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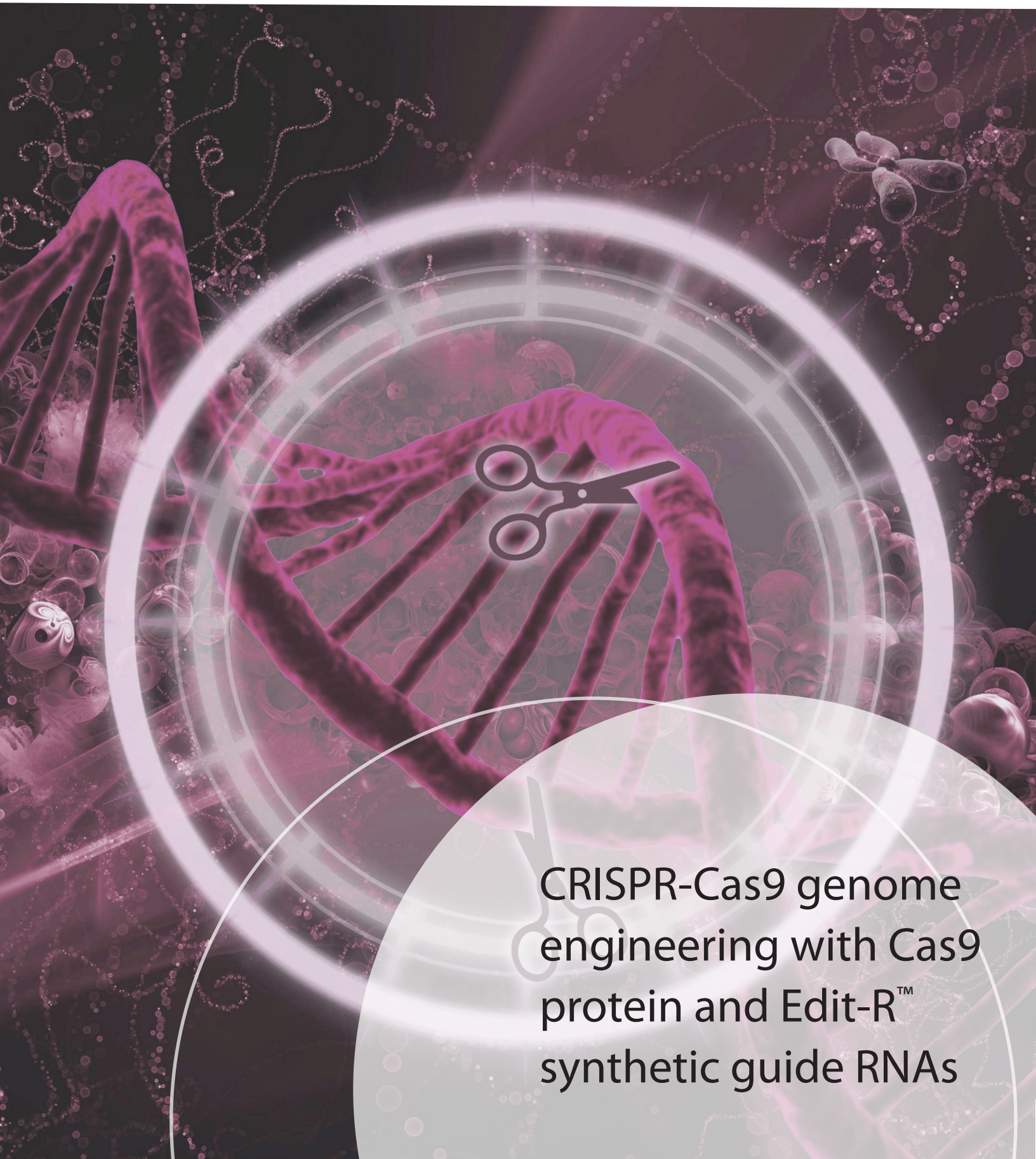

CRISPR-Cas9 genome engineering with Cas9 protein and Edit-R™ synthetic guide RNAs

Contents

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). The pre-crRNA 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 components 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; Figure 1A; Deltcheva, 2011). Alternatively, the crRNA can be fused to the tracrRNA creating a chimeric structure termed a single guide RNA (sgRNA; Figure 1B: Jinek, 2012).

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.

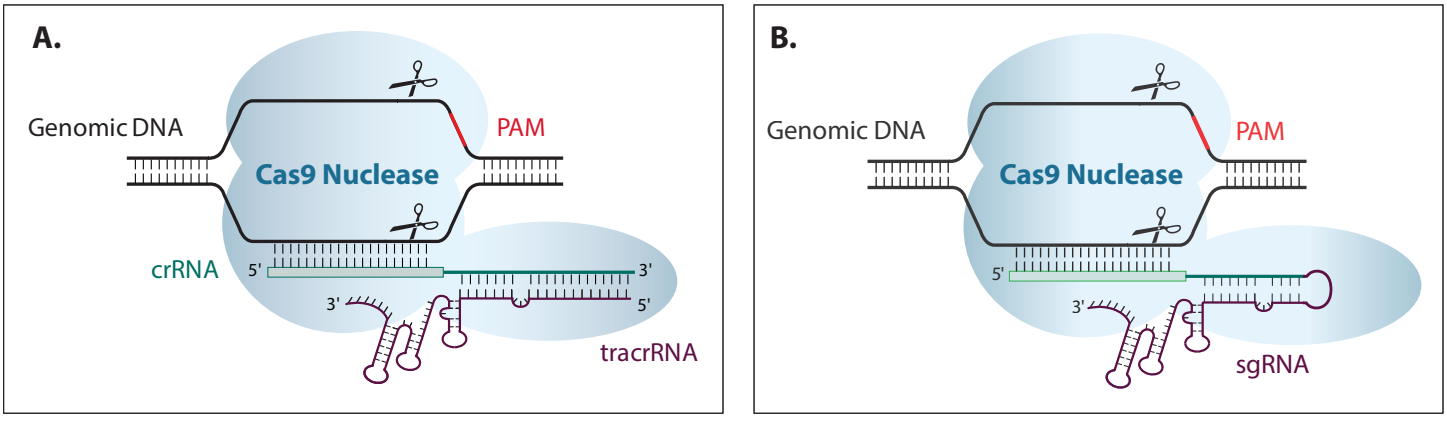

Figure 1. Illustration of CRISPR-Cas9 system. Cas9 nuclease (light blue), programmed by the crRNA (green):tracrRNA (purple) complex (A) or the sgRNA (B), cutting both strands of genomic DNA 5' of the PAM (red).

2 Cas9 nuclease protein NLS and Edit-R synthetic RNAs for gene editing

The CRISPR-Cas9 workflow using Cas9 nuclease protein NLS and Edit-R synthetic guide RNAs include these critical components required for gene editing in mammalian cells based on the natural *S. pyogenes* system: a codon-optimized Cas9 nuclease protein 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 cells of choice using the DharmaFECT™ Duo transfection reagent to perform gene disruption. Figure 2 summarizes the general experimental workflow. Each component is described below.

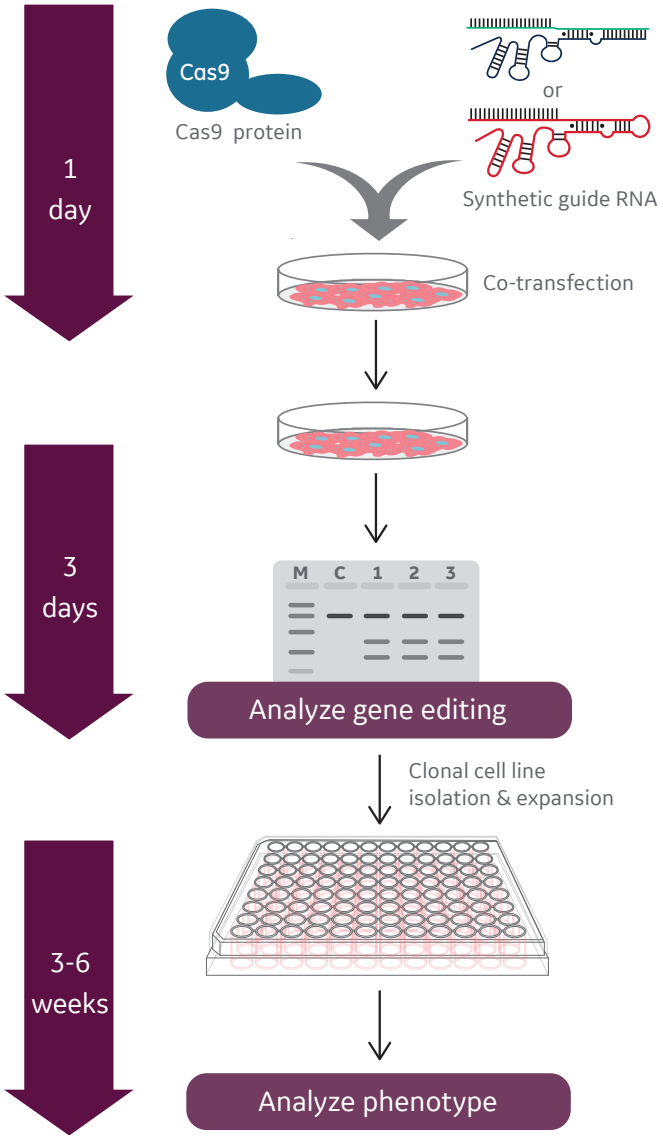

Figure 2. Gene editing with Cas9 Nuclease protein NLS and Edit-R synthetic guide RNA is performed by co-transfecting all components with DharmaFECT Duo transfection reagent (or other DharmaFECT transfection reagent suitable for your specific cells of interest). One may then observe phenotypes directly.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

Cas9 nuclease protein NLS

The Cas9 nuclease protein NLS contains the *S. pyogenes* Cas9 (Csn1) gene with a C-terminal nuclear localization signal (NLS). The Cas9 protein is ready for direct transfection or electroporation.

Edit-R synthetic guide RNA

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 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. The predominant *S. pyogenes* PAM nucleotide sequence is NGG. It is modified for nuclease resistance and can be used with modified or unmodified Edit-R tracrRNA.

Edit-R trans-activating CRISPR RNA (tracrRNA)

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

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

Successful co-transfection of the Cas9 protein and Edit-R 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 8-9. The protocol below assumes that experimental conditions have been optimized as recommended.

Materials required for gene editing using Cas9 nuclease protein NLS and Edit-R synthetic guide RNAs

- Cas9 nuclease protein NLS:

	Concentration		Quantity	
Catalog #	µM	µg/µL	pmol	µg
CAS11728*	20	3.2	250	40
CAS11200*	20	3.2	500	80
CAS11201*	20	3.2	1000	160
CAS11729*	40	6.4	500	80
CAS11730*	40	6.4	2 × 500	160
CAS12205	61.8	10	618	100
CAS12206	61.8	10	3090	500
CAS12207	61.8	10	5 × 3090	5 × 500

* Discontinued.

- Synthetic targeting guide RNA (choose one):
  - » Edit-R synthetic sgRNA
  - » Edit-R synthetic crRNA and tracrRNA oligos
- Non-targeting control guide RNA (choose one):
  1. Edit-R synthetic sgRNA non-targeting control (Cat #U-009501-XX, U-009502-XX, U-009503-XX, U-009504-XX, U-009505-XX)
  2. Edit-R crRNA non-targeting control (Cat #U-007501-XX, U-007502-XX, U-007503-XX, U-007504-XX, U-007505-XX)
- DharmaFECT™ transfection reagents (Cat #T-2001, T-2002, T-2003, T-2004 or T-2010)

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

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

We have found that both DharmaFECT 1 and DharmaFECT Duo transfection reagents work well in most cells. Some cells may require a specific DharmaFECT reagent; see cell type guide: DharmaFECT Cell Type Guide.

- Assay for assessing cell viability such as a 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 (Cat #B-006000-100)

Protocol for co-transfection of Cas9 nuclease protein NLS and Edit-R synthetic guide RNAs

The following is a general protocol using DharmaFECT 1 transfection reagent to deliver Cas9 nuclease protein NLS and Edit-R 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 HeLa 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, HeLa cells should be diluted to 10,000 cells in 0.1 mL of medium for plating at 10,000 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 protein and non-targeting control guide RNA	Negative control: Expression of Cas9 protein without targeting RNA
Cas9 protein and gene-specific guide RNA	Gene editing sample: Expression of Cas9 protein programmed by RNAs for targeted double-strand cleavage in gene of interest
Untransfected	No treatment control sample: Confirmation of cell viability

It is advised that the minimum volume of Cas9 protein working solution required for the experiment be prepared, as storage of diluted Cas9 protein is not recommended.

Co-transfection

5. Prepare a 2.5 µM Cas9 protein working solution from appropriate stock solution as described in table below. These volumes are sufficient for 10 wells with 1 µL/well in 96-well format, including extra volume for pipetting loss.

Protein concentration (µM)	Volume of protein (µL)	Volume of serum free media (µL)	Total volume (µL)
20	1.5	10.5	12
40	0.75	11.25	12
61.8	0.5	11.85	12.35

Prepare 2 µM synthetic guide RNA transfection complex from previously prepared 10 µM stocks (see Appendix).

» For synthetic sgRNA add 2 µL of 10 µM synthetic sgRNA to 8 µL of Tris buffer (total volume is 10 µL).

or

» For crRNA:tracrRNA add 2 µL of 10 µM crRNA and 2 µL of 10 µM tracrRNA to 6 µL of Tris buffer (total volume is 10 µL).

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 50 nM concentration of the synthetic guide RNA and 25 nM of Cas9 protein in the final transfection mixture.

6. Prepare a 8 µg/mL DharmaFECT 1 working solution by diluting 1.6 µL of 1 mg/mL stock DharmaFECT 1 transfection reagent in 198.4 µL serum-free medium and mix gently; this volume is sufficient for 4 wells with 0.4 µg/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 (2 µM)	Cas9 protein working solution (2.5 µM)	DharmaFECT 1 working solution (8 µg/mL)	Total Volume per well
Cas9 protein and non-targeting control guide RNA	46.5	2.5	1	50	100
Cas9 protein and gene-specific guide RNA	46.5	2.5	1	50	100
Untransfected	100	0	0	0	100

Volumes (µL) are for a single well of a 96-well plate of HeLa 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).

7. Add 50 µL DharmaFECT 1 working solution to each sample tube as shown in Table 2 (column 5); this will result in 0.4 µg/well final concentration. DO NOT add DharmaFECT 1 working solution to the untransfected control, which should contain serum-free medium only. This brings the total volume to 100 µL in each tube. Mix by pipetting gently up and down and incubate for 20 minutes at room temperature.
8. 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.
9. Incubate cells at 37 °C with 5% CO_2 for 14-18 hours; replace transfection mix on the cells with regular growth medium without antibiotics.
10. Incubate cells at 37 °C with 5% CO_2 for 72 hours after the initial transfection 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 (Cas9 nuclease protein NLS and Edit-R 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, please see here.

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 Cas9 nuclease protein NLS with with a Edit-R positive synthetic guide RNA control to find conditions that show the highest genomic editing and > 70% cell viability compared to untreated cells.

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 transfection reagent volumes (DharmaFECT 1 is used to illustrate the transfection optimization experiment below). Our recommended ranges for the different components are as follows:

- 2 to 8 µg/mL DharmaFECT 1 transfection reagent (corresponding to 0.1 to 0.4 µg/well of a 96-well plate)
- 50-100 nM synthetic guide RNA
- 25 nM Cas9 protein 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
0.1	A	50 nM			100 nM			50 nM			100 nM		
0.15	B	50 nM			100 nM			50 nM			100 nM		
0.2	C	50 nM			100 nM			50 nM			100 nM		
0.25	D	50 nM			100 nM			50 nM			100 nM		
0.3	E	50 nM			100 nM			50 nM			100 nM		
0.35	F	50 nM			100 nM			50 nM			100 nM		
0.4	G	50 nM			100 nM			50 nM			100 nM		
Untransfected	H	0			0			0			0		

Plate row and column are indicated in bold letters and numbers. A series of DharmaFECT 1 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 synthetic guide RNA concentrations are tested, 50 nM in columns **1-3** and **7-9** and 100 nM 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 2.5 µM Cas9 protein working solution from appropriate stock solution as described in table below.

Protein concentration (µM)	Volume of protein (µL)	Volume of serum free media (µL)	Total volume (µL)
20	12.5	87.5	100
40	6.25	93.75	100
61.8	4	96	100

This is sufficient volume for the entire optimization plate shown in Table 3.

3. Prepare a 2 µM guide RNA transfection mix as described in step 6 of the co-transfection protocol (p.6). Add 160uL of prepared guide RNA to 240 µL of Tris buffer (total volume is 400 µL); this is sufficient volume for entire optimization plate shown in Table 3.

4. In seven separate tubes (or deep well plate), prepare seven DharmaFECT 1 working solutions at 2 to 8 µg/mL (0.1-0.4 µg/well) by diluting 1.4, 2.1, 2.8, 3.5, 4.2, 4.9 and 5.6 µL of 1 mg/mL stock DharmaFECT 1 transfection reagent into 700 µL serum-free medium and mix gently. 700 µL of each DharmaFECT 1 working solution is enough for 12 wells of experimental samples (Table 4). Incubate tubes for 5 minutes at room temperature.

5. For each row of 50 nM synthetic guide RNA samples, in a deep well plate, to one well add serum-free medium (325.5 µL), Cas9 protein working solution (7 µL) and synthetic guide RNA transfection complex (17.5 µL) for a total of 350 µL; this is sufficient for three replicates for 50 nM synthetic guide RNA at each cell density (wells A1-A3 and A7-A9). Then add 350 µL DharmaFECT 1 working solution for a total volume of 700 µL Transfection Mix; this is sufficient for three replicates at each cell density (wells A1-A3 and A7-A9). Repeat for each row (DharmaFECT concentration).

   For each row of 100 nM synthetic guide RNA samples, in a deep well plate, to one well add serum-free medium (308 µL), Cas9 protein working solution (7 µL) and synthetic guide RNA transfection complex (35 µL) for a total of 350 µL; this is sufficient for three replicates for 100 nM synthetic guide RNA at each cell density (wells A4-A6 and A10-A12). Then add 350 µL DharmaFECT 1 working solution for a total volume of 700 µL Transfection Mix; this is sufficient for three replicates at each cell density (wells A4-A6 and A10-A12). Repeat for each row (DharmaFECT concentration)

   DO NOT add Cas9 protein or DharmaFECT 1 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. Given volumes allow for pipetting error.

Given volumes allow for pipetting error.

6. 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.

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	Edit-R synthetic guide RNA (2 µM)	Cas9 protein working solution (25 nM)	DharmaFECT 1 working solution	Total volume per well
50 nM gene-specific Edit-R synthetic guide RNA or positive control synthetic guide RNA with Cas9 nuclease protein NLS	46.5	2.5	1	50	100
100 nM gene-specific Edit-R synthetic guide RNA or positive control synthetic guide RNA with Cas9 nuclease protein NLS	44	5	1	50	100
Untransfected	100	0	0	0	100

7. Incubate the cells at 37 °C in 5% CO_2 for 14 to 18 hours; replace transfection mix on the cells with regular growth medium without antibiotics.
8. Incubate the cells at 37 °C in 5% CO_2 for total of 48 to 72 hours after the initial transfection, before proceeding with analysis.
9. Following transfection, assess the cells for gene editing and cell viability to identify the conditions with highest editing and low cell toxicity. At 48 to 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.

10. Use the optimal determined conditions for your subsequent co-transfection of Cas9 protein with synthetic guide RNA complex.

5 Appendix

Stability and storage

Cas9 nuclease Edit-R protein NLS

Cas9 protein is shipped on dry ice for overnight domestic delivery or priority international for delivery outside of the U.S. Upon receipt, Cas9 protein should be stored at -20 °C. Under these conditions, the reagent is stable for at least one year.

Edit-R synthetic guide RNA

Edit-R synthetic guide RNA 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 RNA 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 resuspended reagents are stable for at least one year. Avoid multiple freeze-thaw cycles. We recommend not exceeding four to five freeze-thaw cycles to ensure RNA integrity.

For a protocol to make 10 µM concentrated stock solutions of synthetic guide RNAs see the Edit-R synthetic guide RNA resuspension protocol.

DharmaFECT Transfection Reagents

DharmaFECT transfection reagents are 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 transfection reagents 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 is the benefit of using Cas9 protein instead of Cas9 plasmid or Cas9 mRNA?

Cas9 protein is a DNA-free option so there is no concern of DNA integration that can happen using plasmid. The protein is immediately available for the cell to use for editing as it does not require transcription (as for plasmid) and translation (as for mRNA). Additionally, because it is quickly turned over by the cell there may be a lower chance of off-targets.

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

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. Synthetic RNAs are quantified by readings at A_{260} using a spectrophotometer.

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

The proprietary 2'-ACE RNA synthesis chemistry has very high coupling efficiencies resulting in RNA oligos of exceptional yield and crude purity. crRNAs are provided deprotected and desalted 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.

What production method and quality control testing is performed for synthetic sgRNA?

Edit-R synthetic sgRNA is purified by C18, and desalted. The synthetic sgRNA is then identity confirmed by MALDI-TOF mass spectrometry and is quantified by readings at A260 using a spectrophotometer. Finally, the appropriate quantity is dried down and packaged in a 1.5 mL tube or 96-well plate. For different purification options and larger amounts of synthetic sgRNA these can be custom ordered through the single stranded RNA synthesis tool.

I resuspended my RNA 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 RNAs?

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 ynthetic guide RNA be subjected to?

We recommend not exceeding four to five freeze-thaw cycles to ensure product integrity.

How should I store my synthetic guide RNAs?

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 (technical@horizondiscovery.com) for additional troubleshooting help.

In what cells can I use the Cas9 nuclease protein NLS and Edit-R 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 synthetic guide RNAs compared to a single guide RNA (sgRNA) expressed from a vector?

The main benefit of using the synthetic RNA components is that multiple RNAs 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 candidates per gene or would like to knockout multiple genes quickly.

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?

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 (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 protein? Or mutant Cas9 protein?

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 guide RNAs, 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.

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