

Strategies For Effective CRISPR-Mediated Gene Modification

December 3, 2018

Presenter:

Ed Davis, Ph.D. 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



GeneCopoeia Products and Services

Functional Genomics & Cell Biology

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



GeneCopoeia Products and Services

Functional Genomics & Cell Biology





GeneCopoeia CRISPR products for cell lines





Outline

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



Outline

CRISPR technologies & applications

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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.



Targeted DNA editing by DSB induction





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 <i>Acidaminococcus sp. BV3L6</i> or <i>Lachnospiraceae bacterium ND2006</i> . 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





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.



Gene knockout-large deletion





Gene mutagenesis-HDR



Modified chromosome

ATCACCTCAACCGGCCAGGATTCCACCACCAGCCAGCGAAGAAGTAGGCAGAACCCCCACGCCCCCAGGACTCCAGGACTCCAGTGTCACTTCG TACTGGAGTTGGCCGGTCCTAAGGTGGTGTTGGTCCGTCGCCTTCTTCATCCGTCTTGGGGGGTCCGCGGGGGGGTCCCTGAGGTCACAGTGAAGCcactccgggatctgggcgggact



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



Gene tagging





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 physiologicallevel expression.



Transgene and marker cassette knocked in

Gene activation or repression



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



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CRISPR technologies & applications

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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.



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Delivery method 1: Cas9/sgRNA plasmid DNA



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



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



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



Coming soon! GeneHero[™] Cas9 nuclease



 Transfection of HEK293 cells using EndoFectin[™] Max



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



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 doit-yourself packaging or ready-to-use particles
- Recommend that you first establish or obtain Cas9-stable cell line



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



GeneCopoeia Technical Note: Cas9 stable cell lines



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 chimeric circle quide RNA (coRNA) meteors a double strand break (DSR) is abareased DNA.

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



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*



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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



Workflow: Preparation



Expresswav to Discovery





Workflow: Preparation



Expressway to Discovery

Workflow: Preparation



GeneCopoeia Expressway to Discovery

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	Product Type:	-				paren.		1.1						
	ORF cDNA		Product	Product ID	Accession	Symbol	Alias	Spe	cies D	lescription				
	CRISPR shRNA Gene qPCR Primers mIRNA target Promoter ORF Knock-in TALE-TF Format: Clone Lerstwiral Particle AAV Particle Species: Human Mouse Rat Reset	ORF cDNA clones	A0448	NM_001291897.1	INS	IDDM, IDDM1, IDDM3 IRDN, MODY10	/2, ILPR, Human		iomo sapiens insulin NS), transcript variant 4, nRNA.					
		ORF cDNA clones	H5101	NM_202003.2	FOXM1	FKHL16, FOXM18, H HFH11, HNF-3, INS-1 MPHOSPH2, MPP-2, PIG29, TRIDENT	IFH-11, 1, MPP2, Hum	uan b a	omo sapiens forkhead ox M1 (FOXM1), ranscript variant 3, mRNA.					
			ORF cDNA. ciones	H9190	NM_021953.3	FOXM1	FKHL16, FOXM1B, H HFH11, HNF-3, INS-1 MPHOSPH2, MPP-2, PIG29, TRIDENT	(FH-11, 1, MPP2, Hum	tan b	Homo sapiens forkhead box M1 (FOXM1), transcript variant 2, mRNA				
			ORF cDNA clones	N0607	NM_001243088.1	FOXM1	FKHL16, FOXM18, H HFH11, HNF-3, INS-1 MPHOSPH2, MPP-2, PIG29, TRIDENT	IFH-11, I. Hum MPP2, Hum	han bi	iomo sapiens forkhead ox M1 (FOXM1), ranscript variant 4, mRNA.				
			ORF cDNA clones	10608	NM_001243089.1	FOXM1	FKHL16, FOXM18, H HFH11, HNF-3, INS-1 MPHOSPH2, MPP-2, PIG29, TRIDENT	FKHL16, FOXM18, HFH-11, HFH11, HNF-3, INS-1, MPHOSPH2, MPP-2, MPP2, PIG29, TRIDENT						
			ORF cDNA clones	T8218	80006192.2	FOXM1	FKHL18, FOXM18, H HFH11, HNF-3, INS-1 MPHOSPH2, MPP-2, PIG29, TGT3, TRIDE	IFH-11, 1. MPP2, Hum NT	tan d	iomo sapiens forkhead ox M1, mRNA (cDNA kone MBC: 10704 MAGE:3833837).				
			ORF cDNA clones	U1376	083113.1	FOXM1	FKHL16, FOXM18, H HFH11, HNF-3, INS-1 MPHOSPH2, MPP-2, PIG29, TGT3, TRIDE	IFH-11. 1. Hum MPP2, Hum NT	san H	luman INS-1 winged-helix omolog mRNA.	c	hat lh	na mon	m 1
			ORF cDNA clones	23073	NM_001042376.2	INS-IGF2	INSIGF	Hum	ham m	lomo sepiens INS-IGEO eadthrough (INS-IGF Or	nline	0	2	1



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	Product Type:			In stores				adarun.					
	ORF cDNA CRISPR shRNA Gene qPCR Primers miRNA target Promoter ORF Knodk-in	Product	Product ID	Accession	Symbol	Alias	Species	Description	- 1				
		CRISPR clones	HTN209749	NM_000207.2	INS	IDDM, IDDM1, IDDM2, ILPR, IRDN, MODY10	Human	insulin	_				
		RNA ne qPCR Primers RNA target smoter RF Knock-In		HTN255592	NM_202002.2	FOXM1	FKHL15, FOXM1A, FOXM1B, FOXM1C, HFH-11, HFH11, HNF-3, INS-1, MPHOSPH2, MPP-2, MPP2, PIG29, TRIDENT	Human	forkhead box M1				
	TALE-TF		CRISPR clones	HTN257953	NM_001185097.1	INS	IDDM, IDDM1, IDDM2, ILPR, IRDN, MODY10	Human	insulin				
	Clone Cone AAV Particle AAV Particle	CRISPR	HTN257954	NM_001185098.1	INS	IDDM, IDDM1, IDDM2, ILPR, IRDN, MODY10	Human	insulin					
		CRISPR	HTN266992	NM_001243069.1	FOXM1	FKHL18, FOXM1A, FOXM18, FOXM1C, HFH-11, HFH11, HNF-3, INS-1, MPHOSPH2,	Human	forkhead box M1					
	Species:		ciones				MPP-2, MPP2, PIG29, TRIDENT						
	Mouse Rat		CRISPR	HTN266993	NM_001243086.1	FOXM1	FKHL16, FOXM1A, FOXM1B, FOXM1C, HFH-11, HFH11, HNF-3, INS-1, MPHOSPH2, MPP-2, MPP2, PIG29	Human	forkhead box M1				
	Reset						TRIDENT			_			
			CRISPR clones	HTN270766	NM_001291897.1	INS	IDDM, IDDM1, IDDM2, ILPR, IRDN, MODY10	Human	insuln				
			CRISPR dones	HTN296566	NM_021953.3	FOXM1	FKHL15, FOXM1A, FOXM1B, FOXM1C, HFH-11, HFH11, HNF-3, INS-1, MPHOSPH2, MPP-2, MPP2, PIG29, TRIDENT	Human	forkhead box M1	C	Chat IIv	ne now	Ļ
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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



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



Smart-Join[™] Blunt-end PCR Cloning Kit

<u>Workflow</u>





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



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



IndelCheck[™] CRISPR indel detection system







Workflow: Preparation

Step 4: Copy number determination

- ✤ For complete knockouts or mutagenesis, might need to modify <u>all</u> 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





Application: Using FISH with a CRISPR knockout

Clone 1

	(500)	500 510	520	530	540	550	560	570	580	590	600	610	620	635
HDAC6 Targeting region NC 018934	C2-4_PREMIX (471)	TICACAGAAGCGAA	TATTAAAAAA	DOAGCOGTIC	COCOCITITATO	COCAATV TAC	AGGCCTCCTC	GAGCGGATGT	ACCCCGAOGA	ACGEOGCOOL	AGSTAAAD	AAGAAAGGCAA	ANTGAAGAAG	CTOGGCCAR
Allele a	C2-6_PREMIX (470)	TICACAGAAGCGAA	TATTAAAAA		*********							AAGAAAGGCAJ	ANTGANGANG	CTOGGCCAA
Allele b	C2-11_PREMIX (469)	TTCACAGAAGCGAA	TATTAAAAA	GGAGCCGTTC	000	********				********	AAAG	AAGAAAGGCAA	MATGAAGAAG	CTOSSCOA
Allele c	C2-7_PREMIX (472)	TTCACAGAAGCGAA	TATTAAAAA	BLAGCOUTIO	COLGCTULATO	CITCRATY				G	AGGTAAAG	AAGAAAGGCAA	AATGAAGAAG	CTOGGCCAN
Allele c	2A-F_PREMIX (470)	TTCACINGNAGCGNAI	TATTAAAAA	GGAGCCGTTC	OCCISC TOTATO	CCCAATC					REGTARAG	AAGAAAGGCAA	ANTGAAGAAG	CTOGGOCAA
Allele d	C2-5_PREMIX (470)	TTCACAGAAACCGAA	TATTAAAAAI	BGAGCOGPTC	constitution	COCAAD TA	G		*******		AG ZARAG	AAGAAAGGEAJ	ANTGAAGAAG	CITOGROCAR
Allele d	C2-8_PREMIX (474)	TICKINGAAGCGAAJ	TATTAAAAA	BUMGCOGTTC	COCGCTCTATO	CCCANTCENE	G			<mark>('6(</mark>	ROGENAAAG	ANGARAGOCAI	ANTGANGANG	CTCGGCCAA
Allele d	2B-F_PREMIX (472)	TTCACAGAAOCGAA	TATTAAAAAA	GGAGCUGTTO	COCOCTCTATO	CUCAATCYAG	G			CG	AGGTAAAG	AAGAAAGGEAA	ANTGAAGAAG	CTOGGOCAR
Allele d	2C-F_PREMIX (470)	TTCRCNGRAGCGAM	TATTABAAAA	GGAGCCETTC	OCCOUNT ATO	COCANTITAG	G				AGGTAAAG	AAGAAAGGCAI	WATGAAGANG	CTOSSOCAA
Allele e HDAC6	Targeting Region NC_018934 (500)	TTCACAGAAGCGAU	TATTAAAAA	GGAGCOGTIC	OCCOCTCTATO	COCAATCING	C			G	AGGTAAAAG	AAGAAAGGCAJ	WATGAAGAAG	CTOGGOCAR
Consensus	Consensus (500)	TICACAGAAGCGAAJ	TATTAAAAAA	GGAGCCGTTC	CCCGCTCTATC	COCAATCTAG				CG	AGGTAAAG	AAGAAAGGCAA	AATGAAGAAG	CTOGGOCAA

Clone 2

	(553)	553	560	570	580	590	600	610	620	630	640	650	660	670	688
HDAC6 Targeting region NC 018934	3-11_PREMIX (487)	TAATAA	GACCOST	TO CERT	CIATOCOCAN	PCTAGAGGCC	TCCTCCGAGC	XIGATGTACCCC	GAGGACGGCC	CCCCGGAGG	MAGNIGAN	CAMATCA.	SANG <mark>CT</mark> E	GOCIAGCAAT	REAAGAAGAAG <mark>ACCTIA</mark>
Allele f	C3-8_PREMIX (516)	GCATTA	TCAATCOG	C <mark>C</mark> AA <mark>CI (</mark> G	COGGGGAGAGO	CGGTTTG	C	STATIGGCCCC	TCTTOCGCTJ	CCTCGCTCA	TGACICOCIG	SCTOSE <mark>T</mark> CG	PTCG CTG	/C <mark>GGCGNOC</mark> GG <mark>1</mark> /	ATCA CTCNCTCA
Allele g	C3-6_PREMIX (487)	TRABAN	IGGA ICCET	T <mark>0000</mark>	*********	*********		*********			MAGAAGAAM	CAMATGA	AGAANCT'C	EGOCIATCANT	SGAAGAAG<mark>AC</mark>UUA
Allele g	3C-F_PREMIX (487)	таалан	IGGACCONT	2 <mark>0:0</mark> 2		0000000				100000	AAAGAAGAAA	CAAAA <mark>T</mark> TA	KAAGCTC	GCCAACCAAT	REAAGAAG <mark>ACCEA</mark>
Allele h G	3-10_PREMIX (487)	TAAAAK	GACCOGT	100000CT	CIATCCGC					GGAGG	'AA <mark>A</mark> GAA <mark>G</mark> AAAI	CARARANCA.	HGANG <mark>CT</mark> C	IG <mark>GOCAADCAAT</mark>	SCAAGAAG <mark>ACCTA</mark>
Allele i	3A-F_PREMIX (487)	TAAAAA	SA CG	Q OPCI							-АА <mark>А</mark> БАЛ <mark>Б</mark> ААЛ	GCNRAA <mark>T</mark> CA	AGAAG <mark>CT</mark> C	IS <mark>GC(AACCAAT</mark>)	SCAN-ANNACCUA
Allele i	3B-F_PREMIX (492)	TRAAAM	GACCET	10 CT							-AA <mark>A</mark> EAA <mark>G</mark> AAA	CANAA CA	KGANG <mark>CT</mark> U	IS GOCKADCAAT	SEAAGAAG <mark>ACITTA</mark>
Allele i	C3-4_PREMIX (488)	TAAAAA	COLOCIE COL	2 <mark>0. d</mark> ant							-AA <mark>A</mark> GAA <mark>G</mark> AAAI	CAAAA IGA	KGANG <mark>CT</mark> C	IGGUGNAGCART I	RGAAGAAG <mark>ACICTA</mark>
Allele i	C3-5_PREMIX (487)	TAAAAA	GACCORT	d de la							AA <mark>A</mark> GAA <mark>G</mark> AAA	CANAATGA	KIANG T C	GGCCAAGCAAT	SEAASPAC <mark>ACE TA</mark>
Allele i	C3-9_PREMIX (488)	TAAAAA	GGAGCCGI	7 <mark>0.0</mark> 0601							-MAGAAGAAA	SCRARATGA	AAAAAC	ISGOOGASCAAT	SGAAGAAG <mark>AC</mark> U I <mark>A</mark>
Allele iHDAC6 Targeting Regio	n NC_018934 (518)	TAALAA	CENCOST	Concerned and	CIATOCOCAA	ICTAG				CGGAGG	AAAGAAGAAM	BANAN TA	A ANALIC	FRECANCEAST	ERAGRAC <mark>ACCTA</mark>
Consensus	Consensus (553)	TAAAAA	JOGADCCGT	TOCCOGCI	200						AAAGAAGAAA	GCAAAATGA	AGAAGCTC	GGCCAAGCAAT	SGAAGAAGACCTA



GeneCopoeia Application Note: FISH with CRISPR



Download from:

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



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Isolate single clones post-transfection/transduction



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





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



Expressway to Discovery



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





HDR-mediated applications using donor plasmids





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





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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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)



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



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 noncanonical 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!



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



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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