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Introduction to CRISPR-Cas9 Techniques

Strategies for the Laboratory and the Classroom

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Dedicated to our students, who are our partners in finding effective ways to teach and learn

Preface

Molecular biology changes at a breakneck pace. From a scientific perspective, the technological advances brokered by molecular biology and genetic engineering have allowed for advances in our understanding of nature and medicine that could only have been dreamed of by the pioneers of the field in the early twentieth century. From a pedagogical perspective, these advances are at once exciting and daunting. While the scientific community's breakthroughs in the last several decades have been remarkable, the amount of education and training needed for the next generation of scientists to join this community continues to rise higher and higher. For the instructors responsible for training these future scientists, there is a constant challenge to not only keep up with the latest advances in molecular biology but also find effective means to present these advances to their students. CRISPR is a classic example of such a technology that has arisen from a little-known bacterial defense mechanism to a central pillar of genetic engineering in just a few short years.

At the 2013 American Society for Cell Biology meeting in New Orleans, I was presenting a research poster and was randomly placed next to Dr. Anil Challa, whose work you will find in a couple of different locations in this book. Anil has dedicated himself toward making biotechnology accessible to undergraduates, and his work in New Orleans dealt with designing a workflow for bringing CRISPR in zebrafish to the undergraduate classroom. At the time, CRISPR was just emerging as a mainstream genetic engineering technique, making Anil's work all the more exciting. This random meeting in New Orleans was the start of a flourishing network of undergraduate educators from institutions of all sizes and missions united in the goal of finding effective ways to introduce CRISPR to the next generation of scientists. The editors of this volume, along with several of its authors, worked to create a series of workshops supported by the U.S. National Science Foundation (Award #s 1823595, 1916486, and 2120417) in which current and future instructors gathered to learn about CRISPR, its applications, and how to effectively present to undergraduates the science and ethics surrounding its use. These workshops have been essential in the building of a community whose strength derives from the diversity of classroom experiences and types represented in its ranks. Through this diversity, our network's membership has been able to benefit from the combined classroom experiences of the group and to obtain new and exciting ideas to bring back to their own students. This book is another

exciting product of this network and serves as a guide for novice and seasoned instructors alike in bringing CRISPR technologies to budding scientists. We hope that you will benefit from the ideas presented here and enjoy using this guide as much as we have enjoyed its preparation.

Hampden-Sydney, VA, USA Michael J. Wolyniak

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Part I

Foundations of CRISPR in the Classroom

CRISPR-Cas9 Techniques: Strategies for the Laboratory and the Classroom

1

Kevin Davies

History was made in October 2020 as two women were named co-winners of a Nobel Prize for the first time. Appropriately, the prize was for chemistry—the two women had struck a serendipitous but tight bond when they first met at a conference in early 2011. Jennifer Doudna, a biochemist at University of California, Berkeley, was awakened in the middle of the night by a congratulatory phone call from a reporter with *Nature.* (Heidi Ledford later praised Doudna's work ethic. "I certainly wouldn't have taken a call from me at that hour!" she tweeted.) The other recipient was Emmanuelle Charpentier, a French microbiologist based in Berlin.

The official announcement from Stockholm hailed the winners for their breakthrough in "rewriting the code of life" by developing a "genetic scissors" that could be programmed to target almost any DNA sequence at will. That this foundational technology, which was published in 2012, should arise from an ancient microbial immune system that itself was only discovered in the early 2000s, made the accomplishment even more remarkable.

Doudna and Charpentier's Nobel success was built on the contributions of a handful of unsung scientists dating back to 2001, when University of Alicante microbiologist Francisco Mojica coined the term "CRISPR", for *Clustered Regularly Interspaced Short Palindromic Repeats*. A few years later, Mojica and his colleagues were the first to report that the DNA segments stitched into these curious repetitive DNA elements had been captured from viruses. What propelled Mojica and his fellow microbiologists was not the lure of personalized medicine or developing a new method for genome editing, but simply the thrill of basic research and the joy of scientific discovery. But the industrial relevance of their discovery soon became apparent. Two years after Mojica's report was published, a group of researchers led by Rodolphe Barangou and Phillipe Horvath, working for a

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yoghurt company, supplied the first experimental proof that CRISPR sequences conferred immune protection to their host bacteria from phage attack.

By the time Charpentier and Doudna crossed paths in 2011 at a conference in Puerto Rico, several key details of the CRISPR machinery had been reported, including the involvement of short RNAs and a CRISPR-associated nuclease protein that cleaved a specific DNA target. Doudna and Charpentier decided to combine their respective strengths in structural biology and microbiology to dissect the molecular basis of CRISPR gene targeting. Doudna was the more senior of the pair, with a bigger lab, better funding (she has been an Investigator with the Howard Hughes Medical Institute since 1997), and a string of major papers to her name showcasing her prowess as a structural biologist and her love of RNA biology. By contrast, Charpentier had struggled to secure funding in Europe and had a lower profile on the international stage but had just published her first senior author paper in *Nature*.

Doudna and Charpentier's landmark paper was published in *Science* a year later in June 2012 [1], the work led by a Czech postdoc in Doudna's Berkeley laboratory, Martin Jínek [2]. The newfound ability to program the CRISPR system to edit a specific DNA sequence using a single-guide RNA as the nuclease's GPS signal laid the foundation for the genome editing revolution. Similar results were published 2 months later by Virginijus Siksnys and colleagues in Lithuania, in which he talked about the potential of CRISPR-Cas9 to perform "DNA surgery" [3].[1]

Although not the first technology to correct DNA sequences—ironically *Nature Methods* hailed earlier technologies for genome editing as its "Method of the Year" in 2011 [4]—CRISPR was easier to work with, much more flexible and far more affordable. That potential has been fully realized over the past decade. While research on the biology and evolution of CRISPR is a major field in its own right, the term "CRISPR" has become almost synonymous with genome editing—both for its scientific and medical potential as well as its ethical controversies.

1.1 The CRISPR Craze

The "CRISPR craze" stems from the extraordinary potential and myriad applications of genome editing, often blurring science fact and fiction. Numerous fields—molecular biology, development biology, microbiology, immunology, plant genetics—have been transformed by advances in CRISPR and genome editing. From editing plants and livestock to eradicating malaria, from rapid diagnosis of infectious diseases to "resurrecting" the woolly mammoth and other extinct species, CRISPR has unleashed the imagination of researchers around the world. But the most immediate and important application is in treating patients with life-threatening genetic diseases. Just 2 months after the CRISPR

[1] Had the Siksnys manuscript been reviewed and published by his first-choice journal *Cell* in the first half of 2012, the history of CRISPR might have been different.

Nobel prize, researchers at CRISPR Therapeutics (a biotech company co-founded by Charpentier) in partnership with Vertex Pharmaceuticals published preliminary results on a small group of sickle-cell disease (SCD) patients [5]. One of these patients was Victoria Gray, a mother-of-three from Forest, Mississippi. Led by her physician, Haydar Frangoul, the experimental CRISPR treatment produced a sustained boost in Gray's fetal hemoglobin levels that many clinicians would consider a cure. Choosing his words carefully, gene editing pioneer Fyodor Urnov hailed the results as "borderline utopian" [6]. Four years after Gray's treatment, dozens of SCD patients have been essentially cured using CRISPR, and the therapy is on the fast track towards regulatory approval at the end of 2023.

Early clinical success is not confined to treating SCD. In 2021, another biotech company, Intellia Therapeutics, published exciting results treating patients with a rare inherited liver disorder, delivering CRISPR *in vivo*—directly into the patient's body—for the first time. Other groups showed promise in treating patients with a hereditary form of blindness and generating CAR-T cells for cancer. Meanwhile, researchers are also advancing new-and-improved forms of genome editing, which can make more precise DNA substitutions without cleaving both strands of the double helix. The development of base editing, pioneered in David Liu's laboratory at the Broad Institute, has quickly shown promise in preclinical experiments treating mouse models of sickle-cell disease and premature aging, as well as a monkey model of heart disease.

1.2 Crossing the Germline

Patients with literally thousands of genetic diseases stand to benefit from some form of CRISPR-based genome editing in the years ahead, if the early potential and clinical success can be sustained. Fixing broken genes in adults or children, targeting the blood, or liver, or muscle, or other tissues, is known as *somatic* gene therapy. While some safety issues remain a potential concern—the possibility of off-target effects, immune responses to the bacterial Cas9 protein—for the most part these have been well characterized and controlled.

But in 2018, the world was shocked by the scandalous work of a young Chinese scientist, He Jiankui ("JK" for short). By editing a gene in human embryos and then implanting those genetically modified embryos, JK crossed a sacrosanct red line. The births of twin girls, Lulu and Nana, sent shockwaves around the world. JK had deliberately altered the human *germline*, changing the genes that these so-called CRISPR babies could pass onto their children and future generations.

The first accounts using CRISPR to edit human embryos, from Chinese researchers, were published in 2015. The ease of use of CRISPR meant it was not hard for JK—a physicist by training—to assemble the necessary technical skills in his lab. JK decided to target the *CCR5* gene, which encodes the major receptor through which HIV gains entry into cells. About 1% of Caucasians possess a 32-base deletion in this gene that prevents production of the receptor and thus renders them resistant to the virus. JK believed that by

disrupting the *CCR5* gene in human embryos using CRISPR, any resulting babies would be immune to HIV—even though his team was performing a standard procedure (sperm washing) that would achieve the desired result without need of genome editing.

JK performed his work in secret, confiding in a small group of scientists including his American PhD and postdoctoral supervisors. In April 2018, he emailed confidants in his "circle of trust" that the first pregnancy was confirmed. However, he had not bargained for some brilliant reporting by science journalist Antonio Regalado, who revealed the pregnancies in a major scoop published on Thanksgiving Sunday, 2018.

Since then, scientists have debated how the world should respond to the birth of the "CRISPR babies". The most important committee report to date was published in 2020 by the National Academy of Sciences and the UK Royal Society [7]. Rather than support a blanket moratorium on human hereditary genome editing (HHGE), the commission concluded that there could be rare medical circumstances in which a case could be made to support HHGE, i.e. when couples with a serious genetic disease such as SCD or cystic fibrosis might wish to have a biologically healthy child. The report also stressed that more research was required to prove that CRISPR editing could be performed safely in human embryos.

1.3 Rewriting the Code

My personal interest in CRISPR arose rather late. I trained as a molecular geneticist but hung up my lab coat after two unproductive postdoctoral fellowships. I joined the editorial staff of *Nature* in 1990 and was appointed the founding editor of *Nature Genetics* in 1992. Over the years, I've written several popular science books with the aim of conveying the medical and societal important of advances in genetics and genomics to a wide audience. With most of my attention focused on genome sequencing, somehow I missed the initial crescendo of interest in human genome editing, including the 2005 report in *Nature*, when Urnov and his colleagues at Sangamo coined the term "genome editing" [8]. I also failed to register the early buzz over Doudna and Charpentier's landmark article in *Science* in June 2012. But as CRISPR began to generate more media coverage, branching out into magazine covers, movies, books and film documentaries, it was impossible not to be swept along by the "CRISPR craze". The sheer range of applications was like something out of a science fiction series—indeed, it didn't take long before CRISPR made its Hollywood debut in the Dwayne Johnson film *Rampage*.

By 2017, I was sold on the idea that CRISPR was the most exciting new technology in genetics and the life sciences since the advent of PCR or Sanger sequencing four decades earlier. I felt compelled to start working on a book on the CRISPR story as well as launch a related scientific journal [9]. *The CRISPR Journal* debuted in 2018 under the editorship of Rodolphe Barrangou (North Carolina State University). The debut issue was marked by a superb review article by the aforementioned Fyodor Urnov on the history of genome editing "B.C."—*before* CRISPR [10].

Table 1.1 Literary resources on CRISPR and genome editing

Francoise Baylis	Altered Inheritance: CRISPR and the Ethics of Human Genome Editing (Harvard University Press, 2019)
Kevin Davies	Editing Humanity: The CRISPR Revolution and the New Era of Genome Editing (Pegasus Books, 2020)
Jennifer Doudna and Samuel Sternberg	A Crack in Creation: Gene Editing and the Unthinkable Power to Control Evolution (Houghton Mifflin Harcourt, 2017)
John H. Evans	The Human Gene Editing Debate (Oxford University Press, 2020)
Henry T. Greely	CRISPR People: The Science and Ethics of Editing Humans (MIT Press, 2020)
Eben Kirksey	The Mutant Project: Inside the Global Race to Genetically Modify Humans (St. Martin's Press, 2020)
Walter Isaacson	The Code Breaker: Jennifer Doudna, Gene Editing, and the Future of the Human Race (Simon & Schuster, 2021)

My book *Editing Humanity: The CRISPR Revolution and the New Era of Genome Editing* was published on October 6, 2020—ironically the eve of the Nobel Prize for Chemistry announcement. There have been several other excellent books on CRISPR and genome editing published over the past few years (see Table 1.1). Also highly recommended is *Human Nature*, an excellent documentary film on the discovery of CRISPR and its applications, released in 2019.

1.4 Next-Generation CRISPR

Over the past few years, representing the journal and reporting for the book, I've attended several major CRISPR conferences, including the annual grassroots CRISPR meeting every June, the Genome Engineering conference hosted at Cold Spring Harbor Laboratory, and the Keystone conferences on genome editing. Each meeting seems to draw bigger and younger audiences, emphasizing how vibrant and exciting the genome editing field is. I am also struck by the intellectual contributions being made by postdocs and graduate students. For example, powerful new forms of precision genome editing capable of engineering point mutations without cleaving the double helix have been driven by key discoveries made by Alexis Komor and Nicole Gaudelli, two postdocs in David Liu's lab at the Broad Institute [11]. Andrew Anzalone followed that by joining Liu's lab as a postdoc and developing prime editing in his first year [12].

A major reason underlying the global sweep of CRISPR is how relatively easy and flexible the technology is. CRISPR experiments do not require million-dollar instruments. Reagents and guides can be ordered online, putting a premium on the ingenuity and creativity of the individual researcher. The CRISPR toolbox continues to grow as new forms of CRISPR-Cas nucleases are either discovered or engineered. Thankfully, the non-profit repository Addgene has been facilitating the economical distribution of key CRISPR reagents to hungry researchers around the world [13].

As the CRISPR toolbox continues to expand, so too will the dazzling applications that innovative scientists come up with. But it all begins with the original CRISPR-Cas9 genome editing system, which did after all win a Nobel Prize! This timely volume—***CRISPR-Cas9 Techniques: Strategies for the Laboratory and the Classroom***—contains a wealth of valuable and accessible material for educators and students eager to share in the fun and flexibility of CRISPR in the classroom [14]. I am convinced that the practical information and ideas in this publication will help and inspire the next generation of scientists to experience the wonders of CRISPR for themselves. The authors of the chapters that follow are dedicated to their students and excited about the potential of CRISPR to captivate students to the wonders of biology in general and genome editing in particular. I hope you enjoy and benefit from the valuable material in this book.

As the chief editor of *The CRISPR Journal*, Professor Rodolphe Barrangou, is fond of saying: "Keep Calm and CRISPR On."

Take Home Message

CRISPR is a Nobel Prize-winning technology for genome editing that, in a less than a decade, has transformed countless fields of biology and medicine. The most spectacular application of CRISPR is the 2023 approval of a highly effective and safe cell therapy for sickle cell disease. The information in this book will assist researchers already versed in working with CRISPR and novices alike.

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Kevin Davies, PhD, is the Executive Editor of *The CRISPR Journal* and *GEN Biotechnology*. He is the author of five books on genetics including *Cracking the Genome* and *Editing Humanity: The CRISPR Revolution and the New Era of Genome Editing* (Pegasus Books, 2020). He is currently writing a new book on sickle-cell disease for Harvard University Press.

Expansions on CRISPR-Cas9 Technology: Innovations for the Future

2

Anil Kumar Challa

Microbes to molecular geneticists are like the *akshayapaatra* in the Hindu mythology or *cornucopia* in the Greek mythology with regards to the bounty of experimental tools and technologies showered upon them. They are the 'vessels' or 'horns' that keep giving, seemingly forever. The number of molecular tools in the toolkit of today's experimental geneticist is fantastic. If an experimentalist from 100 years ago time travels and arrives today, they would be astounded to see the ways in which we can manipulate genetic material using microbial molecular machines and cellular machinery. Not just that, we have used those machines and machinery to further modify, enhance and even create previously non-existent new machines.

2.1 Using the Term "Molecular Machines" to Describe Proteins Can Create a Good Context for Students to Grasp Both Structure and Function of Proteins

One set of molecular machines that have given rise to the first biotechnology revolution in the 1970s, and have now become commonplace in modern biology laboratories, sometimes even in high schools, are the **restriction endonucleases**. These were discovered as key players in bacterial innate immune systems that enable them to fight invading bacteriophages. Restriction endonucleases or simply restriction enzymes (REs) are site-specific protein nucleases that can bind to specific sequences of double stranded DNA and create double-strand breaks (DSBs). REs have contributed to the development of a number of follow-up technologies that have paved the way for sequencing of whole genomes.

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Incidentally, they also formed the foundation for the development of customizable and programmable nucleases (ZFNs and TALENs) that gave molecular geneticists the power to access and engineer specific sites within eukaryotic genomes.

The ability to sequence, catalog and analyze microbial genomes then led to the discovery of the CRISPR-Cas systems. They are adaptive immune systems found in bacteria and archaea that protect them against invading genetic material, such as viruses or plasmids. CRISPR-Cas systems are incredibly diverse in the microbial world. CRISPR-Cas systems have been identified in roughly 40–50% of sequenced bacterial genomes and about 90% of archaeal genomes. The CRISPR Database features 36,605 (36,052 bacteria and 553 archaea) species (https://crisprcas.i2bc.paris-saclay.fr) [1–4].

The diversity of CRISPR-Cas systems is characterized by the presence of multiple CRISPR-Cas types and subtypes, as well as the differences in the Cas protein sequences and structures. Broadly, there are two main classes of CRISPR-Cas systems, which are further divided into six types (I–VI) and numerous subtypes based on the arrangement of their *cas* genes and the presence of specific signature genes.

Class 1 systems (Types I, III, and IV) are characterized by multi-subunit effector complexes, while Class 2 systems (Types II, V, and VI) have a single large effector protein. Each type and subtype have unique features and mechanisms, contributing to the overall diversity of CRISPR-Cas systems [5].

This extensive diversity can be attributed to the constant evolutionary arms race between bacteria and their invading genetic elements, as well as horizontal gene transfer events, which lead to the spread and evolution of CRISPR-Cas systems across different microbial species. The number, sequence, and organization of the spacer sequences represent genetic memory of previous encounters with foreign genetic elements.

Akin to the RE story, discovery of this bacterial system also quickly led to the development of a programmable nuclease technology that made possible efficient and facile *in vivo* DNA sequence manipulation. Multiple CRISPR-Cas systems have been found in diverse microbial species. However, many studies focus on the CRISPR-Cas9 system from a small number of species, and predominantly *Streptococcus pyogenes.*

2.2 Comparing and Contrasting the Restriction-Modification System with the CRISPR-Cas System, Both from a Microbial Physiology Perspective and from a Technology Perspective Can Be Part of a Good Classroom Pedagogy Strategy

The excitement about finding CRISPR as a programmable tool is because of its ability to identify a desired and very specific 20 nucleotide sequence in a given genome using a short RNA guide. To put this in perspective, REs typically recognize sequences of four, six, or eight nucleotides. As a rough estimate, if we assume that each base pair has an equal likelihood of occurring, a specific four-base-pair recognition sequence would occur approximately once every $4^4 = 256$ base pairs, a specific six-base-pair recognition sequence would

Restriction Enzymes - worksheet

Bacterial Species	*Xanthomonas badrii*	*Escherichia coli* RY13	*Haemophilus influenzae* Rc	*Klebsiella pneumoniae* OK8
Enzyme type	Type II, subtype P	Type II, subtype P	Type II, subtype P	Type II, subtype P
Enzyme name	XbaI	EcoRI	HinCII	KpnI
Recognition and cleavage site (⇩)	T⇩CTAG A | |||| | A GATC⇧T	G⇩AATT C | |||| | C TTAA⇧G	GTC⇩GAC ||| ||| CAG⇧CTG	G GTAC⇩C | |||| | C⇧CATG G

Where, how and how many times would EACH of these enzymes recognize and cut this DNA sequence?

```
5′-TGGCTCTAGAGGTACCCGAATTCGATATCATCGTCGACATCGATAAGGAATTCTGAATTCCACATCCACCGGTGCTAGCGGATCC -3′
   |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3′-ACCGAGATCTCCATGGGCTTAAGCTATAGTAGCAGCTGTAGCTATTCCTTAAGACTTAAGGTGTAGGTGGCCACGATCGCCTAGG -5′
```

CRISPR-Cas RNP nuclease - worksheet

Bacterial species	*Streptococcus pyogenes*
Enzyme name	Spy Cas9
Type	Class II, Type II
1° Recognition sequence (PAM)	NGG (N=A, T, G, or C)
Cut site	5′-.....✂...NGG-3′ 3′-.....✂...NCC-5′

Where, how and how many times CAN Spy Cas9 recognize and cut this DNA sequence?

```
5′-...ATGGCAAACCTGCTCTACATTTGGCTTCCGAAAGGCCTGGAGGGCATTTGCACTGTGCCCCAACTCTTTACAAGGAGCTGGTCAA... -3′
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3′-...TACCGTTTGGACGAGATGTAAACCGAAGGCTTTCCGGACCTCCCGTAAACGTGACACGGGGTTGAGAAATGTTCCTCGACCAGTT... -5′
```

Fig. 2.1 This classroom exercise helps to illustrate to students the fundamental differences between how restriction enzymes and CRISPR-Cas RNP nuclease work to cut DNA at specific genomic locations

occur approximately once every $4^6 = 4096$ base pairs, and an eight-base-pair recognition sequence would occur approximately once every $4^8 = 65{,}536$ base pairs. For every recognition sequence a specific enzyme is needed. However, with a single enzyme (Cas9) in the CRISPR system, a large number of recognition sequences can be programmed using short guide RNA molecules. Figure 2.1 illustrates how a relatively simple classroom activity can demonstrate to students the difference the mechanistic and specificity differences between these molecular techniques for precision in genetic engineering.

2.3 Engineering the Nanobiobots—Changing the Landmarks

Once the native CRISPR-Cas systems were discovered and adapted as experimental tools, efforts to engineer them to make them more versatile and efficient were underway. Protospacer Adjacent Motifs (PAMs) are short DNA sequences that are located next to target sequences and play a critical role in the recognition and binding of the CRISPR

system to specific target sequences in the genome. Kleinstiver and co-workers [6, 7] showed that it is possible to modify the PAM recognition of CRISPR-Cas9 nucleases, allowing greater flexibility in targeting specific sequences in the genome. Engineering PAMs significantly increases the number of targetable sequences in the genome and can also enhance the efficiency and specificity of gene editing.

2.4 Engineering the Nanobiobots—Delivering Diverse Payloads

The CRISPR-Cas9 system functions as an RNA-guided nuclease that creates double-stranded breaks in DNA by cleaving two phosphodiester bonds. While it doesn't directly edit genes, it effectively prompts the cell's DNA repair machinery to initiate repair at a specific site.

Interestingly, the true editing capability of the CRISPR-Cas system emerged after the nuclease activity was partially or completely disabled. By mutating specific amino acid residues in one of Cas9's two nuclease domains, scientists developed a modified protein capable of making a single-strand break, or "nick," instead of a double-strand break. This modified protein is known as a "nickase." The nick can be made on either target DNA strand, depending on which nuclease domain is altered [8].

When both nuclease domains undergo specific single amino acid substitutions, the resulting protein can only bind to the target DNA but cannot break the phosphodiester bonds. This "dead" Cas9, termed dCas9, cannot induce double-stranded breaks. These two Cas9 variants, nickase and dCas9, can now be engineered to deliver various payloads, expanding the potential applications of the CRISPR-Cas system [9].

As part of the efforts to image genomic loci in living cells, a CRISPR/Cas system using deactivated/dead Cas9 (dCas9) was developed and optimized [10]. The CRISPR/Cas system's capabilities were extended to sub-cellular imaging by adding a fluorescent protein tag to dCas9 ribonucleoprotein (RNP) complex. Making EGFP the payload on the dCas9 nanobiobot allowed the visualization of the movement and localization of the complex within the cell, enabling the dynamic imaging of genomic loci in real-time and at high resolution in living human cells. This work provided insights into the dynamic nature of gene regulation in human cells.

With the ability to change payloads, several methods could be developed. Gilbert et al. developed a CRISPR-based system for the genome-scale control of gene repression and activation in human cells [11]. The system, called CRISPRi and CRISPRa, uses modified versions of the CRISPR/Cas9 enzyme to either inhibit or activate gene expression at specific genomic loci. CRISPRi uses a dead Cas9 enzyme that is unable to cut DNA, but can still bind to specific genomic loci and recruit proteins that inhibit transcription. CRISPRa uses a Cas9 enzyme that has been modified to bind a transcriptional activator protein, which can stimulate transcription at the target locus. The authors demonstrated the utility of these systems by using them to perturb the expression of thousands of genes in human cells and measuring the effects on cell behavior. They found that CRISPRi and CRISPRa

can be used to effectively control gene expression in a genome-wide manner and can provide insight into the functions of individual genes.

Similarly, protein payloads that can change the methylation status of both DNA and, methylation and acetylation status of DNA binding (histone) proteins are being used to experimentally tinker with the epigenomic landscapes.

In an extraordinary feat of protein engineering, novel CRISPR-based protein payloads have been created that simply edit single bases without causing double-strand breaks in DNA. There are two main types of CRISPR base editors: cytosine base editors (CBEs) and adenine base editors (ABEs). CBEs are created by fusing a dead Cas9 (dCas9) or nickase (Cas9n) with a cytidine deaminase enzyme, which can convert cytosine (C) to Uracil (U), which is then recognized as thymine (T) by the cellular machinery during DNA replication or repair. ABEs consist of dCas9 or Cas9n fused with a lab-evolved adenosine deaminase enzyme, which converts adenine (A) to inosine (I), which is functionally similar to guanine (G) and is recognized as such by the cellular machinery during DNA replication or repair.

Prime editing is a further advancement that enables the precise introduction of various genetic modifications, such as insertions, deletions, and base substitutions, without the need for double-strand breaks (DSBs) or donor DNA templates. Prime editing involves the use of a specially engineered protein called Prime Editor (PE), which is a fusion of Cas9 nickase (Cas9n) and a reverse transcriptase enzyme. The system also employs a unique single-guide RNA molecule called Prime Editing Guide RNA (pegRNA). The pegRNA has two essential parts: the spacer sequence that is complementary to the target DNA, and an extension containing the desired edit, called the primer binding site (PBS). The PBS is crucial for the reverse transcriptase to start copying the edit into the target DNA.

2.5 Phage Genomic Encoded CRISPR-Cas Systems

While CRISPR-Cas systems are encoded in microbial hosts to protect them from viral attacks, recent studies have shown the presence of a variety of CRISPR-Cas systems encoded in phage genomes. Interestingly, all six CRISPR types (types I–VI) found in bacteria are also found in phage genomes. This discovery is quite unexpected and seemingly counter-intuitive. Notwithstanding that, we now have access to this phage-encoded repertoire of CRISPR systems that can be adapted for genome engineering.

2.6 RNA-Guided Endonucleases in Eukaryotes

Over the last few years, studies have found TnpB, a transposon-associated RNA-guided DNA endonuclease, marking a significant discovery of an RNA-guided system. Investigating a specific transposon family, a study revealed that these transposons encode diverse RNA-guided DNA endonucleases, termed OMEGA (obligate mobile element–

guided activity). These nucleases, including TnpB, utilize RNA guides to target specific DNA sequences for cleavage. TnpB is identified as a likely ancestor of Cas12 endonucleases, indicating an evolutionary link to CRISPR-Cas systems. This finding expands the understanding of RNA-guided DNA targeting and introduces TnpB as a promising tool for genetic modification.

While many of these have been studied in prokaryotic lineages, there have been reports of TnpB-like proteins, termed Fanzors, that are widespread in diverse eukaryotic transposable elements (TEs). While there were suggestions about the possibility of TnpB being the ancestor of the eukaryotic transposon-encoded Fanzor (Fz) proteins, recent biochemical studies demonstrate that Fanzors are indeed RNA-guided DNA endonucleases [12]. This exemplifies that RNA-guided endonucleases are present in all three domains of life.

2.7 RNA-Activated Protease Activity

Adding to the many surprises, genes encode proteins that do not have nuclease functions (causing DNA or RNA cleavage) but instead have protein cleavage functions have been found in association with CRISPR systems. These CRISPR-associated proteases have endopeptidase activity that allows them to perform RNA-activated protein cleavage. One specific example, the Craspases, belongs to the type III CRISPR–Cas system with two major components: the nuclease component and a protease component. These Craspases perform a delicate balancing act between their nuclease and protease activities [13–17].

Within a couple of decades, we have learned about a wide variety of biochemical systems that have motivated researchers to create powerful tools for genome manipulation. This tremendous progress stems from the study of microbes, which have proven to be a treasure trove of genetic and molecular insights. Reiterating what was mentioned at the beginning, microbes are akin to the *akshyayapaatra*, or cornucopia, continually providing us with innovative tools that enhance our understanding of the genetic and cellular landscape. This microbial wealth has propelled the advancement of CRISPR technology, paving the way for groundbreaking applications in medicine, agriculture, and biotechnology. As we continue to explore and understand these microbial systems, we unlock further potential for transformative scientific and technological developments.

Questions

1. How different are bacterial innate and adaptive immune systems, in terms of effectiveness and specificity?
2. What are the similarities and differences between restriction endonucleases and RNA-guided nucleases?

Take-Home Message

- CRISPR-Cas systems are part of microbial adaptive immunity with a diverse repertoire, and have been adapted into the genetics toolkit of experimental biologists.
- These systems are being used as experimental tools in research laboratories, diagnostic and therapeutic tools in the clinic.
- The diversity of these systems suggests that there is more to discover and learn, and a greater number of potential tools that will emerge.

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CRISPR for the High School Classroom

3

David Wollert

3.1 Introduction

"Hidden inside some of the world's smallest organisms is one of the most powerful tools scientists have ever stumbled across. It's a defense system that has existed in bacteria for millions of years and it may someday let us change the course of human evolution."

That's the informational blurb associated with a 2015 *Radiolab* podcast episode about CRISPR [1].

During the 2016 Fall semester, I was just finishing up a class lecture on molecular genetics at Chattanooga State Community College. A student with a gleam in her eye approached me after class with a question: "Have you ever heard of CRISPR?" I hadn't. She told me she had just listened to a *Radiolab* podcast about a new gene editing technology that allowed scientists to quickly and easily edit an organism's DNA in whatever way they wanted. Of course, it sounded too good to be true. Nonetheless, I told her I would check it out. A few days later I listened to the podcast. I've been hooked on CRISPR ever since.

Despite the technology being in its infancy, I knew it was a game changer and needed to be introduced into the General Biology and Microbiology curriculum I was teaching at the college. At the time, there weren't many educational resources available. I came across a helpful *YouTube* video created by Paul Anderson (Bozeman Science) that inspired me to develop my own audiovisual and hands-on materials. These materials would eventually be presented at an NABT professional development conference, licensed by Howard Hughes Medical Institute (HHMI), and published in *The American Biology Teacher*. Such was the vacuum and necessity for teaching resources at the time. Fortunately, there are now plenty

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M. J. Wolyniak et al. (eds.), *Introduction to CRISPR-Cas9 Techniques*, Learning Materials in Biosciences, https://doi.org/10.1007/978-3-031-73734-3_3

of CRISPR-related resources available, including wet-labs that allow students to actually perform CRISPR gene editing in the classroom.

3.2 What Is CRISPR?

The story of CRISPR essentially began in 1987 when Japanese scientist Yoshizumi Ishino sequenced a small region of the *E. coli* chromosome. This was a time when DNA sequencing was still a tedious process, and scientists had yet to sequence any complete genomes or compile extensive searchable databases.

Ishino discovered a curious pattern in the *E. coli* DNA, namely a collection of short identical palindromic sequences separated by short stretches of what seemed to be random sequences. His published paper concludes with the guileless statement, "The biological significance of these sequences is not known" [2].

Similar patterns were subsequently discovered in a variety of microorganisms, such that this particular chromosome region acquired the name CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats). In 2005, Spanish researcher Francisco Mojica discovered that the random intervening sequences were not random after all. Rather, they were homologous to bacteriophage DNA (Fig. 3.1), with each spacer sequence being homologous to a particular type of phage [4].

Bacteriophage are viruses that exclusively infect bacterial cells, so scientists were immediately curious as to why bacteria would be housing phage DNA in their chromosomes. One early suggestion was that the CRISPR region was part of an immune defense against the viruses. This suggestion would be proven correct in 2007 by a pair of researchers working for the *Danisco* food corporation in Denmark [5].

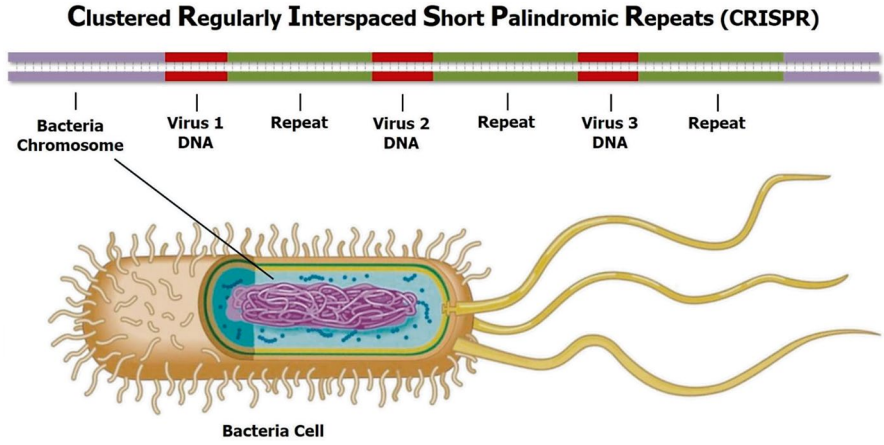

Fig. 3.1 Arrangement of palindromic repeats and viral spacer sequences in the CRISPR region of a bacterial chromosome [3]

Danisco was a major producer of yogurt and had a vested interest in studying the relationship between bacteriophage and bacteria, such as *Streptococcus thermophilus*, used in yogurt production. Rodolphe Barrangou and Philippe Horvath suspected that the CRISPR region of bacterial DNA might be part of an adaptive immune response against bacteriophage infection.

In order to test their hypothesis, they performed an experiment that lends itself well to discussing the scientific method in the classroom. First, they sequenced the CRISPR region of wild-type *S. thermophilus*. Next, they inoculated *S. thermophilus* with a particular bacteriophage to which the culture was susceptible. They spread the culture on a growth plate in hopes that at least a few mutant colonies might survive the infection. Indeed, they found nine mutant colonies that survived.

They then sequenced the CRISPR region of the mutant survivors and were excited to see that the bacteria had added a short spacer of the bacteriophage DNA sequence to their chromosomes. Apparently, the bacteria had somehow "learned" to recognize this particular type of virus. And because the new spacer was incorporated into the chromosome, this recognition capacity would be inherited by all future daughter cells.

The discovery was exciting, but it didn't actually explain *how* the CRISPR system worked to provide immunity against the virus. In 2008, Stan Brouns (working in the lab of Joh van der Oost) discovered that the CRISPR region is transcribed into RNA, so RNA apparently played a role in the immune defense [6].

Yet another clue came from the work of Danish researcher Ruud Jansen, who had discovered protein-encoding genes associated with the CRISPR region. These Cas (CRISPR-associated) genes encode proteins with a variety of helicase and nuclease capabilities [7]. Certainly, the most famous Cas protein is Cas9, discovered by the Russian scientist Alexander Bolotin, while working at the French National Institute for Agricultural Research [8].

Cas9 was isolated from *Streptococcus pyogenes*, a microbe infamous for causing strep throat. Cas9 is a nuclease enzyme capable of cutting (cleaving) DNA. In that sense, it is similar to the restriction enzymes discovered several decades earlier that became staple workhorses of molecular genetics research. But Cas9 differs from restriction enzymes in a remarkable and highly significant way: Cas9 is *programmable.* This would be the pivotal insight worked out by Emmanuelle Charpentier and Jennifer Doudna that eventually led to their 2020 Nobel Prize for the discovery of CRISPR-Cas9 gene editing (Fig. 3.2) [9].

American Jennifer Doudna was an RNA expert working at Cal-Berkley, while Emanuelle Charpentier was a French professor working at Umeå University in Sweden. The two met at a research conference in 2011 that led to their collaborative work with CRISPR-Cas9. Charpentier had discovered an RNA molecule used by Cas9 to direct the cutting of DNA [10]. Doudna had been exploring the function of other Cas proteins and was intrigued by the role RNA played with Cas9.

Fig. 3.2 Emmanuelle Charpentier and Jennifer Doudna. They received the 2020 Nobel Prize in Chemistry "for the development of a method for genome editing." Wikimedia Commons Photo credits: Bianca Fioretti (Hallbauer & Fioretti) and Duncan Hull (The Royal Society)

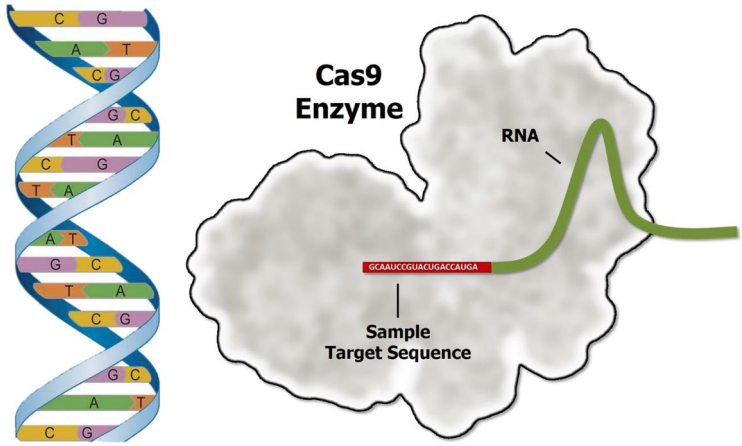

Fig. 3.3 Nuclease capacity of the Cas9 enzyme. Cas9 will cut dsDNA at a location complementary to the 20-nucleotide target sequence specified by the attached RNA [3]

The RNA contained a CRISPR repeat sequence adjacent to a short 20-nucleotide viral spacer sequence. Indeed, it was the 20-nucleotide spacer sequence that guided the Cas9 enzyme to a specific DNA cutting location. By changing which viral spacer sequence was loaded into Cas9, the enzyme could be programmed to cut at potentially any target sequence (Fig. 3.3). Obviously, this was a significant difference between Cas9 and traditional restriction enzymes, which only cut at a short (perhaps six nucleotide) and fixed target sequence. The restriction enzyme *EcoRI*, for example, always and only cuts at GAATTC.

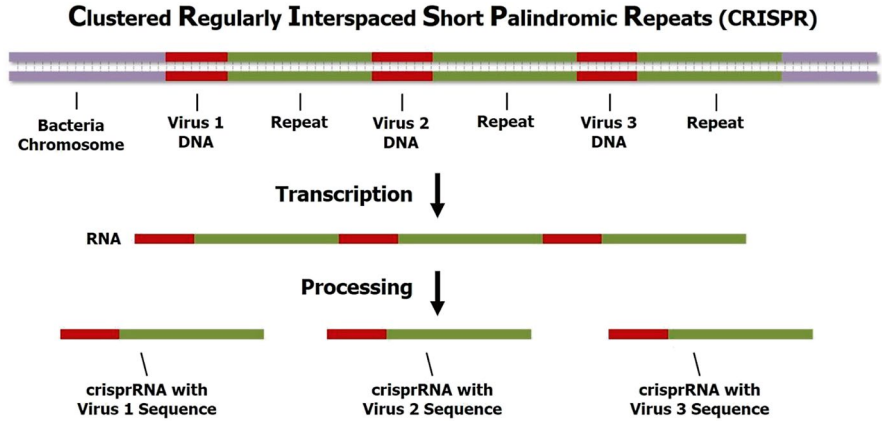

Fig. 3.4 Transcription of CRISPR region of bacterial chromosome. The CRISPR region of the chromosome is transcribed into RNA, which is then cleaved into separate crisprRNA molecules (also called crRNA). Each crisprRNA contains a repeat sequence and a short viral sequence specific to a particular strain of bacteriophage [3]

Doudna and Charpentier proceeded to work out the natural functioning of the CRISPR immune system, which can be summarized as follows (Figs. 3.4 and 3.5):

1) Bacteria incorporate short stretches of bacteriophage DNA into the CRISPR region of the chromosome. Each phage DNA (spacer) sits adjacent to a short palindromic repeat sequence.
2) The entire CRISPR region of the chromosome is transcribed into one long stretch of RNA.
3) The RNA is then broken into multiple fragments of crisprRNAs, with each fragment consisting of a repeat sequence and a spacer (viral target) sequence.
4) Each crisprRNA is loaded into a separate Cas9 protein, thus programming each Cas9 nuclease to seek out and cut DNA matching the viral target sequence.
5) If a virus with DNA matching the target sequence were to enter the bacteria cell, the appropriate Cas9/RNA complex would recognize the viral DNA via complementary base-pairing.
6) Cas9 would then cut (cleave) the invading viral DNA, thus preventing successful infection.

With the CRISPR-Cas9 mechanism finally elucidated, Doudna and Charpentier immediately recognized the potential of harnessing the programmable capabilities of Cas9 in the laboratory. Their goal was to create a crisprRNA of their own design, so as to intentionally target a DNA cutting site of their own choosing. If successful, they could potentially use CRISPR-Cas9 to disable specific genes. The significance of such a tool cannot be overstated, as the disabling of genes has traditionally been the means by which a gene's function is elucidated. If you want to learn the function of your car's alternator, one approach is to disable it and see what effect that has on the car's performance.

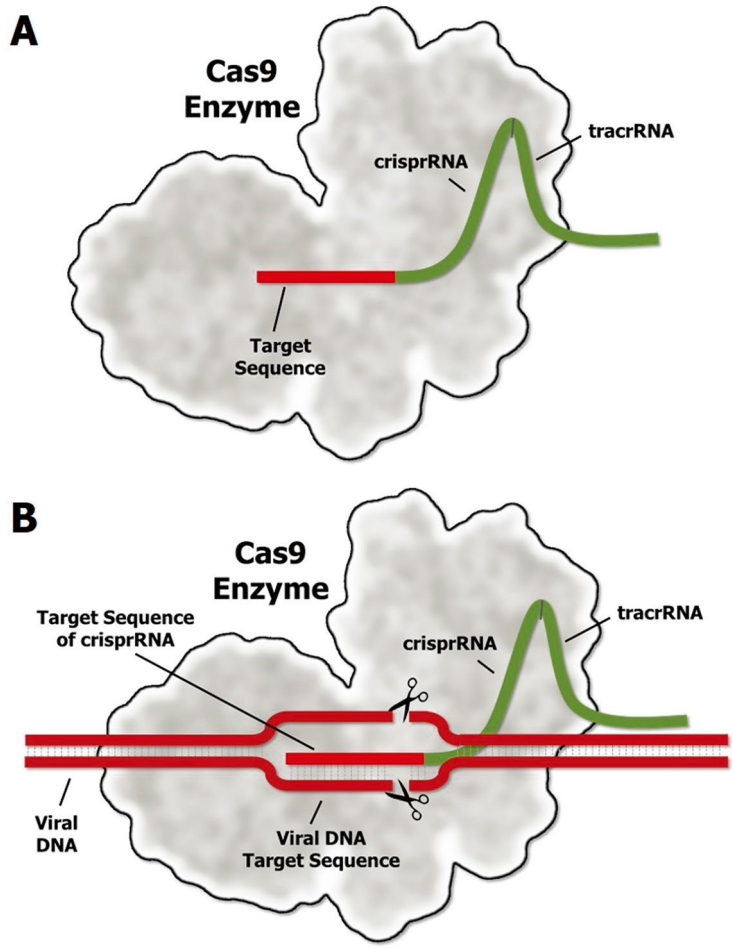

Fig. 3.5 Cas9 enzyme programmed with crisprRNA and tracrRNA. (**a**) crisprRNA is loaded into the Cas9 enzyme by attaching to tracrRNA. (**b**) The programmed CRISPR-Cas9 complex binds to viral DNA that is complementary to the target sequence in the crisprRNA. The enzyme complex then creates a double-strand break, which inactivates the viral DNA [3]

Doudna's lab set up a classic experiment to test the approach. (The experiment is explored in one of the CRISPR paper model activities reviewed later in this chapter and also lends itself well to a classroom discussion of the scientific method.)

Their experiment utilized *E. coli* that had been previously engineered to possess the Green Fluorescent Protein (GFP) gene. Doudna's lab designed and synthesized a crisprRNA (which they called guideRNA) to recognize a 20-base sequence within the GFP gene. They then introduced plasmids into *E. coli* that encoded the Cas9 enzyme and the GFP-targeting guideRNA. The expressed Cas9 protein would combine with the transcribed guideRNA and proceed to cut the GFP gene at the recognized target sequence. Disabling the GFP gene would produce an obvious phenotypic change, as the resulting

colonies of bacteria would no longer produce the green-fluorescent pigment. (Note: Some of the wet-lab classroom activities discussed later in this chapter perform a similar phenotypic color change experiment by disabling the *LacZ* gene.)

The experiment was successful, as immediately evidenced by the growth of non-green colonies. Doudna wanted to confirm that the GFP gene had been cut at the exact location targeted by the guideRNA. To provide evidence of precise cutting, her lab cut the DNA at a second targeted location using a traditional restriction enzyme. If the restriction enzyme and the CRISPR-Cas9 complex each cut at their intended location, it would produce a DNA fragment of a predictable size. Sure enough, the fragments were revealed using gel electrophoresis.

Doudna and Charpentier published their work in 2012, which included yet another guileless remark in the history of CRISPR, "We propose an alternative [gene editing] methodology based on RNA-programmed Cas9 that could offer considerable potential for gene-targeting and genome-editing applications" [9].

The next challenge was to see if CRISPR could be used to edit the DNA of eukaryotic cells, including human cells. Toward this end, Doudna's lab successfully used CRISPR to disable a gene in cultured human kidney cells [11].

Around the same time, Feng Zhang and George Church of the Broad Institute and Harvard were also using CRISPR to edit human cells, but they took the technique a step further [12, 13]. Indeed, Zhang and Church used CRISPR to introduce a *new* DNA sequence at the site of the cut DNA. They did so by exploiting a mechanism of DNA repair known as Homology-Directed Repair (HDR).

Eukaryotic cells (including human cells) have evolved two repair mechanisms for dealing with broken (or cut) DNA: Non-Homologous End Joining (NHEJ) and Homology-Directed Repair (HDR). NHEJ is the simpler of the two processes. The cell essentially uses a few spare nucleotides as a type of glue to connect the broken ends of the DNA back together. The incorporated random nucleotides, however, constitute a mutation. If the NHEJ repair occurs within a gene sequence, the resulting mutation will likely disable the gene. Thus, the gene is *knocked-out* (Fig. 3.6).

Disabling a gene can impair cellular function. Thus, the preferred mechanism of repair is HDR. Recall that most eukaryotic cells are diploid, with each chromosome being part of a homologous pair of similar genetic information. If one member of the pair is somehow broken, the other chromosome can serve as a guiding template for repair. The cell simply copies the appropriate region of the intact chromosome into the broken region of the damaged chromosome. In so doing, the broken chromosome is restored back to a functional sequence.

Jhang and Church attempted to hijack the HDR repair system. In performing their CRISPR experiment, they provided the cell with a piece of *donor* DNA of their own design. The ends of the donor DNA consisted of sequences matching the cut ends of the cell's chromosome. The middle region of the donor DNA, however, consisted of whatever sequence the researchers chose. In other words, they could include a *new* gene sequence in the donor DNA. The assumption was that HDR would recognize the ends of the donor

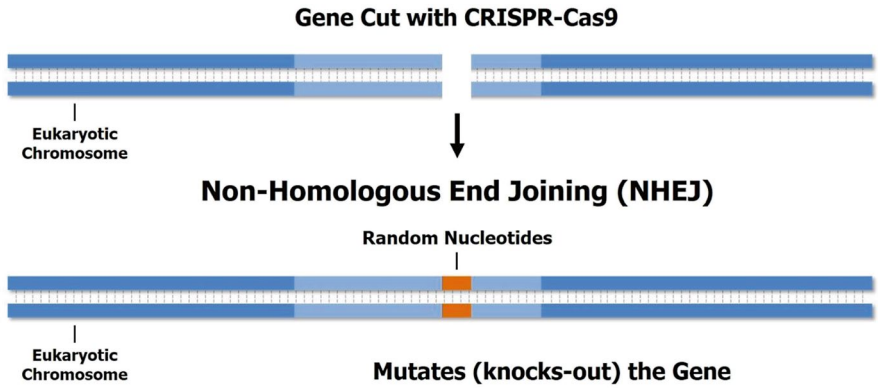

Fig. 3.6 DNA repair by non-homologous end-joining (NHEJ). The cut DNA is repaired by incorporating random nucleotides at the site of the break. These additional nucleotides, however, introduce a mutation into the gene, which disrupts the gene's function [3]

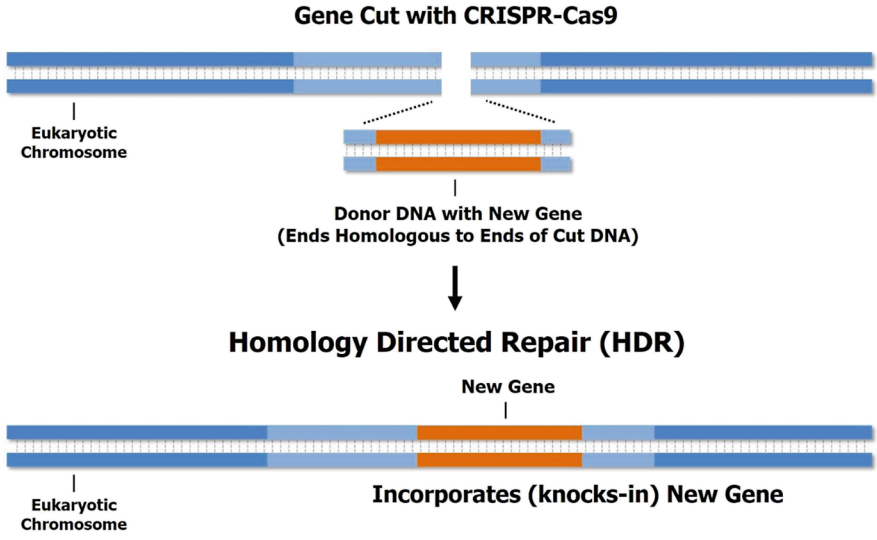

Fig. 3.7 Homology Directed Repair (HDR) of DNA. The cut DNA is repaired by copying the template DNA molecule (donor DNA) into the repair site. The ends of the donor DNA are homologous to the ends of the cut DNA (a requirement for HDR), but the donor DNA also contains a new gene that is smuggled into the repair site [3]

DNA as homologous to the cut DNA and repair the cut by copying the entire donor DNA sequence into the repair site. The new gene could, therefore, be *knocked-in* using a metaphorical Trojan horse (Fig. 3.7). With the proven ability to knock genes in and out of cells, the prospect of using CRISPR to treat genetic disease became a very real possibility.

The applications of CRISPR technology are far-reaching. Since the first demonstrated use of CRISPR to *knock-in* and *knock-out* genes, the technology has gone on to impact

numerous areas of biological research and application. CRISPR has already been used to correct a variety of genetic conditions in cultured human cells, live animal models, and even adult humans. Examples include HIV infection, cancer, blindness, deafness, sickle cell disease, cystic fibrosis, and diabetes, just to name a few [14]. Such treatments are in various stages of development and trials. In 2023, the Food and Drug Administration officially approved the first CRISPR-based treatment for sickle cell disease.

In addition to its many biomedical applications, CRISPR also has the potential to revolutionize the food industry. In 2016, the U.S. Department of Agriculture approved the first CRISPR-edited food item for human consumption—white button mushrooms. Plant biologists at Pennsylvania State University used CRISPR to disable an enzyme that normally causes the mushrooms to brown, thereby extending their shelf life [15].

Scientists have created drought-resistant grain, corn with more kernels, kale without the bitter taste, and tomatoes producing large amounts of GABA, an inhibitory neurotransmitter in the brain, just to give a few examples [16]. Scientists are also using CRISPR to edit a wide variety of domesticated animals, including stronger dogs, meatier chickens, and beefier, hornless cattle [17].

Another fascinating CRISPR application called a gene drive offers the potential to introduce genes quickly and efficiently into large-scale populations [18]. One goal of this technology is to drive the malaria-transmitting *Anopheles* mosquito to extinction [19]. CRISPR applications have even made it onto lists of weapons of mass destruction (WMDs), as government leaders recognize how a similar gene drive technology could be exploited by terrorists with nefarious intentions [20].

The temptation to control nature is ever present and provides an ongoing tension between that which we *can* do and that which we *should* do. This temptation was recognized soon after the genetic code was deciphered in the 1960s. According to Marshall Nirenberg, one of the code-crackers himself, "Decisions concerning the application of this knowledge must ultimately be made by society, and only an informed society can make such decisions wisely."

Current and future applications of CRISPR are too numerous to discuss here, not to mention the many bioethical challenges raised by the technology. A short, but more thorough, overview of CRISPR written for high school teachers and students can be found in *The American Biology Teacher* [3]. Fortunately, there are many resources to help you bring this fascinating topic to your students.

3.3 CRISPR in the High School Classroom

There are many resources available to facilitate teaching CRISPR in the high school classroom including discussion-based approaches, paper model dry-labs, wet-lab simulations, and lab kits that allow students to actually perform CRISPR-based gene editing. A table summarizing the activities and resources discussed in the following pages can be found at the end of the chapter. A teacher's preferred choice of activity will depend upon budget,

equipment, and time constraints, as well as the level of student preparedness. Some activities lend themselves to general high school biology classes, while others may be more appropriate for AP and IB courses. Students will need a basic understanding of DNA structure (particularly complementary base-pairing) and the central dogma of molecular genetics.

The Next Generation Science Standards (NGSS) are an effort to standardize science content taught in schools across the nation [21]. The topic of CRISPR easily aligns with NGSS standards:

Performance Expectations

- **HS-LS1-1.** Construct an explanation based on evidence for how the structure of DNA determines the structure of proteins, which carry out the essential functions of life through systems of specialized cells.
- **HS-ETS1-1.** Analyze a major global challenge to specify qualitative and quantitative criteria and constraints for solutions that account for societal needs and wants.

Disciplinary Core Ideas

- **LS1.A: Structure and Function**
  - All cells contain genetic information in the form of DNA molecules. Genes are regions in the DNA that contain the instructions that code for the formation of proteins, which carry out most of the work of cells. (HS-LS1-1)
  - **Note:** This Disciplinary Core Idea is also addressed by HS-LS3-1.
- **ETS1.A: Defining and Delimiting an Engineering Problem**
  - Criteria and constraints also include satisfying any requirement set by society, such as taking issues of risk mitigation into account, and they should be quantified to the extent possible and stated in such a way that one can tell if a given design meets them. (HS-ETS1-1)

Science and Engineering Practices

- Developing and Using Models
- Constructing Explanations and Designing Solutions

Crosscutting Concepts

- Structure and Function
- Connections to Engineering, Technology and Applications of Science Influence of Science, Engineering, and Technology on Society and the Natural World

3.4 Case Studies

Case studies can be an effective means of bringing CRISPR into the classroom. In addition to overcoming the challenges posed by expensive lab experiments, case studies foster inductive and deductive reasoning while honing problem solving skills. Case studies can also incorporate and connect multiple aspects of a topic. For example, a good CRISPR case study can be used to teach the molecular biology of gene editing, while simultaneously exploring its potential bioethical ramifications.

The National Center for Case Study Teaching in Science (NCCST), hosted by the National Science Teachers Association (NSTA), contains nearly a 1000 peer-reviewed case studies on a variety of topics in all areas of science. The case studies themselves are freely accessible, although a subscription is required for access to teaching notes and answer keys.

The NCCST case studies are well-constructed and written by STEM educators. At the time of this writing, only a few cases focused on CRISPR, but that number will certainly increase in the near future. One such example is discussed below:

3.4.1 Cut It Out! Editing DNA with CRISPR-Cas9

https://www.nsta.org/ncss-case-study/cut-it-out

This four-part case study introduces students to CRISPR using a variety of online resources, including a TED Talk, magazine article, and scientific journals. The resources (links provided) are incorporated into a family narrative involving Duchenne Muscular Dystrophy (DMD). The activity is best suited for an AP biology class, as it covers some advanced topics. The teacher might consider modifying the activity for less advanced classes.

Part I: An inquisitive grandfather and lifelong learner come across Jennifer Doudna's TED Talk on CRISPR. Students watch the video to get a broad overview of CRISPR gene editing.

Part II: The grandfather's daughter (an expectant mother) is meeting with a genetic counselor to discuss the possibility of her child having DMD. The mother learns from an article in a science magazine about a new gene tool that has corrected DMD in mice. Students read the article and get a more detailed explanation of how CRISPR has been used to treat a specific genetic disease.

Part III: A granddaughter is sitting in her biology class and learns how bacteria use CRISPR as an immune defense against viral infection. Students read an article in the journal *Bioessays* to learn about this natural function of CRISPR in prokaryotes.

Part IV: The granddaughter is inspired to research CRISPR further in hopes of talking about it with her expectant aunt. Students read three journal articles to get a deeper understanding of CRISPR. Each section in the case study concludes with a set of discussion questions to help guide the activity.

Advantages

- Cost-effective
- Potentially suitable for general high school class or AP

Disadvantages

- No hands-on experience
- Students read journal articles that may be too advanced for their level of preparation

3.5 Dry-Lab Activities

Hands-on experiments are a great way to engage students and promote kinesthetic learning. Nonetheless, few high school biology teachers operate with an abundance of funding for perishable lab kits and expensive lab equipment. Fortunately, there are cost-effective dry-lab activities that can be used to engage students with CRISPR-based gene editing.

One of the challenges of teaching CRISPR, and molecular biology in general, is the abstract nature of the topic. Molecular biology occurs at a scale inaccessible to even the best light microscopes. For this reason, paper models are an effective hands-on method for teaching CRISPR-based gene editing. Moreover, paper models can present CRISPR at a variety of comprehension levels.

At the most basic level (and perhaps most appropriate for general high school students), a paper model can simulate the Cas9 enzyme cutting DNA at a specific sequence, as depicted by written nucleotide letters or by color-coded regions of paper. At a more advanced level (appropriate for AP biology), paper models can require students to research genes, identify potential target sequences using a sequence database, design custom guideRNAs, account for Protospacer Adjacent Motif (PAM) sequences, and then use their custom paper model to simulate cutting of DNA by Cas9, while perhaps also predicting the size of resulting fragments. Examples of two paper model options are discussed below.

3.5.1 Dry-Lab Activities for Introducing Students to CRISPR-Based Gene Editing

https://online.ucpress.edu/abt/article-abstract/82/5/315/110288/Wet-amp-Dry-Lab-Activities-to-Introduce-Students?redirectedFrom=fulltext

This paper model activity appeared in the May 2020 issue of *The American Biology Teacher (ABT)* and introduces CRISPR by simulating the groundbreaking experiments in which CRISPR was first used to *knock-out* a gene [22]. Students also use the paper model to simulate how CRISPR can be used to *knock-in* a new gene sequence. Access to the linked article requires a subscription to the *ABT* journal, but the article could also be

accessed via interlibrary loan. *MiniOne Systems* has also developed a CRISPR kit that includes this paper activity.

As discussed previously, Doudna's lab designed a guideRNA to specifically target and disable the Green Fluorescent Protein (GFP) gene in a strain of *E. coli*. Their success in cutting the gene was the first example of CRISPR-based gene editing. This paper activity allows students to simulate that famous experiment.

After reading through a short introduction of CRISPR, students cut out a Cas9 enzyme, guideRNA, and a long stretch of bacterial DNA that contains the GFP gene. Students then select an appropriate 20-nucleotide target sequence within the gene and write that sequence on the paper guideRNA. Students use the paper model to kinesthetically simulate loading of guideRNA into Cas9, scanning of bacterial DNA for the target sequence, and cutting DNA at the target location. Students then simulate electrophoresis of the resulting fragments. (Note: As discussed in the next section of the chapter, this activity can be extended into a wet-lab activity in which students perform electrophoresis using predigested fragments of DNA.)

In the second part of the activity, students learn about two methods of DNA repair: Non-Homologous End-Joining (NHEJ) and Homology Directed Repair (HDR). They further learn how HDR can be utilized to introduce new DNA sequences into cuts created by CRISPR. The paper model is used to simulate how DNA can be cut with CRISPR and a new gene introduced via HDR.

A unique advantage of these paper activities is their connection to the actual experiments first used to demonstrate the power of CRISPR. Thus, a history of science components enters into the learning experience. The level of depth presented in these activities lends itself well to the high school classroom, although perhaps best suited for an AP class. The paper model activities can easily transition into a discussion of the many current and potential applications of CRISPR technology, including those with important bioethical considerations.

MiniOne Systems has developed a CRISPR lab kit that includes this dry-lab paper activity, along with materials and reagents for the corresponding wet-lab activity that appeared in the same *ABT* article. The wet-lab activity is discussed under *CRISPR Simulations* later in this chapter.

Advantages

- Presents CRISPR at a basic level suitable for most high school biology classes
- Simulates a famous experiment, which may engage student interest
- Inexpensive (Cost of photocopying materials onto cardstock)

Disadvantages

- Simulation, rather than actual experiment
- Level of depth may be more suitable for AP

3.5.2 Building a Paper Model of CRISPR-Cas9

https://www.biointeractive.org/classroom-resources/building-paper-model-crispr-cas9

This HHMI activity was developed from the paper model activity described above. HHMI licensed and adapted the model to serve as a more general and basic simulation of CRISPR. Students cut out paper models of Cas9, guideRNA and targetDNA. The guideRNA and targetDNA already have sequences written on them, so the concept of Cas9 targeting and cutting of DNA can be explained and simulated quickly and easily.

The activity also simulates the difference between NHEJ and HDR in a basic and general manner, which allows students to understand the processes of *knocking out* a gene and *knocking in* a new sequence.

Once students have used the paper model, the activity continues with a self-paced *Click & Learn* activity that allows students to explore CRISPR using interactive animations and short videos about scientists currently using CRISPR in their research. This activity could be followed by a group or classroom discussion about potential applications and bioethical issues surrounding CRISPR technology.

Advantages

- Presents CRISPR at a basic level suitable for most high school biology classes
- The second part of the activity (self-paced Click & Learn) can be done at home
- Reasonable class time commitment: One to two 50-min class periods
- As with most *BioInteractive* resources, HHMI provides downloadable Google Docs (PDF) that can be modified, along with plenty of high-quality animations and video supplements
- Inexpensive

Disadvantages

- Simulation, rather than actual experiment

3.6 Group Projects

3.6.1 CUT! How Does CRISPR Work?

https://www.sciencebuddies.org/teacher-resources/lesson-plans/how-does-CRISPR-work#:~:text=CRISPR%20harnesses%20the%20natural%20immune,is%20called%20the%20CRISPR%20array

Science Buddies offers an interesting non-traditional approach to teaching CRISPR in the high school classroom. The activity initially introduces students to CRISPR using a

variety of interactive simulations and other resources (links provided). Students then demonstrate their knowledge of CRISPR by constructing a physical model of CRISPR-Cas9 using simple home materials. After completing the physical model, students create a stop-motion animation video depicting and explaining the molecular process of CRISPR-based gene editing. The activity concludes with a guided discussion of ethical challenges posed by CRISPR technology.

This well-designed guided activity consists of three pedagogical stages: Engage, Explore, and Reflect. During the *Engage* component, students review their understanding of genes and genetic disease in a guided discussion format. A video resource presents the *NIH Common Fund Somatic Cell Genome Editing Program*, which is followed by a basic introduction of CRISPR.

The activity continues with an *Explore* component in which students watch a variety of videos that explain and discuss CRISPR in more detail. Once they have learned the basics of CRISPR, they are assigned to groups with the direction to create a physical model of CRISPR and short (2–4 min) stop-motion animation video depicting the CRISPR mechanism.

The group work begins with a guided brainstorming session about constructing the physical model and developing a storyboard for the animation. The lesson plan provides a video resource and storyboarding template to help students with this important step. Indeed, most of the learning about the mechanics of CRISPR will occur in the storyboarding process.

The lesson plan also includes a video resource explaining stop-motion animation. Students are encouraged to use the app *Stop Motion Studio*, which has both iOS and Android versions. After constructing their physical model of CRISPR using inexpensive home materials, they photograph the steps of CRISPR activity and create their animation video. Videos are submitted electronically and viewed by the class.

The activity concludes with a *Reflect* component in which students participate in a discussion regarding the many applications of CRISPR, both current and potential, along with bioethical considerations. The lesson plan provides guidance for the discussion, which also mentions career connections. Finally, students are assessed on their understanding of CRISPR using a provided quiz and grading rubric.

Advantages

- Utilizes non-traditional hands-on approach by having students create stop-motion animation videos
- Engages student creativity and may appeal to less science-oriented students
- Excellent well-designed teaching plan and rubrics provided
- Adaptable to remote learning
- Inexpensive

Disadvantages

- Lengthy class time commitment, although much can be done outside of class if necessary
- Requires students to learn stop-motion animation software

3.7 Wet-Lab Activities

One of the best ways for students to learn science is through hands-on experimentation. Nonetheless, some types of experiments lend themselves well to the classroom, while others present both conceptual and financial challenges. There are numerous lab kits available to science educators covering a wide range of molecular biology topics. Although many of the experiments are simple to perform, the challenge is to avoid a lab experience in which students simply follow a few cookbook instructions without a complete understanding of what is taking place in the tubes and flasks in front of them. They can proudly announce that they have "performed genetic engineering," but they may not be able to *explain* what they did or the protocol involved.

Molecular biology lab kits can also be prohibitively expensive. Many kits are designed for one class of approximately 32 students (8 groups of 4 students). Given the limitations of many high school budgets, teachers often resort to using a single kit as a demo for each of their class sections, rather than having multiple small groups perform the experiment in each class. This approach circumvents the cost issue, but it also makes the experiment less engaging and effective. These limiting parameters also apply to CRISPR lab kits, which are relatively new and essentially come in two forms: CRISPR simulations and actual CRISPR experiments.

3.7.1 CRISPR Simulations

CRISPR simulation kits are generally less expensive and can present CRISPR at a more basic level. A common approach is to have students learn about CRISPR, apply their knowledge to a hypothetical laboratory experiment, and then perform an activity, such as electrophoretic separation of predigested DNA fragments, which is then interpreted in the context of the simulated experiment. No actual gene editing is performed, but students get to perform an experiment and evaluate data as if an actual gene editing experiment had been conducted. This approach allows students to learn and perform specific molecular research techniques (such as pipetting and electrophoresis), while engaging in critical thinking about a simulated experiment. Moreover, simulation lends itself to a wide variety of real-life experimental scenarios, from curing genetic disease to engineering our food supply. An example of this type of activity is outlined below.

3.7.1.1 Wet-Lab Activities for Introducing Students to CRISPR-Based Gene Editing

https://theminione.com/product/crafting-genetics-crispr-lab-kit/

MiniOne Systems has developed a CRISPR simulation kit based upon a wet-lab activity published in *The American Biology Teacher* [22]. The kit also includes the paper model activity published in the same article and discussed previously. Students are introduced to CRISPR and the landmark experiments that demonstrated the extraordinary potential of the new technology.

Students first use a paper model to simulate how Doudna's lab used CRISPR to *knock-out* the GFP gene in bacteria. Recall that Doudna's experimental design allowed researchers to predict in advance the size of DNA fragments that would be generated if CRISPR cut the DNA at precise targeted locations. Thus, in addition to the paper activity, *MiniOne* kit has students perform electrophoretic separation of predigested DNA fragments resulting in a pattern of bands similar to what would have been generated in Doudna's original CRISPR experiment. Students also separate fragments of DNA to simulate how homology-directed repair (HDR) can be used to *knock-in* new gene sequences. *MiniOne* provides two 20-min videos that provide an introduction to CRISPR, including its discovery, myriad applications, and bioethical considerations.

Advantages

- Simulates a famous experiment, which may engage student interest
- Kit includes printed paper activity and all reagents necessary for electrophoresis
- Two 20-min video resources introduce students to CRISPR and its applications

Disadvantages

- Requires molecular biology lab equipment for electrophoresis

3.7.1.2 Using CRISPR to Treat Cystic Fibrosis

https://www.edvotek.com/135

Edvotek has developed a small collection of lab kits that simulate various CRISPR scenarios. This particular kit focuses on a genetic mutation found in cystic fibrosis patients. Students initially learn background about CRISPR and its potential for treating genetic diseases, such as cystic fibrosis.

In Module I of the activity students design and evaluate a unique guideRNA molecule for targeting the cystic fibrosis mutation. The goal is to identify a 20-base target sequence within the CFTR gene, but which will not target other regions of the genome. This step highlights the specificity of CRISPR-targeting (as compared to restriction enzymes), while also introducing the concern of off-target mutations. Once a suitable guideRNA sequence has been identified, a hypothetical experiment is performed in which a Cas9/guideRNA complex is used to cut the targeted region in the DNA of a cystic fibrosis patient.

In Module II, students perform real electrophoresis to separate the fragments of DNA resulting from the hypothetical experiment. The DNA is actually predigested phage DNA designed by *Edvotek* to produce a banding pattern that can be interpreted in light of the hypothetical experiment. This allows students to predict and interpret results generated from a wet-lab activity. The lab activity can then be followed up with a general discussion of CRISPR applications and bioethical considerations.

Advantages

- Built around the topic of cystic fibrosis, so easily relatable to other topics in genetics
- Relatively inexpensive for a molecular genetics wet-lab

Disadvantages

- Requires molecular biology lab equipment for electrophoresis

3.7.1.3 A-Maize-ing Editing: Using CRISPR to Improve Crops

https://www.edvotek.com/210

This *Edvotek* kit simulates CRISPR editing of the SH2 gene in corn in order to improve taste and increase sweetness of the crop. Similar to the previous *Edvotek* lab, students initially learn about CRISPR and then explore its potential to genetically engineer food crops.

In Module I of the activity, students design a guideRNA to target and disrupt the SH2 gene. This gene normally encodes an enzyme that transforms sugar into starch. By disabling the gene, the corn will retain more sugar, which will improve flavor and sweetness.

An interesting component of this particular lab activity is that students design and evaluate their guideRNA using the online BLAST sequence database. This introduction of bioinformatics is a valuable extension of the activity and makes the simulation a bit more authentic.

In Module II, students perform electrophoresis to separate the fragments of DNA resulting from the simulated cutting of SH2 by the custom Cas9/guideRNA complex designed by the students. Again, this allows students to predict and interpret results generated from a wet-lab activity. The lab activity can then be followed up with a general discussion of CRISPR applications and bioethical considerations. This particular lab activity specifically lends itself to a discussion of genetically modified organisms (GMOs).

Advantages

- Built around the topic of genetically engineered food, which should attract student interest
- Introduces students to bioinformatics through its use of BLAST
- Relatively inexpensive for a molecular genetics wet-lab

Disadvantages

- Requires electrophoresis lab equipment

3.7.2 CRISPR Experiments

CRISPR is not the first gene editing technology developed in the laboratory. In the early 2000s, researchers began pioneering Zinc-Finger Nucleases (ZFNs) and Transcription-Activator-Like Effector Nucleases (TALENs) to make precise edits in DNA. These cobbled-together artificial enzymes made it possible to create targeted cuts in DNA, but the process was expensive, tedious, and time-consuming.

Doudna and Charpentier immediately recognized the potential of CRISPR due to its precision, ease of use, and minimal cost. Their landmark paper concludes: "Zinc-finger nucleases and transcription-activator–like effector nucleases have attracted considerable interest as artificial enzymes engineered to manipulate genomes. We propose an alternative methodology based on RNA-programmed Cas9 that could offer considerable potential for gene-targeting and genome-editing applications" [9].

Indeed, gene editing with CRISPR is so quick, easy, and affordable that it is now being done in thousands of labs around the world, as well as in high school and college classrooms. Educational kits in which students actually use guideRNA and Cas9 to *knock-out* genes and/or *knock-in* new sequences are becoming readily available. These kits are a bit more expensive than simulation kits and require a bit more lab equipment, but the prospect of students gaining hands-on experience with this cutting-edge research tool may justify the time and investment.

Most of the CRISPR lab kits currently available are designed around the *LacZ* gene in *E. coli*. A gene in the lac operon *(LacZ)* encodes an enzyme called β-galactosidase (β-gal), which catalyzes the hydrolysis of the sugar lactose into its component sugars. β-gal can also hydrolyze a sugar analog called X-gal, which produces a blue pigment after it is hydrolyzed. Thus, bacteria expressing functional β-gal turn blue when they are grown in the presence of X-gal.

The goal of these lab kits is for students to use CRISPR to cut and disable the *LacZ* gene. Successful cutting of the gene results in cells unable to digest X-gal, which can be recognized phenotypically, as cells will no longer produce blue pigment and appear as white colonies on a growth plate. In this manner, the experiment is analogous to the Doudna lab's disabling of the GFP gene in its landmark experiment.

These kits typically involve a transformation step, which can be challenging for students to perform successfully. Most of the kits require a significant amount of prep time, particularly those involving live cultures. An additional challenge is that cultures must be grown 24–48 h in advance of class and must then be used within 24–48 h to ensure successful editing and transformation. This can present a challenge if a teacher's classes are

spread out over a week. Nonetheless, the kits generally work well and allow students to actually perform CRISPR gene editing. A few examples of such kits are presented below.

3.7.2.1 Out of the Blue CRISPR Kit

https://www.bio-rad.com/en-us/sku/12012608EDU-out-blue-crispr-kit?ID=12012608EDU&gclid=Cj0KCQjwl92XBhC7ARIsAHLl9al39tBd-mIV2bVBDeHw2IGO0-5a2-hXMAfEfDcor6o3SKa2_4c2qEgaAkqaEALw_wcB

This *BioRad* kit essentially operates as described above. Students use CRISPR to edit the *LacZ* gene in *E. coli*, resulting in cells that are unable to digest X-gal, as recognized by a blue-to-white color change. Although CRISPR is used to cut the *LacZ*, the gene is subsequently repaired by HDR using a short piece of donor DNA. The donor DNA introduces a stop codon into the gene, which prevents the synthesis of a functional protein.

The experimental design is robust and lends itself to teaching several aspects of experimental design and molecular genetics, including operons and HDR. However, the depth of contextual knowledge involved may also present a challenge if using the kit in the general high school classroom. The kit can certainly be used successfully in a simple manner that allows students to "do CRISPR," but to fully understand what is taking place requires a deeper understanding of experimental design and molecular genetics. As such, the kit is best suited to an AP course.

Although successful gene-editing is easily confirmed by the blue-to-white color change of the bacterial colonies, *BioRad* offers an optional genotyping extension kit in which students use PCR to confirm the precise gene edit. PCR is performed using primers designed to target the edited region of the DNA. PCR products are then separated by gel electrophoresis with predicted bands confirming the addition of donor DNA at the edited site.

Advantages

- Students get to perform CRISPR-based gene editing
- Uses common lab equipment (including micropipettes)
- Good experimental design
- Relatively time consuming, particularly if adding the genotyping extension activity
- Cost-effective for an actual CRISPR experiment

Disadvantages

- Requires more in depth understanding of molecular genetics to get the full benefit of the lab
- Genotyping extension kit requires a thermal cycler and electrophoresis equipment
- More expensive than simulation kits

3.7.2.2 CRISPR in a Box

https://www.rockland.com/crispr-in-a-box/

Rockland has developed a CRISPR lab activity similar to the *BioRad* kit just described, but with a few key differences. Specifically, CRISPR editing of a *LacZ* gene plasmid is performed *in vitro* using CRISPR-Cas12a ribonucleoprotein (RNP). The cut plasmid is then repaired via HDR in a manner that disables the gene, while also introducing a restriction site to help with subsequent confirmation of the repair. *E. coli* is then transformed with the edited plasmids, with successful editing and transformation revealed by a blue-to-white color change in bacterial colonies. Additional confirmation of the edit can be performed with restriction enzyme digestion and electrophoresis.

Similar to the *BioRad* kit, this experiment is well designed and provides an opportunity to discuss operons and the two mechanisms of DNA repair, NHEJ and HDR. As mentioned above, the CRISPR edit occurs *in vitro*. As such, there are a couple of minprep steps in the protocol that require a benchtop centrifuge capable of 10,000 g. Most high school labs are unlikely to have a centrifuge of this type. Although new centrifuges are prohibitively expensive, older surplus models are available online at very reasonable prices ($150). Similar to the *BioRad* kit, this activity is best suited for an AP course.

Advantages

- Students get to perform CRISPR-based gene editing
- Uses common lab equipment (including micropipettes)
- Good experimental design
- Relatively time consuming, particularly if adding the restriction digest and electrophoresis extension activity
- Cost-effective for an actual CRISPR experiment

Disadvantages

- Requires more in depth understanding of molecular genetics to get the full benefit of the lab
- Requires a desktop centrifuge (and electrophoresis equipment for extension activity)
- More expensive than simulation kits

3.7.2.3 Knockout! A CRISPR/Cas Gene Targeting Lab

https://www.minipcr.com/product/knockout/

miniPCR has also developed a CRISPR lab kit targeting the *LacZ* gene. Their experimental design is simpler than the two previous kits discussed, which makes it more suitable for a general high school class. CRISPR is used to cut the *LacZ* gene in a plasmid, which disables the gene. There is not an HDR step to repair the gene or introduce any type of stop codon or restriction site. The edited plasmid is transformed into *E. coli*, with success revealed by a blue-to-white color change. The simpler protocol should make the overall process easier for students to comprehend.

The *miniPCR* kit includes more study questions and other assessment materials than the *BioRad* and *Rockland* kits. The *miniPCR* kit also includes two paper model activities that can be used to present the mechanism of CRISPR prior to doing the wet-lab activity or as an extension activity after the lab. One paper model focuses on sickle cell anemia, while the other examines shell-coiling in snails. Similar to the *BioRad* and *Rockland* kits, *miniPCR* offers an optional genotyping activity involving restriction digest and electrophoresis. The kit does not include the supplies for electrophoresis (agarose, TAE buffer, etc.), but *miniPCR* offers an inexpensive companion kit for this purpose.

Advantages

- Students get to perform CRISPR-based gene editing
- Uses common lab equipment (including micropipettes)
- Experimental depth and design suitable for a general high school course
- Can be done in a 2-h class period
- Cost-effective for an actual CRISPR experiment

Disadvantages

- Effective transformation efficiency requires students to have good laboratory technique
- Genotyping extension kit requires electrophoresis equipment
- More expensive than simulation kits

3.7.2.4 Chopped! Using CRISPR/Cas9 to Cut DNA

https://www.minipcr.com/product/chopped-crispr-cas9-lab/

Although not designed around the *LacZ* gene and color changes, this kit by *miniPCR* is the simplest and least expensive way to have students actually cut DNA using CRISPR. Students use two different guideRNAs to cut plasmid DNA at two different target locations. The resulting fragments are then separated and visualized using gel electrophoresis to confirm the accuracy and precision of the CRISPR cut. Cleaving of DNA occurs in a cell-free system, so there are no culturing or transformation steps involved. The protocol is short and simple: 1) Cut DNA with CRISPR and 2) View the resulting fragments.

The teacher and student guides provide study and assessment questions. The prelab activities include a paper model for students to learn and simulate exactly what will be happening in the test tube when they perform the wet-lab activity. The kit does not include the supplies for electrophoresis (agarose, TAE buffer, etc.), but *miniPCR* offers an inexpensive companion kit for this purpose.

Advantages

- Students get to cut DNA with CRISPR
- Uses common lab equipment (including micropipettes)
- Experimental design and depth suitable for general high school course

- Can be done in a 2-h class period
- Cost-effective for an actual CRISPR experiment

Disadvantages

- Simple experimental design may not generate quite as much engagement and interest as protocols that involve editing microorganisms

3.7.2.5 The Power of CRISPR

https://innovativegenomics.org/crispr-classroom-kit/

This newly introduced kit from *Innovative Genomics* is an effective compromise between the more complex *LacZ* editing kits and the simplified "cutting DNA in a tube" kit described above. *Innovative Genomics*, in consultation with Jennifer Doudna, was intentional about designing a CRISPR experiment that could be understood and performed at a level commensurate with most high school biology classes. The kit is built around the topic of treating sickle cell disease with CRISPR, while also allowing students to perform CRISPR gene editing in bacteria.

Students inoculate a growth plate with bacteria engineered with the Red Fluorescent Protein (RFP) gene. As such, their initial cultures glow red under fluorescent light. The bacteria also possess a plasmid containing the Cas9 gene, a gene for guideRNA targeting the RFP gene, and the Green Fluorescent Protein (GFP) donor gene. It is important to note that the cells already contain this plasmid, so there isn't a transformation step associated with this lab activity. This makes the overall protocol simpler to perform and results in higher success rates.

Although the bacteria already contain the "CRISPR plasmid", the genes on the plasmid are controlled by various promoters and are essentially turned off until exposed to the proper inducers. Students add the inducers, which leads to plasmid gene expression and initiation of CRISPR editing. The bacteria express the Cas9 protein, which is then loaded with the transcribed guideRNA. The guideRNA targets the RFP gene in the bacteria, which is then cut by Cas9. The cut gene is repaired via HDR, during which the GFP gene donor DNA is copied into the repair site. Thus, the originally red cells begin to produce GFP instead of RFP. Successfully edited cells glow green, rather than red. This visual change and confirmation is exciting for students.

The overall activity is broken down into six lessons, with each lesson intended for a 50-min class period. The teacher guide outlines the units as follows:

- **Lesson 1:** Students are introduced to sickle cell disease and CRISPR. They learn that genes are instructions for protein that determine an organism's traits.
- **Lesson 2:** Students get an initial overview of how CRISPR can edit the DNA of an organism, and then conduct Part 1 of the lab.
- **Lesson 3:** Students conduct Part 2 of the lab and learn more about CRISPR through video.
- **Lesson 4:** Students conduct Part 3 of the lab and solidify their understanding of how CRISPR works by acting out the CRISPR mechanism.

- **Lesson 5:** Students observe the results of the lab and create a model to demonstrate their understanding of how CRISPR works.
- **Lesson 6:** Students read about the risks and benefits of using CRISPR to edit DNA and discuss the ethics of using CRISPR in different circumstances.

The developers have intentionally kept the protocol and conceptual information at a level suitable for a general high school class. For example, the term "guiding sequence" is used, rather than guideRNA, since RNA is not directly included in the NGSS standards. Explanations are kept simple, and the student instruction guides are thorough. By eliminating the transformation step, bacteria can be cultured and refrigerated less stringently without risk of failure. All incubations are done at room temperature.

The kit includes just about everything needed to perform the experiment, including non-perishable manipulatives used for modeling sickle cell disease and the mechanism of CRISPR editing. No expensive lab equipment is necessary. An extensive 156-page teaching manual provides the instructor with plenty of background information, preparation guides, student handouts, study questions, and troubleshooting information. PowerPoint slides and videos are also provided.

Advantages

- Students get to edit DNA in bacteria using CRISPR
- Developed in cooperation with Jennifer Doudna (Nobel Prize winner for CRISPR)
- Extremely thorough 156-page teacher guide, along with PowerPoint slides and videos
- No expensive lab equipment required (kit includes all materials, including manipulatives)

Disadvantages

- Significant class time required (six 50-min periods)
- Expensive, particularly the first time (refill kits are available at lower cost for subsequent use)

3.8 Films

3.8.1 Human Nature

https://www.pbs.org/video/human-nature-hcwiwk/

The tagline for this outstanding documentary film is: *The Story of CRISPR. The Most Important Scientific Discovery of the twenty-first Century.* The 90-min film was released in 2020 and provides an excellent introduction to CRISPR. With effective visuals and compelling storylines, the film weaves together the discovery and science of CRISPR with the personal stories of individuals impacted by the technology.

The film explores the bioethics of gene editing in an open-ended manner that should generate interesting discussions and reflections. Overall, the content is well suited to high school students. Viewers get to hear firsthand from the scientists who developed and pioneered CRISPR, as well as from patients benefiting from its use. Unfortunately, the film's website does not currently provide teaching resources, but there are some study guides available from various web locations, including the *Teachers Pay Teachers* platform.

Advantages

- Film length is 90 min
- Includes interviews with scientists directly involved with discovery of CRISPR
- Personal interest stories with patients impacted by CRISPR
- Excellent graphics and animations
- Good discussion starter
- Explores bioethical questions

Disadvantages

- Film length may be too long

3.8.2 The Gene

https://www.pbs.org/kenburns/the-gene/

This engaging film about genetics is based on Siddhartha Mukherjee's award-winning book, *The Gene: An Intimate History*. The film covers the full history of scientific discovery that led to our current understanding of genes, DNA, and molecular genetics. The film is broad in scope, so gene editing (and CRISPR in particular) is only a small part of the overall film. The 4-h film is divided into two episodes and CRISPR gets a fair amount of attention in the final 25 min of episode 2. This is an excellent film to use when studying genetics in general, but if CRISPR is the intended focus, then *Human Nature* is the better option.

Advantages

- Excellent historical and contemporary overview of genetics
- Interviews with prominent scientists in the field

Disadvantages

- Overall film is 4 h
- CRISPR discussed during final 25 min
- CRISPR information is somewhat broad and oversimplified

3.9 Classroom Discussions

All of the classroom activities outlined in this chapter lend themselves to a follow-up classroom discussion regarding the many applications and bioethical considerations associated with CRISPR. Although popular press tends to focus on using CRISPR to treat genetic disease and potentially create so-called "designer babies," CRISPR applications are far-reaching and impact many areas of biology. A summary of some CRISPR applications along with associated URLs is provided in Table 3.1. Each of these applications lends itself to an interesting discussion. A brief overview of these applications is provided in *The American Biology Teacher* [3].

As mentioned previously, genome editing did not begin with CRISPR. ZFNs and TALENs were developed and explored during the 2000s. However, these techniques were challenging, time consuming, and largely inefficient. With the arrival of CRISPR in 2012, gene editing suddenly became quick, easy, and inexpensive. As a result, the field of gene editing has advanced at a rapid pace and without sufficient time for scientists and society to evaluate the many bioethical concerns inherent to certain applications of the process.

It is worth noting that the discovery of CRISPR stemmed from basic research conducted by an array of unaffiliated scientists from a variety of countries across the world. There are a couple of lessons buried in this fact that can and should be instilled in the minds of high school students, particularly those aspiring to a scientific career.

The first lesson pertains to the importance of funding basic research. There is a popular position that research funding should be explicitly directed at projects with obvious application and/or market potential. CRISPR, however, emerged from a variety of disparate lines of research, ultimately culminating in a revolutionary tool for biotechnology that

Table 3.1 Various applications of CRISPR technology [3]

Application	Reference to explore
Biomedical	
Diabetes	https://www.healthline.com/diabetesmine/could-gene-editing-be-used-cure-diabetes#1
Cancer	https://www.statnews.com/2019/05/02/crispr-targeting-cancer-seeking-go-ahead/
HIV	https://www.sciencemag.org/news/2019/03/curing-hiv-just-got-more-complicated-can-crispr-help
Food biotechnology	
Mushrooms	https://www.foodsafetynews.com/2018/12/the-little-mushroom-that-could-with-a-little-help-from-its-friends/
Soy bean oil	https://www.the-scientist.com/news-opinion/gene-edited-soybean-oil-makes-restaurant-debut-65590
Cattle	https://www.wired.com/story/crispr-gene-editing-humane-livestock/
Basic research	
Gene drives	https://www.synthego.com/blog/gene-drive-crispr
Cellular barcoding	https://www.nature.com/articles/d41586-018-05934-z
Gene targeting with deactivated Cas9	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4922510/

impacts myriad biomedical and agricultural applications. In many ways, CRISPR is a *byproduct* of basic research.

The second lesson pertains to the universal nature of science. In a world encumbered by cultural, political, and religious differences, science provides a universal language that transcends global divisions. The structure of DNA and the central dogma of molecular genetics are the same, whether being studied in China, Germany, Brazil, or the United States. The same holds true for the science of CRISPR. Interestingly, the *bioethics* of various applications of CRISPR present a challenge, in that bioethics are influenced by cultural and philosophical factors, which can not only vary throughout the world, but even within a particular country, such as the United States.

Students can be easily drawn into bioethical discussions regarding CRISPR, but such classroom discussions need parameters to bring structure and coherence to the conversation and to avoid a free-for-all blitz of personal opinions. Bioethics generally operates within four frameworks: 1) Rights and responsibilities, 2) Consequentialism, 3) Autonomy, and 4) Virtue [23]. A brief explanation of these frameworks is provided in Table 3.2 and can serve as a framework for guiding discussions about the bioethical implications of CRISPR.

Table 3.2 Frameworks associated with bioethical decision-making [3]

Bioethical framework	Description	Pertinent example
Rights and responsibilities	The rights of one imply the responsibilities (or duties) of another to ensure those rights.	The imperative to treat cancer patients with available, though perhaps not fully tested, gene-based therapies
Consequentialism	The benefits and harms resulting from an action must be weighed against each other.	Using gene drives to eradicate the *Anopheles* mosquito in an attempt to eliminate malaria
Autonomy	An individual's right to choose for themselves may or may not exceed the benefit of a single decision applicable to everyone.	Editing human embryos and germ cells without consent of the embryo or future generations
Virtue	Decisions should be congruent with what the community accepts as 'good,' such as honesty and kindness.	Creating genetic models of human disease in primates

Take-Home Message

As noted at the beginning of the chapter, CRISPR is a fascinating and potentially controversial topic that will capture the interest of your students. And as reviewed throughout the chapter, there are now plenty of CRISPR-related resources available to bring the topic into your classroom. The resources specifically discussed in this chapter are summarized in Tables 3.3, 3.4 and 3.5.

CRISPR will continue to revolutionize biological research and application. It is already being used in the fields of microbiology, botany, zoology, ecology, biomedicine, agriculture, and just about every other biological discipline. Modifications of the CRISPR system will also allow new applications to emerge. For example, researchers have managed to deactivate the cutting mechanism of Cas9, while retaining its guideRNA programming capacity. As such, deactivated Cas9 (dCas9) can be used as a gene-targeting device for a wide variety of purposes, including diagnostics.

Despite the seemingly positive applications of CRISPR, including the treatment of genetic disease, CRISPR also raises many bioethical questions. The technology is advancing at a faster rate than humanity has been able to process and discern its use. Junjui Huang announced that he had edited human embryos with CRISPR whilst scientists from around the world were meeting to discuss if such an experiment should be performed in the first place [24]. He Jiankui announced the birth of CRISPR-edited twin girls at a time when most countries, including China, were establishing laws and regulations against doing so [25].

Much like the evolution of genetic engineering itself, it's not that such experiments would likely never be performed in the future, but rather that such experiments might benefit from more testing and perfection of the technology prior to implementation. Not surprisingly, opinions regarding CRISPR vary tremendously. And it is for this reason that CRISPR needs to work its way into the high school curriculum. Today's high school teachers are preparing and informing the students that will wield and regulate CRISPR in the years ahead.

Table 3.3 Summary of free CRISPR teaching resources

	Case study (NCCST)	Paper model (ABT)	Paper model (HHMI)	Animation project
Major concepts	CRISPR, genetic therapy, Duchenne muscular dystrophy	Discovery of CRISPR, Gene Knock-out & Knock-in, NHEJ & HDR	CRISPR, Gene Knock-out & Knock-in, NHEJ & HDR	CRISPR, gene therapy, NHEJ & HDR
Materials required	None	Cardstock, scissors, tape	Cardstock, scissors, tape	Assortment of materials for students to create physical modvel (clay, play dough, beads, pipe cleaners, etc.)
Class time (50 min periods)	1	1–2	1–2	3–5
Content level	HS/AP	HS/AP	HS/AP	HS/AP

(continued)

Table 3.3 (continued)

	Case study (NCCST)	Paper model (ABT)	Paper model (HHMI)	Animation project
Additional resources	Downloadable case study	Downloadable paper model and teacher/ student guides	Downloadable paper model and teacher/ student guides, short animations and videos on HHMI website	Downloadable teacher/ student guides, rubric, storyboard. Guides include URLs to web resources
Cost	Free (cost of printing)	Free (cost of printing)	Free (cost of printing)	Free (cost of printing)
Other considerations	Other cases available at NCCST	History of science component	Simpler than the *ABT* paper model	Create stop-motion animation of CRISPR. Creative approach for non-science students.

Table 3.4 Summary of CRISPR simulation teaching resources

	CRISPR simulation (MiniOne Systems)	CRISPR simulation—Cystic Fibrosis (Edvotek)	CRISPR simulation—Edited Crops (Edvotek)
Major concepts	Discovery of CRISPR, Gene Knock-out & Knock-in, NHEJ & HDR	CRISPR, Gene Knock-out & Knock-in, NHEJ & HDR, gene therapy	CRISPR, Gene Knock-out & Knock-in, NHEJ & HDR, food engineering, bioinformatics
Materials required	Microwave Electrophoresis setup	Micropipettes/tips Microwave Electrophoresis setup DNA visualization (white light)	Micropipettes/tips Microwave Microcentrifuge Electrophoresis setup DNA visualization (UV or blue light)
Class time (50 min periods)	2	2	1
Content level	HS/AP	HS/AP	AP
Additional resources	Downloadable teaching guides Two 20-min video resources included	Downloadable teacher/student guides	Downloadable teacher/ student guides
Cost	$144/10 groups	$145/8 groups	$100/8 groups
Other considerations	Includes *ABT* paper model Students design guidRNAs History of science component Video explores CRISPR applications and bioethics	Students design guideRNAs	Students design guideRNAs Introduces students to bioinformatics using BLAST database

Table 3.5 Summary of CRISPR gene-editing lab teaching resources

	Chopped! (miniPCR)	Knockout! (miniPCR)	Out of the Blue (BioRad)	CRISPR in a Box (Rockland)	Power of CRISPR (Innovative Genomics)
Major concepts	CRISPR	CRISPR, NHEJ & HDR, operon, transformation	CRISPR, NHEJ & HDR, operon, transformation	CRISPR, NHEJ & HDR, operon, transformation	CRISPR, gene therapy, sickle cell disease, bioethics
Materials required	Micropipettes/tips Microwave Water bath Electrophoresis setup DNA visualization (UV or blue light)	Micropipettes/tips Microwave Water bath Incubator	Micropipettes/tips Microwave Water bath Incubator Microcentrifuge Electrophoresis setup DNA visualization (UV or blue light)	Micropipettes/tips Microwave Water bath Benchtop centrifuge Electrophoresis setup DNA visualization (UV or blue light)	Standard lab equipment
Class time (50 min periods)	2.5	2.5	5	5	6
Content level	HS/AP	AP	AP	AP	AP
Additional resources	Downloadable teacher/student guides Downloadable slides Electronic prelab	Downloadable teacher/student guides Downloadable slides Downloadable paper models	Downloadable teacher/student guides	Downloadable teacher/student guides	Downloadable teacher/student guides Kit comes with classroom manipulatives Videos
Cost	$125/8 groups	$220/8 reactions	$250/8 reactions	$680/10 reactions	$495/8 reactions $295 refill kit
Other considerations	Less prep time Less class time Optional prelab activity Suitable for HS	Significant prep time Less class time optional PCR extension activity	Significant prep time Optional PCR extension activity	Significant prep time Uses Cas12a Requires benchtop centrifuge (14,000 g)	Significant prep time Simpler experimental design and conceptual depth suitable for HS Refill kits available at lower cost

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CRISPR for Introductory-Level Undergraduate Courses

4

Michael J. Wolyniak

4.1 Introduction

I first encountered CRISPR in the Fall of 2015 after a student of mine came back from Harvard after participating in a former Howard Hughes Medical Institute (HHMI) summer research program that placed students from groups traditionally underrepresented in the STEM disciplines in HHMI investigator laboratories. This student proudly gave a seminar to the department that talked about his gene editing work over the summer, and my reaction was simultaneously one of pride, confusion, and fear. Clearly, I was thrilled that my student received such a top-notch research experience as an undergraduate (he went on to a medical degree from the University of Minnesota and is currently an emergency medicine resident in Newark, Delaware). However, I found myself flummoxed by all the intricacies of what my student had just presented to us as the brave new world of molecular biology. I remember having to have guide RNAs explained to me repeatedly as my student patiently brought me through the process of his research. Finally, I realized how important CRISPR technology was going to be going forward and how critical it was going to be for people like me to "catch up" and figure out how to effectively teach this powerful new technology in our classes.

Like any form of technology, CRISPR has become more accessible as it has become more mainstream. Just as PCR has evolved from an exciting yet rare technology in the 1980s to one of the most commonly used molecular techniques today, so is CRISPR undergoing a similar evolution in accessibility. In a similar evolution, just as I would be committing malpractice to not have my introductory-level biology students practice PCR in the laboratory and learn about the technique in lecture, it is now becoming essential to

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expose our students to CRISPR as part of their foundational studies in the life sciences. But for a technology as relatively novel and complicated as CRISPR, what would qualify as an "introductory" level exploration? What do undergraduate students need to know about CRISPR to best prepare themselves for more advanced biology study and potential careers in STEM fields? This chapter will explore these questions and suggest some strategies for how to best expose introductory level students to the excitement of CRISPR technology, the versatility of its potential, and the potential ethical ramifications of its missuse for society.

4.2 What Do Undergraduates Need to Know?

4.2.1 What Is an Introductory Student?

A good first step for considering how to best introduce CRISPR to introductory level undergraduates would be to define "introductory". This seems like a trivial matter at first, since this term usually refers to students who have had little to no prior college-level biology exposure. However, when I think about the audience that I typically see in my foundational Principles of Biology course, I think about students that in many cases have come from under-resourced high schools that have had limited exposure to how DNA works, let alone how it can be manipulated. I also consider students that may have faced barriers to effectively learning science in high school due to limitations in schools providing equitable educational access to their students. Finally, I think about the struggles that the majority of the students I have in this class have with mastering basic RNA transcription and protein translation. My past experiences have shown me that providing too much detail too quickly on subjects like CRISPR can overwhelm students and make them feel like they do not belong in the life sciences. When I put all this together, it forces me to carefully consider where I would want to place a discussion of CRISPR within my curriculum.

I have found that my students are fascinated by the applications of molecular biology to problems in forensics and that this interest provides an excellent context in which to explore the properties of DNA that enable it to play such a critical role in solving crimes. Likewise, the need to amplify DNA segments at a crime scene to create a DNA profile segues nicely into considering a broad view of how PCR works. Using this philosophy, I see CRISPR as something to be broadly introduced as a molecular tool for students at this level but not necessarily as a DNA-editing engine to be taken apart and explored by the students to master all of its detailed workings. In this context, therefore, the "introductory" undergraduate may in fact be a sophomore or junior who has heard about CRISPR as a molecular tool in earlier classes but would now have had the fundamental exposure to molecular biology necessary to fully comprehend and appreciate the power and potential of CRISPR.

It is important to acknowledge here that no two student bodies are the same. While I offer here my own perspectives based on the students with which I have worked, other

instructors may find themselves with different curricular structures or overall student profiles from what I have described. The point of this section is not so much to define the introductory student with respect to CRISPR but rather to invite the reader to reflect on the students in their own classrooms and to consider the appropriate level in which to introduce a technology as sophisticated as CRISPR to them.

4.2.2 What Are the Fundamentals That Students Need to Know?

Once an instructor has decided WHERE to discuss CRISPR, they now come to the equally-important question of WHAT to teach. As with our previous question, there is no "one size fits all" answer. A skillful use of the backwards design approach to course preparation should be utilized here to adequately reflect on what an instructor wants to convey to their students about CRISPR [1]. When one considers what they want their students to have learned about CRISPR at the end of a given class/module/course, the answer to what to actually teach begins to present itself. For instance, in my Principles of Biology introductory class, I would not expect my students to know much about CRISPR beyond what it is as a tool and what it does in a broad sense. These principles lend themselves naturally to a consideration of how CRISPR SHOULD be used by the scientific community and by society in general, and later on in this chapter. I will describe approaches to this in more detail. For students in my upper-level Molecular and Cellular Biology course, in contrast, I would like them to understand the mechanisms by which CRISPR operates and to be able to design experiments in which CRISPR is an appropriate tool for the given research goals. A more advanced course would also provide a good venue for considering the natural forms of CRISPR in certain bacterial species and the history behind harnessing a natural process into a molecular laboratory tool.

Another important point of reflection for instructors with respect to establishing what to teach with respect to CRISPR would be the context in which the material is meant to be taught. In other words, WHY is an instructor teaching CRISPR? Is it to simply make students aware of an exciting new technology available in the research lab? If so, then a broader consideration in an introductory level course may suffice. Is it to demonstrate some of the bedrock principles in biochemistry molecular biology, such as precise cutting of DNA and the specific actions of enzymes? Then, a more detailed consideration of the "nuts and bolts" of CRISPR is in order. Is it to give students the chance to act like "real scientists" and to implement an experiment of their own? In this case, the instructor has a range of teaching tools available, including hypothesis design and dissemination, the use of databases and bioinformatics for guide RNA construction and selection, or the infusion of an experimental plan into class that gives the student the opportunity to explore CRISPR in an iterative fashion.

Based on this reflection, the answer to the question on "what to teach" an introductory student is directly related to the distinct goals that an instructor has for their students. Later in this chapter, we will consider approaches for looking at CRISPR in both traditional

classroom and laboratory settings, and this will invite the reader to consider the relative importance in their eyes to having their students actually perform CRISPR work in the laboratory as a part of their mastery of "fundamentals". As we will see, one does not need a sophisticated laboratory set-up to effectively demonstrate CRISPR principles to students. In fact, it is quite possible to do an effective job at providing an engaging and fruitful student experience with CRISPR by utilizing with an active learning approach in the classroom with no traditional laboratory component at all.

4.3 Teaching About CRISPR in the Classroom

Teaching about CRISPR in the traditional classroom may take the form of a brief introduction to the subject, a more extensive preparation for an accompanying hands-on laboratory module, or a vehicle to allow students to explore current advances in research. In many cases, limited laboratory infrastructure or financial resources can mean that no laboratory-based CRISPR instruction is possible. Regardless of the context, several tools are available to instructors to make CRISPR accessible and engaging to introductory undergraduate students.

4.3.1 Online Videos

This may seem like an odd place to begin recommendations for a scholarly classroom experience into CRISPR. However, the ability to visualize a process as opposed to seeing static images can make a significant difference in the ability of students to master complex molecular biology concepts like DNA replication, RNA transcription, and protein translation [2]. Sites like YouTube have short but effective videos explaining CRISPR from groups such as the Mayo Clinic (https://www.youtube.com/watch?v=UKbrwPL3wXE), the journal *Nature* (https://www.youtube.com/watch?v=4YKFw2KZA5o), and even the Nobel Prize Committee featuring foundational scientists behind CRISPR like Dr. Jennifer Doudna (https://www.youtube.com/watch?v=KSrSIErIxMQ). A simple Google search for "What is CRISPR?" yields dozens of candidate videos for student consumption, so it is up to the instructor to select which ones best fit the scope of their course. Bear in mind that these videos are not meant to supplant the teaching about CRISPR in the classroom but rather to supplement the work of the instructor with a dynamic and visual presentation of what happens in this process.

4.3.2 Bioinformatics Tools

Dr. Sally Elgin, Professor *Emeritus* at Washington University of St. Louis and founder of the hugely successful Genomics Education Project course-based undergraduate research

experience (CURE) [3], captured the power of bioinformatics tools in the undergraduate classroom when she stated that all you need to do real science was a computer and an Internet connection. Indeed, one of the barriers for many life science instructors when considering the teaching of CRISPR or other molecular biology-based techniques is the significant expense of such techniques to effectively run laboratory exercises. The -omics and sequencing revolutions have greatly expanded the options and tools available to instructors at all types of institutions with undergraduate education missions and have democratized the accessibility of high-quality educational techniques. Even if no wet lab is available, instructors may utilize bioinformatics tools to allow their students the chance to engage with the processes used to generate guide RNAs for CRISPR research as well as to develop a computer-based CURE in which students propose a target gene, justify their choice through the literature, and design the guide RNAs necessary for disruption or editing of the target (Fig. 4.1).

For starters, the instructor will want to decide the target organism in which they would like their students to operate. Once this is established, they will be able to direct their student to an appropriate online platform to identify target genes that can be targeted via guide RNA construction. For vertebrate organisms, Ensembl (https://www.ensembl.org) is an excellent tool for identifying potential genes to study, finding information about what is known about gene product function, and performing alignments between sequences or other comparative genomics activities. Other model systems have their own databases for finding this information, including yeast (https://www.yeastgenome.org), fruit flies

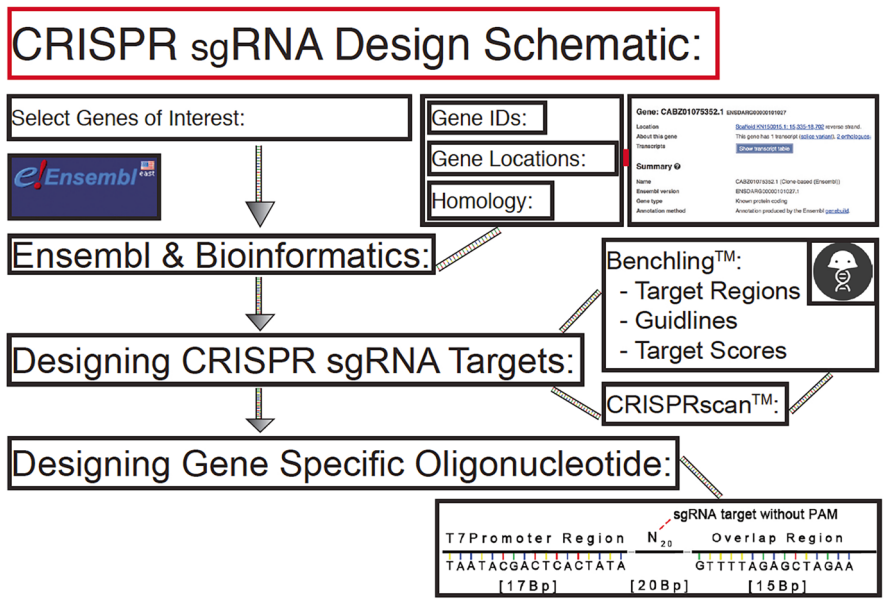

Fig. 4.1 An example of how an instructor may take advantage of different online resources to create an effective bioinformatics-based classroom experience in exploring CRISPR

(https://flybase.org), roundworms (https://www.wormbase.org) and *Arabidopsis thaliana* (https://www.arabidopsis.org).

Once a target gene has been identified, students are now ready to use online tools to propose hypothetical guide RNAs that can be used in a CRISPR experiment. Benchling (https://www.benchling.com/crispr) provides an especially elegant platform for importing target gene DNA into its system and generating candidate guide RNAs; however, many other platforms are available for this purpose depending on the model system in use. This work can be confusing for students at first due to the volume of information they will receive in the form of candidate guide RNAs. However, guide RNA construction is an outstanding way for students to have a CURE-like experience in the classroom without a wet lab companion module. In the process of designing guide RNAs, students will encounter understanding how CRISPR selects its target sites, the location of the target relative to the entire gene, and the potential for off-target cutting that would compromise specificity in a given experiment. Asking students to create a candidate guide RNA for a given target gene and to justify their selection for both the target and the guide RNA requires the student to carefully consider the parameters of their work and to develop a greater understanding of the details that go into a successful CRISPR experiment.

4.3.3 Annotation of Journal Article/Journal Club

An ambitious yet rewarding approach to providing students with insights into CRISPR technology can come from the use of primary literature centered around the use of CRISPR. One could write an entire book on best practices in the use of primary literature in undergraduate coursework and teaching undergraduates how to read and appreciate journal articles, and there are a variety of resources available for instructors who want to learn more about these approaches [4, 5]. The use of a journal article and discussion of this article in a journal club format in class can be an excellent way to engage students with a report on authentic scientific research as well as consideration by the class as to what needs to go into a published scientific journal article. A "slow but steady" approach using multiple class sessions to explore a journal article using CRISPR in detail that requires students to examine figures and tables and interpret what they are trying to say presents a challenging yet accessible way for students to engage with such writing. It is critical for the instructor, of course, to select a journal article for this exercise that will not overwhelm the students with several different experimental techniques and the use of extensive scientific jargon. It is also sensible to have students work in small groups for discerning the message presented by each figure so that they may benefit from each other in working through the often dense language of the paper.

An accompanying approach to this journal club format can be the use of annotation to have students dissect a journal article in detail to explore the strengths and weaknesses of the research under consideration. Here, individual or small groups of students may be tasked with going through passages of the paper with an eye towards identifying

unfamiliar terms that require better definition, explanations of purpose behind individual experiments, and conclusions that the authors draw from each experiment. Students may also be called upon in class to present these ideas to their peers for further discussion. Like with exploring figures and tables, this approach can be daunting to students at first; therefore, it is essential for instructors to provide a strong model of what they expect of their students prior to sending them off to do an assignment like this. However, the results of this approach and with the journal club approach with respect to CRISPR can be twofold: students gain perspective of how CRISPR is being utilized to make scientific breakthroughs in molecular and cellular biology, and students gain confidence in their ability to critically read scientific literature on CRISPR or any other topic going forward. Introducing an approach like this at an introductory level can be a significant amount of work for student and instructor alike but can pay significant dividends down the line in the form of students in advanced coursework who are well-versed in how science is disseminated and are prepared to critically engage with cutting edge work found in journal articles.

4.3.4 Hypothesis Design and Proposal

Another extension of CRISPR-based lessons in the classroom that can provide students with both background into how CRISPR works as well as a taste of an authentic scientific process is a hypothesis design and proposal assignment. This seemingly-simple assignment tasks students with taking their knowledge from the class to propose experiments that can be done with CRISPR to answer a scientific question either generated by the instructor or devised by themselves. Such an assignment also forces students to become more conversant with the primary literature as it requires them to find support for their ideas and to cite these supports in their proposal. For the dissemination portion of the assignment, there is a great opportunity for peer review. Students can share their ideas in small groups in class or in uploaded form to a learning support tool like Canvas or Blackboard to allow peers to comment on each proposal's strengths and weaknesses. For peer review to be successful, it is important for the instructor to lay out ground rules on making constructive and thoughtful suggestions in their comments to ensure that students receive feedback but not discouragement from their peers. Nevertheless, this allows the students to receive feedback from multiple sources and to improve their proposal in its final form. The level of detail that students put into this hypothesis proposal is up to the instructor and is based on their own classroom learning objectives. The proposal could be a standalone assignment or be part of a scaffolded project leading to a full-blown "grant proposal" prepared by the students to be written up and/or presented orally to the class. Regardless of the size of the assignment, the process of designing, defending, and refining an experimental design is critical for undergraduates to experience early in their scientific training, and the study of an emerging mainstream technology like CRISPR provides an outstanding platform in which to provide such a training opportunity.

4.4 Using CRISPR in the Classroom Laboratory

When possible, it is an excellent idea to allow students the opportunity to work with CRISPR technology in a wet lab format. The form that this laboratory experimentation takes, however, will vary from instructor to instructor depending on class learning objectives as well as restrictions in time or resources. A couple of considerations to make before deciding what a successful CRISPR project would like in a given classroom: first, remember that the target audience for the project is introductory-level undergraduates, not expert-level scientists. As such, the experiment that the instructor designs for their students need not be of a high level of sophistication to be engaging and exciting to the students. Second, consider the scope of project that makes the most sense for a given class given its learning objectives and required content to cover. Perhaps the hypothesis design and proposal assignment suggested in the previous section can yield experiments in which students can then conduct their own independent explorations in the lab over the course of an entire semester. However, a project need not take an entire semester and require top-level resources to be impactful to students. Finally, consider the degree of freedom that is appropriate for students in the design and implementation of CRISPR work. Designing lab experiences that take on the high-impact level of a CURE require some level of freedom for the students to design the experiments they will conduct in pursuit of their project. However, allowing every student the freedom to design their own projects free from any instructor parameters can lead to a class that is beyond the management capacity for any instructor and beyond the budget of most institutions. There is a happy medium in which an instructor can provide the students with the scientific authenticity of providing a certain latitude with how experiments can be run but still have all students work towards one or two common research questions or goals. In this section, we will consider just a few of the many laboratory-based CRISPR learning modules that have been developed for use by introductory-level undergraduates.

4.4.1 What System Should Be Utilized for CRISPR Effective Analysis in an Undergraduate Context?

As other chapters of this volume illustrate, there are a wide variety of model systems available to instructors for the demonstration of CRISPR to undergraduates. Clearly, there is no "right" answer to the question of what system to utilize for this purpose. However, there are likely some clear advantages to the use of one system over another for each specific instructor. First and foremost, the instructor's prior training and experience will likely play a major factor in the selection of a model system. As a trained yeast geneticist, for instance, I personally gravitated towards the use of budding yeast (*Saccharomyces cerevisiae*) in my classroom not because it was the most sophisticated way in which I could introduce CRISPR but because it was the most accessible to me as an instructor. I was able to use my familiarity with *S. cerevisiae* to make the laboratory experience for my students as stream-

lined and efficient as possible (forgiving, of course, the usual growing pains with new students to large-scale laboratory projects).

Another obvious consideration in the answering of this question is the resources that are available to the instructor. If an instructor is working at an institution that does not have the capability of doing mammalian cell culture, for instance, then it makes little sense to design a cell culture-based CRISPR laboratory. Along these same lines, the instructor must consider the budget that is available to them and to consider the expense of other laboratory modules that need to be run in a given class. There is a good argument to be made for the pooling of laboratory resources from multiple modules in support of one cohesive laboratory experience that is scaffolded over the entire length of a class; however, instructors do not always have the level of course control necessary to make decisions on what content needs to be covered in a course.

Finally, an important consideration is the amount of time available for the implementation of a CRISPR project that can fit the time limitations of a given course. I learned this firsthand when working with a collaborator of this volume in the design and implementation of a CRISPR-based CURE with the *Arabidopsis thaliana* model system [6]. While bringing a plant-based model system into my class provided a novel laboratory experience for my students, the 4–5 week growth period for the plants required creative course design to provide laboratory activity for the period in which we needed to wait for our CRISPR-modified germlines to mature. Such planning will be necessary for any model system with a life cycle that extends for several weeks; however, there are creative class design options available in which the students in one class do the molecular work that will be brought to fruition and analyzed by the cohort of students in the next offering of the course.

4.4.2 How Much Time Is Necessary to Implement a Successful CRISPR Experiment in the Laboratory?

The answer to the question of how much time should be devoted to doing a CRISPR-based project in the laboratory is largely based not only on the expectations of the instructor but also the limitations imposed on the class by its meeting schedule (Fig. 4.2). Traditionally, the laboratory component of undergraduate classes has met for 1 day a week for 2–4 h. This timeframe allows for most of the molecular biology procedures necessary to successfully design a semester-long project in which guide RNAs are designed, targeted to the appropriate genes in the model system, and assessment in some manner of the results. However, significant limitations can arise from this model as well. If part of the experimental procedure involves the transformation of DNA into bacteria or a PCR reaction or any other process in which there are distinct processing and verification steps, then several laboratory sessions can be taken up one specific step of a long process. In some cases, instructors have looked to utilize part of the lecture portion of the class to complete short steps in the laboratory process (the picking of bacterial colonies to start overnight cultures, for instance); however, this compromises the already limited time available to cover the

Semester Lab Schematic:

Over the course of this semester, you will complete the following phases of the genomic engineering project:

- I. ☐ CRISPR sgRNA Design: Phase I:
  - ☐ a. Select a gene of interest
  - ☐ b. Use Ensembl and Bioinformatics to:
    - ☐ i. Collect gene IDs
    - ☐ ii. Find gene Locations
    - ☐ iii. Learn about the gene's homology
  - ☐ c. Design CRISPR sgRNA targets using Benchling & CRISPRscan
    - ☐ i. Analyze target regions, target scores, and cross-reference data
  - ☐ d. Design gene specific oligonucleotides
- II. CRISPR sgRNA Synthesis: Phase II:
  - ☐ a. Annealing
  - ☐ b. DNA T4 Polymerase
  - ☐ c. In-Vitro Transcription (IVT)
  - ☐ d. DNase I Treatment
  - ☐ e. RNA Purification; Post-RNA Purification HMA & Spectrophotometer Analysis
- III. CRISPR-Cas9 Injection Solution Synthesis: Phase III:
  - ☐ a. Determine the Concentration of Purified RNA via Spectrophotometer
  - ☐ b. Make 500ng/uL Concentrations of Specific RNA for Injection
  - ☐ c. Prepare the CRISPR-Cas9 Injection Solution (combine reagents)
- IV. Zebrafish Embryo Injection Phase IV:
  - ☐ a. Egg Production and Collection
  - ☐ b. Inject One-Cell Stage Zebrafish Embryos
- V. Genotyping Preparation: Phase V:
  - ☐ a. Make 100uM Genotyping Primer Stock Solutions
  - ☐ b. Optimize Annealing Temperature via Gradient PCR
  - ☐ c. Characterization of Gradient PCR through Heteroduplex Mobility Assay (HMA)
- VI. Genotype F_0 Modified Zebrafish Embryos: Phase VI:
  - ☐ a. Phenol Chloroform DNA Extraction and Ethanol Precipitation
  - ☐ b. Genotyping PCR with Modified gDNA from Embryos or Adult Fish
  - ☐ c. Characterization of Genotyping PCR Product through HMA
  - ☐ d. Synthesize TOPO-TA Cloning Characterized PCR Product
  - ☐ e. Plate TOPO-TA PCR Solution on Lysogeny Broth Agar Plates with Kanamycin
  - ☐ f. Characterization of Colonies using PCR and HMA
  - ☐ g. Inoculate Liquid Bacterial Cultures:
  - ☐ h. Isolation of Plasmid DNA from Bacterial Colony via Mini-Prep
  - ☐ i. Characterization of Plasmid DNA using EcoR1 Digestion
  - ☐ j. Determine Concentration of Purified Plasmid DNA via Spectrophotometer
  - ☐ k. Prepare Plasmid DNA for Sanger Sequencing & Analyze Data

Fig. 4.2 An example of a comprehensive, semester-long exploration of CRISPR in the undergraduate laboratory using zebrafish as a model system. Depending on instructor goals, time limitations, and resource limitations, the instructor may pick and choose from this exhaustive list to fashion the CRISPR experience that best fits their specific needs

material necessary in the course. Others have worked with their colleagues to redesign courses so that the laboratory offering is, for instance, 2 days a week for 2 h each instead of 1 day a week for 4 h. In this way, the steps necessary to complete a CRISPR project (or any other molecular biology long term project, for that matter), can be completed in a more efficient fashion with minimal disruption to the lecture portion of the class. It is a good idea to carefully consider each step of the process that is meant to be completed by the students, to remember that each individual step along the way will take significantly longer to complete in the hands of introductory undergraduates as opposed to the instructor, and to plan the overall laboratory portion of the course accordingly.

4.4.3 The "CRISPR in the Classroom" Workflow and Kit

The editors of this volume are proud to have developed a CRISPR workflow that is modular in nature and therefore allows instructors to pick and choose which sections are most appropriate for their classes [7]. The workflow was originally conceived with the use of zebrafish (*Dario rerio)* as a model system; however, it is adaptable for any model system that the instructor chooses to utilize. The versatility of the system is a result of its *in vitro* nature: students utilize purified genomic DNA to set up a potential specific CRISPR-driven cut and SDS-PAGE electrophoresis to visualize results. The modular nature of the workflow allows for the addition of a bioinformatics component at the beginning of the course to target specific genes and design guide RNAs that may be ordered and utilized in the laboratory (Fig. 4.3). It also allows the addition of phenotyping experiments towards the end of the project in which students conduct experiments to assess the effects of targeted gene disruption on the model organism being studied. Under the leadership of Dr. Tiffany Hoage of the University of Wisconsin-Stout and with the generous support of the U.S. National Science Foundation (Award # 2120417), the group has also designed a kit to accompany the use of this workflow that streamlines the process of implementing an *in vitro* CRISPR experiment using the model system and target gene of the instructor's choice. Several other life science supplies companies, including Bio Rad (https://www.bio-rad.com) and Carolina Biological (https://www.carolina.com) also offer kits designed to give students a hands-on experience with CRISPR.

4.4.4 Online Repositories for CRISPR Lab Modules

Several instructors have devised laboratory modules featuring CRISPR that are adaptable to a wide variety of classroom types. In recent years, online repositories for such modules have emerged to provide such instructors with a way to share their modules with the life science education community as well as receive credit for their innovation. These reposi-

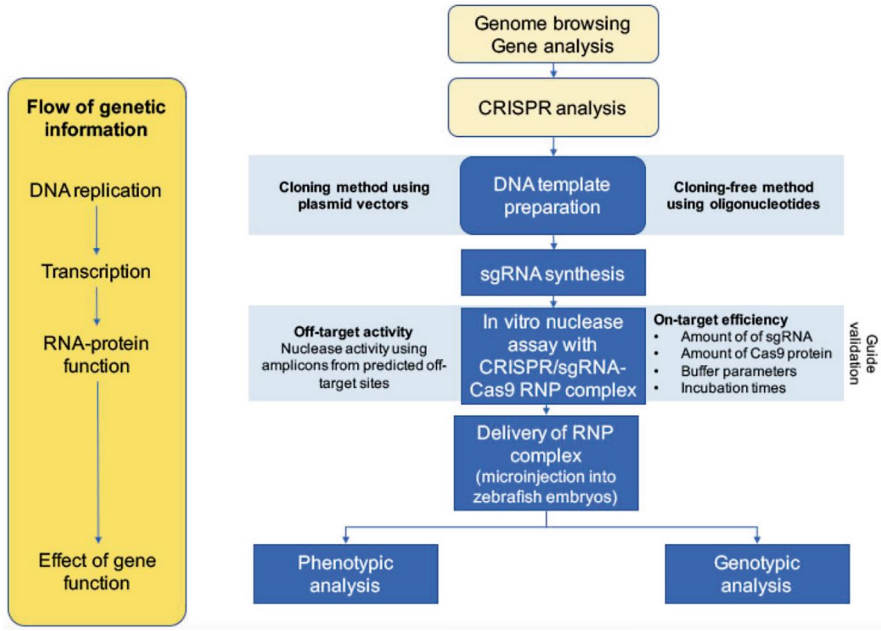

Fig. 4.3 A schematic of the workflow utilized by the "CRISPR in the Classroom" group to examine CRISPR technology in the laboratory in a modular fashion

tories give the community the opportunity to peruse these contributions and select modules that they think could work well for their own specific learning goals in the class.

CourseSource (https://qubeshub.org/community/groups/coursesource/): CourseSource was developed as a way for class modules developed by instructors to receive a level of peer review similar to what is done for traditional journal articles. In this way, CourseSource hopes to support the professional development and advancement of instructors who have put significant amounts of time and effort into developing high-quality classroom activities that can be shared and adapted for used in other classrooms (Fig. 4.4). The modules found here can range from full CUREs to simple laboratory or classroom opportunities that will broadly expose students to CURE as a molecular biology tool.

CUREnet (https://serc.carleton.edu/curenet/index.html): For instructors seeking a more comprehensive laboratory experience for their students, the CUREnet repository collects high-quality CUREs that have been successfully run and assessed by undergraduate instructors. Since CUREs seek to provide as authentic a research experience for undergraduate students as possible, these modules are ideal for instructors who want to not just show students CRISPR but also employ it in the service of answering a larger research question.

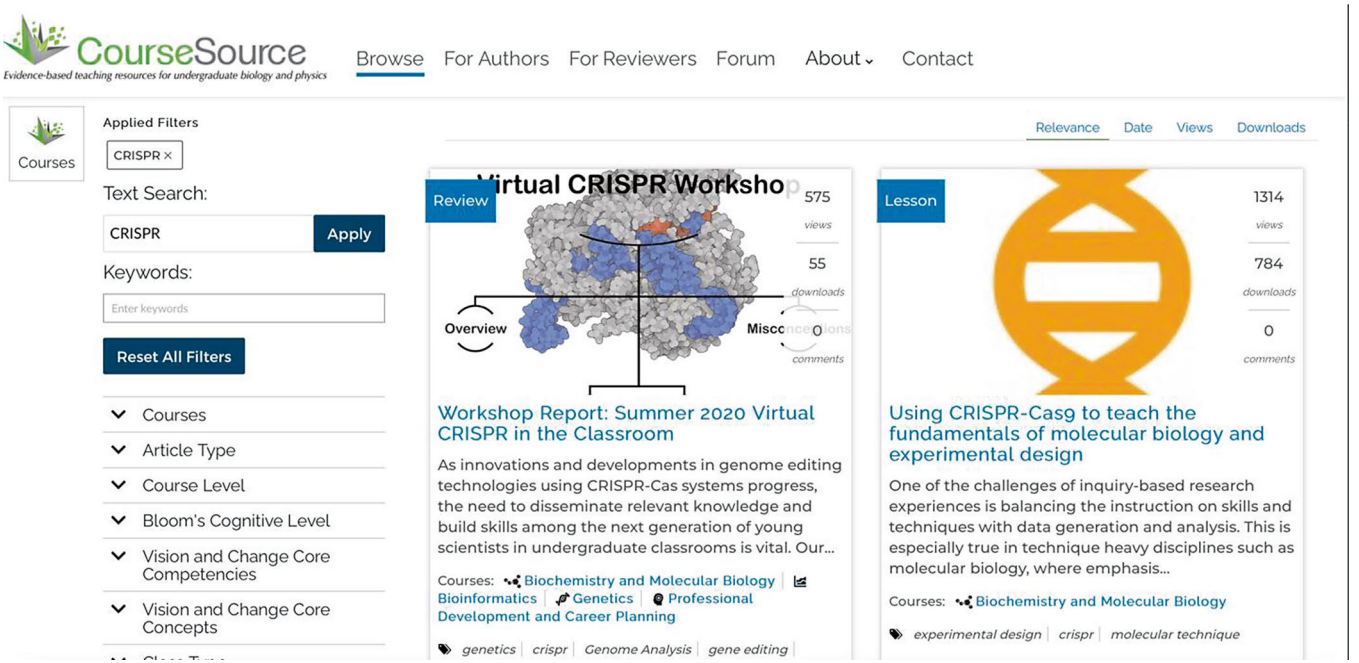

Fig. 4.4 An example of a search for CRISPR-based materials on the CourseSource website

4.4.5 What Happens If (When) Something Doesn't Work?

Even the best of instructors can find that their intricate laboratory plans can go awry with the failure of an individual lab session caused by a simple mistake in preparation. Additionally, it is expected that introductory-level undergraduate students will make mistakes in their time in the laboratory. One of the hardest parts of teaching undergraduate students in the laboratory is the idea that failure is not only expected but also a good thing. Through failure, troubleshooting, and repetition, the scientist improves not only their experiment but their thought process for experimental design and implementation. For CUREs, iteration is considered a vital part of the learning process for this reason [8]. CRISPR, with all of its components and stages, provides many opportunities for students to make mistakes and to suffer setbacks. As instructors design a laboratory experience for their students involving CRISPR, it is essential that they build in time for experimental iteration and troubleshooting and to teach their students the normality of such components in the work of "real" scientists. Likewise, students need to understand that the grade they will receive in the course will derive not from if they achieved a "right answer" in the laboratory but rather by of they have designed and implementated a sound research plan and worked to troubleshoot the inevitable setbacks that will arise during the laboratory process.

4.4.6 Dissemination of Lab Results Via Creating a Scientific Poster

An essential skill for all scientists is the ability to successfully disseminate their findings to their peers. While introductory undergraduates are likely used to the idea of writing a lab report from their previous class experiences, such reports are usually arduous endeavors that are only seen by the authors and the instructor. While scientific writing is clearly a skill that needs to be developed and practiced by these students, the development of a multi-week CRISPR laboratory module, especially in CURE form, lends itself nicely to a scientific poster presentation assignment. For most introductory-level undergraduates, such an assignment will likely be their first encounter with scientific posters despite their prominence as a modality for conveying experimental findings at scientific meetings. Assigning a poster as opposed to or in addition to a laboratory report also encourages students to regularly and carefully document their work since they will need to be able to tall their scientific story on the poster. Most importantly, the poster format allows students to share their work with each other as well as with other members of their community depending on the venue in which posters are presented (Fig. 4.5). This allows all students to appreciate the work that has been done by the class, to discuss findings with each other and get ideas for what improvements to their own scientific work, and to utilize their creativity in the production of an informative yet visually pleasing product.

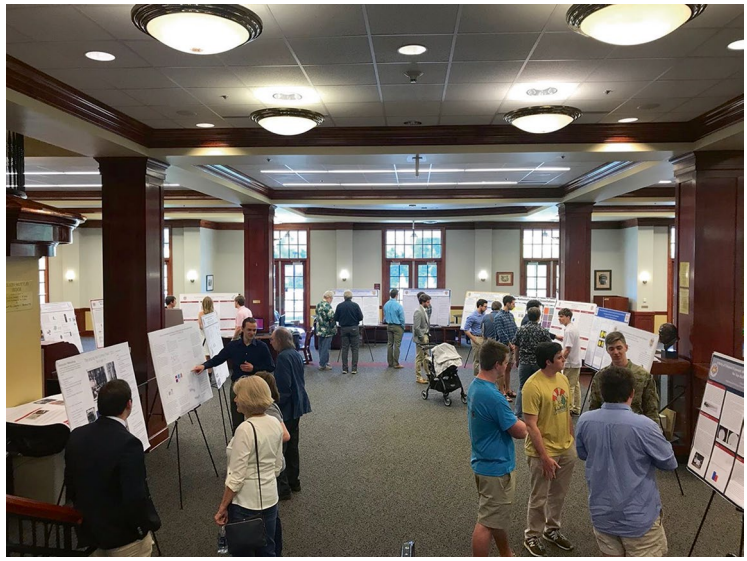

Fig. 4.5 A public poster presentation of laboratory work to the general community allows students to practice their dissemination skills with a generalist audience

4.5 Ethical Ramifications

While many if not most undergraduates have heard of CRISPR due to its increased presence in popular as well as scientific media, it is likely that they are unfamiliar with all of the ways in which the technology can be used (After all, this is the reason it is a topic that requires coverage in the introductory undergraduate classroom). As instructors work to get themselves up to speed with the mechanisms and applications that underly CRISPR, they often lose sight of a crucial opportunity that such lessons present to discuss with students not only HOW such technology is utilized, but when SHOULD it be utilized. I have the privilege of teaching a freshman seminar in Bioethics every other year as part of my reaching repertoire, and this class has never disappointed me as a venue for dynamic and engaging conversation on a host of questions related to the intersection of science and society. In teaching this class, I have found that students are eager to share their ideas on the issues they see in the news and to ask questions about thinks with which they have less familiarity. What follows are some suggestions on how to introduce ideas of the bioethical issues surrounding gene editing in general and CRISPR in particular into the classroom.

4.5.1 Inclusive Ground Rules

When I state that students are eager to share their questions and ideas, it is important to note that this is not something that may happen in a classroom organically. In many cases, students are afraid of looking stupid in front of their classmates and therefore elect to say nothing in class discussions. To encourage a more dynamic classroom as well as a classroom that is welcoming to all students regardless of background, instructors should set ground rules for discussions at the beginning of the course that clearly outlines what is considered constructive and appropriate discourse. By encouraging students to freely share their viewpoints and ideas and strongly discouraging responses that seek to attack or demean, you can slowly but surely give shier students the permission they need to be more open in discussions. In certain cases, this approach will engender ideas in the class that may be seen as abhorrent or, at the very least, disagreeable to the majority of students. The instructor must work in these cases to ask additional questions of the students introducing these ideas to establish their perspective on the matter. Such a approach will avoid simply shutting down discussion of uncomfortable ideas while simultaneously allowing all students the opportunity to reflect on the ideas under consideration. I feel like I have done my job well if I have successfully challenged the preconceived ideas that my students bring to my class, but I do not feel as if it is my job to try and change their ideas if they are unwilling to do so. The introduction of challenging ideas must be followed by adequate time to process and consider the challenge, and the student must arrive at a change of attitudes on their own. With patience and determination, the instructor can succeed in creating a student venue with a free and open exchange of ideas in which diversity of thought makes for a stimulating discussion as opposed to an opportunity for exclusion.

4.5.2 A Case-Study Approach

It comes as no surprise that the best approach to getting a student to understand a topic or idea is to get them to work with it for themselves. Just as a CURE provides an extensive and thorough way for students to engage with a laboratory-based problem, so does a case study provide a tangible and relatable framework in which a student can grapple with the complexities surrounding a bioethical issue. An instructor can simply turn to the news to find announcements of scientific breakthroughs involving CRISPR to find a venue in which to launch a class discussion on the pros and cons of the work being done. For a more refined approach, the National Science Teaching Association (NTSA) recently adopted the classroom case study collection originally curated at the State University of New York at Buffalo from which instructors can peruse and select scenarios that best fit the needs of particular classrooms (https://www.nsta.org/case-studies). This case study collection has several CRISPR-based examples as well as case studies revolving around genetic engineering in a more broad sense.

4.5.3 Reflection Paper Opportunities

In my Bioethics seminar, I require my students to submit a short (1–2 page) reflection paper at the end of each week. In these papers, students may write about anything they wish so long as it falls under the parameters of the course. I grade these papers not on whether I think a student is right or wrong but rather on their ability to express their ideas and to formulate them in a coherent and scholarly manner. Many students will reflect on the topics we have covered in the immediate class periods leading up to the assignment while others will consider topics that they find in the news or in online archives of bioethical questions. These reflection papers allow the student to think more deeply about their thoughts on a given issue and to express ideas in writing that they perhaps were more reluctant to introduce in class conversation. Through my comments, which mostly take the form of questions that I have as a result of what they are writing, I am able to have a dialogue with my students about their ideas and to point them in the direction of additional resources for those who wish to learn more on a given topic. In these papers, I also learn a lot about the misconceptions that students have about CRISPR (primarily, they grossly overestimate the ease in which CRISPR experiments can be done) and in the process find a way to adjust my own teaching to meet the questions that they raise. Taken together, these papers provide a way for students to develop skills in conveying scientific ideas in a relatively informal and low-stakes setting while also obtaining some formative assessment of the direction of class discussion for the benefit of the instructor.

4.5.4 Research Project: An Opportunity to Explore

The centerpiece of this Bioethics seminar is a scaffolded assignment in which students propose a research topic and prepare a short (~10 page) paper in which they use scholarly source material to learn more about their question. The students also prepare a research poster on their topic which they present in a public setting at the College. This assignment is an great example of giving students a controlled level of freedom for pursuing questions like CRISPR in which they may have a great many questions but little opportunity to explore them. By having students go through a proposal phase, the instructor can help the students to refine their ideas and to narrow down their research focus to a question that can be adequately answered in a short research paper. Along the way, the instructor can assign check-in activities like an annotated bibliography, a peer review session in small groups or online, or a draft introductory paragraph or poster framework to keep students working on the assignment at regular intervals. If the instructor wants to focus such a project exclusively on CRISPR, then students could still gave the freedom of identifying specific issues within the use of CRISPR technology that would still allow them to explore in more depth the ramifications of CRISPR-based research. The poster session at the end of the assignment gives the students invaluable scientific dissemination to the general public at the instructor's institution while also giving the institution insight into the work being done in the classroom.

Take-Home Message

My own journey from intimidation to fear to determination to excitement in the face of CRISPR has been, to put it mildly, an eventful one. For myself, who started my academic position in 2009, CRISPR represented the first significant advance in molecular biology technology in which I had no practical experience and the first challenge for me to keep myself current with the advances in my field for the good of my students. In this chapter, I hope that I have conveyed my excitement in the great opportunities available for instructors that embrace this challenge and inspired other instructors to come up with their own classroom innovations to bring CRISPR into the introductory college classroom. A technology like CRISPR is just the sort of topic that can excite a student to seek additional training in molecular biology and join the research community that will continue to expand the use of CRISPR in the years to come.

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CRISPR for Course-Based Undergraduate Research Experiences

5

Jay N. Pieczynski and Maria S. Santisteban

5.1 What Is a Course-Based Undergraduate Research Experience (CURE)?

Coursed based undergraduate research experiences (CUREs hereafter) have grown in popularity in higher education over the last decade and are quickly becoming established as one of many best and higher impact practices in higher education [1–4]. Specifically, CUREs are practices that engage every student enrolled in the course in authentic research, with the defining feature of a CURE being that the answer to the specific scientific inquiry is unknown to both the students and the instructor [5, 6]. CUREs are designed akin to what a student might experience in a traditional independent laboratory undergraduate research experience or internship, albeit expanded to incorporate the entirety of students enrolled in the course. Thus, every enrolled student is provided the opportunity to build some of the scientific skills associated with working in a research lab even though such opportunities might have previously been unattainable or inaccessible. CUREs are a mechanism by which scientific skills can be gained and topics can be reinforced by the act of doing real research in the context of a course.

The rise in popularity of CUREs can be traced to the publication of "Vision and Change: A Call to Action," whereas it was recognized that students need to be active participants in doing science, rather than passive consumers of scientific information [7]. The authors of this seminal document understood that the entirety of the scientific process is important

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and necessary, and that students who develop these skills would be better equipped to tackle scientific questions in the future. Since CUREs involve novel research, they are specifically designed to immerse students in many of the practices associated with the scientific process and authentic research such as hypothesis generation and revision, skill building, troubleshooting, collaboration, and iteration [6]. In addition to learning how to actually "do" science, there are numerous peer reviewed studies demonstrating that CUREs can significantly contribute to positive indicators of student success including improvements in student science identity, increases in feelings of belonging in STEM, a rise in student confidence, increases in diversity in STEM and overall student retention [8–12]. CUREs have also been found important in addressing equality gaps and to overcome inequities intrinsic to the traditional research experiences [5, 13]. For example, traditional research internships might not be feasible for a student who also has many commitments and responsibilities beyond the classroom. By imbedding the research experience directly into the class, this same student can benefit from gaining hands on research experience without the extra time commitment. Additionally, participating in a CURE might be a student's first experience with novel research and act as a launch pad for a future career in science. The rise in popularity in CUREs combined with their positive impacts on students have made them attractive laboratory teaching tools across many disciplines, institute types, and student levels [14–19].

It is important to note that CUREs are not the only laboratory teaching modality available to instructors, and there may be value in using other types of laboratory experiences. Types of labs could be viewed on a spectrum, with CUREs being on one extreme end and demonstrative labs being on the other. Guided inquiry-based laboratories are very similar to CUREs, with the key difference being that results of the lab practice are novel to only the students but known by or at the very least suspected by the instructor [19]. Guided inquiry can be profoundly effective when limited by resources, class size, or time. However, since these guided inquiry labs lack the discovery and relevance aspects of CUREs, recent investigations have focused on the value of these two elements for student's outcomes sometimes with conflicting results. For example, according to some reports students appear not to highly value the opportunity for publishing novel results and others find no significant impact of broadly relevant novel discoveries on students' project ownership [20, 21]. In contrast, others have found that at least for science majors, making broadly relevant novel discoveries that advance knowledge in a field or contribute to a broader on-going research project were significantly and positively related to students' sense of project ownership [9, 11]. Dolan's group reported nevertheless that opportunities for iteration, such as repeating experiments, troubleshooting, and problem-solving, may be more impactful for students than opportunities to make discoveries. Iteration and formative frustration in CURE settings have also been shown to be important to students of all class levels and contribute to student learning [22, 23]. Hence, if the instructor's goal is not for students to make a novel contribution but to increase student motivation and improve learning outcomes, an inquiry-guided experience with ample opportunities for students to struggle, repeat, and reflect may be just as appropriate as a CURE. Further,

demonstration-based verification laboratory experiments (also known as "cookbook labs") are often seen as being on the opposite end of the spectrum when compared to CUREs, where the answer is already known, and the student has little to no input on the design or deployment of the laboratory [24]. Given the positive impacts that CUREs have when compared to other lab modalities, this tends to make demonstrative labs seem archaic, however demonstration labs can provide excellent skill-building platforms, especially when students need in depth exposure to different scientific techniques and approaches to prepare them for future careers.

The introduction of CRISPR-based editing technologies has further pushed the boundaries of what is possible in a CURE both from a teaching, research, and learning perspective. Due to the ubiquitous applications of CRISPR technologies, the speed at which CRIPSR can be utilized, and its emergence as a fundamental skill set, instructors need only to be versed in a few basic molecular biology laboratory techniques and have access to basic molecular biology equipment to implement CRIPSR into their courses at the most introductory level. Given CRISPRs status as a "once in a generation technology," the numbers and possibilities for CRISPR-based CUREs range from completely *in vitro* investigations to utilizing CRISPR in a multitude of model organisms, some of which are discussed in more detail in later chapters in this volume. In the following sections, we will focus on some of the aspects of CUREs from design to deployment, with emphasis on the considerations that need to be made when utilizing CRISPR technology.

5.2 Defining the Scope of Your CRISPR CURE

The initial design or reimagining of a laboratory experience is a daunting task, especially when it involves making wholesale changes to established curricula. In some scenarios, this might even involve a cultural or philosophical shift on behalf of an instructor, the faculty and even the students involved. Just contemplating any major change to an established curriculum and outlining the shear amount of work involved can be somewhat intimidating, if not overwhelming, leading many to continue to follow the status quo. If this new laboratory that you are implementing is a CURE, this will now add even more variables to that equation. If contemplating a CURE, instructors should take a step back and ask themselves, "what are my reasons and motivations for doing a CURE?" Is this CURE to provide students with an authentic research experience that they might not get anywhere else? Am I trying to introduce students to research? Am I using this CURE to supplement my own research or move it forward? Is this CURE to teach students skills that are translatable to a career? Is the CURE to familiarize students in a general education course with the practice and nature of science? Am I trying to motivate students to have agency, claim ownership, and become critical thinkers and learners? Do I want students to develop tolerance for ambiguity and acceptance of failure as key characteristics of a "true researcher"? Is a CURE even the most effective pedagogical technique to foster learning in my students?

If the answer to the questions above point to a CURE being the most effective pedagogy to achieve your teaching goals, then the next step in the process is defining the scope of your CURE. The biggest questions to consider now become, what do you want students to do and learn through this experience? Below are some questions to guide this reflection process:

- What type of scientific question or questions do you think the students in your course can handle?
- What are some scientific questions that you would feel comfortable guiding students through?
- How much time do you have to dedicate to your CURE?
- How long will your CURE last, a few weeks, the entire term, or even longer?
- How will you implement CRISPR into this work?
- What are the learning outcomes that you are striving for and what should students walk away with from this experience? How will you know?
- What resources and knowledge are at your disposal for the students to perform these investigations?
- What lab skills need to be taught for students to engage in this experience and how will those be integrated in the course?
- What is your plan for implementation if students have differing lab experiences?
- How will you avoid and/or mitigate inequities in this experience?
- How will you build enough room for repetition and iteration?

Always keep in mind that as the instructor it is ultimately up to you as to how much independence to offer students in terms of choice, techniques, etc. When student projects move beyond the expertise of the instructor, there can be significant delays in providing meaningful advice while the instructor tries to learn the topic themselves on the fly. This can leave both the instructor and the students feeling frustrated and unaccomplished in the lab.

5.2.1 Developing a "CUREable" Question

Generating good and testable questions is an important skill for all researchers, from the most experienced to the most novice. Setting aside CRISPR for a moment, you'll need to identify a question to address in your CURE. Your CURE question can be as broad or as narrow as you would like it. Allowing overly broad areas for students to explore can be very challenging to manage if you have a lot of groups or multiple sections of the course. On the plus side, it can help you push your own knowledge of the field in new ways and add creativity and new directions to your own research. One important aspect of using CUREs is that you are not necessarily bound by a scheduled set of labs that must match directly with didactic course content to reinforce certain concepts. How do you get started then with designing your CURE question and laboratory course? Even those of us who

routinely teach using CUREs struggle with this question, mostly because there are many unique contextual variables to consider for your unique situation. Some of the most common variables to consider are the resources available at your institute, logistics of your facilities, safety, and the number and experience level of your students. For example, in a low enrollment upper division class comprised of students with strong molecular biology skills, it might be appropriate to empower students, either as small groups or individually, to come up with their own questions or projects in collaboration with the instructor playing the role of guide. However, in a highly enrolled course or one where the students lack specific training such as the introductory or non-majors level, it is likely more feasible to provide a limited number of options for students. Further, depending on the subject matter, the deciding factor on the scope of the CURE might be what the students can accomplish safely in the class. The key here is that regardless of the question(s) being addressed, the investigations must be capable of potentially generating novel data for the science community to make this a true CURE.

The most straight forward path in determining the question your CURE will address is to reflect upon your personal area of expertise and the resources available. From the numerous published and validated CUREs, two variations have arisen. The local model of a CURE is when faculty or a small group of collaborators integrates aspects of their own research interests into the CURE [25–28]. The second basic variation of a CURE is known as the network model, where a large group of institutes contribute knowledge to a broad topic [29]. With regards to the local model, these CURE questions generally begin as either a continuation of a previously established investigation, an offshoot of a previous interesting observation, or as an identified gap in the knowledge. This model has the added benefit that the faculty enthusiasm for their research results in greater enthusiasm for the curricula, as well as the ability to identify talent and maintain an ongoing research program [25–28]. McLaughlin and Coyle (2016) provide a validated pedogeological framework for helping develop questions throughout the process [30]. Using this framework, students and faculty collectively assess what is known about a topic, and then generate a testable question that will directly add to the knowledge surrounding this topic. Importantly, this framework can be expanded or contracted based on the length of the CURE. This methodology of generating questions allows students to have a large amount of input into the CURE, however the major drawback to allowing students to generate their own questions is that the scope of the CURE can become quite broad which can be very exciting but challenging to manage.

In the network model of CUREs, faculty elect to join a national network of other instructors asking individual questions around a core topic with each individual instructor/class focusing on a different aspect of the project. Here, the CURE question becomes a little more focused based on the network's needs, but still maintains the novelty of discovery that defines a CURE. Notable established CURE networks are outlined in Table 5.1. The major benefits of the network model are the availability of resources that the network provides, including training for new members, as well as the built-in collaborations found within the network.

Table 5.1 CURE networks

Resource	Notes	Citation(s)
CUREnet/CUREnet 2 https://serc.carleton.edu/curenet/index.html	Contains multiple CUREs, including those utilizing CRIPSR. CUREnet 2 focuses on building instructional capacity, research, assessment, and sustainability of CUREs	–
CRISPR in the classroom https://qubeshub.org/community/groups/crispr_classroom_network	Focus on utilizing CRIPSR in various undergraduate pedagogies including CUREs	Wolyniak, et al. 2019 [31]
REIL-biology https://rcn.ableweb.org/	Focuses on using CUREs in introductory courses	Spell et al. 2014 [32]
SEA-PHAGES https://seaphages.org/	HHMI sponsored CURE network. It seeks to understand viral diversity and evolution taught as a two-term laboratory course research experience.	Jordan et al. 2014 [33]
The genomics education partnership (GEP) https://thegep.org/	A nationwide collaboration of 200+ institutions that integrates active learning into the undergraduate curriculum through CUREs centered in bioinformatics and genomics	Shaffer et al. 2010 [34] Elgin et al. 2017 [35]
Malate dehydrogenase CURES community (MCC) https://mdh-cures-community.squarespace.com	Biochemistry intensive network CURE	Bell et al. 2020 [36]

5.2.2 Using CRISPR in CUREs

CRISPR gene-editing technologies have the capacity to make many biological questions easier to approach than more traditional methodologies. This also extends to those questions you might ask in a CURE. In fact, many biological questions are now feasible to ask in the undergraduate classroom specifically because CRISPR allows you to conduct these types of investigations at a faster pace. In addition to speed and the expanded scope of projects, other advantages of using CRISPR include introducing students to novel and cutting-edge technologies, the possibility of bringing ethics discussions into the science classroom, and the overall simplicity of the methodologies relative to older gene-editing techniques. A growing number of publications provide guides to start using CRISPR with undergraduates and high school students (Table 5.2; [43–46]. There are many places or strategies to use CRISPR in your CURE and which implementations you chose will depend both on your discipline and on the specific research goals you have for your CURE. How, when, and why to use CRISPR will be dictated based on these goals. Fortunately, CRISPR-based methods are rapidly increasing the types of questions that can be asked in CUREs due to the relative ease of the technique and the availability of reagents. There are many commercially available kits, published protocols/reagents, and web

Table 5.2 Selected examples of CRISPR CUREs

Citation	Brief summary
Bhatt 2018 [37]	Targeted reverse genetics approach to inactivate genes in zebrafish. Student inactive a gene with a known phenotype and a gene with an unknown phenotype
Mills 2021 [38]	Reverse genetic approach to inactivate genes in *Arabidopsis thaliana*. Includes a remote option for virtual students.
Evans 2021 [39]	Reverse genetic approach to inactivate RNA metabolism in *Candida albicans* using CRIPSR. Includes strategies and materials for student engagement during asynchronous laboratory time.
Hastie 2019 [40]	Knock-in endogenous fluorescent tagging and localization of proteins in *C. elegans*
Martin 2020 [41]	Reverse genetic approaches in the traditional model *Xenopus laevis* and an emerging model system *Vanessa cardui* (painted lady butterflies). Includes commentary on choosing a model for CRISPR deployment.
Adame 2016 [42]	Generation of novel mutant alleles using CRISPR-Cas in *drosophila*

Table 5.3 Methods of expression for the CRISPR-Cas9 components. The nucleic acid molecules and/or protein can be introduced using microinjection (worms, fruit flies, and zebrafish), transformation (bacteria, yeast) or transfection (mammalian cell culture)

Expression method	Guide RNA (crRNA + tracrRNA)	Cas9
DNA only	*In vivo* expression from plasmid	*In vivo* expression from plasmid
RNA only	RNA molecule from *in vitro* transcription	RNA molecule from *in vitro* transcription
RNA and protein	RNA molecule from *in vitro* transcription	Protein

resources that provide insight to what is needed to perform a basic CRISPR experiment [47–54]. One straight forward area where CRISPR can be incorporated into your CURE is to use CRISPR as a mechanism to teach molecular biology concepts, tools, and techniques [55]. For example, the various expression systems to generate the guide RNA (Table 5.3)-either introducing the DNA into the organism and letting it produce the gRNA *in vivo* or transcribing the gRNA *in vitro*—present an opportunity to discuss the essential elements of a transcription system. Similarly, the various expression mechanisms used to generate the Cas proteins (either introducing DNA, RNA or the Cas protein itself), lend themselves nicely to discuss the central dogma. Identifying Cas target sites, designing guide RNAs, or verifying gene edits are just a few examples of where CRISPR can be incorporated into the molecular biology portion of a CURE. Importantly, many of these molecular biology techniques provide an opportunity to incorporate the instruction and use of cloning software. CUREs that involve genotype to phenotype analysis, performing knockouts, or genetic manipulations were nearly impossible for students to accomplish in a semester pre-CRISPR or might have required significant instructor time for the CURE to progress forward. With CRISPR, depending on the model system used, knockouts could

be generated in days to weeks leaving more time for in-depth analysis. Keep in mind, any genes students select for targeting with CRISPR should produce easily scored phenotypes [41]. If students wish to focus on essential genes, be certain that a robust and well characterized inducible system is available. If no such system is available, also keep in mind that since CRISPR methods are in their relative infancy, sometimes modifying or developing CRISPR-based protocols can be CUREs unto themselves. For example, others have used CUREs that involve developing protocols and using CRISPR in non-traditional model organisms, such as butterflies or trypanosomes [41, 56, 57]. CRISPR allows the student to take a more active role in the CURE, however, as the instructor you will have to decide the place for CRISPR in your CURE. One area that often helps define the scope of a CRISPR CURE is if the instructor has access to or familiarity with some sort of proteomics or genomics data. These large data sets are prime real estate for asking questions and beginning investigations that yield novel results for students. Furthermore, these investigations have the benefit where even "negative data," furthers our understanding of scientific paradigm. As always, there are limiting factors and barriers for each CURE, however, below are a few approaches that you could work collaboratively with students in a CURE while using CRISPR-based approaches.

Reverse genetic approaches are perhaps the easiest, most popular, and most utilized methodologies in CRIPSR CUREs. Since classical reverse genetics involves a gene to phenotype approach, students and faculty can pick a gene or set of genes to study and then design CRISPR experiments around generating different alleles of these specific genes (Fig. 5.1). The advantage here is that reverse genetic approaches allow the CURE to have more narrow focus and be less open-ended. Examples of reverse genetic CURES include those focusing on a specific biological process, a specific biological pathway or generating various alleles of the same gene [37, 38, 42, 58]. Reverse genetic approaches can also be used in more complex scenarios, such as the identification of genetic interactions, epistatic effects, or suppressor or enhancer mutations. Reverse genetics is also powerful when using CRISPR to endogenously tag proteins for assaying localization. Another reason why reverse genetic approaches are a popular choice for CRISPR CUREs is that reverse genetics can allow for the student to have a large role in all aspects of the CRISPR process, for example, identifying target sequences in genes, designing and producing gRNAs and/or homology directed repair cassettes, transfection/transduction/injection of gRNAs, and verification of mutants both genetically and phenotypically. Furthermore, reverse genetics can be a powerful gene discovery tool when paired with information on differential expression provided by RNAseq. As with any reverse genetic CRISPR-approach, both the student and the instructor should be aware of potential off-target effects and how these might influence the phenotypes produced from this methodology [59].

Classical forward genetics involves observing a scorable phenotype and then determining the allele or alleles associated with that phenotype (Fig. 5.1). Many forward genetic screens utilize chemical mutagens or radiation on a large population to generate these phenotypes. Usually, these approaches are not practical in the teaching lab. However, CRISPR can and has been used to perform such screens [60–62]. The main advantage of CRISPR-based forward screens over mutagenesis is that CRISPR can target known sites

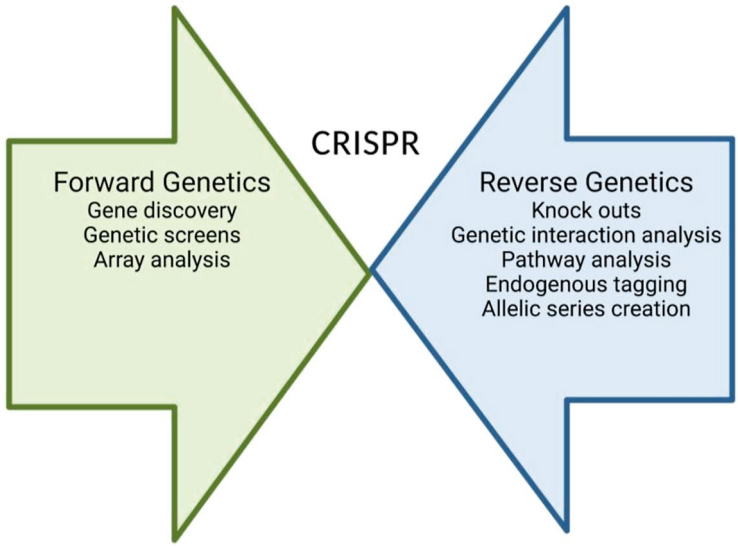

Fig. 5.1 CRISPR as a tool for both forward and reverse genetic approaches. Image created with Biorender

in the genome instead of the random mutations produced by chemical or radioactive mutagenesis. As with all forward genetic screens, identification of targets, both intended and unintended, requires multiple downstream steps to isolate phenotype-specific alleles. Using a library of known gRNA sequences, one could produce several novel alleles using CRISPR. There are two major types of screens: pooled screens and array screens, each with their advantages [63]. Pooled screens utilize mixes (pools) of gRNAs and then screen for phenotypes. The major advantage here is that there are fewer plates to screen initially, but the isolation of individual clones is more time consuming. Array screens utilize single gRNAs, each in different wells of a multiwell plate, allowing one gene knockout per well. Using arrays has the advantage of quicker initial screening for phenotypes but are initially larger and require more initial organization and time. Regardless of screen type, one might argue that the use of CRISPR would make the screening process more streamlined due to the specificity of gRNAs binding to their targets.

One creative and practical way that instructors have and continue to implement CRISPR into CUREs is through methodology development. Some educators have leveraged the fact that CRISPR utilizes evolutionarily conserved mechanisms of DNA repair to apply CRISPR methodologies in new model organisms [38, 57, 58]. Using CRISPR in such a way can be very rewarding for students as any data that they generate in this new model is novel and possibly publishable. These novel findings might even include the methodology used to deploy CRISPR in this new model or contribute to new deployment methodologies in previously established models. Another way that CRISPR CUREs could make a novel contribution to external stakeholders is in reagent development such as producing a cell line or plasmid that would streamline or improve the process of CRISPR-based edits.

In other CUREs, CRISPR might only be part of a larger project. CUREs could be designed that make edits using CRISPR and then students must characterize the effects of the edits on that cell line or organism. Other CUREs might utilize a CRISPR based methodology for sequence identification or genotyping. For example, assays have been developed that utilize CRISPR for diagnostic nucleic acid detection [64–66]. These diagnostic assays were designed for at home, point of care, or field usage and could easily be adapted for the classroom. In many of these assays, researchers use Cas-protein variants to cleave some sort of reporter molecule. Importantly, due to the specificity of CRISPR gRNA binding, reporter cleavage is only possible if there is an exact gRNA-target sequence match. One could therefore develop CRISPR-based assays to detect environmental DNA/RNA in wide range of cases in anything from virus detection to species identification. Using such assays, the applications of CRISPR are limitless and are only bound by the creativity of the user.

5.2.3 What Do You Want Your Students to Learn?

Once you establish a general sense of the question that you would like to address in your CURE and how you might want to use CRISPR, you must decide what the learning outcomes are for your students. In practice, it helps to rely on the principle of backwards design, where the instructor first identifies learning goals and then structures the course to reach those goals. Using backwards design requires that you clearly articulate what skills the students should learn and be able to do at the conclusion of this experience [67]. Importantly, at least some of these outcomes should be attainable for students regardless of laboratory success, however success is defined. Given the open-ended nature of CUREs, the tendency might be to try and accomplish all your learning outcomes, but you will need to keep in mind that these are students whose personal goals might not align with those set forth. It is likely that the more experience you gain with CUREs, the easier it will become to define learning outcomes for students. Below are a few suggested areas of focus that might be relevant to your CURE:

- *Science writing/communication*—write in the style of primary research literature, identify and use sources, communicate data, make figures and figure legends.
- *Technical laboratory skills*—perform certain types of assays/experiments and the theory behind them.
- *Reinforcement of scientific principles*—practical hands-on experience with concepts and principle previously introduced in another part of the course or curriculum.
- *Problem solving/troubleshooting*—address problems and probe likely solutions logically and systematically.
- *Critical thinking and analysis*—analyze data and draw conclusions from data.
- *Experimental design*—design well-controlled experiments/assays that directly address scientific questions.

- *Inquiry specific knowledge*—an in depth understanding of an area of inquiry including background and how data generated contributes to our understanding of an area of study.
- *Scientific metacognition*—how scientific knowledge is generated, tested, and rigorously reviewed.
- *Science identity and agency*—recognizing oneself as a scientist, ownership of research, and the ability to confidently conduct science.
- *Team-based skills*—collaboration, teamwork, and accountability
- *Resilience and grit*—deal with failure and build a growth mindset.

It should be noted that most of these outcomes might not be readily apparent to the students participating in the CURE and might not be specifically spelled out in the syllabus or assignment but are desired outcomes of this type of course [68]. For example, it has been observed that students may remain unaware of their demonstrable gains in problem solving skills after a CURE. Therefore, it becomes essential that the instructor continuously communicates with students the learning outcomes set forth and reinforces them. The reality is that CUREs can be stressful for some students since some students make the false equivalence of learning and getting the "correct" answer. This can also be exacerbated when students assume that being "correct", getting results that match their hypothesis, or getting any results at all is required for receiving a high grade in the course. Not only is this biased, but it also reinforces the unrealistic expectation that science always works. In CUREs, make the focus on the experience and intellectual contribution, rather than the success or failure of the experiment. Students must learn that failure and iteration are major parts of the scientific process and should be appreciated as opportunities to learn [22].

5.2.4 Consider Student Time When Defining Scope

Time is often the rate limiting factor in determining the scope of CUREs. Both student and instructor have a finite amount of time to work on and potentially complete even small aspects of projects. In this section we will focus on student time and will discuss instructor time below. Unlike a research lab, CURE student time is defined by the academic calendar and their own personal class, work, and family schedules. Also, keep in mind that CUREs are centered around the student, and what might take the seasoned researcher only a few minutes might end up taking a student up to an hour of time, or even more when group dynamics come into play. CUREs are a prime example of a case where a "less is more" approach is beneficial; it is always easier to leave a CURE open-ended to move at the pace of the students and the science. Strikingly, we do just this in our own research, but for some reason feel compelled to exhaustively plan out the entire semester's lab activities. Just remember that part of using CUREs is modeling to students how science is best accomplished when we try to limit our biases and objectively interpret data from well-controlled experiments, even if it means exhaustive troubleshooting, repetition, and slowing down in the process.

When defining the scope of their CURE, instructors need to spend a significant amount of energy thinking and reflecting on what students can reasonably accomplish in the time at their disposal. Use these questions to guide you:

- How many lab sessions do my students have in a semester to complete the CURE?
- How long is each lab session relative to the tasks that need to be completed?
- Is there any flexibility in scheduling lab sessions to facilitate your CURE and what would this look like for students?
- Is there any space to allow for repetition?
- Is there an expectation that students spend time on their CURE project outside of formal class time? If so, how will safety be monitored? Who will supervise? How will differences in time students are able to contribute to the project be taken into account?
- Will students be working in pairs/teams where they can split tasks?

The answers to the above questions are complex and will vary from institution to institution, and maybe even from year to year, class to class, or even group to group at your school. Also, implicit in these questions is the hidden curriculum, which is the unwritten rules that govern many of our standard teaching practices including laboratories. Importantly, instructors need to recognize that these unwritten rules will significantly impact many students that would benefit from CURE experiences [5]. For example, if a significant number of students at your institution are commuters, requiring students to tend to laboratory experiments outside of formal class hours is likely to end in a negative experience for all involved. However, one of the major benefits of CUREs where student time can be leveraged is flexibility. Since CUREs closely resemble independent laboratory research experience, you can allow students to have some input into when and how to distribute their time towards the CURE that fits with their schedules.

How do instructors then factor in maximizing student time in their CURE? One strategy for ensuring students can make the most of their time is to employ a "bootcamp," where students can familiarize themselves with common techniques and provides a baseline for completing certain lab tasks. This bootcamp can also be used as a starting point to begin their investigations, such as verifying reagents work as planned. This strategy provides three major benefits: allowing students to begin with some level of lab accomplishment, initiating progress towards teaching student's basic lab skills, and allowing you to expand the scope of your CURE because students will possess certain base knowledge that should allow them to complete future tasks more efficiently.

5.3 Barriers to CUREs

CUREs have been repeatedly demonstrated to be a best practice in teaching [10, 19, 34]. However, we would be remiss if we overlooked barriers to CUREs. In this section we will discuss barriers to CUREs.

5.3.1 Departmental and Institutional Barriers to CUREs

Those wishing to implement CUREs as part of their science courses might experience pushback, especially in terms of the role CUREs have in the science curricula. For some, moving on from traditional verification labs to CUREs might seem like a gigantic departure from established teaching practices that might have existed for decades. The practice of CUREs is still in its infancy, and some colleagues or administrators might even deem them an educational fad that will pass over time. Even more, some instructors might hold the view that the purpose of lab is to reinforce concepts from the didactic parts of a class, and that CUREs cannot accomplish this goal. These barriers will also be magnified if the course is team taught and not all members of the team are comfortable with the teaching dynamics that CUREs bring. There are also budgetary and time considerations that arise with the implementation of CUREs. Additionally, bringing in CRISPR as a new technology will further lead some colleagues/administrators to give pause to the idea. One area of concern that is always raised with CRISPR work is that novice students will work with RNA, and that there is high risk of contamination and degradation with such an unstable reagent, and thus experiments will have a low rate of success. With all these contradictions, it might seem that although CRISPR CUREs sound great in practice, they are not a feasible practice unless the teaching scenario is perfect. That is, a low enrolled class of highly trained and motivated students, a sizable budget, and a large amount of faculty time. However, there is a considerable amount of evidence that suggests that the benefits of teaching CUREs outweigh the perceived barriers that they place on faculty and student development.

One concern that has arisen with the implementation of CUREs is the role of CUREs in curricular development and how this relates to promotion and tenure in academia. Not all institutions recognize curricular updates and implementation of best practices as criteria demonstrating growth in teaching. Therefore, implementing a CURE in a course might be a considerable risk for an instructor whose career depends upon positive teaching evaluations and effectiveness as a teacher. There is a risk in developing and using CUREs when it comes to being evaluated as an effective teacher. Peer instructional evaluators might see CUREs as failing to address essential components and skills of the curriculum and are too tangential to the overall themes of the corresponding lecture course. The worry here is that teaching a CURE would result in less than stellar teaching reviews if the CURE is not "successful." These worries are warranted but can be mitigated by organization and communication to faculty evaluators. It is important to lean on the significant amount of peer reviewed literature that addresses the benefits of CUREs, both for the participating students and the institution. Additionally, instructors can utilize validated student perception instruments to generate data that demonstrates positive indicators of student learning and interest in science. Furthermore, introducing CRISPR in your CURE can have the added benefit of providing definitive evidence that your courses are maintaining currency and relevancy by introducing students to new technologies and methodologies in the class.

Specific student pushback is always a concern when attempting to implement new pedagogical techniques. Students often have the perception that CUREs will require much more of their time compared to verification labs, hence the above section addressing this concerning during CURE development. Also, students might not see the rationale behind the CURE, might perceive the CURE as being more difficult than verification labs, or might have apprehension about the open-endedness of CUREs and low tolerance for ambiguity, all of which can result in lower evaluations. To address student concerns, it is helpful to have clearly defined learning outcomes that are introduced at the beginning of the CURE and continuously reinforced by either communication and/or formative assessments. In essence, you need to demonstrate to the student that the skills and knowledge they are acquiring through the CURE are relevant and can be broadly applied. When it comes to addressing the perceived difficulties of CURE labs compared to traditional labs, it is essential to have a clearly defined grading system in place. This grading system ideally should be based on skill acquisition, cognitive development, and attaining learning outcomes as opposed to achieving the desired laboratory outcome. Keep in mind that there will always be the student that does not "get" the rationale of the CURE and sees the laboratory experience as another checked box. This type of student is one of the reasons many CUREs are deployed as group activities, often with an accountability factor built into the grading scheme.

An argument made against CUREs is that students won't receive proper training and reinforcement of concepts, especially in introductory courses. In reality, student training and motivation is always a concern regardless of the lab scenario. This might make one think that CUREs are only applicable to upper-level courses that have been self-selected for advanced students. However, there is evidence to suggest that using CUREs in introductory courses benefits all students in training [9, 24]. CUREs in introductory courses are especially valuable for students who are unaware of research opportunities due to outside factors such as first-generation status, financial concerns, or cultural norms surrounding research [5]. Student training also relates back to the scope and goals of your CURE. Even the smallest success of a single experiment might be a life altering event for a student in your course, especially one who might not have considered research a viable future path. CUREs in introductory courses provide an excellent training opportunity for all students, not just those who perform independent research later in their careers. In fact, students with early hands-on training in science via CUREs have greater longitudinal outcomes in science, such as an increased 6-year graduation rates and increased likelihood of graduation with a STEM degree [8].

5.3.2 Affordability of CUREs

Affordability is often cited as a major drawback of CUREs. The perception is that with such open-ended questions that CUREs have, there is the potential for costs to skyrocket beyond individual or departmental budgets. These fears are even exacerbated when or if

CUREs become the new normal within a curriculum. While it is true that CUREs have the potential to be very expensive in terms of reagents, equipment and space, the cost of CUREs can be kept minimal by careful planning and organization by the instructor. CUREs often involve a major redesign in curriculum and philosophy, and these changes cannot be made overnight. Instructors must take the time to understand the scope of the project and determine what is realistic based on their budget. This is where leveraging personal expertise and interests come into the fold. It is often substantially cheaper to develop a CURE in an area of active research where many of the reagents and equipment are at the very least familiar to the instructor. This will often prevent you from having to make large purchases. Additionally, it is important to remember that even the most experienced students in a course are still novices compared to the faculty member. This means that the amount of data that students in a CURE are likely to generate is going to be significantly less than what an experienced researcher/instructor can do in the same amount of time. Between the slower pace of the research and the open-endedness of CUREs, it is best to be realistic about what and how much to purchase in preparation of a CURE. If possible, have a basic plan in place but leave room for both iteration and new directions if possible. Also, it is recommended that students performing CUREs work in groups. Not only does group work effectively reduce costs, but it also has the added benefit of incorporating peer learning into the practice.

When utilizing CRISPR in your CURE there are mechanisms to keep the costs minimal. There are several plasmid-based reagents for both guide RNA construction and production that can be purchased from numerous sources. These plasmids typically contain a cloning site for insertion of the crRNA DNA sequence followed by the universal tracrRNA DNA sequence, allowing for a streamlined creation of the full gRNA sequence using traditional cloning methods (Table 5.3). It should also be noted that there are plasmids available for either driving *in vivo* expression of the gRNA from the plasmid directly or for producing guide RNAs via *in vitro* transcription. There are also numerous cost-effective options for Cas enzymes including plasmid-based delivery, purified recombinant Cas proteins, or even model organisms that are already stably expressing the Cas enzyme of choice. Importantly, a number of these reagents can be propagated repeatedly allowing for quick and efficient manipulations at minimal cost to the user. As outlined in Table 5.2 and in future chapters in this volume, there are numerous example instructors who have successfully implemented CRISPR-based CUREs or guided inquiry based CRIPSR labs in various models. Many, if not all of these colleagues are typically more than willing to share both reagents and advice on how to best teach using CRISPR.

5.3.3 Balancing CUREs: Time and Resources

A major concern from those undertaking CUREs in their courses is the amount of time that the faculty member must invest in the project. CUREs do take time, plain and simple,

and each instructor has a different amount to dedicate to their CURE. However, making a few key decisions about your CURE during the planning stages can better help you manage your time during the CURE.

First, understand that your time is a finite resource that must be considered when developing the scope of your CURE. Many of us have great ideas, however executing these ideas might not be feasible given your other commitments. For example, taking time points every 2-h for a day might be appropriate for Ph.D. or post doc work, but what about undergraduates or yourself when you might have to worry about things going on in your personal or professional life? Further, would you or your institute even trust students to perform experiments unsupervised at night or on the weekends? The major question you will have to address is how much of your time are you willing to spend on this investigation and how much will you leave up to the students? The very nature of CUREs would suggest that students should be performing as much of the work as possible to get the most out of the CURE. This will likely mean that you will have to limit the scope of the questions you address and your expectations for results. However, by limiting scope and expectations for results, you can simultaneously reach the goal of having students learn science by doing science all while keeping costs, both time and money, to a minimum.

You will need to structure your CURE in a way that addresses the amount of time you are able to commit to your CURE. What level of structure and order will you have? Will there be parts of the semester or year where you can dedicate a significant amount of time to your CURE and other parts where the CURE will have to run itself? For example, if there is a time intensive and/or highly technical skill, such as microinjection, that is required for your CRISPR CURE you could scaffold your CURE to accomplish that task in a less busy time. One strategy that has been successful for CUREs to maximize faculty time is "backwards" design where the faculty creates reagents in their free time and the students do the analysis and characterization of these reagents in the CURE.

Another popular method for teaching CUREs is making the CURE a continuous project over multiple iterations of a course. For example, in one semester students create novel strains using CRIPSR methods, and in a future course students characterize these strains. This can be adapted to include other courses as well, for example the CRISPR engineering and editing can be done as part of a Genetic/Molecular Biology course and the characterization of strains/organisms could be carried out in a Development/Cell Biology course. This model also lends itself to collaboration, where students at one institute perform one part of a CURE and students at another perform a different aspect of the CURE. Finally, the new strains could also be sent to model organisms' repositories/databases if characterization is out of the scope of the class. This, besides constituting a real contribution to the scientific community, would have the added benefit of enhancing students' motivation and sense of ownership [69].

Another often cited barrier to implementing CUREs in a curriculum is the amount of physical time spent with students mentoring them through experiments. Since students are performing science, it is likely that investigations might diverge from what was intended. This divergence could be for the entire class, a single section of a class, or a group within

a single class. How do you avoid your CURE becoming a major time sink? One solution here is to break your CURE into smaller "mini lab groups" and structure time for different "lab meetings" with these groups. This way, each group has a dedicated time to meet with the instructor and get feedback, ask questions, or get further instructor specific to their current focus in the lab. This strategy is extremely effective if there are multiple sections of the course performing the same CURE or if your class has a large enrollment. Not only does this force students to prepare deliverables for their lab meeting, but it also allows for structured and group specific instruction when needed. If you are concerned about your ability to move around the room addressing different aspects of your CURE at one time, consider enlisting the help of a lab teaching assistant if possible. These teaching assistants can even be students, especially those who might have significant lab experience or might have participated in the CURE before. These extra hands are invaluable in the lab once student work begins to diverge. A lab teaching assistant could also aid with lab preparation.

5.3.4 Resources and Training for CRISPR CUREs

Fortunately, there are a number of opportunities available for training and resources for CUREs in general as well as CRISPR-based CUREs. As mentioned previously, there are CURE network models. These networks also provide training and shared resources for faculty who are new to CUREs, including training workshops and online resources. Lopatto et al. has found that barriers derived from campus infrastructure (such as time for new curriculum development, availability of IT services) are alleviated by a central system that supports a shared investigation making networks invaluable to those wishing to use CUREs [70]. Often included in these networks are specific mentoring programs that provide direct support should questions arise.

5.4 Assessment of CUREs: Are Students Learning?

Obviously, the ultimate goal for any classroom experience is for students to learn, and in this case specifically learn about CRISPR-based technology. Thankfully there are numerous validated assessment tools available to gauge multiple aspects of learning ranging from content and aptitude to identity and feeling of belonging. Auchincloss and colleagues (2014) provide an in-depth and comprehensive overview of CURE assessment strategies and make a number of specific recommendations on how to systematically assess CUREs [6]. This publication was followed by one from Shortledge and Corwin (2016) that provides not only a framework for developing an assessment for your own CURE, but also an extensive list of validated assessment instruments that could be used to assess various aspects of student learning [71].

The most useful feedback from any assessment strategy or tool requires careful planning on the part of the CURE instructor. Using a backward design to develop your CURE

should facilitate the task of assessing it later [67]. First and foremost, the instructor should delineate their scientific research goals and develop corresponding learning objectives; this will inform the selection or development of an appropriate assessment tool. The assessment tool used should closely align with the learning outcomes of the CURE. Failure to do so could ultimately result in difficulties in the interpretation of a CURE as an effective learning tool, a reduction in student learning and engagement, and in the worst-case scenarios could lead to a discontinuation of using CUREs because there is no demonstration of measurable learning gains. Furthermore, having a clear and relevant assessment strategy allows for reflection and growth as an instructor and forces the instructor to think critically about the delivery and effectiveness of their pedagogy. Ultimately, having an aligned assessment will allow CURE instructors to become better overall teachers, but will also potentially allow the CURE to evolve over time.

Take Home Messages

- CUREs are laboratory approaches where students engage in novel research in the context of the classroom.
- CUREs have the capacity to increase interest and retention in science, as well as give students agency in a course.
- Defining the goals and scope of your CURE is essential for reaching student learning outcomes.
- CRISPR-based technologies are easily adaptable and very applicable to CUREs.
  - The methodology, although cutting edge, is relatively simple.
  - For advanced techniques like microinjections, collaborations may be possible.
  - The CURE is scalable from the *in vitro* steps only to the full *in vivo* characterization of phenotypes of strains/organisms. It can also be spread over more than one semester, or to involve more than course.
  - CRISPR CUREs offer a unique opportunity to discuss ethical implications.
- Many barriers to CUREs are easily avoidable by careful organization and planning.

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Part II

Model Systems: Do's and Don'ts

CRISPR in Plants

6

Nicholas J. Ruppel and Dawn Carter

6.1 CRISPR-Cas9 Classroom Approaches Using *Arabidopsis thaliana*

The model organism *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) is commonly used in higher plant-focused molecular-, genetic-, and cellular-themed undergraduate courses. It has several positive attributes amenable to *in planta* studies, most notably a shorter life cycle relative to other model plants, simple growth requirements, and an abundance of genetic and bioinformatic resources available to the academic community. Most professionally-trained plant biologists have experience growing and studying the plant in the research laboratory and can potentially translate this knowledge over to the classroom. By focusing on a plant model, students can be introduced to important topics ranging from translational research in crops to basic science research. As such, instruction with *A. thaliana* has proven ideal in undergraduate education, especially given its suitability for CRISPR-Cas9 genetic manipulation.

In this section, we discuss three approaches that instructors can take when applying CRISPR-Cas9 genetic techniques in *A. thaliana*. As it is standard of this technology, CRISPR-Cas9 can be used as a mutagenesis agent or for genetic element knock-in/replacement purposes.

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6.2 A Focus on Designing Guide RNA Sequences and Cloning into *Agrobacterium* Binary Vectors

This approach entails having students design and build *Agrobacterium tumefaciens* binary vectors containing guide RNA sequences designed to eventually knock-out genes of interest in *Arabidopsis thaliana*. This approach would be suited to a Molecular Biology course and has been employed successfully at Rochester Institute of Technology for Plant Molecular Biology. There are many cloning strategies available, but all will eventually entail cloning the guide sequence into an *Agrobacterium* binary vector. These vectors tend to be large (10 kb+) and typically have low copy numbers. A strategy devised by Kim et al. [1] allows the guide sequence to be cloned directly into a binary vector (pHAtC or pBAtC), without the need to subclone. The strategy uses scarless directional cloning using type IIS restriction enzymes [2], and is very successful, even in novice student's hands. The Kim et al. [1] paper is very amenable to teaching students how to follow methods directly from the paper. We typically have the students read the paper [1] and then devise an experimental plan to fit into their lab sessions. This prompts students to consider how long each step will take, what reagents they will need, and how to fit the work into their allotted class time. For upper level students, this is a valuable skill for graduate school or employment [3].

The design and cloning project can be accomplished in 5–6 weeks for a class that meets once a week for 3 h. Students would start by exploring the TAIR database [4] and selecting a gene of interest for their knock-out experiment. We often suggest some targets that have an observable phenotype [e.g., root mutants such as WEREWOLF [5] or CAPRICE [6]]. Students can then upload the full genomic gene sequence to Benchling [7] or another site that identifies potential guide sequences, such as ChopChop [8]. Benchling [7] allows users to view the exon-intron structure of the gene of interest and narrow the target region. Once they have chosen one or a few sequences, the Kim et al. [1] paper provides clear instructions for designing oligonucleotides that contain the guide sequences together with the necessary overhangs to enable cloning into the binary vector. An alternative is to provide students with pre-designed and pre-made oligonucleotides. Once the assembled guide sequence has been cloned into the binary vector and transformed into competent *E. coli* cells, we typically check for the presence of the inserted sequence via PCR and DNA sequencing. After transformation into competent *Agrobacterium*, the next step would be floral dip transformation of *Arabidopsis thaliana* [9], the process by which genetic material is transferred from the engineered *Agrobacterium* cells to the *A. thaliana* female gametes. Whilst students enjoy dipping their plants, a typical semester will not allow for selection and analysis of transformed seedlings. If the class is taught every year, students could analyze seeds produced by a previous class, or alternative content such as performing an *in vitro* assay [10].

6.3 A Focus on Genetic Transformation and Transgenic Plant Isolation

With this approach, students begin the term by transforming [9] previously-designed CRISPR-Cas9-based plasmid vectors from *Agrobacterium* into *A. thaliana*. These vectors may be instructor-designed and created or originate from student projects (see the last section). Ruppel et al. [11] used this pedagogical approach in an intermediate-level *Genetics* course with 16 students per section that met for one 3-h period per week (see the referenced manuscript's supplemental materials section for laboratory protocols). Generally speaking, the plasmid contains *A. thaliana*-specific targeting guide RNA(s), a Cas9 gene, and plant and bacterial antibiotic selection genes, and may be cloned either by the instructor or purchased from a scientific supply company (e.g., Millipore Sigma [12] has a CRISPR Plant division). Regardless of origin, *A. tumefaciens* liquid cultures are provided to students in the first session for use on flowering *A. thaliana* plants. The floral dip procedures [9] may be repeated during a second session, if desired, to boost plant transformation rates.

Transgenic seeds, once fully developed, collected, and briefly vernalized, are selected according to the plant-specific antibiotic resistance genetic element(s). Typically, selection is done on sterile Murashige and Skoog antibiotic-containing agar media or on soil after spraying the plants with the herbicide bialaphos, depending on the selection elements. The transgenic plant recovery rate can vary depending on transformation protocols but is typically 1–5%. A genetic analysis on the selected plants may be performed depending on the number of plants recovered, when the phenotype is expected to be measurable, and the CRISPR-Cas9-induced genetic penetrance; however, Ma et al. [13] noted that genetic analysis is preferred in second-generation transgenic plants. Waiting until the second generation gives the students a larger population of transgenic plants with which to work as the end of the academic term nears, although doing so likely requires the instructor to incorporate alternative course learning elements while the plants mature. While growing, LeBlanc et al. [14] suggested exposing the first-generation transgenic plants to several successive, short-term high heat conditions to increase the CRISPR-Cas9-induced gene editing penetrance. These heat-treated plants are then grown to seed, with genetic analysis done on their vernalized progeny. If mutagenesis is the goal, given the likely time constraints of proceeding to a second generation, the CRISPR-Cas9 gene target options should be limited to those with a role in early plant development.

6.4 A Focus on Genetic Analysis in Stable Transgenic Lines

An alternative approach when using *A. thaliana* is to provide the students with stably-transformed CRISPR-Cas9 seed lines. The focus here is less on the creation of these lines, and more on the genetic analyses. In our experience running courses that meet once per week for 3 h a session, completion of these procedures can be accomplished in approximately 10 weeks.

Students begin the semester by growing the supplied transgenic plants in parallel to a wild-type line. We have used this approach to study the genes *Phosphoglucomutase* (*PGM*) [15], *Variegated1* (*VAR1*) [16], and *Too Many Mouths* (*TMM*) [17]. The *PGM* gene has a role in plant starch metabolism, with a loss-of-function 'starchless' plant reduced in its capacity for energy storage and, interestingly, unable to properly respond to environmental cues like gravity [15]. The *VAR1* gene has a role in chloroplast development and photosynthesis; loss-of-function *VAR1* mutants result in a plant with green-and-white patterned leaves [16]. The *var1* leaf variegation expressivity is temperature-sensitive, giving the students an opportunity to witness this genetic phenomenon in a plant system. The *TMM* gene has a role in stomatal cell development, which affects gas exchange and cell patterning. Loss-of-function *tmm* mutants contain a higher number of clustered stomata on cotyledons [17].

The students spend the first several weeks of the term becoming familiar with their transgenic plants and gene(s) using primary literature sources and bioinformatic tools, to the point where they can design an experiment to quantifiably distinguish their mutant from the wild-type line. There are numerous useful bioinformatic resources available to instructors who wish to use *A. thaliana* in the classroom [18]. For example, *The Arabidopsis Information Resource* (TAIR) [4] provides a database on the entire *A. thaliana* genome, and the CRISPR-Plant resource can be used to identify CRISPR-Cas9 targets [19]. Full access to the TAIR site requires a subscription, although temporary access can be requested for teaching purposes. With these resources, students at Randolph-Macon Collegenhave identified their gene DNA sequence, gene expression patterns, publication history, and the CRISPR-Cas9 target site used in their plants (as well as every other potential target).

A genotypic analysis can proceed once mutant alleles have been confirmed and the plants are large enough to extract DNA. Several laboratory sessions can be spent PCR-amplifying the wild-type and mutant gene (or an area of the gene that includes the predicted CRISPR-Cas9 target site), running the amplified products on an agarose gel, and gel-purifying the samples. The DNA sequences of these purified products can be generated and aligned; if the CRISPR-Cas9 target sequence is known, the students can search for and focus on this area of the sequence read. Alternatively, if the CRISPR-Cas9 target sequence is not known, students can compare the entire sequence between wild-type and mutant genes. A discussion may follow concerning the impact of the induced mutation, as frameshift mutations are commonly generated by the CRISPR-Cas9 system (Fig. 6.1). At this point, the students have completed a phenotypic and genotypic analysis of their plants.

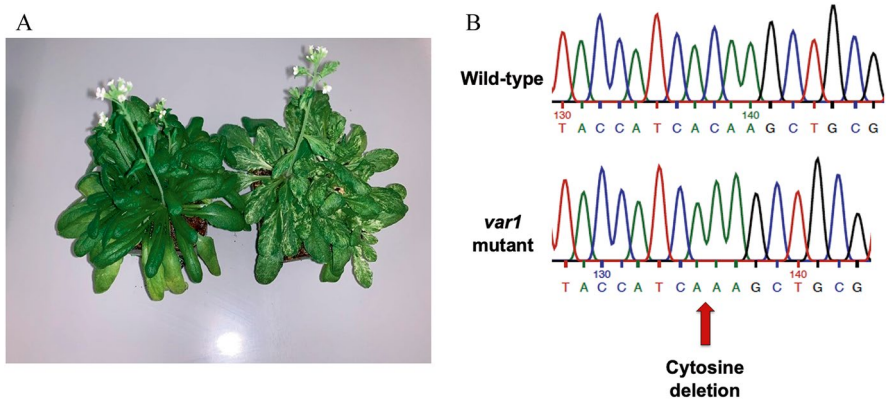

Fig. 6.1 (**a**) Example of an *Arabidopsis thaliana* gene knock-out from a classroom experiment. The target was the Var 1 gene. Wild type plant on left, *var1* mutant on right. (**b**) Sequencing analysis showing a C deletion. This causes a frameshift, resulting in a premature stop codon

6.5 The Challenges and Benefits of *In Planta* Studies

There are several challenges associated with using CRISPR-Cas9 gene editing techniques on *A. thaliana* in the undergraduate classroom. Unlike other model organisms (e.g., *Drosophila melanogaster*, *Escherichia coli*, *Saccharomyces cerevisiae*, etc.), an instructor can only expect to complete 1–2 full *A. thaliana* life cycles during a typical 14-week semester, depending on the applied growth parameters. The moderately protracted growth period has likely limited its overall *in planta* classroom usage, at least for instructors that prefer a multi-generational analysis. On the other hand, instructors who are open to project modularity are more likely to succeed, especially those in which stable CRISPR-Cas9 lines are provided to the students.

To date, there are also few known *A. thaliana*-based courses operating within the course-based undergraduate research experience (CURE) format [20]. This is likely due to the typical course time constraints associated with *in planta* studies. In the past, we have had students design and clone CRISPR-Cas9 gene target constructs for use by future students; these steps are nicely incorporated when active *in planta* studies require several weeks of growth. The cloning and bacterial transformation steps are achievable in a semester, but the subsequent plant transformation and genetic analyses are not. Ultimately, the paucity of student-inquiry options (i.e., CUREs) puts the onus on the instructor to design and create the CRISPR-Cas9 genetic materials.

Take Home Message

Despite these challenges, the use of *A. thaliana* can provide students with a tool to successfully complete a CRISPR-Cas9-based research project. Its use is amenable to various course types, including those focused on bioengineering, genetics, cell and molecular biology, and plant biology, where students do not require significant botanical background knowledge to begin or succeed. The genetic analyses benefit from the potential for studying straight-forward, predictable phenotypes that these students can recognize with minimal training. Also, rearing *A. thaliana* is fairly cost effective, with minimal inputs for grow lights and racks, soil, fertilizer, and pots and flats.

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CRISPR in Zebrafish

7

Anil Kumar Challa

Zebrafish gained popularity as a model system with the work of Dr. George Streisinger [1] in the early 1980s. Over time, a variety of genetic tools and methods were devised to understand embryonic and larval development. While forward genetic tools enabled the discovery of genes (in the classical Mendelian sense) involved in developmental processes through mutant phenotypes, the ability to sequence gene sequences and eventually the genome has opened the avenues of 'reverse' genetics. With the availability and access to gene sequences, there has been a continuous wave of innovations in developing tools, technologies, and methods. CRISPR-Cas based technologies are the latest, and quite powerful, in the genetic toolkit of zebrafish researchers. The key utility of this system is the ability to identify specific short (18–23 bp) sequences in the genome and act on them effectively.

Since the CRISPR-Cas9 system from *Streptococcus pyogenes* (*Spy*) is the most studied and understood, it has been extensively used as a tool in zebrafish research as well. CRISPR targeting of zebrafish gene sequences can be performed with ease in undergraduate laboratory modules. In fact, undergraduate student work can help scientists by generating CRISPR single guide RNA (sgRNA) reagents and validating their activity *in vitro*. These validated reagents can be a useful resource to professional scientists.

While using the system to target specific regions of the genome, the following sequential steps are important.

(a) Purpose of the experiment
(b) Understanding the gene and genomic sequences
(c) CRISPR design, off-target effects and on-target efficiencies

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(d) sgRNA synthesis
(e) in vitro validation (sub-point—PCR amplicon generation, genotyping strategy)
(f) in vivo validation (indels, HMA)
(g) Phenotyping

(a) **Purpose of the experiment**

It is extremely important to have a clear idea about the purpose of the experiment where the CRISPR-Cas9 system is being employed. Broadly, the most common experiment is *gene disruption* to study loss-of-function effects or phenotypes. An increasing number of studies are also interested in modeling human genetic/disease mutations in the zebrafish, especially introducing point mutations or small insertions-deletions (indels) into gene sequences. To aid biochemical and cell biological studies, gene (protein coding) sequences are tagged with peptide epitope (e.g. V5 tag derived from the P and V protein of the simian virus 5 (SV5, a paramyxovirus)), or reporter protein (e.g. green fluorescent protein, GFP) coding sequences at the 5′ or 3′ end of the gene, respectively. Generation of conditional (floxed) alleles using the Cre-lox system (Fig. 7.1) is also of interest; critical exons in genes are flanked by loxp sites, which get excised when the Cre recombinase, provided in trans, recombines the loxp sites.

(b) **Understanding genes and genomic sequence**

Since CRISPR-Cas9 system is used as a reverse genetics tool in which one can learn about the function of a gene from changes in phenotypes, changes in gene expression patterns or changes in functional readouts generated by genomic mutations, a good understanding of the gene or genomic sequence of interest is essential. The current reference genome assembly, GRCz11, is the most recent one and was annotated using an automatic annotation pipeline from ENSEMBL (www.ensembl.org), an online genome browser tool. In this genome assembly, predictions from zebrafish proteins have been given priority over predictions from other non-mamma-

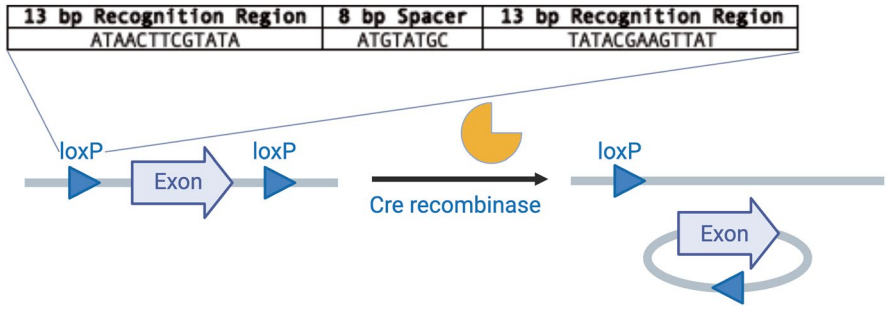

Fig. 7.1 The cre-lox system allows for the generation of conditional mutations via Cre recombinase interacting with *loxP* sequences flanking a target region of DNA and excising the target (adapted from Kim et al. [2])

lian vertebrate species. Several lines of experimental evidence and predictions were taken into consideration. This has a direct bearing on the annotation of gene information like orthologs and paralogs, which can influence decisions on targeting specific gene sequences using the CRIPSR-Cas9 system.

(c) **CRISPR design, off-target effects and on-target efficiencies**

Like all tools, there are challenges and limitations associated with the CRISPR-Cas9 gene editing tool as well. While designing CRISPR experiments, a careful analysis of potential off-target effects is important. This becomes especially important when dealing with members of conserved gene families and proteins with often repeated domains. Prediction of off-target sites in the genome can help design *in vitro* and *in vivo* validation experiments to empirically test if the CRISPR-Cas9 system actually shows off-target activity. Software tools like Benchling are useful and user-friendly in CRISPR design. Unlike off-target effects, predicted on-target efficiencies may not be easy to interpret and have to be empirically tested.

(d) **sgRNA synthesis**

In vitro synthesis (transcription) of sgRNA using double stranded DNA templates and bacteriophage (T3, T7 and SP6) RNA polymerases is a method commonly practiced in laboratories. However, there can be occasional challenges in this process resulting in poor to no synthesis of sgRNA, or lack of nuclease activity due to strong secondary structure formation in the sgRNA. Analyzing the sequence content of the gene-targeting (complementary) RNA in the context of the complete sgRNA can help troubleshoot challenges that we may encounter.

(e) ***In vitro* validation of nuclease activity**

A useful way to find out or predict if the sgRNA-Cas9 RNP complex will have effective nuclease activity *in vivo* is to validate the activity *in vitro*. An *in vitro* approach also has the advantage of being more accessible for instructors with limited infrastructure who do not have the means to utilize *in vivo* systems (zebrafish embryos). A PCR amplified gene fragment containing the CRISPR-targeted sequence can be used as a "substrate" (target) for the CRISPR-Cas9 nuclease activity, which can be visualized by simple gel electrophoresis (Fig. 7.2). In fact, this can be the key experiment that undergraduate students can perform to understand the action of CRISPR-Cas9 system. Once the sgRNA-Cas9 RNP complex binds the target DNA, it initiates a specific double strand break resulting in two fragments of the PCR amplicon. However, often, the RNP nuclease complex continues to bind to the two fragments and hinders the mobility of the complex resulting in the lack of any bands in gels. This is especially pronounced when the nuclease reaction products are analyzed using polyacrylamide gel electrophoresis. To ensure bands of the two fragments resulting from the double strand break caused by the CRISPR-Cas9 RNP complex are visible on a gel, Proteinase K treatment of the sample can be done. This would degrade the Cas9 protein, thereby releasing the cleaved DNA fragments. RNase treatment can also be done prior to Proteinase K treatment to ensure degradation of sgRNA that may hinder clear viewing of the DNA fragments after the CRISPR nuclease reaction.

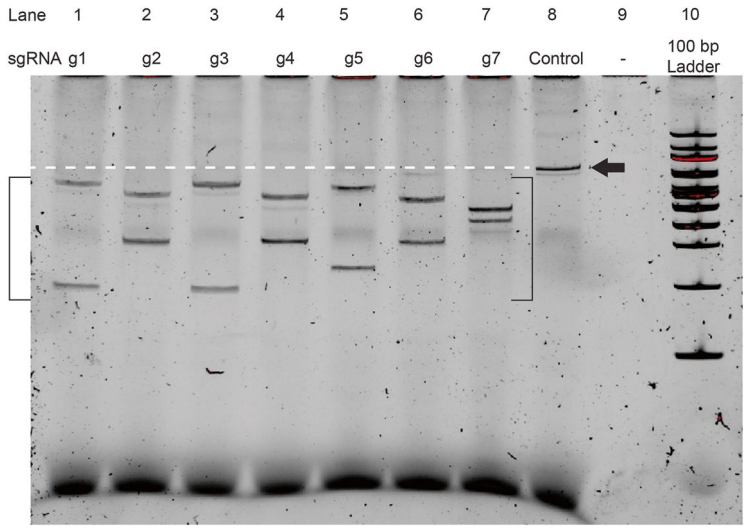

Fig. 7.2 Polyacrylamide gel image showing the results of an *in vitro* CRISPR-Cas9 nuclease assay using PCR products obtained from genomic DNA template and a series of specific sgRNAs (g1-g7). The square brackets indicate DNA fragments resulting from successful cutting of target DNA by the sgRNA-Cas9 RNP complex at specific sites recognized by the respective sgRNA sequences. The black arrow indicates the uncut target DNA fragment ("Control" PCR product) in lane 8; the dotted white line indicates the position of the uncut target DNA in the gel (unpublished data [3])

(f) ***In vivo* validation of CRISPR activity**

Zebrafish is a vertebrate model organism and falls under the United States Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals (Policy). The PHS Policy might be applicable to zebrafish **larvae** from immediately after hatching, which is typically 3 days post fertilization (dpf) under optimal conditions (See Fig. 7.3 for a diagram of the zebrafish life cycle). While this can bring about some constraints for the use of zebrafish in the college classroom, it still allows the use of the zebrafish embryos for up to 3 dpf, by which time several significant and visible developmental events take place.

In vivo validated sgRNAs, along with the Cas9 protein, can be microinjected into 1-cell stage embryos (zygotes) to cause double strand breaks, which are repaired by the cellular machinery that may cause sequence specific modifications and thereby potential loss of function mutations. The genetic mutations thus caused can be analyzed in 1–2 day old embryos (by sacrificing them) via PCR amplification of target gene sequences and visualizing the amplified products by many ways like high resolution melting analysis (HRMA), T7 Endonuclease I assay (T7EI), heteroduplex mobil-

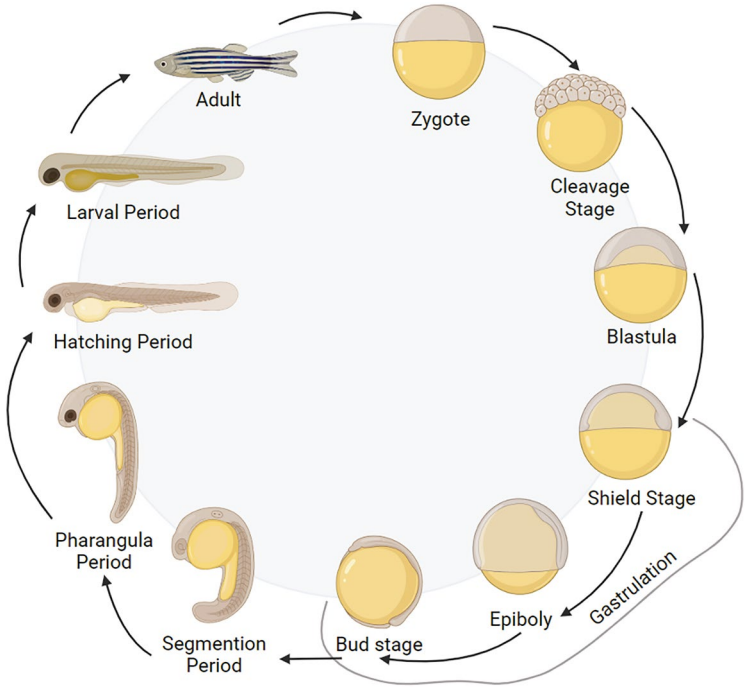

Fig. 7.3 Zebrafish life cycle. *hpf* hours post-fertilization, *dpf* days post-fertilization. Under the rules of the United States Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, the potential experimentation described in this chapter may be done up to 3 days dpf on zebrafish embryos (adapted from Coppola et al. [4])

ity assay (HMA) or fragment analysis using capillary electrophoresis. In all of these, the nature (size, sequence content, etc.) of the PCR amplicons can bring in some challenges. Attention needs to be paid while designing the PCR primers and thereby the PCR amplicons. Of course, in a time when next-generation sequencing technologies are routinely used, whole genome sequencing of injected embryos can be done to reveal a comprehensive view of all the changes that would have possibly happened due to the CRISPR-Cas9 nuclease activity.

(g) **Phenotyping**

Delivery of CRISPR sgRNA-Cas9 RNP complex into zebrafish zygotes is by way of microinjection as alluded to earlier. This process can cause minor damages to the injected embryos leading to deformities of later stage embryos and larvae. We need to discern these injection-derived deformities from non-specific effects, and both from real phenotypes caused by the loss of function of a specific (targeted) gene sequence.

Take Home Message

The zebrafish is a very useful model system that can be used in a college classroom. But it does bring up a few challenges that can be addressed. The zebrafish research community is very collegial and is invested in outreach activities, which can be easily tapped into to obtain help and support by undergraduate instructors and educators. The possibility of collaborating with active researchers to generate and validate CRISPR reagents (by undergraduate students) that can be used in research projects can be a powerful way to engage students with exciting authentic research experiences.

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CRISPR in Drosophila

8

Kumar Vishal, Jeffrey L. Van Zant, and Richard M. Cripps

8.1 Introduction

The fruit fly, *Drosophila melanogaster*, is a genetically amenable model organism that is easy to grow in the laboratory, and has been used for a wealth of genetic experiments in the context of undergraduate teaching. Extensive effort has been put towards developing and elaborating upon CRISPR approaches for manipulation of Drosophila for research applications, and many of these can be transferred to the undergraduate teaching laboratory. Here, we describe the general technical requirements for manipulating Drosophila in the classroom using CRISPR; we provide guidance for obtaining and using CRISPR reagents in the undergraduate laboratory; and we provide examples of teaching scenarios that vary depending upon the duration of the intended class and the technology available to the instructor.

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8.2 Instruction and Facilities

For over a century, the fruit fly, *Drosophila melanogaster*, has been used as a model organism to investigate many areas of biology including, evolution, genetics, and developmental biology. Since Drosophila is readily cultured in the laboratory, has a short generation time, and a substantive embryo production, it remains a common organism for biological research. More recently, *Drosophila* has been used to explore the applications of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based genome engineering. Drosophila has proved to be a reliable model for inducing deletions of varying lengths as well as precise single nucleotide edits [1–3].

Fruit flies are not prohibitively costly, are straightforward to maintain [4], and there are generally few restrictions on their use in laboratories [5]. Culturing fruit flies gives students an opportunity to learn fundamental animal care and development. Keeping flies at 25 °C will accelerate their life cycle, at which temperature the growth of flies from embryo to adult is about 10 days. At 18 °C, development will slow to about 28 days. Fly stocks maintained at 18 °C will require subculturing about every 4 weeks. Growing a population requires the turnover of stocks into fresh bottles or vials about every 14 days. As an adult, a female can produce as many as 100 eggs per day for about 20 days [5]). It is typically more efficient to maintain stocks of individual fly lines large enough for injections rather than ordering fresh flies regularly. However, this may depend on an annual injection schedule and access to equipment such as incubators, CO_2, and appropriate size vials.

The genetics of Drosophila, its relevance to human health and development, and the tools and techniques employed have made Drosophila one of the most important research models in biology. The haploid Drosophila genome has four chromosomes and about 15,500 genes. Sex determination in Drosophila is primarily based on the ratio of X chromosomes to the number of autosomes [6]. Thus, it is complex and not as straightforward as mammalian sex determination, nevertheless the general rule of XX females and XY males still applies. For the maintenance of null mutant flies in a laboratory, balancer chromosomes are used. Balancer chromosomes are engineered and are used to maintain heterozygous flies by preventing meiotic recombination [7]. Students learn a variety of fundamental and advanced genetic tools and techniques through maintaining and crossing Drosophila.

Also, a myriad of biological online resources exists today for Drosophila research. Navigating pertinent websites has become an essential skill today's student must learn. When conducting CRISPR experiments with Drosophila, students will utilize GenBank, FlyBase.org, and primer design sites, among others. FlyBase.org, in particular, offers fundamental tools for any laboratory working with Drosophila [8].

Handling Drosophila in the research laboratory is relatively straightforward, requiring a dissecting (stereo) microscope with a zoom range approximately 8× to 60×; a light source, generally with fiber-optic guides to adjust the angle of illumination; and a source of anesthesia. For the latter, many laboratories use CO_2 delivered to the flies through a porous pad (such as the Flypad; Genesee #59–114). FlyNap (Carolina Biological Supply Co. #173025) can also be used.

8.3 General Practical Considerations

8.3.1 Designing the sgRNA

The single guide RNA (sgRNA) comprises 20 ribonucleotides at the 5′ end that base-pair with the template DNA, followed by 76 ribonucleotides that comprise the remainder of the combined crRNA and tracrRNA. The 20 5′-ribonucleotides are identical in sequence to genomic DNA termed the protospacer, other than for the substitution of T in DNA with U in the sgRNA. In the genomic DNA, the protospacer must be immediately followed by the protospacer adjacent motif (PAM, sequence 5′-NGG) for efficient CRISPR.

Several options are available for deciding upon the sequence of the 20 ribonucleotides that target the sgRNA to its genomic location. For targeting genes that result in visible phenotypes, such as *yellow*, *white* and *ebony*, several existing studies have identified protospacer sequences that function as effective CRISPR targets, and that should work with high efficiency. These are listed in Table 8.1.

If the instructor wishes the students to design their own protospacer sequences, online options are available once a target gene or region has been selected. For effective knockout of a gene, it is recommended that the protospacer lie within the coding sequence and close to the translation start site, and also lie within a constitutive (rather than alternatively-spliced) exon. Alternatively, the instructor may wish to demonstrate how the severity of a mutant phenotype might be impacted by location of the mutation along the length of the gene. In this case, students could target a single gene at multiple positions in the coding sequence, to determine if more 3′ lesions result in a less deleterious phenotype. A suitable gene to target in this approach would be the *white* eye-color gene, since hypomorphic mutants of *white* will show a gradation of eye color from red (wild-type) to white (null mutant).

Gene structures and sequences can be obtained from FlyBase.org [8]. From the main front page, students type a gene name into the "Jump to gene" query at the top right. Once the gene page has loaded, students access the entire transcribed region of the gene by clicking on "Get Decorated FASTA", and also access a JBrowse image using the JBrowse link to the left. JBrowse is a genome browser tool that allows visualization of different genomic features, termed "tracks", and an annotated JBrowse image for the white gene is shown in Fig. 8.1a. In this way, students can use the JBrowse image to identify an exon to target, and then mine the sequence of the exon from the FASTA document.

Table 8.1 Protospacer sequences for commonly-targeted genes

Gene name	Protospacer sequence (5′-3′)	References
yellow	GCGATATAGTTGGAGCCAGC	[9]
	GGTTTTGGACACTGGAACCG	[10]
	GGATGAGTGTGGTCGGCTGT	[10]
white	ATACCATTCCTGCTCTTTGG	[11]
	CAGGAGCTATTAATTCGCGG	[12]
	TAGTTGGCCGCTCCCTGAAC	[12]
ebony	GCCACAATTGTCGATCGTCA	[2]

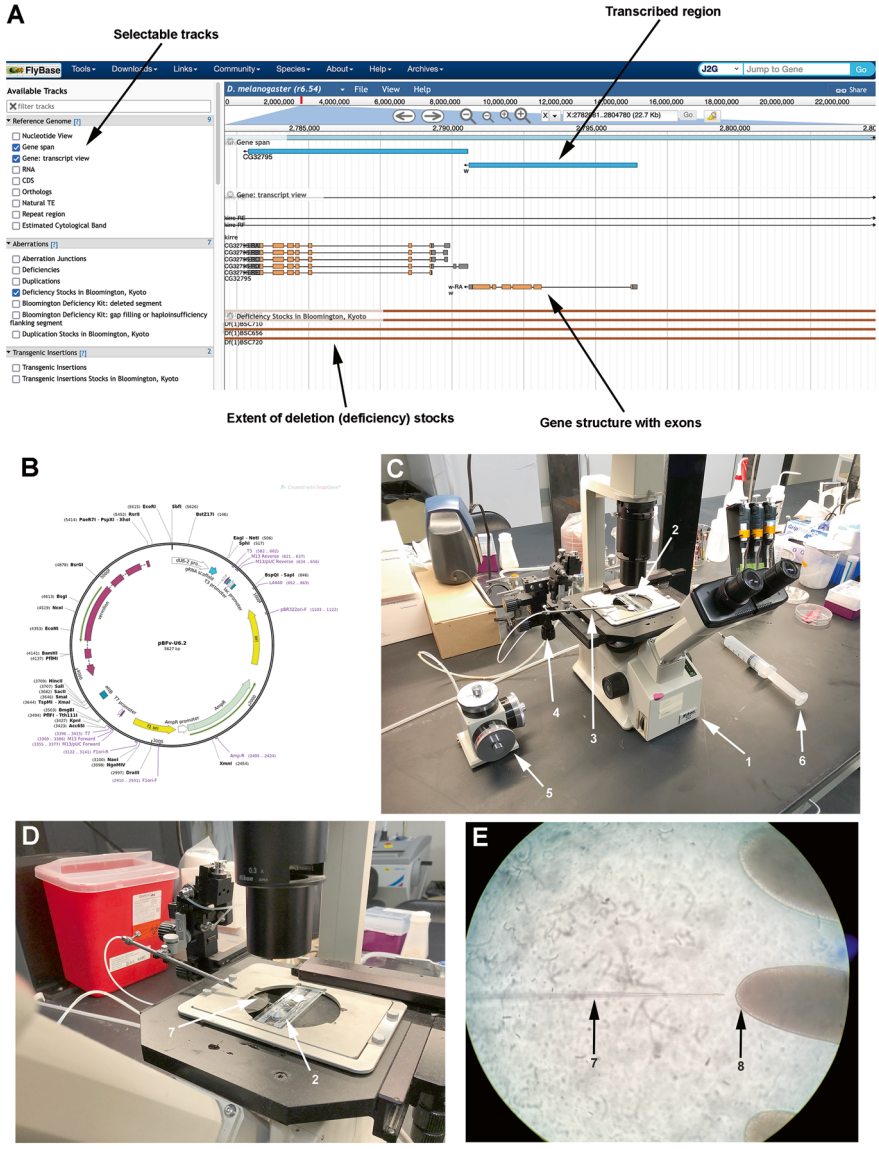

Fig. 8.1 Tools and reagents for performing CRISPR gene editing in Drosophila. (**a**) JBrowse genome viewer image of the *white* gene (*w*) on the Drosophila X chromosome. Individual tracks (such as "Gene span" and "Gene transcript view") can be selected or de-selected using the menu on the left. Blue boxes represent transcribed regions of genes (note that *w* is located within an intron of a larger gene); the transcript view shows the gene structure, with exons as boxes and coding sequence in Salmon. Deficiency lines that can be obtained from the stock center for complementation testing are also shown. Note that the deficiencies span a greater region than that shown. (**b**) Map of pBFv-U6.2, one plasmid that can be used to generate sgRNAs for a target gene. Image obtained from

To identify candidate sgRNAs, we use the CRISPR target finder tool at flycrispr.org [1] (http://targetfinder.flycrispr.neuro.brown.edu/), by pasting into the query box the sequence of the exon of interest and performing the search. The search program provides a list of possible protospacer sequences, and indicates if any similar sequences occur elsewhere in the genome. The existence of other similar sequences should be avoided, since that might cause editing at off-target locations. These sequences can be mapped back to the target exon by the students to ensure that they lie within the coding sequence. If a specific protospacer sequence is selected to be cloned into an sgRNA expression plasmid (see next section), clicking on "Design Experiment" will provide sequences of oligonucleotides to order for cloning the protospacer sequence. The instructor should ensure that the overhangs of the annealed oligonucleotides are compatible with the expression plasmid of choice.

We also usually assess the predicted efficacy of the candidate protospacer at an online source [13] (https://www.flyrnai.org/evaluateCrispr/). We aim for a "score" of 7.0 or better, although weaker scores have been effective. Note that this website also predicts if the protospacer sequence contains a U6 terminator sequence, identified as 5–6 consecutive T nucleotides, that would prevent transcription of full-length sgRNA from a U6:sgRNA plasmid.

Altogether, these steps should identify the protospacer sequence to use. For the in vivo CRISPR described in Strategies 3 and 4 below, the sgRNA is already selected and is expressed ubiquitously in the transgenic animals. It is therefore not discussed in this section.

8.3.2 Synthesizing the sgRNA

Having identified a suitable sequence for the sgRNA, there are several approaches for its synthesis. In one approach, the RNA can be ordered directly from an oligonucleotide synthesis company such as Integrated DNA Technologies, Inc. (IDT). The user simply pastes the 20-nt protospacer sequence that has been selected into the order box, and synthesized sgRNA is returned to the user as a lyophilized powder. The smallest scale of synthesis provides sufficient sgRNA for several rounds of CRISPR. The lyophilized

Fig. 8.1 (continued) AddGene.com and generated by SnapGene software (from GSL Biotech; available at snapgene.com). (**c**, **d**) Standard injection setup. (**e**) Eyepiece view of embryos undergoing injection (photo courtesy Ebru Robinson). Key: 1, Inverted microscope with 10× and 20× objective lenses; 2, Slide carrying embryos stuck to double-stick tape, and covered with oil; 3, Needle holder clamped onto micromanipulators; 4, Coarse control micromanipulators; 5, Fine control micromanipulators; 6, 60-mL syringe attached to needle holder, that provides pressure to squeeze injection mix through the fine needle; 7 (panels **d** and **e**), Injection needle mounted on needle holder; 8 (panel **e**), Posterior end of embryo to be injected

Table 8.2 Available plasmids for generating sgRNAs

Plasmid name	Addgene number	References
pBFv-U6.2	138400	[14]
pCFD3-dU6:3gRNA	49410	[2]
[a]pCFD4-U6:1_U6:3tandemgRNAs	49411	[2]

[a]This plasmid can be used for expression of two gRNAs, see Port et al. [2] for details

sgRNA is resuspended in DEPC-treated water prior to use at a concentration of 1 μg/μl, and diluted to 100 ng/μl in DEPC-treated water for injection into Drosophila embryos.

In a second approach the students generate their own plasmids for sgRNA expression. This requires greater effort from the students, but has the advantage of providing to them CRISPR design and molecular cloning skills. Several plasmids have been created that comprise an ubiquitous U6 promoter, followed by two divergent BbsI sites, followed by the remainder of the sgRNA sequence, and a U6 terminator (plasmids are listed in Table 8.2, and a map of one suitable plasmid is shown in Fig. 8.1b). A complete sgRNA-expressing plasmid can be generated by cutting the parent plasmid with BbsI, which generates non-identical 5′ overhangs. Next, ssDNA oligonucleotides comprising the 20-nt protospacer sequence plus overhangs complementary to the plasmid are phosphorylated, annealed and ligated into the cut plasmid. Finally, the ligation products are transformed into *E. coli* and positive clones identified by sequencing of minipreps. In our experience, the efficiency of creating the correct clones is extremely high (close to 100%). Correct clones are then purified from 50-ml cultures using a midiprep kit such as from Qiagen, and injected into embryos at a concentration of 200 ng/μl.

An important qualification to this approach is that the protospacer must begin with a 5′-G nucleotide in order for the U6 promoter to be active. However, this nucleotide can be substituted into the protospacer sequence in place of a non-G 5′ nucleotide with no significant effect upon CRISPR efficiency. In addition, the protospacer cannot contain five (or more) consecutive T nucleotides, since this sequence comprises a U6 transcription terminator.

We have used each of these two approaches successfully. The former approach (ordering pre-synthesized sgRNAs) requires less laboratory time from the students, but it is more expensive and does not provide experience in molecular cloning. The latter approach uses more steps and can take 2–3 weeks depending upon the class schedule. Moreover, the increased number of steps provides greater opportunities for problems, but provides the students more time to become familiar with molecular techniques that they might use elsewhere.

Other approaches to synthesizing sgRNAs have been used in the research laboratory, but we are not aware of them being used in the classroom for use in Drosophila. One example is to create a dsDNA PCR product that can be directly transcribed by T7 RNA polymerase to generate the sgRNA [10]. Here, the user synthesizes two partially-overlapping oligonucleotides: a “left arm” oligonucleotide containing, from the 5′ end, the T7 RNA polymerase promoter, two G nucleotides as a transcription start site, an 18-nt

protospacer sequence, and part of the remaining sgRNA sequence; and a "right arm" that contains at its 5′ end the reverse complement of the T7 terminator, followed by reverse complemented sgRNA sequence up to the protospacer. These two oligonucleotides are subjected to PCR, where each oligonucleotide is extended on the template of the other to generate a complete dsDNA. Following purification of this product, it can be used in an in vitro transcription reaction using T7 RNA polymerase to synthesize the sgRNA. A qualification to this approach is that the protospacer must begin with two 5′-G nucleotides for efficient transcription by T7 RNA polymerase.

For the in vivo CRISPR described in Strategies 3 and 4 below, the sgRNA is already selected and is expressed ubiquitously in the transgenic animals. It is therefore not discussed in this section.

8.3.3 How to Get the Components into the Embryo

Microinjection directly inserts DNA or RNA into eggs and can be performed on-site. Alternatively, edited plasmids or sgRNA can be shipped to laboratories such as Rainbow Transgenic Flies (Camarillo, California, USA), BestGene Inc. (Chino Hills, California, USA), the Fly Facility (Department of Genetics, Cambridge University, Cambridge, UK), and WellGenetics (New Taipei City, Taiwan).

To conduct microinjections in-house, an inverted microscope with injection capabilities is needed. There are multiple microscope brands to choose from and costs can vary greatly. For injecting reagents into Drosophila eggs, less expensive systems can be used. Microscopes can be purchased and outfitted with injecting capabilities from vendors such as Narishige International USA, Inc. Amityville, NY. A standard setup is shown in Fig. 8.1, panels C and D, and an example of an embryo undergoing injection is shown in Fig. 8.1e.

The protocols for preparing embryos for microinjection are somewhat involved, and are likely best learned through hands-on training. Useful resources for understanding the parameters of microinjection include the original research papers [15], methods papers [16, 17], and video guides (see for example https://www.jove.com/v/20143/microinjection-live-drosophila-embryos-early-delivery-reagents-to).

8.3.4 Fly Lines to Use for Microinjection

For our standard CRISPR class where we knock out genes with unknown phenotypes (Strategy 1 below), we use a transgenic line carrying the *Cas9* gene fused to the germline promoter from the *vasa* gene (*vas-Cas9*) [1], available at the Bloomington Drosophila Stock Center (BDSC) #51323. This construct is carried on the X chromosome, and we target autosomal genes for mutagenesis (chromosomes 2 and 3). The *vas-Cas9* transgene also carries GFP and RFP fused to a promoter for strong expression in the eye, that can be

used to confirm purity of the stock. Since this line already carries the white-eye mutation on the X chromosome, it is not suitable for knocking out *w+* as described in Strategy 2. For this we would use *w+ vasa-Cas9* (BDSC #66554).

8.3.5 How to Characterize the Mutants?

When CRISPR/Cas9 is used to induce a loss-of-function (lof) allele, an expected result may be shorter PCR amplification products when analyzing genomic DNA, however many indels arising from targeting of single target sites will not sufficiently alter the product size from a PCR. Therefore, gel electrophoresis may not be a reliable method of determining CRISPR success. For smaller indels, high-resolution melt analysis (HRMA) might successfully distinguish targeted sites [18], but will not identify the precise changes that have occurred. Detecting mutations can be confirmed by direct sequencing of the CRISPR target site using PCR and DNA sequencing [19]. Cloning the PCR products prior to sequencing removes issues of heterozygosity which can cause difficulty and inconclusive interpretations. Cloning can be conducted using various manufacturer's kits; we use OneTaq polymerase (M0482S, New England Biolabs) for PCR, and the pGEM-T Easy cloning kit (#A1360, Promega Corp.) to generate clones.

8.4 Scenarios for Teaching CRISPR Using Drosophila

This section outlines the approaches that could be taken to teach a practical CRISPR class. The scenarios below are not a complete list of what can be done, but cover both longer-term (Strategy 1) and shorter-term (Strategy 2) classes. Strategies 3 and 4 avoid the need for microinjection, but omit some skills: for example, they do not allow the students to design guide RNAs that they would subsequently use.

8.4.1 Strategy 1: Knock Out Candidate Genes to Generate Stable Mutant Lines

The CRISPR class that we initiated at the University of New Mexico [20] and now offer at San Diego State University covers a full 16-week semester, and is intended to take the students through the entire process of a CRISPR experiment: from identification of protospacers and development of sgRNAs; through microinjection of the CRISPR reagents and developing stable stocks of potential mutant lines; to PCR and sequencing of the mutant lines to identify changes that might have occurred.

The overall design, general principles and genetic crosses of the class are as described [20], and shown in Table 8.3. We have adapted the class over the subsequent years to

Table 8.3 Overall strategy for 16-week CRISPR class (Strategy 1)

Weeks	Activities
1–2	Select genes, design PCR primers
3–4	Extract genomic DNA, PCR, sequence, analyze sequences; order sgRNAs
5	Lectures on CRISPR
6	Inject sgRNAs into embryos
7	Lectures on CRISPR; student presentations describing their selected gene
8	Collect injected G0 adults and cross to balancer stocks
9	Student presentations on CRISPR papers from the literature
10	Screen for ebony offspring in G1; cross to balancer stocks
11	Usually spring break
12	Make stable lines from G1 crosses; freeze flies for genotyping
13	Extract mutant DNA, PCR and sequence
14	Analyze sequences; re-do any DNA extractions
15	Update notebooks and collate all data
16	Student presentations of their results

include three recent innovations. First, before developing the sgRNAs, we sequence the region of the gene that we are targeting. This is to ensure that there are no differences between the reference genome in FlyBase and the genome of the *vas-Cas9* line of flies that we edit (BDSC #51323). This new exercise additionally trains the students in genomic DNA extraction, PCR and sequencing, which they will repeat later in the semester when looking for mutations induced in this sequence.

Second, instead of cloning the protospacer sequence into a plasmid, we order sgRNAs directly from a manufacturer, that can then be injected into embryos. This approach simplifies the experimental design to remove any delays associated with generating the correct clones, although is more expensive and does not provide the students valuable experience in molecular cloning and clone validation.

Third, we use a co-CRISPR strategy, where we target the *ebony* body-color gene alongside targeting the gene of interest [21]. The principal behind this is that if the gene encoding the visible marker has been mutated, the intended mutation has also hopefully occurred. G0 adults are crossed to a multiple-balancer stock that contains two third-chromosome balancers carrying recessive *ebony* alleles. Flies in the G1 generation that show the dark body-color *ebony* phenotype must have inherited a CRISPRed *ebony* allele from the G0 parent, and are then the focus of subsequent crosses to make stable mutant lines. This genetic strategy is shown in Fig. 8.2.

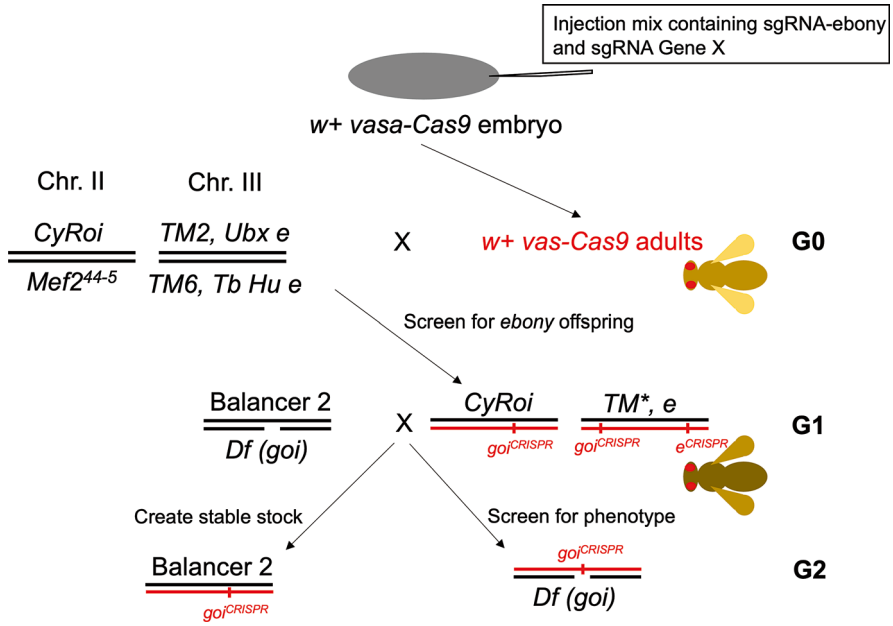

Fig. 8.2 Genetic crosses for Strategy 1. Embryos from the *vasa-Cas9* line (that express *Cas9* in the germline) are injected with sgRNAs, and surviving adults crossed to a multiple-balancer line that is homozygous for the *ebony* mutation on the third chromosome. Offspring showing the *ebony* phenotype in the G1 generation are further crossed to isolate and stabilize the generated mutation. See text for more details

8.4.2 Strategy 2: Knock Out a Single Known Gene to Produce a Visible Phenotype

In this scenario, students would generate mutants for an X-linked visible gene such as *white*, for which mutants show a white eye color instead of the red color of wild-type animals. Students would design sgRNAs for targeting the gene of interest (*white* gene), and generate sgRNA or an sgRNA-expressing plasmid as described above. Next, they will microinject the reagent into *w* + *vasa-Cas9* embryos (source: Bloomington Drosophila Stock Center (BDSC) #66554), that express *Cas9* in the germline. Viable G0 adult females can be crossed to Canton-S (wild type) male flies. In the G1 generation, students will screen for males with white eye color, having inherited the mutant allele from the G0 female parent. These steps are summarized in Table 8.4 and Fig. 8.3.

Having identified mutant males that are hemizygous for the CRISPRed *white* gene, students will perform PCR and sequencing to identify the precise changes arising from mutation of the *white* gene.

The disadvantage for each of Strategies 1 and 2 is that they require specialized equipment and training for microinjection of the sgRNA or the expression plasmid. This can

Table 8.4 Overall strategy for 8-week CRISPR class (Strategy 2)

Weeks	Activities
1	Design PCR primers for *white (w)* gene; order sgRNAs
2	Lectures on CRISPR
3	Inject w-sgRNA into embryos
4	Lectures on CRISPR
5	Collect injected G0 adult females; cross to males
6	Student presentations on CRISPR papers from the literature
7	Screen for white eyed male offspring in G1; freeze for genotyping
8	Extract mutant DNA, PCR and sequence

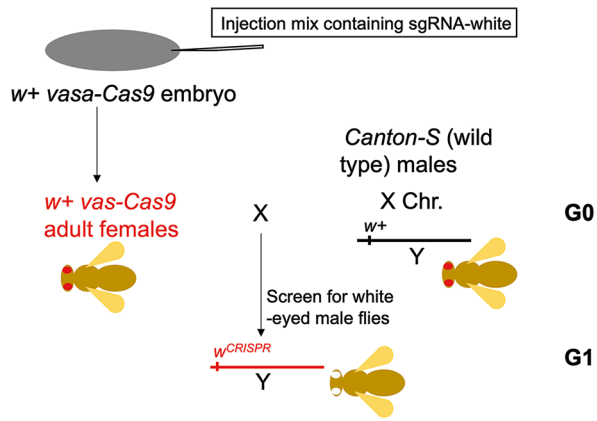

Fig. 8.3 Genetic crosses for Strategy 2. *vasa-Cas9* embryos are injected with an sgRNA targeting the X-linked *white* gene. Surviving females are crossed to screen for white-eyed hemizygous males in the G1. These males can be used for PCR and sequencing to identify the mutations that have occurred

make it challenging for schools without that equipment readily available. To address this, we propose below two additional CRISPR strategies that could be developed.

8.4.3 Strategy 3: Perform CRISPR Genetically, Using Known Reagents

In this scenario, students would cross together fly lines to create a knockout of a given gene during the development of the animal, rather than to generate stable lines. For example, the instructor would generate a fly stock that contains firstly a *Gal4* line expressed in a particular tissue at a particular time, and secondly a *UAS-Cas9* construct; in combination, these two genetic elements will direct the expression of *Cas9* in a stage- or tissue-specific manner. This parental line can then be crossed by the students to *U6:sgRNA* lines that target a given gene such that, in the progeny, there would be in vivo gene editing of the target gene only in the tissue of interest.

Table 8.5 Overall strategy for 6-week CRISPR class (Strategies 3 and 4)

Weeks	Activities
1	Set up genetic crosses
2	Lectures on CRISPR
3	Screen for reduced flight ability in F1 generation
4	Western blot analysis
5–6	Extract mutant DNA, PCR and sequence

There are a wealth of Gal4 lines and UAS-Cas9 lines available at the BDSC (see https://bdsc.indiana.edu/stocks/gal4/index.html, and BDSC stock number 54595, respectively). For sgRNAs, there are currently around 2800 transgenic U6:sgRNA lines available at the BDSC (see https://bdsc.indiana.edu/stocks/genome_editing/sgrna.html) from which the instructor or students could select.

As a guide, we propose combining *fln-Gal4* (an adult flight muscle specific Gal4 available from the authors) and *UAS-Cas9* (BDSC 54595) into a single genetic line. This would enable students to knock down expression of a given gene only in the flight muscles. The *fln-Gal4; UAS-Cas9* line would then be crossed by the students to fly lines carrying *U6:sgRNA* for muscle genes such as for *alpha-Actinin* (*Actn*) or *Zasp52*, for which antibodies are available from the Developmental Studies Hybridoma Bank (BB8/384.1 and 1D3-3E4, respectively). The outcome of the cross should be mutation of that gene only in flight muscle nuclei. Students could then test for effects upon flight ability, perform western blots for the affected protein, and potentially sequence the mutated gene. These phenotypic assays might benefit from scraping out the flight muscles, or isolating top-half thoraces to generate tissue for protein analysis or DNA extraction. This strategy will allow students to generate and analyze mutants in any tissue of interest (for example, nervous system, cardiac system, or stem cells) at any stage of development. Furthermore, this strategy does not require microinjection and is therefore a very simple way of teaching the CRISPR class. More importantly this strategy will require a shorter time window and thus can be designed to teach a short term CRISPR course. The overall design of this class is summarized in Table 8.5 and Fig. 8.3.

An addendum to this class could be to use RNAi to knock down the expression of the same genes, to determine if the same phenotypes are observed. Students would use the same *fln-Gal4* driver, and the instructor could obtain UAS-RNAi lines for candidate genes from either the BDSC or the Vienna Drosophila RNAi Center. RNAi lines targeting *Zasp52* and *Actn* are available and have been shown to work [22, 23].

8.4.4 Strategy 4: Perform CRISPR Genetically, Using Unknown Reagents

This would be essentially the same as Strategy 3 above where a *Gal4; UAS-Cas9* line is generated and crossed by the students to sgRNA lines. However, instead of using a sgRNA to target a gene whose mutation gives a known phenotype, students could perform a genetic screen to identify novel regulators of organ development.

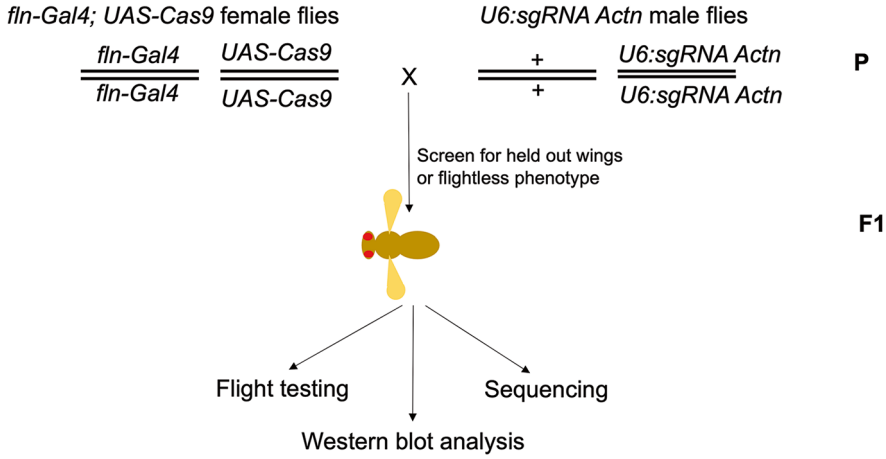

Fig. 8.4 Genetic crosses for Strategies 3 and 4. Flies expressing *Cas9* in the flight muscle are crossed to flies expressing ubiquitously a sgRNA targeting a specific gene (the Alpha-actinin-encoding gene, *Actn*, is shown here). The offspring can be analyzed for flight ability, Alpha-actinin accumulation, and changes to the *Actn* gene

Table 8.6 Summary of CRISPR strategies for Drosophila

Strategy	Details
1	A 16-week program that enables students to go from design through to generating stable mutant lines and analyzing the mutants they generate.
2	An 8-week program where students mutate genes with known mutant phenotypes and observe the phenotypes in the G1 generation.
3 and 4	A 6-week strategy that obviates sgRNA design and the requirements for microinjection, but enables students to observe and characterize mutant phenotypes.

For example, students can use this strategy to knock out components of all known signaling pathways in muscles (using sgRNA reagents from the BDSC) and screen for factors that affect muscle formation and maintenance. The primary screen will be based on either flight-testing the progeny or looking for the "held out wing phenotype" as a visual indicator of muscle defects [24]. The candidates that show muscle defects and/or reduced flight ability will be further monitored using immunohistochemistry. Finally, students will examine protein expression of the candidate genes using western blot and will sequence the mutated gene. This strategy will enable students to unravel novel mechanisms regulating muscle development and muscle homeostasis. More importantly, this strategy can be used in the classroom setting to identify novel factors regulating various tissues during development and aging. This strategy is summarized in Table 8.5 and Fig. 8.4, and a brief comparison of each strategy is shown in Table 8.6.

Take Home Messages

The amenability of Drosophila to genetic manipulation has been extended to CRISPR/Cas9 genome editing, and this technology can be applied to the undergraduate teaching laboratory in a variety of ways as described here.

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CRISPR in Yeast

9

Randi Ulbricht

9.1 Introduction to CRISPR in Yeast

Yeast are especially amenable to classroom laboratories due to their ease of growth, storage, and genetic manipulation. The most common laboratory yeast species is *Saccharomyces cerevisiae*. Yeast strains can be obtained through repository services like American Type Culture Collection (ATCC), EUROSCARF, or Yeast Genetic Resource Center. Specific strains available include haploid wild-type strains like S288C or derivatives like BY4741, that is also haploid but deficient in nutritional marker genes like *his3, leu2* and *ura3*. These auxotrophic mutants are particularly useful with plasmids that express genome editing components, since yeast plasmids commonly contain genes that complement the deficient gene to allow selection on media lacking the essential nutrient. For example, the plasmid pML104 [1] contains Cas9 expressed from a constitutive promoter, as well as a *URA3* gene that allows selection of yeast containing pML104 on media lacking uracil. Yeast can be stored in glycerol stocks (15%–30% glycerol in media) at ultracold temperatures for years, or even decades. They can be grown on agar plates or in liquid culture in simple incubators (without CO_2). The optimal growing temperature is usually around 30 °C, but most strains can grow heartily from 24 °C to 37 °C. Their genome is also simpler than higher eukaryotes, which makes design of CRISPR experiments more tractable for student researchers. The genome of strain S288C is fully annotated and available on Saccharomyces Genome Database (SGD). As a eukaryote, the yeast gene structure is similar to other eukaryotic organisms, however yeast genes have very few introns. The double stranded break DNA repair pathways are also analogous to those in higher eukaryotic organisms, which enables CRISPR/Cas9 gene editing to occur in yeast, similar to those higher systems. There are

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several CRISPR/Cas9 resources available for use in yeast, as well as laboratory activities. The accessibility of the yeast model system and available resources provides an opportunity for students to participate in the entire gene editing process, from design to verification of mutant DNA.

9.2 Experimental Design

9.2.1 Selecting a Target Gene

Instructors can choose from a variety of genes to manipulate in yeast. Edited yeast can be identified based on the presence of a phenotype or selectable growth (Fig. 9.1). For example, the adenine biosynthesis pathway can be targeted to produce a color change. A mutation that disrupts the *ADE1* or *ADE2* gene will lead to the accumulation of a red piment that will turn the yeast pink, which can be observed with the naked eye on rich media [2]. An alternative is to reverse the phenotype by starting with *ade1* or *ade2* mutant yeast (one made by a previous cohort, or one that is purchased from a vendor) and knockout the *ADE4* gene or another gene upstream of *ADE1* or *ADE2* in the adenine biosynthesis pathway [2], therefore, the double mutant will not produce adenine, nor the red pigment. In this case, positive mutants would be observed by growth of white yeast (compared to the pink color of the parent strain). Additional protocols have described the editing of genes involved in amino acid production pathways. Knockout of these genes would result in yeast that cannot survive on media lacking the amino acid produced by the deficient pathway [3]. This type of mutation, where the successful mutant will not grow on the selective media, requires a multistep screening process where the yeast are plated on selective and non-selective media to allow isolation of yeast that do not grow upon selection. Alternatively, undergraduate laboratory activities have been described to produce a *can1* mutant that allows yeast to survive on media containing canavanine [4]. The *CAN1* gene encodes an arginine transporter [5]. Canavanine is a toxic arginine analog. Therefore, knockout mutation of *CAN1* leads to yeast that are not susceptible to canavanine toxicity. In this case, the mutants will grow in the selective media, while the parent strain will not. It is also possible for mutations to include insertion of markers that result in selectable phenotype or visible traits. For instance, including a GFP tag as an in-frame insertion can provide students with the opportunity to use flow cytometry, fluorescence microscopy or spectrophotometry to identify edited cells by the presence of fluorescence. A summary of the potential mutations described here is provided in Fig. 9.1. In a course design that includes a more independent research experience, students may choose their own target of interest and design an accompanying screening method that includes an observable phenotype. A separate chapter of this book addresses CRISPR for course based undergraduate research experiences.

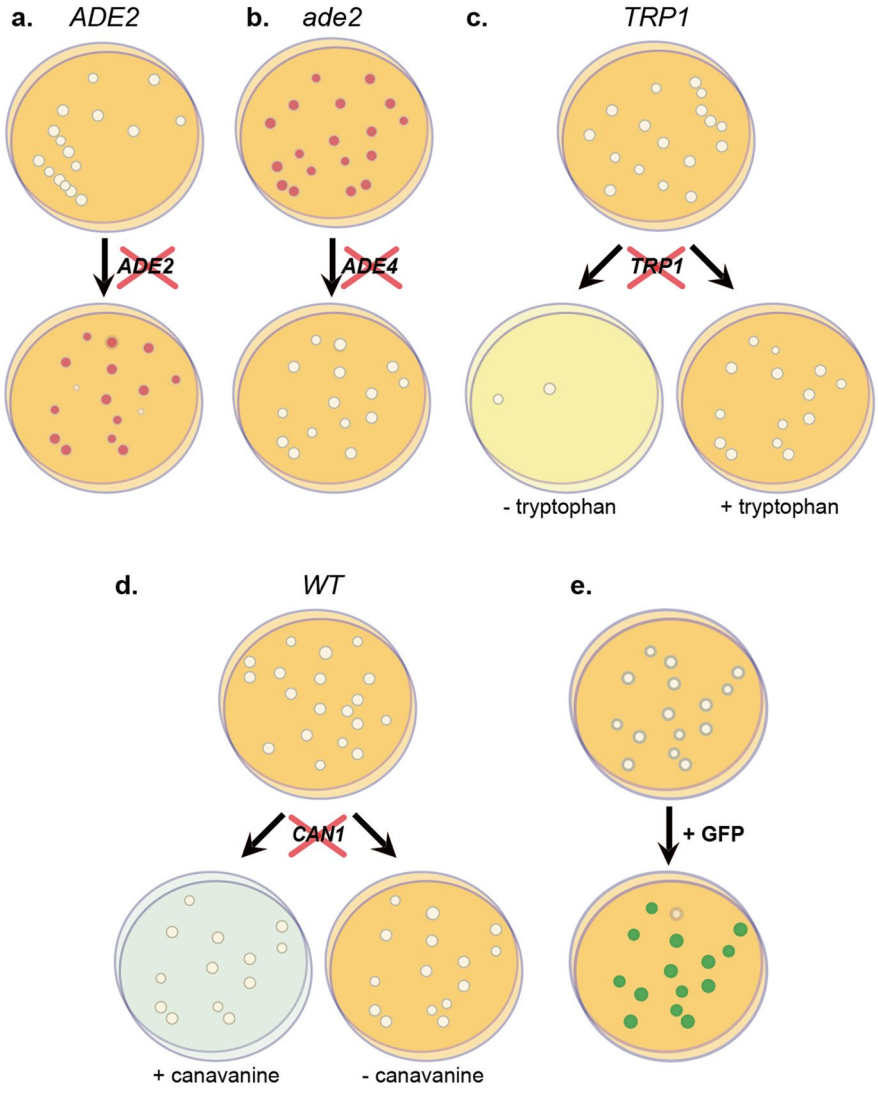

Fig. 9.1 Potential Yeast Mutants. Parent strains (genotype and phenotype shown at top) are altered by gene editing to knockout (red X) or insert genes (+). The resulting mutants can be selected based on growth or phenotype (bottom). (**a**) A null mutation in the *ADE2* gene will cause yeast grown in rich media to develop a red or pink color. (**b**) The *ADE4* gene can be knocked out in a red *ade2* parent strain, allowing yeast to grow white. (**c**) Mutation of genes required for essential amino acid synthesis will prevent yeast from growing in the absence of the essential amino acid. For example, mutation to the *TRP1* gene will prevent mutants from growing on minimal media lacking tryptophan, but the mutants will grow on rich media or media containing tryptophan. (**d**) Mutation of the *CAN1* gene will allow selection of mutants on media containing canavanine. (**e**) Insertion of GFP will allow selection of cells that fluoresce

9.2.2 gRNA Design

The relative simplicity of the yeast genome makes designing a targeting gRNA more straightforward than other eukaryotic organisms. Coding regions are shorter, generally uninterrupted, and lack multiple slicing isoforms, providing a relatively simple landscape from which to choose a PAM and associated gRNA target. gRNA targeting sites should be near the location of the desired mutation. In general, it is suggested that gRNA target the first ~1/3 of the open reading frame to generate knock out mutations. A frameshift or stop codon insertion in the early region of the gene will ensure complete knockout of the gene product. If specific substitutions or in frame insertions are desired, consider where in the gene you would like to edit and choose gRNAs as close as possible to this location.

There are a number of bioinformatic tools available to help instructors and/or students design gRNAs for CRISPR/Cas9 studies in yeast. Some of those that are available at no cost include ChopChop v2 [6], CRISPRdirect [7], CRISPy-web [8], and Yeastriction [9]. Alternatively, provided a set of guidelines for gRNA design, students can retrieve the gene sequence from SGD, copy it into programs like Snapgene or even into a word processing document, to annotate and design gRNA by hand. It is also necessary to verify that the chosen gRNAs are unique and will not produce off-target cleavage events. Some of the available gRNA design tools provide this information, however, students can be guided to search the yeast genome with Basic Local Alignment Search Tool (BLAST) to identify any potential off-target binding locations of the gRNA. gRNA with >17 bp homology to any other genomic location that also has a PAM should be avoided [10].

Some instructors suggest leading students through designing a gRNA target site on one gene, and subsequently allowing them to design another gRNA target independently [11]. In my classroom, together with students, we gather the genome information from SGD and design the first gRNA to that gene together, by hand, to ensure the principles of CRISPR/Cas9 targeting are understood. Independently, students then design at least two additional gRNA to that same gene by hand or with a web tool of their choosing. From the student-designed gRNAs, gRNAs that overlap between groups are chosen for classroom gene editing. Typically, in a cohort of 30–50 students with 2–3 students per group, a total of 3 gRNAs are found to be common between all groups. A dsDNA cassette containing the gRNA gene can be constructed by annealing two complementary oligonucleotides and cloned into the gRNA expression vector. After oligonucleotides are ordered, students or the instructor can clone the gRNA cassettes.

9.2.3 Selecting a Mutation

Expression of the gRNA and Cas9 will result is a dsDNA break that is repaired by the cell's endogenous DNA repair systems. Unlike other yeast species and mammalian cells that rely predominantly on NHEJ, HR is the prominent dsDNA break repair system in *S. cerevisiae* [12]. HR will use a donor template to extend resected DNA ends near the Cas9-mediated cleavage site. A mutation is engineered through a donor template with the

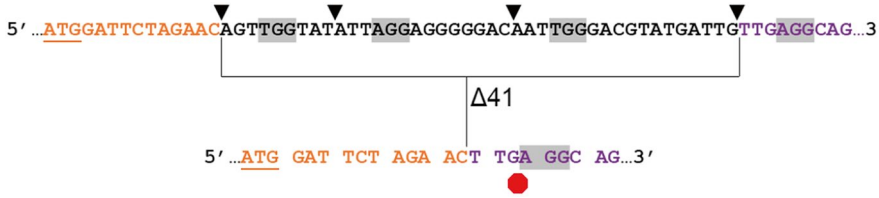

Fig. 9.2 Yeast deletion mutant. The 5′ end of the coding region for *ADE2* gene (top). The start codon is underlined. Potential PAM sequences are shaded gray and corresponding Cas9 cleavage sites are indicated with triangles. The repair template would generate a mutant *ade2* gene (bottom sequence) with a 41 bp deletion between the first and last Cas9 cut site to eliminate three of the four indicated PAM sequences and 17 bp of the target for the downstream PAM. The deletion generates a frameshift and a premature stop codon (red octagon)

desired sequence flanked by homologous regions. HR in yeast is near 100% efficient in providing a frameshift mutation upon cotransfection of a gRNA/Cas9 plasmid with a donor template [13–15]. Mutations can vary from a knockout mutation (premature stop and/or frameshift), insertion of a tag, substitution (for reverse genetics), or a large deletion. Mutations should also alter the target sequence and/or PAM sequence to prevent repeated Cas9 cleavage after successful repair. Because NHEJ is generally lacking in *S. cerevisiae*, if an HR template is not provided or not successful in repairing damage, the DNA damage will be unrepaired and lead to cell cycle arrest. This allows a system where unedited organisms are selected against. Moreover, if the repaired DNA is re-cleaved by continuous expression of Cas9 and the gRNA, the yeast will not survive. Any mutation that eliminates at least three base pairs of homology and/or disrupts the PAM are likely to prevent repeated cleavage, and allow survival [16]. For example, Sehgal et al., [11] inserted three in frame stop codons within the gRNA target sequence and PAM sequence to eliminate repeated cleavage events of the repaired substrate, and produce a knockout of the gene. Repair templates may include large deletions within coding regions to generate frameshifts and knockout mutations that also remove the PAM and/or gRNA targeting sites. In this strategy, gRNA can be targeted to regions within the deletion. This allows use of the same template to repair several individual gRNA-guided cleavages within the same gene (Fig. 9.2). Insertion of a tag or marker could also occur at the Cas9 cleavage site, disrupting the target. Overall, when designing a desired mutation, it is important to consider the effect of the edit on the gene, the location of the mutation, and prioritize disruption of Cas9 cleavage in edited genome.

9.2.4 Designing a Template for Repair

The donor template can be in the form of a single stranded oligonucleotide DNA (ssODNA), linear double stranded DNA (dsDNA) or plasmid. Single stranded templates appear to be more effective than dsDNA in yeast with high expression of Cas9 [1] as well as in some higher eukaryotic systems [17, 18]. A single stranded template that is sense to

the target strand (or complementary to the gRNA) appears to be more efficient than the antisense strand [18], though other studies found that the strand chosen has no effect [17]. The cost of several synthetic ssODNA may be prohibitive for larger classes, however a single template for multiple gRNA from multiple student groups should help reduce costs. Moreover, direct transfection of 90 nt ssODNA (synthesized in a 250 nmole scale) with standard desalting, rather than HPLC or PAGE purification, helps eliminate some time and cost, while not compromising the results, in our experience. dsDNA is often chosen due to cost considerations. In this case, a precise template can be cloned into a plasmid for downstream amplification and long-term storage. The plasmid itself can be used as an HR template [14, 19] or the template can be PCR amplified to create dsDNA donor for use in CRISPR/Cas9 experiments [11]. Complementary ssODNA can be used to make dsDNA or ssODNA can be PCR amplified for cloning.

The minimum length of homology for a repair template in yeast has been found to be around 100 bp [19, 20] and CRISPR/Cas9 dsDNA breaks in yeast have been successfully repaired with a 90 nt ssODNA [1] and dsDNA [13]. Studies in human cells show that asymmetrical design of the template can improve HR efficiency [18]. Homologous sequence extending from the dsDNA break toward the PAM sequence should be at least 50–60 nucleotides long. The arm of homology that is distal to the PAM sequence, should be around 36 bp [18].

The template for repair can be student- or instructor-designed. Allowing students to design the template encourages them to consider types of mutations that will knockout the gene, as well as conceptualize mechanism of dsDNA repair. However, students need significant training in CRISPR and dsDNA repair, as well as specific instruction in order to handle this challenge. Instructor designed templates have the benefit of considering multiple experimental designs, simultaneously. Designing templates that can be used by several groups allows ordering as few ssODNA templates as possible, which helps simplifying preparation of the laboratory and limit cost. In the case of instructor designed templates, students may map the intended mutation on the gene by aligning the template with the gene's wild-type sequence and identifying the discrepancies.

9.2.5 Controlling Cas9 Expression

While Cas9 expression from high copy number plasmids improves the rate of gene editing from CRISPR/Cas9 in yeast [19], and has successfully been used in undergraduate teaching laboratories [3], repeated cleavage events by Cas9 and gRNA will damage the edited organism. Limiting the time frame of Cas9 and/or gRNA expression can help prevent damage. One way to limit Cas9 expression in yeast is to provide a galactose inducible Cas9 [4, 13]. Yeast that contain this plasmid are grown in glucose, where Cas9 is not expressed. They are washed and shifted to galactose for 24 hours. to allow Cas9 expres-

sion. Galactose is provided for a limited amount of time in order to limit the expression of Cas9. The gRNA, provided in an alternative plasmid, is expressed from a constitutive promoter. The presence of both Cas9 and gRNA will allow gene editing to occur. A detailed protocol for use in undergraduate laboratories using the inducible Cas9 is provided [4].

9.2.6 Genotyping

Genotyping yeast is also relatively simple. Clones can be isolated and sub-cultured, genomic DNA isolated, and PCR amplified for Sanger sequencing. Colony PCR can be used to avoid genomic DNA isolation steps, however, these procedures are less reliable than amplifying from purified genomic DNA. The primers for PCR amplification should flank the intended mutation site. Students or instructors can design or locate appropriate PCR primers using programs such as ChopChop V2 [6] and PrimerBLAST from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). If the experimental design includes a large deletion or insertion, PCR amplification will produce DNA fragments with size differences that can be detected by agarose gel electrophoresis. For instance, the 41 bp deletion within the coding region illustrated in Fig. 9.2 will produce a frameshift within the encoded gene. If PCR primers flanking this region amplify a 200 bp product from wild-type yeast, the product from mutated yeast will have band that is 159 bp and can be differentiated on a 2% agarose gel. If smaller insertions, deletions or substitutions are preferred, or the exact genome alteration is desired, Sanger sequencing of the purified PCR product can be accomplished through commercial vendors. The output file for these services can be either FASTA files or electropherogram traces (ab1 files). The ab1 files can be useful and viewed via free programs like FinchTV (Geospiza). Ab1 files are particularly useful when polyclonal modifications exist. Sequence variants can be detected from the overlapping electropherogram peaks. The sequence from the FASTA files can be aligned to the annotated genome sequence with CLUSTAL Omega or other software. If the suspected mutation creates or removes a restriction enzyme site, restriction fragment length polymorphism (RFLP) analysis can be used as an alternative form of genotyping.

9.3 Resources

It is possible for instructors with even limited experience with CRISPR/Cas9 or yeast to implement CRISR/Cas9 gene editing in the classroom. There are multiple detailed, published hands-on classroom laboratory activities available [3, 4, 11, 21]. These publications include learning objectives, protocols, assessments, and experimental tips. The plasmids

Table 9.1 CRISPR plasmids for use in *S. cerevisiae*

Plasmid name	Uses	Markers	Ref.	Addgene #
pCAS	Cas9 expression, gRNA cloning	Kanamycin, G418 resistance	[22]	60847
pML104	Cas9 expression, gRNA cloning	Ampicilin, *URA3*	[1]	67638
pML107	Cas9 expression, gRNA cloning	Ampicilin, *LEU2*	[1]	67638
p415-gal-Cas9	Galactose inducible Cas9	Ampicilin, *LEU2*	[13]	43804
p426-SNR52-gRNA	gRNA cloning	Ampicilin, *URA3*	[13]	43803
pV1382	Cas9 expression, gRNA cloning	Ampicilin, *URA3*	[14]	111436
pVG1	Cas9 expression, ADE2 gRNA, ADE2 PTC repair	Ampicilin, *URA3*	[14]	111444
pCRCT	Cas9 expression, gRNA cloning, repair template cloning	Ampicilin, *URA3*	[19]	60621

Plasmids for use in CRISPR/Cas9 studies in yeast are listed by name. The use of the plasmid includes applicable cloning sites for gRNA (or cloned ADE2 gRNA in the case of pVG1), Cas9 expression from constitutive or inducible promotors, a site for repair template cloning or a previously cloned template for repair of the ADE2 gene that introduces a premature termination codon (PTC). Markers on each plasmid for use in yeast or bacteria are provided, as well as the reference describing each plasmid. The reference number for ordering each plasmid through Addgene is provided

necessary to complete these activities are publicly available (Table 9.1). While the majority of these activities provide the opportunity for hands-on learning, the increased demand for on-line learning has made virtual formats specifically related to gene editing *S. cerevisiae* also available [23].

While experimental design provides an opportunity for high impact, inquiry-based learning, it is often not feasible for students or instructors to design unique CRISPR/Cas9 gene editing in yeast. Instructors may choose from previously validated gRNA and template combinations (Table 9.2) to use as controls and or to ask students to duplicate previous studies. Instructors may even access plasmids containing previously validated donor template DNA and gRNA cassettes (Table 9.1).

Table 9.2 Verified targets for yeast mutants

Gene targeted	gRNA sequence	Template sequence	Ref.
TRP1	CACAGGTAGTTCTGGTCCAT	ATGTCTGTTATTAATTTCACAGGTAGTTCTGGTCCAT**ATd1**TGAAAGTTTGC GGCTTGCAGAGCACAGAGGCCGCAGAATGTGCTCTAGAT	[1]
ADE2	AATTGGGACGTATGATTGTTG	CGGACAAAACAATCAAGTATGGATTCTAGAACAGTTGGTATATTAGGAGGG GGA**T**AATTG**T**GACGTATGATTGTTGAG**TA**AGC**T**AACAGGCTCAACATTAAG ACGGTAATACTAGATGCTGAAAATTCTCC	[11]
CAN1	GATACGTTCTCTATGGAGGA	CCCGACGAGAGTAAATGGCGAGGATACGTTCTCTATGGAGGAT**TAG**ATAGG TGATGAAGATGAAGGAGAAGTACAGAACGCTGAAGTGAA	[13]
ADE2	ATTGGGACGTATGATTGTTG	ATGGATTCTAGAACAGTTGGTATATTAGGAGGGGGACAATTGGGACGTATG A**T**TT**AA**TGAG**AATTC**GCAGCAAACAGGCTCAACATTAAGACGGTAATAC	[14]
ADE2	AGTATGGCGGAATCTGAACAA	GCAGGAGAATTTTCAGCATCTAGTATTACCGTCTAATGTTGAGCCTGTT**d52**G TTCTAGAATCCATACTTGATTTGTTTTGTCCGATTTTCT	RU
ADE4	CCAAACCACTCCAGTAGCTC	GTAGCTATACCAGCTGCATCTTGTCCACGATGTTGTAGAAAAATGCATCCAT CACATAA**d8**CTACTGGAGTGGTTTGGTTTGCTAATACAAT	RU

The sequence of the template used to repair Cas9-mediated cleavage using the indicated gRNA is shown, as well as the name of the targeted gene. Bold regions indicate substituted or inserted nucleotides in the template. Deleted nucleotides are shown by "d" followed by the number of nucleotides deleted from the gene sequence. Templates and guides without a numerical reference were not previous published

9.4 Summary

Working with yeast in the undergraduate classroom allows students hands-on experience and high-impact learning activities with eukaryotic model systems, while also demanding limited resources and little experience by the instructor. The experimental design for CRISPR/Cas9 in yeast is similar to other eukaryotic model systems. The main differences lie in the repair processes, since NHEJ is not readily available to repair Cas9-mediated dsDNA breaks in yeast. However, CRISPR/Cas9 is extremely efficient in yeast due to its prominent use of HR, and the wide variety of Cas9/gRNA expression systems available. Moreover, the rapid growth and easy storage of yeast allow students to participate in the entire process of gene editing, from beginning to end, in only one semester. There are multiple published descriptions of classroom activities that allow undergraduate students to design and implement CRISPR/Cas9 gene editing in yeast. Students can be as involved in the process as the instructor desires. They can be guided to complete every step in the process including gRNA design, donor template design, cloning of gRNA and/or template, yeast transformation, selection of mutants, and genotyping. Conversely, instructors may provide the materials, allowing students to concentrate on selected activities.

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CRISPR in Butterflies

10

Hooi Lynn Kee

10.1 CRISPR in Butterflies: An Undergraduate-Accessible Organism for Gene Editing

Non-traditional model organisms, such as tardigrades and hydra, have become increasingly utilized by researchers to study cell biology and development [1]. The use of CRISPR-Cas technology has allowed researchers to study gene functions in some of these organisms. After surveying non-traditional model organisms that CRISPR-Cas9 technology has developed in, we tested *Vanessa cardui* butterflies as an appropriate organism to develop for undergraduate biology lab experience in my Genetics course in Fall 2019 and Molecular Biology & Biotechnology course in Spring 2021 [2]. We determined that *V. cardui* butterflies and caterpillars have specific advantages for undergraduate teaching, which include: (1) ~1 mm big egg size for CRISPR delivery into embryos by microinjection (2) relatively easy and economical animal husbandry, and (3) the ability to conduct both molecular and phenotypic analysis in an undergraduate lab. Established protocols by the labs of Arnaud Martin (George Washington University), Robert Reed (Cornell University) and Antónia Monteiro (National University of Singapore) have demonstrated the ability to use CRISPR-Cas9 gene editing to study genes that regulate pigmentation, eyespot development, and patterning of butterfly wings of different butterfly species [3–5]. CRISPR-Cas9 in butterflies is a relatively new accomplishment, and only about 22 genes have been targeted in the Lepidoptera butterflies (reviewed in [5]). Thus, there is potential for many new genes to be investigated and new CRISPR mutants to be created and characterized through molecular and phenotypical analysis. Here we describe how integrating CRISPR into the classroom with butterflies provides an opportunity to allow for students to develop

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integration of multiple levels of biological principles of a charismatic and engaging organism.

10.2 Overall Strategy of CRISPR in Butterflies

The overall framework of integrating CRISPR with butterflies in an undergraduate lab experience centers around using CRISPR technology to create a loss-of-function mutant in *V. cardui* butterflies over the course of 4–6 weeks (Fig. 10.1). Students first determine

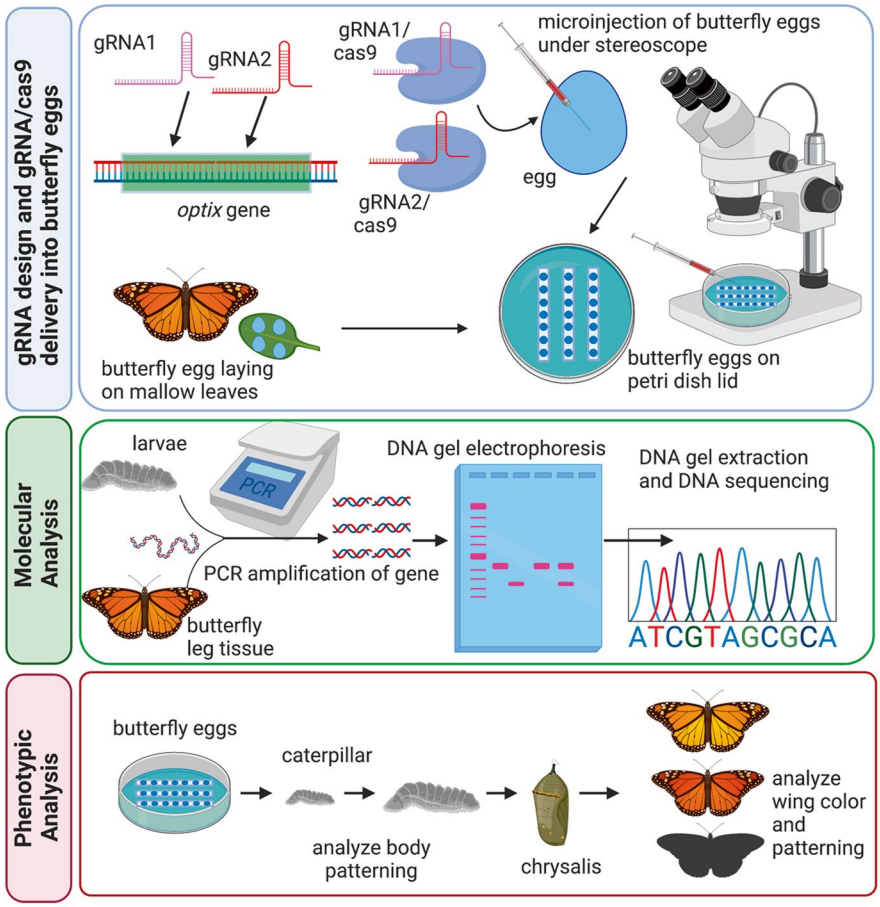

Fig. 10.1 Overall CRISPR in butterfly strategy. Top panel depicts gRNA/Cas9 delivery into butterfly eggs that are collected from mallow leaves. CRISPR delivery into butterfly eggs involves stereomicroscope and microinjection system. Middle panel depicts molecular analysis conducted to determine nucleotide changes from CRISPR activity and DNA repair. Bottom panel depicts rearing CRISPR-injected butterfly eggs through caterpillar and chrysalis stage and analyzing CRISPR effects on butterfly wing color and patterning

how CRISPR is used to target a specific wing patterning/color gene at a specific location using CRISPR tools, specifically guide RNA (gRNA) and Cas9. They then deliver this gRNA/Cas9 complex into butterfly eggs. The butterfly eggs hatch into caterpillars, and caterpillars are reared until butterfly form. Phenotypic analysis is conducted on butterflies to determine the effect CRISPR activity has on butterfly wing pattern and color. While students wait for the butterflies to form for phenotypic analysis, molecular analysis can be conducted on newly hatched caterpillars to characterize nucleotide changes caused by gRNA/Cas9 activity and DNA repair mechanisms. The molecular analysis techniques involve Polymerase Chain Reaction (PCR) to amplify our gene of interest, DNA gel electrophoresis on the PCR products, and subsequent DNA sequencing of PCR products. This process is depicted in Fig. 10.1 and adapted from Martin and Reed labs [4, 6, 7].

10.3 CRISPR Targeting

Using the *optix* gene as an example, either one gRNA/Cas9 or two gRNA/Cas9 complexes can be designed to the gene (Fig. 10.2). gRNA targets the Cas9 to the desired sequence, which is followed by a Cas9 endonuclease cut of the double-stranded DNA. This is followed by a DNA repair process called non-homologous end joining to fix the DNA cut. By using two gRNA/Cas9 complexes, we can try to create a deletion between the two gRNA target sites, resulting in a loss-of-function mutation of the gene (Fig. 10.2a, c). This allows us to visualize whether dual-gRNA/Cas9 activity was successful in our molecular analysis, because we can see a change in PCR product size compared to wild-type. Alternatively, a single gRNA/Cas9 complex can be targeted to the gene, and as a result of Cas9 nuclease activity and DNA repair, mutations like insertion/deletion or substitution are created that lead to a disruption in the coding sequence (Fig. 10.2b). The nucleotide sequence of *optix* is shown in detail (Fig. 10.2c), with gRNA target sequences and PCR primer sequences annotated, and the translated Optix protein sequence.

We describe in this chapter the advantages of using CRISPR technology in butterflies and include key tips and recommendations to do this in an undergraduate lab setting.

10.4 Animal Husbandry and Egg Collection

One of the main advantages to using CRISPR in butterflies in undergraduate lab is the relative ease of animal husbandry and egg collection compared to other model organisms. Caterpillars can be purchased from butterfly farms or online vendors, including Amazon. A batch of 30 caterpillars is the minimum number for rearing to collect butterfly eggs. The caterpillars go through four molt stages before forming a chrysalis, and then emerging from chrysalis form after about a week as a butterfly (Fig. 10.3C–J). The butterflies live for 2–3 weeks and can be housed in mesh butterfly cages indoors. We prefer to purchase from

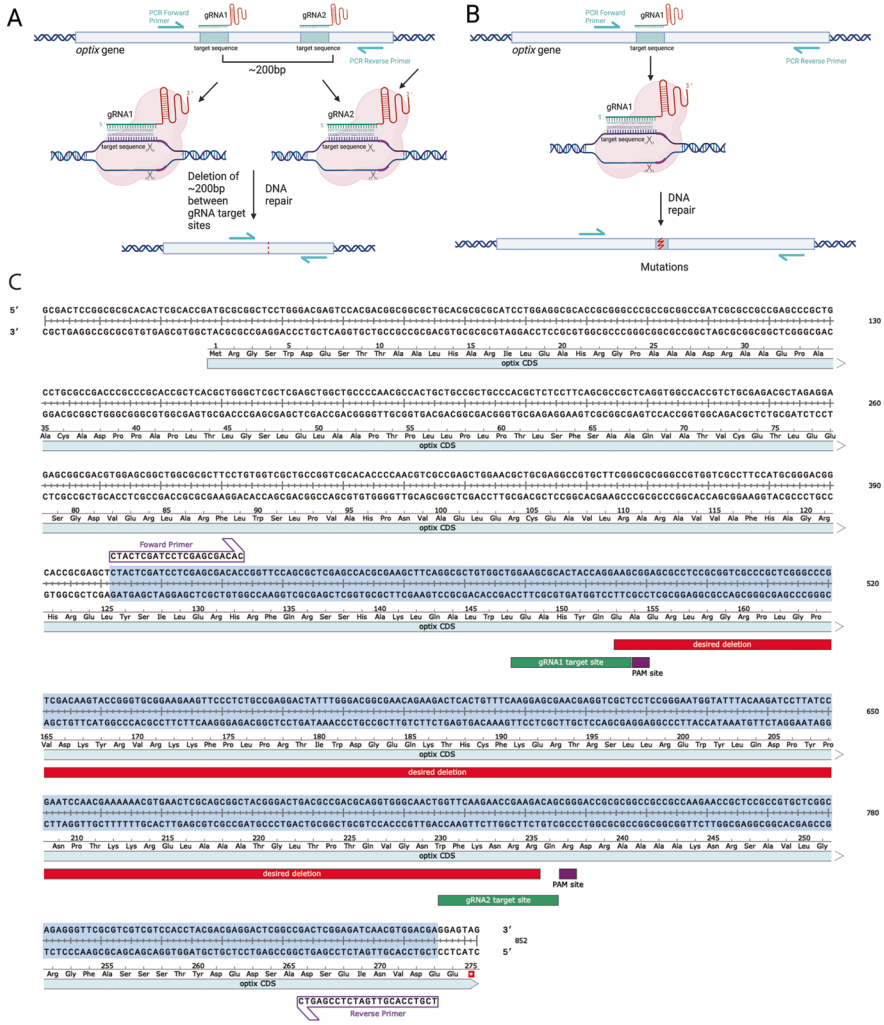

Fig. 10.2 Model of CRISPR targeting strategy. An example is given using *optix* gene, where either (**a**) two gRNA/Cas9 complexes or (**b**) one gRNA/Cas9 complex is targeted to two or one target sites. In (**a**), the goal is to create a 248 bp deletion between the two target sites, leading to loss-of-function of the gene. In (**b**) the goal is to cause substitution, insertion or deletion mutations at the target site, leading to disruption of coding sequence. This strategy is from [2, 6]. (**c**) The *optix* gene is annotated with the coding sequence (CDS, blue), PCR primers (purple), guideRNA1 and 2 target sequences (green), PAM sequences (purple), and desired deletion (red) between two guideRNA target sequences. The PCR product using the forward and reverse primers is highlighted in blue

Carolina Biological Sciences (Catalog #144080) as their stock seems to be the cleanest and most reliable. In our experience, other vendors and farms sometimes can provide a sickly stock that result in caterpillars that do not grow and eat properly after one or two generations of rearing. You can tell a stock is not doing well if the larvae grow slow, barely eat the diet, and produce a pink pigmented frass before failing to pupate as L5 (larvae stage 5) into

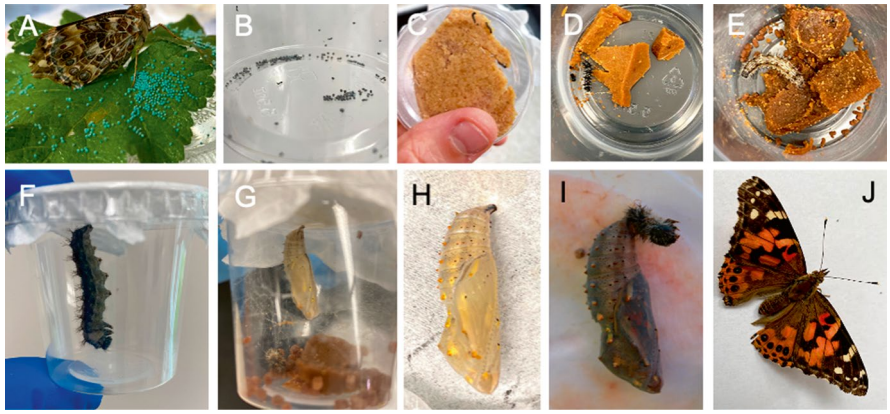

Fig. 10.3 Life cycle of painted lady caterpillars and butterflies. (**a**) Female butterfly laying eggs (blue balls) on mallow leaves. (**b**) Eggs before hatching turn dark. (**c**) Caterpillar hatchlings on artificial food diet in 1 oz plastic cup. (**d**) Caterpillar after molting. The molt that has been shed is the black dots to the left of caterpillar. It is around this Larval 3 stage that you would receive if purchased caterpillars from vendor. (**e**) Caterpillar about 2 weeks after hatching. (**f**) Caterpillar in the J position, hanging from top of cup. (**g**) Newly formed chrysalis with golden, shiny hue. (**h**) Close up of chrysalis after hardening. (**i**) Chrysalis right before hatching, where pigmented wings can be seen. (**j**) Hatched butterfly

chrysalis. Typically, this sickness would appear not in the first generation but in later generations. If you plan to just rear one generation for butterfly egg collection, and then not continue rearing, then purchasing from other vendors or butterfly farms should be fine. We have not had this sickness problem with Carolina Biological Sciences caterpillars thus far. To avoid disease, extra precaution can be taken with eggs that have been laid by the butterflies. Specifically, eggs can be washed with benzalkonium chloride before being reared for caterpillars and butterflies, as this will reduce contamination and diseased animals.

We recommend that instructors purchase and rear one full life cycle of painted lady caterpillars and butterflies and egg collection prior to doing CRISPR in the classroom to develop a sense of the developmental timing at the instructor's lab/institution (Fig. 10.3). Timing is key to ensure that students will have eggs for CRISPR delivery. The timing of caterpillar growth is depending on temperature and humidity. You can grow caterpillars indoors, which makes it easier for instructors as no special animal husbandry is necessary. We recommend growing caterpillars at a temperature of 24–25 °C. At 22 °C, normal lab temperature, the larvae grow slower than at 24 °C, taking about a week longer to reach chrysalis stage. To increase temperature slightly, we house our caterpillars in a makeshift chamber, and use temperature-controlled seedling mats to increase the temperature slightly (Fig. 10.4a). Typically, Carolina will send caterpillars that are in larvae L2 or L3 stage (Fig. 10.3d) in cups with artificial diet. You can purchase additional diet from Carolina or prepare your own diet. We have used Frontier Scientific Painted Lady Diet, but have recently switched to Southland Inc Multiple Species Diet, and prepare the diet using the

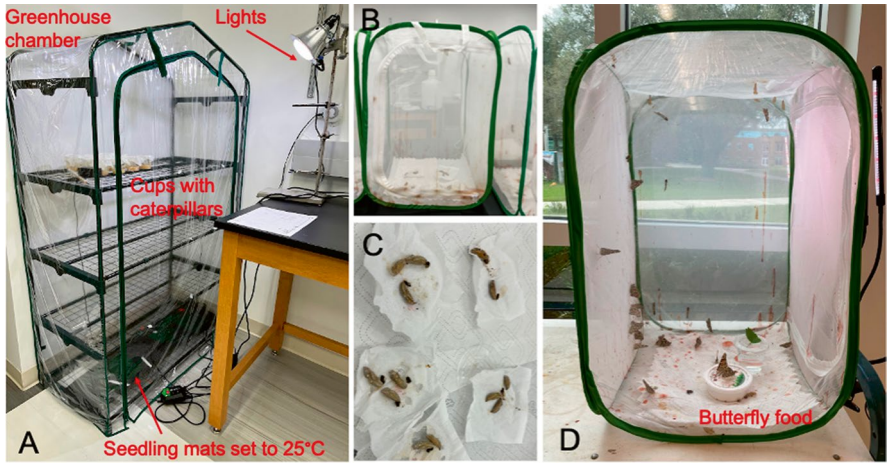

Fig. 10.4 Caterpillar and butterfly husbandry and egg collection. (**a**) Greenhouse shelf with plastic covering is set up with seedlings mats set to 25 °C and plant growth lights set on a timer. Caterpillar cups are set on shelves. (**b**) Butterfly mesh cage housing 20–30 chrysalis. (**c**) Close up of chrysalis set on the bottom of cage. (**d**) Butterfly mesh cage housing butterflies. 50% orange Gatorade in water in plastic cup with cotton ball on top is used as butterfly food

manufacturer's recipe, with the addition of 5 mL of canola oil per 162 g of diet powder and 930 mL water. After preparing the diet, we pour diet into 5 oz Solo plastic cups or 1.25 oz Solo plastic cups with lids, and store in the 4 °C fridge until needed. Before using the diet in cups for caterpillars, we punch small holes in the lid to allow for excess humidity to evaporate and breathing. We make holes by punching lid with forceps, or use a metal rod that we heat up with a Bunsen burner and then punch the holes quickly through the lids. A paper towel or Kimwipe square is used under the lid between the cup and lid. The paper towel allows the caterpillar to form its J-form (Fig. 10.3f) and chrysalis (Fig. 10.3g).

Caterpillars from Carolina take about 1.5–2 weeks to form chrysalis, depending on the temperature. You will see that the caterpillars will molt one or two times and grow quite large. We recommend not putting more than five caterpillars in each 5 oz cup of food. Too many caterpillars in a cup will result in having to change food cups very frequently. As the caterpillars grow larger, they will eat more food. Before forming chrysalides, the caterpillars will stop eating and "J", where they hang from the paper towel or kim wipe (Fig. 10.3f). The chrysalides will form hanging from the paper towel on top (Fig. 10.3g), and after 2 days in chrysalis form when they have hardened, you can move them into a butterfly cage. We just take the Kim-wipes with the chrysalides and lay them on the bottom of a butterfly cage that has been lined with paper towel (Fig. 10.4b, c). Others will hang the paper-towels/Kim-wipes with chrysalides to the top or sides of the butterfly cage with pins, but we have found this is not necessary for emergence of the butterflies. The butterflies will emerge from their chrysalis after a week. You will see the golden chrysalis turning darker over time; particular 1–2 days prior to emergence, orange/black wings can be seen (Fig. 10.3i). The butterflies will emerge, and their wings will be soft at first and harden over the next few days. For food, a cup of 50% orange Gatorade in water in a cup with a cotton ball sticking out from the lid should be placed in the butterfly cage (Fig. 10.4d).

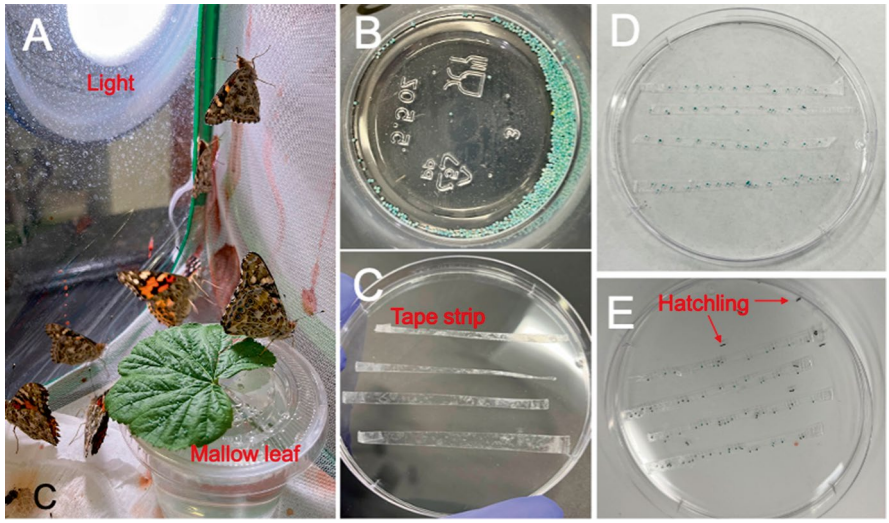

Fig. 10.5 Egg collection and preparation for microinjection. (**a**) External light and mallow leaf on cups are used to stimulate egg laying on leaves. (**b**) Cup full of butterfly eggs from two leaves after 3 h in butterfly cage. (**c**) Prepared 10 cm plate lid with four rows of double-sided tape. (**d**) Using paintbrush, eggs are placed on tape for microinjection. (**e**) After microinjection, caterpillars will hatch from eggs 3–4 days later, and crawl off the tape

After 3–4 days, eggs can be collected from the butterflies (Fig. 10.5a). You can watch for mating to occur between females and males starting about 2 days after emergence, where they come close together and connect the ends of their bodies together. Female adults will produce eggs for the next 7–10 days. To stimulate egg laying, 2–3 leaves from the mallow plant are placed in plastic cups with water and holes for the stems, and placed into the butterfly cage. A light can be placed outside of the cage to stimulate egg laying activity around the cup of leaves. Over the next 3–5 h, females will lay eggs on the leaves. The eggs are small blue balls, less than 1 mm big (Fig. 10.5a, b).

We prepare butterfly eggs for microinjection as follows. Using gloved hands, the butterfly eggs are gently brushed off the leaves into plastic or stainless-steel cups. The lid of a 10 cm petri dish is used because it has a shallower lip than the bottom of a petri dish. Although the plastic lids for the artificial diet could also work. Double-sided tape is cut into 3–4 strips and the strips are placed on the petri dish (Fig. 10.5c). A kimwipe is used to dab the tape slightly to add some fibers to the tape and make the tape a little less sticky. Too much dabbing will make the tape not sticky enough, so it is important to just softly dab the kimwipe once over the tape and not multiple times. Students who dab multiple times, find that once they place the eggs on the tape the eggs do not stick enough, which makes it harder to inject later. Using a clean paintbrush, eggs are moved from the plastic cup onto the tape (Fig. 10.5d). Students find it hard initially to move the eggs from the cups to the tape because of the eggs can bounce around due to static effect. Using clean water to dampen the paintbrush slightly to be able to pick up the eggs can help, but this can also lead to contamination later. We have students move about 50 eggs per plate onto the 3–4 rows of tape. Under a stereomicroscope, students then move the eggs so that the tapered

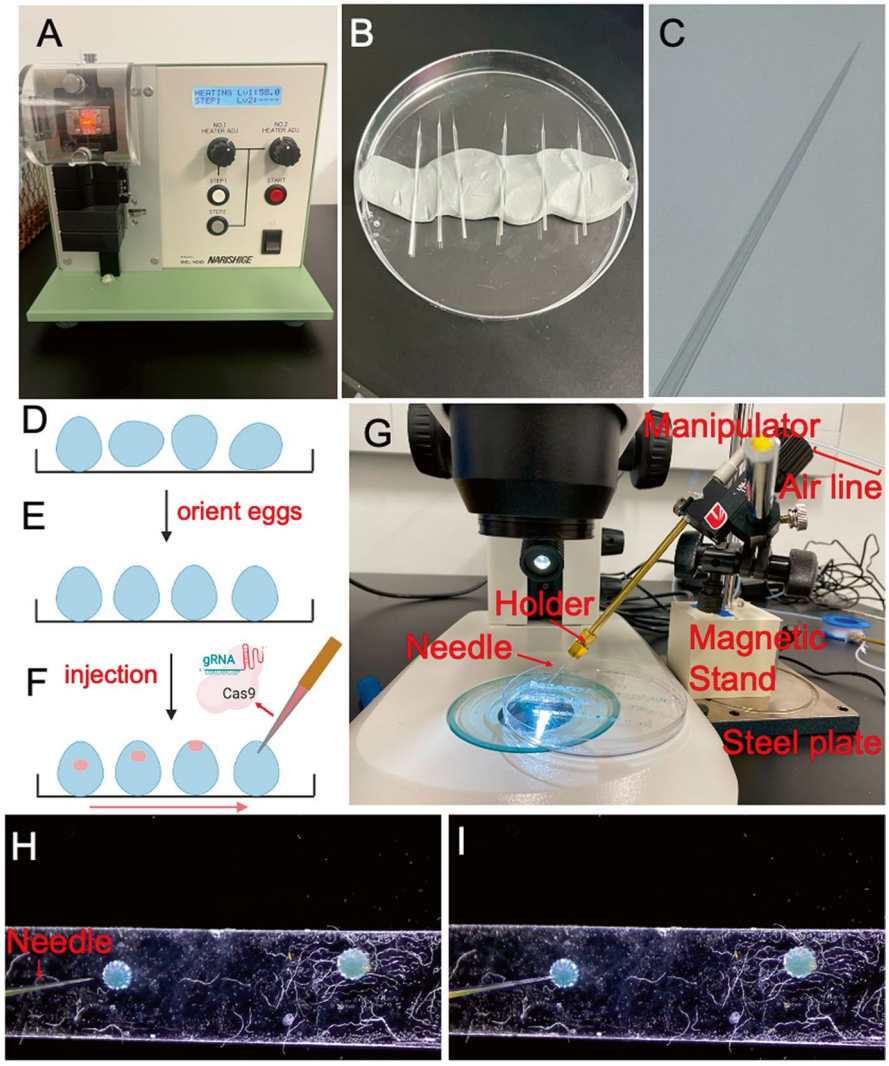

Fig. 10.6 CRISPR microinjection of butterfly eggs set up. (**a**) PC-100 Narishige needle puller used to pull needles (**b**) that are stored on clay in petri dish before use. (**c**) Taper of pulled needle. (**d**) When placed on a plate, butterfly eggs are in various orientation. (**e**) Using paintbrush or blunt end of capillary tube, eggs are moved so that the pointy end is facing up. (**f**) Glass needle delivers CRISPR reagents into individual eggs from left to right. (**g**) Microinjection set-up with important features labeled. (**h**) View of butterfly eggs and needle under the microscope, where two eggs are on top tape. The white threads are KimWipe fibers. The glass needle is positioned above egg before microinjection. (**i**) Needle has been micromanipulated into the egg, and using foot pedal the CRISPR gRNAs and Cas9 have been delivered into egg

end is facing up and the flatter end is facing down (Fig. 10.6d, e). This takes some time for students who are not used to looking under a microscope and using their hand-eye-coordination. We stagger students so that the while there is a group of student microinjecting, the second group of students are working on plating their eggs onto the plate.

10.5 CRISPR Delivery: Microinjection System, Micromanipulator and Microscopes

A major advantage of conducting CRISPR in painted lady butterflies is the size of the butterfly eggs, the ease of collecting many butterfly eggs for a lab course, and the ability for students to delivery CRISPR into the butterfly eggs themselves, rather than having an instructor do the delivery. We felt that it was important that students experienced themselves the activities associated with CRISPR delivery. Butterfly eggs are big (1 mm) in comparison to other model organism embryos like *C. elegans* (40 μm). Thus, they require a simpler set-up with a stereoscope at 2–4× and not an inverted microscope at 40× magnification. We use Zeiss stereoscopes (Fig. 10.6g; Stemi 305 with Stand K Edu) and side LED lights (Navlinge, Ikea) to view the butterfly eggs as 2–4× magnification. CRISPR reagents are delivered using a course air microinjection system (Fig. 10.6g). We use Tritech Research microINJECTOR System that includes the footswitch, pulse-control module and dual-pressure. This microinjector system can use lab air jets, compressed Nitrogen gas or an external air compressor (California Air Tools 2010A Ultra Quiet and Oil-Free 1.0HP 2.0 Gallon Aluminum Tank Air Compressor, Amazon). We have tried both compressed nitrogen gas and external air pressure. It is important to decide which air supply you will be using and purchase the appropriate microinjection system with regulator that works for the air source. We have tried using the single-pressure system and that works fine, but we have found the dual-pressure system to work better for us for providing a balancing pressure. Although we actually dial the balancing pressure to 0 psi (no pressure), and this provides sufficient balancing pressure for us.

The microinjection system will be connected to a pulled glass needle that will contain your CRISPR reagents (discussed in next section). The glass needle is prepared by purchasing borosilicate glass capillary needles with filament from World Precision Instrument (18100F-3). The needle has a capillary inside that allows you to load the CRISPR reagents in fluid from the back. The needles are pulled using a needle puller. Initially, the needle puller we use is the PC-10 Gravity Puller from Narishige, using a "one-stage (or step 1) pull", with No.2 heater set to 58. Narishige does not sell the PC-10 anymore but sells the updated PC-100. We have recently tested the PC-100 and found that the setting for the PC-100 is a one-stage pull, Step 1, with the No 1 heater set to 58 (Fig. 10.6a). The number 58 isn't degrees, it's the measure of the power being applied to the heating element. The taper of the pulled needle should look like the image shown in Fig. 10.6c. For a class of 15–20 students, we try to pull enough needles so that each student has five pulled needles for each injection session. We pull needles 1–2 days before and store the needles on clay in petri dishes (Fig. 10.6b).

The needle is connected to the microinjector but is held up by a micromanipulator (Fig. 10.6g). Three-axis micromanipulators can be expensive, particularly if multiple micromanipulators are needed. We initially used the three-axis micromanipulator from Drummond Scientific Company, and like the feel of this. The course manipulator is the main feature used in the three-axis micromanipulator. Students quickly learn how to use

this micromanipulator. We also tested a more economical one-axis course manipulator from Narishige (UM-1C manipulator and GJ-1 magentic stand with a steel plate from Amazon), and found having only one axis to manipulate is simpler for students. Although the one-axis course manipulator is not as solid and heavy as the three-axis manipulator, but the cost (~$500) of the one-axis manipulator is three times cheaper than the three-axis manipulator with stand (~$1500), making it a more economical option for classroom lab setting.

The needle is angled at a 45-degree angle and adjusted so that the point of the needle is about 1–2 cm away from the butterfly eggs. This allows the student to use the course axis manipulator to push the needle down into the egg (Fig. 10.6h, i). Once the needle is in the egg, the student will use the foot pedal to press down 1–3 times, and then using the manipulator, move the needle out. Students should be able to see the CRISPR reagents enter the egg due to the red labeling dye, or movement of liquid in the tip of the needle. We encourage students to press on the foot pedal again a few times after taking the needle out to try push out any yolk from the egg that has entered the needle. Naturally there will be some yolk liquid that enters the needle and causes what seems like a clog in the needle. However, the needle will unclog itself when back in a new egg. If students are having continuous issues with needle clogging, the instructor can help by sacrificing one egg and using that egg to unclog the needle by just pushing on the foot pedal multiple times until the CRISPR red liquid is now able to leave the needle.

To go from one butterfly egg to the next butterfly egg on the right, we move the plate left (Fig. 10.6f). So, if you are right-handed, you will be holding the micromanipulator with your right hand, and the plate with your left hand, and your foot will be resting on the foot pedal. The most common issue students have during microinjection is breaking their needle during microinjection against their plate or by pushing into the eggs too hard. If their needle is broken too much at the tip, then it will damage the eggs too much or too much CRISPR reagents will be ejected from the tip. We usually load as many needles as possible with the CRISPR reagents and line them up on a piece of tape on a shelf by the scopes, so that when students break a needle, we can quickly come in and change it out for them. The initial breaking of the needle is discussed in the next section.

10.6 CRISPR Delivery: CRISPR Tools—Guide RNA and Cas9

We use synthesized guideRNA from Synthego (CRISPRevolution sgRNA EZ Kit, 1.5 nmol) and purified Cas9 protein from QB3 Macrolab, UC Berkley. The Synthego website is easy to use and requires only a 17–23 nucleotide genome targeting sequence in 5′ to 3′ order (without the PAM sequence). Synthego will add their propriety 80-mer SpCas9 scaffold sequence to create the single guide RNA. Upon receiving the lyophilized gRNA, we resuspend them in 100 µL of low TE (0.1 mM EDTA) and vortex well. The 100 µL is then aliquoted into small aliquots of 2.5 uL aliquotes and frozen at −80 °C til use. This frozen stock is about 500 ng/µL. The Cas9 from QB3 (comes as 65 µg in 10 µL aliquots) is

diluted with 53 μL of Synthego's nuclease free water (or Bombyx injection solution) and 7 μL 0.5% Phenol Red to make a 70 μL solution of Cas9 at 2× concentration of 1000 ng/μL. 5 uL aliquots are made and stored at −80 °C.

Prior to microinjection session, the microinjection CRISPR mixture is prepared using RNAse-free conditions. 5 μL of Cas9 stock and 2.5 μL of each gRNA (if using two gRNAs) is mixed in a RNAase free-tube (final concentration of Cas9 is 500 ng/μL and gRNAs is 250 ng/μL), and the complex was allowed to form at room temperature by incubating for 10 min, before putting on ice. The phenol red provides a red visual marker. Multiple tubes can be prepared if needed, depending on how many students will be injecting. Pulled needles are loaded by pipetting 1 μL of the CRISPR solution onto the back of the needle, with capillary action pulling the liquid to the bottom. A fast way to load 10 needles for class is to have needles taped up against the edge of a shelf with the tip facing down, and then pipet 1 μL onto the back of each needle. The needle is gently screwed into the micromanipulator holder. The micromanipulator needle holder does have an O-tube ring inside to help with the seal, and it is important that the tube is there and not missing or crushed. The needle tip is sealed, so the tip needs to be broken so that a small hole for delivery into the eggs is present. To break the needle, using the course axis manipulator, gently lower the needle and tape the tip of the needle against the plastic lid holding the butterfly eggs. When you lift the needle slightly, press on the foot petal and observe whether small droplet of red liquid comes out of the needle. It is important not to break the needle too much, as you want to retain a fine point for the needle to be able to penetrate the butterfly egg easily.

10.7 CRISPR Gene Targeting of Butterfly Wing Color and Patterning Genes

We have successfully had students use CRISPR against the butterfly color and patterning genes *optix* and *WntA* in lab [2]. Specifically, students have delivered gRNA against these genes into butterfly eggs, and observed mutant phenotypes, and characterized molecular nucleotide changes caused by CRISPR using techniques described in the Molecular Analysis section. *WntA* is a signaling gene that controls wing patterning for butterflies [4]. CRISPR targeting of *WntA* results in abnormal wing patterning, which is fascinating for students to observe. CRISPR targeting of *optix* results in more black pigmentation and less orange pigmentation on *V. cardui* butterfly wings, demonstrating that the normal function of *optix* is to repress black pigmentation and promote orange pigmentation in these butterflies [6]. Our students have also successfully designed and tested their own gRNA against *optix* and *WntA* [2].

We recommend instructors use published gRNA sequences against targeted genes with known CRISPR mutant phenotypes, prior to trying CRISPR with novel genes with students. For example, knockouts of *Spalt* transcription factor can produce loss of wing eyespot [8]. A CRISPR knockout of the long non-coding RNA ivory produced a drastic mutant

phenotype of white-yellow wings [9]. Other pigmentation genes have also been characterized using CRISPR that could be potential targets in class [10].

10.8 CRISPR gRNA Design

If having students design gRNA target sequences, the transcriptome of *Vanessa cardui* is online, thus allowing the visualization of coding nucleotide sequences. However, the annotated genome is not available yet, therefore it important to take into consideration potential intron/exon boundaries, so as to not design a gRNA at one of these boundaries. One way to get around this is to use the genomic annotation of the*Vanessa tameamea* genome to visualize intron/exon boundaries of homologous genes https://www.ncbi.nlm.nih.gov/assembly/GCF_002938995.1/

We use the computer software program called SnapGene to visualize the gene sequence. We have students look for potential protospacer adjacent motif (PAM) sequences of 5′-NGG-3′, and then take the preceding 20 nucleotides as the target sequence. We like to use a paper model to model gRNA binding to target DNA before using SnapGene [11]. Other programs like Benchling can be used as well instead of SnapGene. Martin et al. uses a different strategy, where students first take an orthologous protein sequence from another species and perform a TBLASTN against the transcriptome on www.butterflygenome.org to acquire the most likely ortholog in *V. cardui* [7]. Then students design a gRNA target as described above with the gene sequence.

10.9 Analysis of CRISPR Mutants

In our hands, students obtain about a 10–30% injection success hatching rate. The success rate depends on how well the students microinjected their eggs. We have students attempt to inject 50 eggs in one sitting. Students work in pairs, and each student conducts at minimum two injection sessions so that each pair has collectively injected about 200 eggs. 3–4 injection sessions are ideal, as there is a learning curve to the injection process. The first microinjection session typically results in a low yield of hatchling. If there is time and four injection sessions can be accommodated in the class, having students practice in the first injection session with just phenol red dye is recommended so that students learn the hand-eye-foot coordination of microinjection. Then subsequent injections are done with CRISPR. After microinjection, the plates of eggs are placed in a Tupperware and after 3–4 days, small hatchlings emerge (Fig. 10.5e).

Depending on the goals of the instructor/lab, the hatchlings can be used in two ways: 1) all the hatchlings can be placed in cups of artificial diet and allowed to grow until butterfly stage for phenotypic analysis; or 2) some of the hatchlings are frozen in tubes at −20 °C for molecular analysis and the rest are placed in food for phenotypic analysis. It is important to emphasize that not all injected animals will produce mutant phenotype and CRISPR modifications. Published papers even demonstrate that experienced scientists that injected

1700 painted lady butterfly eggs with CRISPR against *optix* had a 5–19% hatch rate and produced only 9 adults with mutant phenotypes and 13 pupae from chrysalis with mutant phenotypes [6]. An additional study injected 425 eggs with CRISPR against *Wnt* and had a hatch rate of 24.9% and 12 adults with mutant phenotypes (11.3%) [4]. Thus, with novice students, we would expect much less and variability amongst student groups, with each group consisting of a pair of students. In our hands, over the course of two different courses, we have observed that in the first cohort class, all groups obtained at least one phenotypic mutant with one group obtaining four mutants. In the second cohort class, only half the groups obtained at least one phenotypic mutant and one group obtained five phenotypic mutants. Each pair usually has about 30–50 butterflies from two rounds of injections. To increase the number of phenotypic mutants that students can observe, we have had the instructor microinject 200–400 eggs on the side, and the undergraduate lab assistant care for the hatched caterpillars and butterflies. This will yield for us about 5–10 more phenotypic mutants that students can analyze.

10.10 Phenotypic Analysis of CRISPR Mutants

After hatchlings are placed in food cups, students maintain the caterpillars and change their food every 2–3 days, or when necessary. In the beginning 10 hatchlings can be placed in each 5 oz cup of food, but as the caterpillars reach L3 stage, it is best to reduce the number of caterpillars per cup and put five in each cup. If space is not an issue, you can use smaller 1.5 oz cups and place one caterpillar in each cup. Each pair of students should also have a batch of control uninjected animals for phenotypic comparison. It will take about 3–4 weeks to produce butterflies after the hatchlings have formed. Again, the timing depends on temperature. We recommend rearing them at 24–25 °C to speed up development. To keep students on track with animal husbandry, we use a shared online digital lab notebook, where students write notes on when they come in and how the number of animals. This allows the instructor to keep an eye on the animal husbandry being done by students, and also keeps students motivated when they see their peers are working on maintaining their animals. While students are waiting for butterflies to form, we focus on the molecular analysis of CRISPR-mediated targeting, described in the next section.

After butterflies hatch out of chrysalis, students collect the butterflies in plastic cups and store them at −20 °C freezer. In class, students dissect out the wings and compare to uninjected control animals for phenotypic differences. We have focused on two genes associated with wing pigmentation and patterning, *optix* and *WntA*, because mutant phenotypes are easily visualized. For example, CRISPR mutants of *optix* produce butterflies with wings that have increased black pigmentation (Fig. 10.7a). It is important to note that there is a variation in the amount of mutant phenotypes produced in the wings of butterflies. For example, some students will find that half their butterfly wings, like the left side, has a mutant phenotype, while the other half looks like wild-type wings. As we are delivering CRISPR into developing butterfly eggs, it is rare to generate a complete CRISPR knockout. Instead, clonal patches of mutant phenotypes are more likely to develop and be

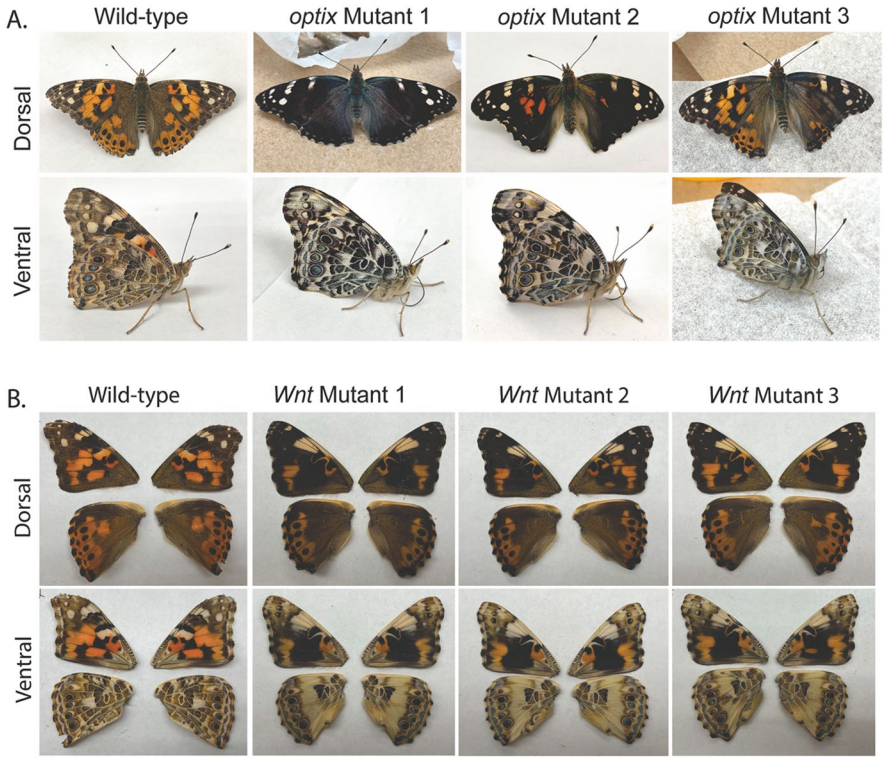

Fig. 10.7 Students' phenotypic analysis of CRISPR *optix* mutants in butterflies. (**a**) Butterfly wings of wild-type and CRISPR *optix* mutants obtained by students. Three different mutants show variation in CRISPR mosaicism. (**b**) Butterfly wings of wild-type and CRISPR *WntA* mutants obtained by students. Figure is modified from [2]

observed. We find that the phenotypes produced and the molecular analysis that we can conduct allows constructive discussion about genotypic-phenotypic relationships.

If instructors want students to choose their own gene of interest, then an important consideration is that appropriate phenotyping assays needs to be developed that can be easily conducted and quantified by students. We recommend if students do test a novel gene, they use *WntA* as a positive control for the CRISPR experiment, which will allow them to still produce CRISPR mutant phenotypes with the positive control, even if they do no produce an easily observable phenotype with their gene of interest.

10.11 Molecular Analysis of CRISPR Mutants

If one of the goals of the lab is for students to analyze whether gRNA/Cas9 targeted their gene successfully, then lab activities should include conducting molecular analysis of CRISPR-d animals. It is important to note that CRISPR delivery into butterfly eggs will

generate mosaic mutants with clonal tissue populations of CRISPR modification within the animals. However, molecular analysis in caterpillars and butterflies is simple and straightforward. We use the Thermofisher's Phire PCR kit for our molecular analysis. The kit comes with a DNA Phire Dilution Buffer and DNA release solution. A single caterpillar hatchling or a tiny piece of butterfly thorax tissue can be placed in 40 μL Dilution Buffer and 1 μL DNA release solution, and lysed at room temperature incubation for 5 min and 98 °C for 2 min. After a minute of centrifugation, 1 μL of the supernatant is then used as the template DNA source for a PCR amplification reaction. We have used both the Phire Polymerase from the kit, but have also used Taq DNA polymerase from NEB. We follow manufacturer's protocol for PCR cycling conditions. It is important to note that the Phire Polymerase utilizes different cycling conditions and temperatures than the standard Taq DNA polymerase PCR conditions. Therefore, if you are testing amplification of a new gene, it is best to utilize the Thermofisher's online tool for temperatures to try for Phire Polymerase and test annealing temperatures of a new set of primers with gradient PCRs. We have tried direct PCR method of Phire PCR kit, where a single caterpillar is placed directly into the PCR reaction. This works, but we find that the dilution method to produce genomic DNA is more reliable and consistent in students' hands. In class, we have each student pair assay one wild-type and seven CRISPR-d injected hatchlings to fill a strip of PCR tubes. We have also had students conduct PCR on butterfly thorax tissue of butterflies with visible mutant phenotypes. As a class, we discuss each group's data, emphasizing that we are working together as a class to determine if CRISPR modification has occurred in any of the mutants we are screening.

Following PCR, 15 μL of the PCR amplicons are run out on an agarose gel. In painted ladies, the CRISPR strategy is to use two guide RNAs to create a loss-of-function deletion within the gene (Figs. 10.2a and 10.8b). For example, with *optix* gene, wild-type PCR amplicon would be 444 bp, and CRISPR mutant with deletion between the two gRNA will produce a DNA band that is ~200 bp (Figs. 10.2a and 10.8b). This allows us to visualize in a DNA gel whether CRISPR-mediated deletion is produced due to dual gRNA targeting (Fig. 10.8d,e). Although we deliver two different gRNA/Cas9 complexes into butterfly eggs, it is possible for only one gRNA target sequence to be targeted by one of the complexes and produce a small insertion or deletion (Fig. 10.8c). If that is the case, then the PCR amplicon will look similar to wild type in size (Fig. 10.8d,e). We used to run only 4 μL of PCR amplicons out on an agarose gel, but in the last iteration of the lab, we ran 15 μL and found that running more volume of PCR product on the gel showed us that more samples had the ~200 bp band (Fig. 10.8e, asterisks), which we confirmed with DNA sequencing was a result of CRISPR targeting.

To analyze the sequence of the gene, DNA bands are cut out from the agarose gel and purified with a gel purification kit (Zymo). The purified PCR amplicon can be sent for DNA sequencing (Eurofins) and then aligned with the reference wild-type sequence using SnapGene software. In our class, we have observed that students do obtain mutant sequences with insertion/deletion/substitutions at or around one gRNA site in multiple hatchlings assayed, showing successfully CRISPR targeting [2]. Students use SnapGene

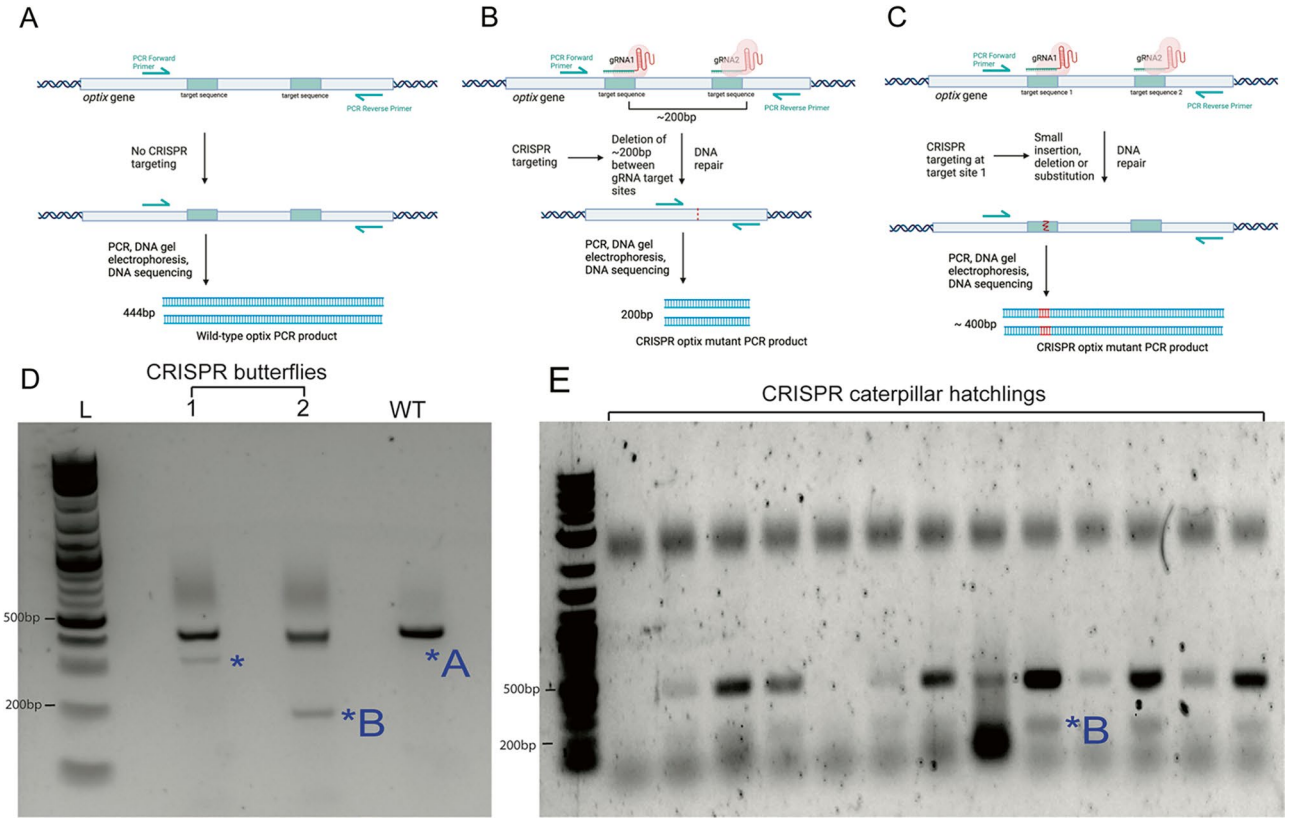

Fig. 10.8 Students' molecular analysis of CRISPR *optix* mutants. Schematic of expected PCR products produced from (**a**) wild-type *optix* gene, (**b**) CRISPR mutant that formed from dual gRNA/Cas9 complex targeting, and (**c**) CRISPR mutant that formed from single gRNA/Cas9 complex targeting. DNA gel electrophoresis of PCR amplified products from (**d**) butterfly tissue and (**e**) caterpillar hatchlings show CRISPR-mediated deletion of ~200 bp (asterisks*B) from CRISPR mutant and wild-type (asterisks*A). Parts of this figure is modified from [2]

software to determine how the nucleotide changes affect the reading frame, amino acids encoded, and protein produced. In the first iteration of this lab experience, we observed the dual gRNA/Cas9-mediated deletion in two hatchlings and two butterfly tissue samples within a class, so this is less frequently observed, but still possible within a class (Fig. 10.8b).

10.12 Other Considerations

All CRISPR edited animals are frozen at −20 °C a few days after hatching. Currently, we do not mate CRISPR edited animals to pass on CRISPR changes to next generations. However, a recent study showed that it is possible to create a G1 CRISPR mutants by mating pooled G0 CRISPR mutants together [9].

If it is not possible to microinject butterfly eggs with CRISPR due to limited time and resources, a scaled down version of the lab can be conducted, as described [11]. Briefly "purified" butterfly DNA can be subjected to PCR amplification and DNA gel electrophoresis to determine if CRISPR targeting was successful in the butterfly *optix* gene. The "purified DNA" is butterfly gene sequences of wild-type and CRISPR mutants in a plasmid, as the plasmid is an easy way for long-term storage for instructors. Further DNA sequence analysis on the computer using SnapGene can be done with prepared DNA sequencing data (as .seq files) to determine which specific nucleotides have been altered and how that affects the protein produced (Fig. 10.9a, b). With this scaled down version students can learn molecular biology techniques associated with CRISPR targeting and analysis. Alternatively, it also possible to use this "purified" butterfly DNA samples as positive controls for PCR if conducting PCR on caterpillar hatchlings.

Take Home Message

- We have used *V. cardui* butterflies as a model organism to target wing patterning and color genes *WntA* and *optix* in undergraduate lab courses.
- Using butterflies allows us to have students deliver gRNA/Cas9 tools into butterfly eggs that can be collected from adult butterflies in the lab.
- Students are successfully able to characterize CRISPR modification through both molecular and phenotypic mutant analysis over the course of 5–8 weeks, depending on the structure of the lab course (Fig. 10.10a–j).

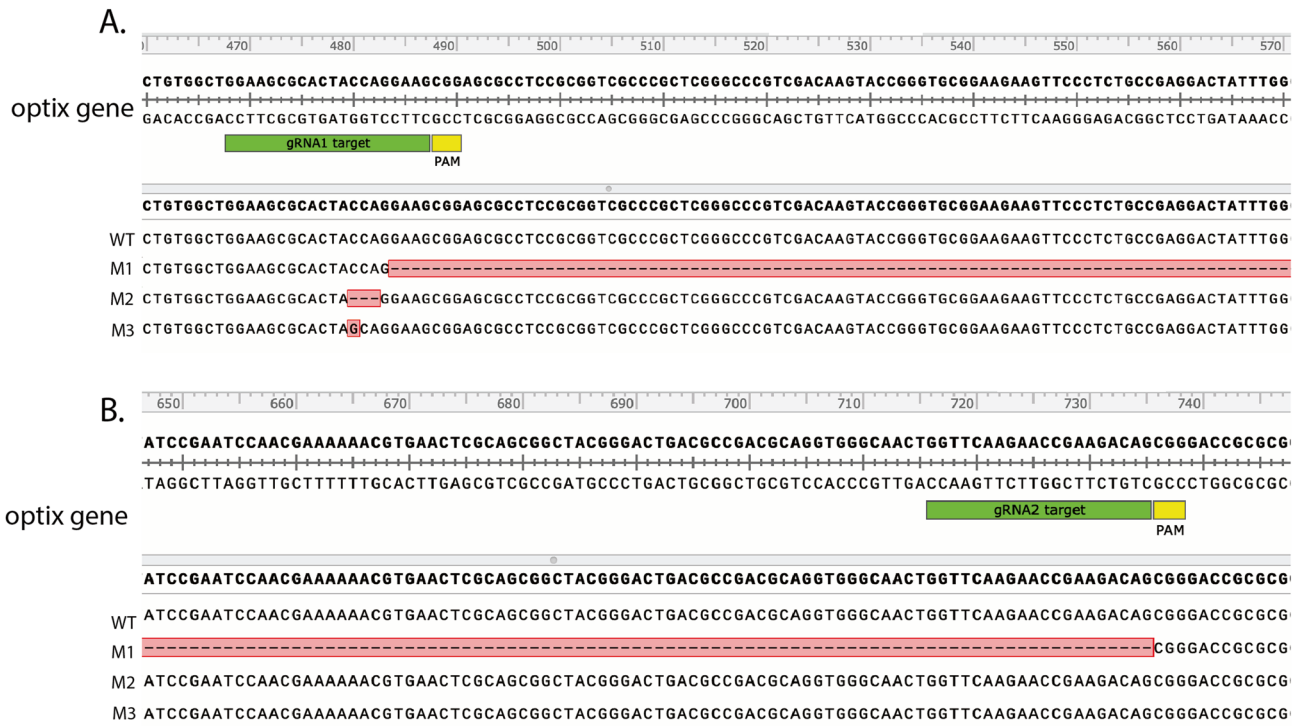

Fig. 10.9 DNA sequence alignment of wild-type and CRISPR mutants of *optix* gene. Visualization of the sequence alignment of PCR-amplified *optix* gene from wild-type and CRISPR mutants. gRNA1 and gRNA2 target sites are labeled in green in the reference *optix* gene sequence (**a**, nucleotides 460–570; **b**, nucleotides 647–748). Numbers at the top indicate nucleotide number. PAM sequence is labeled in yellow. The dashed lines in mutant sequences indicate deleted nucleotides and substitution of nucleotide is also indicated in red. Deletion of three nucleotides in M2 and point mutation in M3 lead to premature stop codon and truncated protein. In M1, 252 nucleotides are deleted resulting in loss of 84 amino acids in the middle of protein. SnapGene allows students to expand alignment view and visualize amino acids affected

Fig. 10.10 Students working on CRISPR in butterflies. (**a**, **b**) Students using paintbrush to place butterfly eggs on tape on petri dish lid. (**c**) Students using pipet tip and stereomicroscope to maneuver eggs into the right position, with tapered end facing up. (**d**) Students in the lab working on caterpillar and butterfly husbandry, which includes changing caterpillar food for caterpillars and placing formed chrysalides into butterfly cages. (**e**) Student microinjecting butterfly eggs with CRISPR using our microinjection system. Student uses the foot pedal to push CRISPR into the butterfly egg with micromanipulator once the needle has been driven into the butterfly egg with micromanipulator. (**f**) Student using the coarse micromanipulator to move the needle with the right hand, and uses her left hand to move the plate, which allows her to move the butterfly eggs, one by one, to the needle to inject. (**g**) A close up of the microinjection process with students two hands. (**h**) Student using forceps and scissors to cut out butterfly wings. (**i**) Students working to analyze butterfly wings for CRISPR phenotypes. (**j**) Student holding up butterflies produced from the lab that have been mounted in a frame

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Part III

Additional Applications of CRISPR in the Classroom

Navigating Computational Resources for the CRISPR Classroom

11

Linnea Andersen, Carlos Goller, Leigh Ann Samsa, and Arnab Sengupta

11.1 Introduction

We define "computational resources for the CRISPR-Cas Classroom" as all software and web applications that assist in the design, execution, and analysis of a CRISPR experiment. It is inclusive of molecular biology sequence management and CRISPR-centric resources such as guide RNA design tools. Throughout this chapter, we use the terms "computational resource", "platform", "software", "app/application", and "(digital) tool" interchangeably to refer to CRISPR-centric computational resources that an instructor might consider using in the classroom. Additionally, "database" and "repository" both refer to curated collections of sequences.

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11.1.1 When Is It Appropriate to Use Computational Resources Within a Course?

General CRISPR-Cas concepts can be communicated and learning can be evaluated using standard teaching and learning techniques. Instructors who only need to teach the general concepts of CRISPR-Cas do not need to ask students to also learn to use various software and applications that are discussed in this chapter. That said, some of these computational resources can help enrich lessons, provide opportunities for *in silico* lab activities, and support student technology literacy with tools used in research. And, the computational resources described herein and those similar enable meeting learning objectives that extend beyond basic concepts and into application. Further, learning new software is a critical and highly individualized skill outside of the scope of the learning outcomes evaluated in most courses. Some students "pick up on it" very quickly while others need time to work through tutorials. Use computational tools wisely and allow ample time for students to build sufficient familiarity with computational resources so that they can focus on learning about or applying CRISPR-Cas technologies. Gaining fluency in navigating and learning new computational tools is a highly applicable professional skill for students who advance into science careers.

Instructors may use computational resources to teach CRISPR-Cas technologies in multiple higher education settings. In this chapter, we consider the following settings: (1) advanced undergraduate or graduate biology (1) lecture and (2) laboratory sections and (3) computational biology courses. These are elaborated upon below.

11.1.1.1 Setting 1: (Molecular) Biology Classroom

In an undergraduate or graduate course of study, CRISPR-Cas technologies are generally not contained within standard curricula but are of high interest to students. Thus, CRISPR-Cas technologies are often introduced in the context of learning objectives related to the relationship between genotype and phenotype, or to biotechnologies, or even genetic engineering. In those contexts, CRISPR-Cas technologies (often only the engineered CRISPR-Cas9 system) are presented as a modern technique for genetic engineering and computational tools are NOT necessary for students to construct a fundamental understanding of the technology. Likewise, in courses for non-majors, central concepts in genetic engineering and CRISPR-Cas technologies can be easily communicated and student understanding evaluated without using any computational resources. Some resources, such as sequence viewers, can be used to enhance learning (e.g., by showing what the gene really "looks" like and where a guide RNA, or gRNA, would bind). In this case, usually static screenshots are sufficient and, while instructors would need to build their own competencies to create custom materials, it is often not necessary for students to learn to use these tools.

11.1.1.2 Setting 2: (Molecular) Biology Lab

In a laboratory setting, computational tools are an excellent choice when students are expected to develop skills in CRISPR-Cas experimental design and interpretation and/or a deeper understanding of CRISPR-Cas technologies. Such laboratory activities would likely appear in advanced genetics or molecular biology courses. As a sequence-based technology, functional and high-level mastery of this technology necessitates that students develop competencies in navigating gene and genome databases, sequence viewers, and other such computation tools to develop a complete understanding of CRISPR-Cas gRNA customization and experimental validation. At the graduate level, students require such competencies to transition to independent work as they design and build custom CRISPR-Cas tools and use them to conduct research.

Computational tools are an invaluable building block for online lab activities (some of which could play a role in an active learning classroom). Science majors expose students to advanced concepts in genetics and genetic engineering, and sequence viewers/manipulators and guide RNA design tools can be used to deepen understanding in these areas. These tools also enable a wide range of "real research world" mini-activities. For example, students can have an interactive experience with genetic engineering through labs that feature CRISPR computational tools.

11.1.1.3 Setting 3: Computational Biology Course

Computational resources are also required when CRISPR-Cas technologies serve as a use case for undergraduate and graduate level computational sciences. In computational biology-type courses, students work at the command line interface where they may perform genome assembly and other sequence technology analytic tasks. For these students, CRISPR-Cas technologies make an exceptional tool for student practice.

Throughout this chapter we point out where an instructor might choose to use some tools over others based on the context of their higher education classroom. Computational resources can be used to ground concepts in tangible biology, present opportunities to practice transferable bioinformatics skills, and aid in building experimental design skills.

11.1.2 Why Are Computational Resources Needed?

CRISPR-Cas biotechnologies are a group of molecular tools originating from the prokaryote immune system, repurposed and re-engineered into powerful molecular biology tools. CRISPR-Cas technologies pair nucleotide-level specificity with effector activity to manipulate the genetic code. In each CRISPR-Cas system, a guide RNA (gRNA) assembly directs an effector Cas protein (or protein complex) to a nucleotide sequence. The result of effector activity is usually detected using a molecular readout such as DNA sequencing or gene expression. Specificity and efficiency of targeting is encoded within the gRNA sequence, and the specific enzymatic action (e.g., double stranded break or C->T swap-

ping) that occurs upon binding is encoded by the effector sequence. *Thus, nucleotide sequence is of the utmost concern in all CRISPR-Cas experiments.*

Fortunately, digital tools are available to aid in viewing and manipulating nucleotide sequences. Figure 11.1 illustrates when and how computational tools are used in a typical experiment using the CRISPR-Cas9 technology as an example. Notably, sequence management tools are used throughout every stage of a typical experiment.

A summary of a typical CRISPR Cas9 experiment is as follows: the experimental objective is to knock out a gene in a cell's genome. The approach is to use CRISPR-Cas9 to create a double stranded break in the DNA which is subsequently incorrectly repaired by the cell's DNA repair machinery, introducing a frameshift mutation that compromises gene function. In a typical CRISPR-Cas9 experiment, a customized single guide RNA (sgRNA) is expressed along with *S. pyogenes* Cas9. Within the sgRNA, 20 nucleotides (nts) provide specificity to DNA target sites; the target site must be followed by the "NGG" protospacer. When co-expressed, the sgRNA forms a complex with Cas9 and guides the complex to the specified nucleotide sequence. Upon binding, the DNA is opened and Cas9 cleaves the DNA backbone 3–4 nt upstream of the protospacer. The efficiency of cleavage, and when performed in a genomic context, the efficiency and efficacy of repair events that result in desired mutations. Since a double stranded break is created at any 20 nt site matching the sgRNA, it is important to select unique sequences. Publicly available genome databases and/or custom genome assemblies provide complete sequence information about the genome of interest (Fig. 11.1a). These are accessed and viewed using genome browsers. Gene annotation tools help identify areas of the genome where a mutation may eliminate gene function. A mock eukaryotic gene is illustrated to emphasize the impor-

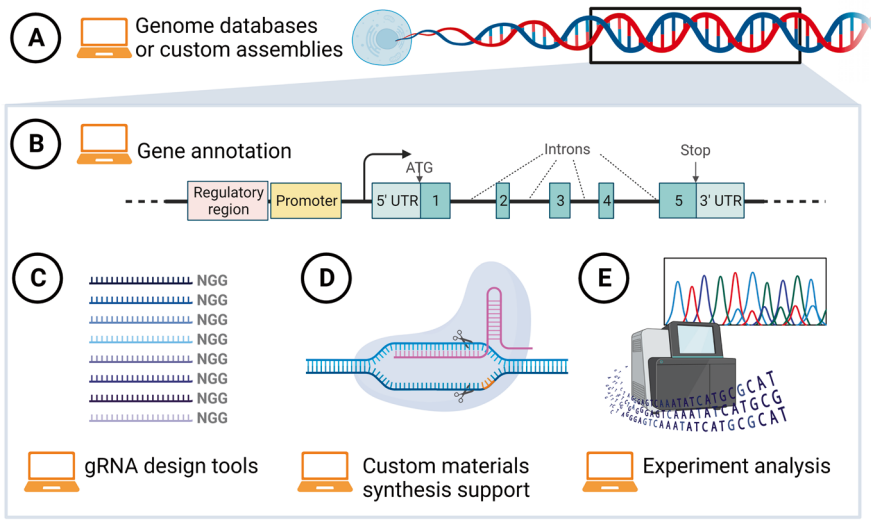

Fig. 11.1 Computational tools used in a typical CRISPR-Cas9 gene knockout experiment (<original to the author>; Created with BioRender.com)

tance of targeting conserved, early exons over other regions of a gene (Fig. 11.1b). Many gRNA design tools are available that provide, for an inputed DNA sequence, lists of possible gRNAs accompanied by on- and off-target effect and efficiency prediction scores. Advanced gRNA design tools enable the user to input information about the Cas effector that will be used, the sgRNA expression system, genome of interest, cloning and other elements that are known to impact efficiency and efficacy (Fig. 11.1c). A wide range of computational tools are available to support custom synthesis of materials necessary for a CRISPR experiment—from oligonucleotide synthesis to cloning and expression (Fig. 11.1d). After molecular tools are generated and expressed in the cell or organism of choice, computational tools are used to analyze data characterizing on-target changes (using Sanger sequencing) and detecting any off-target changes (whole genome sequencing) (Fig. 11.1e).

Classroom and laboratory objectives may focus on any or all of the stages described in Fig. 11.1 and instructors should consider leveraging computational tools as appropriate for their classroom or laboratory needs.

11.1.2.1 Example: (Molecular) Biology Classroom

Learning Framework Learning Goals: How do different types of mutations affect genes and the corresponding mRNAs and proteins? How is genetic information expressed so it affects an organism's structure and function?

Leverage CRISPR-Cas genetic engineering as real world application: Scientists can use CRISPR/Cas9 to make precise double stranded breaks in DNA that lead to mutations that eliminate gene function. To do this, they need to know the structure of a gene so that they can guide Cas9 to a location that is likely to lead to a mutation that will eliminate gene function.

Computational tool(s) leveraged: Genome browsers leverage databases of reference sequences for a wide range of organisms. In these genome browsers, gene annotations show the position of start and stop codons and, for eukaryotes, indicate important exon/intron boundaries and regulatory sequences.

11.1.2.2 Example: (Molecular) Biology Lab

Learning Framework Learning Goals: How do different types of mutations affect genes and the corresponding mRNAs and proteins? How is genetic information expressed so it affects an organism's structure and function? What experimental methods are commonly used to analyze gene structure and gene expression*?*

Leverage CRISPR-Cas genetic engineering as real world application: Scientists can use CRISPR/Cas9 to make precise gene edits and knock out gene function. How do they know that an experiment "worked"?

Computational tool(s) leveraged: Methods to analyze gene structure and expression are leveraged to show that an experiment worked. The most direct method for confirming that an experiment led to a mutation is to use Sanger sequencing to directly assess DNA sequence. If the DNA sample is taken from a pool of cells that have been exposed to the

CRISPR/Cas9 editing agents, scientists expect many different insertions, deletions, and substitutions (indels) at the predicted target site. Computational tools can be used to view a Sanger sequence and assess the extent to which there is evidence of indels in the DNA sample.

11.1.2.3 Example: Computational Biology Course

Learning Framework Learning Goals: Where are data about the genome found (e.g., nucleotide sequence, epigenomics) and how are they stored and accessed? How can bioinformatics tools be employed to analyze genetic information?

Leverage CRISPR-Cas genetic engineering as real world application: Scientists can use CRISPR/Cas9 to make precise gene edits and knock out gene function. Each targeting event where a gRNA:Cas9 complex binds to DNA and creates a double stranded break is associated with a likelihood of on-target and off-target effect, corresponding to creation of insertions, deletions, substitutions, or proper repair (on-target) at the desired target site and (off-target) at sites other than the target site, respectively. A scientist has performed whole genome sequencing on a pool of cells targeted with one of three gRNAs. The scientist sends you unprocessed sequence data from an Illumina HiSeq run and asks you to analyze the data to answer, "Which gRNA performed the best?".

Computational tool(s) leveraged: Answering the scientist's questions involves writing custom scripts to align the data to a reference genome and to count the number of on and off-target mutations evident in the sequence data. Tools leveraged will likely include a coding interface (usually Python), genome databases, and Illumina sequence management tools (for the HiSeq run).

11.2 Navigating Computational Tools for Teaching CRISPR-Cas

Below, we introduce computational tools as they pertain to each element of a CRISPR experiment. Throughout, we provide information that will help instructors select which tools are best suited for their classroom/laboratory needs.

11.2.1 DNA Sequence Tools

11.2.1.1 Sequence Databases

Since CRISPR is a sequence-based technology, a fundamental component of student learning is gaining experience obtaining, exploring, and evaluating sequence data [1–6].

Public repositories:

Genomic sequence data is an important element of any CRISPR experiment. We anticipate that most instructors prefer to use free, publicly available data available for curated repositories. To use this data, students will learn to navigate these databases and their associated browsers. Several such databases and repositories have been established to host and make

Table 11.1 Popular open-access sequence databases[a]

Database	URL	Comments/Description
GenBank	https://www.ncbi.nlm.nih.gov/genbank/	GenBank is a public database of sequence genomic, transcriptomic, and proteomic data that can be submitted by individual research groups from around the world. GenBank data is not curated but does contain submitter-provided annotations. Redundant sequences are commonly found on this database. The advantage of using GenBank is the greater representation of rare species, newly discovered genes, and gene variations.
RefSeq	https://www.ncbi.nlm.nih.gov/refseq/	Genomic DNA, transcript (RNA), and protein sequences available listed as RefSeq on the NCBI database are curated, annotated, and checked for non-redundancy. RefSeq sequences tend to be more reliable as compared to GenBank, however they predominantly represent extensively studied organisms. Novel genome sequences, newly discovered genes, and rare species that are not as widely studied are scarcely available on RefSeq.
Alliance of Genome Resources (Alliance); other species-specific repositories	https://www.alliancegenome.org/	Maintains the following: • FlyBase, *Drosophila spp.* (https://flybase.org/) • Mouse Genome Informatics (MGI), *Mus musculus* (https://www.informatics.jax.org/) • Rat Genome Database (RGD), *Rattus norvegicus* (https://rgd.mcw.edu/) • *Saccharomyces* Genome Database (SGD), *Saccharomyces cerevisiae* (https://www.yeastgenome.org/) • WormBase, *Caenorhabditis elegans* and related nematodes (https://www.wormbase.org/) • Zebrafish Information Network (ZFIN), *Danio rerio* (https://zfin.org/) • Arabidopsis Information Resources (TAIR), *Arabidopsis thaliana* (https://www.arabidopsis.org)

[a]<original to the author>

publicly available genetic sequence data along with various relevant sequence-related information (e.g., metadata, gene structure annotations). These are summarized in Table 11.1.

How to choose the most appropriate public sequence database for your needs:
RefSeq is sufficient for most molecular biology and laboratory classroom needs. The highly curated content is well-annotated and the RefSeq interface includes robust help and tutorial support. GenBank sequences are not as closely curated but can be a great source of unique sequences that contain, for example, differences between strains that are not represented in a reference sequence.

RefSeq might not be sufficient if your classroom has advanced and specialized needs, which is often the case when working with less-widely researched and non-model organisms. The Alliance of Genome Resources (Alliance) maintains many species-specific databases for model organisms. If your organism of interest is not represented in RefSeq or Alliance, start by identifying where the sequence data of interest are published, since there might be only one option for sourcing sequence data. When data are hosted across multiple databases, we encourage instructors and students to consider the species of interest, gene(s) of interest, research question and overall learning objectives. Additionally, students may need to gain experience navigating databases specific to a sector or species of interest. For example, the United States Department of Agriculture (USDA) maintains the Ag Data Commons (https://data.nal.usda.gov/search/type/dataset), a repository of data generated for agriculturally relevant organisms and including omics data.

For computational biology courses in particular, it may be more suitable for students to gain experience navigating one of the more commonly used databases hosted by one of the members of the International Nucleotide Sequence Database Collaboration (INSDC), such as the Annotated/Assembled Sequences database (https://www.ddbj.nig.ac.jp/ddbj/index-e.html) hosted by the DNA Data Bank of Japan (DDBJ, Japan), GenBank® (https://www.ncbi.nlm.nih.gov/genbank/) hosted by the National Center for Biotechnology Information (NCBI, USA), or the ENA Browser (https://www.ebi.ac.uk/ena/browser/home) hosted by the European Nucleotide Archive (ENA, UK).

In some courses students generate or use sequence data generated in-house. Or they might use data generated from unique samples that contain a mutation or other disparity from the greater population. In these instances, the data hosted on a widely used database or repository such as those mentioned above may not be suitable for subsequent CRISPR learning activities such as gRNA design. However, the ability to navigate, interpret, and obtain high-quality information and data from such databases and repositories is a requisite skill in many subfields of biological sciences. Therefore, it may be valuable for students to obtain and compare the reference sequence data (i.e., reference genome) from the organism of interest to the unique data.

11.2.1.2 Sequence Visualization Tools

Sequence browsing, visualization, and scanning tools facilitate a user's conceptualization of the gene of interest and understanding of various features of sequence data that may heavily influence choices made in experimental design. For introductory or overview-level instruction of CRISPR workflows, web-based genome browsers that provide a graphical interface displaying sequence data are sufficient (e.g., for activities such as gathering target gene location information). Many of the repositories described in Table 11.1 provide free, user-friendly, web-based tools to visualize these biological data. For example, NCBI hosts Genome Data Viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/), a platform that enables the visualization of over 1500 NCBI-annotated and select non-NCBI annotated eukaryotic genome assemblies. Similarly, MGI hosts the Multiple Genome Viewer (https://www.informatics.jax.org/mgv/), a tool that allows users to browse and compare multiple mouse genome sequences as well as the genome assemblies for humans and the other

model organisms. Wang et al. provide a comprehensive review of general (i.e., multi-species) and species-specific genome browsers, the vast majority of which are still in use today [7].

11.2.1.3 Sequence Management Tools

When course learning objectives and activities require students to use their own data and/or progress through the stages of designing and potentially carrying out a CRISPR experiment, instructors may prefer to incorporate the use of higher-capacity sequence management applications.

Desktop-, web-, and/or cloud-based applications with additional suites of tools go beyond sequence browsing (visualization) and provide a variety of sequence management and manipulation features. In many cases, these platforms access and incorporate data from the original host institution through known identifiers (e.g., GenBank® accession number) and/or support user uploads. Table 11.2 presents some of the most widely used applications for sequence management and other tasks such as *in silico* testing for the

Table 11.2 Common tools for sequence management, modification, and visualization[a]

Tool	Primary functions	Platform	License	Link
Benchling R&D Cloud	Sequence management, modification, and visualization	Web[b]	Free[e]	https://www.benchling.com/
SnapGene®	Sequence management, modification, and visualization	Desktop	Purchase required[e]	https://www.snapgene.com/
CLC Genomics Workbench	Sequence curation, management, modification, visualization, modeling, and analysis	Desktop[b]	Purchase required[e]	https://digitalinsights.qiagen.com/
NCBI Genome Workbench	Sequence management, alignment, and visualization	Desktop[d]	Free	https://www.ncbi.nlm.nih.gov/tools/gbench/
Ensembl Genome Browser	Sequence acquisition and visualization	Web[c]	Free	https://useast.ensembl.org/index.html
UCSC Genome Browser	Sequence acquisition, management, and visualization	Web[c]	Free	https://genome.ucsc.edu/index.html
Geneious Prime	Sequence acquisition, management, and visualization	Desktop[b]	Purchase required	https://www.geneious.com/prime/

Licensure information is for use in an academic setting (i.e., cost of licenses for commercial or enterprise purposes may vary)

[a]<original to the author>

[b]Cloud-based options available

[c]Desktop-based options available

[d]Web-based options available

[e]Benchling R&D Cloud licensure is offered on different pricing tiers, including an academic version at no cost. Free versions of CLC Genomics Workbench and SnapGene® are available as CLC Sequence Viewer and SnapGene® Viewer, respectively. These free versions have fewer features than the software packages that require license purchase

exploration, identification, and/or visualization of CRISPR components (e.g., gRNAs, homology directed repair, HDR, templates).

11.2.1.4 Tips for Selecting the Right Sequence Visualization and Management Tools for Your Classroom

Less is more. Select ONE tool for your students to use for sequence visualization and management. Your selection should balance project scope, hardware/technology, budget, and institutional technology policy considerations.

Project scope

Consider the tradeoff of time spent accessing and learning new technology and time using that technology to learn about CRISPR/Cas biotechnologies. Also consider lesson/activity-dependent factors such as whether data will need to be shared between students.

Hardware/technology

Web-based applications offer many advantages over desktop software if your students use their own technology devices. If your students use a range of devices from tablets to workstations, using a web-based application will allow for ease of sharing information between devices that support the approved browser necessary to access the application(s). If students will use stand-alone software, we highly encourage instructors to work with their institution's technology programs to ensure that all students can access, download, install, and operate the software (i.e., obtain licensure requisite to facilitate student use).

Accessibility and security

When adopting new web-based tools, instructors need to consider their ability to provide access to computational resources for students. While most web-based tools for CRISPR and software packages are available for several platforms, it is worth noting that students may use tablets and Chromebooks as their primary devices (i.e., commonly used devices may be limited in configurations that support certain softwares, such as local storage). Importantly, instructors must read and provide learners with relevant links to the accessibility and privacy statements of the software used. It is recommended that before using software as part of a course and requiring students to perform activities that rely on web-based tools accessed via required sign-in and/or registration, you speak to your informational technology and accessibility staff on your campus to determine if there are accessibility or student privacy concerns. For example, if students are using an institution-affiliated login for a website, information technology specialists can help determine if there are any potential issues. For accessibility, web-based content may be inaccessible to learners using assistive technology such as screen readers or with limited mobility.

Institutional information technology policies, procedures, and infrastructure

Instructors should also be aware of specific purchasing requirements and restrictions at their institution. If software are purchased from vendors not in the institutional system, the

process may require approvals or, in some cases, justification or an alternative. If group work is desired, the number of active licenses or devices may not be suitable for use of existing computing resources, for example. In these cases, the instructor should think carefully about the computational tasks and assignments so that all learners have an opportunity to engage with the tools and explore the concepts.

Budget
Free software is available but has limited functionality and/or requires establishing accounts that may or may not be in alignment with your institution's information technology policies.

Don't let versioning get in the way!
Software companies including SnapGene may offer free student licenses for instructors who already have a paid subscription. SnapGene's website provides more information and a useful resource for instructors to share and manage student activation of licenses for a course. Free websites and servers hosting CRISPR may behave differently on student devices that are not maintained by institutional information systems such as managed desktop and active directory. Instructors should keep this in mind when developing tutorials. We have found that linking directly to the tutorial materials provided by the developers often is less confusing to students even though these resources may not include instructor or institution specific information.

11.2.1.5 Highlights and Considerations for Recommended Software/ Applications (Table 11.2)

Benchling R&D Cloud (Benchling, USA) is a cloud-based Laboratory Information Management System (LIMS) with a variety of workflows and tools that enable experiment planning and design directly alongside sequence visualization and management. Benchling R&D Cloud is specifically recommended for users seeking a comprehensive interface without having to download an application and/or those that will be working collaboratively on a single platform, as Benchling allows multiple users to keep track of notes, progress, and metadata in a shared, structured environment. It is important to note that of the applications listed in Table 11.2, Benchling R&D Cloud offers CRISPR-related tools that are applicable across study species, a gRNA design tool (https://www.benchling.com/crispr), as well as CRISPR-focused support for educators and other users in the form of "Training kits" and "Application notes."

SnapGene® (Dotmatics, USA) is an intuitive sequence management, annotation and cloning tool that allows students to explore data imported by the user or via NCBI accession number(s). Data can be stored locally (i.e., on the computer) or through the SnapGene Server to enable collaboration and data sharing. One of the most useful functions of SnapGene in the instruction of CRISPR is the ability to readily annotate and modify sequence data. SnapGene Viewer® is offered as a free version of SnapGene®.

CLC Genomics Workbench (Qiagen, Hilden, Germany) is among the more comprehensive of the applications listed in Table 11.2 in terms of the number of tools offered and depth of tool functionality. Among the numerous tasks the desktop-based CLC Genomics Workbench enables users to perform are: sequence visualization, editing, alignment, extraction, organization, quality control, and trimming; de novo genome assembly; RNA-Seq normalization, quantification, and differential expression analysis; primer design; restriction site analysis; variant detection; and the creation of graphics and trees. Data can be stored locally or through the CLC Genomics Server to enable collaboration or the storage, access, and use of high volumes of data. For the purpose of CRISPR instruction, and if technology availability and obtaining a license are not factors, incorporating CLC Genomics Workbench would be beneficial for courses where students are expected to perform several sequence management and analysis tasks beyond designing a CRISPR-experiment (e.g., validation through RNA-Seq or microarray analysis). The CLC Sequence Viewer is offered by Qiagen as a free version of the CLC Genomics Workbench with fewer tools and does not allow for CLC Genomics Server access.

The NCBI Genome Workbench (NCBI) [8] is an ideal desktop-based tool if students will be using data hosted through NCBI, performing tasks such as sequence alignment or creating graphical representation(s) of sequence data, and/or planning to submit sequence data to NCBI at some point in the workflow. A web interface of the NCBI Genome Workbench is offered as the NCBI Sequence Viewer (https://www.ncbi.nlm.nih.gov/projects/sviewer/).

When you need a little more than a genome visualizer, of the applications listed in Table 11.2, the Ensembl Genome Browser (EMBL-EBI) [9] and UCSC Genome Browser (University of California Santa Cruz, USA) [10] are the most similar to other web-based genome browsers and therefore, depending on student experience and coursework scope, may be the best for acquiring sequence data, general genome browsing, and gene exploration. Both browsers allow users to upload their own data, create groups/projects, and save progress, and share between users. Additionally, both browsers offer multiple, similar tools to perform tasks such as identifying homologous sequences. Notably, the Ensembl Genome Browser includes annotation tracks of predicted CRISPR/Cas9 sites for the human and mouse genomes. Similarly, the UCSC Genome Browser hosts an annotation track of CRISPR/Cas9 target sites for the mouse genome.

Geneious Prime (Dotmatics, USA) is a versatile software with user-friendly graphical user interface that allows users to perform a variety of tasks from annotation to Sanger sequencing and next-generation sequencing (NGS) analysis. Geneious Prime offers CRISPR-specific tools: CRISPR site finder and Analyze CRISPR Editing Results to identify target sites of Cas9 and Cpf1 and measure the frequency of variants around the edited site, respectively. Geneious Prime supports multiple-user collaboration via the Shared Databases feature that allows users to share data, workflows, and other documents.

11.2.2 Guide RNA Design

Designing gRNAs for CRISPR-based experiments requires critical thinking skills to make decisions at several steps of the process and therefore is easily incorporated into the teaching environment. Specifically, proper gRNA design represents a student's understanding of gene structure, CRISPR/Cas functioning, evaluation of possible targeting consequences, and other considerations that require knowledge of biological systems. The development of an incredible number of free web-based tools for gRNA prediction and design has made this process highly accessible and a low material (e.g., consumables, reagents) input activity for the classroom. The extent to which a gRNA tool requires critical thinking is largely a function of the number of input parameter options (e.g., guide length, PAM sequences, reference genome and target location) and output metrics used to evaluate potential gRNAs (e.g., efficiency, specificity) that users must interpret when making a selection. Liu et al. provide additional details about computational approaches for scoring guide RNAs; in the classroom setting, we find it is useful to emphasize the significance of guide length, PAM sequences, and target location [11].

11.2.2.1 Overview of Common Tools

The gRNA prediction and design tools presented in Table 11.3 are widely used tools that support users identifying gRNAs across genomes for multiple species (150 or more) and have the option of uploading genome assemblies for organisms that are not currently listed. These tools also allow users to select from multiple Cas effector proteins and corresponding protospacer adjacent motif (PAM) sequences. Additional gRNA design tools mentioned below are included here based upon being popular for specific areas of study (e.g., Genus-specific tools, CRISPR modification-specific tools), user friendliness, ability to order designed reagents, and/or having other features that may be useful for instruction.

The tools listed incorporate genome data from multiple species and support input sequence data uploaded by users. These tools also allow for users to select other Cas effec-

Table 11.3 CRISPR-Cas guide RNA design tools[a]

Tool	Supported genomes and Cas effectors	Comprehensiveness of gRNA evaluation	URL link
Benchling	Few	Moderate	https://www.benchling.com/crispr
Cas-OFFinder	Several	Fair	http://www.rgenome.net/cas-offinder/
ChopChop v3	Several	Very	https://chopchop.cbu.uib.no/
CRISPOR	Many	Very	http://crispor.tefor.net/
GuideMaker	Many (user-defined)	Moderate	https://guidemaker.app.scinet.usda.gov/

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tor proteins (e.g., Cpf1) in addition to Cas9. The number of supported genomes and Cas effectors are indicated as "Few", "Several", and "Many." These distinctions are made *relative to the other tools listed here*, whereby the range of supported genomes is 160 (Few) to 800 (Many) and supported 3 (Few) to 38 (Many) Cas effectors. No distinction is made for GuideMaker, as users can upload any sequence data and define any PAM sequence. The comprehensiveness of gRNA evaluation output of a given tool is indicated as "Fair", "Moderate", or "Very" and is also *relative to the other tools listed here*. Comprehensiveness is considered "Fair" when metrics such as location, position, direction, number of mismatch bases, and potential off-targets are reported; "Moderate" when those metrics and a ranked list of gRNAs are provided; and "Very" when those metrics, a ranked list, and effector-specific scores of specificity and efficiency are provided, possibly alongside other qualifying values or notes. The listed tools are offered as web-based interfaces and, with the exception of Benchling, as command line interface packages and/or available for local (i.e., desktop) installation.

11.2.2.2 Tips for When to Use Specific Recommended Software/ Applications

Instructors and students are likely to find that each gRNA design tool and unique features lends itself to achieving specific CRISPR-experiment and learning objectives. Some of the specific advantages or scenarios in which the tools listed in Table 11.3 are particularly well-suited for are described below and alongside use case scenarios for other tools.

For Sensitive and Thorough gRNA Evaluation:
CRISPOR and GuideMaker are considered among the most sensitive gRNA design tools as both tools report several gRNA evaluation metrics of specificity, efficiency, etc [12, 13]. For example, CRISPOR output includes two widely used efficiency scoring methods as reported by Doench et al. and Moreno-Mateos et al., and in addition to other, Cas-specific metrics when applicable [14, 15].

High Data Volume:
Cas-OFFinder may be particularly useful when processing large amounts of data (e.g., large genome assembly, multiple CRISPR screens) based on the efficiency of algorithms employed to predict gRNAs [16].

Highly Modifiable Guide Parameters:
E-CRISP (http://www.e-crisp.org/E-CRISP/) allows users to search for targets using ENSEMBLIDs or import FASTA sequence and is the most customizable tool mentioned in terms of tailoring guide parameters such as percent specific nucleotide content, excluding targets with poly motifs, intended edit type, and application sensitivity, to name a few [17].

Edit/Mechanism-Specific Workflows:
ChopChop v3 allows users to designate intended edits (e.g., knock-out, repression, etc.) and also supports TALEN-directed mutagenesis [18]. CRISPR-ERA (http://crispr-era.stanford.edu/index.jsp) allows users to designate the desired manipulation as Editing (via nickase or nuclease), Repression, or Activation (hence "-*ERA*") and is compatible with human and model organism genomes and CRISPick (https://portals.broadinstitute.org/gppx/crispick/public), hosted by The Broad Institute of MIT (USA), enables gRNA prediction using human, mouse, and rat reference genomes hosted through NCBI and Ensembl for knock-out, activation, and inference CRISPR-mechanisms [19]. The Synthego CRISPR Design Tool (https://design.synthego.com/) allows users to select from over 120,000 genome assemblies representing over 9000 species to design gRNAs specifically for Cas9-mediated knock-outs. Users of the Synthego tool also have the option to order the designed guides.

11.2.2.3 Experiment Analysis Tools

Validation of CRISPR gene-editing is essential and required for meaningful interpretation of data. To determine whether the designed guide RNA has produced the predicted effect, a number of orthogonal experiments may be performed to test the activity of the targeted gene product. Orthogonal validation is difficult when the edit produced is not easy to analyze, such as a silent or non-disruptive mutation. Additionally, direct sequence-level verification of the gene edit is often desirable. The TIDE software (http://shinyapps.data-curators.nl/tide/) uses Sanger sequencing data to statistically detect and measure small insertions and deletions in sequence (indels) [20]. A multi-sample strategy uses the related TIDE batch software for similar analysis. TIDER is another computational tool for template-based detection and analysis of point mutations as well as small indels. TIDER requires additional experimental steps, specifically transfection of a template oligonucleotide along with the gRNA and Cas9 [21].

Applications that Include Other CRISPR-Relevant Tools
The Benchling gRNA design tool (Table 11.3) is offered through the Benchling R&D Cloud, a cloud-based application that supports other CRISPR experiment design and planning activities. CRISPRscan (https://www.crisprscan.org/) is a web-based platform hosting a suite of tools including searching for gRNAs on genes, predicting possible gRNAs and off-targets, visualizing gRNAs on model organism genomes, generation and ordering of gRNAs to use in the lab [15]. Additionally, some biotechnology product companies have developed suites of tools that can be used for CRISPR instruction and research (Table 11.4).

Table 11.4 CRISPR-Cas tools from product vendor companies[a]

Tool	Features	Requirements	Link
Integrated DNA Technologies CRISPR tools	CRISPR-Cas9 guide RNAs (gRNAs, such as crRNA and sgRNA) creation Analysis of off-target effects against human, mouse, rat, zebrafish, or *C. elegans* genes are available	Web-based	https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM
ThermoFisher Scientific Invitrogen TrueDesign Genome Editor	Design and order custom CRISPR and TALENs materials for mechanism-specific edits in human, mouse, rat, zebrafish, and roundworm, as well as custom primers for subsequent verification experiments	Web-based; requires account	https://apps.thermofisher.com/apps/genome-editing-portal/
GenScript guide RNA design tool	GenCRISPR gRNA Design Tool Design has human and mouse genome information.	Web-based	https://www.genscript.com/gencrispr-grna-design-tool.html

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11.2.3 Teaching and Learning Aids

11.2.3.1 Practical Web Tools for Lab Design

Bringing CRISPR-Cas technologies to the laboratory classroom has never been easier and there are many computational tools to help make custom lab design for CRISPR-Cas experiments simple, reliable, and convenient. Educators have developed courses for undergraduates that use CRISPR technologies in a variety of contexts, including lab-based, online, and at-home kits. There are numerous web-based tools that aid in the design of lab experiments and selection of appropriate reagents. For example, Dahlberg and Groat Carmona list several courses using CRISPR technologies [22]. For one course using CRISPR-Cas9 gene editing in yeast, the instructors used the *Saccharomyces* Genome Database (https://www.yeastgenome.org/) and Benchling's CRISPR analysis tool [23]. A course that used CRISPR-Cas9 for reverse genetic studies in *Arabidopsis thaliana* used the E-CRISP (http://www.e-crisp.org/E-CRISP/) tool for gDNA design for their assigned genes [24].

For a lower-level undergraduate teaching lab, certain vendors offer pre-packaged solutions for hands-on CRISPR experiments that require minimal supplementary resources (Table 11.5). These also come with teaching materials for multiple lessons where students can learn foundation concepts in CRISPR.

Building on a basic framework, subsequent steps will involve added lab sessions that explore different CRISPR applications and validation strategies. Published articles, from both research and pedagogical journals are a good resource for developing CRISPR teaching labs. However, it is often beneficial to work with genes, model systems, and delivery modes that have already been established at the instructor's institution. This makes troubleshooting easier, especially when you are no longer working with turnkey or pre-packaged CRISPR kits. Table 11.6 outlines a general workflow that an instructor may use to design a custom lab activity.

Table 11.5 Turnkey solutions for an undergraduate CRISPR lab[a]

Resource	Description
BioRad *Out of the Blue* CRISPR Gene-editing kit https://www.bio-rad.com/en-us/category/crispr-gene-editing-kits?ID=Q0JG5VTU86LJ	Good resource for conducting a basic CRISPR experiment in the classroom. Minimal resources necessary. Ideal for lower level courses. Extendable to 2–3 lab periods using Genotyping extension kit.
Innovative Genome Institute *The Power of CRISPR* Kit https://innovativegenomics.org/crispr-classroom-kit/	Turnkey kit with minimal outside resources required. Ideal for small colleges, and for lower level courses. Comes with a six-lesson plan for introduction to CRISPR foundations.

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Table 11.6 Design a customized CRISPR experiment for a teaching lab using the following open-source web-based tools[a]

Application	Example resource
Visualize and annotate target sequence	Benchling
Modify the CRISPR target region – Identify new sequence – Select a different guide RNA – Detect Off-target hits	NCBI databases Benchling CRISPR tool CRISPOR
Select the Appropriate Cas Effector	CRISPOR
Design delivery vehicle and strategy (multiplexing)	Benchling, Addgene (for purchasing plasmids)
Order regents	Integrated DNA Technologies, Synthego, GenScript, ThermoFisher Scientific or equivalent

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11.2.3.2 Using "CRISPR 101" Manuals in Lieu of a Textbook

Do you need a textbook to teach a CRISPR course?

Despite being a relatively new field, a small number of well curated books are available. Most of these books are aimed at researchers and may not be the best resource for undergraduate students, however. The field, although rapidly evolving, does have some core fundamentals vital to understanding advanced applications. For classroom use, textbooks provide a familiar structure, especially for undergraduate students. Several commonly used molecular biology and biochemistry textbooks provide a strong foundation on CRISPR fundamentals, but understandably stop short of diving into types of CRISPR-Cas systems. We find several online materials, many available free with registration, to be highly useful study materials in lieu of traditional textbooks for a CRISPR-focused classroom. In coming sections, we will highlight some of the commonly available materials, comparing their strengths and limitations. Overall, the selection of materials highlighted here have proven to be a reliable option as a teaching resource for upper-level undergraduate and graduate students. We conclude that it is not necessary to have a traditional textbook for teaching a CRISPR-focused course. CRISPR handbooks and manuals described here are effective teaching materials alongside current review articles, research papers, and other web-based resources.

Overview of Current Resources

Textbooks focused on CRISPR courses at an undergraduate level are not widely available yet. However, there are some resources that instructors can use for their class. In Table 11.7 we list some of the currently available options. Paid access books are limited and generally aimed at advanced researchers. For undergraduate and graduate students being introduced to CRISPR, the vendor developed options are a reliable alternative.

Table 11.7 Commonly available textbooks and vendor-developed open-source learning materials for a CRISPR classroom[a]

Title	Publisher	Access type	Description/emphasis
CRISPR Gene Editing Methods and Protocols	Springer eBook ISBN: 978-1-4939-9170-9	Paid access; available as eBook	Ideal for advanced users.
Genome Engineering via CRISPR-Cas9 system	Elsevier eBook ISBN: 978-0-12-818140-9	Paid access; available as eBook	Good option for beginner or intermediate research students.
CRISPR 101	Addgene https://www.addgene.org/educational-resources/ebooks/	Free with registration	Comprehensive guide covering design, delivery, and new technologies. Actively used at the NC State Biotechnology program.
CRISPR Handbook	IDT https://go.idtdna.com/TheCRISPRbasicshandbook	Free with registration	Good introductory resource for students. Integrated with IDT product catalog.
CRISPR Handbook	GenScript https://www.genscript.com/CRISPR-handbook.html	Free with registration	Features case studies and current workflows.
CRISPR 101 eBook	Synthego https://www.synthego.com/resources/crispr-101-ebook	Free with registration	19-page resource with useful graphics.
CRISPR-Cas Gene Editing Teaching Resources (including Bioinformatics activity)	BioRad https://www.bio-rad.com/en-us/applications-technologies/crispr-cas-gene-editing-teaching-resources?ID=Q58I0DWDLBV5	No registration required.	Short web-based resource, easy to use and access. Powerpoints, paper activities, and bioinformatics activity available

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These options provide good background information along with description of homology directed repair and nonhomologous end-joining. E-books from Addgene and IDT are the most favorable for exploring a wide array of applications. Additionally, instructors and students may find generative AI tools, such as "GenomeGuide for CRISPR Research" accessible through ChatGPT, useful in answering questions pertaining to the CRISPR-Cas system and/or research guidance. As CRISPR technologies continue to develop rapidly, however, journal articles and recent review papers are a more comprehensive resource of recent advancements.

Do online resources help navigate computational aspects of CRISPR technologies?
One overarching limitation of the CRISPR handbooks and manuals that we will describe below is that none of them provide guidance with the varied computational aspects of the CRISPR workflow. Nonetheless, having a text resource can be valuable for those new to the field. Table 11.7 lists some of the commonly available options for text resources to include as part of a CRISPR course.

11.2.3.3 Student-Teacher Interfaces

Experimental design
Benchling is a powerful open-source resource for sequence management, annotation, and experimental design. SnapGene®, another similar but paid resource, has certain advantages mainly that it is not entirely web-based and therefore has added data security features. SnapGene® also has easier integration with plasmid maps available through Addgene. For teaching a CRISPR class on a budget, Benchling is a good option, especially where working on a web-based platform may be more seamless for students and that also enables cloud-based data and file sharing. In either case, the sequence viewer is an essential student-teacher interface for an advanced CRISPR course. The instructor can use this interface to evaluate student work by reviewing annotations of the sequence files, provide feedback, and direct proper design of delivery vehicles for gRNAs and Cas genes. This becomes particularly useful when students have free rein over choosing multiplex delivery strategies which are challenging to review in the absence of a tool such as Benchling.

Group work for exploring new concepts and applications
The CRISPR field is developing at a fast pace with innovative applications constantly being added to the repertoire of scientists. When designing course content, it can be challenging to devote sufficient time and emphasis to each new application. This is where exploratory group work during class hours can be a useful strategy. Leveraging cloud-based documentation tools, students can work in small groups to explore a novel CRISPR application, highlighting its key features. The main advantage of this approach is that students can work at the same time on an online collaborative document on a cloud-based drive such as Google Drive or Microsoft OneDrive, or similar platforms. Students create a tangible product that can be archived and used as reference material. The use of cloud-based platforms also enables sharing and collaboration in online learning environments and other scenarios in which face-to-face interaction between students and instructor(s) is limited or does not occur entirely. It also allows students to explore their own interests in CRISPR applications. A potential limitation of this strategy is that of accessibility, especially when students design collaged content on slides. One way to overcome this is to provide a template and structure for student input, delineating features including Alt-Text requirements, font size, image resolution, among others.

11.2.3.4 Tips for Lesson Design

CRISPR technologies and applications continue to extend well beyond the initial thoughts of CRISPR technology potential; in parallel, the number of tools available for analysis of CRISPR-related applications is ever-growing. It is now critical that educators incorporate CRISPR-related skills into their lessons to train students to effectively use software tools to design more robust experiments. Designing CRISPR lessons that highlight the use of technology will also address fundamental learning objectives for courses and curricula and requires attention to evidence-supported approaches, inclusion of equitable practices, and selection and appropriate use of accessible technologies. The expansive growth of applications and tools has also resulted in numerous course-based undergraduate and graduate research experiences using CRISPR technologies addressing learning objectives often particular to the course and institutional context (i.e., IT policies, budget constraints, etc.).

Educators incorporating CRISPR activities should think carefully about the affordances and resources needed to engage learners in online and in-person CRISPR activities. In some cases, lessons include demonstrations and troubleshooting that can be facilitated by in-person interactions. Alternatively, some computer tasks require compute time or time for learners to design appropriate approaches. In these cases, an asynchronous online format has the benefit of providing a learner with individual time to process, troubleshoot, and reflect. In all cases, the educator must consider the course learning objectives, students, and available personnel and computing resources to determine which format is best.

Common hurdles related to student use of computational tools:

New users of computational CRISPR tools may lack the familiarity with bioinformatics software and confidence to learn by exploration. The ability of students to feel confident in making appropriate decisions regarding input and/or selection parameters, the use of tools provided by an application, and interpretation of output information to derive meaningful conclusions and genuinely achieve learning is highly dependent on the extent to which students are comfortable with gene sequence analysis and genetic engineering concepts. For example, an entry- or mid-level biology student with no prior exposure to sequence analysis may be rapidly overwhelmed by the vast array of tools available and subsequently distracted from the more specific CRISPR/Cas-focused activity at hand.

To address these hurdles, remember: less is more. When selecting tools, consider reasonable expectations for the course. There is a cognitive cost associated with orienting to and learning to use each new tool. From the student's perspective, a lesson involving a small number of tools in a streamlined workflow is better for their learning than a lesson that uses many different tools, even if those tools are the "best" or "most cutting-edge" tools for each function (i.e., as is the practice of a discipline-based research project). Start small, use tutorials, and demonstrate software use and examples to help students build the confidence to explore.

Troubleshooting alone can be discouraging for learners developing self-confidence and self-regulation. To keep students centered on the importance of learning computational skills, incorporate those competencies into course expectations; design and improve activities that support learners in the development of bioinformatics competencies such as those shared by the Network for Integrating Bioinformatics into Life Sciences Education (NIBLSE) [25, 26].

Assessment and evaluation considerations:
As new CRISPR-related applications are developed and online resources extend the possibilities of engaging students in the use of CRISPR-related training, educators must continuously evaluate the effectiveness of their lessons. For example, to our knowledge concept inventories specifically on CRISPR have not been developed. Similarly, question banks with CRISPR questions are scattered throughout numerous fields including genetics, cell biology, molecular biology, bioinformatics, and computer science! Communities of instructors and publications of lessons utilizing CRISPR-Cas technologies in the classroom are rich sources of advice to successfully engage students in authentic learning.

As instructors prepare lesson plans and craft learning objectives for students to engage with CRISPR-related bioinformatics tools, best practice is to consider assessments and evaluations at the design phase. Reflections and open-ended questions can help evaluate process-oriented thinking, experimental design skills, and identify misconceptions, though these may not be feasible for large enrollment courses. Multiple-choice questions can be crafted to address key objectives for both high and lower order thinking skills yet require careful design and interactive improvement. Regardless of the method of assessment, it is imperative that instructors design their assessments and evaluations to independently evaluate: (1) student skills in using computational tools for CRISPR, (2) student understanding of concepts in biology, (3) student experimental design skills, and (4) student laboratory performance skills. Students who struggle to use computational tools may have a strong grasp on CRISPR and its uses; assessments and evaluations that do not decouple this risk mis-evaluating student learning.

11.2.3.5 Model Activities

In the lab, a CRISPR experiment is indistinguishable from a standard molecular biology experiment. Students learn the same hands-on skills—pipetting microliter volumes, reagent delivery to cells, DNA extraction, etc. CRISPR-Cas experiments distinguish themselves at the experimental design stage. Experimental design is a goal for many instructors, an essential competency for molecular biologists, and is highly amenable to using computational tools and to lessons for online or virtual instruction. We anticipate that as more structured educational materials continue to be developed for CRISPR/Cas technologies, the available lesson plans and laboratory kits will also increase in number. Instructors are encouraged to look online for lesson plans, workflows, and similar materials produced by other educators. Below, we summarize use of computational tools in model activities for *in silico* project design and blended physical and computational lab activities.

In silico project design:
In silico design is an essential part of any application-focused CRISPR course, regardless of the course being offered in-person or online. Similar software and web-based tools can be used in both formats. Students can be introduced to design resources, and they can be expected to pick a design strategy that works for the assigned project. In a techniques-focused laboratory course, it may be preferable to identify one or two design software and prepare detailed guidelines and a step-by-step instructional resource. At NC State University, the CRISPR Technologies course is normally taught using the latter strategy, where we have used CRISPOR—an open-source, web-based, guide RNA design and evaluation software. Along with detailed notes and demonstrations, students are provided a detailed how-to video. In a standard semester, students spend about 1–2 weeks early in the semester to work with CRISPOR and replicate gene site selection and gRNA design procedures; after creating a manuscript-style figure describing the target site and the instructor's selected gRNAs, students conduct hands-on CRISPR experiments during laboratory hours to use the selected gRNA in a CRISPR experiment.

As a model example: Samsa et al. present an *in silico* gRNA design lab activity for advanced undergraduate and graduate biology students, whereby students are prompted to select a research scenario and navigate multiple computational tools and applications to identify and evaluate the gRNAs optimal for achieving the intended experimental goal [27]. Specifically, students navigate NCBI GenBank® to locate the reference genome of the target organism specific to the selected research scenario, utilize SnapGene® for visualization and annotation of the target sequence (i.e., region of interest), and CRISPOR for gRNA design using a web-based software mentioned in Table 11.3 above [27]. The lesson plan provided by Samsa et al. then walks students through evaluating the output gRNAs taking into consideration the ordered ranking provided by CRISPOR, location relative to the target and in the context of the intended edit, predicted gRNA structure, on-target scores, and predicted off-target sites. CRISPOR output (i.e., gRNA sequences aligned to target region) can be downloaded in compatible formats for a number of relevant softwares including SnapGene®, used by Samsa et al., Benchling and Geneious Prime, described above, and others. As mentioned previously, CRISPOR is considered one of the more sensitive gRNA design tools and outputs several metrics for users to consider when evaluating gRNAs, including performance scores developed for specific Cas enzymes [12]. Further, the CRISPOR platform links to a detailed user manual describing these metrics further and provides contact information for user support. The CRISPOR platform itself is relatively simple—users are not overwhelmed by a multitude of tools and options to select from, enhancing the user-friendly intuitiveness of this application. Samsa et al. reports student feedback describing the lab activity and specifically the use of CRISPOR, in combination with NCBI GenBank® and SnapGene®, as an activity that deepened their understanding of these tools and broadly gRNA design for CRISPR/Cas experiments, despite any hurdles encountered in weighing evaluation metrics to select the best gRNA for the intended outcome.

Another model example is Pieczynski and Kee's "*Designer babies?!" A CRISPR-based learning module for undergraduates built around the CCR5 gene* [28]. In this creative module, students perform a wide range of *in silico* tasks to replicate CRISPR-Cas9 editing

of CCR5 while exploring the ethics of the incident of these edits being performed in children [29]. They use SnapGene(R) as their primary tools for exploring the central dogma of molecular biology, bioethics, and molecular biology techniques through *in silico* predictions of a CRISPR-Cas9 gene editing experiment.

Blending hands-on lab and hands-on keyboard:
Hands-on experience with CRISPR is highly valuable, as this technology is not purely computational and requires careful planning of experiments. Mastery of molecular biology techniques through other courses, can offset some of the emphasis on hands-on laboratory work in a CRISPR-focused course. However, validation of CRISPR results is critical, and experience in planning and conducting validation experiments are desirable skills. Depending on the system and organisms used in a particular CRISPR experiment, experimental validation can be extremely complex. In a CRISPR course, it is essential that we convey this complexity to students and involve them in designing, albeit conceptually, orthogonal experiments that validate predicted results. Beyond experimental work, the "hands-on" activity should extend to practical experience of applying CRISPR computational tools in design, on/off-target screening, validation, and visualization. The *hands-on keyboard* experience in using computational tools is as vital to a successful course as any wet-lab experience.

Sankaran, Smith and Roy's "*CRISPR-Cas9 Gene Editing in Yeast: A Molecular Biology and Bioinformatics Laboratory Module for Undergraduate and High School Students*" is an excellent model of blending molecular biology laboratory activities and bioinformatics into a highly applicable lab module [23]. Students use a single application—Benchling—to access genomic sequence data, design gRNAs to knock out ADE2 (causing a red color phenotype), and design cloning activities. The experiment concludes with a visual readout of colony color that can easily be expanded to include sequence analysis of colonies and correlation with colorimetric phenotype. Indeed, for an entirely online course, McDonnell et al. send students at-home kits with all the necessary materials for students to conduct a CRISPR experiment to knock out ADE2 in yeast [30]. Students send samples back to campus for sequencing and use sequence analysis software to explore the different mutations created.

11.2.3.6 Using Computational Tools to Pivot CRISPR-Cas from a Traditional Laboratory Course to an All-Online Course

During the onset of the COVID-19 pandemic in 2020, the laboratory-intensive CRISPR technologies course at NC State University became a fully online course. The workflow outlined by Samsa et al. was incorporated into lab activities of the all-online version of the CRISPR Biotechnology course (BIT 495/595 CRISPR Technologies) at NC State University with similar feedback and thus success in meeting intended learning objectives [27]. The popular use of CRISPOR as a gRNA design tool and observed success of this lab activity for student learning supports the incorporation of CRISPOR as a tool for CRISPR instruction.

The course format change necessitated reimagining the learning outcomes to now focus on the *in silico* design aspects of the CRISPR workflows. Students worked in groups on

scaffolded projects to address specific goals implementing CRISPR-based strategies. Students made critical decisions such as choice of CRISPR-Cas system, guide RNA design, delivery strategy, and validation techniques. This experience highlighted that even in the absence of laboratory resources, a compelling course providing project-based training to upper-level students is achievable.

Teaching a fully online version of a CRISPR-focused course compelled instructors to design carefully thought-out case studies, where students participated in the design, delivery, and validation planning for the CRISPR case-study experiment. Each case study articulated a scenario where a CRISPR technology can be implemented to achieve a particular experimental goal. In an online class, having a framework to apply fundamental concepts to a practical application helps meet creative learning outcomes. The project framework provided to students should specify the CRISPR deletion or edit that is required, the organism(s) in which the experiments will be conducted, and the overall goal or direction of the project. Based on this framework, students built on the foundation from Samsa et al. and created permutations with alternate choices in gRNA design software, different CRISPR-Cas systems, different delivery and validation strategies, and even different target organisms [27].

Take Home Messages

Researchers continue to develop new applications leveraging the power of CRISPR-Cas technologies and improved bioinformatics tools and expanding databases will require novice life science professionals to understand key concepts and processes. To do so, educators are challenged to keep up with changing technologies and new tools. To do so and effectively integrate CRISPR-Cas9 technologies into courses and workforce development, educators and researchers must consider evolving bioinformatics resources and technologies and yet a need to have common learning goals/frameworks for students engaging in CRISPR-related work.

The community using and creating CRISPR lesson plans should address:

- *Core Learning Objectives:* What are the key concepts students need to understand to use these tools effectively?
- *Accessibility*: accessibility of tools to all learners (assistive technology, American Sign Language, captioning) by keeping current through learning communities, educational networks and national initiatives.
- *Hardware Resources:* web-based vs. software and associated data limitations.
- *Communication:* the community of educators using CRISPR-Cas9 technologies should share information through publications, workshops, and special topics such as this issue for instructional applications of CRISPR.

The community of educators has a wealth of information on CRISPR-Cas9 technologies that with these considerations and goals can be incorporated into courses in meaningful ways and sustainably used in future classes.

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Scaffolding CRISPR Lessons to Accommodate Learning Levels and Resource Availability

12

Donna L. Pattison

12.1 Introduction

In the short time between the discovery of CRISPR-Cas systems [1, 2] in 2012 and the awarding of the Nobel Prize in Chemistry in 2020, CRISPR has become a mainstream molecular biology tool for genome editing. It is important that the general public has a basic understanding of the science at the core of genome editing work in order to understand the potential benefits to society both in terms of advancement of knowledge across the life sciences and for the potential applications in human health care, agriculture, and environmental remediation of heavily contaminated industrial sites. A basic understanding of the power and pitfalls of any type of gene editing technology, and in particular of the CRISPR-Cas systems, is necessary for a full understanding of the ethical ramifications inherent in this endeavor.

CRISPR has become a mainstream term, appearing frequently in the news and popular science podcasts [3, 4]. Accordingly, it is appropriate to begin introducing the concept in high school, particularly since for many students, high school graduation will mark the end of their formal education in science. The content is also appropriate in non-majors general biology courses as the topic provides a rich opportunity to discuss the central dogma, viruses, and immunity in a "hot topics" fashion. For those majoring in the life sciences, a deeper, more thorough understanding of CRISPR-Cas systems is important as a basis for their future work as a research scientist and in evaluating and analyzing work in the field.

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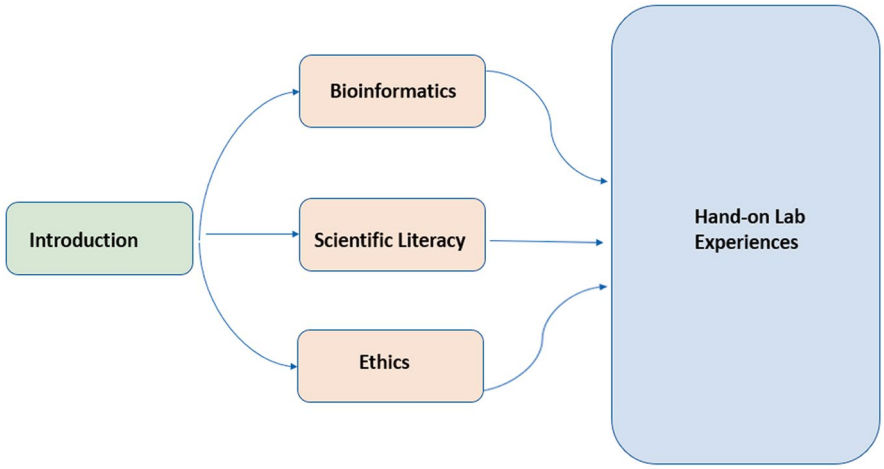

Fig. 12.1 CRISPR can be used as the basis for a module that simply introduces the topic, to providing students with experience in bioinformatics, reading and writing scientific literature, contemplating the ethical considerations of applications of scientific knowledge to a final expansion to hands-on laboratory experiences

12.2 Developing a Well-Scaffolded Learning Plan

CRISPR is a rich topic that can serve as the nucleating force for building a curriculum that not only exposes students to the mechanisms by which CRISPR-Cas systems serve as an adaptive immunity system in bacteria but can also serve as the center pillar for the development of other important skills needed for the workplace. Depending on time and academic level, activities can be developed to help students develop skills in oral presentations, scientific literacy, writing, and bioinformatics. Critical thinking skills can also be engaged through thoughtful discussions on the ethical issues associated with gene editing. While any of these skills can be taught in other contexts, developing a CRISPR-centered module helps students fully grasp the importance and inter-relatedness of these skills. A suggested flow of focus areas for an undergraduate level course is provided in Fig. 12.1.

12.3 Launching Students into CRISPR

An introductory lecture that highlights key terms and acronyms and includes a comparison of the innate immunity system in bacteria provided by restriction enzymes and methylation of chromosomal DNA compared to the adaptive immunity provided by CRISPR-Cas systems is important in setting up context and demonstrating the evolution of knowledge over time. A comparison of some of the key differences between these two defense systems are summarized in Table 12.2.

Table 12.2 Helpful videos for summarizing and reinforcing the basic concepts covered in the introductory lecture on CRISPR-Cas systems

Video	Content	Link
Greene Lab Studios: Cas9: The Enzyme, The RNA, & The Virus	A fun Western-themed very simple description of how Cas9 functions; released in connection with the article *DNA interrogation by the CRISPR RNA-guided endonuclease Cas 9* by Sternberg, Redding, Jinek, Greene, & Doudna in Nature (2014)	https://youtu.be/M739wgbcKuA
CRISPR: Gene editing and beyond by Nature	A summary of how Cas9 works and creative ways to create mutations, influence gene expression, and tagging proteins with fluorescent proteins.	https://www.youtube.com/watch?v=4YKFw2KZA5o&t=2s
Bozeman Science: What is CRISPR	An overview of CRISPR-CAS systems in adaptive immunity with attention to clearly defining terms in the process. Includes a little history and gene editing as well.	https://www.youtube.com/watch?v=MnYppmstxIs
Jennifer Doudna, Science Seminars Behind-the-Scenes Talks by iBiology.org	A more detailed overview of the CRISPR-Cas9 system for the more advanced undergraduate	https://www.snapgene.com/guides/design-grna-for-crispr
Nobel Lectures: Jennifer Doudna and Emmanuelle Charpentier	Jennifer Doudna and Emmanuelle Charpentier share their stories of discovery of CRISPR-Cas systems and the potential for applications.	https://www.youtube.com/watch?v=KSrSIErIxMQ and Nobel Lecture: Emmanuelle Charpentier, Nobel Prize in Chemistry 2020 - YouTube
How to design sgRNA sequences (TaKaRa)	A summary of the key steps in designing guide RNA for CRISPR experiments	https://www.takarabio.com/learning-centers/gene-function/gene-editing/gene-editing-tools-and-information/how-to-design-sgrna-sequences
How Gene Editing Could be Used for Cystic Fibrosis by the Cystic Fibrosis Foundation	An example of potential application of CRISPR-Cas9 for gene editing.	https://www.youtube.com/watch?v=Sp774i6tdzE

12.3.1 Restriction Enzymes

A module in CRISPR can be designed to cover the key concepts students need to know about restriction enzymes. For many years, it was assumed that restriction enzymes were the only defense bacteria had against invading viruses. The harnessing of bacterial restriction enzyme systems made possible the use of recombinant DNA techniques that have advanced our ability to study and understand proteins and the impact of mutations, as well as to produce large amounts of recombinant protein for studies of structure and function and the production of products such as insulin [5, 6].

By approaching the introduction of CRISPR as a compare and contract exercise with restriction enzyme systems (Table 12.1), an instructor can ensure that several key concepts relevant to molecular biology are covered and thoroughly understood. Key points to discuss when introducing restriction enzymes to novice students include:

- Restriction enzyme recognition sites are palindromes. Unlike a word palindrome, such as **RACECAR,** that reads the same forward and backward, for restriction enzyme palindromes, the complementary sequence reads backwards compared to the top sequence. For example, the BamHI site reads:
  - 5′-GGATCC-3′ forward strand
  - 3′-CCTAGG-5′ reverse strand
  - The palindrome is apparent by reading each strand in the 5′–3′ direction.
- Differences between endonucleases, exonucleases, isoschizomers, and neoschizomers and why understanding the differences can be useful in an experimental design context.
- Enzymes with a four-base pair recognition site cut more frequently than enzymes with a six-base pair recognition site.
- The frequency of occurrence of a restriction site can be roughly estimated using the equation $(1/4)^n$=frequency of occurrence of recognition site. $1/(1/4)^n$ yields how many nucleotides will likely contain one recognition site for a given enzyme; n=number of nucleotides in the recognition site).

Table 12.1 Comparison of restriction enzymes to CRISPR-Cas systems

Restriction enzymes	CRISPR-Cas system
Locates cut site using a palindromic recognition site	Locates the cut site using a PAM and match to guide RNA
# per genome for any given enzyme is constant for an organism	Number of sites to any particular guide is determined by how many protospacers (viral segments) have inserted into the genome
Bacterial genomic DNA is methylated preventing cutting by restriction enzymes in vivo	Methylation of genomic DNA does not impact recognition of CRISPRS and protospacers
Deflects a viral invasion	Remembers a viral invasion and prevents future attacks

- The number of restriction sites in any genome can be determined precisely if the full sequence of the genome is known.
- Restriction enzymes can leave either blunt or sticky ends.
- Directional cloning or using two restriction enzymes instead of one is preferred when possible, when designing a cloning experiment.

An instructor can choose which of the above points are covered depending on the level of the student and what other molecular biology related lessons will be covered elsewhere in the curriculum. For example, an instructor might reinforce the concept that the frequency of restriction sites can be estimated mathematically or known precisely if the genome is sequenced by designing an assignment utilizing a tool such as NEBCutter (https://nc3.neb.com/NEBcutter/).

12.3.2 CRISPR-Cas

An explanation of how the CRISPR-Cas9 system in *Streptococcus pyogenes* works provides the basic foundation for understanding the basic CRISPR mechanism. While three different types of CRISPR-Cas systems have been identified (Type I, Type II, and Type III), focusing on the Type II CRISPR-Cas9 system is ideal as a starting point for the classroom as it is well-researched, has a well-understood mechanism, and is one of the most commonly-adopted systems for genome editing [2].

Key points to cover include:

- Definitions and acronyms
  - CRISPR: Clusters of Regularly Interspaced Short Palindromic Repeats
  - Protospacers: segments between the palindromic repeats that are complementary to viral DNA from past viral invasion.
  - Cas: CRISPR-associated proteins
  - PAM: Protospace Adjacent Motif
  - crisprRNA
  - tracerRNA
  - sgRNA
- Enzyme function: Helicase and nuclease activity
- Central dogma of molecular biology: DNA→mRNA→protein
- Mechanism by which the system works
- Adaptive immunity
- Applied applications in research
- Impact of insertions, deletions and frameshift mutations

In explaining how CRISPR-Cas9 systems work in nature, it is important to remind students of the Central Dogma of Molecular Biology. The gene for the Cas9 protein is on the bacte-

rial genome. It is translated to mRNA and then translated to the protein that will complex with CRISPR RNA (crRNA) to seek out matches in invading viral nucleic acid material (either DNA or RNA) [1]. The CRISPR region of the genome and the trans-activating RNA (tracrRNA) region of the genome are transcribed from their DNA separately. The two products anneal to form the crRNA or crRNA complex with the tracrRNA binding to the palindromic repeat regions of the crRNA. This binding creates segments of double stranded RNA which is subsequently cleaved by RNase III yielding crRNAs that contain one spacer sequence from the CRISPR locus on the bacterial genome and a tracrRNA [7]. The crRNA forms a complex with the Cas9 protein. When complexed, the Cas9 protein scans viral DNA for a PAM site. The PAM sequences are typically 2–5 nucleotides in length and vary with the bacterial species and type of Cas protein. For Cas9, the PAM is 5′-NGG-3′. When one is located, the Cas9 uses a helicase activity to unwind double-stranded DNA, check against the protospacer DNA and, if a match between the viral DNA and the protospacer DNA is present, the Cas9 nuclease activity is used to cut the viral DNA, thus inactivating the virus. The lesson can be extended to discuss the naturally occurring CRISPR RNA complex and the use of the chimeric single guide RNAs (sgRNAs) that contain both crRNA and tracrRNA connected with a linker loop to form a hairpin loop that is commonly used in research applications. The chimera allows for the researcher to only produce or order one oligo rather than two [1].

At this point it is helpful to stop and provide students with a video that puts the entire process into motion. This provides reinforcement and clarity to a static slide based or whiteboard/chalk talk. Reinforcement and repetition are both important for students to build confidence in their understanding and for long-term retention of the key concepts [8, 9]. A number of excellent videos can be found online. Table 12.2 provides a few noteworthy options. The session can wrap up with a brief overview of the possible applications for utilizing CRISPR-Cas systems in research to introduce mutations through non-homologous end-joining that leaves errors resulting in frame-shift mutations [10], precise gene editing, knock-in of tags, knock-outs of genes, and clever blocking of transcription through the recruitment of factors that create steric hinderances to the process or enhancement of transcription through the fusion of transcriptional activators to Cas [11–13].

12.4 Reinforcing Concepts

12.4.1 Interactive Simulation

For students to assimilate material into their long-term memory, they need to interact with the material in many different ways. HHMI Biointeractive has an animated interactive activity *CRISPR-Cas9 Mechanism & Applications* that covers how the system works and how it is used (https://media.hhmi.org/biointeractive/click/CRISPR/). Students can click the labels in the tool to access definitions of the labels on elements of the system as they watch the system in action. Under the *How It's Used* section, 20 short videos are available

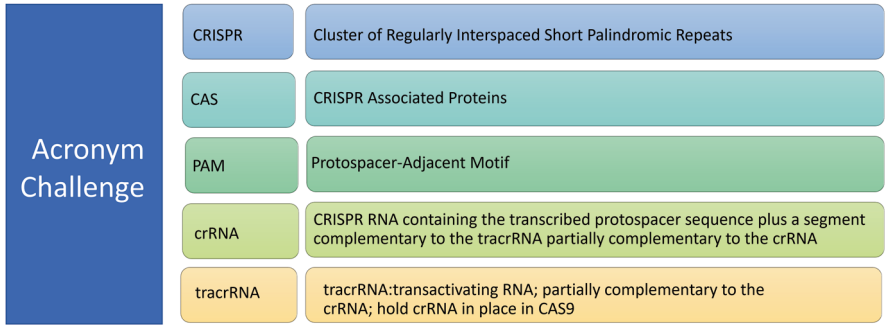

Fig. 12.2 Acronym challenge. Students test their knowledge of the acronyms essential to being able to understand the scientific literature involving CRISPR-Cas systems by filling in the definitions of each acronym on an index card without notes. Think-pair-share can be employed

to explore research, medicine, agriculture and to listen to scientists including Jennifer Doudna and David Liu discuss various aspects of CRISPR. If students will be working for example with the butterfly related CRISPR activities available on the HHMI Interactive page, the discussion of the work by Robert Reed serves as a nice leader into the *Using CRISPR to Identify the Functions of Butterfly Genes and Winging It: Analyzing a Scientific Paper* discussed later in this chapter.

12.4.2 Acronym Challenge

The vocabulary associated with CRISPR-Cas is challenging, entirely new to most students and laden with acronyms. Students often become lost and disinterested in science when they have not mastered the associated vocabulary and acronym soup. A simple way to encourage students to master the acronyms is to hand them each an index card at the start of class as a class warm-up and ask them to define the acronyms (Fig. 12.2). Think-pair share [14] can be utilized to allow students to grapple with what they know and where they have gaps. Working with partners can enliven the classroom but lecture notes should not be allowed. The activity, although simple, helps students avoid the illusion of knowing the topic well when they really do not. Cards can be collected to count towards class participation or other points or students can keep them as a reminder they really need to master the terminology.

12.4.3 Modeling

For the kinesthetic learner, modeling the process of cutting by CRISPR-Cas9 can reinforce the basic process for students. A 2-D paper model activity is available from HHMI Biointeractive (https://www.biointeractive.org/classroom-resources/building-

paper-model-crispr-cas9) that is suitable for high school and introductory level college courses. HHMI's model is based on one developed by David Wollert at Chattanooga State Community College (see his chapter elsewhere in this volume). In the HHMI iteration, students study the use of CRISPR-Cas for gene knockouts and editing.

Bio-Rad also offers a 2-D paper model exercise that reinforces how the CRISPR-Cas9 system works on a very introductory level (https://www.bio-rad.com/webroot/web/pdf/lse/literature/CRISPR%20Cas%209%20Paper%20Model%20Activity.pdf). This exercise also exposes students to the concepts of nonhomologous end joining and homology directed repair. The model uses the lac Z gene coding for β-galactosidase from the lac operon and is a good lead in to a wet-bench lab using the Bio-Rad *Out of the Blue* CRISPR kit. Students can see if they successfully edited the lacZ gene in the wet-bench activity by noting a loss of blue color in colonies grown on media supplemented with X-gal which produces a blue pigment when cleaved by a functional β-galactosidase.

Colorful, reusable kits to review bacterial adaptive immunity and the CRISPR-Cas9 mechanism are available from 3D Molecular Designs (https://3dmoleculardesigns.com/). The kits come with lesson plans as well. Students manipulate the foam pieces to create a CRISPR array in the adaptive immunity kit and to make a cut with the CRISPR Cas-9 kit.

Regardless of the modeling used, allowing students time to talk through the process helps solidify understanding. When students have to explain the process to a classmate all the way through, they are able recognize where they have gaps in understanding or are missing needed vocabulary. It also allows the instructor to address misconceptions students may have.

12.5 Bioinformatics: Oligo Design

Two types of oligo design can be taught in conjunction with a module on CRISPR: PCR primer design and sgRNA design. The oligo or primer design lessons can be stand-alone computer-based activities, or they can be used as a lead-in to wet bench work. For labs choosing to do a validation of cutting with Cas9, target DNA will need to be amplified by PCR for use in class. Students can learn to design the primers for the PCR used to amplify the target and then move on to sgRNA design. Courses where the wet-bench lab is an authentic research experience and guides are introduced into the selected model system would still benefit from amplifying the target by PCR to test the sgRNAs to be sure the target can be cut with Cas9 before moving onto the *in vivo* portions of the project.

12.6 Primer Design for PCR Amplification of the Target Sequence

For basic primer design for PCR, the Primer3web (https://bioinfo.ut.ee/primer3/) website is a good choice as students must consider a number of parameters including primer length, melting temperature, % GC, self-complementary, pair complementary, and maxi-

mum repeated nucleotides (poly-X) among other options [15, 16]. For instructors that are teaching more advanced students and who want to add a biochemistry focus and/or scientific literature focus to the class, an assignment comparing the differences in methodology for calculating melting temperatures based on the three options available in the Primer3 program can form the basis of an in-depth walk through the research that resulted in the salt correction formulas used in the program. Within the Primer3 setup, students must select between these three salt correction formula options [17–19]. These research papers allow an advanced-level consideration of the biochemistry and thermodynamics. The Santa Lucia [18] paper is an excellent example of the use of the Gibbs free energy equation in a clear application rather than just the hypothetical lens from which students tend to view their textbook coverage of the material. The Owczarzy [19] paper provides the advanced biochemistry student context for the importance of magnesium concentrations in PCR reactions. Rather than understanding simply that magnesium is an important cofactor for a successful PCR reaction, the student gains a deeper understanding of the impact of varying the concentrations of Mg^{2+} has on melting temperature. It is an excellent paper in helping students understand how the chemistry behind why the "standard" reaction conditions we use in PCR work and the experimental designs used to generate what is now "common knowledge" for the professionals in the field. To improve the critical thinking and data analysis skills of students, standalone figures from any of these papers can be provided to students and students can be asked to write a short paragraph interpreting the results before being provided the full context of the paper.

12.7 Guide RNA Design

Following an overview of how CRISPR-Cas systems function to provide adaptive immunity, an overview of single guide RNA design, highlighting the value of using a chimera containing both crRNA and tracrRNA linked together into one RNA instead of two can follow. A simple way to orient students to the basics of guide RNA design is the *Using CRISPR to Identify the Functions of Butterfly Genes* activity available on the HHMI Biointeractive site (https://www.biointeractive.org/classroom-resources/using-crispr-identify-functions-butterfly-genes). In this activity, students practice identifying PAM sites and where the Cas9 will cut. The activity then walks students through a simplified version of creating a guide RNA. While the activity is suitable for high school students, it is an excellent introduction for undergraduate students who will be designing sgRNAs using available tools such as ChopChop [20] or Benchling (www.benchling.com). The one caveat is that the answer key is available online. This works best as an in-class activity where students are not able to look up all the answers and skip the actual thinking and learning part of the exercise!

Following some basic practice identifying PAM sites and cleavage sites in the target DNA, undergraduate level students can be led through the steps of designing a single guide RNA. This activity is best suited for upper-level undergraduates who have had prior

coursework covering gene expression and transcription and translation thoroughly or in a Course-Based Undergraduate Research Experience (CURE) course where ample time has been spent covering these basic foundational topics before introducing CRISPR and guide RNA design.

In designing your sgRNA assignment, the instructor has a number of options in the selection of targets for the sgRNA. The first decision to make is what model system will be used (i.e. human, zebrafish, mouse, *Arabidopsis*, etc.). Selecting a model system aligned to current research in your lab or prior work as a graduate student reduces the amount of time it takes to design the activity and makes troubleshooting easier. The second decision to be made is whether all students will be assigned the same gene, students will choose their own gene from a specified genome, or students will be offered a short list of genes for which a guide can be designed. For the purpose of teaching the concept, checking for understanding, streamlining grading, and managing a large class, assigning a specified gene or limiting the option choices makes the class run more smoothly. What you decide may also be determined by any follow-up activity you plan to do at the bench. If the wet-bench activity is more demonstration than active research, having students design guides for the demonstration target creates a smooth flow, however, it is more cost effective to order the oligos you need to create the guides in advance and all students use the same guides in the experiment rather than ordering student created guides (unless the classes have very small enrollments). If the students will be conducting authentic research as a next step, the gene selected will determine what is used for this exercise and student-designed guides will need to be ordered. Typically, students should design multiple guides to a target to ensure that at least one guide actually cuts the target. Four is a good number to use. Sometimes it is difficult to design four good guides to a particular target. This is a good lesson for students as well as they wrestle with the realities of naturally occurring gene sequences. Having students design their own guides builds the following skills:

- Utilizing genome databases to pull sequences
- Gaining familiarity with gene ID and gene location nomenclature
- Recognizing the difference between coding exons (CDSs), untranslated regions and introns in the sequence
- Understanding the on-target and off-target scores
- Identifying the appropriate PAM for the chosen Cas
- Understanding construction of an sgRNA that will serve in place of the crRNA and trRNA complex

If the instructor wishes to use this exercise in an *in vivo* experiment, it is important to know that the cost of ordering the sgRNAs in a fully synthesized form from a company has decreased considerably; therefore, ordering premade guides rather than a specific gene-specific oligo to synthesize the guide in the lab is often worth the time saved at the bench and greatly reduces the number of things that can go wrong at the bench for students. However, working through the sgRNA guide development exercise is important in helping

students fully understand how the CRISPR-Cas systems function in nature and the science behind commercially purchased guides. The exercise deepens understanding of basic concepts in molecular biology.

12.8 The History Lesson

There is often a disconnect between typical science textbook knowledge and the individuals who made the discoveries. When science concepts are simply taught as a series of knowns, the excitement of the pursuit of new knowledge and that this is a very human activity done by ordinary people gets lost. By telling the story of how a groundbreaking discovery was made and the subsequent development of the technology that emanated from the basic science, students gain important insights that impact their ability to see themselves as scientists.

Basic research is critical. Entirely new fields rarely develop from what is already known or accepted as truth about how the universe works. Advances in medicine and technology are built on advances in understanding basic science and biology. Society often demands that work in basic science demonstrates a clear and obvious marketable medical solution or industry application, or the work is not deemed worthy of funding. However, few great advances are made without fundamental knowledge afforded by the simple study of natural and physical systems and how they work. Future advances depend on continued government funding of basic research and this concept is one the next generation of tax-paying citizens needs to understand. The shift from the basic science discoveries of the CRISPR-Cas adaptive immunity systems in bacteria and archaea to the development of gene editing applications was swift but without the basic fundamental research, the applications would not be possible.

A number of biographical and historical type books (Table 12.3) are now available that take readers through the stories of discovery and also address the ethics concerns of those who discovered the systems and conducted the first demonstrations that CRISPR-Cas could be used as a genome editing tool. For more advanced students, book studies can be used to reinforce understanding of the basic science but, importantly, frame science as an activity conducted by regular mortal humans who sometimes find themselves at the junc-

Table 12.3 Selected historical books dealing with the discovery and expansion of CRISPR-Cas9 technology in genetic engineering

Author	Title	Publisher
Kevin Davies	*Editing Humanity: The CRISPR Revolution and the New Era of Genome Editing*	Simon & Schuster
Jennifer Doudna and Samuel H. Sternberg	*A Crack in Creation: Gene Editing and the Unthinkable Power to Control Evolution*	HarperCollins
Walter Isaacson	*The Code Breaker: Jennifer Doudna, Gene Editing, and the Future of the Human Race*	Simon & Schuster

tion of something that significantly shifts our fundamental understanding of nature and how to manipulate it. It helps students see scientists, particularly the ones whose names grace the pages of textbooks and classroom lessons, as ordinary people who worked hard and contributed to our collective knowledge base. The stories of discovery that include the stories of the discoverers and not just the scientific knowledge, can spark interest in science and help students see themselves as future scientists. Sharing the history of science and its key players puts science in the context of a process rather than a collection of facts, highlighting the nature of the work of scientists and hopefully inspiring the next generation to pursue careers in STEM.

When embarking on a book study, it is best to set up a schedule of chapter due dates to keep students on track with their reading. Several options are possible to ensure students are actually doing the reading.

1. A reading guide for each chapter can be assigned to ensure students are actually doing the reading and engaging with the material. A few questions that ask students their thoughts or opinions on events or actions or concepts in the chapter are sufficient.
2. An alternative to a guided questions assignment is to ask students to keep a journal. At the end of each chapter, they can summarize their thoughts, impressions, questions, etc. about the reading. The instructor can provide as much or as little structure as they deem necessary depending on the learning goals.
3. A short quiz at the start of class that asks a few questions students should be able to answer if they did the reading when they arrive in class also helps ensure students do the reading. Be careful not to ask "nit-picking" questions that focus on the small details or students who did the reading will become frustrated. The point is not for students to memorize the chapters. The questions should be on broader events or themes in the chapter that the student who read the chapter will be able to answer in some detail.
4. A class discussion on the chapter that highlights any areas you wish to highlight or for students to consider in more depth can be a lively way to engage students in sharing their thoughts and to help them think more critically about the story.

A book study approach can provide enrichment to a course, whether it is lecture or lab based. A key consideration to keep in mind, however, is the total time outside of class being demanded for the totality of assignments for the class in which this is introduced.

12.8.1 Technology Transfer and Intellectual Property Law

An interesting way to bring this career path to the attention of students is through a discussion of the litigation and outcomes of the lawsuits concerning the patents related to CRISPR [21, 22]. The legal battles surrounding the patents on the applied uses of CRISPR technology have been fierce and heated. The story offers a window into what is patentable, the rules around who is assigned credit for discovery, and the difficulties in trying cases in

court when those making the final judgements have a very limited (and sometimes erroneous) understanding of the science and technology under discussion.

Students that start out as biology majors with the intention of becoming medical doctors or scientific researchers sometimes change their minds once they have had opportunities to shadow practitioners or work at the lab bench. Possible job pathways for these students include working in technology transfer offices at universities and government research institutes and working in patent law to review potential applications or defend/prosecute patent infringement cases.

There are two avenues into work in intellectual property law. Students can pursue this career as a full practicing attorney able to present cases in court after attending law school. Alternately, they may take the patent bar to become a patent attorney without attending law school if they hold a degree in a relevant field of science/technology. Patent attorneys can support intellectual property lawyers but cannot present a case in court. Patent attorneys often review potential patents to ensure the proposed patent meets the criteria for originality and to ensure that the new invention does not infringe on existing patents. It is a potential path for students that have earned an undergraduate degree in biology but have decided they do not want to go to medical school, and they do not like working at the lab bench.

When determining whether an invention is patentable, one must demonstrate that the invention is useful, not immediately obvious (it took some creative thinking to devise it), and novel (not already in use or produced in some form by someone else). Additionally, there are a few things that cannot be patented. One is math algorithms as these are fundamental steps to solve problems and no other way of doing so is likely to yield an accurate and useful result. An algorithm is essentially a procedure on how to do something so no type of algorithm is patentable. Things found in nature are also not patentable. Therefore, the CRISPR-Cas9 system found in *Streptococcus pyogenes* is not patentable. The use of a single guide RNA (which is a modification of the system found in nature but is not itself naturally occurring) is patentable as are other creative bioengineering techniques that modify the basic CRISPR-Cas systems to do new things. Who owns the patent is important as applied technologies can generate modest to extraordinary amounts of revenue. Patents are typically held by the person who thought of the idea. The person who carries out the work does not have to be included on the patent but they may share the revenue. A patent gives the holder(s) the right to exclude other from making, using, or selling the invention for 20 years from the date of the application in the United States.

12.9 Ethics and Case Studies

Case studies (Table 12.4) can be useful and engaging ways to build student knowledge and are particularly powerful as tools to invoke critical thinking about ethical issues where there is not always a clear and easy right or wrong or one-size-fits-all type answer. There are multiple methods that can be employed to roll out a case study.

Table 12.4 Sample case studies that are available for classroom use in helping students understand both CRISPR-Cas9 and the ethical ramifications of its use

Case study	Author and link	Topic	Type
Cut It Out! Editing DNA with CRISPR-Cas 9	Grace A. Farber and Amy T. Hark National Center for Case Study Teaching in Science https://www.nsta.org/ncss-case-study/cut-it-out	Introduces basic concepts of CRISPR-Cas9; frames discussion in the issue of X-linked Duchenne muscular dystrophy	A 4-part interrupted study that moves students from a TED talk, to a science news article, a review paper and a research report.
A CRISPR Human	Andrea M. Henle https://static.nsta.org/case_study_docs/case_studies/crispr_human.pdf	Introduces basic concepts of CRISPR-Cas9; frames discussion in the issue of cystic fibrosis	A 4-part interrupted study
The Case of the Designer Baby	https://capebretonspectator.com/2020/11/11/bioethics-case-study-gene-editing/	A very short read that poses very basic questions about where the line on editing human embryos should be drawn.	A short case presentation with discussion questions
The Ethics of Editing Embryos	https://onlineethics.org/cases/ethics-emerging-technologies-life-sciences/case-genome-editing-ethics-crispr-cas9	A consideration of editing human embryos and related decisions by scientific journals to publish or decline the research for publication	A short case presentation with discussion questions
CRISPR: Next-Gen Mosquito Zapper?	https://www.scu.edu/ethics/focus-areas/bioethics/resources/crispr-next-gen-mosquito-zapper/	Students must weigh the impacts of reducing vs eliminating mosquito populations in a region experiencing a Zika outbreak.	Questions embedded in the case; can be completed in one class session

1. An instructor can simply conduct some case studies entirely as a class discussion after presenting the case or allowing students time to read a short case. Discussions can be full class or small groups can engage with a set of questions and then share out thoughts in a class-wide discussion.
2. Students can read ahead and address short answer questions or write a short essay to share their thoughts as a pre-cursor to a class discussion.
3. A basic case can be shared out in class and discussed then followed by a more detailed research assignment involving a literature search and use of citations to support a paper that thoroughly discusses the issue.

While we tend to think in terms of humans when discussing ethics, gene editing can have ramifications on multiple organisms in an environment. Discussion of the ramifications of genetic modifications of bacteria, insects, viruses, or crops can expand the students understanding of the importance of looking at all possible outcomes of gene editing and not just the short-term desired outcome (see bioethics article https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7129066/pdf/turkjbio-44-110.pdf for possible risks across multiple organisms and scenarios). Providing students a framework for making ethical decisions can serve as a useful tool for discussions and help students become proficient at examining issues logically and thoroughly rather than simply through the lens of personal emotions (https://www.scu.edu/ethics/ethics-resources/a-framework-for-ethical-decision-making/).

A discussion of the debates surrounding the discovery of restriction enzymes and the moratorium on their usage in the early 1970s provides an interesting comparative history. The importance of the Asilomar conference and the decisions scientists made to ensure the responsible conduct of recombinant DNA work was foundational and marks the beginning of both the hopes and dreams for curing genetic disorders as well as serious concerns about potential misuse of the technology [23].

Take Home Message

Regardless of whether you are teaching a high school course, an undergraduate introductory level course, advanced level undergraduate course or specialized research experiences for undergraduates' course, all students benefit from a carefully constructed and well-thought out plan to develop their understanding of a topic. Scaffolding learning to begin broadly and then build in layers of depth allows students time to assimilate the material such that they have a very solid understanding of at least the most basic concepts involved. There are many ways to scaffold learning and the options chosen should reflect your teaching goals. When asked the question, "What do you want students to learn by the time they graduate?", few instructors rattle off a list of facts. Typically, they list skills: scientific literacy, oral and written communication, critical thinking, data analysis, and professional and social skills. A well-constructed CRISPR-Cas9 module can be used as the engine to build these skill sets in addition to bringing students up to date on this newest edition to our gene editing toolkits.

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