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CRISPR-Cas9 protein for genome editing

Getting started guide

ThermoFisher SCIENTIFIC

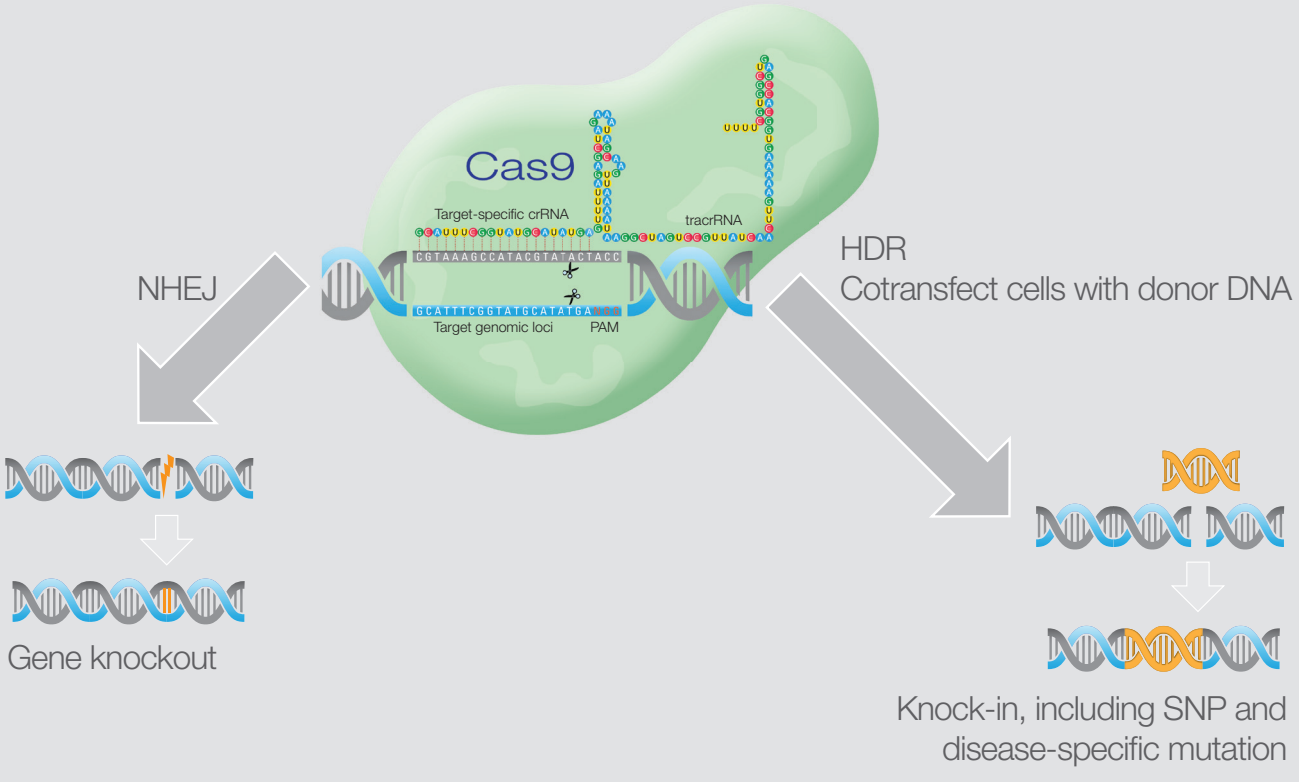
Introduction

Editing endogenous genomic DNA in eukaryotic cells is now possible with CRISPR-Cas9 technology. By taking advantage of a cell's own DNA repair mechanisms, we are able to add, change, or delete almost any genomic DNA sequence.

Cas9 is an endonuclease with the ability to cut double-stranded DNA. It can be directed to a specific genomic locus by a CRISPR guide RNA (gRNA) sequence, and an "NGG" sequence known as the protospacer adjacent motif (PAM) sequence. Cas9 recognizes the PAM site and the gRNA base pairs with the complementary target DNA sequence. When the gRNA/Cas9 complex binds to the target site, the Cas9 introduces a blunt-ended, double-stranded cut, 3 bases upstream of the PAM site. If the purpose of the edit is to knock out a particular gene, then we can capitalize on the cell's nonhomologous end joining (NHEJ) DNA repair pathway. This process is somewhat error prone and can result in the insertion or deletion of a small number of bases, known as "indel" formation. This often causes a reading frame shift and/or premature termination of the protein. When knocking out a gene of interest, it is recommended to target the 5' end of the gene.

To introduce a specific edit into the DNA, such as introducing a single nucleotide polymorphism (SNP) or a disease-specific mutation, then we can exploit the cell's homology-directed repair (HDR) pathway. By introducing a short donor DNA sequence along with the gRNA/Cas9 complex, we can drive the cell to use the donor DNA to repair the double-stranded break. The DNA must contain the desired edit flanked by regions of homology to the target site. When knocking in a specific edit, it is recommended to introduce the double-stranded break within 10 bases of the edit site for maximum efficiency.

The purpose of this guide is to provide a helpful, simple way to get started using CRISPR-Cas9 technology and walk you through the design and workflow of a CRISPR editing experiment.



What you'll need

CRISPR design tool
GeneArt CRISPR Search and Design Tool

gRNA synthesis kit
GeneArt Precision gRNA Synthesis Kit

CRISPR-Cas9 protein
GeneArt Platinum Cas9 Nuclease

CRISPR-Cas9 delivery
Lipofectamine CRISPRMAX Cas9 Transfection Reagent or Neon Transfection System

Detection kit
GeneArt Genomic Cleavage Detection Kit

Things to consider

What type of edit will you be performing?

- Knock out a gene of interest
  - To knock out a gene of interest, you'll want to introduce a double-stranded break in early exons to generate a frame-shift or premature termination.
- Knock in a specific mutation or short DNA sequence
  - To knock in a specific change, such as an SNP or a disease-specific mutation, introduce a double-stranded break within 10 bases of the nucleotides you wish to edit.
- Design gRNAs using our Invitrogen™ GeneArt™ CRISPR Search and Design Tool at **thermofisher.com/crisprdesign**
  - For knockout experiments, search our database of >600,000 predesigned CRISPR gRNAs targeting human and mouse genes.
  - For knock-in experiments, perform a search of your sequence of interest and we will give you the recommended gRNA sequences.
  - Test 2 or 3 gRNAs to identify the gRNA that gives you the highest cleavage efficiency. This will reduce the screening time required to identify your clone of interest.
- If you would like us to design and synthesize your gRNA, please let us know. You can reach us at **GEMServices@thermofisher.com**

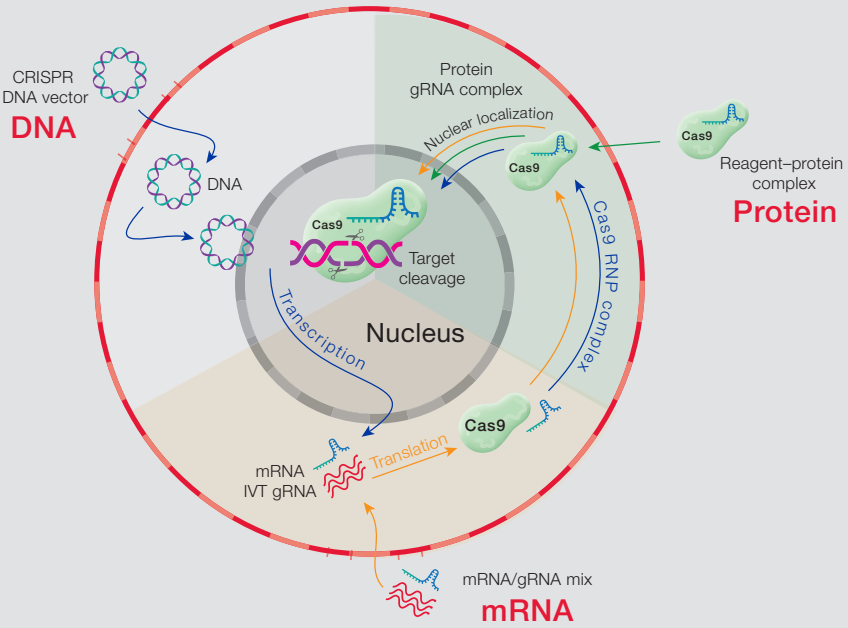
Know your cell line

- If your cell line can accept lipid-based transfection reagents, then we recommend using Cas9 protein with Invitrogen™ Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent
- If your cell line does not transfect well with lipid-based transfection reagents, we recommend using our Invitrogen™ Neon™ Transfection System
- Seeding density, passage number, and type of media can all have an effect on levels of editing efficiency. Consider optimizing these parameters to achieve the highest possible editing efficiency. This can significantly reduce the time required to identify your clone of interest

NOTE: We can provide you with an HPRT control to test and optimize your conditions. Contact us at **GEMServices@thermofisher.com**

Maximum efficiency, minimal off-target cleavage with CRISPR-Cas9 RNP complex

By transfecting Cas9 protein together with the targeting gRNA, we achieve higher cutting efficiencies and lower off-target effects than with traditional CRISPR plasmid formats. With the ribonucleoprotein complex (RNP), you can deliver the active Cas9/gRNA directly into the cell, expediting the time to targeting your edited cells, since you no longer have to wait for the cells to perform the transcription, translation, or complex formation. For a complete list of cell lines with their associated editing efficiencies and conditions tested, please refer to our latest publication [1].



FAQs

Q: What do I do when there is no 5′ NGG (PAM) sequence available?

A: We recommend engineering a TAL effector to edit your desired gene efficiently. We offer Invitrogen™ GeneArt™ PerfectMatch™ TAL effectors. These are TAL effector nucleases that remove the 5′ base constraint and can be designed to target any desired sequence within the genome. For more information, visit **thermofisher.com/tal**

Q: How many guide RNAs do you recommend designing against my desired edit locus?

A: We recommend testing 2–3 gRNAs against each locus being targeted. Testing multiple gRNAs increases the chances of finding a gRNA with high editing efficiency, which will reduce the screening time required to identify your clone of interest.

Q: How long after transfection do you assess mRNA and protein knockdown?

A: We recommend performing the genomic cleavage detection assay 48–72 hours posttransfection.

Q: How precise is the cleavage event?

A: Cleavage is precise. After binding of the Cas9 and gRNA complex to the target genomic sequence, the nuclease activity occurs 3 bases upstream of the PAM (NGG) site.

For a complete list of FAQs, visit **thermofisher.com/crispr101**

Checklist

Cells

- ☐ Culture media (If you don't know what media to use, go to **thermofisher.com/gibco** or contact technical support at **GEMServices@thermofisher.com**)
- ☐ Growth conditions

Components

- ☐ Cas9 protein (Invitrogen™ GeneArt™ Platinum Cas9 Nuclease)
- ☐ Design tool (GeneArt CRISPR Search and Design tool, **thermofisher.com/crisprdesign**) for gRNA design and GCD primer design
- ☐ gRNA (Invitrogen™ GeneArt™ Precision gRNA Synthesis Kit) or custom gRNA synthesis (for more information, inquire at **GEMServices@thermofisher.com**)
- ☐ Donor DNA for knock-in experiments (we can design and synthesize your donor DNA or plasmid. For more information, inquire at **GEMServices@thermofisher.com**)

Delivery based on cell type

- ☐ Lipofectamine CRISPRMAX Cas9 Transfection Reagent for lipid transfection
- ☐ Neon Transfection System for electroporation

Detection and analysis

- ☐ **Detect**—Invitrogen™ GeneArt™ Genomic Cleavage Detection (GCD) Kit to check editing efficiency; primers for GCD, E-gels, gel imager
- ☐ **Clonal isolations**—genotyping and sequencing to validate clones; sequencing to analyze off-target effects
  - Grow cells, expand cells
  - Sort onto 96-well plates, image, and isolate clones
- ☐ **Validate**—GCD or sequencing primers to validate edit by Sanger or next-generation sequencing
- ☐ **Bank cells**—freezing media

Guidelines for CRISPR-Cas9 protein transfection

- For transfections into adherent cells, we recommend using the Lipofectamine CRISPRMAX reagent first, and then trying the Neon Transfection System, if needed.
- For transfections into suspension cells, we recommend using the Neon Transfection System.
- Perform transfections using standard 24-well culture plates. This plate size is convenient when screening different gRNA sequences to identify the most suitable and effective candidate for genome editing.
- Seed cells 24 hours prior to transfection so that they are 30–70% confluent on the day of transfection. Depending on the cell type, this will be approximately 0.4–1.2 x 10^5 cells per well in a 24-well plate format.
- The molar ratio of *in vitro*–transcribed (IVT) gRNA to Cas9 protein to form Cas9 nuclease/RNP complexes is approximately 1.2 to 1. Once formed, the Cas9 nuclease/RNP complexes are stable for up to 3 hours at room temperature.
- While dosage depends on the gRNA target, cell line, and desired cleavage efficiency, the optimal amount of Cas9 nuclease required is usually around 0.5 µg per well of a 24-well plate when using lipid transfection reagents such as the Lipofectamine CRISPRMAX reagent, and 1.0 µg per well of a 24-well plate when using the Neon Transfection System, with the performance plateauing at higher Cas9 amounts for both transfection methods.
- If using different well formats, scale the recommended amounts based on the transfection plate format.
- Set up each reaction—at least in duplicate.
- Prepare at least 0.5x more of the Cas9/gRNA complexes for electroporation than needed to avoid introducing bubbles.

Protocol: Positive control CRISPR-Cas9 protein editing in HEK293FT cells

1 Design

When doing your first editing experiment, we recommend performing this positive control first, or in parallel.

1. Order primers for gRNA synthesis and for GeneArt genomic cleavage detection assay at **thermofisher.com/crisprdesign**
2. HEK296FT cell seeding
   - Grow HEK293FT cells in DMEM medium supplemented with 10% FBS
   - Plate cells on 24-well plates in 500 µL of growth medium
   - Cell seeding numbers/well (x 10^3) = 90
   - Confluence (%) prior to transfection = 75

Order oligos:
thermofisher.com/crisprdesign

2 Make gRNA

All the reagents necessary to perform the *in vitro* transcription and purification are included in the Precision gRNA Synthesis Kit.

1. Assemble the DNA template for gRNA production by PCR using the Thermo Scientific™ Phusion™ High-Fidelity PCR Master Mix, which is included in the GeneArt Precision gRNA Synthesis Kit.
2. Generate the gRNA by *in vitro* transcription using the Thermo Scientific™ TranscriptAid™ transcription kit included in the GeneArt Precision gRNA Synthesis Kit.
3. Remove the DNA template by DNase I degradation.
4. Purify the IVT gRNA using Thermo Scientific™ GeneJET™ purification columns.
5. Measure the purified gRNA concentration.

GeneArt Precision gRNA Synthesis Kit

3

Deliver

Lipid-mediated cell transfection

1. Seed 0.4–1.2 x 10^5 cells/well in a 24-well culture plate one day prior to the experiment so that the culture is 30–70% confluent on the day of transfection.
2. Label and prepare tubes for appropriate experimental and control samples.

NOTE: Cell density at the time of transfection is critical. Testing different cell-seeding densities may be necessary to determine the optimal confluence for transfection.

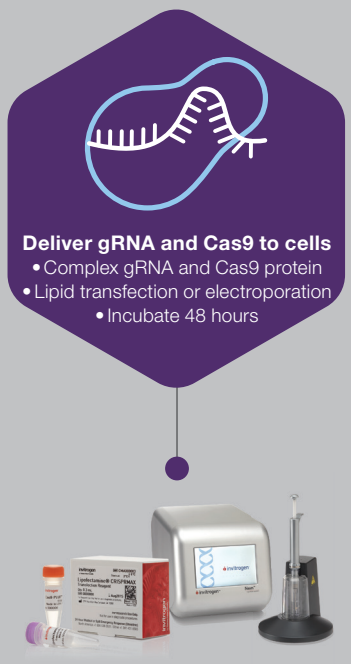
3. Prepare Cas9 nuclease/gRNA RNP complexes by combining the following components and mix well.

Gibco™ Opti-MEM™ I Reduced Serum Medium	25 µL
GeneArt Platinum Cas9 Nuclease	500 ng
gRNA	125 ng
Invitrogen™ Lipofectamine™ Cas9 with PLUS™ solution	1 µL

4. Dilute 1.5 µL of Lipofectamine CRISPRMAX reagent in 25 µL of Opti-MEM I Reduced Serum Medium and incubate at room temperature for 5 minutes.

NOTE: Cas9/RNP complexes are stable for up to 2 hours at room temperature.

5. Add Cas9 protein/gRNA complex with Cas9 Plus solution to diluted CRISPRMAX reagent and mix well by vortexing.
6. Incubate the Cas9 protein/gRNA complex with Cas9 PLUS solution at room temperature for 10–15 minutes to allow formation of the Cas9/gRNA–lipid complexes.
7. Add 50 µL per well of Cas9/gRNA–lipid complexes to the cells to be transfected. Swirl the plate gently to allow mixing of the transfection mixture with the culture medium.
8. Incubate the cells in a humidified incubator at 37°C, with 5% CO_2 for 48 hours.



Lipofectamine CRISPRMAX Cas9 Transfection Reagent

GeneArt Platinum Cas9 Nuclease

Neon Transfection System

Electroporation

For detailed instructions on using the Neon Transfection System, refer to the Neon Transfection System user guide available at **thermofisher.com**

1. On the day of transfection, count the HEK293FT cells in suspension. Alternatively, detach the adherent cells from the culture flask using the Gibco™ TrypLE™ Select Enzyme and then resuspend the cells in growth medium prior to cell counting.
2. To 5 µL of resuspension buffer R, add 1 µL of GeneArt Platinum Cas9 Nuclease (1 µg/µL) and 1 µL of IVT gRNA (240 ng/µL). Gently mix the Cas9/gRNA complex.
3. Incubate at room temperature for 5–10 minutes.

4. Meanwhile, transfer 1 x 10^6 cells to a sterile test tube and centrifuge at 500 x *g* for 5 minutes.
5. Aspirate the supernatant and resuspend the cell pellet in 1 mL of PBS without Ca^{2+} and Mg^{2+}.
6. Centrifuge the cell suspension at 500 x *g* for 5 minutes.
7. Carefully aspirate the entire PBS buffer without disturbing the cells.

NOTE: Air bubbles can cause arcing during electroporation, which lead to lowered or failed transfection. If you notice air bubbles in the tip, discard the sample and carefully aspirate a fresh sample into the tip without introducing any air bubbles.

8. Resuspend the cells with 100 µL of resuspension buffer R.
9. Add 5 µL of the resuspended cells to 7 µL of the Cas9/gRNA complex solution (from Step 2). Mix gently.
10. Carefully aspirate 10 µL of the cell mixture containing Cas9/gRNA complex into the 10 µL Invitrogen™ Neon™ Tip, taking care not to introduce any air bubbles.
11. Electroporate the cells using the Protocol #24 (1600 V, 10 ms, and 3 pulses).
12. Immediately transfer the electroporated cells to a 24-well plate containing 0.5 mL of the appropriate growth medium.
13. Incubate the cells in a humidified incubator at 37°C, with 5% CO_2 for 48–72 hours.

4 Detect

Following 48–72 hours posttransfection, perform a genomic cleavage detection assay. This technique leverages mismatch-specific endonucleases to detect genomic indels incorporated during cellular NHEJ repair mechanisms.

We recommend using the GeneArt Genomic Cleavage Detection Kit (Cat. No. A24372) for performing cleavage efficiency analysis. See the GeneArt Genomic Cleavage Detection Kit User Guide for detailed instructions. For this analysis, you need to design PCR primers that are approximately 150–300 bases away from the target cleavage site on each side and amplify the genomic target of 400–600 bases. The GeneArt genomic cleavage detection assay will cleave the amplified genomic PCR product if a modification has occurred, which can then be visualized on a gel.

References:

1. Liang X et al. (2015) Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *J Biotechnol.* 208:44-53.

GeneArt Genomic Cleavage Detection Kit

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Ordering information

Product	Quantity	Cat. No.
CRISPR protein		
GeneArt Platinum Cas9 Nuclease (1 µg/µL)	10 µg	B25642
GeneArt Platinum Cas9 Nuclease (1 µg/µL)	25 µg	B25640
GeneArt Platinum Cas9 Nuclease (3 µg/µL)	75 µg	B25641
CRISPR gRNA synthesis		
GeneArt Precision gRNA Synthesis Kit	25 reactions	A29377
Custom-synthesized gRNA	Inquire at **GEMServices@thermofisher.com**	
Detection and analysis reagents		
GeneArt Genomic Cleavage Detection Kit	20 reactions	A24372
Transfection reagents		
Lipofectamine CRISPRMAX Cas9 Transfection Reagent	75 reactions	CMAX00008
Neon Transfection System	1 each	MPK5000

Additional resources to help you get started

CRISPR-Cas9 resource	Where to find it
Genome editing learning center	thermofisher.com/genomeedit101
Genome editing support center	thermofisher.com/genomeeditsupport
Genome editing system selection guide	thermofisher.com/genomeeditselect
Delivery format product selection guide	thermofisher.com/crisprtransfection
gRNA design tool	thermofisher.com/crisprdesign
Products for monitoring genome editing	thermofisher.com/detectCRISPR

Resources available from custom services (email: **GEMServices@thermofisher.com**)

- Positive control: 10 µg IVT gRNA targeting safe-harbor human *HPRT* gene validated in HEK293FT cells
- 500 pmols of each GCD primer to detect cleavage at human *HPRT locus*
- Certificate of analysis
- We can do large-scale and high-throughput IVT gRNA design, synthesis, and validation

Find out more at **thermofisher.com/crispr**

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