UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araraquara

"JULIO DE MESQUITA FILHO" SÃO PAULO STATE UNIVERSITY

SCHOOL OF PHARMACEUTICAL SCIENCES OF ARARAQUARA

Development of a toolkit to support the teaching of gene editing technology by CRISPR/Cas

Student: Guilherme Engelberto Kundlatsch

Supervisor: Prof Dr Danielle B. Pedrolli

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Monograph presented to the Graduate Program in Bioscience and Biotechnology Applied to Pharmacy as requirement for obtaining the title of Master of Science

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TÍTULO DA DISSERTAÇÃO: Desenvolvimento de uma ferramenta para suporte ao ensino da tecnologia de edição gênica por CRISPR/Cas

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“The study is the light-bringer of the brave”

Enéa Joaquina de Lima Kundlatsch

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We will continue to move forward.

Abstract

In this work, a tool was developed using laser cutting technology to teach the CRISPR/Cas gene editing technique. CRISPR/Cas is a powerful gene editing tool with wide applications in industry, agriculture and, most importantly, in human health. Although several authors have developed methodologies for its teaching, these works in general require significant infrastructure. To democratize the teaching of this technique, we developed a low-cost (<3 US dollars per student) toolkit that does not require laboratorial infrastructure, and which can be produced locally through digital fabrication tools. MDF-made pieces were designed and manufactured to allow the user to assemble DNA, RNA and polypeptide strands, and to edit DNA sequences using parts representing the CRISPR/Cas system. The toolkit consisting of 186 pieces was named LeDNA, and 10 units were produced for testing. Its impact on high school students was evaluated in comparison and in conjunction with a practical and a theoretical class. A new plasmid was developed for practical classes, allowing the editing of a strain of *Bacillus subtilis* producing green fluorescence to produce red fluorescence. A simplified CRISPR/Cas application protocol was also developed, feasible in educational laboratories with limited access to equipment. The different methodologies were analyzed in three groups of thirty high school students from public schools. A new assessment instrument was created to evaluate the participants' performance and it was applied before the first intervention and after each of the three. LeDNA promoted a significant increase in educational performance both individually and when associated with traditional methodologies.

Keywords: CRISPR/Cas; laser cutting; synthetic biology; education; genome editing

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Summary

1. Introduction

1.1 Identification of the CRISPR/Cas system – an historical overview

The discovery and understanding of the CRISPR/Cas system took decades of research and the work of several researchers around the globe, starting with Ishino et al. in 1987[1]. Studying the regulation of phosphate metabolism, the scientists observed repetitive sequences (DR – Direct Repeats) regularly spaced by 32 nucleotides in the genome of *Escherichia coli* K12. Nakata et al. demonstrated that these regularly spaced sequences (RSDR – Regularly Spaced Direct Repeats) were present in the genome of other microorganisms, such as *E. coli* C600 and Ymel, *Salmonella typhimurium* TA1535 and *Shigella dysenteriae* Sh[2]. Further studies demonstrated the presence of those regions in the genome of more organisms, such as *Mycobacterium tuberculosis*[3,4], *Haloferax mediterranei*, and *Haloferax volcanii*[5,6], with diversity in the size and composition of RSDR even at the strain level.

With the advent of large-scale sequencing, the presence of RSDR in many bacteria and archaea was established through comparative sequence analysis[7]. The greater availability of information about these sequences and the observation of their particular characteristics compared to other repetitive sequences led to reclassification, and they were renamed SRSRs (Short Regularly Spaced Repeats). Finally, Ruud Jansen and colleagues introduced the terminology currently used to name these sequences in 2002: CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)[8].

In 2005, three independent studies pointed to the origin of the spacers in the different CRISPR loci[9–11]. By applying bioinformatics analysis, it was demonstrated that most spacers are derived from bacteriophages and conjugative plasmids. Furthermore, *Streptococcus thermophilus* resistance to phage infections was shown to be directly correlated with the number of spacers at the CRISPR locus, presenting the first direct evidence of a CRISPR-mediated immunity to external genetic elements in bacteria[9].

Simultaneously with the studies on CRISPR loci, *cas* genes were identified, encoding CRISPR-associated proteins. Horvath et al., in 2007, demonstrated the role of Cas proteins in the mechanism of acquired phage immunity[12]. The researchers infected bacteria with phages and observed changes in the loci after infection. They identified insertions of new spacers with homology to the invading bacteriophage in resistant bacteria. In addition, the number of spacers correlated with the level of phage resistance, indicating modifications in the CRISPR

locus in the face of recurrent infections. Furthermore, they demonstrated that the Cas9 protein is directly linked to immunity to external genetic elements. By deleting the protein gene, the phage resistance phenotype was no longer observed.

Another essential element to the system's functioning is observed adjacent to each protospacer. Those conserved motifs are 2-5 base-pair DNA sequences that vary according to the CRISPR-based system and organism. They were initially identified by Bolotin et al. in *Streptococcus* strains[9]. Its universality was demonstrated by Mojica et al.[13], that created the acronym PAM (Protospacer Adjacent Motif).

Finally, another critical element elucidated in this period was the processing of RNA transcribed from the CRISPR loci, with different systems described in different organisms[14,15]. Among the mechanisms described, we can highlight that of *S. pyogenes*, in which the RNA is processed by small RNA encoded in-trans, the CRISPR-associated Csn1 protein, and the endoribonuclease III[16].

These initial findings allowed a general understanding of how CRISPR/Cas immunity works. It can be divided into three steps represented in figure 1: (1) adaptation, (2) expression, and (3) interference[17,18]. In the adaptation stage, there is recognition and fragmentation of invading elements, such as bacteriophages, and incorporation into the CRISPR locus as a new spacer. In the expression step, the CRISPR sequence is transcribed and processed into smaller RNAs, crRNAs, which contain the spacers that have been incorporated. The interference step occurs when there is a subsequent attack from the same invader. The crRNA and the Cas protein recognize the foreign DNA due to sequence homology and break it. This general scheme presents particularities in different organisms. Present in most archaea and many bacteria, the CRISPR/Cas system has significant variability between organisms, highlighting its dynamic character and remarkable modularity, the result of a perpetual race between hosts and invaders[19].

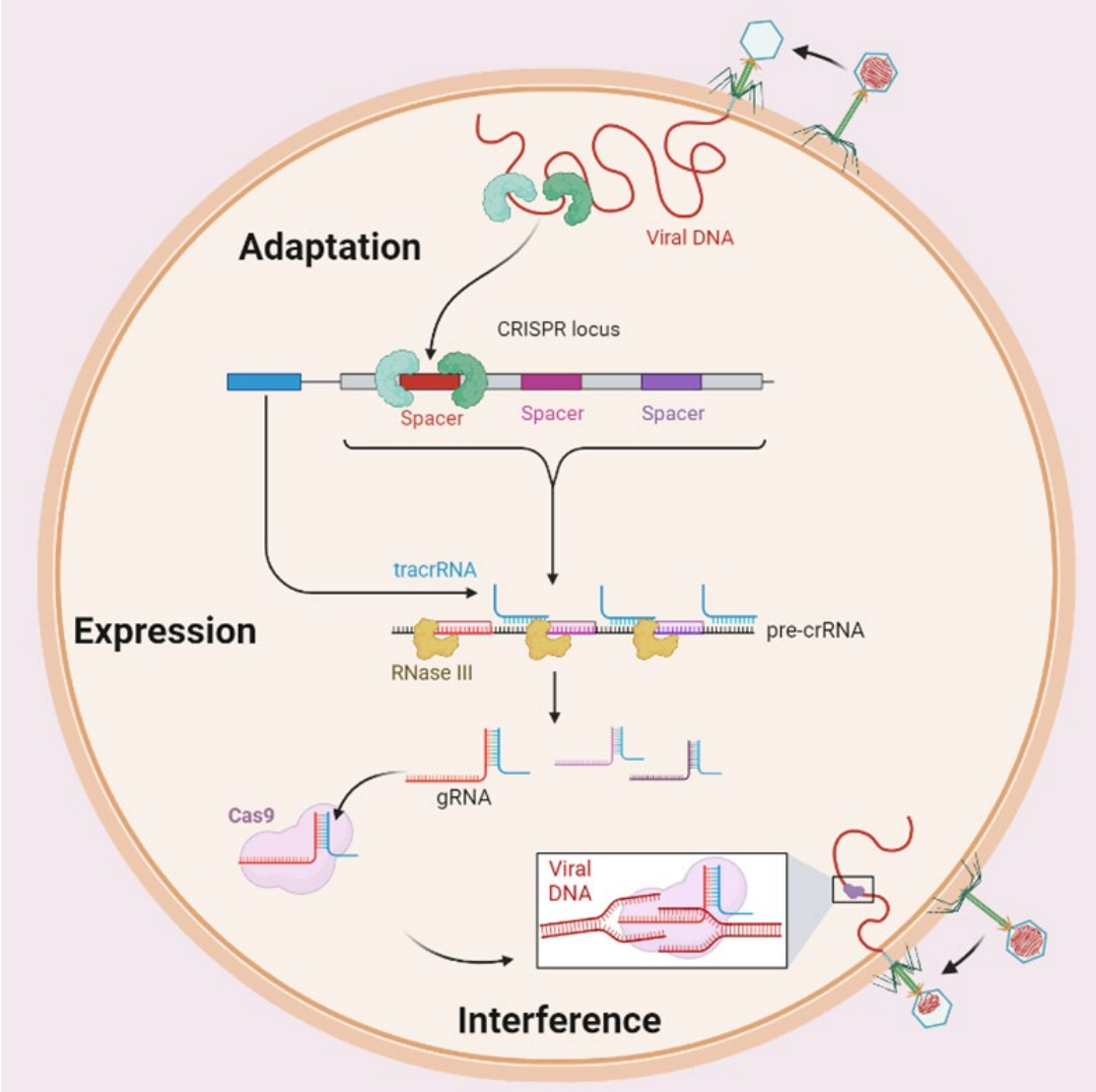


Figure 1. General representation of CRISPR-Cas-based immunity. This figure was generated using the Biorender software based on Agarwal and Gupta[20].

1.2 CRISPR/Cas as a genome-editing technology

An important step toward using CRISPR/Cas as an editing tool was the recombinant expression of the CRISPR/Cas locus of *S. thermophilus* in *E. coli* by Sapranauskas et al.[21]. The resulting strain displayed immunity to bacteriophages that had a DNA sequence matching the spacer sequence in the locus. In the same work, it was demonstrated through site-directed mutagenesis that the PAM nucleotides are essential for CRISPR/Cas activity. Furthermore, it was observed that despite the presence of four Cas proteins at the locus, Cas9 alone was capable of producing interference. Jinek et al.[22] evaluated the activity *in vitro* of Cas9 purified

from *S. pyogenes*, demonstrating that the protein had the ability to cut DNA in a PAM-dependent manner in the presence of tracrRNA and sequence-specific crRNA. They also demonstrated that a single chimeric RNA (called guide RNA – gRNA) comprising the features of both tracrRNA and crRNA was able to guide the Cas9 to catalyze DNA cleavage. Soon after, the efficiency of the technique for editing mammalian[23–25], bacterial[26], and zebrafish[27] cells was demonstrated.

While in the natural system the double-stranded break results in the degradation of the invading genetic element, in the application of the technique for gene editing three outcomes are possible. If the cell is unable to repair the double-stranded DNA break, cell death occurs. To perform the repair, the cell may employ the NHEJ (Non-Homologous End Joining) or the HDR (Homology-Directed Repair) mechanism. In the first case, there is no template for the repair, the strands are reunited with error, and there is a scar in the form of insertion or deletion mutations. In the case of a gene, a shift in the reading frame and the anticipation of stop codons can be introduced, resulting in gene knockout[28]. In the second case, the availability of a DNA sequence that shows homology with the regions adjacent to the cut site allows it to be used as a template for repair[29]. One can use this mechanism to replace a natural sequence in the cell genome with a synthetic one (donor DNA)[30]. In general, through NHEJ a gene of interest is deactivated, while through HDR it is edited. These mechanisms are represented in figure 2.

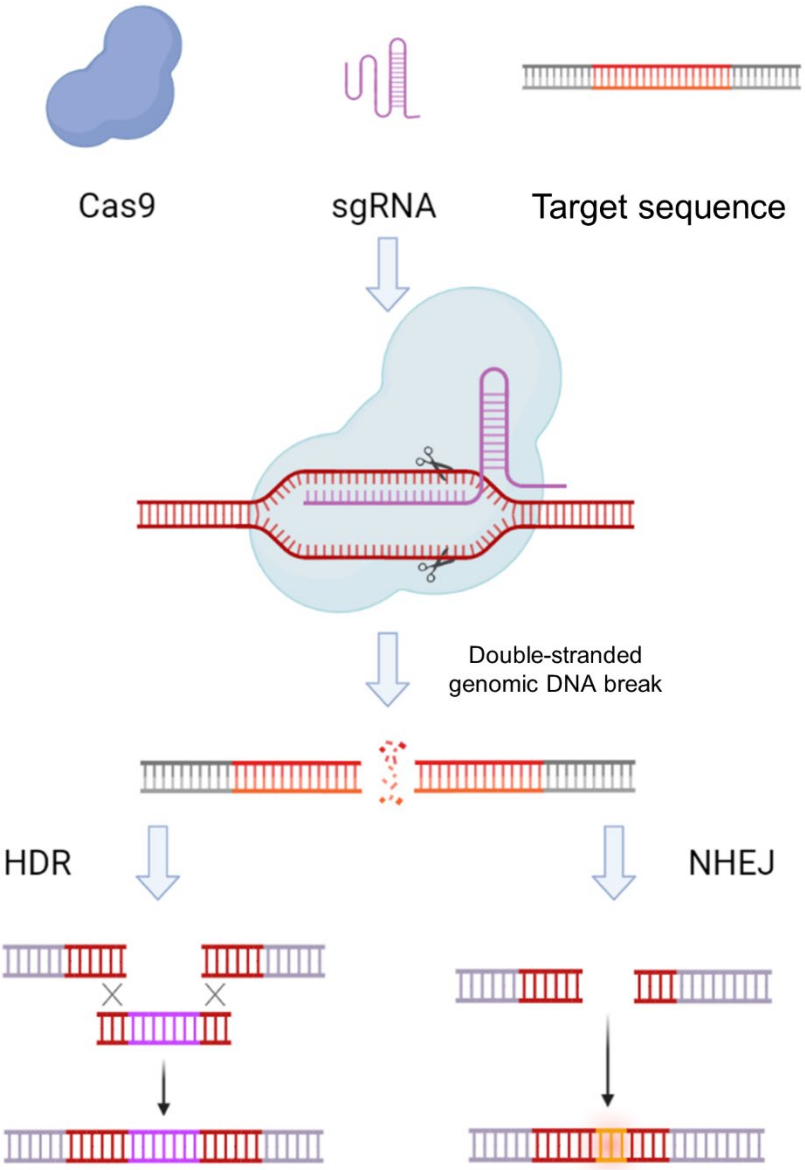


Figure 2. How the CRISPR/Cas gene editing system works. Guided by sgRNA, the Cas9 enzyme breaks the double-stranded DNA. Two different repair mechanisms can be used, depending on the availability or not of a template for the repair. This figure was generated using the Biorender software.

Reprogramming, therefore, the CRISPR/Cas system to redirect it to targets of interest, a powerful technology emerged, with revolutionary effects in multiple sectors[31]. Several studies have demonstrated possible applications of CRISPR/Cas to combat diseases such as cataracts[32], coloretal cancer[33], cystic fibrosis[34], Duchenne muscular dystrophy[35], hemophilia[36], and Huntington disease[37], among many others[17]. Among the applications in human health, we can also list the identification of new therapeutic targets[38], optimization of immunocompatible organisms for xenotransplantation[39], and combatting disease vectors[40]. Agarwal and Gupta

reported that in the first eight years after the development of this editing tool, more than 19,000 articles were published containing the term "CRISPR"[20]. To exemplify some of the results obtained since the publication of the technique, table 1 presents a non-exhaustive list of organisms that were modified through CRISPR/Cas.

Target Species	Application	Reference
Bacteria		
Ashbya gossypii	Production of folic acid and biolipids	Jiménez et al.[41]
Clostridium autoethanogenum	Production of biofuels	Nagaraju et al.[42]
Streptococcus thermophiles	Pro-biotic activity and fermentation	Hao et al.[43]
Streptomyces roseosporus	Auroramycin production	Jia et al.[44]
Yeast		
Aspergillus niger	Galactaric acid production	Kuivanen et al.[45]
Fusarium fujikuroi	Gibberellic acid production	Shi et al.[46]
Corynebacterium glutamicum	γ-aminobutyric acid production	Cleto et al.[47]
Yarrowia lipolytica	Increased synthesis and storage of lipids	Schwartz et al.[48]
Plant		
Golden delicious (Apple)	Increased resistance to fire blight disease	Malnoy et al.[49]
Manihot esculenta (Cassava)	Herbicide resistant	Hummel et al.[50]
Zea mays (Maize)	Drought tolerance	Svitashev et al.[51]

Hordeum vulgare (Barley)	Grain number increase	Kapusi et al.[52]
	Animal	
Ovis aries (Sheep)	Improved meat production	Crispo et al.[53]
Sus domesticus (Pig)	Virus resistance	Whitworth et al.[54]
Bos taurus (Cattle)	Improved animal welfare	Carlson et al.[55]

Table 1. Modified organisms for agricultural and industrial applications using the CRISPR/Cas tool.

1.3 CRISPR/Cas in the classroom

Considering the importance of CRISPR/Cas technology and its wide use, it is not surprising that the teaching of this tool to different audiences has been the subject of analysis by different researchers (Table 2). However, the vast majority of these works evaluated CRISPR/Cas teaching for undergraduate students only. Taking into account the great potential for future impact on society as a whole and the adjacent ethical issues of gene editing[56], it is of interest that teaching on CRISPR/Cas is not only present in the specific training of professionals who will develop and apply the technology, but also in the general training of all high school students who will eventually be affected, directly or indirectly, by its application. Since the vast majority of teaching works on the subject focus on more advanced levels of education, in this work we seek to develop a tool aimed at high school students.

Measuring the success of those educational tools through increased student learning and engagement is a fundamental part of their development[57]. Teaching CRISPR/Cas at the undergraduate level allows the use of assessment tools that require more time from the participants, such as poster presentations[58], lab reports and essays[59–61] and in-lab practical exam[61]. A teaching activity for high school requires a faster form of assessment due to the shorter time available. Ruppel et al.[62] and Waal et al.[63] applied quizzes to assess the learning of technical aspects of CRISPR/Cas and demonstrated significant improvement in student performance. Expanding on their works, we developed a new tool to assess the learning of not only the technical aspects of CRISPR/Cas, but also the understanding of both basic

genetics topics (already included in the high school curriculum) and the applications of this genome editing tool in students' daily lives.

Microorganisms, especially *Saccharomyces cerevisiae* and *Escherichia coli*, are the organisms most commonly used in CRISPR/Cas education (table 2), which is not surprising given their ease of cultivation and genetic manipulation. To act as a control in the evaluation of the toolkit developed in this work, we developed a practical class using *Bacillus subtilis*. This organism was chosen both because it is classified as GRAS (generally regarded as safe) and because of the widely established tools for its manipulation through CRISPR/Cas, requiring even less infrastructure than *S. cerevisiae* and *E. coli* [64].

Biological System	Target Audience	Reference
Arabidopsis thaliana	Undergraduate students	Ruppel et al. [62]
Caenorhabditis elegans	Undergraduate students	Hastie et al. [65]
Cell-free	Undergraduate students	Collias et al. [66]
Danio rerio	Undergraduate students	Bhatt & Challa [58]
Danio rerio	Undergraduate students	Wolyniak et al. [67]
Drosophila melanogaster	Undergraduate students	Adame et al. [68]
Escherichia coli	Undergraduate students	Militello & Lazatin [59]
Escherichia coli	Undergraduate students	Pieczynski et al. [61]
Escherichia coli	High School students & non-scientists	Ziegler & Nellen [69]
Mammalian cell culture	Undergraduate students	Anderson [70]
Saccharomyces cerevisiae	Undergraduate & High School students	Sankaran et al. [71]
Saccharomyces cerevisiae	Undergraduate students	Sehgal et al[60]
Saccharomyces cerevisiae	Undergraduate students	Vyas et al. [72]
Saccharomyces cerevisiae	Undergraduate students	Waal et al. [63]
Saccharomyces cerevisiae	Undergraduate students	Juríková et al. [73]

Streptococcus thermophilus	Undergraduate students	Hynes et al. [74]
Vanessa cardui	Undergraduate students	Thulluru et al. [75]
Vanessa cardui & *Xenopus laevis*	Undergraduate students	Martin et al. [76]

Table 2. CRISPR/Cas educational projects and the corresponding biological models applied

Although it is still the prevailing methodology in Brazilian schools, several authors have already demonstrated the limited effectiveness of a class system based only on lectures for teaching biology in the first half of the last century[77–79]. Experimental teaching, however, faces difficulties in Brazil, since less than half of the country's high schools have a science laboratory[80]. An alternative would be the use of computer simulations. Several authors have reported the positive impact of these methodologies on teaching biotechnology topics[81–84]. Bonde et al. demonstrated a 101% increase in results in learning tests after using a virtual gamified laboratory experience in biotechnology education compared to the traditional methodology[85]. Recently, Pal et al. reported the development of a virtual teaching module for CRISPR/Cas[86]. However, these approaches also come up against infrastructure limitations. In 2019, 68% of Brazilian students reported not having access to the internet at school[87].

Alternatively, noncomputer educational games (e.g., card games, dice games, board games) do not require specific infrastructure and have been successfully applied by various authors in teaching several biology topics (Table 3). However, its use in CRISPR/Cas teaching has not yet been reported.

Subject	Reference
Biochemistry	Rose et al. [88]
Biochemistry	Pennington et al. [89]
Cell Biology	Spiegel et al. [90]
Cell Biology	Carvalho et al. [91]
Evolution	Muell et al. [92]
Evolution	Miralles et al. [93]

Evolution	Luttikhuizen [94]
Genetics	Osier et al. [95]
Genetics	Osier et al. [96]
Immunology	Eckert et al. [97]
Immunology	Steinman et al. [98]
Microbiology	Coil et al. [99]
Molecular Biology	Barnes [100]
Nutrition	Amaro et al. [101]
Physiology	Chaves et al. [102]

Table 3. Projects applying board games to teach different biology topics.

Board games are not the only tool being used to facilitate access to activities that would otherwise be costly and difficult to perform. Lego® bricks have been used to create tools and equipment for biotechnology, serving as a powerful building ecosystem[103]. We can understand this phenomenon in a broader context: the democratization of invention in education through digital fabrication tools[104]. The development of this project arises at the intersection of these three methodologies, inspired by the modularity of Lego® bricks and by the collaborative aspects of knowledge construction of educational board games, employing digital fabrication tools to create a low-cost toolkit and allow its decentralized production in the future.

1.3.1 Makerspaces and laser-cutting

Makerspaces, also called hackerspaces or FabLabs (Fabrication Laboratories), are open spaces for collaboration and collective construction, contributing to the development of technological skills in their users[105]. These spaces have been used in the educational context since the early 2000s, and the ideas behind this movement are based on four-decade-old theoretical and pedagogical pillars: experiential education, constructionism, and critical pedagogy[106]. One of the tools commonly found in these places is laser-cutting machines. The laser cutting process has numerous applications in the automotive, aerospace, shipbuilding, and material manufacturing industries[107]. In the context of makerspaces, it allows rapid and digitally controlled design technology, empowering users to transfer computational designs

into the physical world[108]. The user creates a vectorized image and sends commands to the machine, which guides a laser to cut and/or engrave a material. In general, in digital fabrication spaces, they are used for cutting acrylic or MDF (Medium Density Fiberboard). In this project, we chose MDF as raw material, due to its low cost, aiming to produce a final kit for less than 5 dollars per student. By producing the toolkit through laser cutting we can openly distribute the digital files, allowing educational spaces to manufacture locally our CRISPR/Cas teaching tool.

2. Justification

Despite the several works already developed with gamification and board games, the author is unaware of the existence of any tool that allows the teaching of CRISPR/Cas technology without a laboratory structure. The current project, therefore, aims to fill this gap.

The growing use of digital fabrication technologies in education is based on the work of numerous theorists and educators who have proposed teaching models with a greater focus on experimentation and direct relationships with real objects over the last century[104]. By allowing students to build using prototyping equipment such as 3D printers and laser cutters[109], educators empower students to manipulate the atom. This project aims to reproduce this effect in the process of learning the gene-editing technique CRISPR/Cas. Faced with the inevitable bioethical issues regarding the application of the technique in humans[56] and the proximity of this reality[110], the development of a low-cost tool for its use in the classroom can significantly impact students' education.

3. Objectives

3.1 General objective

To develop, using digital fabrication techniques, a methodology that allows the macroscopic visualization of the molecular mechanisms of CRISPR/Cas technology.

3.2 Specific objectives

- To design MDF (Medium Density Fiberboard) pieces that allow the student to assemble a DNA sequence (deoxyribonucleic acid) of their choice.
- To develop a system that allows the student to reproduce the action of RNA polymerase and simulate the transcription process by inserting pieces representing RNA (ribonucleic acid).
- To create a mechanism that emulates the action of a ribosome, translating the RNA strand and producing a polypeptide.
- To generate a piece that represents the activity of the Cas9 protein, connecting to a guide RNA, designed by the student, and recognizes the target DNA, performing the double-strand break.
- To produce a system that represents the NHEJ repair, silencing the gene initially assembled by the student.
- To create a mechanism that demonstrates HDR repair, using a student-built strand of DNA as a template.
- To develop a protocol for teaching CRISPR/Cas technology in the laboratory, editing the GFP (Green Fluorescent Protein) gene by replacing it with the RFP (Red Fluorescent Protein) gene.
- To develop a methodology to measure the knowledge about CRISPR/Cas and interest in the field to be applied before and after classes.
- To teach different groups of public high-school students, applying: theoretical classes, the toolkit developed in this project and the experimental protocol developed, quantitatively evaluating the learning in each scenario.

4. Methodology

4.1 Design and manufacturing of the toolkit

The pieces were developed using the Inkscape software (version 1.0.2). A 40W laser cutting machine, model SL-320 from CNC Zone, was used for its production. Both the software provided by the machine manufacturer, CorelLaser and an open-source alternative, the K40 Whisperer, were used to send the trajectory instructions. The pieces were produced using MDF (Medium-density fiberboard) rectangular plates with a thickness of 3 mm, supplied by different manufacturers. Each complete kit was produced using eighteen 30 x 20 cm plates. 40% of the power at a speed of 10 mm/s was used to cut the pieces. For the engraving of molecular structures and names, the cutting function was used at a speed of 20 mm/s, reduced to 10% of power, resulting in a "half-cut." A box was also developed and produced to facilitate the storage of the parts, with different compartments to organize the different components. Seven plates of 30 x 20 cm were used to produce each box, using the exact cutting and engraving configurations described above.

4.2 Development of the practical class

A practical class with a simplified protocol was developed to evaluate the performance of the tool proposed in this work. In this class, students transformed a strain of *Bacillus subtilis* expressing the green fluorescent protein (GFP) with a plasmid developed following the methodology described here, aiming to replace this reporter protein with the red fluorescent protein (RFP) in the microorganism genome.

4.2.1 Strains and transformation

Escherichia coli Top10 (Thermo Fischer) was utilized for plasmid construction. Once the assembly was complete, the plasmid was transferred into *E. coli* JM109 (Promega). For the production of the chemically competent cells, solutions of 0.1 M $CaCl_2$ and 0.1 M $CaCl_2$ plus 10% glycerol (v/v) were prepared, sterilized and cooled. *E. coli* precultures were prepared in 5 mL of LB medium (10.0 g/L Tryptone, 10.0 g/L NaCl, 5.0 g/L yeast extract. pH between 7.0 and 7.2) from stock in glycerol at -80°C (incubated at 37°C and 220 rpm for 12 hours). These were inoculated in 50 ml of LB medium at an OD_{600} of 0.03 and incubated at 37ºC and

220 rpm until reaching an OD_{600} of 0.3. Upon reaching the determined OD_{600}, they were transferred to two cooled sterile 50 mL centrifuge tubes, which were centrifuged for 10 minutes at 4000 rpm at 4ºC. The supernatant was discarded and the tubes were immediately placed on ice. The precipitate was resuspended in 10 mL of 0.1M $CaCl_2$ and incubated on ice for 20 minutes. The tubes were centrifuged again for 10 minutes at 3000 rpm at 4ºC and the supernatant was discarded again. The precipitate was carefully resuspended in 1 ml of 0.1M $CaCl_2$ plus 10% glycerol. Aliquots of the resulting solution were made into sterile 1.5 mL microtubes (100µL per tube). Aliquots were stored at -80°C. For the transformation steps, aliquots were thawed on ice. After thawing, 2-5 µL of intact plasmid or 10-20 µL of ligation reaction were added to 50 µL of competent cells, and the mixture was incubated on ice for 30 minutes. After this period, the tube was placed in a thermostated bath at 42ºC for 30 seconds and immediately transferred to ice, where it was incubated for 2 minutes. Subsequently, 250 µL of SOC medium (5 g/L yeast extract, 20 g/L tryptone, 0.548 g/L NaCl, 0.186 g/L KCl, 2.4 g/L $MgSO_4$, 200 g/L glucose were added. pH 7.5) and incubated for 1 hour at 37ºC and 220 rpm. After incubation, the transformed cells were plated at different concentrations on LB-agar medium, plus antibiotic, and incubated at 37ºC for 12 to 18 hours. The colonies that grew isolated on the plates were inoculated in liquid LB medium plus antibiotic and incubated at 37ºC and 220 rpm for 12 to 18 hours.

For gene editing using CRISPR/Cas, a strain of *B; subtilis* expressing the GFP protein, with the plasmid pDR111__GFP(Sp)[111] integrated into the *amyE* locus of the genome, was already available in the laboratory. For the production of the chemically competent cells, precultures were prepared in 5 mL of LB medium (incubated at 37°C and 220 rpm for 12 hours) from the glycerol stock at -80°C. The T Base solution (2.0 g/L $(NH_4)2SO_4$, 18.3 g/L $K_2HPO_4 \cdot 3H_2O$, 6.0 g/L KH_2PO_4, 1.0 g/L Sodium Citrate$\cdot 2H_2O$ was prepared. The OD600 of the pre-inoculum was quantified and a volume of the pre-inoculum was inoculated into 20 mL of SpC medium (20 mL T Base, 0.2 mL of glucose 50% (m/v), 0.3 mL of $MgSO_4 \cdot 3H_2O$ 1 .2% (w/v), 0.4 ml yeast extract 10% (w/v), 0.5 ml casamino acids 1% (w/v)) at an OD_{600} of approximately 0.5. The culture was incubated at 37°C under vigorous aeration (220 rpm) and the OD600 was monitored at 30-minute intervals. When it was observed that the growth reached the stationary phase, 2 ml of this culture were inoculated in 200 ml of SpII medium (200 ml T Base, 2 ml glucose 50% (m/v), 14 ml $MgSO_4 \cdot 3H_2O$ 1.2% (m/v), 2 mL 10% yeast extract (m/v), 2 mL 1% casamino acids (m/v), 1 mL 0.1M $CaCl_2$) preheated to 37°C. The incubation was maintained at 37°C, but at a lower agitation (180 rpm). After incubation for 90 minutes, the culture was transferred to sterile 50 mL centrifuge tubes and centrifuged at 8000

xg for 5 minutes at room temperature. The supernatant was then transferred to a sterile tube and stored. The precipitated cells were resuspended in 12 ml of this supernatant plus 8 ml of 50% glycerol (v/v; sterile solution). Finally, 250 µL aliquots of this mixture of cells were prepared in microtubes, which were stored at -80°C. For the transformation steps, aliquots were thawed at 37°C. 250 µl of cells, 250 µl of SpII medium + EGTA (200 ml of SpII, 4 ml of EGTA (0.1M, pH 8.0)) and 1200 ng of the plasmid to be transformed were added. The mixture was incubated at 37°C and 220 rpm for 1 hour. After this period, between 20 and 100 µL of the transformation were plated on LB-agar medium plus antibiotic. The plates were incubated at 37°C for 18 hours. The colonies that grew isolated on the plates were inoculated in liquid LB medium plus antibiotic and incubated at 37ºC and 220 rpm for 12 to 18 hours.

4.2.2 Plasmid construction

4.2.1 Design and cloning of the sgRNA

The plasmid pJOE8999[60] containing the Cas9 gene was used for the construction of the plasmid for practical classes. The design of the guide RNA sequence was made using analysis tools from the online platforms IDT (https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM) and CRISPR RGEN Tools (http://www.rgenome.net/cas-designer/).

The selection criteria were the score provided by the software for on-target and out-of-frame action, percentage of guanine and cytosine, absence of sequence of transcription termination, and presence of the PAM characteristic of SpCas9 ("NGG") (table 4).

Sequence	Position	Cleavage position (%)	Direction	GC content (% without PAM)	Out-of-frame score	On-target score
GGTGAAGGTGA TGCTACATA*CGG*	100	16.1	+	45.0	67.7	62

Table 4. sgRNA characteristics determined using IDT and CRISPR RGEN Tools online softwares. The PAM is highlighted. It was not part of the oligonucleotide.

Once the guide was defined, the nucleotides "TACG" and "AAAC" were added at the 5' end of the forward and antisense strands, respectively. Those sequences are complementary to the sticky ends in plasmid pJOE8999 after its digestion with the restriction enzyme *BsaI* (figure 3).

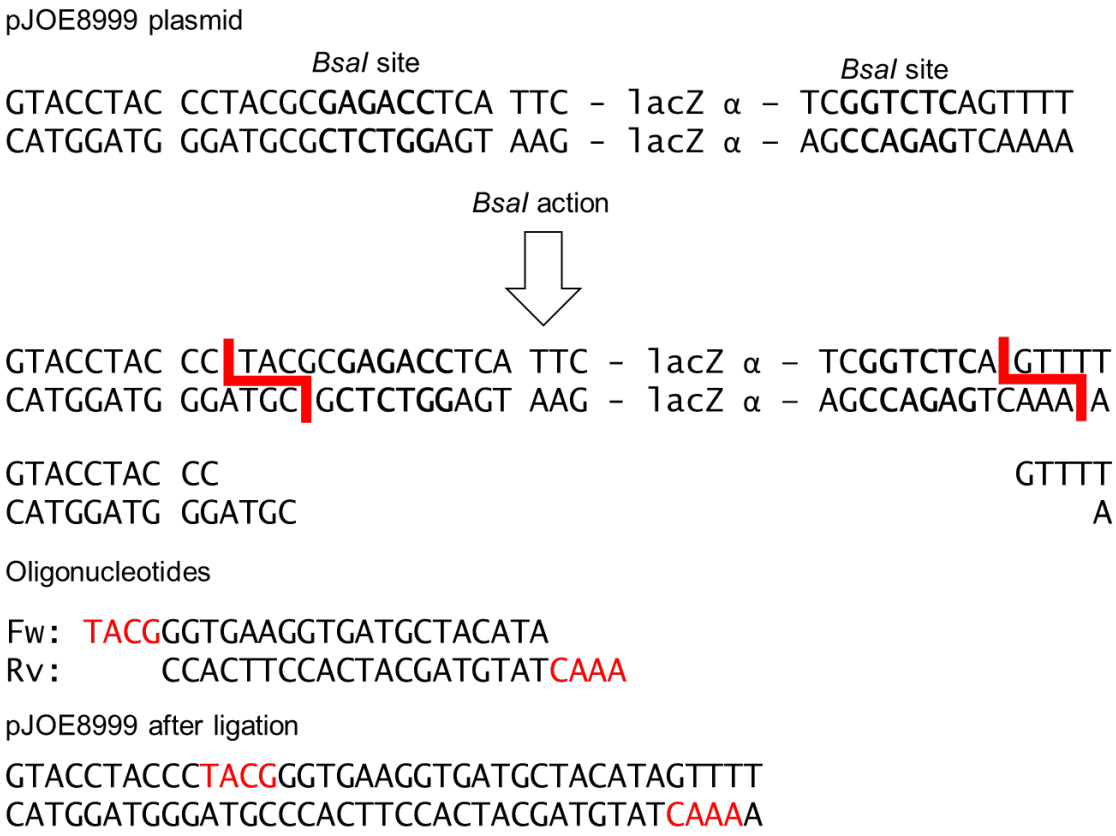


Figure 3. sgRNA ligation to the plasmid after digestion with *BsaI*. The plasmid has two sites for the action of the *BsaI* enzyme. After digestion, two cohesive ends are formed. The oligonucleotide design includes overhang sequences for ligation at these ends.

Once the oligonucleotides were acquired, the plasmid was digested, the guide annealed, and the plasmid cloned and transformed. The oligonucleotides corresponding to the guide RNA were annealed by adding 25 µL of each strand (forward and reverse) to 50 µL of 10 mM Tris-EDTA pH 8.0 (TE). The solution was heated at 98°C in the thermoblock for 10 minutes and naturally cooled to room temperature. 1500 ng of the plasmid was digested with restriction enzyme BsaI-HFv2 (New England Biolabs Inc.), with incubation at 37°C for 4 hours. Then, the reaction was incubated in a thermoblock at 80ºC for 20 minutes for thermal inactivation of the enzyme. The ligation of the guide to the plasmid was performed with 50 ng of plasmid and a ratio of 1:150 (plasmid:insert), calculated using the *Ligation Calculator* (http://www.insilico.uni-duesseldorf.de/Lig_Input.html). The T4 DNA Ligase enzyme (Thermo Fisher Scientific) and a total reaction volume of 10 µL were used. The reaction was incubated at 22°C for 2 hours. The resulting plasmid was then transformed by heat shock into chemically

competent *E. coli* Top10. Cells were plated on LB-agar medium plus 50 µg/mL kanamycin and incubated at 37°C for 18 hours. Colonies transformed by the ligation reaction were inoculated in liquid LB medium plus 50 µg/mL kanamycin and incubated at 37°C and 220 rpm for 12 hours. The plasmid was extracted from 2ml of each inoculum using the PureYield™ Plasmid Miniprep System kit (Promega).

The binding of the insert to the plasmid was confirmed by DNA sequencing, using 300 ng of plasmid, in the 3130 Genetic Analyzer sequencer equipment (Applied Biosystems), using the reagents from the BigDye® Mix Applied Biosystems kit (Thermo Fisher Scientific). First, the samples were prepared through a DNA amplification reaction (PCR), followed by purification before being loaded into the sequencer. The PCR (Polymerase Chain Reaction) was performed with an initial denaturation step of 1 minute at 96°C; followed by 25 cycles comprising the steps of denaturation at 96°C for 10 seconds, annealing for 5 seconds according to the T_m of the primer used (never exceeding 60°C) and extension at 60°C for 4 minutes; closing with a final extension at 60°C for 5 minutes. The product was then purified using the BigDye® XterminatorTM Purification kit (Applied Biosystems). 45 µL of the SAMTM Solution reagent was added, followed by the addition of 10µL of the XterminatorTM solution. Then, the microtube was placed on a shaker for 30 minutes at 2000 rpm, protected from light. After this period, the sample was centrifuged at maximum speed for 10 seconds and 20 µL of the resulting supernatant was pipetted into a 96-well plate specific for sequencing. The plate containing all the samples to be sequenced was placed in a plate centrifuge for 10 seconds at maximum speed and finally placed in the sequencer. The result was analyzed using the Benchling software.

4.2.2 Donor DNA assembly

The objective of the plasmid we developed is to replace the gene that expresses the GFP protein, present in the genome of the modified *B. subtilis* strain, with the RFP protein gene. Therefore, the donor DNA must have the sequences upstream and downstream to GFP, as well as the sequence that expresses RFP (figure 4). Figure 5 presents in more detail the strategy for assembling the donor DNA and the enzyme cutting sites added to the oligonucleotides. The sequences and properties of the final oligonucleotides are described in table 5.

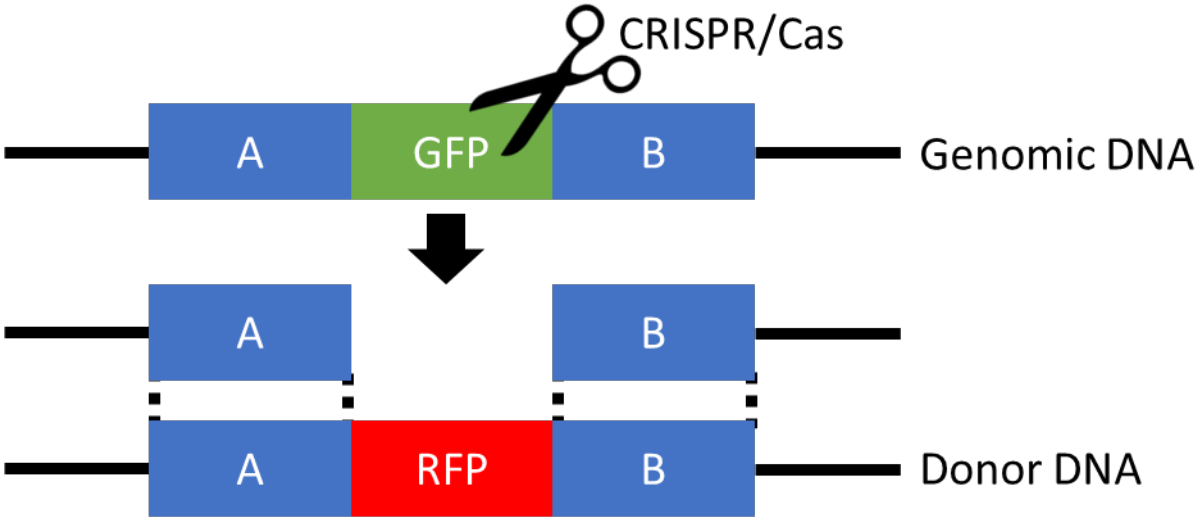


Figure 4. Overview of donor DNA assembly. To occur recombination and, consequently, to the RFP protein gene to be inserted into the genome of the microorganism, two homologous regions are necessary, here called "A" (upstream region, composed of 836 nucleotides) and "B" (downstream region, composed of 836 nucleotides). The primers for amplification of these regions were designed to keep the promoter, the ribosome binding site and the terminator already used in the expression of the GFP gene.

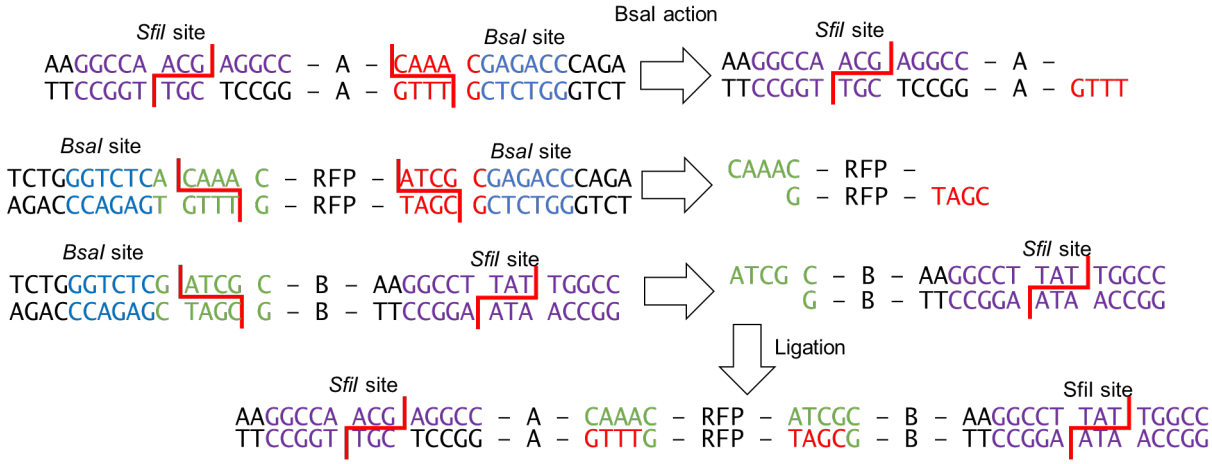


Figure 5. Donor DNA assembly strategy. The *SfiI* enzyme cleavage site is shown in purple, and the *BsaI* cleavage site is in blue. The nucleotides highlighted in red are part of the sequence to be amplified, while the nucleotides highlighted in green were added to the overhangs of the primers to form the sticky ends after digestion by *BsaI*. After the ligation between red and green sites, a sequence not recognized by the enzyme is created. After digestions and ligations, the donor DNA has both homology regions and RFP sequences. It has also sites for digestion with the enzyme *SfiI*, allowing the ligation in the plasmid pJOE8999.

Name	Sequence	GC content*	T_M *	T_M of the most stable hairpin
Fw_upstream	AAGGCCAACGAGGCCCTCCTGAT CCAAACATGTAAGTACCAATAAGG	40.6%	66.0°C	56.9°C
Rv_upstream	TCTGGGTCTCGTTTGTCCTCC TTATTAGTTAATCAGCTAGCTG	39.4%	66.1°C	36.5°C
Fw_RFP	TCTGGGTCTCACAAACATGGC TTCCTCCGAAGACGTTATC	50.0%	65.4°C	45.4°C
Rv_RFP	TCTGGGTCTCGCGATCT ACACTAGCACTATCAGCG	52.0%	65.8°C	37.4°C
Fw_downstream	TCTGGGTCTCGATCGCCAGCT GCTGGTATTACACATGGTATGG	48.1%	66.4°C	40.8°C
Rv_downstream	AAGGCCTTATTGGCCCGGT CTGATAAGAGACACCGGC	59.1%	65.7°C	52.5°C

Table 5. Sequences and properties of oligonucleotides used in the assembly of donor DNA. In yellow are nucleotides added to facilitate binding of restriction enzymes; in blue the *BsaI* site; in purple the *SfiI* site; in green nucleotides added for annealing after digestion, as illustrated in figure 19; in black the nucleotides that anneal with the original sequence to be amplified. *Refers only to nucleotides that anneal to the original sequence.

To amplify the upstream and downstream regions we started by cultivating the target *B. subtilis* strain in liquid LB medium and extracting its genome using the Wizard® Genomic DNA Purification Kit (Promega). 250 ng of the resulting genomic DNA was used as a template for each PCR amplification reaction using Phusion® High-Fidelity DNA Polymerase (New England Biolabs). The standard PCR protocol used was an initial denaturation phase at 98°C for 30 seconds; followed by 30 cycles with denaturation at 98°C for 10 seconds, annealing for 30 seconds (with temperature varying according to the primers used) and extension at 72°C for 30 seconds for every 1000 base pairs of the desired product; ending with a final extension phase of 10 minutes at 72°C. In the case of the upstream region, gradient PCR was performed to evaluate different annealing temperatures, with temperatures ranging from 59°C to 68°C. The annealing reactions performed between 64°C and 68°C for the upstream sequence and 65°C for the downstream sequence were correctly amplified, as confirmed by 1% (w/v) agarose gel electrophoresis. The product was purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega).

To obtain the sequence of the Red Fluorescent Protein (RFP), an *E. coli* Top10 strain carrying the pSB1C3 plasmid containing the gene of interest was cultivated in liquid LB medium with 35 µg/mL of chloramphenicol. The plasmid was extracted from 1.5mL of the culture using the PureYield™ Plasmid Miniprep System kit (Promega). PCR amplification was performed using Phusion® High-Fidelity DNA Polymerase (New England Biolabs), using 25

ng of plasmid DNA and 65°C as annealing temperature, following the protocol described above. Amplification was confirmed by electrophoresis in 1% (m/v) agarose gel. The product was purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega). Finally, the three fragments were joined using the Golden Gate technique[61], using 100 ng of each fragment, T4 DNA Ligase buffer and T4 DNA ligase enzymes (New England Biolabs) and BsaI-HF®v2 (New England Biolabs). The reaction product was then used as a template for a PCR reaction using Phusion® High-Fidelity DNA Polymerase (New England Biolabs) and the outer fragment primers. Three temperatures (64°C, 66°C and 68°C) were evaluated. The products were analyzed on a 1% (w/v) agarose gel stained with SYBR™ Green I Nucleic Acid Gel Stain (Thermo Fischer) and then cut and purified from the gel using the Wizard® SV Gel and PCR Clean-Up kit. System (Promega).

4.2.3 Plasmid assembly and evaluation

The plasmid pJOE8999 + sgRNA and the donor DNA were both digested with *SfiI* enzyme (New England Biolabs), with incubation at 50°C for 3 hours. Ligation was performed with 60 ng of plasmid in each reaction in ratios of 1:1, 1:2 and 1:3 (plasmid:insert), calculated using the Ligation Calculator (http://www.insilico.uni-duesseldorf. de/Lig_Input.html). T4 DNA Ligase enzyme (Thermo Fisher Scientific) and a total reaction volume of 10 µL were used. The reaction was incubated at 22°C for 2 hours. The resulting plasmids, in addition to a control plasmid without the addition of donor DNA, were then transformed by heat shock into chemically competent *E. coli* Top10. Cells were plated on LB-agar medium plus 50 µg/mL kanamycin and incubated at 37°C for 18 hours.

After transformation, colonies that grew in the Petri dish with antibiotic-containing medium were collected and analyzed by colony PCR. The primers used for this analysis and their properties are described in Table 6. The forward primer anneals in a region upstream to the insert, while the reverse primer anneals in a region downstream. Thus, if the plasmid had religated itself without the addition of the insert, an amplification of a sequence of 1138 base pairs would occur. Considering that the insert has 2019 base pairs, the positive result would be a sequence of 3157 base pairs.

Name	Sequence	GC content	Annealing temperature	Annealing temperature of the most stable hairpin
pJOE8999_seq(fw)	GAGACAAACCAA TACGTGAACAAG	41.7%	54.3°C	26.7°C
pJOE8999_seq(rv)	AACCATCACTGT ACCTCCCAAC	50%	56.8°C	14.6°C

Table 6. Sequences and properties of oligonucleotides used in colony PCR analysis.

Colonies that grew were evaluated by colony PCR using Taq DNA polymerase (Thermo Fischer). Positive colonies were cultured in liquid LB medium with 50 µg/mL of kanamycin and incubated at 37°C and 220 rpm for 12 hours. Plasmid was extracted from 2 ml of each inoculum using the PureYield™ Plasmid Miniprep System kit (Promega). The plasmid was then transformed by heat shock into chemically competent *E. coli* JM109, which were plated on LB-agar medium plus 50 µg/mL kanamycin and incubated at 37°C for 18 hours. The colonies that developed were collected, cultivated in liquid LB medium plus 50 µg/mL kanamycin and incubated at 37°C and 220 rpm for 12 hours. The plasmid was once again extracted from 2ml of each inoculum using the PureYield™ Plasmid Miniprep System kit (Promega). In addition, 20% (v/v) glycerol stock was carried out at -80°C of both *E. coli* strains (Top10 and JM109) containing the complete plasmid. This was called pEduc, considering its application. The plasmid was evaluated by digestion and sequencing. 500 ng of the plasmid was digested with SalI and XbaI enzymes at 50°C for 2 hours. The result was evaluated by electrophoresis in a 1% (m/v) agarose gel. For sequencing, four primers were utilized: forward and reverse primers from the RFP sequence, forward from the downstream sequence and reverse from the upstream sequence. A 3130 Genetic Analyzer sequencer equipment (Applied Biosystems) was used, using reagents from the BigDye® Mix Applied Biosystems kit (Thermo Fisher Scientific) and the same protocol described above. Results were compiled and analyzed using the Snapgene software.

4.2.3 CRISPR/Cas editing evaluation

The genome editing efficiency was evaluated as illustrated in figure 6. Competent *B. subtilis* cells were prepared and transformed as described in 4.2.1 using the pEduc plasmid (1). Volumes of 20, 50 and 100 µL of the transformation were inoculated onto plates with LB-

agar medium plus 5 µg/mL kanamycin and 1% mannose (m/v) (2). These were incubated at 30°C for two days to induce Cas9 gene expression and promote gene editing. Isolated colonies were collected from the transformation plates and streaked onto new LB-agar (antibiotic-free) plates for plasmid loss (3). The plates were divided into ten parts and a colony was striated on each part. They were then incubated at 42°C for 12 hours. The isolated colonies that grew were divided in half, half being streaked on plates of LB-agar medium and the other half streaked on plates and LB-agar medium plus kanamycin 5 µg/mL (4). Colonies that grew only in the medium without antibiotic were collected and cultivated in liquid LB medium with the addition of 1% lactose (m/v) to induce gene expression (5). Fluorescence was evaluated using a transilluminator.

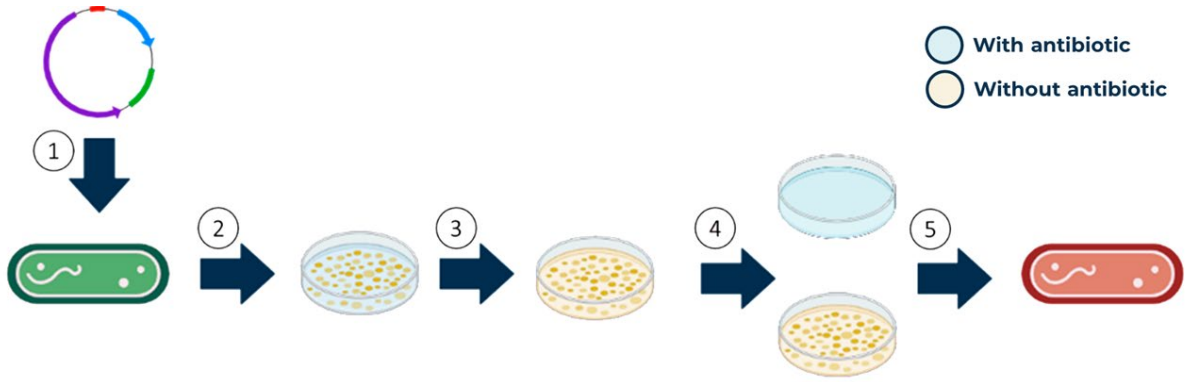


Figure 6. CRISPR/Cas editing evaluation. After transformation (1), cells were plated in a medium containing antibiotic and the cas9 gene expression inducer (2). Then they were transferred to an antibiotic-free medium for plasmid loss (3). Plasmid loss was assessed by the absence of growth in a medium containing antibiotic (4) and positive colonies were cultured in liquid medium for fluorescence analysis (5).

4.2.4 Simplified protocol for the practical class

For the application of the practical class, a protocol was developed that did not require equipment and could be performed in the 90 minutes available for the activity (figure 7). All solutions and media needed were previously prepared in the University Lab. 250 µL of chemically competent *B. subtilis* cells, 250 µL of SpII + EGTA medium, 1200 ng of pEduc and 250 µL of liquid LB medium were aliquoted into separate microtubes for each pair of students (45 pairs participated in the study). A Petri dish with LB-agar medium plus 5 µg/mL kanamycin and a Petri dish with LB-agar medium without antibiotic per pair were also provided. A Pasteur pipette was provided per pair for handling the samples, in addition to toothpicks for striation. For the safety of the participants, each one received a disposable lab coat and gloves.

The students thawed the cells using hand heat (approximately 37°C) and added the thawed cells and SpII + EGTA medium to the tube containing the plasmid (1). The mixture was left on the bench for 45 minutes. While waiting, the students received a plate containing already transformed colonies of *B. subtilis*. They streaked these colonies on plates with LB-agar medium without antibiotic to reproduce the process of plasmid loss after genome editing (2). They then received another plate containing other colonies, which had previously lost the plasmid, and inoculated them in 250 µL of liquid LB medium (3). Then, they plated the transformation that they had performed at the beginning of the activity in a Petri dish with LB-agar medium plus 5 µg/mL kanamycin (4). The plates were stored at room temperature for the growth of the transformed cells and analyzed together with the students the following week. Finally, strains producing GFP and RFP proteins were presented to the students, which were visualized using a transilluminator, to represent the initial and final stages of the procedure performed by them (5).

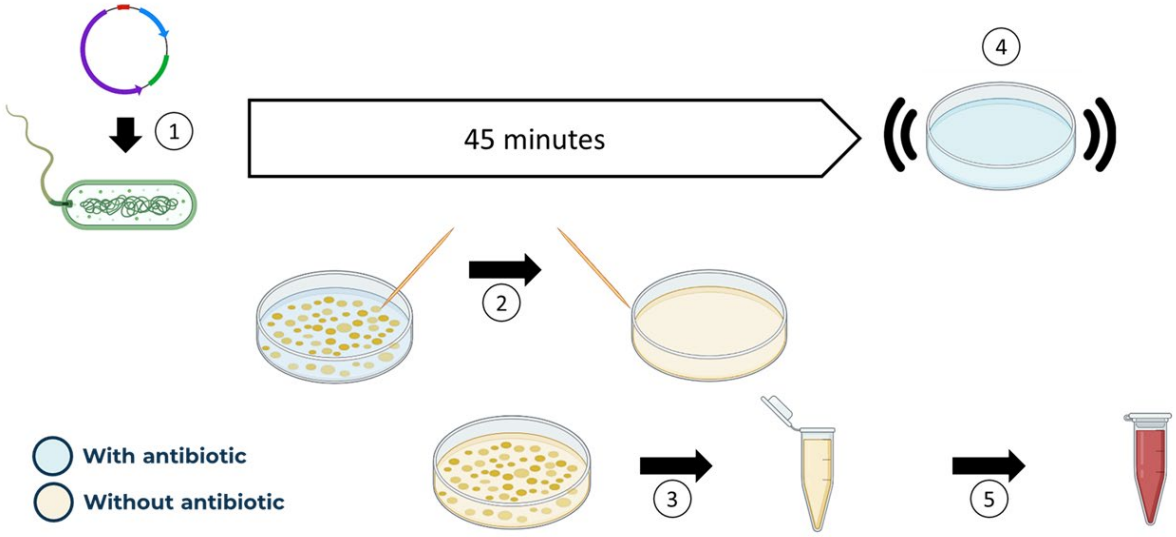


Figure 7. Simplified CRISPR/Cas protocol for practical class. After transformation (1), cells were incubated in the bench. In the meantime, the participants received already transformed cells and streaked them in antibiotic-free medium to simulate the plasmid loss process (2). In the next step they received a new Petri dish containing cells without plasmid and inoculated them in liquid medium (3). Then the students plated the cells they transformed in the first step (4). Finally, the students visualized the fluorescence of cells producing GFP and RFP using a transilluminator.

4.2.5 Biosafety and waste management

To ensure the safety of the participants, the practical classes were held in a public educational institution with a teaching laboratory, and students were provided lab coats and disposable gloves. Although the institution had on-site waste treatment infrastructure, including an autoclave for sterilization of materials, it was decided to transport and treat all waste in our research laboratory, following the usual protocols for genetically modified organisms and residual DNA.

4.3 Development of a methodology for learning assessment

To evaluate the impact of the toolkit proposed in this work, a questionnaire composed of two parts was developed consisting of five and nine questions. The first part, which addresses students' interest in biology, was adapted from Kennedy et al[55]. The second part was developed to measure students' learning outcomes and is divided into three distinct sections, each consisting of three questions. The first section assesses knowledge of genetics topics that are already included in the high school curriculum in Brazil. The second section assesses the technical aspects of how the CRISPR/Cas technology works. Finally, the third section assesses students' understanding of this technology's present and future impacts. Different versions of the questions with minor changes were developed to reduce the interference in the test results of both the successive repetitions and the possibility of sharing answers between the participants. The socioeconomic questionnaires (Appendix 1) and the different versions of the learning assessment questionnaire (Appendix 2) are available in the appendices.

The different questions composing the learning assessment tool were developed to allow students to express knowledge in different ways. For this reason, the scoring system differs between them, as shown in Appendix 3. In all questions, the participant had the option of selecting "I don't know/I would like not to answer."

4.4 Evaluation of the tool proposed in this work

4.4.1 Partner educational institution

The evaluation of the tool developed in the current project was carried out in collaboration with the Escola Estadual de Ensino Integral Sebastião de Oliveira Rocha, located in São Carlos, São Paulo. This institution was selected because of the availability of

infrastructure to hold practical classes and for having previously participated in other projects to teach Biotechnology and Molecular Biology techniques proposed by federal and state universities in the region. All students enrolled in the institution's third year of high school in 2022 participated in the evaluation. The choice for the third-year classes was carried out in a meeting with the school's technical staff, considering the suitability of the topic discussed in the present work to the schedule of classes and the regular curriculum of students. In addition, the new national high school curriculum had already been implemented in the first- and second-year classes but not in the third-year classes. Unlike the previous curriculum, there are no reserved times for laboratory activities in this new curriculum. Participants were already divided into three groups of 30 students each. Thus, 90 students from the aforementioned institution participated in the present work, divided into three groups that correspond to the three classes in which they regularly study.

4.4.2 Activities and assessments

Each of group of students participated in three classes lasting 90 minutes each, one theoretical, one practical, and another with the new toolkit LeDNA, taught at the partner educational institution. Each group participated in one activity per week for three weeks. Before the start of the first intervention, each participant answered the socioeconomic questionnaire and the evaluation questionnaire. After each activity, the participants answered one of the versions of the evaluation questionnaire (the three versions developed are available in Appendix 2), as described in Figure 8.

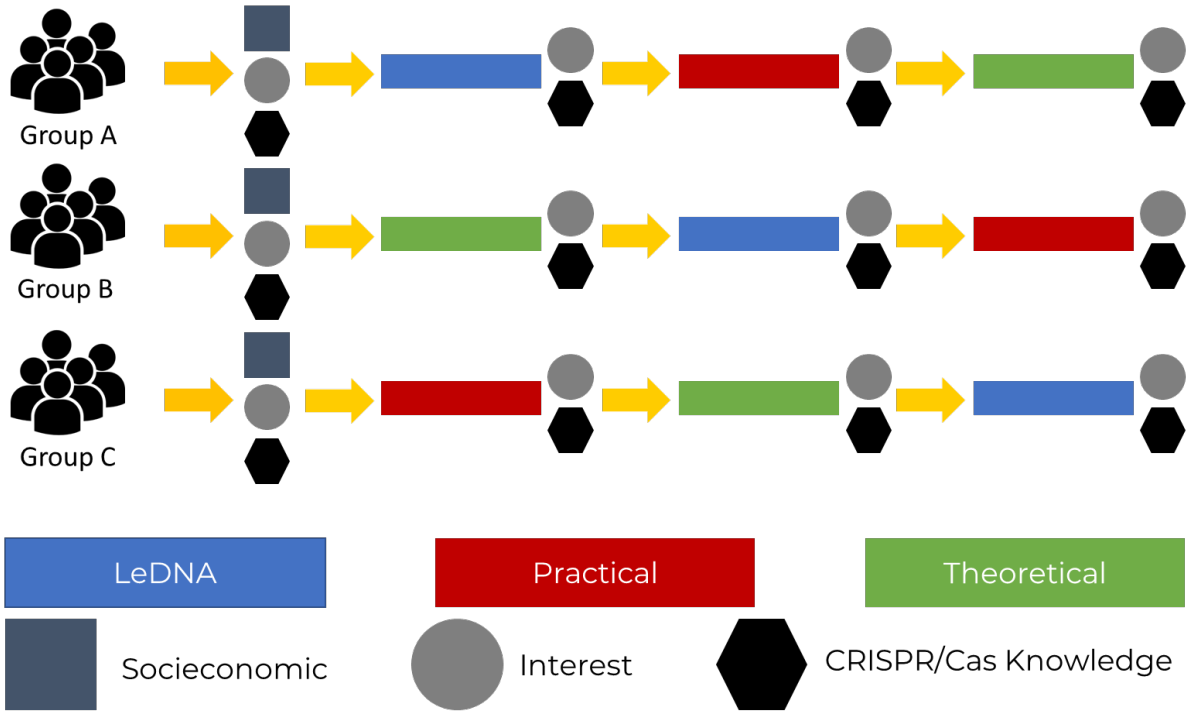


Figure 8. Application of the different interventions in the three study groups. There was a one-week break between each activity. The bars represent the activities and the geometrical shapes de questionnaires.

4.5 Statistical analysis

The test results were adjusted to a Two-Parameter Logistic Model (2PL) of the Item Response Theory using jMetrik[112] software to evaluate the developed assessment instrument. The parameters of difficulty and discrepancy were calculated for each of the questions that fit the model. Finally, the alpha coefficient (or Cronbach's Alpha)[113] was calculated to assess the test reliability. Then, the socioeconomic characteristics of the different groups were evaluated, verifying if they could interfere with the final result of the evaluation. The results of the science attitude survey and the learning assessment tool were then analyzed using the Minitab software. Both the total results and the results per section were evaluated. The Shapiro-Wilk test[114] was used to verify the normality of the distribution of results. ANOVA[115] was performed in cases where the results followed a normal distribution to verify differences between the groups. In the other cases, the Kruskal–Wallis[116] test was used.

5. Results

5.1 Toolkit design

The toolkit developed in this work was named "LeDNA" by combining the words "Lego" and DNA. Its central objective is to allow students to simulate CRISPR/Cas gene editing and observe its impact. Therefore, the toolkit must represent not only the genome editing mechanism but also the final product of the technique, i.e., the modification in the produced protein or its deactivation. We developed pieces representing the sugar-phosphate backbone of both DNA and RNA, DNA and RNA nucleotides, ribosomes, amino acids, hydrogen bonds, and peptide bonds, allowing the user to assemble a double-strand DNA and perform transcription and translation. Pieces representing the Cas protein and the guide RNA were also designed, allowing the user to edit the original DNA strand. The development of each of these items is presented in subsequent sessions.

5.1.1 Sugar-phosphate backbone

A single piece was developed to represent the sugar-phosphate backbone of both double-stranded DNA and single-stranded RNA (figure 9). It is 28 cm wide by 3 cm high, with grooves of 3 mm every 2 cm, allowing the fitting of 12 nucleotides. The toolbox contains four identical copies of the part, two intended for the assembly of the DNA strand, one for the RNA strand, and one for the donor DNA for repair after the action of the Cas protein. For the construction of the guide RNA, a reduced version of the piece was developed, containing the same height and distance between the grooves, but fitting only six nucleotides.

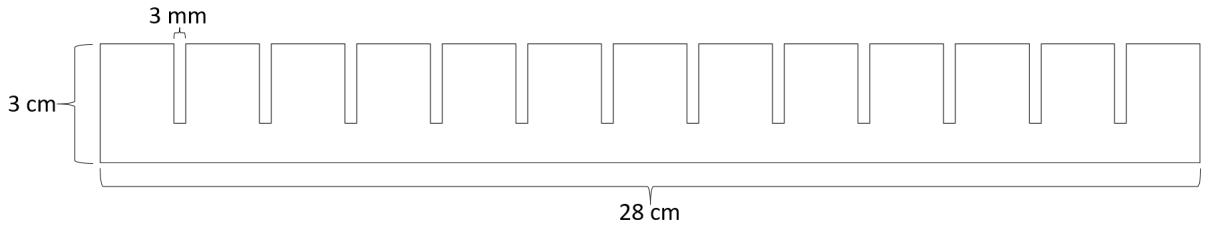


Figure 9. Design of the part representing the sugar-phosphate backbone. The addition of nucleotides occurs perpendicularly. Each gap has the same measure of the thickness of the material used in the production of the parts.

5.1.2 Double-stranded DNA

The following design requirements were established for the development of the pieces representing the DNA structure (figure 10):

1 – To allow the user to form any sequence of their choice on the template strand;

2 – To allow the user to pair only suitable nucleotides (adenine with thymine, cytosine with guanine) to form the antisense strand;

3 – Not to allow system stability in case of single strand construction;

4 – To allow the action of the Cas protein after pairing the guide RNA.

In order to complete criterion 1, the same nucleotide/sugar-phosphate backbone connection was implemented in the four different nucleotides, allowing their organization in any order. Two features were implemented in the development of the parts to fulfill criterion 2. First, the distance from the point of connection to the sugar-phosphate backbone and the extremity of the piece differs between purines (8.89 cm) and pyrimidines (5.76 cm). Thus, once the first purine/pyrimidine pair is correctly fitted, the sugar-phosphate backbones are positioned at the correct distance, and a purine/purine or pyrimidine/pyrimidine pairing is not possible. Second, the connection between the nucleotides occurs according to the number of hydrogen bonds performed by the same. Since the distance from the first bond to the base of the piece differs between those that make two connections – thymine and adenine – (2.08 cm) and those that make three – guanine and cytosine – (1.81 cm), the incorrect pairing is not possible. Combining the two features makes it possible to connect only a purine/pyrimidine pair that forms the same number of hydrogen bonds. To meet criterion 3, the nucleotide/sugar-phosphate skeleton connection point was shifted from the center of the piece by 7.5 mm. Therefore, when the user builds a single-stranded structure, it does not remain in an upright position, tipping over (to the left, in the case of the template strand, to the right, in the case of the antisense strand). Finally, to meet criterion 4, an appendix was added at the top of the piece, allowing the Cas protein to fit if the guide RNA is correctly aligned, as discussed in item 5.1.6. The molecular structure of the compound was also added to the design of the piece, allowing the user to visualize the nucleotide, as well as its name and an indication that it is a piece of DNA, to facilitate its use.

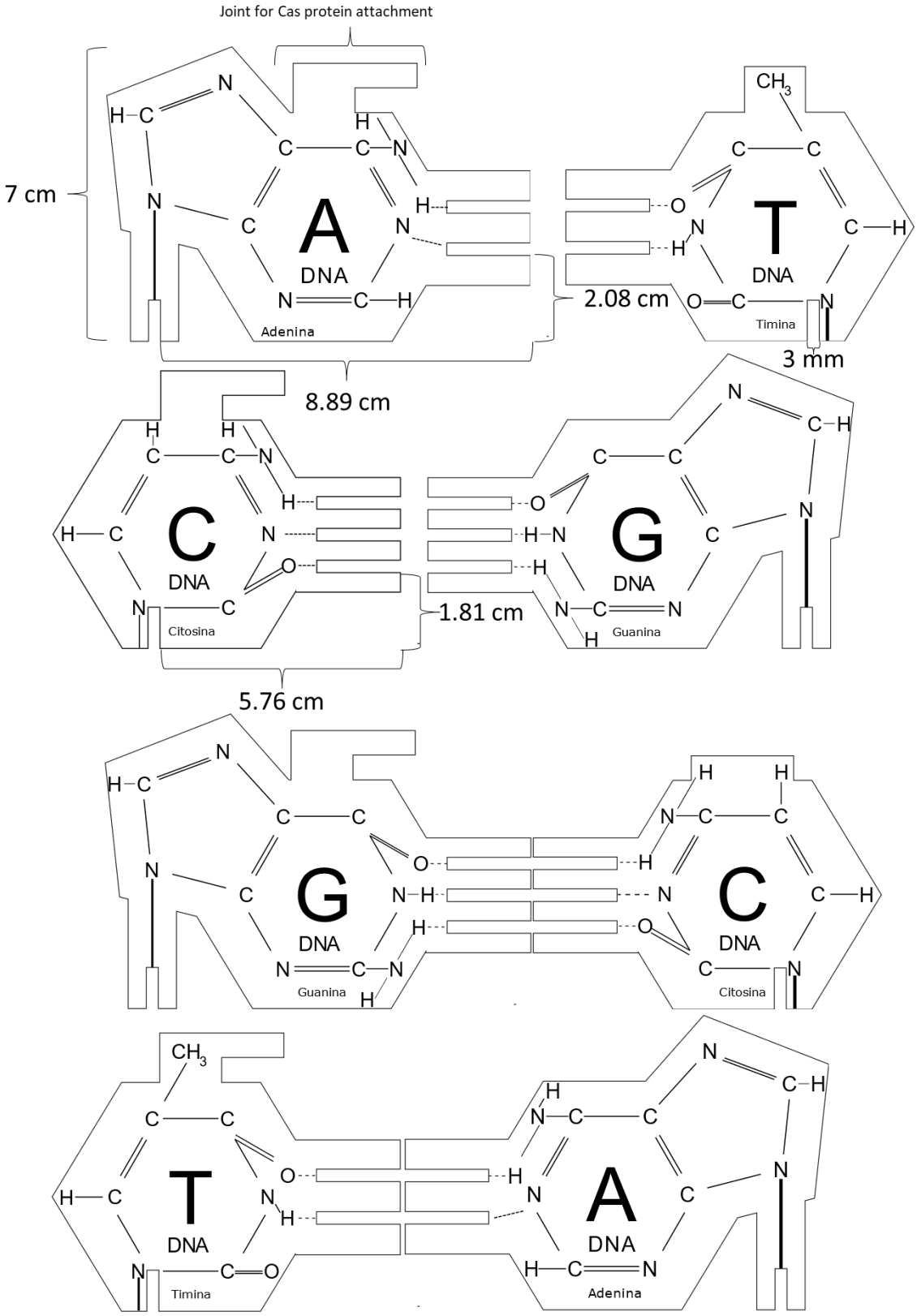


Figure 10. Design of the pieces representing deoxyribonucleotides (DNA). The fitting of the pieces takes place perpendicularly to the sugar backbone, with the hydrogen bonds shown

in figure 5 connecting them. The complete kit contains the following number of copies of these parts: 7 adenines, 7 cytosines, 5 guanines and 8 thymines for the template strand and 8 adenines, 5 cytosines, 7 guanines and 7 thymines for the antisense strand. Part numbers differ due to the encoding of the start codon (ATG) and stop codons (TAA, TAG, and TGA).

5.1.3 Single-stranded RNA

The following design requirements were established for the development of parts representing the RNA structure, shown in figure 11:

1 – To allow the user only the pairing of suitable nucleotides (adenine and uracil, cytosine and guanine) for the formation of the RNA strand;

2 – To transmit the information contained in the DNA strand to form the appropriate polypeptide;

3 – To allow system stability for single-strand construction;

The strategy described in the previous item was employed to achieve criterion 1. Purines and pyrimidines have different distances between the connection to the sugar-phosphate backbone and the piece extremity, as well as different distances between the connection to the hydrogen bond and the base for nucleotides that perform different numbers of hydrogen bonds. To promote the correct fitting of the corresponding amino acid and thus transmit the information established in the formation of the double-stranded DNA by the user, each piece of RNA had a structure added on top. In the case of uracil, this joint is continuous, while in the others, it is segmented, divided into two parts in adenine, into three in guanine, and into four in cytosine (figure 7). In all cases, the distance between the beginning of the first segment and the end of the last one (even when there is only one segment) is the same, ensuring the exact fit for all nucleotides in the piece representing the Cas protein (described in item 5.1.6). Due to these structures added to each nucleotide, gaps in the design of each amino acid, described in item 5.1.5, allow the recognition of the nucleotide present in the DNA template strand. Unlike the pieces previously described for DNA, the sugar-phosphate backbone connection was added in the center of the pieces, allowing the stability of the single strand. Similarly to DNA nucleotides, the chemical structure, and name of each of the nucleotides were engraved. In addition, the term RNA was also included to facilitate the use of the toolkit by the user.

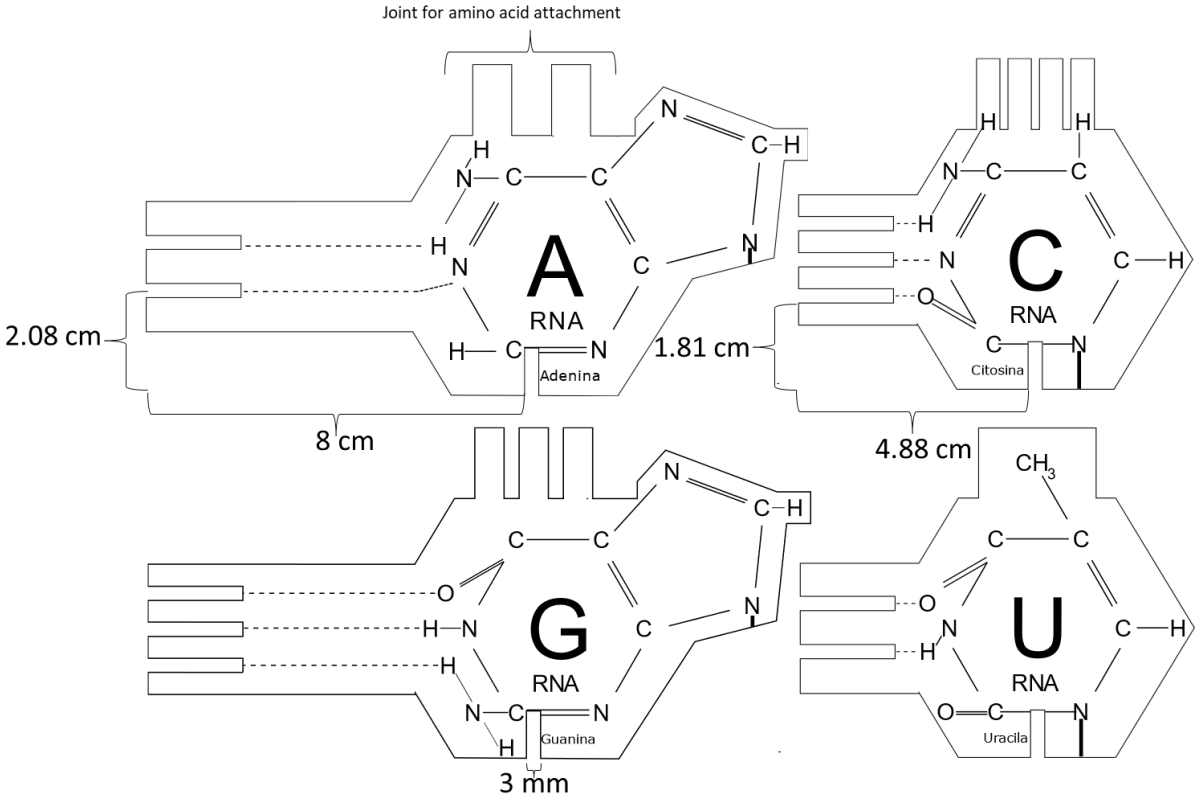


Figure 11. Design of pieces representing ribonucleotides (RNA). As with the DNA pieces, the fitting occurs perpendicularly to the sugar backbone, with the hydrogen bonds, shown in figure 5, connecting them. The complete kit contains the following number of copies of these parts: 8 adenines, 5 cytosines, 7 guanines and 7 uracils. Part numbers differ due to start codon (AUG) and stop codon possibilities (UAA, UAG and UGA).

5.1.4 Ribosome and bonds

Parts representing hydrogen bonds were developed to connect the different nucleotides (figure 12a). Altogether thirty-two parts representing hydrogen bonds are part of the toolkit. This number was determined considering that the user will build a sequence of 12 nucleotides, therefore, of 4 codons. It must start with the start codon (AUG), which requires seven bonds. For the two subsequent codons, the user can choose any nucleotides, so each new trio will need from six (if you only use nucleotides that require two hydrogen bonds, A and U/T) to nine bonds (if you only use those that require three bonds, G and C). Finally, it must include a stop codon, which can be UAA (6 bonds), UAG (7), or UGA (7). Therefore, the maximum number of hydrogen bonds the user may need is seven for the first codon, nine for the second, nine for the third, and seven for the fourth, totaling thirty-two pieces.

For the peptide bond, a different mechanism was employed (figure 12b). Considering that the amino acid pieces are inserted over the already existing RNA strand, it becomes more accessible for the user to also insert the bond over the piece instead of laterally, as in the case of hydrogen bonding. Four copies are part of the toolkit. The last of the four codons the user constructs must always be a stop codon. The piece representing the stop codon does not have a peptide bond slot precisely to stop the polypeptide formation. Therefore, three amino acids will always fit together, so two bonds are needed to connect them. Four copies were added to the toolkit, allowing the formation of two polypeptides composed of three amino acids each to allow the user to observe the polypeptide formed before and after the use of CRISPR/Cas.

The design of the piece representing the ribosome (figure 12c) allows it to fit into any of the four nucleotides since its connection gap is continuous.

It connects into three nucleotides (one codon) to make the codon concept more accessible for the user and make it easier to connect the amino acid correctly. In addition, the side opening allows the piece to be pulled out from under the amino acid after fitting and then used for the addition of the subsequent nucleotide.

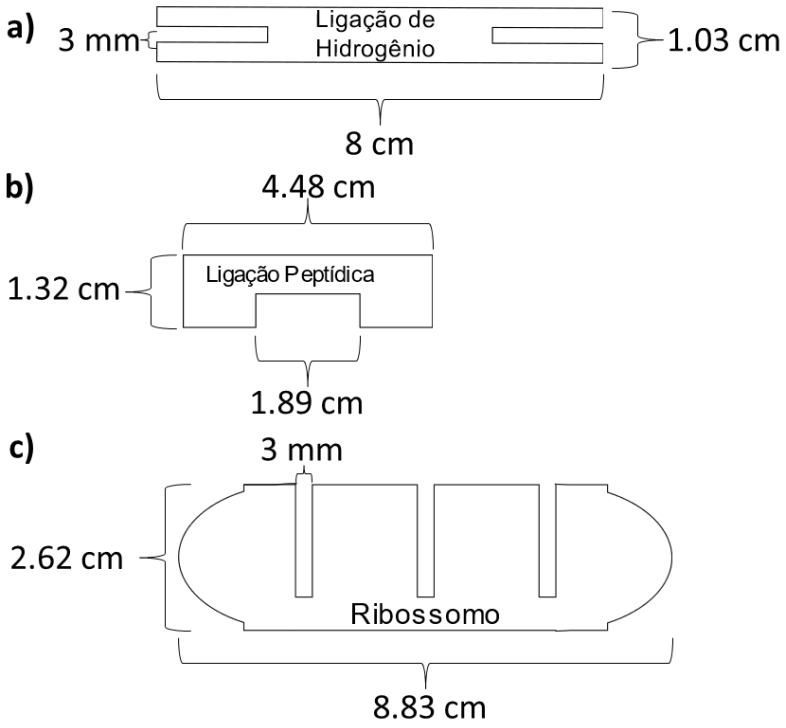


Figure 12. Design of connecting parts. a) Hydrogen bonds (l*igação de hidrogênio* in Portuguese) are used to connect nucleotides. Each kit has 32 copies of the part. **b)** Peptide bonds (l*igação peptídica* in Portuguese) are used to connect amino acids. Each kit has 4 copies of the part, allowing the construction of two polypeptides with three amino acids each. **c)** The ribosome (*ribossomo* in Portuguese) fits over three pieces of RNA (codon), directing

the user to add the corresponding amino acid piece. Their socket spaces are not closed to allow removal from the side for use in the subsequent codon. Each kit contains one ribosome.

5.1.5 Amino acids and stop codons

The following design requirements were established for the development of parts representing the amino acids (figure 13):

1 – To recognize the information transmitted through the RNA sequence;

2 – Since the genetic code is redundant, an amino acid encoded by more than one codon must recognize each one of them;

3 – A stop codon must stop the formation of the polypeptide.

For each of the twenty amino acids, a different design was developed, highlighting the different side chains and, therefore, their different physicochemical characteristics. Each amino acid also has a gap to connect with the peptide bond. To meet criterion 1, each one has a cut pattern that allows it to fit over the correct nucleotide triplet and no other. In the case of amino acids encoded by more than one codon (as described in criterion 2), a separate piece was developed for each of them, conserving the side chain and modifying the cut pattern as necessary. For example, six distinct pieces represent the amino acid leucine, but each one fits into a specific nucleotide sequence (codon) rather than the others (figure 14). In the case of the three stop codons, the gap for connecting with peptide bonds was removed, interrupting the translation process.

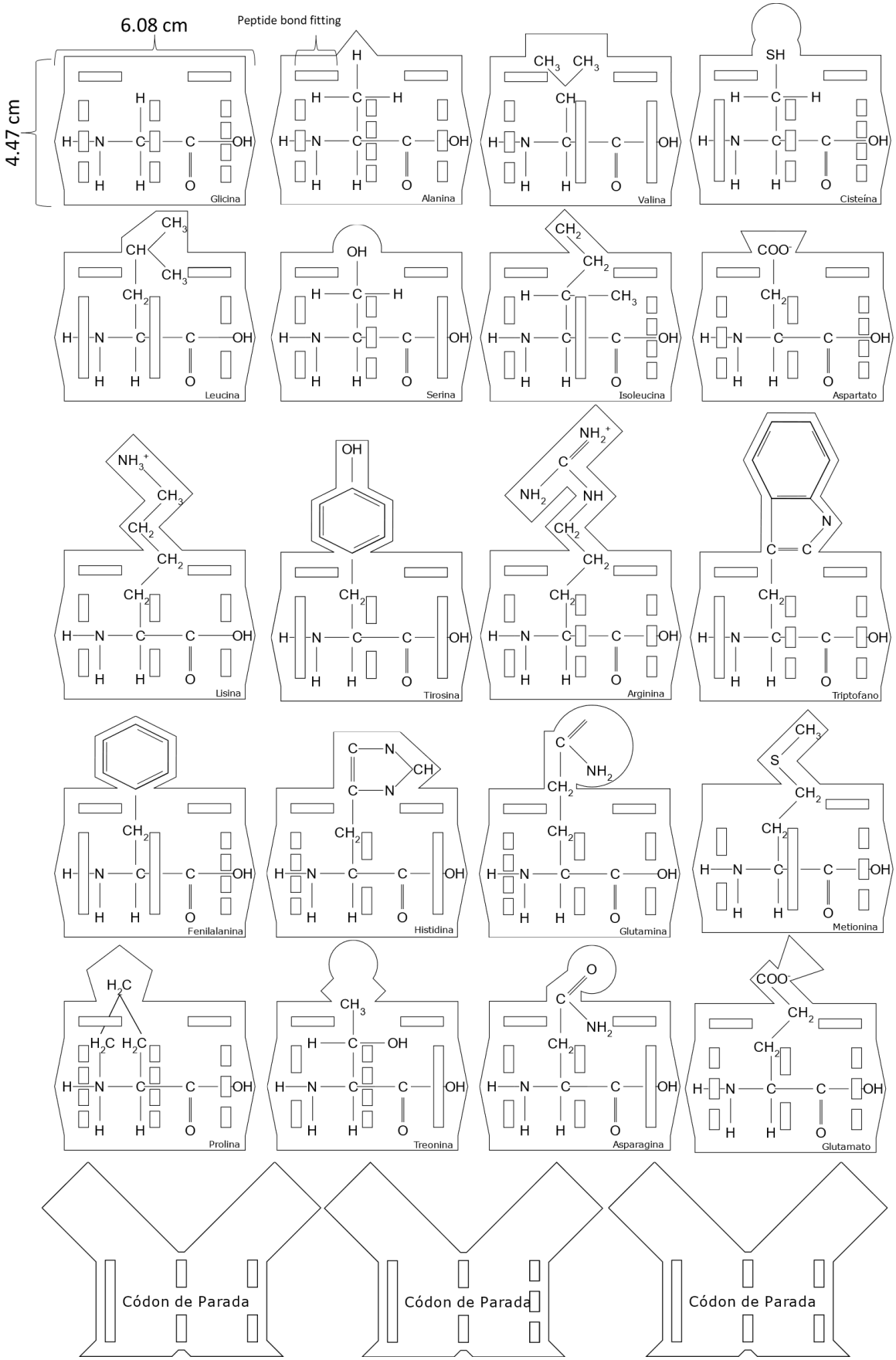

6.08 cm
4.47 cm
Peptide bond fitting
Glicina
Alanina
Valina
Cisteína
Leucina
Serina
Isoleucina
Aspartato
Lisina
Tirosina
Arginina
Triptofano
Fenilalanina
Histidina
Glutamina
Metionina
Prolina
Treonina
Asparagina
Glutamato
Códon de Parada
Códon de Parada
Códon de Parada

Figure 13. Design of pieces representing amino acids and stop codons. A version of each amino acid was made for each codon that is able to encode it, keeping the exact side chain representation and adjusting to the proper fitting pattern. The following number of parts were developed: alanine (4), arginine (6), asparagine (2), aspartate (2), cysteine (2), phenylalanine (2), glycine (4), glutamine (2), glutamate (2), histidine (2), isoleucine (3), lysine (2) leucine (6), methionine (1), proline (4), serine (6), tyrosine (2), threonine (4), tryptophan (1), valine (4), in addition to the three stop codons presented, totaling 64 pieces.

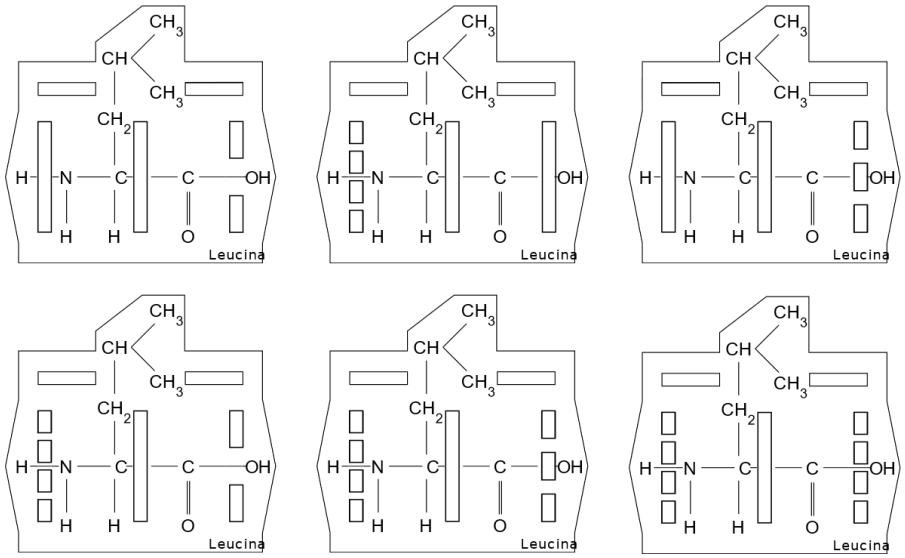


Figure 14. Different models of the amino acid leucine. Amino acids encoded by more than one codon were represented by similar pieces with modifications in the RNA fitting pattern. Only methionine and tryptophan are represented by a single piece in the kit.

5.1.6 Cas protein and guide RNA

For CRISPR/Cas gene editing, the user employs three elements:

1 - The Cas protein;
2 - A guide RNA;
3 - A donor DNA for the repair (optional)

The DNA template is optional as the user may insert nucleotides randomly to represent the NHEJ repair mechanism. The recognition and fitting in the DNA strand occur through interaction with the guide RNA, simulating the mechanism present in nature. The fitting of the guide RNA then promotes cleavage of the DNA by the Cas protein. The design criteria for the Cas9 protein were:

1 – To allow fitting of any user-designed guide RNA;

2 – To cleave the template DNA strand.

The piece was developed with a width of 12 cm and space to fit six RNA nucleotides (Figure 15). The fittings are a continuous gap, allowing any of the four nucleotides to be used. The piece is 10.5 cm long, allowing it to be inserted under the upper joint of the DNA template pieces after pairing with the guide RNA, removing them through a lever mechanism when lifted.

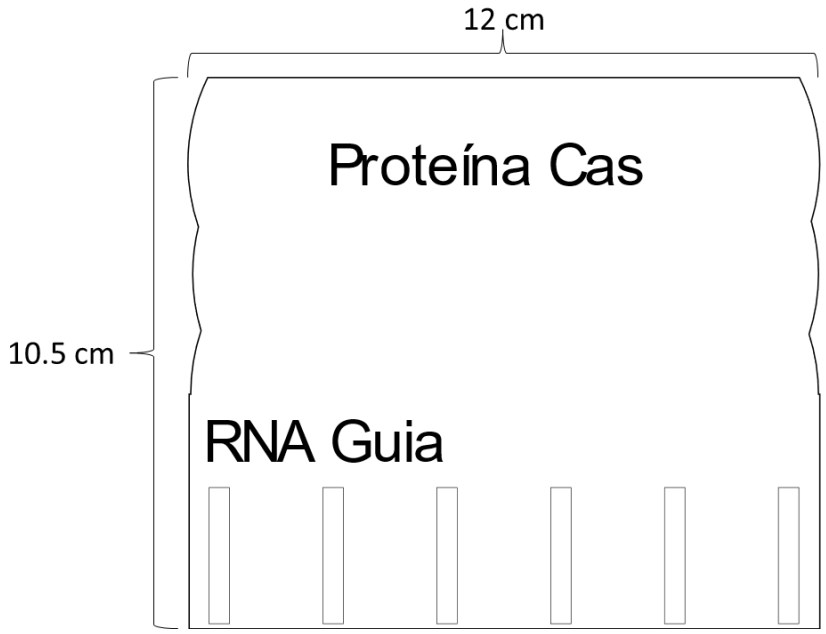


Figure 15. Design of the part representing the Cas protein of the CRISPR/Cas system. At the bottom edge of the image are the nucleotide slots that allow any RNA piece to be added. The top edge of the image is the point on the part that connects under the joint of the template DNA nucleotides and removes them when Cas is lifted by the user. Proteína Cas = Cas protein; RNA Guia = guide RNA.

5.2 Toolkit manufacturing

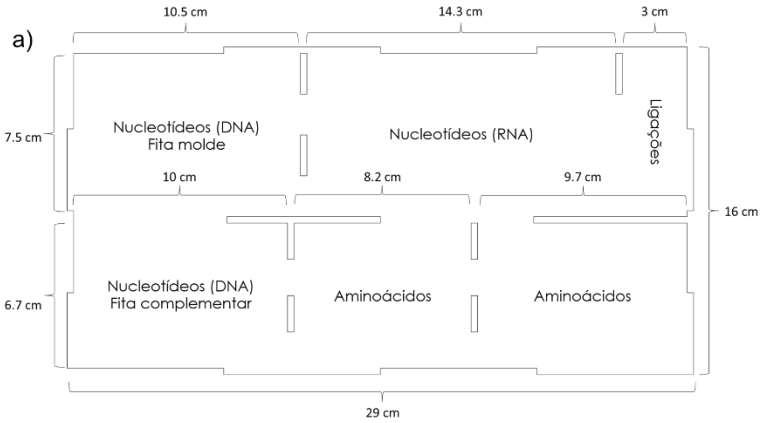
The complete set of kit parts is displayed in figure 16. The process of creating a double-strand DNA sequence, a single-strand RNA and a polypeptide takes around 45 minutes. The edition of the original sequence through CRISPR/Cas, followed by transcription and translation of the modified DNA sequence takes additional 45 minutes. The process can be seen at: https://youtu.be/y2XPC0IKDjY .

Figure 16. Complete LeDNA kit. At the top, each piece is presented individually. At the bottom, assembled DNA and RNA strands are presented and similar pieces are superimposed, to facilitate visualization. The complete set includes 27 nucleotides for template strand DNA, 27 for antisense strand DNA, 27 for RNA, 64 amino acids, 32 hydrogen bonds, 2 peptide bonds, 1 ribosome, 4 sugar-phosphate backbones, 1 sugar-phosphate backbone for guide RNA, 1 Cas protein.

5.3 Development and manufacturing of a storage box for the toolkit

A box was designed and constructed for the correct storage of the different kit parts (figure 17). The 29 x 16 x 11.5 cm box has dedicated compartments for each group of pieces.

a)


10.5 cm
14.3 cm
3 cm
7.5 cm
Nucleotídeos (DNA)
Fita molde
Nucleotídeos (RNA)
Ligações
10 cm
8.2 cm
9.7 cm
16 cm
6.7 cm
Nucleotídeos (DNA)
Fita complementar
Aminoácidos
Aminoácidos
29 cm

b)


LeDNA
unesp
SYNBIO
UNESP AQ4

c)


unesp
SYNBIO
UNESP AQ4

d)


Nucleotídeos (DNA)
Fita molde
Nucleotídeos (RNA)
Nucleotídeos (DNA)
Fita complementar
Aminoácidos
Aminoácidos

Figure 17. LeDNA storage box. The design of the base is represented in **(a)**. The hollow regions between the compartments are used to fit the walls. The different compartments for amino acids have different measurements to accommodate the diversity of the size of the side chains of the pieces. On the lid **(b)** the name of the kit was added. On the front **(c)** the logos of Unesp, the Faculty of Pharmaceutical Sciences and the SynBio Araraquara laboratory were added, in addition to the following phrase "Developed by Guilherme E. Kundlatsch under supervision of Prof. Dr. Danielle B. Pedrolli". The base **(d)** was built following the design shown in a).

In total, ten boxes, each containing a complete set of parts, were produced. Each box requires 7 MDF boards 30 x 20 cm with 3 mm thickness for its production, totaling 25 boards for the production of each complete LeDNA kit.

5.4 Plasmid development for the practical class

For the practical class, we developed a plasmid to replace the gene that expresses the GFP protein, present in the genome of the modified *B. subtilis* strain, with the RFP protein gene. To guide the Cas9 protein, oligonucleotides matching the forward and reverse strand of the gRNA were ligated into the digested plasmid. The sequencing of the resulting plasmid is shown in figure 18. To replace *gfp* with *rfp*, the donor DNA has the upstream and downstream regions of the *gfp* gene, and the *rfp* gene in between. All tree sequences were amplified by PCR (figure 19).

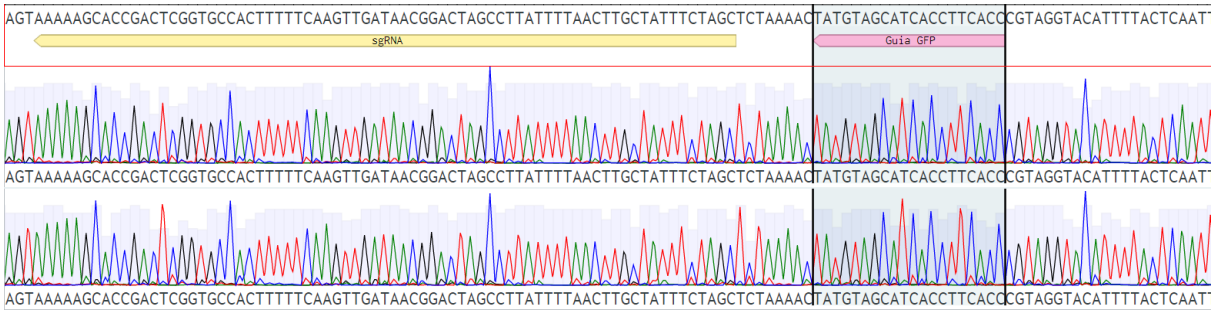


Figure 18. Result of sequencing of plasmid pJOE8999 after addition of the guide. The upper sequence was constructed in silico in Benchling software for cloning design and is the expected sequence. The two lower sequences were obtained by sequencing in duplicate using the same primer. The result demonstrates that the addition of the guide was successful.

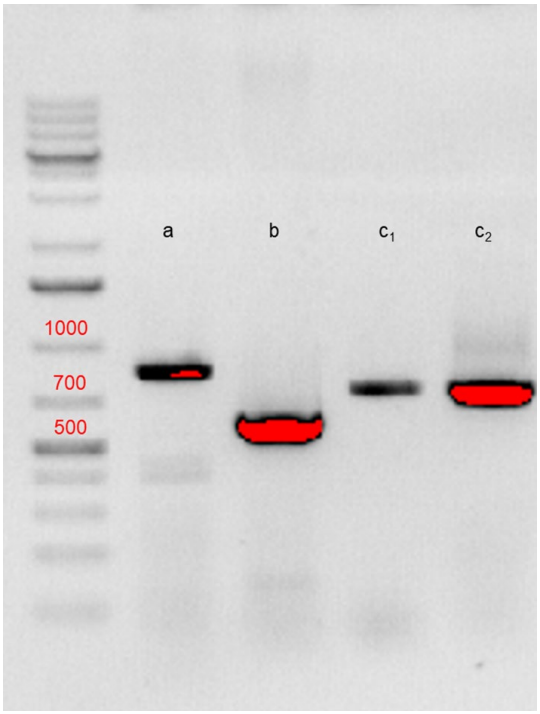


Figure 19. Agarose gel electrophoresis of PCR products from donor DNA sequences. The upstream (a – 836 base pairs), downstream (b – 567 base pairs), and RFP sequences (in duplicate, c1 and c2 – 706 base pairs) were correctly amplified. The number of base pairs of the ladder bands of interest is indicated in red just above each band.

Once the sequences were amplified, they were joined by Golden Gate. The reaction was carried out in duplicate. Different annealing temperatures were evaluated in the final amplification step. The products were analyzed by agarose gel electrophoresis (figure 20) and bands of the expected size were cut from the gel and purified for plasmid assembly.

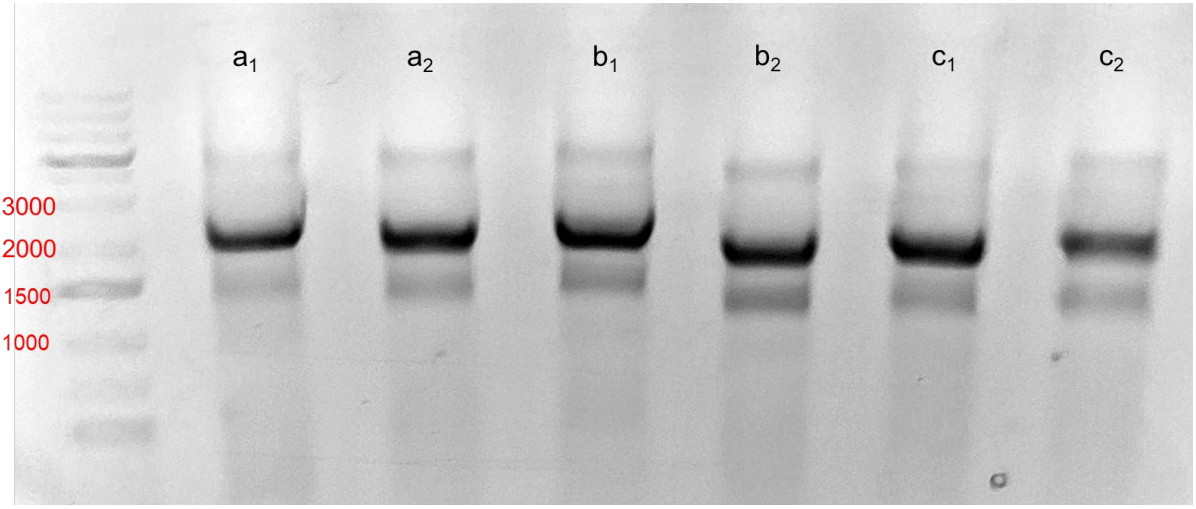


Figure 20. Agarose gel electrophoresis of the PCR product after Golden Gate assembly. The reaction was performed in duplicate and the product of each was used as a template for PCR at three different temperatures: 64°C (a1 and a2), 66°C (b1 and b2), 68°C (c1 and c2). A 2019-base pairs band was expected. This was successfully achieved in all reactions. The number of base pairs of the ladder bands of interest is indicated in red next to each band.

Once the donor DNA sequence was obtained, it was ligated into the plasmid already containing the gRNA through *Sfi*I digestion stick ends. *E. coli* transformants were screened for positive colonies and one was found (figure 21).

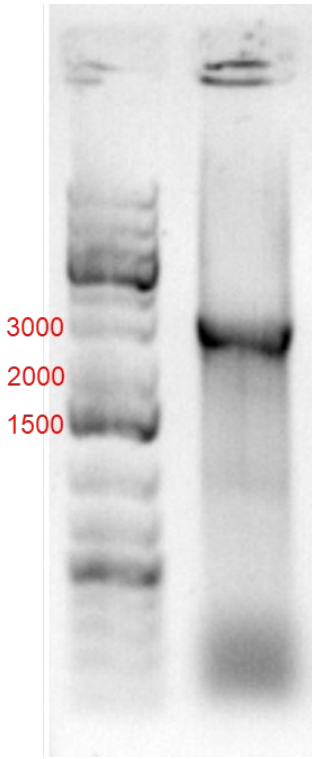


Figure 21. Agarose gel electrophoresis of the colony PCR product after transformation with the complete plasmid. A band of approximately the expected size (3157) indicates that this colony has a plasmid with the insert. Negative colonies are not represented in the image.

The colony that showed a positive result was used as a plasmid source. The complete plasmid was named pEduc, considering its application. Figure 22 is a schematic representation of it.

Figure 22. Schematic representation of the complete pEduc plasmid. Image produced using Snapgene software. The complete plasmid includes the sgRNA, highlighted in pink, and the donor DNA, composed of the upstream sequence (in green), the RFP gene (in red) and the downstream sequence (in green). In addition, it has the other sequences already present in pJOE8999 necessary for the functioning of the CRISPR/Cas system.

To confirm the correct construction of pEduc, the plasmid was sequenced. Results of all sequencing attempts were compiled in the Snapgene software and are simply presented in figure 23.

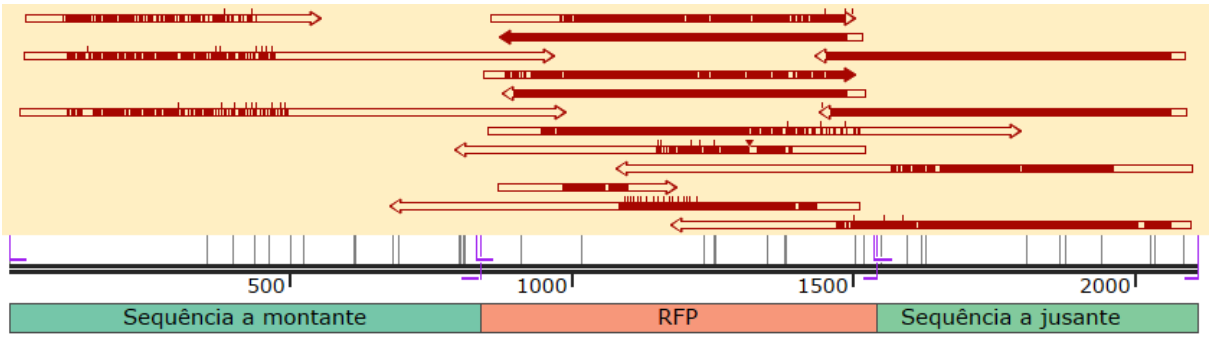


Figure 23. Sequencing of the donor DNA region in the complete plasmid. The different arrows represent different sequencing reactions. The sections in red indicate that the sequenced nucleotide was the expected nucleotide. Sessions with no fill at the beginning and end of the arrows indicate low-quality regions of the sequencing that did not align with the template sequence. The RFP and downstream sequences were confirmed through sequencing.

The sequencing results indicate that the RFP and downstream sequences were correctly added to the plasmid, however, it was not possible to confirm the upstream sequence, especially the region closest to the RFP sequence, which contains the promoter and ribosome binding site for transcription and translation of the protein.

The plasmid was then used to transform a *B. subtilis* strain expressing GFP, in order to validate the practical class protocol. After transformation, the loss of the plasmid was performed. Colonies in which plasmid loss occurred and untransformed control lines were cultured in the presence of lactose to induce reporter protein expression. They were then centrifuged and visualized using the transilluminator, as shown in figure 24.

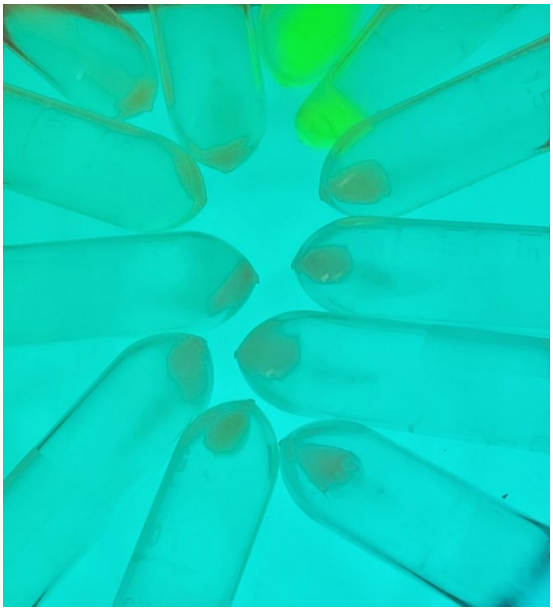


Figure 24. *B. subtilis* after gene editing by CRISPR/Cas. Control strains (at the top of the image) exhibit green fluorescence, while the edited strains do not. However, these do not exhibit the expected red fluorescence.

The objective of the practical class is to replace the gene encoding the GFP protein in the *B. subtilis* genome by the RFP protein gene. The result presented in figure 24 indicates that the editing mechanism is active, with the expression of the GFP gene being turned off. However, it was not possible to visualize the RFP expression. Considering that the sequences of the promoter and the ribosome binding site in the donor DNA were not successfully verified in the sequencing, a possible explanation for the failure would be the presence of a mutation in this region.

5.5 Application of the practical class with high school students

The theoretical, practical and LeDNA kit activities were applied in a public school over three weeks, starting on May 30, 2022 and ending on June 14, 2022. One of the limitations faced was the choice of not using methods of maintaining sterile conditions, such as a Bunsen burner (to avoid risks to the participants, since they wore disposable lab coats) and a laminar flow hood (due to the infrastructure limitations of public high school institutions). This limitation is relevant in two steps of the class protocol, in the plating of the transformed cells and in the striation (simulating the loss of the plasmid). In the case of the plating step, the exposure time of the plates to air was reduced, since the students transferred the transformed cells to the Petri dish (using a Pasteur pipette) and then immediately closed the plate, spreading the cells in the plate through circular movements with the closed plate resting on the table. In the stage in which the students simulated the process of plasmid loss, they streaked colonies already transformed using a toothpick, which resulted in a more extended period of exposure to air and a greater risk of contamination. In addition, unlike the plates used for the transformation step, these plates did not include the addition of antibiotics, which increased the risk of contamination. Figure 25 presents the result obtained by a group of 24 students at this stage.

Figure 25. Petri dishes containing *B. subtilis* streaked by students during the practical class. It is possible to observe the growth of colonies of *B. subtilis* on all plates, with little or no presence of contaminants. Plates contain LB with no antibiotic added.

The plates produced by the students showed a surprisingly small number of contaminations, considering the conditions of the activity. This result demonstrates that this step of the protocol can be adopted even in the absence of methods for maintaining sterile conditions.

The efficiency of the simplified transformation protocol used in the activity was also analyzed. Considering the limitations of infrastructure and class time, the incubation after transformation was modified from 1 hour at 37°C and shaking at 220 rpm to 30 minutes at room temperature without shaking. Figure 26 presents the results obtained by the 3 groups of students who participated in the study.

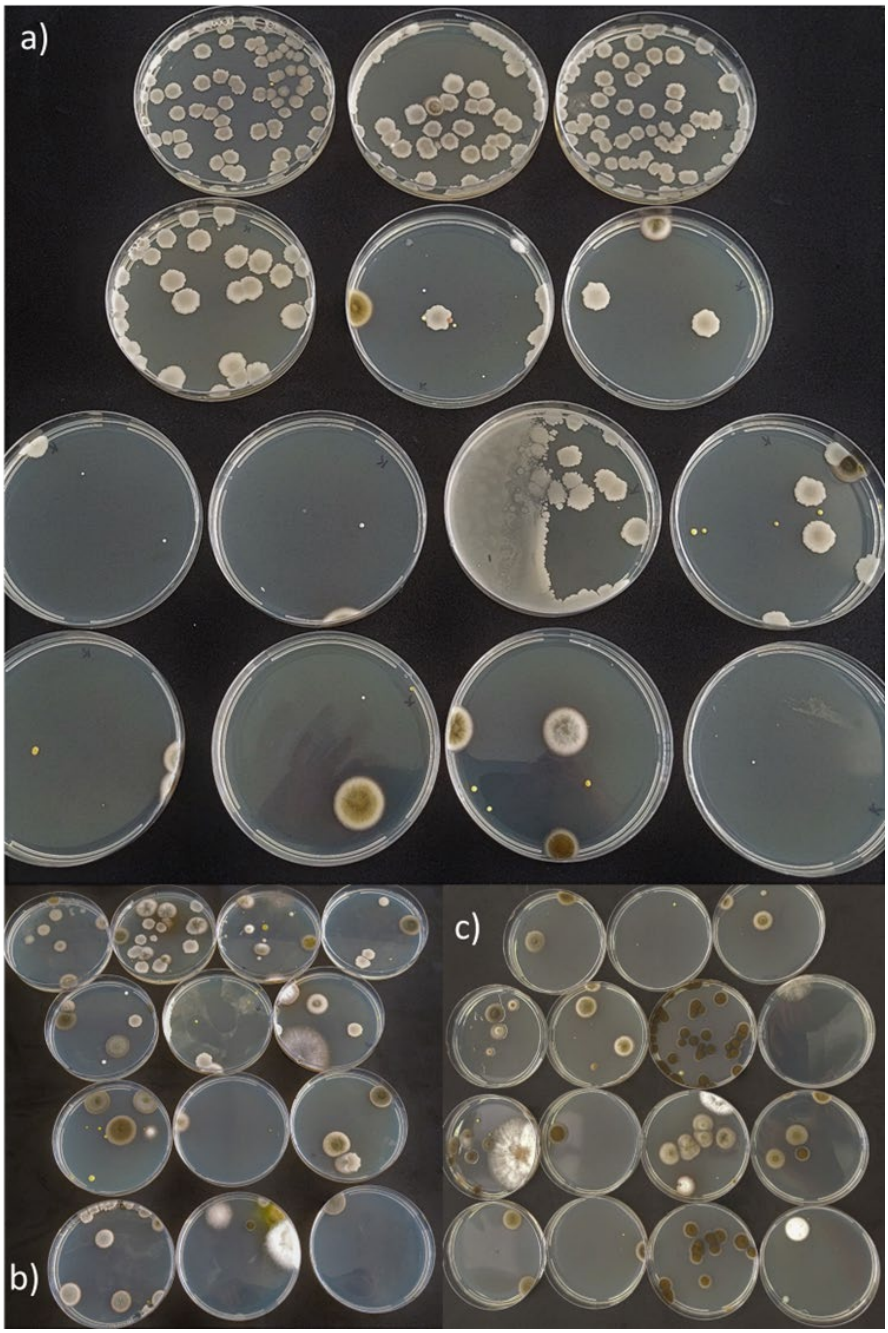


Figure 26. Petri dishes containing *B. subtilis* transformed by the students during the practical class. a) Results obtained by the first group to participate in the practical activity. 8 of 14 plaques (4 negatives not shown) demonstrated growth of *B. subtilis*. Considering the presence of antibiotic in the medium, it is likely that all of them indicate success in the transformation reaction. **b) Results obtained by the second group.** 8 of the 14 plates had colonies of *B. subtilis*, indicating successful transformation. However, in the positive plates there is a much smaller number of colonies of interest and a much greater number of contaminations, when compared to the first group. **c) Results obtained by the third grou.** It was not possible to observe colonies of *B. subtilis* on any plate.

The result obtained by the first group indicates that the protocol proposed in this project is viable, being possible to perform the transformation and obtain a low number of contaminants even with the limitations of time and infrastructure. It is possible that the decrease in the number of positive colonies in the plates over the weeks is related to the storage conditions of the chemically competent cells of *B. subtilis*. While in the laboratory the cells were stored at -80°C; for the practical activity, the cells were stored in the fridge freezer of the teaching laboratory of the partner institution. Its loss of viability, therefore, may be related to the storage temperature. It is concluded that, in future applications of this protocol, the cells must remain stored at -80°C and be transported to the partner institution only when the activity is performed.

5.6 Application of the LeDNA kit

The LeDNA toolkit was applied in seventy-five-minute classes. Participants were divided into groups of 3 to 4 students, and each group received a complete kit. The students started the assembly by building a donor DNA strand, adding the three nucleotides referring to the start codon, and, therefore, the amino acid methionine. Then each group added six nucleotides of their choice. Finally, they were presented with the three stop codon sequences, adding the one of their choices. Then, they were instructed to complete the double-stranded DNA by adding the nucleotides from the antisense strand and the necessary hydrogen bonds. Once the double-stranded DNA was obtained, they assembled a single-stranded RNA from it. These two steps were performed in approximately 45 minutes by all groups, with assistance when necessary.

From the sequence of the messenger RNA formed, the participants performed the assembly of the polypeptide, adding three amino acids and two peptide bonds, in addition to the piece representing the stop codon. Finally, they performed the assembly of the guide RNA and the cleavage of the original DNA strand using the Cas protein, using a donor DNA as a template for the repair. This second stage took approximately 30 minutes. After completing the activity, each participant devoted 15 minutes to solving the evaluation questionnaire. The intervention was carried out in the multimedia room of the partner educational institution, which allowed the use of tables for the use of the kit in groups. Figure 27 shows some of the participants using LeDNA.

Figure 27. Study participants using the LeDNA tool. Students were divided into groups of three to four participants, with one or two groups at each table.

5.7 Application of the CRISPR/Cas theoretical class

A theoretical class of seventy-five minutes was developed, with an additional period of fifteen minutes being reserved for carrying out the assessment activity. The class was divided into three blocks of twenty-five minutes. In the first block, applications of CRISPR/Cas technology were presented, aiming to arouse the interest of the participants. These applications were selected in order to bring genetic editing closer to students' daily lives. Applications in livestock[54,55] and agriculture[117,118] related to students' eating habits were presented. The use of this technology to combat disease-transmitting insects was also

discussed[40], with students reporting cases of family members who were affected by dengue fever and malaria recently. Finally, greater emphasis was given to applications in the health area[119], with emphasis on human gene editing. The subsequent block was dedicated to reviewing topics that students had already studied in high school, including the structure and properties of DNA, RNA and proteins, as well as the mechanisms of transcription and translation. This review was carried out by asking students about these topics, with additional comments when necessary. Finally, the final block was intended to present the operation of the CRISPR/Cas technology, discussing its action within the cell and the components necessary for its application by a researcher. The class was held in the partner institution's multimedia room, with the use of a projector to facilitate the visualization of the molecular mechanisms by the participants.

5.8 Evaluation of the different methodologies

5.8.1 Participants' socioeconomic profile

Before the first intervention, a socioeconomic assessment was conducted with each participant to identify possible differences between the three groups. Participants' age (figure 28), gender (figure 29), family income (figure 30), type of institution attended in the previous school levels (figures 31 and 32), and legal guardian level of education were assessed (figure 33).

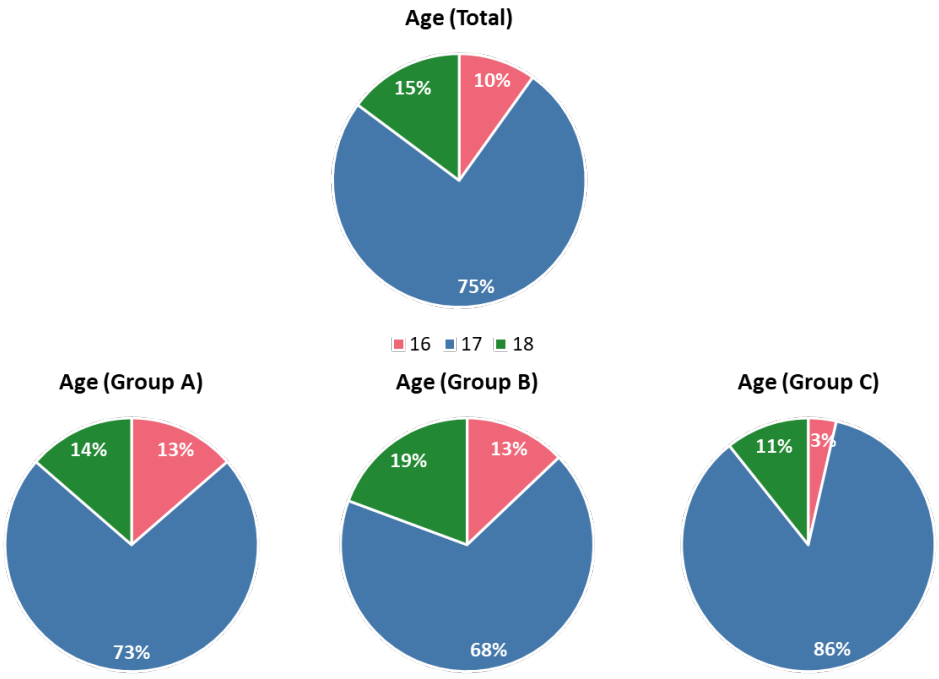


Figure 28. Distribution of study participants by age. For this educational level, students are expected to be 16 (in orange) or 17 (in blue) years old, depending on their date of birth.

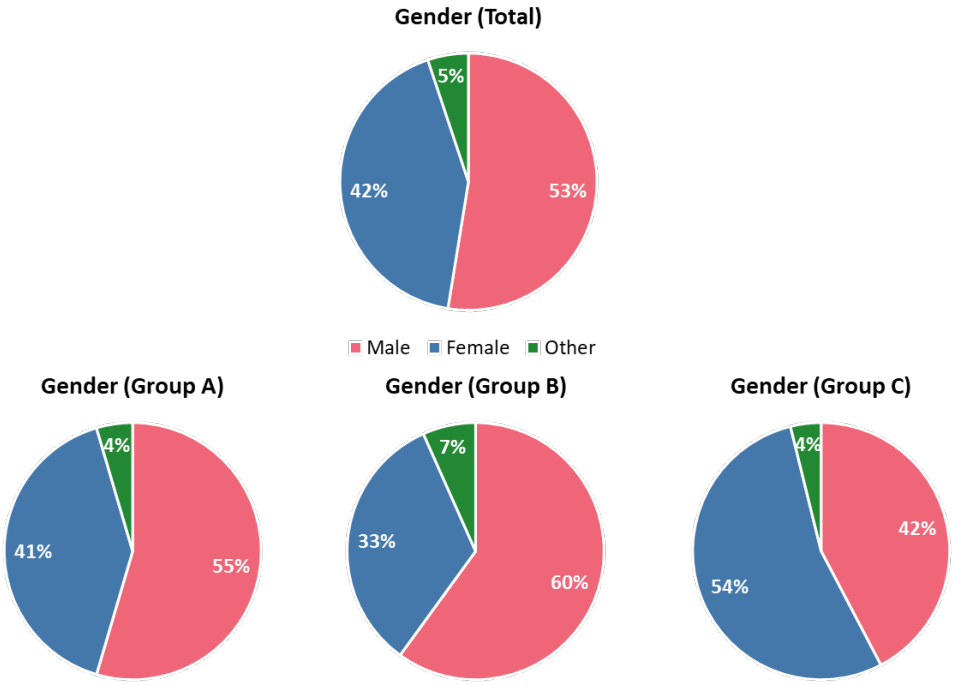


Figure 29. Distribution of study participants by gender. Participants were given the options: male, female, and an open field to declare their gender.

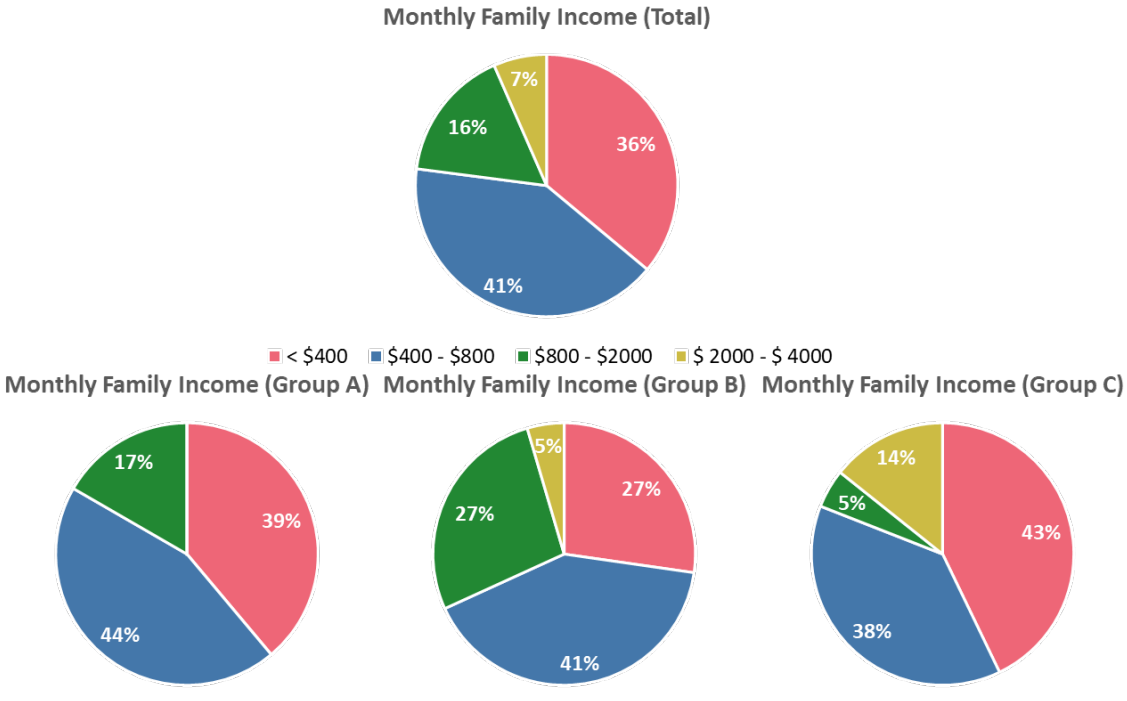


Figure 30. Distribution of study participants by monthly household income (in US dollars). Family income is the sum of the income of each of the components of the family nucleus. The option "more than $4000" was available on the form, but was not marked by any participant.

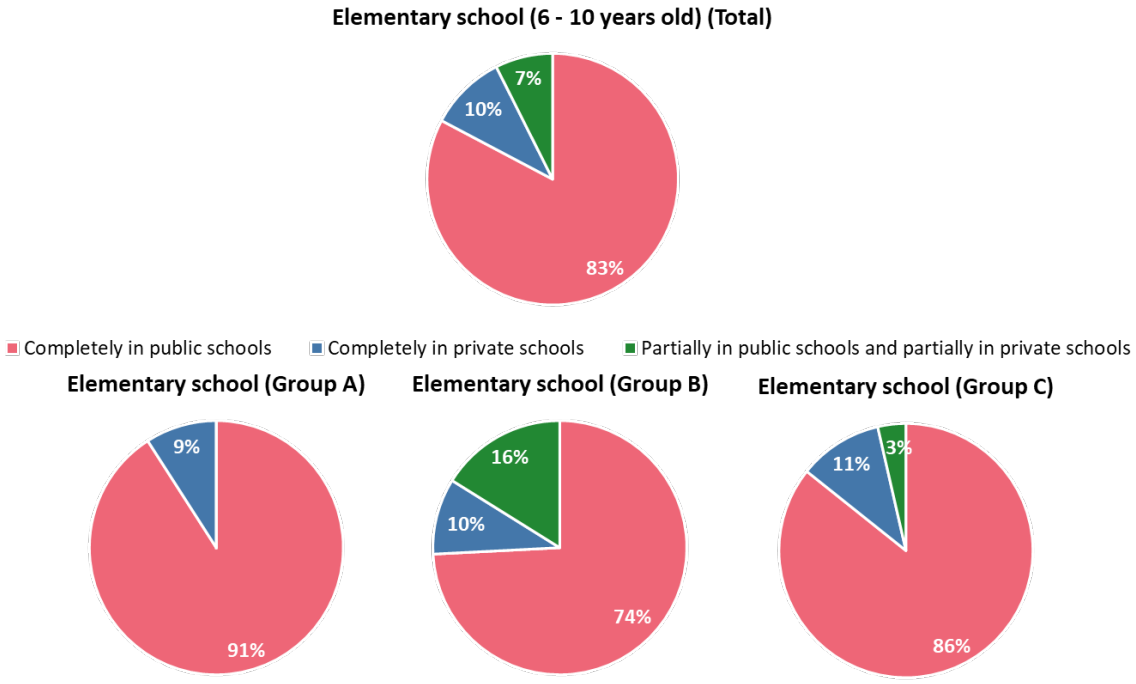


Figure 31. Distribution of participants by type of institution they attended during elementary school. In the Brazilian educational system, elementary school is denominated "Fundamental 1".

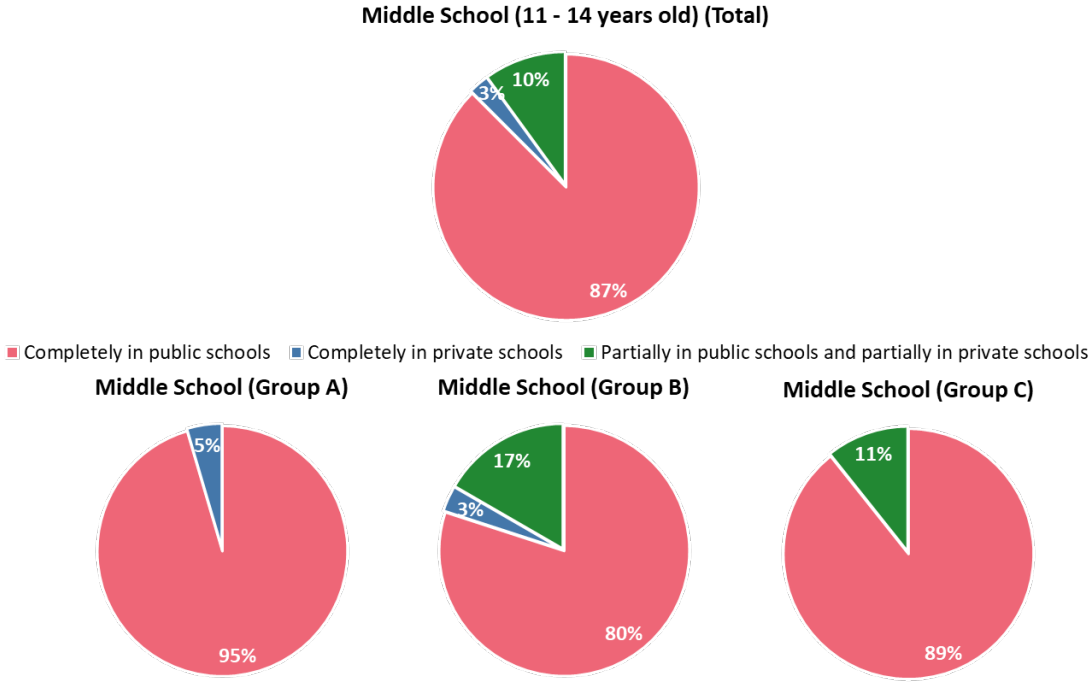


Figure 32. Distribution of participants by type of institution in which they studied during middle school. In the Brazilian educational system, middle school is denominated "Fundamental 2".

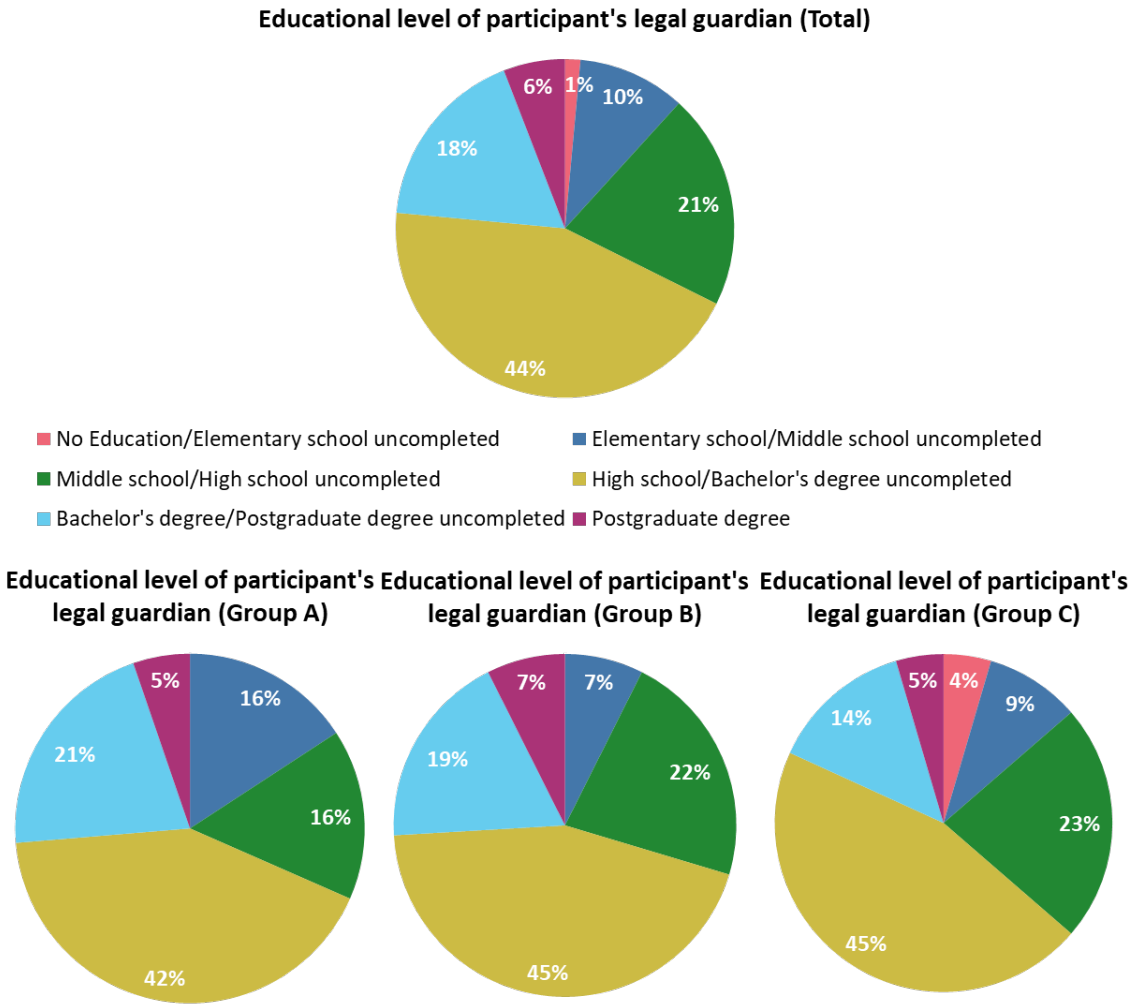


Figure 33. Distribution of participants according to the educational level of their legal guardian. In the case of more than one legal guardian, it was suggested to the participant to provide information regarding the one with the highest level of education.

There are few differences in age, family income and the institutions in which students attended the inner years of their training. It is noteworthy that a total of 77% of the study participants have a monthly family income equal to or less than $800. Group A has a greater number of participants in this income bracket, totaling 83% of the group. In addition, Group A also has a greater number of students who studied only in public schools during elementary school (91%) and middle school (95%). Despite the inferior performance of public institutions in educational evaluations[120], the group performance in the study seems to not have been affected by this characteristic, as will be shown later in the results. It is also noteworthy that 76% of the students' legal guardians did not attend higher education.

Throughout the study, for different reasons, students could not participate in one or more activities. Only the results of students who participated in the three activities of their group were counted in the analysis of the different interventions. In all, 21 students from Group A, 24 from Group B and 17 from Group C participated in all assessment stages.

5.8.2 Attitude towards science

Although the initial goal of this project was teaching the CRISPR/Cas technology, a secondary effect may be the increase of participants' interest in science. Over the last few decades different tools have been developed to measure such interest. Blalock et al. [121] evaluated dozens of instruments, developed over a period of 70 years, to assess such interest and its corresponding psychometric evidence. Among those with adequate reliability according to the authors' criteria, we can list the Test of Science Related Attitudes (TOSRA)[122], Views on Science-Technology-Society (VOSTS)[123], Science Assessment Instrument[124], and Simpson–Troost Attitude Questionnaire[125]. However, all of these are quite extensive, featuring over 60 items. Because of this, Kennedy et al[126] proposed an instrument capable of evaluating multiple aspects with the least possible number of items, more suitable for successive interventions, such as those proposed in the present work.

Participants' interest in science was assessed using the instrument developed by Kennedy et al[126]. To assess data normality, the Ryan-Joiner test was performed using Minitab software. A p-value > 0.1 was obtained, indicating that the data follows a normal distribution (Appendix 6).

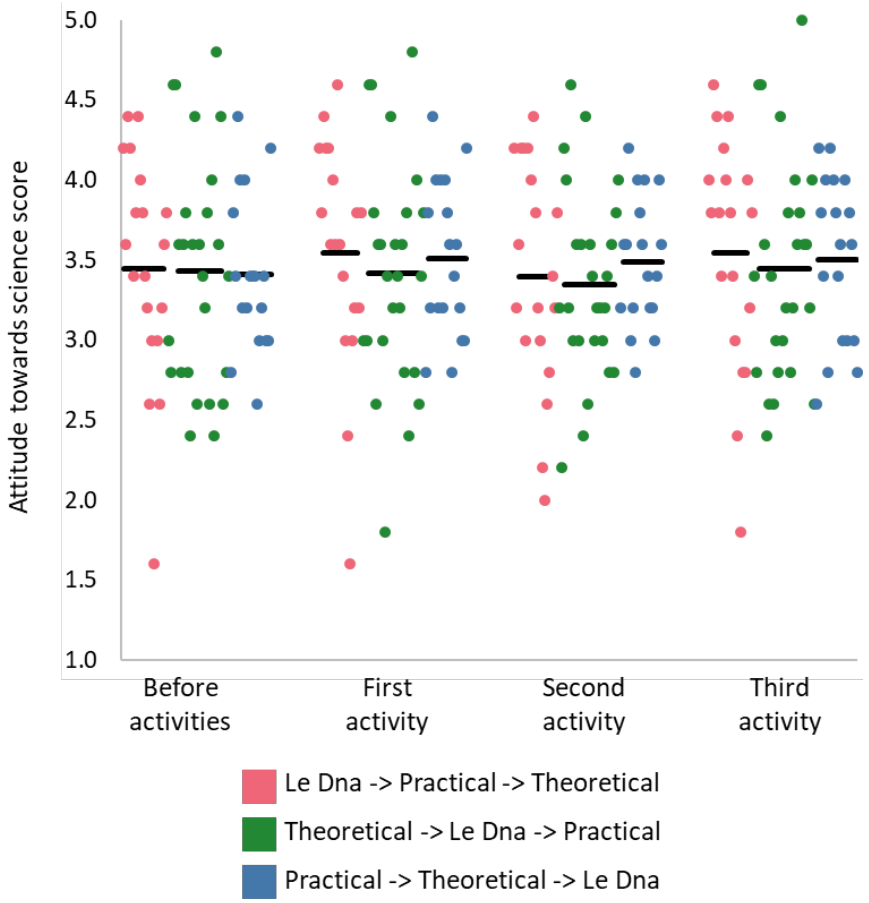


Figure 34. Attitude towards science score per student. Each individual score is represented by a single dot colored according to which group the student belonged to. The results are distributed along the x-axis according to the number each participant received during the study to facilitate visualization. The mean for each group is represented by a black dash. The scores vary from 1 (lowest interest) to 5 (maximum interest). No statistically significant difference was found between the groups (one-way ANOVA F(11,240) = [0.21], p = 0.997).

None of the different interventions was able to change students' perception of science (figure 34). The lack of impact may be explained by the fact that the activities were carried out with students in the last year of high school, when most of them have already decided which career they would like to follow, as observed in the answer to question 9 of the form as will be shown later in table 14.

5.8.3 Statistical validation of the test

To assess students' interest in science, a series of previously validated instruments were available in the literature. However, a similar instrument was not available for the

assessment of CRISPR/Cas learning, therefore we developed one composed by nine questions grouped into three sections of three, to evaluate participants knowledge in Basic Genetics, CRISPR/Cas Mechanism, and CRISPR/Cas Applications. For validation, only responses from students who participated in all activities were accounted, totaling 224 responses. The questions in the first section "Basic Genetics" resulted in a higher frequency of correct answers compared to the other two (Table 7), and presented a higher difficulty parameter (Table 8), which indicates that they are easier questions. This is expected, since they assess topics that students had previously studied in High School.

Question	Score	Frequency	Percentage
1	0	93	41.52%
	0.5	27	12.05%
	1	104	46.43%
2	0	84	37.5%
	0.2	17	7.59%
	0.4	4	1.79%5
	0.6	26	11.61
	0.8	0	0%
	1	93	41.52%
3	0	37	16.52%
	0.5	5	2.23%
	1	182	81.25%
4	0	99	44.20%
	0.33	34	15.18%
	0.66	38	16,96%
	1	53	23.66%
5	0	150	66.96%
	0.5	24	10.71%
	1	50	22.32%
6	0	192	85.71%
	0.5	8	3.58%
	1	24	10.71%
7	0	100	44.64%
	0.33	39	17.41%
	0.66	62	27.68%
	1	23	10.28%
8	0	166	74.11%
	1	58	25.89%
9	0	171	76.34%

1	53	23.66%

Table 7. Analysis of the frequency of responses to the evaluation form. Questions 1, 2 and 3 compose the "Basic Genetics" section, questions 4, 5 and 6 the "CRISPR/Cas Mechanism" section and questions 7, 8 and 9 the "CRISPR/Cas Applications" session.

Question	Difficulty	Discimination
1	0.5848	0.5688
2	0.5071	0.6486
3	0.8237	0.4719
4	0.3957	0.6030
5	0.2768	0.5246
6	0.1250	0.4792
7	0.3428	0.7486
8	0.2589	0.5279
9	0.2366	0.5326

Table 8. Calculated difficulty and discrimination of each test item. The closest the Difficulty parameter is to 1, the easiest the question.

Test results were analyzed by fitting the data to a two-parameter logistic (2PL) item response theory model that describes the relationship between an individual's response to a test item and his or her standing on the construct being measured. All nine questions included in the assessment fit to the model (Table 9). The alpha coefficient (also called Cronbach's Alpha) was calculated as 0.8464, indicating the test's reliability. The analyzes show that the instrument developed for this work is capable of evaluating CRISPR/Cas learning.

Question	p-value
1	0.4168
2	0.1702
3	0.8370
4	0.4890
5	0.5943
6	0.4426
7	0.2616
8	0.2111
9	0.9142

Table 9. Item fit to Two-Parameter Logistic Item Response Model. A p-value larger than 0.05 indicates that the question fits the model.

5.8.4 Learning assessment

To assess data normality, the Ryan-Joiner test was performed using Minitab software. The p-value of 0.028 indicates that the data do not follow a normal distribution (Appendix 6).

Therefore, the Kruskal Wallis multiple comparisons test was used to verify whether the differences in results are significant.

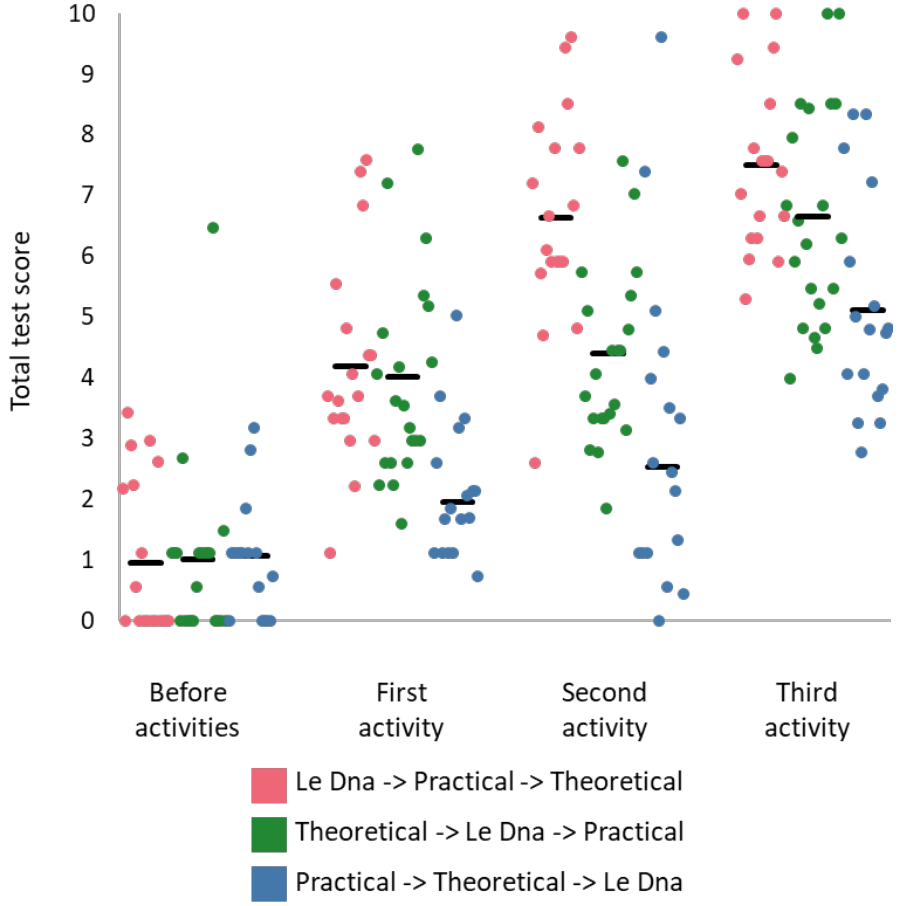


Figure 35. Total score per student. Each individual score is represented by a single dot and the mean for each group is represented by a black dash. The scores vary from 0 to 10.

Group	vs	p-value
Group A: LeDNA -> Practical -> Theoretical	Group A: Initial	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group B: Initial	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group C: Initial	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group A: LeDNA	0.0013
Group A: LeDNA -> Practical -> Theoretical	Group B: Theoretical	0.0002
Group A: LeDNA -> Practical -> Theoretical	Group C: Practical	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group B: Theoretical -> LeDNA	0.0015
Group A: LeDNA -> Practical -> Theoretical	Group C: Practical -> Theoretical	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group A: Initial	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group B: Initial	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group C: Initial	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group B: Theoretical	0.0021
Group B: Theoretical -> LeDNA -> Practical	Group C: Practical	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group C: Practical -> Theoretical	< 0.0001

Group C: Practical -> Theoretical -> LeDNA	Group A: Initial	< 0.0001
Group C: Practical -> Theoretical -> LeDNA	Group B: Initial	< 0.0001
Group C: Practical -> Theoretical -> LeDNA	Group C: Initial	< 0.0001
Group C: Practical -> Theoretical -> LeDNA	Group C: Practical	0.0007
Group A: LeDNA -> Practical	Group A: Initial	< 0.0001
Group A: LeDNA -> Practical	Group B: Initial	< 0.0001
Group A: LeDNA -> Practical	Group C: Initial	< 0.0001
Group A: LeDNA -> Practical	Group C: Practical	< 0.0001
Group A: LeDNA -> Practical	Group C: Practical -> Theoretical	0.0001
Group B: Theoretical -> LeDNA	Group A: Initial	< 0.0001
Group B: Theoretical -> LeDNA	Group B: Initial	< 0.0001
Group B: Theoretical -> LeDNA	Group C: Initial	< 0.0001
Group A: LeDNA	Group A: Initial	0.0001
Group A: LeDNA	Group B: Initial	< 0.0001
Group A: LeDNA	Group C: Initial	< 0.0001
Group B: Theoretical	Group A: Initial	0.0002
Group B: Theoretical	Group B: Initial	0.0001
Group B: Theoretical	Group C: Initial	0.0003

Table 10. Significant total score differences between interventions. Analysis from results shown in Figure 35. Only comparisons with a p-value lower than 0.05 are listed.

Each isolated intervention positively impacted participants' knowledge about CRISPR/Cas, with all groups scoring significantly higher after the first intervention compared to the initial score (table 10). Furthermore, the combination of the three methodologies resulted in superior knowledge scores than any of the interventions individually, as demonstrated by comparing of the results of Group A after three interventions against the three groups after one intervention. In addition, the order of activities did not affect the final result, with the three groups obtaining final scores with no significant difference after three activities. Noteworthy, the application of LeDNA followed by a practical class (Group A) resulted in higher test scores than the application of a practical class alone (Group C) and also than the application of a practical class followed by a theoretical class (also Group C). The theoretical activity followed by the practical class and LeDNA (Group B) also resulted in significantly better results than the application of only the practical combined with the theoretical class (Group C), demonstrating again the ability of LeDNA to impact learning beyond the results obtained by the other methodologies. The results indicate that the tool developed in this work can be combined with traditional methodologies to increase student performance.

5.8.5 Learning per section

In order to better understand the relationships between the different methodologies and optimize the way they can be used together we analyzed the performance of students separately in each of the three sections that compose the test. Each of the sections consists of three questions, totaling 33% of the final test score. To facilitate the interpretation of the data, the values were adjusted from 0 to 10, with 10 indicating the maximum score in that section and therefore 3.33 in the final test score.

5.8.5.1 Basic Genetics

The Ryan-Joiner test resulted in a p-value of 0.027, indicating that the data again do not follow a normal distribution (Appendix 6). As before, the Kruskal Wallis multiple comparisons test was used to verify whether the differences in results are significant.

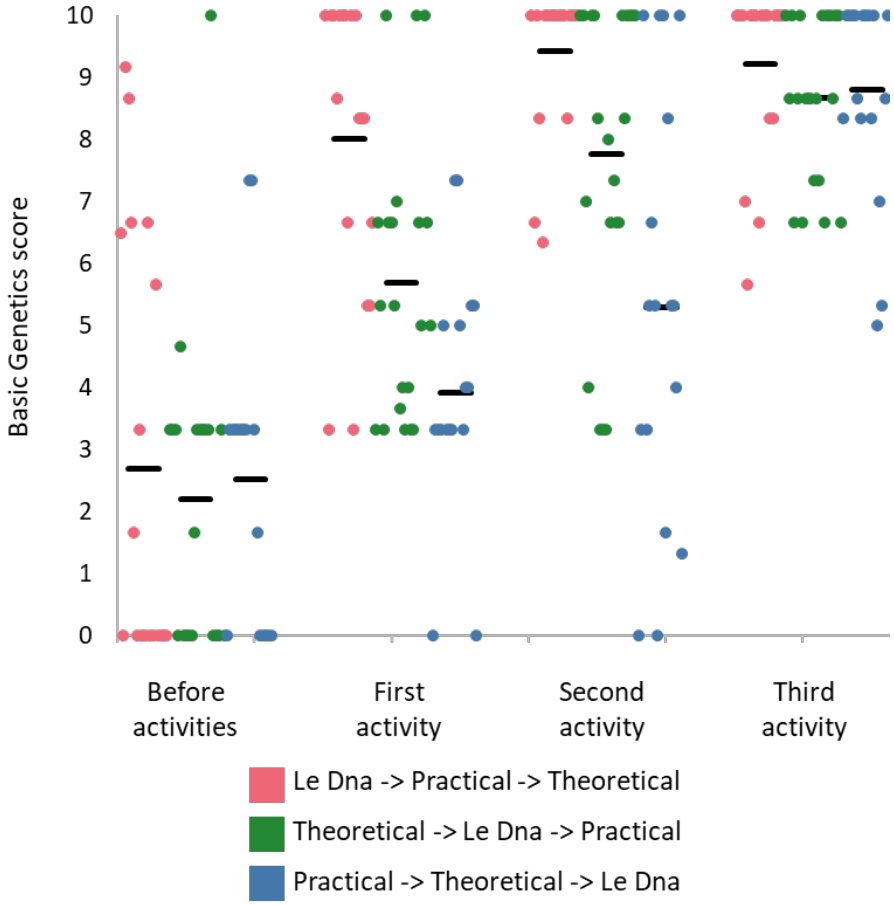


Figure 36. Basic Genetics score per student. Each individual score is represented by a single dot and the mean for each group is represented by a black dash. The scores vary from 0 to 10.

Group	vs	p-value
Group A: LeDNA -> Practical -> Theoretical	Group A: Initial	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group B: Initial	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group C: Initial	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group B: Theoretical	0.0004
Group A: LeDNA -> Practical -> Theoretical	Group C: Practical	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group C: Practical -> Theoretical	0.0006
Group B: Theoretical -> LeDNA -> Practical	Group A: Initial	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group B: Initial	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group C: Initial	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group C: Practical	< 0.0001
Group C: Practical -> Theoretical -> LeDNA	Group A: Initial	< 0.0001
Group C: Practical -> Theoretical -> LeDNA	Group B: Initial	< 0.0001
Group C: Practical -> Theoretical -> LeDNA	Group C: Initial	< 0.0001
Group C: Practical -> Theoretical -> LeDNA	Group C: Practical	< 0.0001
Group A: LeDNA -> Practical	Group A: Initial	< 0.0001
Group A: LeDNA -> Practical	Group B: Initial	< 0.0001
Group A: LeDNA -> Practical	Group C: Initial	< 0.0001
Group A: LeDNA -> Practical	Group B: Theoretical	0.0002
Group A: LeDNA -> Practical	Group C: Practical	< 0.0001
Group A: LeDNA -> Practical	Group C: Practical -> Theoretical	0.0003
Group B: Theoretical -> LeDNA	Group A: Initial	< 0.0001
Group B: Theoretical -> LeDNA	Group B: Initial	< 0.0001
Group B: Theoretical -> LeDNA	Group C: Initial	< 0.0001
Group B: Theoretical -> LeDNA	Group C: Practical	0.0006
Group A: LeDNA	Group A: Initial	< 0.0001
Group A: LeDNA	Group B: Initial	< 0.0001
Group A: LeDNA	Group C: Initial	< 0.0001
Group A: LeDNA	Group C: Practical	0.0004

Table 11. Significant differences in "Basic Genetics" scores between interventions. Analysis from results shown in figure 36. Only comparisons in which a p-value lower than 0.05 was obtained are listed.

Considering that this section of the test assesses aspects that students had studied before, the low starting score was not expected (figure 36 and table 11). We speculate that the poor performance was due to educational difficulties arising from the pandemic, especially in public education institutions in Brazil[127]. At the end of the three activities, all groups achieved a high score in this section (average of 9.22 in group A, 8.66 in group B and 8.80 in group C),

indicating that the interventions were efficient in reversing the adverse scenario. There was no significant difference between the groups after the three activities, indicating that the order is not relevant for teaching basic genetic topics. Combining LeDNA and Practice (Group A) resulted in significantly higher test scores than just the theoretical class (Group B), just the practical class (Group C) and the combination of the latter two (Group C). Although LeDNA was developed with the aim of teaching CRISPR/Cas gene editing, the result demonstrates that it is a useful tool to teach introductory genetics topics, including the central dogma of biology and the structure of biomolecules such as DNA, RNA and proteins.

5.8.5.2 CRISP/Cas Mechanism

This section addresses the molecular mechanisms of the CRISPR/Cas tool using results from questions 4 to 6 in the test. Once again, the Ryan-Joiner test resulted in a p-value < 0.01, indicating that the data do not follow a normal distribution (Appendix 6) and the same evaluation used in the previous session was applied.

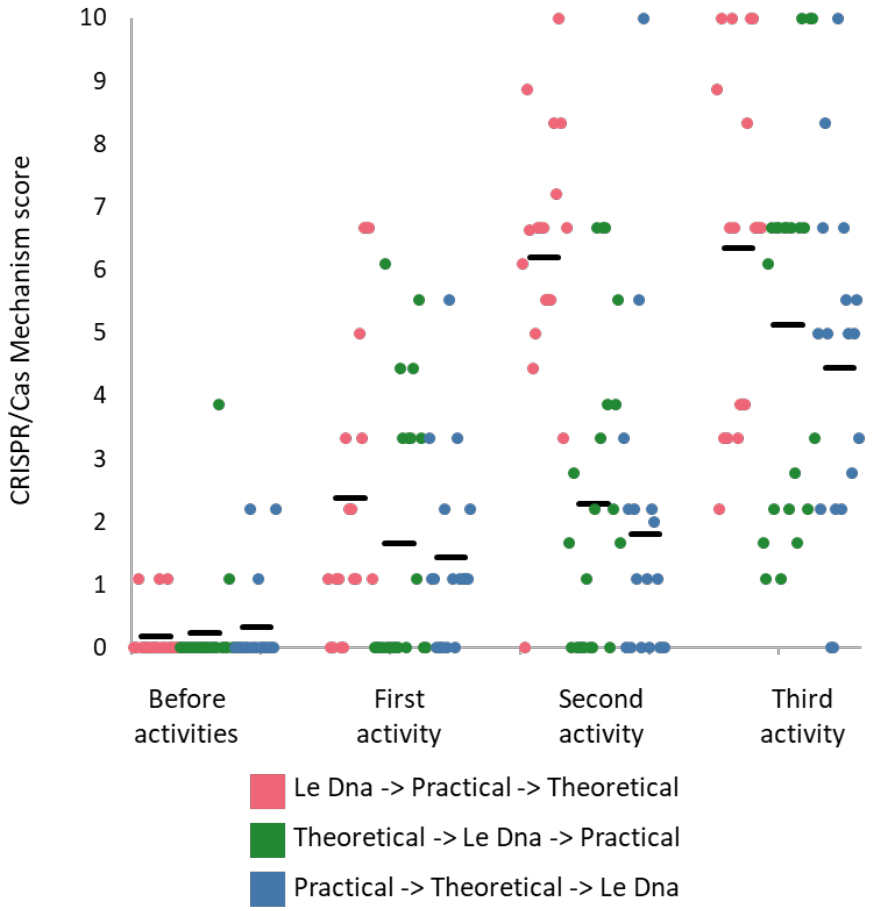


Figure 37. CRISPR/Cas Mechanism score per student. Each individual score is represented by a single dot and the mean for each group is represented by a black dash. The scores vary from 0 to 10.

Group	vs	p-value
Group A: LeDNA -> Practical -> Theoretical	Group A: Initial	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group B: Initial	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group C: Initial	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group A: LeDNA	0.0010
Group A: LeDNA -> Practical -> Theoretical	Group B: Theoretical	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group C: Practical	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group B: Theoretical -> LeDNA	0.0002
Group A: LeDNA -> Practical -> Theoretical	Group C: Practical -> Theoretical	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group A: Initial	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group B: Initial	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group C: Initial	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group B: Theoretical	0.0001
Group B: Theoretical -> LeDNA -> Practical	Group C: Practical	0.0007
Group B: Theoretical -> LeDNA -> Practical	Group C: Practical -> Theoretical	0.0007
Group C: Practical -> Theoretical -> LeDNA	Group A: Initial	< 0.0001
Group C: Practical -> Theoretical -> LeDNA	Group B: Initial	< 0.0001
Group C: Practical -> Theoretical -> LeDNA	Group C: Initial	< 0.0001
Group C: Practical -> Theoretical -> LeDNA	Group B: Theoretical	0.0025
Group A: LeDNA -> Practical	Group A: Initial	< 0.0001
Group A: LeDNA -> Practical	Group B: Initial	< 0.0001
Group A: LeDNA -> Practical	Group C: Initial	< 0.0001
Group A: LeDNA -> Practical	Group A: LeDNA	0.0019
Group A: LeDNA -> Practical	Group B: Theoretical	< 0.0001
Group A: LeDNA -> Practical	Group C: Practical	0.0001
Group A: LeDNA -> Practical	Group B: Theoretical -> LeDNA	0.0004
Group A: LeDNA -> Practical	Group C: Practical -> Theoretical	0.0001
Group A: LeDNA	Group B: Initial	0.0017

Table 12. Significant differences in "CRISPR/Cas Mechanism" scores between interventions. Analysis from results shown in figure 37. Only comparisons in which a p-value lower than 0.05 was obtained are listed.

CRISPR/Cas is not a topic currently discussed in the educational system. Thus, as expected, most students scored zero before the first activity (figure 37 and table 12). Unlike the previous section, in this one the difference in scores was significant only in one of the individual activities (LeDNA Group A), and even in this case only in comparison to the initial results of Group B. In addition, the combinations of LeDNA with theoretical classes (Group B) and theoretical classes with practical classes (Group C) also did not result in an outcome

statistically different from that obtained without any intervention. On the other hand, when combining LeDNA with practical classes (Group A), the result was not only superior to all groups without intervention, but also to all individual interventions and to the combinations of theoretical classes with LeDNA (Group B) and theoretical classes with practice (Group C). The impact of these two activities together (LeDNA and practice) can also be verified by the fact that only when groups B and C finished the third activity their score raised significantly compared to the initial test. As in the previous section, the order of activities did not result in significantly different scores at the end of the three interventions. The result of this session points out the potential of LeDNA to complement a practical class in teaching the CRISPR/Cas mechanism, since the results of the combination of the two activities were significantly different from any isolated activity and from any other combination that did not include the two.

5.8.5.3 CRISPR/Cas Applications

This section addresses the present and future impacts of the CRISPR/Cas technology on participants' daily lives. As in the two previous sections, the Ryan-Joiner test indicated that the data do not follow a normal distribution (p-value < 0.01) (Appendix 6)

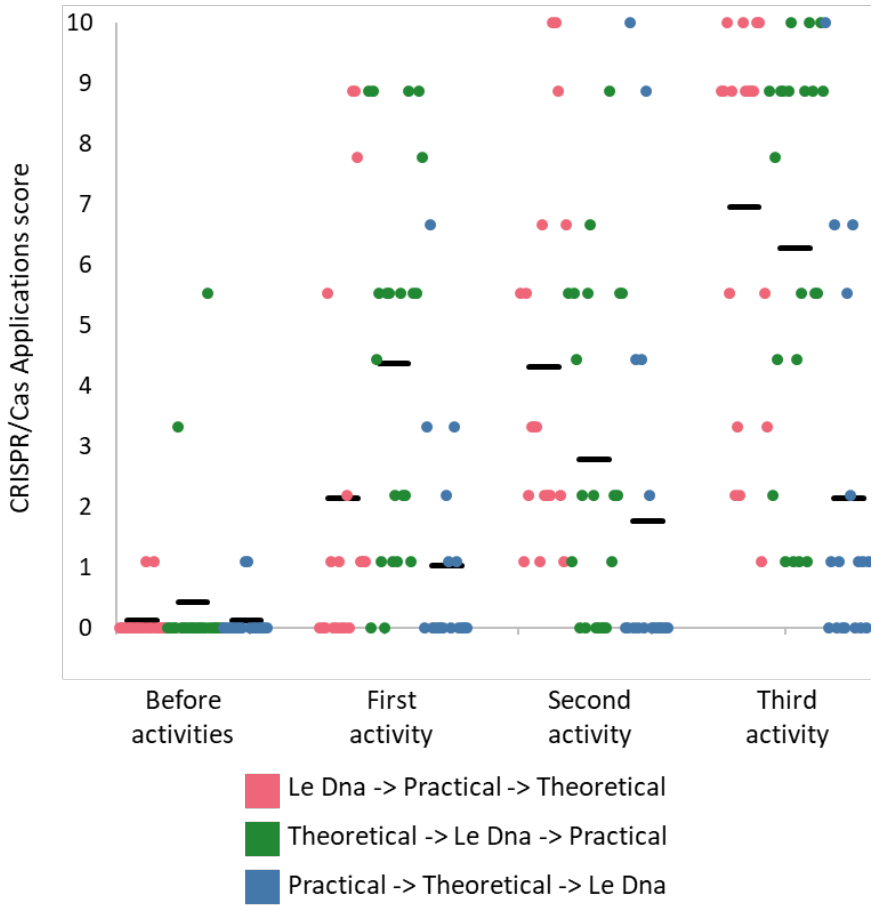


Figure 38. CRISPR/Cas Applications score per student. Each individual score is represented by a single dot and the mean for each group is represented by a black dash. The scores vary from 0 to 10.

Group	vs	p-value
Group A: LeDNA -> Practical -> Theoretical	Group A: Initial	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group B: Initial	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group C: Initial	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group A: LeDNA	0.0001
Group A: LeDNA -> Practical -> Theoretical	Group C: Practical	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group B: Theoretical -> LeDNA	0.0012
Group A: LeDNA -> Practical -> Theoretical	Group C: Practical -> Theoretical	< 0.0001
Group A: LeDNA -> Practical -> Theoretical	Group C: Practical -> Theoretical -> LeDNA	0.0002
Group B: Theoretical -> LeDNA -> Practical	Group A: Initial	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group B: Initial	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group C: Initial	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group A: LeDNA	0.0003
Group B: Theoretical -> LeDNA -> Practical	Group C: Practical	< 0.0001
Group B: Theoretical -> LeDNA -> Practical	Group C: Practical -> Theoretical	< 0.0001

Group B: Theoretical -> LeDNA -> Practical	Group C: Practical -> Theoretical -> LeDNA	0.0006
Group A: LeDNA -> Practical	Group A: Initial	< 0.0001
Group A: LeDNA -> Practical	Group B: Initial	< 0.0001
Group A: LeDNA -> Practical	Group C: Initial	< 0.0001
Group A: LeDNA -> Practical	Group C: Practical	0.0003
Group A: LeDNA -> Practical	Group C: Practical -> Theoretical	0.0007
Group B: Theoretical -> LeDNA	Group A: Initial	0.0014
Group B: Theoretical -> LeDNA	Group B: Initial	0.0017
Group B: Theoretical -> LeDNA	Group C: Initial	0.0017
Group B: Theoretical	Group A: Initial	< 0.0001
Group B: Theoretical	Group B: Initial	< 0.0001
Group B: Theoretical	Group C: Initial	< 0.0001
Group B: Theoretical	Group C: Practical	0.0008
Group B: Theoretical	Group C: Practical -> Theoretical	0.0017

Table 13. Significant differences in "CRISPR/Cas Applications" scores between interventions. Analysis from results shown in Figure 38. Only comparisons in which a p-value lower than 0.05 was obtained are listed.

Once again, it was not expected that the participants could have previous knowledge about the topic to answer the test correctly before the first activity. Unlike the other sections, in this one the order of activities resulted in a significant change (figure 38 and table 13). Group C (practical –> theoretical –> LeDNA) had a significantly lower performance when compared to the other two groups. Among the individual interventions, only the theoretical class (Group B) resulted in significantly higher test scores than the initial results. Noteworthy, the combination of lecture with LeDNA (Group B) is significantly superior only to the initial results. However, when practical activity is added, better results are achieved than LeDNA individually (Group A) and practice combined with theoretical activity (Group C). A similar phenomenon occurs in Group A. No significant performance difference was verified for any group after the first activity with LeDNA. After LeDNA was combined with a practical class, the group reached higher scores than after the practical activity only (Group C) and practical followed by theoretical activity (also Group C). We speculate that the understanding of CRISPR/Cas applications advances after the practical class, however this gain is only effective if preceded by a theoretical or LeDNA class. This result demonstrates once again that the combination of different activities enhances the participants' learning.

To illustrate the participants' perception of CRISPR/Cas at the end of the activities, some of the participants' responses to question 9 are listed in table 14: "Imagine that you travel

35 years into the future and find yourself. What do you think your profession will be? Will it be affected by CRISPR/Cas technology?"

Group	Answer	Career
Group A	"Modifying DNA to make muscle development easier through physical training"	Physical education
Group A	"Making students think faster to increase learning"	Pedagogy
Group A	"Everyone's daily life will be modified by CRISPR/Cas, because it can change the way in which any product is produced"	Computer engineering
Group A	"I will promote the sale of products developed with CRISPR technology"	Advertising and marketing
Group B	"Class materials (books) could be produced differently"	Social sciences
Group B	"In the movie Winter the Dolphin, the character loses his tail and has to wear a prosthesis so as not to harm his spine, in the future with CRISPR/Cas I could modify his DNA so that the tail could grow again"	Marine biology
Group C	"Clothing made by microorganisms modified with CRISPR/Cas can affect my work"	Dance

Table 14. Participants' perception of the impact of CRISPR/Cas on their future careers

7. Conclusions

The CRISPR/Cas gene editing technology promises to transform the medicines that heal us, the nourishment that supports us, and even our own DNA over the next few decades, redefining what it is to be human. LeDNA, the tool developed in this work, expands the accessibility to knowledge about this revolutionary technology, allowing high school students to reproduce molecular phenomena on a macro scale. The user manipulates these otherwise invisible phenomena by building a gene of their choice and reproducing the processes of transcription, translation, and editing by CRISPR/Cas. However, the system's functioning limits the student to only biologically correct interaction so that the user learns about the structure and functioning of biomolecules when building them.

Unlike the vast majority of other tools developed for CRISPR/Cas education, LeDNA does not require any laboratory infrastructure. Although it is not possible to compare the learning gains resulting from the use of LeDNA and other methodologies due to the different assessment instruments used, we demonstrate that the use of this new tool, both individually and in conjunction with theoretical and practical classes, results in significant gains in knowledge acquisition. LeDNA stands out from previously described methodologies due to its low production cost resulting from using MDF as raw material, obtaining a cost of less than 10 US dollars per kit (< 2.50 dollars per student). In addition, the choice of laser cutting as a production technology allows the sharing of the digital files necessary for the production of LeDNA, allowing FabLabs, makerspaces, hackerspaces, and schools that use this tool to locally produce the toolkits, facilitating the dissemination in a way that would be unfeasible for other methodologies. Similar to noncomputational educational games developed for other topics in biology, LeDNA allows students to collaborate with each other, collectively building knowledge. It is therefore situated in an intermediate zone between practical laboratory methodologies and virtual methodologies, combining the advantages of both approaches. While allowing hands-on collective knowledge acquisition (such as laboratory activities), it does not require expensive and complex infrastructure (such as virtual activities).

In this project, we also report a new plasmid and a new protocol for using *B. subitillis* in teaching CRISPR/Cas. This protocol eliminates the need for equipment and can be successfully performed in a period of only 90 minutes. We also developed a new measurement instrument to assess participants' learning, validating it using the Item Response Theory. LeDNA, our new practical methodology, and a traditional class were successfully applied to 90 students in a public school. The analysis of the students' performance throughout the study

demonstrated that the combination of theoretical, practical, and LeDNA classes produces the best final results, indicating complementarity between them, with LeDNA significantly increasing the result of the other methodologies. Also noteworthy are the positive results of using LeDNA individually or in conjunction with theoretical classes, validating the tool as a viable alternative for institutions without laboratory infrastructure.

Although it was conceived for teaching CRISPR/Cas, LeDNA can be expanded in the future to teach other topics in Molecular Biology. For example, the RNA nucleotides could be used to teach RNA interference and the amino acids to teach the structure and mechanisms of action of proteins and enzymes. The use of LeDNA may also be of interest to other target audiences, such as undergraduate and graduate students, who have not yet had contact with gene editing. Overall, LeDNA is a low-cost, open-access validated tool that could be deployed around the globe to democratize CRISPR/Cas education.

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9. Appendix 1 - Socioeconomic Questionnaire

Important: Responses to this form are anonymous and will not be used individually. You are not obligated to answer all the questions and you can hand it in without answering any questions you wish, even without answering any of them.

Number (delivered by the researcher at the beginning of the first activity): _____

My age is: _____ ( ) I'd rather not answer

My gender is: _____ ( ) I'd rather not answer

I attended Elementary School...

( ) Entirely in a public institution
( ) Entirely in a private institution
( ) Partially in a public institution and partially in a private institution
( ) I don't remember/I prefer not to answer

I attended Middle School...

( ) Entirely in a public institution
( ) Entirely in a private institution
( ) Partially in a public institution and partially in a private institution
( ) I don't remember/I prefer not to answer

The family income in your house, adding up all the members of your family is:

( ) Up to 2 minimum wages
( ) From 2 to 4 minimum wages
( ) From 4 to 10 minimum wages
( ) From 10 to 20 minimum wages
( ) More than 20 minimum wages
( ) I don't know/I prefer not to answer

The educational level of the head of my family is:

( ) No education/Incomplete Elementary School
( ) Completed Elementary School complete/Incomplete Middle School
( ) Completed Middle School/Incomplete High School
( ) Completed High School/Incomplete Higher Education
( ) Completed higher education
( ) Graduate (specialization, master's, doctorate)
( ) I don't know/I prefer not to answer

10. Appendix 2 - Evaluation Questionnaire

Questions 1 to 5 assess students' interest in science and biology. Questions 6 to 8 assess the student's knowledge of topics already discussed in the São Paulo High School Curriculum. Questions 9 to 11 assess the student's technical knowledge of CRISPR/Cas technology. Questions 12 to 14 assess the student's understanding of the applications of the same technology. 4 different versions of the questionnaire were developed, covering the same themes.

Participant Number: ___

Part 1 – Mark an X in the option (number or word) that best suits you.

1 – It is very likely that I will continue studying biology after finishing high school.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

2 – I think studying biology is

Chato				Interessante
1	2	3	4	5

3 – I have difficulty completing biology activities.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

4 – I think I'm pretty good at biology.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

5 – Working as a scientist would be interesting.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

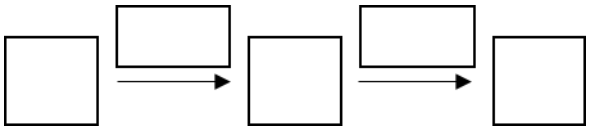
Part 2 – This table can be used to answer subsequent questions.

U	UUU, UUC – Fenilalalanina	UCU, UCC, UCA, UCG – Serina	UAU, UAC – Tirosina	UGU, UGC – Cisteína
	UUA, UUG – Leucina		UAA, UAG – **Códon de Parada**	UGA – **Códon de Parada**; UGG – Triptofano
C	CUU, CUC, CUA, CUG – Leucina	CCU, CCC, CCA, CCG – Prolina	CAU, CAC – Histidina	CGU, CGC, CGA, CGG – Arginina
			CAA, CAG – Glutamina	
A	AUU, AUC, AUA – Isoleucina	ACU, ACC, ACA, ACG – Treonina	AAU, AAC – Asparagina	AGU, AGC – Serina
	AUG – Metionina (códon de início)		AAA, AAG – Lisina	AGA, AGG – Arginina
G	GUU, GUC, GUA, GUG – Valina	GCU, GCC, GCA, GCG – Alanina	GAU, GAC – Aspartato	GGU, GGC, GGA, GGG – Glicina
			GAA, GAG – Glutamato	

6 – What amino acids would be produced from this DNA sequence: **TACGAGTAAATTGCA** ?

☐ I don't know/I prefer not to answer

7 – Organize these terms into the appropriate boxes: Protein, RNA, DNA, Ribosome, RNA Polymerase.



☐ I don't know/I prefer not to answer

8 – Draw a line connecting the nucleotides that pair in the formation of the double-stranded DNA.

Adenine	Adenine
Cytosine	Cytosine
Guanine	Guanine
Thymine	Thymine

☐ I don't know/I prefer not to answer

9 – You are a scientist in your laboratory and you would like to correct a defective gene in a certain organism. Mark with an X which items you would need to insert into the organism.

☐ Ribosome ☐ Mitochondria ☐ Messenger RNA ☐ Guide RNA

☐ CRISPR protein ☐ Cas protein ☐ DNA template ☐ Mitochondrial DNA

☐ I don't know/I prefer not to answer

10 – After the success of your previous experiment, you would now like to disable another gene, rather than modifying it. You repeat the previous steps, but this time you DO NOT include which of the previous items?

☐ Ribosome ☐ Mitochondria ☐ Messenger RNA ☐ Guide RNA

☐ CRISPR protein ☐ Cas protein ☐ Template DNA ☐ Mitochondrial DNA

☐ I don't know/I prefer not to answer

11 – You would like to replace the codon that encodes the amino acid APARTATE with the codon of GLUTAMATE in a certain protein in this part of its gene: ...GTACTGTCG... You used CRISPR/Cas technology to cut the gene, which DNA sequence would you use as model for repair?

☐ I don't know/I prefer not to answer

12 – Please mark the activities for which CRISPR/Cas technology is already used/may be used in the future.

☐ Modifying embryonic cells to avoid hereditary diseases

☐ Developing drought resistant plants

☐ Creating yeasts with greater biofuel production capacity

☐ Chemical synthesis of new antibiotics

☐ I don't know/I wish not to answer

13 – Briefly describe an activity in your daily life that could be impacted by CRISPR/Cas technology.

☐ I don't know/I wish not to answer

14 – Let's say you travel 30 years into the future and find yourself. What do you think your profession will be? Will it be affected by CRISPR/Cas technology? Justify briefly.

☐ I don't know/I wish not to answer

Participant Number: ___

Part 1 – Mark an X in the option (number or word) that best suits you.

1 – It is very unlikely that I will continue studying biology after finishing high school.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

2 – In my opinion studying biology is

Chato				Interessante
1	2	3	4	5

3 – I find it easy to complete biology activities.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

4 – I think I'm great at biology.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

5 – Working as a scientist would be very boring.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

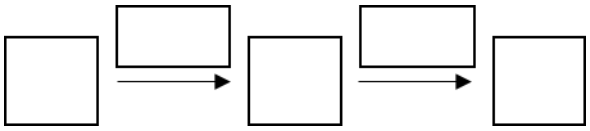
Part 2 – This table can be used to answer subsequent questions

U	UUU, UUC – Fenilalalanina	UCU, UCC, UCA, UCG – Serina	UAU, UAC – Tirosina	UGU, UGC – Cisteína
	UUA, UUG – Leucina		UAA, UAG – **Códon de Parada**	UGA – **Códon de Parada**; UGG – Triptofano
C	CUU, CUC, CUA, CUG – Leucina	CCU, CCC, CCA, CCG – Prolina	CAU, CAC – Histidina	CGU, CGC, CGA, CGG – Arginina
			CAA, CAG – Glutamina	
A	AUU, AUC – Isoleucina	ACU, ACC, ACA, ACG – Treonina	AAU, AAC – Asparagina	AGU, AGC – Serina
	AUA – Isoleucina; AUG – Metionina (códon de início)		AAA, AAG – Lisina	AGA, AGG – Arginina
G	GUU, GUC, GUA, GUG – Valina	GCU, GCC, GCA, GCG – Alanina	GAU, GAC – Aspartato	GGU, GGC, GGA, GGG – Glicina
			GAA, GAG – Glutamato	

6 – What amino acids would be produced from this DNA sequence: TACGGGTGAATCGCA ?

☐ I don't know/I wish not to answer

7 – Organize these terms into the appropriate boxes: RNA, DNA, Ribosome, Protein, RNA Polymerase.



☐ I don't know/I wish not to answer

8 – Draw a line connecting the nucleotides that pair in the formation of the double strand of DNA.

Adenine	Adenine
Cytosine	Cytosine
Guanine	Guanine
Thymine	Thymine

☐ I don't know/I wish not to answer

9 – You are a scientist in your laboratory and you would like to modify a defective gene in a certain organism. Mark with an X which items you would need to insert into the organism.

☐ Mitochondria ☐ Messenger RNA ☐ Guide RNA ☐ Ribosome

☐ RISPR protein ☐ Template DNA ☐ Cas protein ☐ Mitochondrial DNA

☐ I don't know/I wish not to answer

10 – After the success of your previous experiment, you would now like to disable another gene, rather than correct it. You repeat the previous steps, but this time you don't include which of the previous items?

☐ Mitochondria ☐ Messenger RNA ☐ Guide RNA ☐ Ribosome

☐ CRISPR protein ☐ Template DNA ☐ Cas protein ☐ Mitochondrial DNA

☐ I don't know/I wish not to answer

11 – You would like to replace the codon that encodes the amino acid Glutamate with the codon of Aspartate in a certain protein in this part of her gene: ...GTACTTTCG... You used CRISPR/Cas technology to cut the gene, which DNA sequence would you use as model for repair?

☐ I don't know/I wish not to answer

12 – Please tick an X for activities for which CRISPR/Cas technology is already used/may be used in the future.

☐ Creating microorganisms with greater capacity to produce biofuels

☐ Modifying human cells to prevent inherited diseases

☐ Chemical synthesis of new drugs

☐ Developing frost resistant plants

☐ I don't know/I wish not to answer

13 – Briefly describe an activity in your daily life that could be impacted by CRISPR/Cas technology.

☐ I don't know/I wish not to answer

14 – Let's say you travel 20 years into the future and find yourself. What do you think your profession will be? Will it be affected by CRISPR/Cas technology? Justify briefly.

☐ I don't know/I wish not to answer

Participant Number: ___

Part 1 – Mark an X in the option (number or word) that best suits you.

1 – It is very possible that I will continue studying biology after finishing high school.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

2 – In my opinion studying biology is

Chato				Interessante
1	2	3	4	5

3 – It is very easy for me to complete the biology activities.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

4 – I think I'm excellent in biology.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

5 – Working as a scientist would be fantastic.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

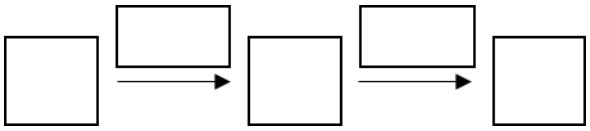
Part 2 – This table can be used to answer subsequent questions

U	UUU, UUC – Fenilalalanina	UCU, UCC, UCA, UCG – Serina	UAU, UAC – Tirosina	UGU, UGC – Cisteína
	UUA, UUG – Leucina		UAA, UAG – **Códon de Parada**	UGA – **Códon de Parada**; UGG – Triptofano
C	CUU, CUC, CUA, CUG – Leucina	CCU, CCC, CCA, CCG – Prolina	CAU, CAC – Histidina	CGU, CGC, CGA, CGG – Arginina
			CAA, CAG – Glutamina	
A	AUU, AUC – Isoleucina	ACU, ACC, ACA, ACG – Treonina	AAU, AAC – Asparagina	AGU, AGC – Serina
	AUA – Isoleucina; AUG – Metionina (códon de início)		AAA, AAG – Lisina	AGA, AGG – Arginina
G	GUU, GUC, GUA, GUG – Valina	GCU, GCC, GCA, GCG – Alanina	GAU, GAC – Aspartato	GGU, GGC, GGA, GGG – Glicina
			GAA, GAG – Glutamato	

5 – What amino acids would be produced from this DNA sequence: TACTTTTCAATTGCA?

☐ I don't know/I wish not to answer

6 – Organize these terms into the appropriate boxes: Protein, RNA Polymerase, RNA, DNA, Ribosome.



☐ I don't know/I wish not to answer

7 – Draw a line connecting the nucleotides that pair in the formation of the double strand of DNA.

Adenine	Adenine
Cytosine	Cytosine
Guanine	Guanine
Thymine	Thymine

☐ I don't know/I wish not to answer

8 – You are a scientist in your laboratory and you want to modify a defective gene in a certain organism. Mark with an X which items you would need to insert into the organism.

☐ Mitochondria ☐ Ribosome ☐ Cas protein ☐ Mitochondrial DNA

☐ CRISPR protein ☐ Template DNA ☐ Messenger RNA ☐ Guide RNA

☐ I don't know/I wish not to answer

9 – After the success of your previous experiment, you would now like to disable another gene, rather than correct it. You repeat the previous steps, but this time you don't include which of the previous items?

☐ Mitochondria ☐ Ribosome ☐ Cas protein ☐ Mitochondrial DNA

☐ CRISPR protein ☐ Template DNA ☐ Messenger RNA ☐ Guide RNA

☐ I don't know/I wish not to answer

10 – Would you like to replace the codon that encodes the amino acid Asparagine with the codon of Lysine in a certain protein in this part of her gene: ...GTATTATCG... You used CRISPR/Cas technology to cut the gene, which DNA sequence would you use as model for repair?

☐ I don't know/I wish not to answer

11 – Please mark activities for which CRISPR/Cas technology is already used/may be used in the future.

☐ Designing microorganisms with greater ethanol production capacity

☐ Developing pest resistant plants

☐ Editing the human genome to prevent inherited diseases

☐ Chemical synthesis of new compounds for the chemical industry

☐ I don't know/I wish not to answer

12 – Briefly describe a day-to-day activity that could be impacted by CRISPR/Cas technology in the future.

☐ I don't know/I wish not to answer

13 – Let's say you travel 15 years into the future and find yourself. What do you think your profession will be? Will it be affected by CRISPR/Cas technology? Justify briefly.

☐ I don't know/I wish not to answer

Participant number: ___

Part 1 – Mark an X in the option (number or word) that best suits you.

1 – It is hardly possible for me to continue studying biology after completing high school.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

2 – In my opinion studying biology is

Chato				Interessante
1	2	3	4	5

3 – It is very difficult for me to complete the biology activities.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

4 – I think I perform well in biology.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

5 – Working as a scientist would be really bad.

Concordo Totalmente	Concordo Parcialmente	Não Concordo/ Nem Discordo	Discordo Parcialmente	Discordo Totalmente

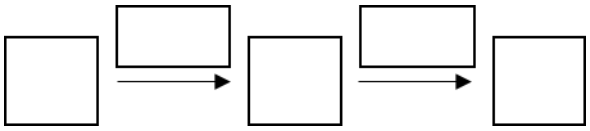
Part 2 – This table can be used to answer subsequent questions

U	UUU, UUC – Fenilalalanina	UCU, UCC, UCA, UCG – Serina	UAU, UAC – Tirosina	UGU, UGC – Cisteína
	UUA, UUG – Leucina		UAA, UAG – **Códon de Parada**	UGA – **Códon de Parada**; UGG – Triptofano
C	CUU, CUC, CUA, CUG – Leucina	CCU, CCC, CCA, CCG – Prolina	CAU, CAC – Histidina	CGU, CGC, CGA, CGG – Arginina
			CAA, CAG – Glutamina	
A	AUU, AUC – Isoleucina	ACU, ACC, ACA, ACG – Treonina	AAU, AAC – Asparagina	AGU, AGC – Serina
	AUA – Isoleucina; AUG – Metionina (códon de início)		AAA, AAG – Lisina	AGA, AGG – Arginina
G	GUU, GUC, GUA, GUG – Valina	GCU, GCC, GCA, GCG – Alanina	GAU, GAC – Aspartato	GGU, GGC, GGA, GGG – Glicina
			GAA, GAG – Glutamato	

5 – What amino acids would be produced from this DNA sequence: TACCTTTCAACTGCA?

☐ I don't know/I wish not to answer

6 – Organize these terms into the appropriate boxes: DNA, Ribosome, Protein, RNA Polymerase, RNA.



☐ I don't know/I wish not to answer

7 – Draw a line connecting the nucleotides that pair in the formation of the double strand of DNA.

Adenine	Adenine
Cytosine	Cytosine
Guanine	Guanine
Thymine	Thymine

☐ I don't know/I wish not to answer

8 – You are a researcher in your laboratory and would like to modify a defective gene in a certain organism. Mark with an X which items you would need to insert into the organism.

☐ Mitochondria ☐ Ribosome ☐ CRISPR protein ☐ Template DNA

☐ Messenger RNA ☐ Guide RNA ☐ Cas protein ☐ Mitochondrial DNA

☐ I don't know/I wish not to answer

9 – After the success of your last test, you would now like to disable another gene, rather than correct it. You repeat the previous steps, but this time you don't include which of the previous items?

☐ Mitochondria ☐ Ribosome ☐ CRISPR protein ☐ Template DNA

☐ Messenger RNA ☐ Guide RNA ☐ Cas protein ☐ Mitochondrial DNA

☐ I don't know/I wish not to answer

10 – Would you like to replace the codon that encodes the amino acid Lysine with the codon of Asparagine in a certain protein in this part of her gene: ...GTATTTTCG... You used CRISPR/Cas technology to cut the gene, which DNA sequence would you use as model for repair?

☐ I don't know/I wish not to answer

11 – Please mark activities for which CRISPR/Cas technology is already used/may be used in the future.

☐ Chemical synthesis of new compounds for the petrochemical industry

☐ Designing yeasts with increased capacity to produce biofuels

☐ Developing disease resistant plants

☐ Modifying the human genome to prevent inherited diseases

☐ I don't know/I wish not to answer

12 – Briefly describe a day-to-day activity that could be impacted by CRISPR/Cas technology in the future.

☐ I don't know/I wish not to answer

13 – Let's say you travel 35 years into the future and find yourself. What do you think your profession will be? Will it be affected by CRISPR/Cas technology? Justify briefly.

☐ I don't know/I wish not to answer

11. Appendix 3 – Learning assessment questionnaire's scoring system.

Section 1 – Basic Genetics

Question 1

a) Which messenger RNA will be produced from this DNA sequence:

Template strand 5'- TACGAGTAAATTGCA - 3'

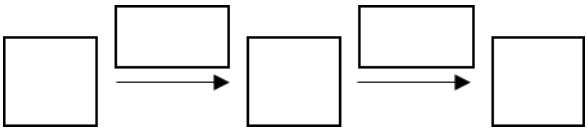
Antisense strand 3'- ATGCTCATTTAACGT - 5'

b) Which amino acids would be produced from the messenger RNA sequence generated in a)?

Scoring: The participant who writes the correct sequence of messenger RNA gets 0.5 points, getting another 0.5 if he describes the correct amino acids.

Question 2

Organize these terms into the appropriate boxes: Protein, RNA, DNA, Ribosome, RNA Polymerase.



Scoring: For each correctly filled box, the participant receives 0.2 points.

Question 3

Draw a line connecting the nucleotides that pair in the formation of the double-stranded DNA.

Adenine	Adenine
Cytosine	Cytosine
Guanine	Guanine
Thymine	Thymine

Scoring: For each item correctly connected the participant receives 0.25 points

Section 2 – CRISP/Cas Mechanism

Question 4

You are a scientist in your laboratory, and you would like to correct a defective gene in a particular organism. Mark with an X which items you need to insert into the organism for genome editing.

☐ Ribosome ☐ Mitochondria ☐ Messenger RNA ☐ guide RNA

☐ CRISPR protein ☐ Cas protein ☐ Template DNA ☐ mitochondrial DNA

Scoring: For each correct item the participant receives 0.33 points. For each incorrect item, the student loses 0.33 points, and the final score of the question cannot be less than 0.

Question 5

After the success of your experiment in the previous question, you would now like to disable another gene instead of modifying it. You repeat the previous steps, but this time you DO NOT include which of the previous items?

☐ Ribosome ☐ Mitochondria ☐ Messenger RNA ☐ guide RNA

☐ CRISPR protein ☐ Cas protein ☐ Template DNA ☐ mitochondrial DNA

Scoring: If the participant selects the correct answer, the student receives 1 point. If the participant selects the correct answer and any additional item, the student receives 0.5 points.

Question 6

You would like to replace the codon that encodes the amino acid ASPARTATE with the codon for GLUTAMATE in a given protein. You used CRISPR/Cas technology to cleave the genome, which DNA sequence would you use as a donor for the repair?

Protein-coding DNA : Template strand 5′- ...GTACTGTCG... -3′

Antisense strand 5′- ...CATGACAGC... -3′

Scoring: The student who describes the correct donor DNA receives 1 point. The participant who describes a donor that also promotes changes other than the desired one receives 0.5 points.

Section 3 - CRISPR/Cas Applications

Question 7

Mark activities for which CRISPR/Cas technology is already used/may be used in the future.

Question 7

Please mark activities for which CRISPR/Cas technology is already used/may be used in the future.

☐ Modify embryonic cells to prevent hereditary diseases

☐ Develop drought resistant plants

☐ Create yeasts with greater biofuel production capacity

☐ Chemical synthesis of new antibiotics

Scoring: For each correct item the participant receives 0.33 points. For each incorrect item the student loses 0.33 points.

Question 8

Briefly describe an activity in your daily life that could be impacted by CRISPR/Cas technology.

Scoring: In this open question, the participant receives 1 point for any answer, as long as it is justified.

Question 9

Imagine that you travel 30 years into the future and find yourself. What do you think your profession will be? Will it be affected by CRISPR/Cas technology? Justify briefly.

Scoring: In this open question, the participant receives 1 point for any answer, as long as it is justified.

11. Appendix 3 - Free and Informed Consent Form

The person you are responsible for is being invited to participate in the research entitled "Development of a toolkit to support the teaching of gene editing technology by CRISPR/Cas", developed by Master's student Guilherme Engelberto Kundlatsch under the guidance of Prof Dr Danielle Biscaro Pedrolli, within the scope of the Postgraduate Program in Biosciences and Biotechnology Applied to Pharmacy, School of Pharmaceutical Sciences, Universidade Estadual Paulista (Unesp).

The main objective of the study is to develop a teaching tool that allows the macroscopic visualization of the molecular mechanisms of CRISPR/Cas gene editing technology and to evaluate its effectiveness when compared to theoretical classes and laboratory classes on the same subject. The invitation to participate was made because he was a high school student, the target audience for the technology developed in the project. Consenting their participation is voluntary, that is, not mandatory, and you have full autonomy to decide whether or not you want them to participate, as well as withdraw your consent at any time. Neither you nor he will be harmed in any way if you decide not to consent to participation, or to withdraw from it. However, it is very important for the execution of the research. The confidentiality and privacy of the information provided by the participant will be guaranteed. Any data that could identify the participant will be omitted in the dissemination of research results, and the material will be stored in a safe place. At any time, during the research, or later, you can request information from the researcher about the participation and/or about the research, which can be done through the means of contact explained in this Term.

The participation of the person for whom you are responsible will consist of attending three different classes of 45 minutes each, one of them theoretical, another laboratory practice and another with the tool developed in this work. Classes will be taught at the educational institution where the participant is already regularly enrolled. During the theoretical class, concepts such as the structure of DNA, RNA and amino acids; production of a protein from a gene; and genetic editing using CRISPR/Cas technology will be presented to the participant through a lecture. During the laboratory practical class, students will carry out a bacterial transformation in a strain provided by the researchers, modifying the color of a microorganism, using the didactic laboratory infrastructure available at the school regularly attended by the student. During the class with the tool proposed in this work, students will manipulate pieces produced in MDF using laser cutting by the researcher, simulating the molecular mechanisms

described in the theoretical class and used in the practical class. In addition, the participant will answer a socioeconomic questionnaire before the start of the first class. They will also answer questionnaires to assess both knowledge about the topics taught and interest in science at four different times, being them before the beginning of the first class and after each of the three classes. All responses collected will be anonymized. The answers will be transcribed and stored in digital files. At the end of the research, all material will be kept on file, under the custody and responsibility of the responsible researcher, for at least 5 years, according to CNS Resolution No. 466/2012. By participating in the research, the student has the benefit of discussing topics already included in the High School curriculum, such as the structure and interactions of DNA, RNA and amino acids, as well as a topic on the frontier of current scientific knowledge, genetic editing by technology. CRISPR/Cas, which can bring about major transformations in areas such as health and agriculture in the coming decades. In all research there are risks. In this, there are some risks that we will seek to minimize. These include possible tiredness, discomfort due to the time spent or anxiety to answer correctly in the stages of filling out the forms, which will be mitigated by emphasizing to the students that the completion is not mandatory and that the answers will not be used for school evaluation. They also include any accidents during the activities, which will be mitigated using the same infrastructure and protocols that students are already familiar with in the school environment, both in the classroom and when using the unit's laboratory infrastructure. They also include time spent in the classroom, damage that will be minimized by properly coordinating research activities with the faculty to adjust them to the planned didactic calendar. There is also the biological risk of handling microorganisms during the practical class in the laboratory. To mitigate it, we will use an organism known to be safe (Bacillus subtilis) and widely used in the food industry, as well as provide personal protective equipment, including disposable lab coats and gloves. We will also have the presence of the school's technical staff, usually present in laboratory classes. Clarifications will be carried out before and during the course of the research regarding the procedures to be carried out and the participant will not have any cost or any financial compensation for the authorization. If any damage occurs as a result of the participation of the person for whom you are responsible, the participant will be entitled to compensation.

The results of this research will be published in conferences and scientific articles and in the dissertation format, keeping all participants anonymous. This term is written in two copies, one for the person responsible for the research participant and the other for the researcher. In case of doubts about the participation of the person for whom you are

responsible, you can contact the responsible researcher through the e-mail g.kundlatch@unesp.br or by phone 16 982021710.

_____

Researcher name and signature

_____

Place and date

_____

Name and signature of the person responsible for the research participant

_____

Place and date

12. Appendix 4 - Free and Informed Assent Form

You are being invited to participate in the research "Development of a toolkit to support the teaching of gene editing technology by CRISPR/Cas", developed by Master's student Guilherme Engelberto Kundlatsch under the guidance of Prof Dr Danielle Biscaro Pedrolli, both from Universidade Estadual Paulista (Unesp).

We want to know if the tool we developed to teach gene editing works worse, equal or better than a practical laboratory class or a theoretical class in the classroom. You are being invited because you are a high school student, the target audience of our work. You don't have to participate in the work if you don't want to and, if you decide to participate, you can withdraw at any time, you won't have any problem. All information you provide will be confidential and private, and any data that could identify you will be omitted from the disclosure of the results of the engagement. You can ask during or after the survey for more information about your participation or the survey, using the contact information at the bottom of the page.

Your participation will be to attend three classes of 45 minutes each, one theoretical, another laboratory practice and another with the tool that we are developing in this project. Classes will be held at the school you attend. During the theoretical class you will learn concepts such as the structure of DNA, RNA and amino acids; production of a protein from a gene; and gene editing using CRISPR/Cas technology. During the practical class in the laboratory, you will perform a genetic modification on a microorganism by changing its color. During the class with the tool that we are developing in this work, you will use MDF pieces produced with laser cutting that simulate the mechanisms presented in the theoretical class and used in the practical class. In addition, you will answer a socioeconomic questionnaire before the start of the first class. You will also answer questionnaires to assess your knowledge about the topics taught and your interest in science at four different times, being them before the beginning of the first class and after each of the three classes. Rest assured, we will anonymize all responses and will not be used to rate you. Paper forms will be converted to digital files. At the end of the research, the files will be kept by the responsible researcher for at least 5 years.

There are two main benefits to participating in the survey. First, topics that are part of your high school curriculum will be discussed, such as the structures of DNA, RNA and amino acids and their interactions. Second, you will be able to learn and experience in practice a recent advance in science, CRISPR/Cas technology, which allows precise gene editing and

has great applications in areas such as health and agriculture. Like all research, this one also has some risks, which we will try to minimize. You may feel tired or anxious answering a form, so let's make it clear that answering is not mandatory and that your answers will not count towards your grade. You can have an accident, such as tripping and falling, during some activity, so we will carry out classes in the space you are already used to and following the procedures you already use, both in the classroom and in the school laboratory. Another risk is the class time that will be used for our activity. The researcher will coordinate the activities with your biology teacher to ensure that they best fit your class schedule.

There is also the biological risk of using a microorganism during the practical class. We will minimize this risk by using a safe bacteria (Bacillus subtilis) used widely in the industry and you will be provided with the appropriate protective equipment, including a disposable lab coat and glove. We will also have the team you are used to in laboratory classes close by. All these clarifications will be repeated before and during the research. You will incur no cost and will not receive any financial compensation. In the event of any damage, the participant will be entitled to compensation.

The results of this research will be published in conferences and scientific articles and in dissertation format, keeping you and your colleagues anonymous. This document has two copies, one for you and one for the researcher. If you or your guardian have any questions, you can contact the researcher by phone 16 98202-1710 and by e-mail g.kundlatch@unesp.br.

_____

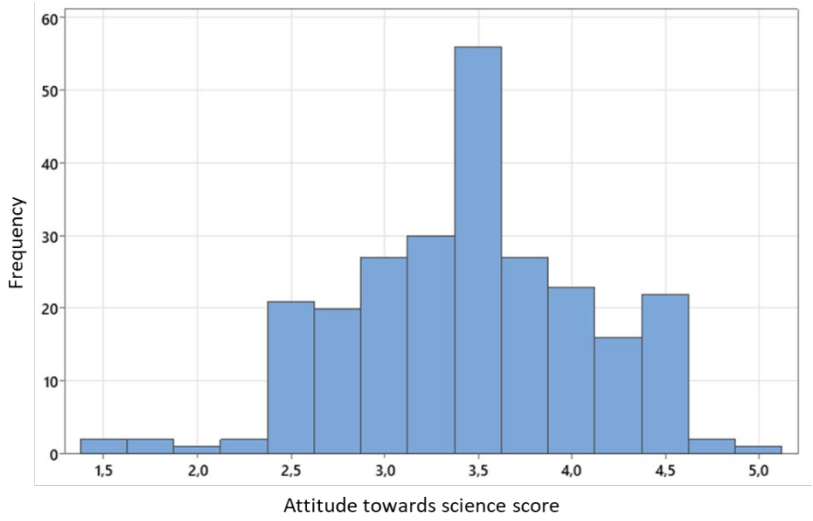
Researcher's signature
São Carlos, June 13, 2022

_____

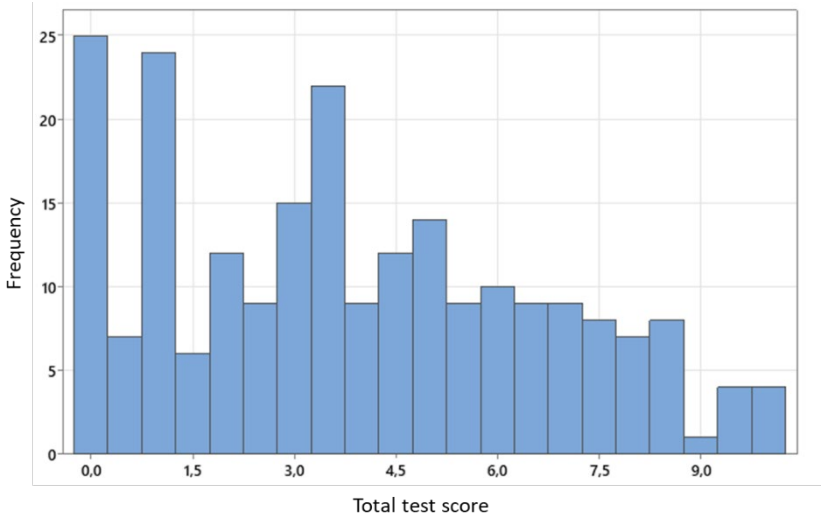
Signature of the research participant
São Carlos, June 13, 2022

13 Appendix 5 – Data frequency distribution

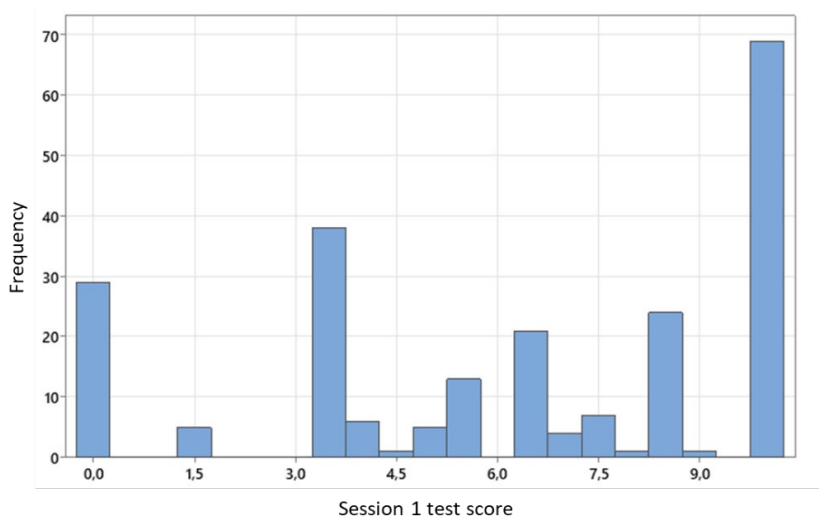
Data normality was assessed using the Ryan-Joiner test and the results are described in the results section. To allow visualization of the distribution of points, the following histograms were created using Minitab software.



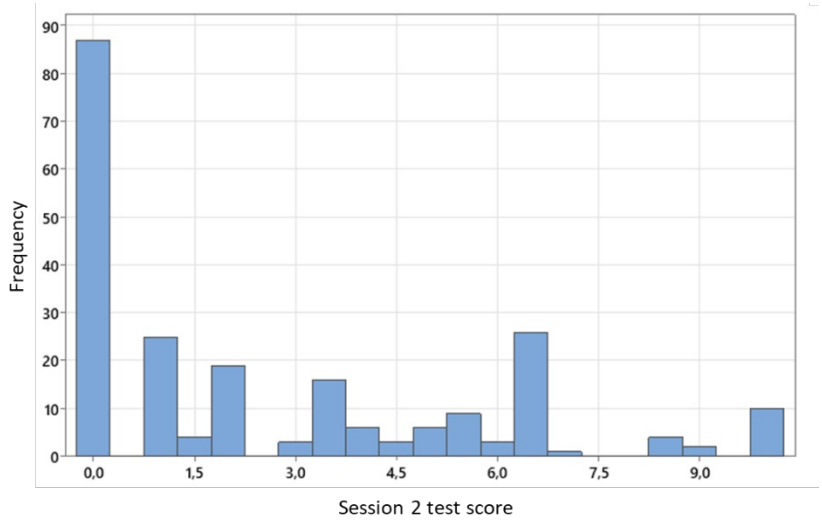
Attitude towards science histogram. Student scores (from 1 to 5) were grouped into 15 intervals.



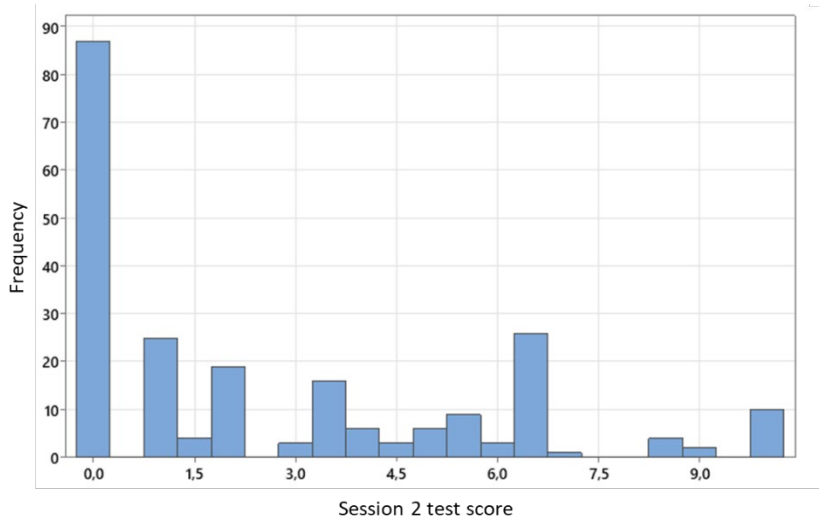
Test score histogram. Scores were grouped into 15 intervals. It is possible to observe that there is no normal distribution, with a higher frequency in the initial intervals.



"Basic Genetics" score histogram. Scores were grouped into 15 intervals. It is possible to observe that there is no normal distribution, with a higher frequency in the final interval.



"CRISPR/Cas Mechanism" score histogram. Scores were grouped into 15 intervals. It is possible to observe that there is no normal distribution, with a higher frequency in the initial interval.



"CRISPR/Cas Mechanism" score histogram. Scores were grouped into 15 intervals. It is possible to observe that there is no normal distribution, with a higher frequency in the initial interval.