Lab 08.08.17: Gibson assembly of toehold and eGFP

1. Aim:

Do a Gibson assembly of toehold and eGFP in order to have plasmid DNA of toehold-eGFP.

2. Materials:

- Material for PCR
- Primers:
  - For toehold:
    - Forward: iG_LacZ_GFP_G-f
    - Reverse: iG_LacZ_GFP_G-r
  - For eGFP:
    - Forward: iG_GFP_GS-f
    - Reverse: iG_GFP_GS-r
- Master mix for Gibson assembly
- DPN1 => cut all methylated cytosines

3. Procedure:

- Followed this table for PCR:

DNA	*Gibson Toehold-GFP*		1.61ng/uL	Forward	B_5'final	Reverse	B_3'final
Component	Concentration	Units	Desired concentration	Units	Volume to add (uL)		Total volume (uL)
water					71.5		100
HF buffer	5	x	1	x	20		
dNTP	10	mM	0.2	mM	2		
DMSO	100	%	5	%	5		

forward primer	100	uM	0.5	uM	0.5		
backwards primer	100	uM	0.5	uM	0.5		
DNA of toehold colony 2	6.74	ng/uL	1	ng	0.148367953		
Phusion polymerase	2000	U/ml	0.75	U/50uL	0.375		

DNA	Gibson Toehold-GFP		1.61ng/uL	Forward	B_5'final	Reverse	B_3'final
Component	Concentration	Units	Desired concentration	Units	Volume to add (uL)		Total volume (uL)
water					71.4		100
HF buffer	5	x	1	x	20		
dNTP	10	mM	0.2	mM	2		
DMSO	100	%	5	%	5		
forward primer	100	uM	0.5	uM	0.5		
backwards primer	100	uM	0.5	uM	0.5		
DNA of yEGFP	3.79	ng/uL	1	ng	0.263852243		
Phusion polymerase	2000	U/ml	0.75	U/50uL	0.375		

					add in 2 tubes :	49.625	

- Programme for PCR: Gibson assembly toehold-eGFP (env. 2h20)
- Agarose gel to check PCR
  => Didn't work

Followed this table to redo eGFP PCR:

DNA	Gibson Toehold-GFP		1.61ng/uL	Forward	B_5'final	Reverse	B_3'final
Component	Concentration	Units	Desired concentration	Units	Volume to add (uL)		Total volume (uL)
water					35.4		50
HF buffer	5	x	1	x	10		
dNTP	10	mM	0.2	mM	1		50
DMSO	100	%	5	%	2.5		
forward primer (iG_GFP_GS_r)	100	uM	0.5	uM	0.25		
backwards primer	100	uM	0.5	uM	0.25		
DNA of yEGFP linear	7.65	ng/uL	2	ng	0.261437908		
Phusion polymerase	2000	U/ml	0.75	U/50uL	0.375		

DNA	Gibson Toehold-GFP		1.61ng/uL	Forward	B_5'final	Reverse	B_3'final
Component	Concentration	Units	Desired concentration	Units	Volume to add (uL)		Total volume (uL)
water					35.1		50
HF buffer	5	x	1	x	10		
dNTP	10	mM	0.2	mM	1		
DMSO	100	%	5	%	2.5		
forward primer (iG_GFP_GS_r)	100	uM	0.5	uM	0.25		
backwards primer	100	uM	0.5	uM	0.25		
DNA of yEGFP plasmid	3.79	ng/uL	2	ng	0.527704485		
Phusion polymerase	2000	U/ml	0.75	U/50uL	0.375		

DNA => make yeGFP linear	Positive control		1.61ng/uL	Forward	B_5'final	Reverse	B_3'final
Component	Concentration	Units	Desired concentration	Units	Volume to add (uL)		Total volume (uL)
water					35.1		50

HF buffer	5	x	1	x	10		
dNTP	10	mM	0.2	mM	1		
DMSO	100	%	5	%	2.5		
forward primer (yeGFP B_3' final)	100	uM	0.5	uM	0.25		
backwards primer	100	uM	0.5	uM	0.25		
DNA of yEGFP plasmid	3.79	ng/uL	2	ng	0.527704485		
Phusion polymerase	2000	U/ml	0.75	U/50uL	0.375		

- We redid PCR of eGFP

DNA	Gibson eGFP		1.61ng/uL	Forward	B_5'final	Reverse	B_3'final			
Component	Concentration	Units	Desired concentration	Units	Volume to add (uL)		Total volume (uL)		Standard PCR	
water					70.8		100		Temperature [°C]	Time [s]
HF buffer	5	x	1	x	20				98ºC	2m
dNTP	10	mM	0.2	mM	2		100		98ºC	30s
DMSO	100	%	5	%	5			45x	72ºC	30s
forward primer	100	uM	0.5	uM	0.5				72ºC	30
backwards primer	100	uM	0.5	uM	0.5				72ºC	7m

DNA of yEGFP	5.8	ng/uL	5	ng	0.862068966				4ºC	
Phusion polymerase	2000	U/ml	0.75	U/50uL	0.375	In each 2 tubes				

- We redid PCR of eGFP again…

DNA	yEGFP for Gibson		**1.61ng/uL**	**Forward**	**B_5'final**	**B_3'final**			
Component	**Concentration**	**Units**	**Desired concentration**	**Units**	**Volume to add (uL)**	**Total volume (uL)**		**Standard PCR**	
water					211.3	300		Temperature [°C]	Time [s]
HF buffer	5	x	1	x	60			98ºC	2m
dNTP	10	mM	0.2	mM	6			98ºC	20s
DMSO	100	%	5	%	15		30x	72ºC	30s
forward primer (iG_GFP_GS-f)	50	uM	0.5	uM	3			71/69/67ºC	30s
backwards primer	50	uM	0.5	uM	3			72ºC	7m
DNA (plasmid yEGFP)	3.79	ng/uL	2	ng	0.527704485			4ºC	
Phusion polymerase	2000	U/ml	0.75	U/50uL	1.125				
					(in each two tubes)				

Add in 2 tubes :	MM	49.625
	Phusion	0.375

- add 1 [ul] of DPN1 to 50 [ul] of PCR product and incubate at 37°C during 1h
- Do a purification using Qiagen purification kit
- Prewarm the incubator at 50°C
- Do Gibson assembly (next day), following this table:
  - Mix all these and incubate for 15 minutes at 50°C

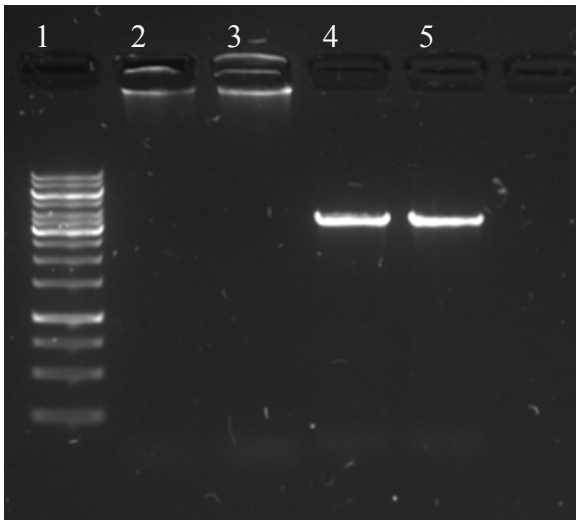
		Fragment conc. [ng/uL]	Final amount of the fragment [ng]
Fragment TH [uL]	**0.38**	132.3	50
Fragment GFP [uL]	**14.08**	7.1	100
Gibson Assembly MM [uL]	**14.46**		
H2O [uL]	**0.00**		

Total [uL]	28.92		

- Make transformation of the plasmid with DH5a competent cells, see protocol: Subcloning EfficiencyTM DH5αTM Competent Cells
    - We made 3 transformations: 5 [ul], 2 [ul] and no DNA
    - We used LB medium
- Select colonies, touch the colony with tip, touch the bottom of a PCR tube with this tip and discard the tip in culturing tube
- Do a colony PCR with phusion and 27 final & rev1 primers
    - Normal PCR but without adding DNA (we amplify DNA from the selected colony)
    - Extension 45-60s
    - Length of PCR products obtained should be 1000bp
    - => choose colony with right band in agarose gel and do glycerol stock
- Do a miniprep

4. Labels:

5. Results:



Lane 1: 1kb ladder
Lane 2: EGFP amplification
Lane 3: EGFP amplification
Lane 4: Toehold+plasmid amplification
Lane 5: Toehold+plasmid amplification

6. Conclusion:

The first PCR amplified the toehold and plasmid but not GFP so it is repeated.

7. Comments (problems, what could be better, …):

8. Lab photos: