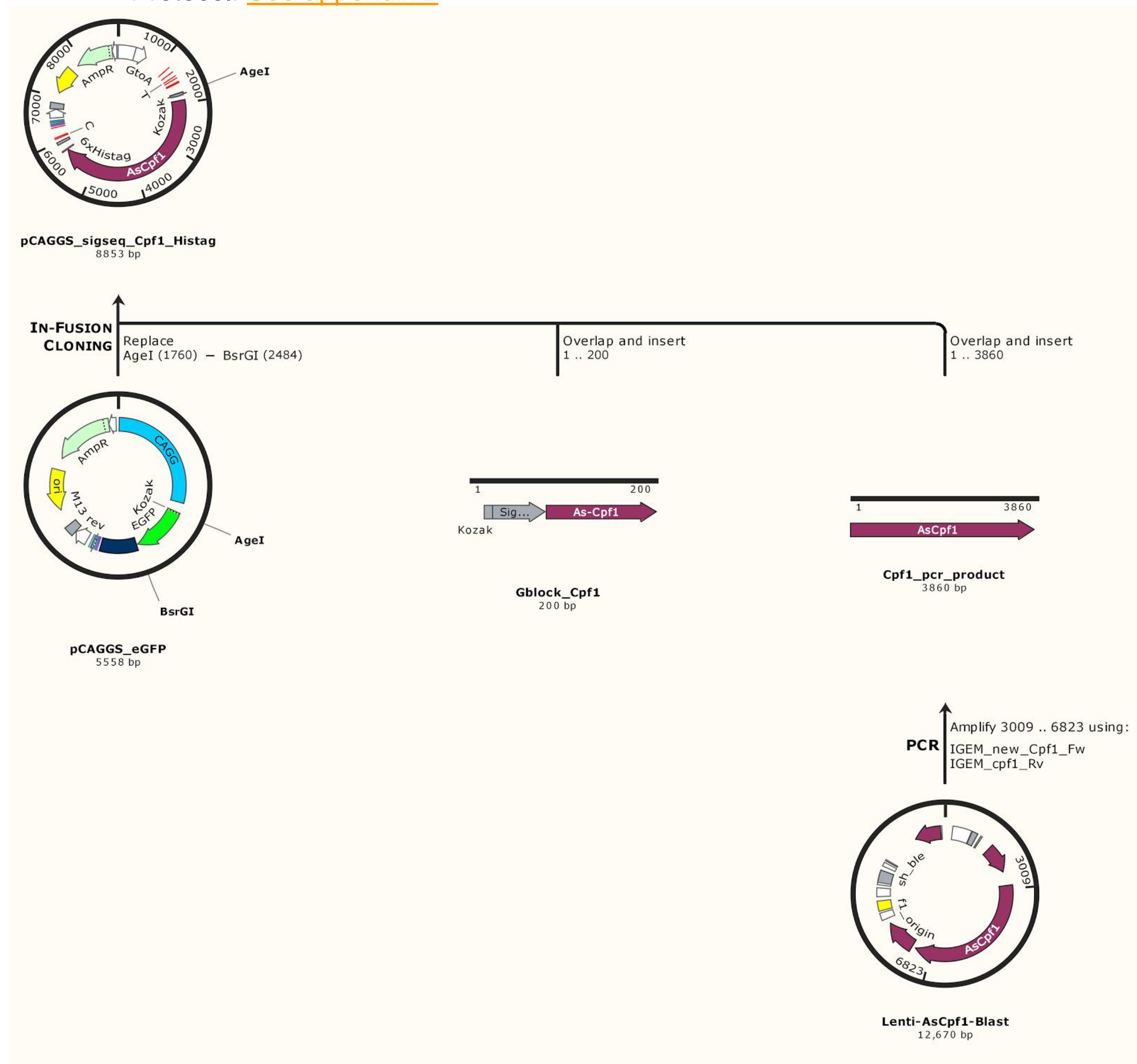
Secreting functional Cas9 and Cpf1

Supplementary info // iGEM Utrecht 2017

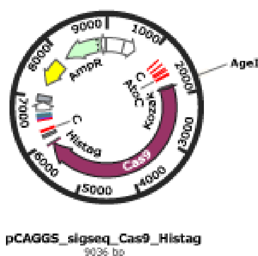
Creating the DNA constructs - **AsCpf1**:

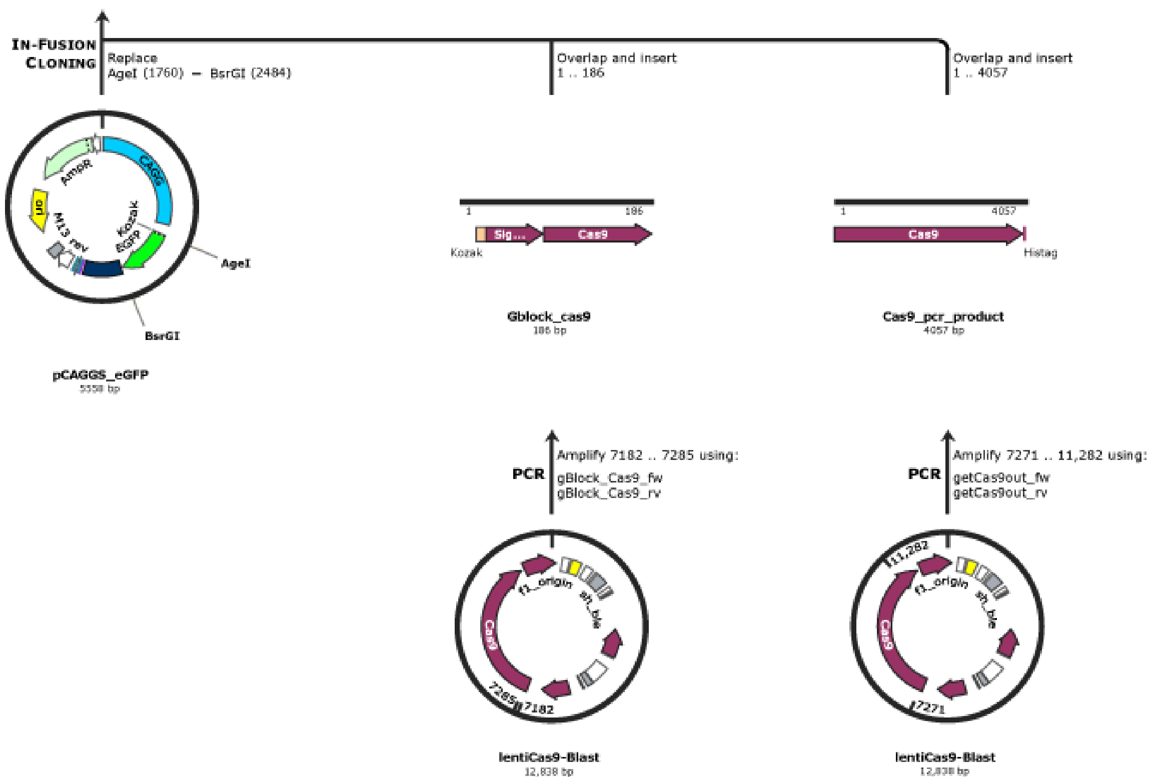
1. PCR was performed using the following plasmid and primers, to create a fragment containing Cpf1 with a C-terminal Histag and overlap region for the final backbone plasmid.
   Plasmid: Lenti-AsCpf1-Blast (from Addgene, nr: 84750)
   Fw primer 5'-3': TCATCGAGGAGGACAAGGCCC
   Rv primer 5'-3': GCCGCTTACTTGTACTTAATGATGATGATGATGATGGCCG CCGCCGTTGCGCAGCTCCTGGATGTAG
   Protocol: See appendix 1
2. gBlock containing a kozak sequence, signal sequence and overlap regions with the backbone and Cpf1 was ordered from IDT. See snapgene file below for sequence
3. In-Fusion Cloning was then performed using AgeI and BsrGI to linearize the backbone plasmid and the two previously created fragments.
   Plasmid: pCAGGS_eGFP
   Protocol: See appendix 2



Creating the DNA constructs - **Cas9**:

1. PCR was performed using the following plasmid and primers, to create a fragment containing Cas9 with a C-terminal Histag and overlap region for the final backbone plasmid.
   Plasmid: Lenti-Cas9-Blast (from Addgene, nr: 52962)
   Fw primer 5'-3': ATTCAAGGTGCTGGGCAACAC
   Rv primer 5'-3': GCCGCTTACTTGTACTTAATGATGATGATGATGATGGCCG CCGCCGTCGCCTCCCAGCTGAGACA
   Protocol: See appendix 3
2. PCR was performed to create a second fragment containing a kozak region, the signal sequence and the first part of Cas9 (without its methionine).
   Plasmid: Lenti-Cas9-Blast (from Addgene)
   Fw primer 5'-3': CCCGGGATCCACCGGTGCCGCCACCATGGCGTGG ACCAGCCTGATTCTGAGCCTGCTGGCGCTGTGCAGCGGCGCGAGCAGCG ACAAGAAGTACAGCATCGGCCTG
   Rv primer 5'-3': CCCAGCACCTTGAATTTCTTGCTG
   Protocol: See appendix 4
3. In-Fusion Cloning was then performed using AgeI and BsrGI to linearize the backbone plasmid and the two previously created fragments.
   Plasmid: pCAGGS_eGFP
   Protocol: See appendix 2





STable 1. Nanodrop results of midiprepped plasmids

Plasmid	**Concentration (ng/ul)**
pCAGGs_sigseq_Cas9_Histag	809,7
pCAGGs_sigseq_Cpf1_Histag	1322
Lenti Cas9	2593,9
Lenti Cpf1	1312,7

Appendix 1: Cpf1 PCR protocol

1. Cpf1 PCR

   Before starting:
   - Thaw DMSO, HF/GC buffer (GC buffer for templates with high GC content), dNTPs.
   - Dilute template to 5-10 ng/uL → LentiCas9-Blast
   - Primer stock at 100uM and working solution at 10uM
     - Example: 88 nmol of primer dilute in 880 uL MiliQ → 100uM
       88 nmol = 0,088 ug
       uM = ug/L
       L = 10^6 uL
       88 nmol/880 ul = 0,088 ug/880 x 10^{-6} L = 100 uM

PCR master mix:

H_2O	118µL
5x HF/GC buffer	40µL
Template	10µL
Fw	10µL
Rv	10µL
DMSO	6µL
dNTP mix	5µL
phusion polymerase	3µL

mix well (vortex before adding polymerase and after adding phusion polymerase only flick the tubs, no vortexing), briefly spin down.
Divide into 8 PCR wells (**about 23µL/well**)
PCR cycles:

98°C		12s	
	|98°C	10s	
42x	|63°C	25s	
	|72°C	58s	(15s/kb; minimum 15s)
72°C		5min	
4°C		---	

For gel extraction make 1% gel, using **thicker and wider** combs.
Collect all reactions in one tube, add 40µL of 6x loading dye.
Load 60µL sample to each slot on the gel.
Go through the gel extraction procedures.
NOTE! Use one column to bind and elute in 20µL

Appendix 2: InFusion protocol

Notes:

- Use 1,5 mL epps for the reaction.
- Amount of DNA is approximately, if your DNA is at 180-220 ng/ul use 1 ul for 200ng. If you need 50 ng and you have 100ng use only 0,5 ul (don't use less volume than 0,5 → dilute your sample)
- Don't exceed maximum volume 5 ul, remember you need 1 ul for the reaction mix, you have 4 ul to play around to introduce samples.
- If necessary dilute your sample in MiliQ or EB buffer (don't dilute all the sample, take a small aliquot in a new tube and add MiliQ to your final concentration)
- Be careful with pipetting, you are only pippeting 0,5 to 2 ul. Make sure you put the drop inside the epp. You want to see all the drops in the epp separately.
- Reaction mix cannot be at RT, it needs to be always at -20ºC. take it out of the freezer last minute, keep it on ice (don't hold the epp on your hands for a long time) and put it right away back to the freezer.
- Start around 2 pm, transformation cannot be done in the morning and the infusion needs to be transform right after.

Cas9 without eGFP

digested **pCAGGS_eGFP**	200ng
new Cas9 no eGFP pcr	200ng
Cas9 gBlock pcr	50 ng
5x infusion mix	1µL
final volume (+ MiliQ)	5µL

flicking to mix, briefly spin down. Do not vortex!! Incubate at
50ºC for 15min (water bath or hot plate)
Keep on ice

Use 2µL for transformation.

Cpf1 without eGFP

digested **pCAGGS_eGFP**	200ng
new Cpf1 no eGFP pcr	200ng
Cpf1 gBlock pcr	50 ng
5x infusion mix	1µL
final volume (+ MiliQ)	5µL

flicking to mix, briefly spin down. Do not vortex!! Incubate at
50ºC for 15min (water bath or hot plate)
Keep on ice

Use 2µL for transformation.

Transformation protocol

a. Pre-warm SOC medium at 37ºC and pre-warm plate (if it's not a new one)

b. Get a box with ice and put Top 10 (from the -80ºC), let them thaw for 15 min

c. In transformation tubes:

  i. Add 2 µL of your reaction

  ii. Add 50µL Top 10 to the tube

d. Flick bottom to mix carefully

e. Keep on ice for 25 min

f. Heatshock 42ºC 30'' in a water bath (do not move the tube, hold it straight)

g. On ice 2 min

h. Add pre-warmed SOC 750 µL

i. 37ºC 225 rpm shaking 45 min

j. Move everything to 1,5 mL epp

k. Spin 3500 rpm 4 min

l. Remove 700 µL media

m. Resuspend pellet with the rest of media

n. Spread on pre-warmed plate until is dry → incubator 37ºC overnight

o. Next morning put plate in the fridge

Miniprep from 10 colonies from each inFusion (Invitrogen kit)

- Day before: At around 15-16 h prepare the miniprep cultures ○ 4 ml media
  - Amp
  - Pick one colony
- Next day: Centrifuge 4 mL at RT, 1200xg for 10 min.
- Pre-heat TE buffer (50-70 ºC)
- Remove media
- Resuspend pellet with 250 ul R3 (with RNase)
- Add 250 L7 (lysis buffer). Mix gently by inverting the tub. Incubate at RT 5 min.
- Add 350 uL N3 (precipitation buffer) and immediately mix.
- Centrifugate 10 min 1200xg.
- Loading sample: 1 column/ 1 sample ○ With vacuum: bind columns to the vacuum. Load the sample (supernatant, just the clear part). Wash column with 500 ul W10 (with ethanol added) and second wash with 700 uL W9 (with ethanol added).

- With centrifuge: place columns in washing tubes. Load the sample into the column. Centrifuge 1 min 12000xg. Remove liquid in the washing tube, place again the column in the washing tube and add 500 ul W10 (with ethanol added). Remove liquid in the washing tube, place again the column in the washing tube and add 700 ul W9 (with ethanol added). Remove liquid in the washing tube.

- Place columns in the washing tube and remove ethanol by centrifuge 2 min at 12000xg.
- Elute the plasmid by introducing the column in a recovery tube (1,5 epp). Add 20 ul pre-heated TE buffer. Incubate column 1 min at RT. Centrifuge 2 min 12000xg.
- Nanodrop:
  - Measure:
    - Clean nanodrop with tissue
    - Open with water
    - Blanc with TE buffer
    - Measure sample

6. Sequencing (5 form each group, is not necessary to do all of them just the ones with highest concentration)
   - 5 ul primer at working solution (10uM)
   - 500 ng DNA from miniprep (maximun 5 uL, minipreps under 100 ng/ul are not good)
   - Add MiliQ to final volum 10 uL if necessary.

8. Maxiprep

Day 1

Transformation from miniprep

Day 2

1. Culture 400mL:
   a. Before lunch → 400mL LB + 400 µL Amp
   b. Pick 5 colonies and throw the tip into the bottle
   c. 37ºC 225 rpm overnight (O/N)

Day 3 → **MAXI-PREP** (for 400mL O/N culture) Invitrogen kit

2. Get 2 bottles from downstairs and split the culture
3. Spin 25 min max g (ground centrifuge)
4. Remove supernatant
5. Get R3 buffer from fridge (ML1)
6. Add 10 mL R3 into one bottle
7. Resuspend by vortexing
8. Move all cell suspension to the other bottle
9. Resuspend by vortexing
10. Move everything into a 50 mL tube (Falcon)
11. Vortex briefly

12. Add 10 mL L7 buffer
13. Revert few times (NO VORTEXING!!!)
14. Leave at room temperature (R/T) for 5 – 10 min
15. Add 10 mL N3 buffer
16. Homogenize by inverting
17. Spin at maxi speed 30 min
18. 10 min before 19 ends add 30 mL EQ1 / column
19. Load all lysate on the column
20. Add 60 mL W8
21. Move the column on top of 50 mL tube
22. Add 15 mL E4 to elute
23. Add 10,5 mL isopropanol into the falcon tube and mix well
24. Pre-cold centrifuge in ML1
25. Spin in ground-top centrifuge at 10.000xg 4ºC for 30 min
26. Carefully remove the supernatant
27. Add 5 mL 70% ethanol
28. Spin at 4000xg 5 min 4ºC
29. Carefully remove supernatant
30. Spin again for 1 min and remove supernatant by pipette
31. Open tube to air dry the pellet (~10 min)
32. Add 400 µL TE and vortex
33. Spin to collect all the solution 34. Pipett solution to epp 1,5 mL 35. Nano-drop lecture:
    a. Dilute 10x: 18 µL TE + 2 µL maxi-prep solution
    b. Measure:
        i. First clean with water
        ii. Blanc with TE buffer
        iii. Measure sample

Appendix 3: Cas9 PCR protocol

1. Cas9 PCR
   Before starting:
   - Thaw DMSO, HF/GC buffer (GC buffer for templates with high GC content), dNTPs.
   - Dilute template to 5-10 ng/uL → LentiCas9-Blast
   - Primer stock at 100uM and working solution at 10uM
     - Example: 88 nmol of primer dilute in 880 uL MiliQ → 100uM
       88 nmol = 0,088 ug
       uM = ug/L
       L = 10^6 uL
       88 nmol/880 ul = 0,088 ug/880 x 10^{-6} L = 100 uM

PCR master mix:

H_2O	118µL
5x HF/GC buffer	40µL
Template	10µL
Fw	10µL
Rv	10µL
DMSO	6µL
dNTP mix	5µL
phusion polymerase	3µL

mix well (vortex before adding polymerase and after adding phusion polymerase only flick the tubs, no vortexing), briefly spin down.
Divide into 8 PCR wells (**about 23µL/well**)
PCR cycles:

98°C		12s	
	|98°C	10s	
42x	|63°C	25s	
	|72°C	1 min 1s	(15s/kb; minimum 15s)
72°C		5min	
4°C		---	

For gel extraction make 1% gel, using **thicker and wider** combs.
Collect all reactions in one tube, add 40µL of 6x loading dye.
Load 60µL sample to each slot on the gel.
Go through the gel extraction procedures.
NOTE! Use one column to bind and elute in 20µL

Appendix 4: PCR gBlocks Cas9

Before starting:

- Thaw DMSO, HF/GC buffer (GC buffer for templates with high GC content), dNTPs.
- Dilute template to 5-10 ng/uL.
- Primer stock at 100uM and working solution at 10uM
  - Example: 88 nmol of primer dilute in 880 uL MiliQ → 100uM
    88 nmol = 0,088 ug
    uM = ug/L
    L = 10^6 uL
    88 nmol/880 ul = 0,088 ug/880 x 10^{-6} L = 100 uM

PCR master mix:

H_2O	118µL
5x HF/GC buffer	40µL
Template	10µL
Fw	10µL
Rv	10µL
DMSO	6µL
dNTP mix	5µL
phusion polymerase	3µL

mix well (vortex before adding polymerase and after adding phusion polymerase only flick the tubs, no vortexing), briefly spin down.
Divide into 8 PCR wells (**about 23µL/well**)
PCR cycles:

98°C		12s	
	|98°C	10s	
42x	|*°C	25s	
	|72°C	15s	(15s/kb; minimum 15s)
72°C		5min	
4°C		---	

* 62ºC for Cas9

For gel extraction make 1% gel, using **thicker and wider** combs.
Collect all reactions in one tube, add 40µL of 6x loading dye.
Load 60µL sample to each slot on the gel.
Go through the gel extraction procedures.
NOTE! Use one column to bind and elute in 20µL