CTS™ Cas9 Proteins

Catalog Numbers A45220, A45221, A54223, A54224, A54285, A54287

Pub. No. MAN0030087 Rev. A.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

Gibco™ CTS™ Cas9 Proteins are recombinant Streptococcus pyogenes Cas9 proteins purified from *E. coli*, for genome editing 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) aids delivery to the nucleus, increasing the rate of genomic DNA cleavage.

The CTS™ proteins are manufactured to meet the standards for Ancillary Materials for Cell, Gene, and Tissue-Based Products including USP <1043>, Ph.Eur. 5.2.12, and ISO 20399 which include aseptic filling, and extensive release testings. It is provided at a 10 mg/mL concentration in a transfection-ready format for electroporation.

Thermo Fisher Scientific offers a variety of CTS™ Cas9 proteins and kits for different applications.

- Gibco™ CTS™ TrueCut™ Cas9 Protein is engineered to deliver maximum editing efficiency.
- Gibco™ CTS™ HiFi Cas9 Protein is a high fidelity Cas9 protein variant engineered to demonstrate superior off-target profiles, while preserving maximum on-target editing efficiency.
- CTS™ HiFi Cas9 + CTS™ Xenon™ Genome Editing Buffer Kit is designed for applications where the highest knock-in efficiency is desired. The CTS™ Xenon™ Genome Editing Buffer is designed to support cells through gene editing during and after electroporation.

Contents and storage

Table 1 CTS™ Cas9 Proteins (Cat. Nos. A45220, A45221, A54223, A54224, A54285, A54287)

Component	Cat. No.	Amount	Concentration	Storage	Shelf life[1]
CTS™ TrueCut™ Cas9 Protein	A45220	2.5 mg	10 mg/mL	-20°C[2]	36 months
	A45221	5.0 mg			
CTS™ HiFi Cas9 Protein[3]	A54223	2.5 mg			12 months[4]
	A54224	5.0 mg			
CTS™ HiFi Cas9 Protein + CTS™ Xenon™ Genome Editing Buffer Kit[5]	A54285	2.5 mg CTS™ HiFi Cas9 Protein 1 × 100 mL CTS™ Xenon™ Genome Editing Buffer	—	-20°C[2] (protein) 2–8°C (buffer)	CTS™ HiFi Cas9 Protein: 12 months[4] CTS™ Xenon™ Genome Editing Buffer: 12 months
	A54287	5.0 mg CTS™ HiFi Cas9 Protein 2 × 100 mL CTS™ Xenon™ Genome Editing Buffer	—		

[1] Shelf life duration is determined from date of manufacture

[2] Occasionally after prolonged storage at –20°C, small, clear, colorless crystals can be observed. The crystals rapidly dissipate after mild vortexing and transfer to 4°C on ice, and the crystals should be dispersed prior to formulation and transfection. No performance differences have been observed.

[3] Storage buffer composition: 10 mM Tris pH 8.0 (HCl), 100 mM NaCl, 200 mM Na_2SO_4, 50% (v/v) glycerol, DNA-free Water for Molecular Biology

[4] Additional real time stability data is being collected and shelf life will be updated. For current shelf life, please consult the product COA.

[5] Do not allow buffers to undergo warming and cooling cycles, or excessive shaking.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
CTS™ OpTmizer™ T-Cell Expansion SFMCTS™, bottle format	A1048501
CTS™ Immune Cell SR	A2596101
CTS™ Dynabeads™ CD3/CD28	40203D
CTS™ DPBS without calcium chloride, without magnesium chloride	A1285601
CTS™ TrypLE™ Select Enzyme	A1285901
TrueGuide™ Synthetic gRNA	thermofisher.com/truedesign thermofisher.com/trueguide
StemFlex™ Medium Kit	A3349401
CTS™ RevitaCell™ Supplement (100X)	A4238401
CTS™ Xenon™ Genome Editing Buffer, bottle format	A4998001
Neon™ NxT Electroporation System	NEON1
Neon™ NxT Electroporation System 10-µL Kit:[1] • Neon™ NxT Resuspension Genome Editing Buffer • Neon™ NxT Resuspension R Buffer	N1025. N1096
Neon™ NxT Electroporation System 100-µL Kit:[1] • Neon™ NxT Resuspension Genome Editing Buffer • Neon™ NxT Resuspension R Buffer	N10025, N10096
CTS™ Xenon™ Electroporation Instrument	A50301
CTS™ Xenon™ SingleShot Electroporation Chamber	A50305
CTS™ Xenon™ MultiShot Electroporation Cartridge	A50306
Optional: GeneArt™ Genomic Cleavage Detection Kit	A24372

[1] The kit contains additional buffers not listed here.

General design guidelines

- Culture conditions (media, supplements, culture vessel, seeding density, media depth, etc.) both pre and post electroporation can affect transfection efficiency, cell health, growth and expansion.
- The efficiency with which mammalian cells are transfected with gRNA varies according to cell type and the delivery option used.
- Genome editing experiments can be designed and reagents ordered directly through the free TrueDesign Genome Editor Software For more information, see **thermofisher.com/truedesign**
- Custom or predesigned TrueGuide™ synthetic gRNAs are available from Thermo Fisher Scientific for research applications. For more information, see **thermofisher.com/trueguide**
- Positive and negative control gRNAs should be used to determine gRNA amount and transfection conditions that give the optimal gene editing efficiency with highest cell viability.

Recommended delivery options

Choosing the right delivery option is critical for transfection and gene editing efficiency.

Cas9 RNP	Neon™ NxT Electroporation System[1]	CTS™ Xenon™ Electroporation System[2]
CTS™ TrueCut™ Cas9 Protein + gRNA, or CTS™ HiFi Cas9 Protein + gRNA	For small research scale (up to 100 µL) or when optimizing for a new cell model or payload type	For large scale electroporation to support preclinical process development and commercial manufacturing (up to 25 mL)

[1] *Neon™ NxT Electroporation System User Guide*, Pub. No. MAN0026677
[2] *CTS™ Xenon™ Electroporation System User Guide*, Pub. No. MAN0025488

If optimization is required for a new cell model or payload type, optimization can be performed using the Neon™ NxT Electroporation System. To ensure scalability between Neon™ NxT and CTS™ Xenon™ systems, use the 100 µL Neon™ NxT tip during optimization.

Electroporation guidelines by application

Payload guidelines

- For **general gene editing applications** (including gene knockout), we recommend using a 1:1 to 1:1.5 molar ratio of Cas9 protein to gRNA as a starting point.
- For **knock-in application**, donor can be added directly to Cas9 RNP (a premixed gRNA-Cas9 protein).
  - ssODN donor: A 1:1 to 1.5:1 molar ratio of donor ssODN to gRNA is recommended for highest knock-in efficiency.
  - dsDNA donor: Further optimization may be necessary to determine the appropriate donor amount since the knock-in efficiency is dependent on the length and format of the donor DNA, homology arm length, toxicity level and cell type.
  - AAV viral donor: Further optimization to determine Multiplicity of Infection (MOI) is required since the knock-in efficiency is dependent on the donor length, homology arm length, toxicity level and cell type.

Buffer guidelines

- For **gene knockout**, we recommend either using Neon™ NxT Resuspension R Buffer, Neon™ NxT Resuspension Genome Editing Buffer, CTS™ Xenon™ Electroporation Buffer or CTS™ Xenon™ Genome Editing Buffer.
- For improved **knock-in** efficiency (HDR %) especially for ssODN donor knock-in, we recommend the use of CTS™ Xenon™ Genome Editing Buffer or Neon™ NxT Resuspension Genome Editing Buffer.
- For ***in cellular* genome-wide off-target screening** (e.g. GUIDE-Seq, TEG-Seq), we recommend using Neon™ NxT Resuspension R Buffer or CTS™ Xenon™ Electroporation Buffer .

IMPORTANT! Do not use Genome Editing Buffer (e.g. Neon™ NxT Resuspension Genome Editing Buffer or CTS™ Xenon™ Genome Editing Buffer) when performing in cellular genome-wide off-target screening as these buffers contain chemicals that interfere with the integration of dsTag to the double stranded break (DSB) sites.

Table 2 Application

Application	Neon™ NxT Electroporation System	CTS™ Xenon™ Electroporation System
Gene knockout (KO)	• Neon™ NxT Resuspension R Buffer, or • Neon™ NxT Resuspension Genome Editing Buffer	• CTS™ Xenon™ Electroporation Buffer , or • CTS™ Xenon™ Genome Editing Buffer
Knock-in (KI)	Neon™ NxT Resuspension Genome Editing Buffer	CTS™ Xenon™ Genome Editing Buffer
In cellular genome-wide off-target screening	Neon™ NxT Resuspension R Buffer	CTS™ Xenon™ Electroporation Buffer

Example protocol for therapeutic cell types

Protocols for the electroporation of T-cells and stem cells are provided in this user guide as examples. Conditions may vary depending upon cell type and the downstream experimental procedure to be performed.

Protocol for T-cells transfection

T-cells are activated and enriched from peripheral blood mononuclear cells (PBMC) isolated from healthy donors.

For optimal editing efficiency, it is recommended to culture the required number of T-cells on Day 0 (or 3 days before performing electroporation).

1 Day 0: Prepare T-cells (3 days before electroporation)

1. **Isolate PBMCs**

   We recommend using the Gibco™ CTS™ Rotea™ Counterflow Centrifugation System for the isolation of peripheral blood mononuclear cells (PBMCs) from an apheresis product and frozen for future use. For instructions on how to prepare the cells, see *CTS™ Rotea™ Counterflow Centrifugation System User Guide* (Pub. No. MAN0018908).

2. **Activate T-cells**

   Thaw enough vials of PBMC for the activation and enrichment of T-cells. Normally the same amount of T-cells can be obtained after 3-day activation from PBMC. For example, 50 million T-cells can be obtained from 50 million PBMC.

   Activate the T-cells (at 1 × 10^6 cells/mL) using CTS™ Dynabeads™ CD3/CD28 beads. For complete protocol details, see *CTS™ Dynabeads™ CD3/CD28 User Guide* (Pub. No. MAN0008945).

3. **Culture and expansion of T-cells**

   Culture the activated T-cells in Gibco™ CTS™ OpTmizer™ T-Cell Expansion SFM supplemented with Gibco™ CTS™ Immune Cell SR) and other components including IL2 as instructed per the product insert.

   Culture the T-cells in a humidified 37°C, 5% CO_2 incubator for 3 days before electroporation.

2 Day 3: Electroporate T-cells

1. **Prepare plate or flask with media.**
   a. Add specified volume of T-cell growth medium (we recommend CTS™ OpTmizer™ T-Cell Expansion SFM). See Table 3 for required culture media volume for the specific system and tip/chamber size.
   b. Place the plate or flask in the 37°C incubator to pre-warm.

2. **Prepare CRISPR Cas9/gRNA complex (and/or donor).**

   If performing in cellular genome wide off-target screening (e.g. GUIDE-Seq or TEG-Seq), proceed to substep 2.2b.

   a. Mix the CRISPR-Cas9 Protein, gRNA, donor DNA (optional) and the appropriate buffer for a specific application (See Table 2) in a fresh, RNase-free microcentrifuge tube according to the payload table (Table 3). Mix well.

   IMPORTANT!
   - Maintain Cas9 protein:gRNA at a 1:1 to 1:1.5 molar ratio. Increasing gRNA usually increases editing efficiency until it is saturated. Ensure that the total volume of the RNP complex (Cas9 Protein + gRNA) does not exceed 20% of electroporation volume.
   - Prepare an additional 20–30% extra volume of sample to account for volume loss during handling. For example, if a 1 mL electroporation volume is required, prepare 1.2 mL (ensure cell numbers are adjusted accordingly).
   - Ensure the cell culture medium does not exceed 50x the electroporation volume. For example, for 1 mL electroporation volume the cell culture medium should not exceed 50 mL.

   b. (For in cellular genome wide off-target screen) Mix the CRISPR-Cas9 Protein, gRNA, dsTag and the appropriate buffer for a specific application (See Table 2) in a fresh, RNase-free microcentrifuge tube/flask according to the payload table (Table 3). Mix well.

   IMPORTANT! We recommend using the 100 µL Neon™ NxT tip for genome wide off-target screen.

c. Incubate the CRISPR-Cas9/gRNA complex and buffer (from substep 2.2a or substep 2.2b) at room temperature for 5–20 minutes

Table 3 Recommended payloads for T-cells

Platform	Neon™ NxT Electroporation System		CTS™ Xenon™ Electroporation System	
Tip/chamber size	10 µL tip	100 µL tip	1 mL SingleShot	5 mL MultiShot
Culture media volume	0.2 mL per well 96-well plate	1 mL per well 24-well plate	30 mL in T-75 flask	180 mL in G-Raz
Cell amount	2–5 × 10^5 per well	2–5 × 10^6 per well	50 × 10^6	5 × 50 × 10^6
Cas9 protein amount	1.25 µg (7.5 pmol)	12.5 µg (75 pmol)	120 µg (732 pmol)	5 × 120 µg (5 × 732 pmol)
gRNA amount	0.24 µg (7.5 pmol)	2.4 µg (75 pmol)	32 µg (969 pmol)	5 × 32 µg (5 × 969 pmol)
ssODN Donor[1] (75–100 bp)	15 pmol	150 pmol	163 µg (320 nM)	5 × 163 µg (5 × 320 nM)
Large dsDNA or AAV viral donor[1,2]	**Optimization required**			
Example: Large dsDNA, CD19 CAR (3.2kb)	2 µg (0.5 pmol)	20 µg (5 pmol)	240 µg (50 pmol)	5 × 240 µg (5 × 50 pmol)
dsTag (for off-target screen)[3]	—	75 pmol	—	—
Buffer volume	5 µL	50 µL	1 mL	5 mL

[1] Any donor (ssOND, large dsDNA or AAV) is only required for knock-in application.
[2] If using long dsDNA or AAV viral donor, optimization is required because the amount depends on the donor size
[3] dsTag is needed to perform in cellular genome wide off-target screen. The amount of dsTag to be added to the RNP should be equivalent to gRNA amount (e.g. 75 pmol for 100 µL tip).

3. **Prepare T-cells for electroporation.**
   a. Perform bead removal: Transfer the appropriate number of cells for removal of magnetic beads. Gently mix T-cells by pipetting. De-bead by placing the tube in magnetic separator for 1–2 minutes. Transfer the supernatant containing the T-cells into a fresh tube.
   b. Count the cell number and calculate the total number of cells required based on the payload (see Table 3 for required cell amount). Centrifuge cells at 100–400 × *g* for 5 minutes at room temperature.
   c. Wash the cells with CTS™ DPBS without calcium chloride, without magnesium chloride 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. Resuspend the T-cells: Aspirate the DPBS and resuspend the cell pellet in the appropriate buffer used in substep 2.2a (see Table 2 for recommended buffer by application) at the specified concentration. For example, to use 200,000 cells/reaction, resuspend the cells at 40,000 cells/µL, then use 5 µL (200,000 total cells) of the resuspended cells per reaction. Gently pipette the cells to obtain a single cell suspension.

4. **Add cells to CRISPR Cas9/gRNA complex.**
   a. Pipette the T-cells from step 2.3 up and down to resuspend any cells that might have settled at the bottom of the tube.
   b. Add 5 µL (for 10 µL Neon™ NxT system) or 50 µL (for 100 µL Neon™ NxT system) of the cell suspension to the RNP (CRISPR-Cas9/gRNA complex and/or donor) from step 2.2".

IMPORTANT! Prepare an additional 20–30% extra volume of sample to account for volume loss during handling. Ensure that the total volume of the RNP complex (Cas9 protein + gRNA) does not exceed 20% of electroporation volume.

2 Day 3: Electroporate T-cells *(continued)*

5. **Electroporate T-cells.**

   a. Use the appropriate electroporation tip or chamber, to aspirate the cell + RNP mix (CRISPR-Cas9/gRNA complex and/or donor) from step 2.4, then electroporate using the T-cells electroporation condition:

   - Neon™ NxT: 1600V / 10MS / 3 pulses
   - Xenon™ SingleShot: 2300V / 3MS / 4 pulses

   IMPORTANT! Avoid creating bubbles that can hinder electroporation.

   b. After electroporation, immediately transfer the contents from the tip or chamber into plate or flask containing pre-warmed growth medium as prepared in step 2.1.

   c. Transfer the plate or flask to a humidified 37°C, 5% CO_2 incubator, then incubate the cells for 48 to 72 hours.

3 Day 6–10: Verify editing efficiency and proceed to downstream applications

After 48 to 72 hours incubation, use a small portion of T-cells for viability and perform the following functional assays depending on your experiment (KI or KO) and sample number:

- Flow cytometry if you have a proper antibody against your targeted gene for KI and KO (e.g. TCR). It is simple and quick assay to measure editing efficiency at function level. We recommend using the Invitrogen™ Attune™ NxT Flow Cytometer. For more information, see **https://www.thermofisher.com/us/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometers/attune-nxt-flow-cytometer.html**.
- NGS based sequencing assay for KI and KO, such as the Ion Torrent™ Targeted Amplicon-seq Validation (TAV). It is suitable for large sample number using 96-well format. The benefit of using sequencing assay is to obtain the sequencing change information at genome level near the targeted region. For more information on CRISPR Validation Tools, see **https://www.thermofisher.com/us/en/home/life-science/genome-editing/crispr-validation.html**
- NGS based sequencing assay for genome-wide off-target screen, such as GUIDE-seq, TEG-Seq or Target-seq, dsTag is required to be co-transfected to the cells. We recommend using 100 µL Neon™ NxT tip for this assay. Thermo Fisher Scientific offers TEG-Seq as a service. For more information about TEG-Seq analysis and service, see **https://www.thermofisher.com/us/en/home/products-and-services/services/custom-services/teg-seq-crispr-off-target-analysis.html**
- GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372) for KO assay. It is a T7 Endonuclease I (T7E1) mismatch assay and suitable for small sample number. For more information and detailed protocols, see the *GeneArt™ Genomic Cleavage Detection Kit User Guide* (Pub. No. MAN0009849).

Protocol for pluripotent stem cells (PSCs) transfection

Protocol for PSCs transfection on the Neon™ NxT Electroporation System. For optimal editing efficiency, it is recommended to culture the PSCs 1–3 weeks before performing electroporation.

1 **1–3 weeks before electroporation: Prepare PSC culture**

1. **Culture PSCs.**

   a. Prepare PSCs using growth medium of choice. We recommend the StemFlex™ Medium Kit. For complete PSCs expansion protocol details, see *StemFlex™ Medium Kit User Guide* (Pub. No. MAN0016431).

   The total cell number required for your experiment will be based on the electroporation scale and platform format of choice and the total number of samples required for your experiment. Utilize cell culture plate surface coating reagent (we recommend rhLaminin-521) for ideal PSC culturing and proliferation. Incubate coated plates at 37°C for at least 2 hours (ideally, 24 hours) before seeding cells

2 **Day 1: Electroporate PSCs**

1. Prepare plate with media

   a. Add specified volume of PSC growth medium (we recommend StemFlex™ Medium supplemented with RevitaCell™ Supplement) into each well. See Table 4 for required culture media volume for the specific system and tip/chamber size.

   IMPORTANT! For optimal recovery after electroporation, we recommend using RevitaCell™ supplemented media right after electroporation and for the following one to two passages.

   b. Place the plate in the 37°C incubator to pre-warm.

2. Prepare CRISPR Cas9/gRNA complex (and/or donor)

   If performing in cellular genome wide off-target screen (e.g. GUIDE-Seq or TEG-Seq), proceed to substep 2.2b.

   a. Mix the CRISPR-Cas9 Protein, gRNA, donor DNA (optional for knock-in application) and the appropriate buffer (See Table 2) in a fresh, RNase-free microcentrifuge tube according to the payload table (Table 3). Mix well.

   IMPORTANT!
   - Maintain Cas9 protein:gRNA at a 1:1 to 1:4 molar ratio. Increasing gRNA usually increases editing efficiency until it is saturated. Ensure that the total volume of the RNP complex (Cas9 protein + gRNA) does not exceed 20% of electroporation volume.
   - Prepare an additional 20-30% extra volume of sample to account for volume loss during handling. For example, if a 100 µL electroporation volume is required, prepare 120 µL(ensure cell numbers are adjusted accordingly).
   - Ensure the cell culture medium does not exceed 50x the electroporation volume. For example, for 100 µL electroporation volume the cell culture medium should not exceed 5 mL.

   b. (For in cellular genome wide off-target screen) Mix the CRISPR-Cas9 protein, gRNA, dsTag and the appropriate buffer (See Table 2) in a fresh, RNase-free microcentrifuge tube/flask according to the payload table (Table 3). Mix well.

   IMPORTANT! We recommend using the 100 µL Neon™ NxT tip for genome wide off-target screen.

c. Incubate the CRISPR-Cas9/gRNA complex and buffer (from substep 2.2a or substep 2.2b) at room temperature for 5–20 minutes.

Table 4 Payloads

Platform	Neon™ NxT Electroporation System	
Tip size	10 µL tip	100 µL tip
Culture media (volume)	0.5 mL per well (24 well plate)	2.5 mL per well (6 well plate)
Cell amount	2–5 × 10^5 per well	2–5 × 10^6 per well
Cas9 protein amount	1.25 µg (7.5 pmol)	12.5 µg (75 pmol)
gRNA amount	0.24 µg (7.5 pmol)	2.4 µg (75 pmol)
ssODN Donor[1] (75-100 bp)	15 pmol	150 pmol
Large dsDNA or AAV viral donor[2]	Optimization required	
dsTag (for off-target screen) [3]	—	75 pmol
Buffer volume	to 5 µL	to 50 µL

[1] ssOND donor is only required for knock-in (KI) editing.
[2] If using long dsDNA or AAV viral donor, optimization is required because the amount depends on the donor size
[3] dsTag is needed to perform in cellular genome wide off-target screen. The amount of dsTag to be added to the RNP should be equivalent to gRNA amount (e.g. 75 pmol for 100 µL tip).

3. **Prepare PSCs for electroporation.**
   a. Detach PSCs before electroporation with CTS™ TrypLE™ Select Enzyme. Rinse PSCs with proper amount (e.g. 2 mL per well if using 6-well plate) of DPBS, aspirate DPBS. Add proper amount (e.g. 1 mL per well if using 6-well plate) of CTS™ TrypLE™ Select Enzyme to cover surface of the well containing PSCs. Incubate for 2–3 minutes in a humidified 37°C, 5% CO_2 incubator.
   b. Gently mix cells and transfer all PSCs to 15-ml or 50-mL tubes (depending on the amount of cells prepared). Centrifuge at 100–400 × *g* for 5 minutes at room temperature. Remove CTS™ TrypLE™ Select Enzyme.
   c. Count the cell number and calculate the total cells required based on the table of payload above. Centrifuge enough cells at 100–400 × *g* for 5 minutes at room temperature. Remove CTS™ TrypLE™ Select Enzyme.
   d. Wash cells with DPBS, no calcium, no magnesium. Add proper amount of DPBS to the PSCs pellets and gently resuspend cells.
   e. Resuspend PSCs in buffer of choice: Aspirate all DPBS (may need to use 20 µL pipette tip to remove all DPBS) and resuspend the cell pellet in the same buffer used in substep 2.2a or substep 2.2b (see Table 2 for recommended buffer by application) at the desired concentration. For example, to use 400,000 cells/reaction, resuspend the cells at 800,000 cells/µL. 5 µL is equal to 400,000 resuspended cells per reaction. Gently pipette the cells to obtain a single cell suspension.

4. **Add cells to CRISPR Cas9/gRNA complex.**
   a. Pipette the PSCs from step 2.3 up and down to resuspend any cells that might have settled at the bottom of the tube.
   b. Add 5 µL (for 10 µL Neon™ NxT system) or 50 µL (for 100 µL Neon™ NxT system) of the cell suspension to the RNP (CRISPR-Cas9/gRNA complex and/or donor) from step 2.2.

IMPORTANT! Prepare an additional 20–30% extra volume of sample to account for volume loss during handling. Ensure that the total volume of the RNP complex (Cas9 protein + gRNA) does not exceed 20% of electroporation volume.

5. **Electroporate PSCs.**
   a. Using the electroporation tip, aspirate the cell + RNP mix (CRISPR-Cas9/gRNA complex and/or donor) from substep 2.4b, then electroporate using iPSCs electroporation condition (1300V / 30MS / 1 pulse).

IMPORTANT! Avoid creating bubbles that can hinder electroporation.

2 Day 1: Electroporate PSCs *(continued)*

b. After electroporation, immediately transfer the contents from the tip into one well of the plate containing pre-warmed growth medium as prepared in step 2.1.

c. Transfer the plate to a humidified 37°C, 5% CO_2 incubator, then incubate the cells fo 48 to 72 hours.

3 Day 2: Change medium

Change medium.

a. Remove old medium and add specified volume of PSC growth medium (we recommend StemFlex™ Medium supplemented with RevitaCell™ Supplement) into each well. See Table 4 for specific volume by system and tip/chamber size.

b. Continue to grow cells for 4–5 days.

4 Day 6–7: Harvest and verify editing efficiency and proceed

1. **Harvest and verify editing efficiency.**

   a. Remove the old growth medium, rinse cells with DPBS and remove DPBS.

   b. Detach cell by adding 0.1 mL CTS™ TrypLE™ Select Enzyme per well (if using 24-well plate) or 0.5 mL per well (if using 6-well plate). Incubate plate in a humidified 37°C, 5% CO_2 incubator for 5 minutes. Add 0.5 mL DPBS per well (if using 24-well plate) or 2 mL per well (if using 6-well plate). Mix and detach all cells. Transfer enough cells to a fresh tube for downstream assay.

2. Use a small portion of PSCs for viability and the following functional assays depending on your experiment (KI or KO) and sample number:

   a. Flow cytometry if you have a proper antibody against your targeted gene for KI and KO (e.g. TCR). It is simple and quick assay to measure editing efficiency at function level. We recommend using the Invitrogen™ Attune™ NxT Flow Cytometer. For more information, see **https://www.thermofisher.com/us/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometers/attune-nxt-flow-cytometer.html**.

   b. NGS based sequencing assay for KI and KO, such as the Ion Torrent™ Targeted Amplicon-seq Validation (TAV-seq). It is suitable for large sample number using 96-well format. The benefit of using sequencing assay is to obtain the sequencing change information at genome level near the targeted region. For more information on CRISPR Validation Tools, see **https://www.thermofisher.com/us/en/home/life-science/genome-editing/crispr-validation.html**.

   c. NGS based sequencing assay for genome-wide off-target screen, such as GUIDE-seq, TEG-Seq or Target-seq, dsTag is required to be co-transfected to the cells. We recommend using 100 µL Neon™ NxT tip for this assay. Thermo Fisher Scientific offers TEG-Seq as a service. For more information about TEG-Seq analysis and service, see **https://www.thermofisher.com/us/en/home/products-and-services/services/custom-services/teg-seq-crispr-off-target-analysis.html**.

   d. GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372) for KO assay. It is a T7 Endonuclease I (T7E1) mismatch assay and suitable for small sample number. For more information and detailed protocols, see the *GeneArt™ Genomic Cleavage Detection Kit User Guide* (Pub. No. MAN0009849).

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: Pub. No. MAN0030087 A.0

Revision	Date	Description
A.0	26 October 2023	Initial release.

The information in this guide is subject to change without notice.