CRISPR/Cas System
Site-Specific Genetic Manipulation

User Manual

Version 2.0

Transposagen Biopharmaceuticals, Inc.
535 W. Second Street, Suite L102. Lexington, KY 40508
info@transposagenbio.com

Table of Contents

1.1 Introduction and Background

Bacteria make use of CRISPRs (Clustered, Regularly Interspaced, Short Palindromic Repeats) together with CRISPR-associated proteins (Cas) as a form of acquired resistance to invading plasmids or viruses. The type II Cas-CRISPR system inserts foreign DNA (protospacers) into the CRISPR sequences, and later transcription produces a CRISPR RNA (crRNA), which anneals to a trans-activating crRNA (tracrRNA).[1,2] The dual-RNA complex interacts with the complementary DNA strand of the target site and recruitment of the Cas9 endonuclease cleaves the DNA within the targeted region.[3] Synthetic single guide RNA (sgRNA) comprised of a fusion of the dual-RNA complex has been shown *in vivo* to cut target DNA.[3, 4] Site-specific cleavage of the target DNA occurs at a location determined by the complementary base pairing of the gRNA and target DNA and a small motif, the protospacer adjacent motif (PAM) shown in Figure 1. In the type II Cas/CRISPR system, the PAM sequence (NGG) is essential for DNA cleavage. This Cas/CRISPR complex will be referred to simply as "CRISPR" throughout this user manual.

CRISPR can be designed against genes of interest to create double-stranded breaks and subsequent indel formation via the Non-Homologous End-Joining (NHEJ) repair mechanism. The insertions or deletions often times result in a frame shift or nonsense mutation, creating a targeted gene knockout. Co-transfection of the Cas/CRISPR system along with a targeting vector can result in the incorporation of the donor sequence through Homologous Recombination (HR) in the cut site, thus resulting in a knock-in mutation as shown in Figure 2.

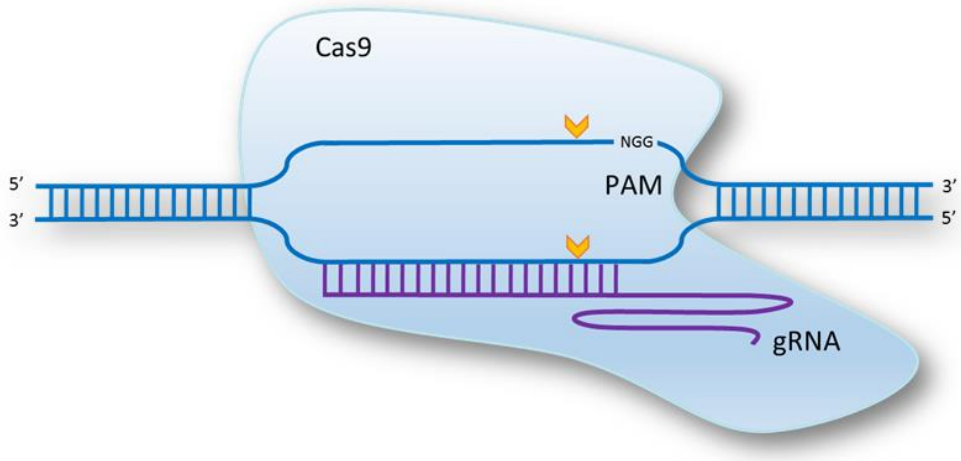

***Figure 1**: Mechanism of Cas/CRISPR directed Mutagenesis. The synthetic gRNA interacts with the complementary sequence within the target region. Recruitment of the Cas9 endonuclease cleaves the DNA 5' of the PAM. Creation of a double-stranded break result in DNA repair mechanism activation and repair of the break via non-homologous end joining (NHEJ) or homologous recombination (HR).*

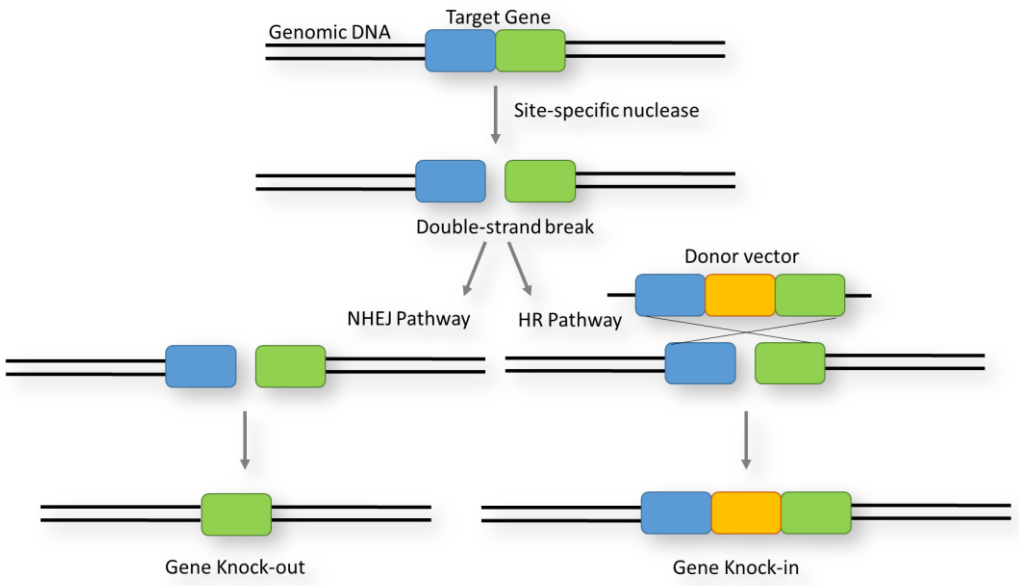

Figure 2. *Mechanism of CRISPR mediated mutagenesis. Knockout mutations are created via non-homologous end joining, which occurs naturally in the cells, causing small deletions or insertions that disrupt the reading frame of the DNA. Knock-in mutations can be created through homologous recombination along with a donor vector that has the desired DNA sequence.*

Transposagen is also able to create targeting vectors for knock-in and gene editing studies. CRISPR technology can be used in conjunction with Transposagen's other core technology, the *piggyBac*™ transposon vectors, which provides the only method for Footprint-Free™ Genetic Engineering down to a single base pair. For additional information, visit the Transposagen website at http://www.transposagenbio.com/gek-footprint-free.

1.2 Design and Construction of CRISPRs

Send an order form according to the instructions provided by your Gene Editing Specialist. Due to some constraints of the CRISPR system, please provide at least 500bp of sequence. After submission of the target sequence, our scientists will design and synthesize your custom vector using methods described in Hwang et al. (2013)[12]. sgRNAs may be placed either into a T7 or U6 driven backbone. Transposagen delivers 10µg – 50µg of sequence verified, transfection-ready plasmids. More information about pricing and ordering can be found on the Transposagen website at http://www.transposagenbio.com/.

Note: Transposagen does not perform cleavage assays/activity assays for standard orders. For an additional fee-for-service, this assay can be performed. Contact your local Gene Editing Specialist or email info@transposagenbio.com for more information.

1.3 Choosing a Donor Plasmid

If you are planning a knock-in or gene editing experiment, you will also need to choose a donor plasmid. In addition to the sequence needed to make the desired mutations, a donor plasmid may contain a selectable marker such as an antibiotic resistance or a fluorescence gene. The use of selection can reduce the number of clones that must be screened for correct insertion of the mutation. Transposagen has many pre-validated selection cassettes available in the Multivector™ series**.** These include, but are not limited to:

Puromycin/TK	Multivector™ Catalog# PB-MV1Puro-TK
Neomycin/TK	Multivector™ Catalog# PB-MV1Neo-TK
GFP/Puromycin	Multivector™ Catalog# PB-MV1CMV-GFP-Puro
Neomycin	Multivector™ Catalog# PB-MV1Neo
Hygromycin/TK	Multivector™ Catalog# PB-MV1Hygro-TK

1.4 Protocol – Introduction of CRISPR into Target Cells in Culture

CRISPRs can be introduced into target cells using the method that is most appropriate for the target cells. Below are example conditions others have used for transfecting cells with site-specific nuclease technologies, including CRISPR. Nucleofection or electroporation usually results in the highest cleavage efficiencies, so it is recommended to use an electroporation or nucleofection delivery method if possible. For gene editing and targeted integration purposes, you will also want to include a donor plasmid in the transfection/ electroporation of the CRISPR into the target cells.

A. Transfection into Cell Lines in Culture

Lipid-based transfection works in many cases, and many researchers choose to use Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions, especially for commonly available cell lines such as CHO, 293, HT1080, HepG2, and NIH 3T3. Transposagen recommends using 500 ng of each CRISPR construct for lipid-based transfections in a 12-well dish ($5x10^5$ cells/well). Appropriate scaling up or down can be made depending on dish size.

B. Transfection of CHO-K1 cells using Amaxa Nucleofector II

1. Mix $1x10^6$ CHO-K1 cells with 1 µg of each CRISPR construct and 100 µL Amaxa Solution T.
2. Transfect cells in an Amaxa Nucleofector II using program U-23
3. Recover into 1.4 ml warm F-12 medium + 10% FCS.
4. Grow as described in Chaderjian et al., 2005[5]

C. Transfection of Mouse Embryonic Stem Cells using Lipofectamine 2000

1. Transfect ES cells using Lipofectamine 2000 (Invitrogen) with 3 µg of each CRISPR construct.
2. 15 hours after transfection, change to ESLX media containing ES-DMEM, 20% ES qualified FBS, 1× nonessential amino acids, 1× nucleosides, 1,000 U/ml ESGRO LIF (Chemicon), 2 mmol/l l-glutamine, 1× Pen/Strep (Invitrogen), and 0.12 mg/ml sodium pyruvate and 0.1 mmol/l BME (Sigma-Aldrich). (Connelley et al., 2010)[6]

D. Electroporation of Cells in Culture

1. We recommend using $2x10^6$ cells in 400 µl OptiMEM (Invitrogen), 10 µg of each CRISPR construct, and 40 µg of donor vector.
2. Electroporation conditions should be optimized for each cell line and may depend on the electroporation instrumentation being used. (Perez-Pinera et al., 2012)[7]

1.5 Creation of Transgenic or Knockout Animals with CRISPR

The CRISPR system has been used in a variety of models for genetic manipulations. Information and/or protocols for particular models can be found in the following publications:

Bacteria: Jiang, W., Bikard, D., Cox, D., Zhang, F. & Marraffini, L.A. (2013)[8]
Danio rerio: Hwang, W.Y. et al. (2013); Cheng et al. (2013)[4,9]
Drosophila melanogaster: Gratz, S.J. et al. (2013)[10]
Mus musculus: Shen, B. et al. (2013); Wang, H. et al. (2013)[11,12]
Saccharomyces cerevisiae: DiCarlo, J.E. et al. (2013)[13]

Transposagen offers custom genetic manipulation of cell lines, rodents, zebrafish, *Xenopus*, and *Arabidiopsis*. Generation of genetically manipulated animals not listed above may be possible, but an established protocol for use of the CRISPR system has yet to be published. Application of the system in a particular model may be used similar to other site-specific gene engineering technologies (e.g., TALENs, ZFNs) where no published reports exist. Below are established protocols for rat genetic manipulation.

A. Creation of Transgenic Rat Spermatogonia with CRISPR using Nucleofector LONZA

We recommend using the protocol described in Izsvák et al. (2010)[14] for creation of transgenic rat spermatogonia. This protocol uses the Amaxa Cell Line Nucleofector Kit L to introduce the DNA.

B. Pronuclear Injection into Rat Embryos

For injection into embryos, we recommend the method published in Guerts et al. (2010)[15]. For more details, we recommend reading the reference. We have summarized the protocol here. The CRISPRs can be delivered to embryos as supercoiled plasmid DNA or as mRNA.

1. Prepare plasmid DNA using the GenElute HP midiprep kit (Sigma-Aldrich) following the manufacturer's instructions.
2. Resuspend in DNA Injection Buffer at 10 ng/µL and store at −20°C until use.
3. Harvest the rat embryos and keep in a 37°C incubator with 5% CO2 until needed.
4. Thaw the prepared DNA and dilute it to the desired concentration using DNA Injection Buffer.
5. Using a microinjector, deliver the CRISPRs to one-cell fertilized embryos. The CRISPRs should be delivered to the pronucleus.

1.6 Cleavage Analysis

A. Enrichment for cells containing Nuclease-Induced Mutations

An effective protocol for determining efficient cleavage has been described by Kim et al., 2011.[17] This method includes an enrichment step for cells containing nuclease-induced mutations. The protocol is summarized here.

1. In this method, a reporter plasmid is also transfected into cells that contains monomeric RFP-enhanced GFP (mRFP-eGFP) as a fusion protein. The eGFP, however is out-of-frame with the mRFP due to a nuclease target site that has been inserted between the two.
2. When the CRISPR has not cleaved the fusion protein, the cells fluoresce only red. If a CRISPR cleaves the fusion protein, the cells will fluoresce both red and green.

3. This method enables one to determine whether a given set of CRISPRs has a high rate of cleavage. Cells can be sorted by FACS analysis or clonal selection, and then further sequenced to verify the presence of the desired genetic changes.

B. CelI Mutation Detection Assay Using Fluorescent Primers

The CelI mutation detection assay is another reliable way to look for genetic changes mediated by CRISPRs (Kulinski et al., 2000).[18] CelI is a nuclease isolated from celery that is stable over a wide range of conditions. It can recognize areas of DNA mismatch and cleave the DNA at those locations.

1. PCR is used to amplify both the normal and mutant alleles, using primers that have a 6-carboxyfluorescein (FAM) tag on one and a 4,7,2',7'-tetrachloro-6-carboxyfluorescein (TET) tag on the other.
2. When the DNA is denatured and then renatured, mismatches occur and the CelI enzyme recognizes the mismatches and cuts them on only one strand.
3. The resulting digested DNA is run on a DNA sequencer such that a digestion product will fluoresce either blue (FAM) or green (TET). The sizes of the two fragments indicate the location of the mismatch, and the sum of the sizes of the fragments equals the entire length of the PCR product.

C. CelI Mutation Detection Assay using PAGE

The CelI Mutation Detection Assay can also be performed without using fluorescent primers (Hibbs et al. Sigma-Aldrich).[19] The reaction works the same way, except that visualization of the fragments is performed using polyacrylamide gel electrophoresis as shown in Figure 3.

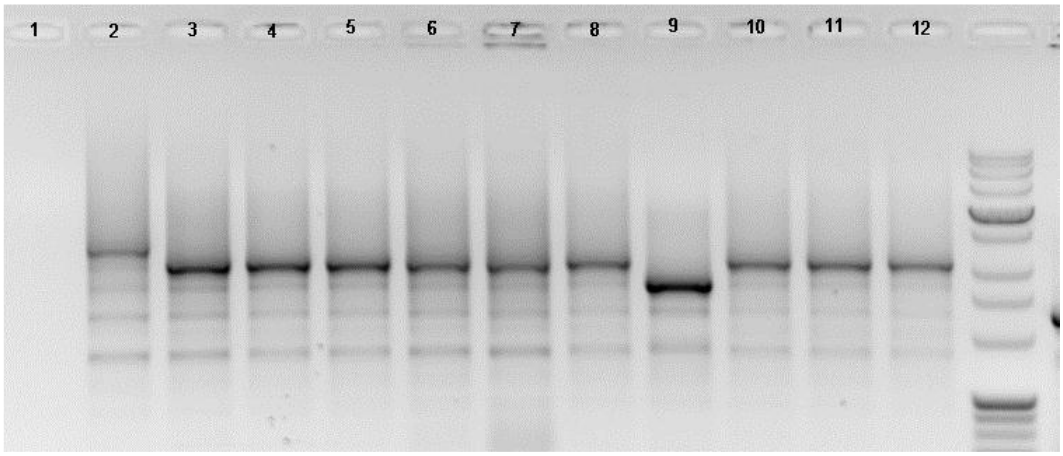

Figure 3*. Individual bacterial colonies were picked, and PCR was used to verify the presence of the correct sized bands. Clones that had the correct band were further sequence verified.*

D. PCR and Sequencing for Cleavage Analysis

Another method for cleavage analysis is to do successive rounds of PCR and sequencing. This method is more time-consuming because often many clones need to be analyzed in order to find one that has the desired cleavage efficiency (Kim et al., 2010).[20]

1. After 2-3 days of culture, isolate genomic DNA from the cells. Perform a PCR using primers that flank the deleted chromosomal sequence, and that result in a PCR product that is less than 1 kb. The Tm should be ~60˚C and the GC content should be 40-60%. Run the PCR for about 30-35 cycles. Then run the PCR out on an agarose gel.

2. The next step is for the PCR product to be sequenced. Subclone the PCR fragment into a blunt vector and then transform into bacterial cells. Pick colonies the next day, run a miniprep, and sequence the inserts using a primer that is included in the vector. Align the sequences with the known wild-type sequence and identify any deletions or insertions within the newly created sequence.
3. Estimate the frequency of the genetic change within the genomic DNA from the original samples by doing nested PCR.
4. Once you have a pair of CRISPRs that have a high cleavage efficiency, use those sequences to further transfect or electroporate the plasmids into your target cells.

E. Cell Line Generation

In general, there are two ways to generate cell lines including knockout cell lines or transgenic cell lines.

1. If you use a homologous recombination vector (donor plasmid) with an antibiotic resistance marker (Neo, puro, etc.) that inserts near the CRISPR cleavage site, you can select for resistant cells to greatly enrich for knockouts or transgenics. However, some copies of the donor vector will undergo random integration, so that not all cells that express the antibiotic resistance will have the desired genetic change. We recommend isolating clones by dilution cloning or single cell sorting. Then use PCR to screen for targeting at the cut site. One primer should anneal within the resistance marker or promoter and one in the genome *outside* the arms of homology. Note that the vector should not contain the entire recognition sequence of the CRISPR to avoid having the vector cleaved inadvertently.
2. The other way is to not use a homologous recombination vector, but rely on nonhomologous end joining to disrupt the coding sequence. Again you'll want to isolate clones, then perform PCR with oligonucleotides that flank the cut site by a couple hundred bases. The PCR product should show a deletion or may sometimes show small insertions. It is also possible to see a much shorter PCR product in some cases. However, both alleles may not have cut, so the sequence results will be noisy near the cut site, and clean before it. If all alleles did cut, the sequence results will be clean but will be different from the wild-type. This method may require screening hundreds of clones to find a clone with the desired genetic change. With either method, if not all alleles are disrupted, another round of transfections may be required.

1.7 Frequently Asked Questions

Q. How do I know where to cut my DNA?

A. The answer depends on your particular application. However, a general design parameter is to BLAST your sequence to be sure it is not repetitive. Also, if you plan to knockout a gene by cutting with CRISPR, a common strategy is to cut in the first coding exon, usually 50-100 bases from the start codon. Please note that this strategy does not necessarily result in completely non-functional gene product(s) and you should do the proper bioinformatics analysis before ordering.

Q. Can I make RNA of the CRISPRs from these plasmid backbones?

A. Yes. There is a T7 promoter for *in vitro* RNA synthesis using kits such as mMESSAGE mMACHINE T7 Ultra kit from Ambion. The vector can be linearized using DraI.

Q. Is the DNA prep I receive suitable for immediate transfection of my cells?
A. Yes, it is endotoxin-free and ready to use.

Q. Do you BLAST or otherwise analyze the requested cut sequence for specificity within the genome?
A. No. It is the responsibility of the end user to determine the suitability of the sequence for his/ her own research purposes. We make no attempt to discern the intended use of the CRISPRs you are ordering.

Q. What is percentage of my transfected cells will actually have the gene-of-interest knocked out or modified?
A. It is difficult to predict the percentage of cells that will be effectively knocked out. There are many factors that affect the outcome:

1. The transfection efficiency
2. The percentage of cells receiving both constructs
3. The cutting efficiency
4. Only a certain percentage of cells are cut at both alleles
5. There may be more than 2 alleles, especially in transformed cell lines
6. Some loci are unavailable, presumably due to chromatin structure

Q. What if my CRISPR doesn't cut?
A. There are many factors that affect the cleavage efficiency of any nuclease (see the answer to the above question). However, we do offer a *limited* guarantee. If, after repeated good faith attempts with proper transfection controls, you do not see any evidence of cleavage activity, we will make another CRISPR to a nearby site from the original sequence you provided to us in your order form free of charge. You will only be responsible to pay for shipping.

Q. If I want to create an animal model or cell line, can you help us with that?
A. Yes! Transposagen offers custom cell line and animal model production. When a custom service order is received we always provide the CRISPRs to the customer for in-house use as the first milestone for the project.

1.8 References

1. R. Barrangou et al., CRISPR provides acquired resistance against viruses in prokaryotes. Science 315, 1709 (Mar 23, 2007).
2. S. J. Brouns et al., Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 321, 960 (Aug 15, 2008).
3. M. Jinek et al., A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816 (Aug 17, 2012).
4. W. Y. Hwang et al., Efficient genome editing in zebrafish using a CRISPR-Cas system. Nature biotechnology 31, 227 (Mar, 2013).
5. W. B. Chaderjian, E. T. Chin, R. J. Harris, T. M. Etcheverry, Effect of copper sulfate on performance of a serum-free CHO cell culture process and the level of free thiol in the recombinant antibody expressed. Biotechnology progress 21, 550 (Mar-Apr, 2005).

6. J. P. Connelly, J. C. Barker, S. Pruett-Miller, M. H. Porteus, Gene correction by homologous recombination with zinc finger nucleases in primary cells from a mouse model of a generic recessive genetic disease. Molecular therapy: the journal of the American Society of Gene Therapy 18, 1103 (Jun, 2010).
7. P. Perez-Pinera, D. G. Ousterout, M. T. Brown, C. A. Gersbach, Gene targeting to the ROSA26 locus directed by engineered zinc finger nucleases. Nucleic acids research 40, 3741 (Apr, 2012).
8. W. Jiang, D. Bikard, D. Cox, F. Zhang, L. A. Marraffini, RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nature biotechnology 31, 233 (Mar, 2013).
9. N. Chang et al., Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. Cell research 23, 465 (Apr, 2013).
10. S. J. Gratz et al., Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. Genetics, (May 24, 2013).
11. Shen et al., Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. Cell research 23, 720 (May, 2013).
12. H. Wang et al., One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153, 910 (May 9, 2013).
13. J. E. DiCarlo et al., Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic acids research 41, 4336 (Apr, 2013).
14. Z. Izsvak et al., Generating knockout rats by transposon mutagenesis in spermatogonial stem cells. Nature methods 7, 443 (Jun, 2010).
15. M. Geurts et al., Generation of gene-specific mutated rats using zinc-finger nucleases. Methods Mol Biol 597, 211 (2010).
16. K. Yusa et al., Targeted gene correction of alpha1-antitrypsin deficiency in induced pluripotent stem cells. Nature 478, 391 (Oct 20, 2011).
17. Kim et al. (2011) Nature Methods, 8(11):941-943.
18. Kulinski et al. (2000) BioTechniques, 29:44-48.
19. Hibbs et al. http://www.sigmaaldrich.com/etc/medialib/docs/Sigma-Aldrich/Posters/1/knockout_cancer_lines_poster.Par.0001.File.tmp/knockout_cancer_lines_poster.pdf
20. Kim et al. (2010) Cold Spring Harb Protoc. 8:August, 1-7.