Transposagen

Better Models. Better Results.™

CRISPR/Cas System

Site-Specific Genetic Manipulation

User Manual, Version 3.06.16

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

Contents

1. Introduction and Background

A. The CRISPR/Cas System

Bacteria make use of clustered, regularly interspaced, short palindromic repeats (CRISPRs) together with CRISPR-associated (Cas) proteins as a form of acquired resistance to invading plasmids or viruses. The type II CRISPR/Cas 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]. Recruitment of the Cas9 endonuclease by the dual-RNA complex begins an interrogation of DNA strands[3]. Once a region of complementation is found, a double-stranded cleavage of the DNA by the Cas9 protein occurs[1,2]. Site-specific cleavage of the target DNA occurs at a location determined by the complementary base pairing of the guide RNA (gRNA) and target DNA and a small protospacer-adjacent motif (PAM), as shown in Figure 1[4]. In the type II CRISPR/Cas system, this PAM sequence (NGG) is essential for DNA cleavage[4]. Synthetic single guide RNA (sgRNA) comprised of a fusion of the dual-RNA complex has been shown *in vivo* to cut target DNA[4,5].

CRISPRs can be designed against genes of interest to create double-stranded breaks and subsequent insertions or deletions (indels) via the non-homologous end-joining (NHEJ) repair mechanism[4–7]. These insertions or deletions often times result in a frame shift or nonsense mutation, creating a targeted gene knockout. Targeted knock-ins can also occur via homologous recombination (HR) when the CRISPR/Cas system is cotransfected with a targeting donor vector and/or a single-stranded DNA oligonucleotide (Figure 2)[8]. CRISPR technology can be used in conjunction with Transposagen's other core technology, the *piggyBac*™ transposase/transposon, which provides a seamless and efficient 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.

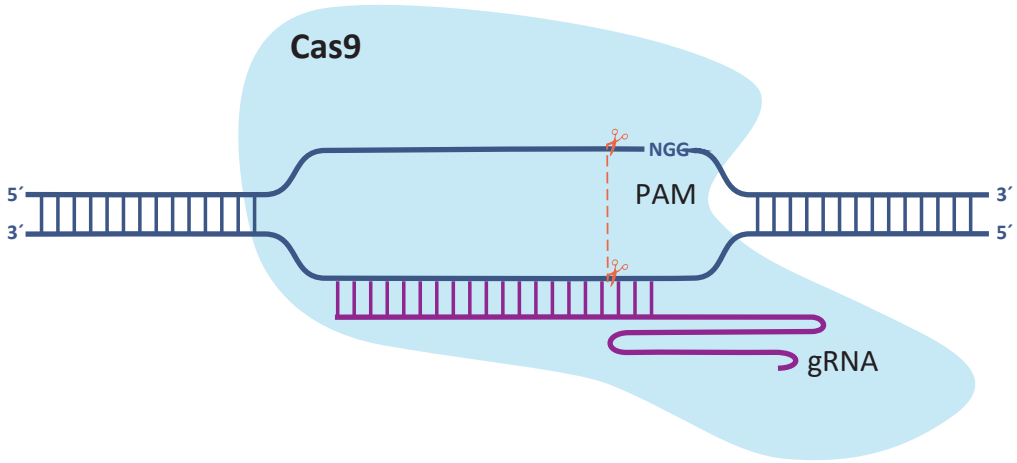

Figure 1: Mechanism of CRISPR/Cas–directed Mutagenesis. *The synthetic gRNA interacts with the complementary sequence within the target region. Recruitment of the Cas9 endonuclease cleaves the DNA 5' of the PAM. Creation of a double-stranded break results in activation of the DNA repair mechanism and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

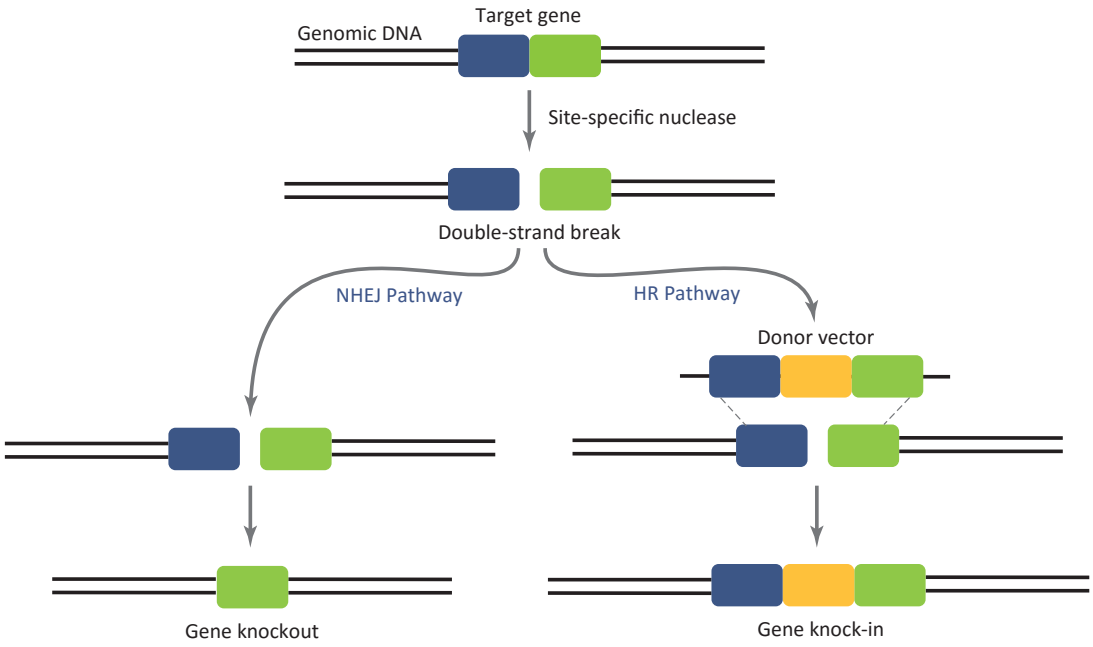

Figure 2: Mechanism of CRISPR/Cas–mediated Mutagenesis. *Knockout mutations are created via non-homologous end joining (NHEJ), which occurs naturally in the cells, causing small deletions or insertions that disrupt the reading frame of the DNA. Knock-in mutations can be created through homologous recombination (HR) that inserts the desired DNA sequence from a donor vector.*

2. Design and Construction of CRISPRs

Transposagen can assist you in the design of your customized CRISPR/Cas9. After you submit your target sequence, we will design and synthesize your custom sgRNA. We offer two expression vectors: a RNA PolIII (U6) promoter driven sgRNA plasmid for expression in cells, and a T7 driven sgRNA plasmid for *in vitro* transcription of gRNA. Transposagen guarantees at least 25 µg of sequence verified, transfection-ready customized plasmids.

A. The CRISPR vectors

Transposagen has the following CRISPR vector and RNA products available for research. These include, but are not limited to:

Catalog #	Description
CCW-001 [SS-wtCRISPR]	Single-Shot CRISPR, the all-in-one CRISPR/Cas9 expression vector. Cas9 expressed by the CMV Promoter, sgRNA expressed by the RNA PolIII U6 promoter.
CCC-001 [wtCRISPR-U6]	Custom CRISPR gRNA expression vector, U6 promoter.
CCC-002 [wtCRISPR-T7]	Custom CRISPR gRNA expression vector, T7 promoter.
CCC-003 [sgRNA]	Custom wtCRISPR sgRNA production.
SWC-001 [Cas9]	Cas9 nuclease expression vector, CMV promoter.
SWC-002 [mCas9]	Cas9 nuclease mRNA.

3. Introduction of CRISPR into Target Cells in Culture

The CRISPR/Cas9 system can be introduced into cells using most established protocols for nucleic acids and/or proteins. Below are example conditions others have used for transfecting cells with site-specific nuclease technologies, including CRISPR. 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. Basic Protocol: Transfection into cell lines in culture

Lipid-based transfection works in many cases, especially in easier to transfect, common cells lines such as CHO, HEK293, HT1080, HepG2, and NIH 3T3. We suggest following the manufacturer's instructions, especially for commonly available cell lines. To optimize for efficient cleavage, we recommend using the maximum amounts of nucleic acid and protein suggested in manufacturer's protocol. The amount of nucleic acids added can be adjusted up or down, depending on cell numbers and specific protocols.

B. Electroporation of cells in culture

Electroporation conditions should be optimized for each cell line and may depend on the instrumentation and/or reagents being used[9]. We recommend using the manufacturer's or lab protocols for introducing nucleic acids into your particular cell type, keeping in mind a few different conditions should be tried to optimize efficacy.

4. Mutation Detection

A. Using heteroduplex cleaving endonucleases

Cleavage detection and efficiency can be measured using many commercially available kits (ThermoFisher Scientific: Cat # A24372; IDT: Cat # 706025) utilizing endonucleases (T7 or Cel1) that cleave mismatches of heteroduplexed DNA caused by indels formed during NHEJ. A typical Cel1 assay result is shown in Figure 3. We recommend following manufacturer's protocols.

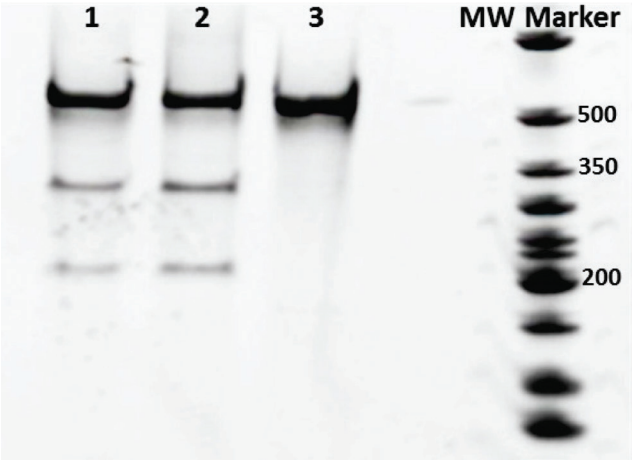

Figure 3: PCR products amplified from AAVS1 targeted sequence separated in a 4–20% PAGE. *PCR products were assayed for indel formation using a Surveyor Mutation Detection Kit (IDT). Lanes 1 and 2 show amplicons of HEK293 gDNA transfected with Cas9 and a custom CRISPR targeting a locus in AAVS1. Lane 3 shows an amplicon of HEK293 gDNA transfected with a GFP plasmid, acting as a cleavage negative control.*

B. Using PCR and sequencing

Successive rounds of PCR and sequencing also enable a quantitative measurement of target cleavage. This method is more time-consuming than the heteroduplex method described above because often many clones are needed for analysis.

1. After 2–3 days of culture, isolate genomic DNA from the cells.
2. Perform a PCR (30–35 cycles) using primers that flank the targeted chromosomal sequence. The PCR product should be less than 1 kb. The T_m should be ~60˚C and the GC content should be 40-60%.
3. Analyze PCR products by agarose gel electrophoresis.
4. Subclone the PCR fragment into a blunt vector (for example, using the Topo® TA Cloning Kit for Subcloning by Thermo Fisher Scientific) and then transform into bacterial cells.
5. Pick colonies the next day, perform a miniprep, and sequence the inserts using a primer that is included in the vector. Alternatively, perform a PCR on the colonies to amplify the cloned PCR fragment and directly sequence the PCR product.
6. Align the sequences with the known wild-type sequence and identify any deletions or insertions within the newly created sequence.
7. Once you have a pair of CRISPRs that have high cleavage efficiencies, use those to further transfect or electroporate the plasmids into your target cells. Multiple rounds of transfection can be performed to ensure that each allele is modified.

5. Cell Line Generation

Note: Transposagen recommends knowing the exact allele copy number before beginning any cell line genetic modification.

A. Using homology-directed repair for targeted cell knock-ins:

The use of Transposagen's *piggyBac*™ system allows for quick and easy Footprint-Free™ targeted knock-ins with edits down to a single base pair. The employment of a HR targeting vector, or donor plasmid, with an antibiotic resistance marker (neomycin, puromycin, etc.) that inserts into the CRISPR cleavage site allows for the selection of modified clones. Resistant cells are greatly enriched for knockouts and/or knock-ins. Some copies of the donor vector will randomly integrate; not all cells that express the antibiotic resistance will have the desired genetic change. Transposagen recommends isolating clones by dilution cloning or single cell sorting. Subsequently run a PCR screen for targeting at the cut site. One primer should anneal within the selection cassette and one in the genome outside the arms of homology.

▸▸ Learn more about Footprint-Free™ Gene Editing at http://www.transposagenbio.com/gek-footprint-free

B. Using non-homologous end joining for targeted cell knockouts:

Cells that have undergone CRISPR/Cas9 cleavage and have indels confirmed via methods described above may be isolated by single cell cloning. PCR should be performed on the clones using oligonucleotides that flank the cut site as described earlier. This PCR product should then be cloned as noted above and several of the subclones sequenced to verify all alleles have been altered to introduce a frameshift mutation. This method may require screening hundreds of clones to find a clone with

the desired genetic change. If cutting is incomplete and some alleles remain unmodified, further transfections may be required until all alleles are modified.

6. 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 use a basic local alignment search tool (BLAST) to evaluate your sequence to be sure it is not repetitive. Also, if you plan to knock out a gene by cutting with CRISPR, a common strategy involves a cut in the first coding exon, usually 50–100 bases from the start codon. The Cas9 nuclease generally blunt cuts the DNA three base pairs 5' of the PAM site. 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 synthetic CRISPR RNA from these plasmid backbones?

A. ***Yes.*** Transposagen offers a T7 promoter vector for *in vitro* RNA synthesis using kits such as the HiScribe™ T7 High Yield RNA Synthesis Kit from New England Biolabs. Transposagen's T7 vector should be linearized using DraI prior to *in vitro* transcription.

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 use BLAST or otherwise analyze the requested cut sequence for specificity within the genome?

A. ***No.*** It is your responsibility to determine the suitability of the sequence for your research purposes. We make no attempt to discern the intended use of the CRISPRs you are ordering. For more information about predicted off-target analysis (through our partnership with KBioBox), contact us at info@transposagenbio.com.

Q. What percentage of my transfected cells will 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:

- The transfection efficiency
- The cutting efficiency
- Only a certain percentage of cells are cut at both alleles
- There may be more than 2 alleles, especially in transformed cell lines
- Some loci are unavailable, presumably due to chromatin structure
- Alleles that have indels must also induce a frameshift mutation to render the gene non-functional

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, some genomic targets and/or cell lines may not be amenable to cutting. Please consult the literature prior to your assay to see if CRISPR/Cas9 has been used in your system. If you are interested in Transposagen's cutting validation services, contact us at info@transposagenbio.com.

Q. If I want to create an animal model or cell line, can you help me 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. For more information, contact us at info@transposagenbio.com.

7. References

1. Barrangou, R. *et al.* CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315, 1709–1712, doi:10.1126/science.1138140 (2007).
2. Brouns, S. J. *et al.* Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* 321, 960–964, doi:10.1126/science.1159689 (2008).
3. Sternberg, S. H. *et al.* DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* 507, 62–67, doi:10.1038/nature13011 (2014).
4. Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821, doi:10.1126/science.1225829 (2012).
5. Hwang, W. Y. *et al.* Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature Biotechnol.* 31, 227–229, doi:10.1038/nbt.2501 (2013).
6. Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823, doi:10.1126/science.1231143 (2013).
7. Mali, P. *et al.* RNA-guided human genome engineering via Cas9. *Science* 339, 823–826, doi:10.1126/science.1232033 (2013).
8. Yang, H. *et al.* One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* 154, 1370–1379, doi:10.1016/j.cell.2013.08.022 (2013).
9. Perez-Pinera, P. *et al.* Gene targeting to the ROSA26 locus directed by engineered zinc finger nucleases. *Nucleic Acids Res.* 40, 3741–3752, doi:10.1093/nar/gkr1214 (2012).

8. Limited Use

Information in this document is subject to change without notice.

DISCLAIMER: TRANSPOSAGEN AND/OR ITS AFFILIATE(S) DISCLAIM ALL WARRANTIES WITH RESPECT TO THIS PRODUCT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, OR NON-INFRINGEMENT. TO THE EXTENT ALLOWED BY LAW, IN NO EVENT SHALL TRANSPOSAGEN AND/OR ITS AFFILIATE(S) BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THE USE OF THIS PRODUCT.

The following Label Licenses govern the specified product uses. Each Purchaser holds the responsibility for determining whether patent or other intellectual property rights held by third parties restrict the use of Products for a particular application.

LIMITED USE LABEL LICENSE FOR CRISPR PRODUCTS: The purchase of this CRISPR product from Transposagen conveys onto the final purchaser ("Limited Licensee") a non-transferable right to use the CRISPR Product solely for research in accordance with all of the following requirements: The Limited Licensee (i) shall not sell or otherwise transfer such CRISPR Products or any material that contains a CRISPR Product in whole or part) to any other person or entity, or use CRISPR Products to perform services for the benefit of any other person or entity, (ii) shall use only the purchased amount of the CRISPR Products purchased and shall use CRISPR Products, components of the CRISPR Products only for its internal research as a research tool and not for any Commercial Purpose, (iii) shall use CRISPR Products in compliance with all applicable laws and regulations, including without limitation applicable human health and animal welfare laws and regulations, (iv) Broad, MIT and Harvard shall provide no warranties of any kind to the Limited Licensee (statutory or implied) concerning the Patent Rights, or CRISPR Products,

including without limitation, as to product quality, condition, description, merchantability, fitness for a particular purpose, non-infringement of intellectual property rights or the absence of latent or other defects, and all such warranties are hereby expressly disclaimed, (v) Broad, MIT and Harvard shall expressly disclaim any warranty regarding results obtained through the use of the CRISPR Products, including without limitation any claim of inaccurate, invalid or incomplete results, (vi) Broad, MIT and Harvard, and their directors, trustees, officers, employees, agents, faculty, affiliated investigators, and students, shall have no liability to the Limited Licensee, including, without limitation, for any loss of use or profits, business interruption or any consequential, incidental, special or other indirect damages of any kind, regardless of how caused and regardless of whether an action in contract, tort, strict product liability or otherwise, (vii) the Limited Licensee shall indemnify, defend and hold harmless the Indemnitees against any liability, damage, loss, or expense (including without limitation reasonable attorneys' fees and expenses) incurred by or imposed upon any of the indemnities in connection with any claims, suits, investigations, actions, demands or judgments arising out of or related to the exercise of any rights granted to the Limited Licensee under the Limited License or any breach of the Limited License by such Limited Licensee, provided that, to the extent the foregoing is not permitted by law, the Limited Licensee agrees, to the extent permitted by law, that it, and not the Indemnitees, shall be responsible for any liability, damage, loss or expense arising out of or related to the exercise of any rights granted to the Limited Licensee under the Limited License or any breach of the Limited License by Limited Licensee, and (viii) the CRISPR Product and its use may be the subject of one or more issued patents and/or pending patent applications (other than the Patent Rights) owned by Broad, MIT and Harvard and the purchase of the CRISPR Product does not convey a license under any claims in the foregoing patents or patent applications directed to the CRISPR Product or use, production or commercialization thereof, except as expressly set forth in the Limited License.

LIMITED USE LABEL LICENSE FOR PIGGYBAC™ VECTORS AND TRANSPOSASE: The purchase of this product conveys to the buyer a limited, non-exclusive, non-transferable license under Transposagen Intellectual Property to use this product and components of this product only to perform internal research for the sole benefit of the buyer for a period of 12 (twelve) months from date of purchase. Following the 12 month trial period, purchaser will be required to obtain a nominal technology license in order to continue to use the *piggyBac*™ technology. If applicable, the buyer may also use standard molecular biology techniques to make additional copies of this product for purposes of internal research for the sole benefit of the buyer. Any buyer that is a for-profit entity may not use this product, its components or materials or cells made using this product or its components to generate any animal without first obtaining additional rights from Transposagen. The buyer cannot sell or otherwise transfer (a) this product, (b) its components, or (c) materials, cells, or organisms made using this product to a third party, or otherwise use this product, its components, or materials, cells, or organisms made using this product or its components for any Commercial Purpose or Development Purpose with the sole exception that buyer may transfer this product, its components, and/or materials, cells, or organisms made using this product or its components to (i) the buyer's legal affiliates, (ii) contract service provider and/or (iii) a scientific collaborator, provided that each such legal affiliate, contract service provider and/or scientific collaborator agrees in writing (1) not to transfer such product, its components, or materials, cells, or organisms made using such product or its components to any third party, and (2) to use such product, its components, and materials, cells, and organisms made using such product and/or its components solely for research as set forth in this limited use label license and not for Commercial Purposes or Development Purposes. If this product is subject to multiple limited use label licenses, the terms of the most restrictive limited use label license shall control.

"Commercial Purpose" means any activity for consideration, including but not limited to: (a) any use, directly or indirectly, in manufacturing, production, production of biomolecules or quality control; (b) any use to provide a service, information, or data for consideration; (c) any use for therapeutic, diagnostic or prophylactic purposes; or (d) any sale, resale, leasing, or licensing, whether or not for research purposes.

"Development Purpose" means any: (a) clinical activity following IND-enabling preclinical toxicological studies or equivalents thereof; (b) activity in any agricultural field trial, including any such field trial that would be subject to regulation by the United States Department of Agriculture if the plants were to constitute genetically engineered organisms under 7 C.F.R. § 340 (or any successor regulation); (c) activity directed towards the submission of data to the United States Department of Agriculture, or any equivalent regulatory agency outside of the United States, in support of an application for clearance, approval or deregulation by such agency; (d) scale-up activities, the primary focus of which is to increase from small-scale to production-scale.

"Transposagen Intellectual Property" means: (a) certain patents and/or patents pending and (b) technical information, trade secrets, protocols, practices, methods, DNA sequences and other knowledge, information and skills controlled by Transposagen ("Transposagen Know-How"), that are necessary and/or useful in buyer's use of this product.

For information on obtaining additional rights to the product for any use not permitted herein, please contact us at info@transposagenbio.com.