USING CRISPR TO TREAT CYSTIC FIBROSIS

Ref.CRIS1

1. EXPERIMENT OBJETIVE

The aim of this experiment is to introduce students to the principles and practice of RNA guide design (gRNA), as well as agarose gel electrophoresis to examine DNA samples.

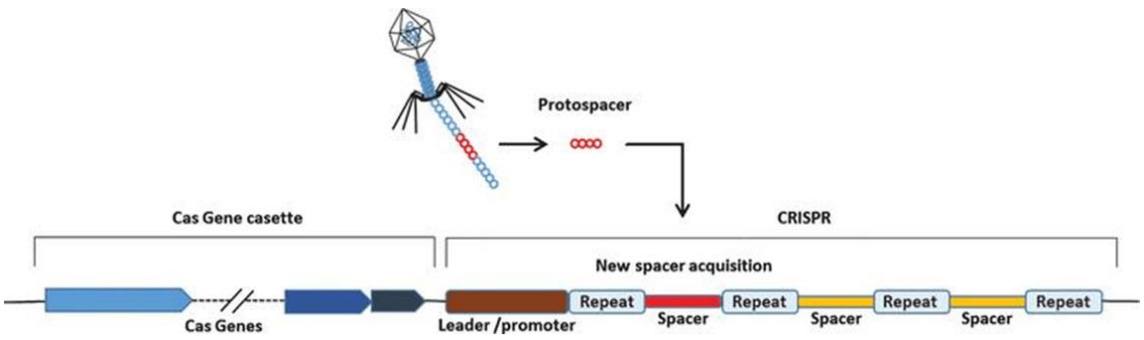
2. INTRODUCTION

2.1 CRISPR

CRISPRs (clustered regularly interspaced short palindromic repeats) are families of DNA sequences in bacteria. These sequences contain DNA fragments from viruses that have attacked bacteria. These fragments are used by the bacteria to detect and destroy DNA from new attacks by similar viruses, and thus be able to effectively defend against them. These sequences play a key role in bacterial defencesystems and form the basis of a technology known as CRISPR / Cas9.

CRISPR are DNA loci that contain short repeats of base sequences. Following each repeat are short segments called "spacer DNA" from previous exposures of other viruses. These sequences are found in approximately 40% of bacterial genomes and 90% of sequenced archaea genomes. These genomes are often associated with Cas genes, which code for CRISPR-related nuclease enzymes. These Cas genes are normally found next to the CRISPR sequences.

The CRISPR / Cas system is a prokaryotic immune system that confers resistance to external agents such as plasmids and phagos. In addition, it provides a form of acquired immunity. CRISPR spacers recognize specific sequences and guide Cas nucleases to cut and degrade these exogenous gene elements in a manner analogous to RNAi in eukaryotic systems.



CRISPR history

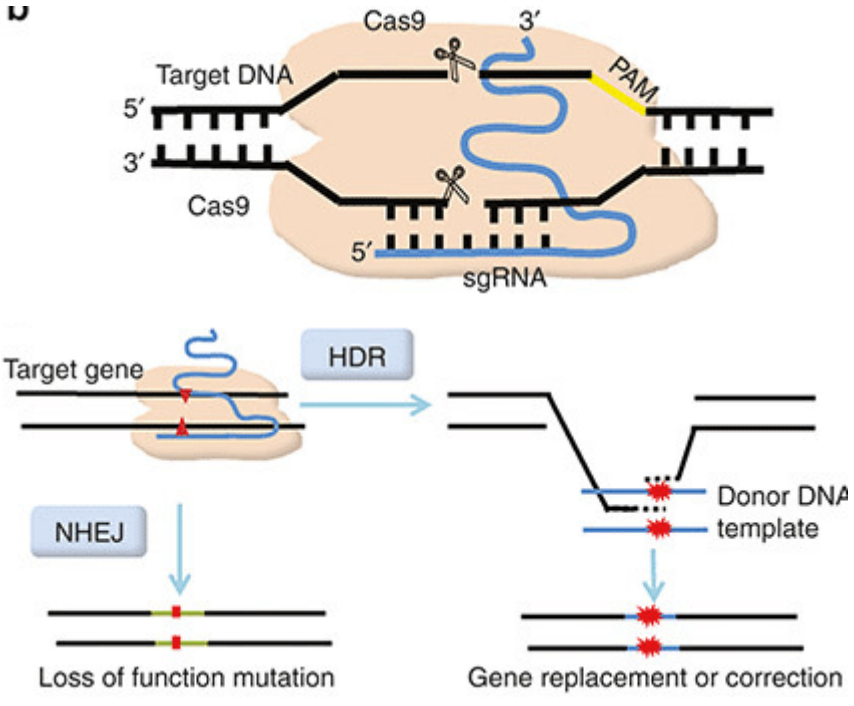
In 1987, Yoshizumi Ishino and his collaborators at the University of Osaka, Japan discovered an area within the bacterial genome that contained 5 identical segments with 29 base pairs. These segments were separated by a 32 base pair block of DNA that they called spacers. Each spacer has a unique configuration. These foreign fragments and spacers became known as short, grouped and regularly interspaced palindromic repeats, or CRISPR. Scientists also discovered that alongside these sequences there were always sequences that encoded the genes for the Cas enzyme.

In 1990, bacterial DNA could be sequenced and CRISPR too. In addition, the spacers were found to share the same sequence among themselves that they called PAM (protospacer adjacent motif). The PAMs are used by the Cas enzymes to recognize the target where the cut is to be made. Different Cas enzymes can recognize different PAM sequences, the most common used Cas9 from *Streptococcus pyogenes*recognizes the PAM sequence5'-NGG-3', where N can beany nucleotide base.

CRISPR has been used in a variety of settings, from fighting viruses to fermentation process. With all these experiments carried out, it was observed that CRISPR can be programmable, that is, that it can be used to cut DNA in the area of interest of the researcher. Thus, becoming a great tool of genetic engineering.

This new CRISPR function allowed the creation of a CRISPR RNA guide (gRNA) that cuts DNA specifically without altering cellular function. Above all, it has been used for digestion of genes, that is, to make DNA cuts and eliminate the region concerned.

cvcHow does CRISPR work? First, the DNA is cut using CRISPR-Cas to create a double stranded break. Next, the cells are given a template DNA strand, containing the correct sequence, which can be incorporate into the cut DNA using homology directed repair (HDR). With HDR, the natural cellular machinery will incorporate the template DNA into the genome at the site of the CRISPR digest. By controlling the template DNA strand, researchers can repair mutated genes or even insert entirely new genes into an organism. CRISPR-Cas systems allow researchers to easily place the new genes precisely where they want them, unlike some of the older methods of gene therapy where the new genes are randomly inserted into the plant or animal genomes.



All these applications are great advantages for biology and genetic engineering, but it has been shown in some studies that these modifications can make a cell become carcinogenic over time, that is, it increases the risk of cancer. Therefore, it is necessary to continue studying this method to make it safe and include it in clinical trials. Today, many research groups are working with CRISPR to try to repair defective DNA in mice, editing genes, and rewriting the genome. New companies are trying to apply this technology to create new cancer treatments.

2.2 CYSTIC FIBROSIS

Cystic fibrosis is a recessive genetic disease caused by the mutation in the CFTR gene. The CFTR gene codes for a protein that is involved in the production of sweat, digestive fluids, and mucus. CFTR mutations cause protein malformation and inactivity. Patients with this mutation have difficulty breathing since the mucus is much denser, fertility problems, and a reduction in life expectancy.

3. EXPOSITION OF FACTS

In this experiment, students will simulate the use of CRISPR-Cas9 to target a genetic mutation found in patient suffering from cystic fibrosis. Students will use the CRISPR technique to investigate a new treatment for cystic fibrosis. For them you will design a guide RNA of the mutated gene region.

4. COMPONENTS

Prepared sample for electrophoresis - A: DNA standard marker - B: gARN 1 - C: gARN 2 - D: gARN 3 - E: gARN 4 - F: gARN 5	Conserve at 4ºC
Utraspec Agarose	Room temperature
Electrophoresis buffer 50x	Room temperature
Flasblue DNA Stain	Room temperature

5. EXPERIMENT PROCEDURES

5 work groups will be carried out.

5.1PCR REACTION

1. Dilute the 50x electrophoresis buffer with distilled water according to the following table:

Gel de agarosa 0,8%				
Size of gel	**Concentrated buffer 50x**	**Distilled water**	**Agarose (g)**	**Total volume**
7 x 7 cm	0.6 ml	29.4 ml	0.23 g	30 ml
7 x 10 cm	1.0 ml	49.0 ml	0.39 g	50 ml
7 x 14 cm	1.2 ml	58.8 ml	0.46 g	60 ml

2. Prepare the tray where the electrophoresis gel will be made, placing the comb and the upper and lower edges.
3. Mix the agarose with the 1x electrophoresis buffer in a 250 ml Erlenmeyer flask
4. Dissolve the agarose by boiling the solution. To do this, place the solution in the microwave for 1 min, carefully extract it from the microwave and mix while turning. If you still see floating particles, reheat it a little more in the microwave.
5. Cool the solution with water. Place the Erlenmeyer flask under the tap water, trying not to let water run down the top, and keep touching the glass until you don't burn it with your hand.
6. Pour the content into the container where the gel will be made and let it cool. Once cold, remove the comb and the edges of the gel. Watching especially when removing the comb not to break any well.
7. Place the gel inside the electrophoresis tray and cover it with 1x electrophoresis buffer.
8. Pipette each of the following samples into each of the wells, trying to place as many as possible. The quantity that comes in each of them is 35 µl. NOTE: not all gels wells have a capacity of 35 µl, there are some that 20 µl is enough. Load the samples from number 1, which is tube A, to number 6, which is tube F.
9. Cover the cuvette and place the electrodes. Observe bubbles coming out of the sides, which indicate that the current is circulating. Run the gel for 20-35 min. NOTE: Make sure that the front of samples that will be a blue line does not disappear at the end of the gel.
10. When the electrophoresis is over. Remove end caps and comb and place it in a cuvette to be able to stain the gel.

5.2 DNA PURIFICACTION OF PCR PRODUCTS

1. Dilute 5ml of Flashblue 10x with 95ml of distilled water and mix well.
2. Take the gel that we have inside a bucket outside the gel and cover it with 1x flashblue and incubate for 5min.
3. Transfer the gel to another bucket and cover it with water. Incubate for 20 min and if possible, place it on a moving surface. If not, make mixtures in the form of circles every so often.
4. Eliminate the water and visualize the different bands that have appeared tinted. NOTE: if when remove water the gel is still blue and the bands are not very visible, put water back until the gel is covered and incubate for 10 more minutes.

Staining trays can be in plastic boxes where the gel will fit without being folded. If there is only one per group there is no problem, when eliminating the Flashblue, several washes are made with water until we see that the plastic box does not have any blue substance and is left to incubate.

6. RESULTS

Observe the different bands that appear stained in the gel.

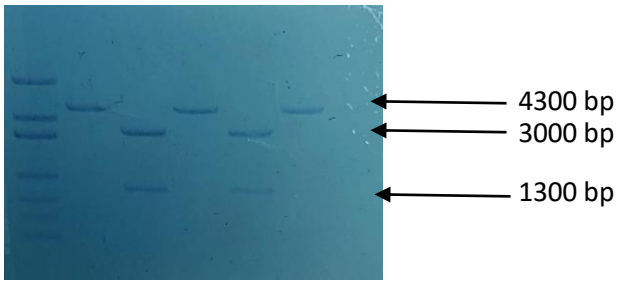


Image: stained agarose gel

1: DNA standard marker(Tube A)
2: Sample 1 (tube B)
3: Sample 2 (tube C)
4: Sample 3 (tube D)
5: Sample 4 (tube E)
6: Sample 5 (tube F)

In this image you can see the agarose gel after staining with the FlashBlue.In it we can see that in sample 1, 3 and 5 only one band with a weight of 4300 bp appears, while samples 2 and 4 They present two bands, one of 3000 bp and the other of 1300 bp. These results indicate that the guide DNAs for the patient's cure are 2 and 4 since they present the gene deletion, since two bands are seen, indicating that the gene of interest has been removed.

7. EXERCISES AND QUESTIONS ABOUT THIS PRACTICE

Next, an exercise and different questions about practice are asked.

EXERCISE

You are given a DNA sequence from the cystic fibrosis patient with the deleted mutated region, it is about complementing the deleted gene region and seeing where in this sequence the Cas9 enzyme could act by cutting DNA and incorporating this sequence into the genome of the patient. Remember that Cas9 acts by cutting on the triple NGG, with N being any of the bases present in the human genome (A, G, C and T).

The sequence is:

5' TACCTAGATGTTTTAACAGAAAAAGAAATATTTGAAAGCTGTTCTGTAAACTGATGGCT

3' ATGGATCTACAAAATTGTCTTTTTC

GCCCCTCAGGCAAACTTGACTGAACTGGATATATATTCAAGAAGGTTATCTCAAGAAACT 3'

Then write the 5 possible sequences, counting from the MAP 20 nucleotides upstream.

Name	Target sequence (spacer)	PAM secuence
Ejemplo	5’ CTTTTTCTGTTAAAACATCT 3’	AGG
gARN1		
gARN2		
gARN3		
gARN4		
gARN5		

QUESTIONS

1. What is the PAM sequence and why is it so important for Cas9 activity?
2. How can CRISPR be used to repair genetic mutations in patients?
3. What do you think about this technique? You think it may be a technique that in the future can be applied to humans and repair any genetic mutation.

For any additional questions or queries, please contact us info@bioted.es