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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 chimeric single guide RNA (sgRNA), makes a double strand break (DSB) in chromosomal DNA (Jinek, *et al*. 2012). Creation of a DSB is the necessary initiation event for most CRISPR-mediated genome editing applications. Alternative variations of SpCas9, such as a nuclease-dead version fused to transcriptional effectors or base editors, Cas9s from other species, or nucleases that cleave RNA, have also been employed for genetic modulation.

Why use a Cas9-expressing stable cell line?

In cell line-based genome editing, Cas9 and sgRNA are typically introduced into cells using transient transfection of either DNA or RNA, or Cas9 protein:sgRNA ribonucleoprotein (RNP) complexes. Frequencies of DSB formation, as measured in insertion/deletion (indel) assays, often exceed 60% (Fu, et al., 2013). Therefore, transfection of cell lines that do not have Cas9 previously integrated is usually sufficient for gene knockouts resulting from nonhomologous end joining (NHEJ), and for applications requiring donor-mediated, homology-directed repair (HDR), including gene knockout, gene mutagenesis, gene tagging, and Safe Harbor transgene knock-in.

There are additional applications that, while it is not required, are enhanced by having Cas9 stably integrated into the genome: 1) gene knockout using lentiviral CRISPR; 2) sgRNA library screening; 3) sgRNA functional validation; and 4) inducible gene editing. Each of these applications is discussed below.

Application 1: Gene knockout using lentiviral CRISPR

While transfection is commonly used for cell line-based genome editing, some cell lines do not transfect well using chemical-, lipid-, or electroporation-based approaches. For these cell lines, researchers often turn to lentiviral-mediated CRISPR delivery, because lentiviruses efficiently

infect a very large number of mammalian cell types. It is possible to express both Cas9 nuclease and an sgRNA from the same lentivirus, as has been shown previously (Sanjana, *et al.*, 2014), or to co-transduce two different lentiviruses, one expressing Cas9 and the other expressing sgRNA. However, the titers of such "all in one" or Cas9-only lentiviral CRISPR viruses tend to be significantly lower than that achieved from sgRNA-only lentiviral vectors due to the large (>4.4 kb) size of the Cas9 gene. It is for this reason that GeneCopoeia recommends, for genome editing using lentiviral CRISPR applications, that scientists transduce a Cas9-expressing stable cell line with sgRNA-only lentiviruses.

GeneCopoeia provides two options for lentiviral-based CRISPR-Cas9 tools. In the first option (the "DIY", or do-it-yourself option), you can purchase lentiviral-based sgRNA-expressing plasmids using our online gene search tool, or by requesting a quote. GeneCopoeia will design and construct the plasmids for you. Once you receive your plasmids, you can package them into lentivirus particles yourself using GeneCopoeia's Lenti-Pac™ packaging kits.

In the second option, GeneCopoeia will package your sgRNA lentiviral plasmids into particles for you. These particles are high titer (>10^8 TU/ml) and ready-to-use. More information is available on the GeneCopoeia lentiviral particle website. In any case, whether you are producing your own lentivirus, or having GeneCopoeia make it for you, you would use your lentiviral particles to infect a cell line stably expressing Cas9, then screen for your genetic modification and phenotype.

Application 2: sgRNA library screening

One of the most important applications for Cas9-expressing stable cell lines is sgRNA library screening. CRISPR sgRNA libraries are collections of hundreds-to-thousands of plasmids, each expressing a unique sgRNA, and are highly useful for forward mutagenesis screens (Sanjana, et al, 2014). GeneCopoeia carries pre-made CRISPR sgRNA libraries that cover several well-studied pathways and gene groups, and also offers custom sgRNA library construction. For more information, please visit our CRISPR sgRNA library page.

CRISPR sgRNA libraries enable researchers to knock out many genes simultaneously in mammalian cells, opening the door to high-throughput drug target identification and validation, phenotypic changes, and reporter assays. Previously, these applications were achieved using short hairpin RNA (shRNA)-based RNA interference (RNAi). However, while shRNA libraries are useful for high-throughput loss-of-function screens, RNAi has several disadvantages compared with CRISPR: 1) RNAi causes knockdown of gene expression, not knockout, so false negatives resulting from residual gene expression can be missed; 2) RNAi acts only on cytoplasmic RNA, and so cannot silence nuclear RNAs like long non-coding RNAs. Conversely, CRISPR makes permanent changes to the genetic code. Thus, CRISPR can make a complete knockout of all alleles of a gene, whether their transcription products are localized to the nucleus or cytoplasm. So, CRISPR sgRNA library screening can be straightforward, provided you have a good assay or phenotype for screening (Figure 1).

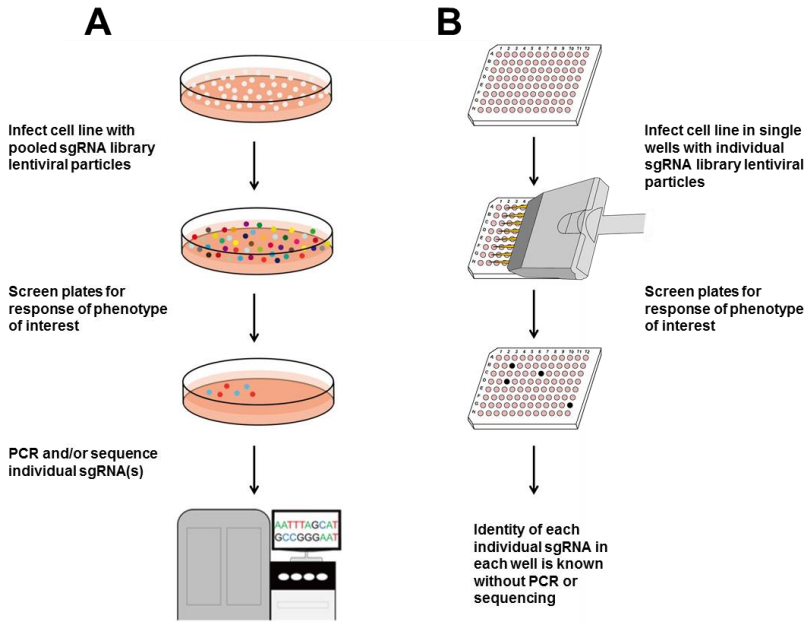

Figure 1. Workflow for CRISPR sgRNA libraries. A. Pooled screen. Cells infected with each sgRNA library pool are screened for the desired readout. Pooled cells are subjected to Sanger sequencing for individual sgRNAs, or deep sequencing to look for over- or under-representation of individual sgRNAs. B. GeneCopoeia sgRNA libraries are available either as pools or individual sgRNAs. Cells are infected with individual sgRNA lentiviruses. Wells are screened for the readout of interest. Individual sgRNAs corresponding to the phenotype of interest are already **known without sequencing.**

CRISPR sgRNA libraries are best suited for lentiviral delivery rather than plasmid transfection, because lentiviruses provide higher delivery efficiency. However, because the titer of Cas9-expressing lentiviruses is lower than sgRNA-expressing lentiviruses, most sgRNA libraries, including GeneCopoeia sgRNA libraries, are built without Cas9 in the vector. We recommend using our sgRNA libraries in Cas9-expressing stable cell lines.

One example of how useful GeneCopoeia's Cas9 stable cell lines are for sgRNA library screening comes from a paper published by Prashant Mali's group at the University of California at San Diego (Shen *et al*., 2017), who used GeneCopoeia Cas9 stable cell lines with combinatorial sgRNA libraries to identify synthetic interactions among 73 cancer-associated genes. Most of these interactions were subsequently validated by drug treatments.

After their initial study, Mali's group went on further to use sgRNA libraries in GeneCopoeia Cas9-expressing HeLa and A549 cell lines to knock out 51 genes encoding oxidative phosphorylation enzymes singly and in pairwise combinations (Zhao, *et al.* 2018). The researchers found, among both cell lines, 25 novel cases in which a single gene knockout had little to no effect on cell growth, whereas a pair of genes caused a significant growth defect. In addition, investigators observed that some single gene knockouts had a more severe growth phenotype in HeLa cells than in A549 cells. This difference in phenotype was due to the revelation that A549 cells, and not HeLa cells, carry a mutation in KEAP1, which encodes an E3 ubiquitin ligase that is mutated in many cases of lung cancer and has been implicated in resistance to chemotherapy. Loss of KEAP1 causes an elevation in the levels of some enzymes involved in oxidative phosphorylation, resulting in resistance to single oxidative phosphorylation gene knockout. The authors concluded that design of anticancer drugs targeting oxidative phosphorylation enzymes needs to be done in consideration of the KEAP1 mutational status of the cancer.

Application 3: sgRNA functional validation

Cell line-based CRISPR genome editing projects typically require several (>3) months of work, including transfection, clone isolation, and screening. While CRISPR typically provides a highly efficient method for genome editing, the individual sgRNAs themselves do exhibit variations in efficiency due to the nature of individual target sites. Therefore, it would be helpful to determine which CRISPR sgRNAs have the highest potential for successful genome editing before doing lots of screening work.

We recommend the mismatch cleavage assay (Qiu, et al., 2004) for sgRNA functional validation in a Cas9 stable cell line. GeneCopoeia's IndelCheck™ mismatch cleavage assay is a cell culture-based assay designed to efficiently detect indels resulting from DSB-mediated NHEJ. The system includes a high-efficiency Taq DNA polymerase for amplifying the target site, as well as T7 Endonuclease I for cleaving heteroduplex DNA produced from annealing CRISPR-modified DNA with unmodified DNA. The basic workflow for the IndelCheck™ mismatch cleavage assay for sgRNA functional validation is illustrated in Figure 1.

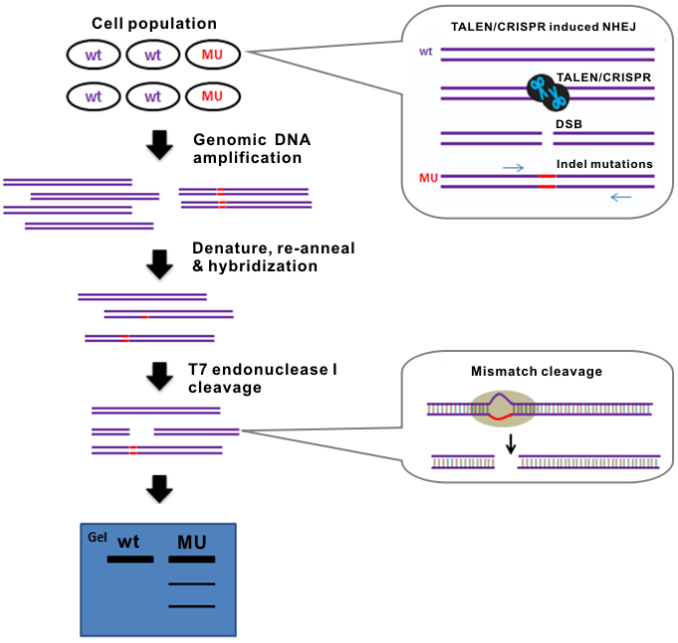

Figure 1. Workflow of the IndelCheck™ mismatch cleavage assay. A population of cells that has been exposed to CRISPR components is subjected to genomic DNA PCR using primers flanking the target site. Denaturation and reannealing of the PCR products creates a mixed population of homoduplex and heteroduplex molecules. The fragments are treated with T7 Endonuclease I, which cuts only the heteroduplex molecules. Cleavage products are detected by standard agarose gel electrophoresis.

Functional validation of CRISPR sgRNAs can be performed in either a commonly used, "model" cell line (*e.g.* HEK293), or in your own cell line. GeneCopoeia has many pre-made human, mouse, and rat Cas9-expressing stable cell lines in widely-used backgrounds (Table 1). Alternatively, you can purchase CRISPR reagents from us that allow you to readily build your own Cas9-expressing stable cell line.

Application 4: Inducible genome editing

CRISPR is commonly used to generate genome modifications in a constitutive fashion, without regard to a specific time or stage in cell growth. Sometimes, though, it is useful to introduce the modification after a particular event has occurred, such as treatment of cells with a compound, or at a specific time during differentiation. Inducible CRISPR (iCRISPR) was first demonstrated

by Gonzalez, et al. (2014), who built an induced pluripotent stem cell (iPSC) model cell line carrying Cas9 that can be expressed after addition of the tetracycline analog doxycycline.

GeneCopoeia Cas9 stable cell lines

GeneCopoeia carries a large suite of human, mouse, and rat cell lines stably expressing the SpCas9 nuclease or a high-fidelity SpCas9 variant (SpCas9-HF1) (Table 1).

Catalog #	Cell line	Cell type	Selection marker	Cas9 integration site
Human cell lines stably expressing *S. pyogenes* Cas9 (SpCas9)				
SL502	HEK293	Embryonic kidney	Puro	AAVS1
SL554	HEK293	Embryonic kidney	Hygro	AAVS1
SL501	NCI-H1299	Lung cancer	Puro	AAVS1
SL533	NCI-H1299	Lung cancer	Hygro	Random
SL504	A549	Lung cancer	Hygro	AAVS1
SL529	H1975	Lung cancer	Hygro	Random
SL536	NCI-H1437	Lung cancer	Hygro	Random
SL561	NCI-H661	Lung cancer	Hygro	Random
SL578	HCC827	Lung cancer	Hygro	Random
SL503	HeLa	Cervical cancer	Hygro	AAVS1
SL514	MCF-7	Breast cancer	Hygro	AAVS1
SL524	MCF-7	Breast cancer	Hygro	Random
SL515	MDA-MB-231	Breast cancer	Hygro	Random
SL516	MDA-MB-468	Breast cancer	Hygro	Random
SL517	T47D	Breast cancer	Hygro	Random
SL535	SK-BR-3	Breast cancer	Hygro	Random
SL544	HCC70	Breast cancer	Hygro	Random
SL547	DU4475	Breast cancer	Hygro	Random
SL558	HCC1428	Breast cancer	Hygro	Random
SL562	CAMA-1	Breast cancer	Hygro	Random
SL563	HCC38	Breast cancer	Hygro	Random
SL574	AU-565	Breast cancer	Hygro	Random
SL565	HCC1500	Breast cancer	Hygro	Random
SL518	HepG2	Liver cancer	Hygro	AAVS1
SL522	SNU-475	Liver cancer	Hygro	Random
SL530	SNU-449	Liver cancer	Hygro	Random
SL531	PLC/PRF/5	Liver cancer	Hygro	Random
SL537	SNU-387	Liver cancer	Hygro	Random
SL538	SNU-423	Liver cancer	Hygro	Random
SL543	C3A	Liver cancer	Hygro	Random
SL557	SK-HEP-1	Liver/ascites cancer	Hygro	Random
SL520	AGS	Stomach/gastric cancer	Hygro	Random
SL526	SNU-1	Stomach/gastric cancer	Hygro	Random
SL527	KATO111	Stomach/gastric cancer	Hygro	Random
SL545	SNU-16	Stomach/gastric cancer	Hygro	Random
SL565	NCI-N87	Stomach/gastric cancer	Hygro	Random
SL521	BXPC-3	Pancreatic cancer	Hygro	Random
SL528	HPAF-II	Pancreatic cancer	Hygro	Random
SL540	CFPAC-1	Pancreatic cancer (derived from metastatic liver)	Hygro	Random
SL570	Panc 10.05	Pancreatic cancer	Hygro	Random
SL523	HT-29	Colon cancer	Hygro	Random
SL525	LoVo	Colon cancer	Hygro	Random
SL539	RKO	Colon cancer	Hygro	Random
SL541	T84	Colon cancer	Hygro	Random
SL542	COLO 205	Colon cancer (derived from metastatic site)	Hygro	Random

SL551	SNU-C1	Colon cancer	Hygro	Random
SL572	HCT116	Colon cancer	Hygro	Random
SL532	LS411N	Caecum cancer	Hygro	Random
SL583	Granta-519	Blood cancer	Hygro	Random
SL546	U-2 OS	Bone cancer	Hygro	Random
SL552	K562	Bone marrow cancer	Hygro	Random
SL555	Jurkat	Peripheral blood cancer	Hygro	Random
SL548	DU145	Prostate cancer	Hygro	Random
SL550	SH-SY5Y	Neuroblastoma	Hygro	Random
SL560	T24	Bladder cancer	Hygro	Random
SL566	HK2	Immortalized kidney cell line	Hygro	Random
SL577	786-O	Kidney cancer	Hygro	Random
SL576	1321N1	Brain astrocytoma	Hygro	Random
SL512	HT1080	Fibrosarcoma	Hygro	AAVS1
SL580	SK-OV-3	Ovary	Hygro	Random
Human cell lines stably expressing high-fidelity S. pyogenes Cas9 (SpCas9-HF)				
SL553	HEK293	Embryonic kidney	Hygro	AAVS1
Mouse cell lines stably expressing S. pyogenes Cas9 (SpCas9)				
SL509	Neuro2a	Neuroblastoma	Hygro	ROSA26
SL559	Neuro2a	Neuroblastoma	Hygro	ROSA26
SL510	Neuro2a	Neuroblastoma	Puro	ROSA26
SL511	Neuro2a	Neuroblastoma	Neo	ROSA26
SL564	C2C12	Muscle (myoblast)	Hygro	Random
SL567	BA/F3	Lymph	Hygro	Random
SL569	NIH-3T3	Embryonic fibroblast	Hygro	Random
SL581	4T1	Mammary gland	Hygro	Random
SL582	CT26	Colon	Hygro	Random
Mouse cell lines stably expressing high-fidelity S. pyogenes Cas9 (SpCas9-HF)				
SL571	Neuro2a	Neuroblastoma	Hygro	ROSA26
Rat cell lines stably expressing S. pyogenes Cas9 (SpCas9)				
SL534	C6	Glioma	Hygro	Random

Table 1. List of GeneCopoeia pre-made human, mouse, and rat Cas9 stable cell lines.

These cell lines provide several benefits and advantages for genome editing work, including:

- **Built-in, stable Cas9 expression.** Minimizes need for co-transfection/transduction of sgRNAs. Ideal for sgRNA library screening, lentiviral CRISPR, sgRNA validation, and inducible CRISPR.
- **Single clone isolation.** GeneCopoeia's Cas9 stable cell lines are derived from single cell clones, providing a uniform genetic background, unlike stable pools, which can vary in transgene expression and carry unwanted background mutations.
- **Functionally validated.** The function of Cs9 is validated for cleavage ability using the IndelCheck™ T7 Endonuclease I assay, assuring high quality.
- **Compatibility.** Compatible with GeneCopoeia Genome-CRISP™ sgRNA clones, sgRNA libraries, and donor clones.

Figure 3. Structure of a stably integrated CRISPR Cas9 nuclease construct. Cas9 or its derivatives is integrated into a chromosome either randomly via lentiviral transduction or by Safe Harbor knock-in under control of a constitutive promoter.

If you don't see your cell line of interest in Table 1, you can use either GeneCopoeia's Cas9 lentiviral clones, lentiviral particles, or Safe Harbor knock-in system to integrate the Cas9 gene yourself. GeneCopoeia's Safe Harbor knock-in system consists of kits containing CRISPR reagents targeting the human AAVS1 and mouse ROSA26 Safe Harbor sites, and can be ordered with many donors for HDR, such as those expressing Cas9 used for the cell lines in Table 1, or any of more than 45,000 human and mouse ORFs. More information about GeneCopoeia's Safe Harbor kits and knock-in ORF clones is available on GeneCopoeia's Safe Harbor Knock-in website.

At GeneCopoeia, our Genome Editing scientists have a wealth of expertise with CRISPR applications in mammalian systems. We start at CRISPR design and deliver sequence-verified plasmid DNA or lentiviral particles. We also offer functional validation kits and services for your CRISPR constructs, construct stable cell lines or transgenic mice containing your CRISPR-mediated modification of interest, and provide scientific consulting services to help you plan your projects.

Want to know more about Cas9 stable cell lines or to place an order? Visit our website, call 1-866-360-9531, or email inquiry@genecopoeia.com.

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