

## CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR Associated protein 9)

### **What general biological, chemical, and/or physical principles and concepts is this technique based on?**

The CRISPR/Cas system is a bacterial and archaeal adaptive immune defense system that cleaves foreign genetic material [1]. The CRISPR locus within the prokaryotic genome has short repeats separated by genetically unique spacers [2]. These spacers consist of genetic material from invading agents such as viruses [2]. To enable immunity, the CRISPR locus is transcribed and processed into mature CRISPR RNA (crRNA) containing the spacer sequence complementary to the foreign genetic material. The crRNA associates with a trans-acting CRISPR RNA (tracrRNA), which is also found in the bacterial genome [1]. Of the three CRISPR/Cas systems, the type II system is the best studied. The Cas9 endonuclease is guided by the crRNA:tracrRNA complex to introduce a double-stranded break in the invading DNA, preventing the survival and replication of the invading agent [1]. The complementary sequence found in the foreign DNA is called a protospacer, and to prevent Cas9 from targeting the host genome, recognition of a short sequence downstream of the protospacer (called a protospacer adjacent motif, or PAM) is necessary for Cas9 cleavage to occur [3].

### **What does this technique 'do'?**

CRISPR/Cas9 is used as a gene editing/genome engineering tool in mammalian cells [1]. The Cas9 nuclease is guided by a single-guide RNA (sgRNA), corresponding to the tracrRNA:crRNA complex in the bacterial immune system. The sgRNA directs Cas9 to a specific genomic site, allowing the introduction of a double-stranded break (DSB) upstream of a PAM (which is a recognition sequence in the target DNA necessary for Cas9 activity). The cell recognizes the DSB and initiates one of two possible repair processes: non-homologous end-joining (NHEJ) or homology-directed repair (HDR), both of which can introduce new mutations into the DNA [4]. NHEJ, which occurs in the absence of a repair template, is error-prone and often results in deletions or insertions at the site of the break [4]. HDR can be induced by adding a DNA template with homology to the regions flanking the DSB, leading to incorporation of the template sequence into the genome [4].

### **What applications is this technique employed for?**

CRISPR/Cas9 is used as a genome editing tool for a variety of purposes in research. The technique is less expensive and far more precise than previously used genome targeting mechanisms because it relies on RNA-based DNA recognition (in contrast to protein-based DNA recognition) [7].

Gene knock-out can occur when the DSB induced by Cas9 leads to repair by NHEJ, which is an error-prone process. The subsequent insertions/deletions in the gene often result in a frameshift or a premature stop codon, rendering the gene dysfunctional [7].

Multiplexed editing is a method to create larger deletions in a target genome - by creating two sgRNAs and thereby inducing DSBs at two genomic sites simultaneously, the region between the breaks is deleted from the genome [7].

Sequence-specific mutagenesis is carried out by the addition of a template DNA which is introduced by HDR. This method can specifically mutate genes and create gene knock-ins. It holds tremendous promise for therapeutic gene editing in the future, opening up the possibility of curing genetic diseases in humans [7].

Gene regulation: A mutated Cas9 with no nuclease activity fused to a transcriptional activator/repressor can alter gene activity [8].

**What questions relating to gene regulation and/or development can be addressed using this technique? Provide two examples (peer-reviewed papers) that use this technique.**

In 2016, Rajagopal *et al.* [5] used a CRISPR/Cas9-based approach to quantify the effects of cis-regulatory DNA on gene expression in mouse embryonic stem cells. The green-fluorescent protein (GFP) gene was fused to a gene of interest using CRISPR. Then, a library of sgRNA was used, in which unique sgRNAs were added to induce various mutations within a 40 kb region around a gene in order to induce loss of function to suspected cis-regulatory elements. Next, fluorescence activated cell sorting (FACS) was used to sort cells based on fluorescence intensity to quantify how gene expression was affected by the alteration of cis-regulatory elements. Finally, the region around the gene of interest was sequenced for all of the sorted cells. The study found that promoters from neighbouring genes, as well as unmarked regulatory elements (that lack typical histone modifications that would normally indicate regulatory function) have unexpectedly large roles in gene expression. The authors conclude that correlative genome annotation without direct perturbation analysis of the genome is insufficient to fully predict cis-regulatory regions.

CRISPR/Cas9 can also be used to induce mutations in cells and to track these lineages throughout development. In 2015, LoTurco *et al.* [6] used the CRISPR/Cas9 system to introduce somatic mutations into neural progenitor cells. sgRNAs were designed to target tumour suppressor genes that have been linked to neurodevelopmental disorders: PTEN, NF1, and p53, and these sgRNAs were introduced alone and in combination into neural progenitor cells. The plasmid used for CRISPR targeting also included the *piggyBac* transposase system, which enabled a eGFP gene to be stably integrated into the CRISPR-targeted cells' lineage. The authors could then track the CRISPR-targeted cells throughout development. Targeted mutation of PTEN correlated with enlarged

neurons and altered excitability, while mutation of NF1 resulted in increased glial cell and decreased neuronal formation. Multiplexed targeting of all three tumour suppressor genes led to the formation of glioblastoma (GBL) tumours. This paper is an example of how targeted gene editing can be used to create specific models of disease and development.

### **7. What critical reagents are required to use this technique?**

A plasmid expressing the appropriate sgRNA is necessary for directing the nuclease to the correct genomic site. Furthermore, a gene encoding the Cas9 nuclease must also be included in the plasmid, as Cas9 is essential for creation of the DSB in the target-site. The PAM close to the target site is needed for Cas9 activity. Lastly, one must be able to screen or select for this plasmid, thus a selectable marker must be included in the plasmid.

If the goal is precise gene editing, a DNA template must also be provided.

A transfection reagent, such as Lipofectamine, is used to transfect the plasmid into the target cell [1].

### **What critical information is required to be able to employ this technique?**

To use the CRISPR/Cas9 system for genomic modification, it is essential to know the sequence of your target site and the flanking regions. This is required for engineering the sgRNA to direct Cas9 to a target site close to a PAM, and necessary for creating a template for HDR. Knowledge of potential off-target recognition is important information regarding the precision of the experiment [1].

### **At least two resources/information sources that you recommend for learning more about this technique (one or more may be developed by your group):**

YouTube video:

Jennifer Doudna (UC Berkeley / HHMI): Genome Engineering with CRISPR-Cas9

<https://www.youtube.com/watch?v=SuAxDVBt7kQ>

Lander, Eric S. "The Heroes of CRISPR." *Cell*, vol. 164, no. 12, 2016., pp. 1828, doi:10.1016/j.cell.2015.12.041.

### **References**

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