Invitrogen™ TrueGuide™ Synthetic gRNA

Catalog Nos. A35506, A35507, A35508, A35509, A35510, A35511, A35512, A35513, A35514, A35515, A35516, A35517, A35518, A35519, A35522, A35523, A35524, A35525, A35526, A35533, A35534

Pub. No. MAN0017058 **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™ TrueGuide™ Synthetic gRNAs are ready-to-use, synthetic gRNAs that both maximize the performance of your CRISPR-Cas9 genome editing experiments and simplify the workflow allowing you to focus on building new models and making new discoveries instead of spending time developing editing tools. TrueGuide™ Synthetic gRNAs are available as a 2-piece crRNA:tracrRNA system for standard editing tasks as well as 1-piece sgRNA and modified sgRNA formats to drive maximum editing efficiency when working with difficult-to-edit and valuable cells such as primary cells, immune cells, and stem cells.

The TrueGuide™ Synthetic gRNAs can be delivered into cells stably expressing Cas9 nuclease or co-delivered with Invitrogen™ TrueCut™ Cas9 Protein v2 (Cat. Nos. A36496, A36497, A36498, A36499) using our recommended delivery reagents (Table 3, page 4).

Table 1. TrueGuide™ gRNA formats

gRNA format	Product*	Amount	Storage**
TrueGuide™ Synthetic sgRNA (1-piece)	TrueGuide™ Modified sgRNA†	1.5 or 3 nmol	After resuspension, store at –20°C.
	TrueGuide™ Synthetic sgRNA	3 nmol	
TrueGuide™ Synthetic crRNA:tracrRNA (2-piece)‡	TrueGuide™ Synthetic crRNA	2 nmol	
	TrueGuide™ Synthetic tracrRNA (includes 5X Annealing Buffer)	5 nmol,20 nmol, or 100 nmol	

* Choose from our pre-designed TrueGuide™ Synthetic gRNAs or order custom-designed TrueGuide™ Synthetic gRNAs that specifically target your sequence of interest at **thermofisher.com/trueguide**.

** Dried oligos are stable at room temperature for 6 months. For long term storage of dried oligos and post-resuspension, store at –20°C.

† Chemical modifications include 2' O-Methyl analogs and 3' phosphorothioate linkages to increase editing efficiency and protect against nuclease degradation.

‡ TrueGuide™ Synthetic crRNA:tracrRNA system requires the purchase of separate TrueGuide™ crRNA and TrueGuide™ tracrRNA products, which are annealed to generate a crRNA:tracrRNA duplex prior to delivery into cells.

TrueGuide™ Synthetic gRNA controls

TrueGuide™ Synthetic crRNA and sgRNA Positive and Negative Controls (see Table 2) are used for the optimization of transfection conditions. For the sequences of positive and negative (non-targeting) TrueGuide™ Synthetic gRNA Controls, see Table 4 (page 11).

Table 2. TrueGuide™ sgRNA and crRNA controls

Product	Catalog No.
TrueGuide™ Synthetic sgRNA Controls	
TrueGuide™ Synthetic sgRNA, Positive Control, AAVS1 (Human)	A35522
TrueGuide™ Synthetic sgRNA, Positive Control, CDK4 (Human)	A35523
TrueGuide™ Synthetic sgRNA, Positive Control, HPRT1 (Human)	A35524
TrueGuide™ Synthetic sgRNA, Positive Control, ROSA26 (Mouse)	A35525
TrueGuide™ Synthetic sgRNA, Negative Control, Human and Mouse	A35526
TrueGuide™ Synthetic crRNA Controls*	
TrueGuide™ Synthetic crRNA, Positive Control, AAVS1 (Human)	A35515
TrueGuide™ Synthetic crRNA, Positive Control, CDK4 (Human)	A35516
TrueGuide™ Synthetic crRNA, Positive Control, HPRT1 (Human)	A35517
TrueGuide™ Synthetic crRNA, Positive Control, ROSA26 (Mouse)	A35518
TrueGuide™ Synthetic crRNA, Negative Control, Human and Mouse	A35519

* Before performing transfections, you must anneal the control crRNA with tracrRNA in 1X Annealing Buffer (final concentration) to prepare the control crRNA:tracrRNA duplex. For your convenience, 5X Annealing Buffer is supplied with TrueGuide™ Synthetic tracrRNA.

Materials required but not provided

- 1X TE buffer, pH 8.0 (Cat. No. AM9849) and nuclease-free water (Cat. No. AM9914G) for gRNA resuspension and dilution
- Human or mouse cell line of choice (wild-type or Cas9-stable)
- Invitrogen™ TrueCut™ Cas9 Protein v2 to transfect cell lines that do not express Cas9 nuclease (Cat. Nos. A36496, A36497, A36498, A36499)
- TrueGuide™ Synthetic sgRNA or crRNA Controls (see Table 2)
- Transfection system to deliver TrueGuide™ Synthetic gRNA into your cells of interest (see Table 3, page 4)

Procedural guidelines

Storage and handling

- Upon receipt, resuspend TrueGuide™ Synthetic gRNA in 1X TE to prepare 100 µM (100 pmol/µL) stock solutions. After resuspension, store the gRNA 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.

Optional: Prepare 5X Annealing Buffer

If you are using the crRNA:tracrRNA format and need additional 5X Annealing Buffer for the annealing and subsequent dilution steps, prepare a solution of 30 mM HEPES, 100 mM Potassium Acetate, and 2 mM Magnesium Acetate in nuclease-free water, then adjust to pH 7.4 using 1 M Potassium Hydroxide.

Prepare working stock of TrueGuide™ Synthetic gRNA

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 is 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™ Sprectrophotometer (or equivalent) or a UV-base plate reader.
5. *Optional:* Aliquot the working stock into one or more tubes for storage.
6. Use working stocks immediately or freeze at –20°C until use.

Anneal crRNA and tracrRNA

Before performing transfections, you must anneal the crRNA with tracrRNA in 1X Annealing Buffer (final concentration) to prepare the crRNA:tracrRNA duplex. Skip this step if you are using the sgRNA format.

1. Mix 10 µL each of the TrueGuide™ crRNA and TrueGuide™ tracrRNA (100 µM) in a PCR tube containing 10 µL of 5X Annealing Buffer and 20 µL of nuclease-free water for a final crRNA:tracrRNA duplex concentration of 20 µM.
2. Mix the contents and anneal in thermocycler:

   95°C for 5 minutes
   95°C to 78°C with –2°C/second ramp rate
   78°C for 10 minutes
   78° to 25°C with –0.1°C/second ramp rate
   25°C for 5 minutes

3. Store the annealed product (crRNA:tracrRNA duplex) at –20°C until required for use. **For further dilution of annealed products, use 1X annealing buffer.**

Transfection guidelines

Cell lines

- For best results, we recommend that you co-transfect human or mouse cells with TrueGuide™ Synthetic gRNA and TrueCut™ Cas9 Protein v2 using our recommended delivery reagents (for most cell types) or the Neon™ Transfection System (for difficult-to-transfect cell lines) (see Table 3, page 4).
- If you wish to transfect TrueGuide™ Synthetic gRNA into cells stably expressing the Cas9 nuclease, you can use an existing Cas9-stable cell line or generate one using the Invitrogen™ LentiArray™ Cas9 Lentivirus (Cat. Nos. A32064, A32069). For more information, see the *Invitrogen™ LentiArray™ Cas9 Lentivirus User Guide* (Pub. No. MAN0016088), available for download at **thermofisher.com**.
- You can also use our custom cell line generation service to generate a Cas9-expressing cell line of your choice (email **GEMServices@thermofisher.com** for more information).

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 3 (page 4) for delivery reagent recommendations.
- For gene editing we find highest editing efficiency with 1:1 molar ratio of gRNA to Cas9 protein. In some cell types such as iPSC and THP1, we have used up to 2 µg Cas9 protein and 400 ng gRNA per well in 24-well format.
- The optimal cell density for transfection varies depending on cell size and growth characteristics. In general, we recommend 30–70% confluence on the day of transfection when using lipid-mediated delivery or 70–90% confluence for electroporation using the Neon™ Transfection System.
- After you have determined the optimal cell number and dosage of Cas9/gRNA that provides maximal gene editing efficiency, do not vary these conditions across experiments for a given cell type to ensure consistency.

  For an overview of the factors that influence transfection efficiency, refer to 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 **thermofisher.com** (Table 2, page 2).
- 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 3. 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 14).

Table 3. Recommended delivery options for TrueGuide™ Synthetic gRNA.

Cas9 format*	Transfection reagent	Electroporation**
Cas9 protein + gRNA (RNP format)†	Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent (Cat. No. CMAX00008)	For maximum efficiency in difficult-to-transfect cell types, use the Neon™ Transfection System (Cat. No. MPK5000) with the TrueCut™ Cas9 Protein v2.
Cas9-stable cells + gRNA	Lipofectamine™ RNAiMAX™ Transfection Reagent (Cat. No. 13778075)	
* For best results, we recommend that you transfect your wild-type cells with TrueCut™ Cas9 Protein v2 + TrueGuide™ Synthetic gRNA (crRNA:tracrRNA duplex or sgRNA) using the Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent or the Neon™ Transfection System. If necessary, you can also transfect your cells with GeneArt™ CRISPR Nuclease mRNA (Cat. No. B25641) + TrueGuide™ Synthetic gRNA using the Lipofectamine™ MessengerMAX™ Transfection Reagent (Cat. No. LMRNA008). ** Use the Neon™ Transfection System 10 µL Kit (Cat. No. MPK1025). † RNP: **R**ibo**N**ucle**o**P**r**otein complex.		

Downstream analysis options

- Invitrogen™ GeneART™ Genomic Cleavage Detection Kit (Cat. No. A24372) for gel based quantification (page 11).
- Ion Torrent™ next generation sequencing for quantification and indel or sequence modification information (page 12).
- Clone isolation and functional validation (page 12).

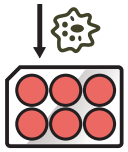
Transfect cells with TrueGuide™ Synthetic gRNA and TrueCut™ Cas9 Protein v2 using Lipofectamine™ CRISPRMAX™ Transfection Reagent

If you are using a cell line that does not stably express Cas9 nuclease, you must deliver the Cas9 nuclease into your target cells along with the TrueGuide™ Synthetic gRNA. For best results, we recommend using the TrueCut™ Cas9 Protein v2 and the Lipofectamine™ CRISPRMAX™ Transfection Reagent. The following protocol is provided as a starting point for transfecting wild-type HEK293 cells with TrueGuide™ Synthetic gRNA and TrueCut™ Cas9 Protein v2 using the Lipofectamine™ CRISPRMAX™ Transfection Reagent. For cell specific transfection conditions, see Appendix A (page 14).

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

Day 0

Step 1 — **Action: Seed cells**

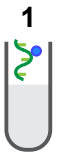


The day before transfection, seed your adherent cells according to the following guidelines so that they are 30–70% confluent on the day of transfection.

	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 — **Action: Prepare Tube 1: Cas9 protein + gRNA solution with Cas9 Plus™ Reagent in Opti-MEM™ I Medium**



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

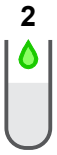
a. If you are using the standard two-piece gRNA format (crRNA:tracrRNA), anneal the TrueGuide™ crRNA and TrueGuide™ tracrRNA in 5X annealing buffer to generate the crRNA:tracrRNA duplex. See page 3 for detailed instructions. For premium one-piece format (sgRNA), skip this step and proceed with Step 2b.

b. Mix the TrueCut™ Cas9 Protein v2, gRNA (crRNA:tracrRNA duplex or sgRNA), Lipofectamine™ Cas9 Plus™ Reagent, and Opti-MEM™ I Reduced Serum Medium in a fresh, RNAse-free microcentrifuge tube according to the following table. Mix well.

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
Cas 9 protein	250 ng (1.5 pmol)	1250 ng (7.5 pmol)	6250 ng (37.5 pmol)
gRNA (crRNA:tracrRNA duplex or sgRNA)*	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

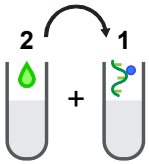
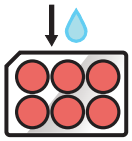
* Add the Lipofectamine™ Cas9 Plus™ Reagent last.

Step 3 — **Action: Prepare Tube 2: Dilute Lipofectamine™ CRISPRMAX™ reagent in Opti-MEM™ I Medium**



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.

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
Lipofectamine™ CRISPRMAX™ Reagent	0.3 µL	1.5 µL	7.5 µL

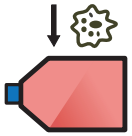
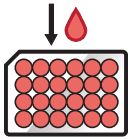
	Steps		Action	Procedure Details
Day 1	4		Incubate Tube 2 for 1 minute at room temperature	Incubate the Lipofectamine™ CRISPRMAX™ Reagent diluted in Opti-MEM™ I Medium (Tube 2) at room temperature for 1 minute. **Do not leave Tube 2 at room temperature for longer than 5 minutes.** **Note:** You can incubate 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.
	5		Mix Tube 1 + Tube 2	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.
	6		Incubate for 10–15 minutes at room temperature	Incubate the Tube 1 + Tube 2 mixture (i.e., transfection complex) for 10–15 minutes at room temperature.
	7		Add the transfection complex to cells and incubate at 37°C	**a.** Add the transfection complex (from Step 6) to your adherent cells at 30–70% confluence according to the following table. **b.** Incubate the cells at 37°C for 2 days.
Days 3–4	8		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. Pick the clones that show the highest cleavage efficiency to use in your downstream experiments. **Note:** We recommend using the GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372) to verify gene editing efficiency in cells transfected with the TrueGuide™ Positive Controls (human AVVS1, CDK4, HPRT1, or mouse Rosa 26) (see page 11).

Reagent	96-well	24-well	6-well
Cas9 protein/gRNA/transfection reagent complex	10 µL	50 µL	250 µL

Transfect cells with TrueGuide™ Synthetic gRNA and TrueCut™ Cas9 Protein v2 using the Neon™ Transfection System

If you are using a cell line that does not stably express Cas9 nuclease, you must deliver the Cas9 nuclease into your target cells along with the TrueGuide™ Synthetic gRNA. For best results, we recommend using the TrueCut™ Cas9 Protein v2. The following protocol is provided as a starting point for transfecting wild-type cells with TrueGuide™ Synthetic gRNA and TrueCut™ Cas9 Protein v2 using the Neon™ Transfection System. For cell specific transfection conditions, see Appendix B (page 15).

IMPORTANT! The following recommendations are for a single well in 24-well format using the 10 µL Neon™ tip. Avoid creating bubbles while electroportion. For details on optimizing the Neon™ electroporation protocol 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**.

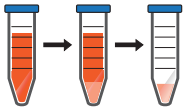
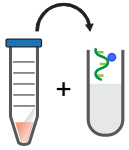
	Steps	Action	Procedure Details
Day 0	1 	Seed cells	1–2 days before transfection, transfer your adherent cells to a new flask with fresh growth medium so that they are 70–90% confluent on the day of transfection.
Day 1	2 	Prepare 24-well plate with media	Add 1000 µL of cell type-specific growth medium into each well of the 24-well plate and place it in the 37°C incubator to pre-warm.
	3 	Prepare Cas9 protein + gRNA in Resuspension Buffer R	**a.** If you are using the standard two-piece gRNA format (crRNA:tracrRNA), anneal the TrueGuide™ crRNA and TrueGuide™ tracrRNA in 5X annealing buffer to generate the crRNA:tracrRNA duplex. See page 3 for detailed instructions. For premium one-piece format (sgRNA), skip this step and proceed with Step 3b. **b.** Mix the TrueCut™ Cas9 Protein v2, gRNA (crRNA:tracrRNA duplex or sgRNA), and Resuspension Buffer R in a fresh, RNAse-free microcentrifuge tube according to the following table. Mix well. **Note:** The amounts provided in the table are for one well of a 24-well plate. 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. **IMPORTANT!** Maintain Cas9 protein:gRNA at a 1:1 molar ratio.

Reagent	Amount per well of 24-well plate
Cas 9 protein*	1250 ng (7.5 pmol)
gRNA (crRNA:tracrRNA duplex or sgRNA)*	240 ng (7.5 pmol)
Resuspension Buffer R†	5.0 µL

† Use high concentration Cas9 protein and ensure that the total volume of the RNP complex (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).

c. Incubate the Cas9 protein + gRNA in Resuspension Buffer R at room temperature for 5–20 minutes.

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

	Steps	Action	Procedure Details
Day 1	4 	Prepare cells in Resuspension Buffer R	**Note:** Prepare extra amount (2X) of cells needed. **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 centrifue 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, in case of iPSC and THP1, we had best results with 80,000 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 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.
	5 	Add cells to Cas9 nuclease + gRNA in Resuspension Buffer R	**a.** Pipette the cells in Resuspension Buffer R (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 the mixture of Cas9 nuclease + gRNA in Resuspension Buffer R from Step 4.
	6 	Electroporate using the cell type-specific Neon™ condition	**a.** Using the 10 µL Neon™ tip, aspirate 10 µL of the cell + Cas9 nuclease + gRNA mix in Resuspension Buffer R, then electroporate using your cell type-specific Neon™ condition (see Appendix B, page 15). **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 1000 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. Pick the clones that show the highest cleavage efficiency to use in your downstream experiments. **Note:** We recommend using the GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372) to verify gene editing efficiency in cells transfected with the TrueGuide™ Positive Controls (human AVVS1, CDK4, HPRT1, or mouse Rosa 26) (see page 11).

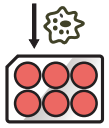
Transfect Cas9-stable cells with TrueGuide™ Synthetic gRNA using Lipofectamine™ RNAiMAX™ Transfection Reagent

The following protocol is provided as a starting point for transfecting U2 OS-Cas9 stable cell line with TrueGuide™ Synthetic gRNA using the Lipofectamine™ RNAiMAX Transfection Reagent. Further optimization of the transfection conditions may be required to transfect other cell lines.

IMPORTANT! Add the reagents in the order indicated. The cell number and other recommendations provided here are starting point guidelines based on the cell types we have tested.

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

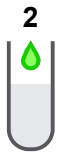
Step 2 — Prepare Tube 1: gRNA + Opti-MEM™ I Medium

a. If you are using the standard two-piece gRNA format (crRNA:tracrRNA), anneal the TrueGuide™ crRNA and TrueGuide™ tracrRNA in 5X annealing buffer to generate the crRNA:tracrRNA duplex. See page 3 for detailed instructions. For premium one-piece format (sgRNA), skip this step and proceed with Step 2b.

b. Mix the gRNA (crRNA:tracrRNA duplex or sgRNA) and Opti-MEM™ I Reduced Serum Medium in a fresh, RNAse-free microcentrifuge tube according to the following table. Mix well.

Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
gRNA (crRNA:tracrRNA duplex or sgRNA)	50 ng (1.5 pmol)	240 ng (7.5 pmol)	1200 ng (37.5 pmol)

Step 3 — Prepare Tube 2: Dilute Lipofectamine™ RNAiMAX™ reagent in Opti-MEM™ I Medium



Dilute the Lipofectamine™ RNAiMAX™ Transfection Reagent in Opti-MEM™ I Reduced Serum Medium in a fresh, RNAse-free microcentrifuge tube according to the following table. Mix well.

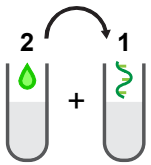
Reagent	96-well	24-well	6-well
Opti-MEM™ I Medium	5 µL	25 µL	125 µL
Lipofectamine™ RNAiMAX™ Reagent	0.3 µL	1.5 µL	7.5 µL

Step 4 — Incubate for 1–5 minutes at room temperature

Incubate the diluted Lipofectamine™ RNAiMAX™ Reagent 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.**

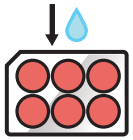
Note: You can incubate the gRNA/Opti-MEM™ I solution at room temperature for longer than 1 minute (we have noticed no change in transfection efficiency when Tube 1 was left at room temperature for up to 30 minutes).

Step 5 — Mix Tube 1 + Tube 2



Add the diluted Lipofectamine™ RNAiMAX™ 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.

<table>
<tr><th colspan="2">Steps</th><th>Action</th><th>Procedure Details</th></tr>
<tr><td rowspan="2">Day 1</td><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>Cas9 nuclease/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. Pick the clones that show the highest cleavage efficiency to use in your downstream experiments.
Note: We recommend using the GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372) to verify gene editing efficiency in cells transfected with the TrueGuide™ Positive Controls (human AVVS1, CDK4, HPRT1, or mouse Rosa 26) (see page 11).</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 in 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.

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 GeneArt™ CRISPR Search and Design tool, available at **thermofisher.com/crisprdesign**.
- To perform the GCD assay for the positive control, you need the primers listed in Table 4 . 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).
- Pick the clones that show the highest cleavage efficiency to use in your experiments. Note that the clone that shows the highest cleavage efficiency may not always be the clone with the highest expression.
- 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 4. 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 (ln)**	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 and crRNA formats; see Table 2, page 2. **Specific to an intron. †Targets 5' exons.

Sequence analysis

- For Sanger sequencing-based editing efficiency analysis, refer to our application note referenced at **thermofisher.com/sangercrispr**.
- If you are experienced in next generation sequencing (NGS) and analysis, you can use barcoded target-specific amplicon primers and perform multiplex analysis using several gRNA-treated samples in parallel. Multiplex analysis using NGS is especially useful when using the custom arrayed plate format for TrueGuide™ synthetic gRNA transfections. For more information on NGS analysis, refer to Ion Torrent™ targeted sequencing solutions at **thermofisher.com/ionapliseqsolutions**.

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. After counting, 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

You can sort single cells per well 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. The following is an example single-cell sorting procedure with 293FT cells.

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 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, 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 transfection conditions using the Lipofectamine™ CRISPRMAX™ Transfection Reagent

The following cell line-specific conditions are provided as a starting point for transfecting wild-type cells with TrueGuide™ Synthetic gRNA and TrueCut™ Cas9 Protein v2 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			Cas9 protein/gRNA (ng/pmoles)			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	1250/7.5	6250/37.5	0.5	2.5	12.5	0.4	2	10
U2OS	Human osteosarcoma	McCoy5A	10	50	250	250/1.5	1250/7.5	6250/37.5	0.5	2.5	12.5	0.3	1.5	7.5
A549	Human epithelial lung carcinoma	DMEM	10	50	250	250/1.5	1250/7.5	6250/37.5	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	2000/12	10000/60	0.8	4	20	0.3	1.5	7.5
K562*	Human leukemia bone marrow	RPMI	10	50	250	250/1.5	1250/7.5	6250/37.5	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	1500/10	7500/50	0.6	3	15	0.3	1.5	7.5
HepG2	Human hepatocellular carcinoma	DMEM	10	50	250	250/1.5	1250/7.5	6250/37.5	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	1250/7.5	6250/37.5	0.5	2.5	12.5	0.3	1.5	7.5
N2A	Mouse brain neuroblastoma	DMEM	10	50	250	250/1.5	1250/7.5	6250/37.5	0.5	2.5	12.5	0.3	1.5	7.5

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

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

The following cell line specific conditions are provided as a starting point for transfecting wild-type cells with TrueGuide™ Synthetic gRNA and TrueCut™ Cas9 Protein v2 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)	Cas9 protein/gRNA (ng/pmoles)	Neon™ electroporation conditions*
Well format	—	—	**24-well**		
HEK293	Human embryonic kidney	DMEM	150	1250/7.5	1150 V/20 ms/2 pulses
U2OS	Human osteosarcoma	McCoy5A	150	1250/7.5	1400 V/15 ms/4 pulses
A549	Human epithelial lung carcinoma	DMEM	120	1250/7.5	1200 V/20 ms/4 pulses
THP1	Human peripheral blood monocyte leukemia	RPMI	200	2000/12	1700 V/20 ms/1 pulse (#5)
K562	Human leukemia bone marrow	RPMI	200	1250/7.5	1700 V/20 ms/1 pulse (#5)
iPSC	Human induced pluripotent stem cells	Essential 8™	80	1500/10	1200 V/20 ms/2 pulses (#14)
iPSC	Human induced pluripotent stem cells	StemFlex™	80	1500/10	1200 V/30 ms/1 pulse (#7)
Human primary T-cell	Healthy donor derived	OpTmizer™ + 2% human serum	200	1250/7.5	1600 V/10 ms/3 pulses (#24)
Jurkat T-cell	Human peripheral blood lymphocyte	RPMI	200	1250/7.5	1700 V/20 ms/1 pulse (#5)
HepG2	Human hepatocellular carcinoma	DMEM	120	1250/7.5	1300 V/30 ms/1 pulse (#8)
N2A	Mouse brain neuroblastoma	DMEM	100	1250/7.5	1400 V/30 ms/1 pulse (#9)

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

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Revision history: Pub. No. MAN0017058

Revision	Date	Description
D.0	13 January 2020	Add Cat. Nos. and correct TrueGuide™ Modified sgRNA product amount.
C.0	26 January 2018	Correct the target-specific crRNA sequence of the Mouse Rosa26 control.
B.0	10 October 2017	Add Cat. Nos. for TrueCut™ Cas9 Protein v2.
A.0	15 August 2017	New user guide.

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