TECHNICAL MANUAL

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INSPIRED CELL SOLUTIONS

Dharmacon™ Edit-R™ CRISPR-Cas9 genome engineering with Cas9 Nuclease mRNA and synthetic guide RNAs

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1 Introduction to the CRISPR-Cas9 system for gene editing

CRISPR-Cas: an adaptive immunity defense mechanism in bacteria and archaea

The CRISPR (clustered regularly interspaced palindromic repeats)-Cas (CRISPR-associated proteins) system is an adaptive bacterial and archaeal defense mechanism that serves to recognize and silence incoming foreign nucleic acids. Upon infection by bacteriophage or other foreign DNA elements, a host organism can incorporate short sequences from the invading genetic material, called protospacers, into a specific region of its genome (the CRISPR locus) between short palindromic DNA repeats of variable lengths. Multiple spacer-repeat units are clustered at the CRISPR locus to form the CRISPR array. The entire locus including the CRISPR array is transcribed by RNA polymerase into a primary transcript, the pre-CRISPR RNA (pre-crRNA), which is then processed into small, mature CRISPR RNAs (crRNAs) such that they include sequences complementary to the foreign, invading DNA. crRNAs guide a multifunctional protein or protein complex (the CRISPR-associated or Cas proteins) to cleave complementary target DNA that is adjacent to short sequences known as protospacer-adjacent motifs (PAMs). Thus, the organism acquires a way to protect itself from subsequent infection (Bhaya, 2011).

A CRISPR-Cas9 platform for mammalian gene editing

Many bacterial and archaeal CRISPR-Cas systems have been identified with diverse sets of mechanisms, Cas proteins and multi-subunit complexes. In particular, the processes and key components of the *Streptococcus pyogenes* CRISPR-Cas9 system have been well-studied and adapted for genome engineering in mammalian cells. In *S. pyogenes*, only three com-ponents are required for targeted DNA cleavage at specific target sites adjacent to a PAM (Jinek, 2012): (1) The endonuclease Cas9, programmed by (2) a mature crRNA processed from transcription of the CRISPR locus/array which complexes with (3) another CRISPR locus-encoded RNA, the trans-activating CRISPR RNA (tracrRNA; Deltcheva, 2011). See Figure 1A for an illustration. Alternatively, the crRNA can be fused to the tracrRNA creating a chimeric structure termed a single guide RNA (sgRNA, Figure 1B).

Upon site-specific double-stranded DNA cleavage, a mammalian cell can repair such a break through either non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ is often imperfect, resulting in small insertions and deletions (indels) that can cause nonsense mutations resulting in gene disruptions to produce gene knockouts (Mali, 2014; Sampson, 2014). This endogenous DNA break repair process, coupled with the highly tractable *S. pyogenes* CRISPR-Cas9 system, allows for a readily engineered platform to permanently disrupt gene function.

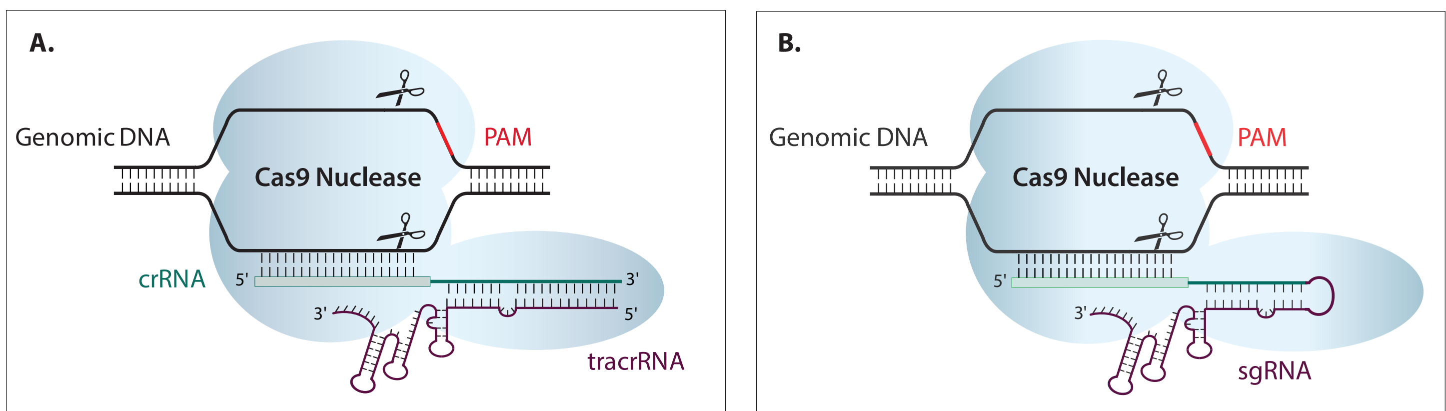

Figure 1. Illustration of CRISPR-Cas9 system. Cas9 nuclease (light blue), programmed by the crRNA (green):tracrRNA (purple) complex (A) or the sgRNA (B), cutting both strands of genomic DNA 5' of the PAM (red).

2 Edit-R Cas9 mRNA and synthetic guide RNAs for gene editing

The Edit-R reagent workflow using Cas9 mRNA and synthetic guide RNAs include these critical components required for gene editing in mammalian cells based on the natural *S. pyogenes* system: a mRNA for translation of a mammalian codon-optimized Cas9 nuclease, and a synthetic guide RNA which can be either a synthetic single guide RNA or a tracrRNA complexed with a gene-specific synthetic crRNA. All components are co-transfected into the mammalian cell of choice using the DharmaFECT™ Duo transfection reagent to perform gene disruption. Figure 2 summarizes the general experimental workflow. Each component is described below.

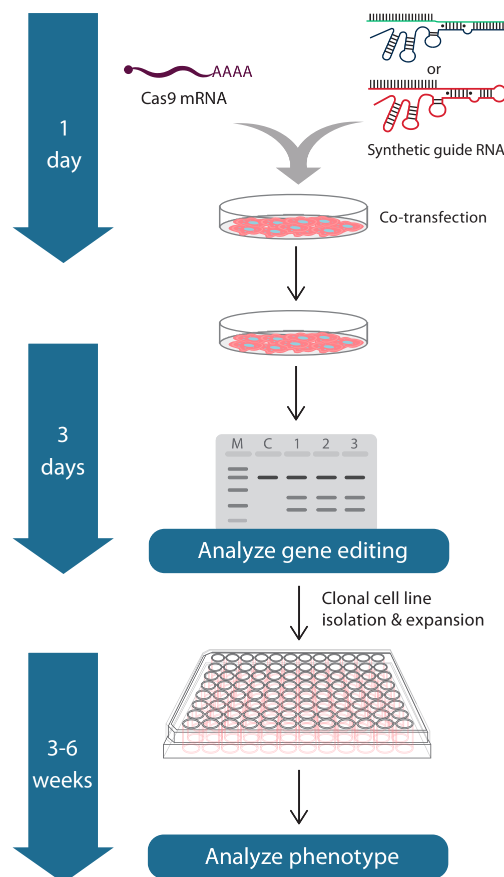

Figure 2. Gene editing with Edit-R Cas9 Nuclease mRNA and synthetic guide RNA is performed by co-transfecting all components with DharmaFECT Duo transfection reagent (or other DharmaFECT transfection reagent suitable for your specific cells of interest). One may then observe phenotypes directly. A DNA mismatch detection assay can be used to estimate gene editing efficiency prior to clonal cell line generation and characterization.

Edit-R Cas9 Nuclease mRNA

The Edit-R Cas9 Nuclease mRNA contains a human codon-optimized version of the S. pyogenes Cas9 (Csn1) gene with a 5′ and 3′ nuclear localization signal (NLS). The Cas9 mRNA is in vitro transcribed, 5′ capped and polyadenylated for translation and nuclear localization of the Cas9 protein. Edit-R Fluorescent Cas9 mRNA has the same components as Edit-R Cas9 Nuclease mRNA but includes a fluorescent protein sequence (either mKate2 or EGFP). It is comprised of a single mRNA strand that is translated into two separate proteins: the fluorescent protein followed by the Cas9 protein.

Edit-R synthetic guide RNA

Edit-R trans-activating CRISPR RNA (tracrRNA)

The Edit-R tracrRNA is a chemically synthesized and HPLC-purified long RNA based on the published *S. pyogenes* tracrRNA sequence (Jinek, 2012). It is modified for nuclease resistance and can be used with modified or unmodified Edit-R crRNA.

Edit-R CRISPR RNA (crRNA)

The Edit-R crRNA is a synthetic RNA, comprised of 20 nucleotides identical to the genomic DNA target site, or protospacer, followed by a fixed *S. pyogenes* repeat sequence that interacts with the tracrRNA. The chosen protospacer sequence in the target genomic DNA must be immediately upstream of a PAM in the genomic DNA. Predesigned crRNAs are available for human, mouse and rat coding genes on dharmacon.com and custom crRNA can be designed and ordered using the Dharmacon CRISPR Design Tool. It is modified for nuclease resistance and can be used with modified or unmodified Edit-R tracrRNA.

Edit-R synthetic single guide RNA (sgRNA)

Edit-R synthetic sgRNA is a 100 nucleotide chimera fusing the crRNA and tracrRNA sequences with a 4 nt tetraloop sequence (Jinek 2012). It is modified for nuclease resistance on both 5′ and 3′ ends of the molecule. Edit-R synthetic sgRNAs can be ordered through the Dharmacon CRISPR Design Tool.

3 Guidelines for co-transfection of Edit-R genome engineering components

Successful co-transfection of the Edit-R components (Cas9 Nuclease mRNA and synthetic guide RNA) and subsequent gene knockout requires careful optimization of delivery conditions for each cell line of interest. For general recommendations on optimizing lipid co-transfection conditions, see pages 8-10. The protocol below assumes that experimental conditions have been optimized as recommended.

Materials required for gene editing using Edit-R Cas9 Nuclease mRNA and synthetic guide RNAs

Edit-R CRISPR-Cas9 materials for gene editing can be ordered at dharmacon.com.

- Edit-R Cas9 mRNA (choose one):
  - » Edit-R Cas9 Nuclease mRNA, 20 µg (1 µg/µL; Cat #CAS11195)
    or
  - » Edit-R mKate2 Cas9 Nuclease mRNA, 20 µg (1 µg/µL; Cat #CAS11859)
    or
  - » Edit-R EGFP Cas9 Nuclease mRNA, 20 µg (1 µg/µL; Cat # CAS11860)
- Synthetic targeting guide RNA (choose one):
  - » Edit-R synthetic crRNA and tracrRNA oligos:
    a. crRNA, predesigned for your gene of interest in a variety of sizes, or designed and ordered using Dharmacon CRISPR Design Tool
    b. tracrRNA, 5, 20 or 50 nmol (Cat #U-002005-XX)
    or
  - » Edit-R synthetic sgRNA, custom ordered using the Dharmacon CRISPR Design Tool

We recommend testing at least three to five guide RNA designs per gene of interest to identify the one with highest editing efficiency that also results in complete knockout of functional protein.

- Non-targeting Control guide RNA (choose one):
  - » Edit-R crRNA Non-targeting Control (Cat #U-007501-XX, U-007502-XX, U-007503-XX, U-007504-XX, U-007505-XX)
    or
  - » Custom synthetic sgRNA non-targeting control ordered using the Dharmacon CRISPR Design Tool
- Dharmacon DharmaFECT Duo transfection reagent (Cat #T-2010-01 (0.2 mL); T-2010-02 (0.75 mL); T-2010-03 (1.5 mL); or T-2010-04 (1.5 mL × 5 tubes))

Additional materials required

Transfection experiments require standard cell culture reagents and instruments appropriate for maintenance of cells. The following additional materials are required but not supplied:

- Multi-well tissue culture plates or tissue culture dishes
- Antibiotic-free complete medium: Cell culture medium (including serum and/or supplements) recommended for maintenance and passaging of the cells of interest without antibiotic
- Flow cytometer (if performing fluorescent enrichment)
- Assay for assessing cell viability such as (Resazurin cell viability reagent)
- Assay(s) for detecting gene editing events in a cell population
- 10 mM Tris pH 7.4, nuclease-free buffer (Tris buffer) solution (Dharmacon, Cat #B-006000-100)

General protocol for co-transfection of Edit-R Cas9 Nuclease mRNA and synthetic guide RNAs

The following is a general protocol using DharmaFECT Duo transfection reagent to deliver Edit-R Cas9 Nuclease mRNA and synthetic guide RNA into cultured mammalian cells. Exact reagent amounts and parameters for co-transfection should be empirically determined through careful optimization in your cells of interest prior to experimentation (see Transfection optimization section on page 8). The protocol below describes delivery conditions in HEK293T cells in 96-well plate format and is given for illustrative purposes only. Reagent volumes can be calculated for including replicate samples as necessary.

Three different types of samples are recommended for a gene editing experiment (Table 1). All steps of the protocol should be performed in a laminar flow cell culture hood using sterile technique.

Cell plating

Optimal cell number for plating will vary with growth characteristics of specific cells and should to be determined empirically.

1. Trypsinize and count cells.
2. Dilute cells in antibiotic-free complete medium to the appropriate stock density. For example, HEK293T cells should be diluted to 20,000 cells in 0.1 mL of medium for plating at 20,000 cells/well in a 96-well plate.
3. Plate 0.1 mL of cell suspension into each well of a 96-well plate.
4. Incubate cells at 37 °C with 5% CO_2 overnight.

Table 1. Recommended samples for a gene editing co-transfection experiment.

Sample	Explanation of sample
Cas9 Nuclease mRNA with non-targeting control synthetic guide RNA	Negative control: Expression of Cas9 nuclease without targeting guide RNA
Cas9 Nuclease mRNA with gene-specific synthetic guide RNA	Gene editing sample: Expression of Cas9 nuclease programmed by guide RNAs for targeted double-strand break in gene of interest
Untransfected	No treatment control sample: Confirmation of cell viability

After each reagent addition, mix the contents of each tube by pipetting gently up and down.

Co-transfection

5. Prepare a 100 ng/µL Cas9 mRNA working solution by thawing Cas9 mRNA on ice and adding 2 µL of 1 µg/µL stock solution of Cas9 mRNA to 18 µL of Tris buffer. Verify the mRNA concentration using UV spectrophotometry at 260 nm and adjust the volume if necessary to obtain 100 ng/µL.

   On initial use, aliquot remaining Cas9 mRNA stock or working solution into convenient volumes and store all aliquots at -80 °C to minimize the number of freeze-thaw cycles (see FAQs).

6. Prepare guide RNA reagents for transfection.
   For crRNA and tracrRNA:

   a. Prepare a 10 µM crRNA stock solution by adding the appropriate volume of Tris buffer to crRNA. Verify the RNA concentration using UV spectrophotometry at 260 nm and adjust the volume if necessary to obtain 10 µM.

   b. Prepare a 10 µM tracrRNA stock solution by adding the appropriate volume of Tris buffer to tracrRNA. Verify the RNA concentration using UV spectrophotometry at 260 nm and adjust the volume if necessary to obtain 10 µM.

   c. Prepare a 2 µM crRNA:tracrRNA transfection complex by adding 2 µL of crRNA and 2 µL of tracrRNA to 6 µL of Tris buffer (total volume is 10 µL).

   For synthetic sgRNA:

   a. Prepare a 2 µM synthetic sgRNA stock solution by adding the appropriate volume of Tris buffer to the sgRNA. Verify the RNA concentration using UV spectrophotometry at 260 nm and adjust the volume if necessary to obtain 2 µM.

7. In a 1.7 mL tube (or deep well plate) prepare for each sample to be transfected as described in Table 2 (columns 2-4) for a final 25 nM concentration of the synthetic guide RNA and 200 ng/well of Cas9 mRNA.

8. Prepare a 50 µg/mL DharmaFECT Duo working solution by diluting 5 µL of 1 mg/mL stock DharmaFECT Duo transfection reagent in 95 µL serum-free medium and mix gently; this volume is sufficient for 10 wells with 0.5 µL/well in 96-well format. Incubate for 5 minutes at room temperature.

Table 2. Preparing transfection samples for gene editing experiment in a 96-well plate format.

Sample	Serum-free medium	Synthetic guide RNA transfection complex (2 µM)	Cas9 mRNA working solution (100 ng/µL)	DharmaFECT Duo working solution (70 µg/mL)	Growth Medium	Total Volume per well
Cas9 Nuclease mRNA with non-targeting control synthetic guide RNA	6.75	1.25	2	10	80	100
Cas9 Nuclease mRNA with gene-specific synthetic guide RNA	6.75	1.25	2	10	80	100
Untransfected	20	0	0	0	80	100

Volumes (µL) are for a single well of a 96-well plate of HEK293T cells to be transfected. It is recommended to prepare sufficient sample volumes for the total number of replicates and to account for pipetting error. Exact reagent amounts for co-transfection in other cell lines of interest should be empirically determined through careful optimization prior to experimentation (see "Transfection optimization" Section on page 8).

9. Add 10 µL DharmaFECT Duo working solution to each sample tube as shown in Table 2 (column 5); this will result in 0.5 µg/well final concentration. DO NOT add DharmaFECT Duo working solution to the untransfected control, which should contain serum-free medium only. This brings the total volume to 20 µL in each tube. Mix by pipetting gently up and down and incubate for 20 minutes at room temperature.

10. Prepare transfection medium by adding 80 µL antibiotic-free complete medium to each sample to bring the total volume in each tube to 100 µL (columns 6 and 7).

11. Remove medium from the wells of the 96-well plate containing cells and replace with 100 µL of the appropriate transfection medium to each well.

If using Edit-R Fluorescent Cas9 mRNA, we suggest enriching for positive fluorescent cells using FACS 24 hours after transfection. Refer to the protocol "Using Dharmacon Edit-R Fluorescent Cas9 mRNA for enrichment of transfected cells" for more information.

12. Incubate cells at 37 °C with 5% CO_2 for 72 hours before proceeding with gene editing analysis.

Take an aliquot of the cells for analysis of gene editing (see "Gene editing assay recommendations"). Enough cells must be retained after gene editing confirmation for clonal cell line generation.

Gene editing assay recommendations

The most commonly used method for detection of indels in a cell population is a DNA mismatch detection assay (Guschin, 2010; Reyon, 2012; Cong, 2013). This assay can be performed on either purified genomic DNA or whole cell lysate using T7 Endonuclease I.

When edited cells are expanded and clonal populations are obtained, the most commonly used method for characterizing gene editing is Sanger sequencing (Reyon, 2012).

Guidelines for electroporation of Edit-R genome engineering components

Successful electroporation of the Edit-R components (Edit-R Cas9 Nuclease mRNA and synthetic guide RNA), and subsequent gene knockout, requires careful optimization of delivery conditions with appropriate electroporation reagents and parameters for each cell line of interest. For a general electroporation protocol see: Edit-R Cas9 Nuclease mRNA Electroporation—Protocol.

4 Transfection optimization

To obtain the highest transfection efficiency of the Edit-R CRISPR-Cas9 components with minimal effects on cell viability, we recommend carefully optimizing transfection conditions for each cell line. Perform transfection optimization using the Edit-R Cas9 Nuclease mRNA with a positive control crRNA and tracrRNA or design a positive control synthetic sgRNA using the Dharmacon CRISPR Design Tool, to find conditions that show the highest genomic editing and > 70% cell viability compared to untreated cells. For transfection optimizations with Edit-R Fluorescent Cas9 Nuclease mRNA, observe fluorescence 24 hours after transfection and determine the conditions with the highest fluorescence intensity and > 70% cell viability compared to untreated cells. The transfection optimization can be easily performed in a 96-well format allowing for testing of multiple transfection conditions while using low amounts of the mRNA and other reagents. The identified conditions can be subsequently scaled up as necessary for transfection in a larger tissue culture dish.

The optimization experiment should include at least two cell densities (in the range of 60 to 80% confluency at the time of transfection) and a range of DharmaFECT Duo transfection reagent volumes. Our recommended ranges for the different components are as follows:

- 20 to 80 µg/mL DharmaFECT Duo transfection reagent (corresponding to 0.2 to 0.8 µL/well of a 96-well plate)
- 100 to 200 ng of Cas9 mRNA per well of a 96-well plate

The following is a description of a transfection optimization protocol in a 96-well plate performed in triplicate (suggested experimental layout in Table 3).

Table 3. Transfection optimization layout in a 96-well plate format.

Transfection Reagent (µg/well)		Cell Density 1						Cell Density 2					
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
0.2	A		100 ng			200 ng			100 ng			200 ng	
0.3	B		100 ng			200 ng			100 ng			200 ng	
0.4	C		100 ng			200 ng			100 ng			200 ng	
0.5	D		100 ng			200 ng			100 ng			200 ng	
0.6	E		100 ng			200 ng			100 ng			200 ng	
0.7	F		100 ng			200 ng			100 ng			200 ng	
0.8	G		100 ng			200 ng			100 ng			200 ng	
Untransfected	H		0			0			0			0	

Plate row and column are indicated in bold letters and numbers. A series of DharmaFECT Duo transfection reagent concentrations are tested in rows **A-G**. Two different cell densities are tested in the left and right halves of the plate. Two Cas9 mRNA concentrations are tested, 100 ng/well in columns **1-3** and **7-9** and 200 ng/well in columns **4-6** and **10-12**. Cells in row **H** are not transfected.

1. Plate cells in a 96-well tissue culture plate one day prior to transfection such that the cells are 60 to 80% confluent on the following day. It is recommended to test at least two cell plating densities.
2. Prepare a 100 ng/µL Cas9 mRNA working solution by thawing Cas9 mRNA on ice and adding 14 µL of 1 µg/µL stock solution to 126 µL of Tris buffer. This is sufficient volume for entire optimization plate shown in Table 3. Verify the mRNA concentration using UV spectrophotometry at 260 nm and adjust the volume if necessary to obtain 100 ng/µL.
3. Prepare a 2 µM synthetic guide RNA transfection complex by adding 46 µL of crRNA stock solution and 46 µL of tracrRNA stock solution to 138 µL of Tris buffer or 46 uL of sgRNA stock solution to 184 uL of Tris buffer (total volume is 230 µL); this is sufficient volume for entire optimization plate shown in Table 3. See step 6 for preparation of stock solutions in the “General protocol for co-transfection of Edit-R Cas9 Nuclease mRNA and synthetic guide RNA" above.
4. In seven separate tubes (or deep well plate), prepare seven DharmaFECT Duo working solutions at 20 to 80 µg/mL (0.2-0.8 µL/well) by diluting 10, 15, 20, 25, 30, 35 and 40 µL of 1 mg/mL stock DharmaFECT Duo transfection reagent into 500 µL serum-free medium and mix gently. This is sufficient for 12 wells (one row) shown in Table 3. Incubate tubes for 5 minutes at room temperature.
5. In a deep well plate, to one well add serum-free medium (45.5 µL), Cas9 mRNA working solution (7 µL) and synthetic guide RNA transfection complex (17.5 µL) for a total of 70 µL; this is sufficient for three replicates for 100 ng Cas9 mRNA at each cell density (wells A1-A3 and A7-A9). Then add 70 µL DharmaFECT Duo working solution for a total volume of 140 µL Transfection Mix; this is sufficient for three replicates at each cell density (wells A1-A3 and A7-A9). Repeat for all 100 ng Cas9 mRNA samples shown In Table 3.

   For the 200 ng Cas9 mRNA samples, add serum-free medium (38.5 µL), Cas9 mRNA working solution (14 µL) and synthetic guide RNA transfection complex (17.5 µL) for a total of 70 µL; this is sufficient for three replicates for 200 ng Cas9 mRNA at each cell density (wells A4-A6 and A10-A12). Then add 70 µL DharmaFECT Duo working solution for a total volume of 140 µL Transfection Mix; this is sufficient for three replicates at each cell density (wells A4-A6 and A10-A12). Repeat for all 200 ng Cas9 mRNA samples shown In Table 3.

   DO NOT add Cas9 mRNA or DharmaFECT Duo working solution to the untransfected control wells (row H), which should contain serum-free medium only. Mix by pipetting gently up and down and incubate for 20 minutes at room temperature. **Note:** Given volumes allow for pipetting error.
6. Prepare transfection medium by adding 560 µL antibiotic-free complete medium to each sample (from step 5) to bring the total volume in each tube to 700 µL.

Table 4. Volumes (µL) for single 96-well samples. The amounts are shown per ONE well of a 96-well plate; for triplicate wells multiply all values by 3.5 to have sufficient volume for three wells and to account for pipetting error.

Sample	Serum-free medium	Synthetic guide RNA transfection complex (2 µM)	Cas9 mRNA working solution (100 ng/µL)	DharmaFECT Duo working solution	Growth Medium	Total Volume per well
100 ng Edit-R Cas9 Nuclease RNA with gene-specific or positive control synthetic guide RNA	6.5	2.5	1	10	80	100
200 ng Edit-R Cas9 Nuclease mRNA with gene-specific or positive control synthetic guide RNA	5.5	2.5	2	10	80	100
Untransfected	20	0	0	0	80	100

7. Remove medium from the wells of the 96-well plate containing cells and replace with 100 µL of the appropriate transfection medium (created in step 6) to each well.

If using Edit-R Fluorescent Cas9 mRNA, we suggest observing fluorescence 24 hours after transfection to determine conditions. To best observe fluorescence, we recommend replacing growth media with imaging media (1xHBSS, 20 mM HEPES, 16.8 mM glucose).

8. Following transfection, assess the cells for gene editing and cell viability to identify the conditions with highest editing and low cell toxicity. At 72 hours post-transfection, perform a mismatch detection assay to estimate gene editing efficiency and a cell viability assay to determine the highest lipid concentration that has minimal cell toxicity (> 70% of cell viability is preferred).

Although cell viability of 70% or higher is ideal, good editing efficiency can sometimes be achieved under transfection conditions with lower cell viability.

9. Use the optimal determined conditions for your subsequent co-transfection of Cas9 mRNA with synthetic guide RNA. We recommend a concentration of synthetic guide RNA between 25 nM to 100 nM (typically 25 nM synthetic guide RNA is sufficient for efficient gene editing).

5 Appendix

Stability and storage

Dharmacon Edit-R Cas9 Nuclease mRNA

Cas9 mRNA is shipped on dry ice for overnight domestic delivery or priority international for delivery outside of the U.S. Upon receipt, Cas9 mRNA should be stored at -80 °C. Under these conditions, the reagent is stable for at least one year; each thaw can reduce functionality, so freeze-thaws should be avoided as much as possible. At first thaw, aliquot Cas9 mRNA stock or working solution into RNase-free tubes at convenient volumes and store at -80 °C to minimize the number freeze-thaws prior to each experiment.

Dharmacon Edit-R Synthetic guide RNAs

Dharmacon Edit-R Synthetic guide RNAs reagents are shipped as dried pellets at room temperature (23 °C). Under these conditions, they are stable for at least four weeks.

Upon receipt, synthetic guide RNAs should be stored at -20 °C to -80 °C. Under these conditions, the reagents are stable for at least two years.

Always resuspend RNA in nuclease-free solutions, such as Tris buffer. In solution and stored at -20 °C, the aliquoted reagents a restable for at least one year.

DharmaFECT Duo transfection reagent

DharmaFECT Duo transfection reagent is shipped on ice packs. If the ice melts during transit there is no risk to the product. Stability testing demonstrates no loss in product functionality if it is subject to warm temperatures during shipping. (see the DharmaFECT Stability Testing Product Bulletin for additional details).

Upon receipt, store DharmaFECT Duo transfection reagent at 4 °C. DO NOT FREEZE. DharmaFECT transfection reagents are labeled with a date of manufacture and are good for 24 months when stored as recommended following that date.

Frequently asked questions

What quality control testing is performed for the synthetic tracrRNA and crRNAs?

The Edit-R tracrRNA is purified by HPLC and evaluated by MALDI-TOF mass spectrometry and HPLC to confirm the length and purity. Synthesized crRNAs are evaluated with MALDI-TOF mass spectrometry. All synthetic RNAs are quantified by readings at A260 using a spectrophotometer.

Do crRNAs synthesized using Dharmacon™ 2'-ACE chemistry need to be HPLC-purified for gene editing *in vitro*?

The proprietary Dharmacon 2'-ACE RNA synthesis chemistry has very high coupling efficiencies resulting in RNA oligos of exceptional yield and crude purity. crRNAs are provided desalted and deprotected to the end user. Additional crRNA purification is not necessary for gene editing experiments. Internal testing and successful gene editing experiments were performed using desalted and deprotected crRNAs.

I resuspended my crRNA in a buffer and there is a slight yellow tint to the solution. Is there something wrong?

No. Deprotection of the bases during oligonucleotide synthesis uses a dithiolate derivative. Sometimes small quantities of this material remain in the sample (thus the yellow tint), but it will have no significant negative effect on editing experiments or cell viability.

What is the stability of the Edit-R synthetic guide RNA?

Dried RNA oligonucleotide pellets are stable at room temperature for two to four weeks, but should be placed at -20 °C or -80 °C for long-term storage. Under these conditions, the dried synthetic guide RNA will be stable for at least one year. Maintaining sterile, RNase- and DNase-free conditions is always recommended as a critical precaution.

How many freeze-thaw cycles can the Edit-R Cas9 Nuclease mRNA and synthetic guide RNAs be subjected to?

We recommend not exceeding two to three freeze-thaw cycles for Cas9 mRNA, and four to five freeze-thaw cycles for synthetic guide RNA to ensure product integrity. On initial use, thaw the Cas9 mRNA on ice and aliquot remaining stock into convenient volumes and store all aliquots at -80 °C to minimize the number freeze-thaws cycles prior to each experiment.

How should I store my synthetic guide RNA?

RNA oligonucleotides should be stored at -20 °C or -80 °C in a non-frost free freezer, either as a dried pellet or resuspended in an RNase-free solution buffered to pH 7.4 to help with stability during freeze-thaw cycles. We recommend that RNA oligonucleotides be resuspended to a convenient stock concentration (see the Dharmacon Edit-R synthetic guide RNA resuspension protocol) and stored in small aliquots to avoid multiple freeze-thaw cycles. RNA oligonucleotides should not go through more than four to five freeze thaw cycles. If degradation is a concern, the integrity of the RNA oligonucleotides can be evaluated on an analytical PAGE gel.

What is the formula for spectrophotometric quantification of synthetic crRNA, tracrRNA and sgRNA??

To quantify RNA, use Beer's Law: Absorbance (260 nm) = (ε)(concentration)(path length in cm), where ε, epsilon, is the molar extinction coefficient (provided on the Product Transfer Form supplied with the crRNA or synthetic sgRNA order; the extinction coefficient for tracrRNA Cat# U-002005 is 757800). When solved for the unknown, the equation becomes: Concentration = (Absorbance, 260 nm) / [(ε)(path length in cm)]. When a standard 10 mm cuvette is used, the path length variable in this equation is 1. If a different size of cuvette is used, e.g., a 2 mm microcuvette, then the path length variable is 0.2.

I see a fair amount of cell death after transfection of my cells. What can I do about this?

Extensive cell death following transfection is an indication that delivery conditions need to be further optimized. Basic parameters to consider when optimizing transfection include transfection reagent and cell-specific conditions such as the amount of transfection reagent, the lot/batch of transfection reagent, duration of transfection, cell passage number and cell density at transfection. Often decreasing the amount of lipid present during transfection and/or the total duration of transfection will help minimize the toxic effect to the cells. Additionally, it is not uncommon to observe some variability from one tube of transfection reagent to another, and this may also represent a source of experimental variability. If the problem persists, we recommend that other transfection reagents be considered or you may contact Technical Support (ts.dharmacon@horizondiscovery.com) for additional troubleshooting help.

In what cells can I use the Edit-R Cas9 Nuclease mRNA and synthetic guide RNAs?

You can use these components in any mammalian cell type that is amenable to transfection or electroporation.

What are the benefits of using two synthetic RNAs (tracrRNA and crRNA) compared to a single guide RNA (sgRNA) expressed from a vector?

The main benefit of using the synthetic RNA components with the synthetic tracrRNA and crRNA is that that multiple crRNAs can be ordered and transfected within a few days. This allows for rapid screening of several target sites within a single gene or knocking out several genes quickly, as long as your cells are transfectable. Expressed sgRNAs require individual cloning of each target sequence into an expression plasmid, growing and selecting several clones to QC by sequencing, then prepping and purifying DNA suitable for transfection. This can be very time-consuming when you wish to test several crRNA candidates per gene or would like to knockout multiple genes quickly.

Can I use a different transfection reagent other than DharmaFECT Duo to deliver the Edit-R components into my cells?

We have validated our protocols and demonstrated successful gene editing using DharmaFECT Duo to efficiently deliver the Edit-R components. Thus we cannot predict the co-transfection ability of other transfection reagents, nor can we troubleshoot experiments performed with any reagent other than DharmaFECT Duo. However, other suitable transfection reagents could be utilized provided co-transfection conditions are carefully optimized for each cell line of interest.

My DNA mismatch detection assay shows a low percentage of editing. Why is that?

Mutation analysis assays, which utilize mismatch-specific DNA endonucleases such as T7EI, rely on PCR amplification of a genomic DNA target site and subsequent observation of cleavage, commonly by gel electrophoresis. While these assays are a straightforward approach for detecting insertions and deletions (indels) introduced by CRISPR-Cas9 gene editing, sensitivity varies between endonucleases and generally underestimates editing efficiency for several reasons:

1. Cas9 cleavage followed by DNA repair through non-homologous end-joining (NHEJ) results in deletions, insertions, and mutations of various sizes. Mismatch DNA endonuclease cleavage can produce smeared bands on a gel which are not easily visualized or quantified.
2. Mismatch DNA endonuclease digestion can lead to non-specific cuts that degrade the PCR product and reduce the intensity of the desired bands, especially at longer incubation times.
3. If the CRISPR-Cas9 gene editing generates large inserts or deletions, primer binding sites can be impacted and the mutation thus will not be detected by the DNA mismatch assay.

Can the Edit-R system be used for gene knockout in non-mammalian organisms?

Edit-R Cas9 mRNA has been demonstrated to be active in *Danio rerio* (Microinjection of zebrafish embryos using Edit-R Cas9 Nuclease mRNA - AppNote). Functionality in other non-mammalian species remains to be determined, and will be partly dependent on the following:

1. Ability to deliver components into cell type of interest
2. Compatibility of Cas9 codon translation (Edit-R Cas9 is human codon optimized)
3. Potential differences in endogenous genome repair mechanisms/efficiency

Can I use the Edit-R synthetic RNA components with my own Cas9 nucleases? Or mutant Cas9 nucleases?

The repeat component of the crRNA sequence and the entire tracrRNA sequence are derived from the *Streptococcus pyogenes* CRISPR-Cas9 system, so they very likely can be used with another *S. pyogenes*-derived Cas9 component that is suitably optimized and sufficiently generates active Cas9 protein. However, this will need to be empirically tested based upon the components in question.

Can the ploidy of my cell line affect the results of my CRISPR-Cas9 gene editing experiment?

When using a CRISPR-Cas9 system to disrupt gene function, it is important to know as much as you can about the gene you are targeting and your cells of interest. In particular, the ploidy of your cells, the gene copy number and the presence of any SNPs are important considerations. In normal diploid cells, obtaining mutations on both alleles may be required for a complete knockout and an observable phenotype. Virtually all cancer lines and many immortalized cell lines exhibit aneuploidy and as such, mutation of multiple alleles may be necessary. The presence of SNPs and multiple genomic locations should also be taken into account when designing crRNAs, as they can affect whether a complete knockout can be achieved. Expansion of clones from single cells followed by sequence validation to verify the desired mutation on all alleles is the most rigorous approach for confirming complete gene knockout.

What filters are recommended for observing mKate2 or EGFP?

mKate2 has an excitation maximum of 588nm and emission maximum of 633nm. Recommended Omega Optical filter sets are QMAX-Red, XF102-2, Texas red, or similar. EGFP as an excitation maximum of 489nm and emission maximum of 509nm. Recommended filter sets are EGFP, FITC, and other green dyes. Recommended Omega Optical filter sets are QMAX-Green, XF100-2, XF100-3, XF115-2, and XF116-2.

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