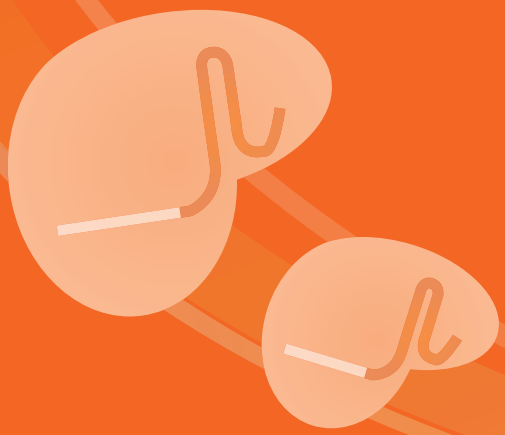
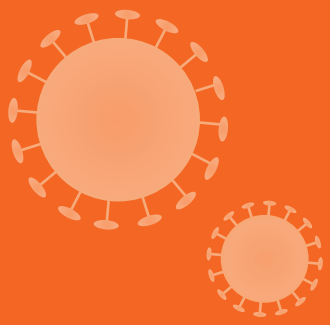
CRISPR Cas9

An Introductory Guide for Gene Knockout





Applied Biological Materials Inc.
#1-3671 Viking Way
Richmond, BC
V6V 2J5 Canada

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For Technical Support: technical@abmGood.com

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*A. CRISPR Products and Services Offered by **abm***

As a leader in genetic engineering and viral vector technology, **abm** offers the most comprehensive portfolio of the latest CRISPR technologies for editing, tagging, silencing, or activating any gene in a variety of research models. To learn more about CRISPR products and services, visit **www.abmGood.com**.

***Figure 1.** From design to delivery to validation, **abm** offers CRISPR products and services to meet every project need.*

CRISPR Plasmids
Pre-designed dual vectors and All-In-One vectors of Cas9 and sgRNA, ready for transfection or lentiviral packaging

Cas9 Double Mutant (dCas9) Cloning Vectors
Explore our dCas9-SAM Activation Library or let your creativity run wild: Attach anything from GFP and epitope tags to histone or methylation modifiers and much more (Cat. No. K014)

CRISPR Proteins
Footprint-free delivery of Cas9 nucleases, nickases, and double mutants (dCas9) protein

CRISPR sgRNA Vector Library
Pre-designed for loss-of-function or activation of target genes in any human, mouse, or rat molecular pathway

CRISPR sgRNA Viral Library
Pre-designed sgRNA lentivirus, adenovirus, or AAV for efficient transduction into any model system

Mammalian Cell Line Gene Editing
Generate a custom Knockout (Cat. No. C208) or Knock-in (Cat. No. C408) in mammalian cells or use Cas9-expressing stable cell lines for multi-gene manipulation analysis

CRISPR Genome-wide Knockout Cell Lines
Ready-to-use knockout cell lines for any human, mouse, or rat gene

Validation of CRISPR Gene Editing
PCR-based Genomic Cleavage Detection Kit (Cat. No. G932) for detecting CRISPR editing in gDNA, Sanger sequencing (Cat. No. C199), or Next Generation CRISPR Validation Service (Cat. No. IA00100/200) through targeted amplicon sequencing of the Cas9 target site

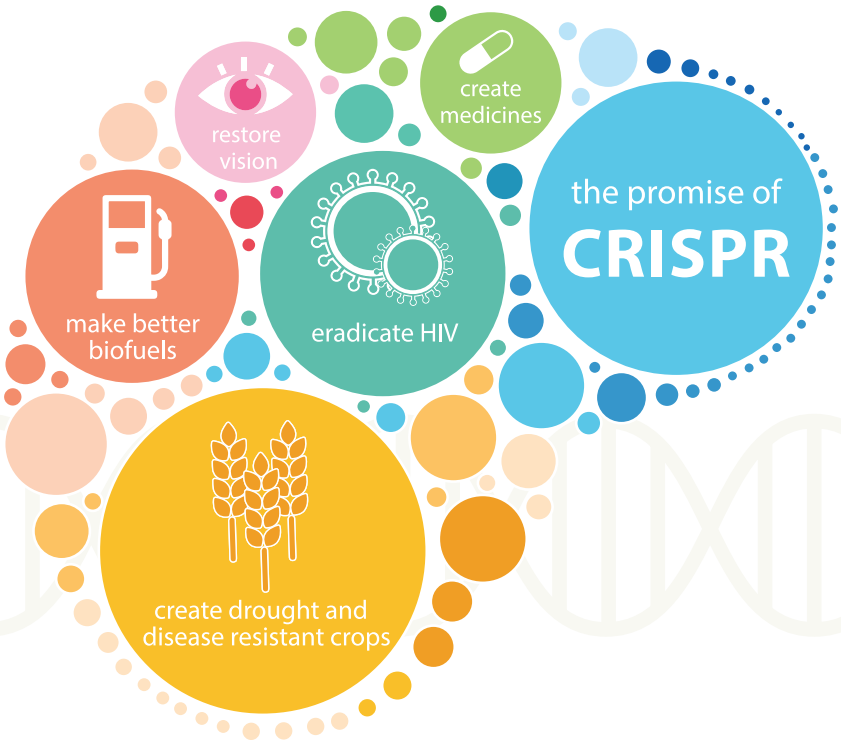
Accessory Products
Anti-Cas9 Antibody (Cat. No. Y300079) for western blot validation studies

B. Introduction to CRISPR Technology:

The Biotech Breakthrough of the Century

The ability to manipulate DNA in a test tube (*in vitro*) using modifying enzymes and PCR technology completely changed the way life science research and medical practices have been carried out over the last 40 years. The ability to engineer DNA *in vivo* (genome editing), however, is the ultimate dream of all scientists—a dream that is already becoming tangible due to the discovery of CRISPR. The advent of the efficient genome editing CRISPR technology (for DNA manipulation *in vivo*) will not only help to provide tremendous insights into the basis of many diseases (including cancers) but also enable efficient and reliable development of novel targets for future medical interventions (Hsu et al., 2014). There is now a real possibility that we will be able to correct genomic defects or enhance a particular genetic functionality in the near future.

Over the last 20 years, scientists have been searching tirelessly for an efficient genome editing technology. Excitement began with the discovery of Zinc Finger technology,



followed by TALEN methodologies. Now with the discovery of CRISPR, this decade is sure to be dominated by gene editing. Although it still may not be the ultimate genome editing technology scientists have been searching for, CRISPR's proven efficiency, speed, and ease of use has made it unquestionably the most monumental life science discovery of the new century so far (Doudna et al., 2014). This handbook outlines guidelines for CRISPR sgRNA design and the experimental procedures needed to achieve a specific gene knockout in a mammalian cell system. These guidelines can easily be modified or adapted for other specific CRISPR applications such as site-specific Knock-in in eukaryotic systems.

i. The Story of CRISPR's Discovery and Development

This CRISPR system was first brought to light in 1987. Nakata and colleagues were studying the *aminopeptidase in alkaline phosphatase isozyme conversion* when they discovered curious repeat and non-repeat sequences downstream of the ***iap*** gene (Ishino et al., 1987). It was only in 2002 that these repeat arrays were given the name CRISPR or Clustered Regularly Interspaced Short Palindromic Repeats (Jansen et al., 2002). At the time, their function was completely unknown. In 2005, Mojica and colleagues revealed that these sequences, or "spacers", actually contained DNA from bacteriophages (Mojica et al., 2005). Shortly after this discovery, Bolotin et al. also observed the presence of endonuclease-encoding Cas genes in close proximity to these CRISPR structures, strongly suggesting that foreign DNA degradation may be a primary function of CRISPR/Cas (Bolotin et al ., 2005). It was Koonin et al. that first proposed that spacers produce short RNA guides that can complex with endonucleases and target viral DNA for degradation (Koonin et al., 2006). Additionally, just upstream of the "protospacers"(target genomic sequences on the foreign DNA) are conserved motifs called protospacer adjacent motifs (PAM). These PAM sites are now proven to be the preferential target sites for Cas endonucleases (Horvath et al., 2008, Deveau et al., 2008), allowing the system to discern between self and non-self DNA (Mali et al., 2013). By the end of the early 2000s, the significance of CRISPR as an immune defense strategy against foreign DNA invasion in bacteria was rapidly becoming self-evident.

By 2010, three CRISPR systems had been identified in bacteria: Type I, II and III. Because of its relative simplicity, Type II CRISPR interference would eventually become the system adapted for genome editing in mammalian cells (Sapranaus-kas et al., 2011) (Figure 1).

The use of the CRISPR Cas system as a gene editing tool began in 2013, with the observation that Type II CRISPR systems from *Streptococcus thermophilus* (StCas) and *Streptococcus pyogenes* (SpCas9) could be engineered to edit mammalian genomes (Mali et al., 2013, Cong et al., 2013). To further adapt the system for mammalian cells, a two-vector system was optimized (Mali et al., 2013). The two major components include: (1) a Cas9 endonuclease and (2) the crRNA-tracrRNA complex. When co-expressed, the Cas9 and the crRNA-tracrRNA form a complex that is recruited to the target DNA

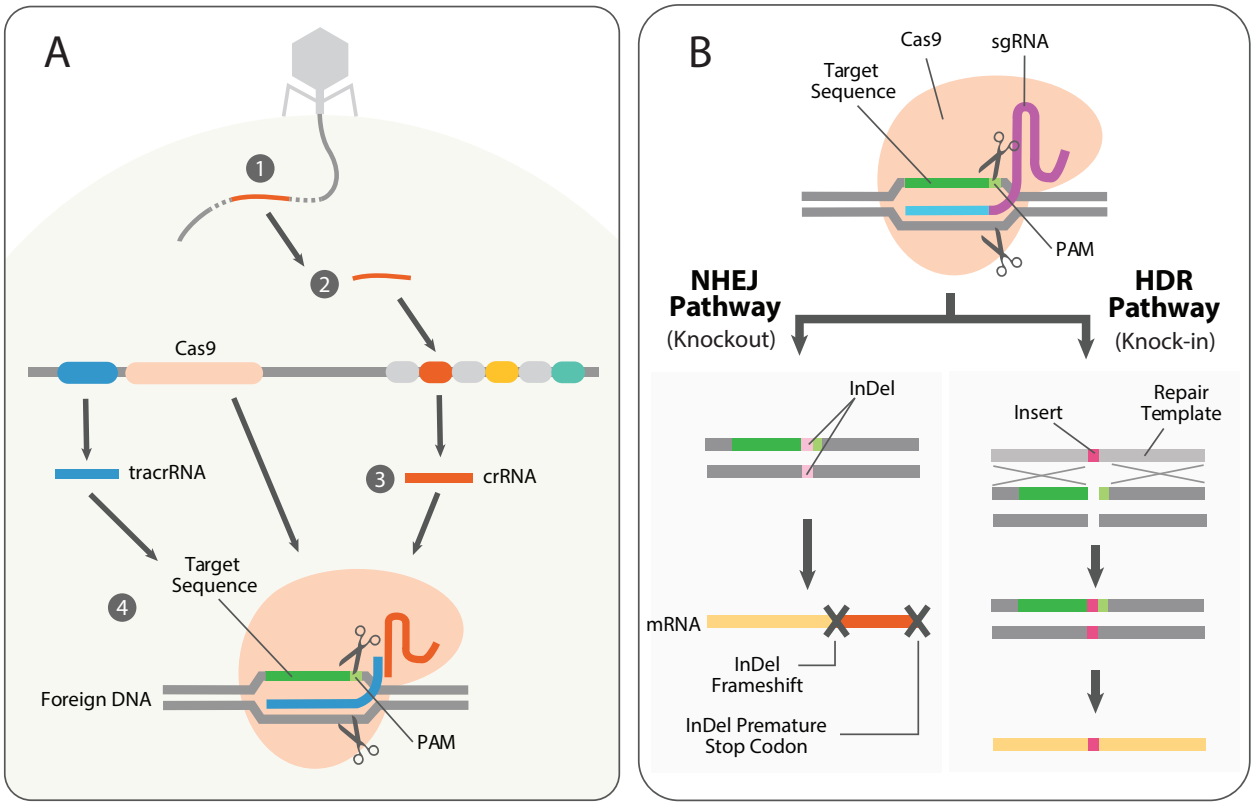


Figure 2: *Mechanism of CRISPR-mediated immunity in bacteria*

(A) CRISPR-based immunity in bacterial systems. During the immunization phase, foreign viral DNA is captured and cleaved by endogenously expressed Cas9 endonucleases (Jiang et al., 2015), and it is subsequently incorporated as repeat "spacers" into the bacterial CRISPR loci. Upon re-infection, the spacers are transcribed into small RNA guides called pre-CRISPR RNA (pre-crRNA). The Cas9 endonuclease and trans-activating crRNA (tracrRNA) then binds to the pre-crRNA to form a mature complex required to target the viral DNA for destruction.

(B) The CRISPR system adapted for gene editing. The CRISPR system used for genome editing requires an endonuclease and an engineered single guide RNA (sgRNA) designed to target the gene of interest. The target DNA sequence must be adjacent to the PAM site. Guided by an sgRNA, Cas9 will dock at the target site and make a double-stranded break. The cell will repair the double-stranded break either via the Non-Homologous End Joining (NHEJ) repair pathway or the Homology Directed Repair (HDR) mechanism. The NHEJ pathway often results in frameshift insertion or deletion (InDel) mutations that effectively knock-out gene activity. In the HDR mechanism, a DNA repair template can be provided to knock-in desired sequences into the break site via homologous recombination.

sequence, and cuts the DNA to form a double-stranded DNA break. This two-part bacterial crRNA and tracrRNA can be combined to form a chimeric single guide RNA (sgRNA) with the same ability to guide Cas9 to target gene sequences (Jinek et al., 2012). These components can then be delivered to mammalian cells via ribonucleoprotein (RNP) transfection or lentiviral, adenoviral, and AAV transduction.

abm offers a variety of products for the delivery of Cas9 and sgRNA. Vectors are available either with Cas9 and sgRNA separately in a two vector (Dual Vector) system or together in a single vector (All-in-One Vector) system for the ultimate flexibility in experimental set-up. Ready-to-transduce viral particles are offered in lentiviral, adenoviral, and AAV for easy delivery into any cell type. **abm** also offers the industry's best selection of Cas9 proteins and kits for the *in vitro* transcription of sgRNA.

ii. Why CRISPR is a Cut Above Other Genome Editing Tools

Scientists are now able to harness CRISPR Cas9's site-specificity for genome editing purposes. Two important advantages of the CRISPR Cas9 system are:

(1) it has remarkable versatility when it comes to working in cells as well as directly in the embryos of multiple species (e.g. from primates to malaria parasites to wheat), and

(2) its simplicity and extremely low cost of implementation.

A modified version of the CRISPR Cas9 enzyme can be used for the Cas9 paired nickase method. This method uses a Cas9 nickase, a version of the Cas9 enzyme which generates a single-strand DNA break (a nick) instead of a double-stranded DNA break. The cell will quickly repair such single-stranded breaks via the HDR pathway using the intact complementary DNA strand as a repair template. Using a pair of Cas9 nickases targeted within 20 bp of each other is an effective strategy for minimizing the risk of off-target cutting, as both nickases must now cut the DNA (one nickase targeting opposite strands of the target site) in order to create a double-stranded break and complete the genome editing process. One off-target cut by a nickase is rapidly repaired with no ill effects. **abm** offers the Cas9 nickase with NLS, no NLS and as the D10A or H840A protein for genome editing.

A catalytically inactive Cas9 protein (Cas9 double mutant or dCas9) can also be used for DNA interference or—when fused to an activator complex such as the Synergistic Activation Mediator (SAM)—be used to increase gene expression. dCas9 can be fused to any effector molecule—from repressors to epigenetic modifiers. It is possible to boost the expression of a gene of interest by several thousand-fold using **abm'**s dCas9-SAM Library.

Finally, when provided with a repair template, the CRISPR system via the HDR pathway can even be used to knock-in genes or reporters and also tag or mutate endogenous proteins. These various techniques showcase CRISPR Cas9 as a powerful RNA-guided platform for sequence-specific control of gene expression. Such targeted control of gene expression may prove particularly useful in novel drug design.

In contrast with previous genome editing tools such as Zinc Finger nucleases (ZFNs) and Transcription activator-like effector nucleases (TALENs), the CRISPR Cas9 and sgRNA editing system does not rely on protein design. The CRISPR system only requires a few simple DNA constructs to encode the gRNA and Cas9, as well as a donor template for homology directed repair if a knock-in is being performed. In addition, multiple genes can be edited simultaneously and in a systematic manner. This simplicity means that the use of the CRISPR Cas9 system for genome editing research requires considerably less time and resources compared to ZFNs or even TALENs, while also demonstrating an improved reliability.

	ZFNs	TALENs	CRISPR Cas9
DNA Binding Domain	Cys2-His2 DNA Binding Protein	Conserved Amino Acid Repeated Motif	Single Stranded sgRNA
DNA Cleavage Domain	FokI Restriction Endonuclease	FokI Restriction Endonuclease	Cas9 Endonuclease
Guiding Mechanism	Protein Guided	Protein Guided	RNA Guided
Ease of Design	★	★★★	★★★★★
Minimized Off-target Effects	★★★★★	★★★★★	★★★
Multiplexibility	★★	★★	★★★★★

Table 1. *A comparison of current genome editing technologies*

Adapted from *CRISPR/Cas9 for genome editing: progress, implications and challenges.* Zhang, Feng, Wen, Yan and Guo, Xiong. 1, s.l. : Human Molecular Genetics, March 17, 2014, Vol. 23.; CRISPR-Cas system for editing, regulation and targeting genomes. Sander, Jeffry D and Joung, J Keith. 4, s.l. : Nature Biotechnology, March 2, 2014, Vol. 32.; and ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Gaj, Thomas, Gersbach, Charles A and Barbas III, Carlos F. 7, s.l. : Cell, July 2013, Vol. 31, pp. 397-406.

C. CRISPR Knock-out Protocol at a Glance

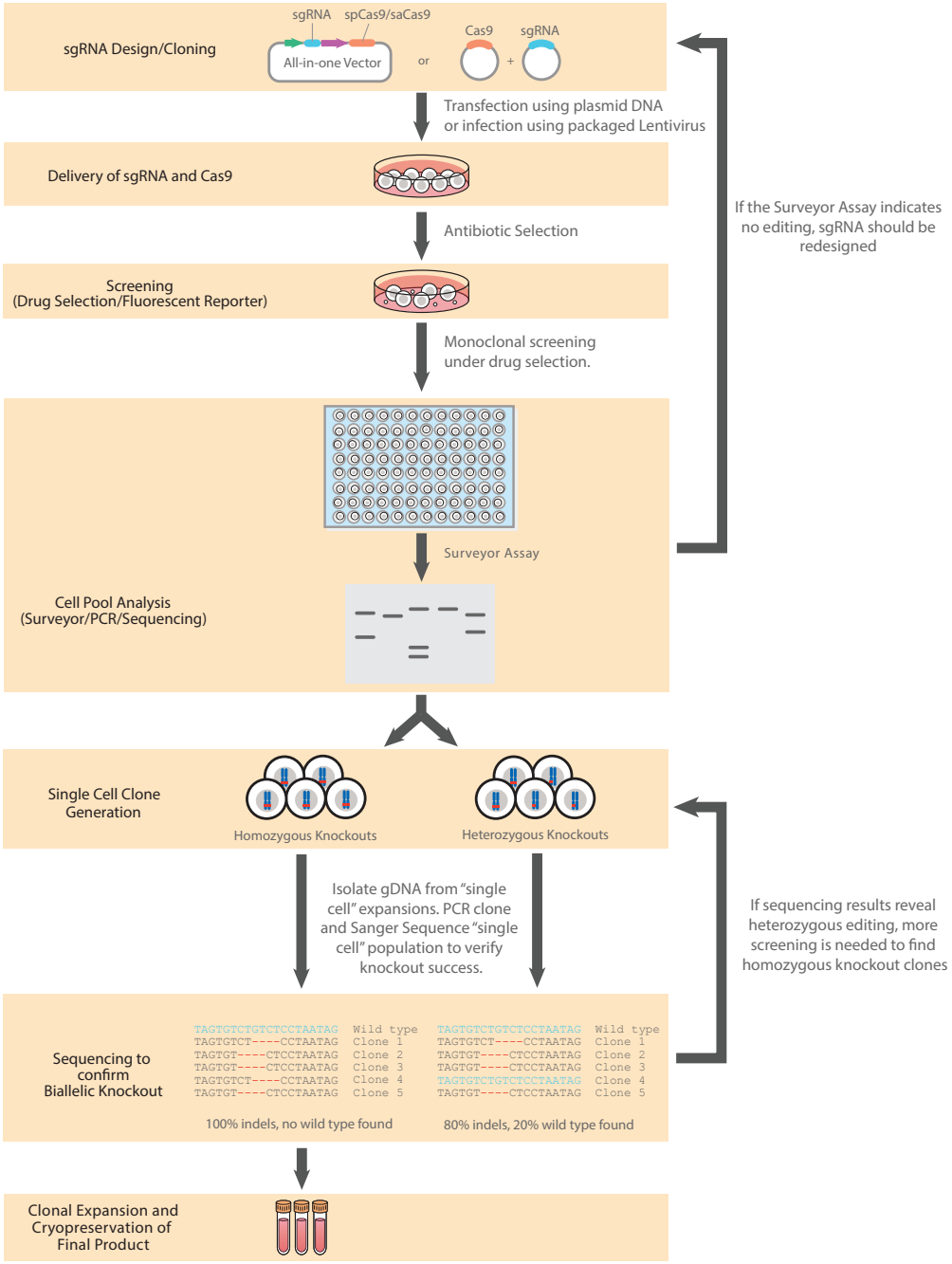


***Figure 3: abm**'s recommended workflow for generating and validating a knockout stable cell line using CRISPR technologies.*

The general workflow described above can be followed to achieve bi-allelic homozygous knockout of any locus in any cell type. In many cases, both copies of a target gene must be functionally silenced in order to achieve a complete functional knockout, therefore we recommend confirming the exact genotype of your edited cells using sequencing methods. Heterozygous editing (where only one of two alleles has been successfully edited) can lead to the continued production of functional protein and an incomplete knockout.

D. sgRNA Design Considerations

The first step of a CRISPR project begins with sgRNA design. **abm** selects sgRNAs based on three important features. The selected sgRNA:

1. Have the fewest potential off-targets (with 0, 1, and 2 nucleotide mismatches).
2. Have the highest predicted cleavage efficiency.
3. Target the earliest possible exons. For genes with multiple splice variants, care must be taken to ensure that a constitutive exon is targeted if the goal is to knockout all splice variants.

This maximizes the effects of frameshift and nonsense mutations that disrupt gene expression. sgRNA target sequences are designed to be immediately upstream of a Protospacer Adjacent Motif (PAM) which has a sequence of 5′-NGG-3′ for spCas9 and is necessary for Cas9 to cleave the DNA at the target position (it is important to note that the smaller saCas9 from *Staphylococcus aureus* relies on a different PAM sequence, 5′-NNGRR(N)-3′). sgRNA target sequences are synthesized and cloned into the pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro or GFP vector (the All-in-One Vector System) or pLenti-U6-

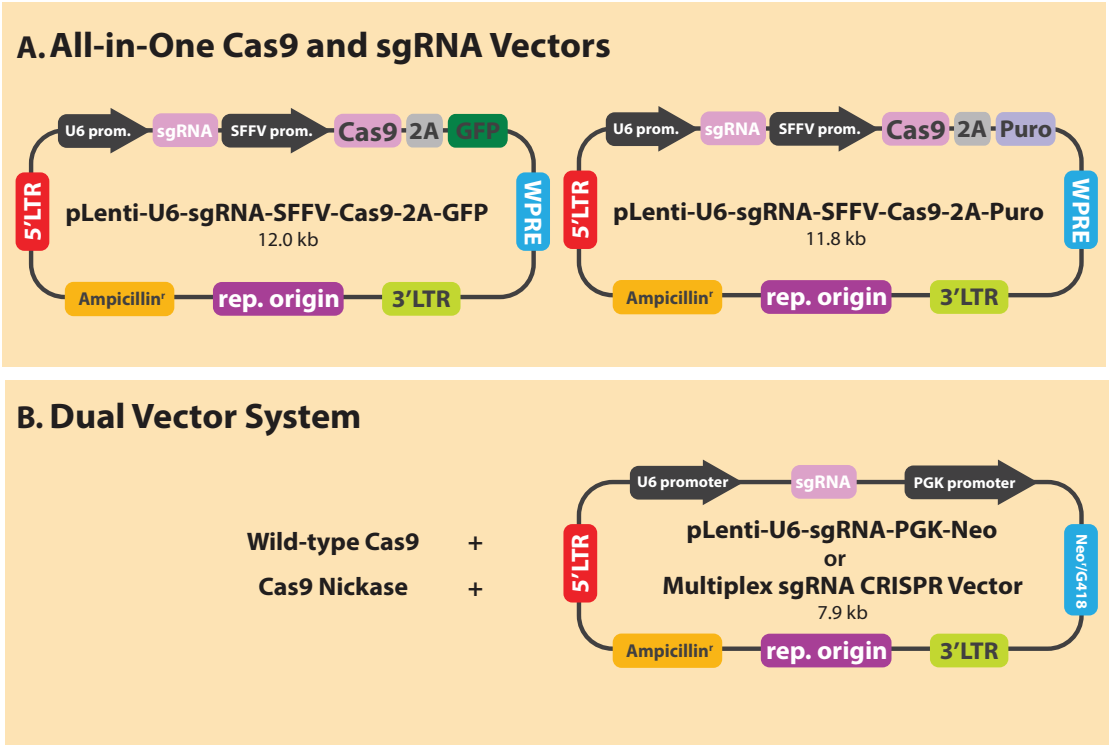


Figure 4. *abm's All-in-One and Two Vector Systems*

(A) **abm**'s All-in-One and (B) Dual Vector Cas9 and sgRNA Vector Systems. sgRNAs from **abm** are typically designed to target early exons. This maximizes the effects of frameshift and nonsense mutations that disrupt gene expression. For genes with multiple splice variants, care must be taken to ensure that a constitutive exon is targeted if the goal is to knockout all splice variants.

sgRNA-PGK-Neo vector (the sgRNA only vector that can be used in a Dual Vector system along with a Cas9 construct). We also offer multiplex sgRNA vectors which contain up to 4 sgRNAs in a single vector. Although other vectors are available, this guide will focus on the pLenti vector systems mentioned above. The vector is then transformed and amplified in a standard subcloning efficiency *E. coli* strain such as ProClone™ DH5α Competent Cells (Cat. No. E003) for transfection or packaging into lentiviral particles.

Depending on your project requirements, the sgRNA may be delivered together with Cas9 in a single plasmid (All-in-One Vector) or separately in a two plasmid (Dual Vector) format.

An All-in-One vector system offers an easy, one-step transfection with increased transfection efficiency and 1:1 sgRNA and Cas9 expression. **abm**'s All-in-One vector is designed with sgRNA expression driven by the U6 promoter, a strong constitutive Pol III promoter suitable for expression of small RNAs. Depending on the required downstream application, an SFFV promoter drives expression of a Cas9-2A-Puromycin or GFP cassette, enabling either downstream screening or tracking and sorting of Cas9-expressing cells (Figure 4A).

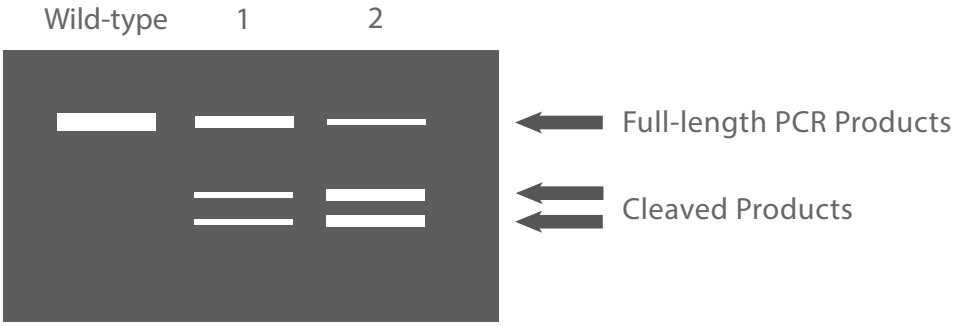
The Dual Vector systems are suitable for multiplex experiments as a pool of several different sgRNA vectors can be transfected alongside Cas9-expressing constructs. **abm**'s Dual Vector system comprises a Cas9-expressing construct and a separate construct carrying the sgRNA (driven by the U6 promoter) as well as the Neomycin resistance (controlled by a PGK promoter) for downstream screening (Figure 4B). Our multiplex sgRNA CRISPR vector is also available, which allows for the expression of up to 4 sgRNAs from a single construct.

In addition, Cas9 expression can be tightly controlled in a time-sensitive and reversible manner by a cumate inducible system, a unique system offered by **abm**.

i. sgRNA Target Validation

If you are designing your own sgRNAs or would like to test the cleavage efficiency of the sgRNAs supplied in our vector or virus sets to see which works best in your system, we recommend pairing our GeneCraft-R Express CRISPR sgRNA Synthesis Kit (S. pyogenes) (Cat No. G948), which allows quick and easy sgRNA synthesis, with our Cas9 purified protein (Cat. No. K108). Simply PCR amplify the region of genomic DNA (see section F. i. for gDNA PCR Primer Design), incubate the PCR amplicon together with the synthesized sgRNA and the Cas9 protein, and visualize the product on an agarose gel.

The result will be similar to that of a Genomic Cleavage Detection Assay (Cat. No G932), where the sgRNA with the strongest bands for the cleaved products would likely perform the best for your CRISPR knockout experiment.



ii. Plasmid Amplification Protocol

Once the sgRNA construct(s) have been designed and prepared, they must be transformed and amplified for direct transfection into cells or for packaging into a viral system. Plasmid DNA from **abm** is supplied in 10 mM Tris (unless otherwise requested) and intended for direct transformation into ProClone™ DH5α Competent Cells (Cat. No. E003). Resistance genes included are usually ampicillin, kanamycin, or spectinomycin, but can also include tetracycline or chloramphernicol in some cases. Refer to the table below for recommended antibiotic concentrations:

KanR	50 µg/ml Kanamycin	TetR	12.5 µg/ml Tetracycline
AmpR	100 µg/ml Carbenicillin/Ampicillin	CamR	50 µg/ml Chloramphenicol
SpecR	50 µg/ml Spectinomycin		

1. Transformation

1.1 On wet ice, thaw an aliquot of ProClone™ DH5α Competent Cells (Cat. No. E003) at 50 µl per transformation reaction. Once cells are thawed, flick the tube gently to mix.

1.2 Add 1 µl of plasmid DNA (10 ng/µl) to 50 µl of competent cells.

1.3 Swirl pipette tip to mix. DO NOT pipette up and down as this can damage the cells.

1.4 Incubate the DNA and the cell mixture on ice for 30 min.

1.5 Heat-shock the mixture for exactly 45 sec. in a 42°C water bath.

1.6 Place the mixture immediately back on ice for an additional 2 min to cool down, then add 150 µl of sterile Blank LB broth (containing no antibiotics).

1.7 Allow the cells to recover for 1 hr at 37°C in a shaking incubator (set at 240 rpm); at the same time, pre-heat your plates in a 37°C incubator.

1.8 Spread the entire volume of cells on an LB agar plate containing the appropriate antibiotic (refer to table of recommended antibiotic concentrations), and leave the plate agar-side down in the 37°C incubator for 10 min.

1.9 Reverse the plate to agar-side up in the 37 °C incubator and leave it overnight (around 16 hrs) to allow colonies to form. If colonies are too dense, plate 1 µl of cells in a 100 µl pool of LB on a fresh LB + antibiotic plate.

1.10 Screen clones the next day using miniprep and restriction enzyme digestion.

2. Plasmid Purification (Miniprep)

2.1 Pick 2 colonies from each plate into separate culture tubes containing 3-4 ml of LB broth with antibiotic.

2.2 Incubate cultures overnight (16-18 hr) at 37 °C on a shaker set at 240 rpm.

2.3 Perform minipreps on the cultures using the Column-Pure Plasmid Miniprep Kit (Cat. No. D504) and elute the plasmids in 40 µl of elution buffer.

3. Plasmid Confirmation (Restriction Enzyme Digest)

3.1 Perform restriction digest using 5 µl of miniprep (~300 ng) with KpnI (Cat. No. E054) to confirm screening.

5 µl Miniprep DNA
2 µl 10X Universal Restriction Enzyme Reaction Buffer (Cat. No. E204)
0.25 µl KpnI (Cat. No. E054)
17.75 µl ddH_2O
20 µl Total Volume

3.2 Incubate the digestion reaction at 37°C for 30 min.

3.3 Run the digest products on 1% agarose gel to visualize the band size.

3.4 For the **pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro Vector**, two bands at 5.5 and 6.3 kb will be observed. For the **pLenti-U6-sgRNA-PGK-Neo Vector**, a single band at 7.8 kb will be observed.

3.5 Once the correct clone(s) are obtained, a midiprep or maxiprep can be performed to obtain sufficient DNA for transfection or lentiviral packaging (depending on the method to be used for Cas9 and sgRNA delivery). For AAV Vectors, refer to the plasmid maps to help select the appropriate digestion enzyme.

E. sgRNA and Cas9 Delivery into Target Cells

The mode of sgRNA and Cas9 delivery will depend largely on the desired expression levels you would like to achieve. For transient expression of sgRNA and Cas9 (and therefore transient gene editing), you may choose to simply transfect your cells with sgRNA and/or Cas9 vectors or proteins. Transient sgRNA and Cas9 expression is particularly useful for reducing off-target effects as the longer the Cas9 and sgRNA persist in the cell, the higher the risk of off-target cleavage and cytotoxicity. **abm** offers constitutively expressing Cas9-expressing cell lines whose cleavage activity can be activated by the delivery of transient sgRNA vectors (Table 2). Refer to Table 3 for a breakdown of the advantages of several common delivery methods to aid in planning for gene editing projects.

Cas9-expressing Cell Lines	Cat. No.
293T	T3251
293	T3252
A549	T3253
HeLa	T3254
HepG2	T3256
MCF7	T3257
K562	T3258
U87- MG	T3259
HT1080	T3260
Jurkat	T3261
A375	T3262
RCS	T3290
MDCK	T3299

***Table 2. abm**'s ready-to-use, constitutively expressing Cas9 stable cell lines cover popular tumor model cell lines for easy CRISPR-mediated gene knockout of any human, mouse, or rat gene.*

Expression System	Components of the System	Application
Lentivector Plasmid DNA	• SFFV is the promoter used for driving constitutive Cas9 expression while the U6 promoter is used for sgRNA • Contains either Puromycin for selection or GFP for identifying/ enriching positive cells • Packaging plasmids must also be used to make lentiviral particles (Cat. No. LV003 for 2nd Generation Packaging Mix, Cat. No. LV053 for 3rd Generation Packaging Mix)	• Stable expression of Cas9 and/or sgRNA in a mammalian cell line
Lentiviral Transduction	• Cas9 and sgRNA can be present together in an All-In-One vector or separate as a Dual Vector system. • Can contain GFP to identify and enrich positive cells • Ready to use viruses are available as 10^7 IU/ml, with higher titers available upon request	• Stable expression of Cas9 and or sgRNA in a wide variety of mammalian cell lines • Useful for difficult-to-transfect cell types • Can be used *in vivo* • A popular choice for conducting genome-wide screens using CRISPR Cas9
AAV Transduction	• Only compatible with All-in-One saCas9 and sgRNA (Cat. No. C307) or sgRNA-only AAV Vectors (Cat. No. C303 or C305) • Serotypes AAV1 - AAV11 as well as AAVDJ, and AAVDJ-8 are available for tissue-specific delivery	• Transient or stable expression of sgRNA and/or saCas9 • Infects dividing and non-dividing cells • AAV is the least toxic method for *in vivo* viral delivery • saCas9-only AAV can be used with transgenic mice for expressing Cas9
Cas9-sgRNA Ribonucleoprotein (RNP) Complexes	• Purified Cas9 protein and sgRNA transcribed *in vitro* (using our EasyScribe T7 Transcription Kit, Cat. No. E081) are combined to form a Cas9-sgRNA complex • The Cas9-sgRNA Ribonucleoprotein is delivered to cells using cationic lipids	• Transient expression of CRISPR components • Limited off-target effects since sgRNA and Cas9 protein are degraded within the cell

Table 3. *Advantages and applications of various common delivery tools offered by* ***abm*** *(adapted from Addgene's Blog: CRISPR 101 series).*

i. Transfection and Transduction Procedure

Depending on the application, proceed with a transfection or a transduction of the vector or virus into the cell line of interest. The following transfection and transduction procedures may serve as general guidelines for each delivery method.

1. Transfection Procedure

1. 1 Approximately 18-24 hours prior to performing the transfection, plate 1 - $3x10^5$ adherent cells (in 2 ml appropriate culture medium complete with serum and antibiotics if they are normally used) into each well of a 6-well plate. Incubate the cells at 37°C in a CO_2 incubator until the cells are 50-70% confluent.

1. 2 The next day, set up the transfection reaction. For each transfection sample, prepare the DNAfectin™ 2100 (Cat. No. G2100) and DNA complexes as follows (per well of a 6-well plate):

a) Add 2 µg each of Vector (sgRNA and Cas9 vectors or All-in-one vector) into 100 µl of serum-free, antibiotic-free media.

b) Vortex DNAfectin™ 2100 thoroughly prior to use. Then, add 12.0 µl of DNAfectin™ 2100 into serum-free, antibiotic-free media.

c) Mix the DNA solution from step a) and the DNAfectin™ =2100 solution from step b), and mix gently to ensure uniform distribution.

d) Incubate for 20 minutes at room temperature to form the DNAfectin™ 2100-DNA complexes. Complexes are stable at room temperature for 3 - 5 hr.

1. 3 Add 800 µl of serum-free and antibiotic-free media to the DNAfectin™ 2100-DNA complexes.

1. 4 Aspirate the growth media from the cells to be transfected.

1. 5 Dropwise, add 1 ml of DNAfectin™ 2100-DNA complexes per well of cells.

1. 6 Incubate the cells for an additional 4-6 hr at 37°C, 5% CO_2.

1. 7 After incubation, add 100 µl of 10% FBS to directly into each well. Incubate the cells at 37°C in a CO_2 incubator for a total of 18-24 hr.

1. 8 Passage cells at 1:10 (or higher dilution) into fresh growth medium 24 hours post transfection and monitor the cells for the next 1-2 days before adding selection drug.

Note: To monitor the success of transfection, it is highly recommended to perform this transfection step with the appropriate GFP control vector in parallel.

2. Packaging and Transduction Procedure

The transduction efficiency of mammalian cells varies significantly under different experimental conditions. This includes virus concentration, exposure time to the virus, and growth area of the well or plate used for the infection.

Day 1: Seed cells one day before viral infection to achieve 20-30% density on day of infection. Incubate the cells at 37°C, 5% CO2 overnight.

Note: It is possible to use other plate formats for transduction. In this case, the amount of cells should be adjusted depending on the growth area of the well/plate.

Day 2: You may need to optimize the Multiplicity of Infection or MOI (the number of virus particles/cell) of the virus you are using to achieve the highest transduction efficiency. Typically, the lentivirus can be used at an MOI of 1, 5, 10, and 50.

e.g. On the day of infection, cells should be at 20% density. If the virus titer is 10^7 IU/ml, the following volumes of virus can be added to 10^5 cells to achieve the target MOI:

Table 4. *Volume of virus needed to achieve the target Multiplicity of Infection (MOI)*

Volume of virus needed	Target MOI
10µl	1
50µl	5*
100µl	10
500µl	50

**An MOI of 5 yields a good transduction efficiency for most cell lines*

$$\text{MOI} = \frac{\text{Volume of virus needed X Titer of virus}}{\text{cells/well at time of infection}} = \frac{\text{10µl X 10,000 IU/µl}}{\text{100,000 cells}} = 1$$

Therefore, to infect at an MOI of 1, use 10 µl of 10^7 IU/ml of virus to infect cells that are at 20% density. If the transduction efficiency of the target cells is low, add **abm's** ViralPlus Transduction Enhancer (Cat. No. G698) at a 1:100 ratio (or at the optimized dilution ratio determined for the transduction). Keep the infected cells at 37°C, 5% CO_2 for incubation overnight until ready for the drug selection step.

Note: If you plan on using sequencing as the primary method of validation and have multiple sgRNA for 1 gene target, it is recommended to infect each sgRNA separately in different wells.

Note: Include one transduction well with a positive GFP control virus (Cat# LVP690). Leave one well of uninfected cells as an additional drug selection control.

Day 3: Incubate the cells for another 24-48 hr at 37°C, 5% CO_2 to allow cells to recover. If the pLenti-U6-sgRNA-SFFV-Cas9-2A-GFP vector is being used, observe cells for infection signal at this time.

3. Killing Curve Determination

For stable cell line generation, it is important that stably-transfected/transduced cells can be selected. This is accomplished by the addition of antibiotic drugs to the culture medium—if the expression system carries a drug resistance gene, only the stably-transfected/transduced cells will survive. Generation of a drug killing curve will help determine the minimum amount of the selection-drug required to kill non-transfected/non-transduced cells.

Note: Check to see which drug is to be used for selection. In **abm**'s All-in-One system (pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro Vector), Puromycin (Cat. No. G264) is used whereas in the sgRNA only system (pLenti-U6-sgRNA-PGK-Neo Vector) Neomycin/Geneticin (Cat. No. G418) is used.

Day 1: Plate the parental cells into a 6-well plate at low density (around $2x10^5$ cells/well) in recommended culture conditions.

Day 2: Prepare medium containing a range of antibiotic concentrations. It is recommend to prepare 0, 0.2, 0.4, 0.6, 0.8 and 1 µg/ml of Puromycin (Cat. No. G264). Add the different concentrations of the drug to each well.

Note: Some cell lines may require a higher range of antibiotic concentration and thus each cell line should be tested individually in order to find the optimal concentration of antibiotic for selection.

Day 3 onwards: Feed cells every 2-3 days with freshly prepared selection media and observe cells under the microscope every day for cell death.

Most cells should be killed at 1 µg/ml. If this is not the case, try a higher range of antibiotic concentrations such as 1, 1.2, 1.5, 1.8 and 2 µg. It is important to identify the antibiotic concentration at which >95% of cell death is observed. This will be the minimum antibiotic concentration to use for selection.

>95% cell death should occur within 1-4 days for Puromycin and 7-14 days for Neomycin/Geneticin.

Once the optimal drug concentration is observed, repeat the assay one more time to ensure that the selected drug concentration is indeed accurate.

Note: The drug sensitivity is highly dependent on cell density. Thus, when the selection drug is added, the cell density should be similar to the one used in your killing curve determination.

4. Drug Selection

Screening of cells via drug selection is a crucial step in the generation of a monoclonal population of cells containing a homologous gene edit. Two rounds of screening are required:

1) An initial screening which determines the population of cells that contain a genome edit and

2) A monoclonal screening which isolates single cell clones that contain the desired edit.

The following protocol covers both rounds of screening, with the final product being a clonal population containing the homologous edit. Note that the All-in-One sgRNA and Cas9 vector confers Puromycin (Cat. No. 6264) resistance whereas the sgRNA-only vectors confer Neomycin/Geneticin (Cat. No. G418) resistance.

4. 1 48-72 hrs after transduction, add the selection drug in the concentration determined in the Killing Curve step.

4. 2 Incubate the cells with the selection drug (an additional 2-3 days for Puromycin and an additional 7-14 days for Neomycin/Geneticin). Non-transduced cells will die and successful colonies will survive as they harbour the gene for resistance against the selection drug.

4. 3 To test the success of the genomic edits at this stage, use **abm**'s Genomic Cleavage Detection Kit (Cat. No. G932). This kit is a simple PCR-based assay that can inform on which clonal pools are suitable candidates for further isolation and characterization of the knockout (see section F for the assay concept and section F.ii for complete protocol details).

4. 4 Prepare for monoclonal selection using single cell dilution. Gene editing in the monoclones selected in this step can be characterized by Sanger sequencing (Cat. No. C199). Refer to section F.iii for protocol details.

4. 5 Select clonal pools for further isolation of monoclones based on the results of the Genomic Cleavage Detection Kit. The following steps will describe how to isolate single cells from the original clonal pool using two dilution series.

4. 6 First, add 100 µl of culture media (containing the selection drug at the concentration determined in the killing curve) to each well of the 96-well plate.

4. 7 Add 200 µl of initial cell suspension into well A1 (Figure 5).

Note: The starting cell density of a clonal pool is approximately 20,000 cells/ml which equates to 4,000 cells in well A1. After completing the first and second dilution series, well A12 should have approximately 1 cell/well.

4. 8 Proceed with the first dilution series (refer to Figure 5, red arrow). Make a 1:2 dilution series vertically by adding 100 µl from well A1 to B1. Mix well and then add 100 µl from well B1 to C1. Repeat until the last well (H1) is reached. Remove 100 µl from the last well to keep the total volume the same (100 µl) for all wells.

4. 9 Add 100 µl of media to each well of column 1 to make the volume 200 µl in total for each well.

4. 10 Proceed with the second dilution series (refer to Figure 5, blue arrows). Using a multichannel pipette, transfer 100 µl of cell suspension from column 1 to column 2 and so forth until column 12 is reached. Again, remove 100 µl from each well to keep the total volume consistent (100 µl) in each well.

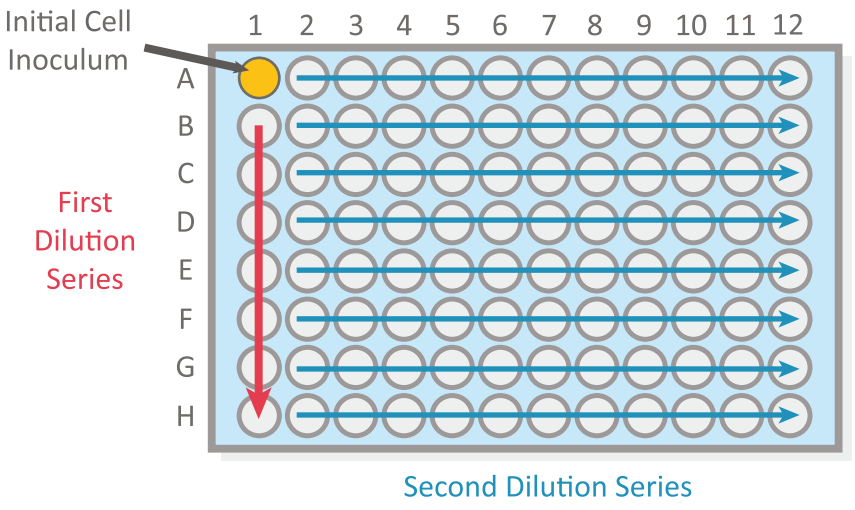


Figure 5. *Illustration of first and second dilution series for monoclonal isolation.*
After completion of the first and second dilution series, the wells on the right-hand side will have single cell clones as they have been diluted to a single cell level.

4. 12 Add 100 µl of fresh media to each well in this plate so that the total volume in each well is 200 µl. Incubate the plate at 37°C, 5% CO_2.

4. 13 It may require approximately 2 weeks for clones to appear.

4. 14 Allow individual monoclones to grow to form a monolayer and transfer cells to a 24-well plate followed by a 6-well expansion. The genomic DNA isolated from each of the resulting expanded clones can be used for further gene editing validation experiments using the Genomic Cleavage Detection Kit (Cat. No. G932) and/or Sanger sequencing (Cat. No. C199) as described in section F.

Note: It is recommended to keep each of the clones in culture while conducting validation studies as back up.

F. Validation of CRISPR Gene Editing

This section focuses primarily on two methods that are commonly used to screen for stable cell line knockouts – a PCR-based method (The CRISPR Genomic Cleavage Detection Kit, Cat. No. G932) and a Sanger sequencing method (Cat. No. C199). In addition, two other validation strategies, a Next Generation Sequencing method (Cat. No. IA00100) and a Western Blot method, are also briefly touched upon.

The CRISPR Genomic Cleavage Detection Kit (Cat. No. G932) is designed to be an easy yet effective way to verify the success of genomic editing as well as track the genomic editing process. This method uses a strategy similar to the Surveyor Assay, in which the DNA of CRISPR-edited cells is used as a template in PCR reactions that amplify the target region of interest. In this assay, the edited DNA is denatured and re-hybridized, a process that produces mismatches in the double-stranded DNA.

A specially engineered detection enzyme recognizes and cleaves the heteroduplex DNA strands 3' to the mismatch site, producing two bands that are detectable after gel analysis. This assay is sensitive to mutations that occur as low as 1 in 32 copies and is able to detect mismatches caused by up to 12 nucleotide indels (Figure 6).

Sanger sequencing (Cat. No. C199) can determine precise sequence information in a timely and cost-effective manner and can be used to follow the progress of monoclonal isolation during the first and second rounds of screening by drug selection. The Sanger sequencing genotyping assay can be used to select for positive clones with the desired homologous gene edit.

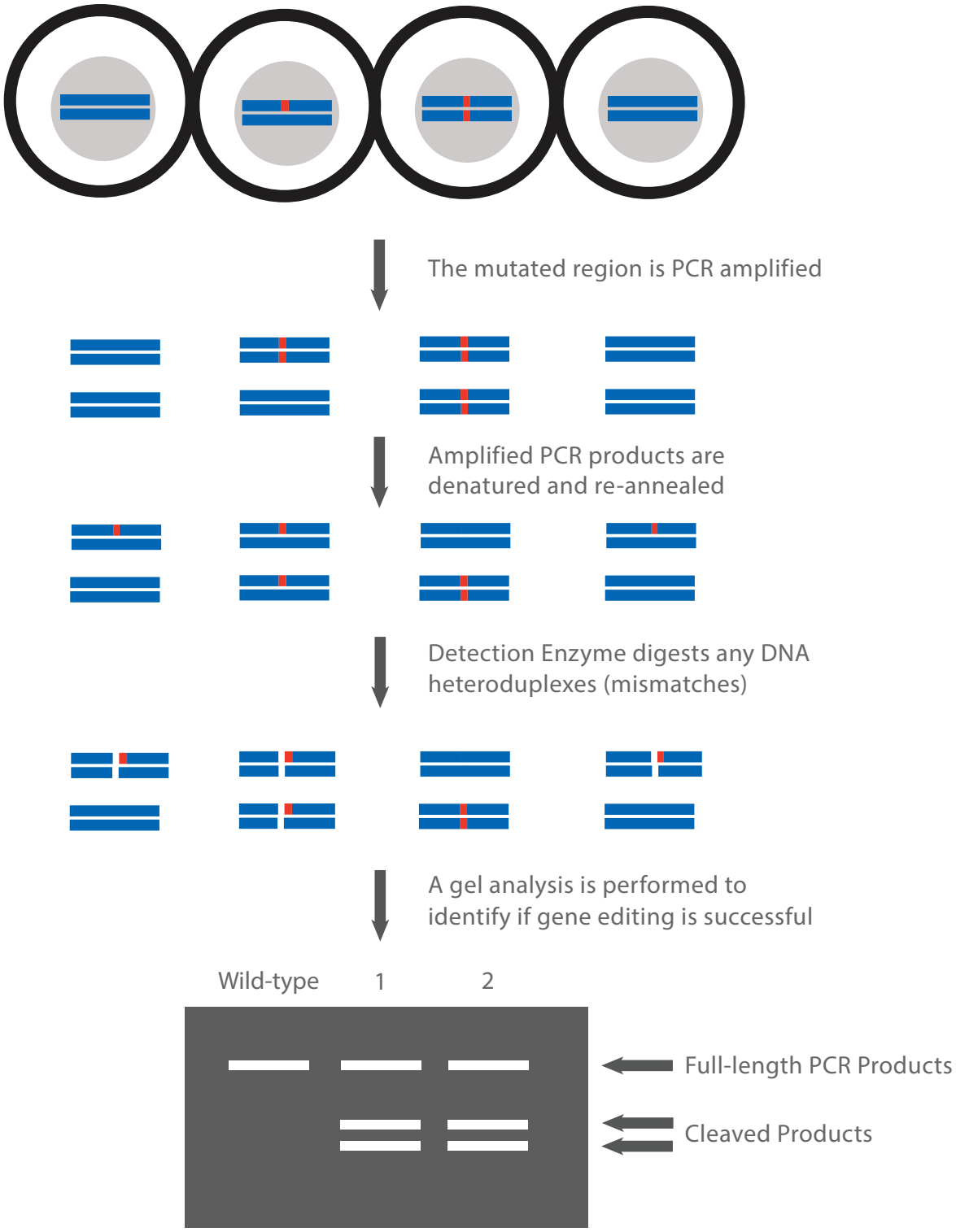


Figure 6. *Detecting gene editing efficiency using the CRISPR Genomic Cleavage Detection Kit (Cat. No. G932).*

i. gDNA PCR Primer Design

The following PCR primer design protocol is suggested for users intending to use the same PCR products for both the Surveyor Assay (Genomic Cleavage Detection Kit, Cat. No. G932) as well as the Sanger sequencing (Cat. No. C199) method of validating genome editing.

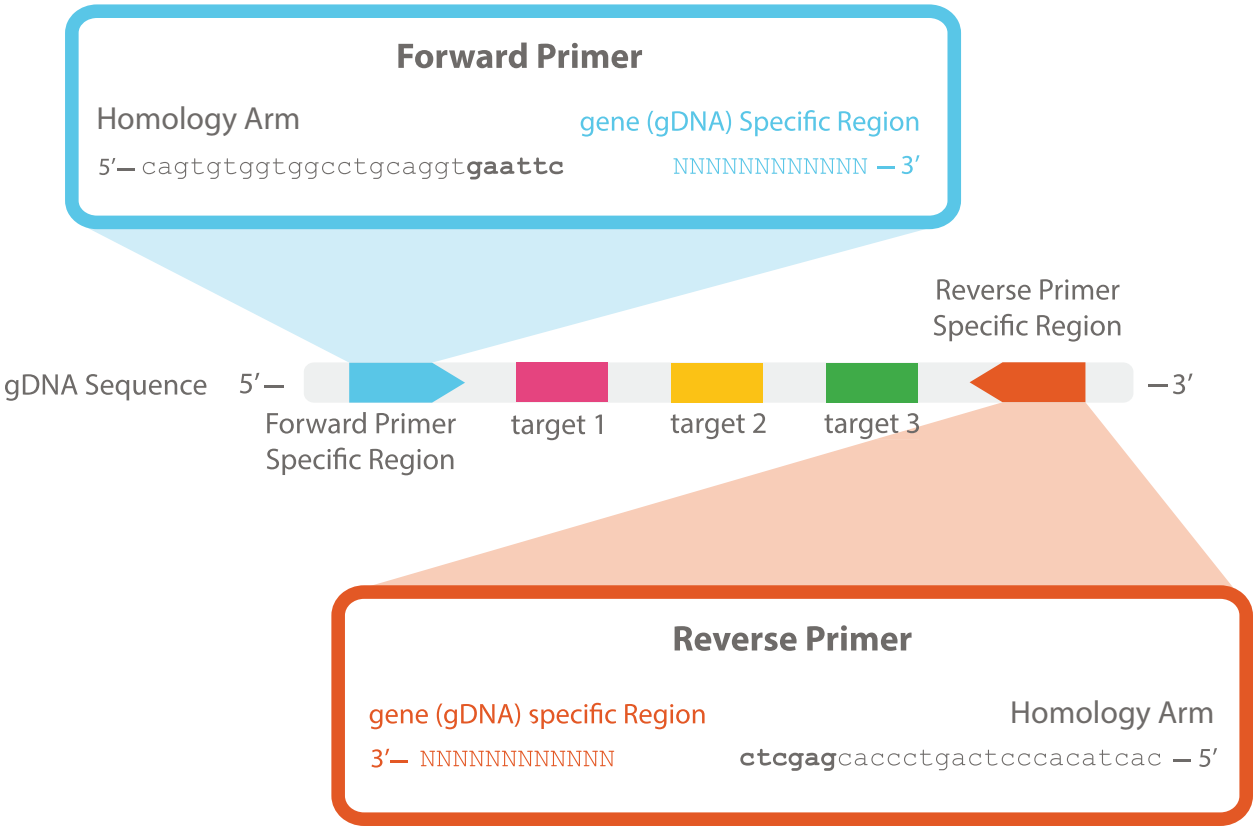


Figure 7. *A schematic describing the overall gDNA PCR primer design*

Primers should be spaced approximately 1 kb from each other in the gDNA, with the sgRNA cut site off-centre between them. The asymmetrical distances on either side of the target site make it easier to identify two distinct product bands when using the CRISPR Genomic Cleavage Detection Kit (Cat. No. G932). Both the forward and reverse primers must have a 20 nt homology arm complementary to the cloning vector. For the purpose of demonstrating the primer design and sequences, abm uses pLenti-III-HA (Cat. No. LV022) linearized with EcoRI and XhoI, though primers can be designed to be used with any vector.

For Example: Forward Primer 5′- cagtgtggtggcctgcaggtgaattcgaattatccgaatggc -3′
Reverse Primer 5′- gtgatgtgggagtcagggtgctcgagattagctattgcgagatc -3′

where blue is the constant homology arm and the red indicates gene specific regions.

ii. Protocol for Using the CRISPR Genomic Cleavage Detection Kit (Cat. No. G932)

As described in the introduction of section F, the CRISPR Genomic Cleavage Detection Kit relies on DNA mismatches between non-edited and edited DNA. A detection enzyme then cleaves mismatched DNA which produces two distinct gel products that confirm gene editing (Figure 6).

Table 5. *Components of* ***abm****'s Genomic Cleavage Detection Kit (Cat. No. G932)*

Product	Quantity	Cat. No./Part No.
Cell Lysis Buffer	1.25 ml	G932-1
Protein Degrader	50 µl	G932-2
Detection Enzyme	13 µl	G932-3
10X Detection Buffer	50 µl	G932-4
Control Primer and Template	10 µl	G932-5
2X PCR Bestaq™ MasterMix	1 ml	G464
Nuclease-free H2O	1 ml	RT-0

1. Cell Lysis

1. 1 Prepare cell pellets to contain $5x10^4$ to $2x10^6$ cells.

1. 2 Add 2 µl of Protein Degrader to 50 µl of Cell Lysis Buffer in an Eppendorf tube. Mix well.

1. 3 Add 50 µl of Protein Degrader/Cell Lysis Buffer mixture to the cell pellet and resuspend.

1. 4 Incubate the tube at 68°C for 15 min, followed by 95°C for 10 min.

1. 5 Start PCR amplification step immediately following cell lysis. Otherwise, store lysates at -20°C.

2. PCR Amplification

2. 1 Prepare for PCR amplification. DNA sample preparation, reaction set-up and subsequent reactions should be performed in separate areas to avoid cross-contamination. Please note that primers should be designed such that the cleavage site is not in the middle of the amplicon, enabling identification of two distinguishable product bands after the cleavage reaction (refer to section F.i gDNA PCR Primer Design). For high GC content reactions, include 5-10% DMSO.

2. 2 Thaw 2X PCR Bestaq™ MasterMix (Cat. No. G464) and primers on ice. Mix solutions thoroughly.

2. 3 Set-up the following reactions in sterile PCR tubes on ice. Mix well and centrifuge briefly.

Table 6. *Components for setting up PCR amplification reactions*

Component	Positive	Sample	Negative	Final Concentration
Control Primer & Template	1 µl	-	-	1X
Cell Lysate	-	2 µl	-	Variable
Primer Mix	-	1 µl	1 µl	200 nM
2X PCR Bestaq™ MasterMix	25 µl	25 µl	25 µl	1X
Nuclease-free H_2O	24 µl	22 µl	24 µl	-

2. 4 Perform the PCR amplification as described in Table 7.

Table 7. *Settings for the PCR amplification cycles*

Step	Temperature	Time	Cycle(s)
Enzyme Activation	95°C	10 min	1
Denaturation	95°C	30 sec	40
Annealing	45 - 72°C (55°C for control)	30 sec	
Extension	72°C	3.5 kb/min (1 min for control)	
Final Extension	72°C	5 min	1
Hold	4°C	Hold	1

2. 5 To check PCR amplification, load 5 µl of PCR product on a 1% gel. Proceed to the next step (the cleavage assay) when a clean product band of desired size is obtained, with no significant non-specific amplification.

2. 6 To prepare for the cleavage assay, set-up the following reactions in sterile PCR tubes on ice. Mix well and centrifuge briefly.

Table 8. *Components for setting up the cleavage assay*

Component	Reaction Volume
PCR Product	1- 6 µl
10X Detection Buffer	1 µl
Nuclease-free H_2O	up to 9.5 µl

2. 7 Perform re-annealing reaction using the cycle settings described in Table 9.

Table 9. *Settings for the PCR re-annealing cycle*

Stage	Temperature	Time	Temperature/Time
1	95°C	5 min	-
2	95-85°C	-	-2°C/sec
3	95°C	-	-0.1°C/sec
4	4°C	Hold	-

3. Gel Analysis of Cleavage Products

3. 1 To prepare for the gel analysis of the cleavage products, add 0.5 µl Detection Enzyme to all samples containing positive and control reactions. Mix well and centrifuge briefly.

3. 2 Incubate at 37°C for 1 hr.

3. 3 Vortex samples and spin everything down.

3. 4 Load 10 µl of each sample along with a DNA ladder and loading dye on a 1-2% agarose gel and run the gel at low voltage for approximately 30 min.

3. 5 View the resulting gel on a UV transilluminator and obtain a gel picture using a gel imaging system. The presence of two smaller cleavage products below the predicted wild-type band indicates that gene editing has occurred.

3. 6 Using gel analysis software, you may also determine the relative amount of DNA contained in each band. This data can be used to calculate the cleavage efficiency using the following equation:

Cleavage efficiency = 1-(1-fraction cleaved)$^{1/2}$

3. 7 If desired, positive samples identified from the CRISPR Genomic Cleavage Detection Kit can be further validated for genomic editing using Sanger sequencing methods (refer to section F.iii).

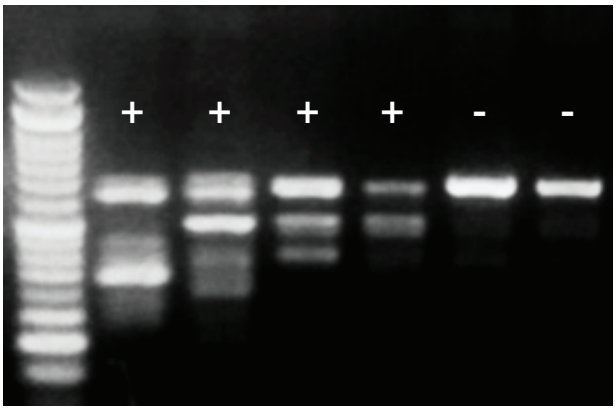


Figure 8. *Example of expected results after using the CRISPR Genomic Cleavage Detection Kit (Cat. No. G932).*

Samples labeled (+) on the gel have been edited whereas samples labeled (–) have not been edited and are identical to the wild type control sample.

iii. Protocol for Using Sanger Sequencing for Validating CRISPR Gene Editing (Cat. No. C199)

1. CRISPR Sample Preparation for Sanger Sequencing

As PCR products from a pool of cells may contain some edited and some non-edited templates, it is challenging to analyze the results from a direct Sanger sequencing of PCR products. For this reason, the PCR products must be cloned into a vector before sequencing so as to ensure a single template is being sequenced and results are easier to interpret.

1. 1 Linearize 3 µg of cloning vector with the appropriate restriction enzymes (as an example, abm uses pLenti-III-HA, Cat. No. LV022, but any vector can be used as long as primers are designed with compatible homology arms).

Incubate at 37°C for 1 hr 30 min, then gel purify the vector and elute in 30 µl.

1. 2 Perform a Ligation-free cloning reaction (Cat. No. E001 or E002) with linearized cloning vector and gDNA PCR samples that are identified as positive from the CRISPR Genomic Cleavage Detection Kit (G932) as follows:

2 µl	Cloning vector
1 µl	Individual PCR product
4 µl	5x Ligation-Free Cloning MasterMix
13 µl	Nuclease Free H_2O
20 µl	Total Volume

Incubate at 4°C for 30 mins*

Note: Only set up Ligation-free reactions 30 min before transformation into competent cells as the procedure is most successful if transformation occurs immediately after.

1. 3 Transform Ligation-free cloning reaction

a) Add 60 µl competent cells (e.g. ProClone™ DH5α Competent Cells, Cat. No. E003) to Ligation-free cloning reaction. Swirl pipette tip to mix. DO NOT pipette up and down as this can damage the cells.

b) Incubate at 4°C for 30 min.

c) Heat shock for 45 s at 42°C.

d) Add 150 µl of sterile Blank LB (containing no antibiotic) and shake in the incubator at 37°C (180 rpm) for 1 hr.

e) Plate cells on Kanamycin (Cat. No. G022) plates.

f) Place in incubator with agar side down (lid up); after 5 min, flip the plate upside down and incubate overnight at 37 °C.

1. 4 Select 5 colonies for miniprep in LB+Kanamycin (50 µg/ml). Cut the minipreps using EcoRI (Cat. No. E048) and XhoI. Expect the PCR product sized band to be excised. If the PCR product is cut internally by EcoRI/XhoI, expect to observe multiple smaller bands.

1. 5 Once successfully cloned minipreps are identified, set aside an aliquot of each of the 5 clones from each gDNA PCR product to be sequenced by Sanger sequencing with the CMV Forward primer (5'-CGCAAATGGGCGGTAGGCGTG-3') if pLenti-III-HA (Cat. No. LV022 was used as the vector). Be sure to use an appropriate sequencing primer that binds upstream of your insert.

2. Analyzing CRISPR Sanger Sequencing Results

2. 1 Import the Sanger sequencing results into the same alignment program as the raw RNA/DNA sequence.

2. 2 Align sample sequences with the sgRNA target and reference sequences.

2. 3 Insertions or deletions at the sgRNA target site that cause a frameshift mutation resulting in an early stop codon will knockout the gene. Only two variations of InDels should be observed for monoclonal bi-allelic knockout and both should be observed. To see if mutations cause an early stop codon, translate the mutated sequence using the ExPasy translation tool or similar.

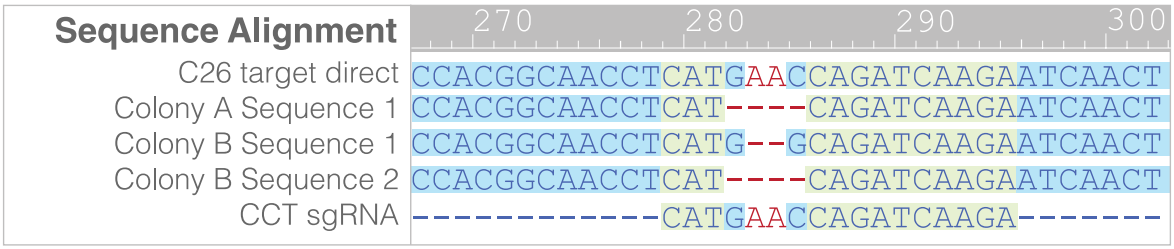


Figure 9. *Comparing edited sequences with reference sequences to identify frameshift mutations*
In this image, there are two frameshift mutations (4 nt and 2 nt deletions) compared to the reference and sgRNA target sequences ("C26 target direct" and "CCT sgRNA").

2. 4 **a)** If a clone appears monoclonal with frameshift mutations in both alleles, sequence more miniprep samples from the transformation plate until you have a minimum of 10 sequencing results from a single gDNA PCR product.

b) If it has been longer than 1 week since the transformation, perform a new Ligation-free cloning (refer to section F.iii) reaction and transformation with the same gDNA PCR product to obtain more colonies to screen.

2. 5 If the wild-type sequence is still detected in the 10 Sanger sequencing results, perform another round of single cell dilution to isolate monoclonal bi-allelic knockouts and repeat drug selection (refer to section E.iv).

iv. Next Generation CRISPR Validation (Cat. No. IA00100)

To characterize the gene edits of larger sample sizes (of more than 100 clones) and analyze any potential off-target edits, Next Generation Sequencing (NGS) is an excellent validation option that enables rapid and precise sequencing results at affordable prices. NGS screening analysis can confirm that your cells are monoclonal bi-allelic knockouts, whether any fraction of the wild-type genotype is left in your sample, and what indels are present in what proportions.

abm's Illumina Certified Next Generation CRISPR Validation Service (Cat. No. IA00100) involves a two-step PCR amplification process that amplifies the sgRNA target site from the gDNA and adds the sequencing adaptors and barcodes required for multiplexing samples. The final sequencing-ready library is then sequenced. The resulting sequencing data is analyzed using the CRISPResso software program which generates a series of graphs for each sample quantifying editing frequency and any potential off-target effects. Sequencing data can be used to determine if the Indels in all alleles cause frameshift mutations cause frameshift mutations or result in an early stop codon.

Next Generation Sequencing can be used to evaluate knockout at each stage of the selection process and monoclonal isolation, providing a robust profile of Indels in the sample.

Note that if Next Generation Sequencing services are needed to validate gene edits or detect off-target effects, a set of unedited control cells will need to be prepared for comparison purposes.

v. Western Blot Validation

For quick and easy confirmation that the resulting knockout stable cell line is no longer producing the functional protein, a Western blot analysis can be carried out. After transfection, single cell dilution and expansion of monoclonal populations, a Western blot analysis can be performed to determine if the resulting cell population is a bi-allelic (both copies of the target gene functionally silenced) or a mono-allelic knockout (only one copy of the target gene functionally silenced). On the Western blot results, a 100% knockout of the protein of interest would correspond to a bi-allelic knockout whereas a 50% knockout would correspond to a mono-allelic knockout.

Use of anti-Cas9 antibody (Cat. No. Y300079) is also a quick strategy for confirming successful transfection/ transduction of Cas9 into the cell line of interest.

G. CRISPR Knockout Case Study

The following case study will demonstrate how to use CRISPR to develop a bi-allelic LIF knockout in Mouse Colon Carcinoma cells and validate genome editing using the Genomic Cleavage Detection Kit (Cat. No. G932) and Sanger Sequencing (Cat. No. C199) as well as Next Generation Sequencing (Cat. No. IA00100) methods.

Phase 1: Cas9 and sgRNA Delivery

- Three sgRNA were designed against mouse LIF locus (Mus musculus, NM_008501). Software analysis was performed to ensure the sgRNA had no predicted off-targets binding sites. The selected sgRNA design was then cloned into the pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro All-in-One lentivector (Figure 10).
- Recombinant Lentiviruses were packaged using **abm**'s second generation Lentiviral packaging system. A multiplicity of infection (MOI) of 5 was used to transduce the cells.

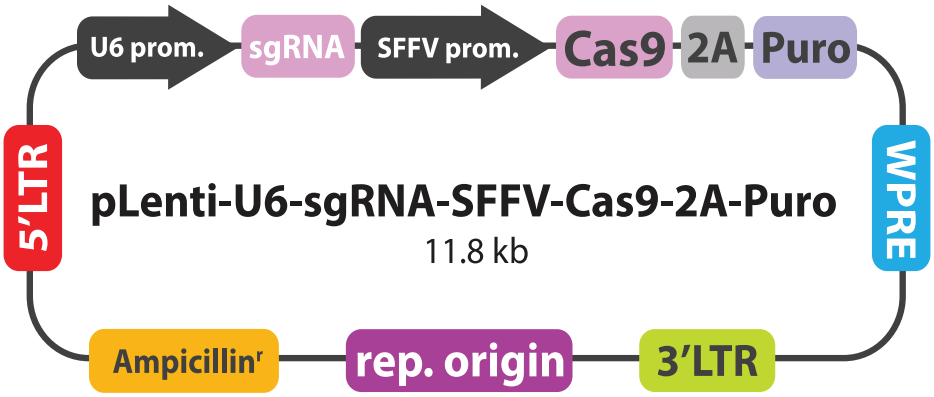


Figure 10. *pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro lentivector is an all-in-one vector for co-expressing sgRNA and Cas9 in mammalian cells.*

Expression of sgRNA is driven by the U6 promoter, a strong constitutive Pol III promoter. An SFFV promoter drives expression of the Cas9-2A-Puro cassette. By using the Cas9-2A-Puro cassette, cells can be directly screened for expression of Cas9, as they will be resistant to Puromycin.

Phase 2: First Round of Colony Screening for Edited Clones

- Cell colonies are isolated after puromycin selection. Genomic DNA was extracted and the surveyor assay was performed to confirm genomic editing of the LIF locus.
- A single band in a Surveyor assay (Genomic Cleavage Detection Kit, Cat. No. G932) at the wild-type (WT) size indicates no editing has occurred; two smaller bands (that add up to the length of the WT) indicate editing has taken place.

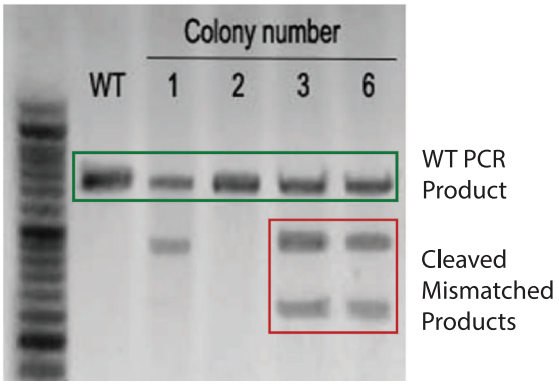


Figure 11. *The surveyor assay indicated that Colony 3 and 6 were edited; colony 2 was not edited; and colony 1 was inconclusive.*

Phase 3: Sequence Analysis of the Edited Colonies

- PCR products from Colonies 3 and 6 were further analyzed via Sanger Sequencing to determine the nature of the knockout (Figure 12).

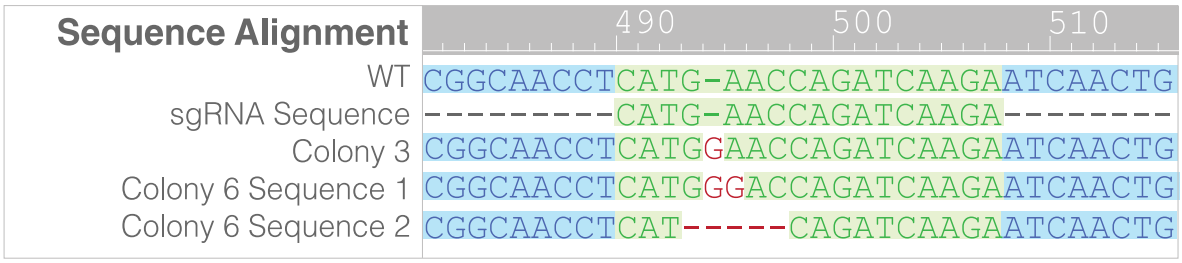


Figure 12. *For colony 3, only one mutant sequence was detected, indicating that these cells are likely only heterozyotic knockouts. In colony 6, two different mutant sequences were detected.*

Phase 4: Second Round of Selection for Monoclonal Bi-allelic Knockout Clones

- Colony 6 was serial diluted into 96 well plates for monoclonal selection. Genomic DNA was extracted from these clones (i.e. 6a, 6b..), PCR amplified, cloned and sequenced.
- Of the colony 6 clones, sequencing showed that only clone 6a had a frameshift mutation in both alleles (Figure 13). A frameshift mutation disrupts the open reading frame, resulting in nonsense mediated decay of mRNA transcript.

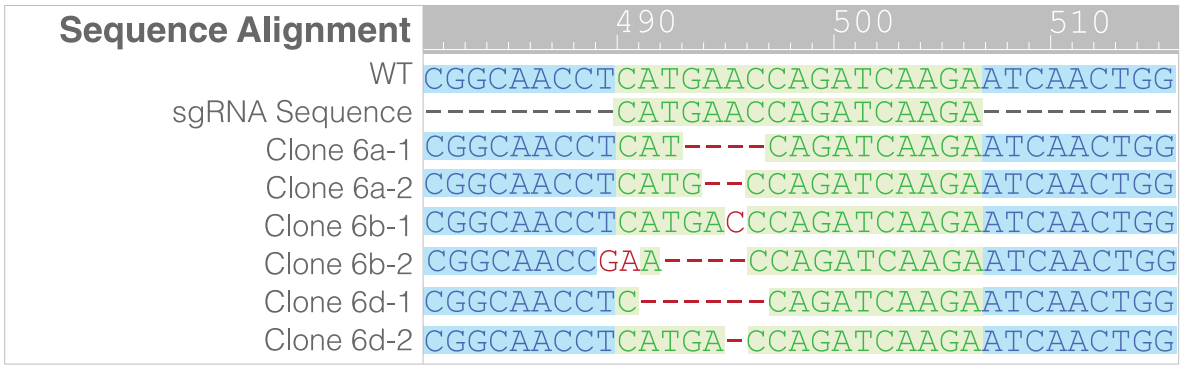


Figure 13. *Clones 6a, 6b and 6d all showed bi-allelic editing. Only clone 6a had frame shift mutations in both alleles. No WT sequences were detected in all subclones.*

- Further sequencing of clone 6a confirmed that only two mutant alleles were present, the 2 bp and 4 bp deletions, and that no WT or other mutations were detected (Figure 14).

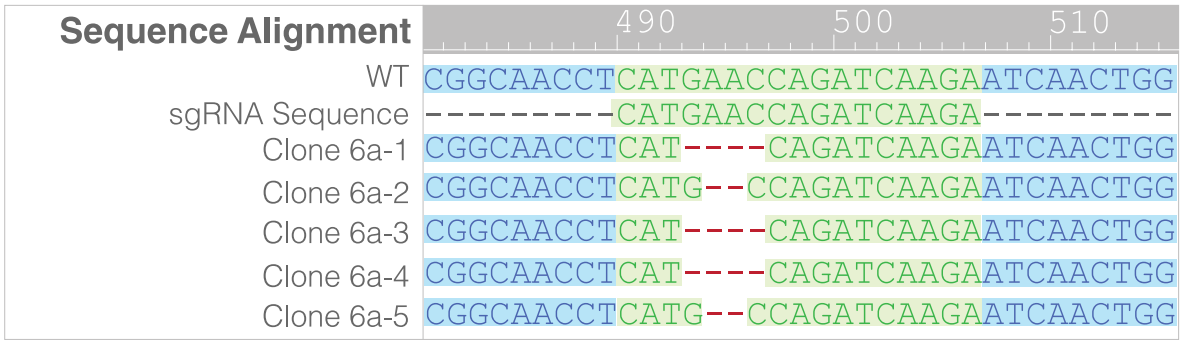


Figure 14. *Further sequencing of 6a confirmed bi-allelic knockout. No WT sequences were detected.*

Phase 5: Confirmation of Knockout by Next Generation Amplicon Sequencing

- With Next Generation Sequencing hundreds of thousands of alleles can be sequenced at once, resulting in a more robust dataset. By contrast, Sanger sequencing is only feasible for 1-100 clones and therefore it can miss a large proportion of the population.
- Next Generation Sequencing was performed at each stage of selection to evaluate knockout (Figure 15). Before editing, only WT sequences were observed. After the first round of selection, colony 6 showed a mixture of edited (70%) and WT (30%) sequences. Finally, after monoclonal selection, clone 6a showed only edited sequences with no WT alleles present.

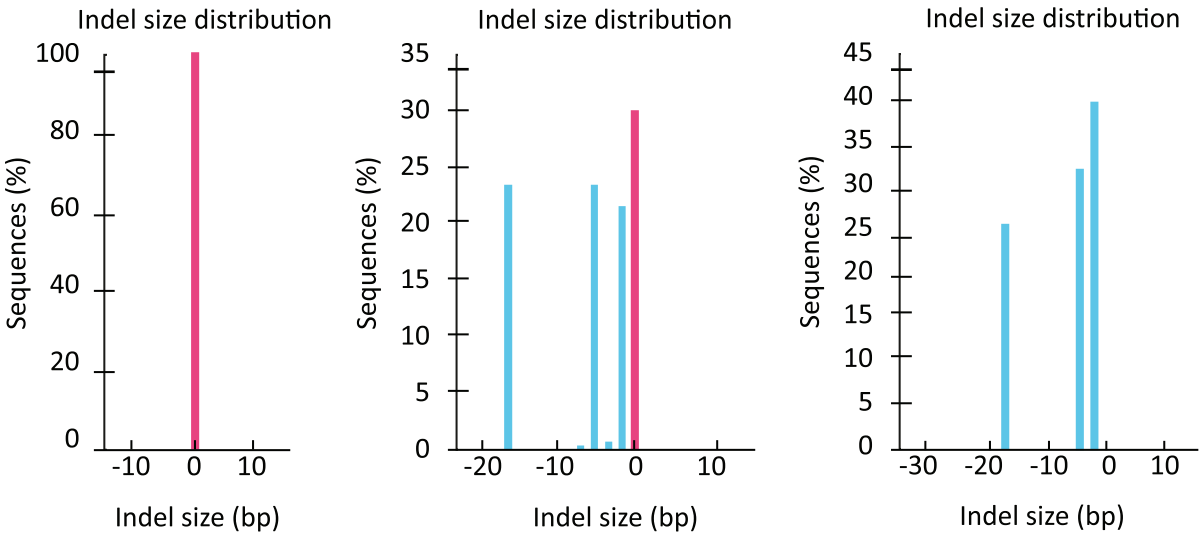


Figure 15. *An example of Next Generation Sequencing for CRISPR Knockout screening.*

A) Before knockout, only WT sequences are detected. B) After Cas9 and sgRNA delivery, the first round of selection shows a mixed distribution of InDel and WT sequences. C) After the second round of selection, only knockouts remain.

H. Conclusion

Because of CRISPR's versatility and relatively low barrier of entry, the cost of gene editing is more accessible than ever before—making large-scale multiplex experiments easy and affordable for any lab to do. The CRISPR Cas9 system and its variations have the potential to not only revolutionize basic research across a wide variety of fields, but also the landscape of medicine itself. It is very possible that in the near future, CRISPR will become a staple in gene therapy, enabling the correction of detrimental genetic mutations that cause everything from cancer to Parkinson's disease.

These guidelines focus only on the use of CRISPR in the generation of stable knockouts—the span and breadth of CRISPR applications far surpasses what can be covered in one handbook, with applications in epigenetic modification, gene therapy, gene circuits, and more. As our understanding of CRISPR in the mammalian system grows, we will be able to perfect the effectiveness of the technology and expand its applications. We are only limited by our own imaginations.

For a more in-depth exploration of the CRISPR system and its applications, visit our Knowledge Base: https://www.abmgood.com/marketing/knowledge_base.php

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