invitrogen USER GUIDE

TrueCut™ Cas9 Proteins

Catalog Nos. A36496, A36497, A36498, A36499, A50574, A50575, A50576, A50577

Pub. No. MAN0017066 **Rev.** D.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

Invitrogen™ TrueCut™ Cas9 Proteins are used for genome editing applications with CRISPR technology. Cas9 protein forms a very stable ribonucleoprotein (RNP) complex with the guide RNA (gRNA) component of the CRISPR-Cas9 system. Incorporation of nuclear localization signals (NLS) aid its delivery to the nucleus, increasing the rate of genomic DNA cleavage. It is cleared rapidly, minimizing the chance for off-target cleavage when compared to plasmid systems (Liang *et al*., 2015). The Cas9 nuclease has been tested in a wide variety of suspension and adherent cell lines and has shown superior genomic cleavage efficiencies and cell survivability compared to plasmid-based CRISPR systems.

Two types of TrueCut™ Cas9 Proteins are available for selection, depending upon the requirements of your particular experiment:

- **TrueCut™ Cas9 Protein v2** is a recombinant *Streptococcus pyogenes* Cas9 (wt) protein that is the preferred choice for most CRISPR genome editing procedures where the highest level of editing efficiency is required.
- **TrueCut™ HiFi Cas9 Protein** is an engineered high fidelity Cas9 protein which is ideal for experiments that are sensitive to off-target events, while still maintaining a high level of editing efficiency.

Table 1. Contents and storage

Product	Catalog No.	Concentration	Amount	Storage
TrueCut™ Cas9 Protein v2	A36496	1 µg/µL	10 µL (10 µg)	–20°C
	A36497		25 µL (25 µg)	
	A36498	5 µg/µL	20 µL (100 µg)	
	A36499		100 µL (500 µg)	
TrueCut™ HiFi Cas9 Protein	A50574	1 µg/µL	10 µL (10 µg)	
	A50575		25 µL (25 µg)	
	A50576	5 µg/µL	20 µL (100 µg)	
	A50577		100 µL (500 µg)	

Storage and handling

- Store TrueCut™ Cas9 Protein v2 and TrueCut™ HiFi Cas9 Protein at –20°C until required for use.
- Maintain RNAse-free conditions by using RNAse-free reagents, tubes, and barrier pipette tips while setting up your experiments.

Materials required but not provided

- TrueGuide™ Synthetic gRNAs (see **thermofisher.com/trueguide**)
  or
  GeneArt™ Precision gRNA Synthesis Kit (Cat. No. A29377)
- Lipofectamine™ CRISPRMAX™ Cas9 Transfection Kit (Cat. Nos. CMAX00001, CMAX00003, CMAX00008, CMAX00015, CMAX00030) (for most cell lines)
  or
  Neon™ Transfection System (Cat. Nos. MPK5000, MPK1025, MPK1096) (for highest transfection efficiency in challenging cell types including suspension cell lines)
- GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372)
- Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985-062)
- 1X TE buffer, pH 8.0 (Cat. No. AM9849) and nuclease-free water (Cat. No. AM9914G)

Prepare working stock of TrueGuide™ Synthetic gRNA

If TrueGuide™ Synthetic gRNA is being used, resuspend the gRNA (sgRNA, crRNA, or tracrRNA) in
1X TE buffer to prepare 100 µM (100 pmol/µL) stock solutions.

1. Before opening, centrifuge each TrueGuide™ Synthetic gRNA tube at low speed (maximum RCF 4,000 × *g*) to collect the contents at the bottom of the tube, then remove the cap from the tube carefully.
2. Using a pipette and sterile tips, add the required volume of 1X TE buffer to prepare 100 µM (100 pmol/µL) stock solutions.
3. Vortex the tube to resuspend the oligos, briefly centrifuge to collect the contents at the bottom of the tube, then incubate at room temperature for 15–30 minutes to allow the gRNA oligos to dissolve.
4. Vortex the tube again to ensure that all the contents of the tube are resuspended, then briefly centrifuge to collect the contents at the bottom of the tube.
5. (*Optional*) Check the concentration of the resuspended oligos using the NanoDrop™ Spectrophotometer (or equivalent) or a UV-base plate reader.
6. (*Optional*) Aliquot the working stock into one or more tubes for storage.
7. Use working stocks immediately or freeze at –20°C until needed for use.

(*Optional*) Generate gRNA by *in vitro* transcription

If using *in vitro* transcribed gRNA with TrueCut™ Cas9 Protein v2 or TrueCut™ HiFi Cas9 Protein in CRISPR-Cas9-mediated genome editing, the GeneArt™ Precision gRNA Synthesis Kit is recommended for preparation of the gRNA. For detailed instructions on how to generate full length gRNA, see the *GeneArt™ Precision gRNA Synthesis Kit User Guide* (Pub. No. MAN0014538), at **thermofisher.com**.

Transfection guidelines

General CRISPR/gRNA transfection guidelines

- The efficiency with which mammalian cells are transfected with gRNA varies according to cell type and the transfection reagent used. See Table 2 (page 3) for delivery reagent recommendations.
- For gene editing (including gene knockout) editing efficiency is highest with a 1:1 molar ratio of gRNA to TrueCut™ Cas9 Protein v2 or TrueCut™ HiFi Cas9 Protein. In some cell types such as iPSC and THP1, we have used up to 2 µg TrueCut™ Cas9 Protein v2 and 400 ng gRNA per well in 24-well format.
- For HDR knock-in editing, a 1.5:1 molar ratio of donor ssODN to gRNA or TrueCut™ Cas9 Protein v2 or TrueCut™ HiFi Cas9 Protein is recommend for highest knock-in efficiency. The donor can be added directly to RNPs (a premixed gRNA-Cas9 protein). If using a dsDNA donor, further optimization may be necessary to determine the appropriate donor amount, since the toxicity level is dependent on the length and format of the donor DNA and cell type.
- The optimal cell density for transfection varies depending on cell size and growth characteristics. In general, use cells at 30–70% confluence on the day of transfection with lipid-mediated delivery, or 70–90% confluence for electroporation using the Neon™ Transfection System.
- After the optimal cell number and dosage of Cas9/gRNA and/or donor that provides maximal gene editing efficiency is determined for a given cell type, do not vary conditions across experiments to ensure consistency.

  For an overview of the factors that influence transfection efficiency, see the "Transfection Basics" chapter of the *Gibco™ Cell Culture Basic Handbook*, available at **thermofisher.com/cellculturebasics**.
- Use the TrueGuide™ Positive Controls (human AVVS1, CDK4, HPRT1, or mouse Rosa 26) and negative control gRNA (non-coding) to determine gRNA amount and transfection conditions that give the optimal gene editing efficiency with highest cell viability. The TrueGuide™ Positive and Negative sgRNA and crRNA Controls are available separately from Thermo Fisher Scientific. For more information, refer to **thermofisher.com/trueguide**.
- The cell number and other recommendations provided in the following procedures are starting point guidelines based on the cell types we have tested. For multiple wells, prepare a master mix of components to minimize pipetting error, then dispense the appropriate volumes into each reaction well. When making a master mix for replicate wells, we recommend preparing extra volume to account for any pipetting variations.

Recommended delivery options

- Choosing the right delivery reagent is critical for transfection and gene editing efficiency. See our recommendations in Table 2. For more information on transfection reagents, see **thermofisher.com/transfection**.
- For cell line specific transfection conditions using the Lipofectamine™ CRISPRMAX™ Transfection Reagent or the Neon™ Transfection System, see the Appendix (page 13).
- For best results, perform electroporation and transfection of cells using both TrueCut™ Cas9 Proteins and TrueGuide™ Synthetic gRNA.

Table 2. Recommended delivery options for TrueCut™ Cas9 Protein v2 and TrueCut™ HiFi Cas9 Protein.

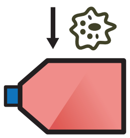
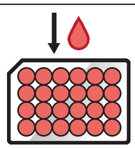
Cas9 format	Electroporation*	Transfection reagent
TrueCut™ Cas9 Protein v2 + gRNA	For maximum efficiency in difficult-to-transfect cell types or for HDR knock-in editing, use the Neon™ Transfection System.	For transfection, including large scale editing (i.e., in 96-well or 384-well plates), use the Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent.
TrueCut™ HiFi Cas9 Protein + gRNA		

* Use the Neon™ Transfection System 10 µL Kit (Cat. No. MPK1025 or MPK1096).

Transfect cells with TrueCut™ Cas9 Protein v2 or TrueCut™ HiFi Cas9 Protein and gRNA using the Neon™ Transfection System

The following protocol is provided as a starting point for transfecting cells with TrueCut™ Cas9 Protein v2 or TrueCut™ HiFi Cas9 Protein and gRNA using the Neon™ Transfection System. For cell specific transfection conditions using TrueCut™ Cas9 Protein v2 or TrueCut™ HiFi Cas9 Protein and TrueGuide™ Synthetic gRNA, see Appendix A (page 13).

IMPORTANT! The following recommendations are for a single well in 24-well format using the 10 µL Neon™ tip. For multiple wells, prepare a master mix of components with extra volume to minimize pipetting errors, then dispense the appropriate volumes into each well. Avoid creating bubbles while mixing and dispensing. For details on optimizing the Neon™ electroporation conditions and scaling down/up for different plate formats, refer to the *Neon™ Transfection System User Guide* (Pub. No. MAN0001557), available for download at **thermofisher.com**.

Steps			Action	Procedure Details
Before starting	1		Prepare cells	**Adherent cells** 1–2 days before transfection, transfer your adherent cells to a new flask with fresh growth medium so that they are 70–90% confluent on the day of transfection. **Primary T cells** Prepare the required number of T cells [1] 3 days before performing electroporation using the appropriate protocol [2]. [1} For primary T cells and iPSCs, this is typically double the amount used for adherent cells (e.g., 200,000 cells/well of a 24-well plate). [2} Typically, T cells are enriched from PBMCs cultured with IL-2 and antibody conjugated magnetic beads (e.g., Dynabeads™ Human T-Expander CD3/CD28, Cat. No. 11141D). For optimal editing efficiency it is recommended to culture T cells under these conditions for 3 days before performing electroporation using the Neon™ Transfection System.
Day 1	2		Prepare 24-well plate with media	Add 500 µL of cell type-specific growth medium into each well of the 24-well plate and place it in the 37°C incubator to pre-warm.

Transfect cells with TrueCut™ Cas9 Protein v2 or TrueCut™ HiFi Cas9 Protein and gRNA using the Neon™ Transfection System, continued

Steps	Action	Procedure Details

Day 1

3 

Prepare TrueCut™ Cas9 Protein + gRNA in Resuspension Buffer (Buffer R or Buffer T)

a. Mix the TrueCut™ Cas9 Protein, gRNA, and Resuspension Buffer R or T (depending on cell type) in a fresh, RNAse-free microcentrifuge tube according to the appropriate table below. Mix well.

Note: Always prepare reaction mixtures with reagent volumes for 1–2 extra reactions so there is sufficient volume to completely fill the Neon™ tip without bubbles, e.g. prepare 3-4 reactions if you do 2 reactions per sample.

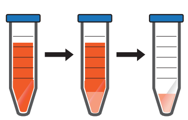
IMPORTANT! Maintain TrueCut™ Cas9 Protein:gRNA at a 1:1 molar ratio. Use high concentration TrueCut™ Cas9 Protein and ensure that the total volume of the RNP complex (TrueCut™ Cas9 Protein + gRNA) does not exceed 1/10th of the total reaction volume (e.g., 1 µL of Cas9 protein + gRNA in 10 µL total reaction volume).

For TrueGuide™ gRNA, use the following table:

Reagent	For TrueGuide™ gRNA	For *in vitro* transcribed gRNA (IVT gRNA)
	Amount per well of 24-well plate	Amount per well of 24-well plate
TrueCut™ Cas9 Protein v2 or TrueCut™ Hi Fi Cas9 Protein	1250 ng (7.5 pmol)	1000 ng (6.1 pmol)
TrueGuide™ gRNA	240 ng (7.5 pmol)	—
IVT gRNA	—	200 ng (6.1 pmol)
Resuspension Buffer R or T	to 5 µL	to 5 µL
ssOND Donor (75–100 bp)*	11.25 pmol	9.15 pmol
Long dsDNA donor*	Varies**	Varies**

* ssOND and dsDNA donor are required for knock-in editing.
** Optimization is required because the amount depends upon the size of the dsDNA.

b. Incubate the TrueCut™ Cas9 Protein + gRNA in Resuspension Buffer R or T (and donor for HDR knock-in editing) at room temperature for 5–20 minutes.

4 

Prepare cells in Resuspension Buffer (Buffer R or Buffer T)

Note: Prepare extra amount (2X) of cells needed to account for variability in pipetting and cell counting.

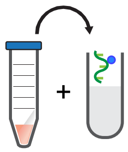
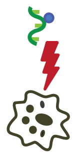
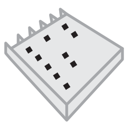
a. If you are using suspension cells, remove an aliquot and determine viable cell count. If you are using adherent cells, detach the cells from the culture flask using Gibco™ TrypLE™ Dissociation Reagent, resuspend the cells in an appropriate volume of growth medium, then determine viable cell count.

b. Transfer the appropriate amount of cells into a 15-mL centrifuge tube, then pellet the cells by centrifugation at 100–400 × *g* for 5 minutes at room temperature.

Note: Optimal cell number used for electroporation varies depending on the cell type. For example, with iPSC and T cells, best results were obtained with 100,000 and 200,000 cells per electroporation respectively.

c. Wash the cells with PBS without Ca^{2+} or Mg^{2+} using the same volume as original cell volume, then pellet the cells by centrifugation at 100–400 × *g* for 5 minutes at room temperature.

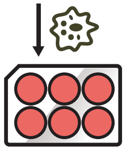
d. Aspirate the PBS and resuspend the cell pellet in Resuspension Buffer R or T (depending on cell type) at the desired concentration. For example, to use 100,000 cells/reaction, resuspend the cells at 2.0×10^7 cells/mL, then use 5 µL of the resuspended cells per reaction. Gently pipette the cells to obtain a single cell suspension.

	Steps		Action	Procedure Details
	5		Add cells to TrueCut™ Cas9 Protein + gRNA in Resuspension Buffer	**a.** Pipette the cells in Resuspension Buffer (from Step 4) up and down to resuspend any cells that might have settled at the bottom of the tube. **b.** Add 5 µL of the cell suspension to TrueCut™ Cas9 Protein + gRNA in Resuspension Buffer from Step 3. **Note**: If preparing a 3X reaction to perform a 2X Neon electroporation, the total volume should be 30 µL.
	6		Electroporate using the cell type-specific Neon™ condition	**a.** Using the 10 µL Neon™ tip, aspirate 10 µL of the cell + TrueCut™ Cas9 Protein + gRNA mix in Resuspension Buffer, then electroporate using your cell type-specific Neon™ condition (see Appendix A, page 13). **IMPORTANT!** Avoid creating bubbles that can hinder electroporation. **b.** After electroporation, transfer the contents of the Neon™ tip immediately into one well of the 24-well culture plate containing 500 µL of pre-warmed growth medium (from Step 2).
Days 3–4	7		Verify editing efficiency and proceed to downstream applications	**a.** After incubation, remove the culture medium and rinse cells with 50–500 µL of PBS. **b.** Use a portion of the cells to perform the genomic cleavage detection assay. **Note:** We recommend using the GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372) or NGS-based targeted amplicon-sequencing to verify gene editing efficiency in cells transfected with the TrueGuide™ Positive Controls (human AVVS1, CDK4, HPRT1, or mouse Rosa 26). See page 9 for more details on downstream analysis options.

Transfect cells with TrueCut™ Cas9 Protein v2 or TrueCut™ HiFi Cas9 Protein and gRNA using Lipofectamine™ CRISPRMAX™ Transfection Reagent

The following protocol is provided as a starting point for transfecting cells with TrueCut™ Cas9 Protein v2 or TrueCut™ HiFi Cas9 Protein and gRNA using the Lipofectamine™ CRISPRMAX™ Transfection Reagent. For cell specific transfection conditions using TrueCut™ Cas9 Protein v2 or TrueCut™ HiFi Cas9 Protein and TrueGuide™ Synthetic gRNA, see Appendix B (page 14).

IMPORTANT! Add reagents in the order indicated. Prepare TrueCut™ Cas9 Protein/gRNA/Cas9 Plus™ reagent solution (Tube 1) before diluting the Lipofectamine™ CRISPRMAX™ Reagent (Tube 2).

	Steps		Action	Procedure Details
Day 0	1		Seed cells	See below
Day 1	2	1 	Prepare Tube 1: TrueCut™ Cas9 Protein + gRNA solution with Cas9 Plus™ Reagent in Opti-MEM™ I Medium	See below

Day 0 – Step 1: Seed cells

The day before transfection, seed your adherent cells according to the following guidelines so that they are 30–70% confluent on the day of transfection.

	96-well	24-well	6-well
Cell density per well	8,000–18,000 cells	40,000–90,000 cells	250,000–450,000 cells
Final volume of media per well	100 µL	0.5 mL	2 mL

Day 1 – Step 2: Prepare Tube 1: TrueCut™ Cas9 Protein + gRNA solution with Cas9 Plus™ Reagent in Opti-MEM™ I Medium

IMPORTANT! Always prepare the TrueCut™ Cas9 Protein/gRNA/Cas9 Plus™ reagent solution (Tube 1) before diluting the Lipofectamine™ CRISPRMAX™ Reagent (Tube 2).

Mix the TrueCut™ Cas9 Protein, gRNA, Lipofectamine™ Cas9 Plus™ Reagent, and Opti-MEM™ I Reduced Serum Medium in a fresh, RNAse-free microcentrifuge tube according to the appropriate table below. Mix well.

IMPORTANT! Add the Lipofectamine™ Cas9 Plus™ Reagent last.

For TrueGuide™ gRNA, use the following table:

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
TrueCut™ Cas9 Protein v2 or TrueCut™ Hi Fi Cas9 Protein	250 ng (1.5 pmol)	1250 ng (7.5 pmol)	6250 ng (37.5 pmol)
TrueGuide™ gRNA	50 ng (1.5 pmol)	240 ng (7.5 pmol)	1200 ng (37.5 pmol)
Lipofectamine™ Cas9 Plus™ Reagent	0.5 µL	2.5 µL	12.5 µL
(*Optional*) ssOND Donor (75–100 bp)*	2.25 pmol	11.25 pmol	56.25 pmol
(*Optional*) Long dsDNA donor*	Varies**		

For *in vitro* transcribed gRNA (IVT gRNA), use the following table:

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
TrueCut™ Cas9 Protein v2 or TrueCut™ Hi Fi Cas9 Protein	200 ng (1.2 pmol)	1000 ng (6.1 pmol)	5000 ng (31 pmol)
IVT gRNA	40 ng (1.2 pmol)	200 ng (6.1 pmol)	1000 ng (31 pmol)
Lipofectamine™ Cas9 Plus™ Reagent	0.5 µL	2.5 µL	12.5 µL
(*Optional*) ssOND Donor (75–100 bp)*	2.25 pmol	11.25 pmol	56.25 pmol
(*Optional*) Long dsDNA donor*	Varies**		

* ssOND and dsDNA donor are required for knock-in editing. If high efficiency knock-in editing is necessary, use the Neon™ Transfection System.

** Optimization is required because the amount depends upon the size of the dsDNA.

<table>
<tr><th colspan="2">Steps</th><th>Action</th><th>Procedure Details</th></tr>
<tr><td rowspan="5">Day 1</td><td>3</td><td>Prepare Tube 2: Dilute Lipofectamine™ CRISPRMAX™ reagent in Opti-MEM™ I Medium</td><td>Dilute the Lipofectamine™ CRISPRMAX™ Transfection Reagent in Opti-MEM™ I Reduced Serum Medium in a fresh, RNAse-free microcentrifuge tube according to the following table. Mix well.
<table>
<tr><th>Reagent</th><th>96-well</th><th>24-well</th><th>6-well</th></tr>
<tr><td>Opti-MEM™ I Medium</td><td>5 µL</td><td>25 µL</td><td>125 µL</td></tr>
<tr><td>Lipofectamine™ CRISPRMAX™ Reagent</td><td>0.3 µL</td><td>1.5 µL</td><td>7.5 µL</td></tr>
</table></td></tr>
<tr><td>4</td><td>Incubate Tube 2 for 1 minute at room temperature</td><td>Incubate the Lipofectamine™ CRISPRMAX™ Reagent diluted in Opti-MEM™ I Medium (Tube 2) at room temperature for 1 minute. Do not leave Tube 2 at room temperature for longer than 5 minutes.
Note: You can incubate the gRNA/Opti-MEM™ I solution at room temperature for longer than 1 minute. We have observed no change in transfection efficiency when Tube 1 was left at room temperature for up to 30 minutes.</td></tr>
<tr><td>5</td><td>Mix Tube 1 + Tube 2</td><td>Add the diluted Lipofectamine™ CRISPRMAX™ Reagent (Tube 2) to the gRNA/Opti-MEM™ I solution (Tube 1) and mix well by pipetting.
Note: For high-throughput setup (e.g., 96-well format or others), always add the contents of Tube 2 into Tube 1 because you can prepare Tube 2 as a bulkier master mix.</td></tr>
<tr><td>6</td><td>Incubate for 10–15 minutes at room temperature</td><td>Incubate the Tube 1 + Tube 2 mixture (i.e., transfection complex) for 10–15 minutes at room temperature.</td></tr>
<tr><td>7</td><td>Add the transfection complex to cells and incubate at 37°C</td><td>a. Add the transfection complex (from Step 6) to your adherent cells at 30–70% confluence according to the following table.
<table>
<tr><th>Reagent</th><th>96-well</th><th>24-well</th><th>6-well</th></tr>
<tr><td>CRISPR-Cas9/gRNA/transfection reagent complex</td><td>10 µL</td><td>50 µL</td><td>250 µL</td></tr>
</table>
b. Incubate the cells at 37°C for 2 days.</td></tr>
<tr><td>Days 3–4</td><td>8</td><td>Verify editing efficiency and proceed to downstream applications</td><td>a. After incubation, remove the culture medium and rinse cells with 50–500 µL of PBS.
b. Use a portion of the cells to perform the genomic cleavage detection assay.
Note: We recommend using the GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372) or NGS-based targeted amplicon-sequencing to verify gene editing efficiency in cells transfected with the TrueGuide™ Positive Controls (human AVVS1, CDK4, HPRT1, or mouse Rosa 26). See page 9 for more details on downstream analysis options.</td></tr>
</table>

Guidelines for verification of editing efficiency

Verification of gene editing efficiency

- Before proceeding with downstream applications, verify the gene editing efficiency of the control target and select the condition that shows the highest level of editing efficiency for future screening experiments.
- To estimate the CRISPR-Cas9-mediated editing efficiency in a pooled cell population, use the GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372), or perform Ion Torrent™ next generation sequencing or a Sanger sequencing-based analysis.
- While the genomic cleavage detection (GCD) assay provides a rapid method for evaluating the efficiency of indel formation following an editing experiment, next generation sequencing (NGS) of the amplicons from the edited population or Sanger sequencing of amplicons cloned into plasmids give a more accurate estimate of the percent editing efficiency and indel types for knockout and HDR knock-in editing.

GeneArt™ Genomic Cleavage Detection (GCD) Assay

- After transfections, use the GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372) to estimate the CRISPR-Cas9-mediated cleavage efficiency in a pooled cell population.
- You can design and order target-specific primer sets for the GCD assay through our TrueDesign Genome Editor, available at **thermofisher.com/crisprdesign**.
- To perform the GCD assay for the positive control, you need the primers listed in Table 3. We recommend using Invitrogen™ Custom DNA Value or Standard Oligos, available from **thermofisher.com/oligos**, for target specific primer sets needed for the GCD assay.
- You can set up the GCD assay in a 96-well plate format and analyze multiple gRNA-treated samples in parallel on a 2% E-Gel™ 48 agarose gel (48-well).
- For more information and detailed protocols, see the *GeneArt™ Genomic Cleavage Detection Kit User Guide* (Pub. No. MAN0009849), available for download at **thermofisher.com/GCDManual**.

Table 3. Target sequences for the positive and negative control (non-targeting) TrueGuide™ Synthetic gRNA sequences.

TrueGuide™ Synthetic Guide RNA Controls*		Primers for the GeneArt™ Cleavage Detection (GCD) Assay	
Locus	Target-specific crRNA sequence	Forward GCD primer	Reverse GCD primer
Human AAVS1**	5′-GCCAGUAGCCAGCCCCGUCC-3′	5′-GAATATGTCCCAGATAGCAC-3′	5′-GTTCTCAGTGGCCACCCTGC-3′
Human HPRT (In)**	5′-GCAUUUCUCAGUCCUAAACA-3′	5′-ACATCAGCAGCTGTTCTG-3′	5′-GGCTGAAAGGAGAGAACT-3′
Human CDK4†	5′-CACUCUUGAGGGCCACAAAG-3′	5′-GCACAGACGTCCATCAGCC-3′	5′-GCCGGCCCCAAGGAAGACTGGGAG-3′
Mouse Rosa 26**	5′-CUCCAGUCUUUCUAGAAGAU-3′	5′-AAGGAGCGAGGGCTCAGTTGG-3′	5′-GGTGAGCATGTCTTTAATCTACCTCG-3′
Negative control (non-targeting)	5′-AAAUGUGAGAUCAGAGUAAU-3′	N/A	N/A

*Available in TrueGuide™ Synthetic sgRNA format (see **thermofisher.com/trueguide**).

**Specific to an intron. †Targets 5' exons.

Sequence analysis

- For next generation sequencing (NGS) based editing efficiency analysis, you can specifically amplify the edited region and barcode amplicons by pooling all amplicons in a single tube and performing sequencing using various NGS platforms such as the Ion Torrent™ Targeted Amplicon-seq Validation (TAV). For more information on NGS analysis, refer to Ion Torrent™ targeted sequencing solutions at **thermofisher.com/ionapliseqsolutions**.
- For Sanger sequencing-based editing efficiency analysis, refer to our application note referenced at **thermofisher.com/sangercrispr**.
- Use the SeqScreener Gene Edit Confirmation App on Thermo Fisher™ Connect to determine the spectrum and frequency of targeted mutations (see Pub. No. MAN0019454 at **thermofisher.com**. for details).

Guidelines for clone isolation and validation

After you have determined the cleavage efficiency of the pooled cell population, isolate single cell clones for further validation and banking. You can isolate single cell clones from the selected pool using limiting dilution cloning (LDC) in 96-well plates or by single cell sorting using a flow cytometer.

Limiting dilution cloning (LDC)

- Based on the editing efficiency and estimated cell viability, you can estimate the number of single clones needed to obtain a desired knock-out (KO) clonal cell line.

  For example, if you desire a homozygous KO with mutations in both copies of a gene and the resulting GeneArt™ cleavage detection efficiency was 50%, then the probability of having both alleles knocked out in any cell is 25% (0.5 × 0.5 = 0.25).

  If the probability of an indel leading to frame shift is 2/3, then the chance of having a homozygous KO is ~11% per cell [(0.5 × 0.5) × (0.66 × 0.66) = 0.11].
- We recommend performing limiting dilution by targeting 0.8 cells/well, which requires you to resuspend the transfected cells (post-counting) at a density of 8 cells/mL in complete growth medium, then transferring 100 µL of this to each well of a 96-well plate.

  If you plate at least ten 96-well plates in this manner and expect only 20% of cells to survive, then the probability of having homozygous KO clones in the 192 surviving cells will be 19–21 cells (192 × 11%).
- Note that single cell clone survivability varies by cell type. Some cells that do not like to remain as single cells need to be plated at a low density to get well separated colonies, which will then have to be manually picked for further screening.

Example LDC procedure using 293FT cells

1. Wash the transfected cells in each well of the 24-well plate with 500 µL of PBS. Carefully aspirate the PBS and discard.
2. Add 500 µL of TrypLE™ cell dissociation reagent to the cells and incubate for 2–5 minutes at 37°C.
3. Add 500 µL of complete growth medium to the cells to neutralize the dissociation reagent. Pipette the cells up and down several times to break up the cell aggregates. Make sure that the cells are well separated and are not clumped together.
4. Centrifuge the cells at 300 × *g* for 5 minutes to pellet.
5. Aspirate the supernatant, resuspend the cells in an appropriate volume of pre-warmed (37°C) growth medium, then perform a cell count.
6. Dilute the cells to a density of 8 cells/mL of complete growth medium. Prepare a total of

50 mL of cell suspension at this cell density and transfer to a sterile reservoir.

Note: You can also perform a serial dilution to get a better estimate of cell density.

7. Using a multichannel pipettor, transfer 100 µL of the cell suspension into each well of 96-well tissue culture plates until the desired number of plates is seeded. Make sure to mix the cells in between seeding the plates to avoid the formation of cell aggregates.

   Note: In general, we seed ten 96-well plates to achieve a large number of clones. Number of plates to seed depends on the editing efficiency of pooled cell population and viability of cells post single cell isolation.

8. Incubate the plates in a 37°C, 5% CO_2 incubator.
9. Scan the plates for single cell colonies as soon as small aggregates of cells are visible under a 4X microscope (usually after first week, depending on the growth rate of the cell line).
10. Continue incubating the plates for an additional 2–3 weeks to expand the clonal populations for further analysis and characterization.

Example single cell sorting procedure in a 96-well plate using flow cytometer

Single cells can be sorted into a 96-well plate format using a flow cytometer with single cell sorting capability. After sorting and expanding the single cell clones, analyze and characterize the clonal populations using suitable assays.

1. Wash the transfected 293FT cells in each well of the 24-well plate with 500 µL of PBS. Carefully aspirate the PBS and discard.
2. Add 500 µL of TrypLE™ cell dissociation reagent and incubate for 2–5 minutes at 37°C.
3. Add 500 µL of complete growth medium to the cells to neutralize the dissociation reagent. Pipette the cells up and down several times to break up the cell aggregates. Make sure that the cells are well separated and are not clumped together.
4. Centrifuge the cells at 300 × *g* for 5 minutes to pellet.
5. Aspirate the supernatant, then wash the cell pellet once with 500 µL of PBS.
6. Resuspend 1×10^6 cells in 1 mL of FACS buffer, then add propidium iodide (PI) to the cells at a final concentration of 1 µg/mL. Keep the resuspended cells on ice.
7. Filter the cells using suitable filters before analyzing them on a flow cytometer with single cell sorting capability.
8. Sort PI-negative cells into a 96-well plate containing 100 µL of complete growth medium. If desired, you can use 1X antibiotics with the complete growth medium.
9. Incubate the plates in a 37°C, 5% CO_2 incubator.
10. Scan the plates for single cell colonies as soon as small aggregates of cells are visible under a 4X microscope. Colonies should be large enough to see as soon as 7–14 days (usually after first week, depending on the growth rate of the cell line). You can perform image analysis to ensure that the colonies are derived from single cells.
11. After image analysis, continue incubating the plates for an additional 2–3 weeks to expand the clonal populations for further analysis and characterization.

Characterize edited clones

You can analyze the single cell clones for purity and the desired genotype (homozygous or heterozygous allele) by various molecular biology methods such as genotyping PCR, qPCR, next generation sequencing, or western blotting.

Supporting tools

At Thermo Fisher Scientific, you can find a wide variety of tools to meet your gene editing and validation needs, including Invitrogen™ LentiArray CRISPR and Silencer™ Select RNAi libraries for screening, primers for targeted amplicon sequencing, antibody collection for knock-out validation, and ORF collections and GeneArt™ gene synthesis service for cDNA expression clones that can be used for rescue experiment reagents.

Appendix A: Cell line specific electroporation conditions using the Neon™ Transfection System

The following cell line specific conditions are provided as a starting point for transfecting cells with TrueGuide™ Synthetic gRNA and TrueCut™ Cas9 Proteins using the Neon™ Transfection System 10 µL Kit. Further optimization of the electroporation or nucleofection conditions may be necessary for best results.

Cell type	Source	Media	Number of cells/10-µL reaction (× 10^3)	TrueCut™ Cas9 Protein/gRNA/ssODN (ng/pmoles/pmols)	Neon™ electroporation conditions*
Well format	—	—	**24-well**		
HEK293	Human embryonic kidney	DMEM	150	1250/7.5/11.25	1150 V/20 ms/2 pulses
U2OS	Human osteosarcoma	McCoy5A	150	1250/7.5/11.25	1400 V/15 ms/4 pulses
A549	Human epithelial lung carcinoma	DMEM	120	1250/7.5/11.25	1200 V/20 ms/4 pulses
THP1	Human peripheral blood monocyte leukemia	RPMI	200	2000/12/18	1700 V/20 ms/1 pulse (#5)
K562	Human leukemia bone marrow	RPMI	200	1250/7.5/11.25	1700 V/20 ms/1 pulse (#5)
iPSC	Human induced pluripotent stem cells	Essential 8™	80	1500/10/15	1200 V/20 ms/2 pulses (#14)
iPSC	Human induced pluripotent stem cells	StemFlex™	80	1500/10/15	1200 V/30 ms/1 pulse (#7)
Human primary T-cell	Healthy donor derived	OpTmizer™ + 2% human serum	200	1250/7.5/11.25	1600 V/10 ms/3 pulses (#24)
Jurkat T-cell	Human peripheral blood lymphocyte	RPMI	200	1250/7.5/11.25	1700 V/20 ms/1 pulse (#5)
HepG2	Human hepatocellular carcinoma	DMEM	120	1250/7.5/11.25	1300 V/30 ms/1 pulse (#8)
N2A	Mouse brain neuroblastoma	DMEM	100	1250/7.5/11.25	1400 V/30 ms/1 pulse (#9)

* Recommendations for the Neon™ electroporation settings are based on the culture conditions tested.

Appendix B: Cell line-specific transfection conditions using the Lipofectamine™ CRISPRMAX™ Transfection Reagent

The following cell line-specific conditions are provided as a starting point for transfecting cells with TrueGuide™ Synthetic gRNA and TrueCut™ Cas9 Proteins using the Lipofectamine™ CRISPRMAX™ Transfection Reagent. Further optimization of the transfection conditions may be necessary for best results.

Cell type	Source	Media	Cell seeding density/well (× 10^3) one day before transfection			TrueCut™ Cas9 Protein/gRNA/ssODN (ng/pmoles/pmols)			Lipofectamine™ Cas9 Plus™ Reagent/well (µL)			Lipofectamine™ CRISPRMAX™ Reagent/well (µL)		
Well format	—	—	96-well	24-well	6-well	96-well	24-well	6-well	96-well	24-well	6-well	96-well	24-well	6-well
HEK293	Human embryonic kidney	DMEM	18	90	450	250/1.5/2.25	1250/7.5/11.25	6250/37.5/56.25	0.5	2.5	12.5	0.4	2	10
U2OS	Human osteosarcoma	McCoy5A	10	50	250	250/1.5/2.25	1250/7.5/11.25	6250/37.5/56.25	0.5	2.5	12.5	0.3	1.5	7.5
A549	Human epithelial lung carcinoma	DMEM	10	50	250	250/1.5/2.25	1250/7.5/11.25	6250/37.5/56.25	0.5	2.5	12.5	0.3	1.5	7.5
THP1	Human peripheral blood monocyte leukemia	RPMI	10	50	250	400/2.4/3.6	2000/12/18	10000/60/90	0.8	4	20	0.3	1.5	7.5
K562*	Human leukemia bone marrow	RPMI	10	50	250	250/1.5/2.25	1250/7.5/11.25	6250/37.5/56.25	0.5	2.5	12.5	0.3	1.5	7.5
iPSC*	Human induced pluripotent stem cells	Essential 8™	8	40	200	300/2/3	1500/10/15	7500/50/75	0.6	3	15	0.3	1.5	7.5
HepG2	Human hepatocellular carcinoma	DMEM	10	50	250	250/1.5/2.25	1250/7.5/11.25	6250/37.5/56.25	0.5	2.5	12.5	0.3	1.5	7.5
MDA-MB231	Human epithelial (breast) adenocarcinoma	DMEM	10	50	250	250/1.5/2.25	1250/7.5/11.25	6250/37.5/56.25	0.5	2.5	12.5	0.3	1.5	7.5
N2A	Mouse brain neuroblastoma	DMEM	10	50	250	250/1.5/2.25	1250/7.5/11.25	6250/37.5/56.25	0.5	2.5	12.5	0.3	1.5	7.5

*Use the Neon™ Transfection System for higher editing efficiency.

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Revision	Date	Description
D.0	26 August 2021	Update of Neon transfection protocol. Addition of donor DNA amounts for knock-in experiments. TrueCut HiFi Cas9 Protein line extension.
C.0	26 January 2018	Correct the target-specific crRNA sequence of the Mouse Rosa26 control.
B.0	27 September 2017	Correct the transfection reagent name in the table in Step 3 on page 5.
A.0	30 August 2017	New user guide.