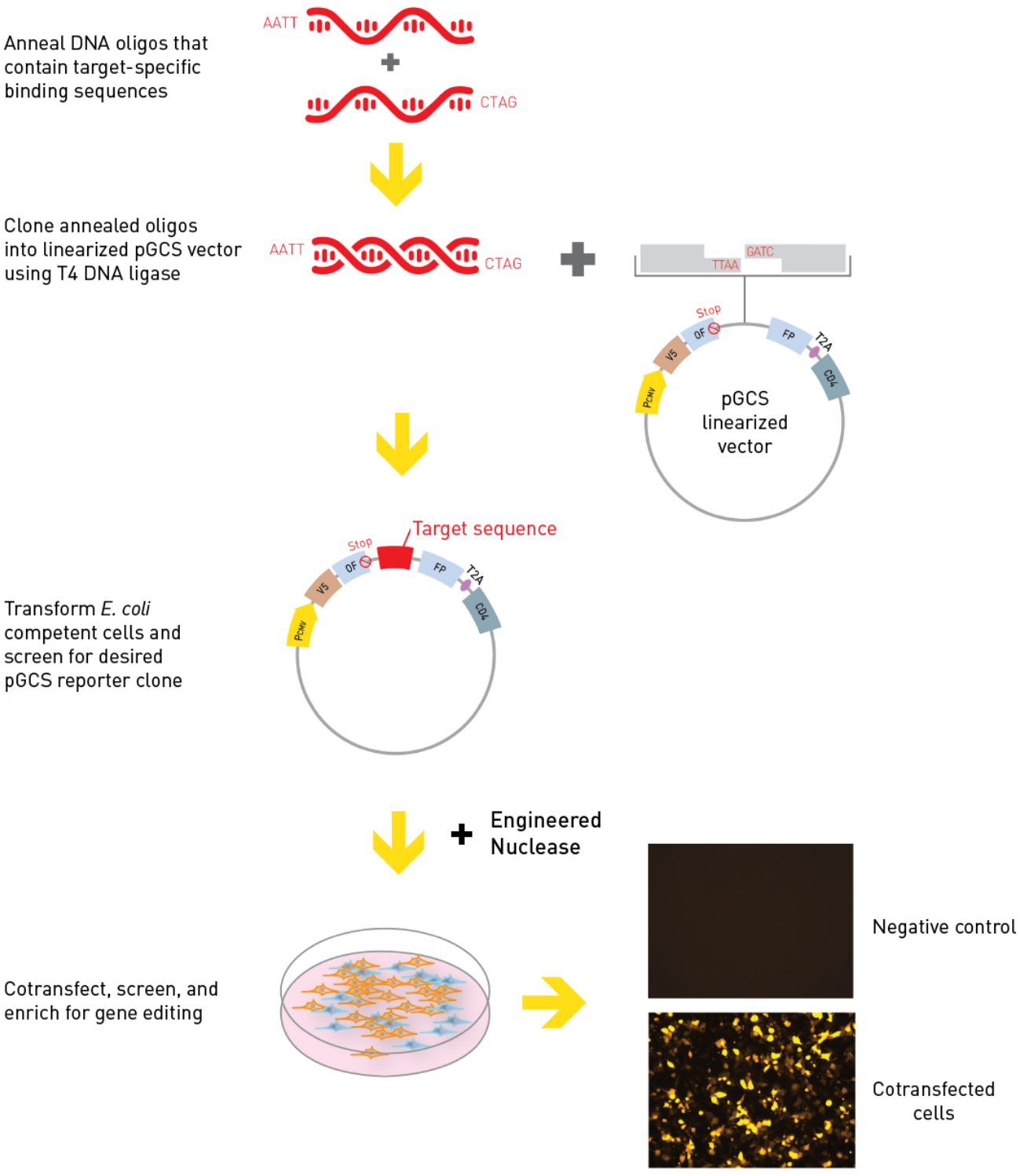
| 5 | Analyze transformants for the presence of insert by sequencing. | 15 |
| 6 | Prepare purified plasmid DNA and transfect the cell line of choice. | 16 |
| 7 | Analyze results. | 17 |
| 8 | Enrich for nuclease-modified cells. | 18 |

**Figure 3** Generation of reporter plasmid and its use in genomic cleavage selection.



# Design single-stranded DNA oligonucleotides

## Introduction

To use the GeneArt® Genomic Cleavage Selection Kit, you will first need to design two single-stranded DNA oligonucleotides with suitable overhangs to complement the linearized vector. One is the forward strand oligonucleotide (or top strand) and the other is its reverse complement (reverse strand oligonucleotide or bottom strand). The oligonucleotides contain the specific target sequence of your engineered nuclease. You will then anneal the forward and reverse strand oligonucleotides to generate a double-stranded oligonucleotide (ds oligonucleotide) suitable for cloning into the linearized vector provided in the kit.

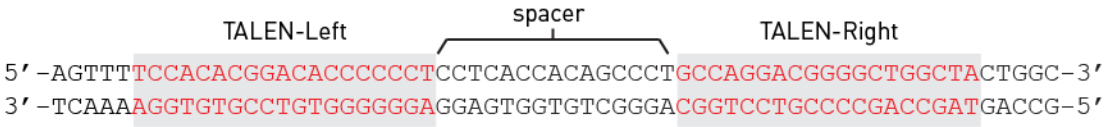
The design of the single-stranded (ss) oligonucleotides is critical to the success of both the cloning procedure and the cleavage of the endonucleases. Use the general guidelines provided in this section to choose the target sequence and to design the ss oligonucleotides.

## Oligonucleotide design guidelines for TALEN or ZFN

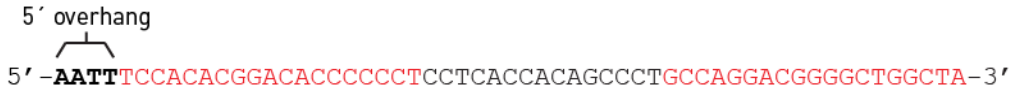
Choose the target sequence on the genomic DNA. The target sequence contains the binding sequences of the TALEN™ or ZFN pair and the corresponding spacer sequence. The length of target sequence ranges from 60 to 70 nucleotides depending on the number of TAL repeats or Zinc fingers and spacer between the two binding sites.

For example:

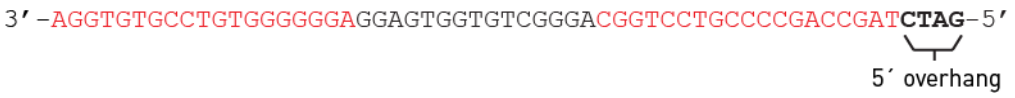
### Genomic DNA targeted by an AAVS1 TALEN pair



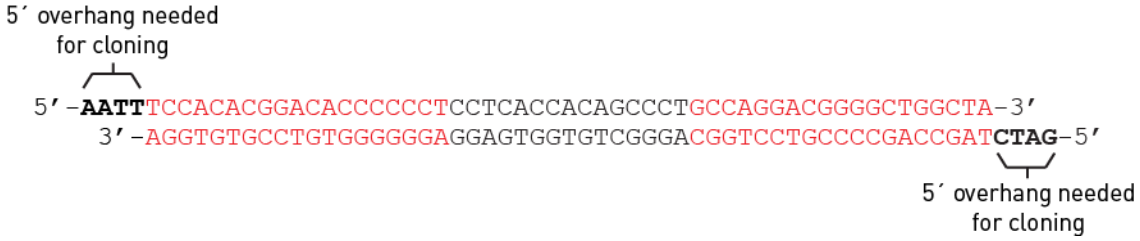

**Top strand oligonucleotide:** Add AATT to the 5′ end of the oligonucleotide. The AATT is complementary to the overhang sequence, TTAA, in the linearized GeneArt® Genomic Cleavage Selection Vector, pGCS.




**Bottom strand oligonucleotide:** The bottom strand oligonucleotide should be the reverse complement of the target sequence. Add CTAG to the 5′ end of the oligonucleotide. This sequence is complementary to the overhang sequence, GATC, in the linearized pGCS vector.



**Annealed ds oligonucleotide:** Annealing the two single-stranded oligonucleotides results in a double-stranded oligonucleotide with compatible ends for cloning into the linearized GeneArt® Genomic Cleavage Selection Vector, pGCS.

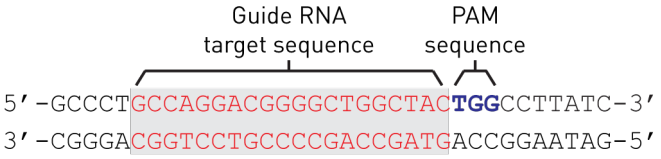



## Oligonucleotide design guidelines for CRISPR guide RNA

Choose a sequence containing the target sequence of 19 to 20 nucleotides in length and the proto-spacer adjacent motif (PAM) sequence (NGG for spCas9).

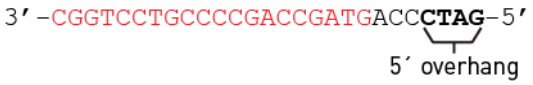
For example:

### The bottom strand of Genomic DNA targeted by an AAVS1 guide RNA

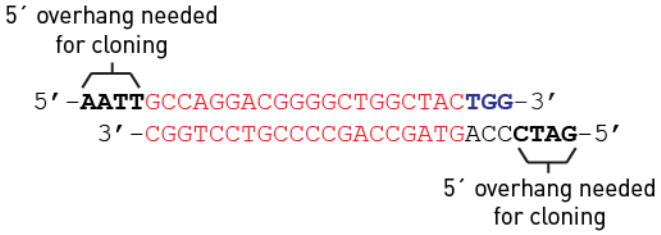



**Top strand oligonucleotide:** Add AATT to the 5′ end of the oligonucleotide. The AATT is complementary to the overhang sequence, TTAA, in the linearized GeneArt® Genomic Cleavage Selection Vector, pGCS.


**Bottom strand oligonucleotide:** The bottom strand oligonucleotide should be the reverse complement of the target sequence. Add CTAG to the 5′ end of the oligonucleotide. This sequence is complementary to the overhang sequence, GATC, in the linearized pGCS vector.




**Annealed ds oligonucleotide:** Annealing the two single-stranded oligonucleotides results in a double-stranded oligonucleotide with compatible ends for cloning into the linearized GeneArt® Genomic Cleavage Selection Vector, pGCS.

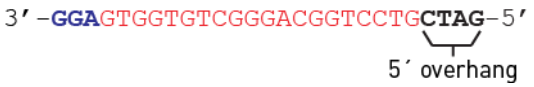


If the guide RNA is designed to target the upper strand, follow the example here:

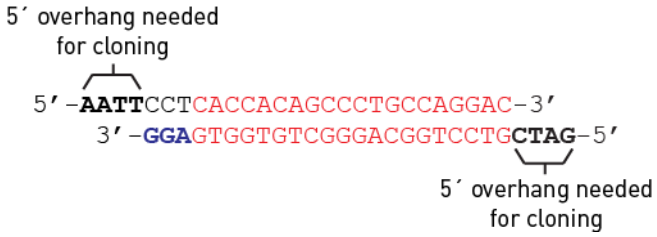
### The upper strand of Genomic DNA targeted by an AAVS1 guide RNA


**Top strand oligonucleotide:** Add AATT to the 5′ end of the oligonucleotide. The AATT is complementary to the overhang sequence, TTAA, in the linearized GeneArt® Genomic Cleavage Selection Vector, pGCS.


**Bottom strand oligonucleotide:** The bottom strand oligonucleotide should be the reverse complement of the target sequence. Add CTAG to the 5′ end of the oligonucleotide. This sequence is complementary to the overhang sequence, GATC, in the linearized pGCS vector.




**Annealed ds oligonucleotide:** Annealing the two single-stranded oligonucleotides results in a double-stranded oligonucleotide with compatible ends for cloning into the linearized GeneArt® Genomic Cleavage Selection Vector, pGCS.



# 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 5 µM to a working concentration of 5 nM.

## Materials required

- Forward strand oligonucleotide (100 µM in water or TE Buffer)
- Reverse strand oligonucleotide (100 µM in water or TE Buffer)
- 5 µM stock of ds Cloning Control Oligo (thaw on ice)
- 5× Ligation Buffer
- DNase/RNase-Free Water
- 1.5 mL sterile microcentrifuge tubes
- Thermocycler or 95°C heat block

## Annealing procedure

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

| | |
|---|---|
| Forward strand oligonucleotide (100 µM) | 1 µL |
| Reverse strand oligonucleotide (100 µM) | 1 µL |
| 5X Ligation Buffer | 4 µL |
| DNase/RNase-Free Water | 14 µL |
| 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 5 µM ds oligonucleotides.

3. Place the PCR tubes in a thermocycler and perform the following cycles:

| Temperature | Ramp rate | Time |
|---|---|---|
| 95°C | — | 5 minutes |
| 95°C–85°C | –2°C/second | — |
| 85°C–25°C | –0.1°C/second | — |
| 4°C | — | hold |

   **Note:** If using an Applied Biosystems® Verite Thermocycler, the above ramp steps correspond to 75% (for –2°C/second) and 5% (for –0.1°C/second) ramp rates, respectively.

   If you do not have a thermocycler, incubate the tube at 95°C for 4 minutes in a heat block. Remove the heat block with tube inside from the heater, and let it cool to 25°C or room temperature.

4. Remove the PCR tubes from the thermocycler or heat block. Centrifuge the tubes briefly (~5 seconds) and mix gently.
5. For long-term storage, keep the 5 µM ds oligonucleotide stock solution at –20°C.

### Prepare 50 nM ds oligonucleotide working solution

After the single-stranded oligonucleotides and the Cloning Control Oligos are annealed, perform a100-fold serial dilution of the 5 µM ds oligonucleotide stock to prepare a 50 nM ds oligonucleotide working solution (100-fold dilution).

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

| | |
|---|---|
| 5 µM ds oligonucleotide stock | 1 µL |
| DNase/RNase-Free Water | 99 µL |
| Total volume | 100 µL |

2. Vortex to mix thoroughly.
3. For long-term storage, keep the 50 nM ds oligonucleotide stock solution at –20°C.

### Handling ds 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 50 nM working solution becomes heated, prepare new diluted solutions, or re-anneal the oligonucleotides.

# Ligation reaction

## Introduction

Once you have generated your ds oligonucleotide and prepared the appropriate stock solutions, clone the ds oligonucleotide into the linearized GeneArt® Genomic Cleavage Selection Vector, pGCS.

## Materials required

- Double-stranded oligonucleotide (50 nM in 1× Ligation Buffer; thaw on ice before use)
- Double-stranded control oligonucleotide (50 nM in 1× Ligation Buffer; thaw on ice before use)
- Linearized GeneArt® Genomic Cleavage Selection Vector pGCS (thaw on ice before use)
- 5× Ligation Buffer (supplied with kit)
- DNase/RNase-Free Water (supplied with kit)
- T4 DNA Ligase (supplied with kit)

## Controls

We recommend including the diluted ds Cloning Control Oligo supplied with the kit as a positive control in your ligation experiment.

If you wish to include a negative control, set up a separate ligation reaction omitting 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 PCR tube:

| | |
|---|---|
| 5× Ligation Buffer | 4 µL |
| Linearized pGCS vector | 2 µL |
| ds oligonucleotide (50 nM) | 2 µL |
| DNase/RNase-Free Water | 11 µL |
| T4 DNA Ligase | 1 µL |
| Total volume | 20 µL |

2. Mix reaction well by pipetting up and down.

   **Note:** The presence of PEG and glycerol in the 5× 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 the reaction for 10 minutes at room temperature (25–27°C).

   **Note:** 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 competent *E. coli* cells", page 14.

   **Note:** You may store the ligation reaction at –20°C.

# Transform competent *E. coli* cells

## Introduction

Once you have completed the ligation reaction, transform One Shot® TOP10 chemically competent *E. coli* with the resulting Genomic Cleavage Selection 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.

## Materials required

- Ligation reaction (from step 4, page 13)
- *Optional:* pUC19 control (supplied with kit)
- One Shot® TOP10 chemically competent *E. coli* cells
- S.O.C. Medium (warm to room temperature before use)
- LB plates containing 100 µg/mL ampicillin (two for each transformation; pre-warm to 37°C for 30 minutes before use)
- 42°C water bath
- 37°C shaking and non-shaking incubator

## Transform One Shot® TOP10 *E. coli* cells

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 13) 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 minimal effect on transformation efficiency.
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 0.5–10 µL of the transformation reaction plus an appropriate volume of SOC on a pre-warmed LB agar plate containing 100 µg/mL ampicillin.
9. Incubate the plates overnight at 37°C.

   **Note:** We recommend plating two different volumes to ensure that at least one plate has well-spaced colonies. If you are transforming the pUC19 control, plate 5–50 µL of the transformation on pre-warmed LB plates containing 100 µg/mL ampicillin.
10. An efficient ligation reaction may produce over a hundred colonies in total. Pick 4–10 colonies for analysis (see "Analyze transformants", page 15).

# Analyze transformants

## Confirm positive clones

Confirm the identity of the ds oligonucleotide insert in positive transformants by sequencing. Analyze each Genomic Cleavage Selection 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.

## Confirm positive clones

1. Pick 4–10 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. Sequence the Genomic Cleavage Selection construct using the pBGH-pA-F Forward Primer (supplied with kit) and confirm the presence of the correct insert in the correct orientation.
4. Proceed to “Transfect mammalian cells”, page 16.

# Transfect mammalian cells

## Methods of transfection

Methods of transfecting plasmids into the mammalian cell lines include calcium phosphate (Chen & Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated techniques (Felgner *et al.*, 1989; Felgner & Ringold, 1989), and electroporation (Chu *et al.*, 1987; Shigekawa & 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) or Lipofectamine® 3000 Reagent (Cat. no. L3000-015).

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 of phenol and sodium chloride contamination. 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 transfections in a standard 6-well plate:

- Use Lipofectamine® 3000 reagent to transfect most cell lines.
- Seed the cells so that they are ~70% confluent on the day of transfection.

  **Note:** Seeding density varies with cell type.
- Use 3 µg of DNA for transfection:

  For TALEN pair:

  Genomic Cleavage Selection vector: 1 µg
  TALEN-Left expression plasmid: 1 µg
  TALEN-Right expression plasmid: 1 µg

  For CRISPR/Cas9:

  Genomic Cleavage Selection vector: 1 µg
  gRNA expression plasmid: 1 µg
  Cas9 expression plasmid; 1 µg
- Results will vary depending upon cell type and passage number, and optimization of lipid:DNA concentrations may be required for best results.
- 1: 1 ratio of the Genomic Cleavage Selection vector and nuclease expression plasmids works well for most transfections; however, titering down the amount of Genomic Cleavage Selection vector could reduce background.

## Controls

We recommend that you always include a negative control (co-transfection of GeneArt® Genomic Cleavage Selection Vector along with an empty vector or an irrelevant nuclease expression vector) in your experiments to evaluate your results. The control oligonucleotides included in the kit contain the target sites for the safe-harbor locus AAVS1 TALEN pair (also known as PPP1R12C locus on human chromosome 19). If you want to include a positive control in your transfection, you can obtain our off-the-shelf validated TAL nuclease pair or CRISPR guide RNA expression plasmid by contacting Technical Support (see page 26).

# Analyze the results

### Microscopy analysis

In most case, you can identify positive cleavage by significantly higher number and expression level of OFP in the test samples compared to the negative control transfection samples. In most cell lines under most conditions, you can observe OFP expression under a microscope 24 hours post-transfection, with the intensity of OFP expression increasing with longer incubation times. You can take fluorescent images of OFP expressing cells using a fluorescence microscope.

We recommend using GeneArt® Genomic Cleavage Detection kit (Cat. A24372) to verify the cleavage on the endogenous genomic locus.

### Flow cytometry analysis

You can measure the percentage and the mean fluorescence intensity (MFI) of OFP expressing cells using a flow cytometer and calculate the fold difference between the test sample and negative control sample.

1. Harvest and centrifuge the cells using conditions appropriate for the cell type.
2. Aspirate the supernatant and re-suspend the cell pellet in fluorescence activated cell sorting (FACS) buffer (we recommend 25 mM HEPES, 1 mM EDTA, 1% FBS) to an appropriate concentration (e.g. $0.5 \times 10^6$–$1 \times 10^6$ cells/mL).
3. Pass cells to be analyzed through a cell-filter tube.
4. Analyze the negative control (GeneArt® Genomic Cleavage Selection vector co-transfected with an irrelevant CRISPR/TALEN or empty expression vector) and the test samples using a flow cytometer. Gate for OFP expression using the negative control.
5. Calculate the fold difference between the test sample and negative control sample as follows:

$$\text{Ratio MFI} = \frac{\text{number of OFP positive cells} \times \text{MFI of OFP positive cells in test sample}}{\text{number of OFP positive cells} \times \text{MFI of OFP positive cells in negative control sample}}$$

# Enrich for nuclease-modified cells

## Introduction

Populations of cells co-transfected with the Genomic Cleavage Selection vector and nuclease expression plasmid(s) can be enriched using fluorescence activated cell sorting (FACS), or using Dynabeads® CD4 magnetic beads.

## Enrichment by FACS

Consider the following guidelines when enriching for OFP-positive cells using FACS:

- Harvest transfected cells and resuspend in FACS buffer. For example, a 0.2-µm sterile-filtered FACS buffer (1× PBS containing 1 mM EDTA, 25 mM HEPES, 1% FBS) works well for adherent cell lines 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 depends on the downstream application of choice, but RPMI media with 2% FBS, and FACS buffer (above) are viable options.

## Enrichment using Dynabeads® CD4 magnetic beads

We recommend the following protocol when enriching for OFP-positive cells using Dynabeads® CD4 magnetic beads (Cat. no. 11331D):

### Harvest cells

1. Harvest live transfected cells and centrifuge at 400 × *g* for 5 minutes at 4°C. Decant the supernatant and resuspend the cells in 2 mL of Buffer I (0.2-µm sterile-filtered PBS with 0.1% BSA, 2 mM EDTA).
2. Centrifuge at 400 × *g* for 5 minutes at 4°C.
3. Wash twice with 2 mL of Buffer I.
4. Resuspend the cells in an appropriate volume of Buffer I.
5. 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) at 4°C.
2. Transfer 25 µL 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 µL 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 µL of Buffer I.
7. Proceed to "Incubate cells with Dynabeads® CD4 magnetic beads", page 19.

### Incubate cells with Dynabeads® CD4 magnetic beads

A 1:1 bead to cell ratio is recommended (25 µL of beads ~ $10^7$ 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 µL of Buffer I.
6. Incubate on a mixer for 2 minutes at 4°C. Repeat steps 3–6 for 5 times.
7. After the final wash, resuspend the cells and beads in 100 µL of Buffer II (0.2-µm sterile-filtered RPMI with 2% FBS).
8. Add 10 µL of DETACHaBead® CD4 (Cat. no. 11331D).
9. Incubate on a mixer at room temperature for 45 minutes.
10. Place the tube on a magnetic separator for 1 minute.
11. Transfer the supernatant (containing CD4 positive cells) to a fresh tube.
12. Resuspend the beads in 500 µL of Buffer II.
13. Place the tube on a magnetic separator and add the supernatant to the tube containing the recovered CD4 positive cells.
14. Repeat steps 13–14 for 3 times to obtain the maximum yield of recovered CD4 positive cells.
15. Bring the tube containing recovered CD4 positive cells to a final volume of 4 mL.
16. Centrifuge at 400 × *g* for 6 minutes at 4°C.
17. Resuspend enriched cells in a solution appropriate for your downstream application (e.g. staining solution, flow cytometry buffer, media).

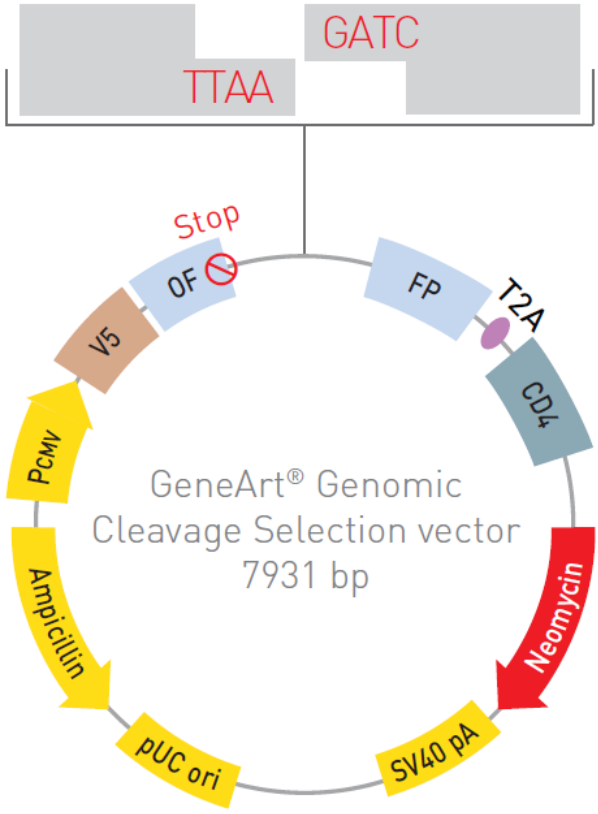
# Appendix A: Troubleshooting

| 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 4 nucleotides on the 5′ end required for cloning into the GeneArt® Cleavage Selection Vector:<br>• Top strand oligonucleotide: include AATT on the 5′ end.<br>• Bottom strand oligonucleotide: include CTAG on the 5′ end. |
| | ds oligonucleotides were degraded | • Store the 50 nM ds oligonucleotide stock in 1× Ligation Buffer at –20°C.<br>• Avoid repeated freeze/thaw cycles. Aliquot the 50 nM ds oligonucleotide stock and store at –20°C. |
| | Oligonucleotide annealing reaction was inefficient | • Ensure that the annealing reaction was performed as directed (page 11).<br>• If ambient temperature is >25°C to 27°C, incubate the annealing reaction in a 25°C incubator. |
| No OFP observed | Oligos designed incorrectly | • For TALEN target, make sure to include both TAL binding sites and spacer region.<br>• For CRISPR target, a PAM sequence should be included. |
| | Transfection efficiency is too low | Optimize transfection conditions. We recommend using Lipofectamine® 2000 and Lipofectamine® 3000 for the best results. |
| | Cell line dependent | • OFP does not express well in certain cell lines, we recommend using 293FT cells as a workhorse cell line to test the cleavage activity. |
| Too much OFP background | Plasmid contamination | • Pick single clones when culturing the Cleavage Selection plasmid.<br>• We do not recommend culturing the bacterial cells for more than 18 hours to avoid recombination of the plasmid.<br>• Verify the plasmid sequence. |
| | Cell line and target dependent | • Reduce the amount of the Genomic Cleavage Selection vector that is included in the co-transfection.<br>• Re-test the transfection in 293FT cells. |
| Results do not match data from GeneArt® Genomic Cleavage Assay | Locus dependent | We recommend using GeneArt® Genomic Cleavage Detection kit (Cat. A24372) to verify the cleavage on endogenous genomic locus. |

# Appendix B: GeneArt® Genomic Cleavage Selection Vector

## Map of pGCS

The figure below shows the features of the GeneArt® Genomic Cleavage Selection vector. The pGCS vector is supplied linearized at nucleotides 1262 and 1323 with 4 base pair 5′ 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 26).



# Features of pGCS

The GeneArt® Genomic Cleavage Selection Vector (7931 bp) contains the following elements. All features have been functionally tested and the vector fully sequenced.

| Feature | Benefit |
|---|---|
| F1 origin of replication | Origin of replication. |
| TK pA | Polyadenylation signal. |
| V5 | This epitope is recognized by the Anti-V5 Antibody |
| CD4 | Reporter gene for bead based enrichment. Can be used for monitoring transfection efficiency when stained with a fluorescently labeled anti-CD4 antibody. |
| OFP | Reporter gene for FACS-based sorting. The fluorescent protein can also be used for monitoring transfection efficiency. |
| BGH-pA | Bovine Growth Hormone and Polyadenylation signal serves as a sequence for the termination of transcription. A polyadenine tail is added to the 3' end to protect the mRNA from hydrolytic enzymes and to provide stability. |
| 2A peptide linker | A self-cleaving peptide linker connecting OFP reporter gene to the N-terminal end of CD4 Following translation, the two proteins flanking the 2A peptide are separated from each other. |
| CMV promoter | Allows expression of OFP and CD4 reporter genes. |
| Ampicillin resistance gene | Allows selection of the plasmid in *E. coli*. |
| pUC origin of replication (*ori*) | Permits high-copy replication and maintenance in *E. coli*. |

# Appendix C: Ordering Information

## Introduction

The products listed in this section may be used with the GeneArt® Genomic Cleavage Selection Vector. For more information, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (see page 26).

## Ordering oligonucleotides

Custom oligonucleotides for use with the Genomic Cleavage Selection Vector can be ordered from Life Technologies. For additional details, visit our website at **www.lifetechnologies.com/oligos** or contact Technical Support (see page 26).

## Additional products

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

| Item | Quantity | Catalog no. |
|---|---|---|
| GeneArt® Genomic Cleavage Detection kit | 20 reactions | A24372 |
| T4 DNA Ligase | 100 units | 15224-017 |
| One Shot® TOP10 Chemically Competent *E. coli* | 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<br>1.5 mL | 11668-027<br>11668-019 |
| Lipofectamine® 3000 | 0.75 mL<br>1.5 mL | L3000-001<br>L3000-015 |
| 293FT Cell Line | $3x10^6$ cells | R700-07 |
| Dynabeads® CD4 Positive Isolation Kit | 5 mL | 11331D |
| HulaMixer™ Sample Mixer | 1 unit | 15920D |

# Appendix D: Safety

## General safety

**WARNING! GENERAL SAFETY.** Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and support" section in this document.

## Chemical safety

**WARNING! GENERAL CHEMICAL HANDLING.** To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

# Biological hazard safety

WARNING! **BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Safety equipment also may include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:

  **www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf**

- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:

  **www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf**

# Documentation and 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/sds**.

**Note:** For the SDSs of chemicals not distributed by Thermo Fisher Scientific, contact the chemical manufacturer.

## Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support.**

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