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Genome Editing: How Do I Use CRISPR?

Presenter:

Ed Davis, Ph.D.
Senior Application Scientist
GeneCopoeia, Inc.

February 22, 2017

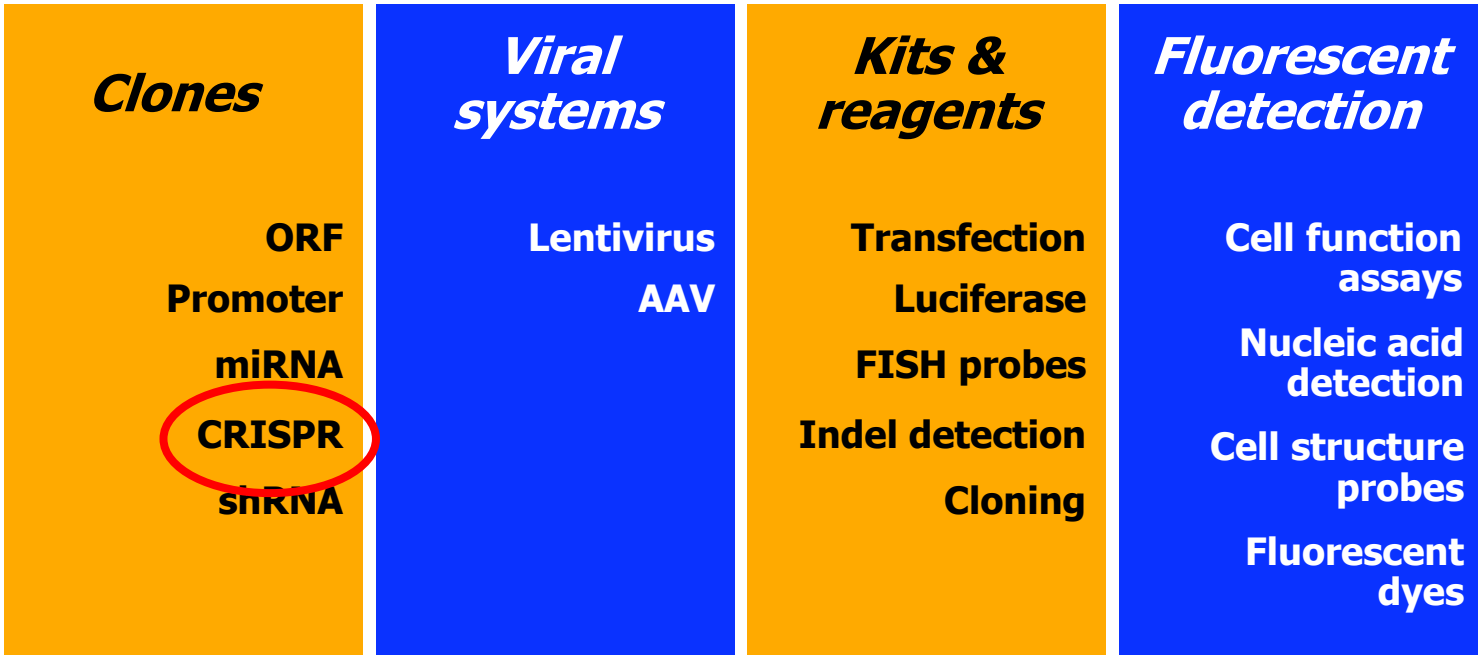
Goals of this presentation

- Show steps to take after receiving genome editing plasmids from GeneCopoeia

- Q & A

GeneCopoeia products & services

Functional Genomics & Cell Biology



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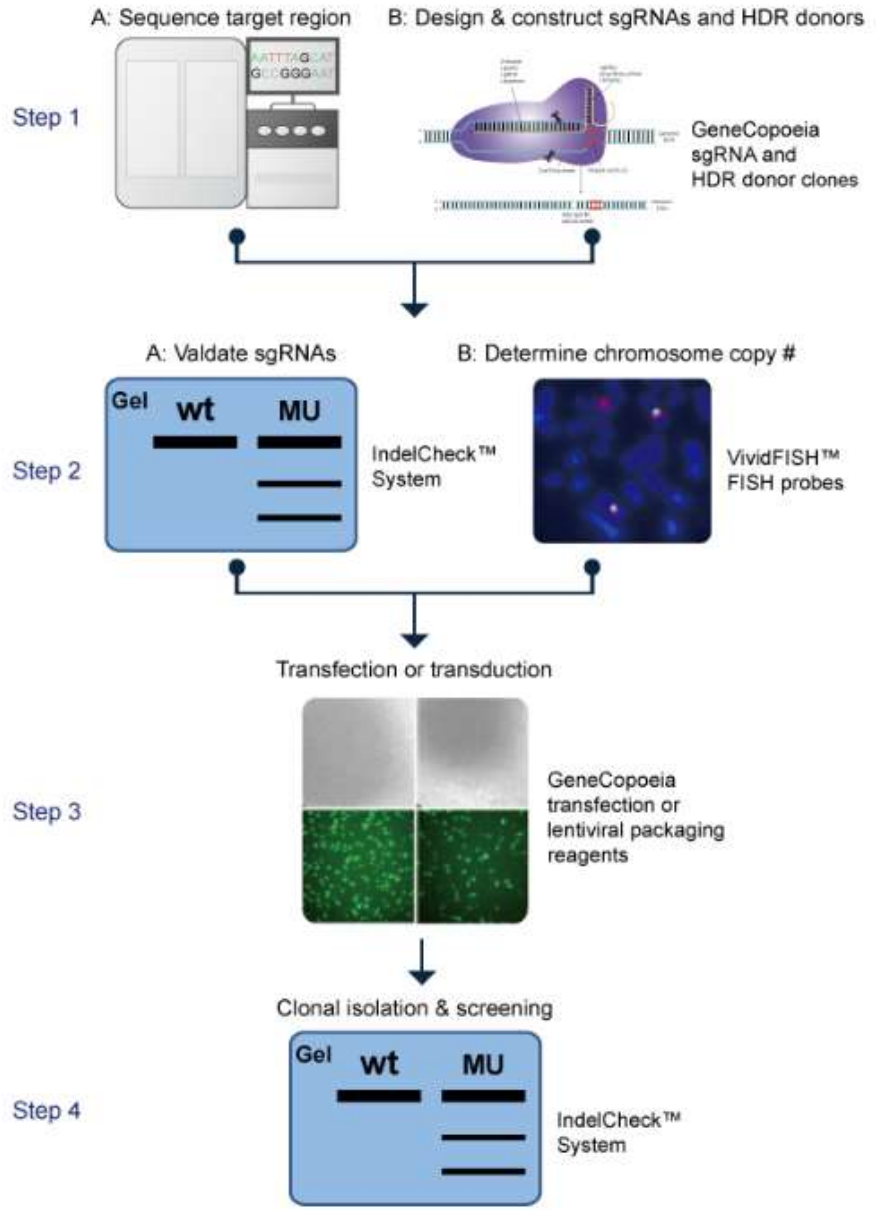
GeneCopoeia CRISPR products for cell lines



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Outline

- Genome editing technologies & applications
- Downstream workflow
- Functional validation
- Transfection or transduction
- Clonal isolation
- Screening for edited clones
- Things to look out for

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CRISPR or RNAi?

Knock down vs. Knock out

Method	Change expression level	Knock down	Knock out	Change genetic code	Clone isolation required?
CRISPR	✓		✓	✓	✓
RNAi	✓	✓			

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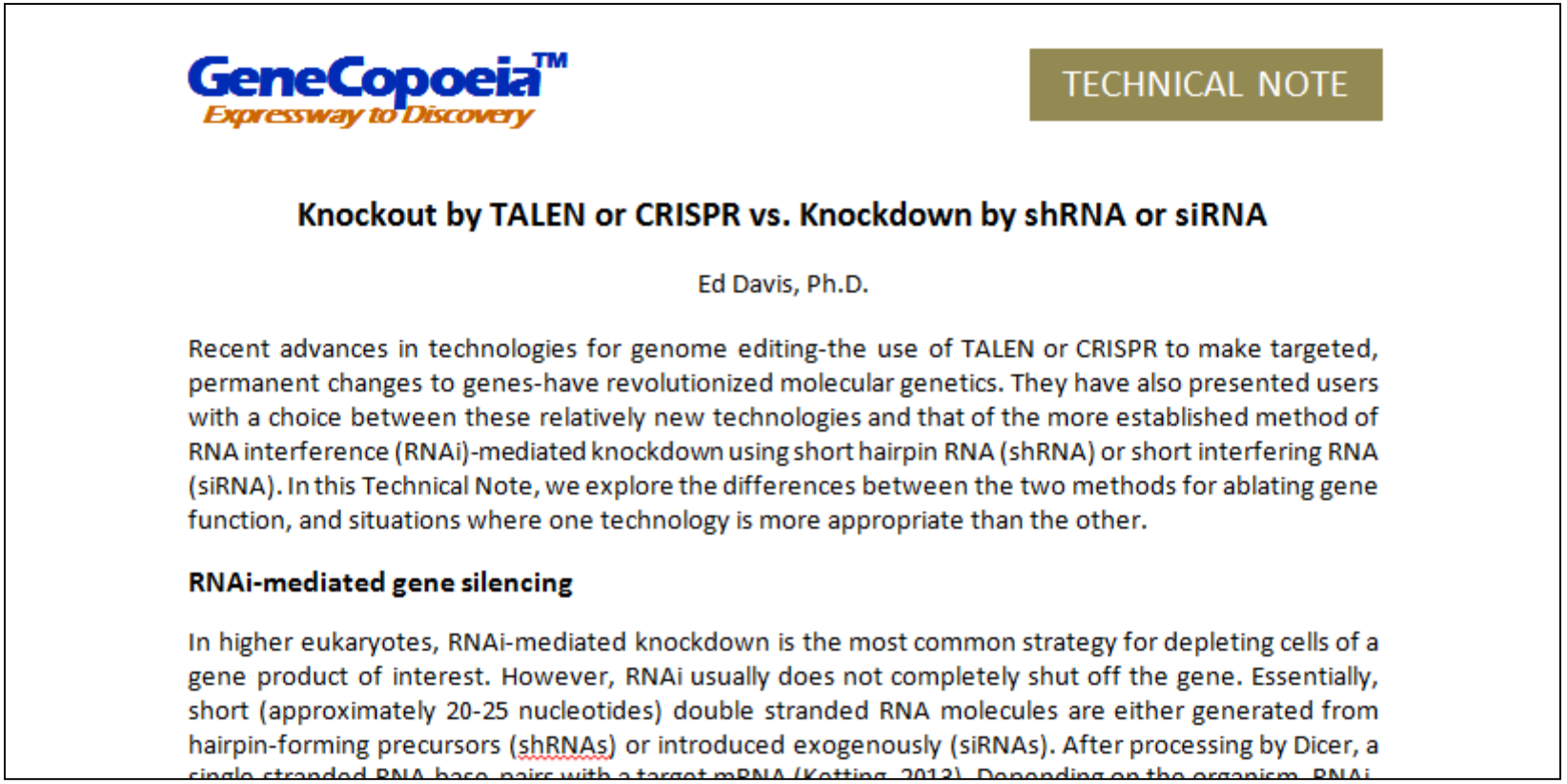
CRISPR or RNAi?

GeneCopoeia Technical Note: Knockdown vs. Knockout



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TECHNICAL NOTE

Knockout by TALEN or CRISPR vs. Knockdown by shRNA or siRNA

Ed Davis, Ph.D.

Recent advances in technologies for genome editing-the use of TALEN or CRISPR to make targeted, permanent changes to genes-have revolutionized molecular genetics. They have also presented users with a choice between these relatively new technologies and that of the more established method of RNA interference (RNAi)-mediated knockdown using short hairpin RNA (shRNA) or short interfering RNA (siRNA). In this Technical Note, we explore the differences between the two methods for ablating gene function, and situations where one technology is more appropriate than the other.

RNAi-mediated gene silencing

In higher eukaryotes, RNAi-mediated knockdown is the most common strategy for depleting cells of a gene product of interest. However, RNAi usually does not completely shut off the gene. Essentially, short (approximately 20-25 nucleotides) double stranded RNA molecules are either generated from hairpin-forming precursors (shRNAs) or introduced exogenously (siRNAs). After processing by Dicer, a single stranded RNA base pairs with a target mRNA (Kotting, 2013). Depending on the organism, RNAi

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http://www.genecopoeia.com/wp-content/uploads/2014/02/Technotes_Knockdown_vs_knockout.pdf

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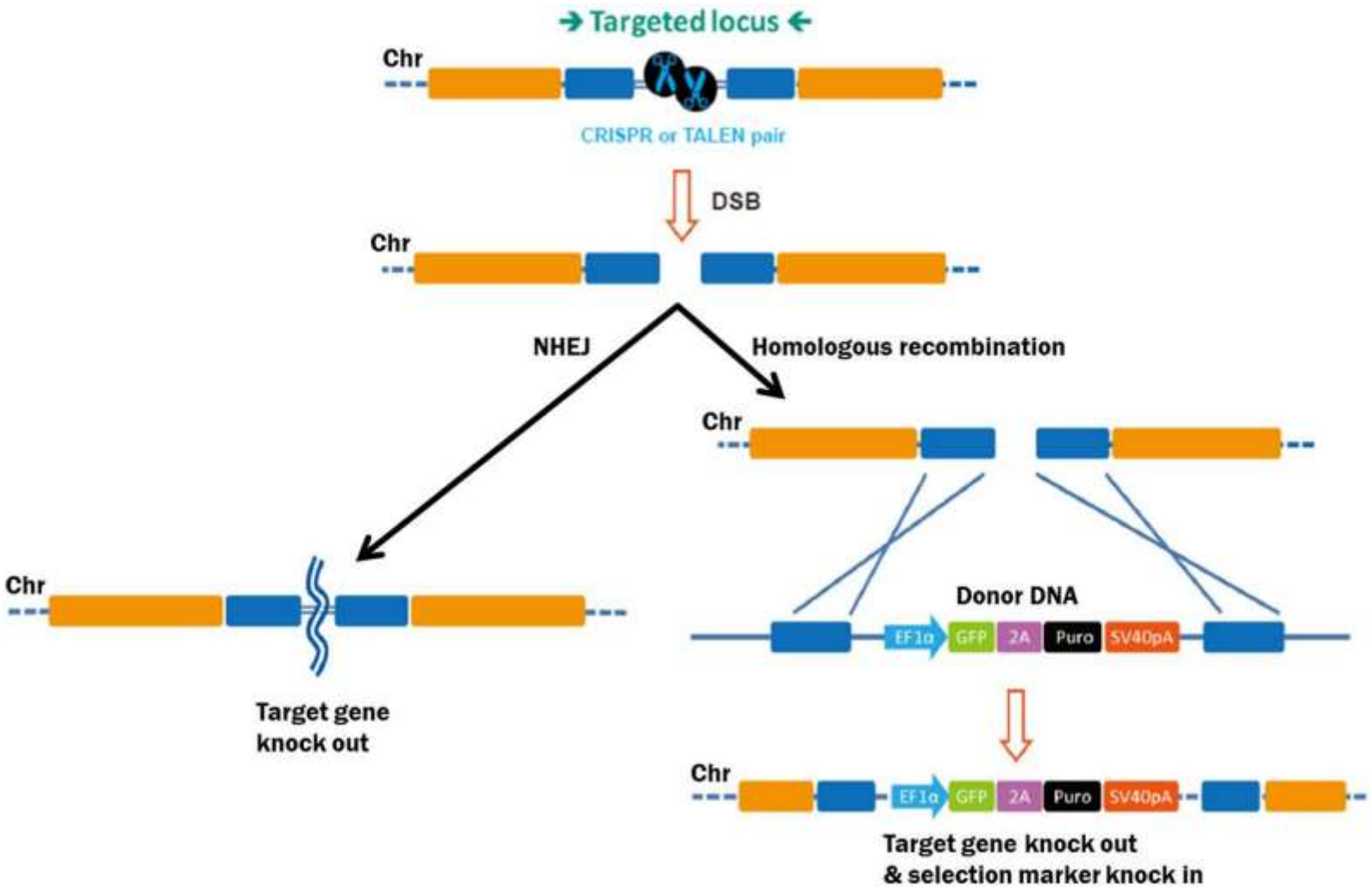
Targeted DNA editing by DSB induction



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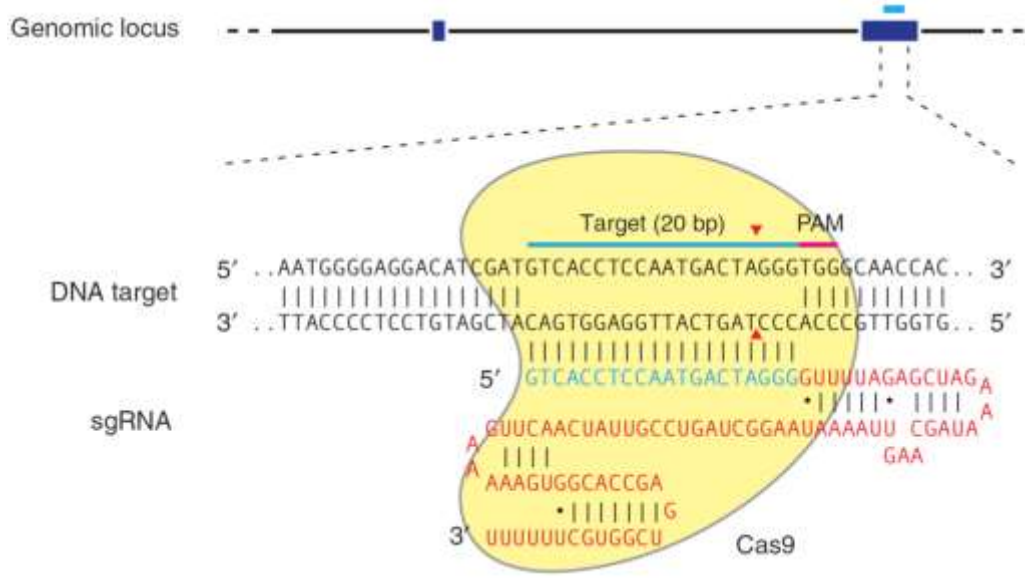
CRISPR genome editing technology

CRISPR-Cas9: RNA-guided endonuclease



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

- 20 nt single guide RNA (sgRNA) guides Cas9 nuclease to target site.
- Requires NGG “PAM” site immediately downstream of sgRNA target sequence.
- Cas9-RNA complex makes DSB 3-4 nt upstream of PAM.
- Target almost any gene in any cell

Applications for genome editing

Application	Example	Technology	Reference
Gene knockout	Knockout SRY, UTY genes in cultured dells	TALEN via NHEJ and plasmid donor	Wang, et al. (2013). Nature Biotech. 31, 530
Gene knockout	Knockout of tet genes in transgenic rats	CRISPR via NHEJ	Li, et al. (2013). Nature Biotech. 8, 684
Correction of disease mutations	Cured heritable cataracts in transgenic mice	CRISPR via oligonucleotide donor	Wu, et al. (2013). Cell Stem Cell 13, 659
Engineered disease resistance	Knockout CCR5 gene to cure patients of HIV	ZFN via NHEJ	Perez, et al. (2008). Nat Biotech 26, 808
Forward mutagenesis screens	Identified genes in mismatch repair pathway by selecting for 6-thioguanosine resistance	CRISPR via NHEJ	Wang, et al. (2014). Science 343, 80
In-frame fusion tagging	C-terminal tag Sox2p with V5	CRISPR via oligonucleotide donor	Yang, et al. (2013). Cell 154, 1370
Transgene knock-in	Sox2, Oct4 ORFs KI into human AAVS1 “Safe Harbor”	TALEN via plasmid donor	GeneCopoeia internal data

Outline

- Genome editing technologies & applications
- Downstream workflow
- Functional validation
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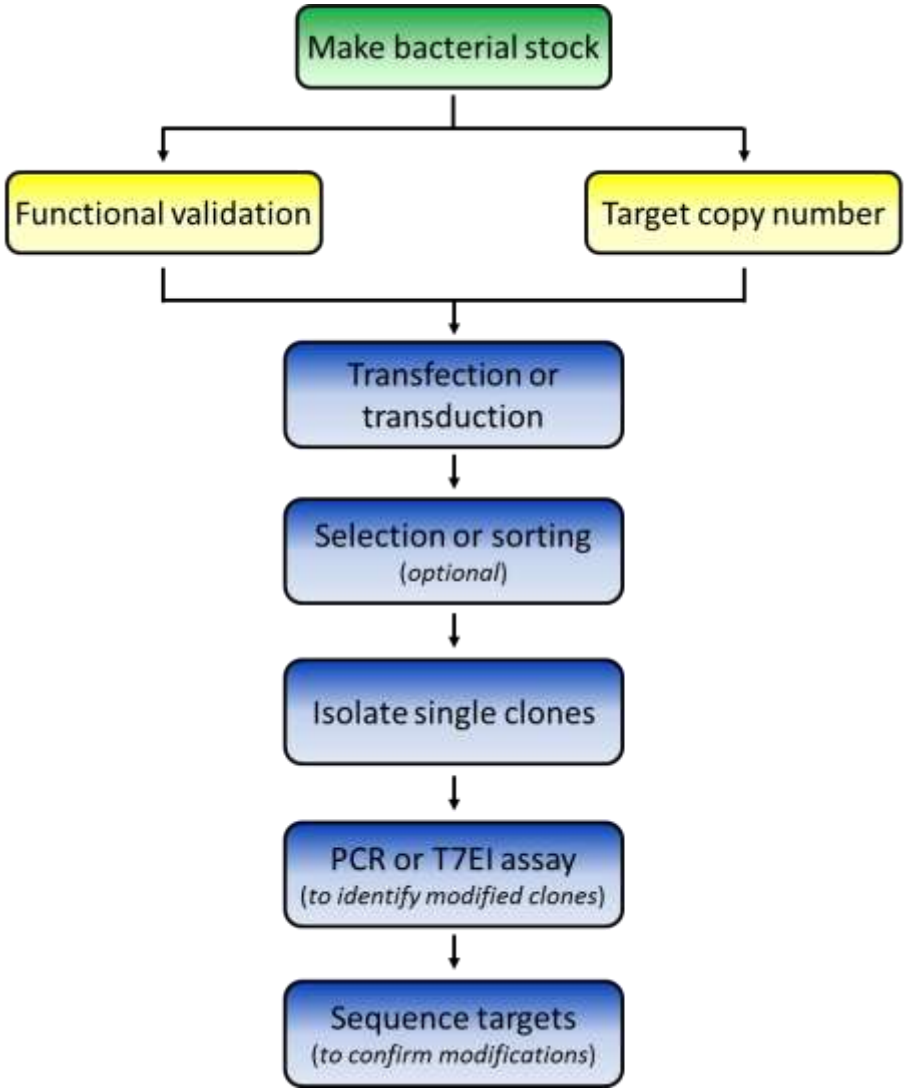
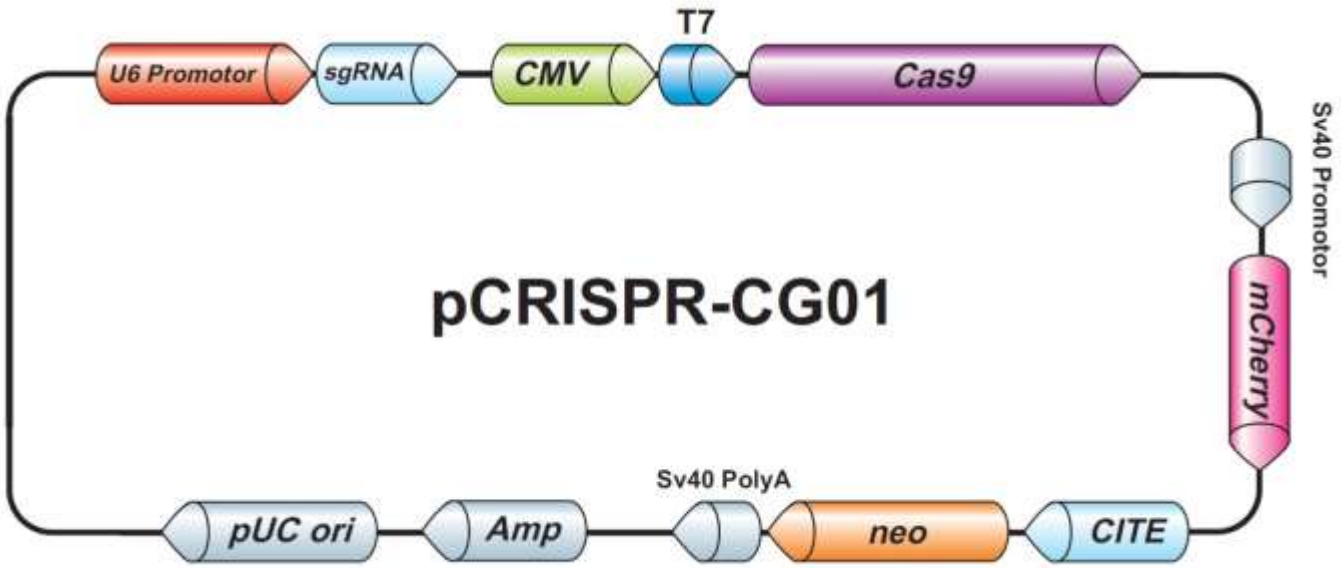
Overview of “downstream” work



Step 1: Make bacterial stock



- Necessary to make renewable stock without re-ordering DNA preps
- Plasmids are circular & can transform E. coli using standard methods
- Plasmids carry ampicillin-resistance gene for selection

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Step 2a: Target analysis

Sequence target in your cell line

- Recommended! By default, sgRNA targets are designed using the NCBI reference sequence. A polymorphism could decrease or eliminate CRISPR activity.

Step 2b: Functional validation

Why do functional validation?

- Cell culture-based genome editing projects can take 3 or more months to complete
- Not all CRISPR sgRNAs are created equal! Test first before undergoing long genome editing project to avoid wasting time and expense.
- Recommend cell culture based strategy using T7 Endonuclease I assay.

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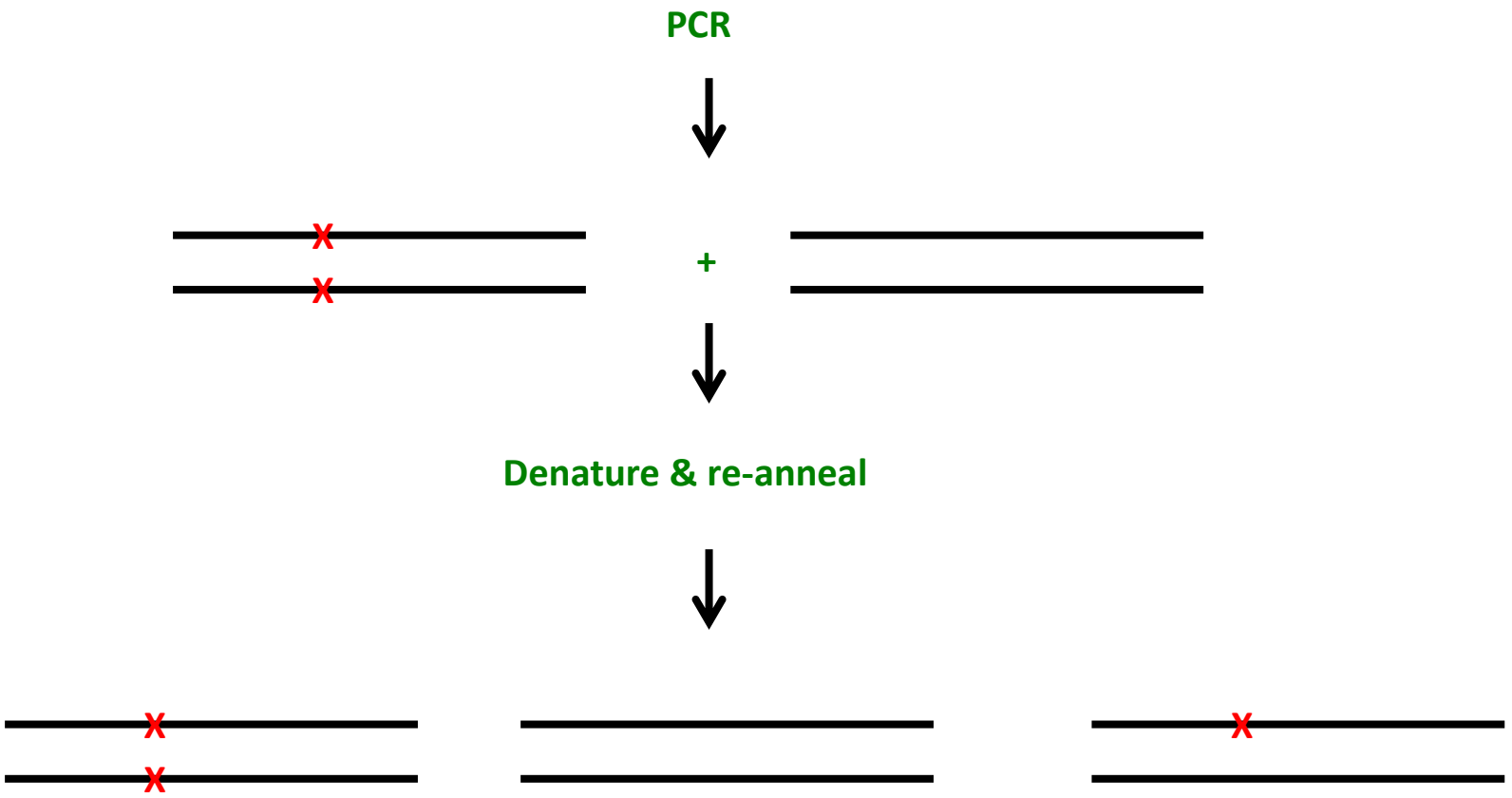
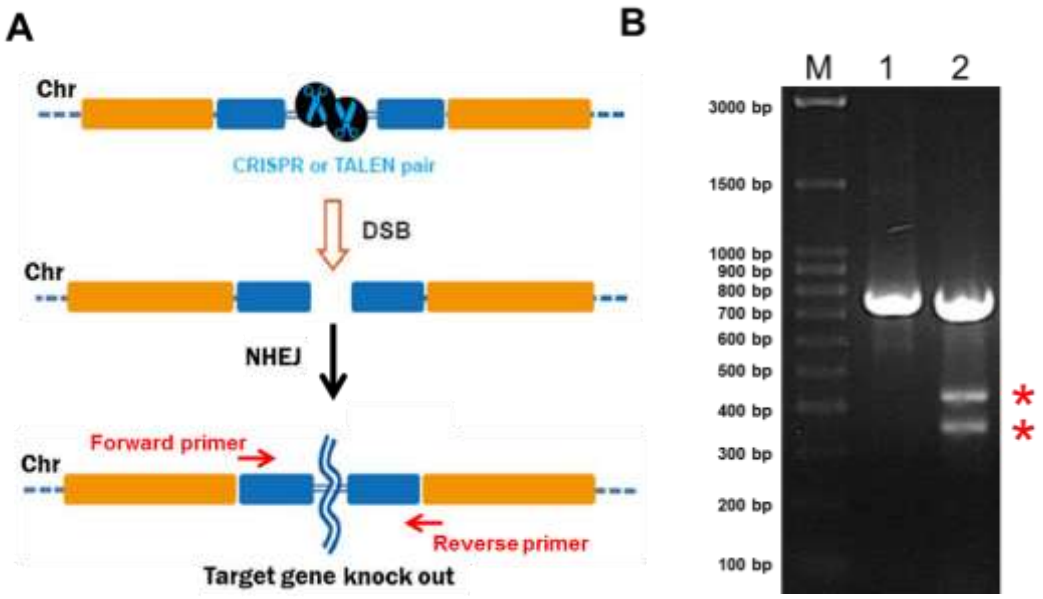
Step 2b: Functional validation



Step 2b: Functional validation

IndelCheck™ T7 Endonuclease System



- Contains PCR and T7 endonuclease I reagents
- Purchase with or without target-specific primers

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Step 2b: Functional validation

GeneCopoeia functional validation services

- Additional service available for human and mouse sgRNAs
- GeneCopoeia will transfect human (HEK293) or mouse (Neuro2A) cells with custom sgRNA plasmids
- GeneCopoeia will isolate DNA from bulk population of cells and perform T7 Endonuclease I assay
- Customer is provided with validation report

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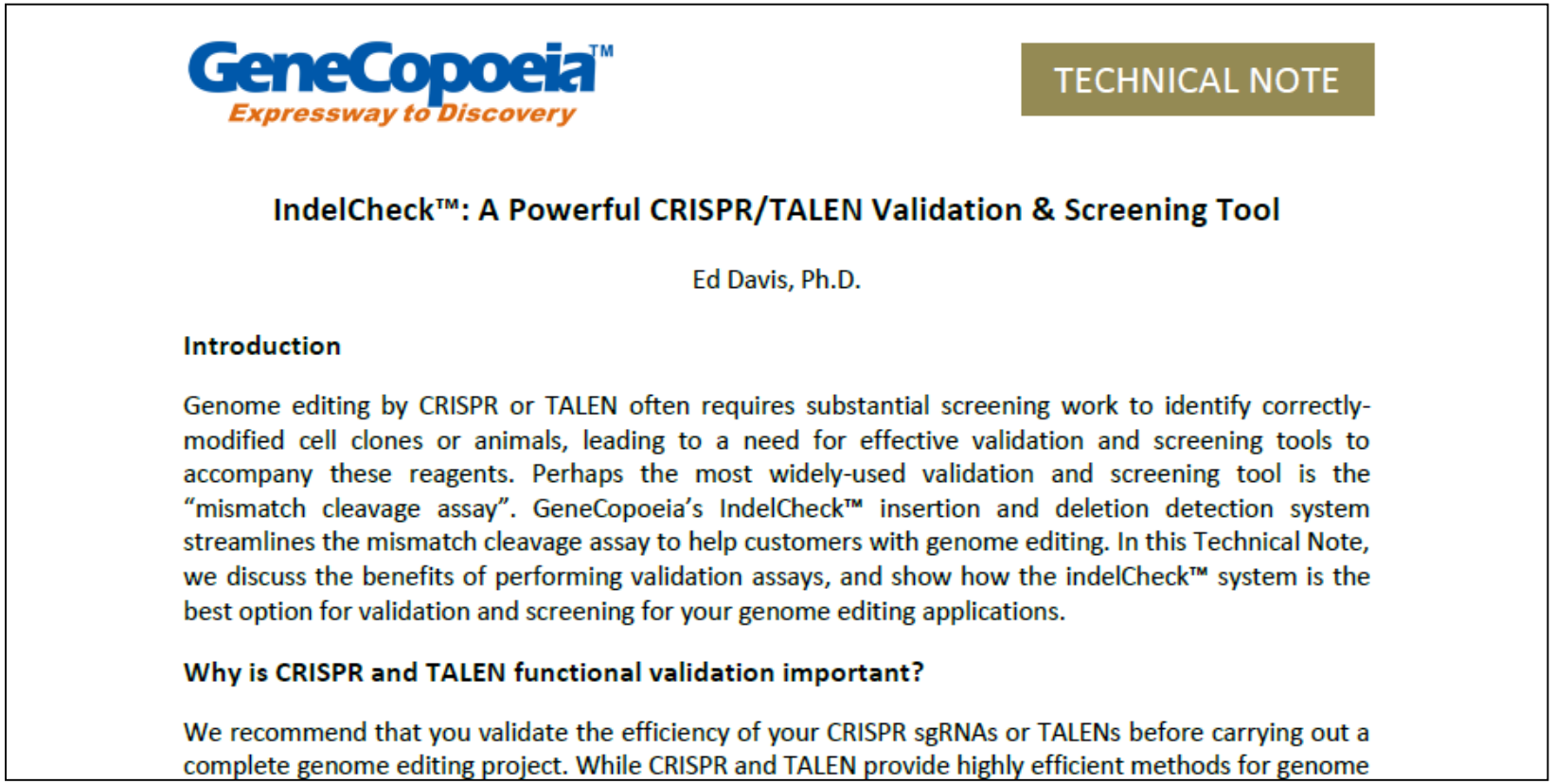
Step 2b: Functional validation

GeneCopoeia Technical Note: IndelCheck™ kit



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TECHNICAL NOTE

IndelCheck™: A Powerful CRISPR/TALEN Validation & Screening Tool

Ed Davis, Ph.D.

Introduction

Genome editing by CRISPR or TALEN often requires substantial screening work to identify correctly-modified cell clones or animals, leading to a need for effective validation and screening tools to accompany these reagents. Perhaps the most widely-used validation and screening tool is the "mismatch cleavage assay". GeneCopoeia's IndelCheck™ insertion and deletion detection system streamlines the mismatch cleavage assay to help customers with genome editing. In this Technical Note, we discuss the benefits of performing validation assays, and show how the indelCheck™ system is the best option for validation and screening for your genome editing applications.

Why is CRISPR and TALEN functional validation important?

We recommend that you validate the efficiency of your CRISPR sgRNAs or TALENs before carrying out a complete genome editing project. While CRISPR and TALEN provide highly efficient methods for genome

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http://www.genecopoeia.com/wp-content/uploads/2015/07/GeneCopoeia-Technical-Note-IndelCheck-system-07-2015.pdf

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Step 2c: Copy number determination

Why do 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, one publication showed that double allele occurs more frequently than single allele (Gonzalez, et al., 2014. Cell Stem Cell 15, 1)

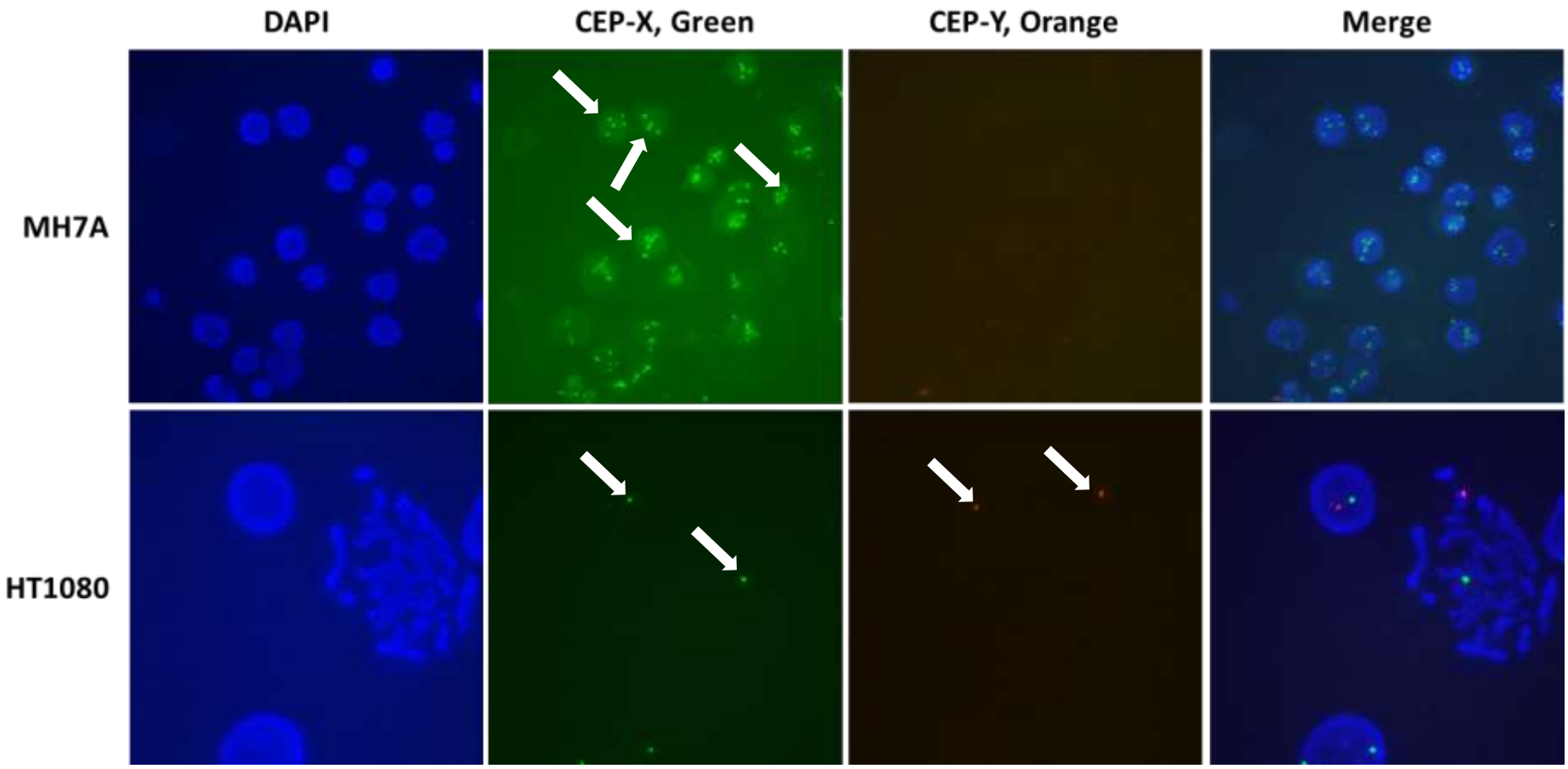
Step 2c: Copy number determination

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

Step 2c: Copy number determination

Application: Using FISH with a CRISPR knockout



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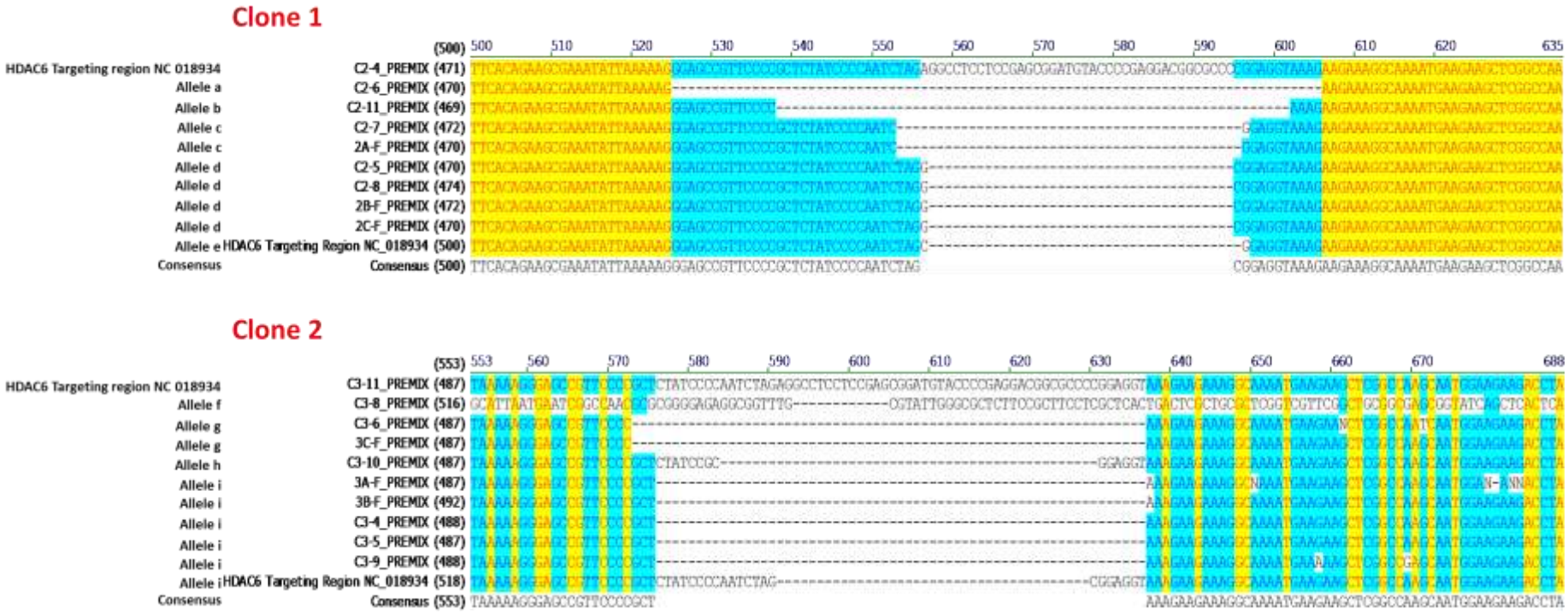
Step 2c: Copy number determination

Application: Using FISH with a CRISPR knockout



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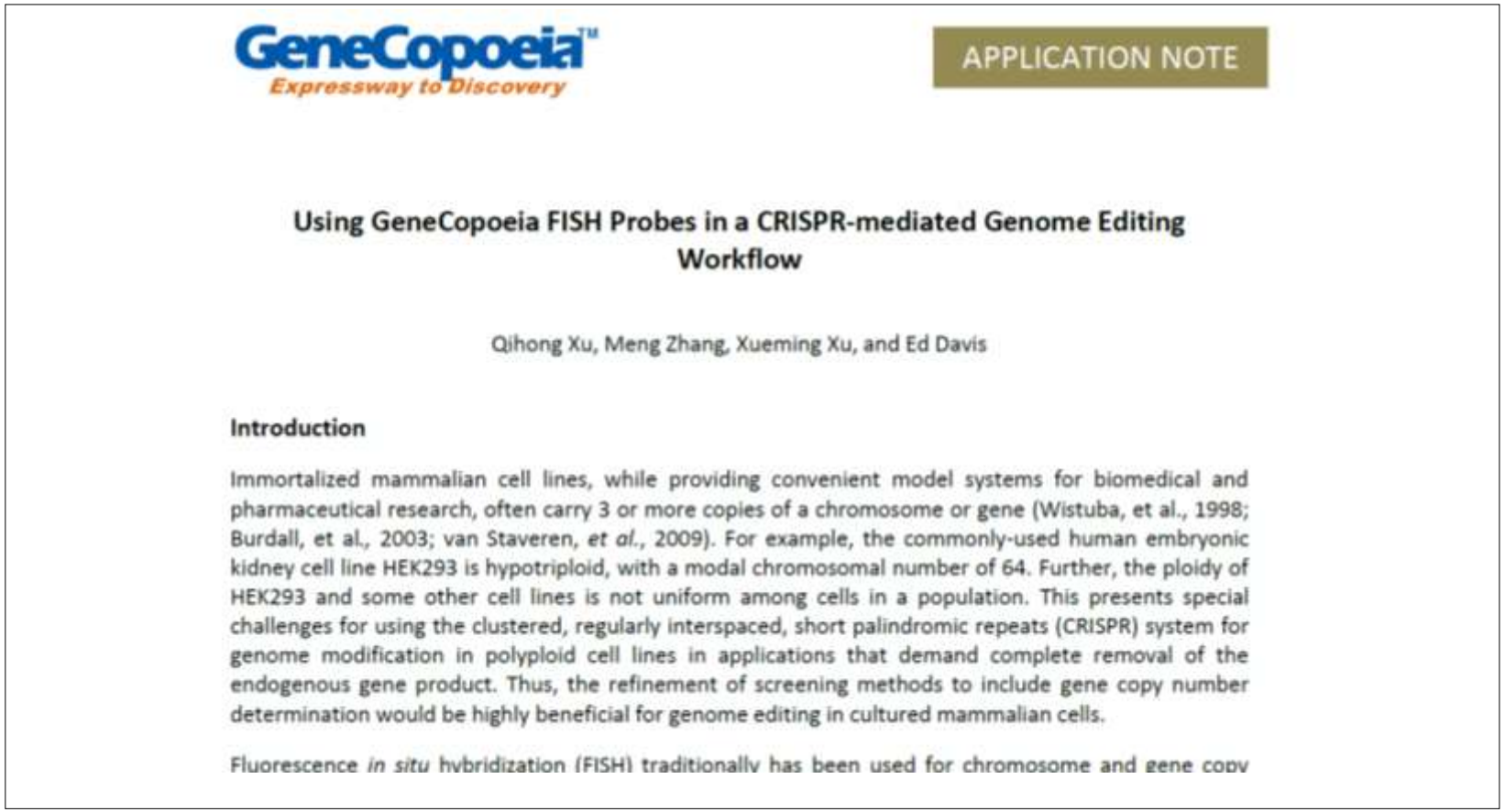
Step 2c: Copy number determination

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

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

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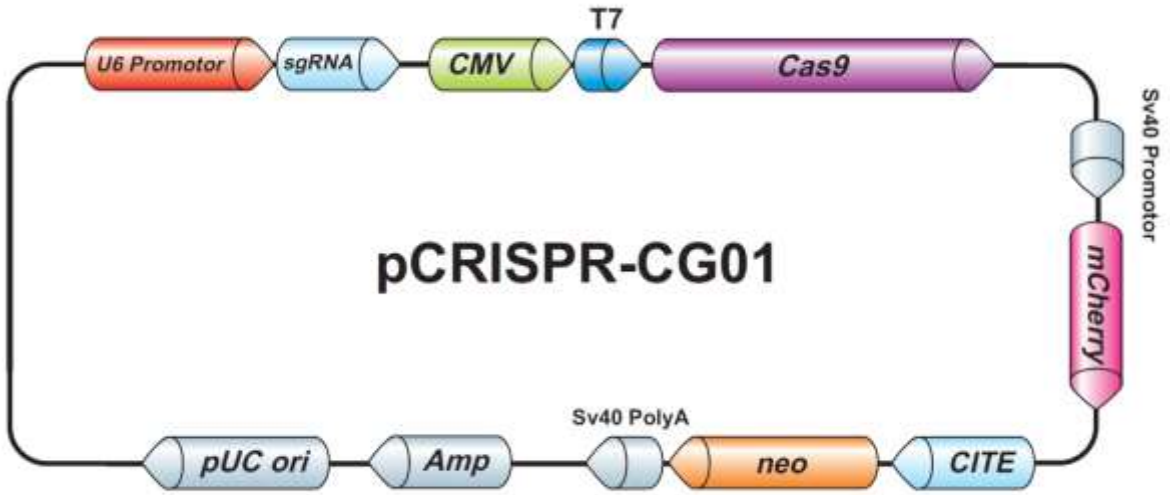
Step 3: Transfection or transduction

Transfection



- Plasmids can be transfected with standard methods
- Use GeneCopoeia Endofectin™ transfection reagents, or whichever works for you
- Carry selectable markers and reporter genes

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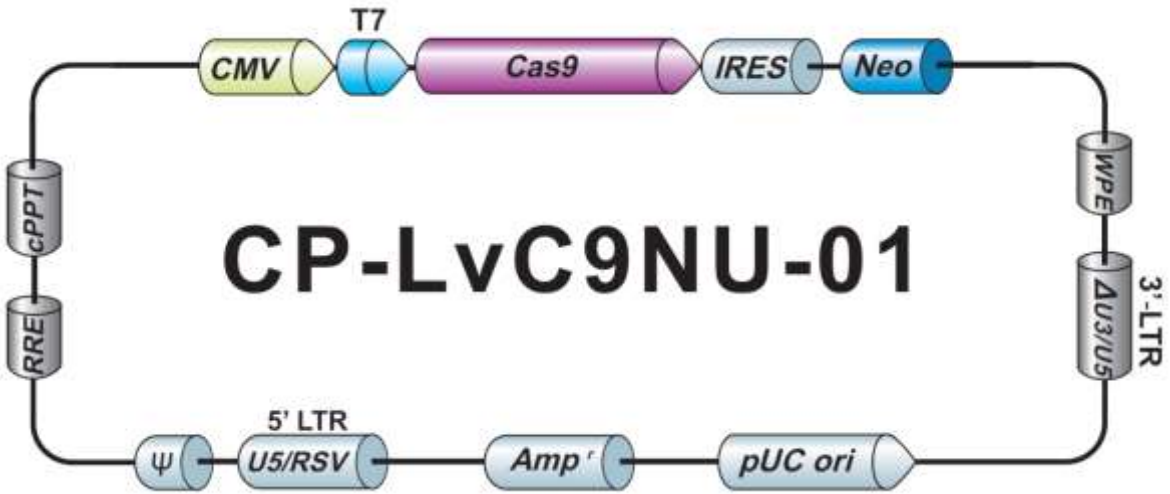
Step 3: Transfection or transduction

Transduction



- Plasmids are compatible with 3rd generation packaging cells
- Use GeneCopoeia Lentifect™ packaging reagents, or whichever works for you
- Carry selectable markers and reporter genes

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Step 4: Clonal isolation

Isolate single clones post-transfection

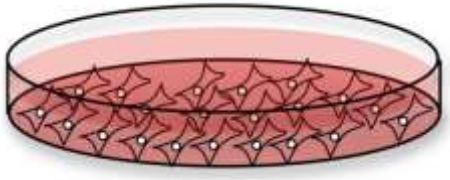

Plate for single colonies
and pick off dish

OR

Do serial dilutions in
multi-well plates

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

Outline

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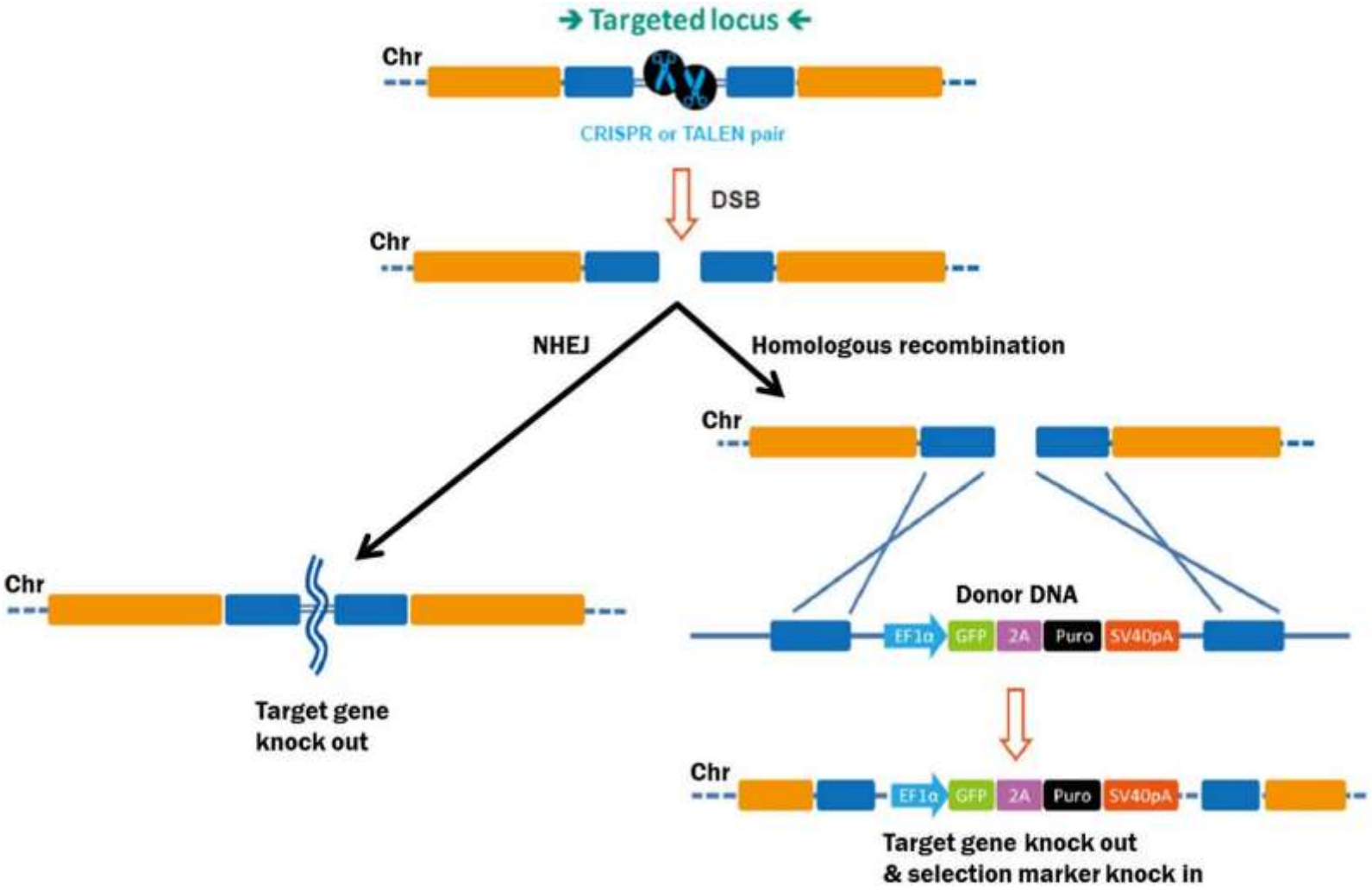
Step 5: Screening

IMPORTANT:

Connect genotype to phenotype!

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

Step 5: Screening



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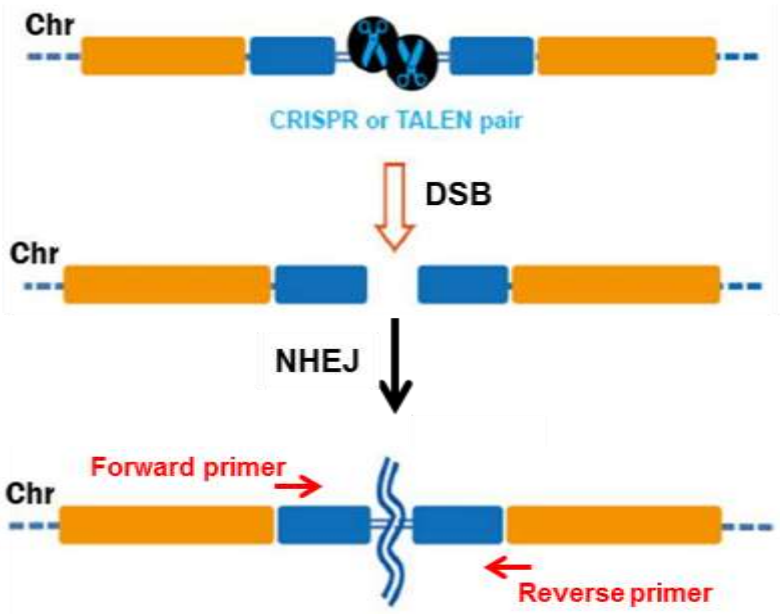
Step 5: Screening

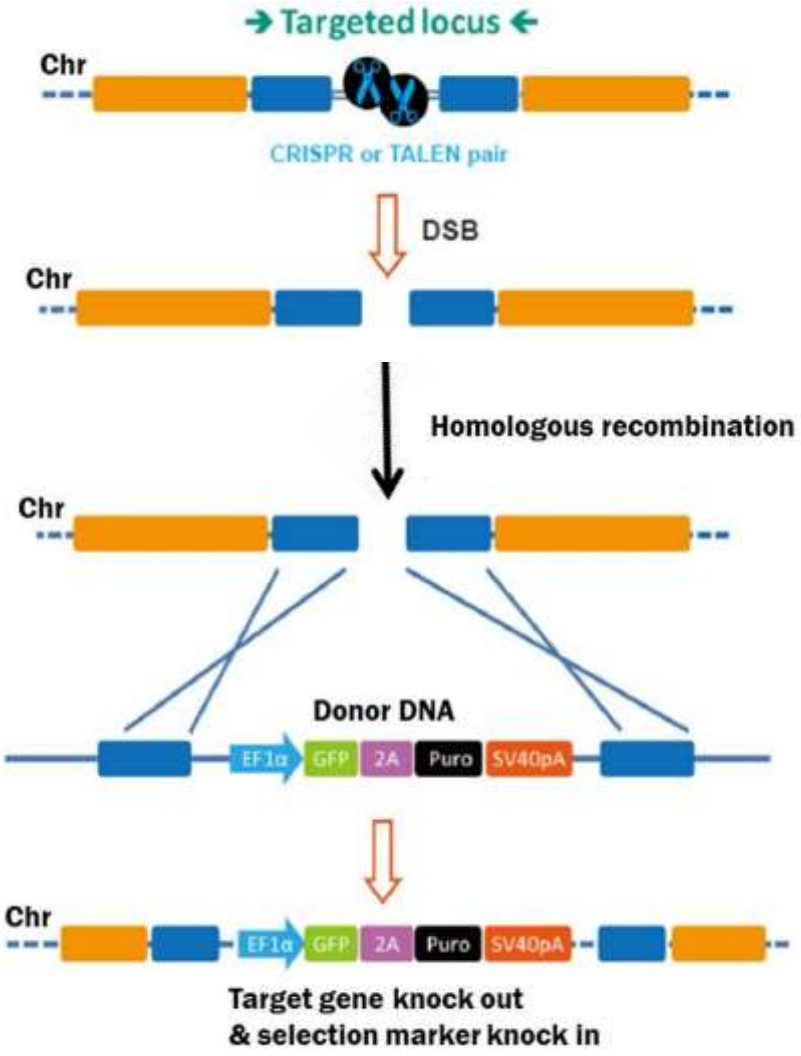
NHEJ-mediated mutations (knockouts)



- 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
- Re-screen positive clones by direct sequencing

Step 5: Screening

HDR-mediated applications using donor plasmids



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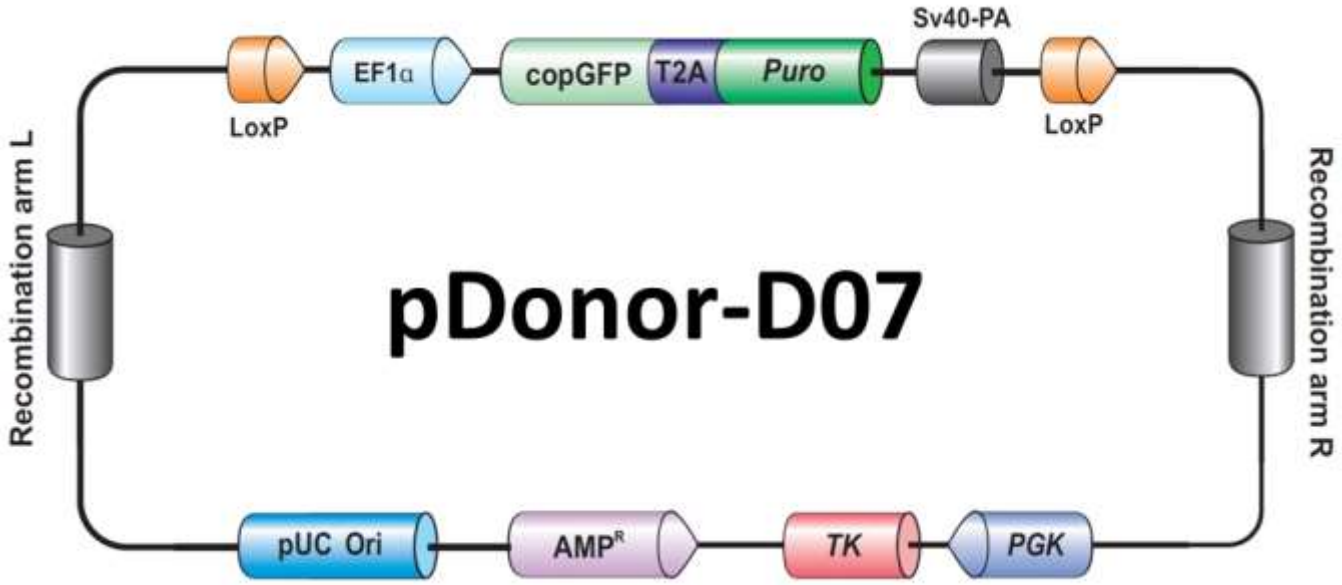
Step 5: Screening

HDR-mediated applications using donor plasmids



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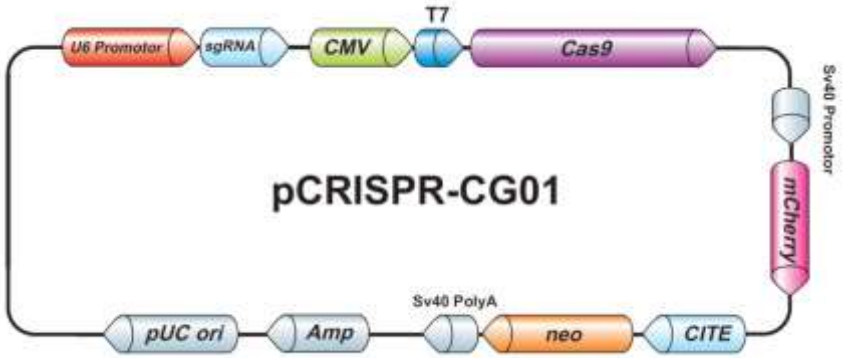
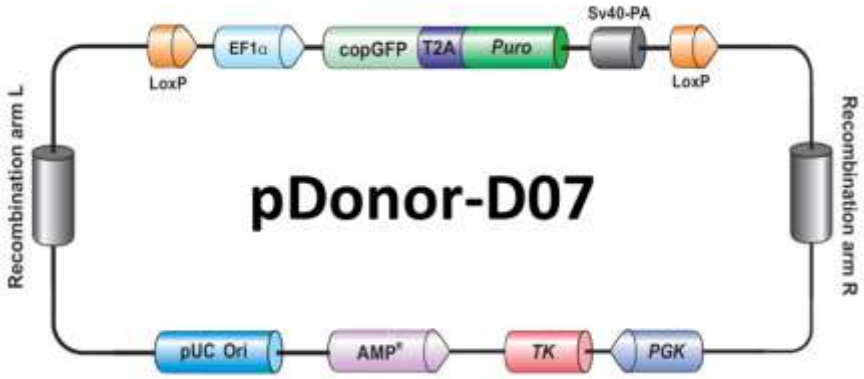
Step 5: Screening

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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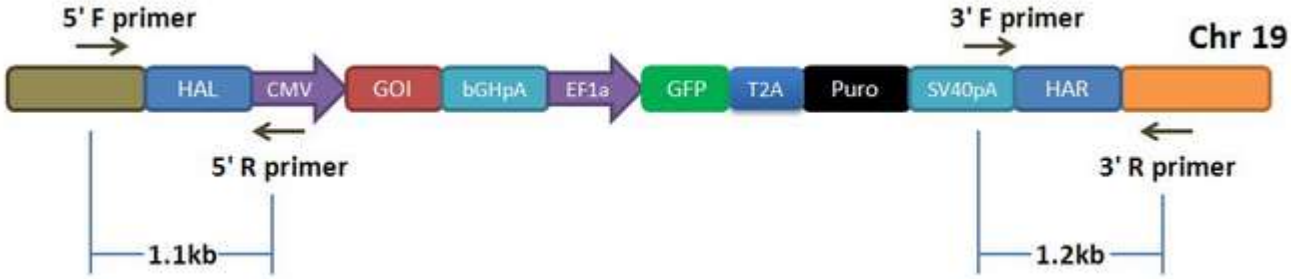
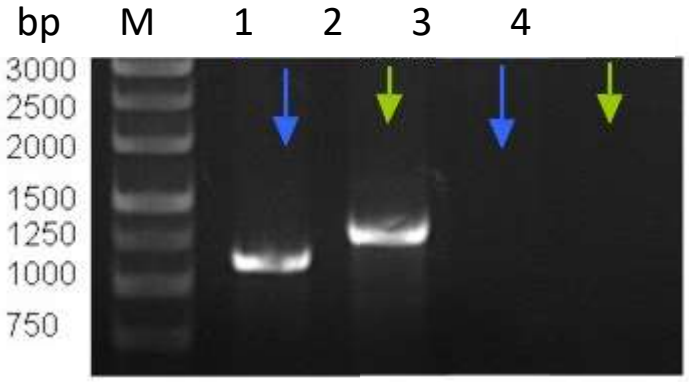
Step 5: Screening

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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Things to look out for!

Transfection efficiency

- Percentage of cells that take up plasmids influences modification rate
- Expect to screen 2x as many clones for a cell line with 40% transfection efficiency as you would for one with 80% transfection efficiency
- Recommend pre-determining transfection efficiency, or use reporter genes on GeneCopoeia plasmids

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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Summary

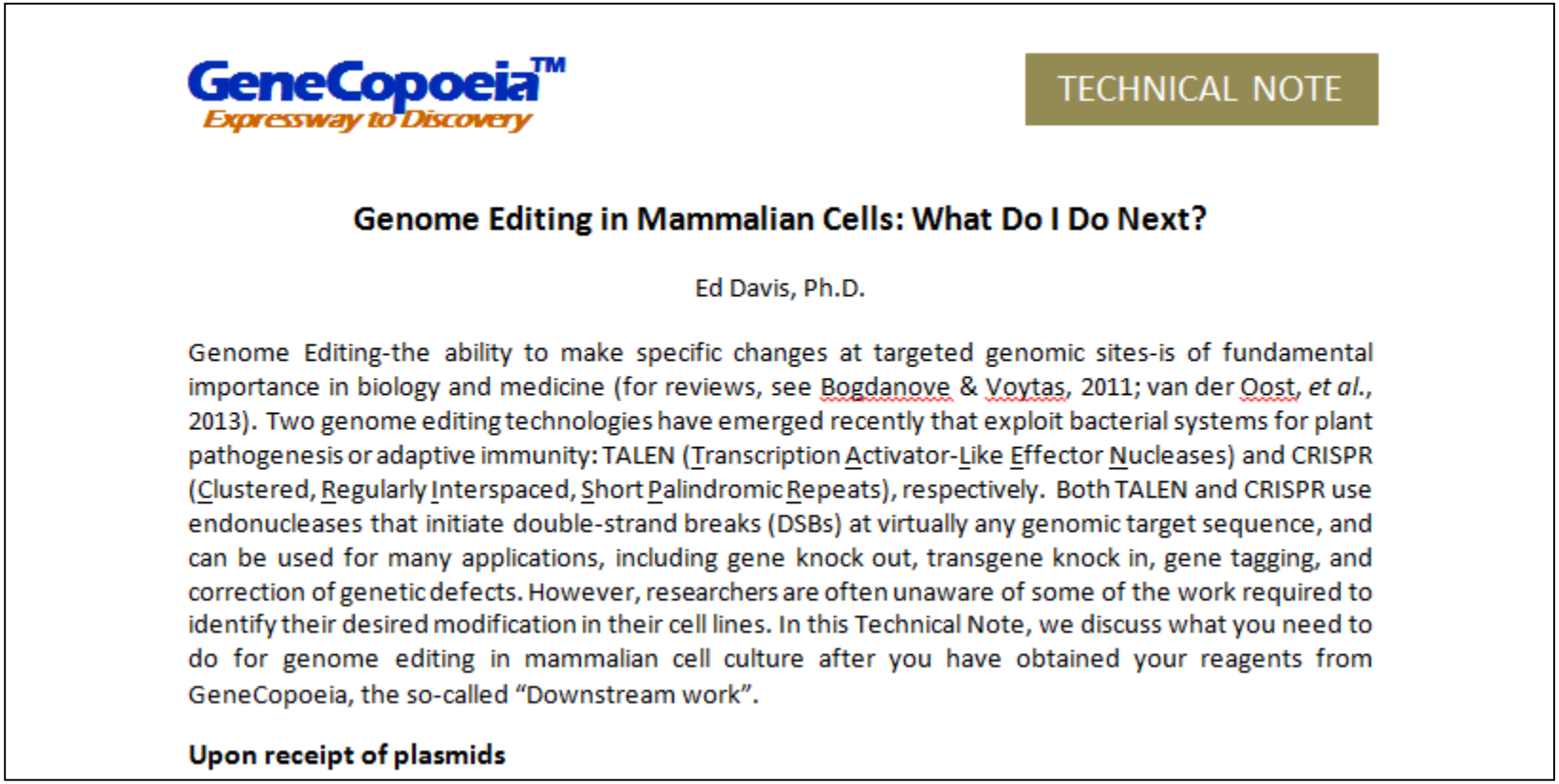
- The workflow for genome editing experiments must be carefully considered, and differs dramatically from that of RNAi

- 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

GeneCopoeia genome editing services

GeneCopoeia Application Note: Downstream work



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TECHNICAL NOTE

Genome Editing in Mammalian Cells: What Do I Do Next?

Ed Davis, Ph.D.

Genome Editing-the ability to make specific changes at targeted genomic sites-is of fundamental importance in biology and medicine (for reviews, see Bogdanove & Voytas, 2011; van der Oost, *et al.*, 2013). Two genome editing technologies have emerged recently that exploit bacterial systems for plant pathogenesis or adaptive immunity: TALEN (Transcription Activator-Like Effector Nucleases) and CRISPR (Clustered, Regularly Interspaced, Short Palindromic Repeats), respectively. Both TALEN and CRISPR use endonucleases that initiate double-strand breaks (DSBs) at virtually any genomic target sequence, and can be used for many applications, including gene knock out, transgene knock in, gene tagging, and correction of genetic defects. However, researchers are often unaware of some of the work required to identify their desired modification in their cell lines. In this Technical Note, we discuss what you need to do for genome editing in mammalian cell culture after you have obtained your reagents from GeneCopoeia, the so-called "Downstream work".

Upon receipt of plasmids

Download from:

http://www.genecopoeia.com/wp-content/uploads/2015/07/Downstream-work-07.pdf

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Upcoming webinar!

Applications For Cas9 Stable Cell Lines

Wednesday, March 22, 2017 12:00 pm ET

Register here:

https://attendee.gotowebinar.com/register/2505334381520479747

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Thank you!

If you have any additional questions, please call

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Email: edavis@genecopoeia.com

Or visit us on the web:

www.genecopoeia.com

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