Strategies For Effective CRISPR-Mediated Gene Modification

December 3, 2018

Presenter:

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Senior Application Scientist
GeneCopoeia, Inc.

Goals of this presentation

- Help guide you though basic considerations, steps, and potential problems for using CRISPR to modify genes in cell lines
- Present CRISPR-based tools from GeneCopoeia for modifying genes in cell lines
- Q & A

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GeneCopoeia Products and Services

Functional Genomics & Cell Biology

Clones	*Viral systems*	*Stable Cell Lines*	*Kits & reagents*	*Fluorescent detection*
ORF	Lentivirus	CRISPR-Cas9 stable cell lines	Transfection	Cell function assays
Promoter	AAV	Labeled cancer cell lines	Luciferase	Nucleic acid detection
miRNA		Cancer biomarker mutant cell lines	FISH probes	Cell structure probes
CRISPR			Indel detection	Fluorescent dyes
shRNA			Cloning	

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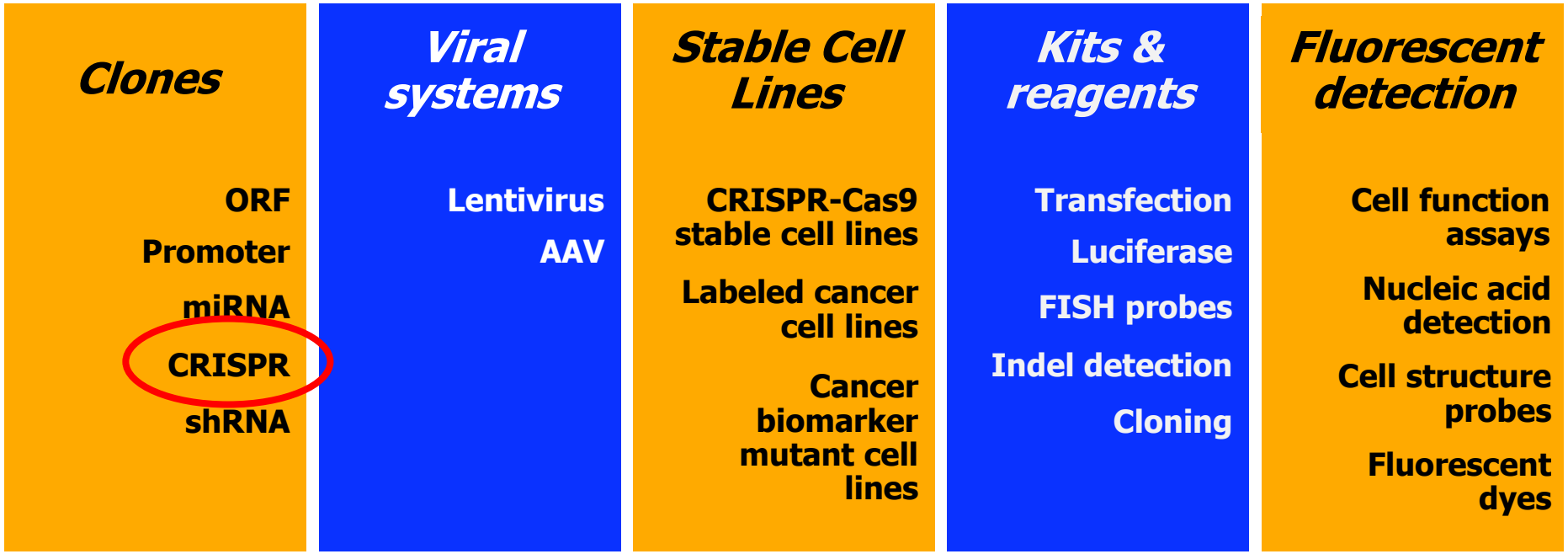

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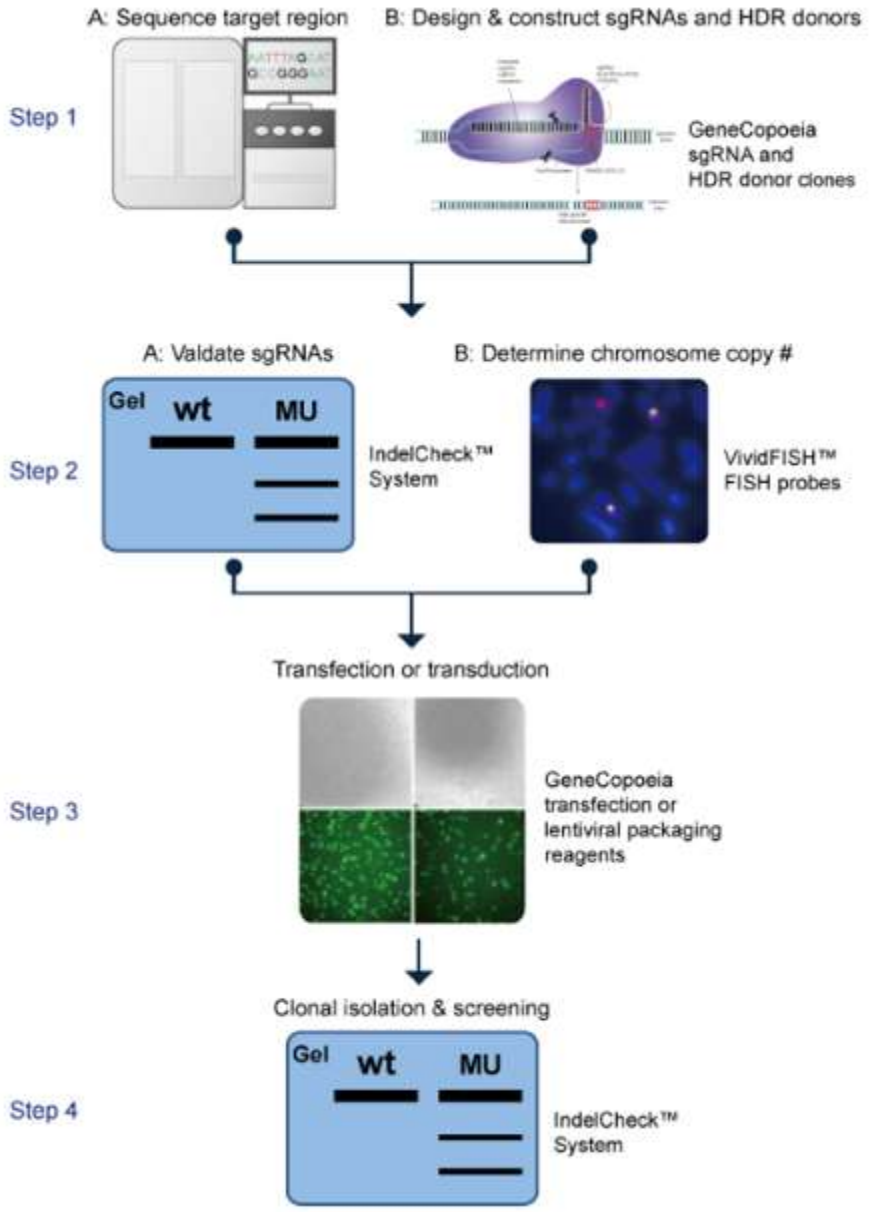
GeneCopoeia CRISPR products for cell lines



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Outline

- CRISPR technologies & applications
- Strategy considerations
- Delivery methods
- Preparation
- Screening
- Things to look out for

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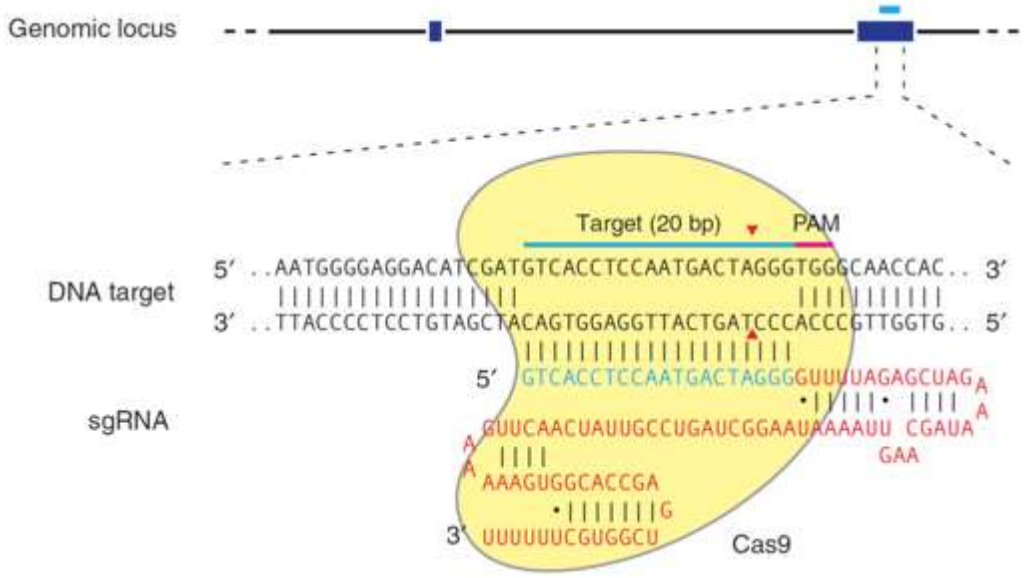
CRISPR-Cas9 genome editing technology

CRISPR-Cas9: RNA-guided endonuclease



Ran, et al. (2013). Nature Protocols 8, 2281

- 17-20 nt single guide RNA (sgRNA) guides Cas9 nuclease to target site.
- Requires NGG “PAM” site immediately downstream of sgRNA target sequence.
- Cas9-sgRNA complex makes DSB 3 nt upstream of PAM.

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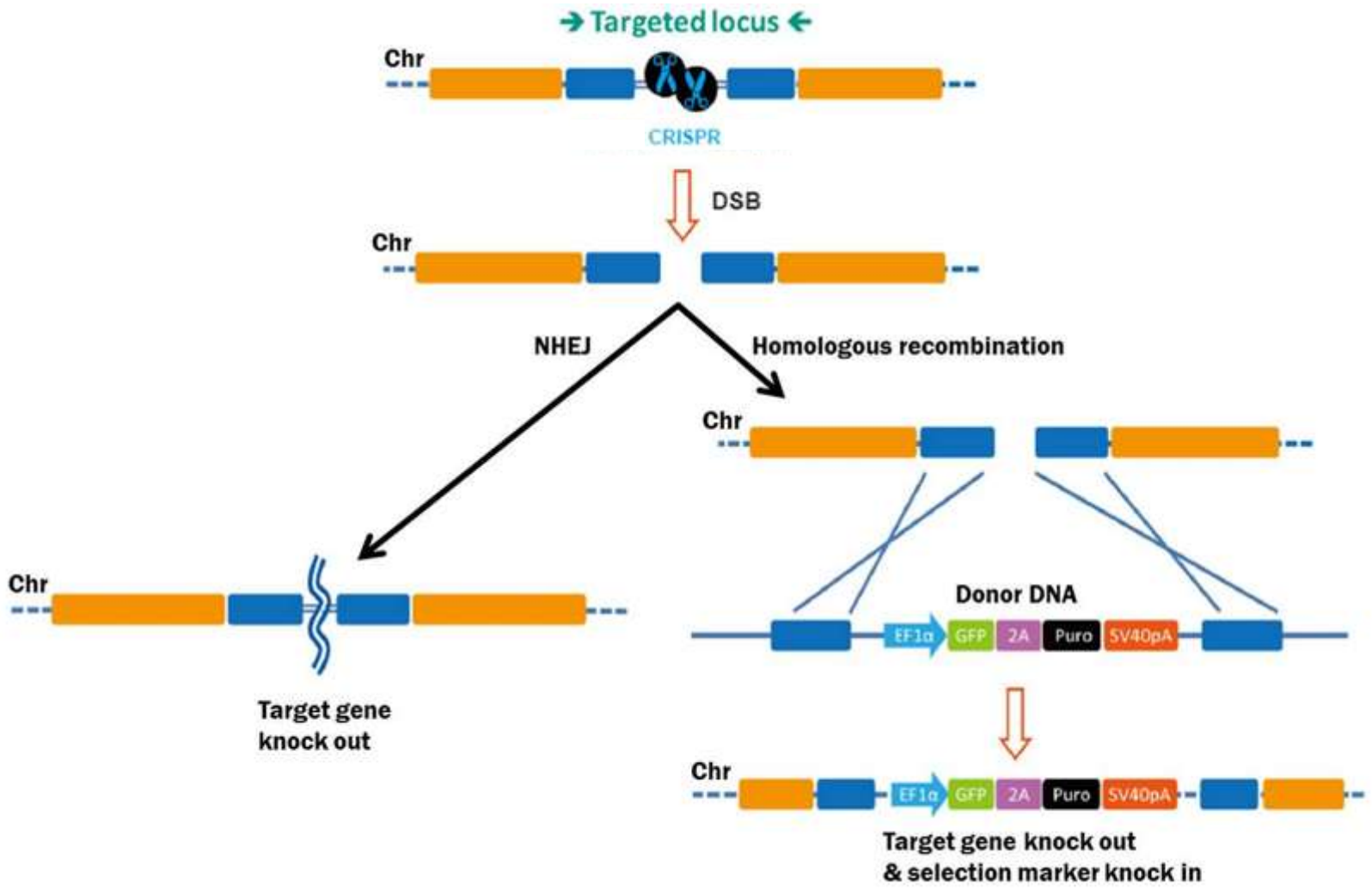
Targeted DNA editing by DSB induction



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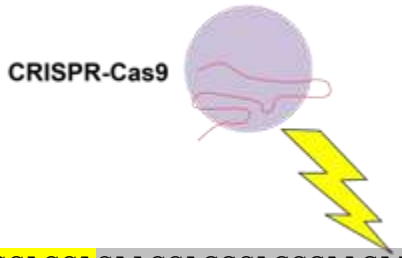
CRISPR-Cas9 genome editing technology

Technological variants

Technology	Description	PAM	Applications
SpCas9	Unmodified Cas9 from *Streptococcus pyogenes*	NGG	Gene knockout, knock-in, tagging, mutagenesis
SpCas9-NG	Mutagenized SpCas9 with relaxed (NG) PAM requirement	NG	Gene knockout, knock-in, tagging, mutagenesis
Cas9-HF, eSpCas9	Mutagenized, high-fidelity SpCas9 for reduced off-targeting	NGG	Gene knockout, knock-in, tagging, mutagenesis
HiFi Cas9	Mutagenized, high-fidelity SpCas9 for reduced off-targeting	NGG	Gene knockout, knock-in, tagging, mutagenesis
Cpf1 (Cas12a)	Nucleases from *Acidaminococcus sp. BV3L6* or *Lachnospiraceae bacterium ND2006*. Smaller than SpCas9, no requirement for tracrRNA, staggered DSB, lower off-targeting.	T/CTTN (5')	Gene knockout, knock-in, tagging, mutagenesis
SaCas9	Cas9 from *Staphylococcus aureus*. Smaller than SpCas9, usually used with adenoassociated virus (AAV)	NNGRRT	Gene knockout, knock-in, tagging, mutagenesis
dCas9	Nuclease-dead Cas9 for tethering to effectors	NGG	Gene mutagenesis, chromatin modification, transcriptional activation/repression
Cas13	Nuclease from *Leptotrichia wadei*. Targets RNA. 20-28 nt spacer	None	RNA knockdown or mutagenesis

CRISPR-Cas9 applications

Gene knockout-frameshift



ATGACCTCAACCGGCCAGGATTCCACCACAACCAGGCAGCGAAGAAGTAGGCAGAACCCCCAGTCGCCCCCTCAGGACTCCAGTGTCACTTCGgtgaggccctagacccgccctgatgaggg

ATGACCTCAACCGGCCAGGATTCCACCACAACCAGGCAGCGAAGAAGTAGGCAGAACCCCCAGTCGCCCCCTCAGGACTCCAGTGTCACTTCGgtgaggccctagacccgccctgatgaggg

→ (Multiple of 3) +/- 1 indel-frameshift

ATGACCTCAACCGGCCAGGATTCCACCACAACCAGGCAGCGAAGAAGTAGGCAGAACCCCCAGTCGCCCCCTCAGGACTCCAGTGTCACTTCGgtgaggccctagacccgccctgatgaggg

→ (Multiple of 3) +/- 2 indel-frameshift

ATGACCTCAACCGGCCAGGATTCCACCACAACCAGGCAGCGAAGAAGTAGGCAGAACCCCCAGTCGCCCCCTCAGGACTCCAGTGTCACTTCGgtgaggccctagacccgccctgatgaggg

→ Multiple of 3 indel-in frame

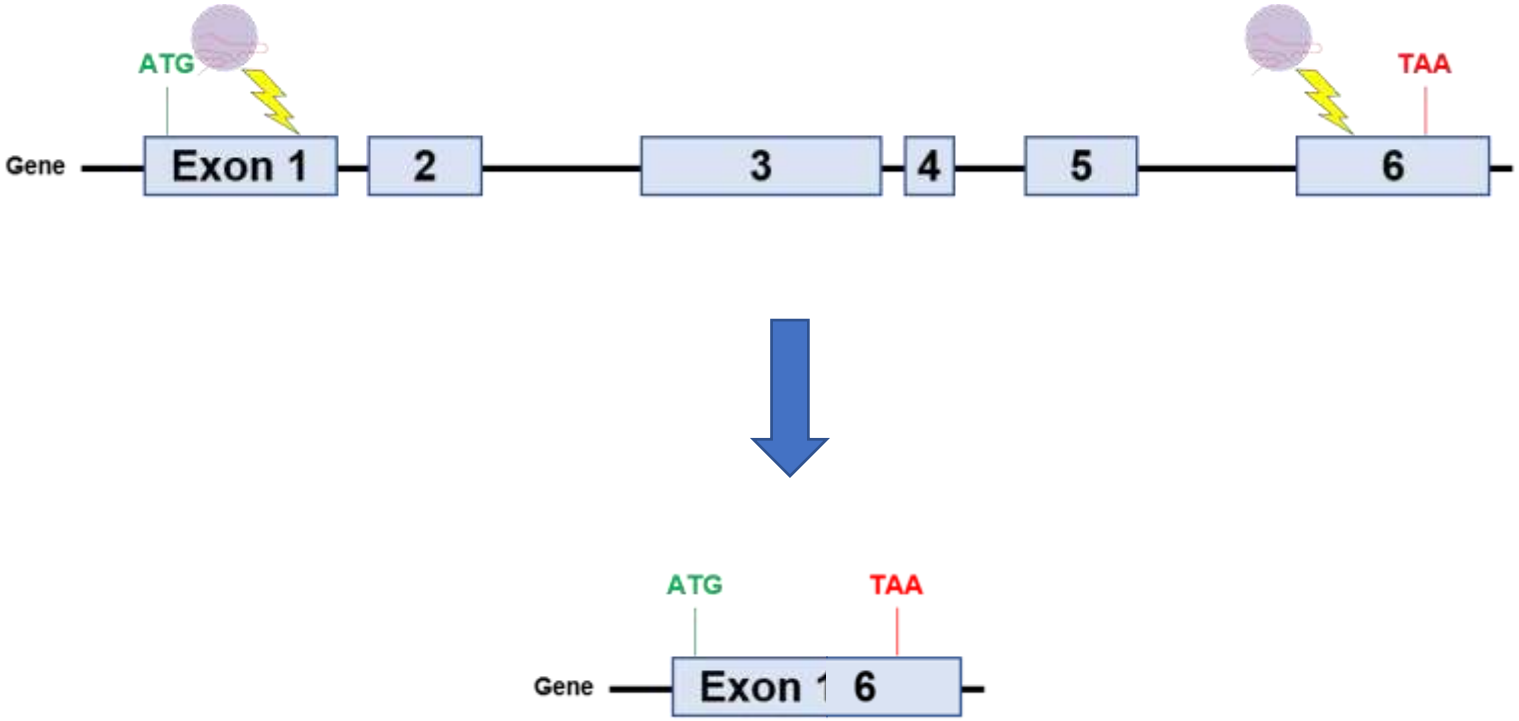
CRISPR-Cas9 applications

Gene knockout-frameshift

- NHEJ-mediated indels occur stochastically, with unpredictable lengths
- In general, 2/3 of indels will cause a frameshift. In most cases this will knock the gene out.
- In general, 1/3 of indels will be in-frame. This may or may not knock the gene out. It could also lead to the production of a protein with a new or altered function.
- Typically, each chromosome will contain a different indel (or no indel). So, only 4/9 of diploid cells with 2 indels will contain 2 frameshift indels.

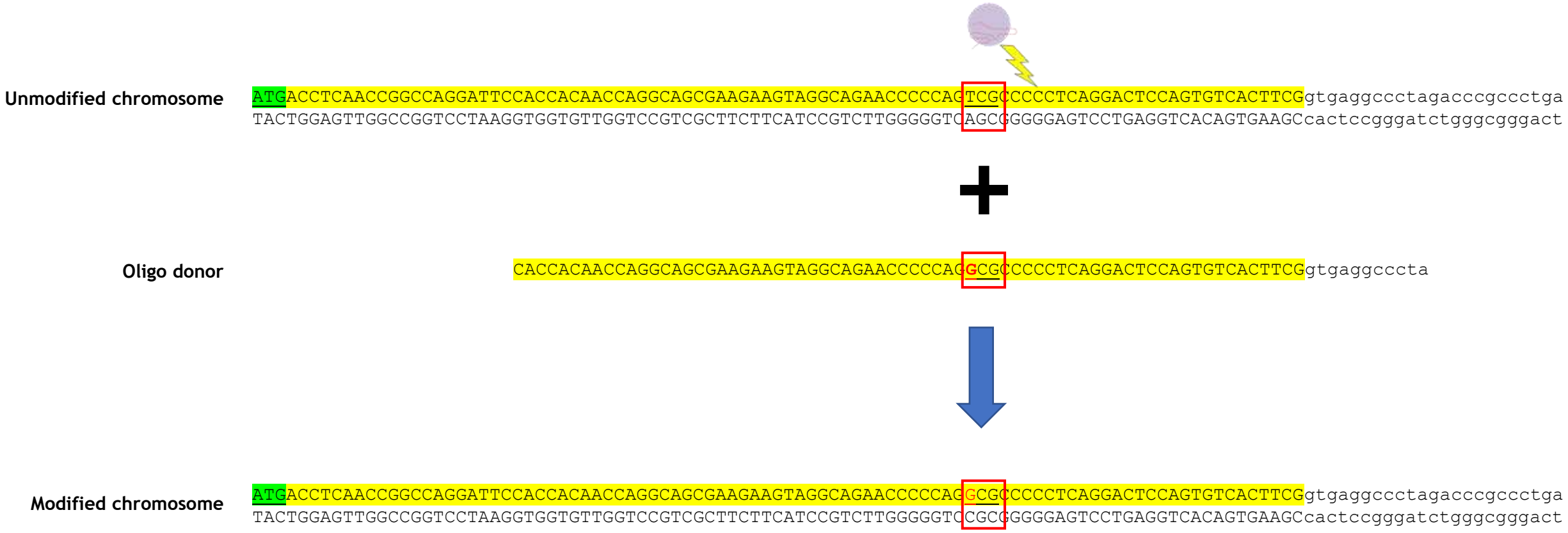
CRISPR-Cas9 applications

Gene knockout-large deletion



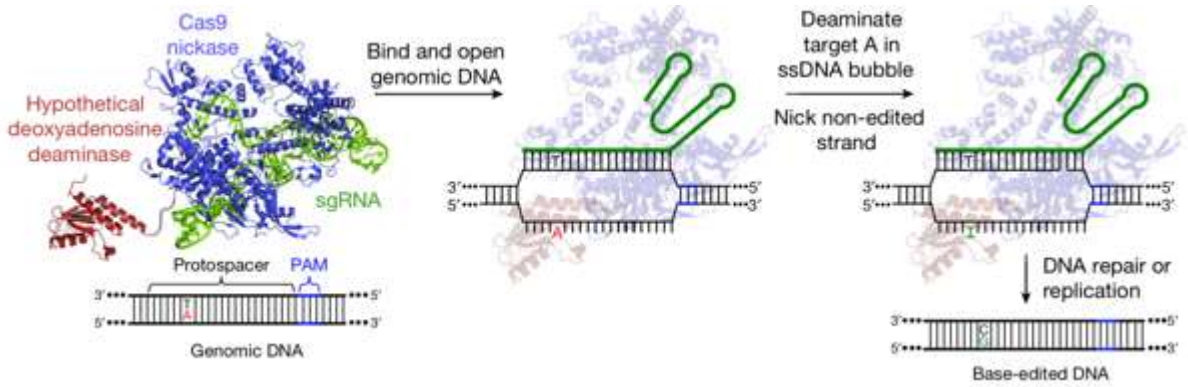
CRISPR-Cas9 applications

Gene mutagenesis-HDR



CRISPR-Cas9 applications

Gene mutagenesis-Base editors



Gaudelli, et al. (2017). Nature 551, 464

- Cas9 nickase fused to cytidine and adenine deminases
- Permit changing of bases without DSBs
- Can change C to T, G to A, A to G, and T to C

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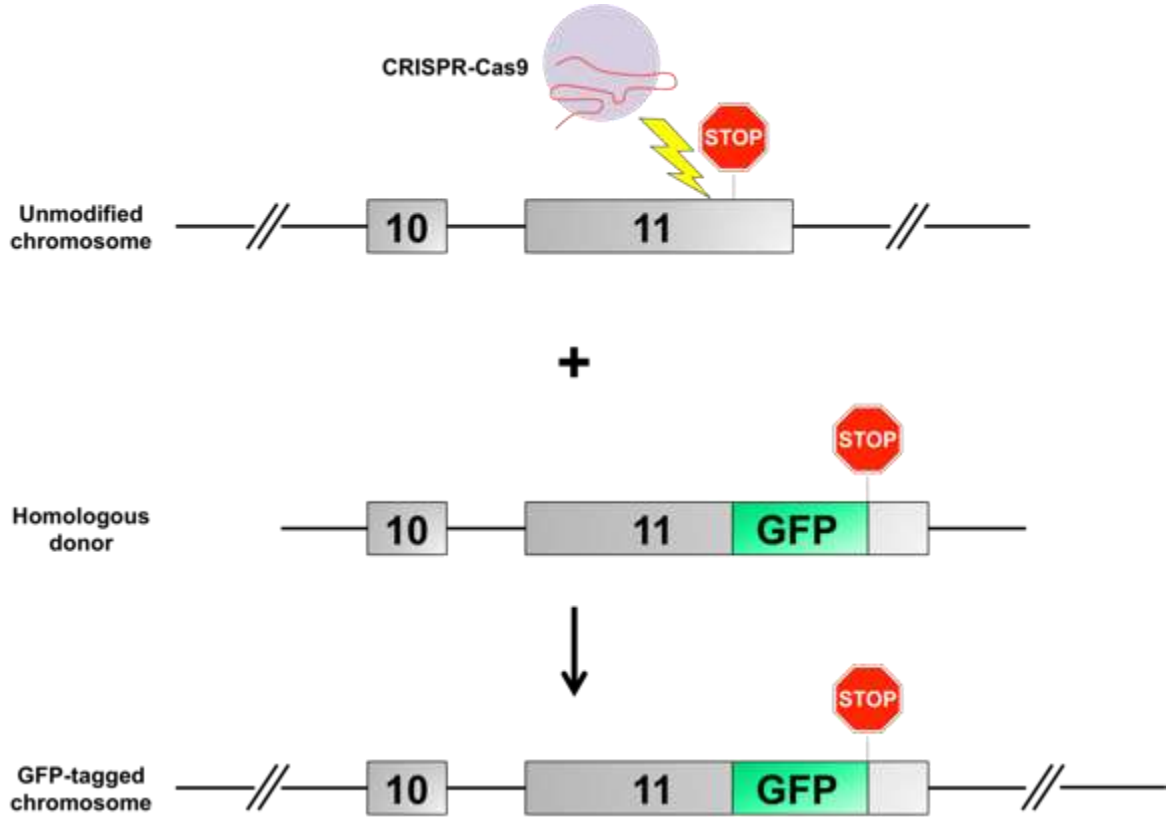
CRISPR-Cas9 applications

Gene tagging



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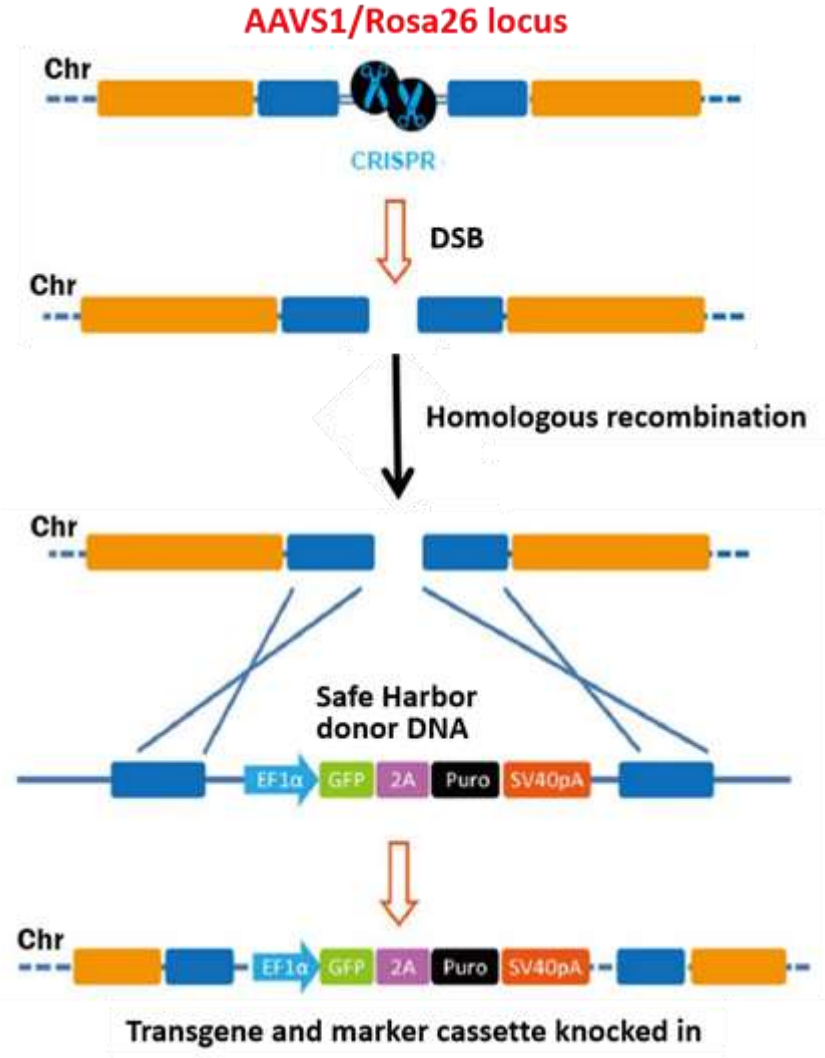
CRISPR-Cas9 applications

Gene knock-in (transgenesis)



Features

- Human AAVS1 & mouse Rosa26 sites ensure transcription-competency of the transgenes & present no known adverse effects on cells
- Safe Harbor integration provides low copy number of transgene & close to physiological-level expression.

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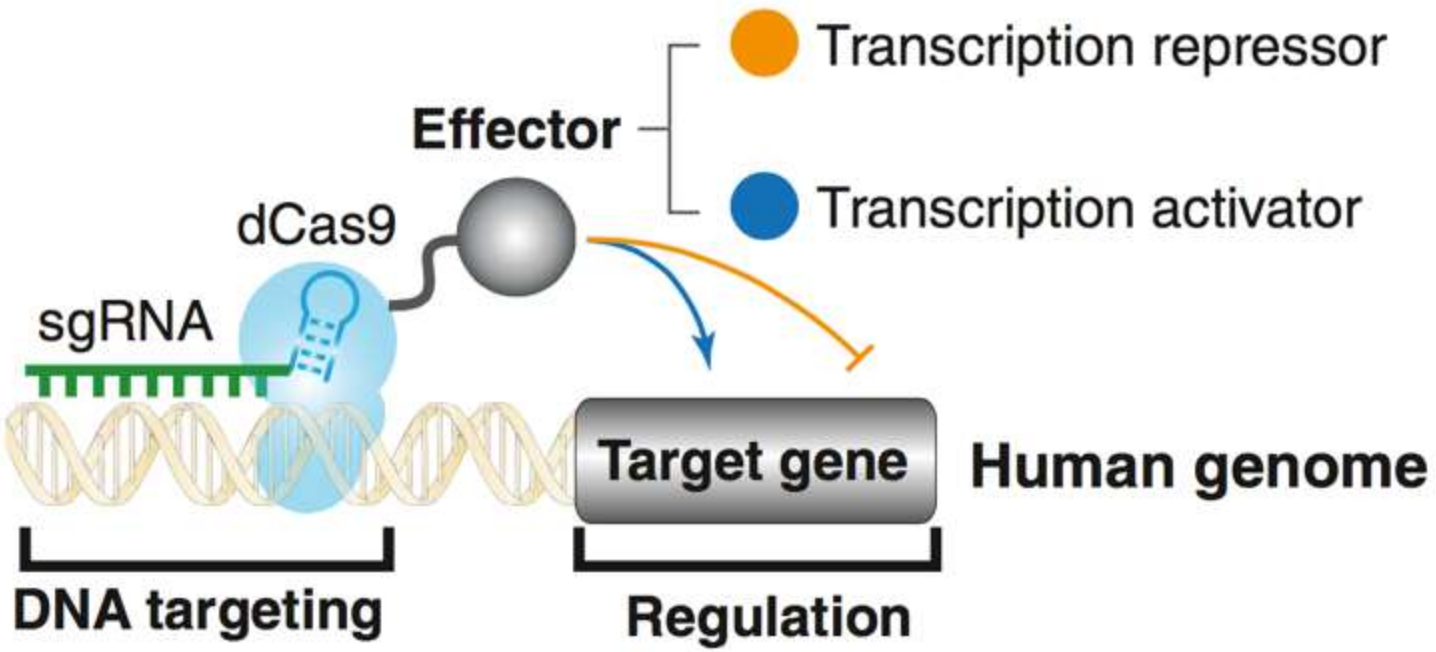
CRISPR-Cas9 applications

Gene activation or repression



Gilbert, et al. (2013). Cell 154, 442

Outline

- CRISPR technologies & applications
- Strategy considerations
- Delivery methods
- Preparation
- Screening
- Things to look out for

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CRISPR-Cas9 genome editing technology

Considerations before you start

- What type of application are you doing (gene knockout, mutagenesis, etc.)? This decision can effect the efficiency and the degree of difficulty screening for the modification.
- What type of cell line are you using (primary, immortalized)?
- Can the cell line be transfected? If it does not transfect well, or not at all, then you might need to use lentivirus for delivery. However, lentivirus cannot be used for HDR applications.
- Other cell line considerations: Growth rate, growth characteristics (adherent vs. suspension growth), target gene copy number.

Outline

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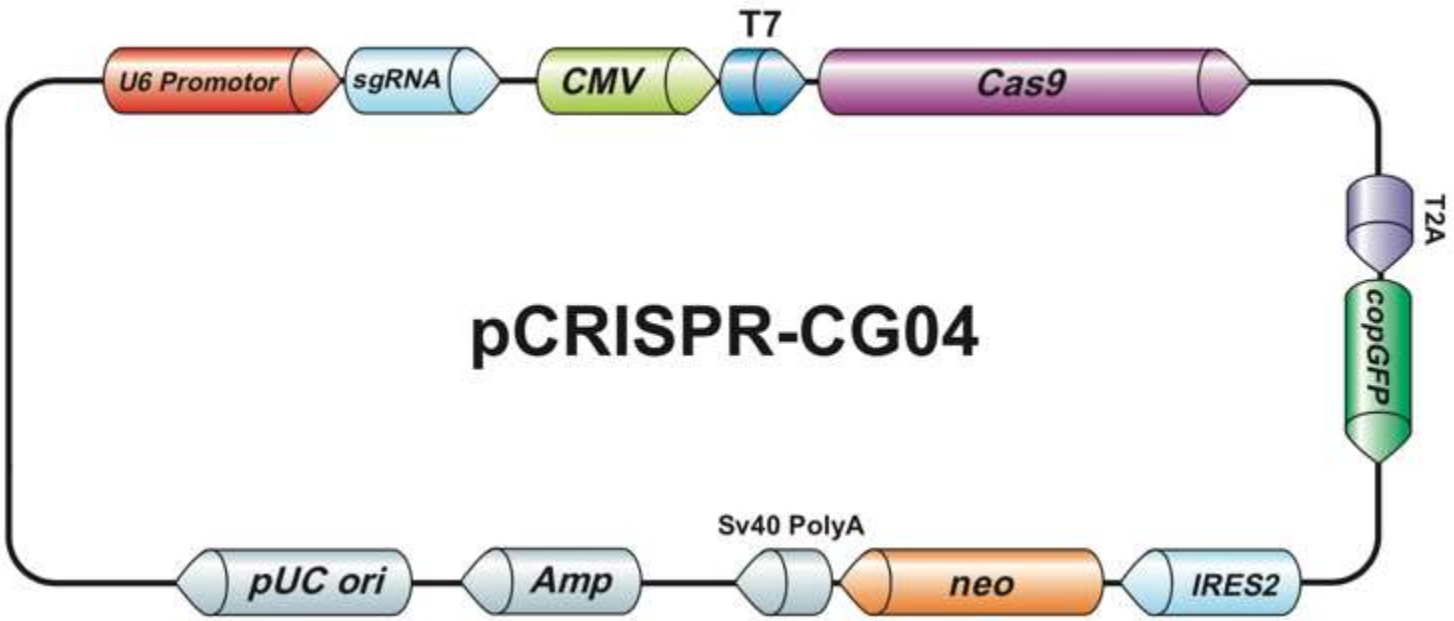
Delivery method 1: Cas9/sgRNA plasmid DNA



- GeneCopoeia Cas9 + sgRNA "All-in-one" plasmid
- Fluorescent reporter allows for sorting
- Selection marker can be used for stable integration
- Cas9 needs to be transcribed and translated

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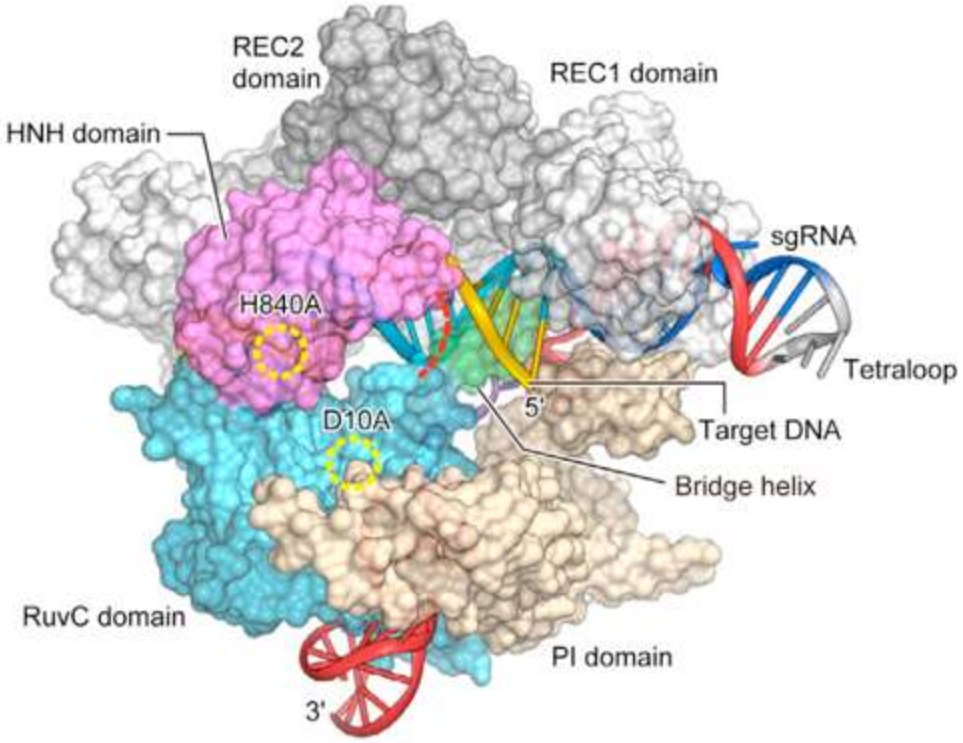
Delivery method 2: Cas9 mRNA + sgRNA

- Co-transfect *in vitro* transcribed Cas9 mRNA with sgRNA
- sgRNA can either be encoded on plasmid, *in vitro* transcribed, or synthetic
- Advantage: Plasmid-free, so cannot integrate
- Cas9 still needs to be translated

CRISPR-Cas9 genome editing technology

Delivery method 3: Cas9 ribonucleoprotein (RNP)



Nishimasu, et al. (2014). Cell 156, 935

- Pre-form complex of Cas9 protein and sgRNA in vitro to form RNP
- sgRNA can be either chemically synthesized or *in vitro* transcribed
- Transfect cells with RNP complex using either lipid transfection reagent or electroporation
- Efficiency of indel formation is generally similar to that of plasmid transfection (Kim, et al., 2014. Genome Research)
- Lower off-targeting compared with plasmid transfection (Kim, et al., 2014. Genome Research)
- Does not integrate, unlike plasmids

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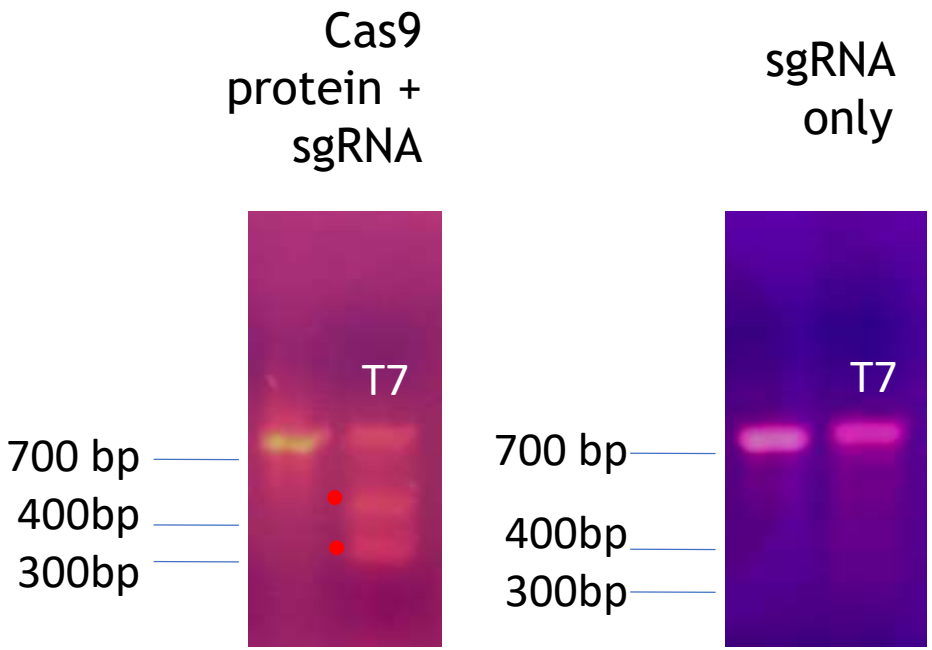
Coming soon! GeneHero™ Cas9 nuclease



- Transfection of HEK293 cells using EndoFectin™ Max

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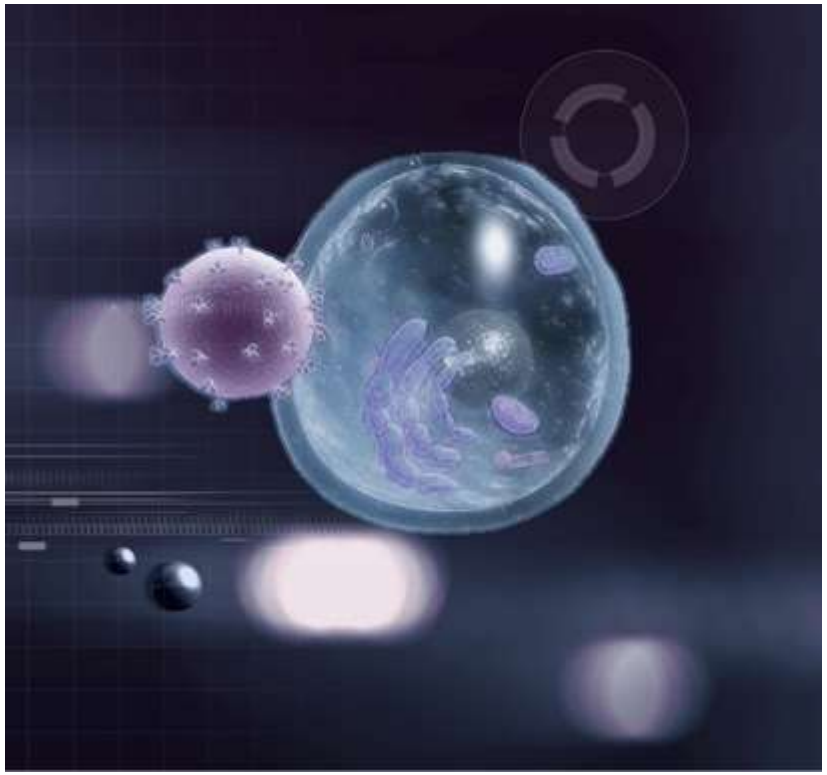
Delivery method 4: Lentivirus



Why lentivirus?

- DNA transfection not always possible or practical. Some cell lines difficult or impossible to transfect.
- Most mammalian cells support infection by engineered lentivirus

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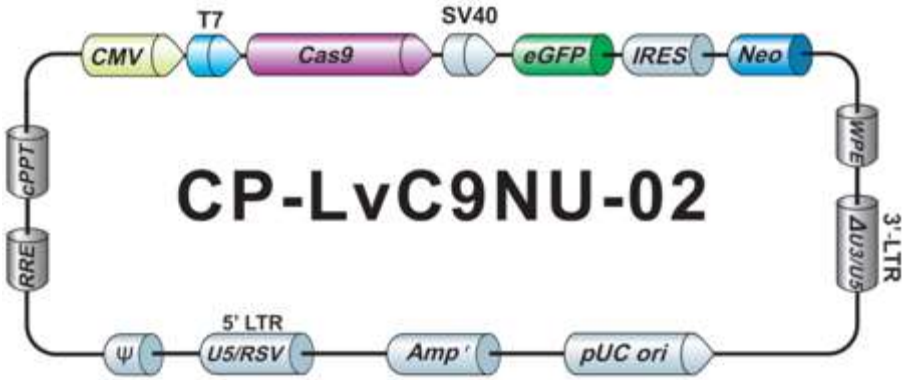
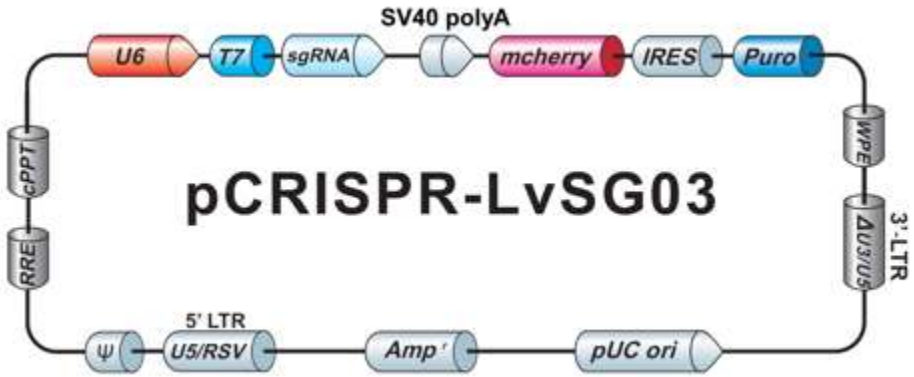
Delivery method 4: Lentivirus





Features

- 2-component system: 1) Cas9 lentivirus + sgRNA lentivirus
- Cannot be used for HDR
- Leads to stable selection
- GeneCopoeia provides either plasmids for do-it-yourself packaging or ready-to-use particles
- Recommend that you first establish or obtain Cas9-stable cell line

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CRISPR-Cas9 genome editing technology

GeneHero™ Cas9 stable cell lines

Features

- Cell lines with Cas9 stably integrated in the genome
- >70 pre-made cell lines available in human, mouse, and rat cell lines
- Functionally validated for Cas9 activity
- Ideal for lentiviral CRISPR applications

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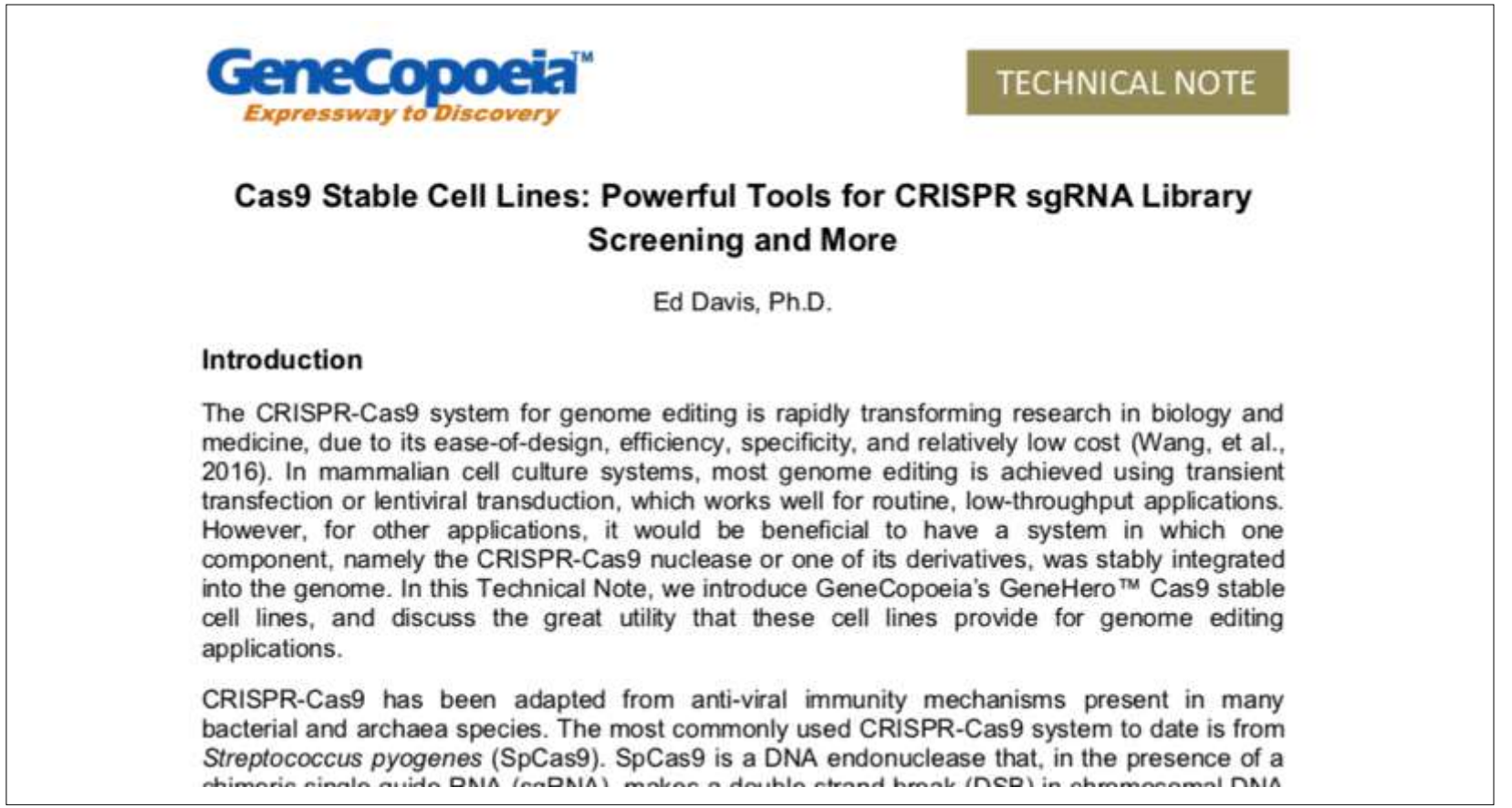
CRISPR-Cas9 genome editing technology

GeneCopoeia Technical Note: Cas9 stable cell lines



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TECHNICAL NOTE

Cas9 Stable Cell Lines: Powerful Tools for CRISPR sgRNA Library Screening and More

Ed Davis, Ph.D.

Introduction

The CRISPR-Cas9 system for genome editing is rapidly transforming research in biology and medicine, due to its ease-of-design, efficiency, specificity, and relatively low cost (Wang, et al., 2016). In mammalian cell culture systems, most genome editing is achieved using transient transfection or lentiviral transduction, which works well for routine, low-throughput applications. However, for other applications, it would be beneficial to have a system in which one component, namely the CRISPR-Cas9 nuclease or one of its derivatives, was stably integrated into the genome. In this Technical Note, we introduce GeneCopoeia's GeneHero™ Cas9 stable cell lines, and discuss the great utility that these cell lines provide for genome editing applications.

CRISPR-Cas9 has been adapted from anti-viral immunity mechanisms present in many bacterial and archaea species. The most commonly used CRISPR-Cas9 system to date is from *Streptococcus pyogenes* (SpCas9). SpCas9 is a DNA endonuclease that, in the presence of a

Download from:

https://www.genecopoeia.com/wp-content/uploads/2018/11/Cas9-Stable-Cell-Lines.-Powerful-Tools-for-CRISPR-sgRNA-Library-Screening-and-More.pdf

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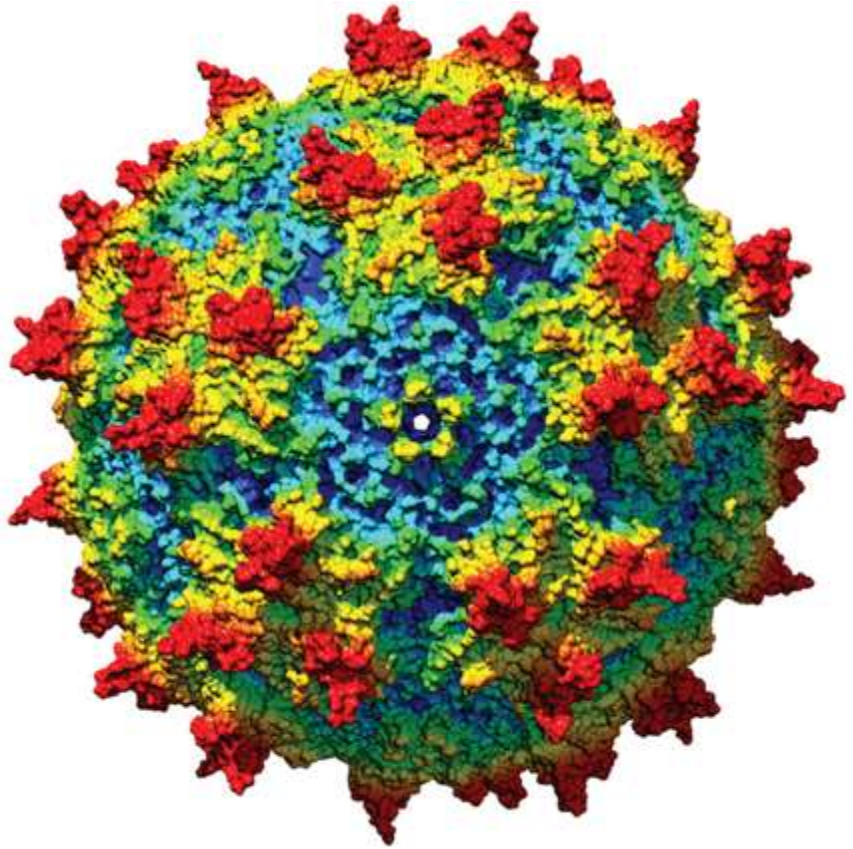
Delivery method 5: Adenoassociated virus (AAV)



- Identified as co-isolate of adenovirus (Ad)
- Single stranded DNA genome of 4.7 kb
- Limited genome size favors use of smaller Cas9 (e.g. SaCas9)
- Engineered AAV does not integrate into genomic DNA
- Infects dividing & non-dividing cells
- More frequently used *in vivo*

Outline

- CRISPR technologies & applications
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- **Preparation**
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CRISPR-Cas9 genome editing technology

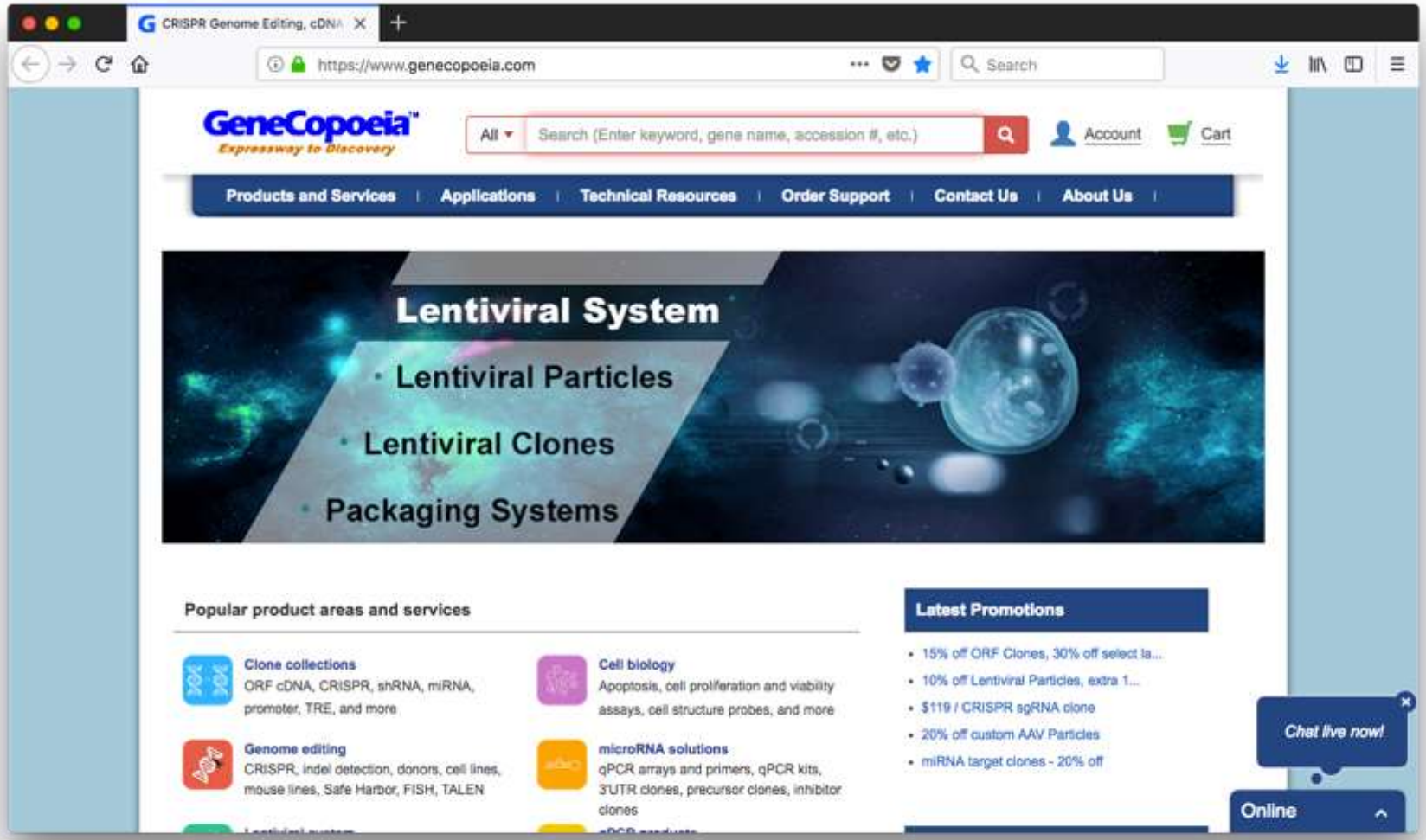
Workflow: Preparation

- Step 1: Choose your application (gene knockout, mutagenesis, etc.) This decision can affect the efficiency and the degree of difficulty in screening for modification.

- Use GeneCopoeia's website to choose targets for knockout, or contact us for custom sgRNA design and donor design, if applicable

CRISPR-Cas9 genome editing technology

Workflow: Preparation



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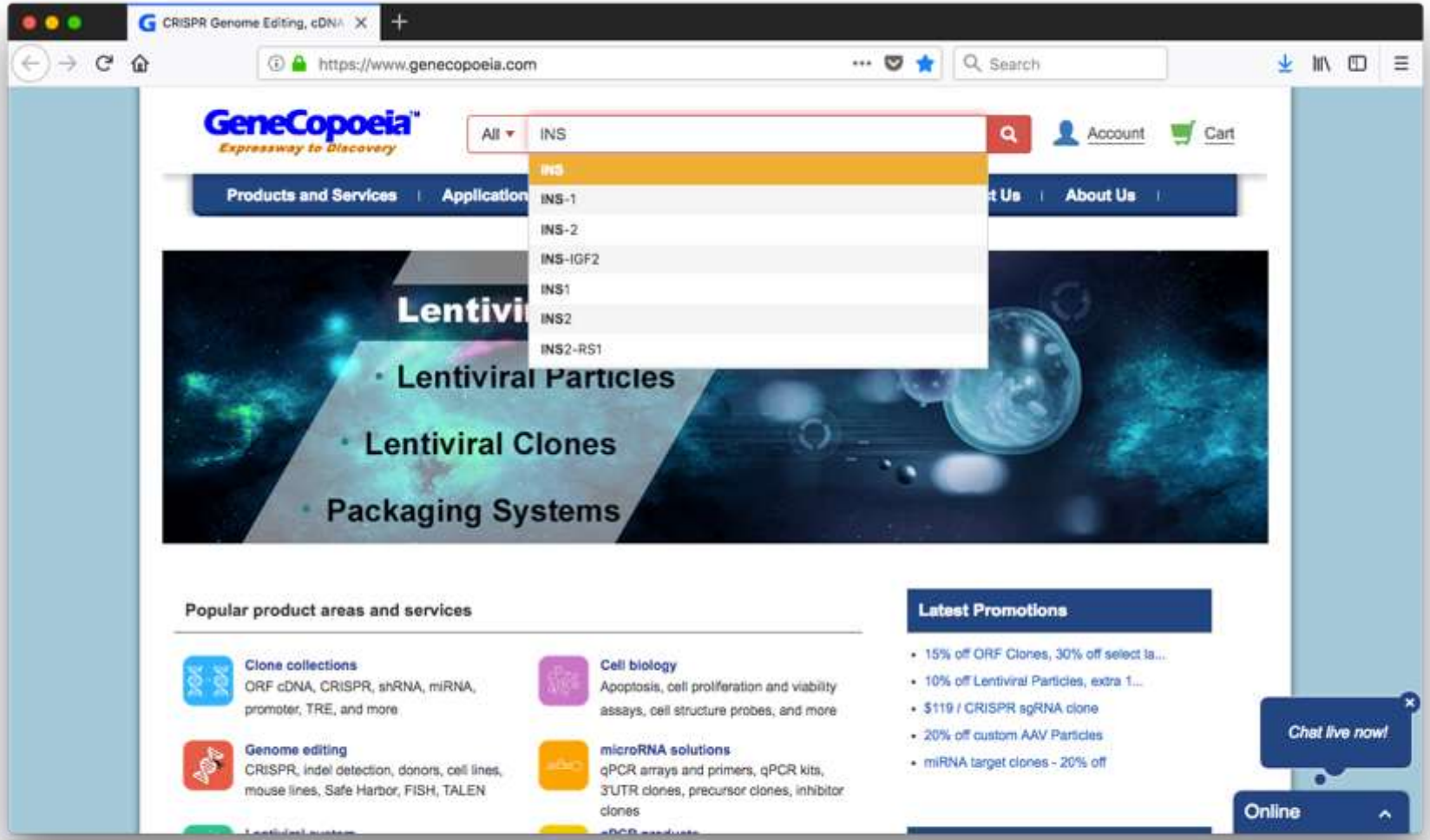
Workflow: Preparation



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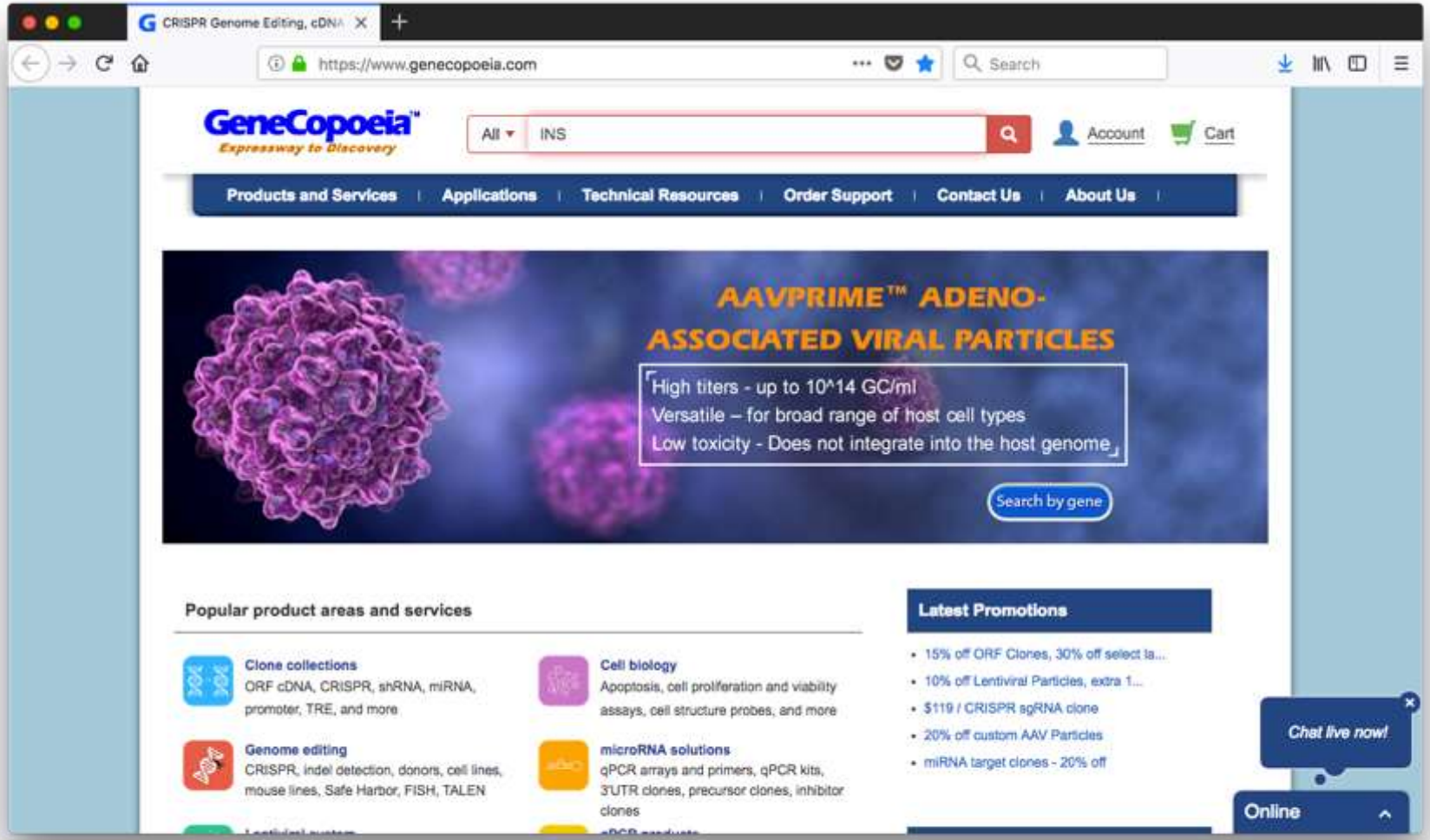
CRISPR-Cas9 genome editing technology

Workflow: Preparation



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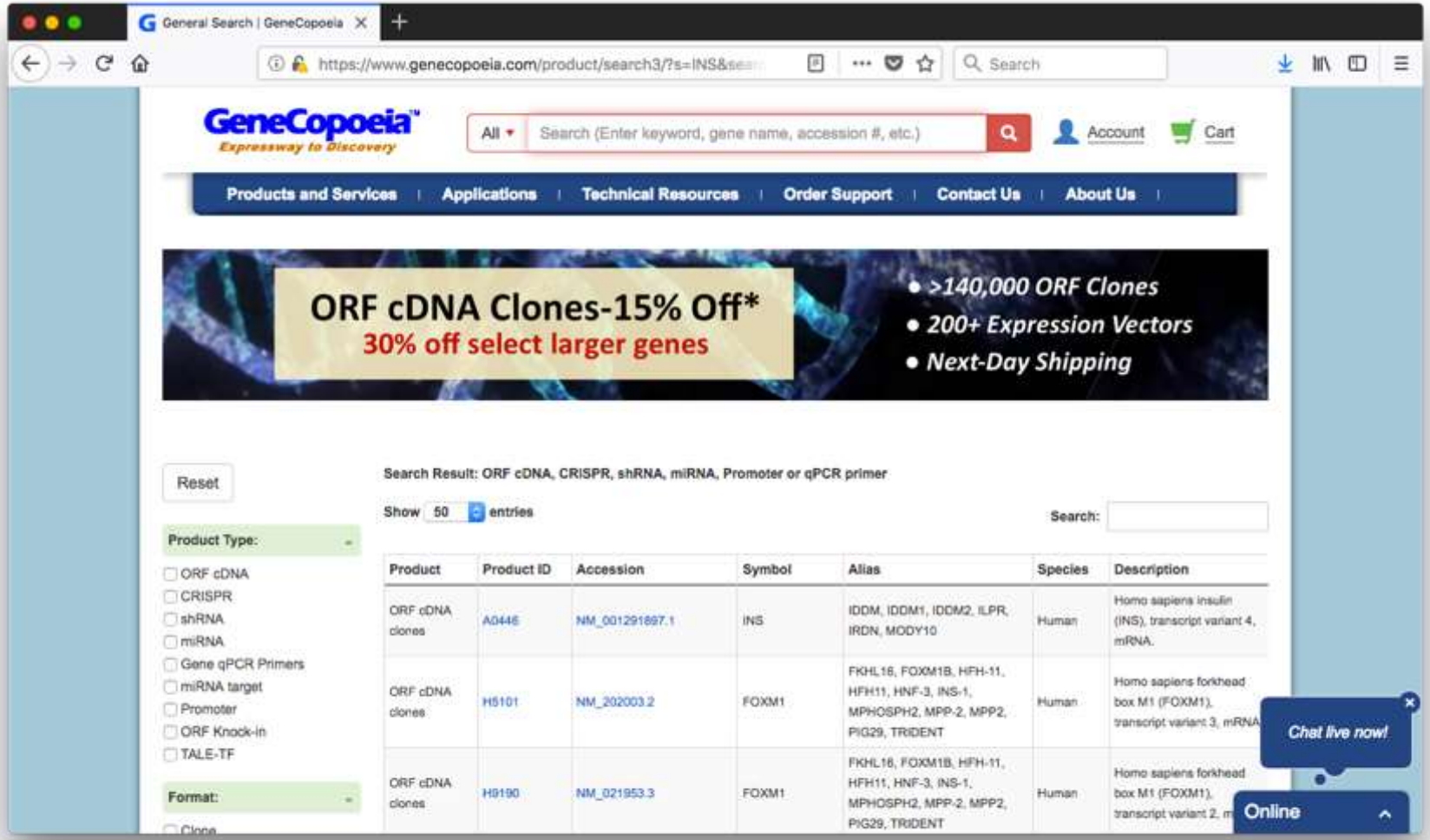
CRISPR-Cas9 genome editing technology

Workflow: Preparation



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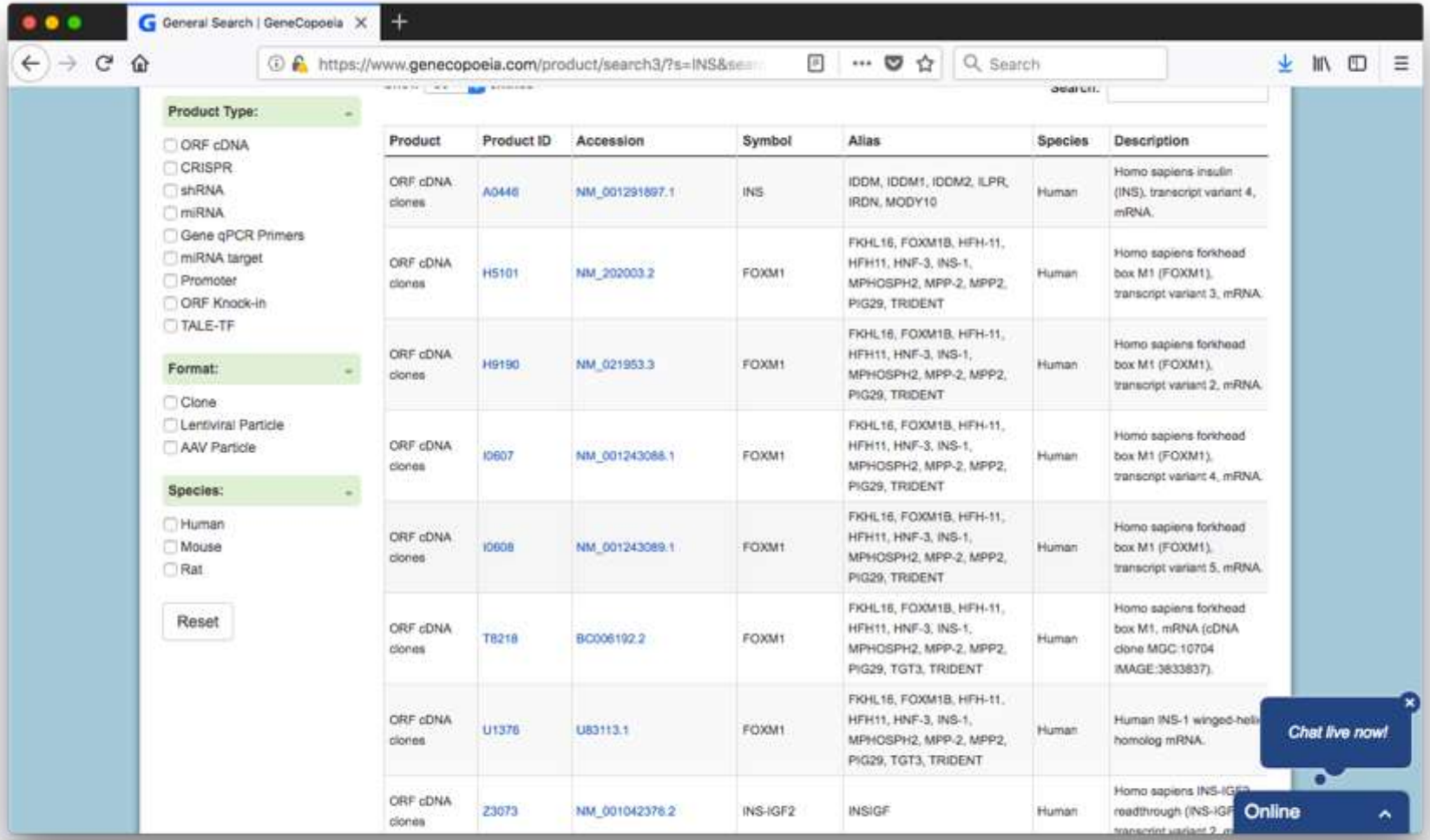
CRISPR-Cas9 genome editing technology

Workflow: Preparation



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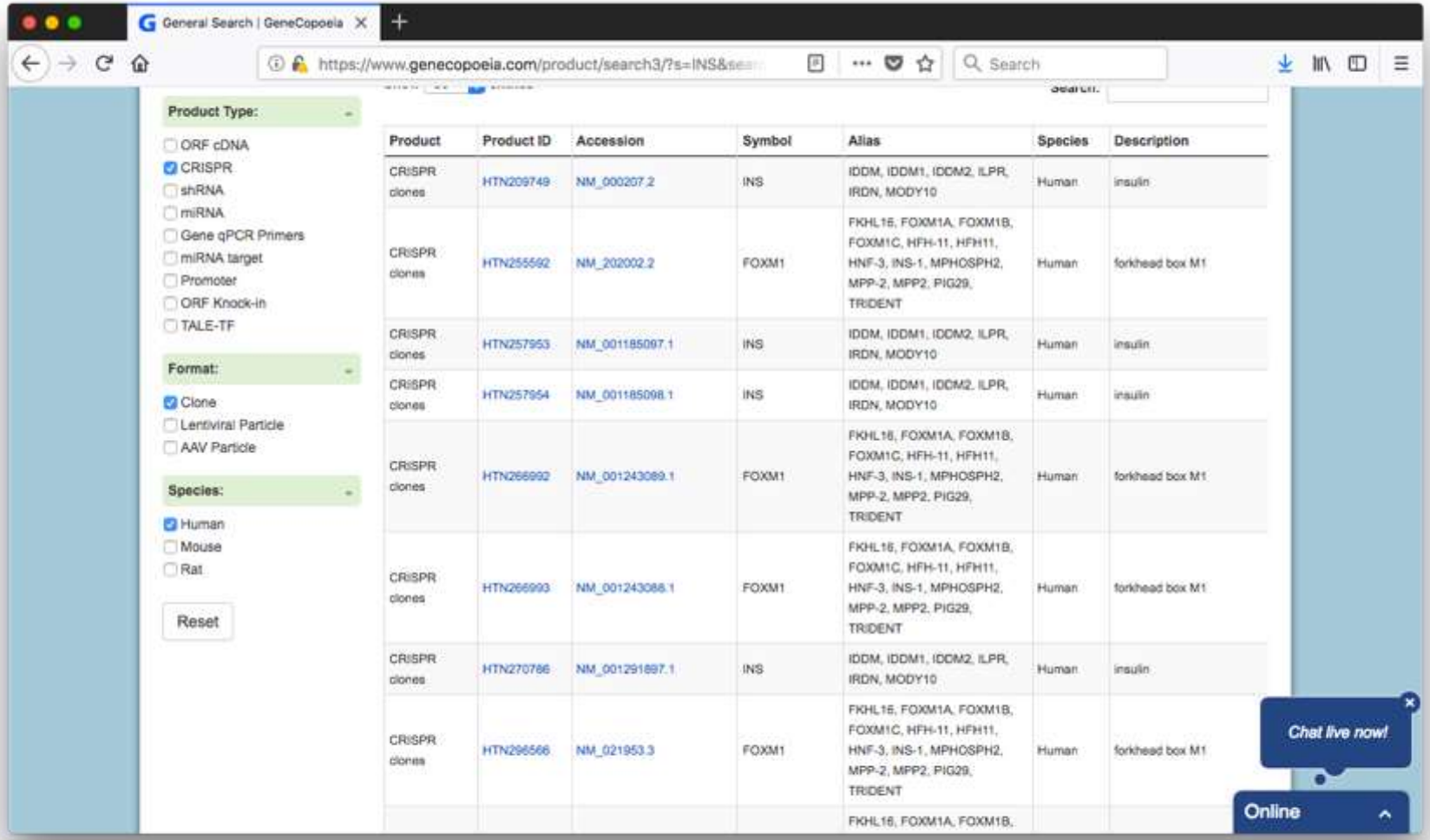
CRISPR-Cas9 genome editing technology

Workflow: Preparation



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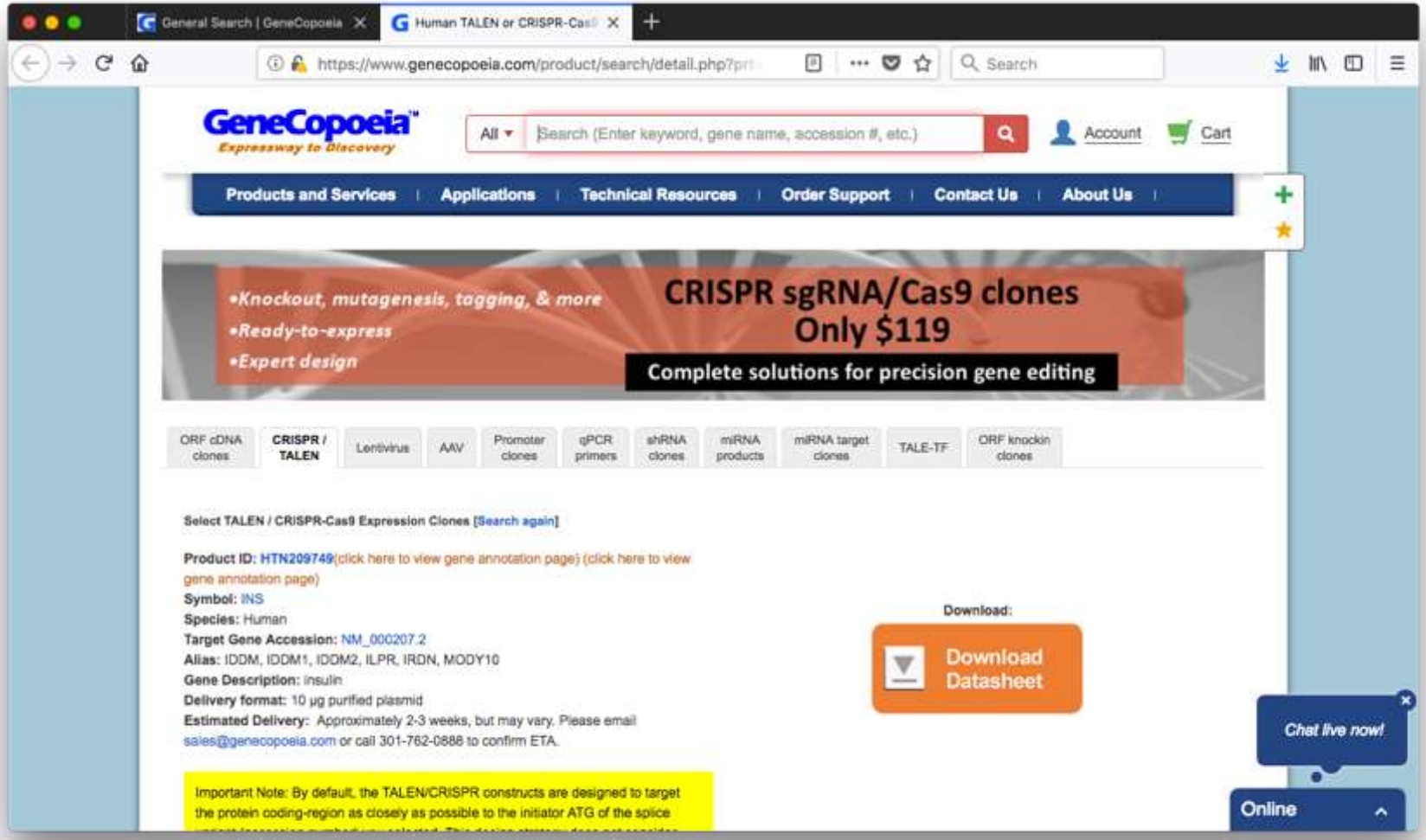
CRISPR-Cas9 genome editing technology

Workflow: Preparation



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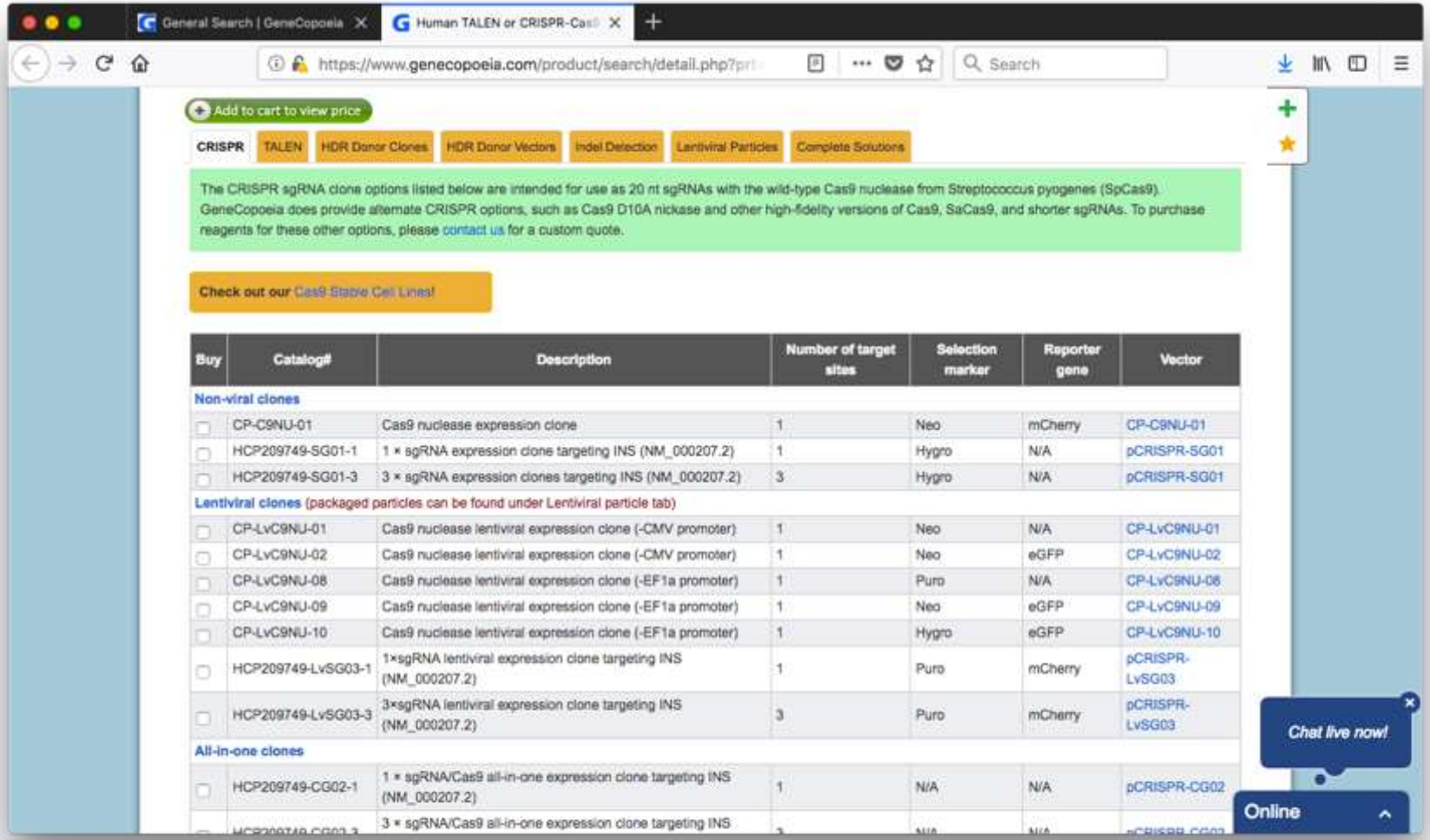
Workflow: Preparation



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Workflow: Preparation

Step 2: Determine the DNA sequence of the sgRNA target site in your cell line

- Necessary to ensure highest possible nucleotide match. Might need to do custom sgRNA synthesis

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CRISPR-Cas9 genome editing technology

Smart-Join™ Blunt-end PCR Cloning Kit

Features

- Convenient method for high efficiency cloning of blunt-end PCR products
- Higher positive ligation rate and true positive colonies by the elimination of self-ligating products
- Cloning of blunt-end PCR products without additional sequences or restriction sites
- Ideal for sequencing regions of genomic DNA

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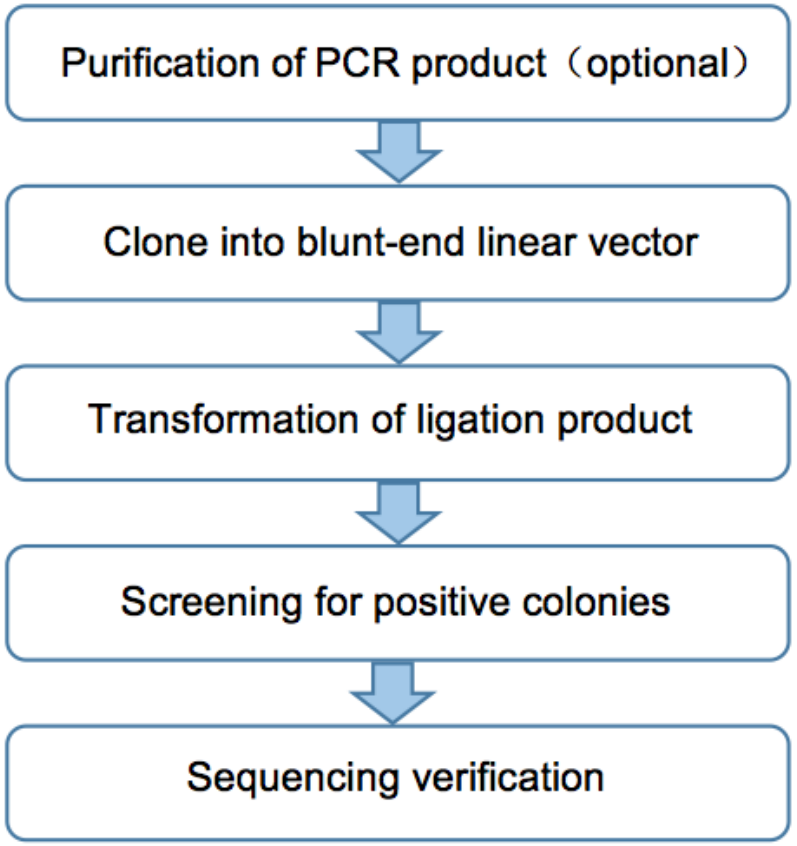
CRISPR-Cas9 genome editing technology

Smart-Join™ Blunt-end PCR Cloning Kit

Workflow



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CRISPR-Cas9 genome editing technology

Workflow: Preparation

Step 3: Validate sgRNA function in your cell line

- Not all sgRNAs are created equal. It is the best practice to try multiple target sites and pre-validate the best one

CRISPR-Cas9 genome editing technology

IndelCheck™ CRISPR indel detection system

Features

- Useful for 1) CRISPR sgRNA functional validation; 2) Screening for positive clones
- 3-component system: 1) Target site PCR kit; 2) T7 Endonuclease I kit; 3) Blunt-end PCR cloning kit
- No genomic DNA isolation is required

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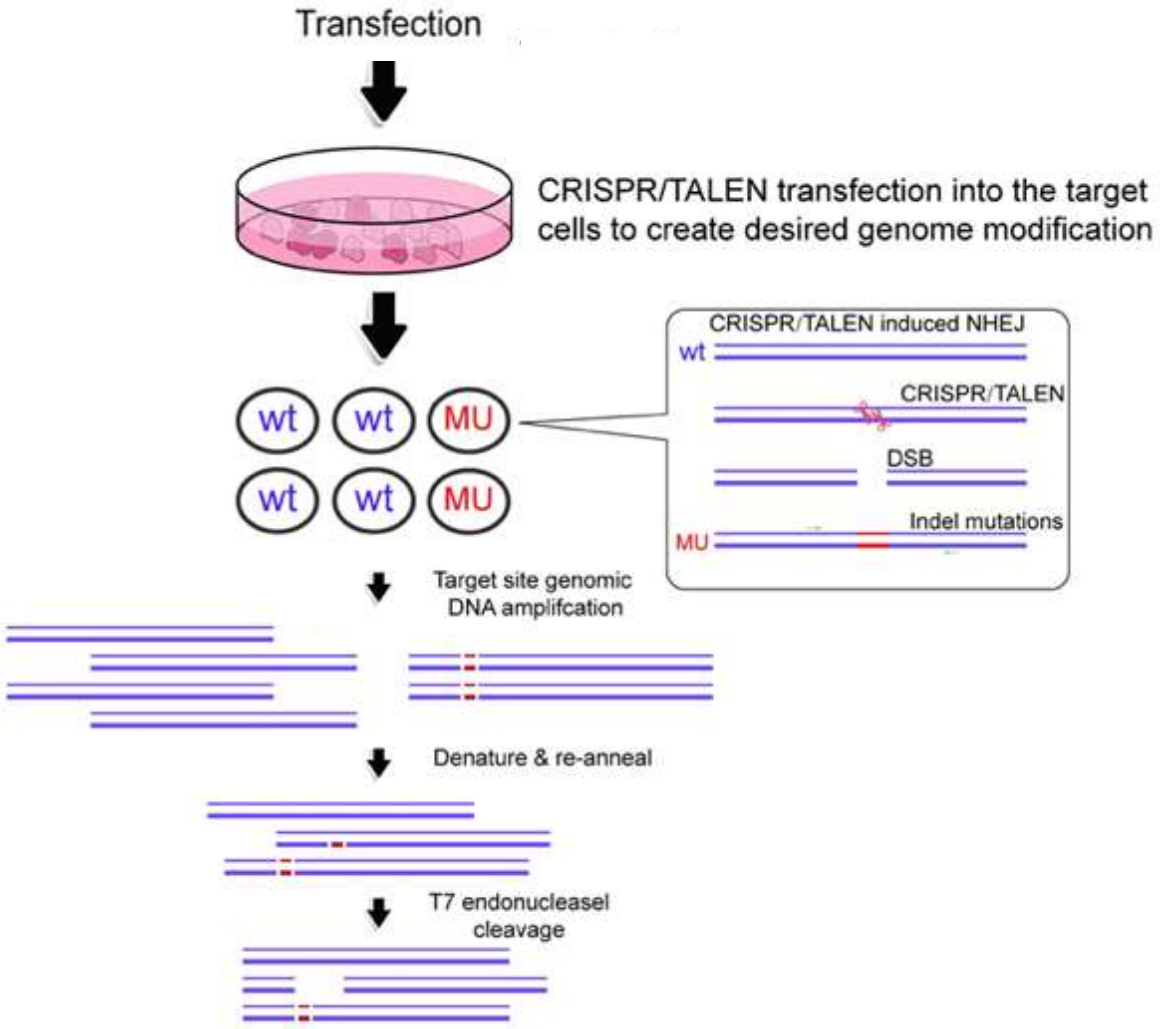
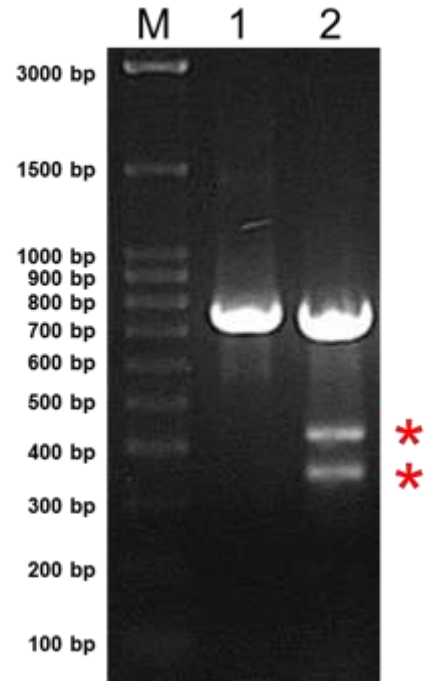
IndelCheck™ CRISPR indel detection system





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CRISPR-Cas9 genome editing technology

Workflow: Preparation

Step 4: Copy number determination

- For complete knockouts or mutagenesis, might need to modify all alleles
- Not all cell lines are diploid. Some lines, like HeLa, have 3, 4, or more genomes
- Would expect multi-allele modification to be more difficult to achieve than single allele
- However, double allele occurs more frequently than single allele (Gonzalez, et al., 2014. Cell Stem Cell 15, 1)

CRISPR-Cas9 genome editing technology

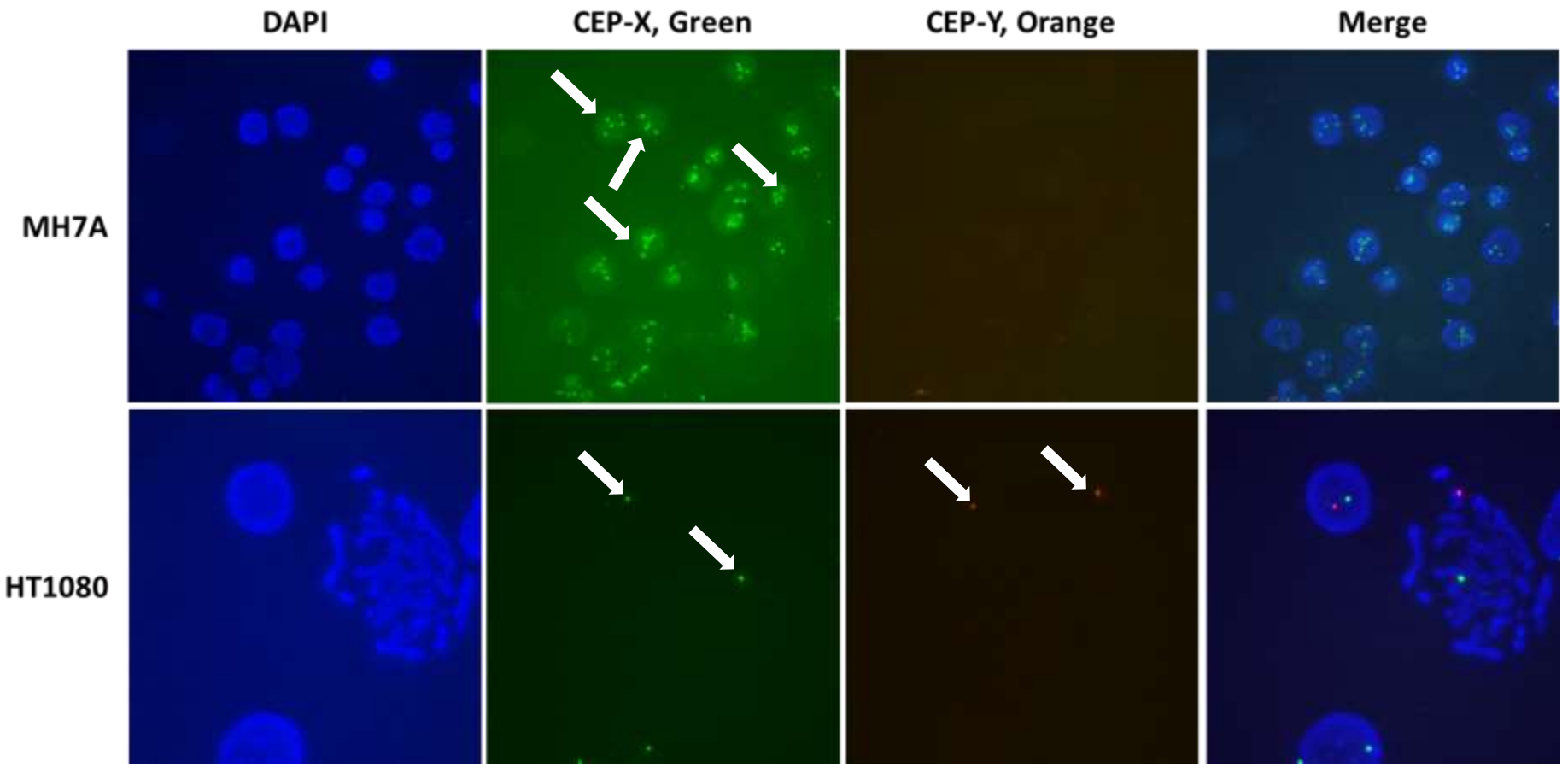
Application: Using FISH with a CRISPR knockout

Project: Knock out HDAC6 gene in human MH7A cells

- HDAC6 (NCBI geneID:10013): Located on X chromosome (Xp11.23)
- MH7A cells: Human immortalized synovial fibroblast line
- HDAC6 copy number: Unknown

CRISPR-Cas9 genome editing technology

Application: Using FISH with a CRISPR knockout



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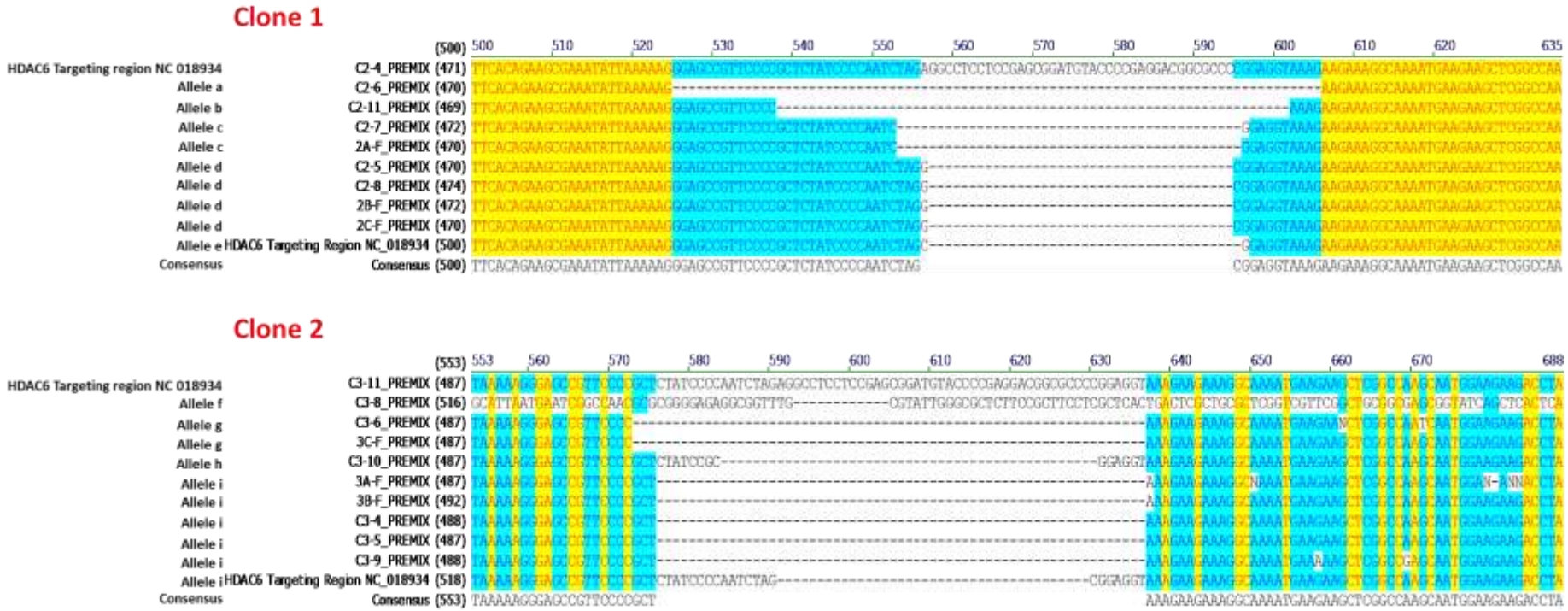
CRISPR-Cas9 genome editing technology

Application: Using FISH with a CRISPR knockout



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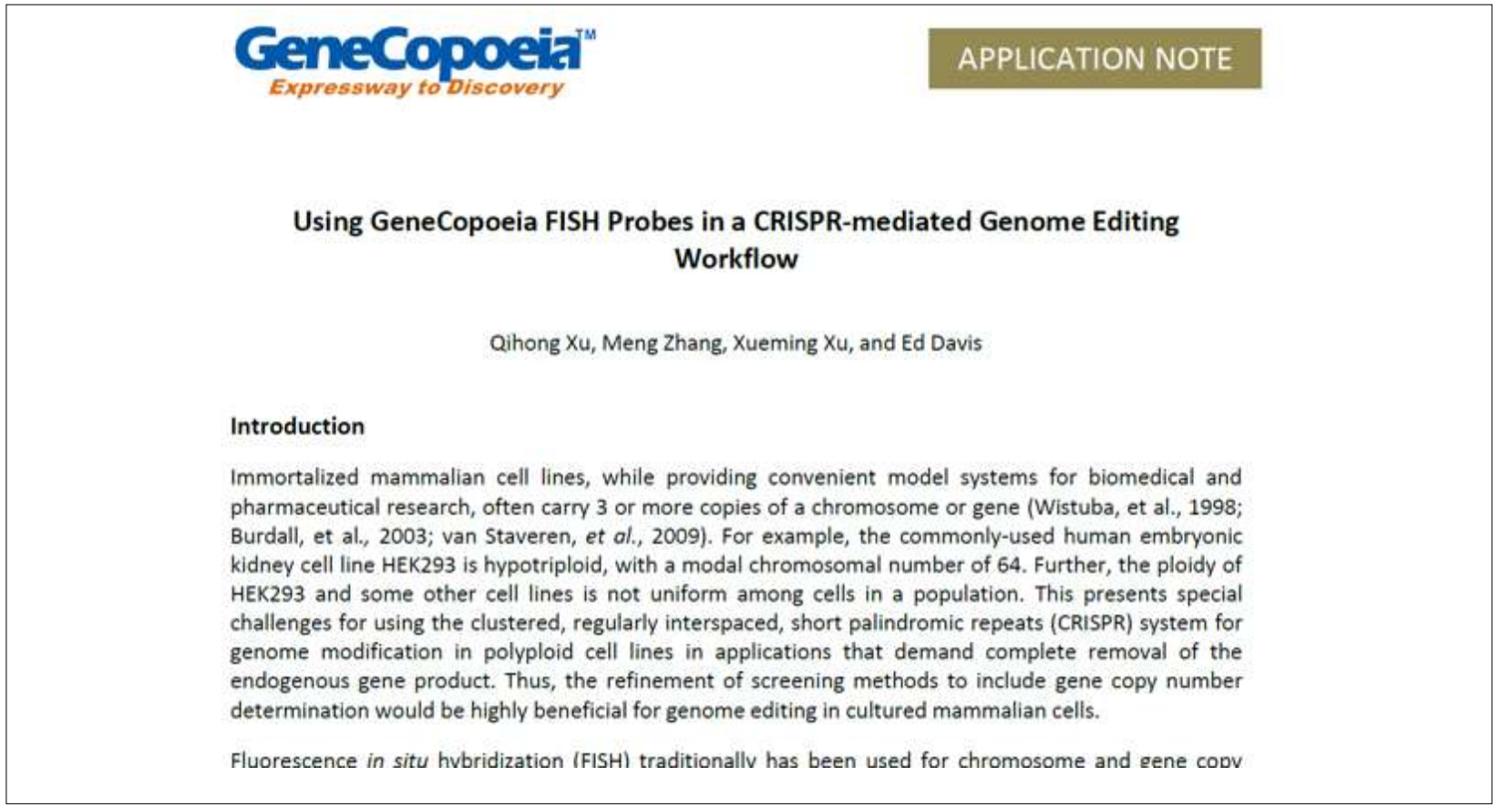
CRISPR-Cas9 genome editing technology

GeneCopoeia Application Note: FISH with CRISPR



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APPLICATION NOTE

Using GeneCopoeia FISH Probes in a CRISPR-mediated Genome Editing Workflow

Qihong Xu, Meng Zhang, Xueming Xu, and Ed Davis

Introduction

Immortalized mammalian cell lines, while providing convenient model systems for biomedical and pharmaceutical research, often carry 3 or more copies of a chromosome or gene (Wistuba, et al., 1998; Burdall, et al., 2003; van Staveren, *et al.*, 2009). For example, the commonly-used human embryonic kidney cell line HEK293 is hypotriploid, with a modal chromosomal number of 64. Further, the ploidy of HEK293 and some other cell lines is not uniform among cells in a population. This presents special challenges for using the clustered, regularly interspaced, short palindromic repeats (CRISPR) system for genome modification in polyploid cell lines in applications that demand complete removal of the endogenous gene product. Thus, the refinement of screening methods to include gene copy number determination would be highly beneficial for genome editing in cultured mammalian cells.

Fluorescence *in situ* hybridization (FISH) traditionally has been used for chromosome and gene copy

Download from:

https://www.genecopoeia.com/wp-content/uploads/2016/02/FISH-for-CRISPR.pdf

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Outline

- CRISPR technologies & applications
- Strategy considerations
- Preparation
- Delivery methods
- Screening
- Things to look out for

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Screening

Isolate single clones post-transfection/transduction

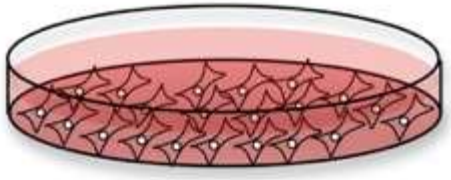
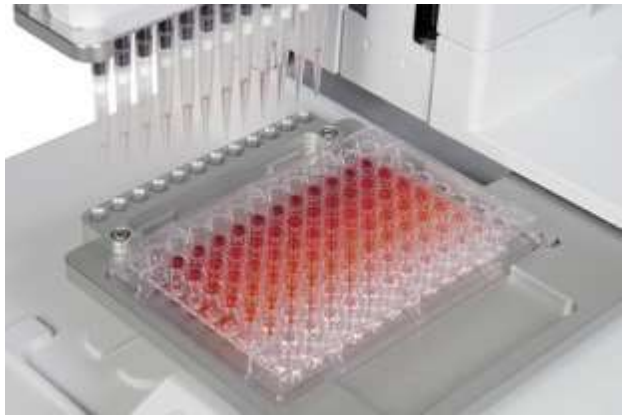


Plate for single colonies and pick off dish

OR

Fluorescence sorting

OR



Do serial dilutions in multi-well plates

- Minimizes potential effects of unwanted modifications resulting from random insertion, cell division or off-targeting

Screening

Isolate single clones post-transfection/transduction

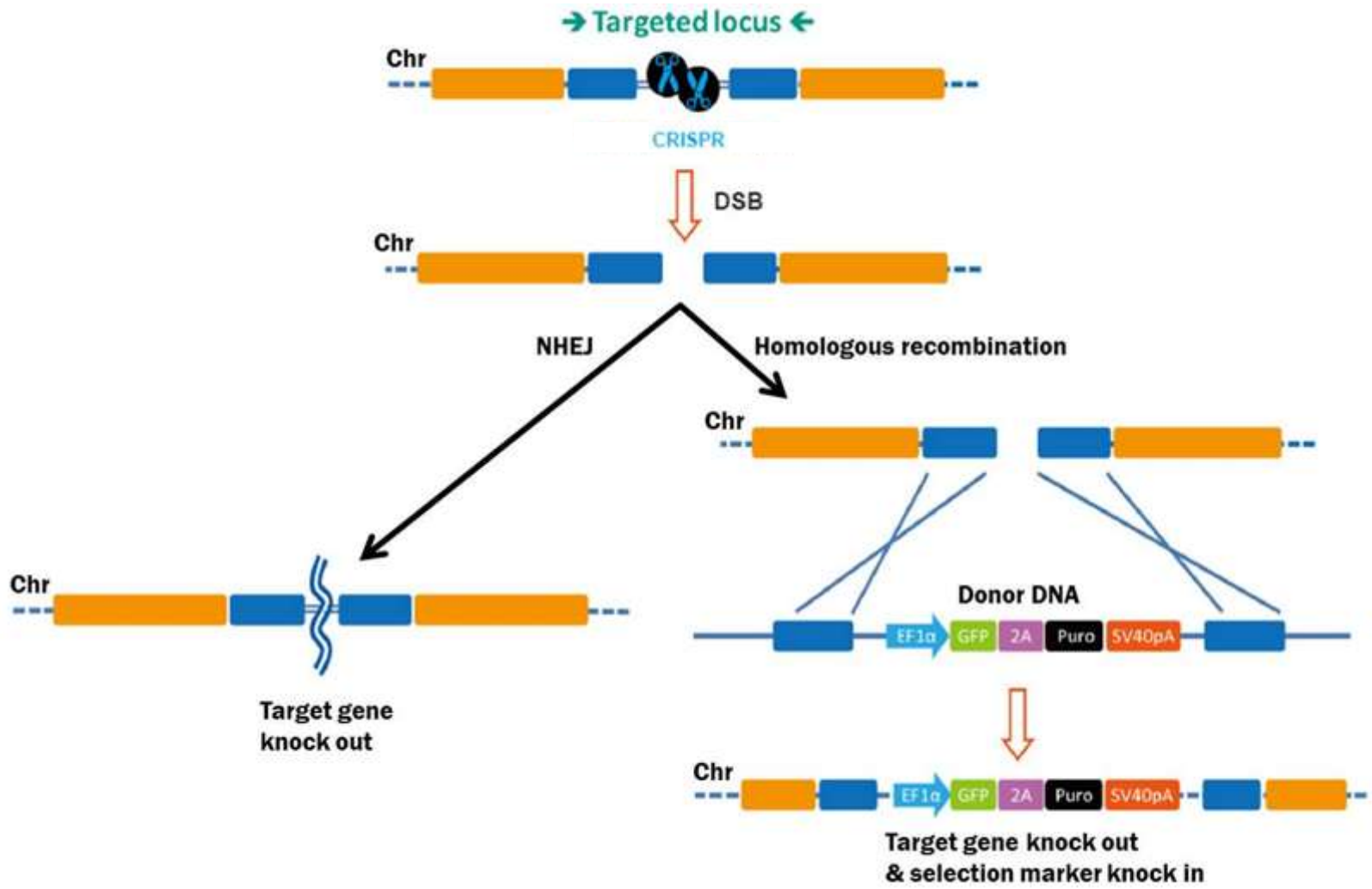
IMPORTANT:

Connect genotype to phenotype!

(or, check the chromosome first before doing functional assays for your target)

Screening

Isolate single clones post-transfection/transduction



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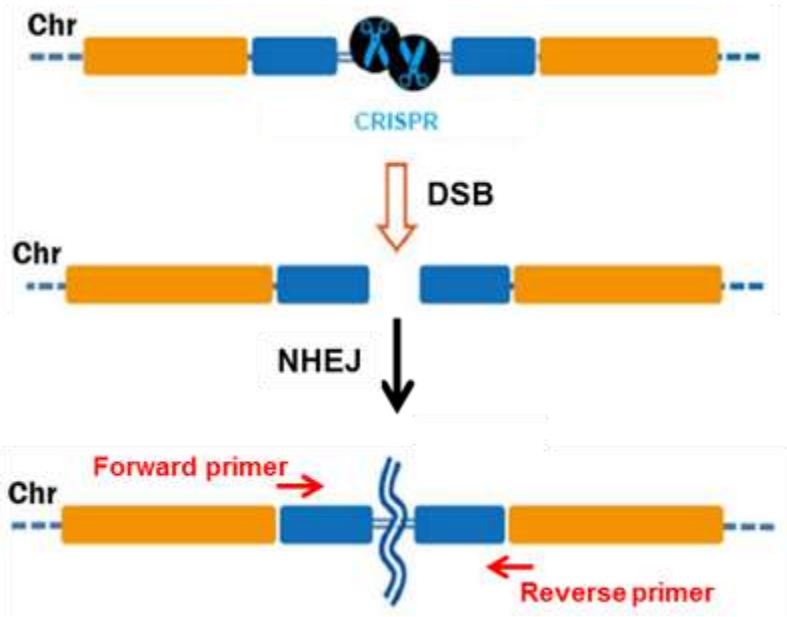
Screening

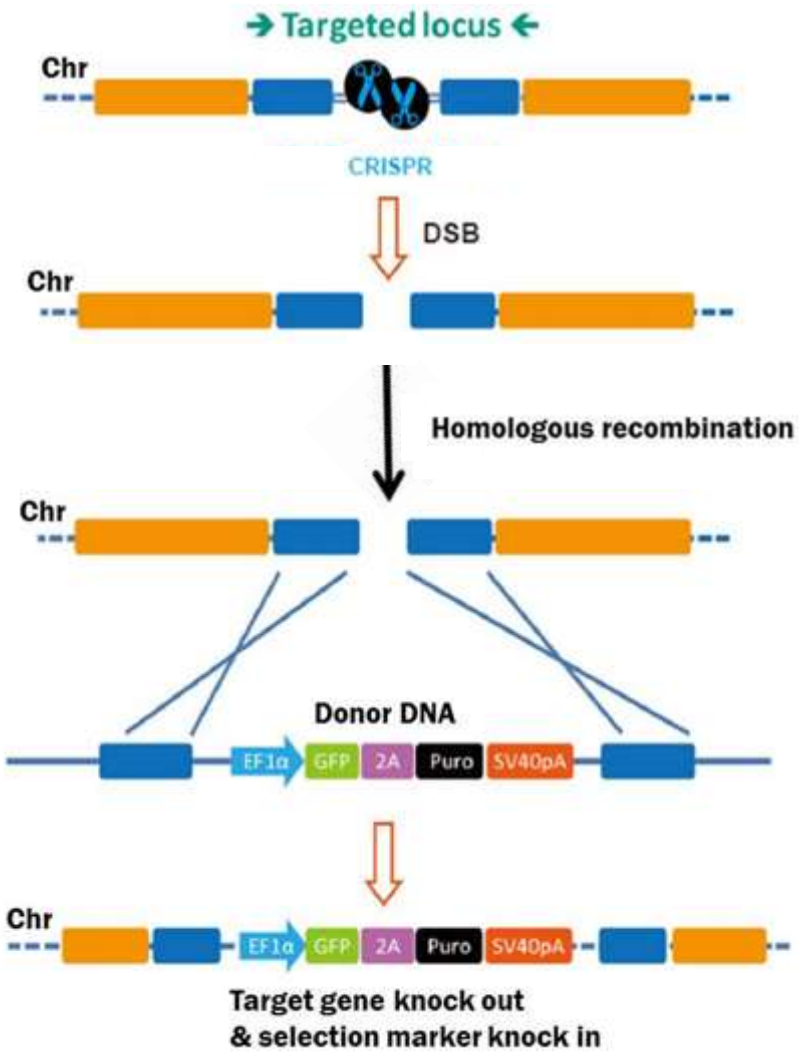
NHEJ-mediated mutations (knockouts)

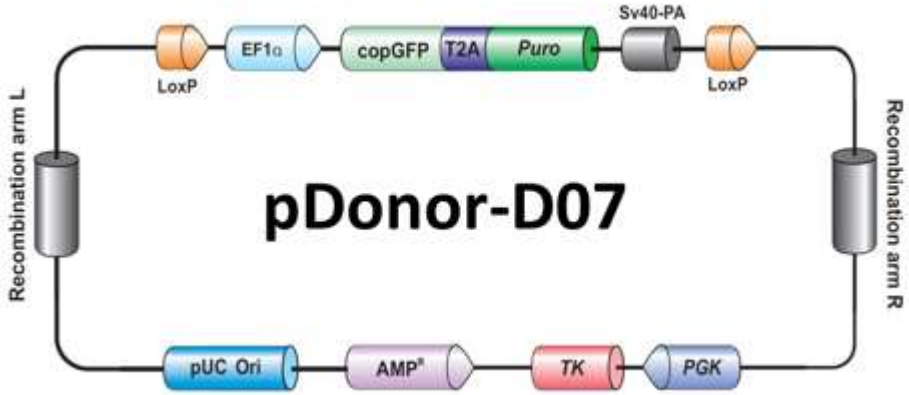


- Isolate clones without selection 2-3 days post transfection/transduction
- Generate PCR products using primers flanking the DSB site (can use same primers as used for validation)
- Screen PCR products by T7 Endonuclease I assay OR direct sequencing. Use GeneCopoeia's IndelCheck system
- Re-screen positive clones by direct sequencing

Screening

HDR-mediated applications using donor plasmids





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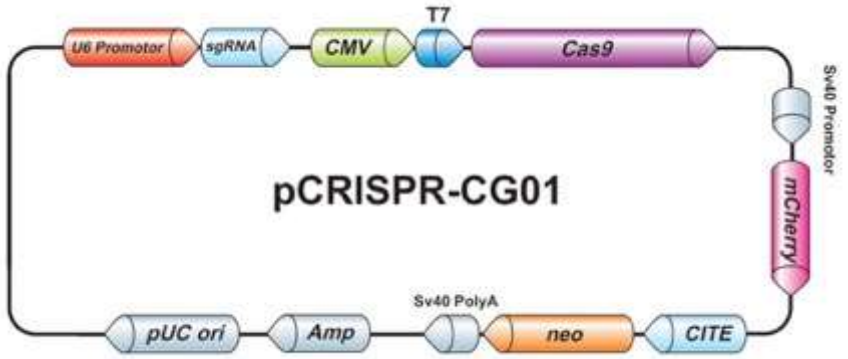
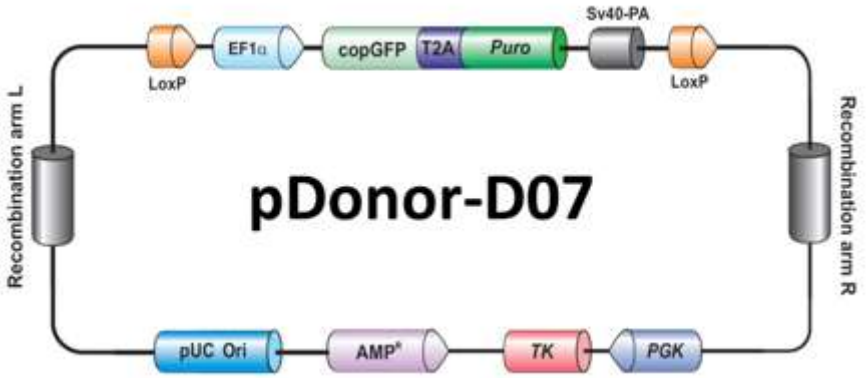
Screening

HDR-mediated applications using donor plasmids





- Co-transfect Cas9, sgRNA, and donor plasmids
- 2-3 days post transfection, apply selection for donor selection cassette, or sort cells expressing fluorescent reporter
- Isolate single clones
- Screen single clones for correct integration by PCR

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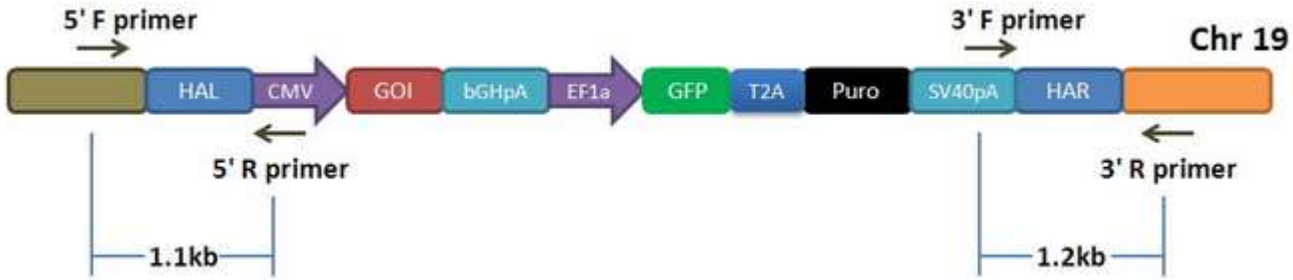
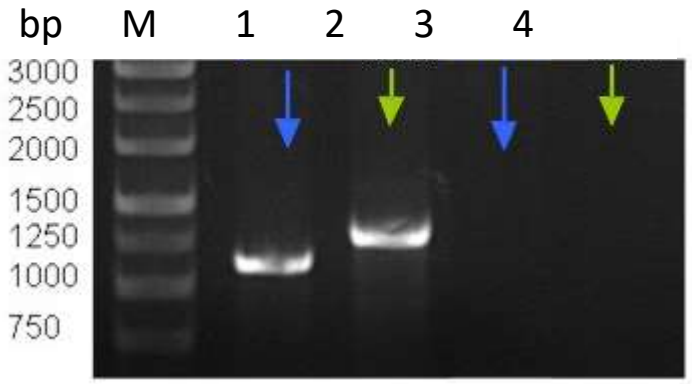
Screening

HDR-mediated applications using donor plasmids

Junction PCR





Will only get PCR products if donor has integrated at the correct site

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Outline

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Things to look out for!

Gene copy number

- For complete knockouts or mutagenesis, might need to modify all alleles
- Not all cell lines are diploid. Some lines, like HeLa, have 3, 4, or more genomes!
- Would expect multi-allele modification to be more difficult to achieve than single allele
- However, one publication showed that double allele occurs more frequently than single allele (Gonzalez, et al., 2014. Cell Stem Cell 15, 1)

Things to look out for!

Cleavage efficiency

- Efficiencies of indel formation by CRISPR usually 5%-70%
- Expect to screen 2x as many clones for a CRISPR sgRNA with 40% cutting efficiency as you would for one with 80% cutting efficiency
- Selection for modifications using a donor can help screening

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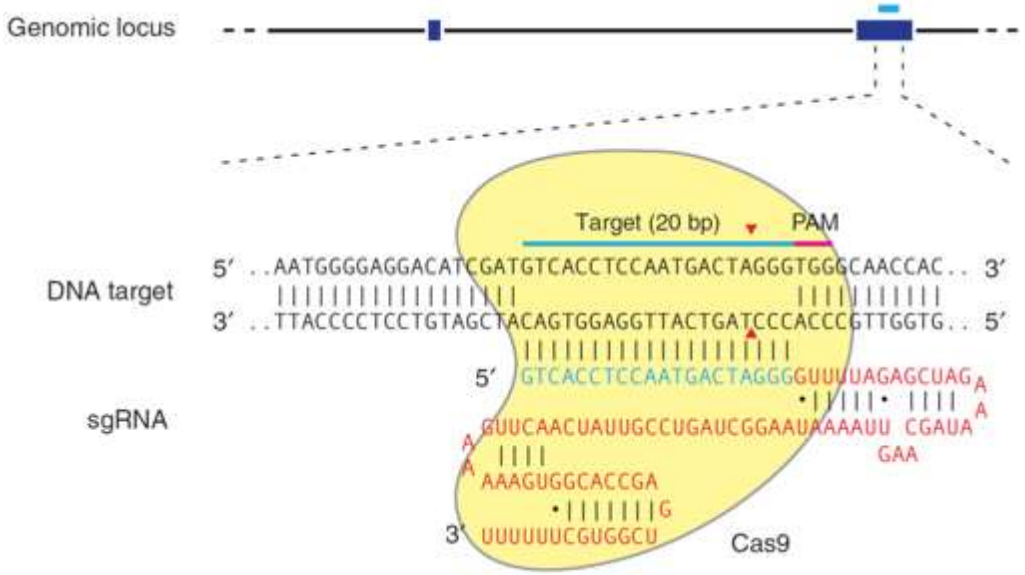
Things to look out for!

Off-targeting



Ran, et al. (2013). Nature Protocols 8, 2281

- CRISPR sgRNAs can tolerate up to 5 mismatches with chromosomal target
- CRISPR sgRNAs can also recognize non-canonical NAG PAM
- Mismatch tolerance and non-canonical PAM recognition can lead to cleavage at other, non-target, or “off-target” sites

- Off-target modifications can have adverse consequences on research results or patient safety!

Things to look out for!

Off-targeting

- Off-targeting should be addressed. GeneCopoeia designs sgRNAs with the lowest possible predicted off-target potential. However, this is a prediction, not experimentally determined.
- Whole genome next-generation sequencing (NGS) is the most comprehensive, unbiased approach. However, this is expensive.
- Can also use targeted, NGS methods such as Digenome-seq and VIVO
- Alternatively, can sequence predicted off-target sites. This is an inexpensive method but is biased.

Summary

- The workflow for CRISPR genome editing experiments must be carefully considered, for aspects such as type of modification you are using, the delivery method, the cell line you are using, etc.

- Other considerations must be taken into account for successful genome editing experiments, such as transfection efficiency, copy number variation, and cleavage efficiency

- GeneCopoeia offers many products and services, including plasmid design & construction, functional validation, validation and screening kits, and transgenic mice, to meet your genome editing needs

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Thank You!

If you have any additional questions, please call

1-866-360-9531 x227

Email: edavis@genecopoeia.com

Or visit us on the web:

www.genecopoeia.com

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