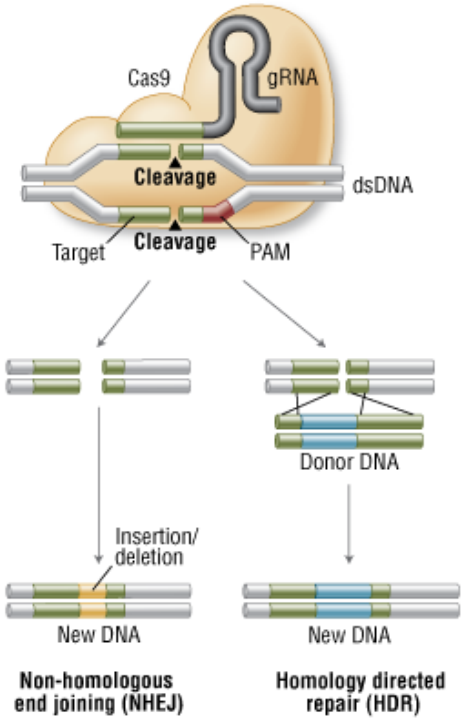
Biosafety Guide to CRISPR

Overview of CRISPR Systems

Adaptive immunity is achieved in many bacteria and archaea species through clustered regularly interspaced short palindromic repeats (CRISPR) systems, which use a combination of CRISPR RNAs (crRNAs) and CRISPR-associated (Cas) proteins. These CRISPR systems may require several Cas proteins (class 1) or only one (class 2). Several class 2 CRISPR systems have been experimentally adapted for directed genome editing purposes. The most common of these is the CRISPR/Cas9 system.

There are two essential components of class 2 CRISPR-based genome editing systems: a guide RNA (gRNA; also called a single guide RNA or synthetic guide RNA, sgRNA) and a non-specific Cas endonuclease. There are two important elements of a sgRNA. The first is a scaffold sequence, which is required for the interaction of the sgRNA with the nuclease. The second is an approximately 20 nucleotide targeting sequence. By changing the targeting sequence, the user defines the site in the genome that will be cleaved by the Cas nuclease. Any targeting sequence can be used, provided it lies immediately upstream of a Protospacer Adjacent Motif (PAM). In bacteria, the scaffold sequence and targeting sequence may be found in separate crRNAs that interact through base-pairing, but for experimental purposes these elements are combined into one sgRNA.

The Cas nuclease binds to the scaffold sequence of the sgRNA. The Cas:sgRNA complex binds to the target gene via the complementary targeting sequence, and the nuclease activity of the Cas enzyme creates a double strand break 3-4 nucleotides upstream of the PAM sequence. Non-homologous end joining repair pathways within the cell will repair the double strand break, but often lead to small nucleotide insertions or deletions at the cleavage site. This typically results in a loss-of-function of the target gene, but the strength of the "knockout" phenotype needs to be validated by the user.



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Homology directed repair can be used to create specific changes in the target gene, from single nucleotide changes to large insertions ("knock-in" mutations). For homology directed repair, a third component – a repair template lacking the PAM sequence but containing the desired changes – must be delivered to the cell along with the Cas nuclease and sgRNA. Mutant forms of Cas9 that cleave only one DNA strand ("nickases") can be used with dual sgRNAs to enhance specificity but are less efficient.

The required PAM sequence will depend on the species of Cas9 used. Cas9 from *Streptococcus pyogenes* is the most common Cas9 nuclease used, although other species, mutant forms of Cas9, or other related Cas nucleases can be used. For example, the Cas9 from *Staphylococcus aureus* is often used when adeno-associated viral packaging is desired because it is significantly smaller in size. Another common alternative is CRISPR from *Prevotella* and *Francisella* 1 (Cpf1). It should also be noted that in bacterial species that have endogenous CRISPR systems, genome editing may be achieved by introducing crRNAs or sgRNAs without an exogenously supplied Cas enzyme.

Biosafety Concerns for the Use of CRISPR Systems

There are distinct biosafety concerns that arise with the use of CRISPR systems in sexually reproducing organisms, cells, and microbes. Because research projects are unique, specific experiments may have additional risks that are not discussed here.

Gene drive

Gene drive becomes a concern when CRISPR/Cas9 or other CRISPR system is used to the edit the genome of a sexually reproducing organism; it does not apply to asexual microbes or cells grown in culture. Gene drive promotes the spread of a genetic trait or modification through a population because it is inherited more often than Mendelian segregation would predict. This can have significant ecological or evolutionary impact if introduced into a wild population.

Gene drive occurs when the genome editing machinery incorporates into the genome as a cassette that is then able to copy itself into the other allele, converting a heterozygous state to a homozygous one. CRISPR can lead to gene drive if both of the following conditions are met:

- All components required for genome editing can be incorporated into the genome and are subsequently inherited together
- The genome editing components are introduced into embryos or germ line cells

If gene drive is identified as a potential risk, additional precautions may be needed. These can include, but are not limited to:

- Use of organisms that are incapable of surviving outside of the laboratory environment
- Physical containment measures that prevent escape or release of modified organisms
- Use of laboratory strains that cannot reproduce with wild counterparts

Oncogenic potential

If genome editing leads to the inactivation of tumor suppressors or activation of oncogenes in human cells, then oncogenesis becomes a possibility in the event of exposure. This is a concern when:

- Genome editing components are delivered by a method that can enter human cells (e.g., viral vectors that can infect human cells)
- All components required for genome editing are likely to be introduced in a single exposure event, such as a needle stick or mucosal splash

- Genome editing is designed to target human oncogenes/tumor suppressors or can target human oncogenes/tumor suppressors due to homology

Genome editing in microbes

If CRISPR systems are being used to edit microbial genomes, then it should be considered whether such editing changes the risks associated with the microbe. For example, will the pathogenicity, virulence, transmission, tropism, or antibiotic resistance of the microbe be altered?

Use of CRISPR Systems for Purposes Other Than Genome Editing

CRISPR-directed cleavage of a wild type sequence can be used as a selection tool to enrich mutant variants in a bacterial population, or vice versa. Similarly, introduction of a "CRISPR array", or set of sgRNAs that target many sites in a bacterial genome for cleavage, may be used to kill a specific species of bacteria in a mixed population. These applications use intact genome editing machinery, but the end goal is death rather than genetic modification of a microbe.

In addition, mutations in Cas9 have been identified that abolish its nuclease activity without disrupting its ability to bind specifically to a target gene based on a sgRNA target sequence ("nuclease dead" Cas9, or dCas9). This has led to the development of numerous non-editing applications of CRISPR systems. By fusing dCas9 to a transcriptional repressor or activator and targeting it to the promoter region, a gene of interest may be repressed (CRISPR-mediated interference, or CRISPRi) or up-regulated (CRISPR-mediated activation, or CRISPRa) without any change in genomic sequence. Other examples include:

- Fusion of dCas9 to a fluorescent marker to label specific genomic regions for detection in live cells
- Purification of a specific genomic sequence by fusing dCas9 to an epitope tag for immunoprecipitation
- Fusion of dCas9 to enzymes that methylate or demethylate DNA in order to induce epigenetic modifications at specific genomic sites

These non-traditional uses of CRISPR systems are based on mutants that lack nuclease activity. Without this activity, there may be no risk of gene drive. Other risks (e.g., oncogenic potential) may still exist, however, and should be addressed. For example, using viral delivery to achieve targeted inactivation of a human tumor suppressor gene by CRISPRi poses a similar risk to using CRISPR/Cas9 to generate a knockout of the gene.

Adding CRISPR to a Bio-ARROW Protocol

Guidance for completing the Genome Editing section in Bio-ARROW can be found on the Genome Editing and Gene Drives KB page. Please contact the Office of Biological Safety at 608-262-2037 or biosafety@fpm/wisc.edu if you have questions or need assistance.