

Product Information

Custom CRISPR Products

Catalog Number **CRISPR**

Product Description

CRISPR/Cas systems have evolved within bacterial and archaeal organisms as a defense against invading viruses and plasmids. Recently, the type II CRISPR/Cas system from the bacterium *Streptococcus pyogenes* has been engineered to function in eukaryotic cells using two molecular components: a single Cas9 protein and a non-coding guide RNA (gRNA).¹⁻⁷ The Cas9 endonuclease can be programmed with a single gRNA, directing a DNA double-strand break (DSB) at a desired genomic location (Figure 1). Similar to DSBs induced by zinc finger nucleases (ZFNs), the cell then activates endogenous DNA repair processes, either non-homologous end joining (NHEJ) or homology-directed repair (HDR), to heal the targeted DSB.

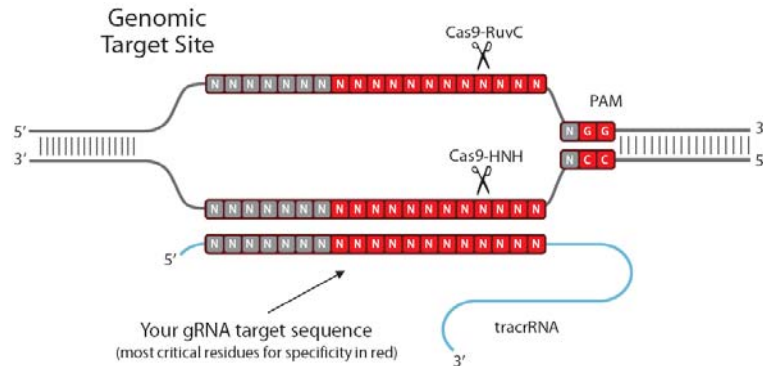
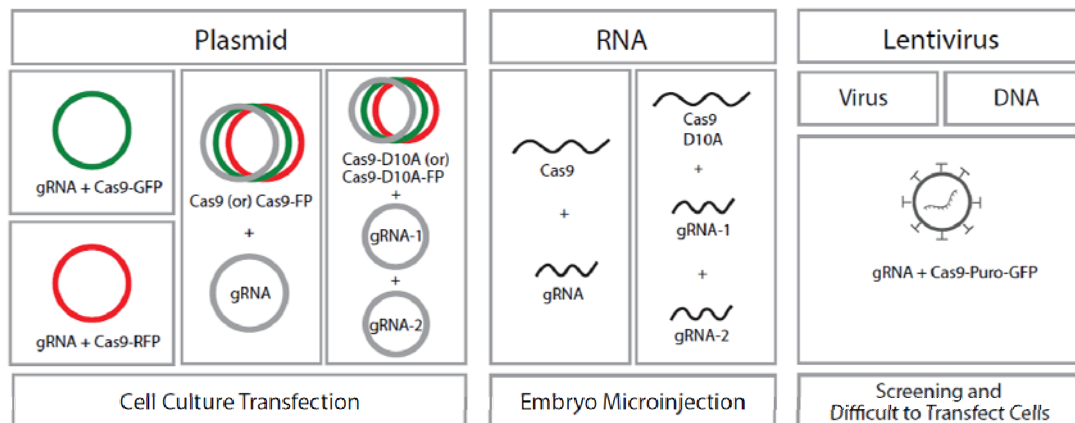


Figure 1. Schematic of a CRISPR/Cas-targeted double-strand break.

The major Sigma CRISPR product classes covered in this technical bulletin include:

1. Single vector CRISPR reagents (U6-gRNA/Cas9-**GFP** or U6-gRNA/Cas9-**RFP**).
2. Dual vector CRISPR reagents (U6-gRNA plasmid + separate Cas9 plasmid with or w/out FP).
3. CRISPR Cas9-D10A nickases (i.e. RuvC minus) and paired gRNAs for reduced off-target activity.
4. RNA-only formats for embryo microinjection (includes Cas9 nucleases and paired nickases).
5. Lentiviral particles (U6-gRNA/Puro-Cas9-**GFP**).

Sigma or User Generated CRISPR Target Design



The primary Sigma product number covered by this technical bulletin is:

CRISPR for:

- Custom gRNA expression plasmids (including All-in-One and gRNA-only plasmids)
- T7-generated gRNA
- Lentiviral particles

Compatible Sigma Products include:

Products for expression of Cas9 nuclease and Cas9-D10A nickase

CAS9P-1EA for the Cas9 plasmid

CAS9D10AP-1EA for the CRISPR Cas9-D10A Nickase plasmid

CAS9MRNA-1EA for injection and transfection-ready Cas9 mRNA

CAS9D10AMRNA-1EA for injection and transfection-ready CRISPR Cas9-D10A nickase mRNA

Products for expression of Cas9 nuclease and Cas9-D10A nickase with fluorescent protein reporters

CAS9GFPP-1EA for the Cas9-2A-GFP plasmid

CAS9RFPP-1EA for the Cas9-2A-RFP plasmid

CAS9D10AGFPP-1EA for the Cas9D10A-2A-GFP plasmid

CAS9D10ARFPP-1EA for the Cas9D10A-2A-RFP plasmid

gRNA positive and negative controls

CRISPR01-1SET for the CRISPR Human EMX1 Positive Control Plasmid

Includes CMV-Cas9 expression plasmid

Includes U6-gRNA plasmid (EMX1-s4) targeting: 5' GAGTCCGAGCAGAAGAAGAAGGG

CRISPR02-1SET for the CRISPR Nickase Human EMX1 Positive Control Plasmid

Includes CMV-Cas9-D10A expression plasmid

Includes U6-gRNA plasmid (EMX1-s4) targeting: 5' GAGTCCGAGCAGAAGAAGAAGGG

Includes U6-gRNA plasmid (EMX1-as4) targeting: 5' GCCGTTTGTACTTTGTCTCCGG

Mismatch detection primers for EMX1 positive controls for use with CEL-I or T7EI assays (not supplied):

Forward EMX1 primer: 5' -ATGGGAGCAGCTGGTCAGAG

Reverse EMX1 primer: 5' -CAGCCCATTTGCTTGTCCCT

Amplicon size: 507 bp

CRISPR06-1EA - CRISPR Universal Negative Control 1

CRISPR07-1EA - CRISPR Universal Negative Control 2

CRISPR08-1EA - CRISPR Universal Negative Control 3

Lentiviral positive and negative controls

CRISPR11 - CRISPR-lenti Human Positive Control DNA

CRISPR11V - CRISPR-lenti Human EMX1 Positive Control Transduction Particles

CRISPR12 - CRISPR-lenti Non-targeting Control Plasmid

CRISPR12V - CRISPR-lenti Non-targeting Control Transduction Particles

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Though the lentiviral transduction particles produced are replication incompetent, it is highly recommended that they be treated as Risk Group Level 2 (RGL-2) organisms.²⁹ Follow all published RGL-2 guidelines for handling and waste decontamination. Also, use extra caution when using lentiviral transduction particles that express CRISPR-targeting genes involved in cell cycle control, e.g., tumor suppressor genes.

Storage and Stability

Store CRISPR plasmid at $-20\text{ }^{\circ}\text{C}$ immediately upon arrival. Store CRISPR RNA and mRNA at $-80\text{ }^{\circ}\text{C}$ immediately upon arrival. Please avoid repeated freeze thawing of the plasmid or RNA. The reagents can be stored at $-20\text{ }^{\circ}\text{C}$ or $-80\text{ }^{\circ}\text{C}$ for up to 12 months.

Practice aseptic technique to avoid DNase contamination of the components. Keep reagent vials and sample tubes closed when not in use.

Note: Unless specified via custom requests, Sigma CRISPR plasmid products are delivered as mini-prep aliquots, which may not be suitable for transfection into particular cell types. For best results, we advise maxi-prepping plasmids using endotoxin-free DNA purification kits prior to transfection.

All-in-one Cas9-reporter Vectors for High Efficiency Single Cell Cloning

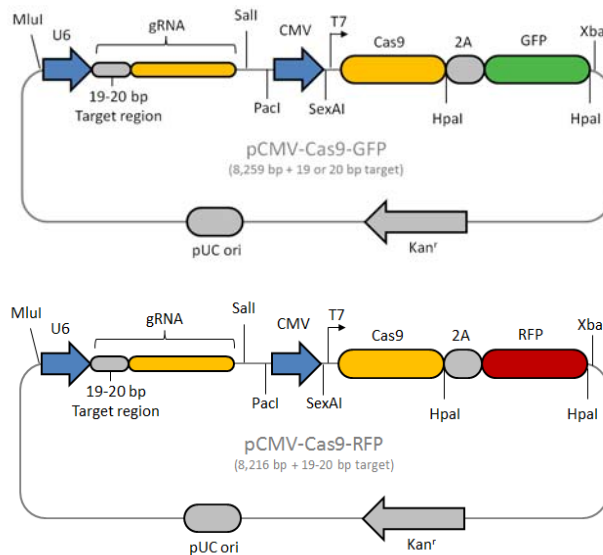
To expedite cell engineering workflows, Sigma offers a CRISPR/Cas subcloning service which delivers targeted CRISPR/Cas plasmids with the following features (Figure 3):

- A single vector format including the Cas9 protein expression cassette and gRNA. This maximizes the chances of successful cellular delivery of all necessary CRISPR/Cas components.
- GFP (or RFP) is co-expressed from the same mRNA as the Cas9 protein via a 2A peptide linkage, enabling tracking of transfection efficiency and enrichment of genome editing activity in cell populations via fluorescence activated cell sorting (FACS).
- The human U6 promoter is used to drive gRNA expression, while CMV promoter drives expression of Cas9 and GFP or RFP proteins. The human U6 promoter was chosen since it has proven to be superior to H1 promoter in previous RNAi applications.⁸
- A T7 promoter sequence is localized immediately upstream of Cas9 cDNA sequence, allowing for *in vitro* Cas9-GFP mRNA synthesis, if desired.

All Sigma CRISPR plasmid DNA is supplied at $20\text{ ng}/\mu\text{L}$ in $50\text{ }\mu\text{L}$. A total of $1\text{ }\mu\text{g}$ of plasmid DNA is supplied and is intended for transformation into *E. coli* to generate a working stock.

All-in-one plasmid maps and features.

The Cas9 protein implemented in these vectors contains both HNH and RuvC activities enabling the creation of double strand breaks. Cas9 is linked to EVROGEN™ TagGFP2 or TagRFP fluorescent proteins. TagGFP2 is the improved variant of TagGFP, a mutant of the *Aequorea macrodactyla* GFP-like protein.^{19,20} TagGFP2 possesses bright green fluorescence with excitation/emission maxima at 483 and 506 nm, respectively. TagRFP is a monomeric red (orange) fluorescent protein generated from the wild-type RFP from sea anemone *Entacmaea quadricolor*.²¹ It possesses bright fluorescence with excitation/emission maxima at 555 and 584 nm, respectively. In both GFP and RFP vectors, the 2A-FP encoding sequence is flanked by two *Hpa*I restriction sites, which allows removal or replacement of the 2A-FP element. The *Xba*I site can be used to linearize the vector for production of Cas9-FP mRNA via *in vitro* transcription using T7 RNA polymerase.



CRISPR Cas9-D10A Nickases and Paired gRNAs for Reducing Off-target Acitivity

Recent evidence indicates off-targeting by CRISPR endonucleases is a significant concern for applications requiring high specificity.^{9,10} To address this problem, Sigma has developed paired nickase technology to expand CRISPR DNA recognition tracts to lengths similar to those of ZFNs and TALENs. While we have found that both D10A and H840A-type Cas9 mutations can result in active paired nickases, our experience suggests that paired nickases based on the Cas9-D10A mutant (i.e. RuvC minus) are most reliable. These results have been independently confirmed.¹¹⁻¹³ Our development work also suggests that for both D10A and H840A-type Cas9 mutants, the critical factor in producing active paired nickases is the positioning of gRNAs in a 5'-to-5' orientation. A quick way to verify the correct 5'-to-5' orientation is to ensure that the PAMs of chosen paired gRNA sites, as viewed on the sense strand, are in a 5'-CCNN₁₉₋₂₀...N₁₉₋₂₀NGG-3' format (see Figure 4). Our data indicate that 5'-to-5' PAM spacings ranging from 30 to 150 bp can result in paired nickase activity useful for genome editing workflows (i.e. indel rates > 1%).

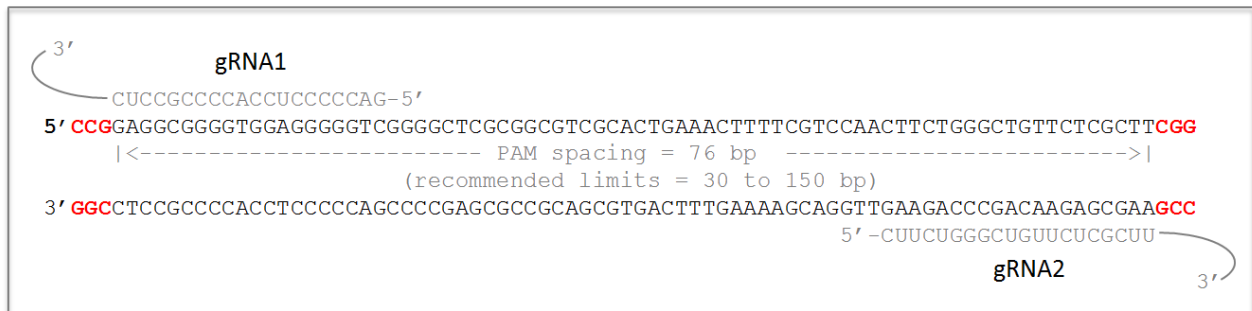


Figure 4. For optimal Cas9-D10A paired nickase functionality, gRNAs should be designed in a 5'-to-5' orientation with PAM spacing between 30 and 150 bp. For the above paired gRNAs, the following targeting sequences would be used by Sigma for cloning into U6-gRNA vectors: gRNA1-5'GACCCCTCCACCCGCCTC, gRNA2-5'CTTCTGGGCTGTTCTCGCTT (this pair targets the VEGFA locus).

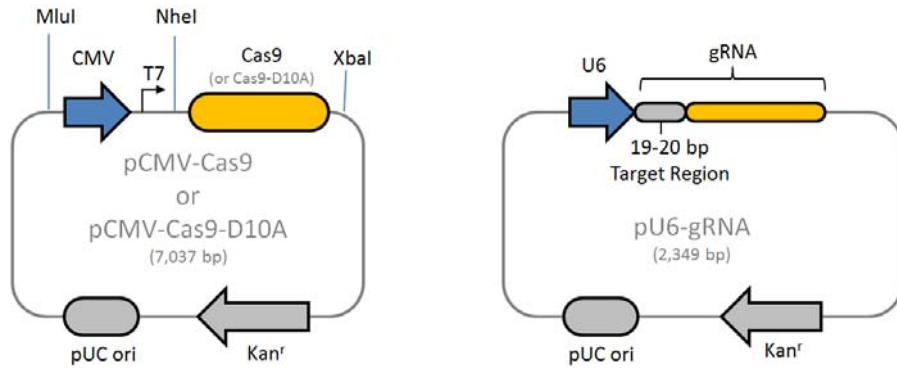
To simplify the use of paired nickases, Sigma has on-line tools which allow access to a library of pre-designed paired nickases. Please check Sigma's on-line CRISPR product offerings for the latest design sets and custom CRISPR and donor design services. We recommend testing 3-4 sets of paired nickases to maximize chances of finding an active pair.

For plasmid-based delivery of paired CRISPR nickases, we recommend the use of a three plasmid system comprised of the Cas9-D10A expression vector and two U6-driven gRNA vectors (Figure 2). The reason for this is two-fold:

1. In our experience, the three plasmid system has worked more reliably than single plasmid formats at producing highly active paired nickases.
2. The three plasmid system allows for flexible titration of gRNA and Cas9-D10A plasmids to optimize genome editing rates. For example, if one gRNA in a paired nickase has low activity, but a valuable location, you can independently increase the plasmid transfection levels of that gRNA in attempts to boost activity.

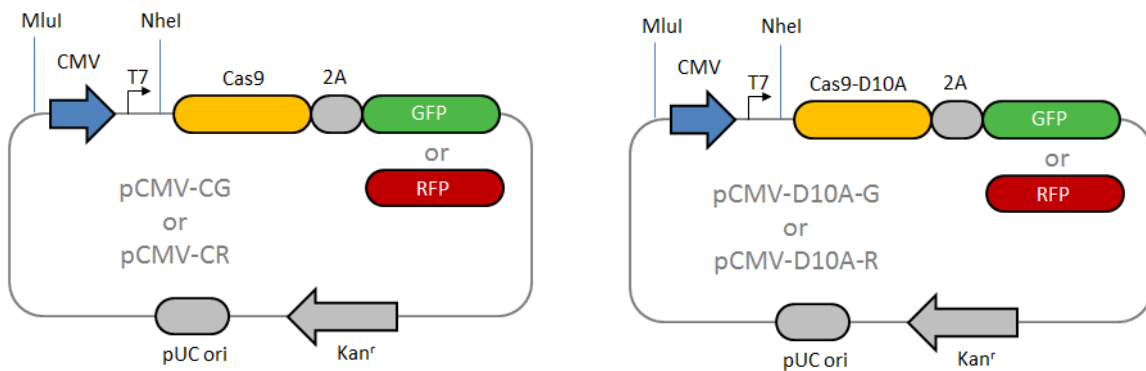
For initial experiments, we recommend:

- $\geq 1.0 \mu\text{g}/\mu\text{L}$ and $\leq 5 \mu\text{L}$ of Cas9 or Cas9-D10A plasmids (per 0.5 to 1 million cells nucleofected)
- $\geq 1.0 \mu\text{g}/\mu\text{L}$ and $\leq 5 \mu\text{L}$ of U6-gRNA plasmids (per 0.5 to 1 million cells nucleofected)



The Cas9 and Cas9-D10A expression plasmids use the CMV promoter for strong transient expression of Cas9. Alternate promoters can be substituted by replacement of CMV using MluI and NheI. Also, the Cas9 and Cas9-D10A expression plasmids can be linearized using XbaI for T7-based mRNA production.

The Cas9 and Cas9-D10A expression plasmids can also be provided as 2A-fusions to GFP and RFP as shown below:



A product number summary is provided at the beginning of this document to guide choices of various Cas9 plasmid formats.

RNA-only CRISPR Formats for Embryo Microinjection

It is well-established in the literature that knockout animals can be generated via microinjection of ZFN mRNA and donor constructs¹⁴⁻¹⁶, and recently these methods have been extended using CRISPR systems.¹⁷ Sigma offers RNA-only CRISPR formats for both single-site and paired nickase formats (Figure 2). The use of RNA vs. plasmid constructs avoids complications with promoter-embryo compatibility as well as the possibility of random integration of nuclease and gRNA-expressing plasmids into the host animal genome.

Sigma CRISPR RNA is supplied at concentrations of 500 ng/μl (50 μl Cas9 mRNA and Cas9-D10A mRNA, capped and polyA-tailed) and 200 ng/μl (20 μl gRNA). Typical microinjection concentrations used in the literature are in the ranges of 20-200 ng/μl for Cas9 mRNA and 10-50 ng/μl for gRNA.

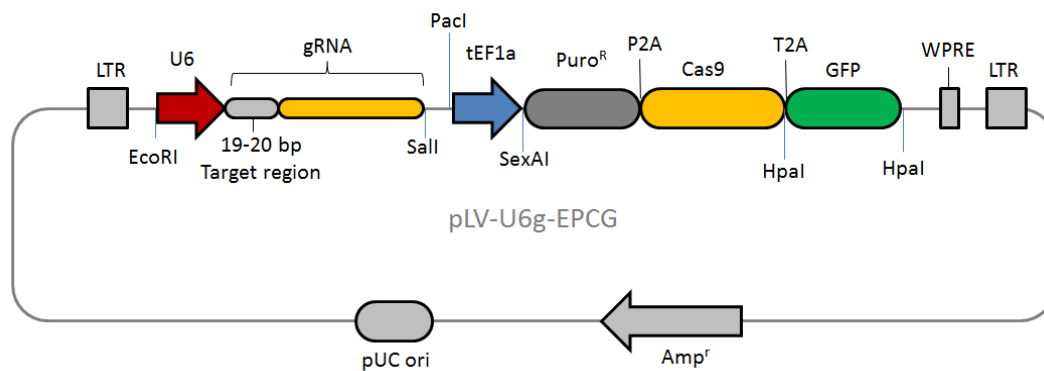
While Sigma CRISPR RNA was not developed for robust application to cell culture, we have seen some success in detecting CEL-I activity using RNA-only formats for both CRISPR nucleases and paired nickases. RNA-only delivery formats may be favorable for cell types which are sensitive to double stranded DNA (such as dendritic cells¹⁸) or when promoter-cell incompatibilities exist.

If you suspect that the promoter used to drive Cas9 in your plasmid is not functional in your cell type, a quick possible solution may be to substitute Cas9-mRNA. Since the human U6 promoter used to drive gRNA has robust performance across different cell types (based on experience with shRNA), try co-transfection Cas9-mRNA or Cas9-D10A-mRNA along with U6-gRNA plasmids. If Cas9-mRNA does not work, then try to modifying your plasmid with an alternate promoter to increase Cas9 expression (see below for plasmid maps, restriction sites, etc.).

Note: while Sigma implements the same high quality RNA synthesis procedures that have been successful in many mRNA-based ZFN transgenic projects, we highly recommend centrifuging your final, concentration-adjusted CRISPR RNA samples immediately prior to microinjection to ensure all particulate material has been removed which may result in clogging of microinjection needles.

Lentiviral CRISPR Formats

Certain cell types are difficult to transfect using lipid reagents, electroporation, or nucleofection. To enable genome editing in these cell types, Sigma has built upon capabilities within MISSION lenti-shRNA products to develop an all-in-one lenti-CRISPR vector containing both gRNA and Cas9 elements (see vector map below). A unique feature of the all-in-one vector (pLV-U6g-EPCG) is a Cas9 ORF flanked by puro and GFP elements, providing multiple options for monitoring stable cell populations which are expressing CRISPR components.



Previous genome editing workflows implementing ZFNs and TALENs primarily utilized transient modes of nuclease expression. A unique feature of lentiviral delivery is the option for highly efficient chromosomal integration of CRISPR components. This allows for longer term CRISPR expression and drug-based enrichment of CRISPR-expressing cell populations^{27,28}. Furthermore, drug selection provides a gentler alternative for clonal isolation versus 96-well or FACS-based single cell cloning where some cell types experience significant levels of apoptosis due to rapid and extreme isolation.

A key experimental parameter to estimate when using lentivirus is multiplicity-of-infection (MOI). When comparing MOI values among different publications, protocols, datasets, etc., it is important to determine what methods were used for MOI calculation. MOI is specifically defined as the number of transducing units (TU) per cell. However, TU is broadly defined in many ways depending on vector type, including:

1. Count of fluorescent cells post-transduction via FACS (for lenti-vectors expressing fluorescent proteins).
2. Drug selection and counting of viable colonies (i.e. colony forming units or CFU).
3. ELISA assays which measure lentivirus associated p24 core protein. Note: this can measure total p24 in the supernatant, but some methods attempt to quantitate only p24 within intact particles.

In our experience with MISSION shRNA vectors, titers based on bulk p24 measurement (meaning free p24 + particle-associated p24) are approximately 20-fold higher than titers measured by counting of viable puromycin-resistant colonies. Similar comparisons with all-in-one lenti-CRISPR vectors yield a p24:CFU ratio of approximately 200 to 400 (dependent upon cell type). This reduction in CFU-based titer is most likely due to the large size of the Cas9 ORF (typically 3-4 kb). When produced via 96-well formats, Sigma's all-in-one lenti-CRISPR vector has been characterized to yield minimum titer of 1×10^6 TU/ml as measured by bulk p24 (free and particle-associated). Higher titer formats are available upon custom request.

96-well protocol for viral titers $\geq 1 \times 10^6$ (p24).

For first time users of CRISPR lentivirus, we advise you conduct two preliminary experiments: (1) determine the sensitivity of your cells to puromycin (kill curve), and (2) determine CFU/ml by transducing cells at various dilutions of virus (see Tip No.5 below). Once these experiments have been completed, they will inform your MOI calculations needed for the following suggested 96-well protocol.

Note: Lentiviruses are quite labile. Multiple freeze-thaw cycles and prolonged exposure to ambient temperatures will decrease the lentiviral titer. Thaw lentiviral particles on ice. Keep them stored on ice when not in use.

Day 1

- Seed cells into a 96-well at approximately 50% confluency (~3,000 to 5,000 cells).

Day 2

- 1) Calculate the volume (μL) needed for your target MOI based on previous experience, or first pass CFU measurements (see Tip No.5 below).
- 2) In a separate test tube, add polybrene to the media at a final concentration of 8 $\mu\text{g}/\text{ml}$.
- 3) Add the appropriate volume of virus to the media/polybrene solution. A typical transduction has 10-15 μL of virus with the final volume of 50 μL made up of media/polybrene.
- 4) Remove media from the 96-well and add the virus/media mixture.
- 5) Mix on a slow rocker 5-15 min then place at 37 $^{\circ}\text{C}$ for 24 hours.

Day 3

- Change the media.

Day 4+

- 1) At 48 hours post-transduction, add puromycin to the media at a concentration determined via kill curve (see Tip No.5 below).
- 2) Select on puromycin for the desired amount of time (typically 4 to 14 days). Less active gRNA designs may require the longer incubation times to achieve maximal knockout.
- 3) Perform the desired phenotypic assays, genotyping assays, or single cell cloning operations.

Tips for Cell Engineering using Sigma CRISPR plasmids, RNA, and virus

1. CRISPR design and specificity

CRISPR endonucleases have shown wide variation in their activity, even among multiple CRISPRs designed within close genomic proximity.² For this reason, we highly recommend that you test 3 to 4 CRISPR nucleases (or paired nickases) that target different DNA sequences. Since size of the DNA target site for CRISPR systems is significantly smaller than that required for CompoZr™ ZFNs (e.g., 30–36 bp), care must be taken during design to ensure minimal off-target breaks elsewhere in the genome. Recent evidence indicates off-targeting by CRISPR endonucleases is a significant concern and some guidance for avoiding off-target activity is beginning to develop.^{9,10} If you feel your particular application requires enhanced specificity, paired CRISPR nickases may be used (Figures 2 and 4). Many on-line tools are available for CRISPR design; however, Sigma has applied its core capabilities in specific ZFN design to the CRISPR/Cas system to create an *in silico* collection of genome-wide CRISPR target sequences for both single site and paired nickase applications. Please check Sigma's on-line CRISPR product offerings for the latest CRISPR design sets and custom design services for ZFNs, CRISPR, and related donor DNAs.

2. Delivery of CRISPR plasmids for cell culture applications

Note: Unless specified via custom requests, Sigma CRISPR plasmid products are delivered as mini-prep aliquots, which may not be suitable for transfection into particular cell types. For best results, we advise maxi-prepping plasmids using endotoxin-free DNA purification kits prior to transfection.

Previous experience with many ZFN projects has shown nucleofection is a robust delivery method for a wide variety of cell types. For initial experiments in human cells, we advise nucleofecting maxi-prepped CRISPR plasmid DNA into well-validated cell types such as K562 or U2OS to assess double strand break activity or donor integration levels. These cell lines have been shown to respond with high levels of homologous recombination²², making them ideal for testing compatibility of ZFN and CRISPR nucleases with newly designed donor constructs before moving to a more challenging or unknown cell type. For mouse and rat cell culture testing, neuro2A and C6 are suitable cell types for initial CRISPR experiments. A good starting point for dosage experiments is:

- 2–8 µg of CRISPR plasmid (for single plasmid Cas9-FP linked vectors)
- ≥ 1.0 µg/µL and ≤ 5 µL of Cas9 or Cas9-D10A plasmids combined with ≥ 1.0 µg/µL and ≤ 5 µL of U6-gRNA plasmids.

(All dosages above assume 0.5 to 1 million cells nucleofected)

Since Sigma's single vector format contains a GFP (or RFP)-linked expression cassette for Cas9, transfected cells can be subsequently inspected by microscopy or FACS to monitor transfection efficiency (Figure 5) prior to performing genotyping assays or single cell cloning efforts.

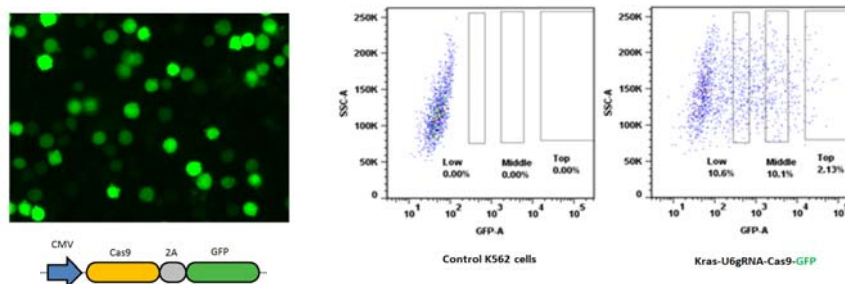


Figure 5. Monitoring Cas9-GFP expression cassette activity via microscopy or FACS.

3. Monitoring CRISPR double strand break activity

Several published protocols exist for monitoring double strand break (DSB) activity. The most rapid, flexible, and economical assay is the mismatch cleavage assay, which can detect the variety of insertions and deletions (indels) generated by NHEJ activity in eukaryotic cells. The most common protocols use the CEL-I enzyme (a.k.a. Surveyor nuclease)²³ and T7 endonuclease I (T7EI).²⁴ If sufficient bioinformatics and deep sequencing resources are available at your institution, deep sequencing can also be used to detect and quantitate indel activity in CRISPR-treated cell populations.

If, for a particularly favored CRISPR design, you cannot detect DSB activity despite many attempts, consider enriching the cell population for Cas9-FP expression as described in the next section.

4. Single cell cloning and genotyping

Since some Sigma CRISPR plasmids implement an FP-linked Cas9 expression cassette, fluorescence-activated cell sorting (FACS) can be used to isolate cell populations with significantly increased frequencies of Cas9-induced modifications (Figure 6). This FACS-enrichment approach is particularly useful in scenarios where delivery efficiencies and/or Cas9 expression levels are low or undetectable. Furthermore, the FP co-expression approach has advantages over surrogate reporters including:

- Cas9-FP linked expression does not require the extra step of cloning of artificial target sites into surrogate reporter plasmids.
- A reduction in the total amount of transfected dsDNA. In many cases, certain cell types are sensitive to high amounts of dsDNA, and adding additional dsDNA mass in the form of a surrogate reporter plasmid to existing CRISPR and donor vector constructs can result in cell toxicity.
- The option to use “dsDNA free” approaches by implementing Cas9-FP mRNA with *in vitro* transcribed gRNA (Figure 2), and single stranded oligos (ssODNs).

The Cas9 and FP coding regions are linked by a small sequence encoding a 2A peptide. The 2A peptide is a “self-cleaving” peptide, which allows production of two individual proteins from one transcript and utilizes “ribosomal skipping” rather than proteolytic cleavage mechanism to generate two individual proteins.^{25,26}

Cells can be harvested for FACS according to commonly used protocols. We suggest cells be sorted into fractions with low, medium, and high FP expression levels. Cell populations with the highest FP expression have been shown to enrich genome edits with the extent of the “high” fraction ranging from 1–30% of the total cell population. The optimal size of the “high” and “medium” populations may vary, depending on genomic loci, cell types, delivery methods, and other experimental variables. A good start is to divide the cell population into three low, medium, and high fractions each of which comprises 1–20% of the total cell population.

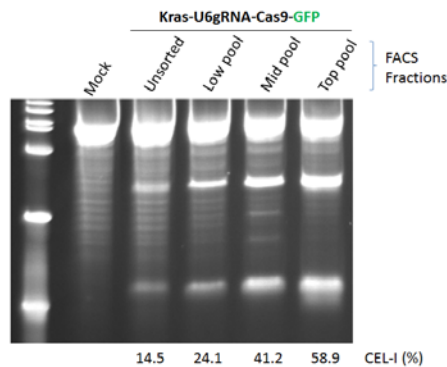


Figure 6. Enrichment of CRISPR/Cas-induced CEL-I activity in human K562 cells via FACS using a single Cas9-GFP vector targeted to the human KRAS locus.

5. Measurement of Colony Forming Units (CFU) in Lentiviral CRISPR applications

Note: this protocol is intended for low-titer virus with bulk p24 ELISA measurements ranging from 1×10^5 to 1×10^6 TU/ml. Resulting CFU measurements will vary depending on cell types and cell culture conditions.

Required Reagents and Equipment

- Lentivirus, target cells, and culture medium
- Hexadimethrine bromide (Polybrene) (2 mg/mL stock); Product No. H9268
- Puromycin (10 mg/mL stock); Product No. P9620
- Crystal Violet Solution; Product No. HT90132
- Dulbecco's Phosphate Buffered Saline; Product No. D8662
- 6-well cell culture treated plates
- 15 mL conical vials
- 5% CO₂, tissue culture incubator

Prior to CFU assay

Obtain a puromycin kill curve roughly as follows:

1. Plate 1.6×10^4 cells into wells of a 96-well plate with 120 μ L fresh media.
2. The next day add 0.5–10 μ g/mL of puromycin to selected wells.
3. Examine viability every 2 days.
4. Culture for 10–14 days. Replace the media containing puromycin every 3 days.
5. The minimum concentration of puromycin that causes complete cell death after 3–5 days should be used for that cell type.

Day 1: Seeding of cells

- Seed 10^5 cells per well in fresh media to one 6-well plate. Place plates in an incubator set to 37 °C, 5% CO₂ for 24 hours.

Day 2: Lentiviral Transduction

- 1) Thaw one vial of lentiviral particles (25 μ L) at room temperature until ice crystals disappear. Mix by gently tapping the tube several times. Store the lentiviral stock on ice. **Note:** Lentiviruses are quite labile. Multiple freeze-thaw cycles and prolonged exposure to ambient temperatures will decrease the lentiviral titer.
- 2) Prepare 10 mL of media containing polybrene (final concentration 8.0 μ g/mL).
- 3) Prepare 2.0 mL ten-fold serial dilutions over a range of 10^{-2} to 10^{-6} in 15 mL conical vials with virus. Mix gently by inverting the tubes five to ten times.
 - i) Mix 20 μ L lentivirus with 1980 μ L polybrene containing media to achieve 10^{-2} dilution.
 - ii) Mix 200 μ L of the 10^{-2} dilution with 1800 μ L polybrene containing media to achieve 10^{-3} dilution.
 - iii) Mix 200 μ L of the 10^{-3} dilution with 1800 μ L polybrene containing media to achieve 10^{-4} dilution.
 - iv) Mix 200 μ L of the 10^{-4} dilution with 1800 μ L polybrene containing media to achieve 10^{-5} dilution.
 - v) Mix 200 μ L of the 10^{-5} dilution with 1800 μ L polybrene containing media to achieve 10^{-6} dilution.
- 4) Remove medium from all wells of the 6-well plate. Add 1.0 mL of polybrene containing complete growth media to one well on one 6-well plate as negative control. Add 1.0 mL of each of the lentivirus dilutions to the remaining wells of the plate.
- 5) Place plate in an incubator set to 37 °C, 5% CO₂ for 24 hours

Day 3: Media change

- Remove the media containing lentiviral particles from the wells. Add 2.0 mL fresh media (without polybrene) to each well. Incubate at 37 °C with 5% CO₂ for 24 hours

Day 4: Puromycin selection

- Remove media from wells. Add fresh media containing puromycin.

Day 5-8: Continued puromycin selection

- Replace media containing puromycin as necessary during the selection process (usually every 2 to 3 days).

Day 9: Stain and counting of colonies

Note: the untransduced control cells should completely die after 4-day selection by puromycin.

- 1) Remove media and gently wash each well with Dulbecco's PBS. Add 1.0mL of crystal violet solution and incubate 10 minutes at room temperature. Remove crystal violet solution. Wash twice with 3.0 mL Dulbecco's PBS.
- 2) Invert plate on paper towels and let dry about 1 hour.
- 3) Count the blue-stained colonies using a microscope at a magnification of 40X.
- 4) Calculate by multiplying the number of colonies per well by the dilution factor. The lentiviral titer is defined here as Colony Formation Units per milliliter (CFU/mL)

References -

For the most up to date genome editing reference list, please visit our website:

<http://www.sigmaaldrich.com/life-science/zinc-finger-nuclease-technology/zfn-references.html>
<http://www.sigmaaldrich.com/life-science/zinc-finger-nuclease-technology/zfn-references.html>
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