22.03.26

Experimental Design and Workflow

1) Integration of Hyg Resistance Cassette

Yeast cells will first be engineered to contain the hygromycin resistance gene (Hyg).

- A PCR product containing the Hyg cassette, flanked by homologous sequences at both ends, will be generated.
- The PCR product will be transformed into yeast cells.
- Cells will be plated on hygromycin-containing media to select for successful integration.

2) Replacement of hyg with nat Using CRISPR-Cas9

In the second stage, the hyg cassette will be replaced with a nourseothricin resistance gene (nat).

- A plasmid expressing Cas9 and gRNA targeting the hyg locus will be introduced.
- The plasmid also confers nat resistance, allowing selection for its presence.
- Successful editing depends on CRISPR-mediated cleavage of the hyg locus, enabling homologous recombination with the nat cassette.

Selection Strategy

- Selection for plasmid uptake will be performed on -URA media, ensuring only transformed cells survive.
- Only cells in which CRISPR successfully cleaves the hyg locus will allow nat integration.

Plating Scheme

1. **First plating**: –URA (selection for plasmid presence)
2. **Second plating**: YPD (non-selective growth)
3. **Final selection**: plates containing **hygromycin and nourseothricin**

Expected Results

- Ideally, ~80% replacement efficiency is expected.
- Correct clones should be:
    - **Positive for nat resistance**
    - **Negative for hyg resistance**

22.03.26

PCR

PCR Products

- Pag32: yeast plasmid with hyg resistance: 1729 bp
- Pag25: yeast plasmid with nat resistance: 1273 bp

Both PCR products are generated using templates with identical:

- Promoter: TEF (Translation Elongation Factor)
- Terminator

Primers are designed such that:

- The 3′ end anneals to the template
- The 5′ end contains homologous overhangs ("tails")
- These overhangs correspond to the target insertion site

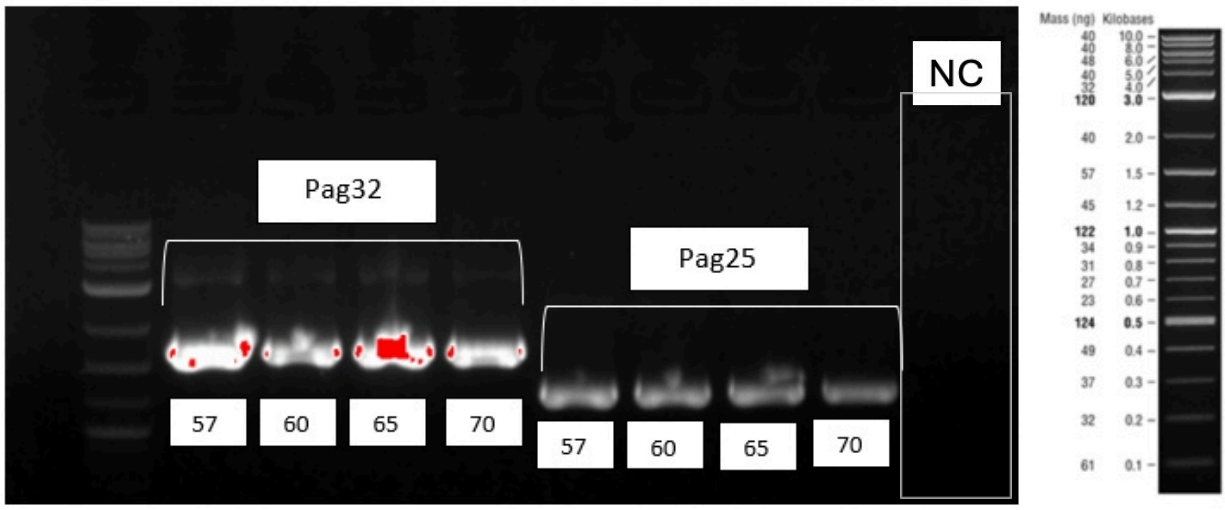
	X1 (uL)	X5 (uL)
PANTA MIX	12.5	62.5
F: FAG-413-20r-p	1	5
R: Rev-pAG-413-20	1	5
Pag 25 NatR / Pag 32 HygR	0.2	1
UPW	10.3	51.5

Cycle setting:

Temp (C)	Time (sec)	
98	30	
98	10	x25
GRAD (57-70)	5	
72	10	
72	60	

22.03.26

Results



1`% gel agarose, 120V ,40min

Explanation: It can be observed that all four temperatures for each of the two plasmids produced the expected results. Therefore, it was decided to combine the four tubes for each plasmid. It can also be seen that the negative control behaved as expected.

Mini Prep

Plasmids

1. **Control plasmid (pCAS2)-** Contains Cas9 but lacks the 20 bp targeting sequence
2. **Targeting plasmid (Pcy5h)-** Contains Cas9 and gRNA targeting the hyg locus

NucleoSpin Plasmid EasyPure, Mini kit was used for two plasmids, pCAS2 and pCYSH.

Plasmid	Concentration (ng/uL)
pCAS2	87
pCYSH	96

26.03.26

Transformation

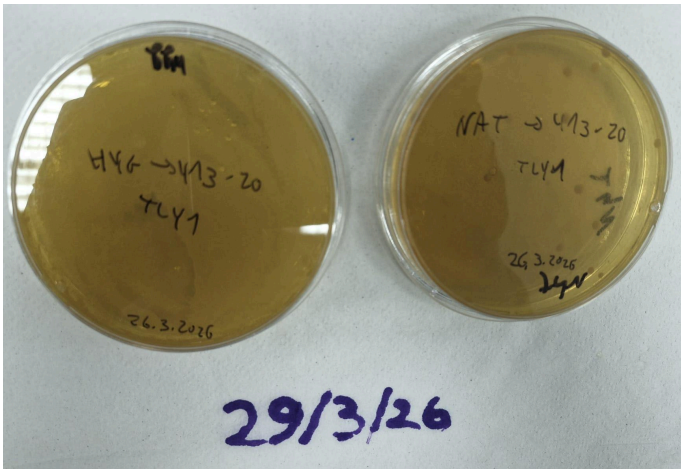
hygR and natR PCR products from Pag32 and Pag25, respectively, were transformed into yeast from S. *cerevisiae*, Type I WT, according to our transformation protocol (Daniel's one).

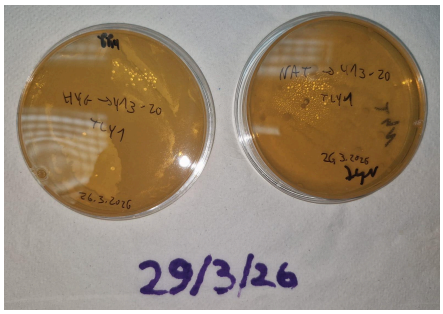
Initial 600nm O.D of the culture: 0.35.
Culture's shared volume for both transformations: 30ml.
Amount of PCR product used in each transformation: 4 µL (with 20 µL DDW).
Recovery time before plating: 2.5 h
After recovery, the transformants were plated on hyg+/nat+ YPD plates (according to the antibiotic resistance gene they were transformed with).
Plates were left in incubation at 30°C for 3 days (will be checked on 29/03)

29.03.26

hygR+ and natR+ yeast colonies grew on their appropriate antibiotic-positive plate:







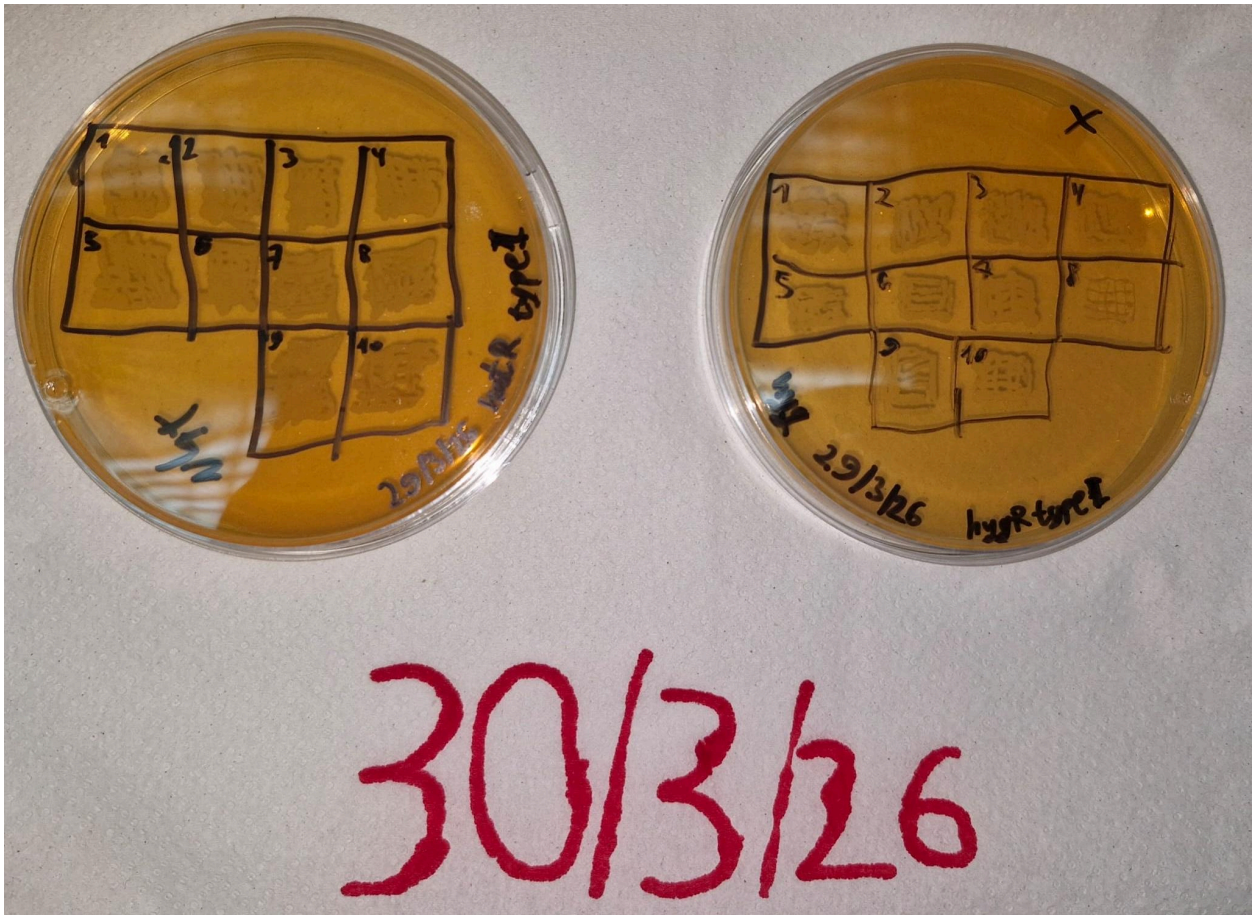
The biggest colonies were transferred into patches on another antibiotic-appropriate plate.

In addition, E.coli starters carrying pAC-dCas-54 and pDD-SCA-16 for Miniprep on the following days.

Another E.coli which we planned to have but did not grow on the plate: pAC-dCas-53 (negative control), we still need to get it.

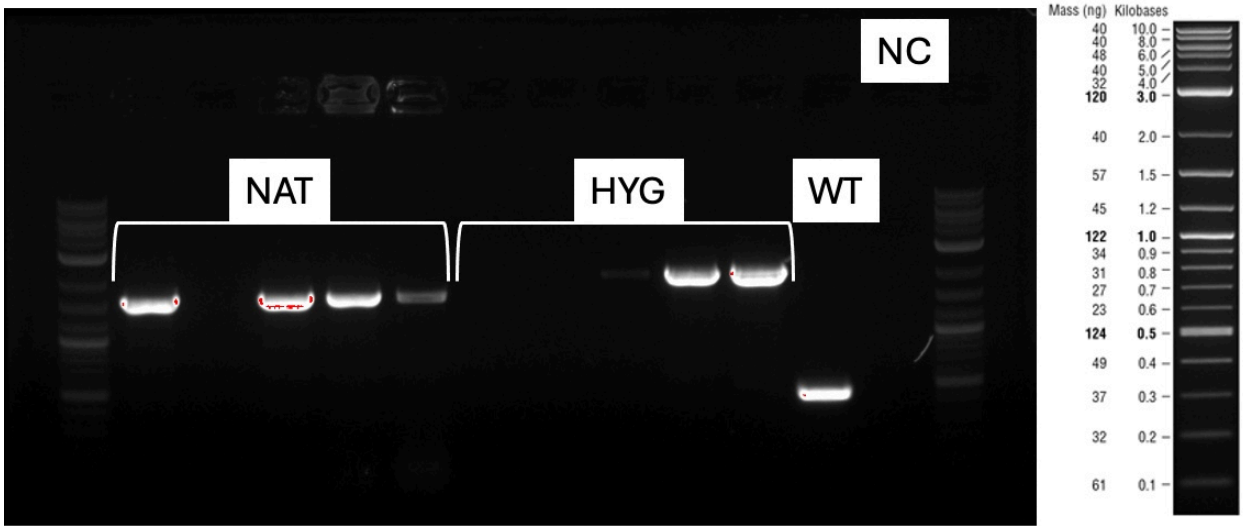
30.3.26

Genotyping



Verifying Transformation success by PCR genotyping of patches 1-5 of each plate (nat / hyg).
Expected length:

- Wild Type - ~460 bp
- Hyg insertion - ~1800 bp
- Nat insertion - ~1600 bp



1% agarose, 120V, 40min, NC = negative control.
Colonies 1,3,4,5 were positive for NAT, colonies 4,5 for HYG

Gibson Design

We need dCas and hyg cassette plasmids:
Build the dCas plasmid based on the pCas2 backbone, pAC-dCas9 (dCas9), and pDD-TetR (VP16). Each has 2 primers homologous to the next part.

- Design each primer as described before, and add an overhang (10~15bp) according to the appropriate next primer.
    - Maintain correct directions (3’ and 5’)
- Primers always have homology at the 5’ end, so they can’t self-amplify - forward primer starts (5’) with reverse primer sequence homology.
    - Homology by temperature - Tm should be comparable (>20bp).

6.04.2026

Mini Prep

Plasmids

1. Pdd-sca

2. **Pac-53**

3. Pac-54

NucleoSpin Plasmid EasyPure, Mini kit was used for two plasmids, pCAS2 and pCYSH.

Plasmid	Concentration (ng/uL)
pddsca	416
pac-53	90
pac-54	24

Transformation

Preparing glycerol stock

Taking equal volume (800 µl) of 50% glycerol and yeast, final conc. 25% glycerol in -80ºC.

- Hyg colony number 4 (serial #100)
- Nat colony number 1 (serial #101)

Primer design for hyg cassette TF

Designed the plasmid ”pCERNAL-8xSCA”, a genome-integrative plasmid with 8x hyg targeting sites near a GAL10 promoter and GFP, based on an existing tet cassette plasmid pDD-8xTetO-GAL(200)-Envy.

For genome integration, we need to linearize the plasmid before transformation.

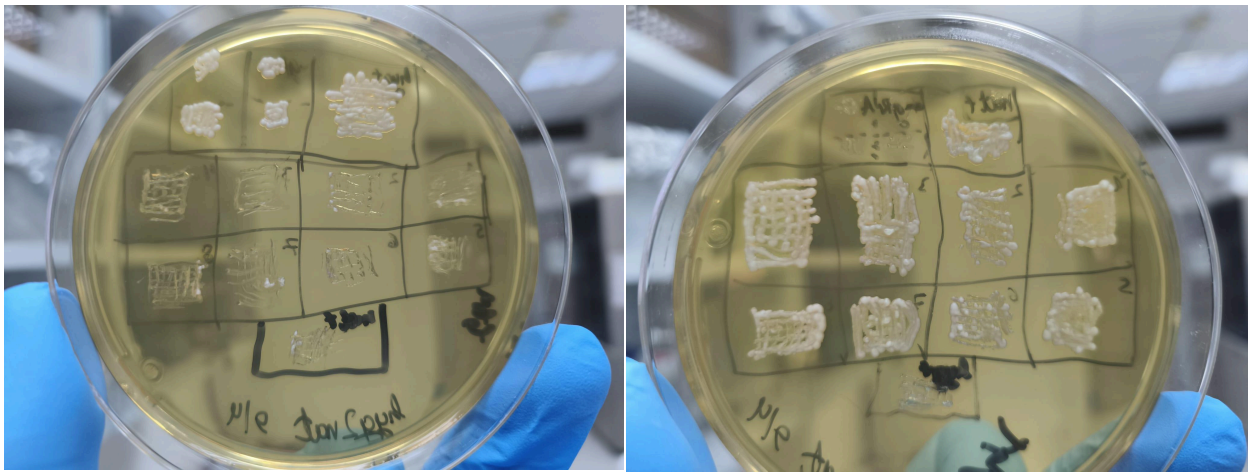
Transformation and crisper assay

Transforming hygR yeast with Cas9 plasmids - pCY5H or pCas2 (negative control, without gRNA), seeding on URA-negative plates.

10.04.2026

Marker Switch Rep 1 Results

The Results were exactly as expected:
Controls: **Positive-** in both nat and hyg plates, the strain, which was inserted with the appropriate antibiotic resistance gene, grew on the plate.
Negative 1- In both the nat and hyg plates, the strain that was inserted with the other antibiotic resistance gene did not grow on the plate.
Negative 2- Colonies of yeast that were transformed with natR+pCas2 (negative control, without gRNA) grew on the hyg plate but not on the nat plate.
Results: hygR Yeast that was transformed with natR and Cas9 plasmid pCY5H (with gRNA) was able to undergo homologous recombination repair and grew on the nat plate.



Photos 10/04: left plate- hyg+YPD, right plate- nat+YPD. On the upper left are colonies of hygR yeast that were transformed with natR+pCAS2 (Cas9 with no gRNA). On the upper right are positive controls with natR/hygR appropriate yeast strains (4 duplicates- different colonies)). The following 8 squares are the duplicates (different colonies) which were transferred to both the hyg and nat plates in the same order. The bottom square in each plate is a negative control with the strain of the opposite antibiotic resistance plated on each plate (hygR on nat+ plate, and natR on hyg+ plate).

8/8 colonies, which were transferred from the YPD plate after the transformation, grew on the nat plate (we chose only big and distinct colonies this time), meaning a transformation+CRISPR efficiency of 100%, which is unrealistic. We will transfer dozens of **random** colonies instead of just a few distinct ones.

$$Efficiency(\%) = \left(\frac{number\ of\ edited\ alleles\ checked}{number\ of\ total\ alleles\ checked}\right) \times 100 = \frac{8}{8} \times 100 = 100\%$$

13.04.2026

Transformation

Transforming hygR yeast with Cas9 plasmids - pCY5H or pCas2 (negative control, without gRNA), seeding on URA-negative plates, was transformed into yeast from S. *cerevisiae*, Type I WT, according to our transformation protocol (Daniel's one).

Amount of PCR product used in each transformation: 4ul (with 20ul DDW).
Recovery time before plating: 2h
After recovery, the transformants were plated on hyg+/nat+ YPD plates (according to the antibiotic resistance gene they were transformed).
Plates were left in incubation at 30°C for 2 days (will be checked on 15/04)

16.04.2026

Growth of colonies was still unsatisfactory. 20 patches for each transformant (pCas2 or pCY5H) on YPD plates. The plate went back to the incubator (30°C) to try and save the repeat.

19.04.2026

PCR-Gibson Assembly

To make pCERNAL-8xSCA-Envy and pCERNAL-dCas9-VP16 in Gibson assembly, 4 components need to be amplified first using PCR: SCA-16, VP-16, Dcas9, and pCAS.
The reaction was made using a gradient for the Ta:

Column Reference	21 / 31	22 / 32	23 / 33	24 / 34
Annealing Temp	57°C	60°C	65°C	70°C

Component	x1 Volume (µL)	x5 Volume (µL)
PCR Mix (Phanta Max)	12.5	62.5
Forward Primer	1.0	5.0
Reverse Primer	1.0	5.0
Template DNA	1.0	5.0
UPW (Ultra-Pure Water)	10.5	52.5
Total	25.0	125.0

Reaction Number	Reaction Target	Length (bp)	Template DNA	Forward Primer	Reverse Primer
1	SCA-16	441 bp	pDP-SCA-16	Fr SCA-pCERNAL	Rev SCA-pCERNAL
2	pCAS	5,744 bp	pCas2	Fr-pCas-pCERNAL	Rev-pCas-pCERNAL
3	dCAS	4,131 bp	pAC-dCas9-53	Fr-dCas9	Rev-dCas9
4	TetR-VP-16	543 bp	pDD-tetR-VP16-4	Fr-VP16x2	Rev-VP16x2

PCR Table 1

PCR-Gibson Assembly Results

We only tried to do reactions 2 and 3 since they have a similar length. The reaction didn't work at any temperature of the gradient. It might have been a technical issue. We need to try again.

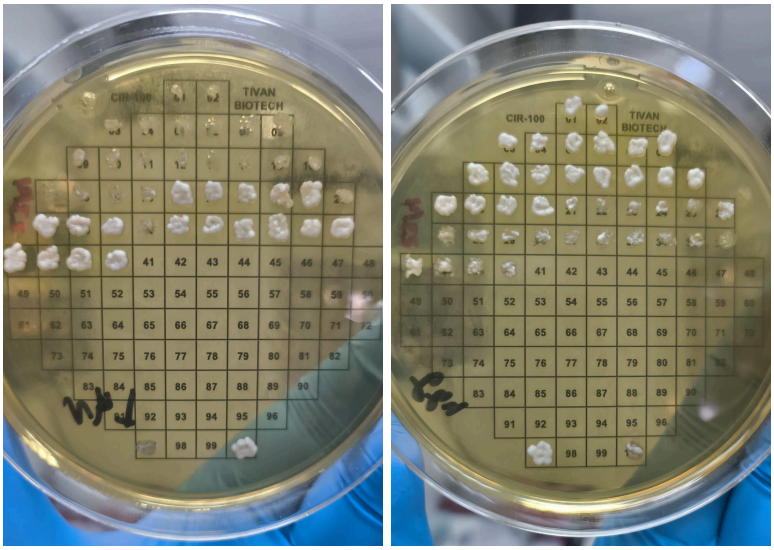
24.4.2026

Marker Switch Rep 2 Results

The Results were exactly as expected:
Controls: **Positive-** in both hyg and nat plates, the strain, which was inserted with the appropriate antibiotic resistance gene, grew on the plate (colony 97 and colony 100, respectively).
Negative 1- In both nat and hyg plates, the strain that was inserted with the other antibiotic resistance gene did not grow on the plate (colony 97 and colony 100, respectively).
Negative 2- colonies 1-20: colonies of yeast that were transformed with natR+pCas2 (negative control, without gRNA) grew on the hyg plate but not on the nag plate.
Results: colonies 21-40: hygR yeast transformed with the natR and Cas9 plasmid pCY5H (with gRNA) underwent homologous recombination repair and grew on the nat+ plate, but not on the hyg+ plates.

18/20 colonies (21-40, minus colonies 26 and 30), which were transferred from the YPD plate after transformation, grew on the nat+ plate (we chose only large, distinct colonies this time), and not on hyg+, indicating a transformation+CRISPR+sgRNA efficiency of 90%.

$$Efficiency(\%) = \left(\frac{number\ of\ edited\ alleles\ checked}{number\ of\ total\ alleles\ checked}\right) \times 100 = \frac{18}{20} \times 100 = 90\%$$

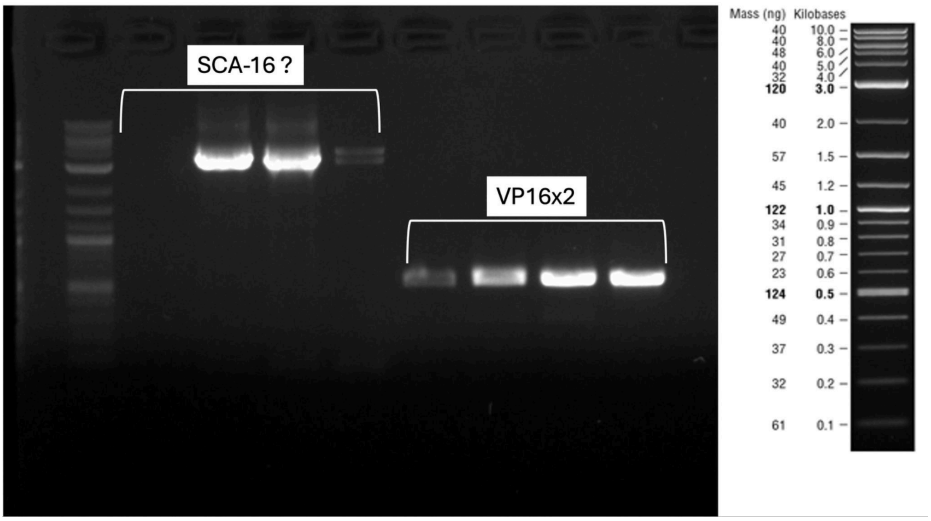


Photos 24/04: Right plate- hyg+YPD, left plate- nat+YPD. The random transformation colonies were plated in YPD (from URA- plates) in patches for recovery and then transferred to both plates. Colonies 1-20 are transformants of pCAS2 (Cas9 with no gRNA), colonies 21-40 are transformants of PCY5H (Cas9 with gRNA), colony 97 is hygR (control), and colony 100 is natR (control).

26.4.2026

PCR-Gibson 1&4 Results

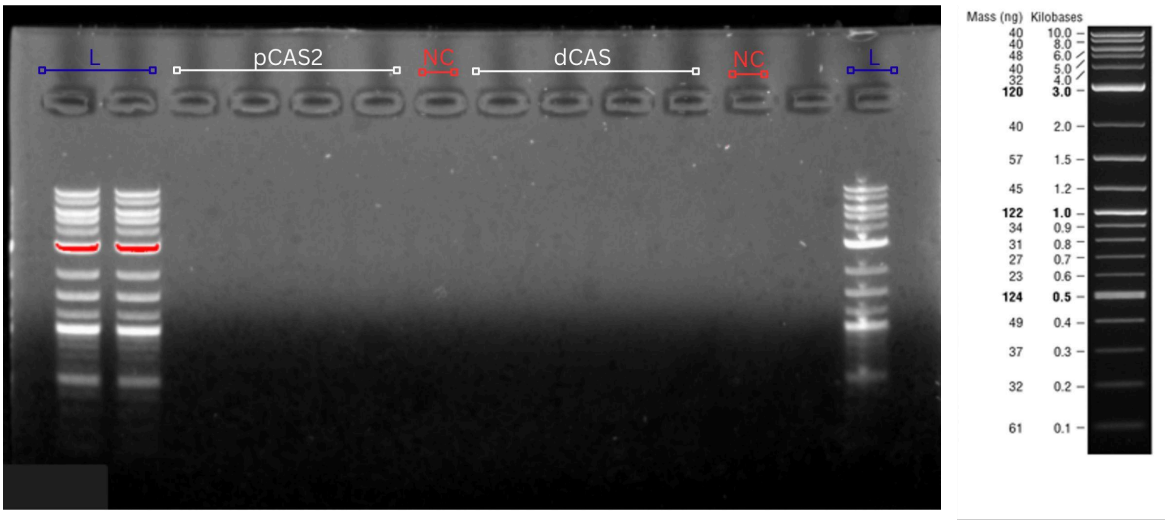
Did reactions 1 and 4 since they should have similar lengths (elongation step set to 5 sec). Reaction 1 results are not as expected. The primers seem to favor a bigger product (3+3.5kbp) instead of the desired product, which is only 441bp. Rev primer has two bands on the plasmid. Reaction 4 (VP16x2) went well, with some non-specific product at 65ºC. Purified PCR product of VP16x2 at 70ºC with a final concentration of 58 ng/µl.



28.4.2026

PCR-Gibson Results

We did PCR reactions 2 and 3 (from PCR table 1), as the PCR reactions should have similar lengths and the same setup, 19.4.
Gel electrophoresis confirmed the ladder (L), but experimental lanes (pCAS2, dCAS) showed no visible bands. The negative control (NC) also yielded no signal, confirming there was no contamination.



30.4.2026

PCR-Gibson

Re-did reactions 2 and 3 (PCR table 1). Used new primer stock, used dCas9-54 in addition to dCas9-53 (our target should be on both), and re-checked template concentrations. PCR had 30x elongation steps (5 more than previously). And the temperature gradient was set to; 55ºC, 58ºC, 64ºC, and 68ºC. Did not have time to run gel electrophoresis.

Transformation rep 3

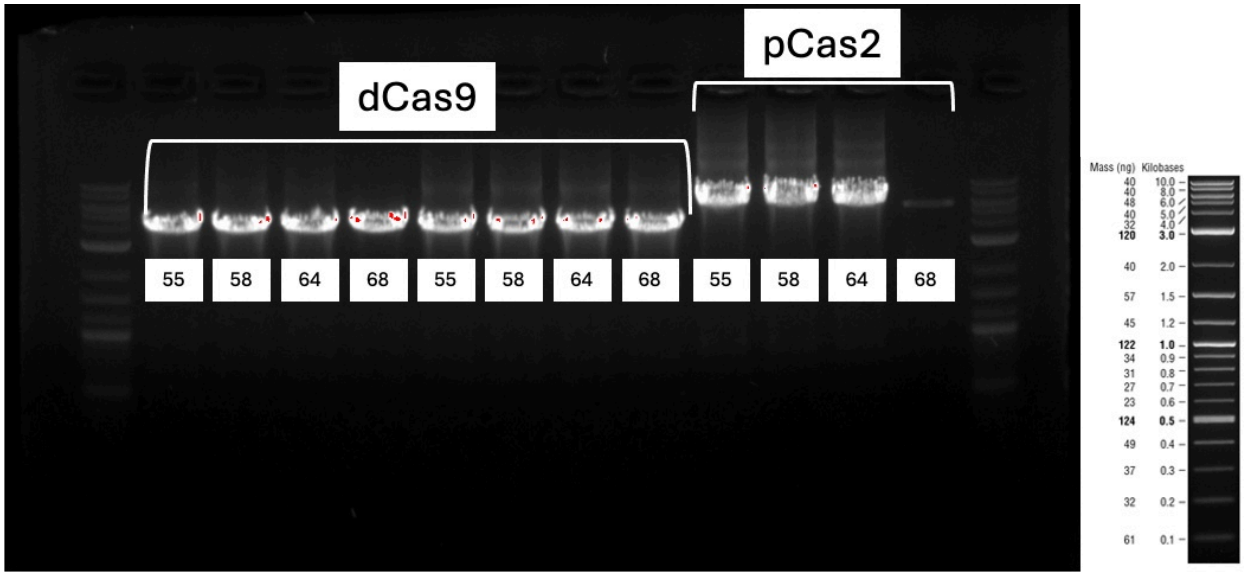
Rep 3 of the antibiotic switch assay, plated on URA^- plates.

3.5.2026

Making rep 3 patches on hyg and nat plates. 1-33 are pCas2 , 34-66 are pCY5H. Had many small colonies on URA-; next time, seed less on ura- plates (maybe 100µl?). Also did another PCR for creating new NAT products (pAG25), same primers, concentrations and settings as in 22.03.2026, but the temperature range was 60, 65, 68, 70.

PCR-Gibson 2&3 Results

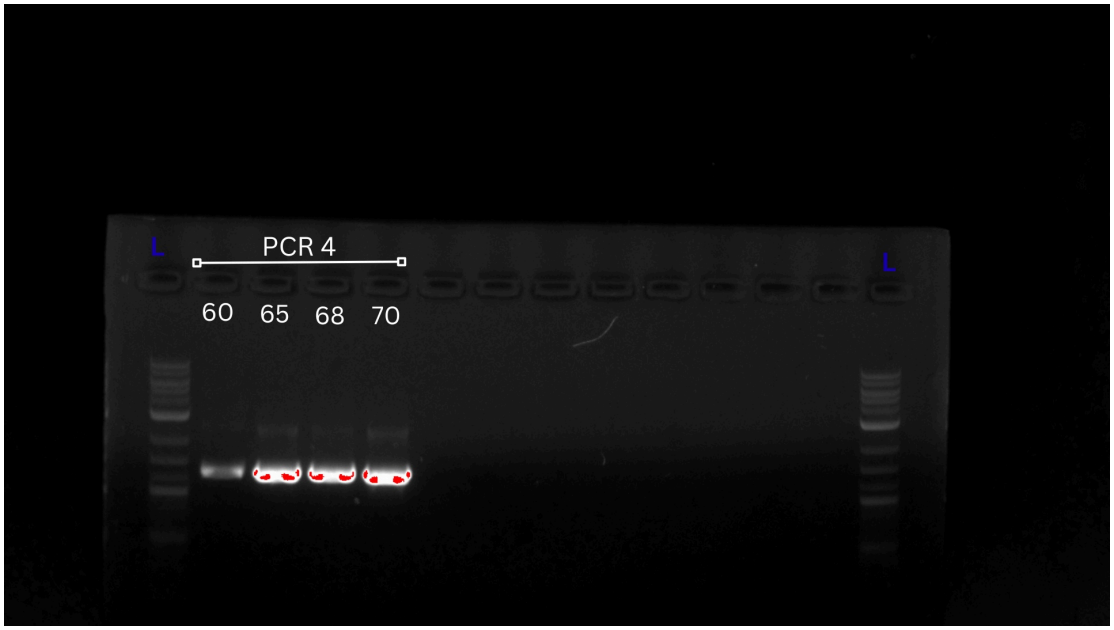
Re-doing the reaction 2&3 was successful, had more elongation steps and careful manual work. Needs PCR purification and then is ready for Gibson – constructing pCERNAL-dCas9-vp16.



4.5.2026

PCR-Gibson 4 Results

Analysis of the natR PCR results via gel electrophoresis confirms successful amplification. The reaction was tested across four annealing temperatures (60 to 70) to determine optimal conditions. Wells 2 through 5 show clear bands at the target size for all temperatures, with optimal bands between 65 to 70. The DNA ladders are in the peripheral wells for size verification.



PCR-Gibson 2-4 DNA purification & Gibson future reaction calculations

We successfully isolated and purified the DNA fragments from the PCR reactions. Visual inspection and initial measurements indicated high purity with no significant protein contamination. DNA concentrations were measured for each fragment to determine the molarity required for the subsequent assembly.

PCR	DNA concentration (ng/uL)	DNA bp	Fmol
2	253	5744	35.6
3	106	4131	33.3
4	58	543	-
4 - diluted 1:5 (future Gibson reaction)	11.6	543	34.7

Following purification, we calculated the volumes required for a 10 µL Gibson Assembly reaction. We settled on an equal ratio of the fragments. The reaction design includes 5 mu/l of Gibson Assembly Master Mix, with the remaining volume comprised of the DNA fragments and water to reach the final 10 mu/l reaction volume. The DNA concentrations and molecular weights (bp) were used to calculate the fmol values for each fragment. The final reaction composition is detailed in the tables below.

Master mix	5 ul
Pure water	2.7 ul
PCR2	0.5 ul
PCR3	0.8 ul
PCR4	1 ul
Total	10

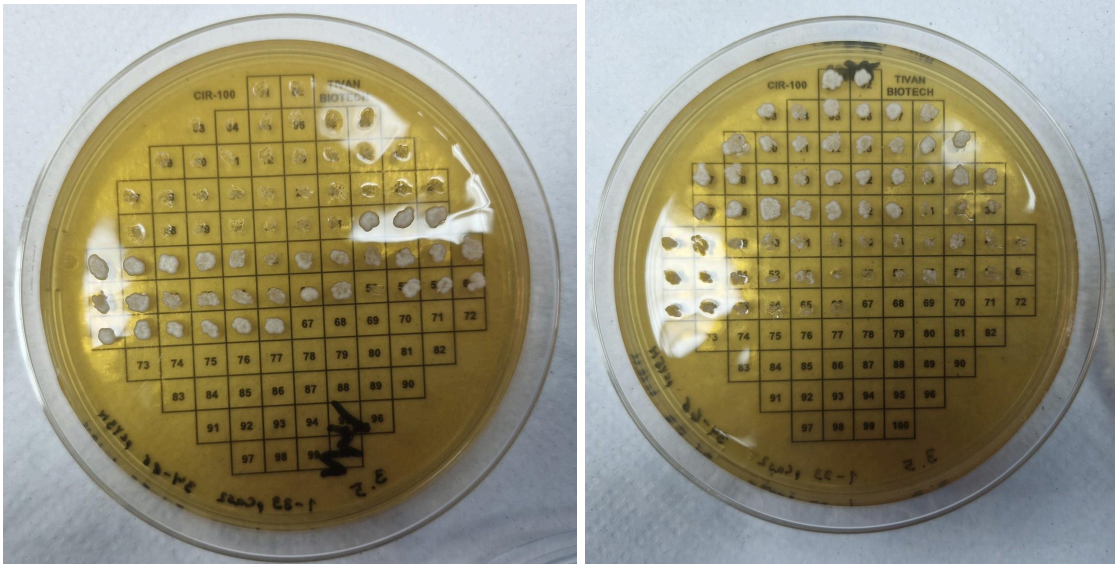
4.5.2026

Marker Switch Rep 3 Results

The Results were exactly as expected:
Controls: **Positive-** in both hyg and nat plates, the strain, which was inserted with the appropriate antibiotic resistance gene, grew on the plate (colony 97 and colony 100, respectively).
Negative 1- In both nat and hyg plates, the strain that was inserted with the other antibiotic resistance gene did not grow on the plate (colony 97 and colony 100, respectively).
Negative 2- colonies 1-33: colonies of yeast that were transformed with natR+pCas2 (negative control, without gRNA) grew on the hyg plate but not on the nag plate (minus colony 34, which did not grow on either plate).
Results: colonies 34-66: hygR yeast transformed with the natR and Cas9 plasmid pCY5H (with gRNA) underwent homologous recombination repair and grew on the nat+ plate, but not on the hyg+ plates.

31/33 colonies (34-66, minus colonies 42 and 57), which were transferred from the URA-, grew on the nat+ plate (we chose only large, distinct colonies this time), and not on hyg+, indicating a transformation+CRISPR+sgRNA efficiency of 94%.

$$Efficiency(\%) = \left(\frac{number\ of\ edited\ alleles\ checked}{number\ of\ total\ alleles\ checked}\right) \times 100 = \frac{31}{33} \times 100 = 94\%$$



Photos 4/5/26: Left plate- nat+YPD.Right plate- hyg+YPD. The random transformation colonies were plated in YPD (from URA- plates) in patches for recovery and then transferred to both plates. Colonies 1-33 are transformants of pCAS2 (Cas9 with no gRNA), colonies 34-66 are transformants of PCY5H (Cas9 with gRNA).

5.5.2026

pCY5H Transformation rep 4

Yael S.

Protocol

The natR PCR product from Pag25 was transformed into S. *cerevisiae* Type I WT yeast.

Grown until 600nm O.D of the culture: 0.46.
After washing, the pellet was resuspended in 90 ul DDW.

Transformation 1 pCY5H	Transformation 2 pCAS2 (Negative control)
0.5ul pCY5H plasmid (87 ng/ml)	1ul pCAS2 plasmid (130 ng/ml)
10ul natR PCR product (wasn't cleaned)	10 µl natR PCR product (wasn't cleaned)
13.5 µl DDW	13 µl DDW

After recovery, the transformants were plated on URA^- YPD plates.
Plates incubated 30°C for 3 days (will be checked on 08/05).

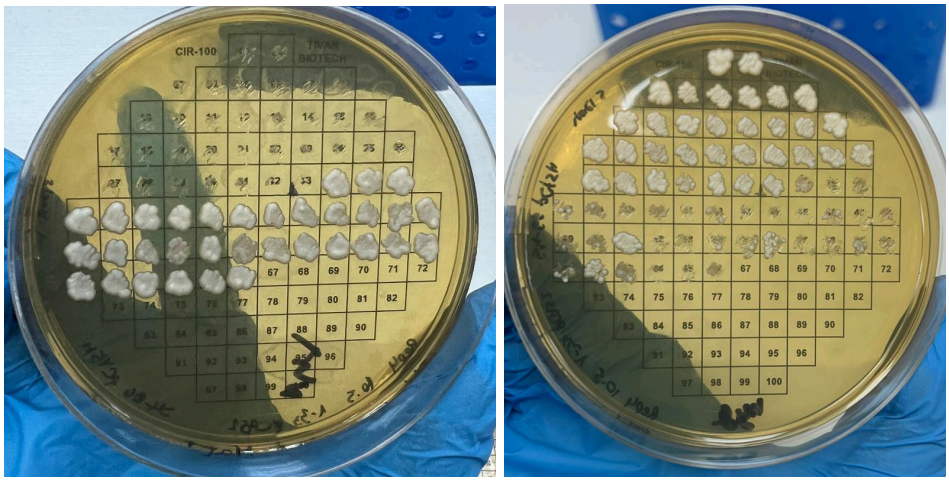
10.5.2026

Yael S.
After recovery, the transformants were plated on hyg+/nat+ YPD plates.
Patches 1-33 pCas2
Patches 34-66 pCY5H
Plates were left in incubation 30°C for 2 days

12.5.2026

Marker switch rep 4 results

NAT switch was successful (100%), but multiple colonies grew on both - notable are 51, 56, 62. Colonies exhibiting growth on both media were classified as incomplete marker switches and excluded from the count of successful edited alleles.



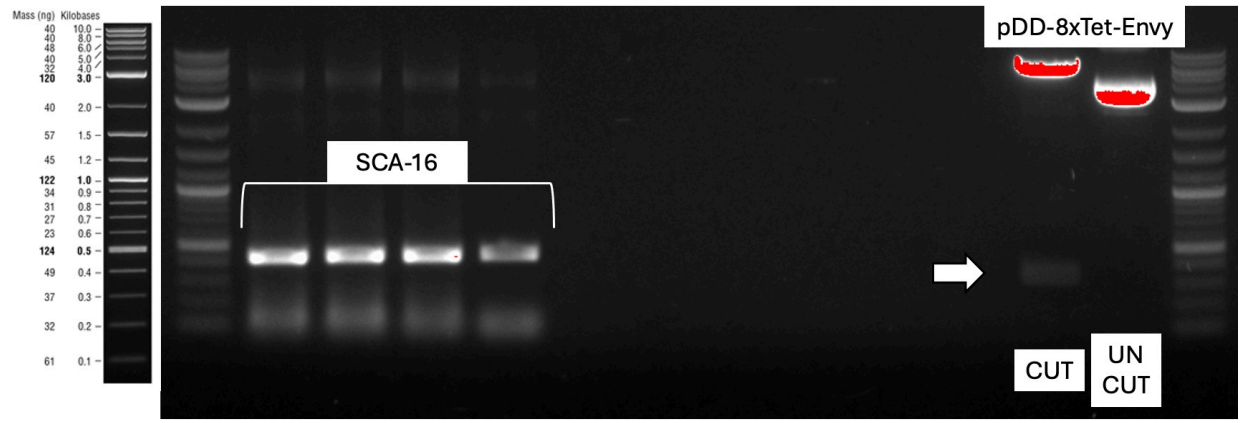
Photos 12/5/26: *Plates from rep 4 after incubation: Left plate- nat+YPD.right plate- hyg+YPD, Colonies 1-33 are transformants of pCAS2, colonies 34-66 are transformants of PCY5H.*

$$Efficiency(\%) = \left(\frac{number\ of\ edited\ alleles\ checked}{number\ of\ total\ alleles\ checked}\right) \times 100 = \frac{30}{33} \times 100 = 91\,\%$$

PCR-Gibson 1 new primers results

Same as 19.4.2026 but with new primers; **Fr2 SCA-pCERNAL, Rev2 SCA-pCERNAL**. Expecting SCA-16 product at **445bp**. Also prepared the backbone by cutting an 8xTet out and cleaning results.

pCERNAL-dCas9-VP16	pCERNAL-8xSCA-Envy
pCas (Backbone, PCR2)	pDD-8xTetO-Envy (backbone) 51ng/µl
dCas9 (PCR3)	8xSCA (PCR1) 118ng/µl
VP16x2 (PCR4)	

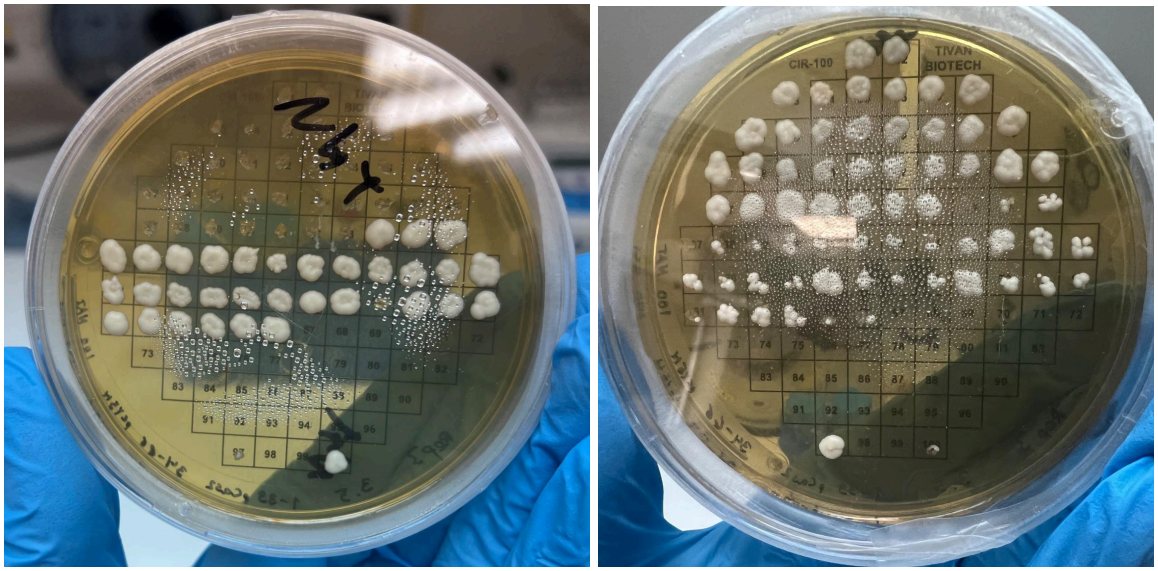


Gel Electrophoresis *results for PCR-gibson reaction 1, SCA-16 and its backbone, cutted with HpaI, PvuII. Arrow highlighting 8xTet section cut from the plasmid.*

All necessary Gibson parts are ready for both pCERNAL-dCas9-VP16 and pCERNAL-8xSCA-Envy

Marker switch rep 3 results 2

After one more day in incubation, the alleged hygR- colonies managed to grow on the hyg plate. natR control also grew on the hyg plate, so it could be depletion of hyg or that hyg isn't a very strong selector. NAT plate efficiency has risen to 97% (previously 94%, one additional colony managed to form).



Plates from rep 3 after 1 more day in incubation: Left plate- nat+YPD.Right plate- hyg+YPD. Colonies 1-33 are transformants of pCAS2, colonies 34-66 are transformants of PCY5H, colony 97 is hygR (control), and colony 100 is natR (control).

Also replenished URA and AMP plates (19 each) and liquid LB and YPD.
– S. Yair

19.5.2026

Gibson Reaction

Used NEBio calculator, aiming for a total quantity of 100 femtomoles (fmol) in each reaction, with a ratio of 1:2 backbone: insert.
Gibson 2 (G2) - for the three-part construct, we aim for equal quantities (fmol) totaling to 100, so 33 for each. To **create pCENRAL-dCas9-VP16** (AMP^R).

PCR	DNA bp	DNA concentration (ng/uL)	Quantity (fmol)	Volume (µL)
2 (pCas)	5744	253	35.6	0.5
3 (dCas)	4131	106	33.3	0.8
4 (VP16x2)	543	58	–	–
4 - diluted 1:5	543	11.6	34.7	1
H_2O	–	–	–	2.7
Mix (2x)	–	–	–	5
Total	–	–	–	10

Gibson 1 (G1) - aiming for the 1:2 ratio, creating **pCERNAL-8xSCA-Envy** (AMP^R + KAN^R).

PCR	DNA bp	DNA concentration (ng/uL)	Quantity (fmol)	Volume (µL)
1 (8xSCA)	445	118	–	0
SCA-16 Dilution 1:5	445	23.6	77.5	0.9
Backbone	6206	51	26.7	2
H_2O	–	–	–	2.1
Mix (2x)	–	–	–	5
Total	–	–	–	10

After 45 minutes (reaction time), transformed DH5α with products (G1, G2) and seeded on AMP plates.

Validation: after transformation and colony growth, perform patch and colony PCR before sequencing.

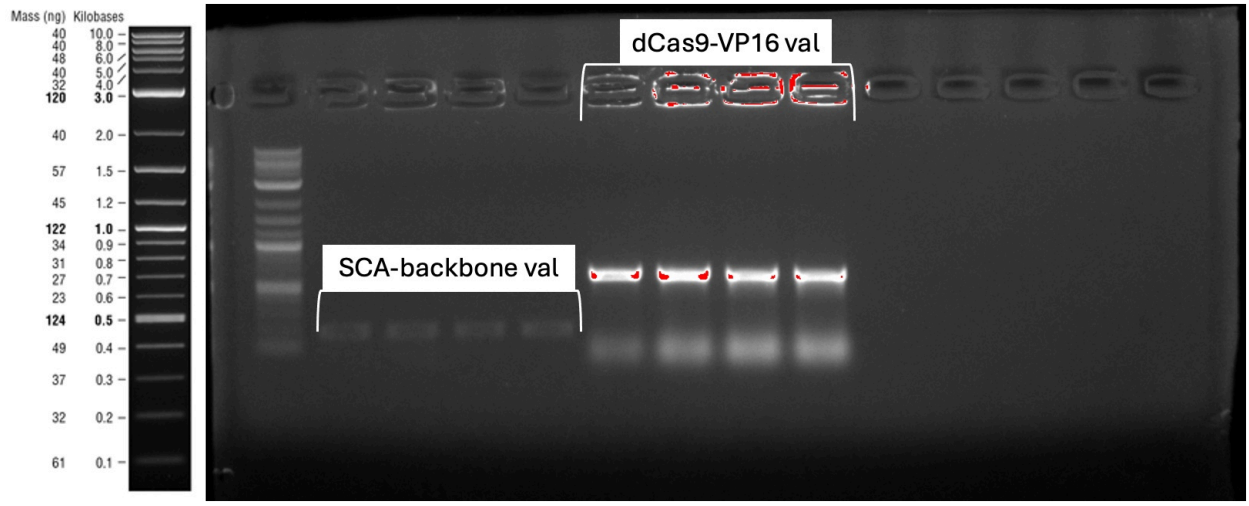
	Forward primer	Reverse primer	Expected bp
pCERNAL-dCas9-VP16	Fr-Cas9-Val	Rev-VP16-Val	626
pCERNAL-8xSCA-Envy	Fr2-SCA-pCERNAL	Rev-Gal10pr-Val	496
pCERNAL-8xSCA-Envy	Fr-SCA-Val	Rev-Gal10pr-Val	193

– S. Yair

25.5.2026

Gibson Validation

PCR and gel electrophoresis to validate Gibson assembly and transformation success.
Respective colonies were also made into patches for further sequencing if positive.
Decided to use Fr-SCA-Val instead of Fr2-SCA-pCERNAL. These reactions test for a section of each plasmid to evaluate whether we should sequence the complete plasmids.



Gel Electrophoresis *results of validation PCR for both Gibson reactions.*

All reactions were successful, with meaningful noise in the pCERNAL-dCas9-VP16 samples.
Decided to continue with miniprep starter - preparing to send it to sequencing on Thursday.
– S. Yair

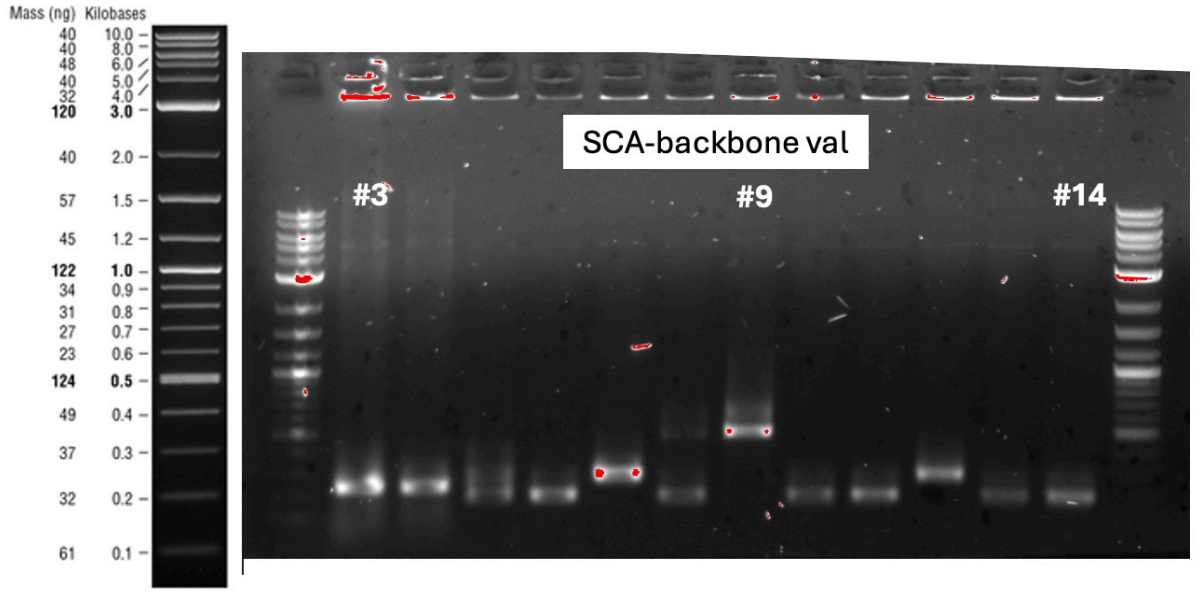
2.6.2026

SCA Gibson Validation

Sequencing results from pCERNAL-8xSCA-Envy came back, missing most SCA (colony 2 had ⅜). Decided to validate and sequence more colonies, 3-14, and different primers to catch the full SCA length - expected 496 bp.

	1x Reaction (µL)	13x
Rapid Taq	5	65
Fr2-SCA-pCERNAL	0.4	5.2
Rev-Gal10pr-Val	0.4	5.2
H_20	4.2	54.6
Total	10	130

Table for the E. coli Gibson validation reactions (rapid taq genotyping~)



Got good results for one colony (#9), will continue with sequencing.

dCas9-VP16 sequencing results

Received sequencing results for pCERNAL-dCas9-VP16, both colonies are good. Will continue with colony #1.

Tomorrow - starters for colony #9 (SCA) and colony #1 (dCas9)
Thursday - miniprep, making stocks from #1, preparing #9 for sanger (500ng in 15µl+1µl primer)

4.6.2026

sgRNA design

Talked with computational lab (Tal) on the required sgRNAs - ordered and designed 6 variants from IDT, most with a small loop size and with homology - for assembly through Gibson:

- Normal hyg targeting sgRNA
- Closed 1 - a closed control sgRNA with normal loop size (14nt)
- Open 1 - an open control sgRNA
- Open 3 - a slightly different open control
- mCherry 1 - an already described sgRNA responsive to mCherry
- mCherry 4 - mCherry responsive sgRNA with long loop (26nt)

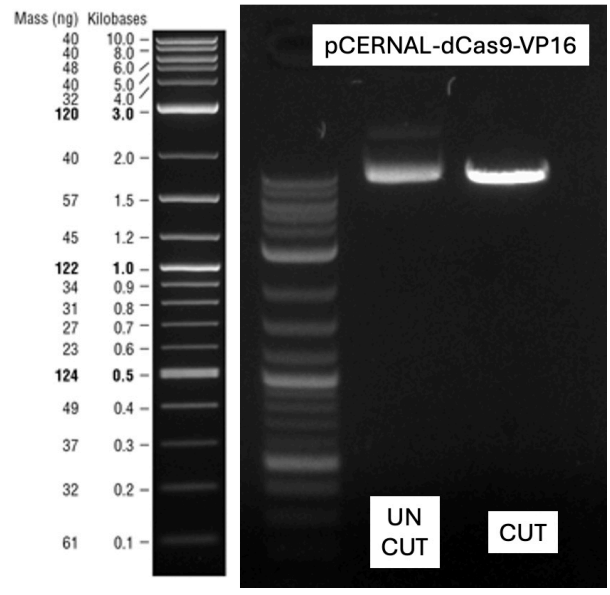
pCERNAL-dCas9-VP16 is kept in our Box as VP I (245.5ng/µl) and VP II.
The stocks in the -80ºC are numbered 430.

9.6.2026

Sending SCA again for sequencing - using the correct primer this time.

Cutting pCERNAL-dCas9-VP16

Cutting pCERNAL-dCas9-VP16 with a single cutter, StuI, as preparation for sgRNA Gibson. Then clean up for StuI inactivation and Gel check.



Gel electrophoresis 1%, showcasing pCERNAL-dCas9-VP16, both uncut and cut with StuI. In the uncut column, multiple bends are visible (supercoiled, relaxed), while the cut column shows a clear bend of the linear

Final concentration of cut pCERNAL-dCas9-VP16 is 57.3 ng/µl.

11.6.2026

SCA #9

Sanger results came back bad, doing restriction to verify insert length - expected 384bp. If it's good, it will be sent to Plasmidsaurus for sequencing.

	Total (15 µl)
Template (147 ng/µl)	10
Cut Smart Buffer	1.5
H_2O	2.5
HpaI	0.5
PvuII	0.5

16.6.2026

Cutting pCas2

Cutting pCas2 (live Cas9) with a single cutter, StuI, as preparation for sgRNA Gibson.
Final concentration of cut pCas2 is 48 ng/µl.

18.6.2026

Planning sgRNAs Gibson

Diluting Oligos and gBlocks: adding 10x TE, incubating 20 min at 50ºC. gBlock are at 10ng/µl, oligo is 100µmol/µl→ dilution by 10^{-3} and take 1.5µl.
Gibson aimed for a 1:5 ratio for small sequences (less than 200), about 100 total with Oligo higher (150fmol).
Gibson on pCERNAL-dCas9-VP16:

	DNA length (bp)	DNA conc. (ng/µl)	Quantity (fmol)	Volume (µl)
gBlock	131,143	10	100	0.8, 0.9
Backbone (dCas9-VP16)	10326	57.3	20	2.2
H_2O	-	-	-	2, 1.9

Mix	-	-	-	5
Total				10

Gibson on pCERNAL-dCas9-VP16 with oligo:

	DNA length (bp)	DNA conc. (ng/µl)	Quantity (fmol)	Volume (µl)
Oligo	77	100 µmol/µl		
Oligo Diluted (1µl to 999µl)	77	100 fmol/µl (~4.7ng/µl)	150	1.5
Backbone (dCas9-VP12)	10326	57.3	20	2.2
H_2O	-	-	-	2.1
Mix	-	-	-	5
Total				10

Gibson on pCas2:

	DNA length (bp)	DNA conc. (ng/µl)	Quantity (fmol)	Volume (µl)
gBlock	131,143	10	100	0.8, 0.9
Backbone (pCas2)	9816	48	20	2.5
H_2O	-	-	-	1.7, 1.6
Mix	-	-	-	5
Total				10

Gibson (7 total), transformation of DH5alpha, seed on plates, patches (5 each), validation PCR and miniprep (2 for each) → sequencing (Sanger).

22.6.2026

sgRNA Gibson assembly

Did Gibson assembly for dCas9 with sgRNAs; closed 1, open 1, open 3.

25.6.2026

Cloning plasmids

Cloning open 1, open 3, and closed 1 plasmids into bacteria for amplification.

30.6.2026

Starter for colony 9 of pCERNAL-8xSCA-Envy, need to replenish plasmid stocks for glycerol stocks and transformations.

Gibson assemblies

Creating pCERNAL-dCas9-VP16 plasmid with unmodified hyg spacer, and Creating pCas2 (live Cas9) plasmids with closed, open1 and open 3 spacer structures - through Gibson assembly, with the oligo template for the unmodified and gBlock for the modified structures, as planned in 18.6.
Plating on AMP

2.7.2026

Transforming Yeast pCERNAL-8xSCA-Envy

SCA 9 purified with nucleospin® - 400ng/µl, 50µl.
Cutting with SrfI, then straight to transforming yeast.

	Total (30 µl)
Template (400 ng/µl)	4
H_2O	22
Cut Smart Buffer	3
SrfI	1

Taking 4µl (~213ng) for transformation:

	Volume (µl)
polyethylene glycol - PEG 3350	240
Boiled salmon sperm (10mg/ml)	10
LiAc 1M	36

Yeast	70
DNA (~200ng)	4
Total	360

7.7.2026

PCR validation gRNA

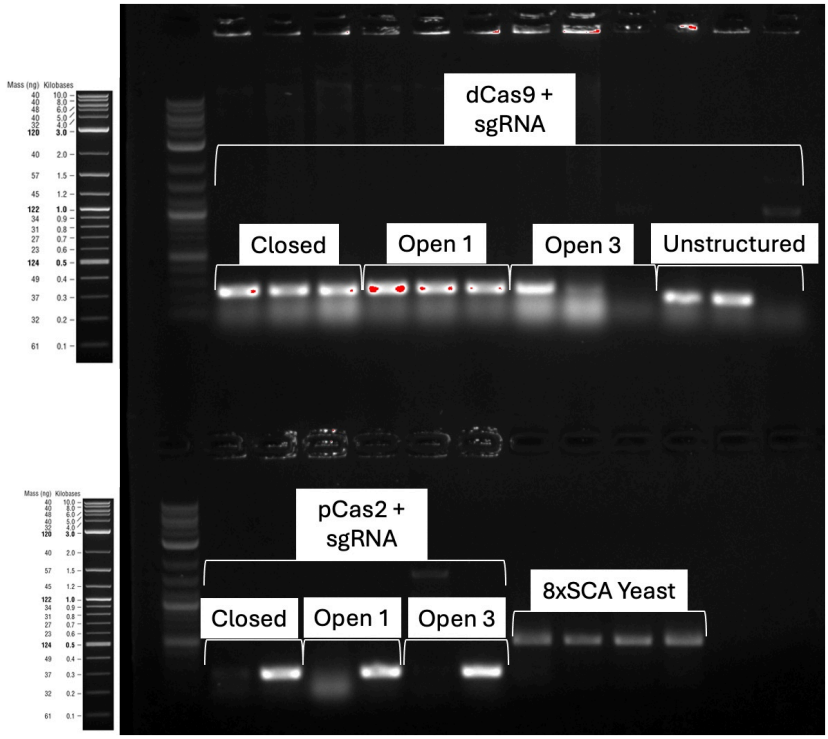
Patches and Validation for the 4 dCas9 and the 3 live Cas9 plasmids with un/structured hyg sgRNAs (closed, open 1, open 3):

Template	Fr Primer	Rev Primer	Expected length (bp)
Yeast 8xSCA	Fr2-SCA-pCERNAL	Rev-GAL10pr-Val	496
pCERNAL-dCas9-VP	Fr-gRNA-Val	Rev-hyg-gRNA-Val	186 (unstructured) 240
pCas2	Fr-gRNA-Val	Rev-hyg-gRNA-Val	246

9.7.2026

Genotyping 8xSCA

Patches and Validation for the Yeast 8xSCA. Thermocycler set to annealing 57ºC, extension 8 sec, 30 cycles.



1% Agarose Gel *- showcasing the great success of our transformations and Gibsons*

12.7.2026

dCas9 starter for hyg (2x), closed, open 1, open 3 + pCas2 starter closed, open 1, open 3.

13.7.2026

Cleaned up pCERNAL-dCas9-VP16 with the 5 sgRNA variants (2xHyg) and the 3 pCas2 variants.
Sent a total of 8 plasmids to Sanger sequencing.

15.7.2026

Sanger results sgRNA

Appeared to have had a human issue. For dCas9 plasmids: 2 hyg colonies, open 1 and open 3 were correct. For pCas2, only open 3 was received.

sgRNA (supposed):	pCERNAL-dCas9-VP16	pCas2
Hyg (unstructured)	Hyg	N/A
Closed	Open 3	Open 3
Open 1	Open 1	Open 3
Open 3	Open 3	Open 3

The current assumption is that the Gibson reaction itself was incorrect. Will repeat after Exams.

16.7.2026

Transforming Yeast w/ sgRNAs

Clean up for another batch of pCERNAL-dCas9-VP16, and transform yeast with pCERNAL-dCas9-VP; one empty and three variants of sgRNA - Hyg, Open 1 and Open 3. Plating on -URA plates.
Made glycerol stock for 8xSCA yeast (#102)

19.7.2026

Patches of pCERNAL-dCas9-VP yeast without a gRNA (empty), and with Hyg, Open 1 and Open 3. Overall 5*4 patches.

20.7.2026

Preparing FACS

Making SC and SC -URA, vacuum filtration, and setting them to grow. Need 3 repeats of hyg, open 1, open 3 + 1 repeat of positive control (TLY22), WT, empty. Overall 12 tubes.

21.7.2026

Made glycerol stocks for pCas2+gRNA open 3 (bacteria) and also pCERNAL-dCas9-VP+Hyg, Open 1, Open 3 (both plasmids carrying bacteria and yeast).

FACS

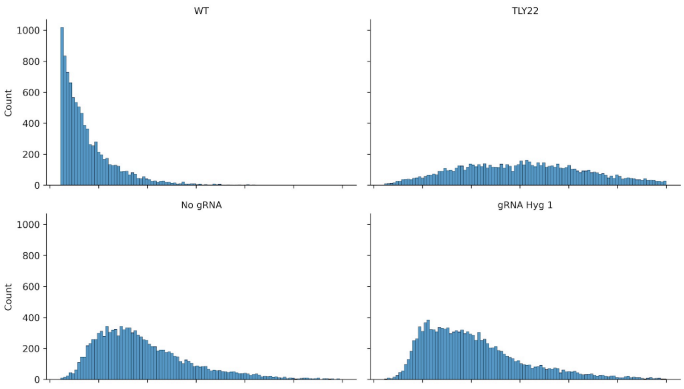
Recommended cell amount for yeast is 250-300K cells, calculated required volume (based on O.D. 600nm of 1 = ~10 million cells) to be 300µl at 0.1 OD.

The following protocol was performed (was short on time and had a delay):

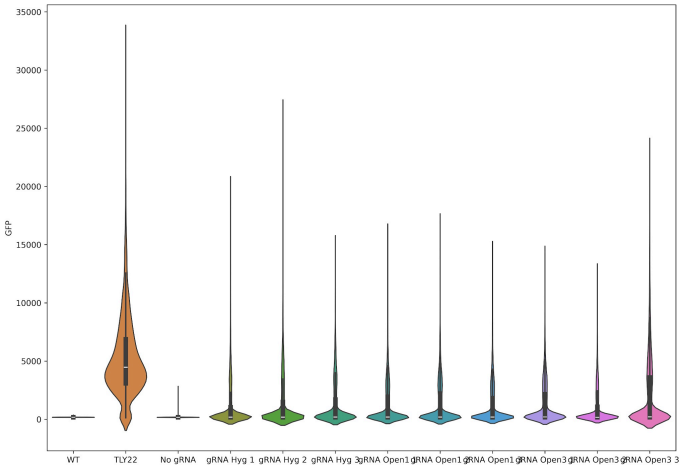
- Overnight starter
- Diluted to 0.15~0.2
- Incubated ~1 hour (attempted to reach log state, didn't have enough time)
- Diluted to 0.1 and transferred 300µl to Eppendorf tubes
- Incubated again for ~30 minutes (due to delays in FACS)

Results:

<u>Cell Size</u> - two distinct populations were noticed during FACS, gating was used to remove the smaller one (still not sure what they were, no bacteria were found in the samples when viewed under the microscope). Additionally, different cell size distribution between treatments was noticed, possibly due to the truncated incubation time and skewed results. Some cells might be in stat while others are in log.



<u>Fluorescence</u> - Both WT and yeast without gRNA had essentially zero GFP signal (negative controls). TLY22, a tetR-based system similar in architecture to our dCas9-VP system, had expectedly high signal (positive control). When removing WT signal from test samples, GFP levels reached approximately half that of TLY22.



Discussion:

Some points to consider for next test: 1) Had a lot of very small cells/something during analysis, which might improve with Incubation - we should give cells more time to reach log (even 4 hours). And maybe test both log and stat 2) instead of TLY22, use TLY18, which has GFP under a strong natural yeast promoter - more useful for other people to compare to and understand. Larger points - dCas9 is not codon optimized.

10.8.2026

Repeating sgRNA Gibsons

Gibson and transformation into DH5alpha. Then plated them on AMP overnight.
pCERNAL-dCas9-VP with Closed/mCherry 1:

	DNA length (bp)	DNA conc. (ng/µl)	Quantity (fmol)	Volume (µl)
Closed / mCherry 1	131	10	100	0.8
Backbone (dCas9-VP16)	10326	57.3	20	2.2
H_2O	-	-	-	2
Mix	-	-	-	5
Total				10

pCERNAL-dCas9-VP with mCherry 4:

	DNA length (bp)	DNA conc. (ng/µl)	Quantity (fmol)	Volume (µl)
mCherry 4	143	10	100	0.9
Backbone (dCas9-VP16)	10326	57.3	20	2.2
H_2O	-	-	-	1.9
Mix	-	-	-	5
Total				10

pCas2 with Closed:

	DNA length (bp)	DNA conc. (ng/µl)	Quantity (fmol)	Volume (µl)

gBlock	131	10	100	0.8
Backbone (pCas2)	9816	48	20	2.5
H_2O	-	-	-	1.7
Mix	-	-	-	5
Total				10

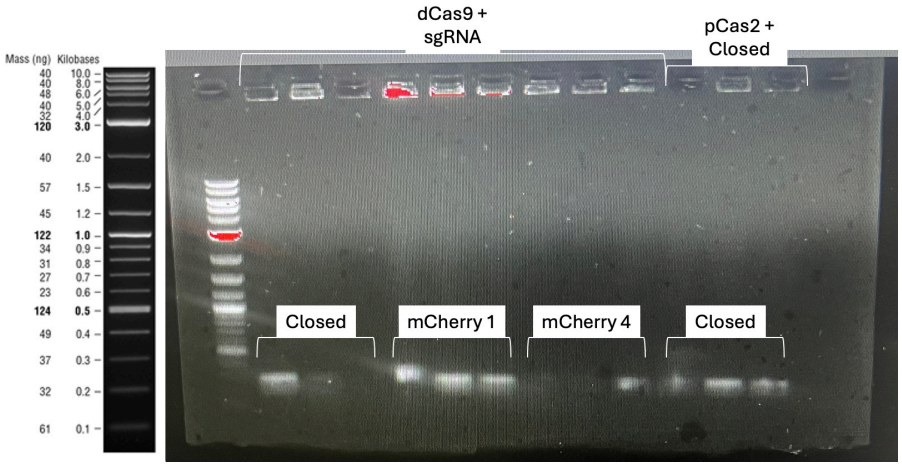
11.8.2026

Colony PCR for Gibson repeat

57ºC annealing, 8sec extension for 30 cycles with the following primers:

Template	Fr Primer	Rev Primer	Expected length (bp)
pCERNAL-dCas9-VP + sgRNA closed	Fr-gRNA-Val	Rev-hyg-gRNA-Val	240
pCERNAL-dCas9-VP + sgRNA mCherry 1	Fr-gRNA-Val	Rev-hyg-gRNA-Val	240
pCERNAL-dCas9-VP +sgRNA mCherry 4	Fr-gRNA-Val	Rev-hyg-gRNA-Val	252
pCas2 + closed	Fr-gRNA-Val	Rev-hyg-gRNA-Val	240

mCherry 4 has a shortened spacer - last 3' nucleotide mismatched on the reverse primer (A on spacer extension, G on primer).



Gel Electrophoresis 1% for validation - *positive for colony #1, #2 in closed / #2, #3 in mCherry 1 / #3 in mCherry 4 / #1, #2, #3 for pCas2 + closed.*

12.8.2026

- ☑ ~~Miniprep dCas9+closed (#1, #2), dCas9 + mCherry 1 (#2, #3), dCas9 + mCherry 4 (#3), pCas2 + closed (#2, #3)~~
- ☑ ~~Send all to Sanger (with Fr-gRNA-Val)~~

16.8.2026

Sanger results - closed rep 2

pCERNAL-dCas9-VP Results:
Closed (#2) has a deletion (19nt stem/anti-stem sequence that includes 9nt of the spacer).
Successfully validated mCherry 1.
mCherry 4 is Open 3, and also needs a new Rev-primer.
pCas2:
closed (#2) - is Open 3.

Suspected Open 3 contamination sources are the gBlock tubes or pre-cut plasmids batch (or sigma water).

Could re-make plasmids batch - would be beneficial for future topologies
gBlocks would be harder to replace - the only source of contamination here would be during diluting them right when we received the gBlocks

Re-preparing samples for validation and sanger:

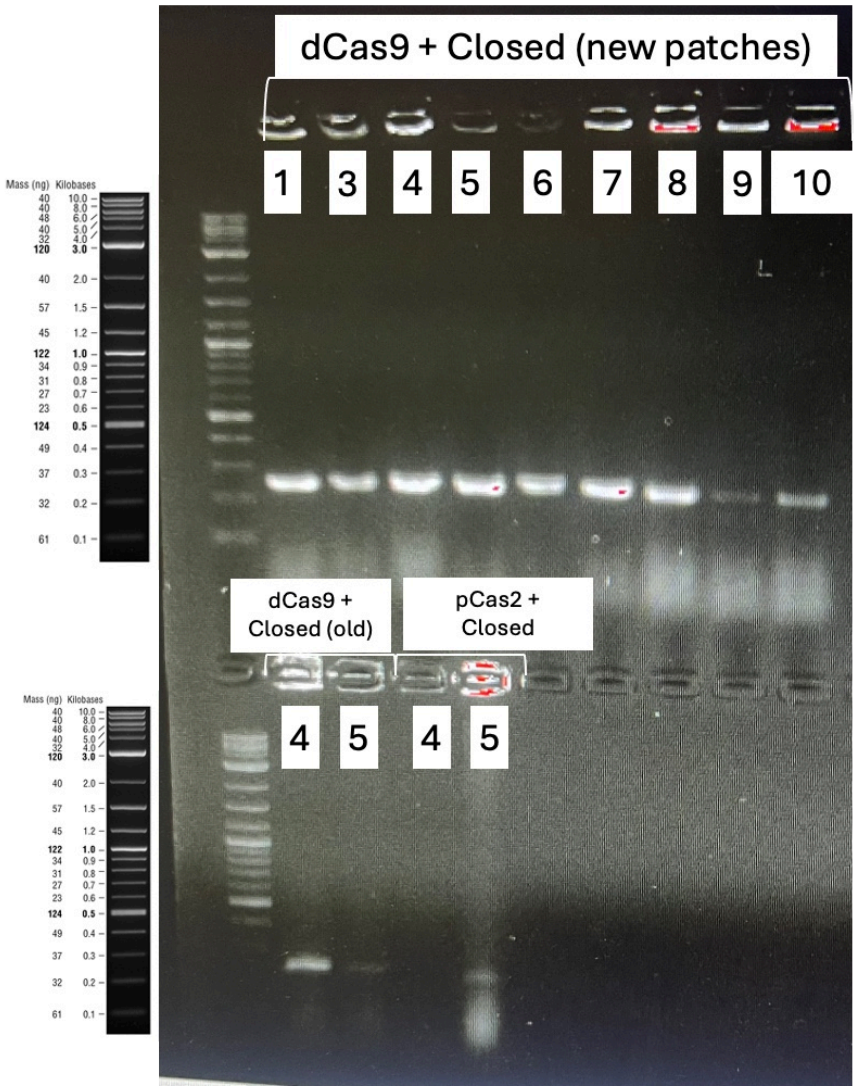
Patches and Starters for 10 new colonies of pCERNAL-dCas9-VP + Closed
Starters for 4 older patches of pCERNAL-dCas9-VP + Closed
Starters for 4 older patches of pCas2 + Closed

17.8.2026

Colony PCR and Minipreps

Setting 13 reaction in gel - 9 from new pCERNAL-dCas9-VP plate, 4 from older plate (2 dCas, 2 pCas2)
57ºC annealing, 8 sec extension for 30 cycles:

Template	Fr Primer	Rev Primer	Expected length (bp)
pCERNAL-dCas9-VP + sgRNA closed	Fr-gRNA-Val	Rev-hyg-gRNA-Val	240
pCas2 + closed	Fr-gRNA-Val	Rev-hyg-gRNA-Val	240



Gel electrophoresis 1.5% - *Validation PCR for closed sgRNA, from two plates(aug-16th, aug-11th).*

19.8.2026

Sanger results

pCas2+closed #3, and 6 out of 7 colonies of dCas9 (old and new) are open 3 again.
pCERNAL-dCas9-VP #10 (new) doesn't align to anything ?
Still have (new) #3, #4, #5, #7, #9 that we can send.
Starter for last week mCherry 1 colony #2 for glycerol stocks.

20.8.2026

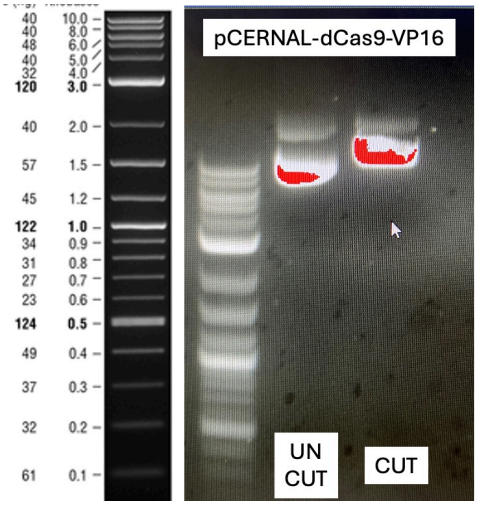
Miniprep and #436

Made Glycerol stocks of pCERNAL-dCas9-VP+mCherry 1 numbered #436.
Sorted yeast boxes - discarded old failed attempts of closed gibsons and empty vector plasmid.
Purified a new batch (>700ng/µl) of pCERNAL-dCas9-VP plasmids (empty vectors, without gRNA).

24.8.2026

Making pCERNAL-dCas9-VP + Closed

Cutting pCERNAL-dCas9-VP16 at the sgRNA integration site with StuI - as preparation for gibson.



Gel electrophoresis 1% - loading 300ng of both cut and un-cut plasmids; 5µl cut plasmid with 1µl loading dye, 0.4µl uncut plasmid with 4.6µl H_2O and 1µl loading dye.

Did have some bends at very large molecular weight, could be un-cut products. But most seem good.

While the gel runs clean-up with PCR clean-up®.

Followed by Gibson for Closed sgRNA:

	DNA length (bp)	DNA conc. (ng/µl)	Quantity (fmol)	Volume (µl)
Closed	131	10	100	0.8
Backbone (dCas9-VP16)	10326	82	20	1.55 (127ng)
H_2O	-	-	-	2.65
Mix	-	-	-	5
Total				10

DH5-alpha transformation and plating.

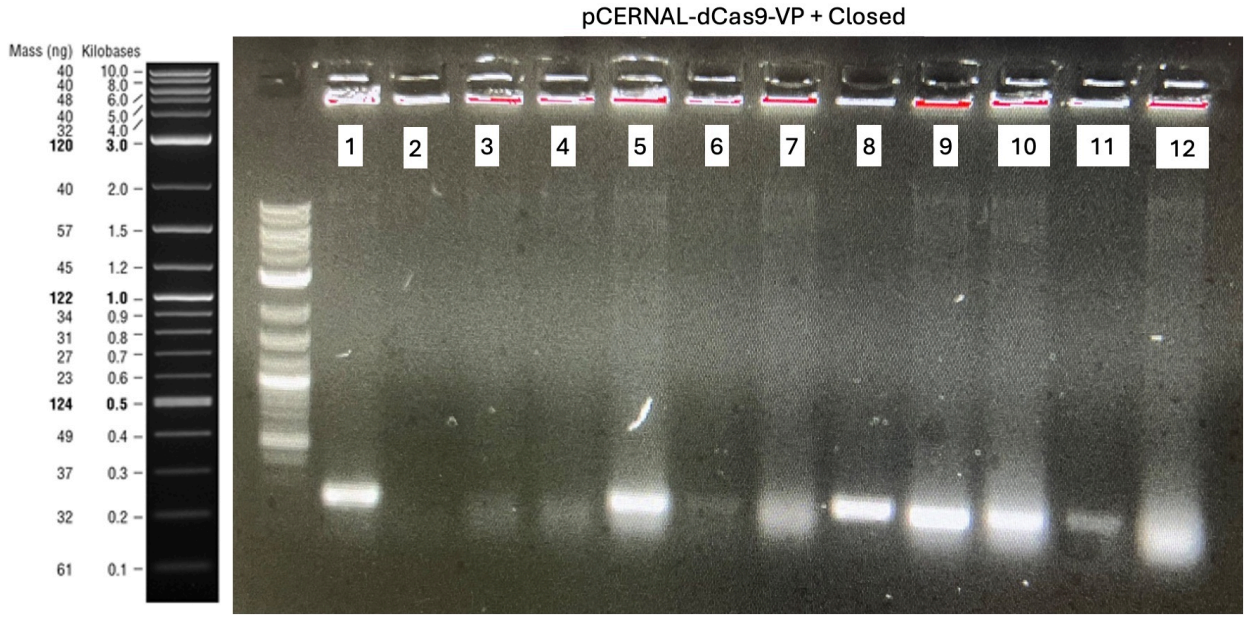
27.8.2026

Colony PCR - New Closed batch

Using Fr-gRNA-Val and Rev-hyg-gRNA-Val mix to amplify a 240nt section from 12 colonies; 57ºC annealing, 6 sec extension and 30 cycles - verified in a 1% gel

	Volume (µl)
Rapid Taq	5
Fr + Rev Primers mix (0.4mM)	0.4
H_2O	4.6
Total	10

All except colony 2 were positive - some had better signal then others, decided to proceed with 4 strong and 2 weak, maybe the self-folding is problematic and stronger gel bends indicate mutations?



Taking colonies 1, 4, 5, 8, 9, 11 for miniprep and sanger.

31.8.2026

Mini-prep and sending to Sanger

Got good concentrations (>500) and sent to sequencing, with the Fr-gRNA-Val primer.

3.9.2026

Sanger Results Closed Rep 3

The resulting Gibsons still had some contamination with open 3 - pointing to source gBlock contamination, but less than previous iterations.
Those without open 3 were unsuccessful restrictions, with one gibson with mutation.
Potential ways forward:

1. Repeat Gibson with a larger fragment - could a short sequence self fold during exonuclease activity?
2. Proceed with mCherry 1 sgRNA switches to get some results.

7.9.2026

Planning new gBlocks

For mCherry Switches experiments:
pRS425 + mCherry Plasmids (Ready in iGEM 2022 box). We have plates of -Leu, -Ura and both.

- Need to make -leu, and -ura -leu, liquid media
- Transformation - #102 with mCherry 1 switch (2026 Box), pRS425 (2022 Box) and both.

For New gBlocks Closed:

- XbaI enzyme - double cutter, give extra time, heat inactivation 65C for 20min
- Cutting pCERNAL-dCas9-VP with XbaI.

Plan For FACS with mCherry 1 switch:

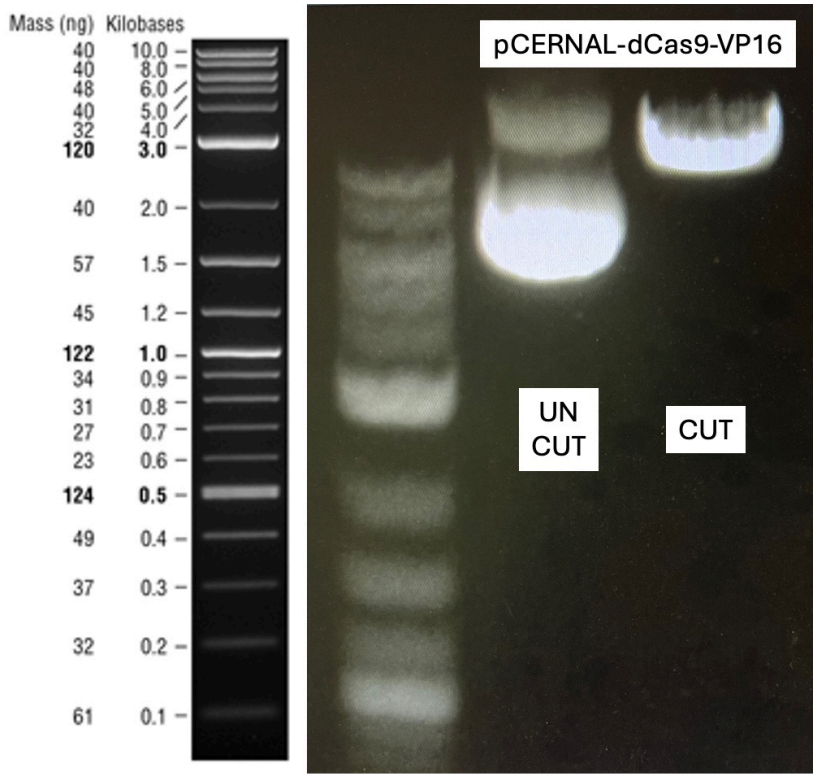
- ☑ ~~Day 0 - Starter for yeast 102 (YPD), 103 (-URA), 104 (-URA).~~ ~~Sep 9, 2026~~
- ☑ ~~Day 1 - Transformation:~~ ~~Sep 10, 2026~~ ~~**+ liquid SC -URA -LEU**~~
  - ☑ ~~#102 - with 1. Switch (**-URA**) and 2. Switch + trigger (-URA -LEU).~~
  - ☑ ~~#103 - with trigger (-URA -LEU).~~
  - ☑ ~~#104 - with trigger (-URA -LEU).~~
    - ☑ ~~Two days growth on plates (URA -LEU all except **#102-1 only -URA**).~~
- ☑ ~~Day 3 - Starter for transformants,102, 103, 104, 105, TLY18 and WT.~~ ~~Sep 14, 2026~~
  - ☑ ~~Pick 3 colonies of 102-1, 102+T. One colony of 103+T, 104+T. mark them on the plate.~~
  - ☑ ~~Liquid **SC**: TLY18, WT~~
  - ☑ ~~Liquid SC -**URA**: 102-1, 103, 104, 105~~
  - ☑ ~~Liquid SC -**URA -LEU**: 102-2, 103+T, 104+T~~
  - ☑ ~~Overnight.~~
- ☑ ~~Day 4 - Early dilutions for starters and 3-5 hours recovery~~ ~~Sep 15, 2026~~

- [x] ~~WT, TLY18, empty (103), empty + trigger (103+T), Normal (104), normal + trigger (104+T), Open 1 (105), switch (3 repeats, 102-1), switch + trigger (3 repeats, 102+T). Total of 13.~~

10.9.2026

Transformations and XbaI cutting

Cutting pCERNAL-dCas9-VP with XbaI at 42C - protocol is 37°C - was still successful:



Transforming yeast with dual plasmids

	Volume (µl)
polyethylene glycol - PEG 3350	240
Boiled salmon sperm (10mg/ml)	10
LiAc 1M	36
Yeast	50
DNA (~250ng) of mCherry 1 switch (0.1 conc,)	2.5
DNA (~200ng) of trigger	0.4

Complete with H_2O	21.1
Total	360

Transforming with trigger only:

	Volume (µl)
polyethylene glycol - PEG 3350	240
Boiled salmon sperm (10mg/ml)	10
LiAc 1M	36
Yeast	50
pRS426+mCherry (~100ng) + H_2O	0.2 + 23.8
Total	360

Transforming with switch only:

	Volume (µl)
polyethylene glycol - PEG 3350	240
Boiled salmon sperm (10mg/ml)	10
LiAc 1M	36
Yeast	50
mCherry 1 switch (~100ng, 0.1x Conc.) + H_2O	1 + 23
Total	360

Plated and waiting for colonies.

14.9.2026

mCherry Switch FACS Starters

Colonies successfully grew on all plates - including double transformations. 3x biological replicates for mCherry 1 switch and Switch+Trigger. Made patches for each.

15.9.2026

Running the FACS

Came early to dilute the overnight growth and reach the log stage. After 4 hours of growth, ran the FACS.

- Still had the two populations - a very small and uniform cell population and another larger with variance population. The Larger population is within the range of previous experiments Daniel ran.
- Leaving only larger cells gets rid of a significant portion of cells (up to 85% in some cases).
- Switch 1 presented the same fluorescence, switch 2 presented decrease in fluorescence (almost half) and switch 3 presented 10% increase in flourescenc
- Switch seems open regardless of trigger

Sample (no mCherry)	Mean GFP (a.u.)	Median	Fold over WT	% GFP-positive	% in bright mode (>500)
No gRNA (should match switch OFF)	175.8	175	1.01	0.9	0.1
Open1 (open guide)	584.4	217	3.35	57.8	36.8
gRNA (open guide)	535.2	195	3.07	49.3	29.7
Switch-1	581.2	220	3.34	60.1	35.7
Switch-2	587.9	223	3.37	61.2	34.2
Switch-3	590.1	223	3.39	60.8	35.9

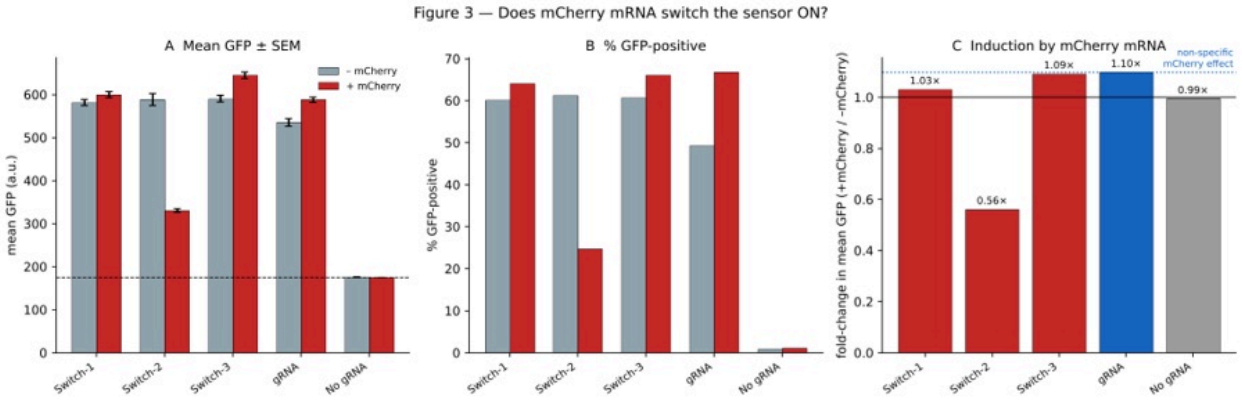


Figure 1. Mean GFP, % GFP-positive, and fold-change on mCherry addition. The dotted blue line in panel C is the non-specific effect measured on the open gRNA control; no switch clears it.

The experiment failed at the blocking step, not at the sensing step. Until the closed switch is measurably darker than an open guide, the mCherry response cannot be evaluated at all: a construct that is already fully ON has no room to be turned on. The 3-9% changes seen on mCherry addition are within, and below, the non-specific effect measured on the always-open control.

Gibson Plan Closed v3 (XbaI)

Gibson Plan for Closed 1 (XbaI):

	DNA length (bp)	DNA conc. (ng/µl)	Quantity (fmol)	Volume (µl)
Closed version 3	684	20	71.2	1.5
Backbone (dCas9-VP16, XbaI)	9776	61.5	20	2
H_2O	-	-	-	1.5
Mix	-	-	-	5
Total				10

– transform DH5alpha, Plate on LB AMP.

16.9.2026

Gibson Closed v3

Gibson was performed as planned, leading to transformation to DH5alpha, Plated on LB AMP

23.9.2026

Patches and Starters

The DH5alpha stock was contaminated, most of the colonies had visible mCherry expression.
Took 12 colonies without visible mCherry for starters and patches.
Colony PCR to verify Gibson success.

Planning forward - Checking this Closed 1 v3 sgRNA, new topology closed sgRNA, and primers
Rev-TEFpr-Val - sits after the XbaI site (downstream of gBlcok) and can be used for validation.
Fr-gRNA-Val - still fits all constructs.
Design newer Rev-gRNA-HYG-Val2: “CTGATGCAGCTCTCGGAGTTTTAGA”

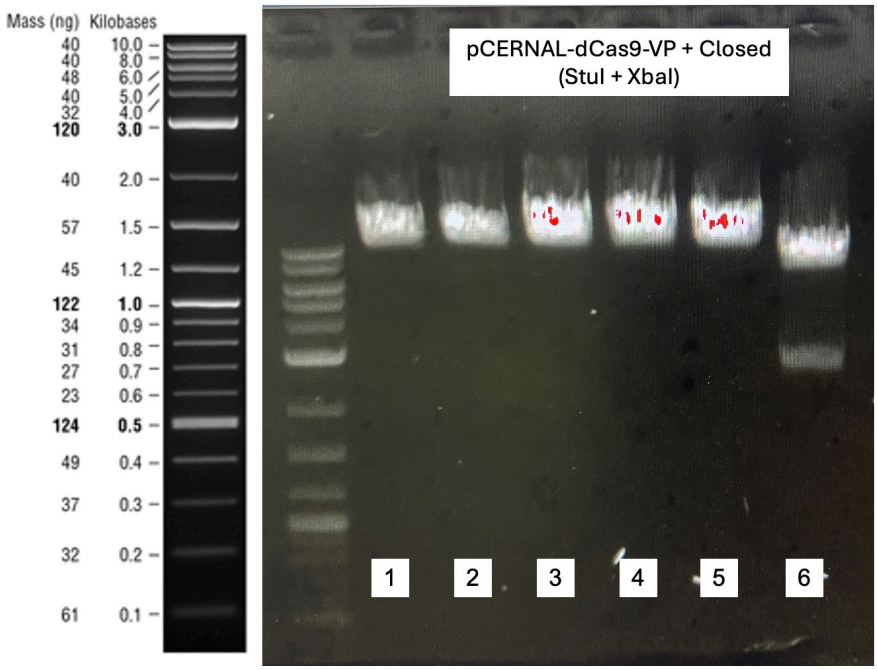
24.9.2026

Fingerprinting verification of Closed

Mini-prep the plasmids and did restriction fingerprinting, sent the plasmids to plasmidsaurus for sequencing.

	Lengths
Template	Per reaction
Restriction 1	0.5
Restriction 2	0.5
rCutSmart	1.5
H2O	Per reaction
Total	15µl

	Expected Lengths
pCERNAL-dCas9-VP	2685, 7641
pCERNAL-dCas9-VP + Closed	10400 (linear)



Gel Electroporesis 1% - *Colony #6 appears to have the empty plasmid, without the closed sgRNA.*

27.9.2026

Transforming Closed (XbaI)

Changing media on yeast starter - trying to get to the log stage for transformation. Then Washing, and transforming:

	Volume (µl)
polyethylene glycol - PEG 3350	240
Boiled salmon sperm (10mg/ml)	10
LiAc 1M	36
Yeast	50
DNA (~100ng) + H_2O	24
Total	360

then plated on -URA plates – we need to make new ones – waiting for the sequencing results from plasmidsaurus to decide which will continue.

29.9.2026

Closed 1 Sequencing Results

Received Closed 1 (XbaI) results from Plasmidsaurus. Colonies 1,3,4 are good – point mutations in unrelated areas and are not expected to cause any issues. Colony 2 had multiple mutations along the gBlock and in its homology region. Colony 5 had a point mutation in the tRNA that serves as a promoter for the sgRNA, thus deemed unsuccessful. Colony 6 was indeed an empty vector / failed gibson (as was assumed after fingerprinting in 24.9).
Conclusion: extending the gBlock and using XbaI is recommended.

Set 5 patches for each of the good colonies, and starters of bacteria colony #3 for the glycerol stocks.

30.9.2026

Glycerol stocks & Starters

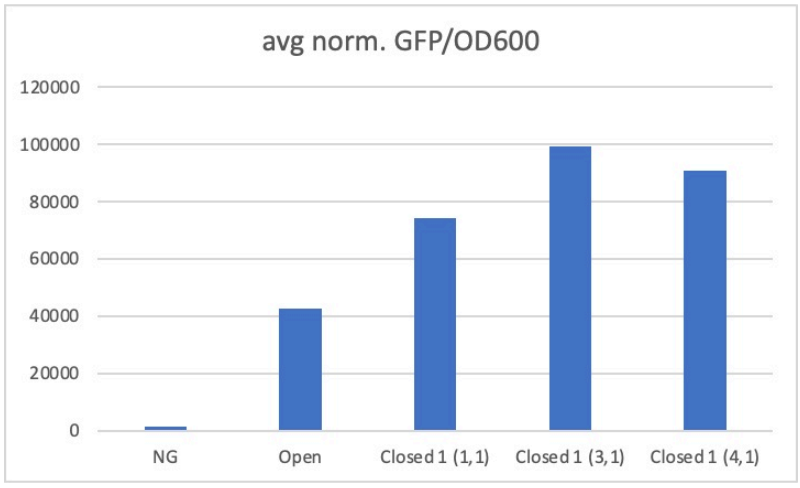
Made glycerol stocks of bacteria with Closed 1 (#433).
Set starters for plate reader tomorrow - 102 (8xSCA), 105 (Open 1 sgRNA) and Closed 1 (x3).

1.10.2026

PlateReader for Yeast

Run the closed variants in the plate-reader. Took 200µl of each sample - SC, 102, 105, and the three closed variants, and set 2 technical replicates for each sample except for the SC. Yeast starters were at about O.D.600nm=0.4 when loading. Results were normalized in the following way:

- Norm. O.D.600nm and GFP for each sample were calculated by reducing the media from each sample.
- Norm. GFP was divided by norm. O.D.600nm (to obtain GFP/OD).
- The average of the two technical replicates (GFP/OD) was calculated and used for graphing.



All variants were higher than no guide, open guide and specifically Closed 1 (bacterial colony 3, patch 1) was highest - possibly noise, will run FACS after newer candidates are ready.

5.10.2026

Closed C22-C25

Gibson for new closed variants - they include the anti-scaffold section and fit into XbaI cut plasmids.
Resuspend gBlocks in 25µl TE to reach final conc. of 20 ng/µl according to IDT protocol, then follow the plan for the 4 switches and 1 closed (5 tubes total):

	DNA length (bp)	DNA conc. (ng/µl)	Quantity (fmol)	Volume (µl)
Switch S22 Switch S23 Switch S24 Switch S25 Closed C24	703 711 708 725 708	20	73.9 73.06 73.37 71.65 73.37	1.6 1.6 1.6 1.6 1.6
Backbone (dCas9-VP16, XbaI)	9776	61.5	20	2
H_2O	-	-	-	1.4
Mix	-	-	-	5
Total				10

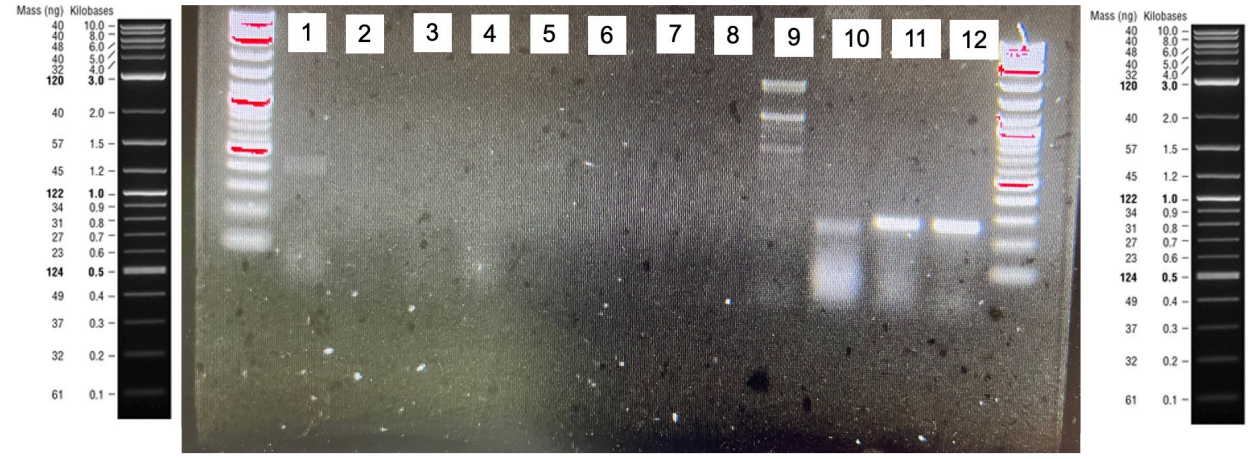
After Gibson, Transformed DH5alpha and plate on AMP.
Also made -URA plates, Kan plates and AMP plates.

6.10.2026

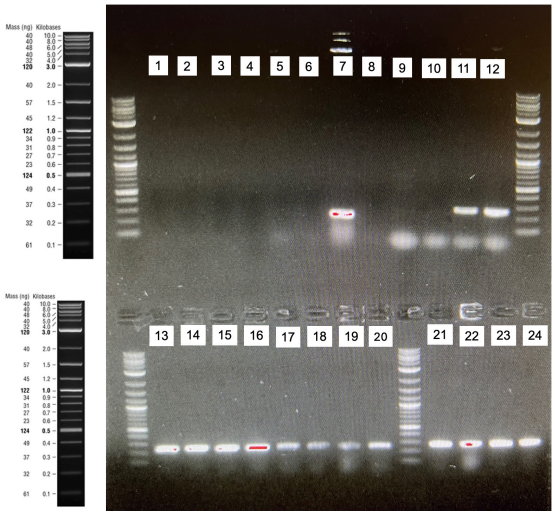
PCR Validation

Validating yesterday's gibson/transformations
Using Fr-gRNA-Val and Rev-hyg-gRNA-Val mix to amplify a 241nt section from 4 colonies of each transformation; 57ºC annealing, 8 sec extension and 30 cycles.

	Volume (µl)
Rapid Taq	5
Fr + Rev Primers mix (0.4mM)	0.4
H_2O	4.6
Total	10



Gel electrophoresis - 1 to 8 are toeholds for bacteria / 9, 10 are colonies 1,2 of Closed C24 / 11, 12 are colonies of 1, 2 of Switch S24.



Gel electrophoresis - wells 1 to 8 are toeholds for bacteria. well 9, 10 are colonies 3,4 of Closed C24. wells 11, 12 are colonies of 3, 4 of Switch S24. wells 13-16 are S22. wells 17-20 are S23. wells 21-24 are S25.

Set starters of colonies #4 of each except Closed #2.

7.10.2026

Miniprep and sanger of new architecture

Miniprep and sent to sanger sequencing using Fr-gRNA-Val. Starter of switch S23 #4 didn't grow, so sent the remaining 4 – S22, S24, S25 and C24.

11.10.2026

Transformations

Transforming yeast with the pre-made plasmids, without sequencing confirmation to save time?
Rounded all DNA samples to 0.4µl (up to 4 times higher)

	Volume (µl)
polyethylene glycol - PEG 3350	240
Boiled salmon sperm (10mg/ml)	10
LiAc 1M	36
Yeast	50
Total DNA (~100ng) + H_2O	24 (0.4 + 23.6)
Total	360

Plated on -URA.
Got and prepared gBlocks for closed at 20µl.

13.10.2026

Starters

Set 20 falcons for the FACS – WT, TLY18, TLY102, TLY111, TLY104, TLY105, TLY107, TLY108 and 3x od each; C24, S22, S24, S25.
Sanger results came back - couldn't sequence the spacer, the closed might be too closed for sanger?

15.10.2026

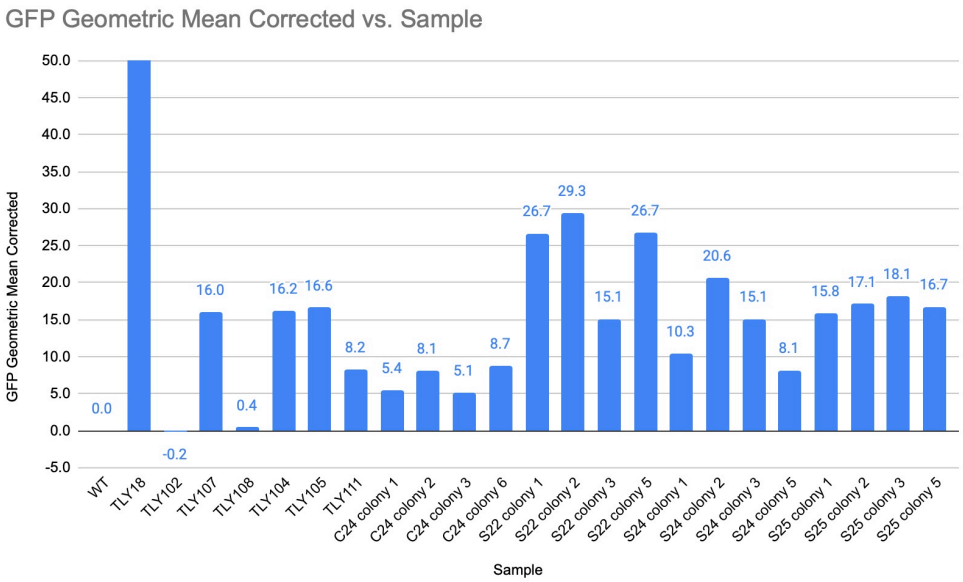
FACS 1st & 2nd gen sgRNA

Re-run the 1st-gen Closed and Switch variants with the 2nd-gen Closed and switches variants.
High-level summary:

- Positive Controls (PC) reach about 16 GFP-units(?).
- Closed (both 1st and 2nd-gen) are about half of PCs (5-8).
- Switches vary by architecture - some are as low as closed (8), others as high as double the PCs (29)
    - Variance was noted even within the same architectures, worth a deeper look?
- 1st-gen switches again exhibit a NOT Gate phenotype/logic.

Thoughts:

1. 1st-gen Closed 1 (TLY111) is leaking, not open - it's less than positive controls, open, 1st-gen switch (without trigger).
2. 2nd-gen Closed C24 - similar performance to 1st-gen Closed 1, comparable architectures of switches (C24 and S24) seems to both have lower GFP levels.
3. 2nd-gen switches display a lot of variance, both by architecture and by colony:
    a. Within S24 - Lowest reached GFP levels comparable to closed (lowest colonies of S24 reached highest colonies of C24), while highest reached PC levels.
    b. Within S22 - Highest reached double the positive controls, with one colony reaching about the same as PC.
    c. Only the 2nd-gen switch S25 was consistent among the switches, with similar GFP levels to the positive control.
4. 1st-gen Switch (TLY107, TLY108) - again displayed a logical NOT Gate behavior, dropping to negative control levels.



Next tasks:

- [ ] gBlocks C22 order

FACS:

- [ ] **SC** WT, TLY102, TLY18
- [ ] **SC –URA** Closed 1 (TLY111), TLY105, C24, Switch S22, S24, S25
- [ ] **SC –URA –LEU** TLY108 (old switch + trigger),