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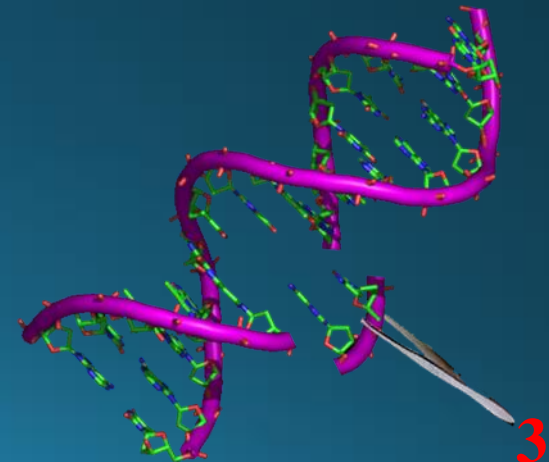
*Title: Using the CRISPR/Cas9 technology to attenuate virulence factors and pathogenicity of bacteria*

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# Outline

- 1. Introduction*
- 2. Mechanisms of Gene Editing*
- 3. Platforms of genome-editing technology*
- 4. CRISPR-Cas technology*
- 5. Major steps in CRISPR genome editing*
- 6. Conclusion and future perspectives*



- Genome editing can be achieved in vitro or in vivo by delivering the editing machinery in situ.
- Gene editing is quite extraordinary technique because of its capability to alter DNA by utilizing engineered nucleases called as **molecular scissors**.
- Genome editing is the process of precisely modifying the nucleotide sequence of the genome.

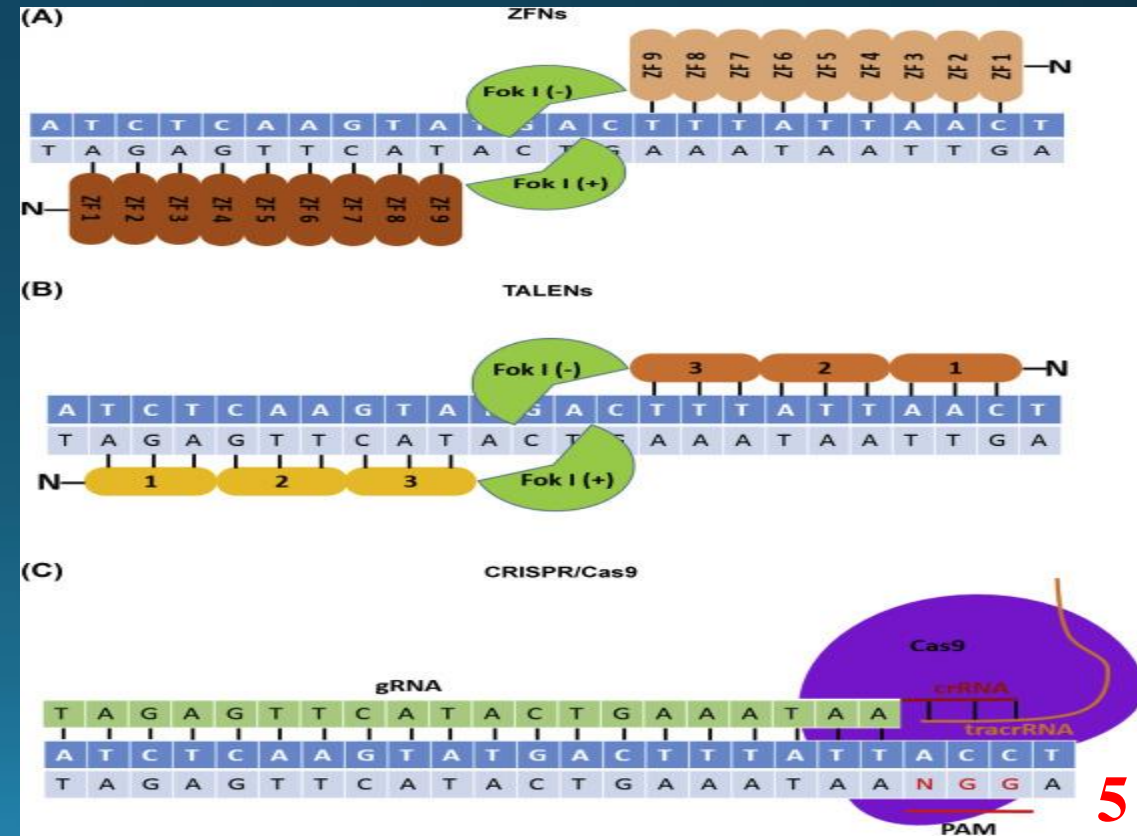


# Genome editing technology

Targeted DNA alterations begin from the generation of nuclease-induced double-stranded breaks (DSBs), which leads to the stimulation of highly efficient recombination mechanisms of cellular DNA.

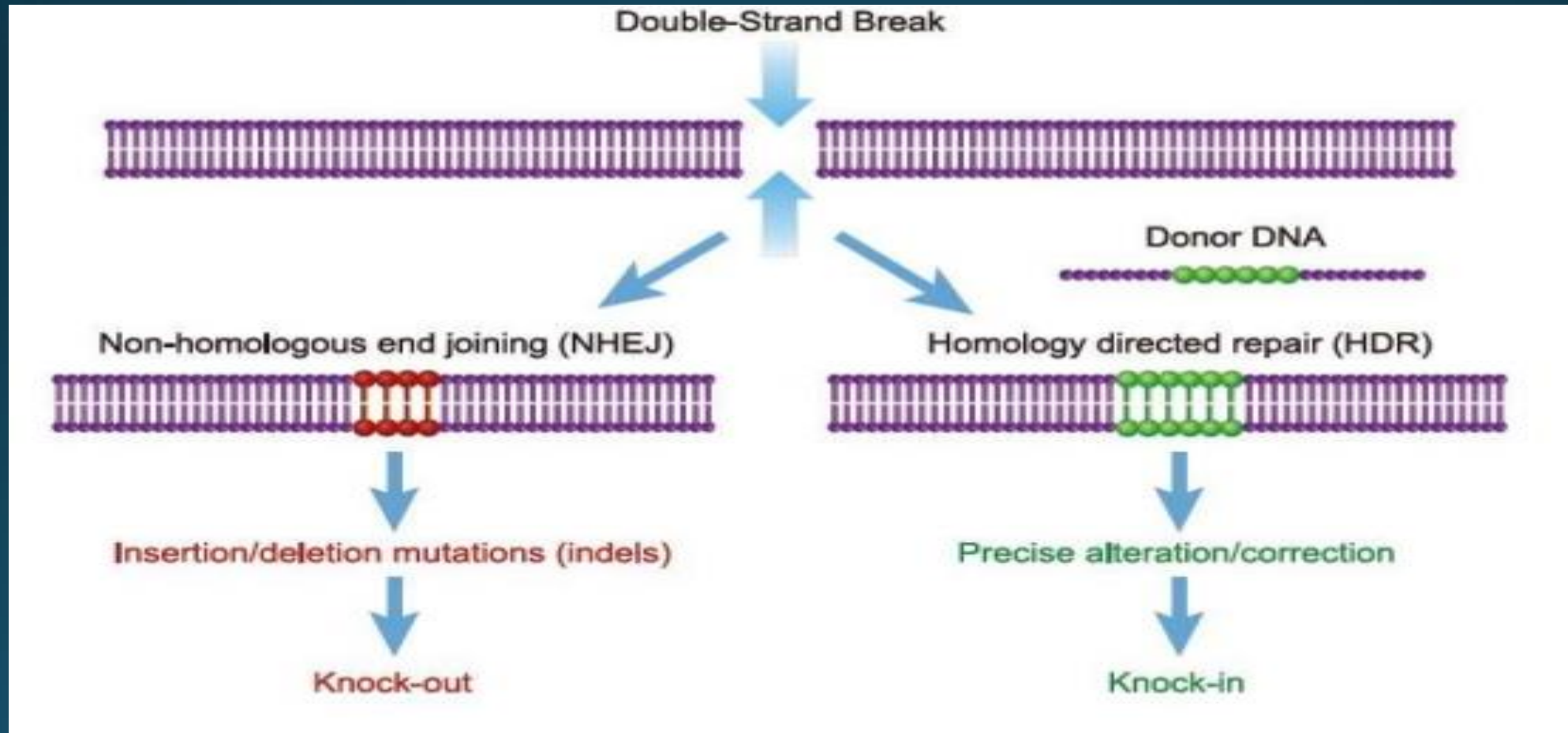
Nuclease-induced DNA DSBs:

- 1-Homology-directed repair (HDR)
- 2-Nonhomologous end-joining (NHEJ)



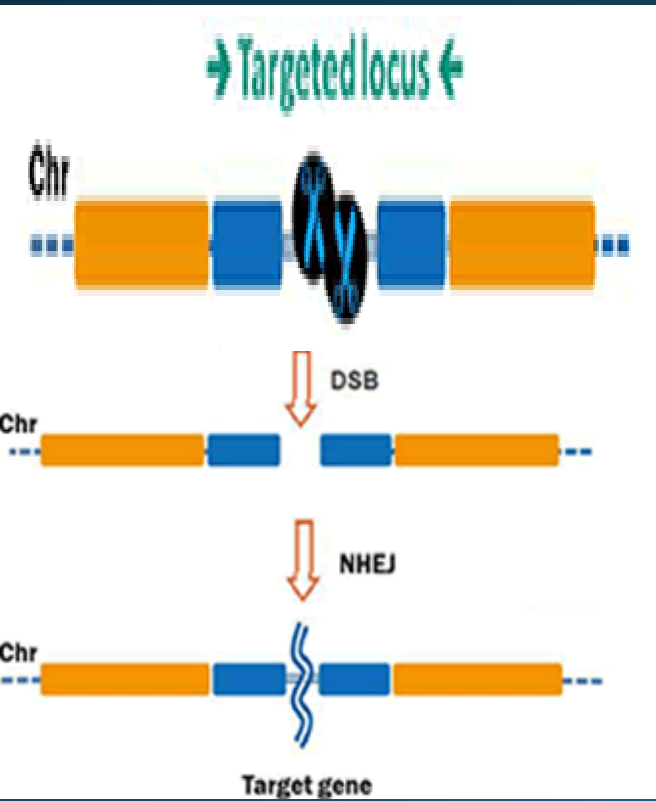
# Mechanisms of Gene Editing

Foundational to the field of gene editing was the discovery that targeted DNA double strand breaks (DSBs) could be used to stimulate the endogenous cellular repair machinery.

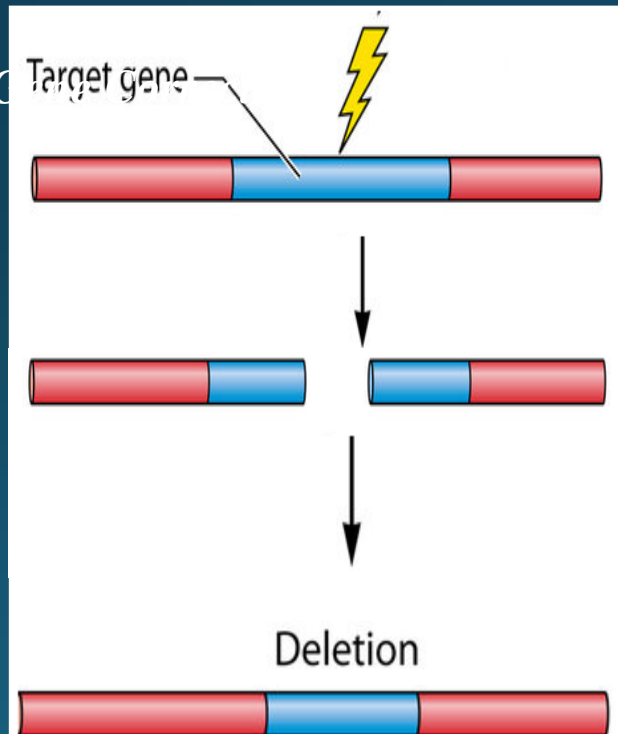


# Types of genetic changes

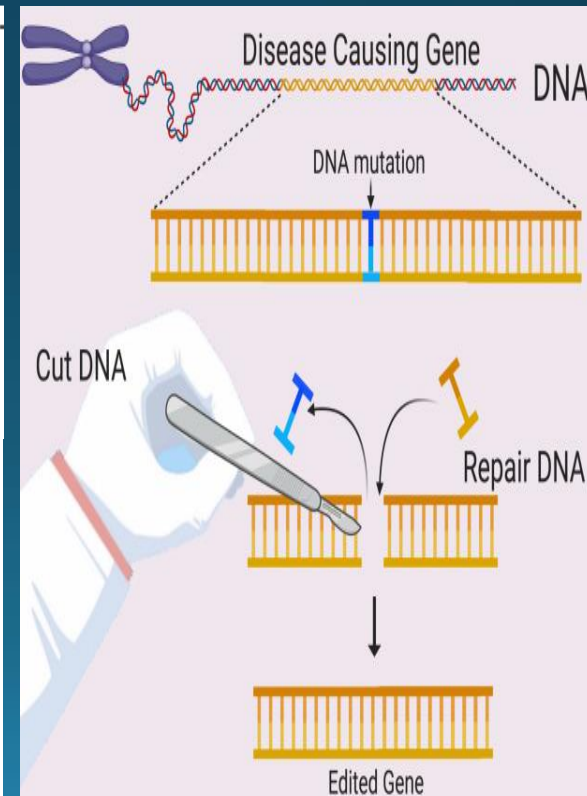
## Gene Knockout



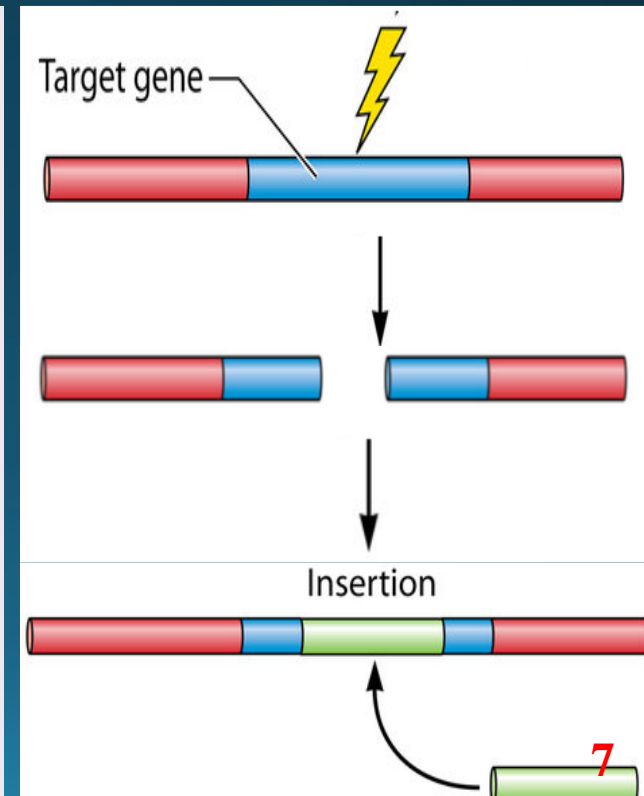
## Gene Deletion



## Gene Correction



## Gene Insertion



# Platforms of genome-editing technology

Previous technology

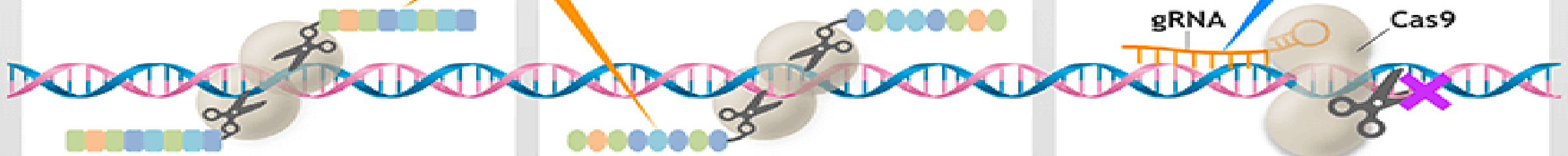
ZFN

TALEN

CRISPR-Cas9

A string of DNA recognition protein motifs is designed and fused with cleaving enzyme

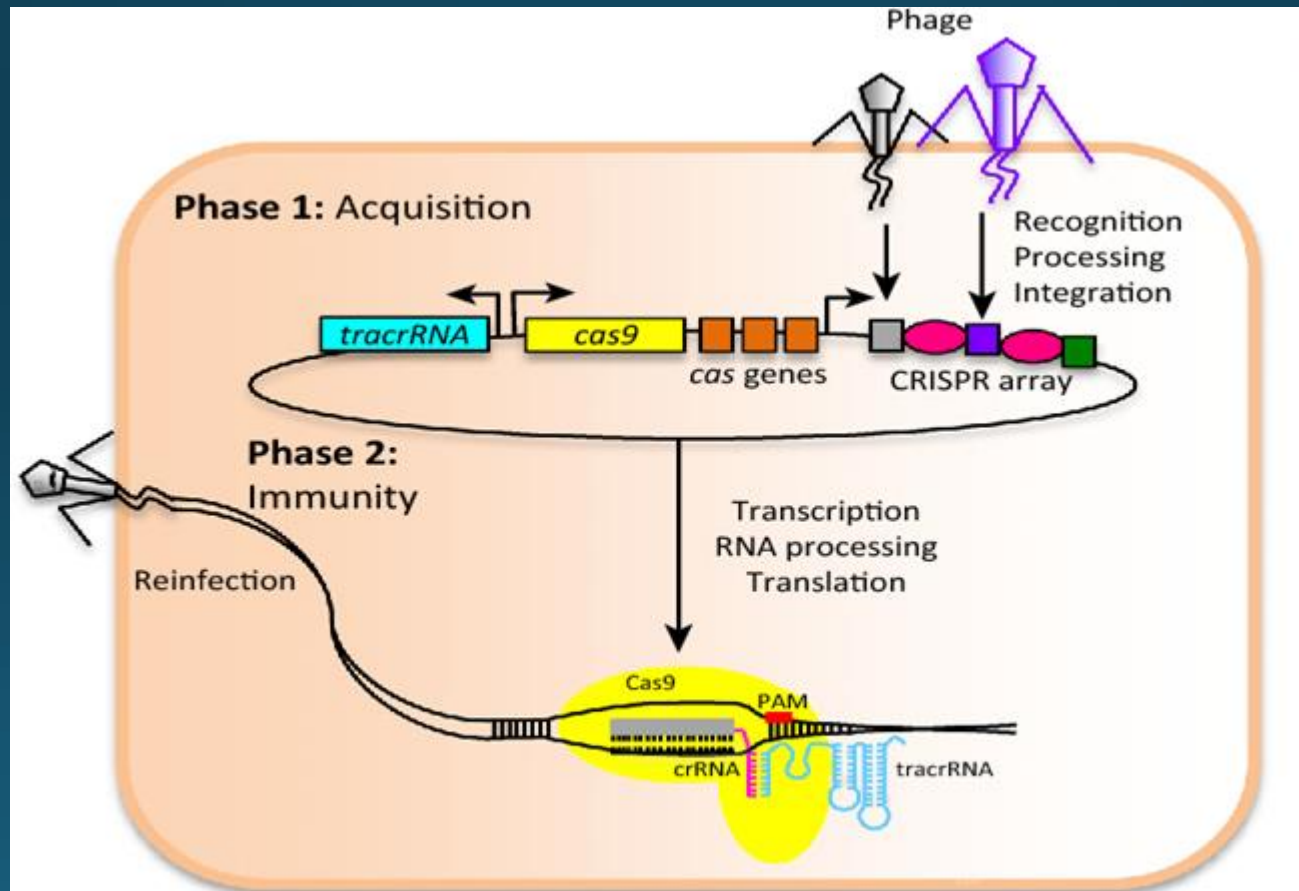
Easy to design and generate gRNA that binds to target sequence. Cas9 is off the shelf.



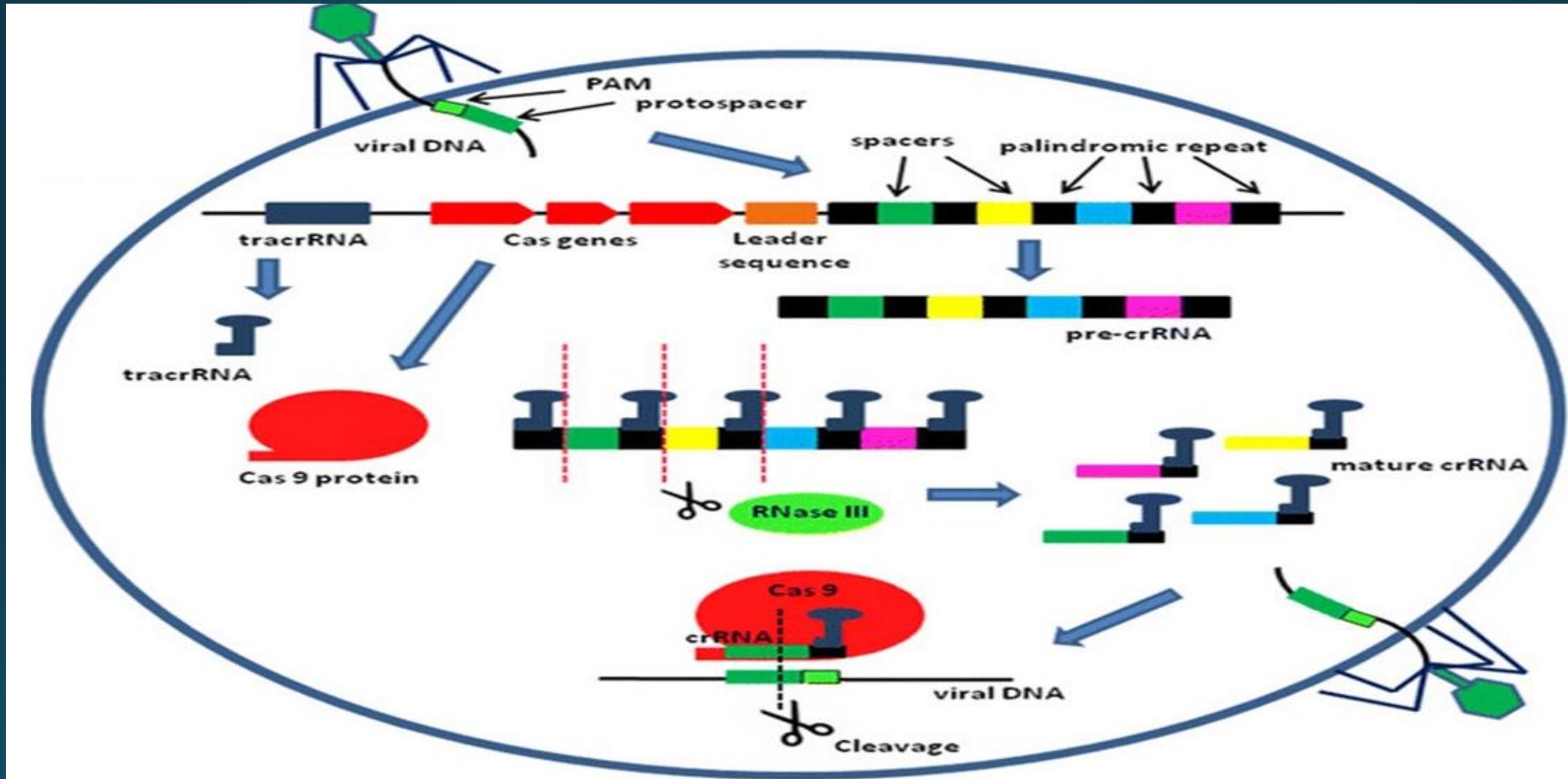


## The CRISPR-Cas immune system

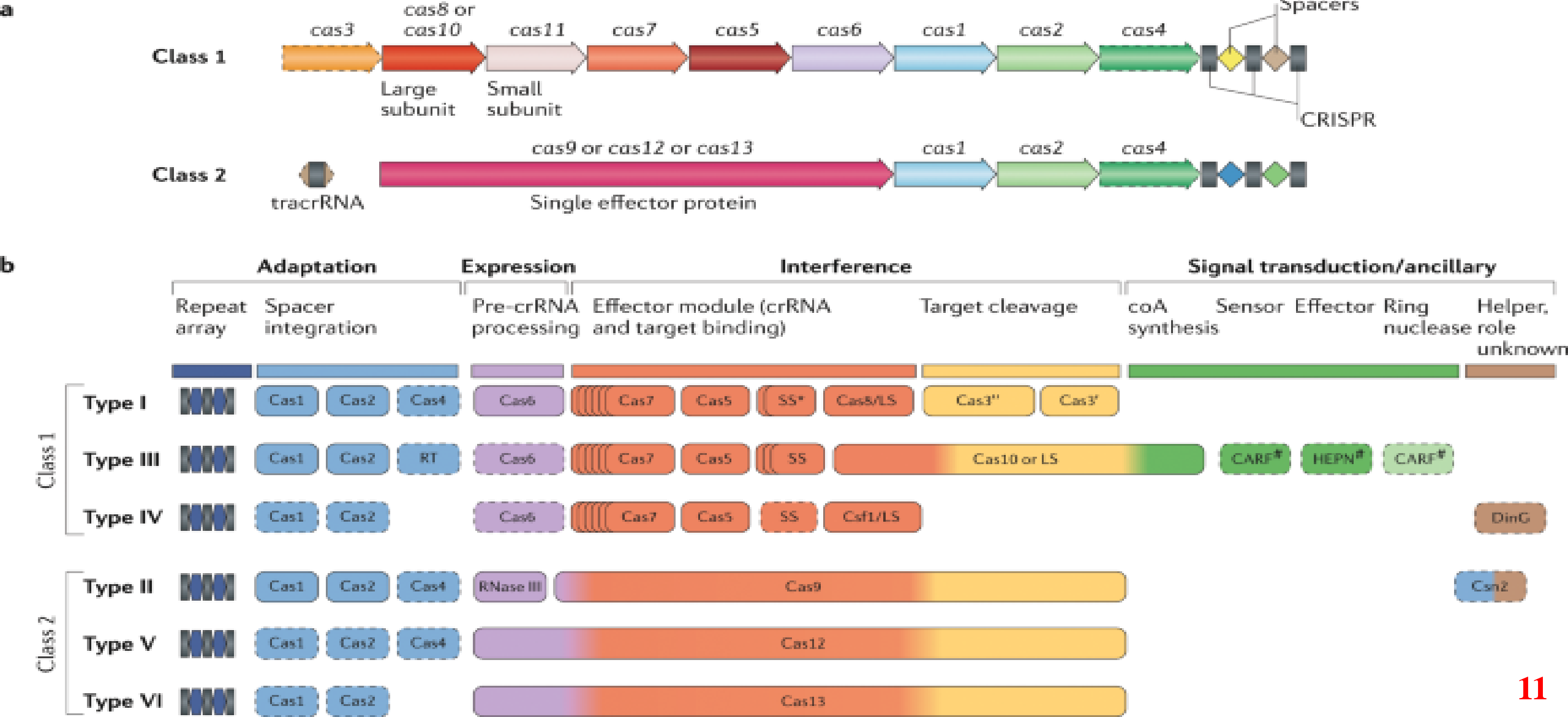
CRISPR (clustered regularly interspaced short palindromic repeats) were firstly identified in the *Escherichia coli* genome in 1987 as an unusual sequence element consisting of a series of 29-nucleotide repeats separated by unique 32-nucleotide “spacer” sequences.

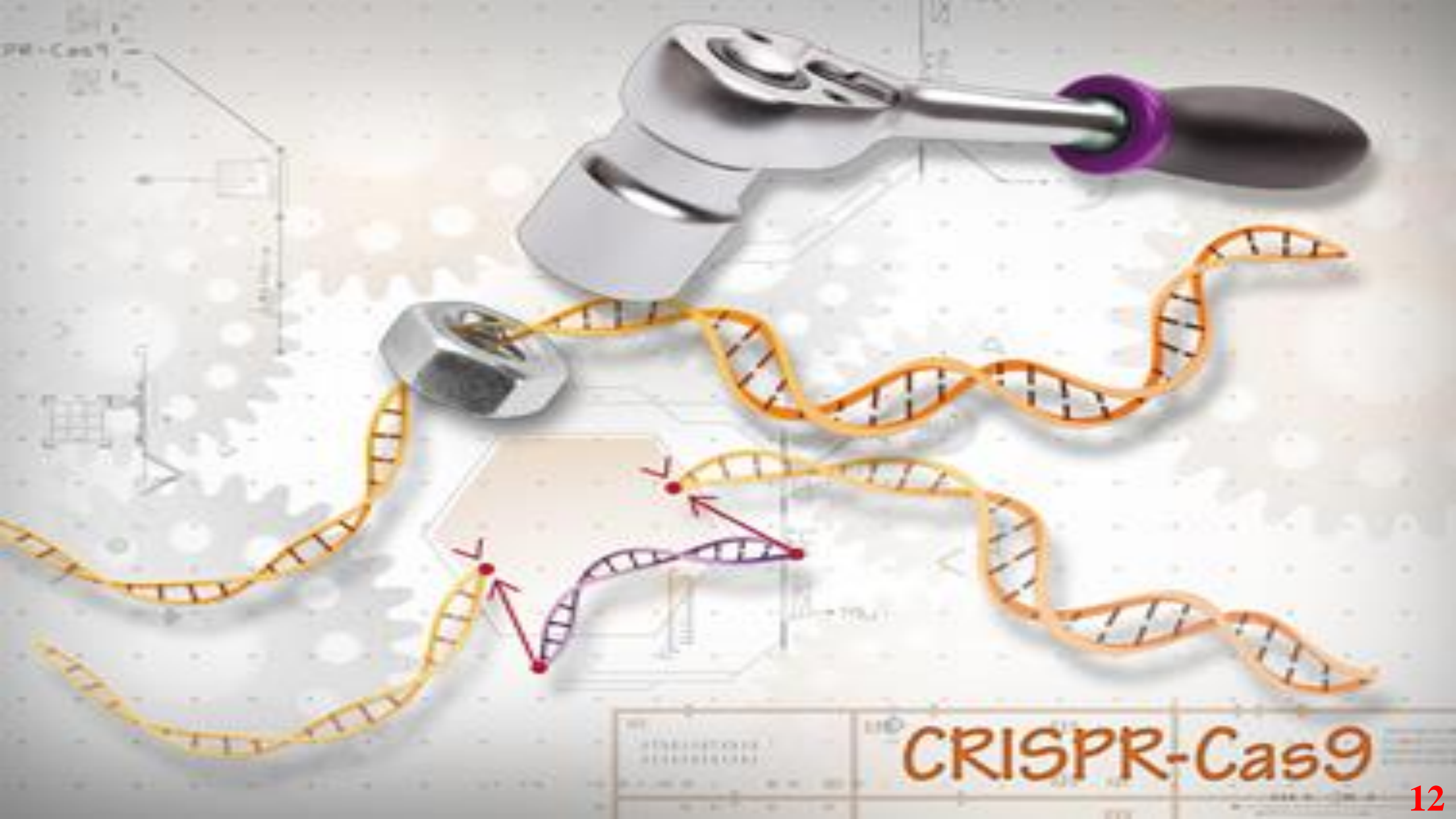


# *Mechanisms of the acquired immune system mediated by the CRISPR-Cas system*



# Evolutionary classification of CRISPR–Cas systems



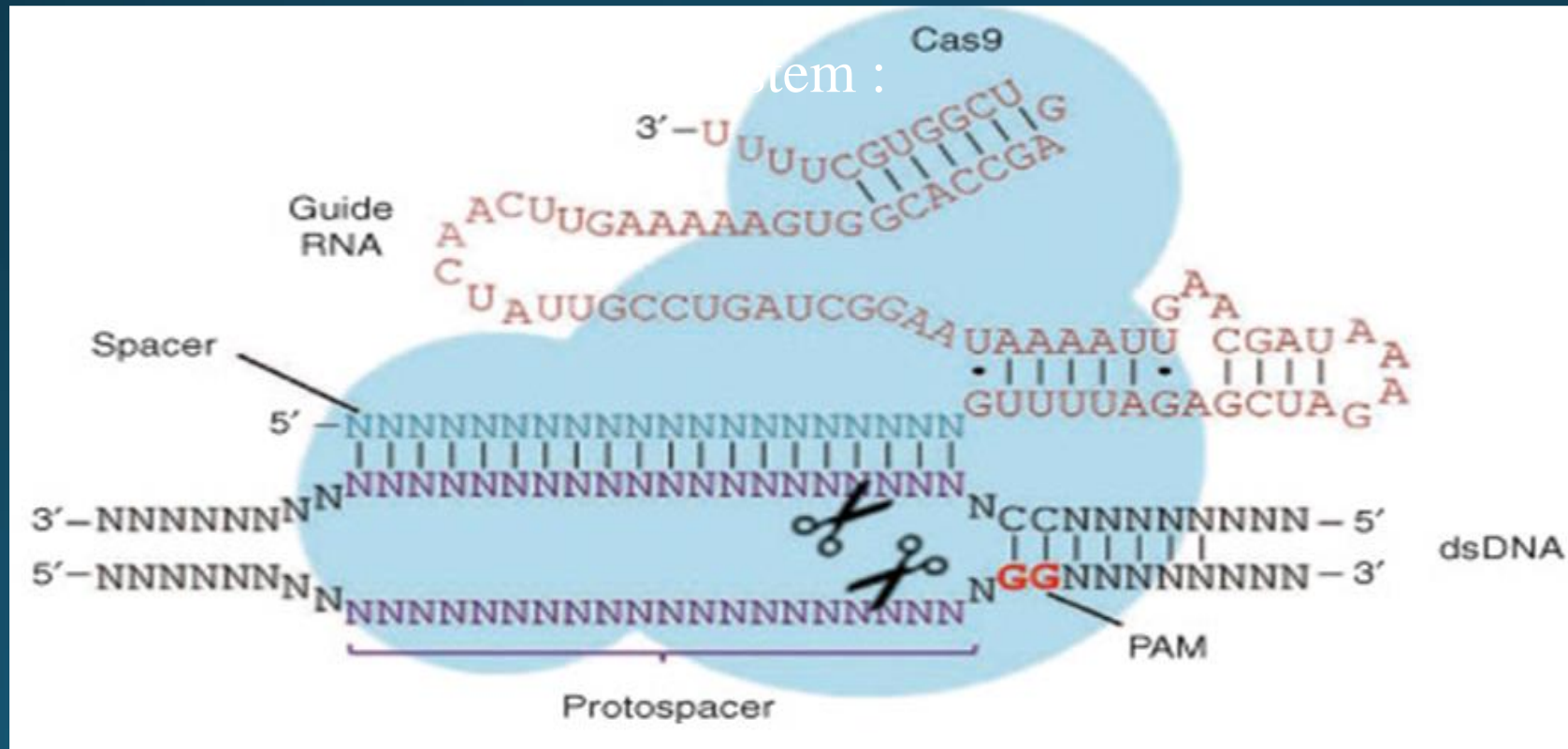


CRISPR-Cas9

# CRISPR-Cas technology

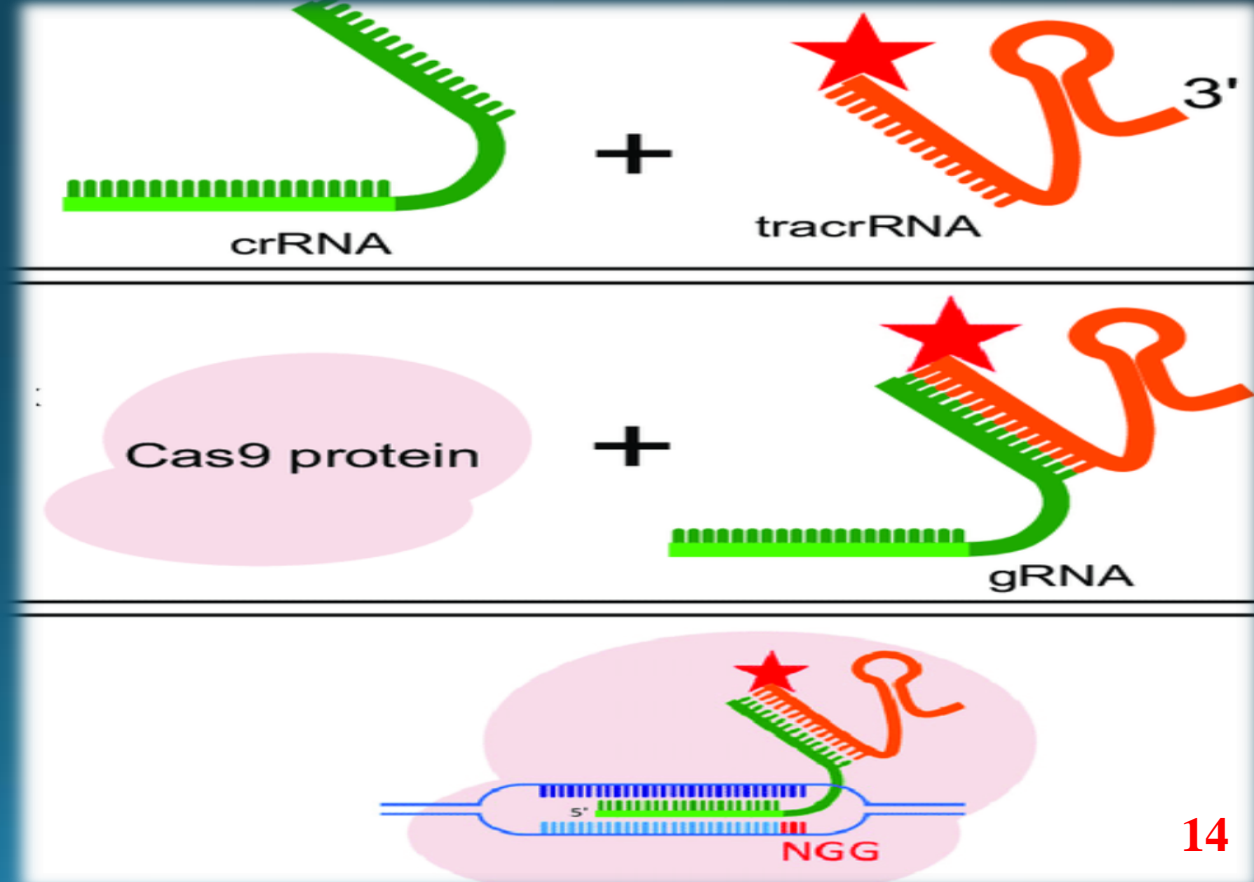
- 1) Cas9 protein (RuvC and HNH)
- 2) CRISPR RNA (crRNA)
- 3) Trans-activating crRNA (tracrRNA)

❖ CRISPR-Cas9 system :



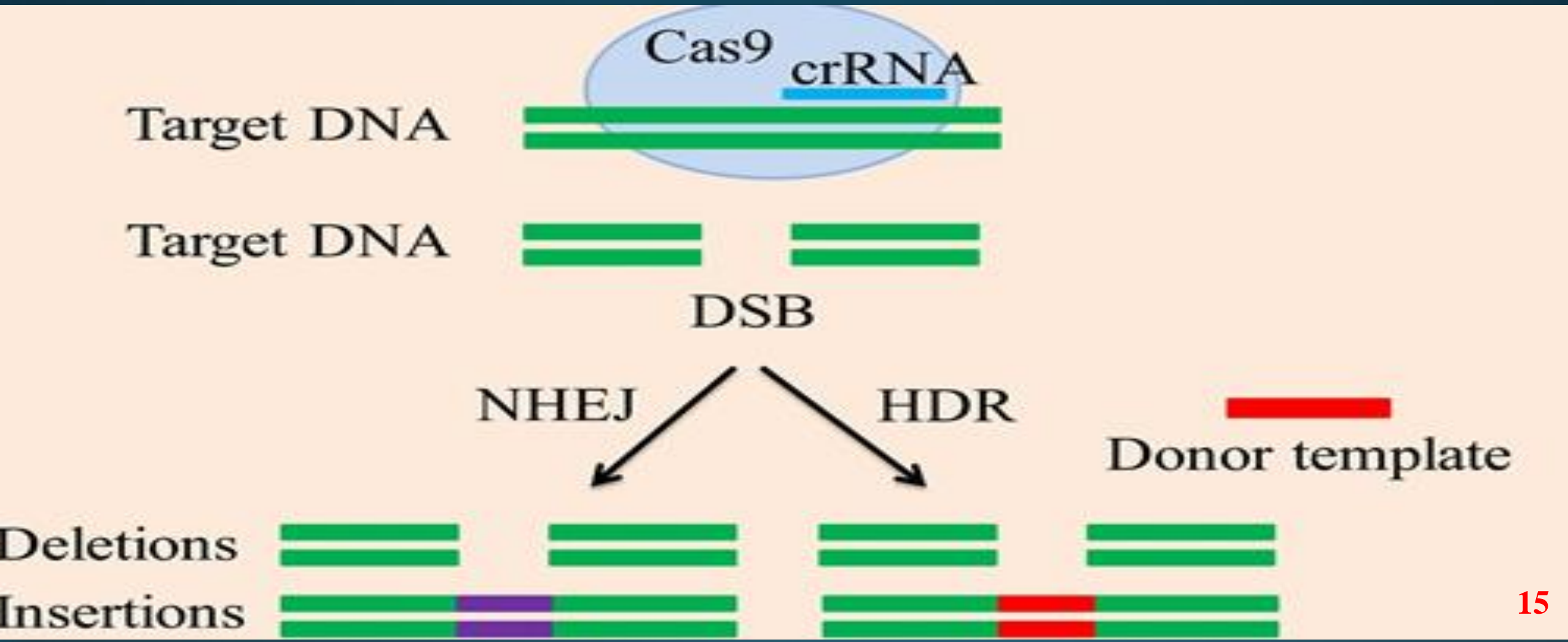
# CRISPR-Cas technology

Doudna, Charpentier and colleagues showed through in vitro DNA cleavage experiments that this system could be reduced to two components by fusion of the crRNA and tracrRNA into a single guide RNA (gRNA).

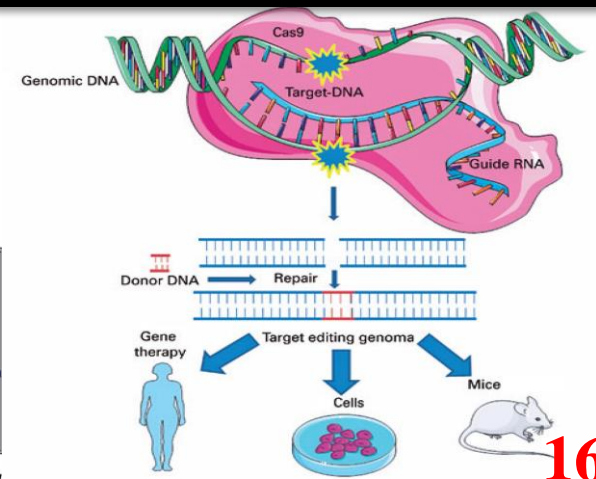
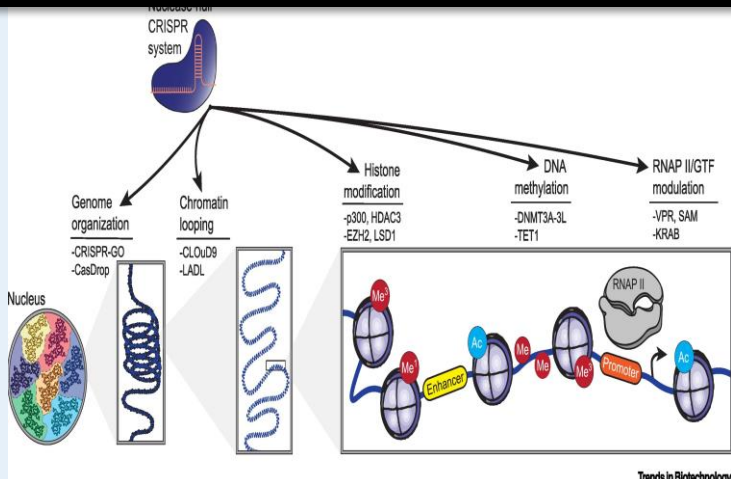
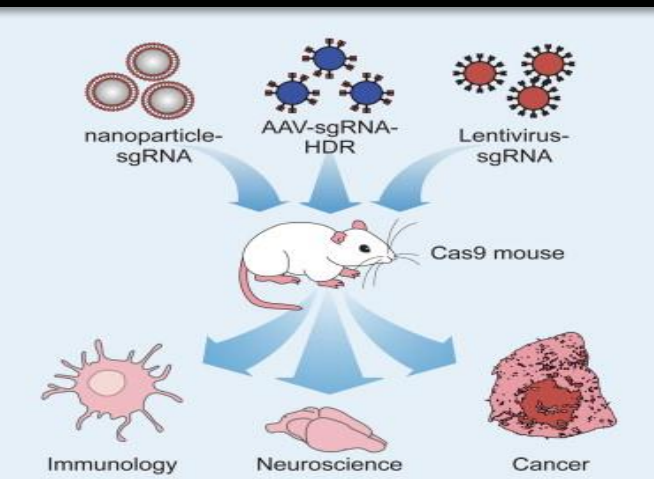
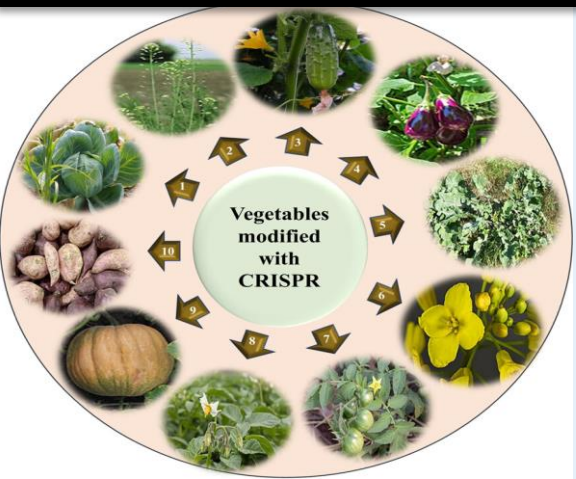
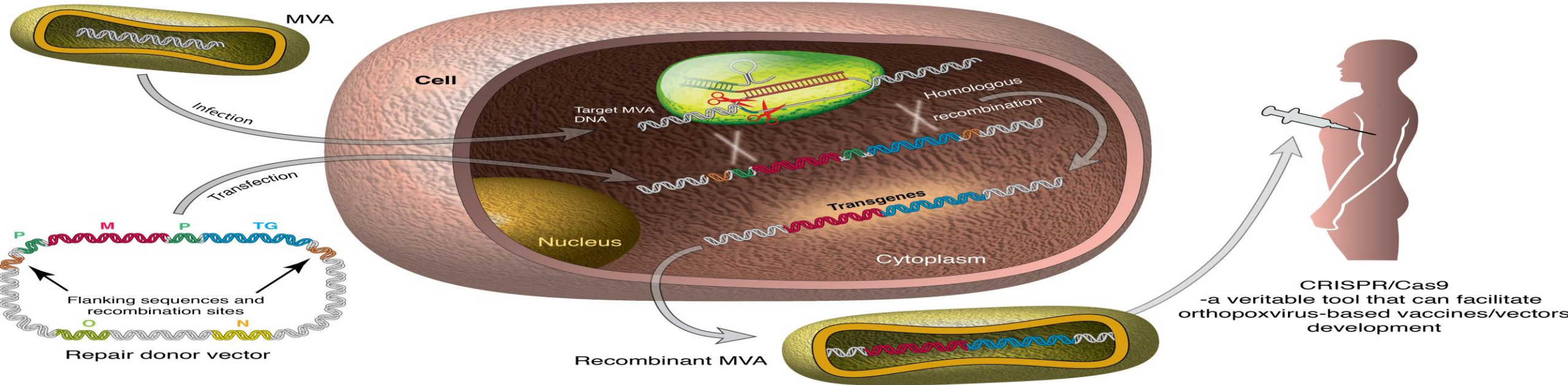


## CRISPR-Cas technology

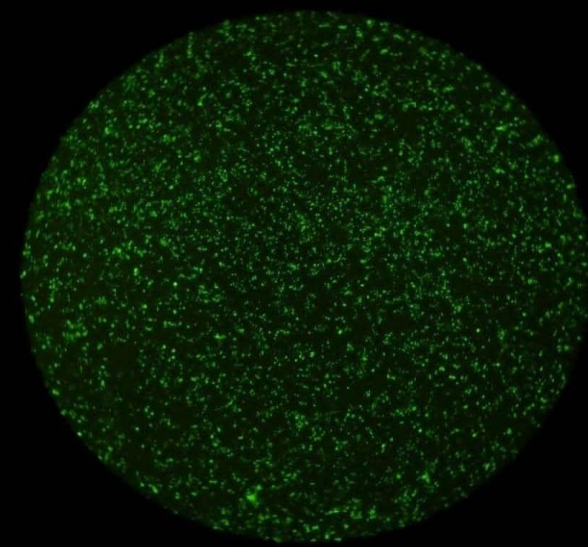
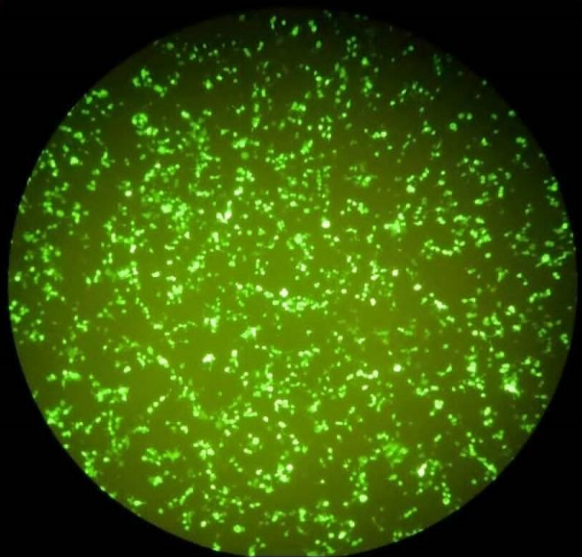
- CRISPR-Cas9 system bases on simple base pairing rules between a specific guide RNA (gRNA) and the target genome site, offers simple yet effective methods of genome editing.



# Applications of crispr-cas9 technology







# Using the CRISPR/Cas9 technology to attenuate virulence factors and pathogenicity of bacteria

## 1- CRISPR-Cas Bioinformatics

Databases hosted on this site



## 2- CRISPR-Cas Experiments



### Bacterial Virulence Factors



# CRISPR-Cas bioinformatics

## Step 1: Obtaining the required gene sequence

Log in

Nucleotide



**Using Nucleotide**  
[Quick Start Guide](#)  
[FAQ](#)

**Nucleotide**  
[Submit to](#)  
[LinkOut](#)  
[E-Utilities](#)

Search for a gene  
   
e.g. *ftsZ* or *uridine*\*

- [Search for a gene](#) - type the name of a gene or other
- [Find a genome](#) - click in the 'browse a genome' box and type a name to find matching genomes
- [View full list of all Ensembl Bacteria species](#)
- [Access Ensembl Bacteria programmatically](#)

**What's New in Release 57**  
In June 2023, Ensembl Bacteria released new gene predictions for almost all of the genomes it hosts. This was done in collaboration with the EMBL-EBI metagenomics team and utilised a pipeline combining [Prokka](#), [cmScan](#), [EggNOG](#) and [InterProScan](#). We have retained the community-submitted annotations for 115 species that are in our pan-taxonomic

## ClustalW2

[Input form](#) | [Web services](#) | [Help & Documentation](#) | [Bioinformatics Tools FAQ](#)

Tools > Multiple Sequence Alignment > ClustalW2

ClustalW2 is a general purpose DNA or protein multiple sequence alignment program for **three or more** sequences. For the alignment of two sequences please instead use our [pairwise sequence alignment tools](#).

### Please Note

The ClustalW2 services have been retired. To access similar services, please visit the [Multiple Sequence Alignment tools](#) page. For protein alignments we recommend [Clustal Omega](#). For DNA alignments we recommend trying [MUSCLE](#) or [MAFFT](#). If you have any questions/concerns please contact us via the feedback link above.



# CRISPR-Cas bioinformatics

## Step 2: gRNA Oligonucleotide Design

Home Instructions Scoring About Updates Submissions Contact FAQ

sg.idtdna.com/site/order/designtool/index/CRISPR\_SEQUENCE

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## CRISPR-Cas9 guide RNA design checker

Assess on- and off-targeting potential of protospacer designs of your own or from publications before ordering guide RNAs (gRNAs, such as crRNA and sgRNA) that are synthesized using our Alt-R gRNA modifications. For HDR experiment designs, please see the following HDR design tool.

Target   
RefSeq/ENSEMBL/gene ID or genomic coordinates.

In   
[Add new species.](#)

Using   
Change default PAM and g

Paste Target Options Reset Options

**Find Target Sites!**

Search for predesigned gRNA Design custom gRNA CRISPR-Cas9 gRNA checker

Species

Input format

Paste/Type input Upload file

Enter up to 99 FASTA Sequences.  
Please enter sequences in standard FASTA formatting.

```
>ExampleFASTA
ATGCGCTATGCGACTAGCTAGTGACTAGCTA
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This field is required.

CHECK

CLEAR AND RESET

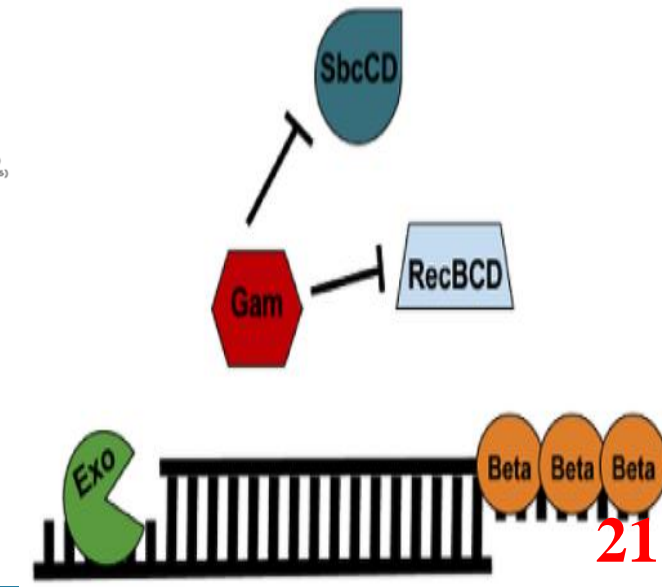
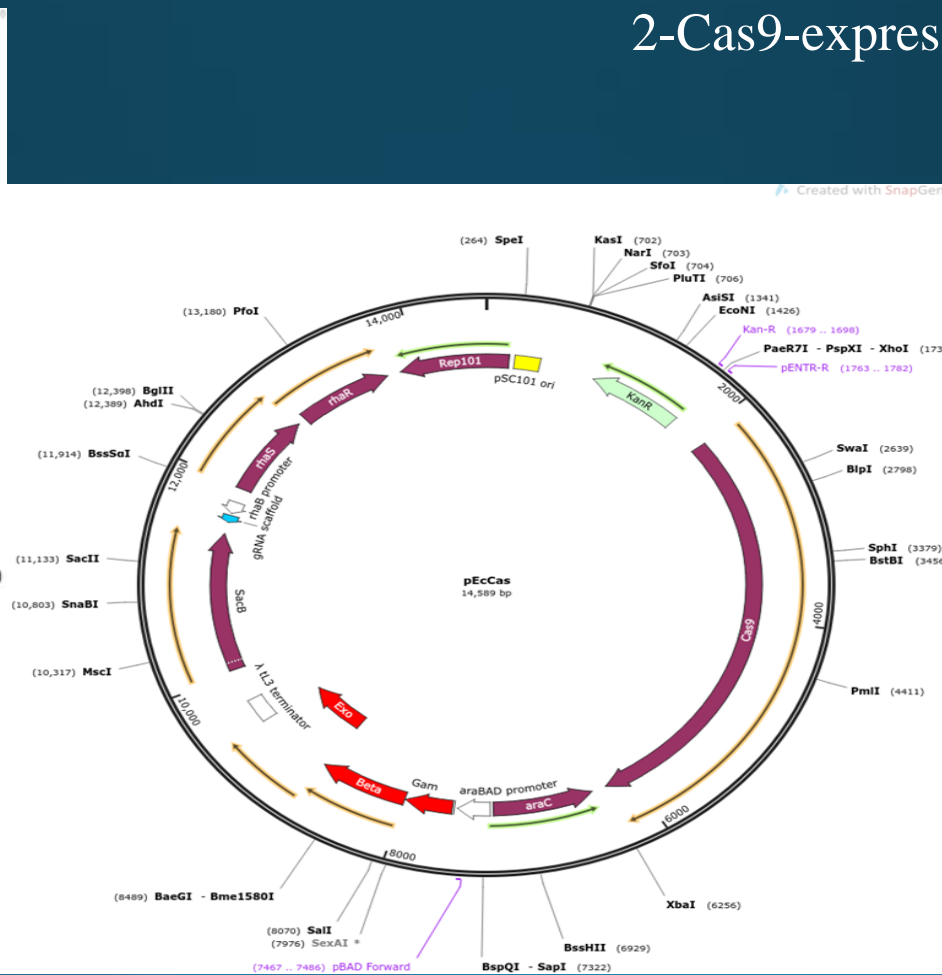
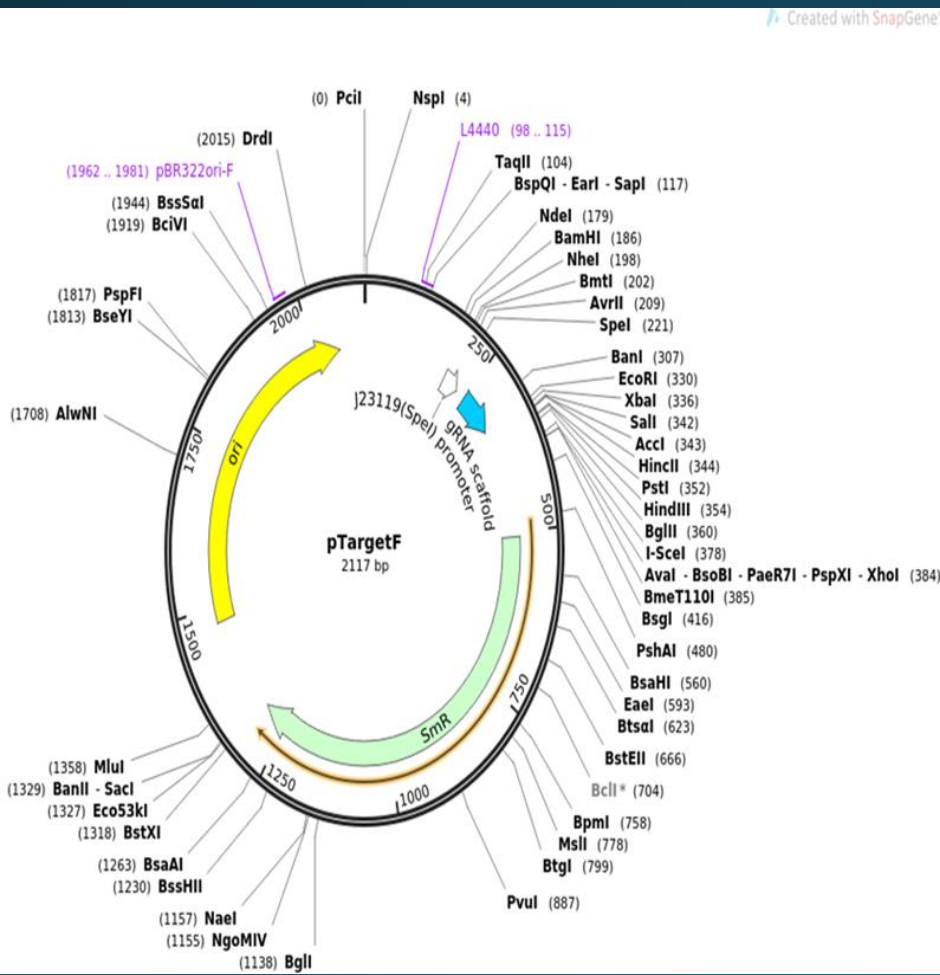
# CRISPR-Cas bioinformatics

## Step 3: Primer Design

1-gRNA expressing plasmid

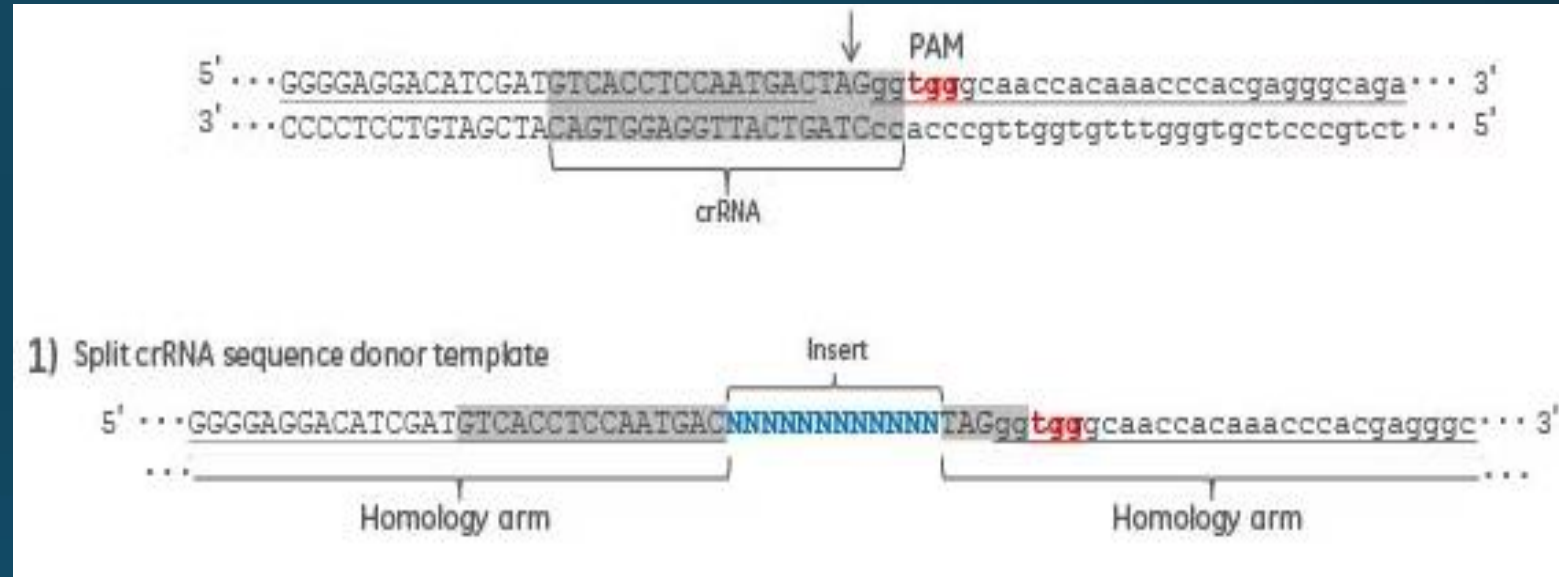
1- Confirmation of Positive Clones by colony PCR ( Plasmid backbone)

2-Cas9-expressing plasmid



## Step 3: Primer Design

2- Construction of HDR oligo fragment



3- Confirmation of the recombination

4- Confirmation of the Sequencing

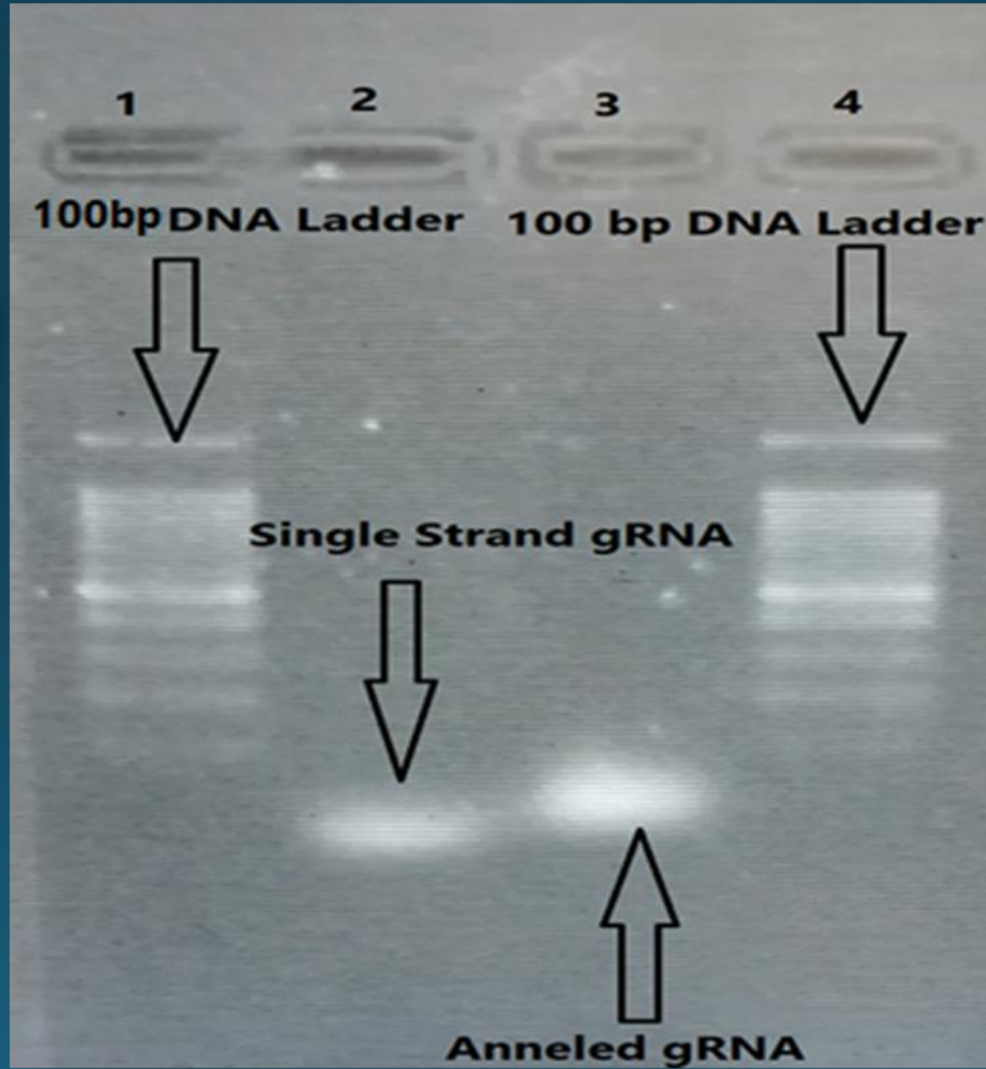
# *CRISPR-Cas Experiments*



# CRISPR-Cas Experiments

## Step 1: gRNA oligo annealing

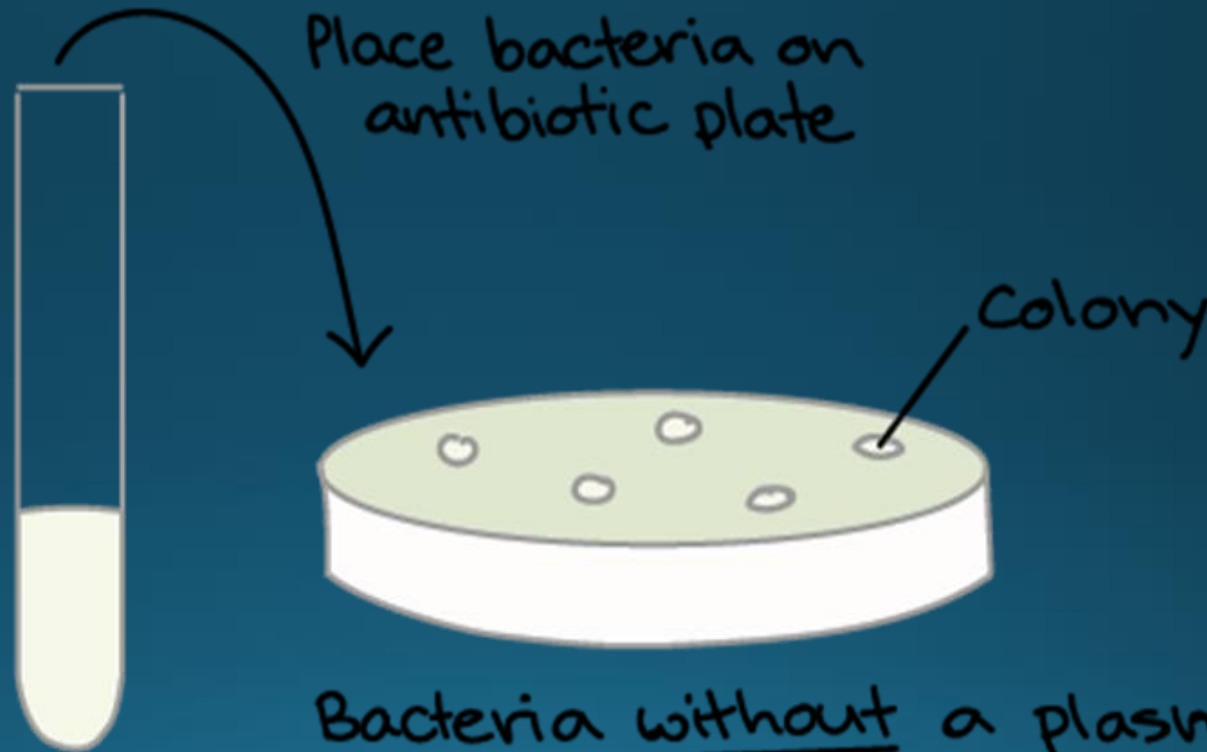
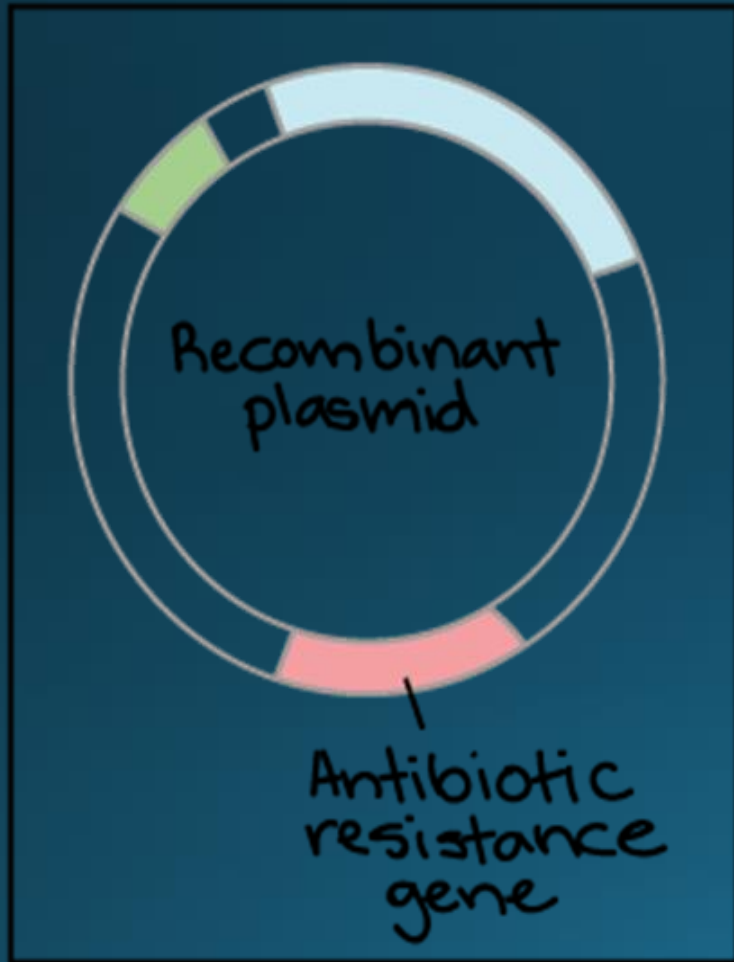
- Anneal two single-stranded DNA oligonucleotides to produce double-stranded DNA fragments.





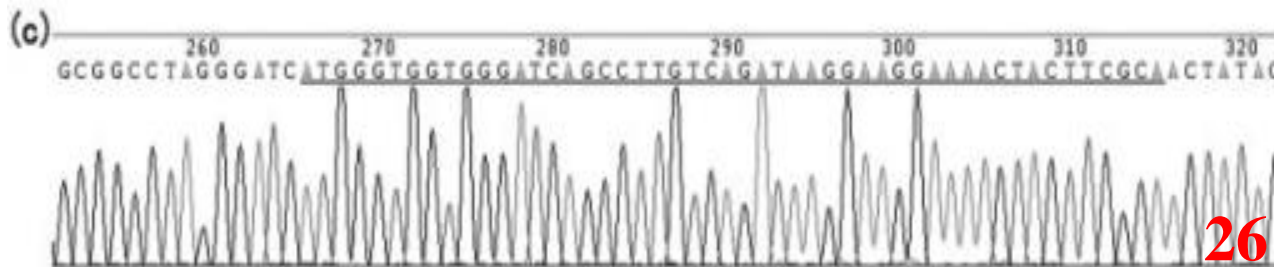
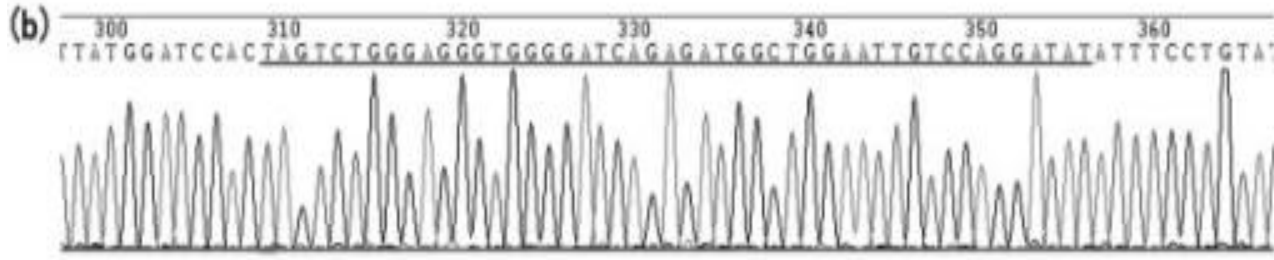
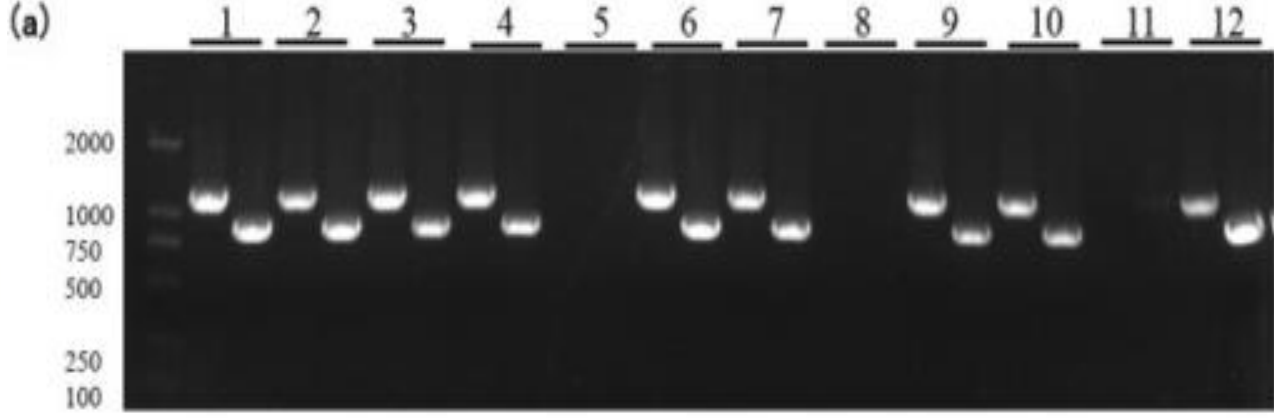
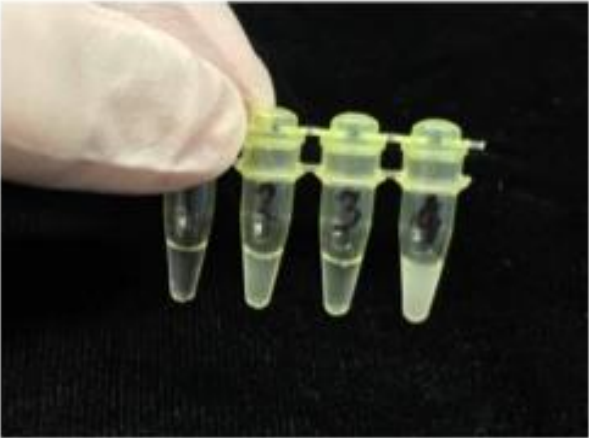
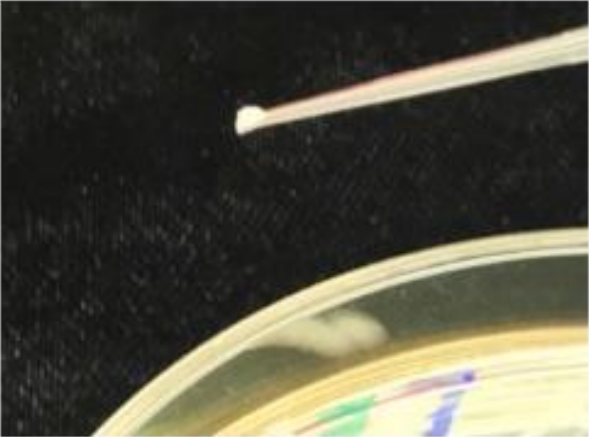
# CRISPR-Cas Experiments

## Step 2: Transformation



Bacteria without a plasmid die.  
Each bacterium with a plasmid makes a colony.

# Step 3: Confirmation of Positive Clones by colony PCR and Sequencing

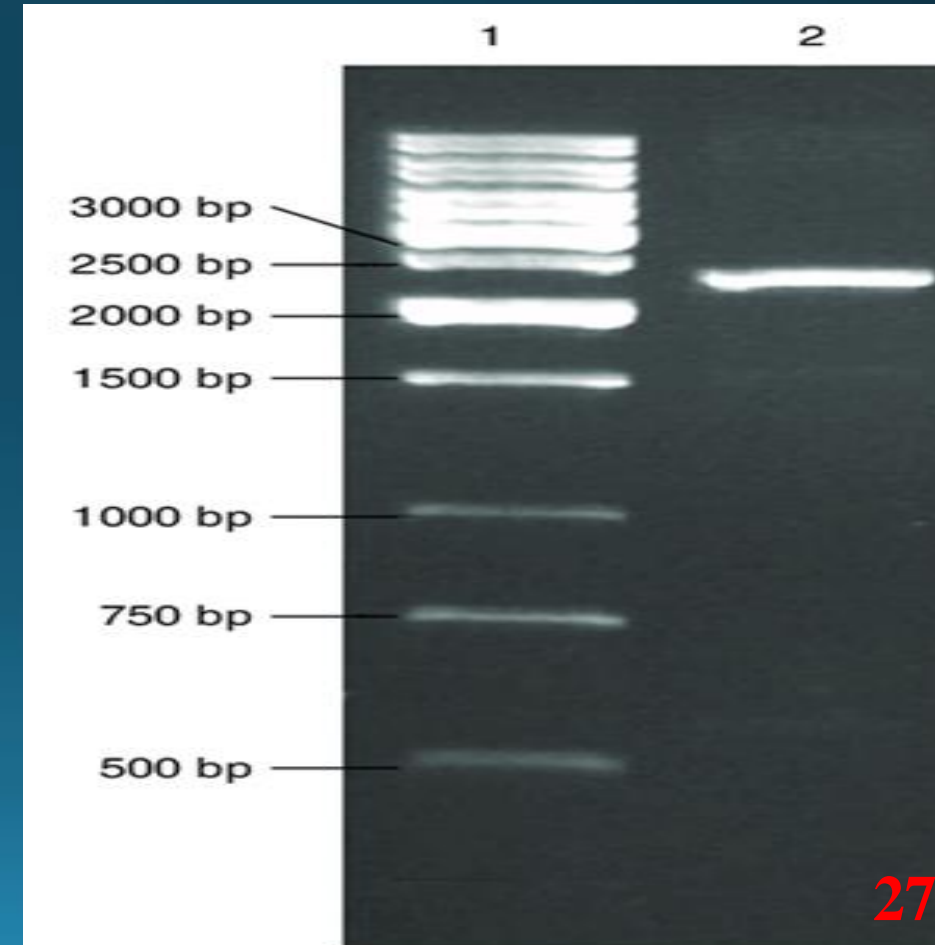
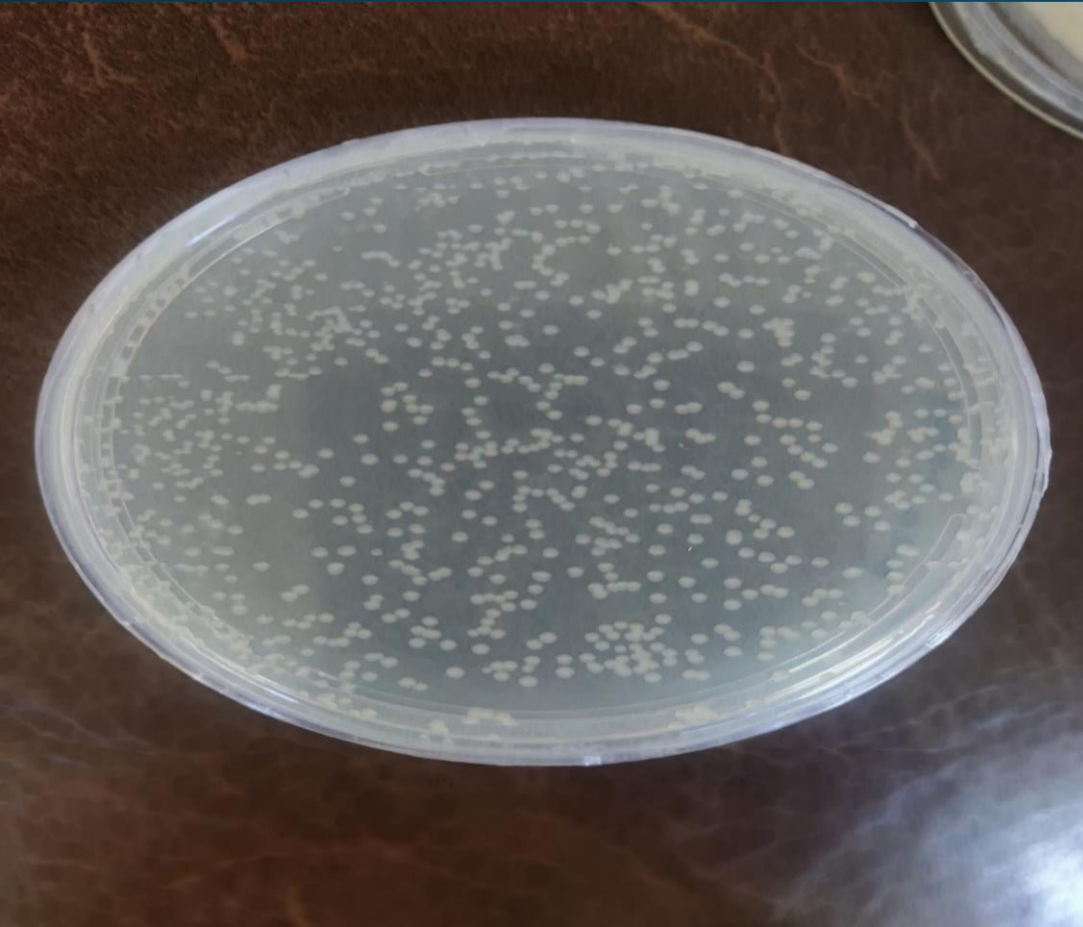


# CRISPR-Cas Experiments

**Step 4:** Introducing cas9 plasmid into bacterial cells through electroporation  
Confirmation of Positive Clones by colony PCR



Generation of bacteria carrying pCas9



# CRISPR-Cas Experiments

*Step 5: induction with arabinose and Preparation of competent cell*



# CRISPR-Cas Experiments

## Step 6: Construction of HDR oligo fragment

overlap extension pcr



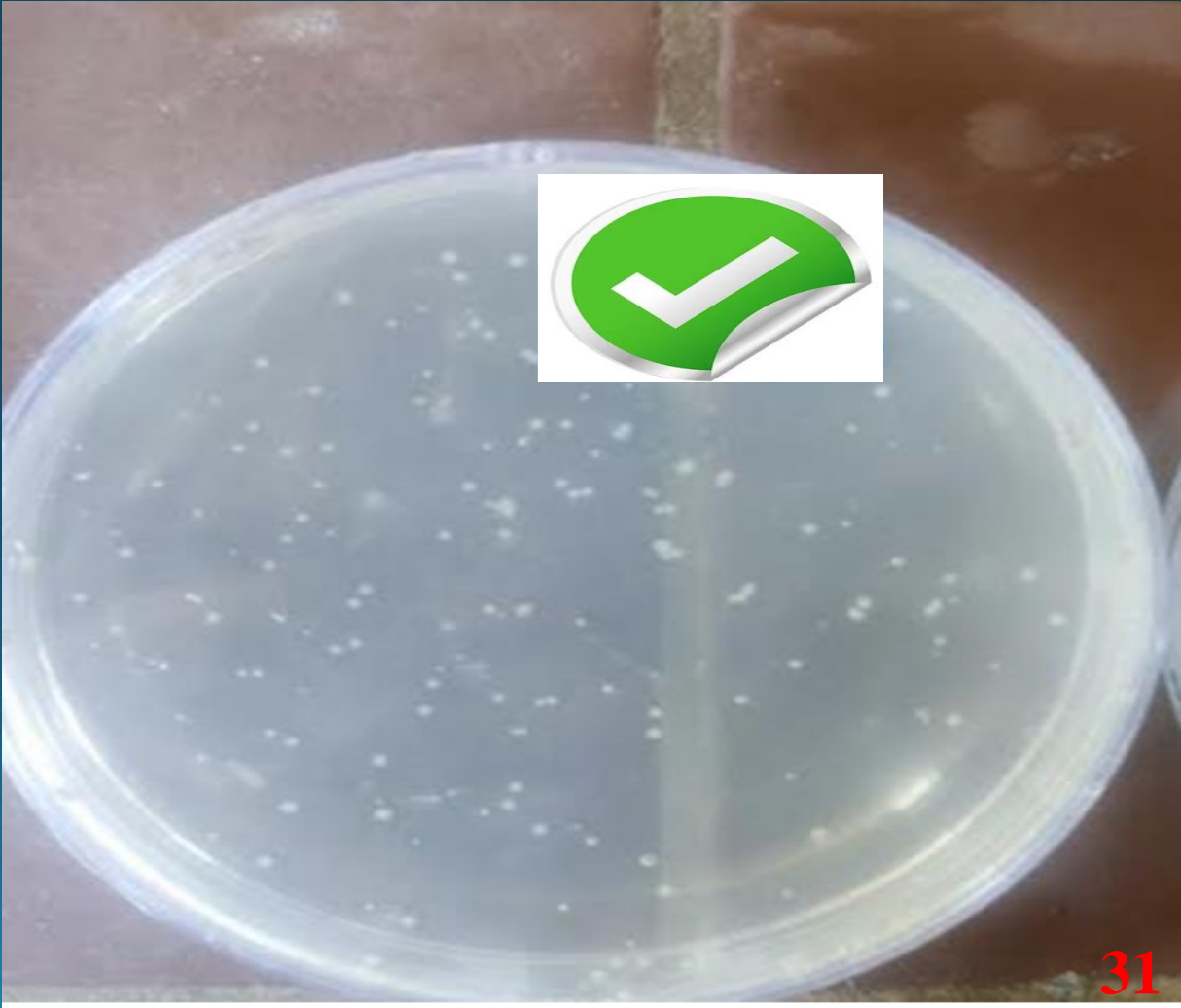
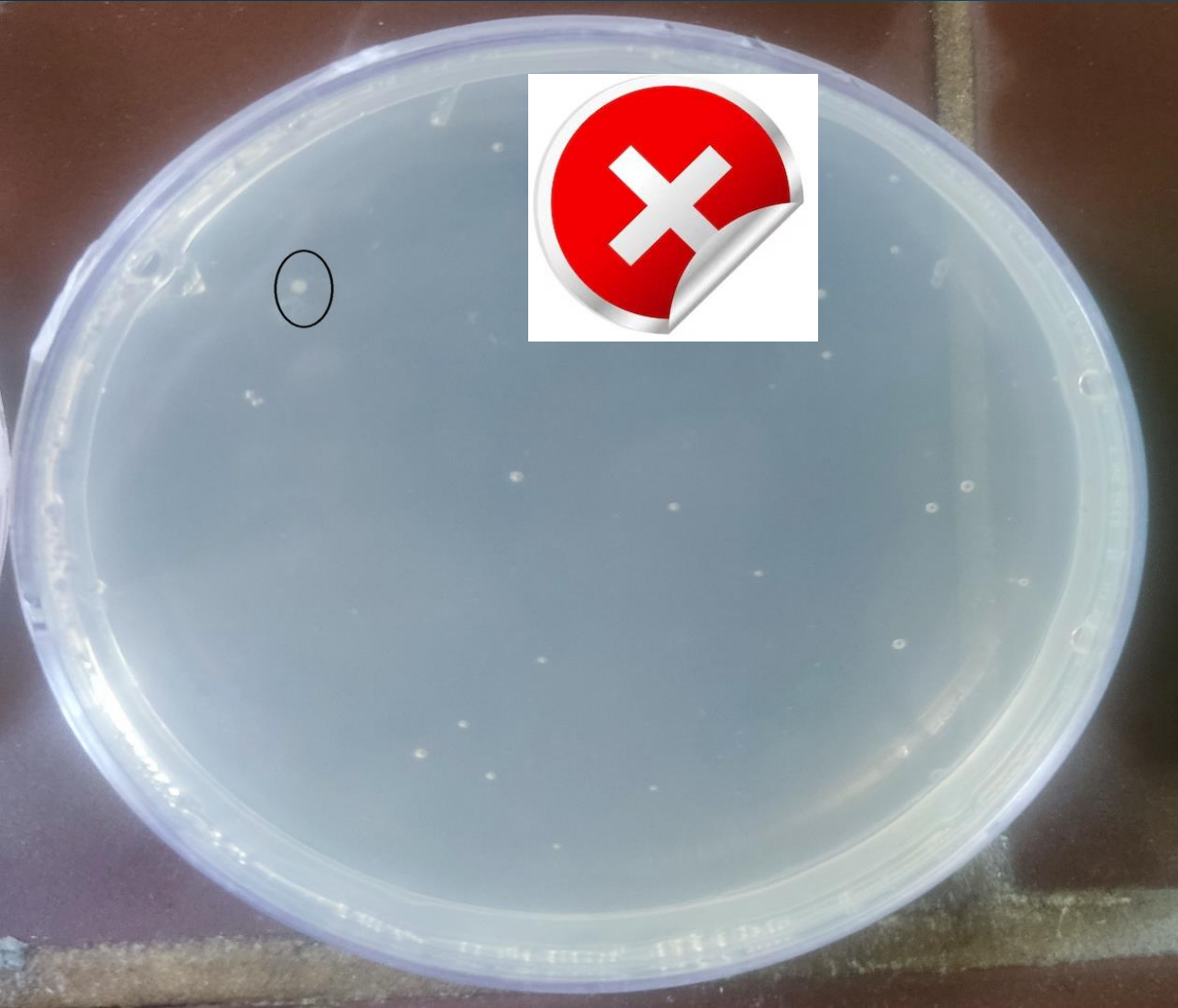
# CRISPR-Cas Experiments

## Step 7: Transfer of sgRNA plasmid and HDR fragment to Cas9 Competent Cells



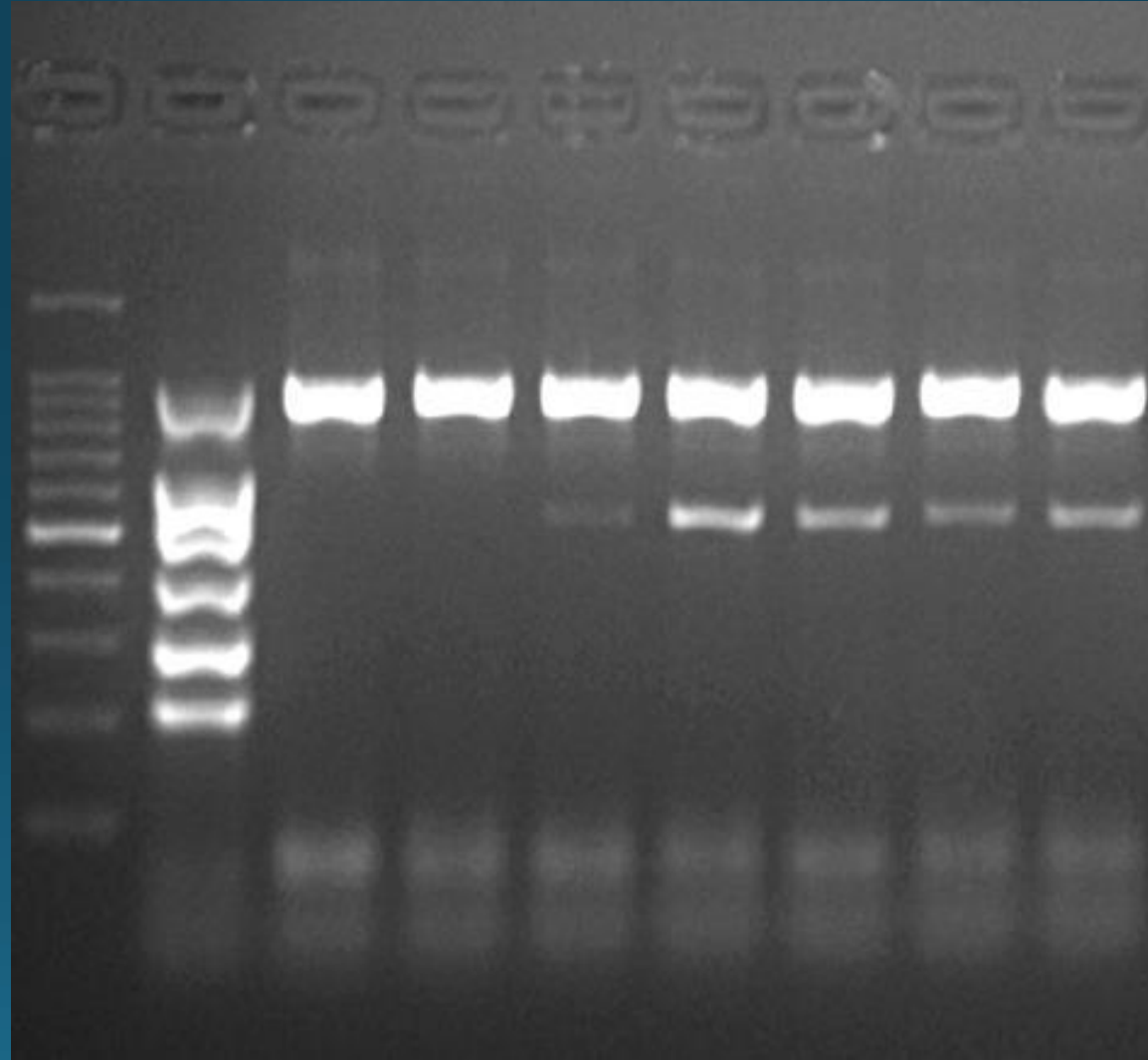
*CRISPR-Cas Experiments*

Con.



# *CRISPR-Cas Experiments*

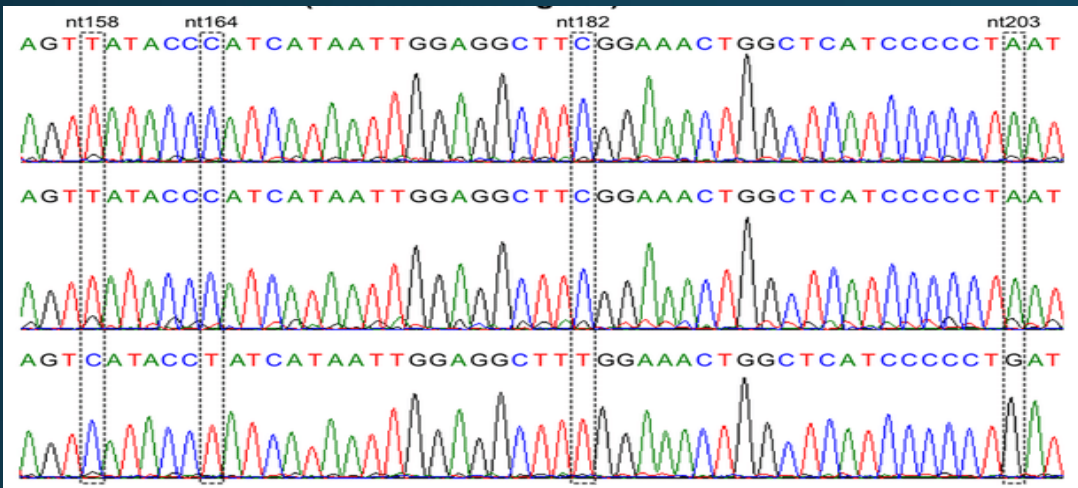
*Step 8: Screening of mutant strains by colony PCR*



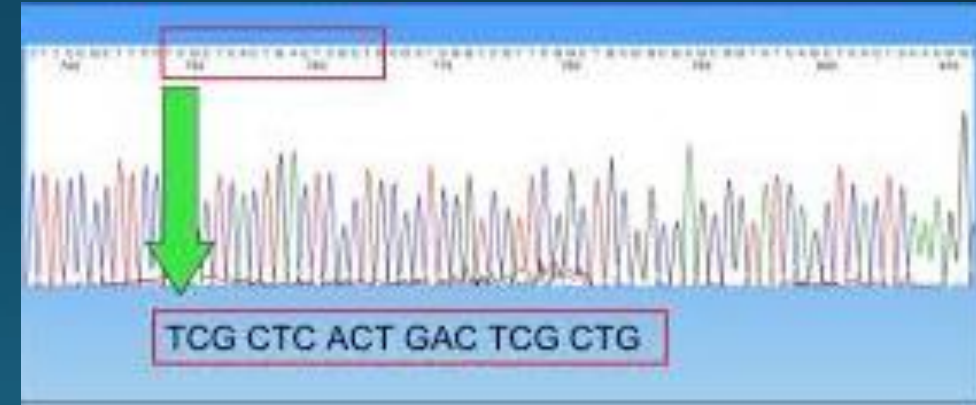
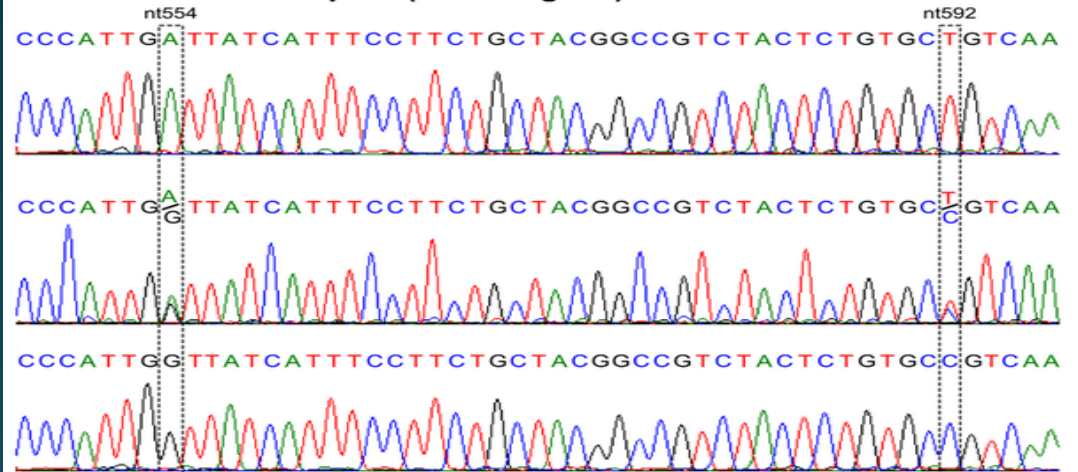


# CRISPR-Cas Experiments

## Step 9: Analysis of modified bacterial genetic structures



*rhodopsin* (nuclear gene)



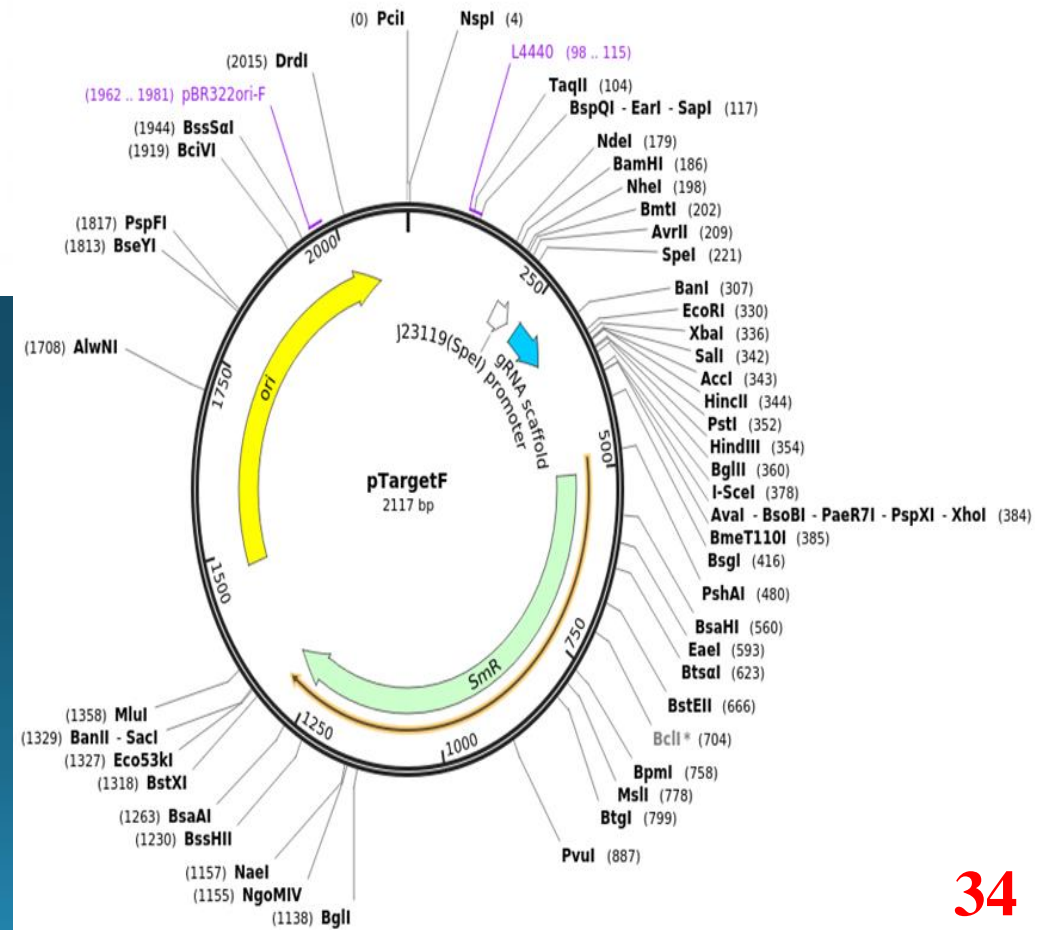
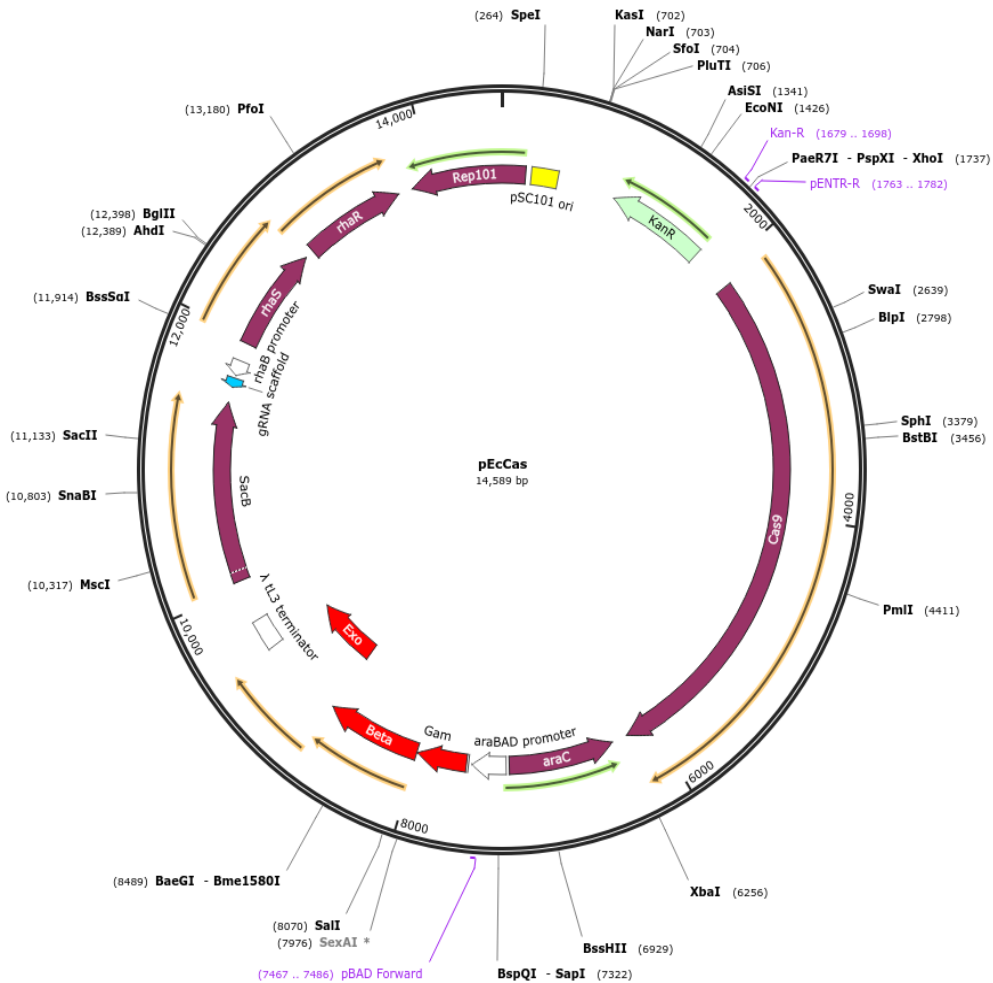
# CRISPR-Cas Experiments

## Step 10: Plasmid curing

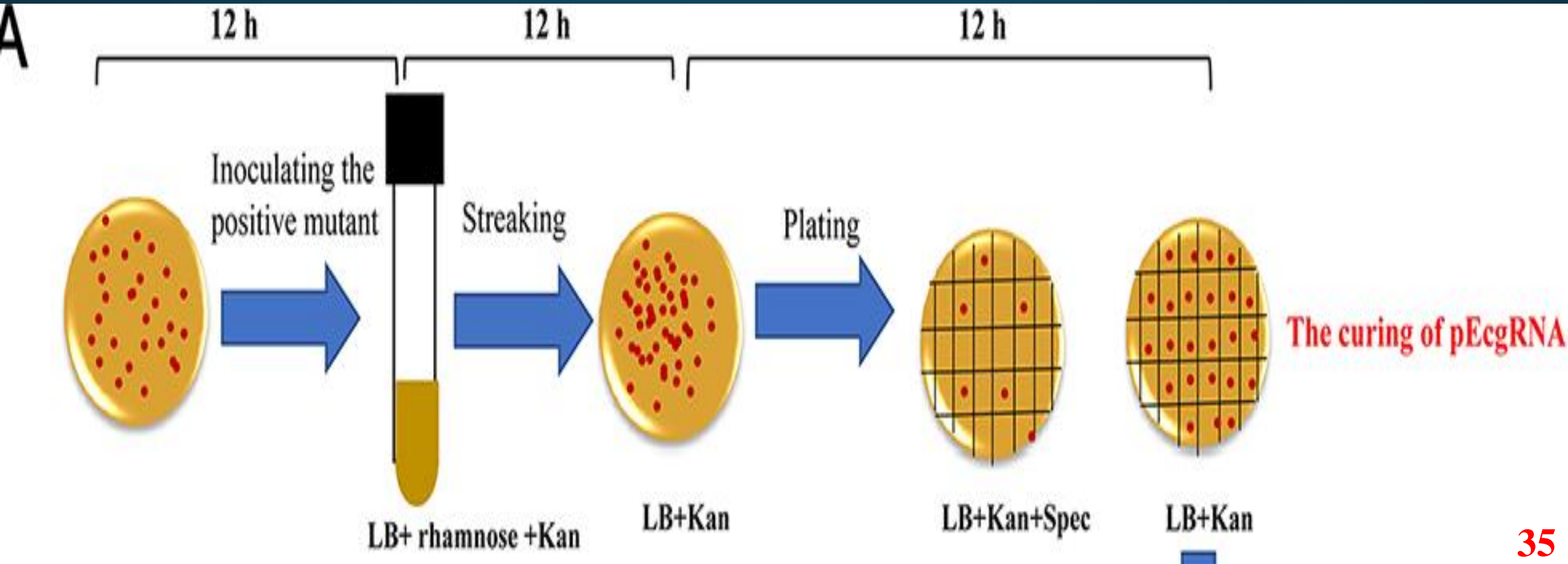
Created with SnapGene®



Created with SnapGene®



## Step 11: Replica plating



## *CRISPR-Cas Experiments*

*Step 12: induction with arabinose and Preparation of competent cell*

*Step 13: Screening of mutant strains by colony PCR*

*Step 14: Analysis of modified bacterial genetic structures*

# *Additional Tests*

*Western blot*



*Cellular assays*



*in vivo test*



## *Conclusion and future perspectives*

Through the implementation of the CRISPR Cas9 strategy, the modified bacteria can find applications in various research contexts. This includes potential use as a live-attenuated vaccine candidate, exploration of the impact of the target gene on bacterial pathogenicity, assessment of the gene's influence on bacterial morphology, and more. This strategy opens avenues for studying the functionality of the bacterium genes and even holds the potential for the creation of novel industrial strains.



*Thanks for your attention*

