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# Modular Approaches to Introducing CRISPR/Cas9 Genome Engineering Technology into Biology Labs

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CRISPR-Cas9 system as a genetic tool has emerged as one of the most important advances in the past 20 years. As this technology is currently making and will continue to make significant impacts in research across the life sciences, it is imperative that undergraduates become conversant in the workings of CRISPR-Cas9 system. In this workshop, we present how the CRISPR-Cas9 system can be introduced in a variety of modules using bioinformatics, *in vitro* studies and in vivo analysis (with zebrafish as one of the models) to develop gene knockouts that can be used for a variety of research questions. Using a workflow developed largely by undergraduate student researchers, we share our experiences with making CRISPR-Cas9 system accessible to undergraduate students at all levels. This workflow and experimental details can be effectively used to introduce, as well as strengthen fundamental ideas in the Central Dogma of Molecular Biology in addition to developing important basic skills in DNA science.

**Keywords**: CRISPR-Cas9; Central Dogma of Molecular Biology; active learning; zebrafish

## Introduction

The meteoric rise of CRISPR-Cas9 technology as a driving force in molecular biology research and its potential impact on society has created a need to develop tools to introduce this technology to undergraduate students in an engaging and accessible manner (Sander and Joung 2014). An increasingly common approach to effectively engage students in the scientific process is the integration of authentic research projects into coursework; however, this can seem like a daunting challenge when considering a novel technology like CRISPR-Cas9. Thus, there exists a need to develop laboratory course modules that can expose undergraduates to how CRISPR-Cas9 technology works and that may be ethically used to specifically mutate target genes in model systems. Several groups have worked to develop such educational tools (Bhatt and Challa 2017; Hynes *et al.*, 2017; Trudel *et al,* 2017; Adame *et al.*, 2016); however, the need still exists for models that can be flexibly adapted for different lengths of course time as well as for introductory level courses where students have little prior experience with molecular biology techniques.

Here we present a workflow that enables students to engage in the construction and testing of CRISPR-Cas9 materials in the pedagogical context of the Central Dogma of Molecular Biology using zebrafish (*Danio rerio)* as a model organism. The workflow is flexible for use in classrooms with varying levels of resources and infrastructure, and can be utilized as either a full semester research experience or a shorter laboratory module based on a specific objective. Importantly, the workflow can be utilized independent of the availability of a zebrafish facility with an entirely *in vitro* focus.

## Procedure

The detailed workflow is presented in the Student Outline. In the first portion, students focus on bioinformatics to select a gene (or set of genes) for targeting within a chosen genome. Using genome browsers (e.g. ENSEMBL) students can query a variety of features of a gene, including chromosomal location, orthologs and paralogs, gene structure (exons and introns), and protein features. Subsequently, optimal CRISPR-Cas9 targets can be selected using online tools like Benchling (www.benchling.com). Finally, PCR primers are designed to amplify a region of the gene encompassing the target site.

 

The first part of the "wet lab" exercises deal with synthesizing a single guide RNA (sgRNA), complexing it with the Cas9 protein, and validating the nuclease activity of this ribonucleoprotein complex *in vitro* using a PCR amplicon carrying the target sequence. In the second part, ribonucleoprotein complexes showing *in vitro* nuclease activity are injected into zebrafish embryos. Developing embryos are observed using light microscopy to analyze any phenotypic defects. Subsequently, genomic DNA from these embryos is obtained and used as a template for PCR amplication and detection of mutations - insertions-deletions ('indels') – using gel electrophoresis. Once 'indels' are detected, the PCR amplicons can be cloned and sequenced to identify the specific sequence changes.

The workflow can provide students with a relatable framework to discuss the Central Dogma of Molecular Biology and flow of genetic information in two distinct ways - the first relates to the experimental protocols aimed at sgRNA synthesis using the principles of DNA replication and RNA transcription followed by a functional readout of the ribonucleoprotein complex's activity. The second way relates to potential phenotypes resulting from CRISPR-Cas9 complexes injected embryos since mutations in individual cells in the injected embryos are copied by replication to their daughter cells.

## Workflow Modularity Provides Flexibility for Use in the Classroom

The workflow can be divided into modules that can be done sequentially or independently. For example, a laboratory exercise can focus only on *in vitro* nuclease activity to demonstrate the ability of various sgRNAs to cleave a target DNA fragment. This activity can also include the testing of off-target effects of the sgRNAs which by themselves can yield valuable experimental results.

The modules up until the *in vitro* nuclease activity can be done independent of a zebrafish facility. The only component that is needed is zebrafish genomic DNA, which can be obtained by requesting established zebrafish labs. In order to perform embryo injections, one needs to have access to a zebrafish facility. Since the zebrafish is a vertebrate, an IACUC approved animal study protocol is necessary to maintain and breed a zebrafish colony. An approved animal study protocol will be needed if live embryos in developmental stages beyond 3 days post fertilization (dpf) and up to 5 dpf are utilized (https://oacu.oir.nih.gov/sites/default/files/uploads/arac-guidelines/zebrafish.pdf). However, collaborations with labs having approved zebrafish facilities can enable the adoption of modules focusing on phenotypic and genotypic analysis; phenotypic analysis can be done by obtaining fixed (injected) embryos and genotyping can be performed on genomic DNA preparations of injected embryos.

If the laboratory modules are done sequentially from the beginning, generation of a double stranded template can be achieved either through a traditional plasmid cloning method, or a cloning-free method. The choice of the method can be based on the desired learning goals of a given course. The cloning method has a few more steps in comparison to the cloning-free method; the cloning method can be used to introduce plasmids vectors, DNA ligation, bacterial transformation, and selection. Having fewer steps, the cloning-free method enables an emphasis on several of the downstream steps and facilitates progression to further steps in the experimental workflow.

## Conclusion

We describe a versatile and effective model for the introduction of CRISPR-Cas9 technology into an undergraduate classroom. The workflow can effectively be scaffolded with discussions on the Central Dogma of Molecular Biology and flow of genetic information and thereby ground the activities into a foundational course in molecular biology. Modularity is another key feature of this workflow, allowing flexibility to instructors who wish to include CRISPR-Cas9 based experiments under constraints of available laboratory time. In the absence of direct access to a zebrafish facility, collaborations with labs working with zebrafish can develop a more comprehensive laboratory experience for students. The collaborators can help with microinjections, fixation of injected embryos for phenotypic analysis, and preparation of genomic DNA for genotypic analysis. The modules done in isolation or in sequence will allow students to master underlying concepts and appreciation for the scientific process. However, it is important to note that the workflow enables instructors to provide an engaging and authentic laboratory experience for their students regardless of their potential time and resource limitations.

# Student Outline

## Objectives

- To gain experience with CRISPR-Cas9 technology
- To design an experiment that allows work with bioinformatics and molecular biology tools
- To better understand the Central Dogma of Molecular Biology

## Introduction

*What is Genomic Engineering?*

The nature of the human body, our components, and how we function has been a subject of fascination for thousands of years. Ancient philosophers questioned the nature of our reality and abstract concepts regarding our soul and mind. Artists and scholars from the Renaissance further pioneered our understanding of concepts like anatomy and physiology.

Today, scientists are building on a foundation of knowledge that encompasses thousands of years of scientific progress, and because of this, they have developed a field that has the potential to be both enlightening and dangerous: Genomic Engineering.

Genomic engineering is a complex process of modifying the biological code, DNA and RNA that runs our bodies. Everyone has within them billion upon billions of base pairs that act as the blueprint from which every function in our body is dependent upon. Through cutting edge research techniques, we can now selectively make changes to this code, which in turn can modify the structure and function of cells, tissues, and organs in our bodies. Currently these research techniques are only being used in model systems, but in the near future scientists and doctors may very well use refined approaches to engineer our very existence as a species.

*Why is Genomic Engineering Research Important?*

Genomic engineering represents the forefront of scientific medical research. By studying its mechanisms and processes, students can gain a better understanding of how the body works and how we will be able to change the body in the future. Science is always changing and it is important that Hampden-Sydney students stay at the forefront of scientific discoveries.

*CRISPR-Cas9*

An exciting new way to perform genomic engineering can be found with CRISPR-Cas9, a natural immune system found in bacteria to fend off bacteriophage invasion that has been adapted for use in laboratories in gene editing. Using the workflow provided, you will create your own CRISPR-Cas9 based gene knockout that we can examine for mutation and, ultimately, phenotypic change in zebrafish.

## Methods and Data Collection

*Part A: Selecting a Gene to Target*

The instructor will introduce you to zebrafish and what types of gene mutations that would be interesting to create. Then, you will follow the workflow to design a targeted mutation in a gene of interest that you can study in zebrafish. Consider what phenotypes you would expect in zebrafish that did not have a working copy of your target gene when making your targeting decisions.

*Part B: Testing to Determine Whether Your Targeting Works In Vitro*

Before you test your potential gene mutation in zebrafish, we want to see if you have designed CRISPR molecules that can actually create the specific mutation you predicted. We will use an *in vitro* system to see via gel electrophoresis if your CRISPR molecules are able to specifically cut zebrafish DNA of interest.

*Part C: Looking at Your CRISPR-Based Mutation In Vivo*

Once we confirm the specific targeted cutting of your CRISPR molecules, we will work to generate zebrafish with your gene mutations. Then, you will be able to observe the development of these zebrafish to see if your predictions about the effects of your gene disruption were correct.

## Discussion

Over the next few weeks, you will have the opportunity to gain, using various bioinformatics tools, a structural perspective on the molecular basis of genetically-inherited diseases. As you saw in your introductory genetics course, human genetically-inherited diseases are caused by DNA sequence variations. Although disease-causing DNA sequence variations can occur in both non-coding and coding regions of the genome, the majority of characterized mutations occur in coding regions. Since they are in the coding region of genes, these mutations often affect the structure and function of proteins. For this laboratory exercise, we will focus on genetically-inheritable diseases that are caused by this type of mutation. More specifically, we will focus on genetically-inheritable diseases that result from a missense mutation. Recall that a missense mutation is a change in the nucleotide sequence of a gene, where one or more nucleotides is/are replaced by another. This mutation results in a new codon, which causes a different amino acid to be inserted into the growing polypeptide chain during translation. For this laboratory exercise, you will be asked to work with your laboratory partner. You and your laboratory partner will be guided in the use of various bioinformatics tools to study the effects of disease-causing mutations on protein structure and function. We will specifically focus on different levels of protein structure and how they intimately relate to one another in the formation of the final, fully-folded protein. At the end of this exercise, you and your laboratory partner will orally present your results to the other members of your laboratory session via a 10-minute Power Point presentation.

## Materials

You will need a computer with Internet access to do the bioinformatics activities. If you intend to do the *in vivo* portions of the workflow, access to live zebrafish and a zebrafish facility will be required. If you are doing the *in vitro* portion only, you will need zebrafish genomic DNA for the CRISPR diagnostic testing experiments.

## Notes for the Instructor

This workflow is designed to be modular in nature. Thus, it is possible to pick and choose which portions are most relevant to your particular course and how much time you wish to devote to the overall project. Clearly, it is paramount to make these determinations before proceeding into the actual project with students.

The work form this workflow is designed to give students an authentic research experience. Therefore, we recommend that one of your assessment tools be a poster presentation or an equivalent opportunity for students to share their work with the public. As dissemination of data is a critical part of the scientific process, we feel that providing an opportunity to do so with this project will further enhance the level of engagement your students will have with this work.

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## Mission, Review Process & Disclaimer

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