TECHNICAL MANUAL

horizon™
INSPIRED CELL SOLUTIONS

Dharmacon™ Edit-R™ CRISPR-Cas9 gene engineering with Cas9 nuclease expression plasmids and synthetic guide RNAs

Contents

1 Introduction to the CRISPR-Cas9 system for gene engineering

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).

Engineering a CRISPR-Cas9 platform for mammalian genome 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 S*treptococcus 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 protospacer adjacent motiff (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.

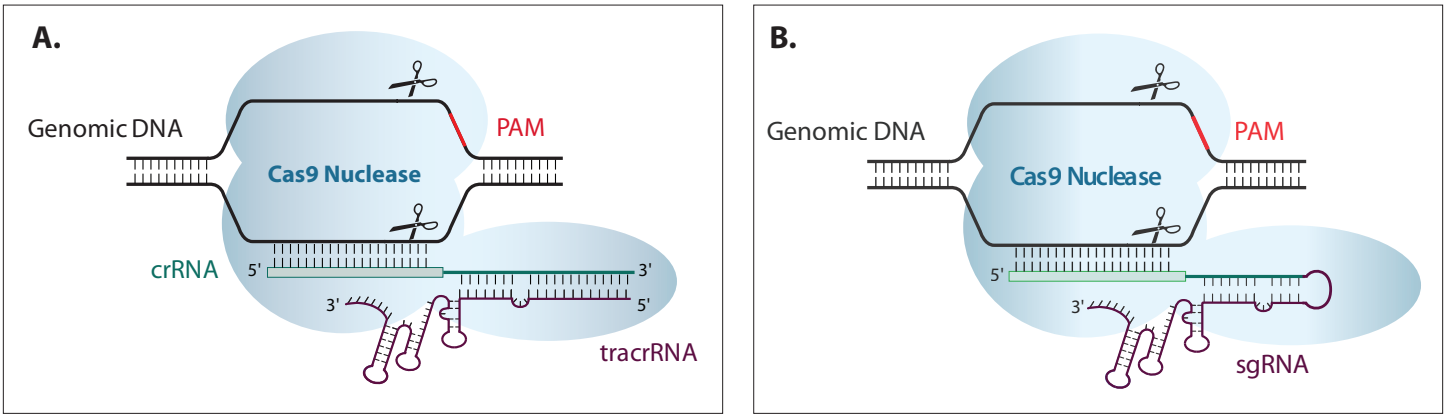


Figure 1. Illustration of CRISPR-Cas9 system. Cas9 nuclease (light blue), programmed by the crRNA (green):tracrRNA (purple) complex (A) or the sgRNA (B), cutting both strands of genomic DNA 5' of the PAM (red).

2 Edit-R gene engineering with Cas9 expression plasmids and synthetic guide RNAs

The Edit-R reagent workflow using Edit-R Cas9 nuclease expression plasmid and synthetic guide RNAs includes these critical components required for gene editing in mammalian cells based on the natural *S. pyogenes* system: a plasmid for expression of a codon-optimized Cas9 nuclease protein and either (a) a synthetic tracrRNA with a synthetic crRNA designed to the gene target of interest or (b) a synthetic single guide RNA designed to the gene target of interest. 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.

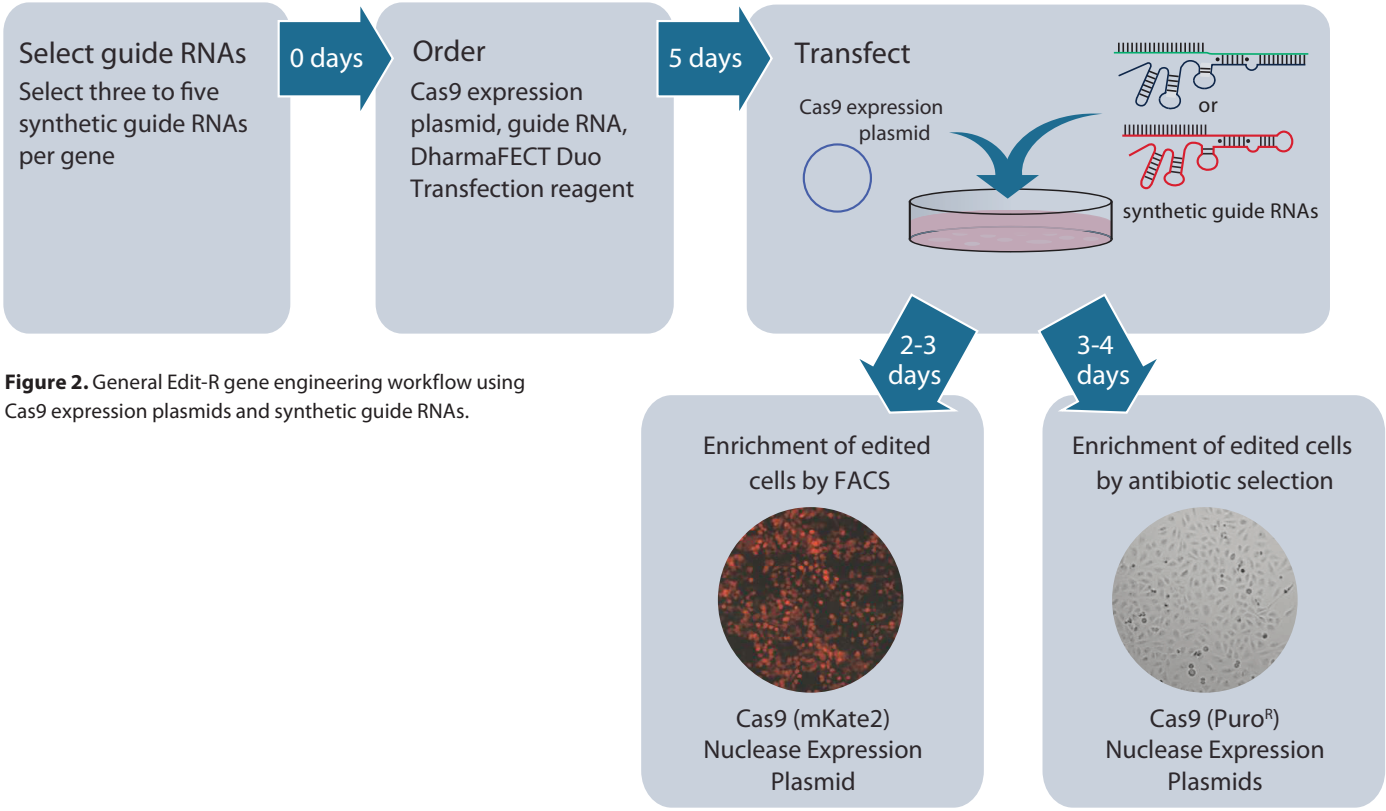


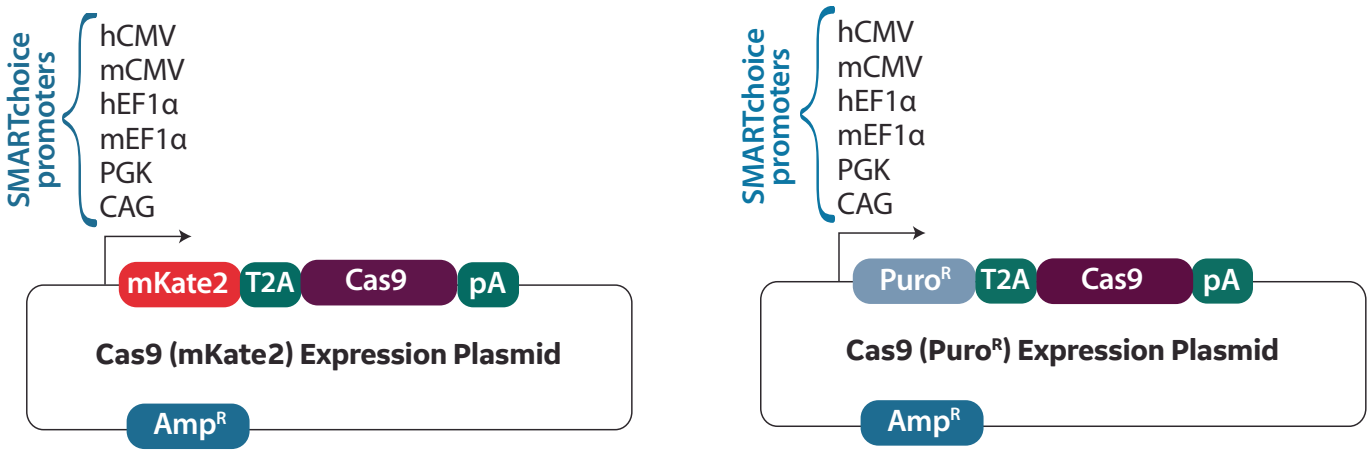
Figure 2. General Edit-R gene engineering workflow using Cas9 expression plasmids and synthetic guide RNAs.

Edit-R Cas9 Nuclease Expression plasmids

The Edit-R Cas9 Nuclease Expression plasmids contain a human codon-optimized version of the *S. pyogenes* Cas9 (Csn1) gene and either the mKate2 fluorescent reporter (Evrogen, Moscow, Russia) or the puromycin resistance marker (Puro^R) under the same promoter **(Figure 3A)**. Placing the mKate2 or puromycin resistance marker at the 5' end of the Cas9 coding region rather than the 3' end ensures that no extra amino acids are added to the C-terminus, thus preserving the full Cas9 endonuclease activity.

SMARTchoice™ promoter options are available **(Figure 3B)** so one can choose the plasmid with the most active promoter for specific cells of interest. All Cas9 nuclease expression plasmids are supplied as dried down, endotoxin-free plasmid DNA, ready for transfection.

A Cas9 expression plasmid utilizing the hCMV promoter and blasticidin resistance marker is also available (see Appendix page 11 for vector map and recommendations on using this plasmid).



Vector element	Utility
Cas9	Human codon-optimized *S. pyogenes* Cas9 nuclease for cleavage of targeted DNA when programmed with synthetic guide RNAs complex
mKate2	Monomeric red fluorescent protein for visual tracking of transfection and cell sorting (excitation and emission maxima 588 and 633 nm, respectively)
T2A	Self cleaving peptide allows for simultaneous expression of mKate2 and Cas9 protein from a single transcript
Amp^R	Ampicillin resistance gene for vector propagation in *E. coli* cultures
pA	Bovine growth hormone (BGH) polyadenylation signal

Vector element	Utility
Cas9	Human codon-optimized *S. pyogenes* Cas9 nuclease for cleavage of targeted DNA when programmed with synthetic guide RNAs complex
Puro^R	Puromycin marker confers antibiotic resistance in mammalian cells for selection of transfected cells
T2A	Self cleaving peptide allows for simultaneous expression of puromycin and Cas9 protein from a single transcript
Amp^R	Ampicillin resistance gene for vector propagation in *E. coli* cultures
pA	Bovine growth hormone (BGH) polyadenylation signal

Figure 3A. Vector elements of the Edit-R Cas9 Nuclease Expression Plasmids.

Promoter	Description
hCMV	human cytomegalovirus immediate early promoter
mCMV	mouse cytomegalovirus immediate early promoter
hEF1α	human elongation factor 1 alpha promoter
mEF1α	mouse elongation factor 1 alpha promoter
PGK	mouse phosphoglycerate kinase promoter
CAG	chicken beta actin hybrid promoter

Figure 3B. SMARTchoice promoter options for Cas9 Nuclease Expression Plasmids.

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). The Edit-R tracrRNA has been tested for efficient editing in multiple mammalian cell types.

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, determined by the end user, must be immediately upstream of a PAM in the genomic DNA. The predominant *S. pyogenes* PAM nucleotide sequence is NGG **(Figure 1)**. Pre-designed crRNAs are available for human, mouse and rat coding genes on dharmacon.horizondiscovery.com and custom crRNA can be designed and ordered using the Dharmacon CRISPR Design Tool.

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 gene engineering components

Successful co-transfection of Edit-R components (Cas9 Nuclease Expression plasmid and synthetic guide RNA) and subsequent gene knockout requires careful optimization of delivery conditions with DharmaFECT Duo Transfection Reagent for each cell line of interest. For general recommendations on optimizing co-transfection conditions, see on pages 9-11. The protocol below assumes that experimental conditions have been optimized as recommended.

Edit-R materials required for gene engineering using Cas9 expression plasmids and synthetic guide RNAs

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

- Cas9 Nuclease Expression plasmid with promoter and reporter/marker of choice, 120 µg dried
  - Cas9 Nuclease Expression plasmids with mKate2 fluorescent reporter
    - Edit-R hCMV_mKate2-Cas9 Expression Plasmid DNA (Cat #U-004100-120)
    - Edit-R mCMV_mKate2-Cas9 Expression Plasmid DNA (Cat #U-004200-120)
    - Edit-R hEF1α_mKate2-Cas9 Expression Plasmid DNA (Cat #U-004300-120)
    - Edit-R mEF1α_mKate2-Cas9 Expression Plasmid DNA (Cat #U-004400-120)
    - Edit-R PGK_mKate2-Cas9 Expression Plasmid DNA (Cat #U-004500-120)
    - Edit-R CAG_mKate2-Cas9 Expression Plasmid DNA (Cat #U-004600-120)
  - Cas9 Nuclease Expression plasmids with Puro^{R} mammalian antibiotic selection marker
    - Edit-R hCMV_Puro^{R}-Cas9 Expression Plasmid DNA (Cat #U-005100-120)
    - Edit-R mCMV_Puro^{R}-Cas9 Expression Plasmid DNA (Cat #U-005200-120)
    - Edit-R hEF1α_Puro^{R}-Cas9 Expression Plasmid DNA (Cat #U-005300-120)
    - Edit-R mEF1α_Puro^{R}-Cas9 Expression Plasmid DNA (Cat #U-005400-120)
    - Edit-R PGK_Puro^{R}-Cas9 Expression Plasmid DNA (Cat #U-005500-120)
    - Edit-R CAG_Puro^{R}-Cas9 Expression Plasmid DNA (Cat #U-005600-120)
  - Cas9 Nuclease Expression plasmid with Blast^{R} mammalian antibiotic selection marker, 120 µg dried (Cat #U-001000-120); see Appendix for vector map and recommendations on using this plasmid
  - (Optional) mKate2 Transfection Optimization Plasmid, 120 µg dried (Cat # U-003000-120); see Appendix for vector map and recommendations on using this plasmid
- Synthetic targeting guide RNA (choose one):
  1. 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 the Dharmacon CRISPR Design Tool
     b. tracrRNA, 5, 20 or 50 nmol (Cat #U-002005-XX)

     or
  2. Edit-R synthetic sgRNA, custom ordered using the Dharmacon CRISPR Design Tool
- Non-targeting Control guide RNA (choose one):
  1. Edit-R crRNA Non-targeting Control (Cat #U-007501-XX, U-007502-XX, U-007503-XX, U-007504-XX, U-007505-XX)

     or
  2. Custom synthetic sgRNA non-targeting control 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.

- 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
- Assay for assessing cell viability such as Resazurin cell viability reagent
- Assay(s) for detecting gene engineering events in a cell population
- (Optional) Puromycin antibiotic
- (Optional) Blasticidin S antibiotic
- (Optional) Flow cytometer, for FACS enrichment
- 10 mM Tris pH 7.4 nuclease-free buffer (Tris buffer) solution (Cat #B-006000-100)

General protocol for co-transfection of Edit-R Cas9 expression plasmids and synthetic RNAs

The following is a general protocol using DharmaFECT Duo Transfection Reagent to deliver Edit-R Cas9 Nuclease Expression plasmid, 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 9). The protocol below describes delivery conditions in HEK293T cells in 24-well plate format and is given for illustrative purposes only.

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 100,000 cells in 0.5 mL of medium for plating at 100,000 cells/well in a 24-well plate.
3. Plate 0.5 mL of cell suspension into each well of a 24-well plate.
4. Incubate cells at 37 °C with 5% CO_2 overnight.

Co-transfection

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

Sample name	Purpose
Cas9 Nuclease Expression plasmid only with a Non-targeting control synthetic guide RNA	Negative control: Expression of Cas9 nuclease without targeting RNAs
Cas9 Nuclease Expression plasmid with gene-specific synthetic guide RNA	Gene engineering sample: Expression of Cas9 nuclease programmed by RNAs for targeted double-strand break in gene of interest
Untreated	No treatment control sample: Confirmation of cell viability

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

5. Three different samples are recommended for a gene engineering experiment **(Table 1)**.
6. Prepare a 100 ng/µL Cas9 plasmid working solution by adding 1.2 mL Tris buffer to 120 µg plasmid. Verify the DNA concentration using UV spectrophotometry at 260 nm, and adjust the volume if necessary to obtain 100 ng/µL.
7. Prepare 2 µM synthetic guide RNA transfection complex from previously prepared 10 µM stocks (see Appendix)
   a. 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).
   or
   b. For synthetic sgRNA add 2 µL of 10 µM synthetic sgRNA to 8 µL of Tris buffer (total volume is 10 µL).
8. In 1.7 mL tubes prepare samples to be transfected as described in Table 2 (columns 2 - 4) for a final 25 nM concentration of the guide RNA and 1 µg/well of Cas9 expression plasmid.
9. Prepare a 60 µg/mL DharmaFECT Duo working solution by diluting 120 µL of 1 mg/mL stock DharmaFECT Duo in 2 mL serum-free medium and mix gently. Incubate tubes for 5 minutes at room temperature.

Table 2. Preparing transfection samples for gene editing experiment in a 24-well plate format.

Sample Name	Serum-free medium	Working guide RNA solution (2 µM)	Working Cas9 plasmid solution (100 ng/µL)	Working DharmaFECT Duo solution (60 µg/mL)	Growth medium	Total volume per 24-well
Cas9 Nuclease Expression plasmid with Non-targeting control synthetic guide RNA	35	5	10	50	400	500
Cas9 Nuclease Expression plasmid with gene-specific synthetic guide RNA	35	5	10	50	400	500
Untreated	100	0	0	0	400	500

Volumes (µL) for single 24-well samples of HEK293T cells. For replicates, please prepare sufficient sample volumes for the 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 9).

10. Add 50 µL DharmaFECT Duo working solution to each sample tube as shown in **Table 2 (column 5)**. This will result in 3 µg/well final DharmaFECT Duo 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 100 µL in each tube. Mix by pipetting gently up and down and incubate for 20 minutes at room temperature.
11. Prepare transfection medium by adding 400 µL antibiotic-free complete medium to each sample to bring the total volume in each tube to 500 µL (columns 6 and 7).
12. Remove medium from the wells of the 24-well plate containing cells and replace with 500 µL of the appropriate transfection medium to each well.
13. Incubate cells at 37 °C in 5% CO_2 for 48 to 72 hours before proceeding with gene editing analysis.

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

If you cannot detect gene editing or the editing level in your assay is low, you can consider enriching the cell population as described in the next section.

Enrichment for gene editing events

Cell populations with increased frequencies of Cas9-induced gene engineering events can be achieved by two different methods, depending on the Edit-R Cas9 expression plasmid used during the experiment:

a. **mKate2-Cas9 expression plasmid for enrichment by FACS analysis**
Use cells transfected with mKate2-Cas9 expression plasmid 48 to 72 hours post-transfection. We recommend performing the transfection experiment in a larger tissue culture dish so that a sufficient number of cells can be harvested for FACS analysis according to commonly used protocols. We recommend sorting the cells into fractions with low, medium, and high mKate2 expression levels. Ensure that each fraction has enough of the total cell population such that desired fractions can be easily expanded for downstream applications and testing.

   mKate2 is a monomeric far-red fluorescent protein with excitation maximum of 588 nm and emission maximum of 633 nm. Recommended Omega Optical filter sets are QMAX-Red and XF102-2 or equivalents. mKate2 can also be detected using Texas Red filter sets or similar.

b. **Puro^R-Cas9 expression plasmid for enrichment by puromycin selection**
For enrichment by puromycin selection, use cells transfected with Puro^R-Cas9 expression plasmid. The appropriate puromycin concentration is specific to each cell line and should be determined experimentally prior to selection using a kill curve. We recommend splitting cells 24 to 48 hours post-transfection prior to starting the puromycin selection. Incubate cells in selection medium for an additional 2 to 4 days.

   Since antibiotics are most effective when cells are actively dividing, it is best to split cells such that they are not more than 25% confluent.

Gene editing assay recommendations

The most commonly used method for detection of indels in a cell population is a mismatch detection assay such as T7 Endonuclease I

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

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 mKate2-Cas9 Expression Plasmid with the most active promoter for your cells to find conditions that show highest fluorescence intensity (excitation and emission maxima 588 and 633 nm, respectively) 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 plasmid. The identified conditions can be subsequently scaled up for transfection in a larger tissue culture dish based on the surface area.

DharmaFECT Duo transfection regent is optimized for delivering both RNA and plasmid into cells. If another transfection reagent is used, careful optimization is recommended to confirm both the RNA and plasmid components are delivered efficiently to the cells.

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												

Plate row and column are indicated in bold letters and numbers. A series of DharmaFECT Duo concentrations are tested in rows **A-G**. Two different cell densities are tested in the left and right halves of the plate. Two plasmid 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.

The optimization experiment should include at least two cell densities (in the range of 60 to 80% confluency) 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 (corresponding to 0.2 to 0.8 µL/well of a 96-well plate)
- 100 to 200 ng of plasmid per well of a 96-well plate

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

1. Plate cells in a 96-well tissue culture plate one day before the 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 Cas9 plasmid working solution at 100 ng/µL by adding 1.2 mL nuclease-free, Tris buffer to 120 µg plasmid. Verify the DNA concentration using UV spectrophotometry at 260 nm, and adjust the volume if necessary to obtain 100 ng/µL.
3. In separate tubes, prepare seven DharmaFECT Duo working solutions (20 to 80 µg/mL) 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. The final DharmaFECT Duo amount is 0.2 to 0.8 µg/well of a 96-well plate. Incubate tubes for 5 minutes at room temperature.
4. To a deep well plate, add serum-free medium and Cas9 plasmid working solution for a total of 10 µL (Table 4). Then add 10 µL DharmaFECT Duo working solution for a total volume of 20 µL Transfection Mix for each well. This is repeated for each DharmaFECT Duo working solution. Mix by pipetting gently up and down and incubate for 20 minutes. DO NOT add plasmid or DharmaFECT Duo working solution to the untransfected control wells, which should contain serum-free medium only.

Table 4. Preparing samples for 96-well transfection optimization.

Sample Name	Serum-free medium	Working Cas9 plasmid solution (100 ng/µL)	Working DharmaFECT Duo solution (20-80 µg/mL)	Growth medium	Total volume per 96-well
100 ng Edit-R mKate2-Cas9 Nuclease Expression Plasmid	9	1	10	80	100
200 ng Edit-R mKate2-Cas9 Nuclease Expression Plasmid	8	2	10	80	100
Untreated	20	0	0	80	100

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.

5. Prepare transfection medium by adding 80 µL antibiotic-free complete medium to each sample from Step 4 to bring the total volume in each tube to 100 µL.
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 5) to each well.
7. Following transfection, assess the cells for mKate2 expression and cell viability to identify the conditions with highest mKate2 expression and low cell toxicity.
   a. At 24 to 48 hours examine the cells by microscopy to determine conditions with highest fluorescence intensity.
   b. At 48 to 72 hours post-transfection, perform 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.

If after performing transfection optimization, the efficiency is low in your cell line of interest, consider performing enrichment in the subsequent gene editing transfection, as per the protocols provided in the "Enrichment for Gene Editing Events" section, see page 9.

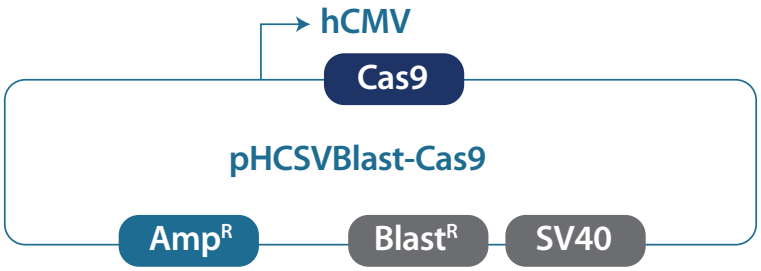
8. Use the optimal determined conditions for your subsequent co-transfection of Cas9 Nuclease Expression Plasmid with guide RNA. We recommend concentration of guide RNA between 25 nM to 100 nM (typically 25 nM is optimal for efficient gene editing). The optimized transfection conditions can be scaled up based on the tissue culture surface area.

mKate2 fluorescence intensity decreases when the Edit-R mKate2-Cas9 expression plasmids are co-transfected with increasing concentration of crRNA:tracrRNA complex.

5 Appendix

Cas9 Nuclease Expression plasmid with blasticidin selection marker

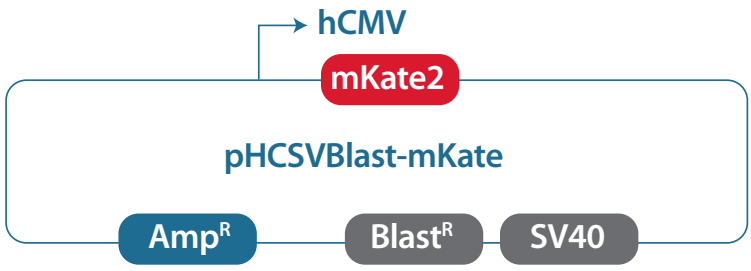
The Blast^R-Cas9 Nuclease Expression plasmid expresses a human codon-optimized version of the *S. pyogenes* Cas9 (Csn1) gene from a human cytomegalovirus (hCMV) promoter, and blasticidin resistance (Blast^R) under the control of the simian virus 40 (SV40) promoter **(Figure 5)**. The endotoxin-free plasmid DNA is provided in ready-to-transfect amounts.



Vector Element	Utility
hCMV	Human *cytomegalovirus* promoter for strong, constitutive expression of transgenes in mammalian cells
Cas9	Human codon-optimized *S. pyogenes* Cas9 nuclease cleaves targeted DNA when programmed with synthetic guide RNAs
SV40	Simian virus 40 promoter drives constitutive expression of blasticidin resistance (Blast^R) gene
Blast^R	Blasticidin resistance marker confers antibiotic resistance in mammalian cells for enrichment of transfected cells
Amp^R	Ampicillin resistance gene for vector propagation in *E. coli* cultures

Figure 5. Vector elements of the Edit-R Blast-Cas9 Nuclease Expression Plasmid.

This plasmid can be used in gene engineering experiments where blasticidin resistance is required for the experiment. A compatible mKate2 Optimization plasmid is available where the mKate2 fluorescent marker has replaced the Cas9 nuclease **(see vector map in Figure 6)**. This plasmid could be used for optimization of transfection conditions in a similar manner as described in "Transfection Optimization" Section, see **(pages 10-11)**.



Vector Element	Utility
hCMV	Human *cytomegalovirus* promoter for strong, constitutive expression of transgenes in mammalian cells
mKate2	Monomeric red fluorescent protein for visual tracking of transfection (excitation and emission maxima 588 and 633 nm, respectively)
SV40	Simian virus 40 promoter drives constitutive expression of blasticidin resistance (Blast^R) gene
Blast^R	Blasticidin resistance marker confers antibiotic resistance in mammalian cells for enrichment of transfected cells
Amp^R	Ampicillin resistance gene for vector propagation in *E. coli* cultures

Figure 6. Vector elements of the Edit-R mKate2 Transfection Optimization Plasmid.

Stability and storage

Edit-R Cas9 Nuclease Expression plasmid

Plasmid DNA reagents are shipped as dried pellets at room temperature (23 °C). Under these conditions, they are stable for at least four weeks. Upon receipt, plasmid DNA should be stored at -20 °C to -80 °C. Under these conditions, the reagents are stable for at least one year.

Always dissolve plasmid in nuclease-free solution, such as nuclease-free 10 mM Tris pH 7.4.

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 one year.

Always resuspend RNA in nuclease-free solutions, such as 10 mM Tris pH 7.4. In solution and stored at -20 °C, the aliquoted reagents are stable for at least two years

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 after prolonged storage at warm temperatures (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.

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

Edit-R synthetic sgRNA is synthesized using Dharmacon 2'-ACE chemistry, purified by size exclusion, deprotected, 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.

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 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 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 plasmids, and synthetic guide RNAs be subjected to?

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

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 (Table 5) 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.

Table 5. Making stock solutions of synthetic guide RNA.

synthetic guide RNA amount (nmol)	Volume (µL) of 10 mM Tris buffer to be added for desired final concentration	
	100 µM Stock	10 µM Stock
1	10	100
5	50	500
10	100	1000
20	200	2000
50	500	Exceeds tube volume*

* When the volume exceeds the tube volume make the 100 µM stock and dilute it 10 times to obtain the 10 µM stock.

Delivery of my package was delayed and my Edit-R Cas9 Nuclease Expression plasmid, mKate2 Transfection Optimization plasmid, tracrRNA and/or crRNAs have been at room temperature for a week. Are they OK to use?

Samples are shipped as a dried pellet and are stable for two to four weeks at room temperature. All RNA oligonucleotides and plasmid DNA should be placed at -20 °C or -80 °C upon receipt.

How are the Edit-R Cas9 Nuclease Expression plasmids, synthetic tracrRNA and synthetic crRNAs shipped?

The Edit-R Cas9 Nuclease Expression plasmid DNA, mKate2 Transfection Optimization plasmid DNA, tracrRNA and crRNAs are all provided as dried pellets and shipped at room temperature.

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.

How should I store the Edit-R Cas9 Nuclease Expression plasmids?

Dried plasmid DNA and DNA resuspended in buffer should be stored at -20 °C or -80 °C. Multiple freeze-thaw cycles should be avoided and strict DNase- and RNase-free conditions should be maintained to prevent degradation of nucleic acids.

My DharmaFECT Duo transfection reagent arrived and the ice was melted. Is the reagent still ok to use?

All DharmaFECT formulations, including DharmaFECT Duo reagent, have undergone substantial stability and functional testing, showing excellent performance even following storage at less-than-ideal conditions. The results are detailed in a product bulletin available for download.

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 Expression plasmids, and synthetic guide RNAs?

You can use these components in any mammalian cell type that is amenable to transfection and where the promoter options for Cas9 expression we provide are active.

How do I choose between the various Edit-R Cas9 expression plasmids?

To select one of the Edit-R Cas9 expression plasmids, consider the following:

1) If you prefer to select Cas9-expressing cells using Blasticidin, and the human CMV promoter is active in your cells of choice, you can choose the Cas9 nuclease expression plasmid with BlastR mammalian antibiotic selection marker (Dharmacon Cat #U-001000-120).

2) If you have access to a FACS instrument and prefer to sort cells based on expression of a fluorescent marker, you can choose one of six Cas9-mKate2 nuclease expression plasmids. Because the Cas9-mKate2 nuclease expression plasmids are offered with a choice of six different promoters, you must choose one based on known promoter activity in your cells of choice.

3) If you do not have access to a FACS instrument, or you simply prefer to carry out antibiotic selection via Puromycin treatment, you can choose one of six Cas9-PuroR nuclease expression plasmids. Since these are also offered with a choice of six different promoters, you should choose the one with an active promoter in your cells.

How do I select the Cas9 expression plasmid with the correct promoter for my cells?

We provide Cas9 plasmids with multiple promoter options for driving efficient expression of Cas9 nuclease fused to either the mKate2 reporter gene or puromycin resistance. We strongly recommend using an Edit-R Cas9 expression plasmid with a promoter which you know is active in your cells, either by your own experimental observations, or through references in the published literature. If such information is not available, testing several Cas9-mKate2 expression plasmid candidates and observing the comparative mKate2 fluorescence intensity can allow you to make an empirical selection.

When should I use the Edit-R mKate2 Optimization plasmid?

The mKate2 Optimization plasmid is similar to the Edit-R Cas9 nuclease expression plasmid with the Blasticidin resistance marker, where the mKate2 fluorescent gene sequence has replaced the Cas9 nuclease gene. The mKate2 Optimization plasmid can be used to perform transfection optimization in a similar manner as described in the "Transfection Optimization" section of the technical manual. However, if you are working with a Cas9 nuclease expression plasmid, then we recommend performing transfection optimization with a corresponding Cas9-mKate2 expression plasmid under the control of a promoter you know to be active in your cells.

Can I use the Edit-R synthetic guide RNAs components with my own preferred Cas9 nuclease expression plasmid?

We have validated the use of Edit-R synthetic guide RNAs and achieved efficient gene editing utilizing the Edit-R Cas9 expression plasmids in mammalian cell lines. Thus we cannot predict the efficacy of, nor can we troubleshoot experiments performed with, any other Cas9 nuclease expression vector. However, 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 successfully be used with another *S. pyogenes* Cas9 nuclease expression plasmid, as long as the expressed Cas9 sequence is suitably codon-optimized and is under the control of a promoter which is active in your cells of choice. Additionally, you must be able to efficiently co-transfect your plasmid DNA with the synthetic RNAs.

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 sequences 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.

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

DharmaFECT Duo is specifically optimized for co-transfection of plasmid DNA and small RNA. 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 assay shows a low percentage of editing. Why is that?

Mutation analysis assays which utilize mismatch-specific DNA endonucleases such as T7EI (NEB 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.

Will selecting edited cells by treatment with Puromycin or Blasticidin increase editing efficiency?

Puromycin- or Blasticidin-selection can enrich for cells in which editing has occurred and increase % editing; however, the degree of improvement greatly depends on optimized delivery conditions. If the transfection efficiency of the Edit-R components is high, then antibiotic selection will show a modest improvement in editing efficiency. However, if the transfection efficiency is low, antibiotic selection improves editing efficiency to a greater degree.

Can the Edit-R system be used for gene knockout in non-mammalian organisms, such as bacteria or worms?

We have designed the Edit-R Cas9 plasmids for expression (and thus have only tested) in mammalian cells. Thus we cannot predict the efficacy of using Edit-R synthetic RNA components, nor can we troubleshoot experiments performed in non-mammalian systems. However, 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* Cas9 nuclease expression plasmid, as long as the expressed Cas9 sequence is suitably codon-optimized and is under the control of a promoter which is active in your cells of choice. Additionally, you must be able to efficiently co-transfect your plasmid DNA with the synthetic RNAs.

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

We have validated the use of Edit-R synthetic tracrRNA and crRNAs and achieved efficient gene editing utilizing the Edit-R Cas9 expression plasmids in mammalian cell lines. Thus we cannot predict the efficacy of, nor can we troubleshoot experiments performed with, a mutant Cas9 nuclease expression vector or Cas9 mRNA. However, 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. Additionally, you must be able to efficiently co-transfect your Cas9 mRNA or plasmid DNA with the synthetic RNAs.

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.

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