Protocol: Moritz Sorg

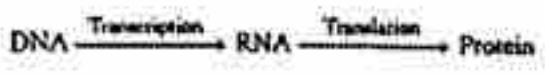
11.12.2018

Merry CRISPR II

Introduction:

Among the seminal discoveries in biology in the twentieth century were the chemical nature and the three-dimensional structure of the genetic material, **deoxyribonucleic acid, DNA**. The sequence of the monomeric subunits encodes the instructions for forming all other cellular components and provides a template for the production of identical DNA molecules to be distributed to progeny when a cell divide. The continued existence of a biological species requires its genetic information to be maintained in a stable form, expressed accurately and reproduced with a minimum of errors. A segment of a DNA molecule that contains the information required for the synthesis of a functional biological product, is referred to as a **gene**. A cell typically has many thousands of genes (altogether the genome), and DNA molecules, not surprisingly, tend to be very large.

A major role for many sequences of DNA is to encode the sequences of **proteins**, the workhorses within cells, participating in essentially all processes. Some proteins are key structural components, whereas others are specific catalysts (termed **enzymes**) that promote chemical reactions. To produce proteins, the information stored in the genome must first transcribed into another type of **ribonucleic acid**, called **RNA**. The RNA can be then subsequently translated into proteins by the cell.



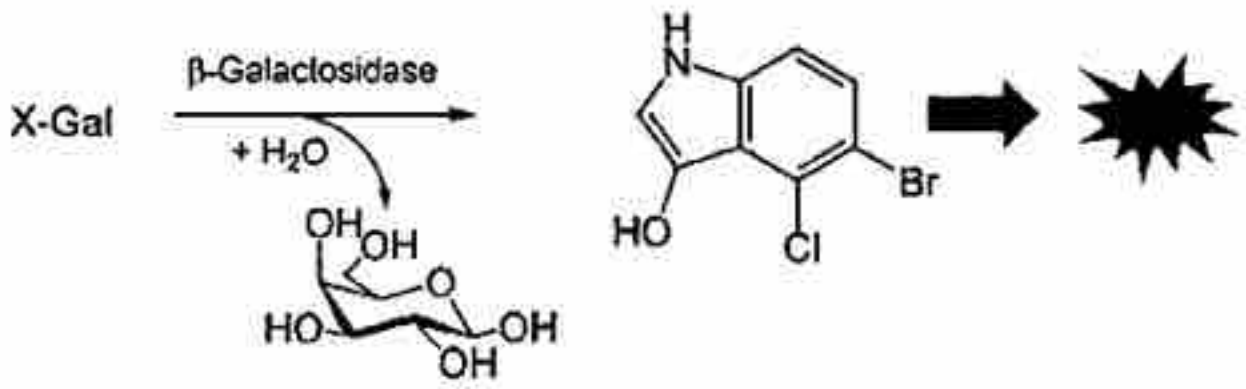
Both processes, transcription and translation are suitable targets to interfere with the protein production in cells. Many antibiotics for example are inhibiting the translation (or less frequent the transcription) machinery and prevent bacteria from producing **any** proteins leading to growth arrest and cell death. Although very successful, the most used antibiotics are not very specific but target very abundant features of bacteria. Only recently, new tools such as RNA interference or CRISPR-dCas9 were developed which allow to target and inhibit **very specific** genes without altering the whole cell.

CRISPR-Cas genes encode proteins that function originally as an adaptive immune system against infectious viruses and plasmids. Immunity is mediated by Cas nucleases and small RNA guides (gRNA), which specify a cleavage site within the genome of the invader. In type II CRISPR-Cas systems, the RNA-guided Cas9 nuclease cleaves the DNA but it can be

reprogrammed to not cut the DNA anymore (so called deactivated dCas9). These dCas9 is still able to bind to the targeted gene and blocks the transcription by sterically interfere with the transcription process.

The *E. coli* strain K12-MG1655 has a native copy of the *lacZ* gene in its genome. This gene encodes for the protein ß-Galactosidase (bacterial equivalent of the human Lactase), an enzyme which catalyzes the cleavage of β-galactosidases (e.g. disaccharides such as lactose or other, more complex structures) into monosaccharides through the breaking of a glycosidic bond.

The aim of this experiment is to inhibit the gene expression of this *lacZ* gene using CRISPR-dCas9 interference. First, we have to insert DNA coding for the gRNA (which target lacZ) as well as the gene coding for the dCas9. X-Gal is a specially designed molecule which turns into blue when cut by the ß-Galactosidase. In the absence of the enzyme, there is no color reaction.



Chemically competent cells

In molecular biology, **transformation** is the genetic alteration of a cell resulting from the uptake and expression of exogenous (foreign) DNA. Transformation can occur naturally in some bacteria e.g. *Bacillus*, *Haemophilus*, *Neisseria* and *Streptococcus* spp (naturally competent). In most bacteria transformation can only occur in cells 'permeabilized' to DNA by artificial methods. The ability of a cell to take up DNA is dependent on the cell being in a particular state referred to as competence. Competence is related to the presence of receptors for DNA on the surface of the cell and the presence of transformation-specific proteins. Treatment of cells with high concentrations of ice-cold $CaCl_2$ in conjunction with heat shock heat induces artificial competence.

Although this transformation process has been well established for years, there is still a lack of scientific explanation of the fundamental mechanisms responsible.

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Day1: **Transformation of CRISPR-dCas9 plasmids into MG1655**

Equipment:	box of ice
	Water bath at 42°C
	Shaker at 37°C
	Incubator at 37°C
	Sterile work place

Strains &:	competent MG1655
chemicals	Sterile Lysogeny Broth (LB)
	Lysogeny Broth Agar (LA)
	Ampicillin (Amp) stock (100mg/ml)
	Chloramphenicol (Cm) stock (25mg/ml)
	DNA plasmids pdCas9
	DNA plasmid pgRNA_crlacZ1 or _crlacZ2
	Sterile cell culture plates (Petri dishes)
	Sterile glass beads or loops for plating
	Sterile tips for the pipette

Work always sterile! (near a flame or in a sterile bench)

Preparation of LA plates:

Melt the LA in the microwave (with loose lid!!) completely and let it cool down to ~60-70 °C (rule of thumb: if you can hold the bottle with gloves on, it is cold enough). **You need 20 ml LA per plate.** When cooled down, add the appropriate amount of antibiotics/chemicals and poor the plates:

2x LA plates with **NO** Antibiotics

2x LA plates with **Amp100** (=final conc: 100µg/ml → 1 µl stock solution per 1ml LA)

2x LA plates with **Cm25** (=final conc: 25 µg/ml → 1µl stock solution per 1ml LA)

2x LA plates with **Amp100** and **Cm25**

Let the plates solidify before using them (minimum 30 min). Also, **label the plates before using them** for the transformation. [strain; plasmid(s); medium; antibiotic(s) →e.g.: MG1655; wt; LA; (noAB)] Label the bottom of the plates, not the lid.

1. Thaw four aliquots of competent MG1655 cells on ice.
2. Incubate the aliquots with plasmid DNA on ice for 15 min.
   I. No plasmid → negative control
   II. 1 µl of pgRNA plasmid → provides Amp resistance
   III. 1 µl of pdCas9 plasmid → provides Cm resistance
   IV. 1 µl of pdCas9 **and** 1 µl pgRNA → provides Amp and Cm resistance
3. Heat shock at 42°C for 1 min.
4. Incubate on ice for 5 min.
5. Add 1 ml LB to each sample and incubate at 37°C for 1 h, shaking at 200rpm.
6. Plate out 100 µl on LA with the respective antibiotics (using sterile glass beads or loops)
   I. No plasmid → LA plate with **NO** antibiotic; **negative control**
   II. pgRNA plasmid → LA plate with **Amp100**
   III. pdCas9 plasmid → LA plate with **Cm25**

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IV. pdCas9 and pgRNA → LA plate with **Amp100** and **Cm25**

7. Centrifuge the remaining cells at 8000 rpm for 5 min.
8. Discard the supernatant and resuspend the pellet in 100 µl LB.
9. Plate out 100 µl on LA with the respective antibiotics
   I. No plasmid → LA plate with **NO** antibiotic; negative control
   II. pgRNA plasmid → LA plate with **Amp100**
   III. pdCas9 plasmid → LA plate with **Cm25**
   IV. pdCas9 and pgRNA → LA plate with **Amp100** and **Cm25**
10. Incubate at 37°C O/N. IMPOTANT: The plates must be placed upside down in the incubator.

Day2: Activation of the CRISPR-dCas9 system and Inhibition of lacZ expression

Strains &: chemicals	- Lysogeny Broth Agar (LA) - Ampicillin (Amp) stock (100mg/ml) - Anhydrotetracycline (aTc) stock (1mM) - Chloramphenicol (Cm) stock (25mg/ml) - X-Gal stock (20mg/ml) - transformed strains Sterile tooth picks or loops to restreak colonies

Work always sterile! (near a flame or in a sterile bench)

Preparation of LA plates:

Melt the LA in the microwave (with loose lid!!) completely and let it cool down to ~60-70 °C. **You need 20 ml LA per plate.** When cooled down, add the appropriate amount of antibiotics/chemicals and poor the plates:

1x LA with **aTc1** (=final conc: 1µM → 1µl stock solution per 1ml LA) and **X-Gal** (=final conc: 0.1mg/ml → 5µl stock solution per 1ml LA)

2xLA with **aTc1**, **X-Gal** and **Amp100**

2xLA with **aTc1**, **X-Gal** and **Cm25**

3xLA with **aTc1**, **X-Gal**, **Amp100** and **Cm25**

Let the plates solidify before using them (minimum 30 min). Also, **label the plates** before using them. You can restreak (=plate) more than one colony on one plate. Divide the plates in quarters (you can draw lines on the bottom) →each quarter one colony.

11. Take out the plates from the incubator. Choose the plates where you can identify single colonies, pick them with a sterile tooth pick (or loop) and restreak them on LA plates containing X-Gal, aTc and the respective antibiotics.
    I. MG1655 control → LA with **aTc1** and **X-Gal**

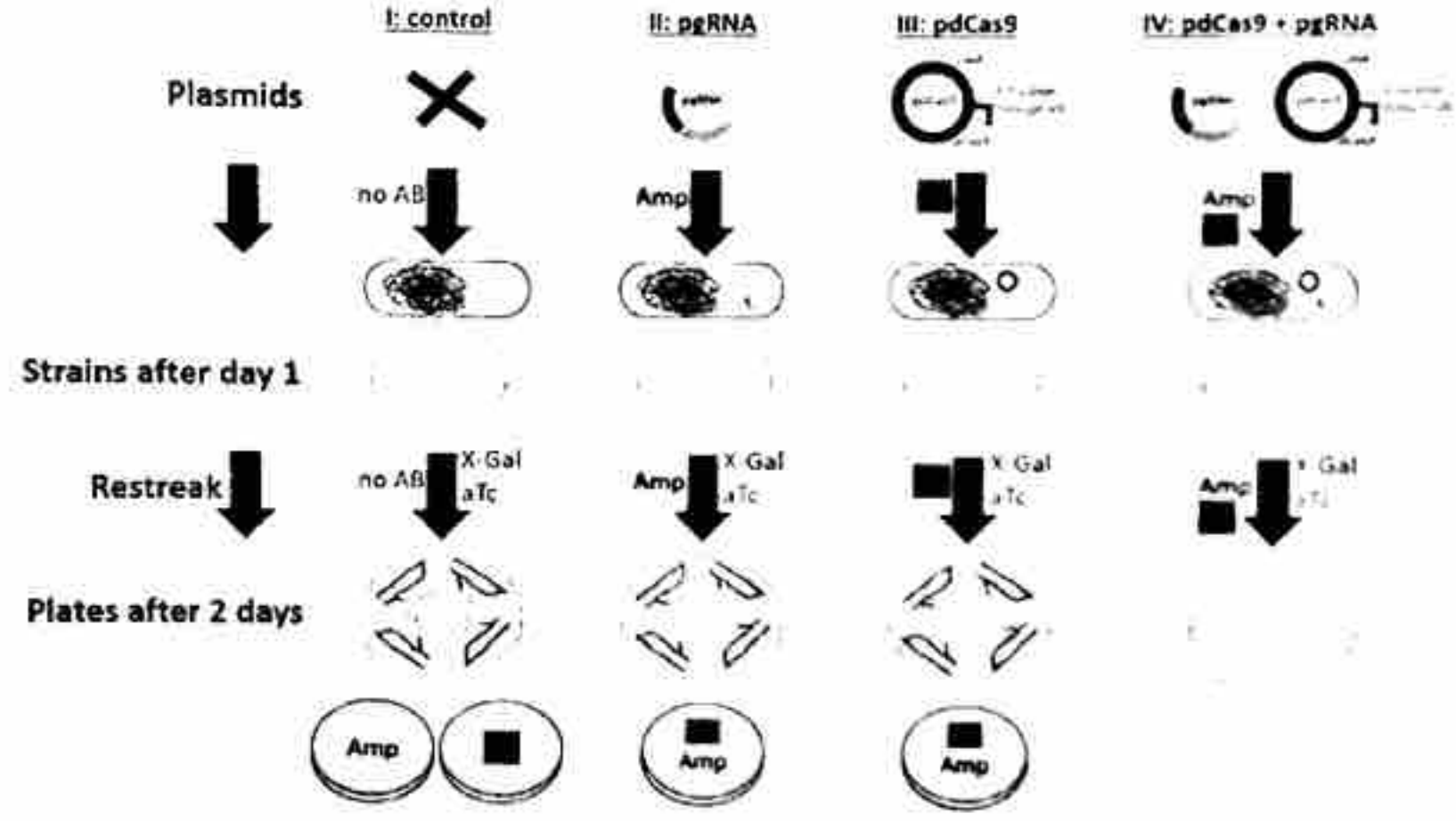
→ LA with **aTc1**, **X-Gal** and **Cm25**

II. MG1655_pgRNA
→ LA with **aTc1**, **X-Gal** and **Amp100**
→ LA with **aTc1**, **X-Gal**, **Amp100** and **Cm25**

III. MG1655_pdCas9
→ LA with **aTc1**, **X-Gal** and **Cm25**
→ LA with **aTc1**, **X-Gal**, **Amp100** and **Cm25**

IV. MG1655_pdCas9 + _pgRNA
→ LA with **aTc1**, **X-Gal**, **Amp100** and **Cm25**

12. Incubate at 37°C O/N. IMPOTANT: The plates must be placed upside down in the incubator.



Expected results:

MG1655 control

The control cells should be susceptible to Ampicillin as well as to Chloramphenicol and should not grow in media containing these antibiotics. aTc has no impact on the cells. The cells produce ß-Galactosidase and turn blue when plated on LA with X-Gal.

MG1655_pgRNA

The uptake of pgRNA plasmid results in an Ampicillin resistance (but no resistance to Chloramphenicol). The cells producing continuously gRNA's, but this has no impact on the cells in absence of dCas9. aTc has no impact on the cells. The cells produce ß-Galactosidase and turn blue when plated on LA with X-Gal.

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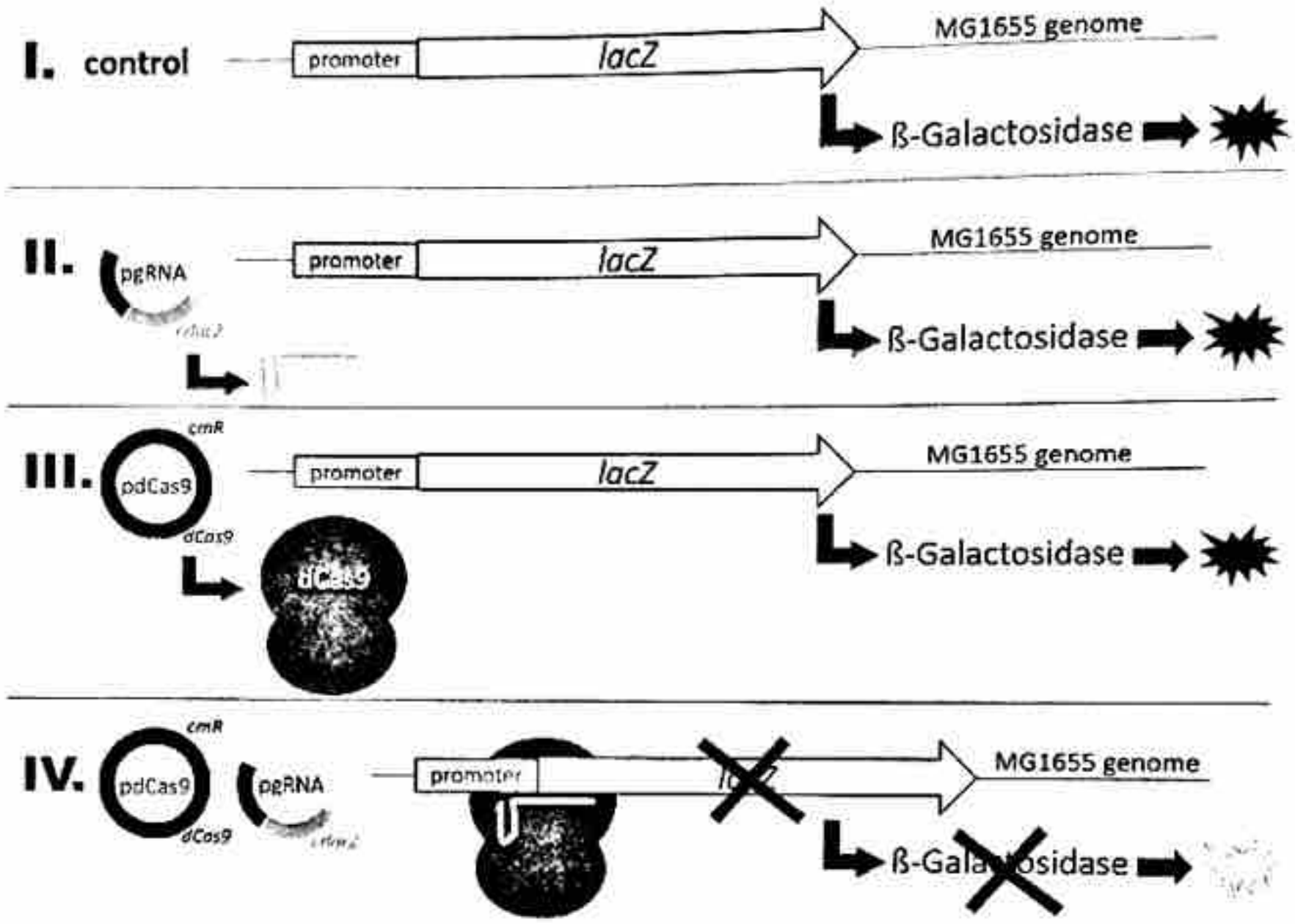

MG1655_pdCas9

Cells who received the pdCas9 plasmid, are resistant to Chloramphenicol but still susceptible to Ampicillin. aTc triggers the expression of dCas9 but without the expression of a guiding RNA, the system is incomplete and not affecting the lacZ expression. The cells produce ß-Galactosidase and turn blue when plated on LA with X-Gal.

MG1655_pdCas9; _pgRNA

The uptake of both plasmids results in a resistance to Ampicillin as well as to Chloramphenicol. The cells producing continuously gRNA's and upon the addition of aTc, dCas9 is produced. The gRNA forms a complex with the dCas9 and directs it to the *lacZ* gene in the genome where it binds and inhibits the expression of *lacZ*. Inhibition of *lacZ* leads to the absence of ß-Galactosidase in the cells. Without the ß-Galactosidase, the cells cannot catalyze the blue-staining reaction of X-Gal and subsequent, are white when plaiting on LA with X-Gal.