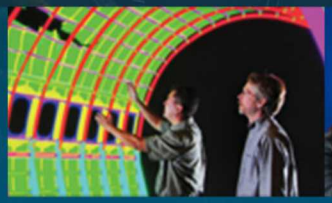
SAND2019-7447PE

Sandia National Laboratories

MAC Internship SNL: CERES Project



PRESENTED BY

1/c Val Hines

UNITED STATES COAST GUARD ACADEMY

U.S. DEPARTMENT OF ENERGY | NNSA

Sandia National Laboratories is a multimission laboratory managed and operated by National Technology & Engineering Solutions of Sandia, LLC, a wholly owned subsidiary of Honeywell International Inc., for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-NA0003525.

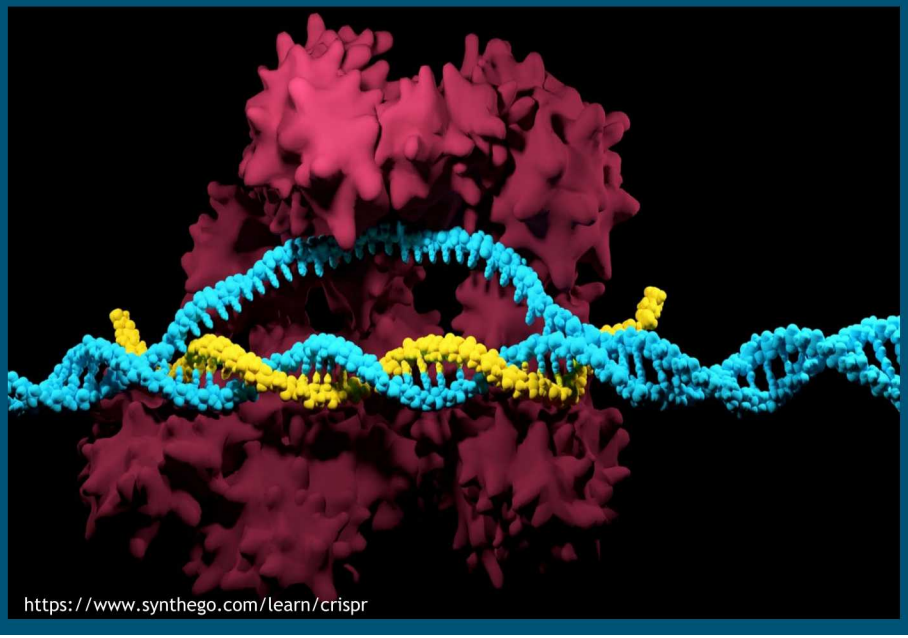
CERES Project Overview/CRISPR Background

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

CERES: CRISPR Engineering for Rapid Enhancement of Strains

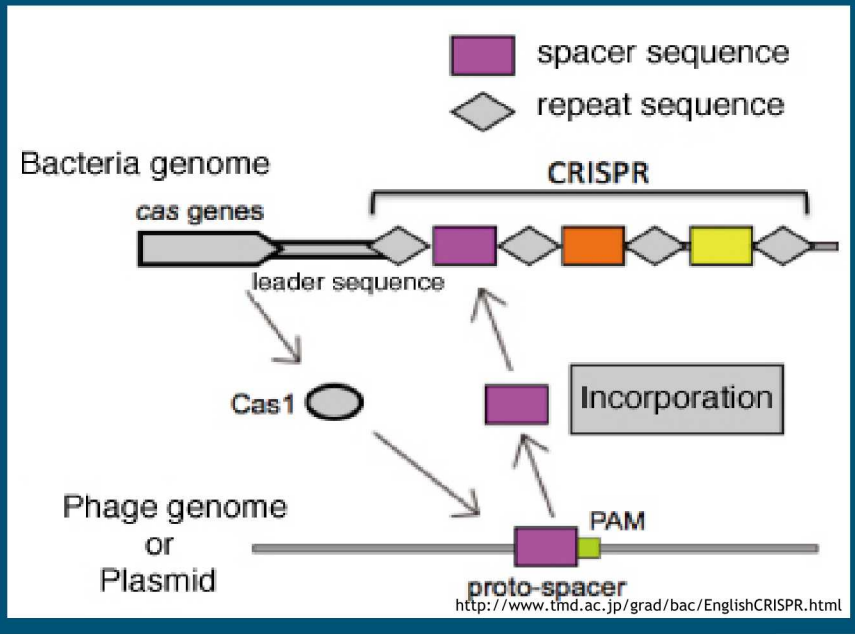
Designing gRNA sequences → Insert gRNA into the E. coli plasmid → Confirm plasmid was inserted (PCR and DNA sequencing) → Prepare the plasmid for 7002 → Insert plasmid into 7002 → gRNA inhibition cpcB= ABS 635nm; Ypet= ABS 730nm & FLUOR 530nm

SPACERS = spacer DNA-> Each spacer = unique
CAS genes= CRISPR ASSOCIATED GENES→ Make CAS Proteins→ Will often be nucleases = cut DNA.



https://www.synthego.com/learn/crispr

Spacers associated with bacteriophage DNA. When phages inject DNA into bacteria → CAS protein will cut it and destroy infection before it can insert itself into genome.
Spacers = like white blood cells→remember infectious attacker so can fight in future



http://www.tmd.ac.jp/grad/bac/EnglishCRISPR.html

CERES Project: Contributions and Big Picture Connection

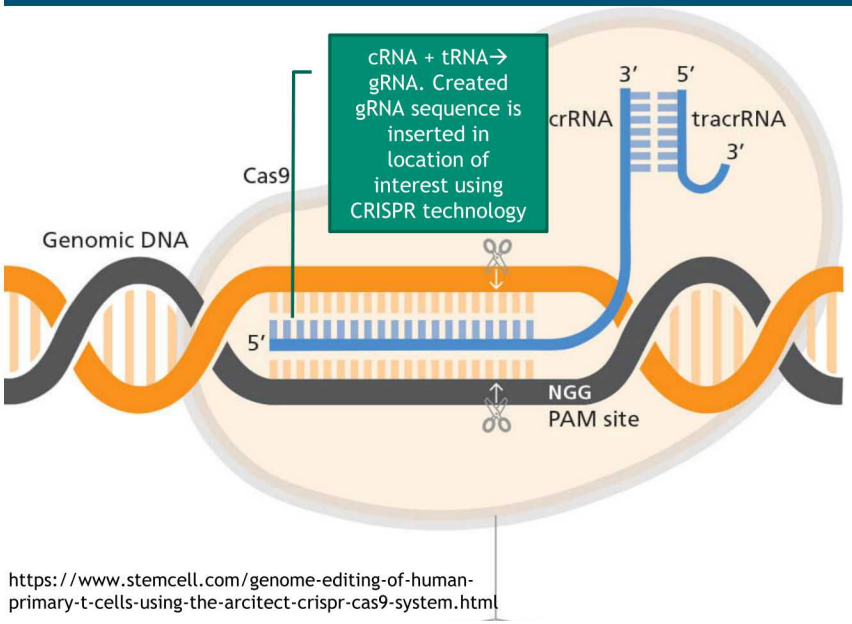
CERES: CRISPR Engineering for Rapid Enhancement of Strains

Designing gRNA sequences → Insert gRNA into the E. coli plasmid → Confirm plasmid was inserted (PCR and DNA sequencing) → Prepare the plasmid for 7002 → Insert plasmid into 7002 → gRNA inhibition cpcB= ABS 635nm; Ypet= ABS 730nm & FLUOR 530nm

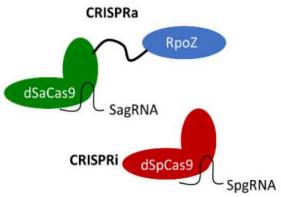
Use CRISPR-engineered technology to insert created gRNA in 7002.

Synechococcus sp. PCC 7002

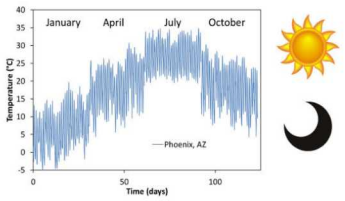
Use cyanobacteria because it is a photosynthetic organism that will use CO_2 in order to create biomass→ achieved higher biomass production through specific gRNA insertion genetic engineering→ lead to biofuel production



https://www.stemcell.com/genome-editing-of-human-primary-t-cells-using-the-arcitect-crispr-cas9-system.html



1. Establish orthogonal CRISPRi/a in 7002



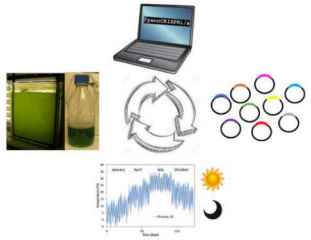
4. Transform and select under realistic production conditions

2. Identify CRISPRi/a targets

5. Characterize CRISPRi/a strains

Figure by Anne Ruffing

3. Construct genome-wide gRNA libraries



6. Iterate to improve

Prepare the Plasmid for Insertion into 7002

- Inoculated *E. coli* colonies with Y1-Y12 and C1-C6 plasmids
  - Control is the parent plasmid

Where "C" = cpcB gene and "Y" = Ypet gene

- ***Plasmid Purification (Miniprep):***
  - *E. coli* with desired DNA plasmid is pelleted, resuspended in resuspension buffer, lysed, and neutralized before spinning the column down to remove the "snot"—all of the *E. Coli* material that is not DNA.
  - The supernatant with DNA is moved to a binding column and eluted with water.
  - Goal is to elute pure DNA and then confirm that the DNA has the desired plasmid based on band sized on a gel electrophoresis run.
- ***Linearization/Digestion:***
  - "Cut" the plasmid at AatII cut site using mixture of AatII and Cut-Smart buffers.
  - Plasmid will linearize→ Facilitates insertion into 7002.

https://www.thermofisher.com/order/catalog/product/K0503

Results

- Ran two separate purifications and subsequent linearizations of Ypet and cpcB samples to prepare for transformation.
  - First pre-transformation concentrations were very low.
  - Second pre-transformation concentrations were higher with minimal outliers (Table 1).

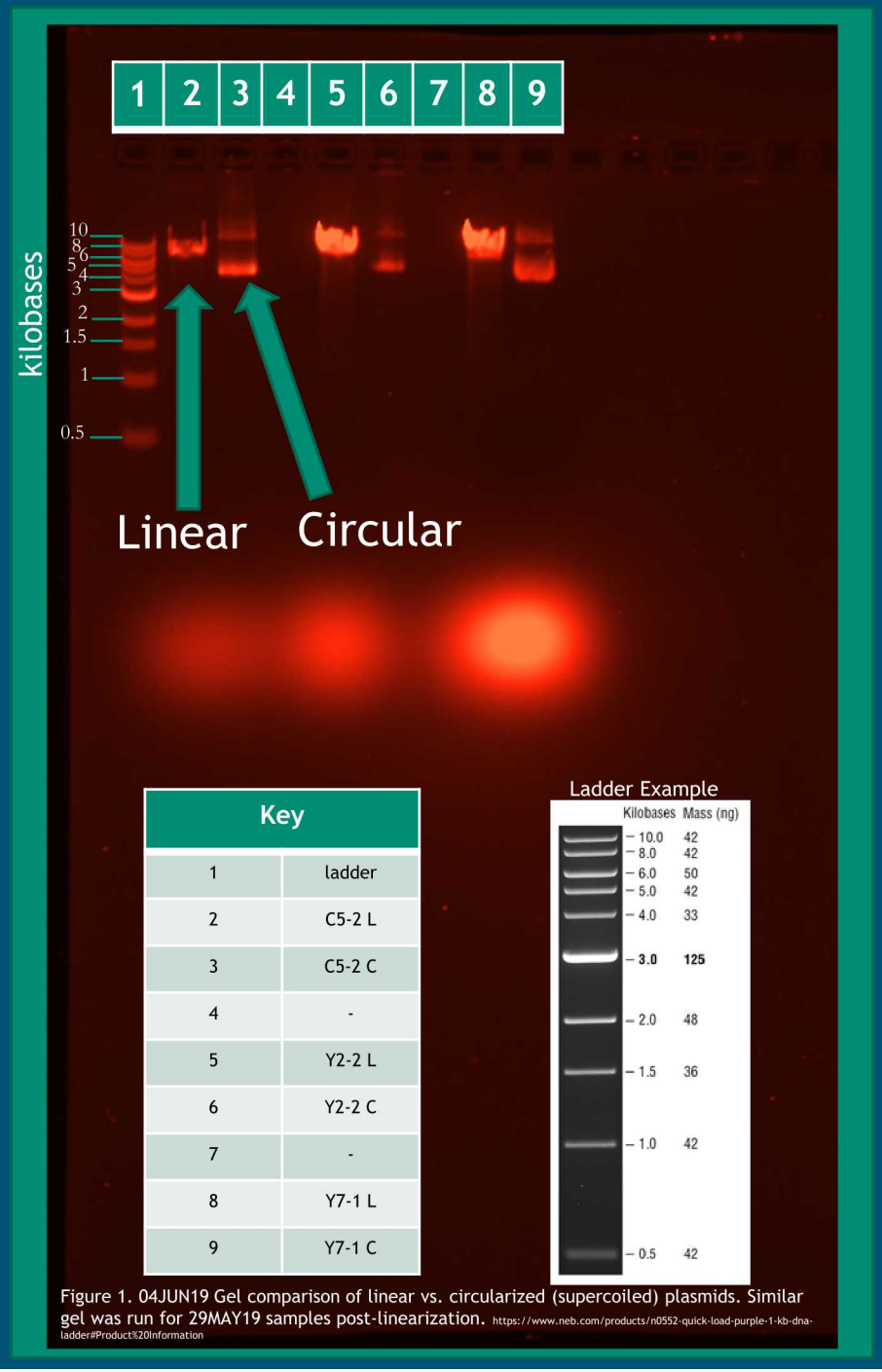


Figure 1. 04JUN19 Gel comparison of linear vs. circularized (supercoiled) plasmids. Similar gel was run for 29MAY19 samples post-linearization. https://www.neb.com/products/n0552-quick-load-purple-1-kb-dna-ladder#Product%20Information

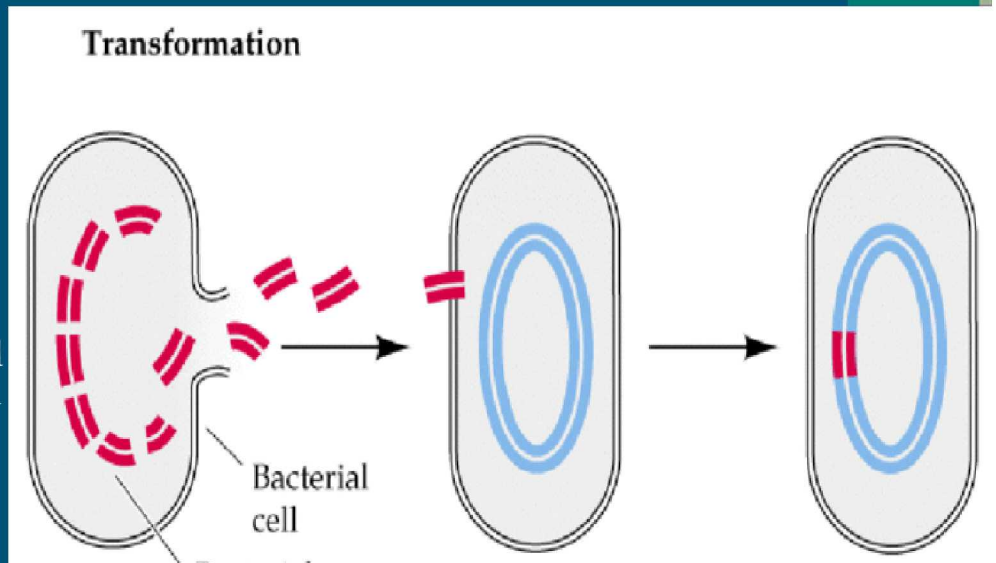
Ypet and cpcB Candidate Concentrations Post-Linearization

Y1-1	357.1
Y2-1	18
Y3-2	260.9
Y4-1	297.1
Y5-1	323.1
Y6-1	52.8
Y7-1	467
Y8-1	12.3
Y9-2	212.4
Y10-1	224.3
Y11-1	127.9
Y12-1	111
C1-8	134.6
C2-3	227.8
C3-23	148.4
C4-22	231.1
C5-2	270.2
C6-5	138
pN1SD	96.5
Y2-2	513.9
Y6-2	527.4

Table 1. Concentrations of Linearized gRNA candidates 29MAY19. Outliers highlighted in yellow. Samples used in gel highlighted in green

Insert gRNA into 7002

- Transform linearized plasmid into 7002 strains→ Then spread on A+/Spectinomycin (Sp) plates to select for genome integration
  - Only colonies with the inserted plasmid (with the Sp resistance gene) should grow on plate.
- ***Transformation:***
  - Amount of each sample from linearization to add to test tube for transformation is calculated based on desired addition of 1 ug of DNA and nanodrop concentrations to determine volume of sample to add (Table 1).
  - Plasmid is added to specific strain of 7002 in a test tube and then incubated at 30C 60 uE light, and 150rpm shaking for 24 hours.
    - Cyanobacteria cells are naturally competent and therefore incubation will prepare the cells for insertion of plasmid (i.e. no heat shock is needed).
- ***Streak, Restreak, and Colony PCR***
  - Post incubation, sample is pelleted resuspended in 50uL of test tube solution, and then spread on A+/Sp40 plates.
    - Colonies are streaked 2 more times (for a total of 3) in order to select for only modified genomes (those with inserted plasmid).
  - Use colony PCR to confirm integration into genome.
    - If integrated band on gel will be 4.4 kb
    - If wild type band will be 1.2 kb



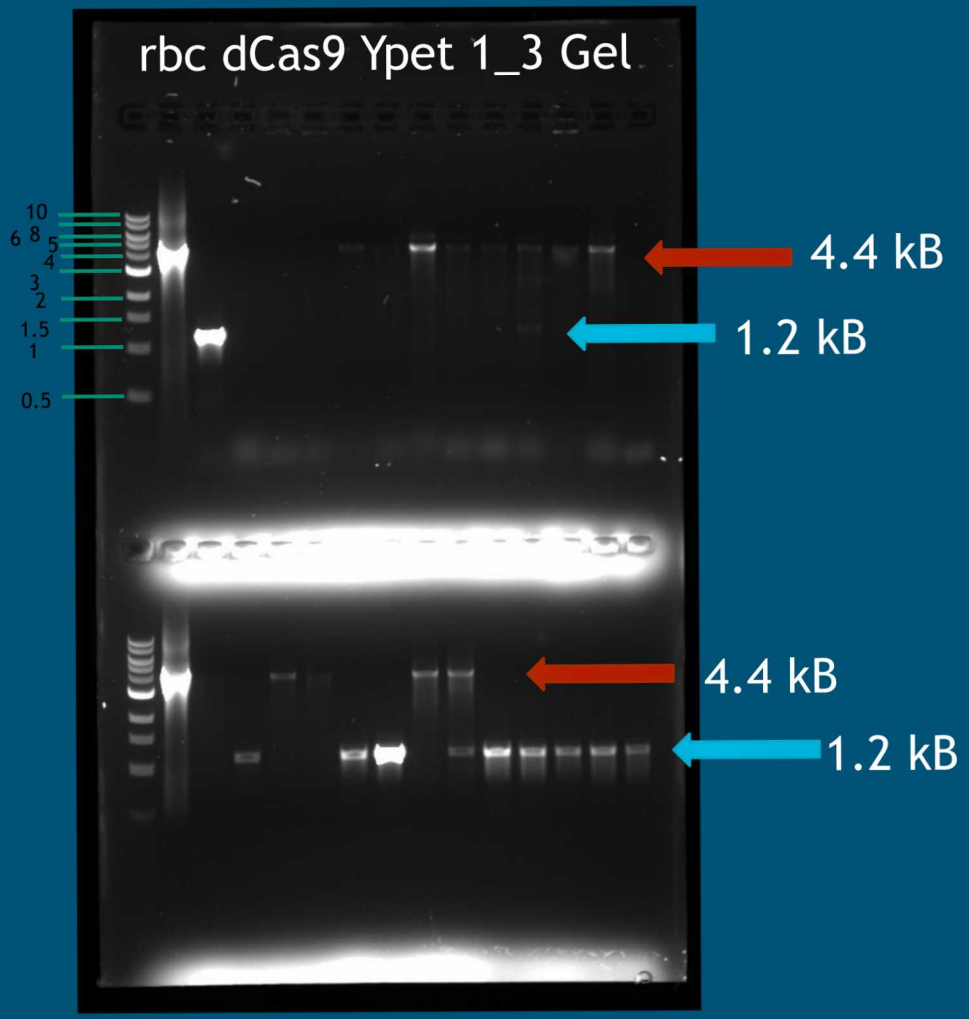
https://bio.libretexts.org/Bookshelves/Microbiology/Book%3A_Microbiology_(Boundless)/7%3A_Microbial_Genetics/7.11%3A_Genetic_Transfer_in_Prokaryotes/7.11B%3A_Bacterial_Transformation Permission pending from Sinaur Associates, Inc.

Used for amplification→ Exponential process to copy gene of interest

PCR: Polymerase Chain Reaction

1. ***Steps***
   1. ***Melt*** (2 DNA Strands separate)
   2. ***Anneal*** (reaction cooled, primers attach to specific location (H-bonding) in genome)
   3. ***Elongation*** (pull base pairs from solution and binds them→ Rxn heated so Taq DNA polymerase can add bases)

Results: Post-PCR Gel and Transformation Streak and Re-streak

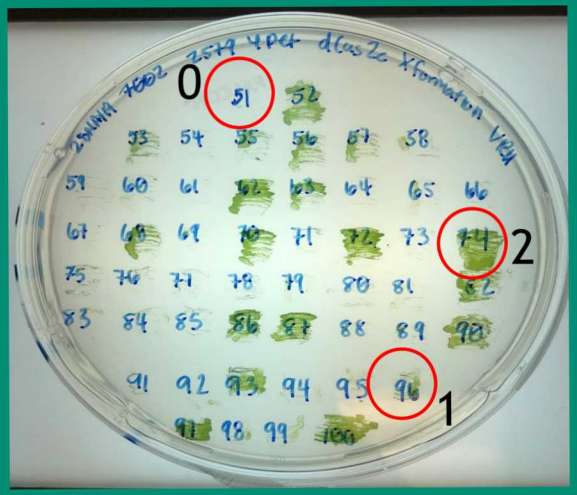


Gel from post colony PCR. Bands identified above were identified as positive for insertion of plasmid due to location of band at the 4.4 kB position.

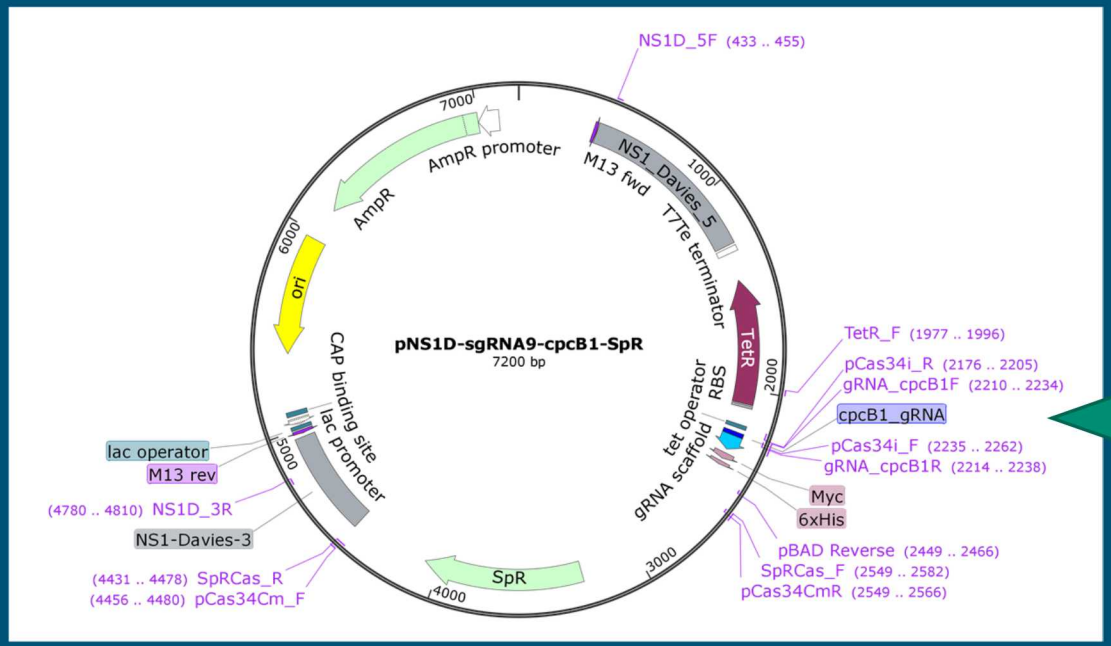
- There were 151 successful colonies across all three strains on the original streaked transformations.
  - The most successful strain was 7002-2579 with 119 successful colonies.
- 57.62% of the 151 re-streaks grew successfully.

Table 2. Growth of Streaked Transformations

Ranking	*Qualifier*	*Occurrence*	*Percentage*	Total Growth
0	No Growth	64	42.38	57.62
1	Some Growth	31	20.53	
2	High Growth	56	37.09	



Measure activity of gRNA and dCas inhibition in strains of 7002

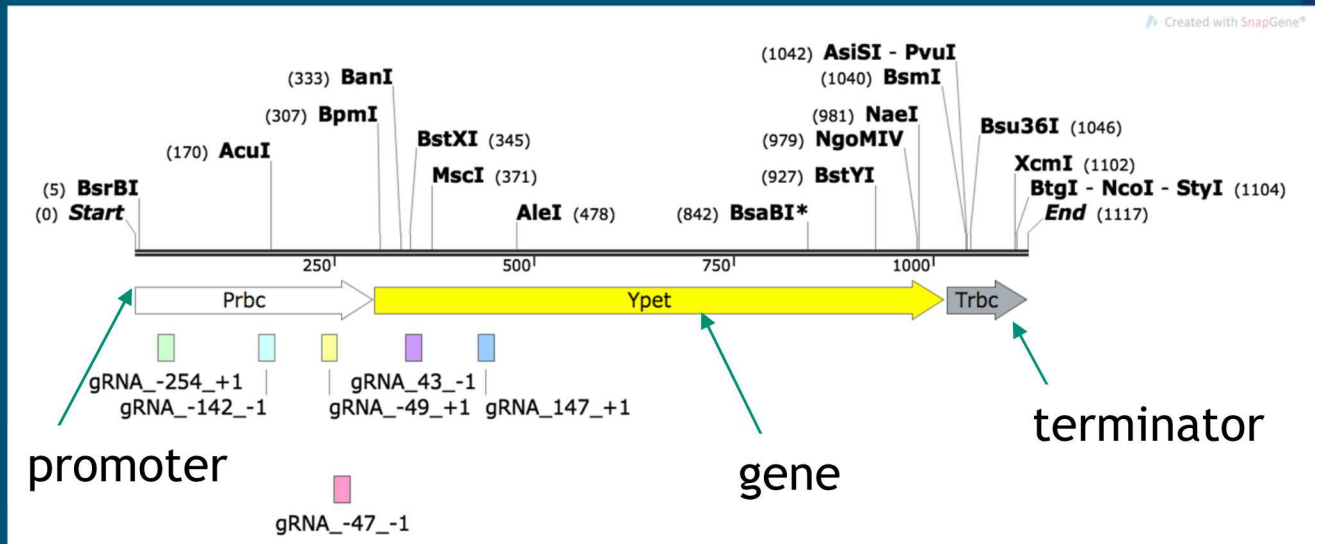


cpcB

- a gene involved in phycocyanin production and known photosynthetic pigment with absorbance at 635 nm.

Ypet

- a yellow fluorescent protein gene that fluoresces with emission at 530 nm and excitation at 480-490nm.



Results: cpcB Absorbance Initial and 48-Hour

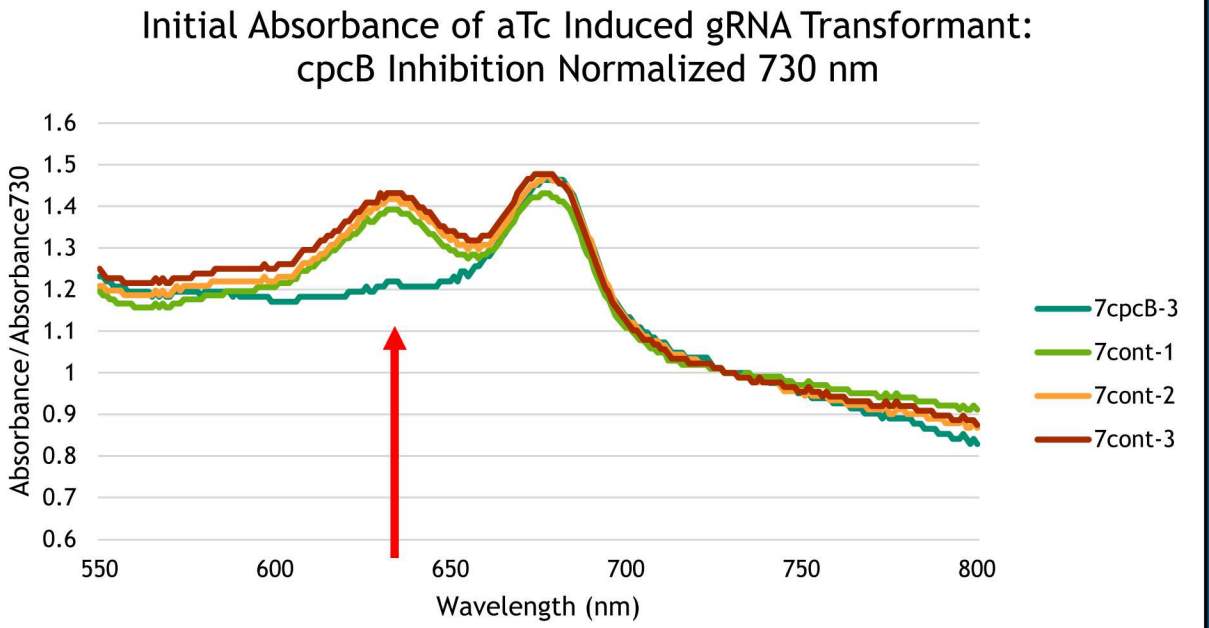


Figure 2: Initial Absorbance of cpcB.

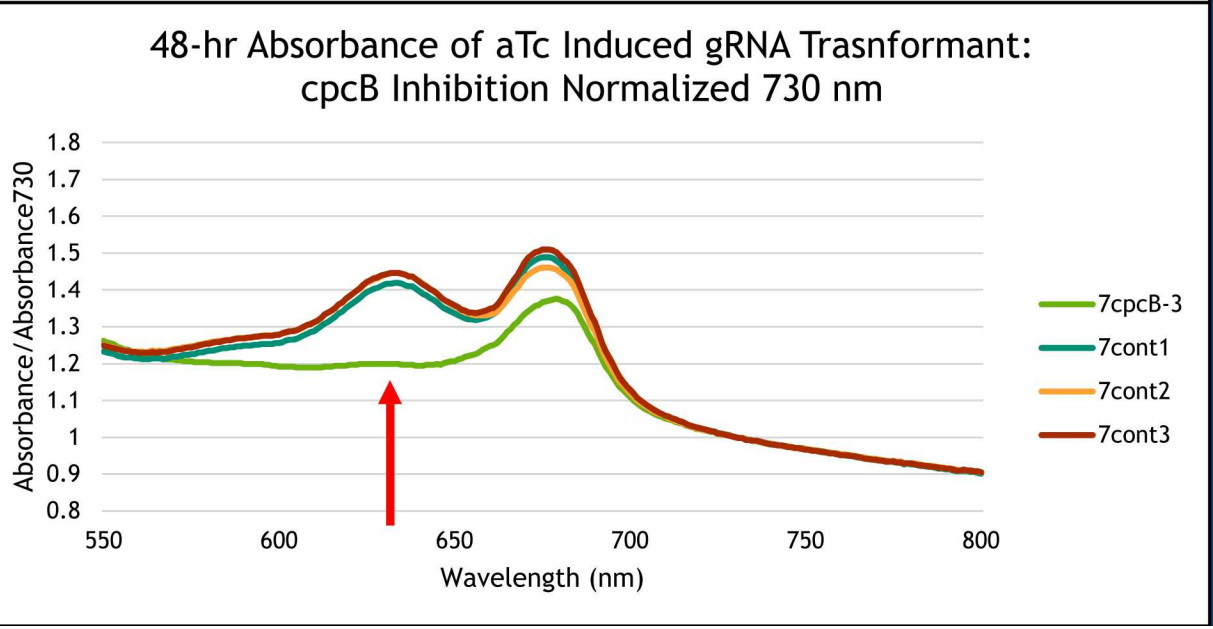


Figure 3: 48-hour absorbance of cpcB.

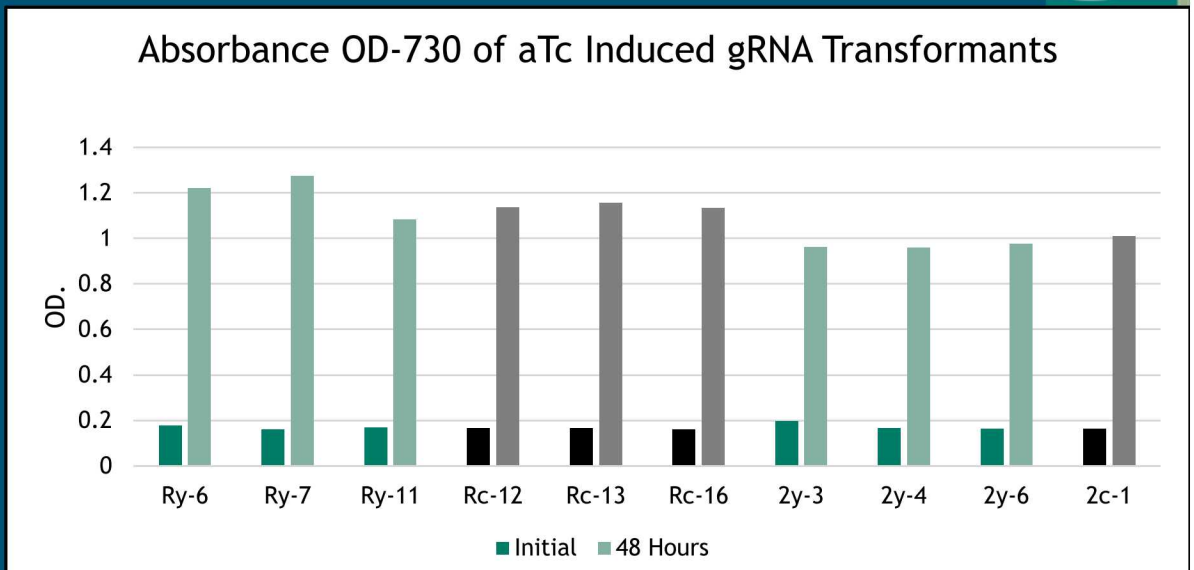
Key
7cpcB - 7002 cpcB
7cont – 7002 control

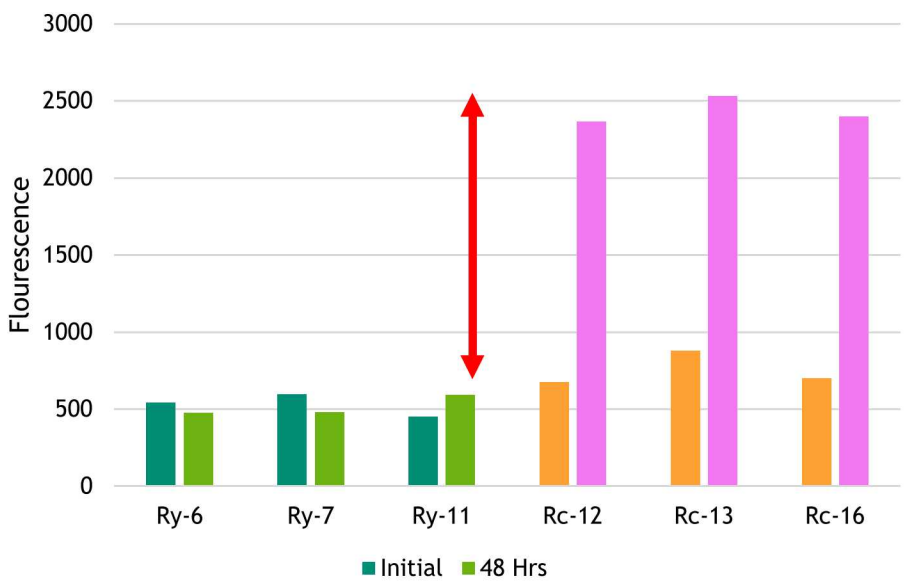
Results: Ypet Absorbance and Fluorescence Initial and 48-hour

Figure 4: Initial and 48-hour absorbance of Ypet

Figure 5 &6: Initial and 48-hour fluorescence of Ypet

KEY
Ry – rbc Ypet
Rc – rbc control
2y – 2579 Ypet
2c – 2579 control





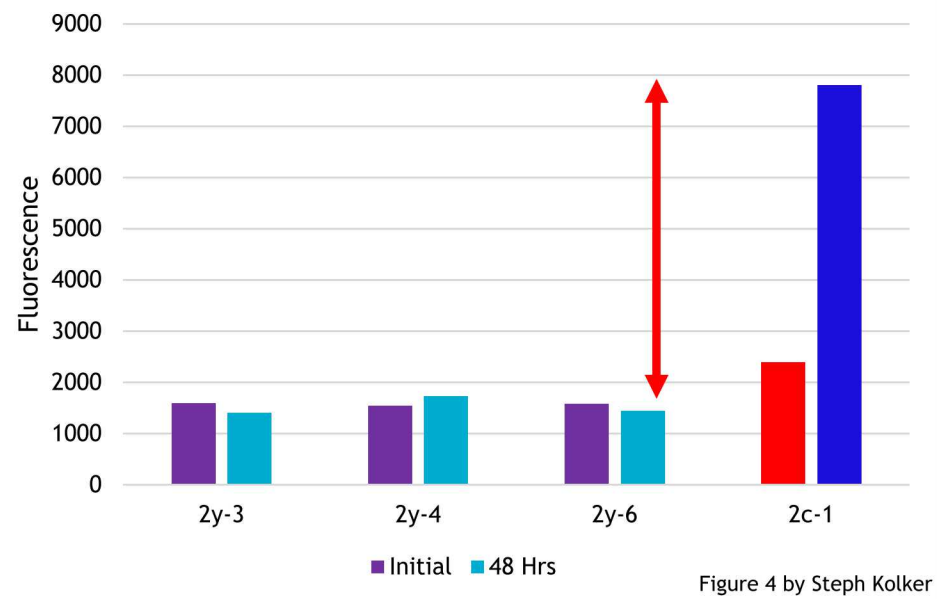
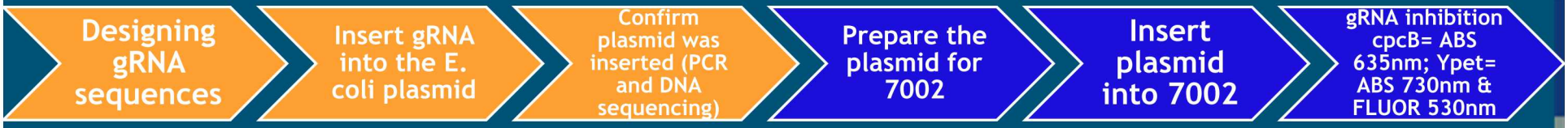


Figure 4 by Steph Kolker

Summary and Future Directions

- Focus was on last 3 steps:
  - ***Prepare the plasmid***: Plasmid Purification/Isolation/Miniprep and Linearization experiments
  - ***Insert the plasmid into 7002***: Transformation, streak, re-streak, and colony PCR confirmation
  - ***gRNA inhibition measurements***: Use plate reader to obtain absorbance and fluorescence measurements
    - 730nm and across spectrum of 550nm to 800nm for cpcB.
    - absorbance at 730 nm and fluorescence at excitation wavelength of 480-490nm and emission at 530 nm taken for Ypet



Future Directions:

- CRISPRa (activation)
- gRNA library
- Select specific gRNA to increase biomass productivity.

Acknowledgements

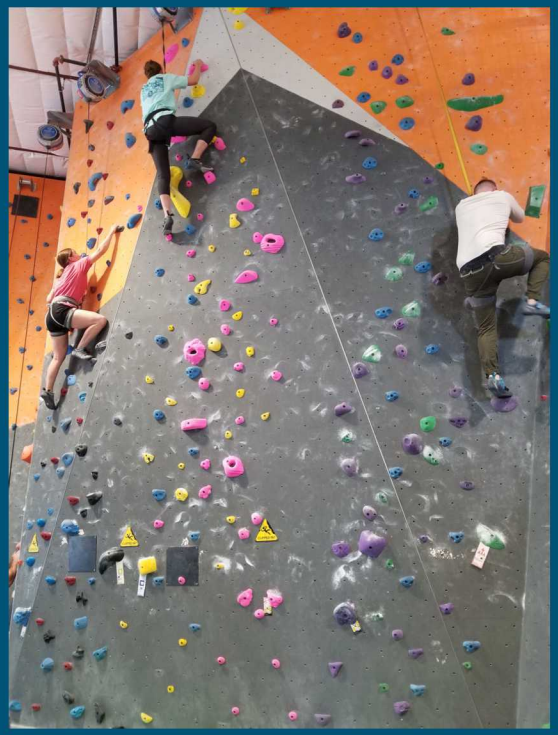
- Anne, Chuck, Jesse, Steph, and Gus
- Staci, Jaime, and the Sandia National Labs MAC Internship Program
- CDR Daffler and USCGA Science and Engineering Departments
- My chauffeur, Pat
- Dr. Gray and USCGA Department of Chemistry

Sandia National Laboratories

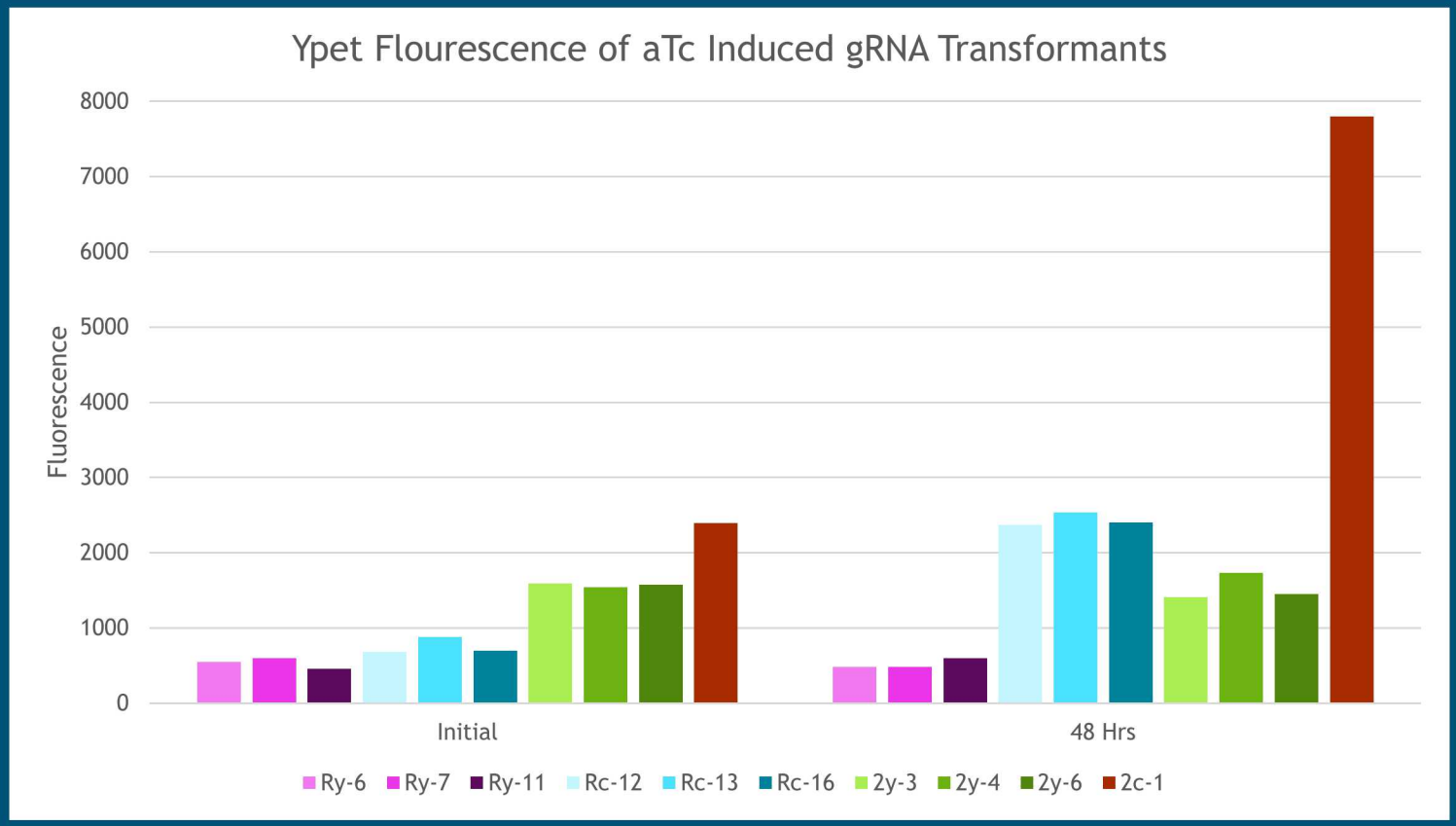
UNITED STATES COAST GUARD ACADEMY

"Non-Technical" Knowledge

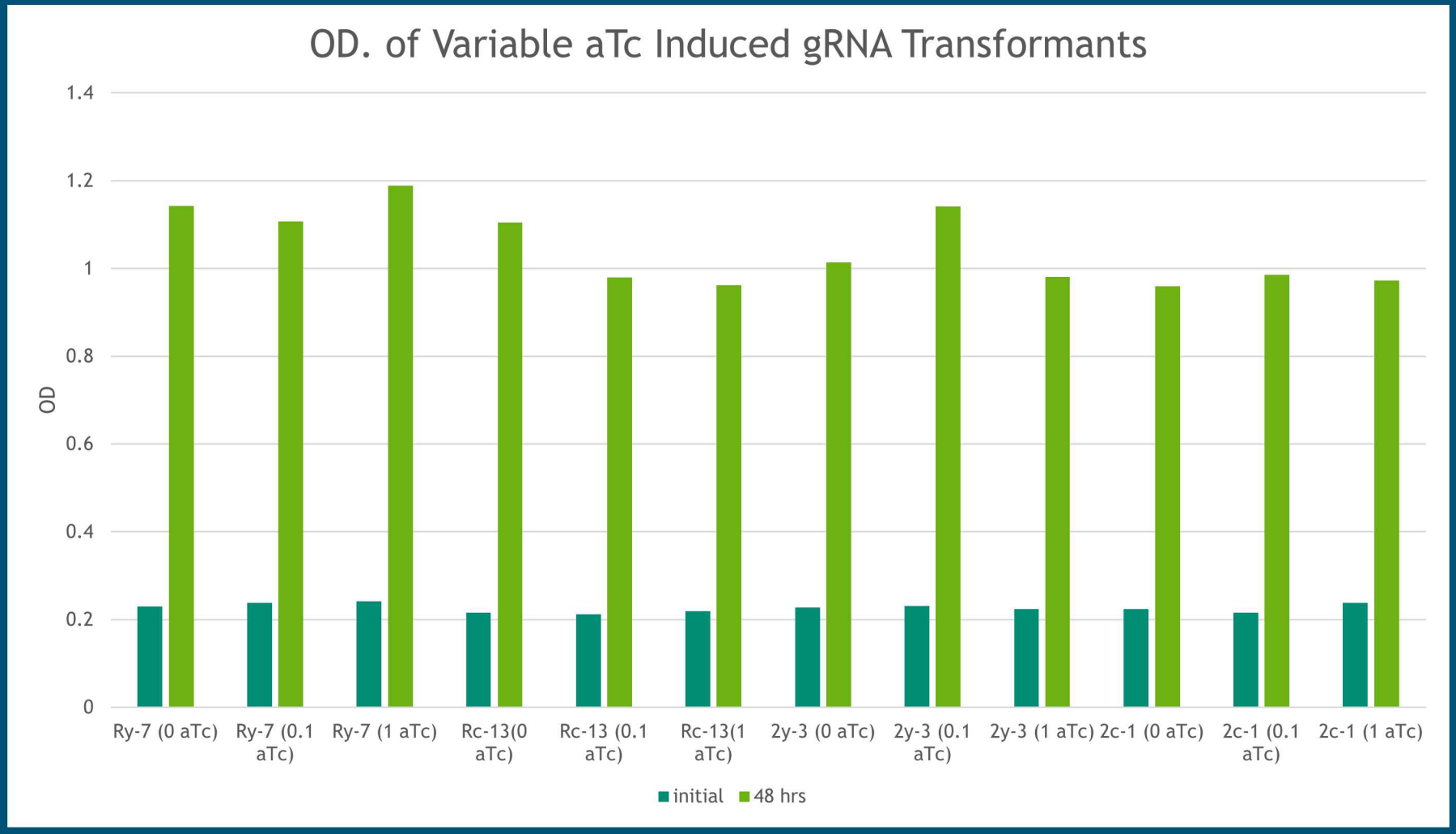
- Cap up vs cap down debate—cap sideways
- Heat the gel for 1 minute 33 seconds
- Horizontal vs. vertical tape
- Jesse's coffee grinder takes ~100 turns
- Save gloves
- Protocols are better explained with sound effects
- Phages make more sense with faces



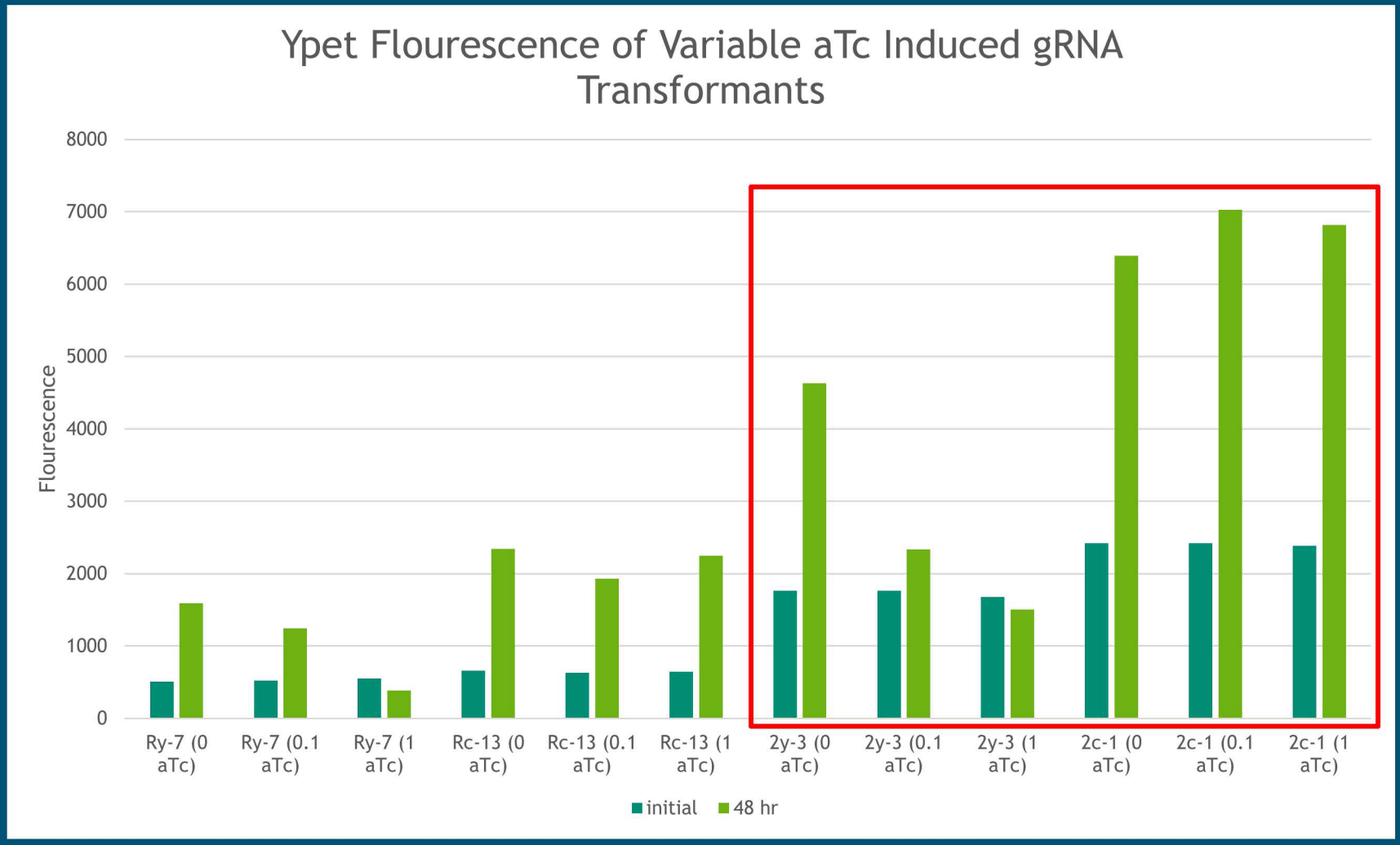
Initial vs. 48-Hour Fluorescence (Different Visualization)

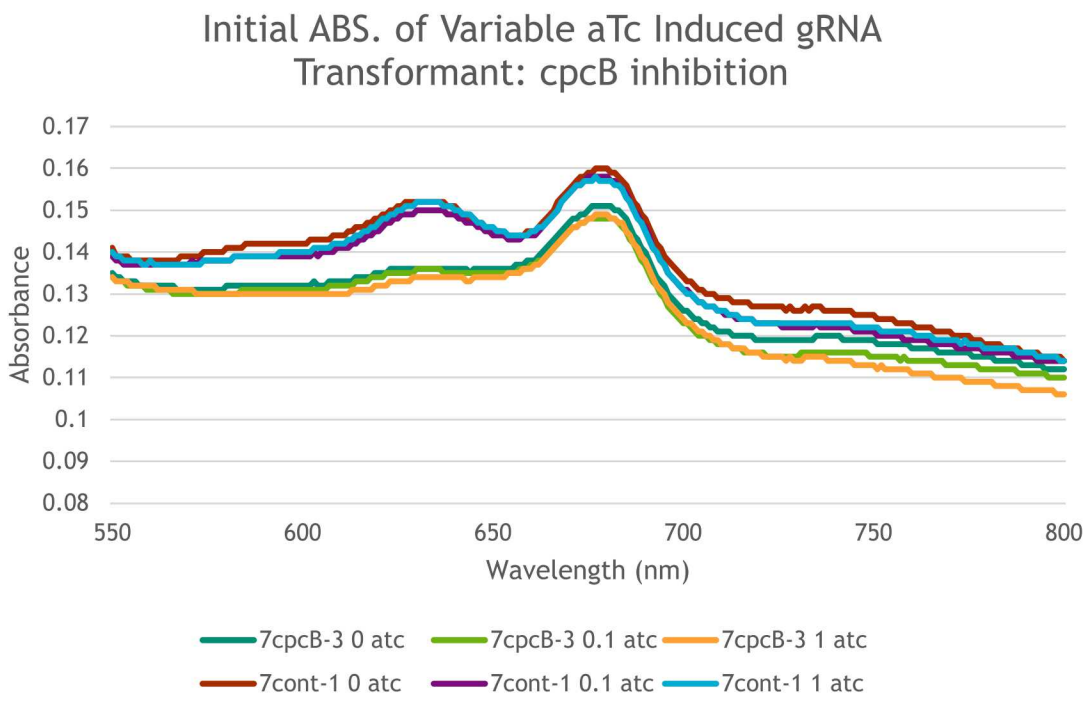


Ypet aTc Diff Concentrations: Absorbance



Ypet aTc Diff Concentrations: FL





cpcB aTc Diff Concentrations: Absorbance Initial and 48-hr