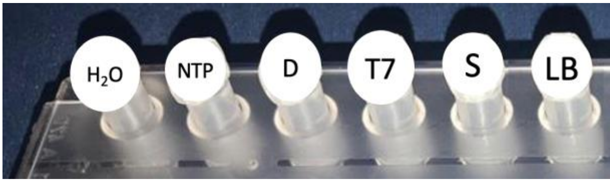
Erasmus+

Student Protocol CRISPR

modern biotechnology at school

1. gRNA-Synthesis



1.1 Pipet following reagents in your empty tube S for gRNA-synthesis:

Tube	Content	Vol.
H_2O	Nuclease free H_2O	16 µL
NTP	NTP Buffer mix	10 µL
D	DNA Duplex	2 µL
T7	T7 RNA Polymerase	2 µL
Total Volume (S)		***30 µL***

1.2 As the blank control for gRNA-synthesis, pipet 5 µL out of your tube S in a new tube marked with S_{t0} and put it on ice.

1.3 Incubate your tube S at 37 °C for 1 h.

2. Gel Electrophoresis Check of the gRNA Produced

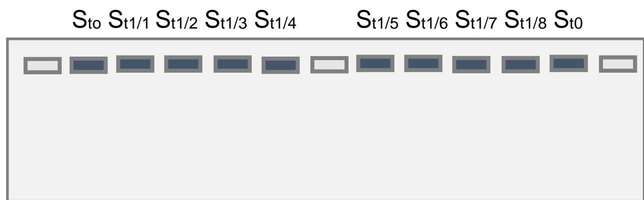
2.1 Open gel cassette and moisten the wells with H_2O_{dist}.

2.2 Aspirate excess water using the kitchen roll.

2.3 Pipet 5 µL out of your gRNA synthesis tube (S) in a new tube marked with S_{t1} and your group number. Keep tube (S) on ice.

2.4 Add into both tubes S_{t0} and S_{t1} 1 µL of loading buffer (LB).

2.5 Pipet according to the following scheme 6 µL of gRNA synthesis tubes $S_{t1/1}$ – $S_{t1/8}$ and two groups S_{t0} in the wells of the gel:

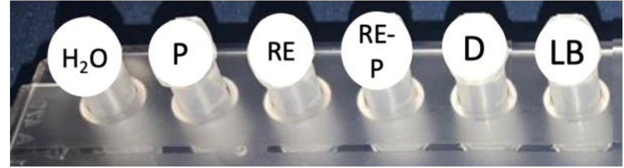


2.6 Connect FlashGel dock to the power supply and start gel electrophoresis at 180 V. Watch progress of electrophoresis by switching on the UV lamp on the dock.

Task: Evaluate your gel regarding gRNA synthesis.

3. Restriction Digestion to linearize Plasmid pBR322

3.1 Pipet restriction digestion ingredients according to the following table into empty tube D:



Tube	Content	Vol.
H_2O	Nuclease free H_2O	15 µL
P	pBR322 (2,5 µg)	5 µL
RE	*PstI* (2,5 units)	2.5 µL
RE-P	10x Restriction Buffer	2.5 µL
Total Volume (D)		***25 µL***

3.2 Incubate tube D at 37 °C for 15 minutes.

4. Proof of Restriction Digestion by Gel Electrophoresis

4.1 Open gel cassette and moisten the wells with H_2O_{dest}.

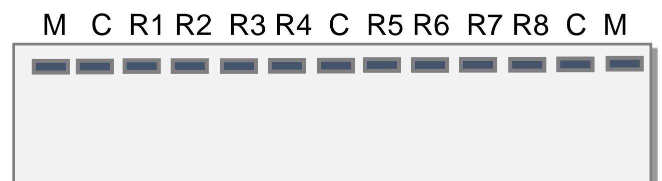
4.2 Aspirate excess water using the kitchen roll.

4.3 Pipet for blank control 1 µL loading buffer (LB), 1 µL of undigested plasmid (P) and 4 µL H_2O in a new tube (C).

4.4 Pipet for proving successful digestion 1 µL loading buffer (LB), 1 µL H_2O and 4 µL restriction digestion from tube D in a new tube (R). Keep tube (D) on ice.

4.5 Pipet according to the following pipetting scheme in each well
- 4 µL marker (M),
- 6 µL undigested plasmid (C)
- 6 µL of restricted samples (R)

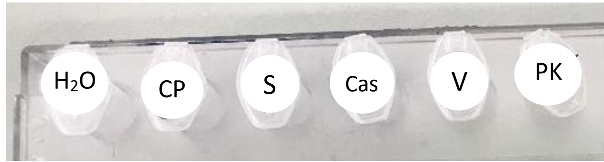
of each 8 work groups **R1 – R8** into the wells of the gel:



4.6 Connect FlashGel dock to the power supply and start gel electrophoresis at 180V. Watch progress of electrophoresis by switching on the UV lamp on the dock.

Task: Evaluate your gel regarding restriction digestion of plasmid pBR322 by *PstI*.

5. Cleavage of linearized Plasmid pBR322 by Nuclease Cas9



5.1 **Pipet samples 1 – 3 according to following table:**

Sample	**1**	**2**	**3**
	$pBR322_{lin}$	$pBR322_{lin}$ + Cas9 (- gRNA)	$pBR322_{lin}$ + Cas9 + gRNA
H_2O (Nucle-ase free)	24 µL	23 µL	22 µL
CP (Buffer)	3 µL	3 µL	3 µL
S (synthe-sized gRNA)	0 µL	0 µL	1 µL
Cas9 Nuclease	0 µL	1 µL	1 µL
Mix by tapping the tubes and incubate for 10 minutes at room temperature			
D (Digest-ed plasmid)	1 µL	1 µL	1 µL
H_2O (Nucle-ase free)	2 µL	2 µL	2 µL
Mix by tapping the tubes and incubate for 10 minutes at 37 °C			
PK (Protein-ase K)	1 µL	1 µL	1 µL
Sample Vol.	**31 µL**	**31 µL**	**31 µL**
Mix by tapping the tubes and incubate for 10 minutes at room temperature			

6. Proof of Cas9 Cleavage in linearized Plasmid ($pBR322_{lin}$) by Gel Electrophoresis

6.1 Pipet 8.5 µL out of your cleavage samples 1 – 3 in 3 new tubes and mark the tubes again with 1, 2 and 3.

6.2 Add to each of the 3 new tubes 1.5 µL of loading buffer (LB) and mix by snipping.

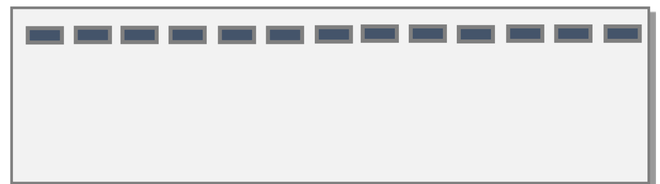
6.3 Open your gel cassette and moisten the wells with H_2O_{dist}.

6.4 Aspirate excess water using the kitchen roll.

6.5 Pipet according to the following pipetting scheme in each well of your gel cassette
- 4 µL marker (M),
- 10 µL of your samples 1 - 3

M 1/1 1/2 1/3 2/1 2/2 2/3 3/1 3/2 3/3 4/1 4/2 4/3

M 5/1 5/2 5/3 6/1 6/2 6/3 7/1 7/2 7/3 8/1 8/2 8/3



6.6 Connect FlashGel dock to the power supply and start gel electrophoresis at 180 V. Watch progress of electrophoresis by switching on the UV lamp on the dock.

Task: Evaluate your gel regarding nuclease Cas9 cleavage.