

Ideal Properties of Genome Editing Tools:



High specificity (zero off-target effects)

High efficiency



Pos/Neg selection unnecessary

Exponential enhanced HR

Zero/low toxicity

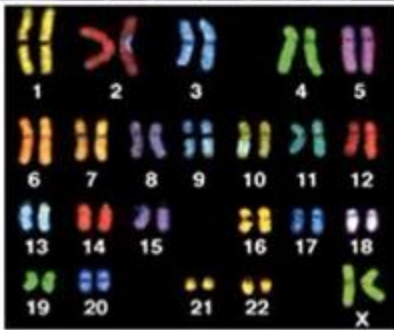
Easy to design/construct

Low cost

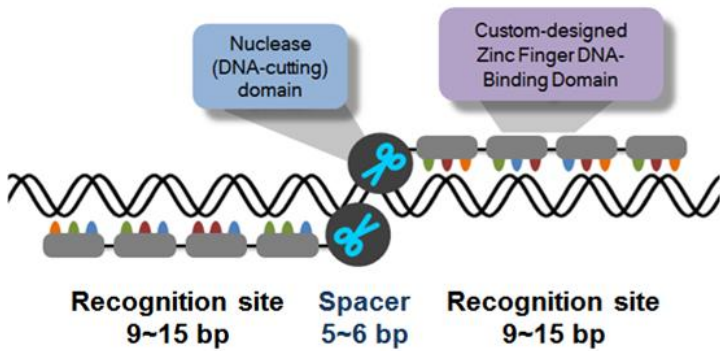
Broad application base

Any site in genome

Any organism

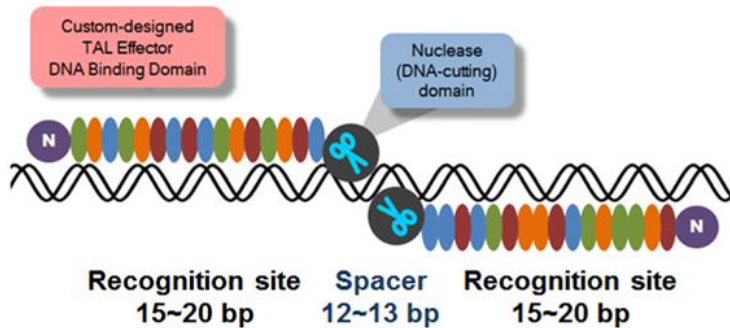


Engineered Nucleases :



Zinc Finger Nucleases (ZFNs)

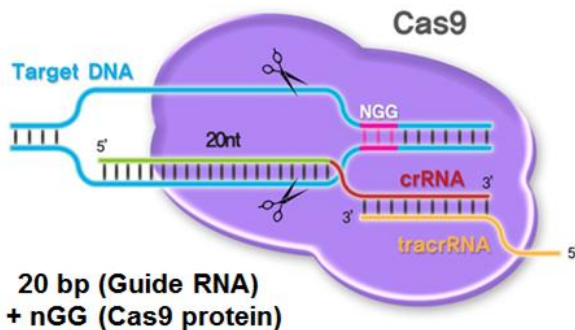
- Represent the first generation of engineered nucleases
- DNA binding module: Zinc fingers
(each module recognizes 3 bp of target sequence)
- DNA cleavage domain: Fok I restriction enzyme nuclease domain
(requires dimerization for cleavage)
- Widely proven in many cells and organisms
- Relatively lower resolution of target sequence programmability
- Relatively lower specificity



TAL Effector Nucleases (TALENs)

- DNA binding module: TAL effector unit
(each module recognizes 1 bp of target sequence)
- DNA cleavage domain: Fok I restriction enzyme nuclease domain
(requires dimerization for cleavage)
- High resolution of target sequence programmability
- High specificity and low toxicity

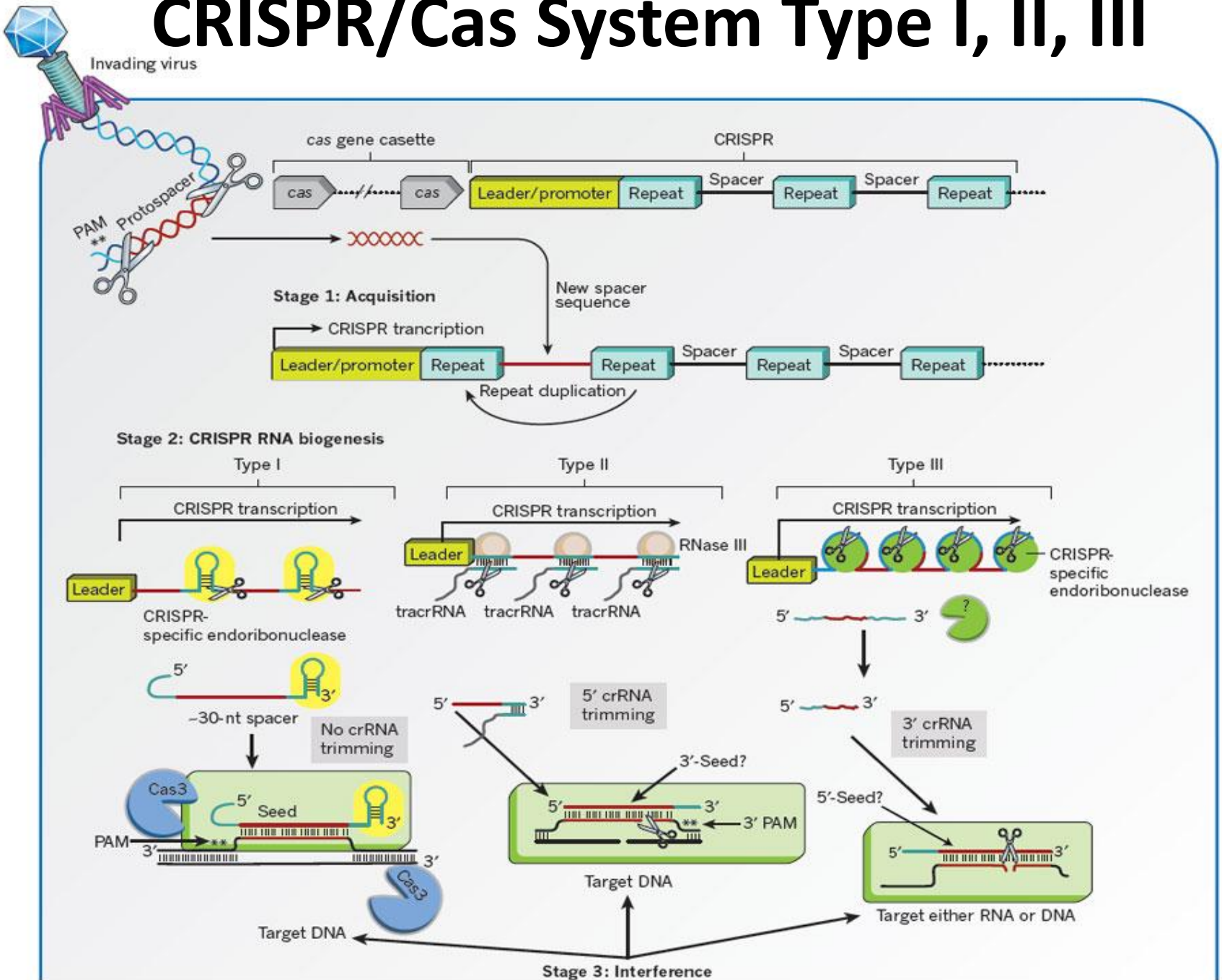
CRISPR/CAS



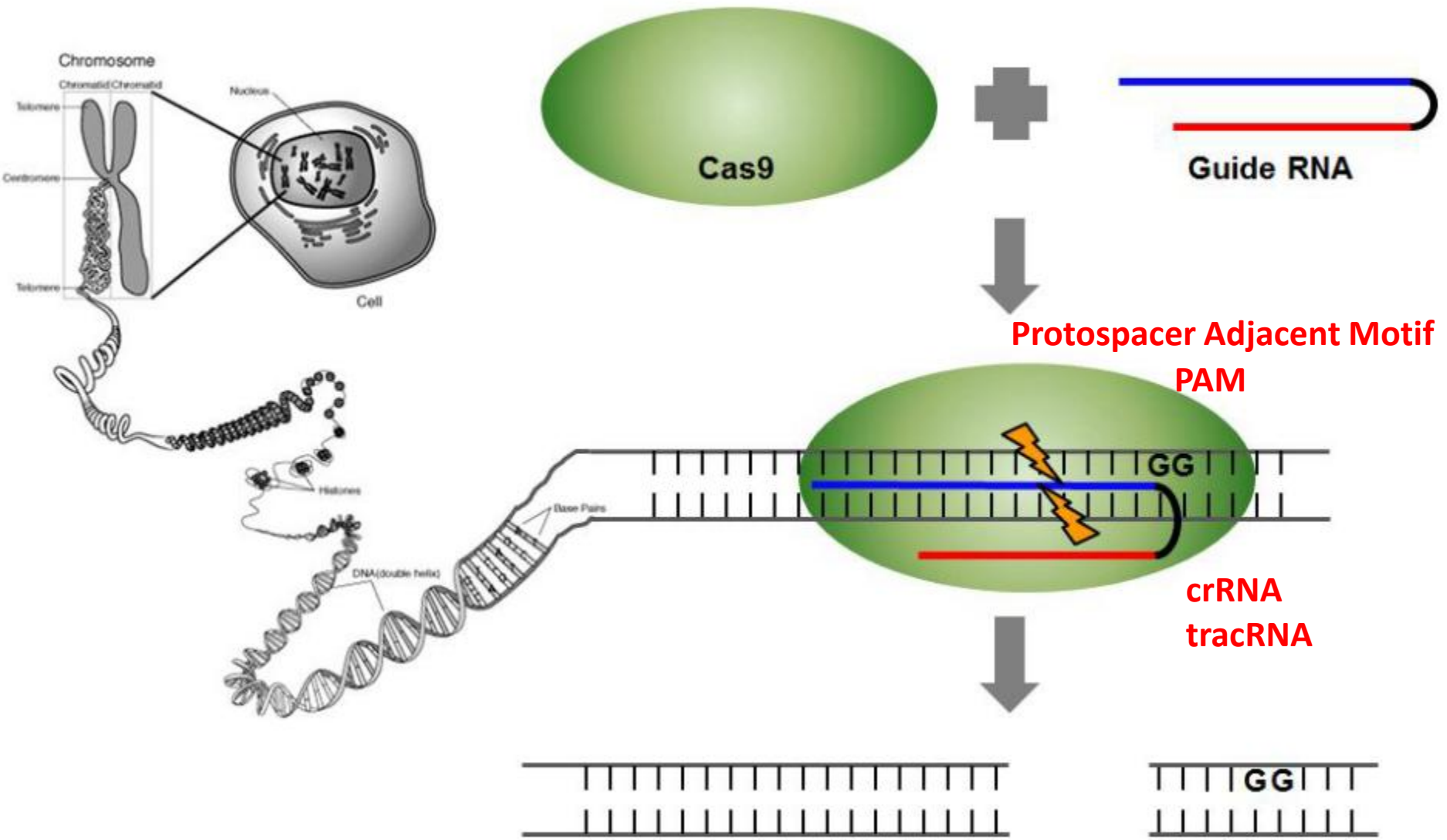
RNA-Guided ENdonucleases (RGENs)

- DNA binding module: Guide RNA that hybridizes to the target DNA (1:1 nucleotide base pairing)
- DNA cleavage module: Cas9 protein (contains two nuclease domains)
- High resolution of target sequence programmability
- High specificity and low toxicity

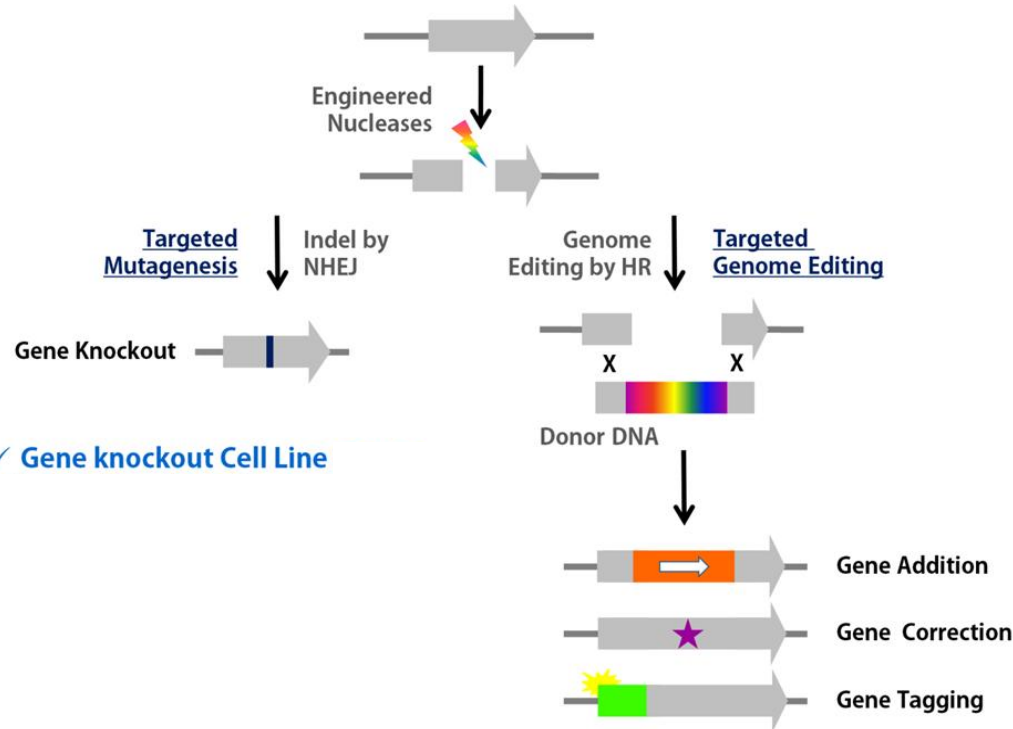
CRISPR/Cas System Type I, II, III



RNA-Guided ENdonuclease (RGEN)



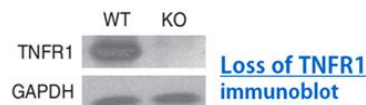
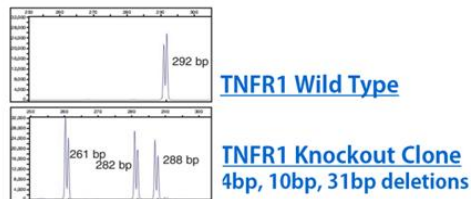
Cell Engineering Using Engineered Nucleases



✓ **Gene knockout Cell Line**

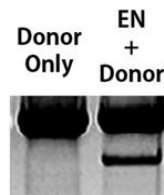
✓ **Gene Knock-in Cell Line**

Establishment of gene knockout cell line by targeted mutagenesis (NHEJ-mediated indels)



Loss of TNFR1 immunoblot

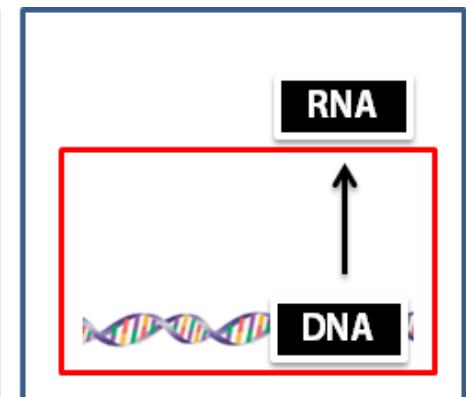
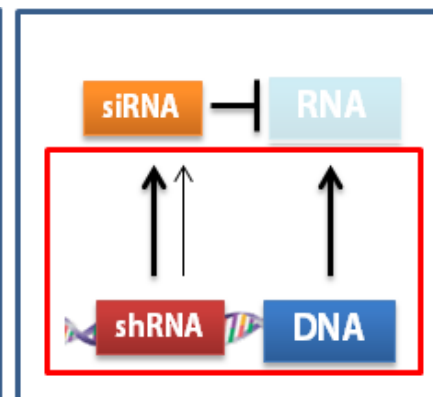
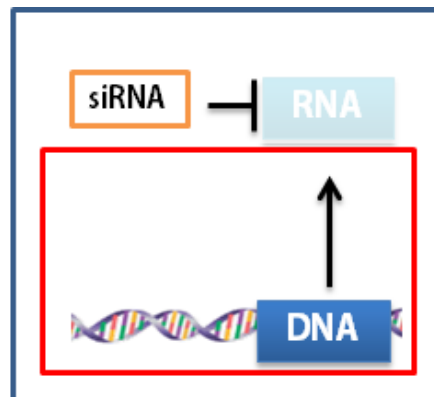
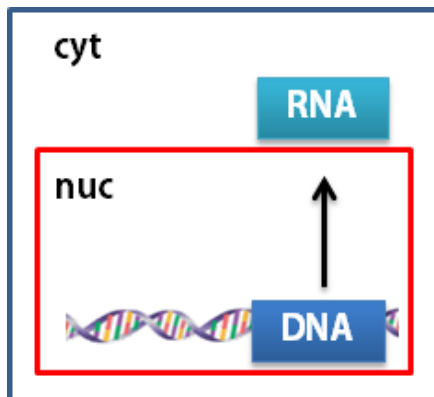
Introduction of specific sequence into target site in cultured cells (HR-mediated genome editing)



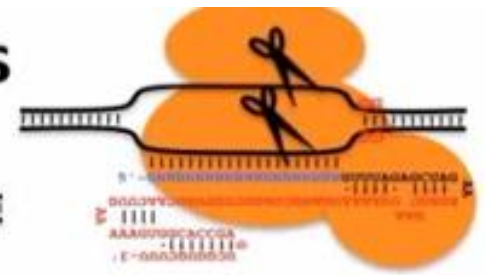
XbaI digestion of amplified target locus (3 days after electroporation)

Knockdown vs Knockout

	siRNA	shRNA	EN
	Knockdown		Knockout
Effect	Reduce		Eliminate
	Quantitative (50-90%)		0%
Duration	Transient	Permanent	Permanent
Stability	N/A	Fluctuate*	Stable
Specificity	Low	Low	High [#]



Comparison of Site-Specific Nucleases



Biallelic Targeting in 2n Cells

Generate Large Deletions

Mutagenesis of 1-Cell Zygote

HDR with ssODN

Multiplex Targeting

TALENs



?

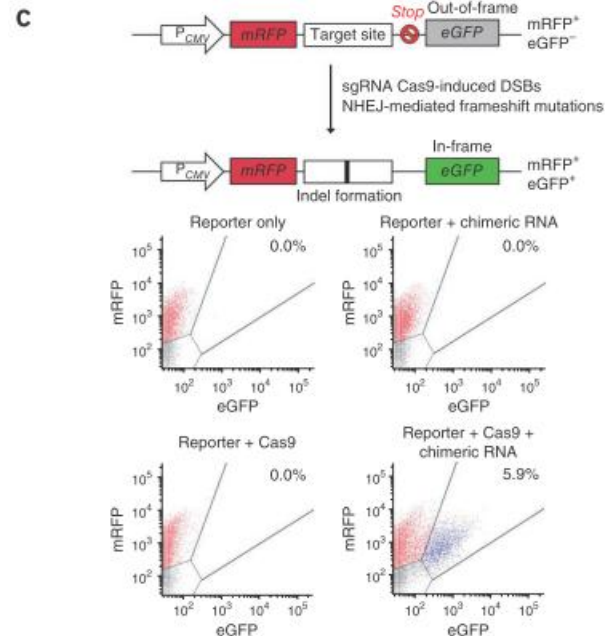
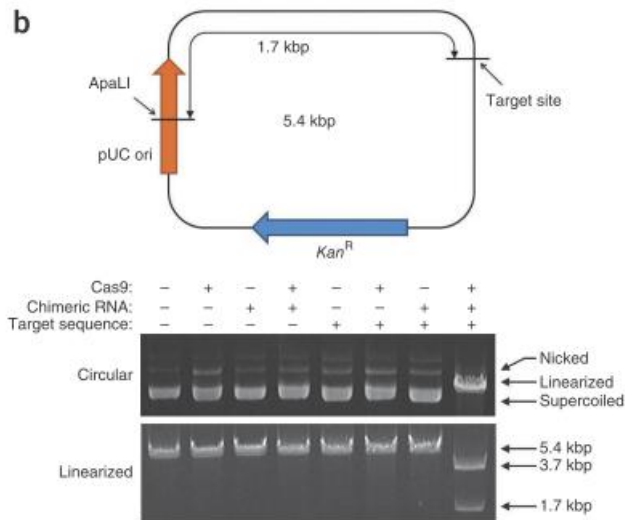
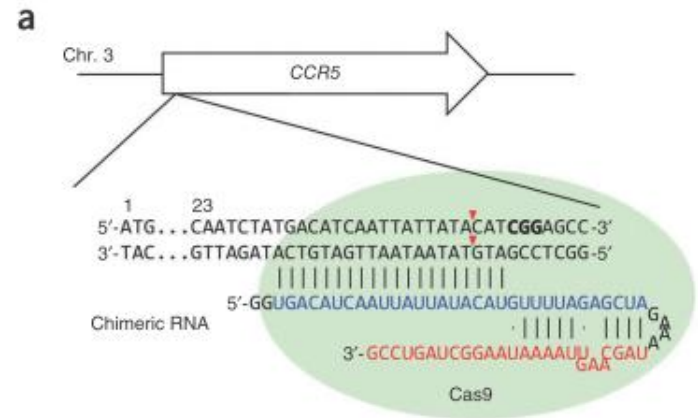
CRISPR/CAS

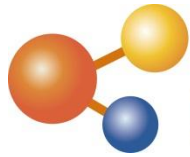


Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease

Seung Woo Cho¹⁻³, Sojung Kim¹⁻³, Jong Min Kim^{1,2} & Jin-Soo Kim^{1,2}

We employ the CRISPR-Cas system of *Streptococcus pyogenes* as programmable RNA-guided endonucleases (RGENs) to cleave DNA in a targeted manner for genome editing in human cells. We show that complexes of the Cas9 protein and artificial chimeric RNAs efficiently cleave two genomic sites and induce indels with frequencies of up to 33%.

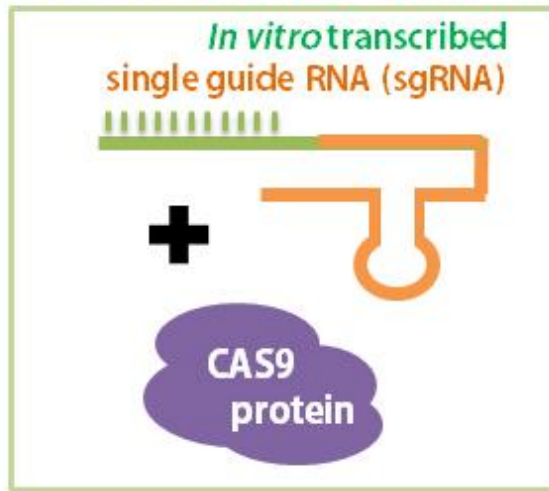




BIOTOOLS ToolGen

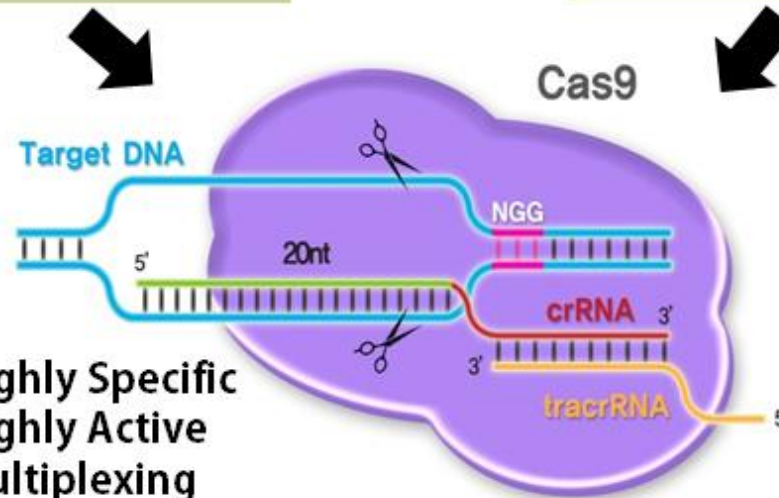
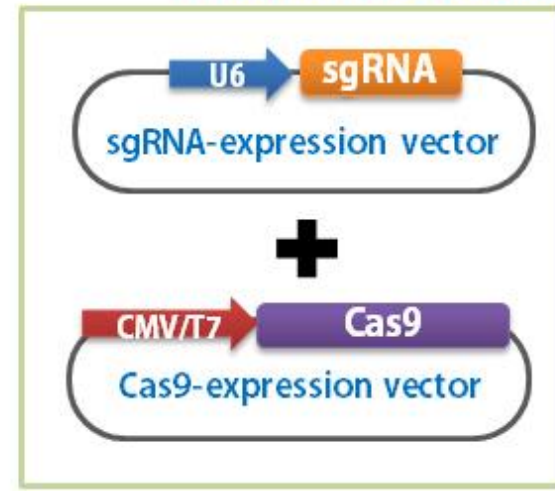
Active RGENs (aRGENs)

- Ready-to-inject
 - : Perfect for KO animal production by embryo injection
- Direct delivery into cultured cells



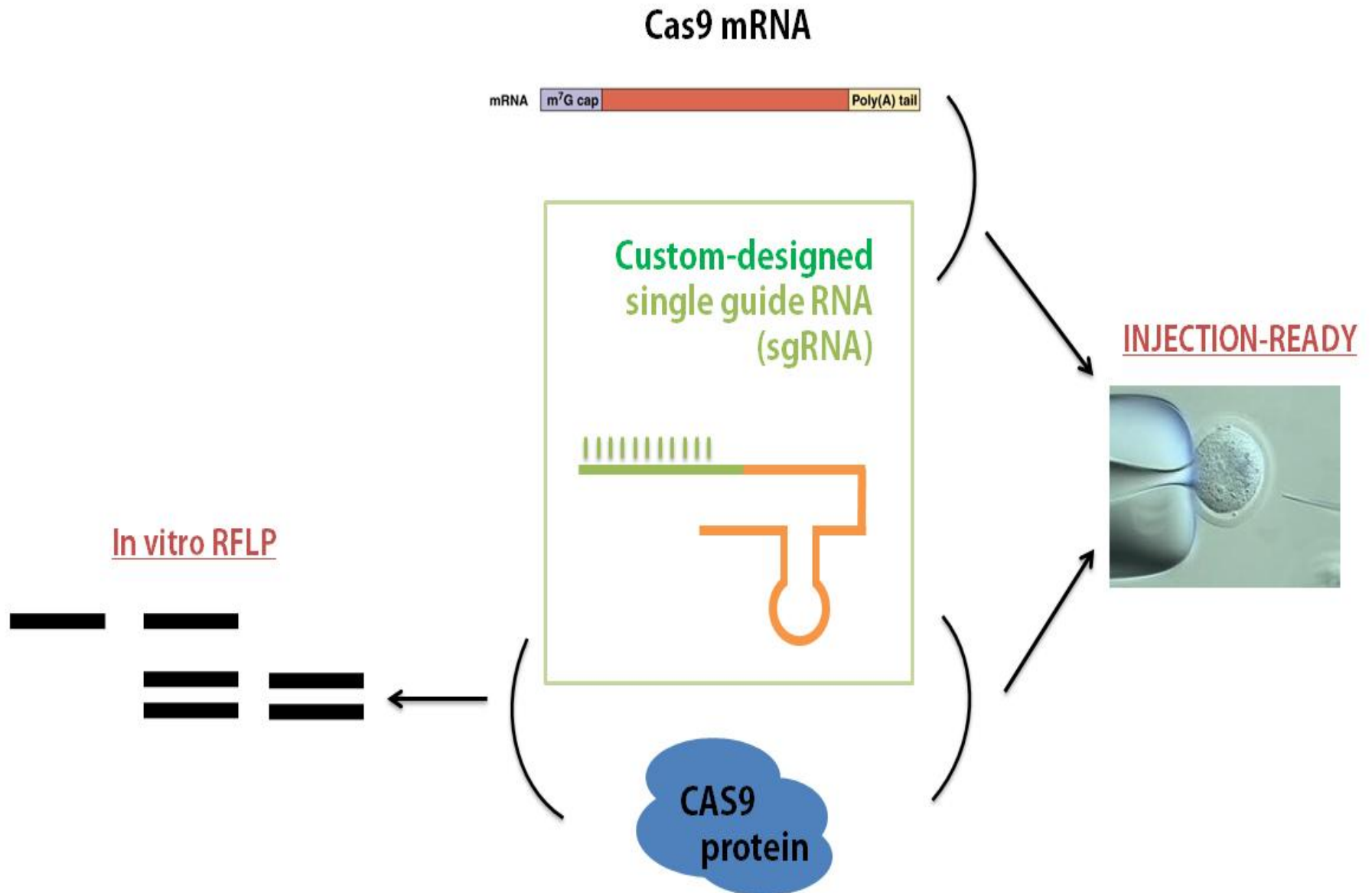
DNA-directed RGENs (dRGENs)

- Ready-to-transfect (plasmid-based system)
 - : Compatible with general transfection protocols



- Highly Specific
- Highly Active
- Multiplexing

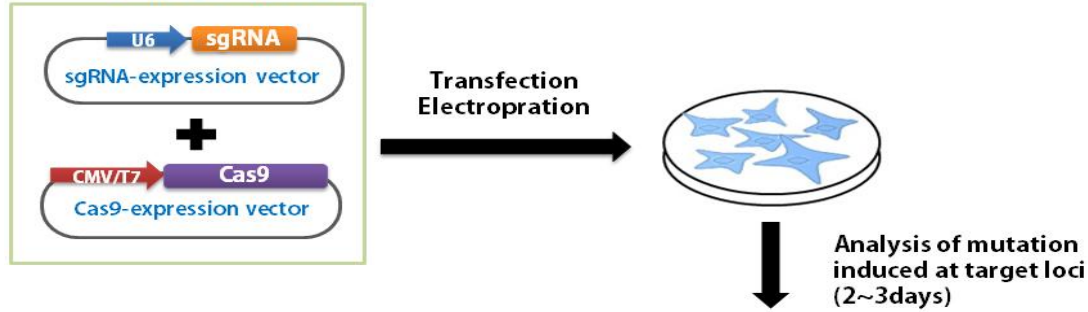
1. Active RGENs: for animal sample



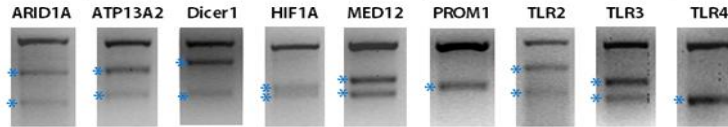
2. DNA-direct RGENs: for cell line

DNA-directed RGENs (dRGENs)

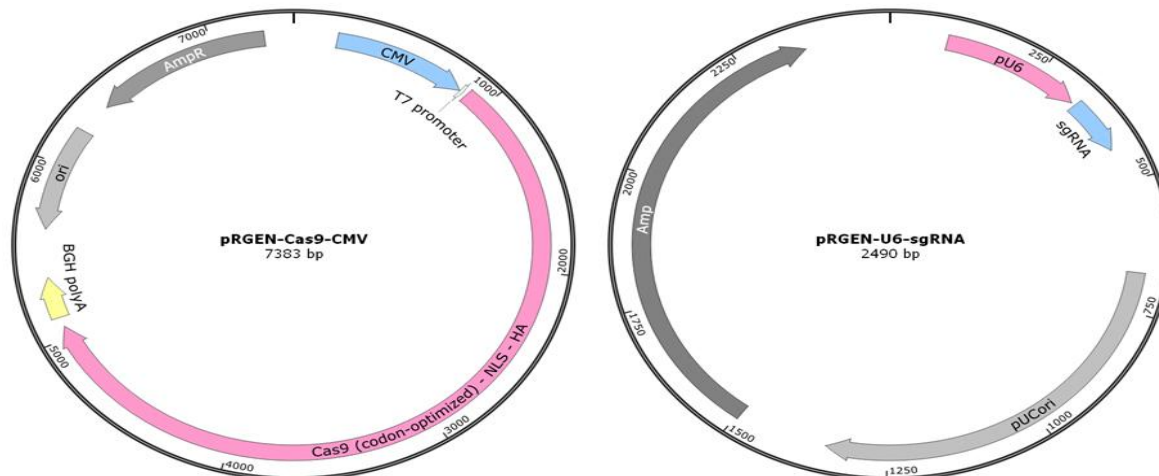
- Ready-to-transfect (plasmid-based system)
: Compatible with general transfection protocols



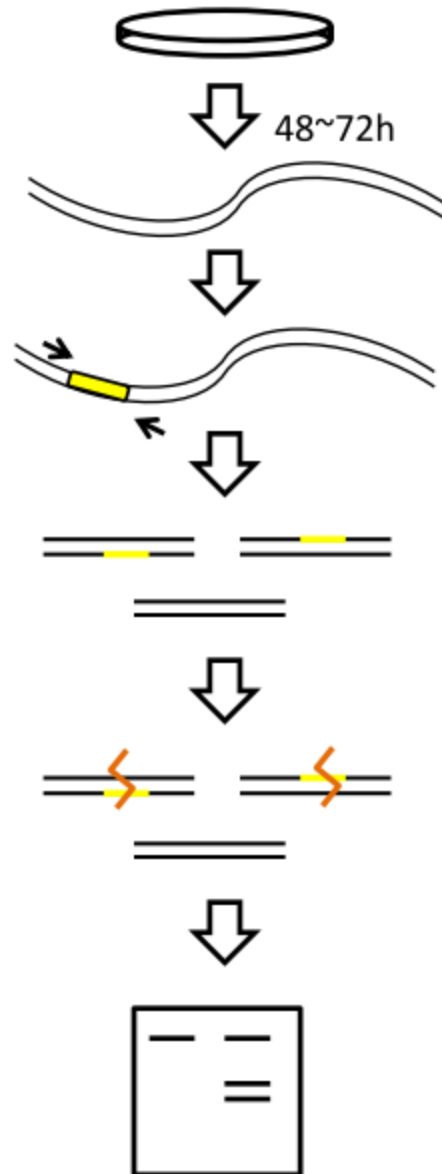
Analysis of dRGEN-induced mutation by a T7E1 assay (293T cells)



dRGEN Expression Vectors



T7E1 protocol



1. Nucleases(ZFN, TALEN) Transfection.

2. Genomic DNA prep.

3. Target site PCR.

4. Denature/Reannealing

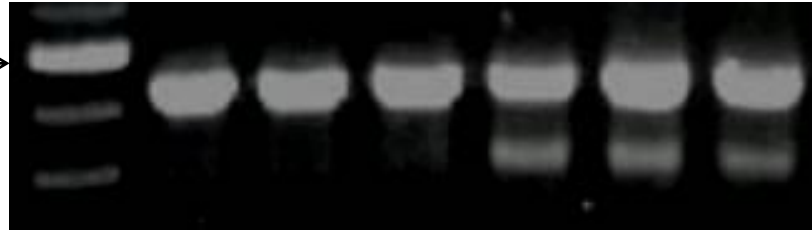
5. T7 endonuclease I reaction.

6. Agarose gel eletrophoresis

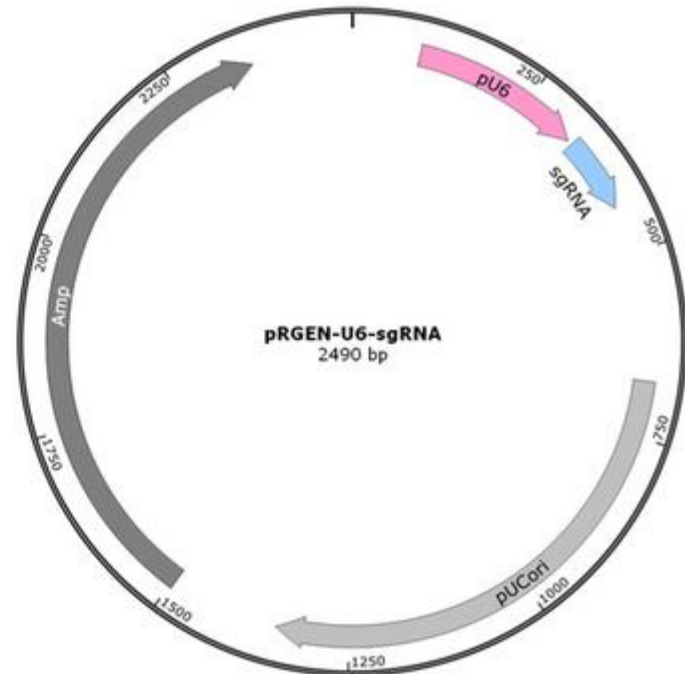
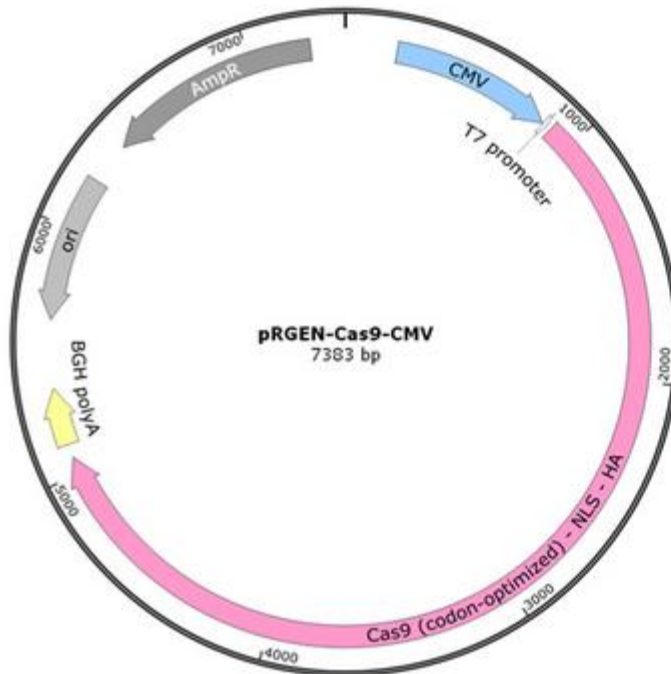
Cas 9 and sgRNA

— — — + + +

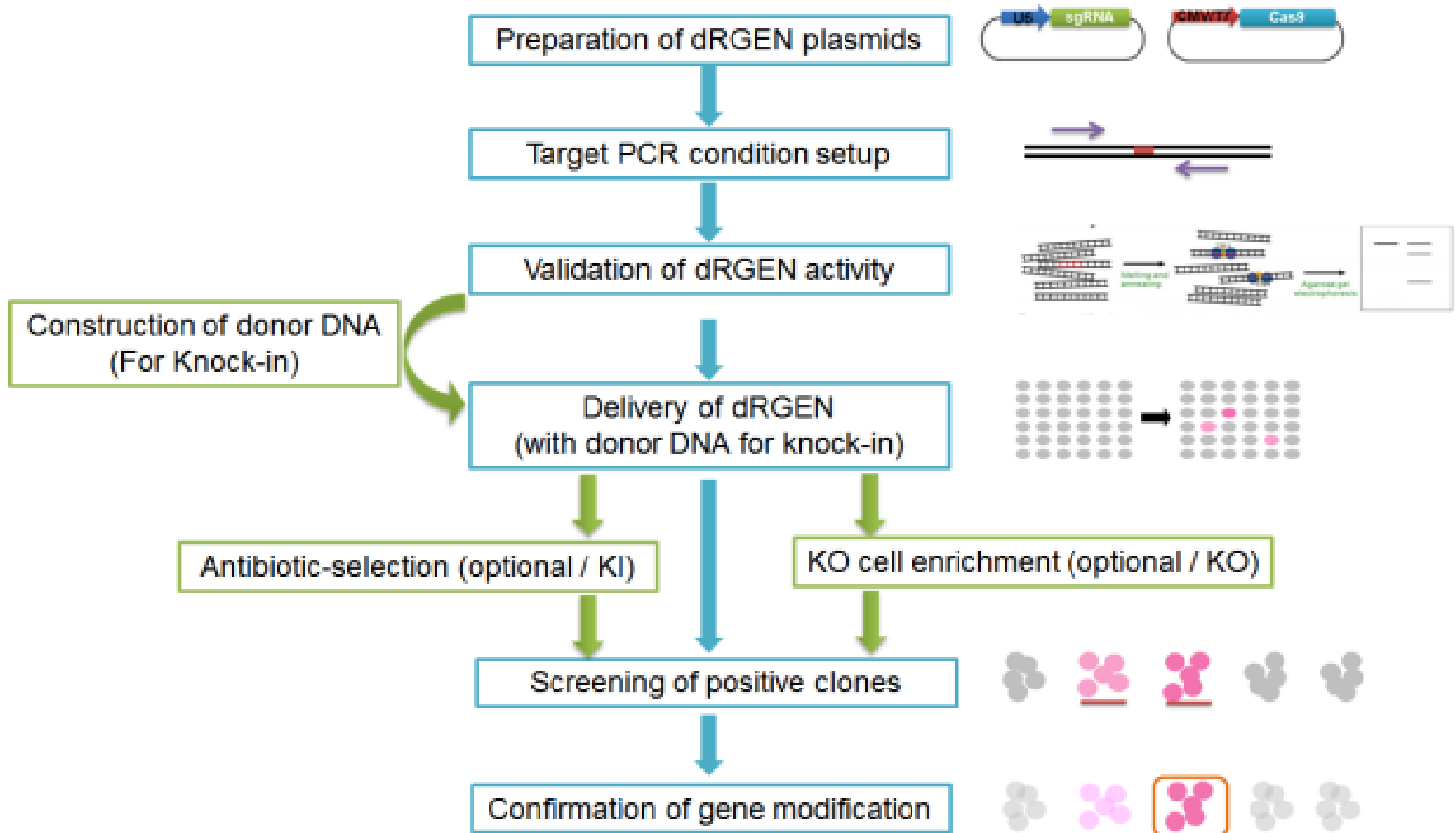
500 bp →

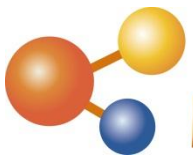


dRGEN Expression Vectors

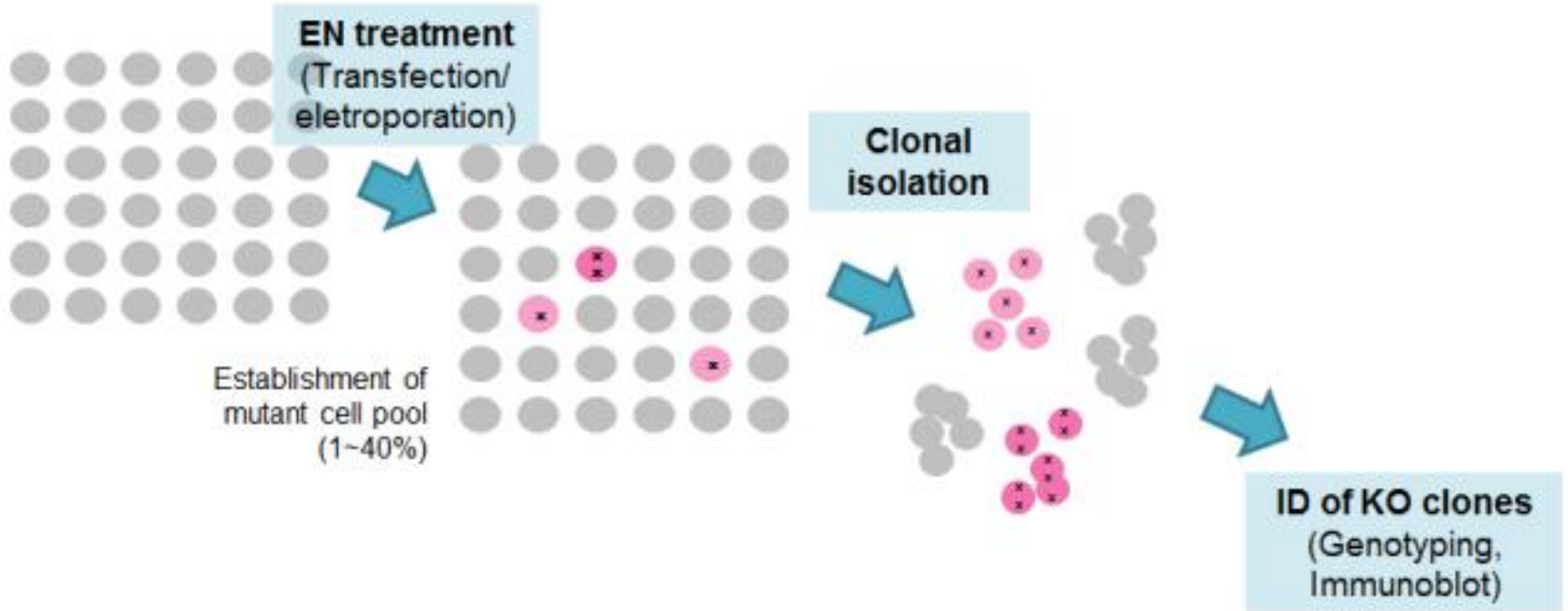


A. Procedure overview





E. Gene Knockout Cell Establishment



- Fluorescence Surrogate Reporter (pRG2S)



- Cell Surface Surrogate Reporter (pMRS)

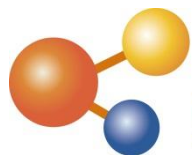


- Hygromycin Surrogate Reporter (pHRS)



Product and Services

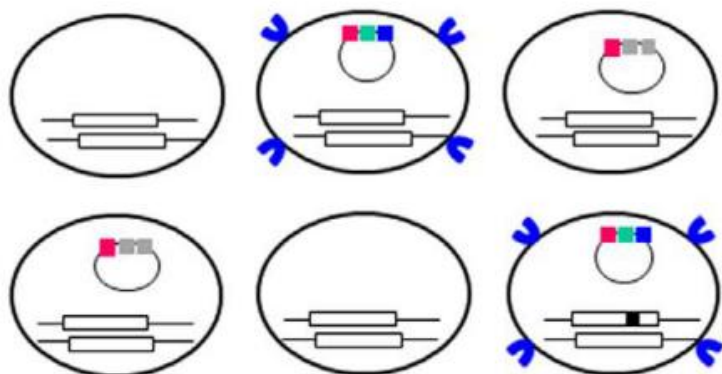
- Empty surrogate reporter vector
 - Linearized surrogate reporter vectors (10 reactions)
 - Protocol for designing and constructing surrogate reporter
- Surrogate reporter synthesis service (Custom-synthesized surrogate reporter plasmid with reporter plasmid containing the target site of your choice)
 - Target sequence analysis (with stop codon editing, if needed)
 - Reporter construction
 - Sequence verification
 - Turnaround time: 2 weeks



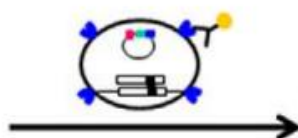
BIOTOOLS

B

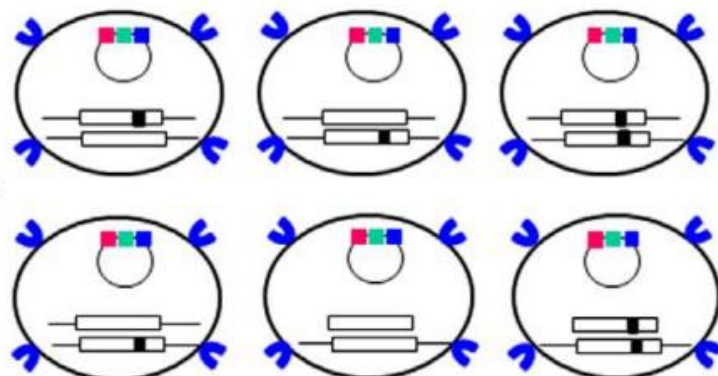
Unseparated cells



Magnetic separation

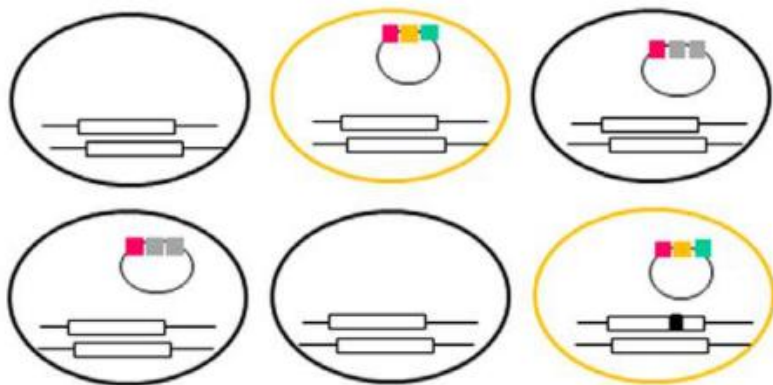


RFP⁺ GFP⁺ H-2K^{k+} cells



B

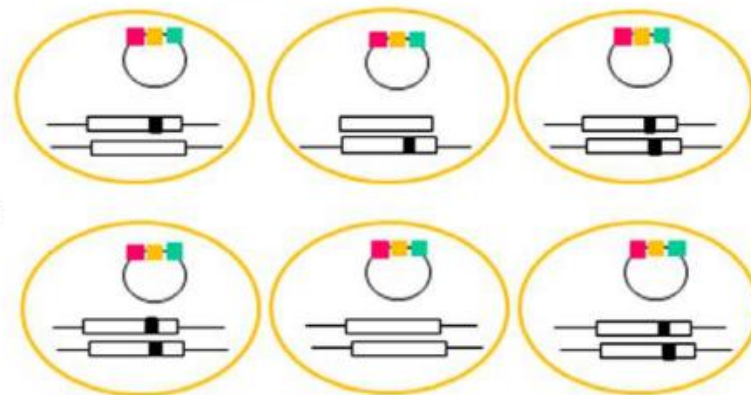
Unselected cells



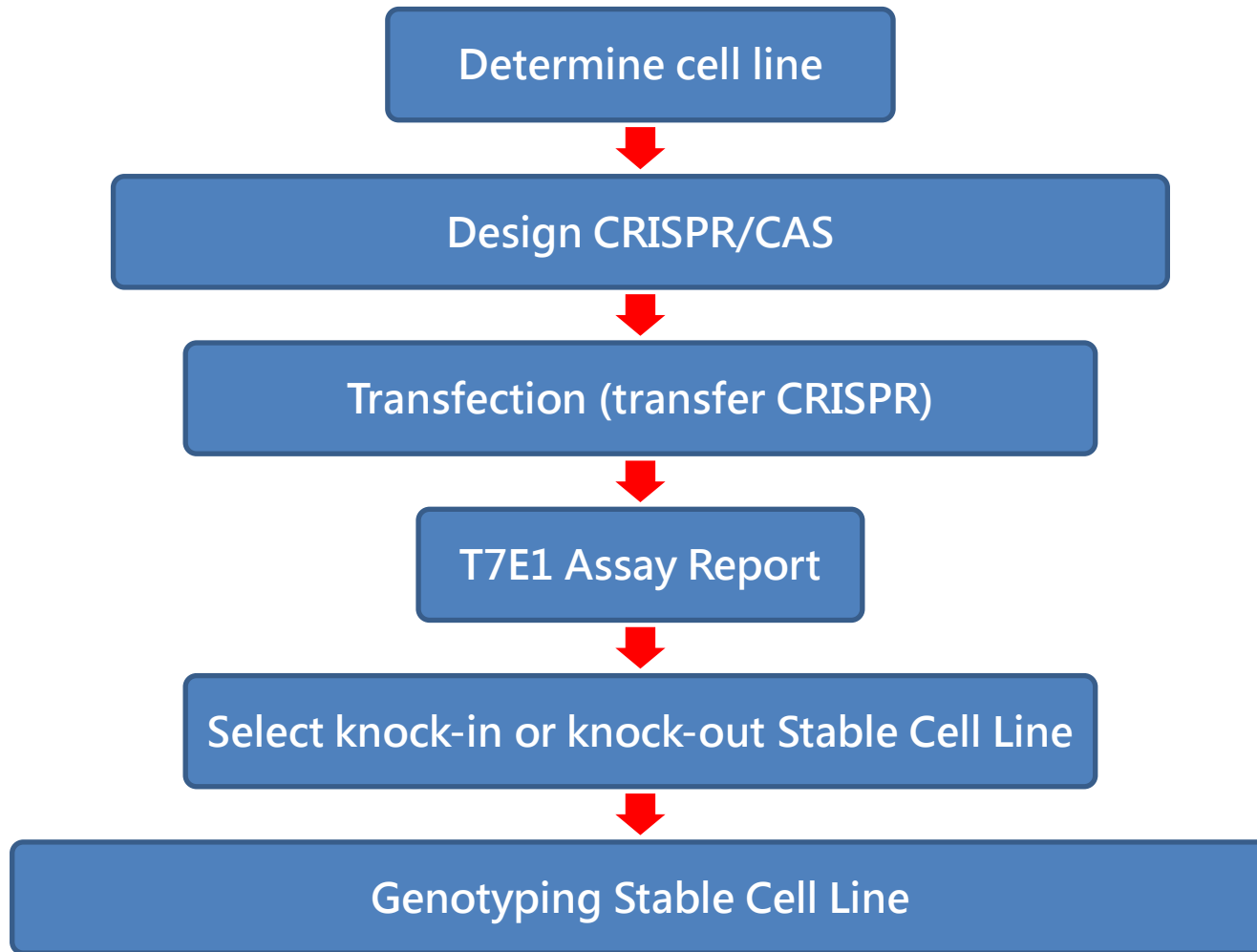
Hygromycin selection



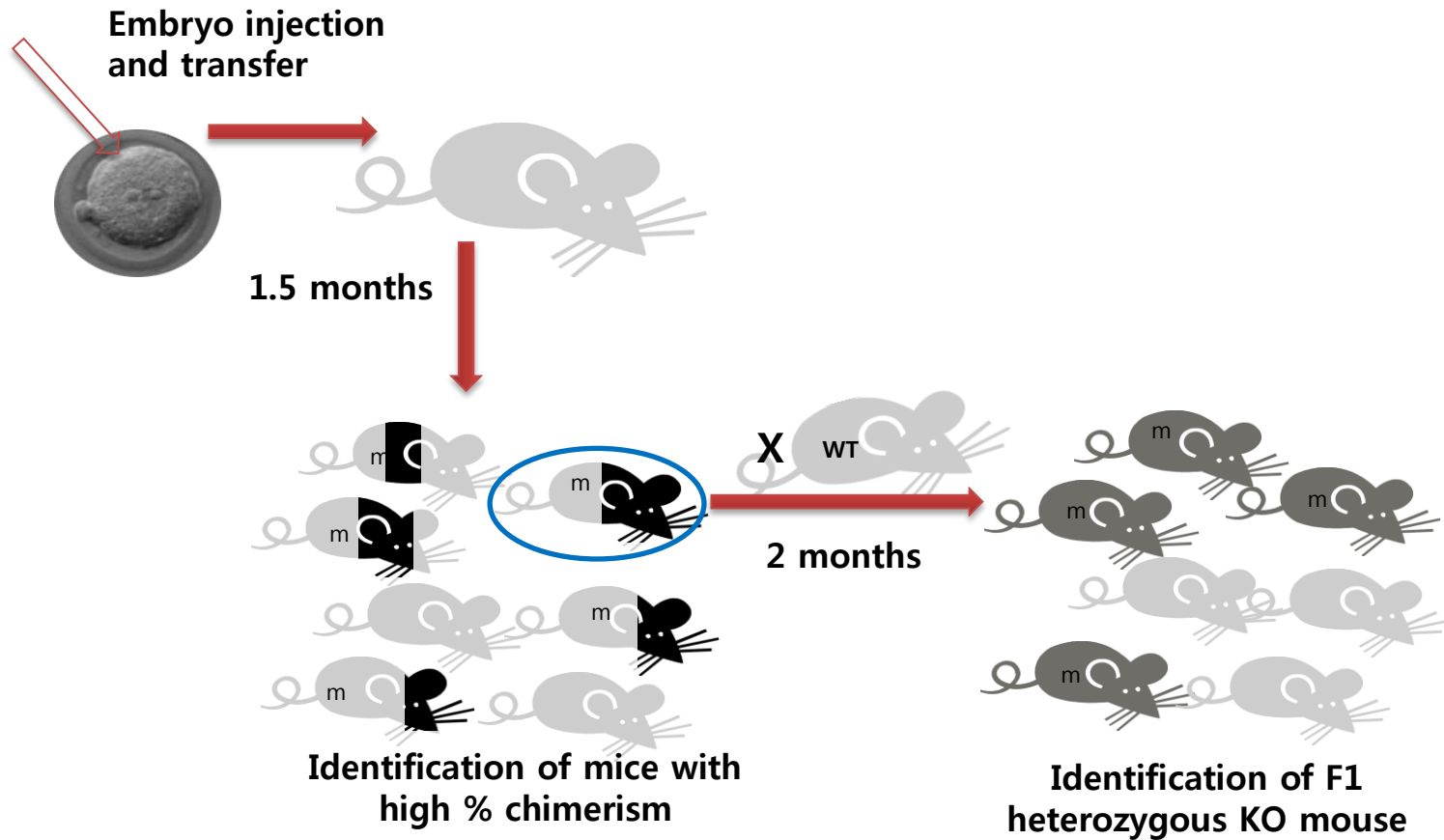
RFP⁺ Hygro^{R+}GFP⁺ cells



Service: *in vitro*



EN
In RNA
enzyme form
Development of
aRGEN)



Service: *in vivo*



Determine inbred mouse strain



Design CRISPR/CAS



Microinjection of RGENs into mouse embryos



Transfer Embryos into the oviducts of pseudopregnant foster mothers

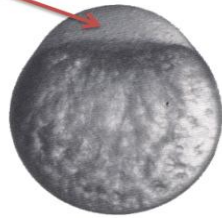


Produce founders

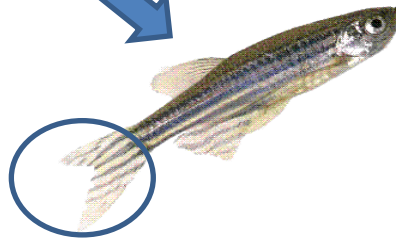


Genotyping of Chimera /Heterozygous /Homozygous founders

EN
In RNA
enzyme form

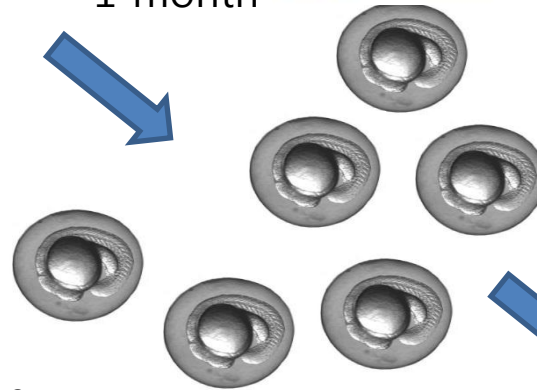


1.5~2 month



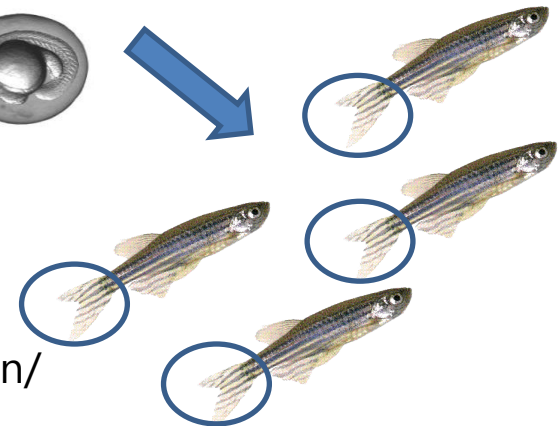
Genotyping
(Somatic mutation/
F0 Chimerism)

1 month



Genotyping
(Germline mutation/
Founder)

1.5~2 month



Genotyping
(Somatic mutation/
F1 Hetero KO)



Service: *in vivo*



Design CRISPR/CAS



Microinjection of RGENs into zebrafish embryos



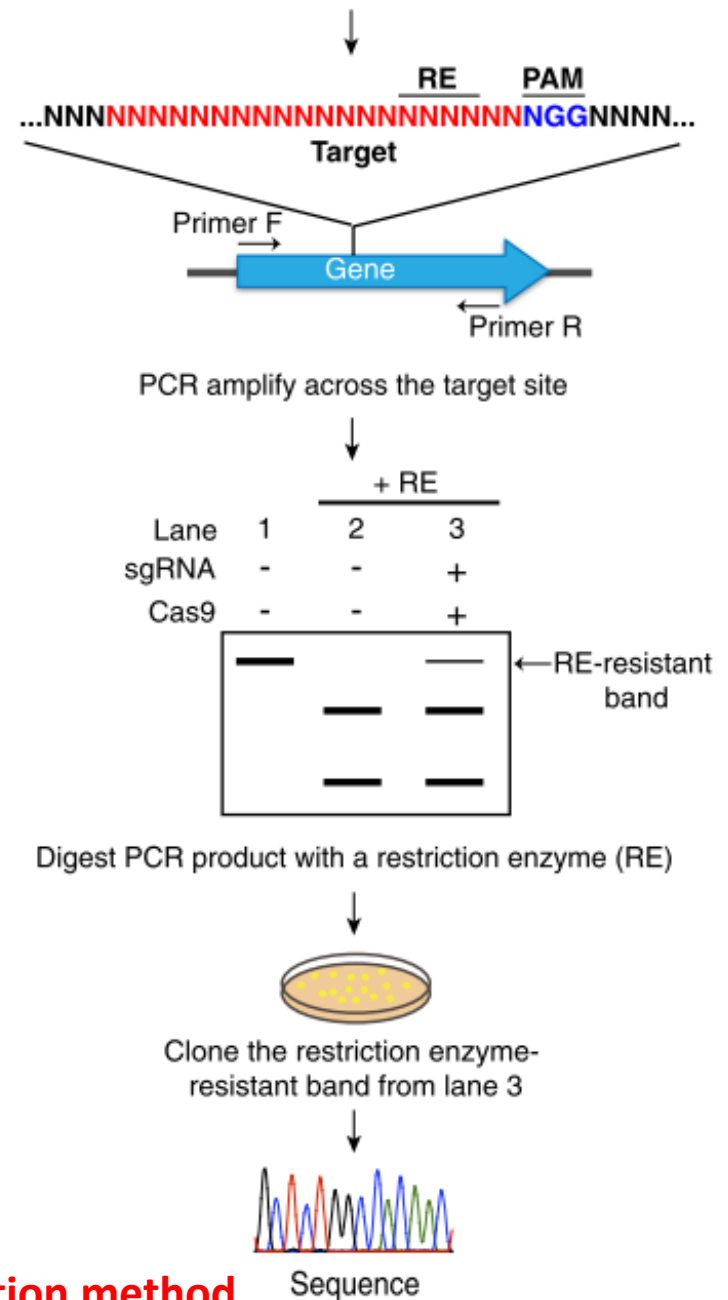
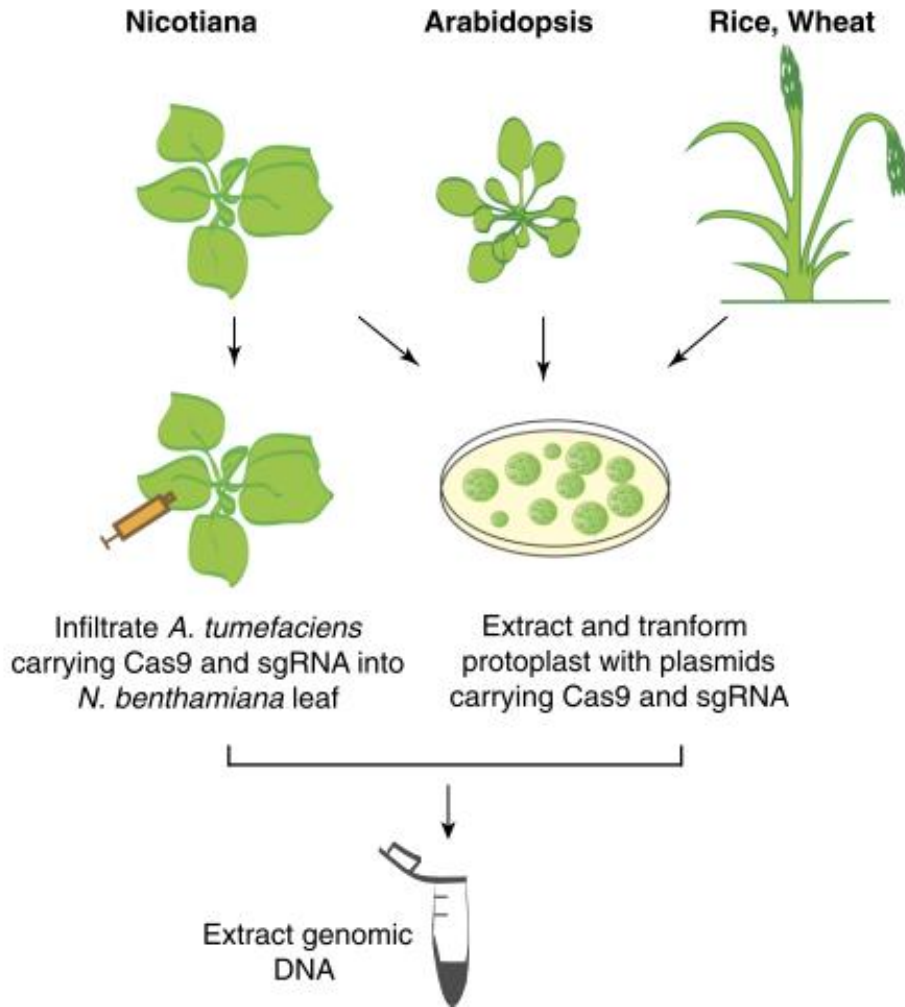
Analysis CRISPR/Cas efficiency



Produce larvae



Genotyping of Chimera /Heterozygous /Homozygous adults



- (i) protoplast transformation and
- (ii) leaf tissue transformation using the agroinfiltration method.

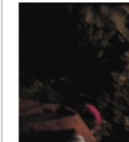


CRISPR technology leaps from lab to industry

Scientists launch company to develop the therapeutic potential of gene-snipping enzymes.

Helen Shen

03 December 2013



Precision gene editing paves way for transgenic monkeys

Despite political challenges, engineered primates could be better disease models than mice.

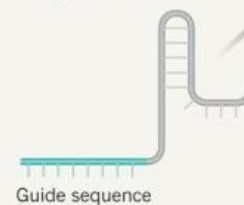
Helen Shen

06 November 2013

CRISPR CUTS

Scientists have developed a precise way to disable genes.

1 Engineer a section of RNA to target a specific region of DNA.

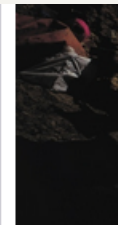
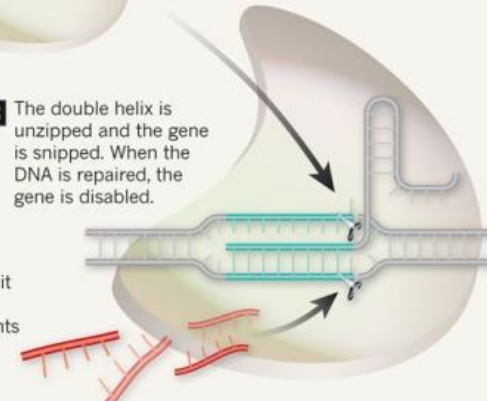


DNA-cutting enzyme

2 The RNA sequence, linked to a DNA-cutting enzyme, finds the right spot on the genome.

3 The double helix is unzipped and the gene is snipped. When the DNA is repaired, the gene is disabled.

4 While the DNA is broken, it is vulnerable to strategic insertions of small amounts of engineered DNA that could alter the gene.

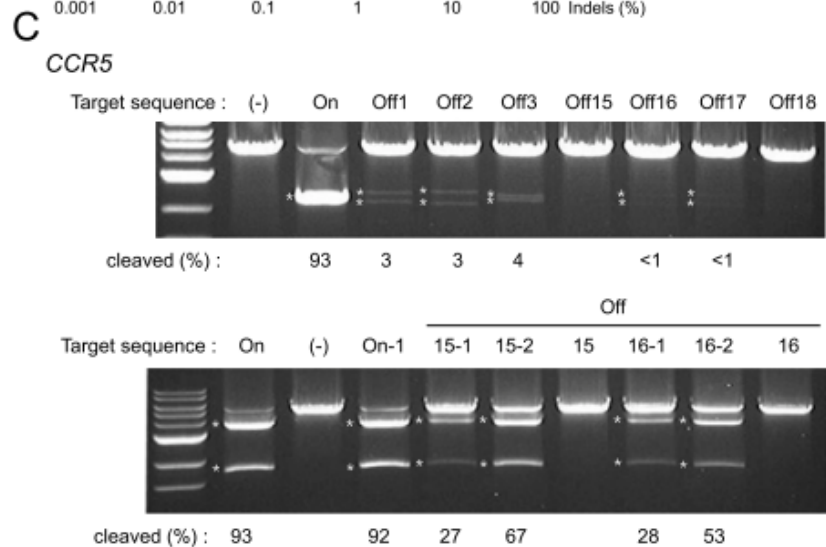
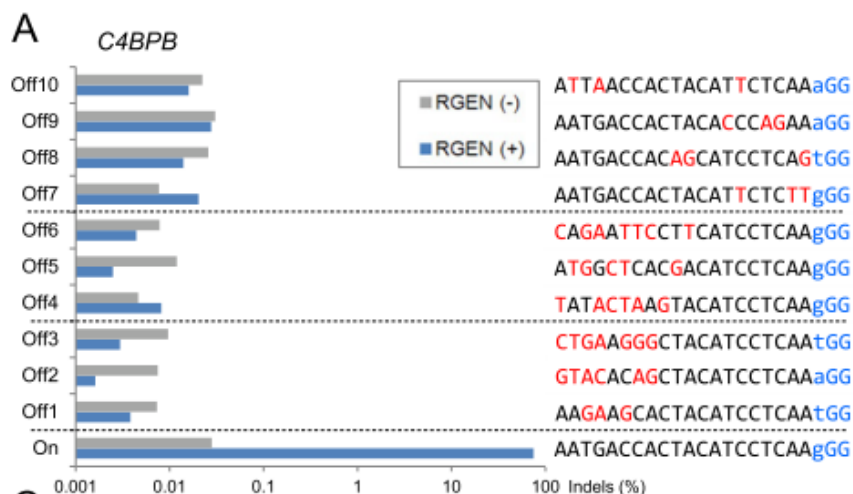


Ancient DNA

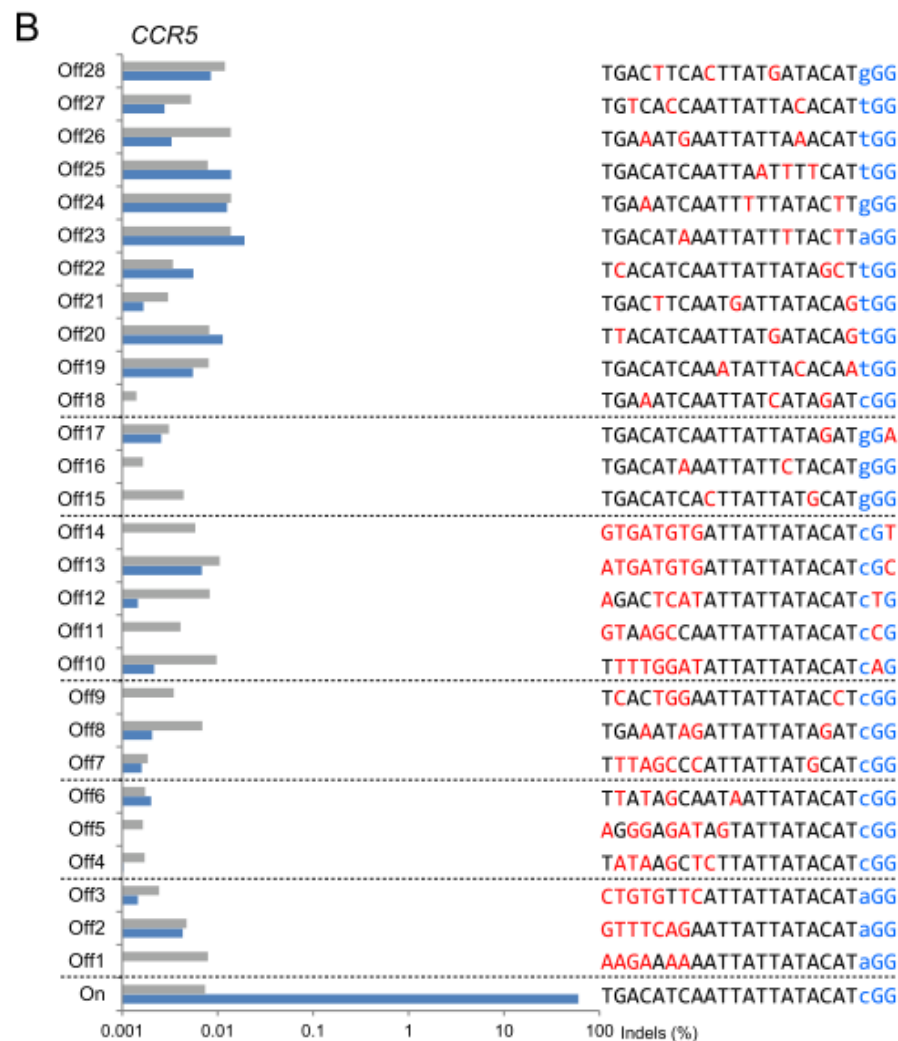


RGENs have recently been shown to support efficient genome engineering in human cells, zebrafish and bacteria ([Cho et al. 2013. Nature Biotech .](#), [Cong et al. 2013. Science](#) , [Hwang et al. 2013 Nature Biotech .](#), [Jiang et al. 2013 Nature Biotech .](#), [Mali et al. 2013. Science](#)).

In addition, the use of RGENs allows multiplexing of genome engineering processes (e.g., knockout of multiple genes in a cell at one time). RGEN applications will expand to various plant and animal cells/organisms soon.



On	TGACATCAATTATTATACATcGG	On-1	TGACATCAATTATTATACATgGG
Off15	TGACATCACTTATTATGCATgGG	Off16	TGACATAAATTATTCTACATgGG
Off15-1	TGACATCAATTATTATGCATgGG	Off16-1	TGACATCAATTATTCTACATgGG
Off15-2	TGACATCACTTATTATACATgGG	Off16-2	TGACATAAATTATTATACATgGG



dRGEN

SgRNA vector

Cas9 WT vector

Cas9 EGFP/Hygro and RFP/puro vector

Cas9 D10A vector

Surrogate reporter

aRGEN

SgRNA mRNA

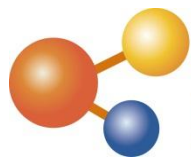
Cas9 mRNA

Recombinant Cas9 protein

Talen

Talen

Surrogate reporter



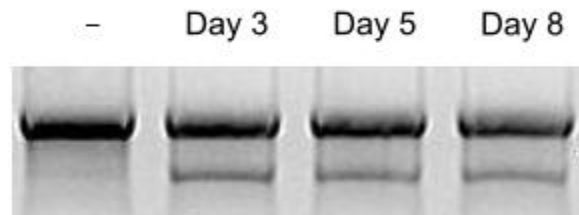
BIOTOOLS

- ✓ **Highly Efficient**
- ✓ **Highly Specific**
- ✓ **Multiplexing**
- ✓ **More options to use**
- ✓ ***in vivo* and *in vitro***

RGENs are not cytotoxic

Nature Biotechnology 31, 230 – 232 (2013) doi:10.1038/nbt.2507

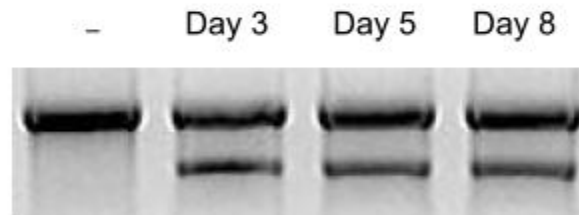
CCR5



Indels (%)

11 11 11

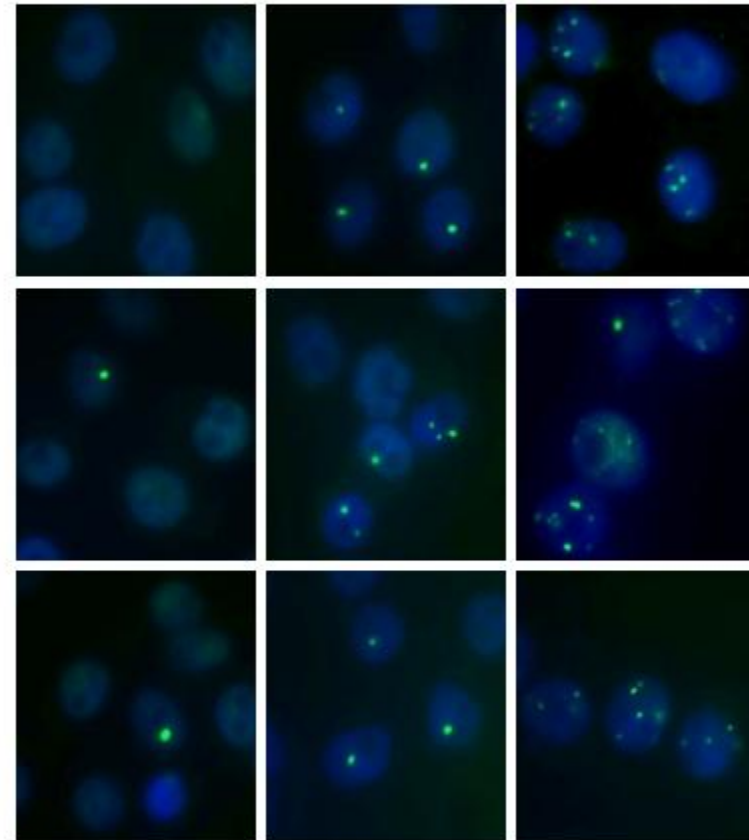
C4BPB



Indels (%)

20 19 18

No treatment RGEN Etoposide



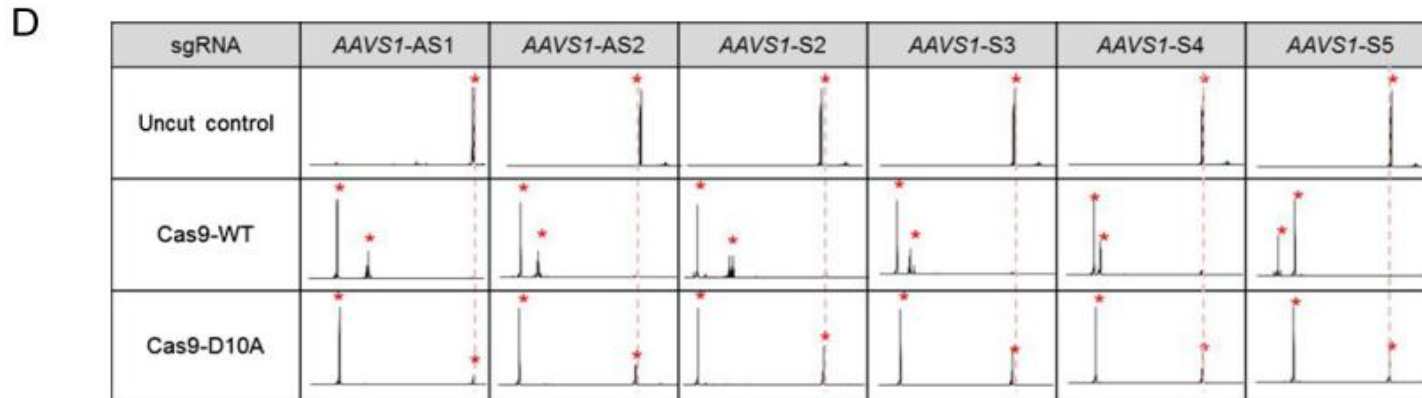
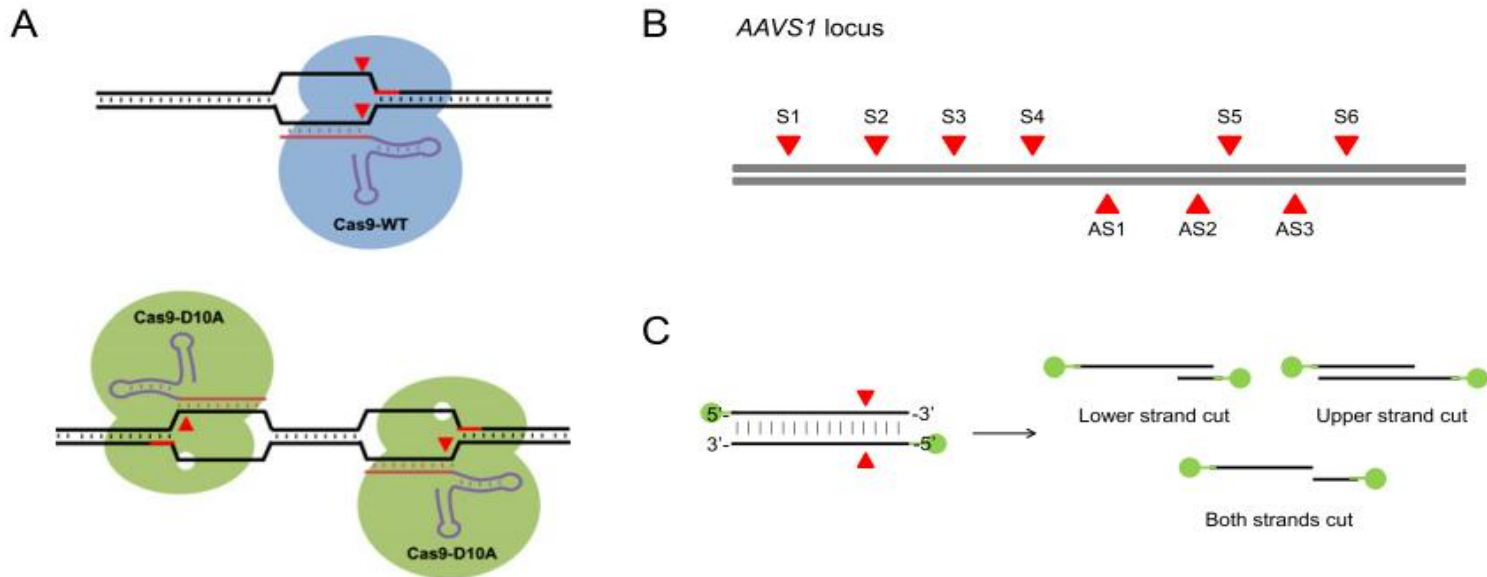
0.2 ± 0.1
(n = 51)

0.8 ± 0.1
(n = 66)

4.9 ± 0.5
(n = 59)

- RGEN-induced mutations are stably maintained.
- RGENs induce ~1 DSB per cell.

Unlike Cas9 nucleases that cleaved both strands of DNA substrates, Cas9 nickases composed of guide RNA and a mutant form of Cas9 in which a catalytic aspartate residue is changed to an alanine (D10A Cas9) cleaved only one strand, producing site-specific nicks



- **2-plasmid system vs 1-plasmid system for dRGEN**

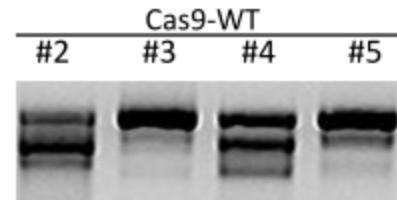
Unlike plasmids for dRGEN from Systembio is all-in-1 vector system which contains Cas9 and guide RNA expression cassette in one plasmid, our expression system is composed of separate expression vectors for Cas9 protein and guide RNA.

We see that the high level of sgRNA expression in cell is critical for efficient genome engineering. For all-in-1 vector system, the ratio of expression cassette for sgRNA and Cas9 protein will be always 1:1. However, for 2-plasmids system allows varying the ratio of each expression cassette and will be able to optimize the genome engineering efficiency in various cell lines.

RGEN transfection in HeLa cells: T7E1 results

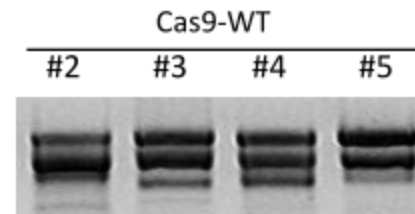
Confidential Information

Low Dose
Cas9 800ng
sgRNA 200ng



Indels (%)	48	3.5	37	20
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High Dose
Cas9 200ng
sgRNA 800ng

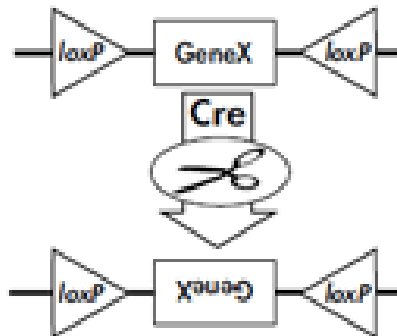


Indels (%)	56	50	44	34
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- sgRNA plasmids but not Cas9 plasmid are limiting.

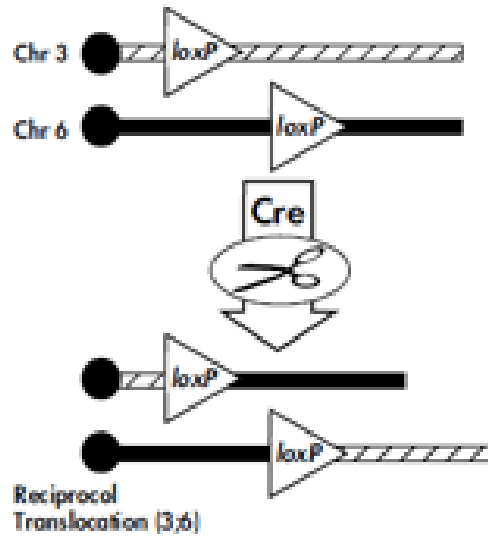
A

Inversion



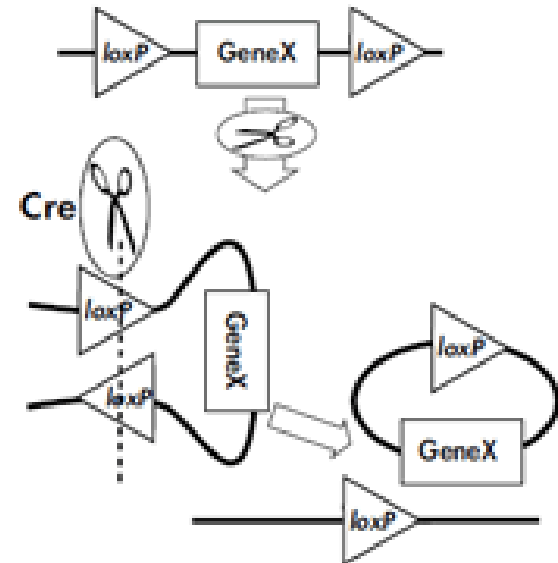
B

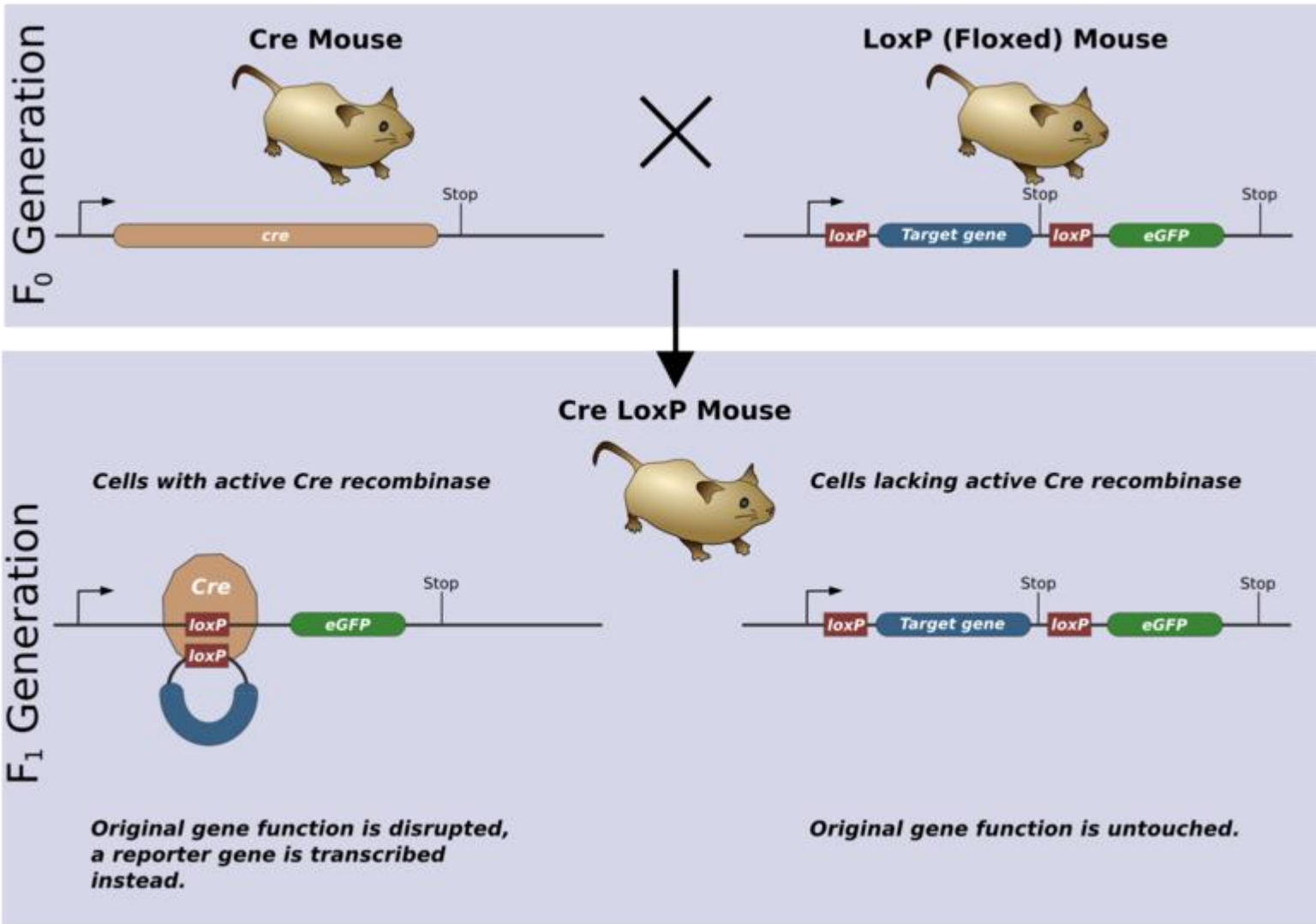
Translocation



C

Deletion





One-Step Generation of Mice Carrying Reporter and Conditional Alleles by CRISPR/Cas-Mediated Genome Engineering

