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| Tips and Tools

# CRISPR/Cas9 gene targeting plus nanopore DNA sequencing with the plasmid pBR322 in the classroom

**Röbbe Wünschiers,[1] Robert Maximilian Leidenfrost,[2] Hauke Holtorf,[3] Bernd Dittrich,[4] Thomas Dürr,[5] Jürgen Braun[6]**

**AUTHOR AFFILIATIONS** See affiliation list on p. 5.

**ABSTRACT** Both nanopore-based DNA sequencing and CRISPR/Cas-based gene editing represent groundbreaking innovations in molecular biology and genomics, offering unprecedented insights into and tools for working with genetic information. For students, reading, editing, and even writing DNA will be part of their everyday life. We have developed a laboratory procedure that includes (i) the biosynthesis of a guide RNA for, (ii) targeting Cas9 to specifically linearize the pBR322 plasmid, and (iii) the identification of the cutting site through nanopore DNA sequencing. The protocol is intentionally kept simple and requires neither living organisms nor biosafety laboratories. We divided the experimental procedures into separate activities to facilitate customization. Assuming access to a well-equipped molecular biology laboratory, an initial investment of approximately $2,700 is necessary. The material costs for each experiment group amount to around $130. Furthermore, we have developed a freely accessible website (https://dnalesen.hs-mittweida.de) for sequence read analysis and visualization, lowering the required computational skills to a minimum. For those with strong computational skills, we provide instructions for terminal-based data processing. With the presented activities, we aim to provide a hands-on experiment that engages students in modern molecular genetics and motivates them to discuss potential implications. The complete experiment can be accomplished within half a day and has been successfully implemented by us at high schools, in teacher training, and at universities. Our tip is to combine CRISPR/Cas gene targeting with nanopore-based DNA sequencing. As a tool, we provide a website that facilitates sequence data analysis and visualization.

**KEYWORDS** CRISPR/Cas9, MinION, nanopore, DNA sequencing, plasmid analysis, web service

In 2012, Jennifer Doudna and Emmanuelle Charpentier described a programmable, RNA-guided endonuclease (1). This enzyme, known as Cas9, induces targeted double-strand breaks in DNA. Using billion-year-old cellular repair machinery, the cell can be manipulated to edit specific nucleotides or insert complete genes. While the former allows, e.g., for the repair of genetic diseases, the latter can introduce new functions to cells. In 2018, Chinese scientist He Jiankui prematurely became the first ever to edit human-fertilized egg cells (2, 3). Clearly, gene editing holds promise for curing genetic diseases and developing crops that can withstand pests and climatic challenges (4). Being applied in crop breeding and human diagnostics, CRISPR/Cas affects us all.

The CRISPR revolution would not have been possible without efficient and economical DNA sequencing. Being a laborious and expensive process until recently, the UK-based company Oxford Nanopore Technologies released in 2014 its first portable sequencing device that employs protein nanopores for DNA sequencing (5, 6). Their device, named MinION, costs less than $1,000 and fits in the palm of your hand.

Gaining insight into the effects of genetics by reading DNA and associating DNA sequences with phenotypes is the foundation of DNA editing, such as with the

**Editor** Megan K. Barker, Simon Fraser University, Burnaby, Canada

Address correspondence to Röbbe Wünschiers, wuenschi@hs-mittweida.de.

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CRISPR/Cas gene editing system. Both methods, DNA sequencing and gene editing, lay the groundwork for future-oriented fields like medicine, breeding, conservation biology, genetic engineering, and the modern bioeconomy (7). We have, therefore, combined them into one teaching activity.

## Classroom activities

"CRISPR in the classroom" is the title of an article in The New York Times published in 2022 (8). There, the author states that "A decade after CRISPR started to become a major tool in genetic research, a new generation of scientists is growing up with the technology. Even high school students are able to run CRISPR experiments." In fact, not only enthusiastic teachers but also some institutions (9) and companies have set out to develop easy-to-use experiments, BIO-RAD's "Out of the Blue CRISPR Kits" being probably the best known (10, 11).

However, these experiments are often (i) either coupled to running research projects that provide both bio-material and infrastructure or (ii) involve culturing and transforming living organisms, which requires microbiology laboratory equipment and at least a biosafety level (BSL) 1 laboratory space. Most classroom-suitable CRISPR experiments use either *Saccharomyces cerevisiae* (12–15) or *Escherichia coli* (16, 17) and confirm the gene edit phenotypically or at the molecular level using PCR and agarose gel electrophoresis. The targeting guide RNA (gRNA) is always pre-ordered from a service provider.

Apart from CRISPR, several authors present curricula for the integration of nanopore DNA sequencing in teaching (15, 18–20). This is an important development because DNA, as barcode for biodiversity and diagnostics, is growing fast. In addition, MinION-based DNA sequencing is becoming as readily available as it was in the past, e.g., with Raspberry Pi computers or fuel cell kits. The biggest hurdle here is no longer the price but the bioinformatics effort involved.

In contrast to all these lab curricula, we combine both gene editing and DNA sequencing into one classroom activity. Therefore, unlike other approaches, we have developed a setup (Fig. 1) that

- does not rely on living organisms or a biosafety laboratory,
- introduces gRNA synthesis *in vitro*,
- targets pBR322 plasmid DNA for Cas9/gRNA-based gene editing,
- enables PCR-free edit conformation by agarose gel electrophoresis and nanopore-based DNA sequencing with the MinION device, and
- provides an online website that manages bioinformatic analysis of the sequencing data.

## Target level and learning goals

The presented activities require a basic understanding of DNA and RNA composition, the genetic code, and enzymes. Therefore, these activities are most suitable for secondary or further education levels, starting at around 15 years of age. We have successfully conducted the described activities both in high schools and as part of teacher training programs (train the trainers). For the theoretical background, we refer to other resources [see references (4, 10, 21–23)]. An example of full-term curriculum can be found in reference (24).

We divided the practical work into six activities as outlined below. The supplemental instruction file includes observation and reflection that suggest learning goals addressing different BioSkills (25):

- Process of Science → Activity 1, e.g., transfer metabolic knowledge to an *in vitro* experiment; transfer of bacterial immune defense system to biotechnological applications.

- Quantitative Reasoning → Activity 2, e.g., understanding that plasmid DNA might not be fully digested; calculating the needed substrates of nucleotides for *in vitro* transcription or gRNA:Cas9 ratio; understanding sequencing errors and relation to the amount of reads.
- Modeling → Activity 4, e.g., understanding the difference between four signal states (single nucleotides) in Sanger and over 1,000 states (pentameres) in nanopore sequencing; training of neural network to read pentamere signals.
- Interdisciplinary Nature of Science → Activities 3 and 5, e.g., data processing and visualization; understanding MinION as lab-on-a-chip device with microfluidics and -electronics; utilization of molecular properties for separation and visualization.
- Communication and Collaboration → Activity 6, e.g., bioinformatics as bridging science and skill; sharing skills during the collaboration of individual group members; visualizing and communicating experimental results.
- Science and Society → e.g., transfer of the potential of DNA diagnostics and CRISPR/Cas-based gene editing to personal and precision medicine.

## Technical prerequisites

No living microorganisms are involved in the presented activities, eliminating the need for a BSL laboratory. However, the equipment of a classical molecular biology laboratory is required.

The MinION DNA-sequencing device plugs directly into a Type-A USB 3.0 port on a desktop or laptop computer. The computer should have a 1 terabyte solid state disk drive, at least 16 gigabyte of RAM, and an Intel i7 or Ryzen 5 processor or higher, respectively. Alternatively, DNA sequencing can be outsourced to a service provider.

To simplify sequence data analysis, we have developed a website that fully automates the analysis and visualization of sequencing results. For experienced instructors and

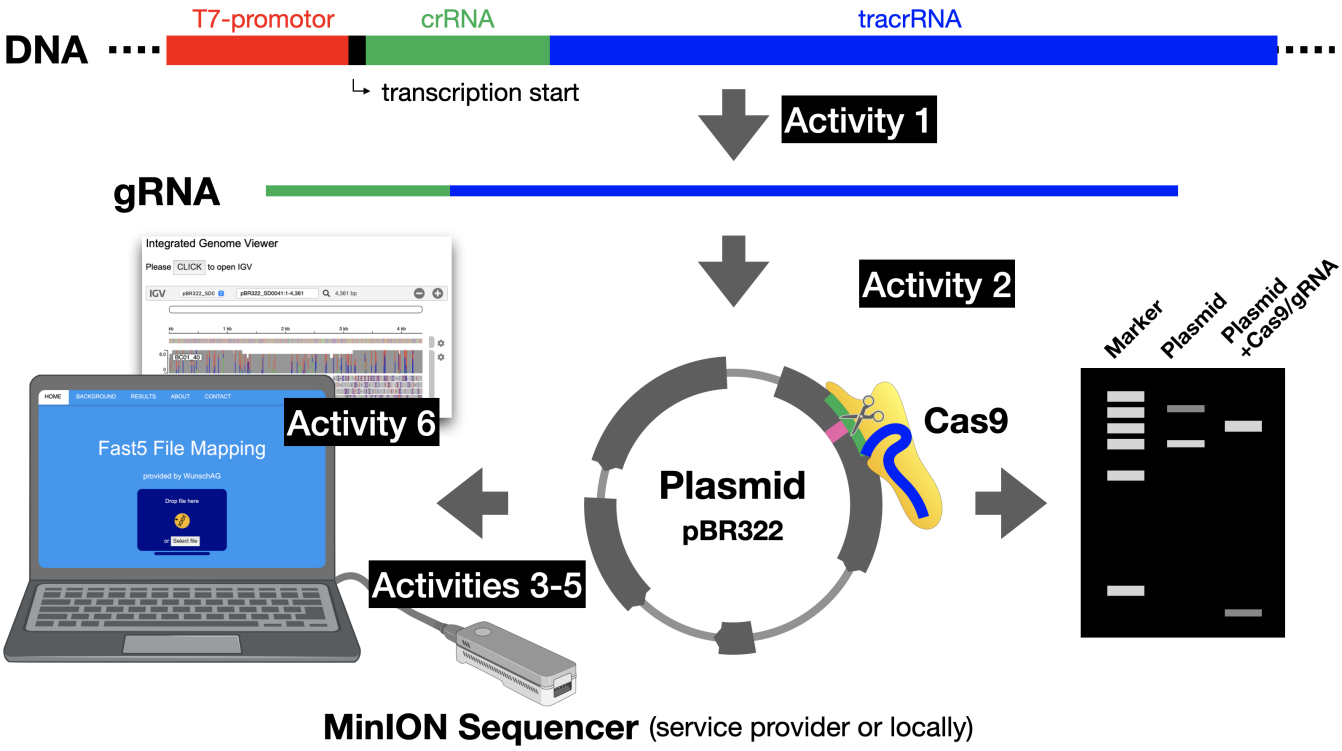


**FIG 1** Overview of T7 RNA polymerase-based biosynthesis of gRNA from a DNA template, subsequent digestion of plasmid pBR322 with the Cas9/gRNA complex, visualization by agarose gel electrophoresis, sequencing of the linearized plasmid and data analysis at our website.

students who prefer a deeper understanding, we also offer instructions for setting up a virtual Linux machine running the necessary software.

### Costs

The activities we propose bring modern biotechnology into the classroom and provide students with valuable hands-on experience in this rapidly developing field. Assuming access to an equipped molecular biology laboratory, an approximate initial investment of $2,700 is necessary. The material costs for each experiment group amount to approximately $130. Costs can be significantly reduced through collaboration between institutions.

MinION-based sequencing of the cut plasmid can also be handed over to a service provider. This eliminates the need to purchase a sequencer and reduces the sequencing cost to approximately $15. The returned Fast5 file can still be uploaded to our website for data analysis and visualization.

## PROCEDURE

We divided the experimental procedures into separate activities to facilitate customization (Fig. 2). In the supplemental material, we describe all activities in detail, provide suggestions for discussion topics, and list material and consumables with their approximate net prices. For all described activities, general laboratory safety instructions apply.

### Activity 1: gRNA synthesis—ca. 90 min

- *In vitro* transcription of gRNA from DNA template by T7 RNA polymerase.

### Activity 2: pBR322 digestion with Cas9 and gRNA—ca. 60 min

- Combine gRNA, pBR322 plasmid DNA, and Cas9 endonuclease.
- Observe native, nicked, and cut plasmid DNA by agarose gel electrophoresis.

### Activity 3: Sequencing library preparation—ca. 20 min

- Fragment plasmid DNA by a transposase enzyme.
- Couple motor protein to direct DNA to the nanopore.
- Attach DNA barcodes to distinguish individual groups.

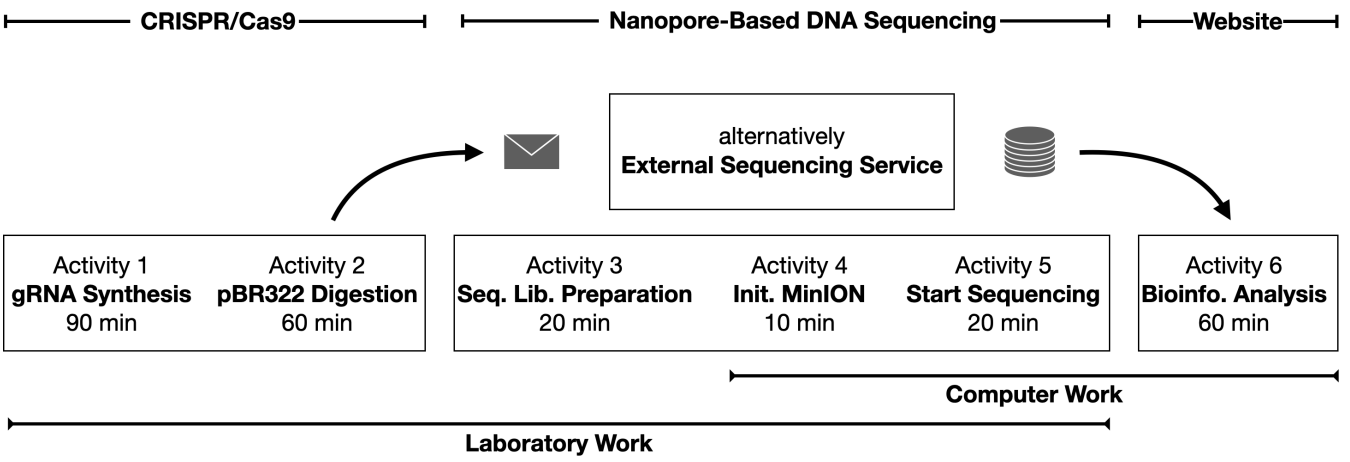


**FIG 2** Schedule of the experimental activities.

### Activity 4: Connecting the MinION and checking the flow cell—ca. 10 min

- Prepare MinION device.

### Activity 5: Priming, loading the flow cell, and sequencing—ca. 20 min

- Prepare sequencing flow cell and load DNA.
- Initiate DNA sequencing.

### Activity 6: Bioinformatic sequence analysis—ca. 60 min

- Upload Fast5 file delivered by MinION (or an external sequencing service) to our website (https://dnalesen.hs-mittweida.de).
- Reads are mapped onto pBR322 plasmid sequence.
- The result is visualized with Integrated Genome Viewer (26) in the web browser.
- Alternatively, follow our instructions to setup software locally.

## CONCLUSION

We developed an experiment with minimal laboratory requirements that can be performed within half a day. All steps neither require complicated handling nor entail unusual risks. We have successfully performed the described activities in high schools and universities outside of laboratories. High school students attested that they acquired a much deeper and vivid understanding of modern laboratory work, the "magic of microliters," the symbiosis of experimental and computational biology, and the field of bioengineering.

We hope that our work will contribute to the teaching of modern molecular biology and stimulate reflection on genetic engineering.

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## AUTHOR AFFILIATIONS

[1]Biotechnology and Chemistry, University of Applied Sciences Mittweida, Mittweida, Germany
[2]Division 4: Hazardous Substances and Biological Agents, Federal Institute for Occupational Safety and Health, Berlin, Germany
[3]Merian-Schule, Freiburg, Germany
[4]Käthe-Kollwitz-Schule, Bruchsal, Germany
[5]Augusta-Bender-Schule, Mosbach, Germany
[6]Johanna-Wittum-Schule, Pforzheim, Germany

## AUTHOR ORCIDs

Röbbe Wünschiers http://orcid.org/0000-0002-2824-4707
Robert Maximilian Leidenfrost http://orcid.org/0000-0003-0494-2612

## ADDITIONAL FILES

The following material is available online.

### Supplemental Material

**Supplemental Instructions (jmbe00187-23-S0001.pdf).** Descriptions of all activities in detail and suggestions for discussion topics and list material and consumables with their approximate net prices.

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