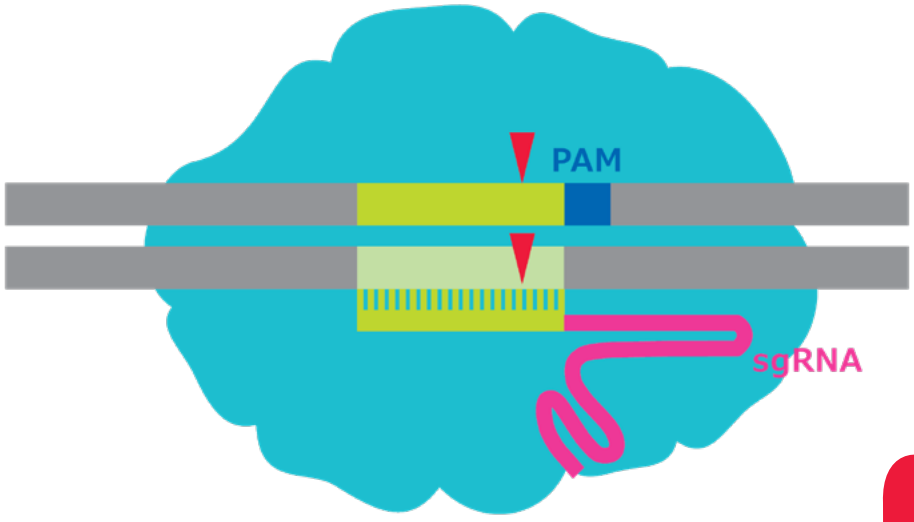
Instructions For Use

CRISPR Ribonucleoprotein (RNP)



The life science business of
Merck operates as MilliporeSigma
in the U.S. and Canada.

SigmaAldrich.com

Sigma-Aldrich®

Table of Contents

Protocols Section

For research use only, not for use in diagnostics procedures

Product Warranty

We guarantee the performance of all SygRNA® synthetic guide RNAs. If your synthetic gRNAs do not yield detectable cleavage at the intended target site, we will provide you a one-time replacement, free of charge.

To qualify for this guarantee, please send an image or sequencing data from a single experiment demonstrating detectable cleavage using one of our available positive controls, side-by-side with the negative results from your predesigned SygRNA® CRISPR. To receive your replacement, simply email CRISPR@sial.com and include sample data from a representative experiment (T7E1, TIDE, or NGS).

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided. We shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

Ordering Information

SigmaAldrich.com/OrderCRISPR
SigmaAldrich.com/CRISPR
SigmaAldrich.com/RNP

For a list of our worldwide offices please go to:

www.sigmaaldrich.com/site-level/corporate/worldwide-offices.html

For customer support please go to:

www.sigmaaldrich.com/customer-service.html

Technical Services Information

To contact technical service:

www.sigmaaldrich.com/technical-service-home.html

CRISPR Use License Agreement

This Product and its use are covered under a license between The Broad Institute, Inc. (BROAD) and Sigma-Aldrich Co. LLC (SIGMA) and are the subject of one or more of the following patents controlled by BROAD, the Massachusetts Institute of Technology (MIT), or the President and Fellows of Harvard College (HARVARD): U.S. Patent Nos. 8,697,359; 8,771,945; and any substitutions, divisions, continuations, reissues, renewals, re-examinations or extensions, and corresponding foreign patent applications and patents (the “Patent Rights”).

BEFORE OPENING OR USING THIS PRODUCT, PLEASE READ THE TERMS AND CONDITIONS SET FORTH IN THIS LICENSE AGREEMENT. YOUR USE OF THIS PRODUCT SHALL CONSTITUTE ACKNOWLEDGMENT AND ACCEPTANCE OF THESE TERMS AND CONDITIONS. If you do not agree to use this Product pursuant to the terms and conditions set out in this License Agreement, please contact Sigma Technical Services within ten days of receipt to return the unused and unopened Product for a full refund; provided, however, that custom-made Products may not be returned for a refund.

The purchase of this Product conveys to you (the “Buyer”) the NON-TRANSFERABLE right to use the purchased Product for Licensed Research Use (see definition below) subject to the conditions set out in this License Agreement. If you wish to use this Product for any purpose other than Licensed Research Use, you must first obtain an appropriate license (see information set out below).

This Product may not be used for any purpose other than Licensed Research Use. Your right to use this Product for Licensed Research Use is subject to the following conditions and restrictions:

1. "Licensed Research Use" means any use for research purposes, with the following exceptions: (i) Buyer may not sell or otherwise transfer the Product (including without limitation any material that contains the Product in whole or part) or any Related Material to any other third party (except that you may transfer the Product, including without limitation an material that contains the Product in whole or in part, or any Related Material to a bona fide collaborator or contract research person or entity), or use the Products or any Related Material to perform services for the benefit of any other person or entity; (ii) Buyer may use only the purchased amount of the Product and components of the Product, and shall use any Related Material, only for your internal research within the Field, and not for any Commercial Purposes; (iii) Buyer shall use the Product and any Related Material in compliance with all applicable laws and regulations, including without limitation applicable human health and animal welfare laws and regulations; (iv) BROAD, MIT and HARVARD shall provide no warranties of any kind to the Buyer (statutory or implied) concerning the Patent Rights, the Product or any Related Materials, including without limitation, as to product quality, condition, description, merchantability, fitness for a particular purpose, noninfringement of intellectual property rights or the absence of latent or other defects, and all such warranties are hereby expressly disclaimed; (v) BROAD, MIT and HARVARD shall expressly disclaim any warranty regarding results obtained through the use of the Product or Related Materials, including without limitation any claim of inaccurate, invalid or incomplete results; (vi) BROAD, MIT and HARVARD, and their directors, trustees, officers, employees, agents, faculty, affiliated investigators, and students, shall have no liability to the Buyer, including, without limitation, for any loss of use or profits, business interruption or any consequential, incidental, special or other indirect damages of any kind, regardless of how caused and regardless of whether an action in contract, tort, strict product liability or otherwise; (vii) the Buyer shall indemnify, defend and hold harmless BROAD, MIT and HARVARD, and their current and former trustees, directors, officers, faculty, affiliated investigators, students, employees, and agents and their respective successors, heirs and assigns (the "Indemnities") against any liability, damage, loss, or expense (including without limitation reasonable attorneys' fees and expenses) incurred by or imposed upon any of the Indemnitees in connection with any claims, suits, investigations, actions, demands or judgments arising out of or related to the exercise of any rights granted to the Buyer hereunder or any breach of this License Agreement by such Buyer; and (viii) the Product and its use may be the subject of one or more issued patents and/or pending patent applications owned by BROAD, MIT and HARVARD and the purchase of the Product does not convey a license under any claims in the foregoing patents or patent applications directed to the Licensed Product or use, production or commercialization thereof.

2. For purposes of Section 1 above, the following definitions shall apply:
   "Commercial Purposes" means (a) the practice, performance or provision of any method, process or service, or (b) the manufacture, sale, use, distribution, disposition or importing of any product, in each case (a) or (b) for consideration, e.g., a fee, or on any other commercial basis.

   "Field" means use as a research tool for research purposes; provided, however, that notwithstanding the foregoing, the Field shall expressly exclude (a) any *in vivo* and ex vivo human or clinical use, including, without limitation, any administration into humans or any diagnostic or prognostic use, (b) any *in vivo* veterinary or livestock use, or non-research agricultural use, or (c) the manufacture, distribution, importation, exportation, transportation, sale, offer for sale, marketing, promotion or other exploitation or use of, or as, a testing service, therapeutic or diagnostic for humans or animals.

   "Related Materials" means (a) any progeny, modification or derivative of a Product, or (b) any cell or animal made or modified through use of a Product, or any progeny, modification or derivative of such cell or animal.

3. Your right to use the Product will terminate immediately if you fail to comply with these terms and conditions. You shall, upon such termination of your rights, destroy all Product, Related Materials, and components thereof in your control, and notify SIGMA of such in writing.

4. SIGMA makes no representations or warranties of any kind concerning the Patent Rights, the Product or any Related Materials and hereby disclaim all representations and warranties, express or implied, including without limitation warranties of merchantability, fitness for a particular purpose, noninfringement of intellectual property rights of BROAD, MIT and HARVARD or third parties, validity, enforceability and scope of the Patent Rights, validity of any claims, whether issued or pending, and the absence of latent or other defects, whether or not discoverable. In no event shall SIGMA or their directors, officers, employees and agents, be liable for incidental or consequential damages of any kind, including without limitation economic damages or injury to property and lost profits, regardless of whether SIGMA shall be advised, shall have other reason to know, or in fact shall know of the possibility of the foregoing.

For information on purchasing a license to this Product for purposes other than Licensed Research Use, contact your local SIGMA sales representative, who will refer you to the proper licensing representative, or in the USA call 800-325-3010.

Materials Provided

Component	Concentration	20 µM Working Solution	30 µM Working Solution	Resuspension Solution	Catalogue No.
CRISPR sgRNA unmodified	3 nM	Add 150 µL Tris solution to 3 nM lyophilized RNA to a final concentration of 20 µM or 20 picomoles per µL	Add 100 µL Tris solution to 3 nM lyophilized RNA to a final concentration of 30 µM or 30 picomoles per µL	10 mM Tris-containing buffer of pH between 7 and 8.**	VC40007
CRISPR sgRNA Modified*	3 nM	Add 150 µL Tris solution to 3 nM lyophilized RNA to a final concentration of 20 µM or 20 picomoles per µL	Add 100 µL Tris solution to 3 nM lyophilized RNA to a final concentration of 30 µM or 30 picomoles per µL	10 mM Tris-containing buffer of pH between 7 and 8.**	VC40007

* Note: The term "Modified" in this context represents stabilizing 2' 0 methyl & phosphothioate modifications added to the ends of synthetic RNA. Modifications are only available in HPLC purification and are intended to increase stability toward exonucleases found in cell environments (Hendel et al. 2015).

** Note: Resuspend sgRNA to a concentration of 20 or 30 µM (20 or 30 picomoles per µL) in 10 mM Tris containing buffer (1:100 dilution of T2663 in nuclease free water, W4502). Resuspension solution not included.

Related Products

Cas9 Protein Products

Catalogue No.	Description
PECAS9	PURedit™ Cas9 Protein with high efficiency mutations from Streptococcus pyogenes, recombinant, expressed in *E. coli*, 3X NLS
CAS9PL	Cas9 Plus Protein, a high fidelity Cas9 variant from Streptococcus pyogenes, recombinant, expressed in *E. coli*, 3X NLS
CAS9GFPPRO	Cas9-GFP Protein from *Streptococcus pyogenes*, fused with enhanced GFP, recombinant, expressed in *E. coli*, 3X NLS
ECAS9GFPPR	eSpCas9-GFP Protein from *Streptococcus pyogenes* with mutations conferring enhanced specificity, fused with enhanced GFP, recombinant, expressed in *E. coli*, 3X NLS
CAS9PROT	Cas9 Protein from *Streptococcus pyogenes*, recombinant, expressed in *E. coli*, 1X NLS
ESPCAS9PRO	eSpCas9 Protein from *Streptococcus pyogenes* with mutations conferring enhanced specificity, recombinant, expressed in *E. coli*, 1X NLS
DCAS9PROT	dCas9-3XFLAG™-Biotin Protein from *Streptococcus pyogenes* with D10A and H840A mutations, recombinant, expressed in *E. coli*, 1X NLS
CAS9D10APR	Cas9-D10A Nickase Protein from *Streptococcus pyogenes* with D10A mutation, recombinant, expressed in *E. coli*, 1X NLS

Storage Conditions

Component	Temperature	Notes
sgRNA Storage	–20 °C	Handle under nuclease free conditions. Store sgRNA stock solutions in a non-frost-free freezer and avoid multiple freeze-thaw cycles. Store at –70 °C long term.
Cas9 Protein	–20 °C	Aliquot Cas9 protein solution and store at –20 °C in a non-frost-free freezer for up to one month. Store at –70 °C long term.

Additional Required Materials

Instrumentation and Equipment

- Incubator, CO_2
- Hemocytometer
- Thermocycler
- Benchtop centrifuge
- Gel electrophoresis power supply
- Nucleofector™

Molecular Biology and Cell Culture Reagents

- Hank's Buffered Salt Solution (Cat. No. H6648)
- ISCOVE's Medium (Cat. No. I3390)
- L-glutamine (Cat. No. G7513)
- Fetal Bovine Serum (Cat. No. F4135)
- Penicillin-streptomycin Solution (Cat. No. P4333)
- GenElute™ Mammalian Genomic DNA (Cat. No. G1N10)
- Ethanol (Cat. No. 459844)
- Cas9-GFP protein (5 µg/µL) (Cat. No. CAS9GFPPRO)
- SygRNA® Synthetic sgRNA (100 µM) (Cat. No. HScontrol_AAVS1)
- TransIT-CRISPR® transfection reagent (Cat. No. T1706)
- Opti-MEM™ I Reduced Serum Medium (Thermo-Fisher 31985062)
- Expand™ High Fidelity PCR System (Cat. No. 11732650001)
- dNTPs (Cat. No. D7295)
- Molecular grade water (Cat. No. W4502)
- T7 Endonuclease I-based Mutation Detection Kit (Cat. No. T7E1001)
- Tris-Borate-EDTA Buffer (Cat. No. T4415)
- DirectLoad™ Wide Range DNA Ladder (Cat. No. D7058)
- Gel Loading Buffer (Cat. No. G2526)

Before You Begin

The type II CRISPR-Cas (clustered regularly interspaced short palindromic repeats—CRISPR-associated proteins) system is essential in the adaptive immunity of bacteria and archaea where it is employed to inactivate foreign genetic material. The system consists of two non-coding RNAs, the CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA) that work in concert with the Cas9 endonuclease. The system can be further reduced to two components by encoding the crRNA and tracrRNA into a single gRNA (sgRNA) (Jinek et al. 2012).

Once loaded with the sgRNA, Cas9 undergoes a conformational change that enables it to bind DNA at cognate sequences called protospacer-adjacent motifs (PAM). PAMs are ubiquitous in the genomes of numerous species and have the sequence 'NGG,' where N is any base. The crRNA, which is typically 19 to 20 bp in length, confers the sequence specificity of the CRISPR system and must contain a PAM at its 3' end.

As the sequence of the sgRNA can be programmed, Cas9 nuclease activity can be directed to any DNA sequence in the format 5'-N19-20 bp-NGG (Sander and Joung 2014). For this reason, CRISPR is well suited for precision genome editing. The system is further reduced to two components by encoding the crRNA and tracrRNA into a single gRNA (sgRNA) (Jinek et al. 2012). The entire complex consisting of the bound sgRNA and Cas9 nuclease is often referred to as a ribonucleoprotein (RNP).

Once the Cas9-gRNA complex binds to a PAM sequence, a "seed" sequence at the 3' end of the gRNA targeting sequence begins to anneal to the target DNA. If the seed and target DNA sequences match, the gRNA will continue to anneal to the target DNA in a 3' to 5' direction. When annealed at the target location, the two nuclease domains of Cas9, HNH and RuvC, then cleave the DNA strands that are complementary and non-complimentary to the crRNA sequence, respectively (Nishimasu et al. 2014).

When Cas9 creates a double-strand break (DSB) it triggers the cell's DNA repair mechanisms. The most active DNA repair pathway for DSBs is non-homologous end joining (NHEJ); however, this pathway frequently results in the insertion or deletion (indels) of a few bases at the site of repair. While, in some cases, the insertions or deletions do not lead to frameshift loss-of-function mutations, the chances of a mutation that disrupts the translational reading frame of a coding sequence are increased considerably (Sander and Joung 2014). Commonly researchers refer to this form of mutation as 'knocked-out' (KO) if its function is disrupted.

Alternatively, homology-directed repair (HDR) or homologous recombination occurs when a double-stranded break in DNA is repaired through homologous DNA—introducing a 'donor' or 'template' DNA, which encodes regions of homology to either side of the double-strand break. In between the homology arms, researchers can include any desired sequences that will then also be integrated during the repair process. Once integrated, this new genetic material is said to be 'knocked in.' Targeted nuclease-induced DSBs significantly increase the frequencies of HDR (Sander and Joung 2014, Baker et al., 2016).

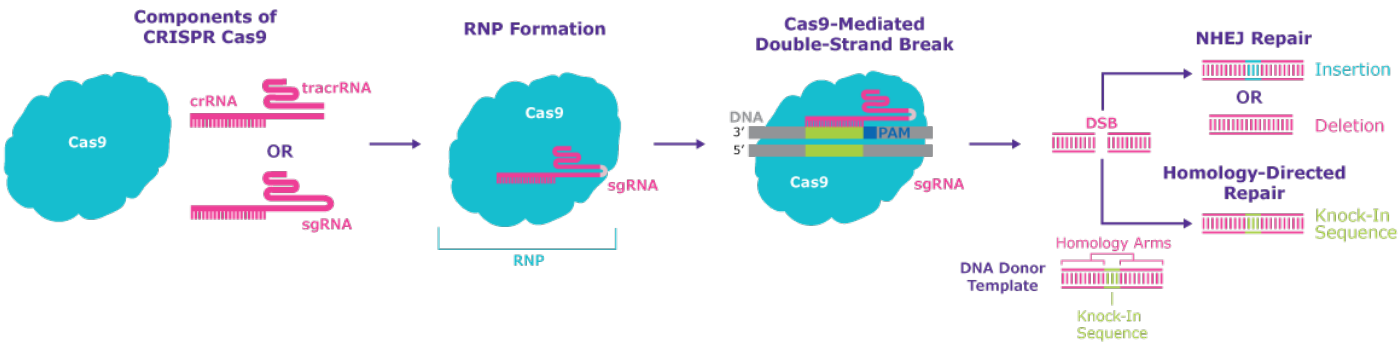


Fig.1 Overview of CRISPR sgRNA and recombinant Cas9 RNP formation leading to DNA double strand break and subsequent editing by common DNA repair pathways non-homologous end joining (NHEJ) and homology-directed repair (HDR).

To use CRISPR as a programmable gene-editing tool, the CRISPR components must be delivered to the nucleus of the cells. Although plasmid-based expression systems for Cas9 and sgRNAs are effective, they result in the active CRISPR components being present in the cells at high quantities and for extended periods, which increases the opportunity of Cas9 to cut somewhere else in the genome (off-target sites).

Ways to reduce potential off-target effects include limiting the cell's exposure to the active Cas9 nuclease, making use of enhanced versions of Cas9 (PURedit™ Cas9 and Cas9 Plus), or targeting the cut-site with a pair of Cas9 nickases (CAS9D10AP) so that off-target cuts create single-strand nicks instead of double-strand breaks, which are then precisely repaired by the cells repair machinery. Limiting the cell's exposure to the nuclease can be achieved by introducing Cas9 into cells as purified protein. This approach offers the distinct advantage of rapid action and the quick clearance, resulting in fewer off-target effects. The use of CRISPR sgRNA and Cas9 recombinant proteins has rapidly become the genome engineering modality of choice for researchers around the world because of its ease of use, high on-target editing and low off-targeting profile.

Protocol Section

Selecting Guide RNA Targets for Gene Disruption and Precise Repair

We offer free gRNA selection and design tools as well as pre-designed gRNAs designed for gene KO for both human and mouse species.

In case your genome of interest is not available within our design tool we can help you select through our custom service or we are happy to accept and synthesize sgRNA from your own designs and preferred gene editing selection tool.

Guidelines for selecting gRNAs for functional gene KO

- gRNA aligns to every transcript within the protein coding portion (CDS region).
- Avoid the first 90 bp.
- Best mismatch score possible, 3 bp or more. Reduce stringency to fit above rules if necessary. (Specificity score, Hsu et al 2013) and within the first $^2/_3$ of the CDS.
- Best efficiency (Doench, Fusi et al 2016) score try and select for >40 if available.
- Do not overlap (this helps to ensure SNPs and chromatin are not interfering with DSB efficiency).
- No more than 4 Ts in a row (important for U6 promoter expression, not critical for RNPs).
- As a practice we recommend testing 3 gRNAs per target.

Guidelines for selecting gRNAs for precise editing

- gRNA sequence is as close as possible to the point mutation desired.
- Include PAM silencing mutations if possible.
- Best mismatch scores possible, 3 bp or more. Reduce stringency to fit above rules if necessary. (specificity score, Hsu et al 2013).
- Best efficiency score; try and select for >40 if available.
- Novel restriction enzymes if possible, for screening desired precise repair mutation.

Where possible we display efficiency scores in our selection tool from the 2016 algorithm based on the following publication:

Doench, J., Fusi, N., Sullender, M. et al. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat Biotechnol 34, 184–191 (2016). https://doi.org/10.1038/nbt.3437

Where possible we display scores in our selection tool from the 2016 algorithm based on the publication:

Hsu, P., Scott, D., Weinstein, J. et al. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 31, 827–832 (2013). https://doi.org/10.1038/nbt.2647

The following protocols outline methods for selecting, delivering, and evaluating sgRNAs with recombinant Cas9 and Cas9 D10A nickase in immune cells, iPS cells, microinjection, electroporation for animal models, and standard laboratory cell lines.

General Guidelines for Preparation of RNPs

When forming RNPs for gene editing, we recommend using Sigma-Aldrich® Cas9 recombinant proteins. Below are general recommendations for our recombinant Cas9 proteins complexed with sgRNA, however, for detailed information specific for each Cas9 variant, we recommend downloading the product information on each Cas9 protein product page. Recombinant Cas9 protein and sgRNAs are resuspended to a concentration of 30 µM in either supplied resuspension solution or 10 mM Tris buffer at a pH of 7.5. Complex into a 4 µL mixture at 3:1 (sgRNA: Cas9 protein) and incubate at room temperature for 5–15 minutes immediately before use.

Note: For Cas9D10A nickase RNPs are formed separately (each complexed with one of the paired sgRNAs), and delivered into cells simultaneously.

Cas9 protein	(1 µg = 6 picomoles): 5 µg/µL = 30 µM
sgRNA	(1 µg = 30 picomoles): 0.5–1 µg/µL = 15–30 µM
tracrRNA	tracrRNA (1 µg = 36 picomoles): 30 uM = 0.8 µg/µL
crRNA	crRNA (1 µg = 75 picomoles): 30 µM = 0.4 µg/µL

CRISPR Chemically Synthesized single guide RNA (RNP) Nucleofection Protocol in Any Cell Line

Prepare Synthetic RNAs and Cas9

- Resuspend the lyophilized synthetic gRNA(s) to a final concentration of 30 µM or 30 picomoles per µL in 10 mM Tris pH 7.4 buffer [Dilute 1 M Trizma Hydorchloride Solution (Cat. No. T2663) to 10 mM with nuclease free water (Cat. No. W4502)]. A lower concentration of synthetic RNA may be required if using lower amounts of Cas9 protein.
- Resuspend the lyophilized Cas9 protein in nuclease free water with 20% glycerol (Cas9 protein is formulated in 20 mM Hepes, 150 mM KCl, and 1% sucrose, pH 7.5 before lyophilization).
- If a lower concentration of Cas9 protein is desired, dilute the Cas9 protein with Dilution buffer (20 mM Hepes, 150 mM KCl, 1% sucrose, pH 7.5) immediately before use. NOTE: Discard the diluted protein after use. Diluted protein cannot be stored at –20 °C and freeze-thawed in the absence of glycerol.
- For short term storage of Cas9 protein (within one month), aliquots can be stored at –20 °C, –70 °C for long term storage.

Prepare Nucleofector™ Solution and Cells

- Find Lonza Nucleofector™ Kit that works on your cell line (Use the “Cell Line” Drop-down menu on the left hand navigation found here.
- Warm Amaxa Cell Line Nucleofector™ Kit solution to room temperature.
- Add entire supplement to Nucleofector™ Solution.
- Obtain enough cells for approximately 250k cells per well in a 12-well plate (final volume per well will be 1 mL).
- For suspension cells: Plate at a density of approximately 2.5 x 10^5 cells/mL.
- Spin cells down and aspirate off medium.
- Resuspend cells in Nucleofector™ Solution, with supplement added, at a volume of 100 µL per well.
- If using PURedit™ Cas9 or Cas9 Plus, add the appropriate amount of transfection buffer to the cell suspension solution. For detailed information, see the product information sheets.

Nucleofection with Synthetic RNA/Cas9 Protein CRISPR Complexes

- Place 0.5 mL of Complete Medium to each well of a 12-well plate.
- Add between 18 and 90 picomoles each of the synthetic guide RNAs to sterile tubes on ice. If using Cas9 Nickase, keep paired sgRNAs separated.
- Add between 1 and 5 µg of Cas9 protein to each synthetic guide RNA, mix gently and incubate at room temperature for 10–15 minutes, forming RNP complexes. If RNP samples are stored on ice after complexing, warm the samples to room temperature for 5–10 minutes prior to transfection.
- Add 100 µL of resuspended cells in Nucleofector™ solution to the RNP mix and pipet gently to mix completely. If using Cas9 Nickase, add 100 µL of resuspended cells in Nucleofector™ solution to one of the Nickase sgRNAs and pipet gently to mix completely. Then pipet the entire mix to the other paired Nickase-RNP complex and pipet gently to mix completely.
- Transfer cell/RNP complex suspension to certified cuvette. Close cuvette with cap.
- Select the appropriate Nucleofector™ Program. For K562 cells from ATCC, select T-016.
- Insert the cuvette with cell/RNP complex suspension into the Nucleofector™ Cuvette Holder and apply the selected program by pressing the X-button.
- Take the cuvette out of the holder once the program is finished.

Distribute the nucleofected cells to each well

- Immediately add 400 µL of complete medium to the cuvette and gently transfer the sample into the appropriate well of the prepared 12-well plate. Use supplied pipettes and avoid repeated aspiration of the sample.
- Incubate for 24–72 hours. Follow the recommended growth conditions for each specific cell line.
- Harvest cells and assay as required.

CRISPR Chemically Synthesized single guide RNA (RNP) Nucleofection Protocol in a CD8+ Primary T Cell Line

Prepare Synthetic RNAs and Cas9 protein(s)

- Resuspend the lyophilized synthetic RNAs to a final concentration of 30 µM or 30 picomoles per µL in 10 mM Tris pH 7.4 buffer [Dilute 1 M Trizma Hydorchloride Solution (Cat. No. T2663) to 10 mM with nuclease free water (Cat. No. W4502)]. A lower concentration of synthetic RNA may be required if using lower amounts of Cas9 protein.
- Resuspend the lyophilized Cas9 protein in nuclease free water with 20% glycerol (Cas9 protein is formulated in 20 mM Hepes, 150 mM KCl, and 1% sucrose, pH 7.5 before lyophilization).
- If a lower concentration of Cas9 protein is desired, dilute the Cas9 protein with Dilution buffer (20 mM Hepes, 150 mM KCl, 1% sucrose, pH 7.5). NOTE: Discard the diluted protein after use. Diluted protein cannot be stored at –20 °C and freeze-thawed in the absence of glycerol.
- For short term storage of Cas9 protein (within one month), aliquots can be stored at –20 °C; –70 °C for long term storage.

Prepare Nucleofector™ Solution and Cells

- Warm Amaxa Human T Cell Nucleofector™ Kit (Cat. No. VAPA-1002) solution to room temperature.
- Add entire supplement to Nucleofector™ Solution.
- Obtain enough cells for approximately 250k stimulated cells per well in a 12-well plate (final volume per well will be 1 mL).
- For suspension Primary T-cells: Plate at a density of approximately 2.5 x 10^5 cells/mL.
- Spin cells down and aspirate off medium.
- Resuspend cells in Nucleofector™ Solution, with supplement added, at a volume of 100 µL per well. If using PURedit™ Cas9 or Cas9 Plus, add the appropriate amount of Transfection buffer to the cell suspension solution. For detailed information, see the product information sheets.

Nucleofection with Synthetic RNA/Cas9 CRISPR complexes

- Place 0.5 mL of Complete Medium, with all added interleukins to each well of a 12-well plate, depending on your previous stimulated cell culture.
- Add Dynabeads™ (ThermoFisher Cat. No. 11362D) 1 : 0.25–0.5 ratio of cells : beads, depending on your previous stimulated cell culture.
- Add between 18 and 90 picomoles each of the paired synthetic guide RNAs to a sterile tube on ice. If using Cas9 Nickase, keep paired Nickase guides separated.
- Add between 1 and 5 µg of Cas9 protein to each synthetic guide RNA, mix gently and incubate at room temperature for 10–15 minutes, forming RNP complexes. If RNP samples are stored on ice after complexing, warm the samples to room temperature for 5–10 minutes prior to transfection.
- Add 100 µL of resuspended cells in Nucleofector™ solution to the RNP mix and pipet gently to mix completely. If using Cas9 Nickase, add 100 µL of resuspended cells in Nucleofector™ solution to one of the Nickase sgRNAs and pipet gently to mix completely. Then pipet the entire mix to the other paired Nickase-RNP complex and pipet gently to mix completely. Transfer cell/RNP complex suspension to certified cuvette. Close cuvette with the cap.
- Select the appropriate Nucleofector™ Program. For Primary T-cells from ATCC, select T-020 or T-023
- Insert the cuvette with cell/RNP complex suspension into the Nucleofector™ Cuvette Holder and apply the selected program by pressing the X-button.
- Take the cuvette out of the holder once the program is finished.

Distribute the nucleofected cells to each well

- Immediately add 400 µL of complete medium to the cuvette and gently transfer the sample into the appropriate well of the prepared 12-well plate. Use supplied pipettes and avoid repeated aspiration of the sample.
- Incubate for 24–72 hours. Follow the recommended growth conditions for each specific primary cell line.
- Harvest cells and assay as required.

CRISPR Chemically Synthesized single guide RNA (RNP) Protocol for Microinjection of Animal Models

Recommendations for RNA handling and general considerations:

- Use dedicated, clean workspace—RNA only
- Use dedicated pipettes and reagents—RNA only
- Use barrier pipette tips
- Use RNAse-free plasticware
- Keep RNA on ice or at 4 °C while handling/centrifuging
- Spin RNPs at high RPM just before injection to remove particulate matter that may clog the needle

Preparation of RNPs for microinjection

* Due to the variability in microinjection protocols below are suggested parameters based on compiled protocols and publications (see references). Optimization of volumes and concentrations may be required for best results:

- Resuspend the lyophilized sgRNAs to a final concentration of 30 µM in 10 mM Tris pH 7.4 buffer [Dilute 1 M Trizma Hydorchloride Solution (Cat. No. T2663) to 10 mM with nuclease free water (Cat. No. W4502)]. A lower concentration of sgRNA may be required if using lower amounts of Cas9 protein.
- Resuspend the lyophilized Cas9 protein to a concentration of 30 µM of nuclease free water with 50% glycerol (Cas9 protein is formulated in 20 mM Hepes, 150 mM KCl, and 1% sucrose, pH 7.5 before lyophilization).

RNP mix for microinjection:

- Combine sgRNA and Cas9 into appropriate injection buffer (typical injection buffer is 10 mM Tris pH 7.4, 0.25 mM EDTA and filtered through an Anotop .02 µM syringe)
- Cas9 to a final concentration of 6 µM
- sgRNA to a final concentration of 6.6 µM
- Centrifugation step to remove particles before microinjection

Suggested Donor DNA plasmid preparation for microinjection (for targeted integration projects only):

- Perform endotoxin-free plasmid DNA preps.
- Perform stability test to confirm DNA is not contaminated with RNase.

Plasmid Donor
- Once DNA quality is confirmed, dilute donor in microinjection buffer along with RNP. Recommended final concentration of donor DNA plasmid is 1 ng/uL. (It is recommended to store donor plasmid, resuspended in water, at –20 °C, 100–1000 ng/uL, then dilute a small aliquot in microinjection buffer for each use.)

Single stranded oligo donor (ssODN)
- Once DNA quality is confirmed, dilute donor in microinjection buffer along with RNP. Recommended final concentration is 0.5–2.5 µM.

Supporting documents—Injection of RNA encoding targeted nucleases is well established in the literature. A few key examples:

- First example rapid gene knockout via injection of ZFN mRNA: Geurts, Aaron M., et al. "Knockout rats via embryo microinjection of zinc-finger nucleases." Science 325.5939 (2009): 433-433.
- First example of directed mutations via ZFN and donor plasmid injection: Cui, Xiaoxia, et al. "Targeted integration in rat and mouse embryos with zinc-finger nucleases." Nature biotechnology 29.1 (2011): 64-67.
- First example of ZFN and single strand oligo DNA donor: Meyer, Melanie, et al. "Modeling disease mutations by gene targeting in one-cell mouse embryos." Proceedings of the National Academy of Sciences109.24 (2012): 9354-9359.
- Repeat of ZFN, oligo, and donor plasmid approach as above references, but with CRISPR nucleases: Wang, Haoyi, et al. "One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering." Cell 153.4 (2013): 910-918.
- Manipulating the Mouse Embryo: A Laboratory Manual ISBN 978-1-936113-01-9

CRISPR Chemically Synthesized Single guide RNA (RNP) Protocol for Electroporation of Animal Models

Prepare Cas9 and sgRNA

- Resuspend the lyophilized sgRNAs to a final concentration of 30 µM in 10 mM Tris pH 7.4 buffer [Dilute 1 M Trizma® hydrochloride solution (Cat. No. T2663) to 10 mM with nuclease free water (Cat. No. W4502)]. A lower concentration of sgRNA may be required if using lower amounts of Cas9 protein.
- Resuspend the lyophilized Cas9 protein to a concentration of 30 µM of nuclease free water with 50% glycerol (Cas9 protein is formulated in 20 mM Hepes, 150 mM KCl, and 1% sucrose, pH 7.5 before lyophilization).

RNP mix for electroporation:

- Cas9 to a final concentration of 6 µM
- sgRNA to a final concentration of 6.6 µM
- Incubate the Cas9-sgRNA for 10 minutes at room temperature

If adding a donor template:

- 10 µM ssODN (approx. 100–130 nt).
- For short term storage of Cas9 protein (within one month), aliquots can be stored at –20 °C; –70 °C recommended for long term storage. NOTE: Discard any diluted protein after use. Diluted protein cannot be stored at –20 °C and freeze-thawed in the absence of glycerol.

Electroporation of Embryos with Cas9-sgRNA complexes

- Prepare KSOM-AA drops, ~20 µL, and cover with mineral oil, incubate at 37 °C, 5% CO_2 for a minimum of 30 minutes
- Prepare 2 mL Opti-MEM I in 35 x 10 mm petri dish, incubate at 37 °C with 5% CO_2 for a minimum of 30 minutes
- Collect zygotes from superovulated donors, wash thoroughly in M2 drops
- Divide 50–80 embryos in each KSOM-AA drop
- Just before electroporation, make 2 x 150–200 µL Opti-MEM I drops (from step 1) in a 60 x 15 mm petri dish
- Wash 50–80 zygotes in 2x 150–200 µL Opti-MEM I drops. Use glass pipette filled with KSOM and 2 air bubbles (no mineral oil!) to manipulate embryos in Opti-MEM (embryos are very sticky in this medium)
- Mix 10 µL Opti-MEM I (from step 1) with 10 µL Cas9-sgRNA mix
- Transfer 50–80 embryos into Opti-MEM I and Cas9-sgRNA mix (use as little media as possible, try not to exceed 10% of total slide volume of 20 µL)
- Transfer Opti-MEM and Cas9-sgRNA mix (as much as you can) with 50–80 embryos to electroporation slide. Use empty new glass pipette (no KSOM) each time to transfer embryos
- Make sure to align embryos in a single file between the electrodes of the slide
- Electroporate using the following BioRad GenePulser Xcell settings:
  - 30 V
  - 3 ms Pulse
  - 100 ms Interval
  - 7 pulses
- Remove electroporated embryos and wash in equilibrated KSOM-AA,
- Incubate at 37 °C in 5% CO_2
- Wash slide with MQ water between electroporation and air dry
- Transfer 20–25 electroporated embryos (same day) or 20 divided embryos (next day) per pseudopregnant females.

CRISPR Chemically Synthesized single guide RNA Using X-tremeGENE™ 360 Transfection Reagent

- Allow X-tremeGENE™ 360 Transfection Reagent, Cas9 protein, guide RNA, and diluent to equilibrate to between 15 °C and 25 °C
- Briefly vortex the X-tremeGENE™ 360 Transfection Reagent vial
- Dilute a 12 µM guide RNA stock solution with appropriate diluent, such as serum-free medium, to a final concentration of 12 nM per well using a sterile tube; mix gently by pipetting.
- If using 2-part crRNA + tracrRNA, combine at a 1:1 molar ratio and incubate for 10 minutes between 15 °C and 25 °C to anneal. Then add to tube containing the appropriate diluent.
- Add a 6 µM Cas9 protein stock solution to the guide RNA at a final concentration of 6 nm per well; mix gently by pipetting
- A 2:1 ratio of guide RNA to Cas9 protein is a recommended starting point. Further optimization may be required
- Incubate the mixture for 10 minutes at between 15 °C and 25 °C to allow RNPs to form
- Add X-tremeGENE™ 360 Transfection Reagent to the RNP mixture; mix gently by pipetting
  Note: The amount of X-tremeGENE™ 360 Transfection Reagent needed may vary by cell type. See ‘Guidelines for preparing reagent: CRISPR-RNP complexes for various culture vessel sizes’ below, to determine component amounts corresponding to the surface area of the cell culture vessel being used
- Incubate the transfection reagent: RNP complex mixture for 15 minutes between 15 °C and 25 °C

- Remove the culture vessel from the incubator; removal of growth medium is not necessary
- Add the transfection complex to the cells in a dropwise manner
- Gently shake or swirl the wells or flasks to ensure even distribution over the entire plate surface. If available, use a rotating platform shaker for 30 seconds at low speed for mixing 96-well plates
- Once the transfection reagent: RNP complex has been added to the cells, there is no need to replace with fresh medium as may be required with other transfection reagents
- Following transfection, incubate cells for 24 to 72 hours
- The duration of incubation will depend on many factors, including the transfected material, the cell type being used, the cell medium, cell density, and the target gene(s)
- After the incubation period, measure the efficiency using an assay appropriate for your system.

Guidelines for preparing reagent: CRISPR-RNP complex for various culture vessel sizes

Culture vessel	Surface area [cm^2]	Total volume of medium [mL]	Suggested amount of transfection complex to add to each well [µL]	Cas9 protein (6 µM stock) 6 nM final [µL]	sgRNA (12 µM stock) 12 nM final [µL]	Amount of X-tremeGENE™ 360 Transfection Reagent [µL]
96-well plate (1 well)	0.3	0.1	10	0.1	0.1	0.2
48-well plate (1 well)	1.0	0.3	30	0.3	0.3	0.6
24-well plate (1 well)	1.9	0.5	50	0.5	0.5	1.0
12-well plate (1 well)	3.8	1.0	100	1.0	1.0	2.0
35 mm dish	8.0	2.0	200	2.0	2.0	4.0
6-well plate (1 well)	9.4	2.0	200	2.0	2.0	4.0
60 mm dish	21	5.0	500	5.0	5.0	10
10 cm dish	55	10	1,000	10	10	20
T-25 flask	25	6.0	600	6.0	6.0	12
T-75 flask	75	20	2,000	20	20	40

CRISPR Chemically Synthesized single guide RNA (RNP) Nucleofection Protocol for Human Pluripotent Stem Cells

The protocol presented here is provided by our Advanced Cell Engineering Services. The partner you can trust to create and deliver customized cellular models for your drug discovery and disease model needs. Our scientists have decades of experience in genome editing and engineering cell lines. We excel in completing the most challenging projects—having engineered over 200 different genes in over 150 various parental cell lines. Our team utilizes the most innovative technologies in the industry for clone identification, enabling high success rates for modifying even the most complex cell lines and targets.

Materials

Extracellular Matrix Coating

- Pluripotent cell types require the use of tissue culture plates coated with a matrix to support cell attachment and expansion. We recommend using one of the below matrix coatings:
  - Stem Cell Qualified ECM Gel: Cat. No. CC131
  - ECM Gel from Engelbreth-Holm-Swarm murine sarcoma: Cat. No. E1270
  - Matrigel® hESC-Qualified Matrix, 354277 LDEV-free: Corning® 354277
  - Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham: Cat. No. D6434

Below are general guidelines for the coating of 6-well plates using the Stem Cell Qualified ECM Gel Matrix (CC131). All procedures should be performed under aseptic conditions in a biological safety cabinet.

- Thaw the ECM Gel Matrix on ice or in a 2–8 °C fridge one day before use. Once thawed, maintain ECM Gel on ice at all times and use pre-cooled medium and pipettes to avoid gelling of the product.
- Target coating concentration is 50 µg/mL. Dilute the ECM Gel Matrix with cold medium according to the lot specifications and certificate of analysis. Scale up or down according to the volumes required.

  Note: The recommended dilutions range from 1:25 to 1:80, however more concentrated ECM Gel may be used if desired.
- Add 1 mL of the diluted ECM to each well of a 6-well plate or 5 mL to one T75 flask. Swirl the culture vessel to spread the ECM evenly across the growth surface. It is important that the growth surface of each culture vessel is completely and evenly coated. Cells will not attach properly where there is insufficient ECM coating.
- Store in a 2–8 °C fridge overnight or at least 2 hours in the fridge before use. Alternatively, plates can be coated for 30 minutes in a 37 °C incubator. If not used immediately, parafilm wrap the ECM coated culture plates and store at 2–8 °C until ready to use. Use the ECM coated culture plates within 3–4 days.
- Prior to seeding the cells, bring the plate to room temperature for 10–15 minutes, remove the coating solution and add 2 mL/well (6-well plate) of human ES/iPSC growth media. Cells can now be plated onto the newly coated plates.
- Prepare synthetic gRNAs and Cas9 or Cas9-Nickase protein

  Note: This protocol is based on using 1 x 10^6 cells per nucleofection. This protocol is for two samples. For more samples, adjust the following protocol as necessary. Where targeted integration is desired, donor molecules must be designed and included in the transfections as well, and will require specific detection assays to determine integration efficiency. These methods will be governed by overall cleavage efficiency of the nuclease, distance between the cut site to the desired mutation site, and local sequence composition. For best results, it is highly recommended that the end user optimizes donor designs based on these criteria.

Materials specific for iPS Cells

- P3 Primary Cell 4D-Nucleofector™ X Kit L: Lonza V4XP-3024
- 4D-Nucleofector™ X Unit: Lonza AAF-1002X
- mTeSR™ 1 culture media: Stem Cell Technologies 85850
- Accutase® Solution: Cat. No. A6964
- ROCK Inhibitor (Y-27632): Cat. No. SCM075

Protocol

- Resuspend the lyophilized synthetic RNAs to a final concentration of 30 µM or 30 picomoles per µL in 10 mM Tris pH 7.4 buffer [Dilute 1 M Trizma® hydrochloride solution (Cat. No. T2663) to 10 mM with nuclease free water (Cat. No. W4502)]. A lower concentration of synthetic RNA may be required if using lower amounts of Cas9 protein.
- Resuspend the lyophilized Cas9 protein in nuclease free water with 50% glycerol (Cas9 protein is formulated in 20 mM Hepes, 150 mM KCl, and 1% sucrose, pH 7.5 before lyophilization).
- If a lower concentration of Cas9 protein is desired, dilute the Cas9-Nickase protein with dilution buffer (20 mM Hepes, 150 mM KCl, 1% sucrose, pH 7.5).

  NOTE: Discard the diluted protein after use. Diluted protein cannot be stored at –20 °C and freeze-thawed in the absence of glycerol.
- For short term storage of Cas9 protein (within one month), aliquots can be stored at –20 °C; –70 °C for long term storage.
- Enzymatically remove hiPS cells from plate surface with Accutase® containing 10 uM Rock Inhibitor (RI) (Y-27632) methodology. This should result in roughly 1.5 to 2 x 10^6 cells per confluent well of a 6-well plate.

- Transfer cell suspension volume that achieves 2 x 10^6 cells to a 15 mL conical tube and centrifuge at 200 x g for 5 minutes.
- Open Lonza P3 kit. Add 200 µL of P3 solution to a clean 1.5 mL Eppendorf tube. Then add 44 µL of P3 supplement to the 200 µL of P3 solution.
- Gently mix. Aliquot 50 µL to an Eppendorf tube. Repeat for a 2nd Eppendorf tube.
- For tube #1, add 2 µL pmaxGFP plasmid (2 µg total, Lonza kit) as nucleofection control.
- For tube #2 (and subsequent tubes), add 2–10 µg of pre-paired RNP from above.
- Retrieve cells from centrifuge and aspirate media from cell pellet.
- Add 100 µL of P3 supplemented solution to cell pellet and gently resuspend. If using PURedit™ Cas9 or Cas9 Plus, add the appropriate amount of Transfection buffer to the cell suspension solution. For detailed information, see the product information sheets.
- Aliquot 50 µL of cell suspension to tube #1, mix by pipetting up and down 2–3 times. Transfer all contents to a labelled Lonza 4D cuvette.
- Repeat with tube #2.
- Conduct nucleofection with either of the two below conditions.
  a. P3 solution: CM100 (20–50% survival, >90% nucleofection efficiency)
  b. P3 solution: CM130 (50–75% survival, >75% nucleofection efficiency)
- After nucleofection, let the cuvettes incubate for 10 minutes. This step increases the nucleofection efficiency, but incubation past 10 minutes begins to increase cell death.
- At 10 minutes, transfer cell suspension to mTeSR™ with ROCK Inhibitor.
  a. For maximal recovery when nucleofecting with CM100, it is advisable to plate all 1 x 10^6 cells onto 1 well of a ECM coated plate.
  b. For CM130 conditions, it is advisable to plate in either 1 or 2 wells of a 6-well plate.
- Incubate overnight in a 37 °C, 5% CO_2 humidified incubator.
- Change media with mTeSR™ (no ROCK Inhibitor supplementation) and continue culturing iPS normally.
- Harvest cells and assay as required.

Assessing the Cleavage Efficiency of the Targeted Nuclease

- Following transfection of the nuclease reagents, cells should be incubated for 24–72 hours before assessment of nuclease activity. The T7 Endonuclease Detection Assay is a well-known method for detecting genome editing events from CRISPR (Cat. No. T7E1001-1KT). The protocol for this kit can be found in the product information sheet.
- Additional sequence-based methods for assessing the rate of mutation are also available. TIDE utilizes sanger-based sequencing in conjunction with a specially developed algorithm that identifies major induced mutations in the projected editing site. Other methods utilized targeted next-generation sequencing methods and alternative web-based software for calculating the rate of targeted mutation. An excellent summary and resource of these above methods can be found on the following page **SigmaAldrich.com/T7E1** and in the following publication:

  Sentmanat, M.F., Peters, S.T., Florian, C.P. et al. A Survey of Validation Strategies for CRISPR-Cas9 Editing. Sci Rep 8, 888 (2018). https://doi.org/10.1038/s41598-018-19441-8

Clone Isolation and Screening

- It is highly recommended that clonal isolates be generated for genotypic screening to obtain pure populations of either knock-out cells or SNP converted cells. Limiting dilution and FACS are among the most common methods for cloning. We recommend optimization of the cloning conditions for your desired cell line prior to attempting gene editing.

Troubleshooting

Type of problem	Problem Definition	Solution
Cell Type	gRNAs for my cell type are not currently available in your webtool	Please contact our technical services team. We can provide designs for custom genomes for an additional fee.
No or low indels detected in cells/embryos	No SpCas9 protein source	Cas9 proteins proteins need be added to form CRISPR-RNP complexes; i.e. use Sigma-Aldrich® Cas9 proteins. GFP proteins aid in visual detection of RNP in your cells or embryos with no loss in activity compared to WT.
	sgRNAs are degraded	Store sgRNA in proper conditions. The sgRNAs have been shown to withstand several rounds of freeze-thaws without a decrease in activity, but aliquoting the sgRNA into smaller quantities upon resuspension will ensure the highest, consistent activity.
		Under normal cell culture conditions, synthetic RNA modifications are not required; however, for certain cell lines, this may be necessary. Stabilizing modifications are available on all synthetic gRNAs
	sgRNAs not paired for nickase applications	Paired sgRNAs are needed for Cas9 nickase-RNPs. Using our CRISPR Design tool, optimally paired sgRNAs can be chosen for your gene of interest.
	Improper amounts or ratios of Cas9 proteins and sgRNAs	The ratio of Cas9 proteins to sgRNA is important. See the protocol for recommended ratios or the product information sheets supplied for each Cas9 protein on the product pages.
No or low indels detected in cultured cells	Cells died or look unhealthy after harvest with RNP	For any transfection reagent or nucleofection, the protocol should be optimized for each cell line used. Refer to the manufacturer's protocol for further assistance. Make sure the cells are of proper confluency and growth rate
	The transfection or nucleofection is not working or causes excessive cell death.	
	Incorrect cell type	Use correct cell type for your targets (i.e., mouse cell line for mouse gRNA targets)
No or low indels detected in animal embryos following microinjection	Microinjection	The microinjection method is highly variable and often results in inconsistent gene editing. Test RNP complex editing in a mouse cell line to determine efficacy of Cas9 protein and sgRNA sequences. Optimize the microinjection method using references in this protocol or use electroporation.

References and Further Reading:

1. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., Zhang, F. (2013) Multiplex Genome Engineering Using CRISPR/Cas Systems. Science 339:6121, 819–823.
2. Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., Sander, J.D. (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nature Biotechnology 31, 822–826.
3. Fu Y, Sander JD, Reyon D, Cascio V, and Joung KS. 2014. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nat Biotechnol. 32(3), 279–284.
4. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier E. (2012) A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 337, 816–821.
5. Kim S, Kim D, Cho SW, Kim J and Kim JS. 2014. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res. 24, 1012–1019.
6. Mali, P., Aach, J., Stranges, P.B., Esvelt, K.M., Moosburner, M., Kosuri, S., Yang L., Church Church, G.M. (2013) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nature Biotechnology 31, 833–838.
7. Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M. (2013) RNA-Guided Human Genome Engineering via Cas9. Science 339, 823–826.

MSDS Information

Material Safety Data Sheet

Date Printed: 28.11.2019

Date Updated: 16.08.2016 Version 2.0 According to Regulation (EC) No. 1907/2006

1. Product and Company Information
   Product Name Product Number
   Company
   Technical Phone # Fax
   OLIGONUCLEOTIDES N/A
   Sigma-Aldrich Company Ltd. The Old Brickyard
   SP8 4XT New Road, Gillingham GB
   44-(0)-1747-833000 44-(0)-1747-833313
   Emergency Phone #
   CAS # None
   44-(0)-1747-833100

2. Composition/Information on Ingredients Product Name
   OLIGONUCLEOTIDES
   Formula Molecular Weight Synonyms

3. Hazards Identification
   EC no
   Annex I Index Number
   Not a hazardous substance or mixture according to Regulation (EC) No. 1272/2008.

4. First Aid Measures
   After Inhalation: If inhaled, remove to fresh air. If breathing becomes difficult, call a physician.
   After Skin Contact: In case of contact, immediately wash skin with soap and copious amounts of water.
   After Eye Contact: In case of contact with eyes, flush with copious amounts of water for at least 15 minutes.
   Assure adequate flushing by separating the eyelids with fingers.
   After Ingestion: If swallowed, wash out mouth with water provided person is conscious. Call a physician.

5. Fire Fighting Measures
   Extinguishing Media Suitable
   Water spray. Carbon dioxide, dry chemical powder, or appropriate foam.
   Special Risks Specific Hazard(s) No data available
   Special Protective Equipment for Firefighters
   Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.

6. Accidental Release Measures Procedure(s) of Personal Precaution(s)
   Exercise appropriate precautions to minimize direct contact with skin or eyes and prevent inhalation of dust.
   Methods for Cleaning Up
   Sweep up, place in a bag and hold for waste disposal. Avoid raising dust. Ventilate area and wash spill site after material pickup is complete.

7. Handling and Storage
   Directions for Safe Handling: Further processing of solid materials may result in the formation of combustible dusts. The potential for combustible dust formation should be taken into consideration before additional processing occurs.Provide appropriate exhaust ventilation at places where dust is formed.
   Storage: Conditions of Storage Keep tightly closed.

8. Exposure Controls/Personal Protection Engineering Controls
   Safety shower and eye bath. Mechanical exhaust required.
   General Hygiene Measures: Wash thoroughly after handling.
   Personal Protective Equipment: Respiratory Protection
   Respiratory protection is not required. Where protection from nuisance levels of dusts are desired, use type N95 (US) or type P1 (EN 143) dust masks. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).
   Hand Protection: Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices.
   Wash and dry hands
   Eye Protection: Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

9. Physical and Chemical Properties

   9.1 Information on basic physical and chemical properties
   - a) Appearance
   - b) Odour
   - c) Odour Threshold
   - d) pH
   - e) Melting point/freezing point
   - f) Initial boiling point and boiling range
   - g) Flash point
   - h) Evaporation rate
   - i) Flammability (solid, gas)
   - j) Upper/lower flammability or explosive limits
   - k) Vapour pressure
   - l) Vapour density
   - m) Relative density
   - n) Water solubility
   - o) Partition coefficient: noctanol/water
   - p) Auto-ignition temperature
   - q) Decomposition temperature
   - r) Viscosity
   - s) Explosive properties
   - t) Oxidizing properties

   9.2 Other safety information
   No data available

10. Stability and Reactivity

10.1 Reactivity
No data available

10.2 Chemical stability
Stable under recommended storage conditions.

10.3 Possibility of hazardous reactions
No data available

10.4 Conditions to avoid
No data available

10.5 Incompatible materials
Strong oxidizing agents

10.6 Hazardous decomposition products
Form: solid

Hazardous decomposition products formed under fire conditions. Nature of decomposition products not known.
Other decomposition products—No data available

In the event of fire: see section 5

11. Toxicological Information

11.1 Information on toxicological effects

Acute toxicity

Oral: No data available Inhalation: No data available Dermal: No data available

Skin corrosion/irritation
No data available

Serious eye damage/eye irritation
No data available

Respiratory or skin sensitisation
No data available

Germ cell mutagenicity
No data available

11.2 Carcinogenicity

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.

Reproductive toxicity
No data available

Specific target organ toxicity—single exposure
No data available

Specific target organ toxicity—repeated exposure
No data available

Aspiration hazard
No data available

Additional Information
RTECS: Not available

12. Ecological Information

12.1 Toxicity
No data available

12.2 Persistence and degradability
No data available

12.3 Bioaccumulative potential
No data available

12.4 Mobility in soil
No data available

12.5 Results of PBT and vPvB assessment
PBT/vPvB assessment not available as chemical safety assessment not required/not conducted

12.6 Other adverse effects
No data available

13. Disposal Considerations

13.1 Waste treatment methods Product
Offer surplus and non-recyclable solutions to a licensed disposal company.
Contaminated packaging
Dispose of as unused product.

14. Transport Information

14.1 UN number
ADR/RID: -

14.2 UN proper shipping name
ADR/RID: Not dangerous goods IMDG: Not dangerous goods IATA: Not dangerous goods

14.3 Transport hazard class(es)
ADR/RID: -

14.4 Packaging group
ADR/RID: -

14.5 Environmental hazards
ADR/RID: no

14.6 Special precautions for user
No data available

IMDG: -

IMDG Marine pollutant: no

IATA: no

15. Regulatory Information

15.1 Safety, health and environmental regulations/legislation specific for the substance or mixture.
This safety datasheet complies with the requirements of Regulation (EC) No. 1907/2006.

15.2 Chemical safety assessment. For this product a chemical safety assessment was not carried out

16. Other Information

Warranty

We make no warranties of any kind or nature, express or implied, including any implied warranty of merchantability or fitness for any particular purpose, with respect to any technical assistance or information that we provide. Any suggestions regarding use, selection, application or suitability of the products shall not be construed as an express or implied warranty unless specifically designated as such in a writing signed by an officer or other authorized representative of our company.

Liability

We shall not in any event be liable for incidental, consequential, indirect, exemplary or special damages of any kind resulting from any use or failure of the products. All sales are subject to our Terms and Conditions of Sale or an agreement between the parties governing the purchase and sale of the products, if applicable.

Merck KGaA
Frankfurter Strasse 250
64293 Darmstadt, Germany

SigmaAldrich.com

MK_UG6430EN Ver. 1.0
39371
01/2022