25/05/2026

Kan: 1:4000 (for liquid starter, use 1:2000)
Amp: 1:1000

Transformation

Protocol followed:

1. Centrifuged the plasmids for 1 minute at 6000 g.
2. Added TE: 20 mL of 10mM TE (+EDTA), (DNA conc: 200ng/ml)
3. #1plasmids Vortexed + spinndown
4. Incubation at 50°C for 15 minutes
5. #2 plasmids vortexed + spin down
6. Took out frozen competent bacteria and put them in ice
7. #1 bacteria gentle spin down
8. Inserted 0.5 microliter of plasmid into 1-5
9. Waited 20 minutes of incubation on ice
10. 2 minutes of incubation at 42 °C
11. 2 minutes of incubation on ice
12. Incubated for 1 hour with SOC
13. Plated 120 µL on LB plates with appropriate antibiotics and placed them in the incubator

Notes:

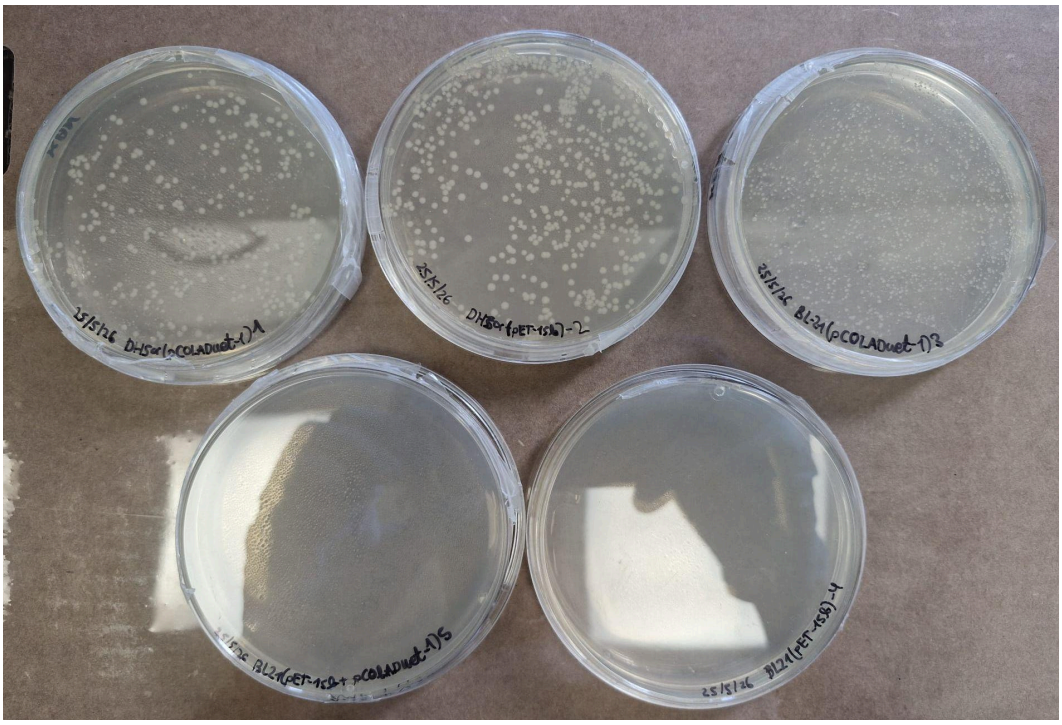
#1 Antibiotic Resistance:

- pET-15b: amp resistance
- pCOLADuet-1: kan resistance

#2 plates contents:

1 - DH5α + pCOLADuet-1
2 - DH5α + pET-15b
3 - BL21 + pCOLADuet-1
4 - BL21 + pET-15b
5 - BL21 + pET-15b + pCOLADuet-1

26/05/2026



After incubation at 42 °C, some of the plates had colonies in them, and some did not:

Plate Number	Plate	Colonies
1	DH5α pCOLADuet-1	V
2	DH5α pET-15b	V
3	BL21 pCOLADuet-1	V
4	BL21 pET-15b	X
5	BL21 pET-15b + pCOLADuet-1	X

28/05/2026

Glycerol Stock

Colonies from the plates that did manage to grow (from 26/5) were picked from the plates to a Falcon vial (27/5) starter with LB. 1ml from the culture was transferred to a cryo 2ml tube and mixed with 1ml 50% glycerol. The stocks were added to the catalog table in the drive.

Mini-prep

NucleoSpin Plasmid EasyPure, Mini kit was used for two plasmids.

Plasmid	Concentration (ng/ul)
pCOLDuet-1	76.8
pCOLDuet-1	65.5
pET-15b	71.6
pET-15b	36

04/06/2026

PCR + Gel + Restriction

PCR

PCR temperature gradient for pCOLDuetB & pCOLDuetGFP

We made 3 plasmids:

- pCOLDuetB-1: pCOLDuet-1 without a toehold and reporting gene (GFP)
- pCOLDuetGFP: pCOLDuet-1 without a toehold.
- pET-15b: Empty (no mCherry)

2 PCR reactions (pCOLDuetBB & pCOLDuetGFP)
For each, we repeated 5 times, doing a PCR temperature gradient.

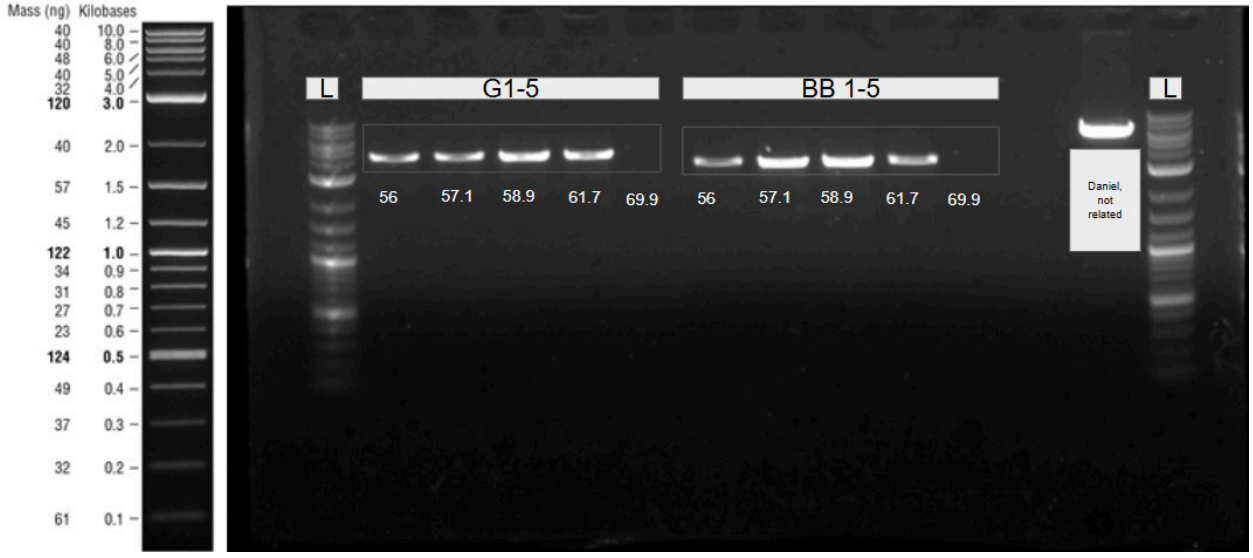
Table for a single reaction

Component	X1 (uL)	X5 (uL)

PANTA MIX	12.5	62.5
F1: FR-pCOLDuet-1-BB	1	5
F2: FR-pCOLDuet-1-GFP	1	5
R: Rev-pCOLDuet-1-BB	1	5
DNA (pCOLDuet1)	1	5
UPW	10.5	52.5

Gel Results:
Order:

- Ladder
- G1-5, should be 4053 bp
- BB1-5, should be 3297 bp
- Daniel hitchhiking on our gel, not related to us
- Ladder



It appears temperatures 56 to 61.7 work, while 69.9 doesn't. Therefore, we stored tubes 1-4 and disposed of tube 5 for each. The band's bp size seems to be borderline compared to the expected size. We would further verify the results in a future experiment.

<u>Concentrations found:</u>
pCOLDuet-GFP: 26.6 ng/ul
pCOLDuet-BB: 27.2 ng/ul

Restriction

Component	Amount (uL)
H2O	14
pET-15b + Mcherry (72 ng/ul)	30
NcoI	0.5
BsrGi	0.5
Cutsmart	5

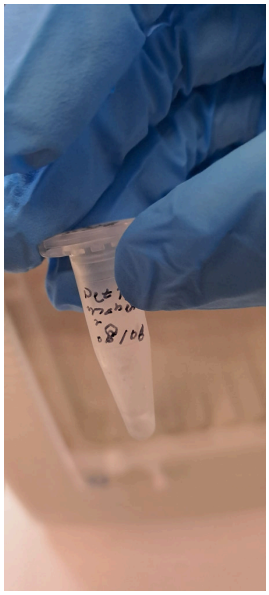
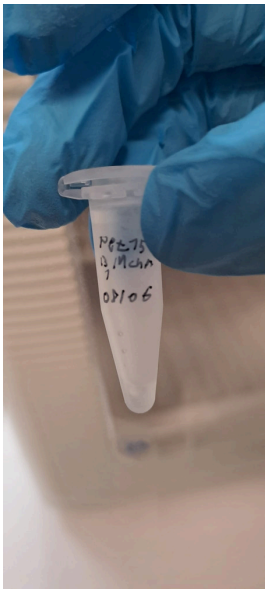
- 1 hour at 37°C
- 20 minutes at 80°C

We performed restriction and will validate the results in a future experiment (gel)

08/06/2026

Mini-prep

The NucleoSpin Plasmid EasyPure Mini kit was used for two samples of pET-15b.
Due to issues with the NanoDrop's computer, we had to postpone the measurement.
For now, the extractions are stored in the freezer, in the "Igem 2026 Bacteria Stuff" box, under the name "pET-15b mCherry, 08/06 - 1 / 2", as shown in the pictures below:


09/06/2026

NanoDrop

NanoDrop was performed as a follow-up to the mini-prep performed on 08/06/2026. The results are as follows:

Plasmid	**Concentration** (ng/uL)
pET-15b mcherry, 08/06 - 1	82
pET-15b mcherry, 08/06 - 2	57

10/06/2026

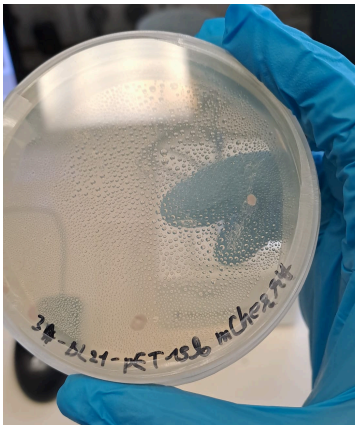
Transformation

To complete the E. *coli* POC set-up (BL21 pET-15b and BL21 pET-15b+ pCOLADuet-1), another round of transformation was performed, with the same protocol, but this time with a duration of the heat shock (42 °C) of 1 minute, with the transformants plated in plate 2 being heat shocked for 2 minutes.

Plate Number	Plate	Antibiotic selection	Colonies
1	BL21 pET-15b + pCOLADuet-1	Amp+Kan	X
2 (2-minute heat shock)	BL21 pET-15b + pCOLADuet-1	Amp+Kan	X
3	BL21 pET-15b	Amp	V (3 colonies)

14/06/2026

Prepared Amp+Kan plates, starter pET15b + mCherry, sent plasmids to Plasmidsaurus



Prepared for future experiment Amp+Kan plates (50mg/ml, 10mg/ml)

Prepared the plasmids pET15b + mCherry and pCOLADuet-1 to be sent to Plasmidsaurus and left the samples at their mailbox.

Set a starter of pET15b+mCherry; the original plate had a limited number of colonies, which were already visibly red. This raised

some tough questions. Hopefully, some of them will be answered by the pladmidsaurus reading.

15/06/2026

Transformation

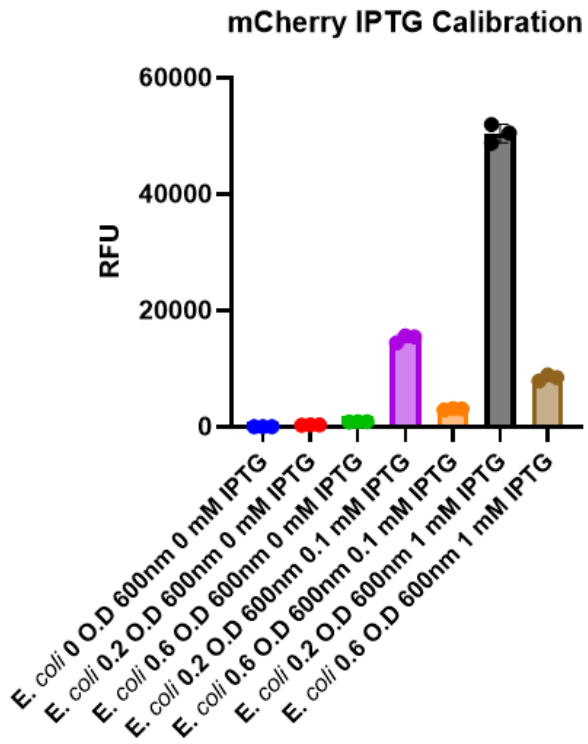
To complete the E. *coli* POC set-up with BL21 pET-15b+ pCOLADuet-1, another round of transformation was performed, with the same protocol, but this time with a heat shock duration (42 °C) of 0.5 minutes and 2 minutes.

pET-15b mCherry check in the plate reader

To check the leakiness of pET-15b and calibrate the IPTG induction. 3 concentrations of IPTG (0, 0.1 mM, and 1 mM) were tested for mCherry fluorescence induction in E. *coli* BL21 pET-15b at two initial OD600 values (0.21 and 0.63), with a 2h incubation at 37 °C. PBS as a control. mCherry fluorescence and final O.D 600nm were measured in the Tecan Spark plate reader (LAA lab): cells were washed in PBS and divided into three wells each.

Sample	600nm O.D	IPTG mM
0	0	0
1	0.21	0
2	0.63	0
3	0.21	0.1
4	0.63	0.1
5	0.21	1
6	0.63	1

0	1	2	3	4	5	6
0	1	2	3	4	5	6
0	1	2	3	4	5	6



Conclusion for pET-15b, mCherry, IPTG, and bacteria concentration

It seems that it is best to work with an initial E. coli concentration of 0.2-0.3 O.D. at 600nm before the 2-hour induction period, and a concentration of 1 mM for the inducer IPTG.

Conclusion

It seems that using 0.2 O.D 600nm initial bacterial concentration with 1mM IPTG is the better option (between the surveyed options), as it has the best on/off ratio (the black bar compared to the red bar)

14/06/2026

Transformation Results

No colonies grew on both plates.

14/06/2026 - Competent Preparation

Chemically Competent E. coli Protocol (TSS Method)

Preparation (before Day 3)

- Autoclave 100 mL LB in a 500 mL Erlenmeyer flask.
- Prepare 5 mL TSS (see recipe below).
- Prepare 100 Eppendorf tubes, label with strain name, and place at 4°C.
- Have ready: 1 LB plate (no antibiotics), at least 10 mL LB for TSS and starter.

Day 1

- Streak E. coli on LB plate.
- Incubate overnight at 37°C.

Day 2

- Pick a single colony, transfer to 2 mL LB starter in a 14 mL tube.
- Incubate overnight at 37°C with shaking.

Day 3

- Transfer 0.5 mL from starter into a 100 mL LB flask. OD600 should be under 0.1.
- Grow at 37°C with shaking to OD600 = 0.3–0.5.
- Transfer culture to 50 mL tubes.
- Incubate on ice for 20 min.

Important: from here on, keep everything at 4°C and cells on ice at all times.

- Centrifuge at 3000 RPM for 10 min at 4°C.
- Discard supernatants carefully and completely.
- Resuspend cells in 5 mL pre-chilled TSS. Vortex gently for full resuspension.
- Incubate 15 min on ice.
- Dispense 50 µl aliquots into pre-chilled Eppendorf tubes.
- Freeze aliquots at −80°C.

TSS Buffer (5 ml total)

Component	Quantity
PEG 3350/8000	0.5 g
$MgCl_2$ (1 M)	150 µl
DMSO	250 µl

LB to 5 ml

- Filter, sterilize.
- Store at 4°C for up to 1 week.

18/06/2026

BL21 Pet-15b Competent Bacteria

We decided to make the BL21 pET-15b competents and insert pCOLADuet-1 with different inserts (RNA switches) into it, since pET-15b seems to be the “heavier” transformation to do, since pCOLADuet-1 transformation yielded many colonies, and pET-15b only yielded a few, and only after the second try of transformation into BL21. About 50 competent stocks were made.

22/06/2026

Transformation of pCOLADuet Green into BL21 Pet-15b Competent Bacteria

We performed the transformation of pCOLADuet toehold (green) into BL21 Pet-15b competent bacteria. We transferred 100 µL of them to dual plates containing Amp + Kan. The bacteria were left to incubate for the rest of the night (a total of 4 plates).

The remainder of the bacteria that completed the transformation were stored in the 4C refrigerator.

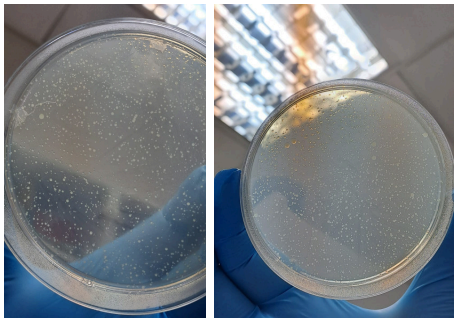
During the transformation, we used 2 different sources for the same plasmid, one with ‘high’ concentration (76.8 ng/uL, **labeled as HC**) and one with ‘low’ concentration (65.5 ng/uL, **labeled as LC**). For each of those, we administered 2 different heat shock duration treatments: 1 minute and 2 minutes (labeled as 1 Min and 2 Min).

**The plating results are described in the log on 23/06/2026*

23/06/2026

Patching

The plates from the transformation performed on 22/06/2026:



We continued to sample from each plate colony in different sizes (large, medium, and small), patched them onto a double plate (Amp + Kan) as described in the table below, and put them in the incubator.

1	2	3	4	Large Colony
5	6	7	8	Medium Colony
9	10	11	12	Small Colony
Plate original treatment: HC, 2 min	Plate original treatment: HC, 1 min	Plate original treatment: LC, 2 min	Plate original treatment: LC, 1 min	

25/06/2026

Toehold POC

The bacteria were diluted to 0.2 O.D 600nm for every sample in LB. The samples were then incubated with IPTG 1mM for 2.5 hours at 37 °C with shaking, then suspended in PBS after centrifuging at 4000 × g for 3 minutes, and placed in the wells of a 96-well plate (black walls).

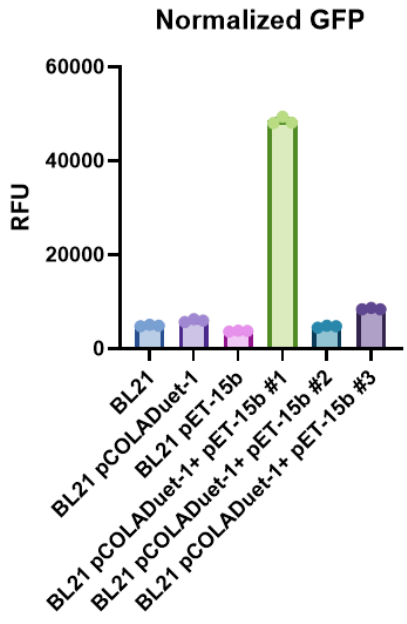
Sample	Genotype	Phenotype on plate kan+amp	Initial 600nm O.D	IPTG mM
0	Blank (PBS)		0	0
1	BL21		1.86	0/1

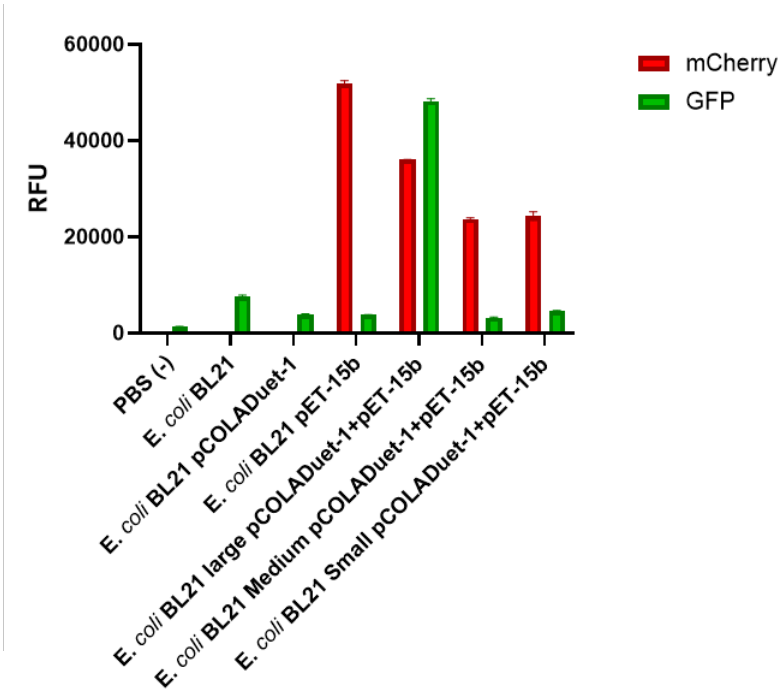
2	BL21 pCOLADuet-1		1.48	0/1
3	BL21 pET-15b		1.73	0/1
4	BL21 pCOLADuet-1+ pET-15b	Large	1.47	0/1
5	BL21 pCOLADuet-1+ pET-15b	Medium	1.38	0/1
6	BL21 pCOLADuet-1+ pET-15b	Small	0.27	0/1

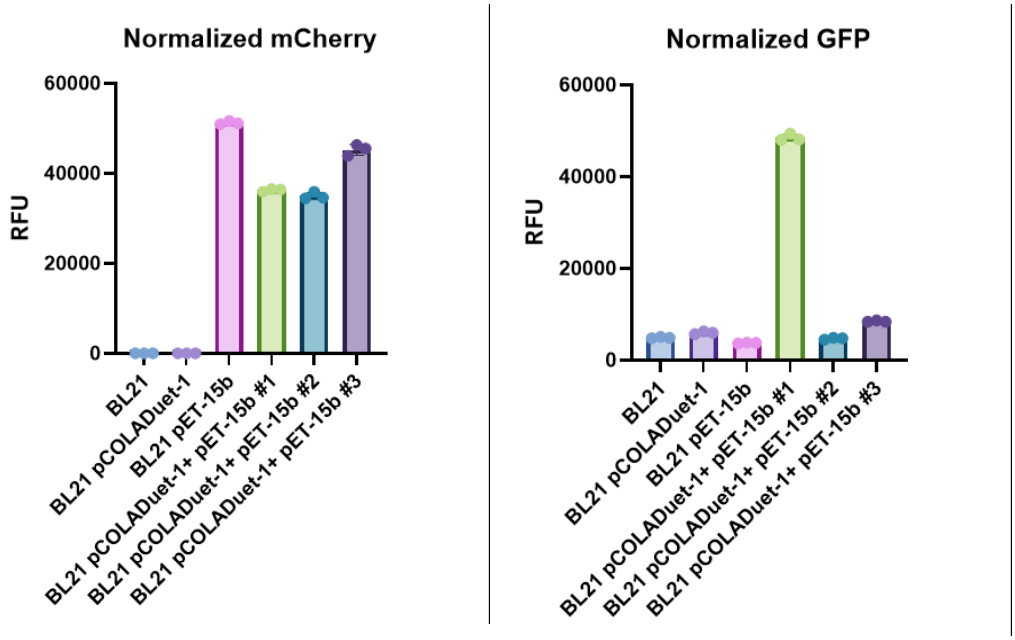
Plate Reader:

0	1IPTG	2IPTG	3IPTG	4IPTG	5IPTG						
0	1IPTG	2IPTG	3IPTG	4IPTG	5IPTG						
0	1IPTG	2IPTG	3IPTG	4IPTG	5IPTG						

The results (next page):







Results and discussion:

Overall, it seems that the restoration of Green et al. 2014 worked as planned, although we intended to add samples without IPTG as a control, which we forgot to include.
The big graph is not normalized to the concentration of the bacteria (600nm O.D.) in the well, which is a more accurate measure, as can be observed by the relatively large noise of the GFP without normalizing the bacteria concentration compared to the normalized graph.
It seems that the large colony (#1) on the double antibiotic plates was the most successful and was the only colony that was able to produce GFP in such magnitude. The relevant colony was made into a glycerol stock as a control for future experiments and for replication of the POC experiment, this time with and without IPTG.

29/06/2026

Toehold POC second repetition + IPTG controls

- The bacteria were diluted to 0.2 O.D 600nm for every sample in LB and the appropriate antibiotics (amp - 1:2000, kan - 1:4000).
- The samples were then incubated with or without IPTG 1mM for 3 hours at 37 °C with shaking.
- The samples were centrifuged at 4000 × g for 3 minutes; the supernatant was removed, and each pellet sample was suspended in PBS (300 µL).
- 100 µL of each sample was placed in the wells of a 96-well plate (black walls).

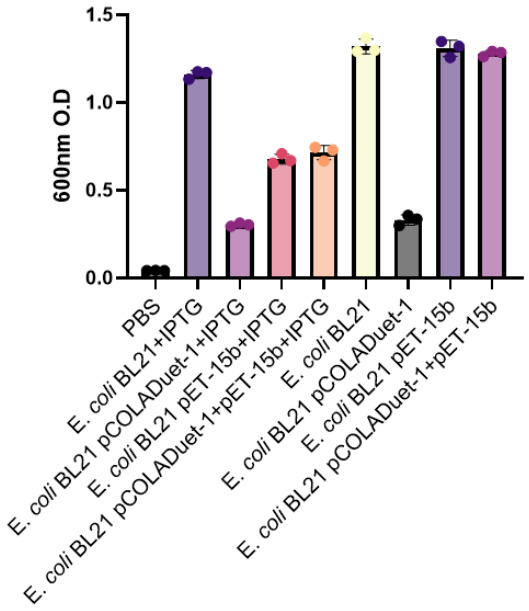
Sample	Genotype	Phenotype on plate kan+amp	Initial 600nm O.D	IPTG mM
0	Blank (PBS)		0	0
1	BL21			0/1
2	BL21 pCOLADuet-1			0/1
3	BL21 pET-15b			0/1
4	BL21 pCOLADuet-1+ pET-15b	Large		0/1

Plate Reader:

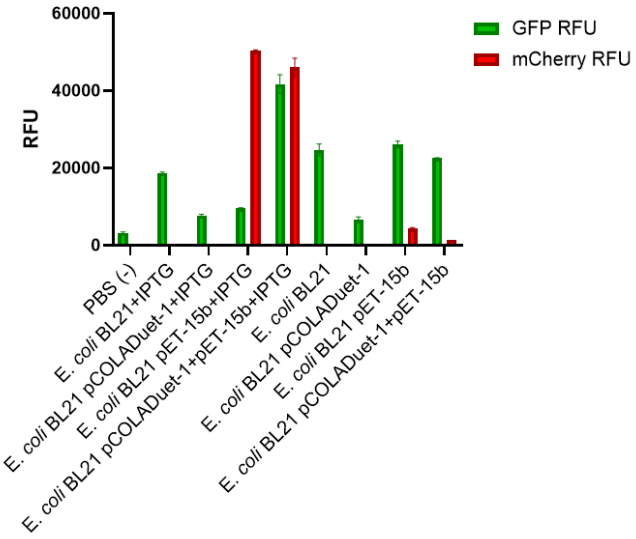
0	1IPTG	2IPTG	3IPTG	4IPTG	1	2	3	4			
0	1IPTG	2IPTG	3IPTG	4IPTG	1	2	3	4			
0	1IPTG	2IPTG	3IPTG	4IPTG	1	2	3	4			

Results:

600nm O.D: It seems that something might be wrong with the colony we chose to represent the E. *coli* BL21 pCOLADuet-1, as it might disrupt the on/off results. We will check the other colony and a more viable one.

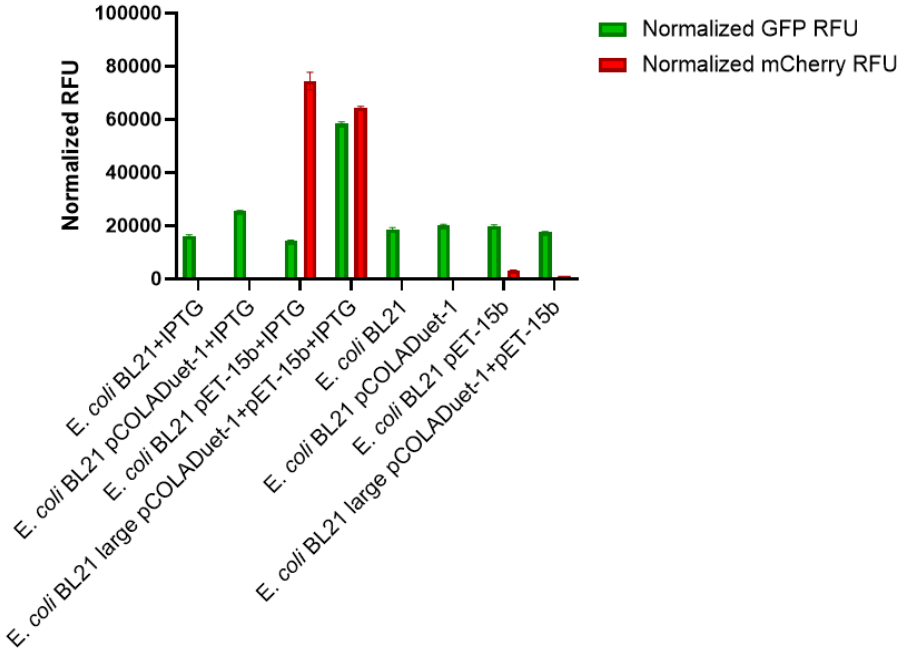


Raw:



Normalized by O.D 600nm: GFP

ON/OFF: 2.3



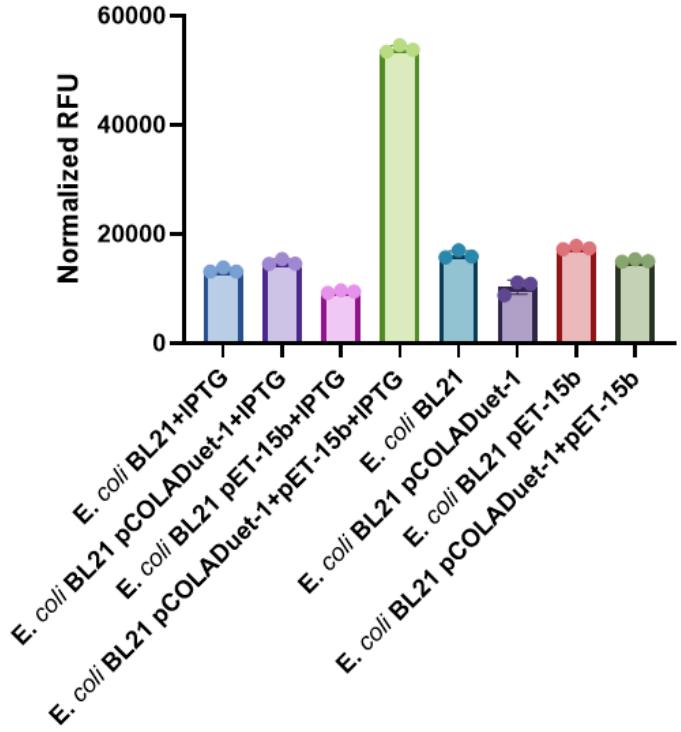
Normalized by O.D. 600nm and autofluorescence subtracted: GFP

ON/OFF: 3.63

On/OFF ratio with lousy autofluorescence normalization: 27.31

Pet+Duet+IPTG/Duet ON/OFF: 3.63

Pet+Duet+IPTG/Duet ON/OFF with lousy autofluorescence normalization: 23.41


Normalized RFU
60000
40000
20000
0
E. coli BL21+IPTG
E. coli BL21 pCOLADuet-1+IPTG
E. coli BL21 pET-15b+IPTG
E. coli BL21 pCOLADuet-1+pET-15b+IPTG
E. coli BL21
E. coli BL21 pCOLADuet-1
E. coli BL21 pET-15b
E. coli BL21 pCOLADuet-1+pET-15b

13/06/2026

GIBSON Planning

We want to make 3 plasmids:

pCOLADuet1-positive-control-sensorA-GFP
pET-15b-Empty
pET-15b-mCherryCS

Gibson 1 (G1) - aiming for the 1:2 ratio, creating pCOLADuet1-positive-control-sensorA-GFP.
Vector isert: 1:2-3 (25fmol:≈ 75fmol)

PCR	DNA bp	DNA concentration (ng/uL)	Quantity (fmol)	Volume (µL)
positive sensor A Gblock	180	10	90.2	1
Backbone (pCOLADuet-GFP	4053	26.6	21.3	2
H_2O	–	–	–	2
Mix (2x)	–	–	–	5
Total	–	–	–	10

Gibson 2 (G1) - aiming for the 1:5(because the insert is an oligo) ratio, creating pET-15b-Empty.

PCR	DNA bp	DNA concentration (ng/uL)	Quantity (fmol)	Volume (µL)
pET15-b-Empty	80	100nM	150	1.5
Backbone pET-15b-Restricted	6287	43.2	22.3	2
H_2O	–	–	–	1.5
Mix (2x)	–	–	–	5
Total	–	–	–	10

Gibson 3 (G1) - aiming for the 1:2 ratio, creating pET-15b-mCherryCS.

PCR	DNA bp	DNA concentration (ng/uL)	Quantity (fmol)	Volume (µL)
mCherryCS	774	20	62.9	1.5
Backbone pET-15b-Restricted	6287	43.2	22.3	2
H_2O	–	–	–	1.5
Mix (2x)	–	–	–	5
Total	–	–	–	10

14-15/07/2026

Gibson + Transformation

The reactions described yesterday were made today. Transformations were made the following day.

21/07/2026

Validation PCR

Validation PCR for colonies from the transformation plates of the new plasmids:

1. pCOLADuet1-positive-control-sensorA-GFP
2. pET-15b-Empty
3. pET-15b-mCherryCS

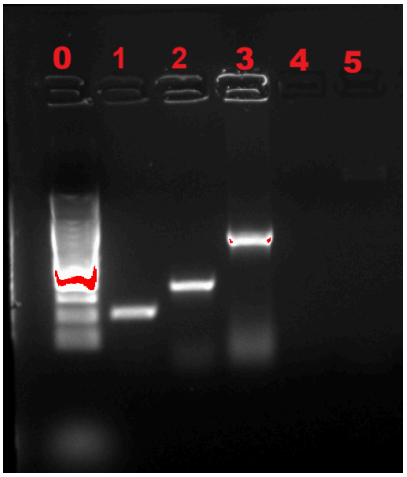
Reaction	1	2	3	4	5
Rapid Taq Mix	5ul	5ul	5ul	5ul	5ul
UPW	4.2ul	4.2ul	4.2ul	4.2ul	4.2ul
F1: Fr-pCOLADuet-PS-A-Val	0.4ul			0.4ul	
F2:Fr-pET-15b-mCherryCodS-Sanger		0.4ul			0.4ul
F3: Fr-pET-15b-mCherryCodS-val			0.4ul		
R1:Rev-pCOLADuet-PS-A-Val	0.4ul			0.4ul	
R2:Rev-pET-15b-mCherryCodS		0.4ul	0.4ul		0.4ul

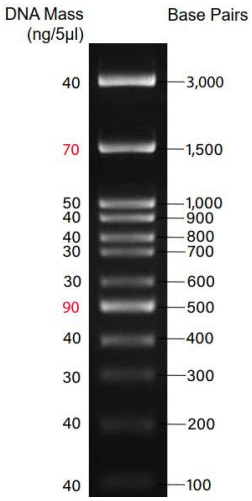
DNA (colony from plate)	pCOLADuet1-positive-control-sensorA-GFP	pET-15b-Empty	pET-15b-mCherryCS	x	x

Results:

It seems that the Gibson reaction and the PCR validation worked as planned:

- 0: ladder (?BioHelix 100bp DNA ladder?)
- 1: pCOLADuet1-positive-control-sensorA-GFP
- 2: pET-15b-Empty
- 3: pET-15b-mCherryCS
- 4: Negative control pCOLADuet-PS-A-Val
- 5: Negative control: pET-15b-mCherryCodS-val





22/06/2026

Mini prep and Sanger sequencing

Miniprep was performed to the three transformants, only pET-15b-Empty and pET-15b-mCherryCS came out with sufficient concentrations. The two were sent to Sanger sequencing using the Sanger primers and the reverse primer and came out successfully as intended.

28/07/2026

pCOLADuet positive control, Sanger

Mini-prep for pCOLADuet-positive resulted in 97ng/µl, starter was 20ml. Sent two identical samples of pCOLADuet-Positive to Sanger sequencing, one sample (called R+C) with primer: Rev-pCOLADuet-PS-A-Val, the other (S+C) with primer: Fr-pCOLADuet-PS-A-Sanger.

05/08/2026

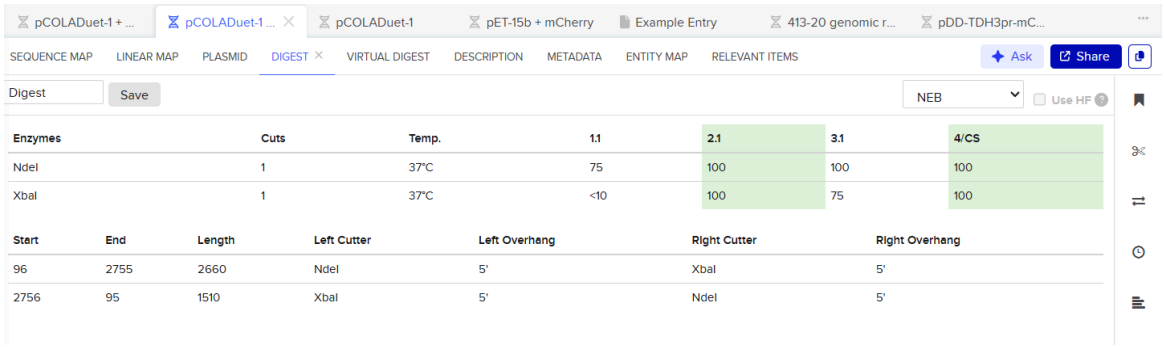
pCOLADuet positive control: Slicing + Gel
mCherryCS fluorescence(IPTG+-)

Slicing + Gel:

pCOLADuet positive control (as a validation) was mixed as mentioned below:

Element	Control (ul) - C	Plasmid (ul) - P
plasmid	5	5
cutsmart	-	1.5
Puer water	10	7.5
NdeI	-	0.5
XbaI	-	0.5

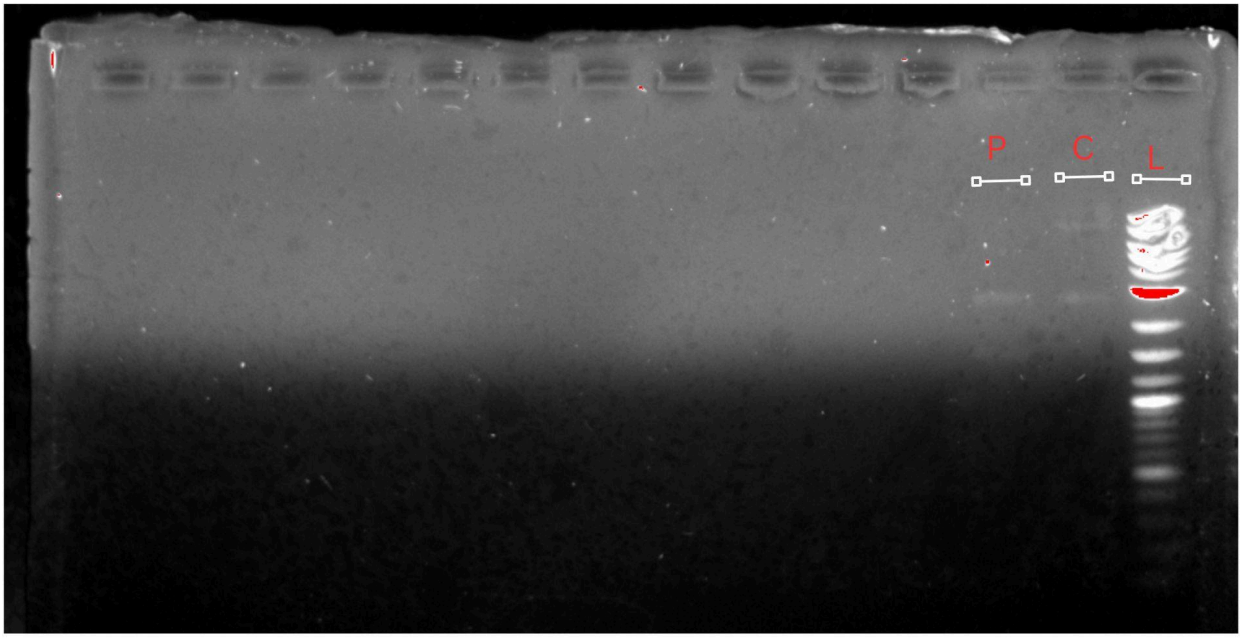
The mixes were incubated for 3 hours. Later, they were mixed with 3 µL of 6x loading dye, and 18 µL was loaded onto the Gel. The expected length of a successful cut by both enzymes was calculated using BenchLink:



Enzymes	Cuts	Temp.	1.1	2.1	3.1	4/CS
NdeI	1	37°C	75	100	100	100
XbaI	1	37°C	<10	100	75	100

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
96	2755	2660	NdeI	5'	XbaI	5'
2756	95	1510	XbaI	5'	NdeI	5'

The expected lengths match the bands seen in the gel, though the low signal might indicate the concentration of the plasmid is lower than we initially measured.



pCOLADuet positive control - IPTG activation:

The bacteria were treated in the following steps:

- The bacteria were diluted to 0.1 OD and then incubated for 1 hour.
- Diluted again to 0.2 OD.
- Incubated for 2 hours with IPTG and without it.
- OD was measured again (Listed in the table below)
- The samples were centrifuged at 4000 × g for 3 minutes; the supernatant was removed, and each pellet sample was suspended in PBS (300 µL).
- 100 µL of each sample was placed in the wells of a 96-well plate (black walls).

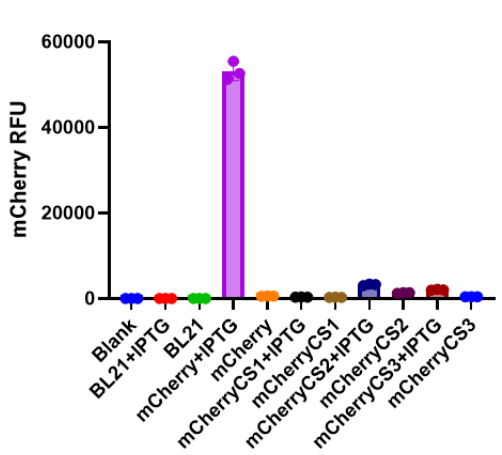
Sample	Genotype	Amp?	Pre Read 600nm O.D	IPTG mM
0	Blank (PBS)	0		0
1	BL21	0	1.21 I - 1.21	0/1
2	Pet15b Mcherry	1	1.23 I - 1.12	0/1
3	CS1	1	1.32 I - 1.37	0/1
4	CS2	1	1.23	0/1

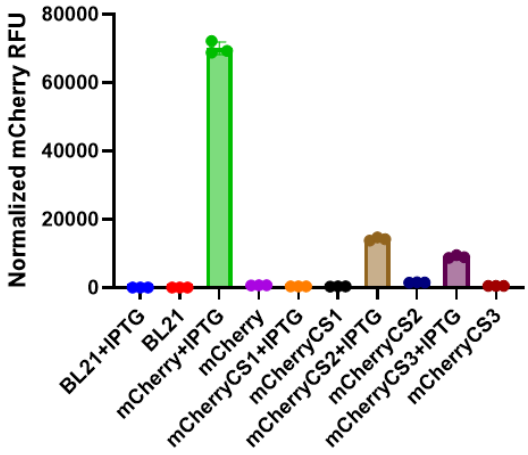
			I - 0.35	
5	CS3	1	1.24 I - 0.33	0/1

Plate Reader:

0	1IPTG	1	2IPTG	2	3IPTG	3	4IPTG	4	% I	5	
0	1IPTG	1	2IPTG	2	3IPTG	3	4IPTG	4	5 I	5	
0	1IPTG	1	2IPTG	2	3IPTG	3	4IPTG	4	5 I	5	

Results by 600nm O.D. from the plate reader:





13/06/2026

GIBSON Planning

We want to make 5 plasmids:

pCOLADuet1-R4-1
pCOLADuet1-R4-2
pCOLADuet1-R4-3
pCOLADuet1-R4-4
pCOLADuet1-R4-5

Gibson 1-5 (G1-5) - aiming for the 1:2 ratio, creating pCOLADuet1-pCOLADuet1-R4-1.
Vector isert: 1:2-3 (25fmol:≈ 75fmol)

PCR	DNA bp	DNA	Quantity (fmol)	Volume (µL)

		concentration (ng/uL)		
Gblock R4-1-5	180	10	90.2	1
Backbone (pCOLADuet-GFP	4053	26.6	21.3	2
H_2O	–	–	–	2
Mix (2x)	–	–	–	5
Total	–	–	–	10

11/08/2026

Gibson + Transformation

The reactions described yesterday were made today. Transformations were made the following day to DH5a.

13/08/2026

R4 MiniPrep + Sanger

Starters for pCOLADuet-R4-2 and pCOLADuet-R4-4 did not grow. Sent 3 remaining for sanger sequencing with Rev-pCOLADuet-PS-A-Val or Fr-pCOLADuet-PS-A-Sanger:

Sanger Tube names	pCOLADuet-R4-1	pCOLADuet-R4-3	pCOLADuet-R4-5
Rev-pCOLADuet-PS-A-Val	R4-1-V	R4-3-V	R4-5-V
Fr-pCOLADuet-PS-A-Sanger	R4-1-S	R4-3-S	R4-5-S

Here's today's entry in your notebook style, everything scaled 1:5 from the 14/06 TSS prep. I kept Amp in the growth steps since this strain carries pET-15b-mCherryCS.

18/08/2026

Competent Preparation — BL21 pET-15b-mCherryCS

Chemically competent cells were prepared from the BL21 pET-15b-mCherryCS strain, scaled down 1:5 from the standard protocol. Amp (1:2000) was included in all growth steps

Day 1

10 ml LB+Amp starter in a 50 ml tube.

Incubated overnight at 37°C with shaking.

Day 2

Transfer 0.1 ml from starter into the 20 ml LB+Amp 50 ml flask.

Grow at 37°C with shaking to OD600 = 0.37

Incubate on ice for 20 min.

Centrifuge at 3000 RPM for 10 min at 4°C.

Discard supernatant carefully and completely.

Resuspend cells in 1 mL pre-chilled TSS. Vortex gently for full resuspension.

TSS Buffer (1 mL total)

Component	Quantity
PEG 3350 50%	200 µL
$MgCl_2$ (1 M)	30 µL
DMSO	50 µL
LB	to 1 mL

Filtered.

Incubate 15 min on ice.

Dispense 50 µl aliquots into pre-chilled Eppendorf tubes (~20 aliquots).

Freeze aliquots at −80°C, green box, second shelf.

Patch plating — BL21 DH5a patches

Because we weren't able to grow R4-2 and R4-4 DH5a, and the first round of sequencing for the other plasmids didn't work, we plated 8 patches for each transformant plate of variants, only R4-1/3/5 grew, while R4-2 and R4-4 didn't. We need to repeat the Gibson + DH5a for them.

11/08/2026

Gibson

We're attempting to make 2 plasmids that we didn't manage to make the last time:

pCOLADuet1-R4-2
pCOLADuet1-R4-4

Gibson 1+2 (G1-5) - aiming for the 1:2 ratio, creating pCOLADuet1-pCOLADuet1-R4-2/4.
Vector isert: 1:2-3 (25fmol:≈ 75fmol)

PCR	DNA bp	DNA concentration (ng/uL)	Quantity (fmol)	Volume (µL)
Gblock R4-2/4	180	10	90.2	1
Backbone (pCOLADuet-GFP	4053	26.6	21.3	2
H_2O	–	–	–	2
Mix (2x)	–	–	–	5
Total	–	–	–	10

Transformation

The 3 other plasmids (R4-1/3/5 + pCOLADuet-Positive-Sensor-A (which came back from sequencing as true)) are being transformed into E. *coli* BL21 MCH (pET-15b with mCherry) and E. *coli* BL21 MCHCS (pET-15b with mCherryCS (recoded)).
The double plasmid transformations (R4-1/3/5 + pCOLADuet-Positive-Sensor-A) were plated on "kan 10+amp 50" LB plates.
The DH5a pCOLADuet1-R4-2/4 were plated on "kan 10" plates.

25/08/2026

Miniprep + Transformation

We performed miniprep in accordance with the Plasmid EasyPure protocol for the samples written in the table below:

Source	Colony	Concentration (ng/uL)
R4-2	#5	66
R4-2	#1	107.1
R4-4	#1	118.7
R4-4	#6	126.7

* all of the samples had standard NanoDrop figures.

Transformation

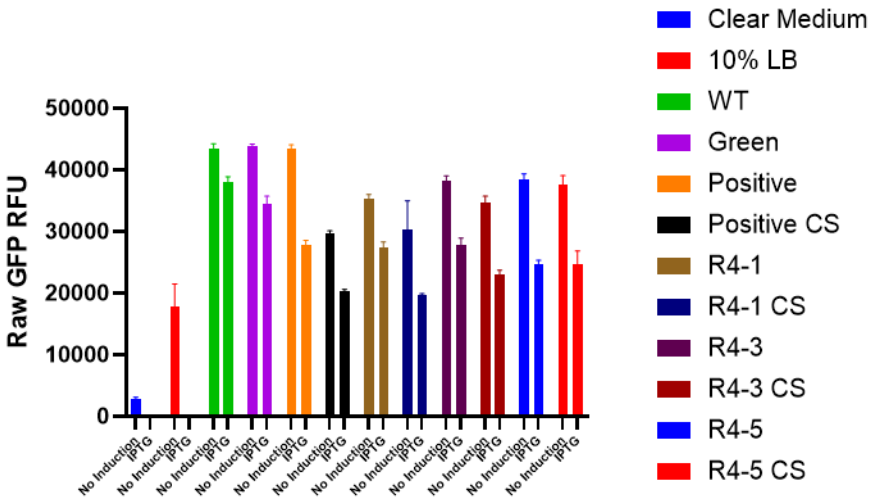
We then used the DNA extraction for transformation for the following sources: R4-2#1, R4-4#6. Each of those we transformed into BL21+MCH/MCH-CS and then plated them on amp+kan plates

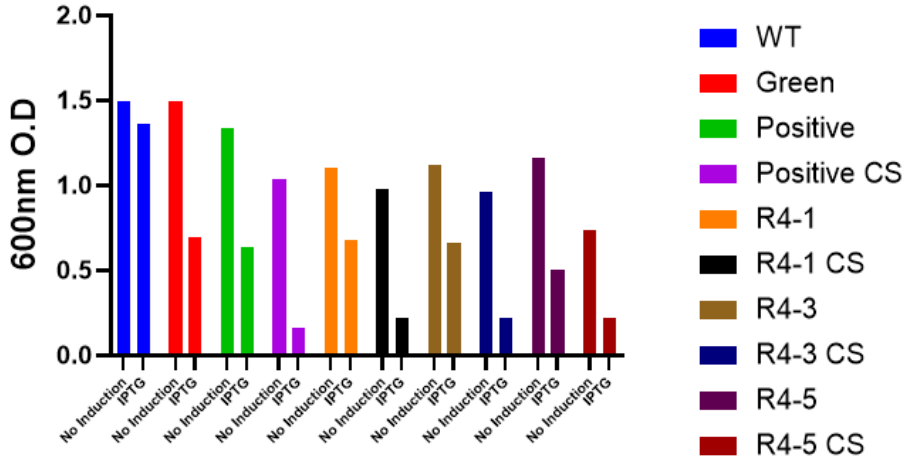
26/08/2026

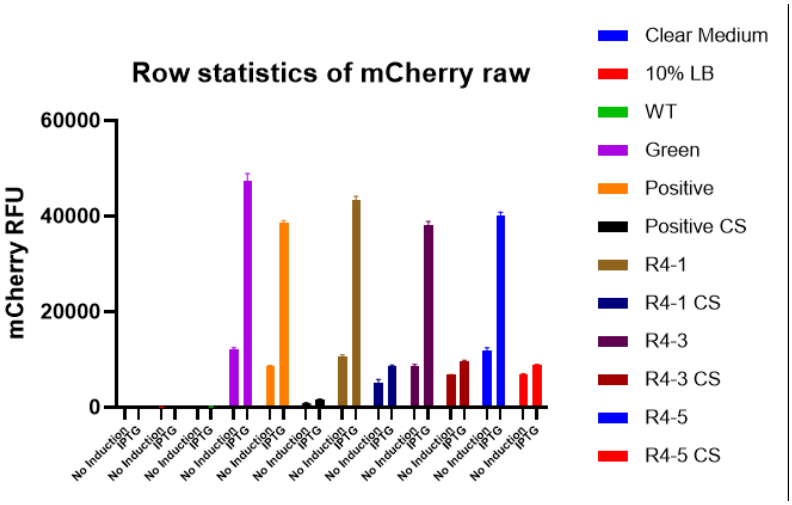
CERNAL Toeholds (R4-1,3,5) + IPTG controls+ Positive+mCherry CS

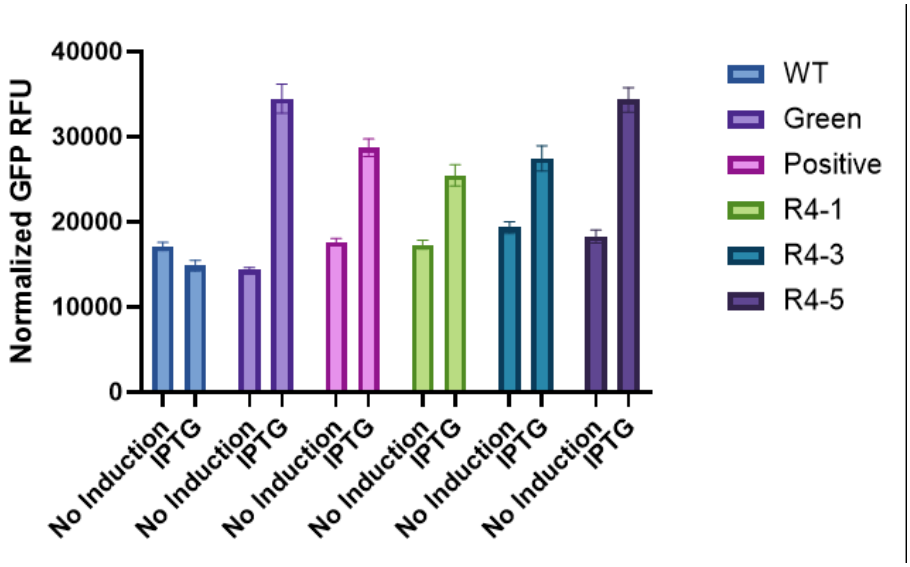
We performed a test experiment for our original toeholds (R4-1, R4-3, and R4-5) with and w/o IPTG, mCherry/mCherry-CS, and original positive controls. Starters were incubated O/N, then concentrations were measured at 600nm O.D. The samples were diluted to 0.2 600nm O.D. in Neidhardt MOPS medium (clear medium), incubated with and without 1 mM IPTG for 2 h, then measured again by spectrophotometry, plated in a 96-well plate, and measured again for 600nm O.D., GFP fluorescence, and mCherry fluorescence. The plate table map: x=IPTG

0	1	2	3	4	5	6	7	8	9	10
0	1	2	3	4	5	6	7	8	9	10
0	1	2	3	4	5	6	7	8	9	10
0	1X	2X	3X	4X	5X	6X	7X	8X	9X	10X
0	1X	2X	3X	4X	5X	6X	7X	8X	9X	10X
0	1X	2X	3X	4X	5X	6X	7X	8X	9X	10X

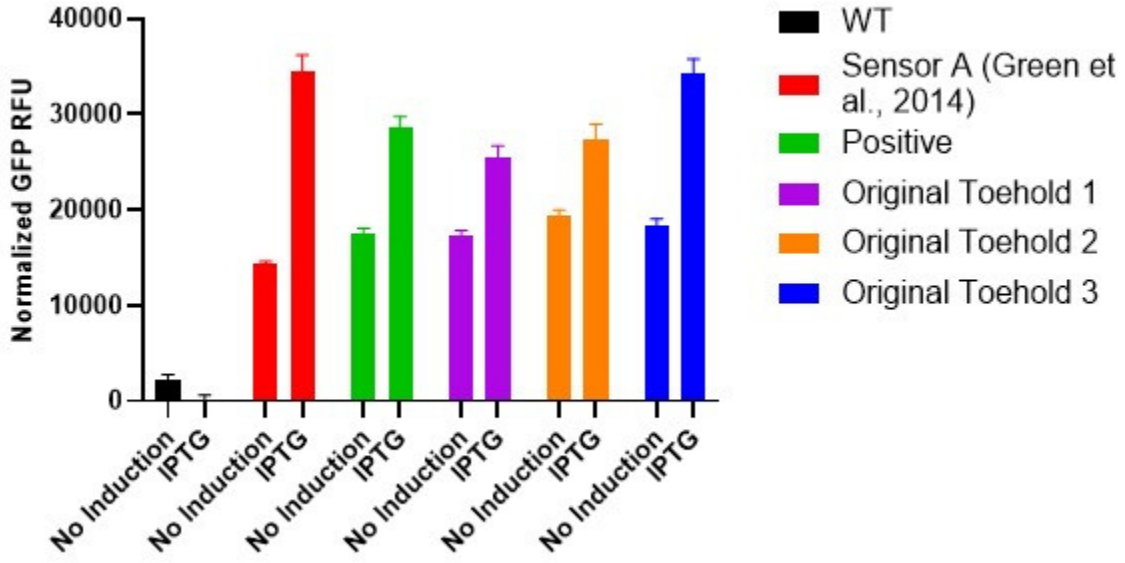

Raw GFP RFU
50000
40000
30000
20000
10000
0
No Induction
IPTG
Clear Medium
10% LB
WT
Green
Positive
Positive CS
R4-1
R4-1 CS
R4-3
R4-3 CS
R4-5
R4-5 CS


600nm O.D
2.0
1.5
1.0
0.5
0.0
No Induction
IPTG
WT
Green
Positive
Positive CS
R4-1
R4-1 CS
R4-3
R4-3 CS
R4-5
R4-5 CS


Row statistics of mCherry raw
mCherry RFU
60000
40000
20000
0
No Induction
IPTG
Clear Medium
10% LB
WT
Green
Positive
Positive CS
R4-1
R4-1 CS
R4-3
R4-3 CS
R4-5
R4-5 CS



This graph
It seems that 10% LB has GFP fluorescence, next time, we should incubate completely in Neidhardt MOPS medium. mCherry CS doesn't seem to function



02/09/2026

CERNAL Toeholds (R4-1-5) + IPTG controls+ Positive
R4-2 + R4-2 Sanger

The day before (01/09/2026), we prepared 3ml starters as described below, using Neidhardt MOPS medium (clear medium) instead of LB:

Starter	antibiotics
BL21 WT	-
Green	amp + kan
Positive control	amp + kan
R4-1:5	amp + kan

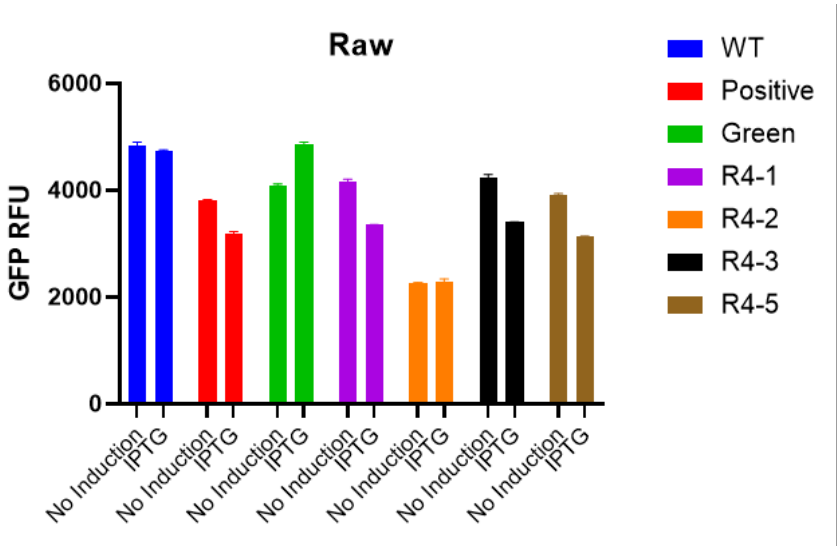
The starter of R4-4 patch 1 didn't show any growth and therefore was dropped from the rest of the experiment below:

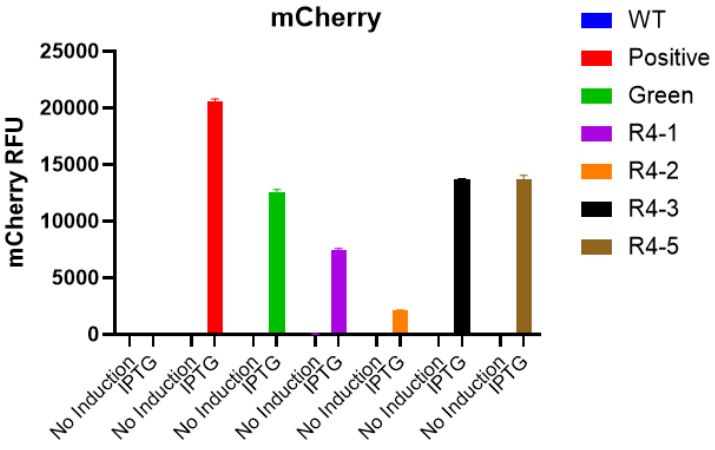
1. The starters were diluted to 0.2 OD600; they were then incubated for 2 hours.
2. The starters were diluted again to 0.2 OD600; this time each sample was divided to samples with and without IPTG at 1 mM. They were then incubated for 2 hours.
3. The samples were then plated in a 96-well plate and measured again for 600nm O.D., GFP fluorescence, and mCherry fluorescence.

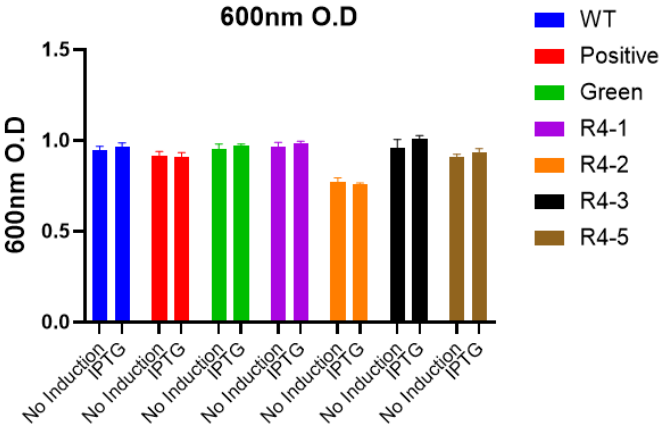
Plate map:

Bl21	Bl21	Bl21	Bl21 + IPTG	Bl21 + IPTG	Bl21 + IPTG
PC	PC	PC	PC + IPTG	PC + IPTG	PC + IPTG
Green	Green	Green	Green + IPTG	Green + IPTG	Green + IPTG
1	1	1	1 + IPTG	1 + IPTG	1 + IPTG
2	2	2	2 + IPTG	2 + IPTG	2 + IPTG
3	3	3	3 + IPTG	3 + IPTG	3 + IPTG
5	5	5	5 + IPTG	5 + IPTG	5 + IPTG

The experiment led to several unexplained results, including the negative control BL21 allegedly producing GFP without producing mCherry. The experiment needs to be redone for better clarity. You can view the raw results and their analysis here.
Just medium samples are missing.







R4-2 #1 + R4-4 #6 were sent for Sanger sequencing.

06/09/2026

R4-2 + R4-4 sequencing results: R4-2 is incorrect (possibly a sequencing error), R4-4 is the correct sequence.

10/09/2026

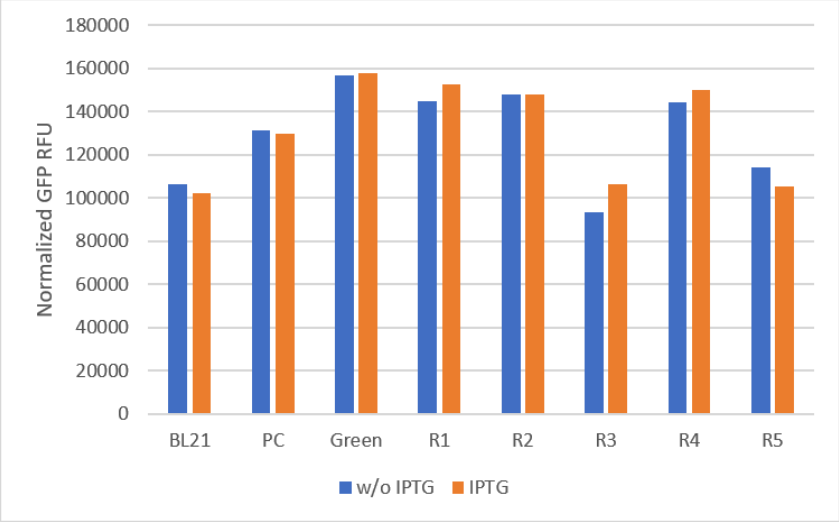
CERNAL Toeholds (R4-1-5) + IPTG controls

we prepared 3ml starters as described below, using Neidhardt MOPS medium (clear medium) instead of LB:

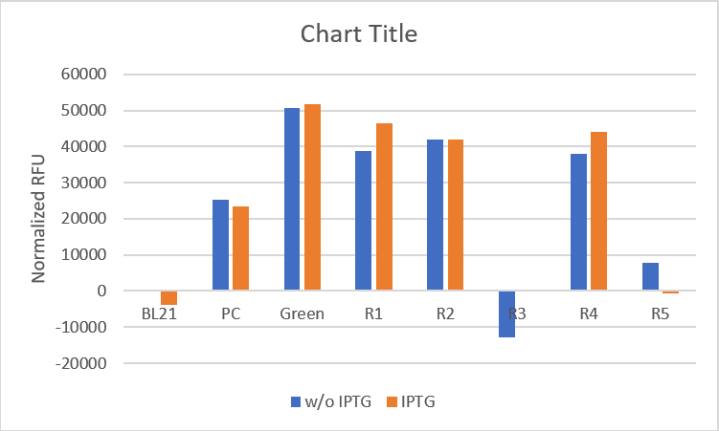
Starter	antibiotics
BL21 WT	-
Green	amp + kan
Positive control	amp + kan
R4-1:5	amp + kan

4. The starters were diluted to 0.2 OD600; they were then incubated for 1.5 hours.
5. The starters were diluted again to 0.2 OD600; this time each sample was divided to samples with and without IPTG at 1 mM. They were then incubated for 3 hours.
6. The samples were then plated in a 96-well plate and measured again for 600nm O.D., GFP fluorescence, and mCherry fluorescence.

Prewash (medium omission)

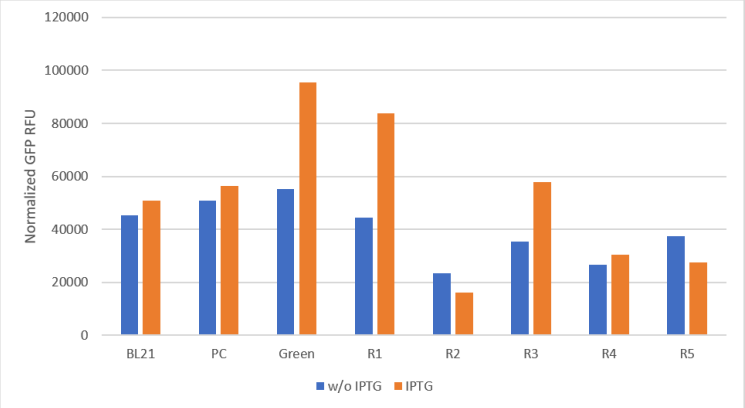


Prewash (medium and BL21 omission)

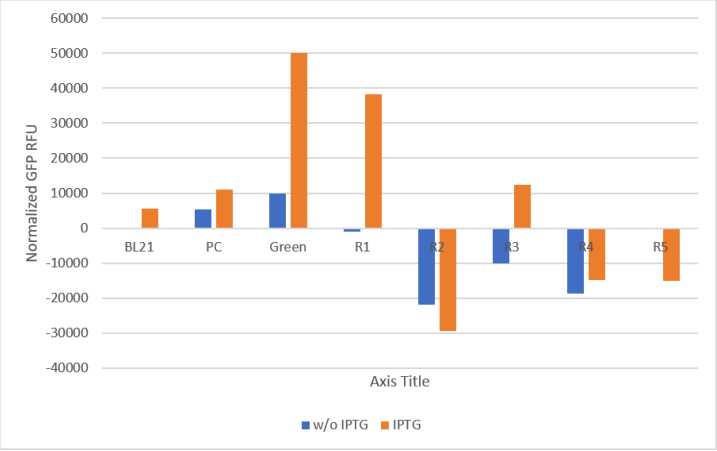


We then decided to wash the Neidhardt MOPS with PBS and then measure again:

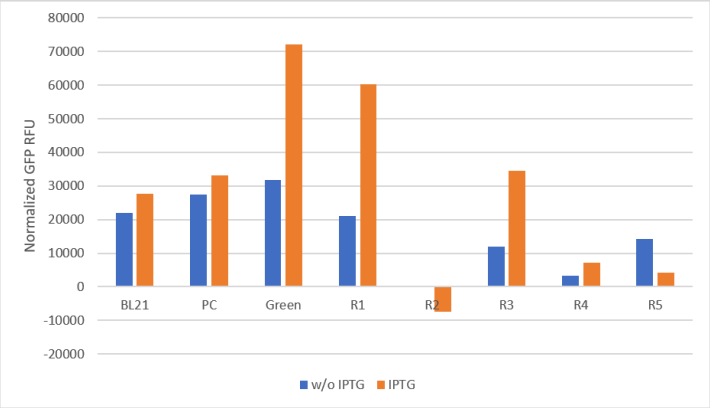
After Wash (medium omission)



After Wash (medium and BL21 omission)



After Wash (medium and R2 omission)



GFP	on/off
BL21	1.253982
PC	1.204971
Green	2.270348
R1	2.871014
R2	-3E+15
R3	2.871389
R4	2.251653
R5	0.293115

14/09/2026

CERNAL Toeholds (R4-1,2,3,4,5) + IPTG controls+ Positive

We performed a test experiment for our original toeholds (R4-1-5) with and w/o IPTG, mCherry, and the original positive controls. Starters were incubated O/N, then concentrations were measured at 600nm O.D. The samples were diluted to 0.2 600nm O.D. in Neidhardt MOPS medium (clear medium), incubated for 1.5 hours, and then with and without 1 mM IPTG for 3 h, then measured again by spectrophotometry, plated in a 96-well plate, and measured again for 600nm O.D., GFP fluorescence, and mCherry fluorescence. The plate table map: x=IPTG

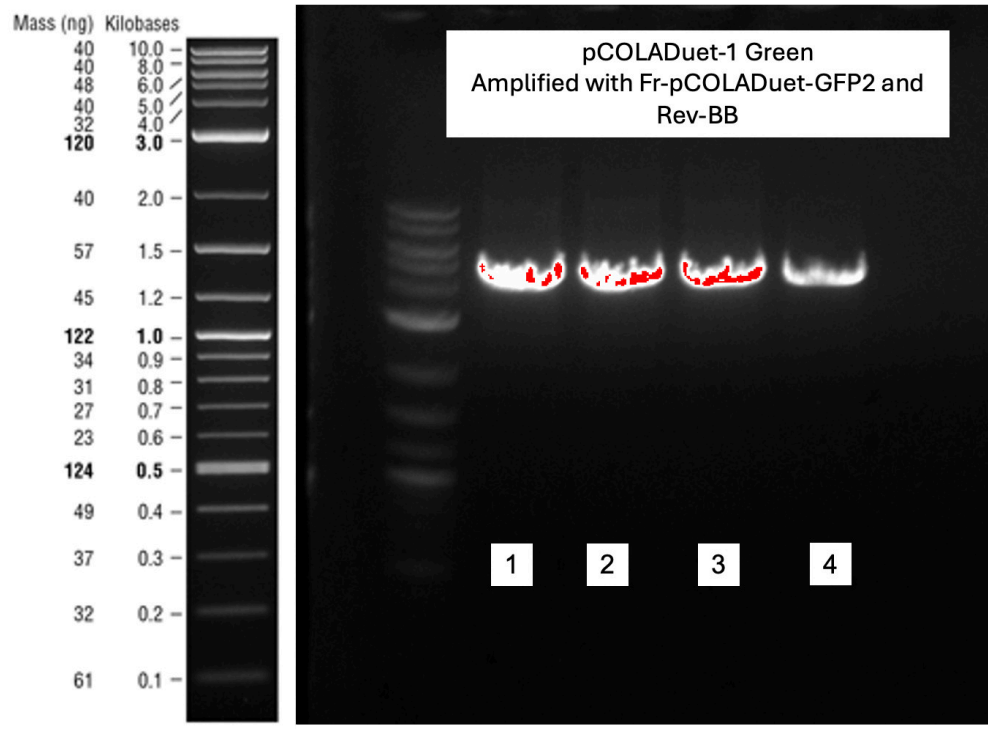
BL21	BL21	BL21	BL21X	BL21X	BL21X
PC	PC	PC	PCX	PCX	PCX
Green	Green	Green	GreenX	Green X	GreenX
R1	R1	R1	R1X	R1X	R1X
R2	R2	R2	R2X	R2X	R2X
R3	R3	R3	R3X	R3X	R3X
R4	R4	R4	R4X	R4X	R4X
R5	R5	R5	R5X	R5X	R5X
MOPS	MOPS	MOPS			

15/09/2026

PCR pCOLADuet with Fr-GFP2

Performed PCR to amplify the backbone, pCOLADuet-1 Green, using the new Fr-pCOLADuet-GFP2 with Rev-BB. Using PhantaFlash with minimal plasmid (less than 1ng per

reaction), 25sec elongation, 30 cycles over a gradient of temperatures; tube 1 at 56℃,2 at 58.5℃, 61℃, 64℃ and tube 5 at 68℃.



Gel electrophoresis 1% - *Amplification was successful until about 64℃ (lane 4), with 68℃ being completely negative*.

After combining tubes and clean-up, conc. was about 160 ng/µl.

– Shoat Y.

16/09/2026

GIBSON + transfomations

We want to make 8 plasmids:

pCOLADuet1-C1
pCOLADuet1-C2
pCOLADuet1-C3
pCOLADuet1-C4
pCOLADuet1-C5
pCOLADuet1-C6
pCOLADuet1-C7
pCOLADuet1-C8

Gibson 1-5 (C1-8) - aiming for the 1:2 ratio, creating pCOLADuet1-pCOLADuet1-R4-1.
Vector isert: 1:2-3 (25fmol:≈ 75fmol)

PCR	DNA bp	DNA	Quantity (fmol)	Volume (µL)

		concentration (ng/uL)		
Gblock C1-8	160	10	101.4	1
Backbone (pCOLADuet-GFP2(backbone)	4053	164	64.62	0.5
H_2O	–	–	–	3.5
Mix (2x)	–	–	–	5
Total	–	–	–	10

After the reactions, we transformed the plasmids into DH5a and plated them on LB +kan plates.

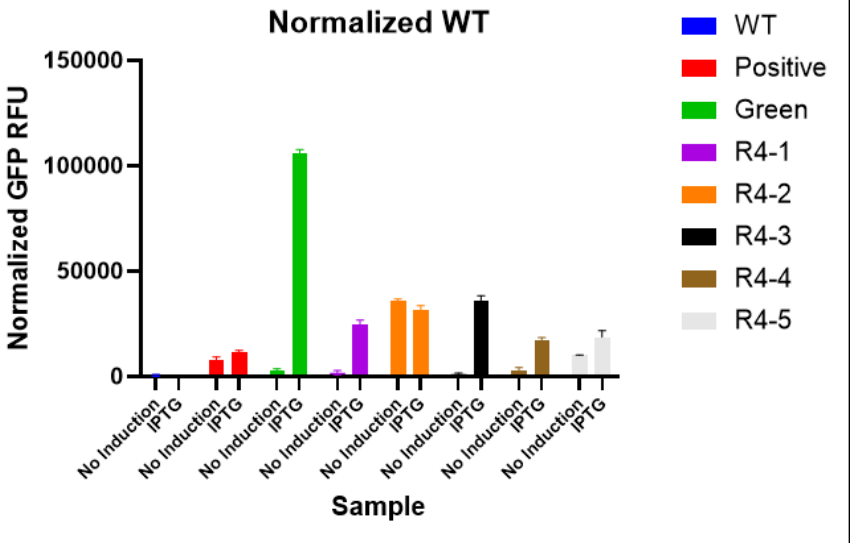
CERNAL Toeholds Prep (R4-1-5) + IPTG controls Rep 4

We prepared 3ml starters as described below, using Neidhardt MOPS medium (clear medium) instead of LB:

Starter	antibiotics
BL21 WT	-
Green	amp + kan
Positive control	amp + kan
R4-1:5	amp + kan

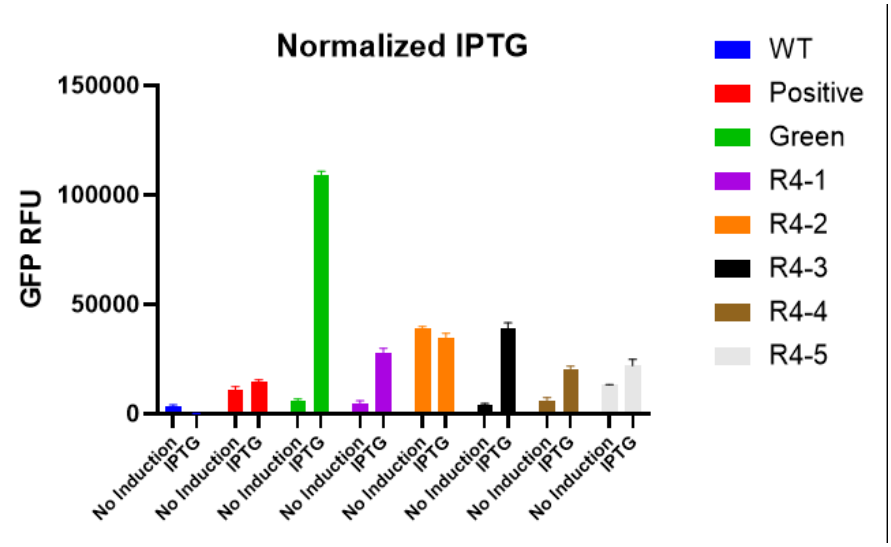
7. The starters were diluted to an OD600 of 0.2 and incubated for 1.5 hours.
8. The starters were diluted again to 0.2 OD600; this time, each sample was split into samples with and without 1 mM IPTG. They were then incubated for 3 hours.
9. The samples were then washed in PBS and then plated in a 96-well plate and measured again for 600nm O.D., GFP fluorescence, and mCherry fluorescence.

Results:
When normalized by IPTG WT (BL21):

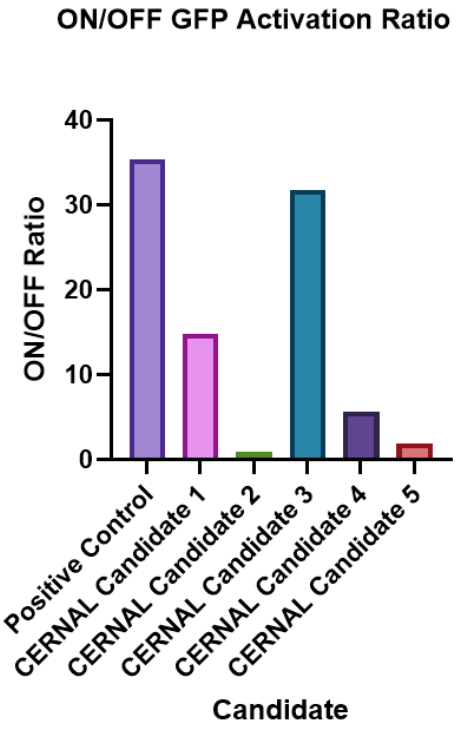


	AVG1	AVG2	ON/OFF
WT	3192.5	2E-12	8E-16
PC	11036	14549	1.3184
GR	6190.5	109250	17.648
1	4848.1	27688	5.7111
2	39108	34511	0.8824
3	4317.3	38921	9.0151
4	6166.3	20073	3.2553
5	13226	21930	1.6581

When normalized by IPTG WT (BL21):



	AVG1	AVG2	ON/OFF
WT	-1E-12	-3193	3E+15
PC	7843.2	11357	1.448
GR	2997.9	106057	35.377
1	1655.6	24496	14.796
2	35916	31318	0.872
3	1124.8	35728	31.765
4	2973.7	16880	5.6765
5	10034	18738	1.8675



17/09/2026

CERNAL new round of positive control + IPTG

We prepared 3ml starters as described below, using Neidhardt MOPS medium (clear medium) instead of LB:

Starter	antibiotics
BL21 WT	-
Positive control	amp + kan

10. The starters were diluted to an OD600 of 0.2 and incubated for 1.5 hours.
11. The starters were diluted again to 0.2 OD600; this time, each sample was split into samples with and without 1 mM IPTG. They were then incubated for 3 hours.
12. The samples were then plated in a 96-well plate and measured for GFP fluorescence and mCherry fluorescence.
13. OD was not measured because we accidentally used a black plate, which doesn't allow for OD measurement. In addition, the BL21 was probably contaminated. Therefore, no meaningful results can be drawn from this experiment.

21/09/2026

Apparently, the DH5α competent bacteria used on 16/09 were contaminated with E.coli kan+ and mCherry-expressing (unlike what our plasmid should induce).

23-24/09/2026

We tried moving the switch assay to M1 medium (RCVBN), but for some reason, only some of the E. *coli* strains were not able to grow in it.

24/09/2026

We performed another transformation of pCOLADuet-1 C1-C8 (NOT candidates) in bought DH5a (donated by another lab), plated on LB+kan.

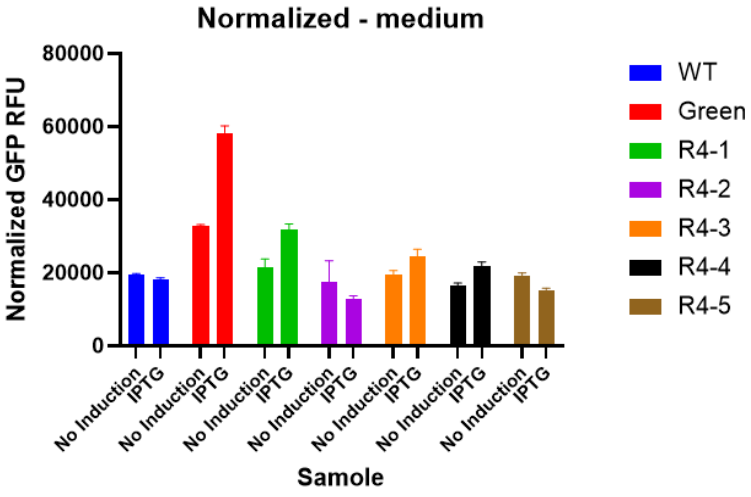
25/09/2026

The colonies have grown on the plates, the bigger colonies were transferred to other LB+kan plates to create patches, and also for starters (miniprep) and colony PCR for validation

28/09/2026

Starters for transformants of the Gibson plasmids NOT grown (but C7 and C8) each from colony one of the patch plates, and mini-prep was performed
PCR reactions (validation for NOT gates Gibson C1-C8), and two colony PCR reactions (7 patch 1, and 8 patch 2)

Normal1	on/off
BL21	0.931354
Green	1.776914
1	1.49146
2	0.731956



3	1.24942
4	1.331879
5	0.79185

Normal1	on/off
BL21	0.806883
Green	2.259049
1	2.195069
2	0.033278
3	1.699254
4	2.426495
5	0.40373

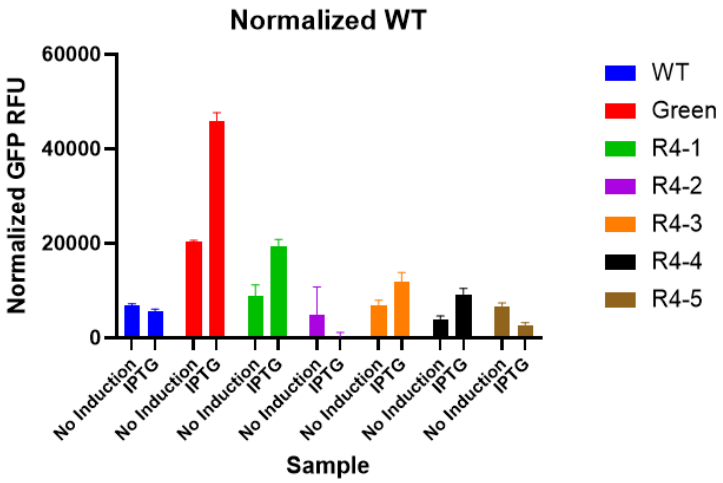


Table for a single reaction

Component	X1 (uL)	X10 (uL)
PANTA MIX	12.5	125
F: Fr-pCOLADuet-PS-A-Sanger	1	10
R: Rev-pCOLADuet-PS-A-Val	1	10
DNA (from colonies)	1	1
UPW	9.5	95

1.10.2026

Miniprep and purified C7 (patch #1) and C8 (patch #2) plasmids and got about ~70ng/µl of plasmid each.

3.10.2026

NOT plasmid sequencing results

Plasmidsaurus sequences for the 6 plasmids pCOLADuet C1-C6 came back:
C1- As expected
C2- Almost as expected, 1 point mutation in KanR.
C3- Almost as expected, 1 point mutation in KanR.
C4- Almost as expected, 1 point mutation in GFP- silent: GAG to GAA = Glu.
C5- Almost as expected, 1 point mutation in LacI.
C6- As expected.

5.10.2026

Gibson R5 CERNAL Toeholds

PCR	DNA bp	DNA concentration (ng/uL)	Quantity (fmol)	Volume (µL)
Gblock R5-21-25	160	10	101.4	1
Backbone (pCOLADuet-GFP2(backbone)	4053	164	64.62	0.5
H_2O	–	–	–	3.5
Mix (2x)	–	–	–	5
Total	–	–	–	10

The products were then transformed into DH5a (5 candidates).

NOT plasmids-MCH transformants patches transferring

All transformants grew on kan+amp plates and were transferred into patches on different plates.

Toeholds R4 kinetics

To check whether there are more optimal measurement points, and whether it varies for each candidate, we diluted every candidate to 0.2O.D 600nm, and let it reach the log phase (1 h incubation) in Reinhardt MOPS and then again to 0.2O.D 600nm with and without IPTG and plated them in a 96-well plate, and measured 600nm O.D., GFP, and mCherry fluorescence in 10-minute intervals continuously for 3 hours.

Because of the Reinhardt MOPS tendency to cause E.coli autofluorescence for GFP, results were not as successful as intended.

Construct	ON/OFF (total)	ON/OFF (specific)	Leak vs background	Relative leak	Welch *p*	Read as
Green Toehold	1.43	**7.9**	1.05×	13%	0.03	Works; true ratio is higher than this
Toehold1	1.02	n.d.	1.04×	n.d.	0.32	No switching
Toehold2	1.45	3.3	1.18×	31%	0.009	Confounded by growth arrest
Toehold3	1.03	n.d.	1.09×	n.d.	0.034	No switching
Toehold4	—	—	—	—	—	Invalid — wells never grew
Toehold5	0.9	n.d.	1.19×	n.d.	0.043	No switching

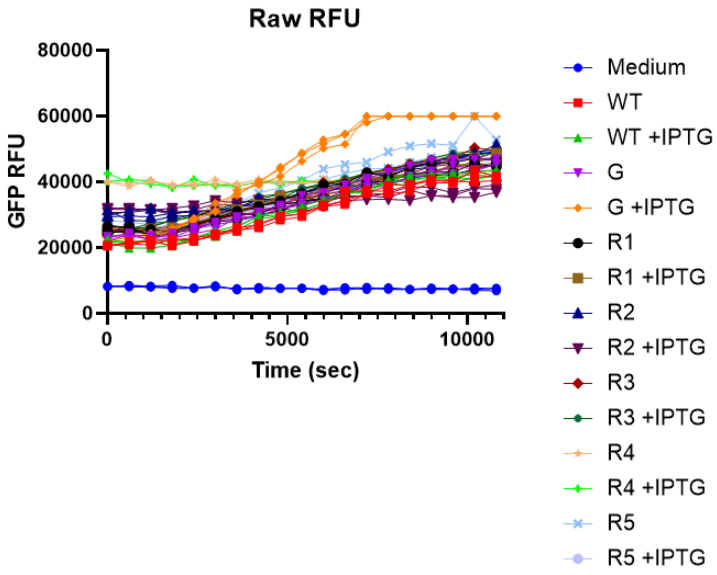


Figure 1 — Growth. Toehold4 wells never grew; Toehold2 +IPTG arrests at ~0.17

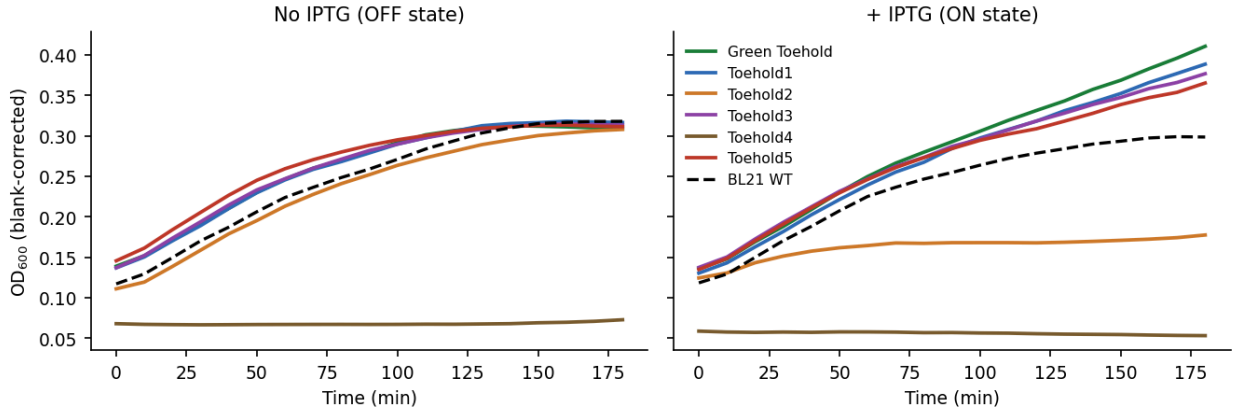


Figure 2 — Input arm: IPTG-driven mCherry. Induction works in every construct except Toehold4

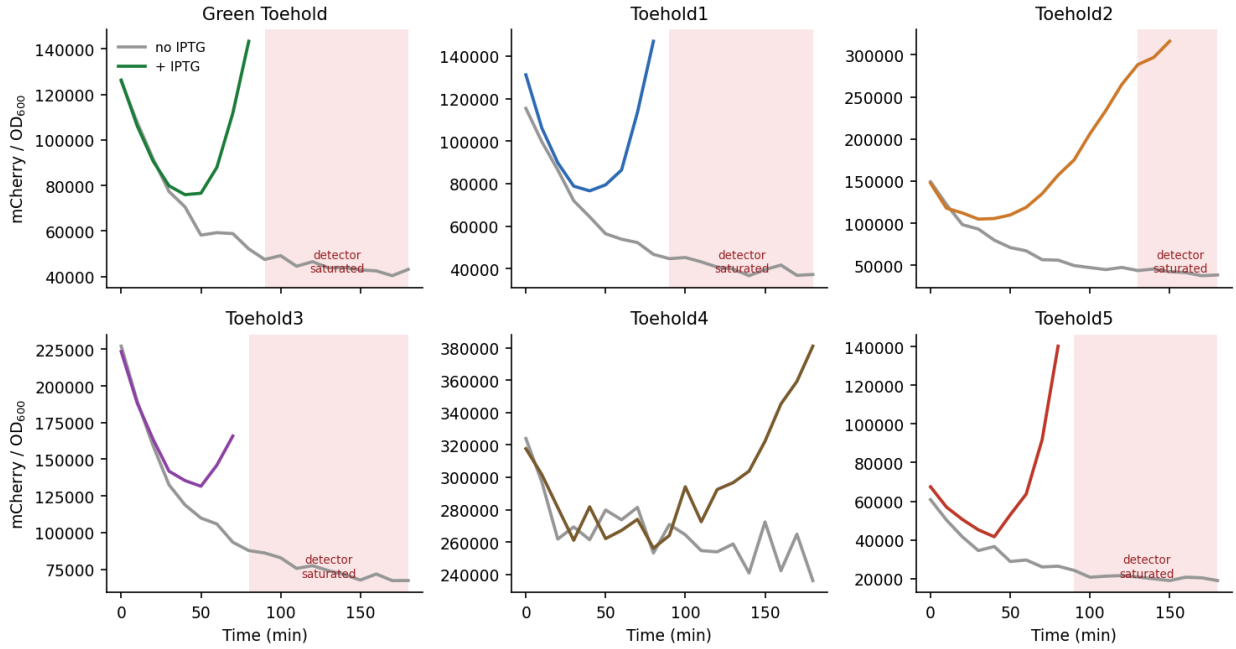


Figure 3 — Output arm: GFP. Only Green Toehold rises above BL21 autofluorescence on IPTG

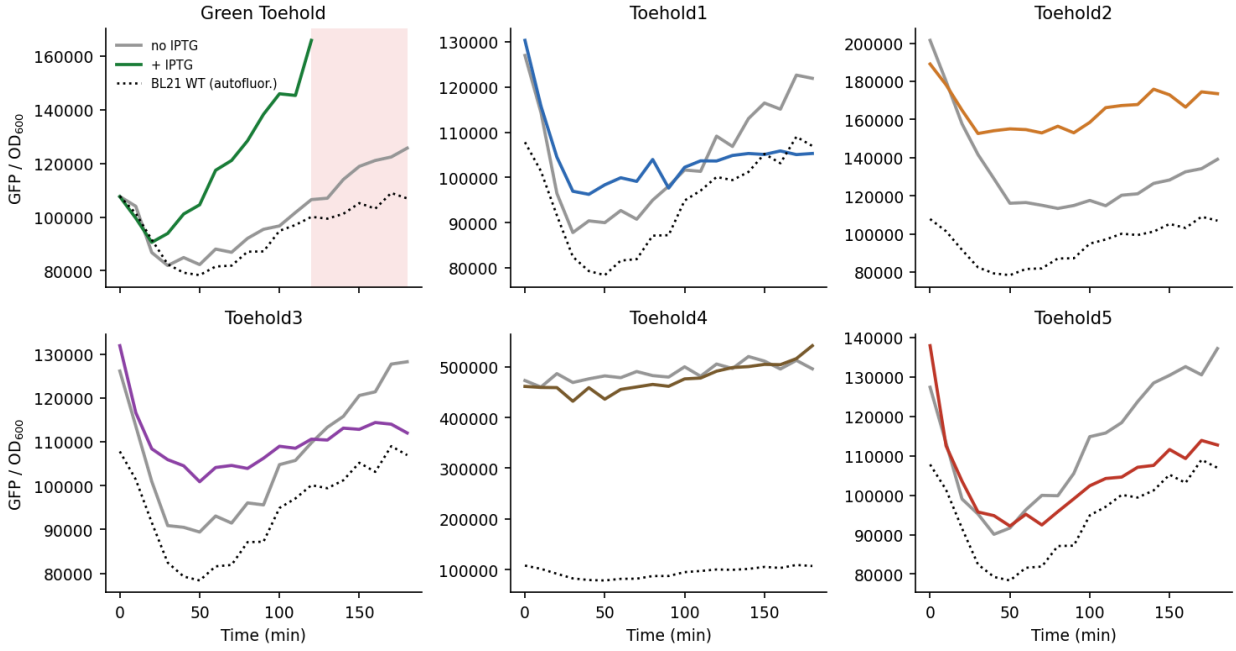
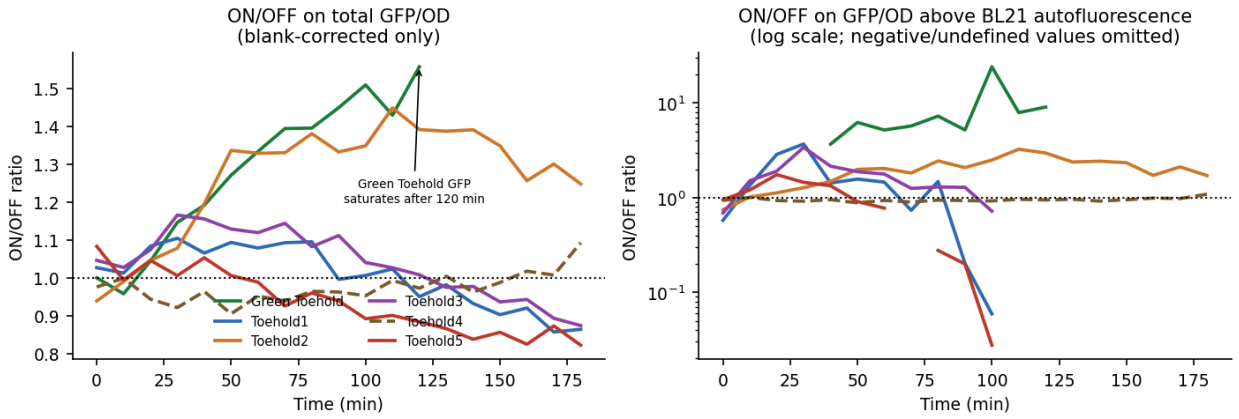


Figure 4 — ON/OFF ratio over time

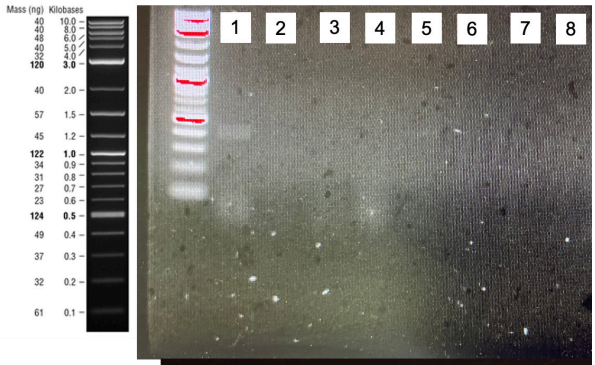


6.10.2026

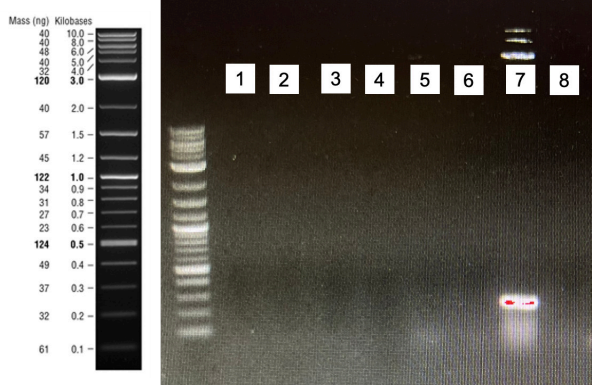
Colony Validation

To verify that the DH5a pCOLADuet-R5-21-25 are positive for the Gibson products and can be sequenced, validation PCR amplifying the whole switch:

	Volume (µl)
Rapid Taq	5
Fr + Rev Primers mix (0.4mM) Fr-pCOLADuet-PS-A-Sanger Rev-pCOLADuet-PS-A-Val	0.4
H_2O	4.6
Total	10



Gel electrophoresis - 1, 2 are colonies 1,2 of R5-21 / 3, 4 are colonies 1,2 of R5-23 / 5, 6 are colonies 1,2 of R5-24 / 7, 8 are colonies 1,2 of R5-25.



Gel electrophoresis - 1, 2 are colonies #3,4 of R5-21 / 3, 4 are colonies #3,4 of R5-23 / 5, 6 are colonies #3,4 of R5-24 / 7, 8 are colonies #3,4 of R5-25.

Colony #3 of R5-25 had a smaller product then expected - between 300-200bp vs the expected 400-500bp

11.10.2026

Gibson (Second time) R5 CERNAL Toeholds

To verify the failure of the validation PCR, the whole reaction was performed again:

PCR	DNA bp	DNA concentration (ng/uL)	Quantity (fmol)	Volume (µL)
Gblock R5-21-25	160	10	101.4	1
Backbone (pCOLADuet-GF P2(backbone)	4053	164	64.62	0.5
H_2O	–	–	–	3.5
Mix (2x)	–	–	–	5
Total	–	–	–	10

The products were then transformed into DH5α (5 candidates) and plated on LB+kan plates.

13.10.2026

NOT-C7-C8

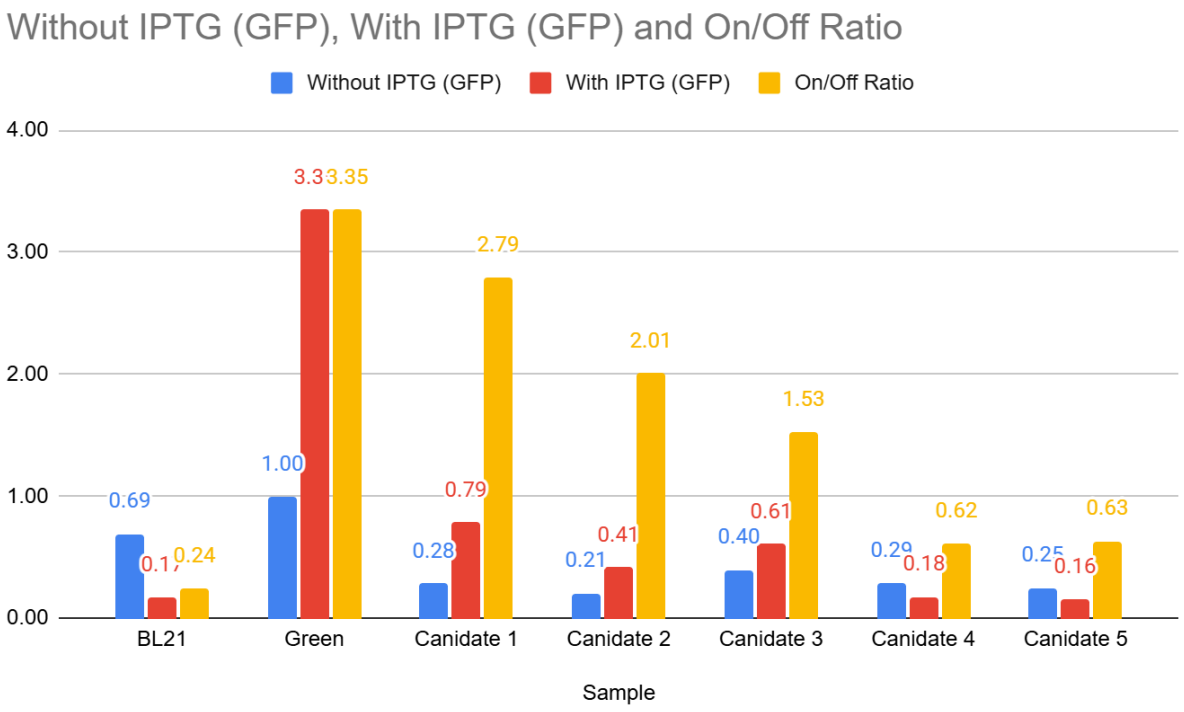
Sequencing results from Sanger sequencing came back for NOT candidates C7 and C8, both came out as intended (positive).
We transferred DH5α to patches on LB+kan plates, and picked one colony (colony 1) for each candidate as a starter in LB+kan for mini-prep the following day.

Flow cytometry

We measured our toehold candidates and Green's toehold performance via flow cytometry. We first followed the same procedure as in the plate reader protocol (ie starters in LB, dilution to OD 0.2, incubation for 2 hours, and second dilution to OD0.2 and incubation with IPTG for 2 hours). We then diluted the samples in PBS in a 3:1 ratio, same as Alexander Green did in this article ().

We applied the same gating and analysis pipeline as Green and got the following results:





GFP median without lowest results compensation:

Sample	GFP Median Without IPTG	GFP Median With IPTG
BL21	3.66	3.13
Green	2.96	6.31

Candidate 1	3.25	3.75
Candidate 2	3.17	3.38
Candidate 3	3.36	3.57
Candidate 4	3.25	3.14
Candidate 5	3.21	3.12

GFP median with lowest results compensation:

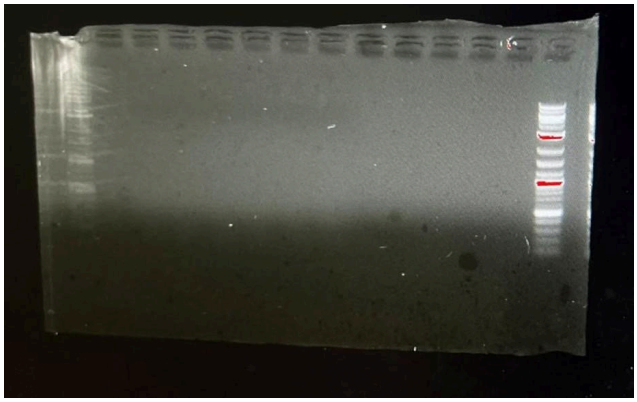
Sample	GFP Median Without IPTG	GFP Median With IPTG	On/Off Ratio
BL21	0.69	0.17	0.24
Green	1	3.35	3.35
Candidate 1	0.28	0.79	2.79
Candidate 2	0.21	0.41	2.01
Candidate 3	0.40	0.61	1.53
Candidate 4	0.29	0.18	0.62
Candidate 5	0.25	0.16	0.63

14.10.2026

The starters for R5-21 to R5-25 (5 samples + negative control) didn't grew in the LB+kan starters, so we decided to do colony PCR to the patches (which were small as well, due to the use of toothpick instead of streaking stick), the colony was first diluted in UPW before loading to the PCR reaction as 1ul DNA template.

	Volume (µl)
Rapid Taq	12.5
Fr-pCOLADuet-PS-A-Val	1
Rev-pCOLADuet-PS-A-Val	1
H_2O	9.5
Total	25ul

Apparently, diluting the already small amount of cells was fatal to the reactions, and there were no bands of any length: —=no sample in well,(-)=negative control.
0—21-22-23-24-25-(-)-------0



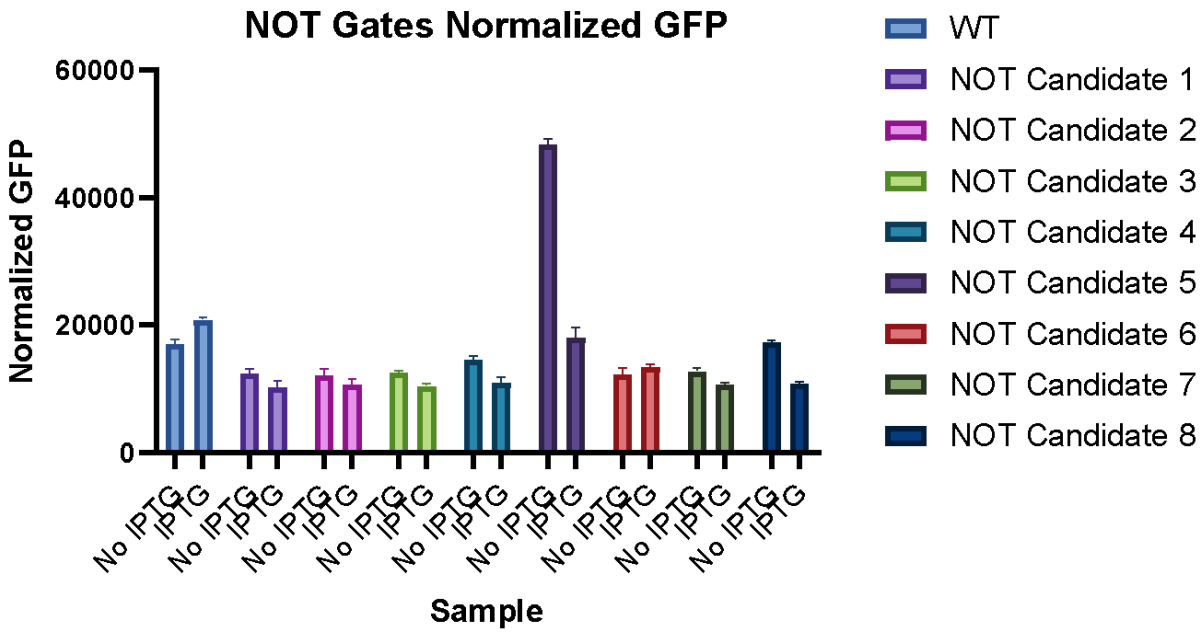
The colonies were incubated for another day in the incubator (37C), and new starters were picked for mini-prep the following day.

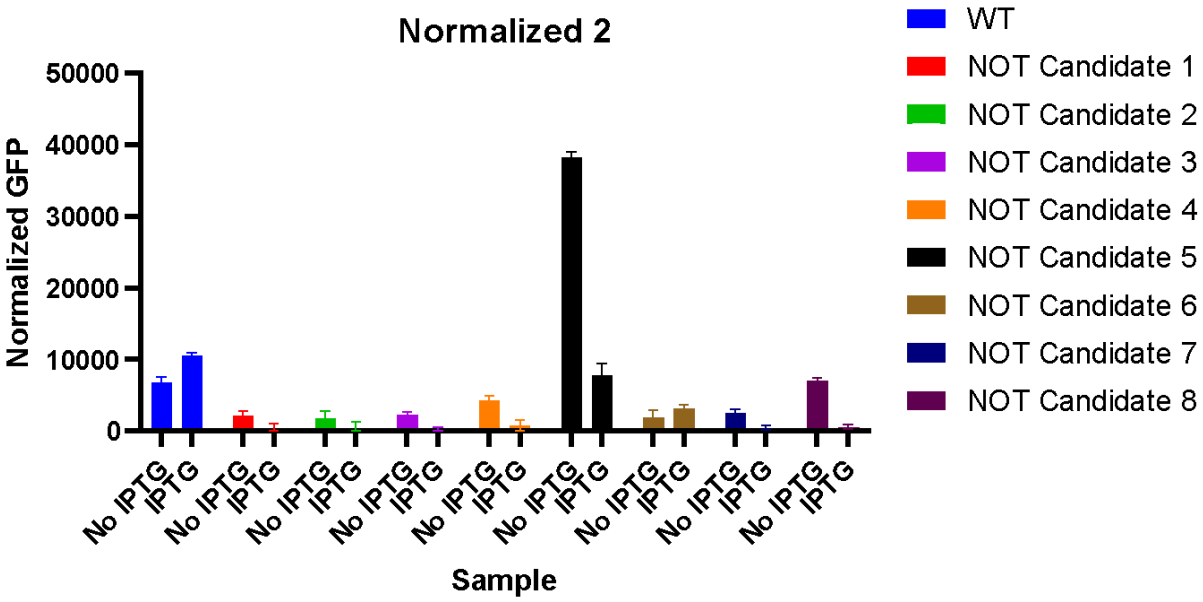
19.10.2026

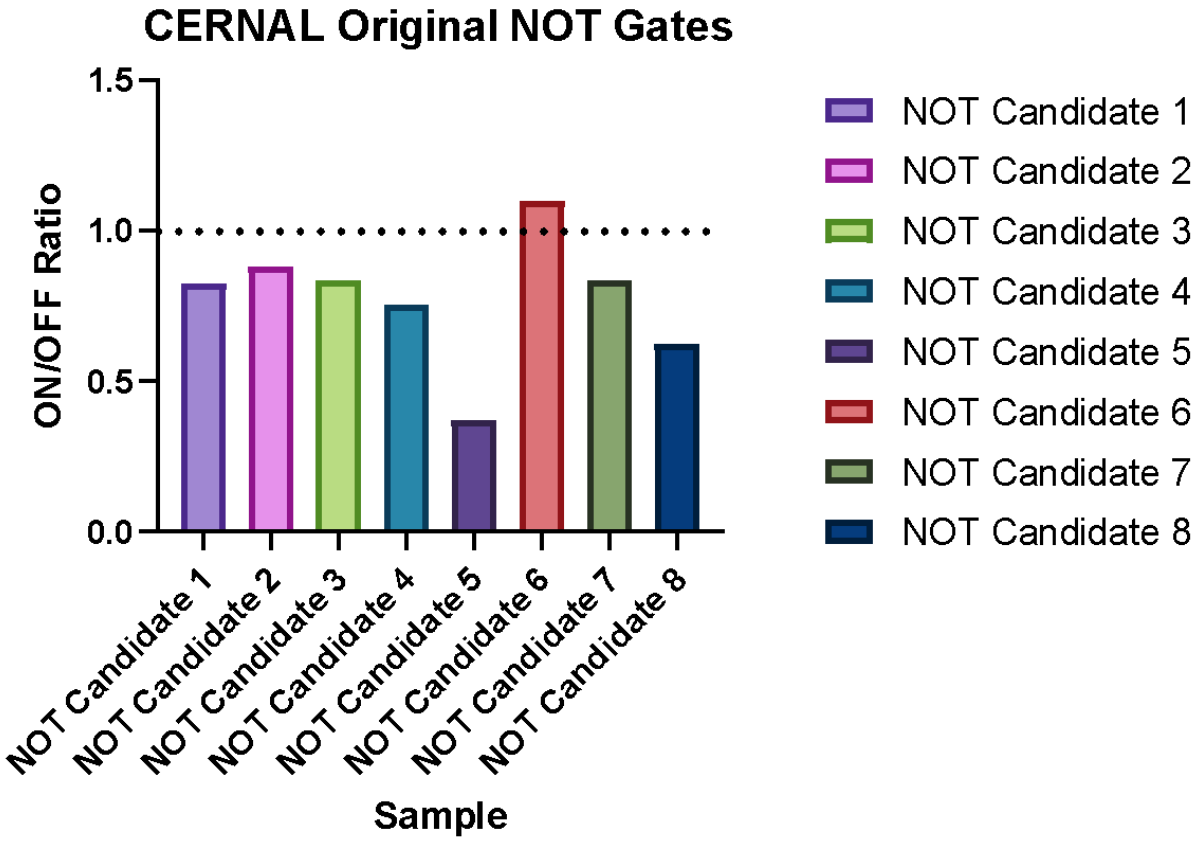
Not gate - Kinetics and plate reader experiment

Each condition was grown overnight in a starter culture with antibiotics (except for the BL21 control), both with and without IPTG induction (1mM). We centrifuged and washed equal amounts of the bacterial cultures with 300 µL of PBS. Next, 100 µL of each bacterial suspension was transferred to a 96-well plate, and a plate reader measured GFP and mCherry fluorescence levels.

The results are summarized below:


NOT Gates Normalized GFP
Normalized GFP
60000
40000
20000
0
No IPTG
IPTG
Sample
WT
NOT Candidate 1
NOT Candidate 2
NOT Candidate 3
NOT Candidate 4
NOT Candidate 5
NOT Candidate 6
NOT Candidate 7
NOT Candidate 8


Normalized 2
Normalized GFP
50000
40000
30000
20000
10000
0
No IPTG
IPTG
Sample
WT
NOT Candidate 1
NOT Candidate 2
NOT Candidate 3
NOT Candidate 4
NOT Candidate 5
NOT Candidate 6
NOT Candidate 7
NOT Candidate 8



The same overnight cultures were diluted to an optical density (OD_{600}) of 0.2, incubated for 1 hour, and subsequently transferred to a 96-well plate in triplicate. Each replicate was induced with 1 mM IPTG. The cultures were then grown at 37 °C with continuous shaking, and OD_{600} GFP and mCherry measurements were taken over the following 3 hours.

Figure 2 — Input arm. IPTG induces mCherry in every candidate except Candidate 2

