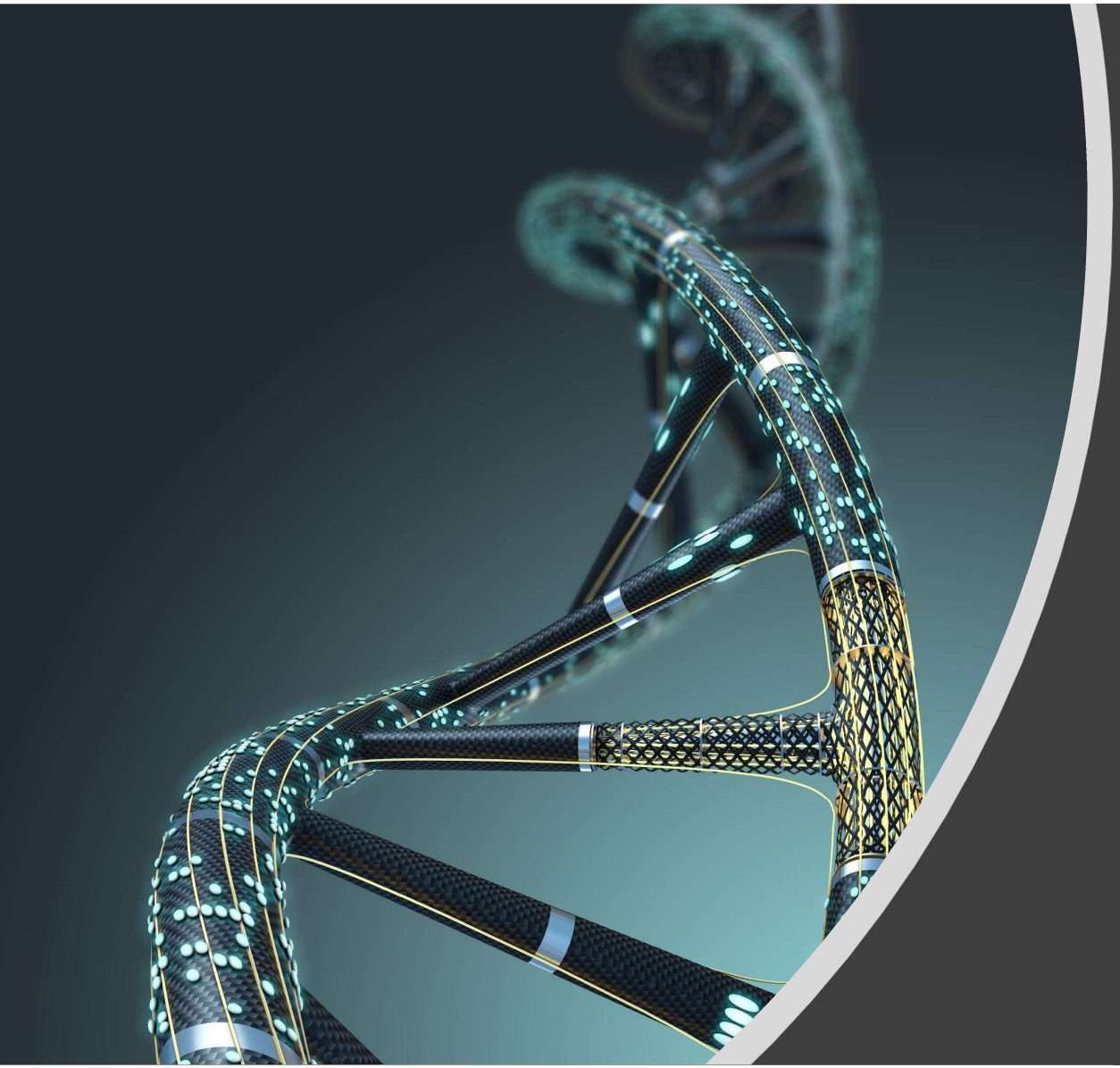
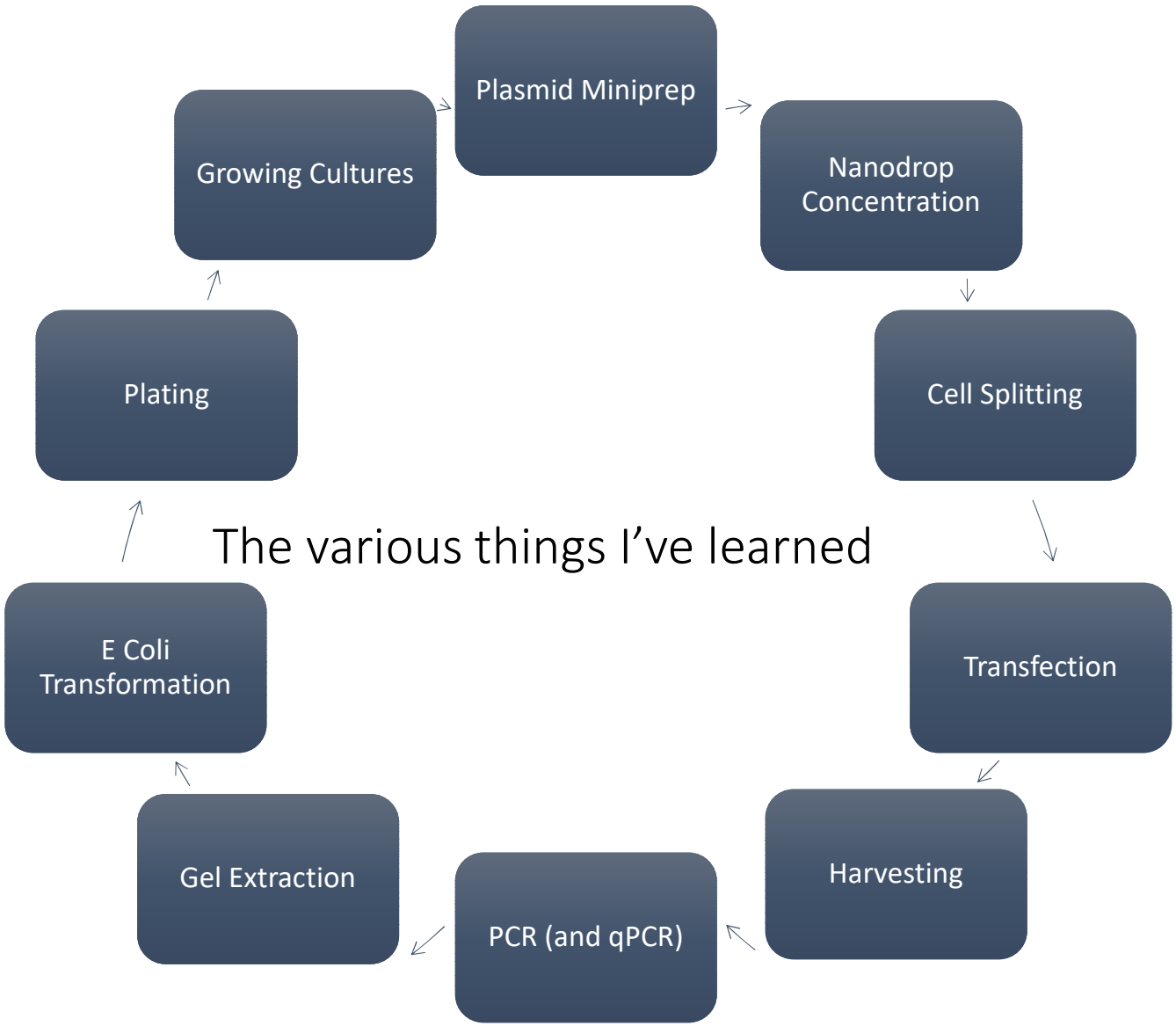


Reddy Lab Rotation

Gabriel Kennedy

03/01/23

Let me start with some personal background

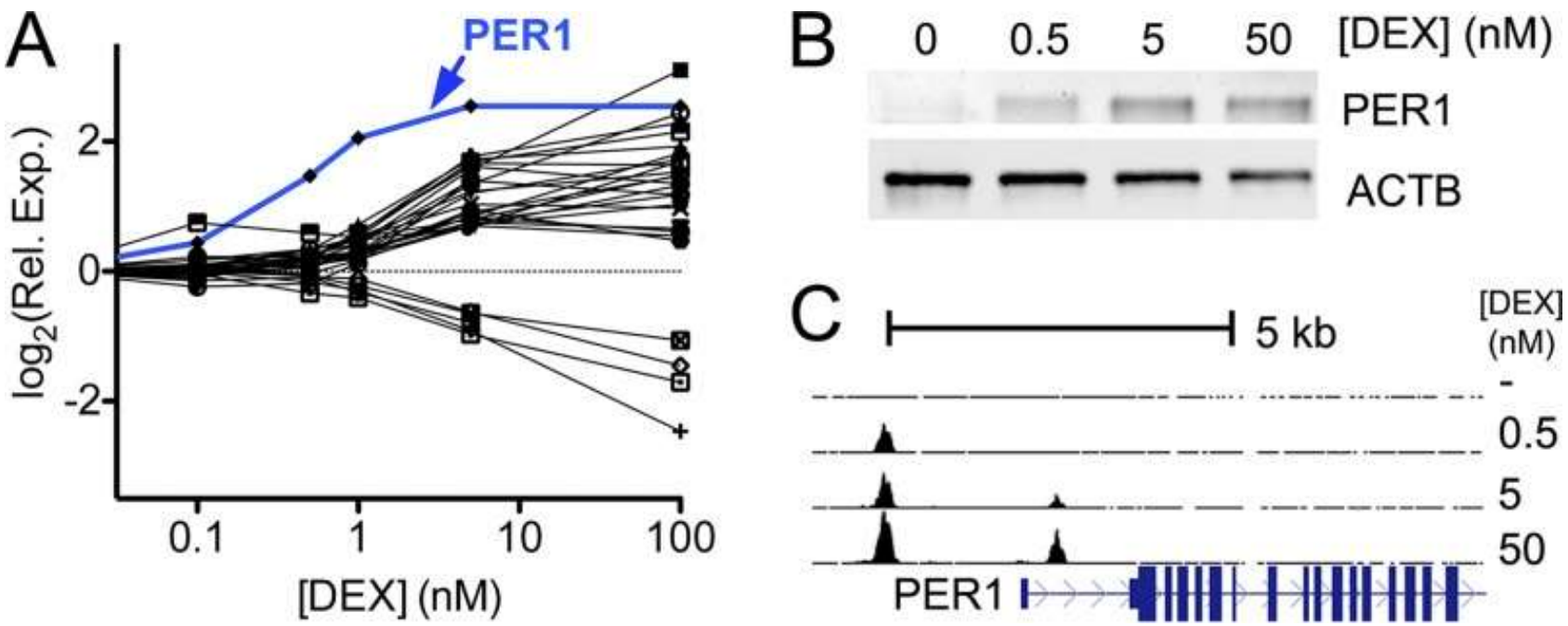

Plasmid Miniprep
Nanodrop Concentration
Cell Splitting
Transfection
Harvesting
PCR (and qPCR)
Gel Extraction
E Coli Transformation
Plating
Growing Cultures
The various things I've learned

Additionally

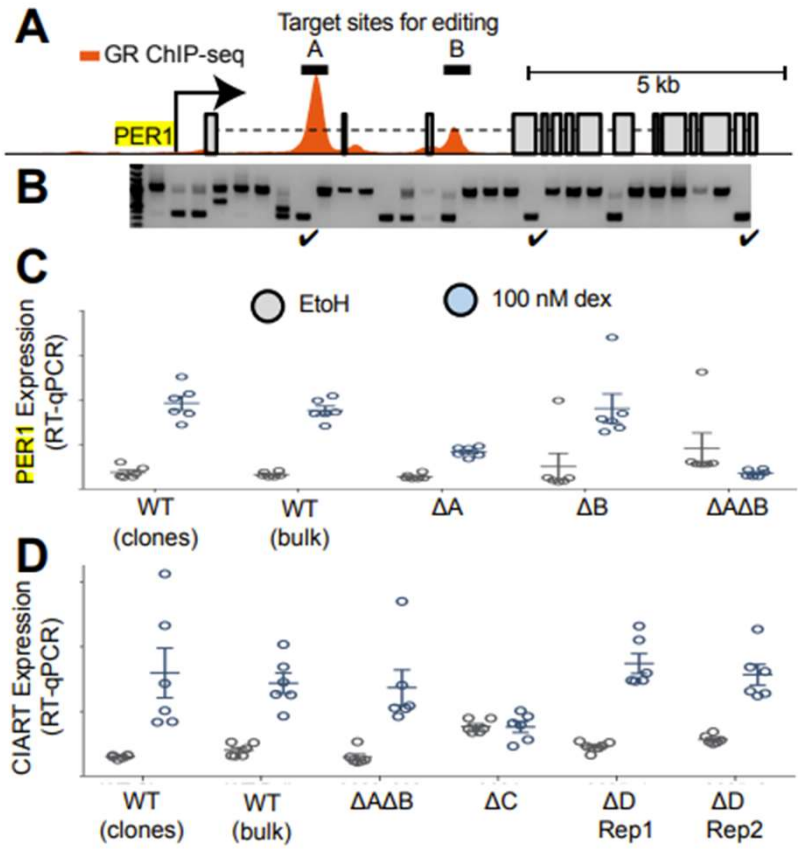
- Reading plasmid maps
- Sending colonies to Genewiz
- Pipetting
- Parafilm
- Gel Creation (Agar and its wonders)
- Designing and ordering guides
- Basic Wet Lab methodologies
- Microscopy
- And more

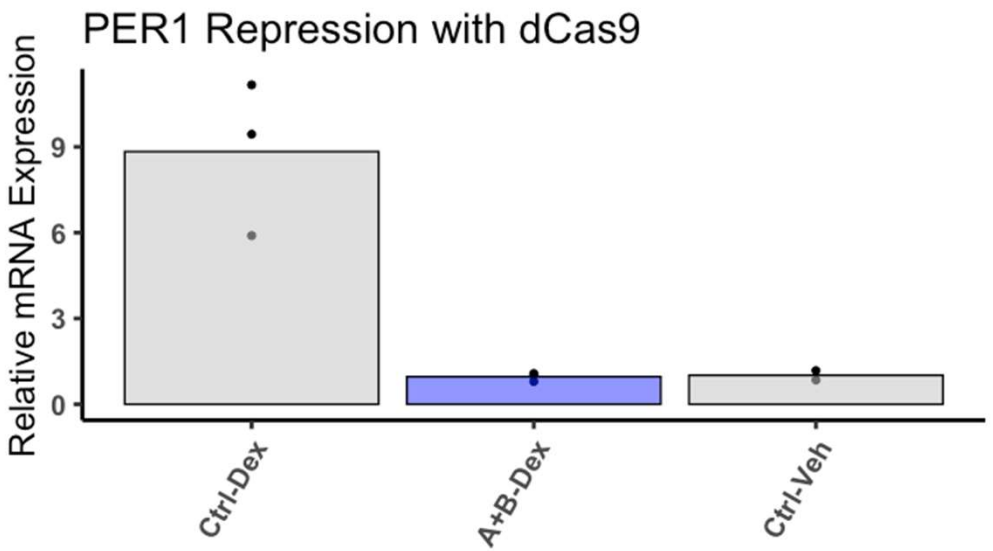
Thank You!

Now let’s get into the actual presentation.



PER1 Glucocorticoid response





PER1 Glucocorticoid response

Primary Goals

- Test Cas12 ability to make precise genomic deletions
- Test critical regions of the enhancers to determine what is necessary for the glucocorticoid response

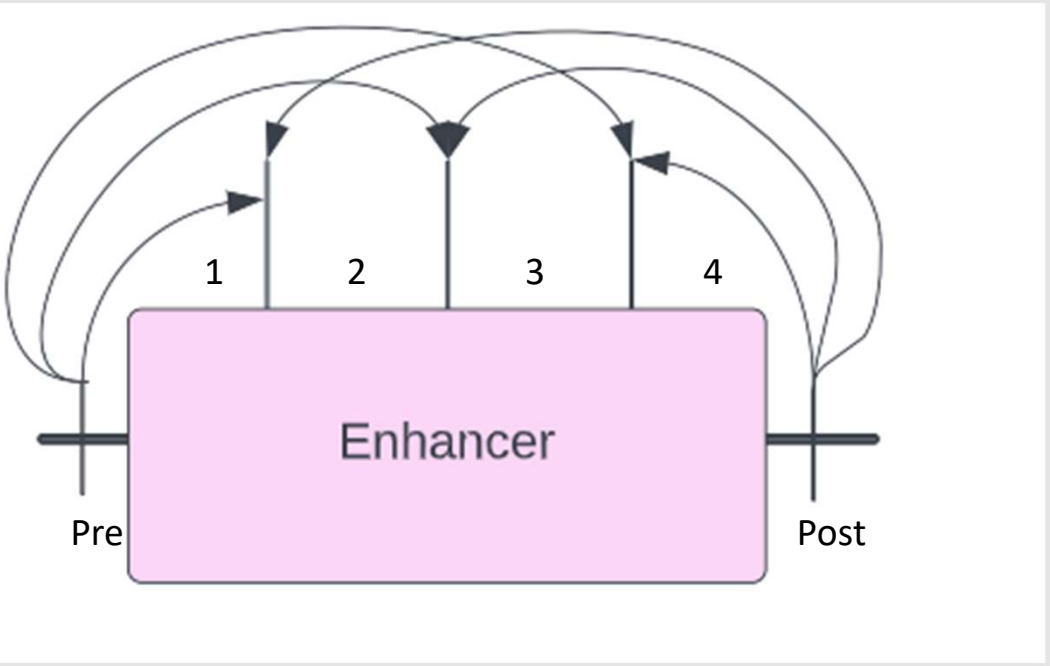
Approach

So how are we planning on testing this

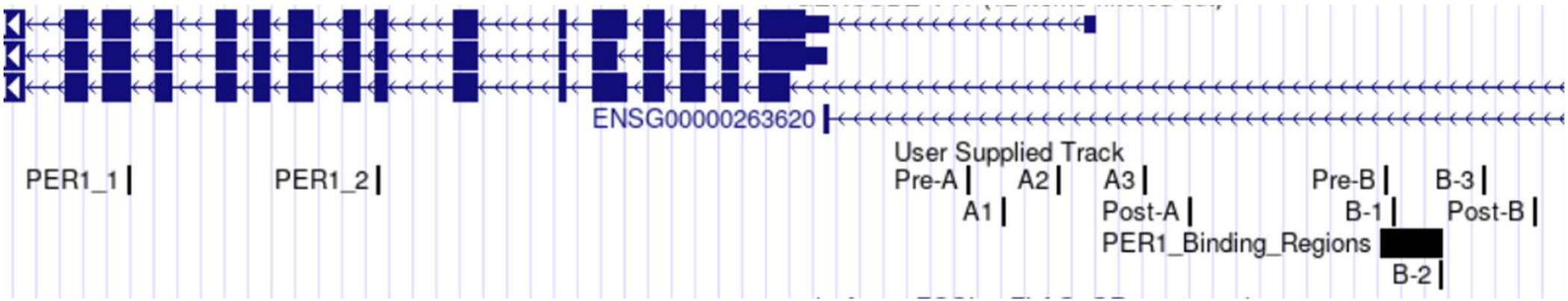
Well, first…

…we should figure out where we want to cut

Where to Cut?

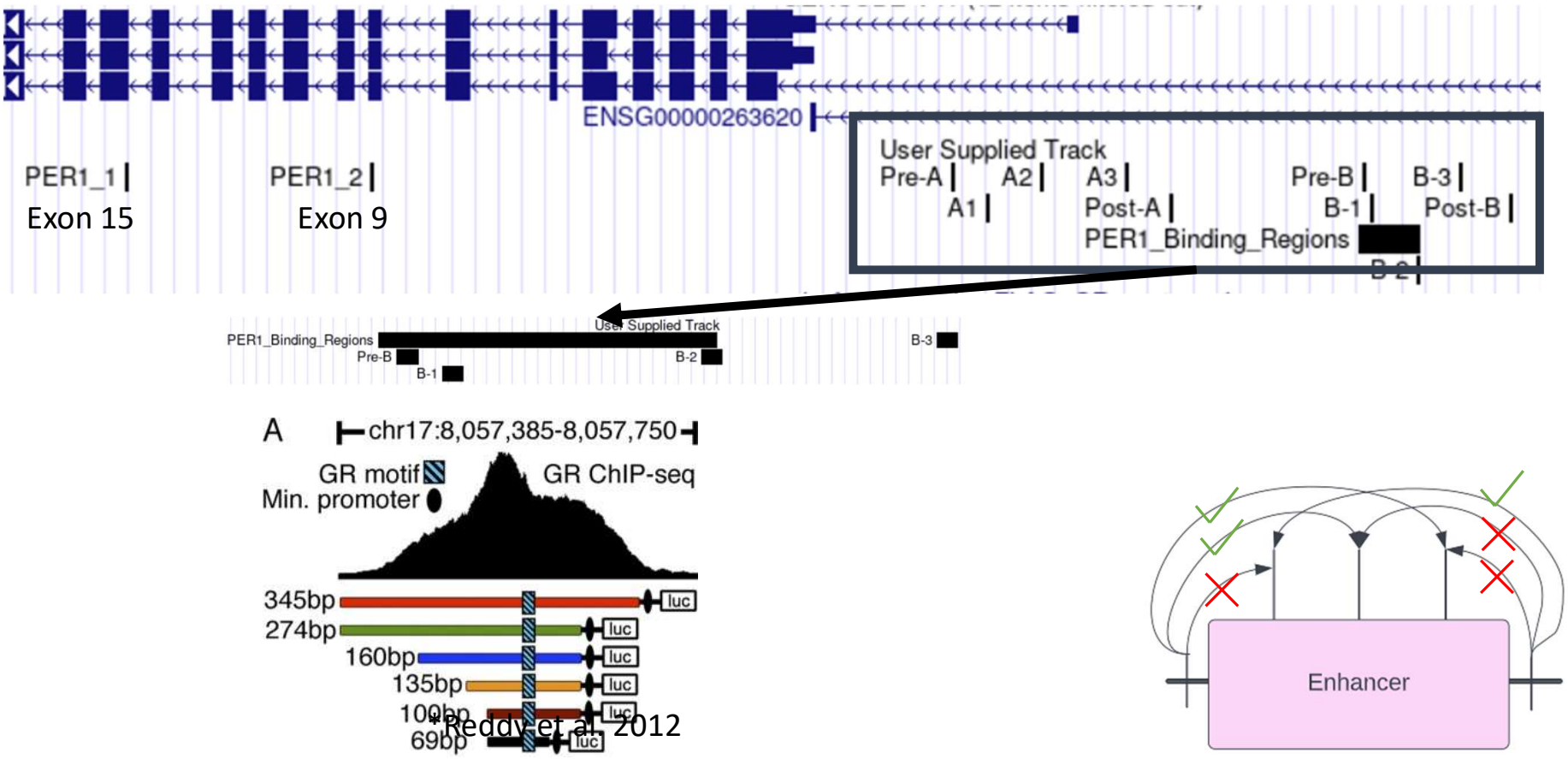


- The PER1 gene
- The enhancers
- A non-targeting control (ACTB)
- Sub-regions of the enhancer
  - In short, I split each enhancer into 4 regions using three internal points
  - Note for later: B2 means enhancer B, region 2



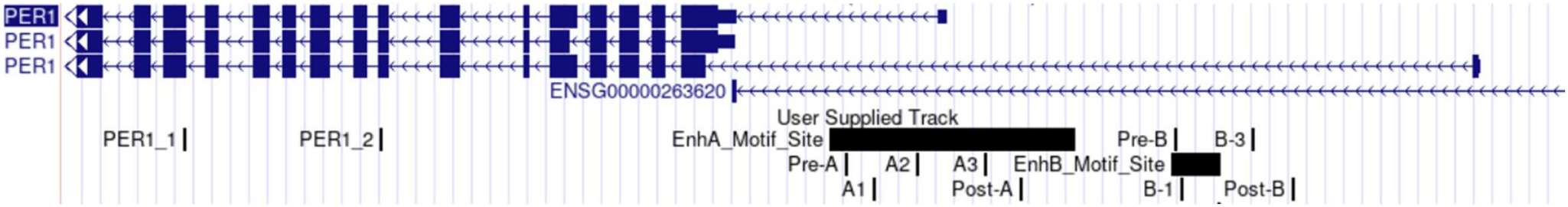
- So, I designed guides for all the locations shown above
- Note: My listed enhancer A and enhancer B are reversed from the commonly accepted order

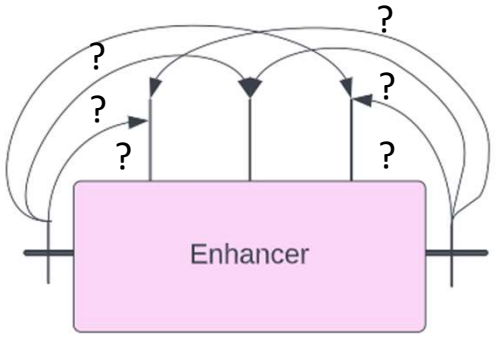
Experimental Design



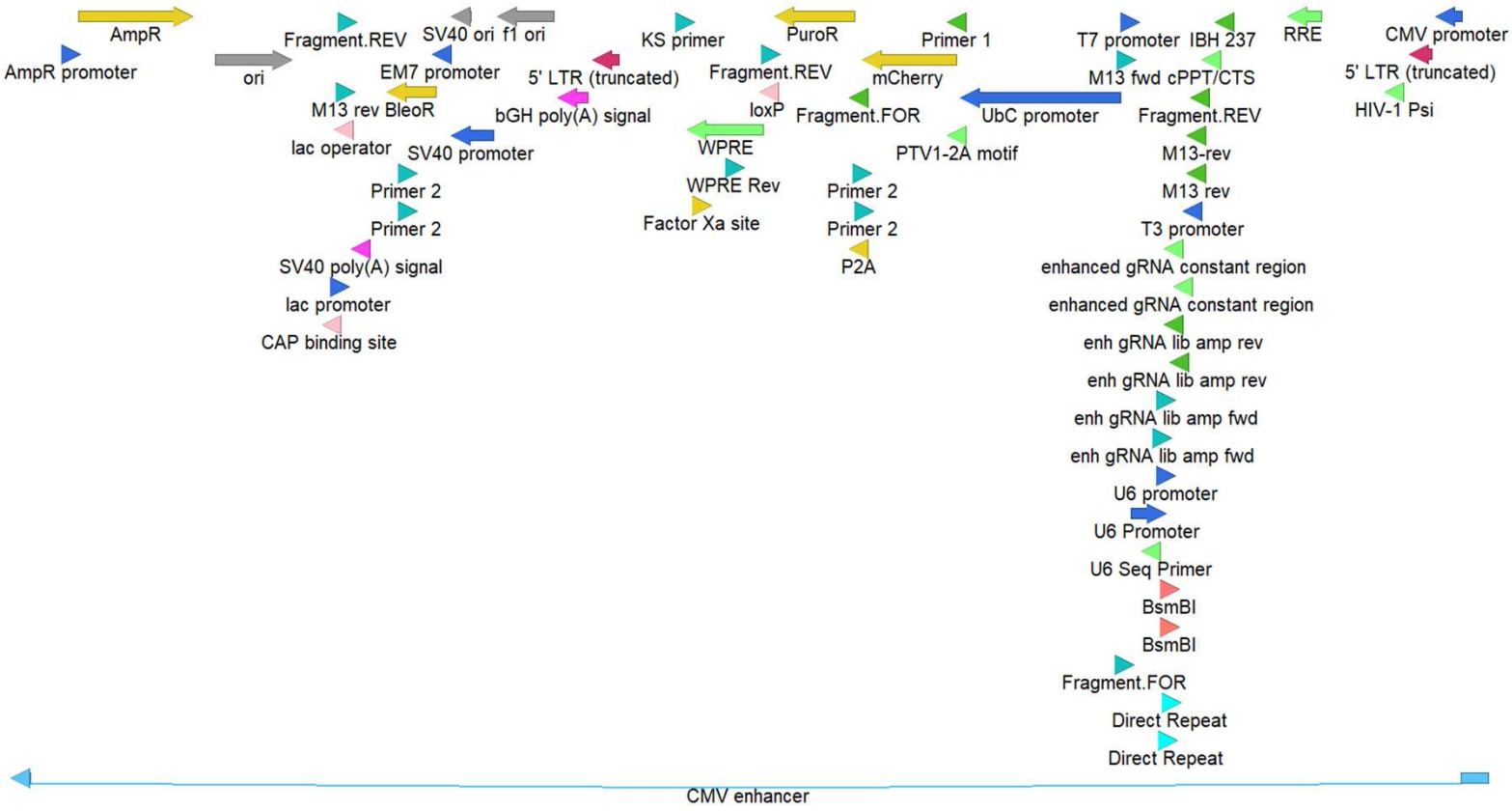
*Reddy et al. 2012

Enhancer B Expectations

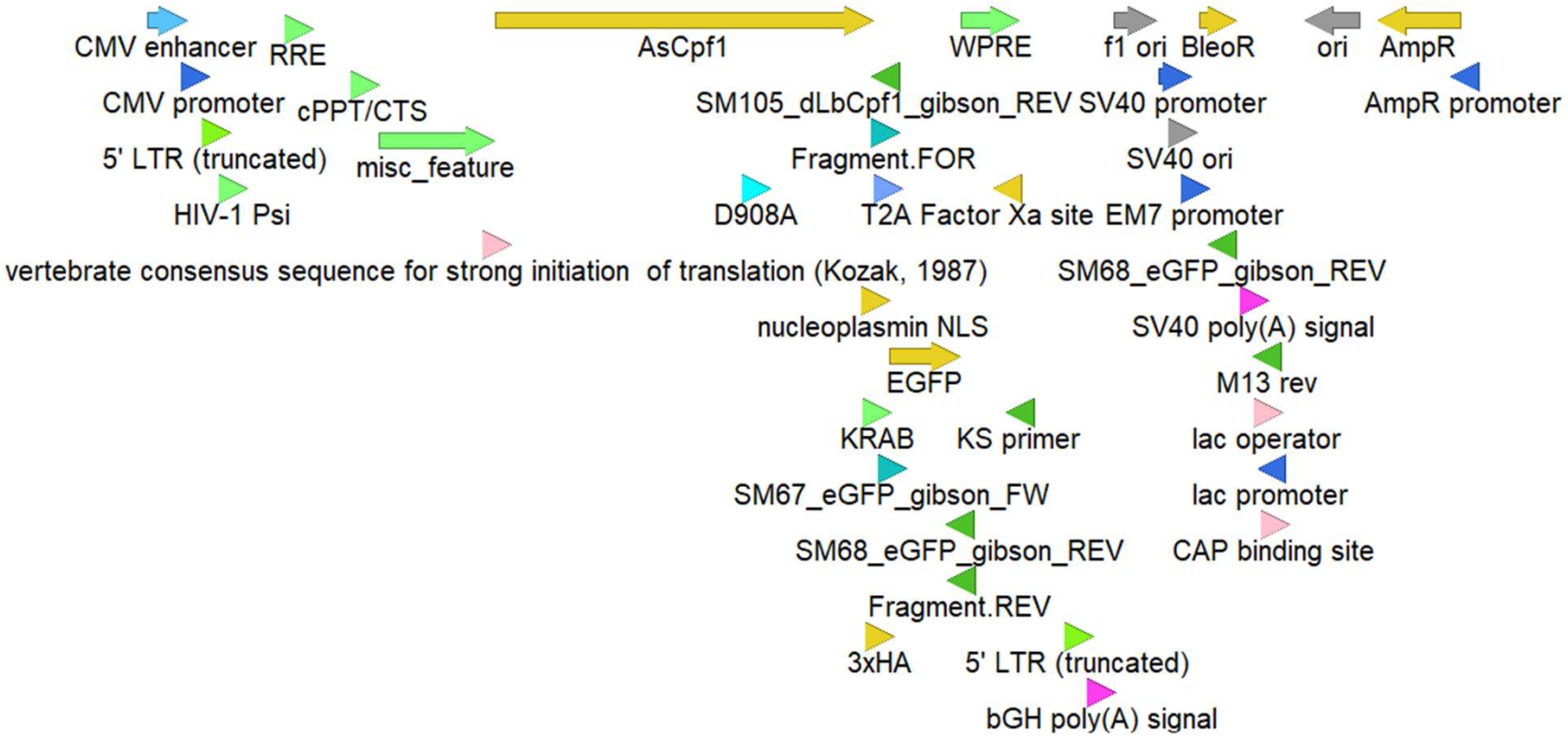




Enhancer A Expectations



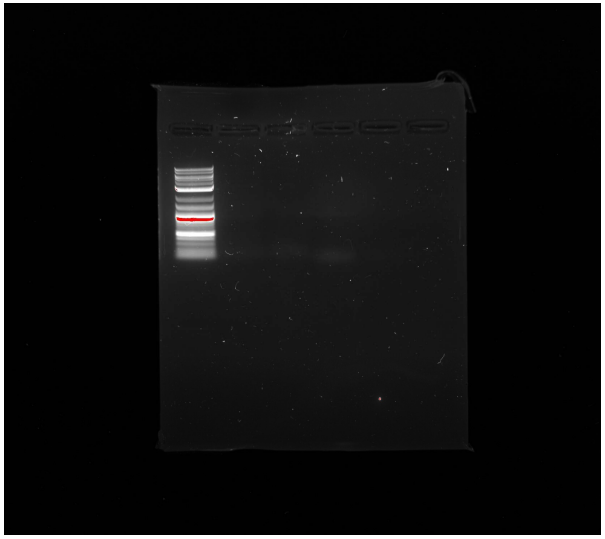
Plasmid Map - gRNA

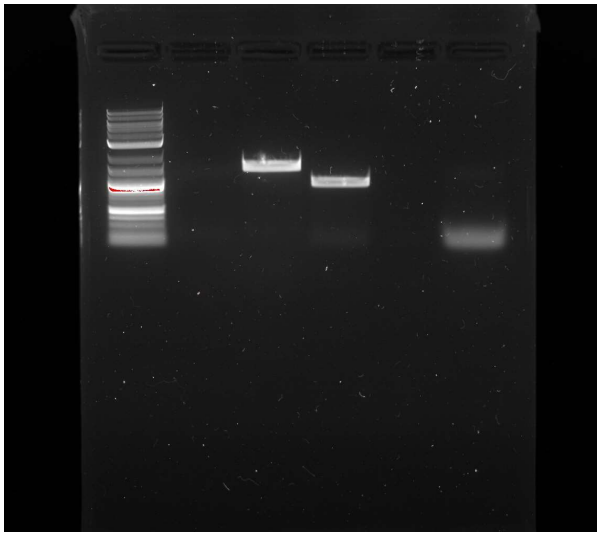


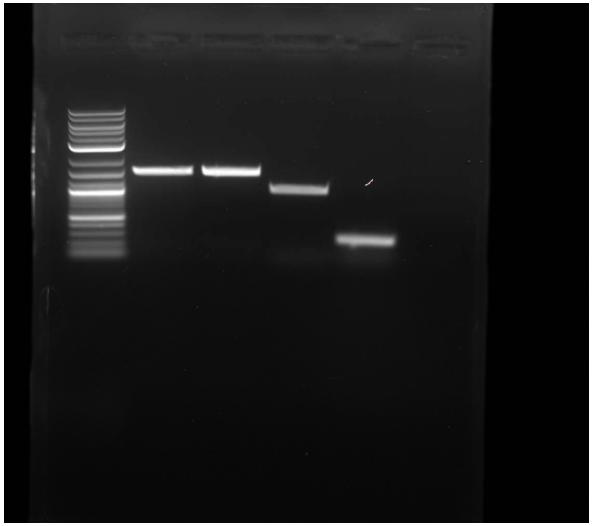
Plasmid Map – dAsCas12 contruct

Methods

- Cell Lines:
  - A549
  - 293T
- Transfection:
  - Lipofectamine
- Measurements:
  - PCR-based Screening
    - Examine cut efficiency
  - qPCR tests
- Selection:
  - Puromycin

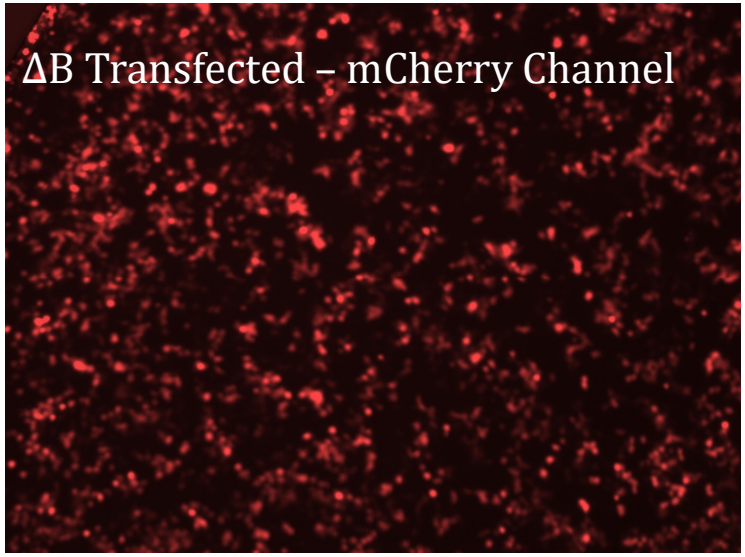


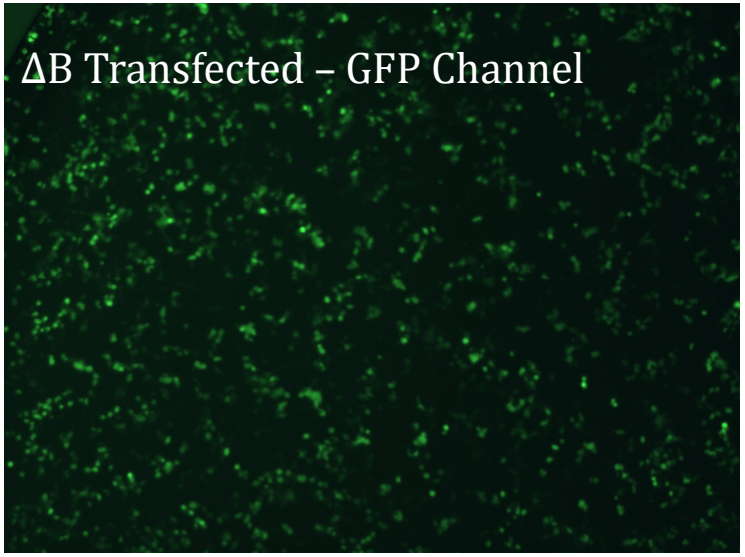




Validating the Genomic Primers

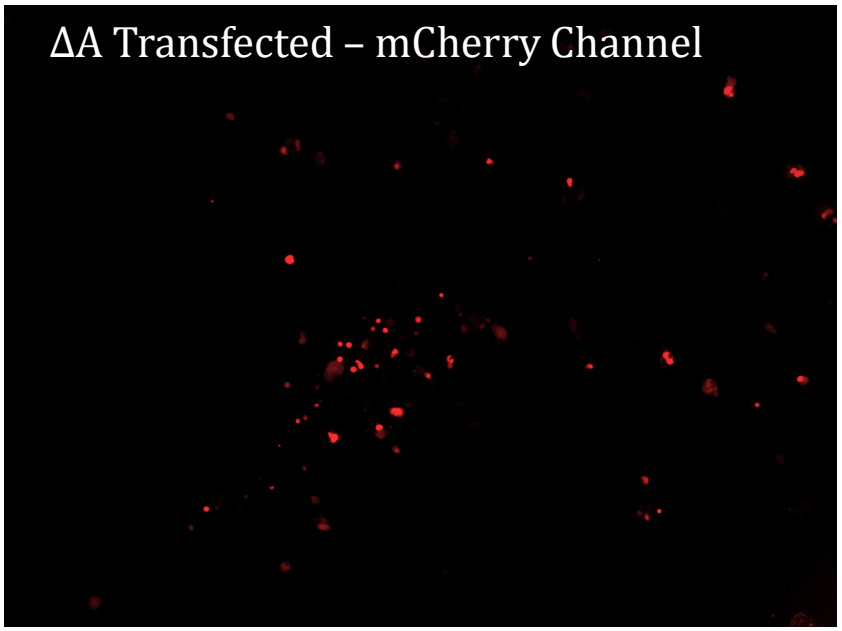
- Tested several times
  - 1: 1 kb ladder
  - 2: Genomic Primer A (Option 1) ~1.5 kb
  - 3: Genomic Primer A (Option 2) ~1 kb
  - 4: Genomic Primer B (Only Option)
  - 5: Control
- Genomic primers created using Primer Blast

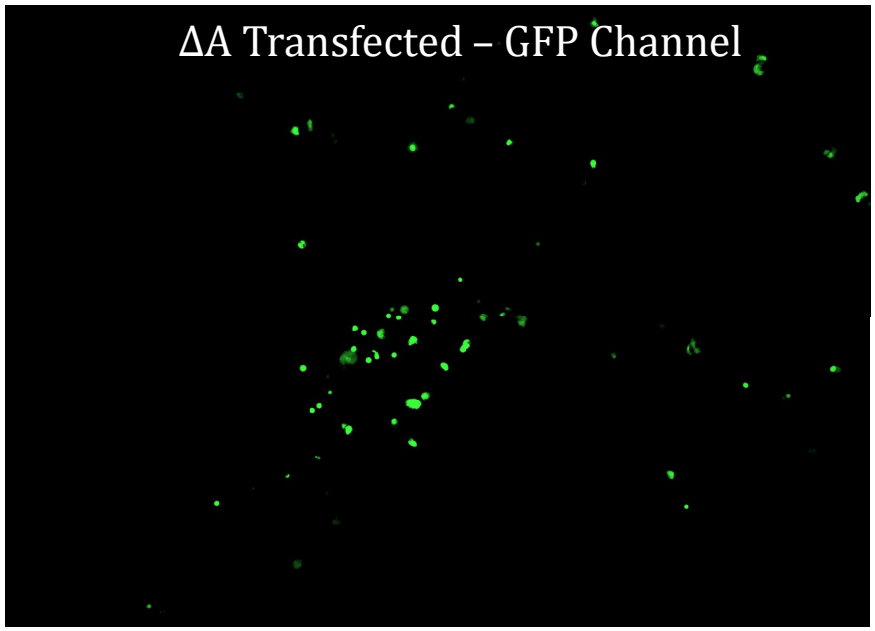




Transfection Efficiency

These images shows transfection efficiency before selection



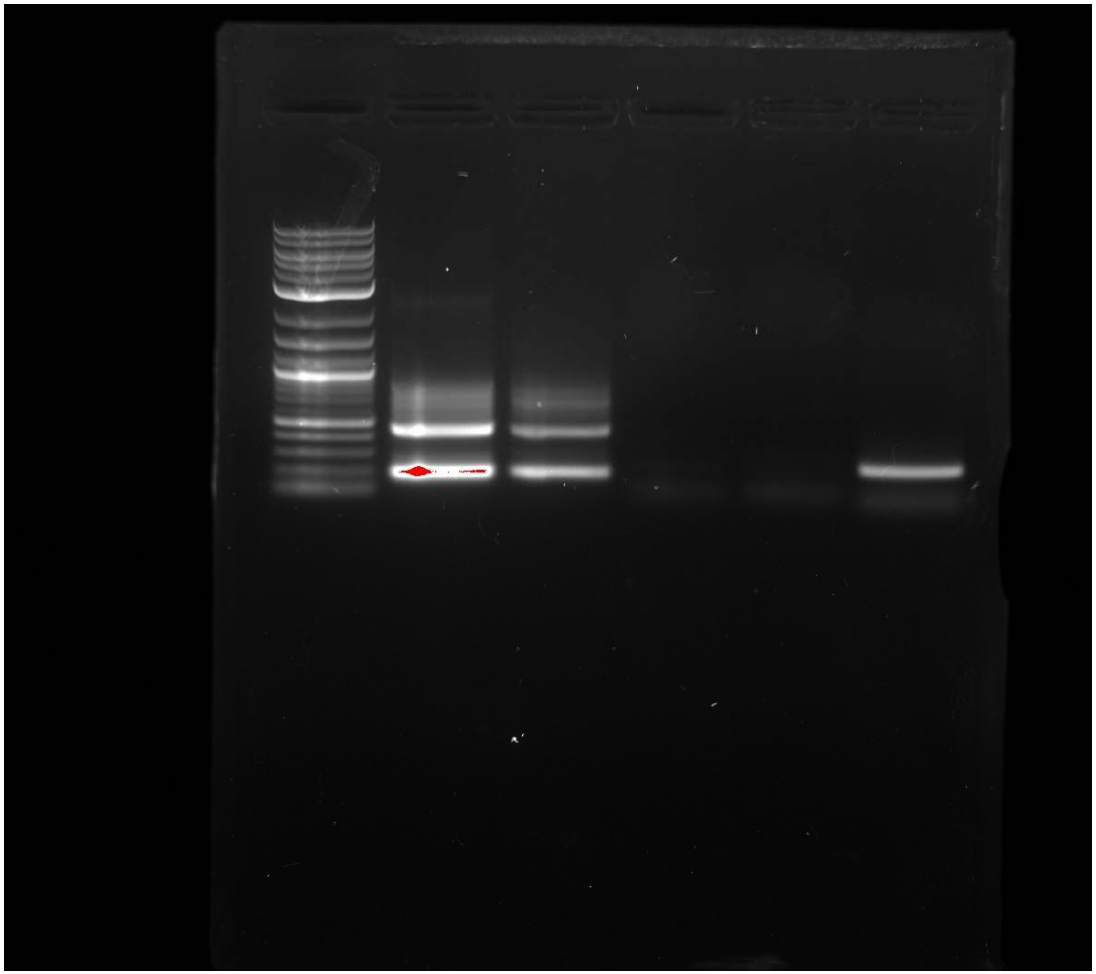


- These images show transfected cells after selection and 48 hours had passed
- Cells were split and Puromycin was added at a 10,000x concentration

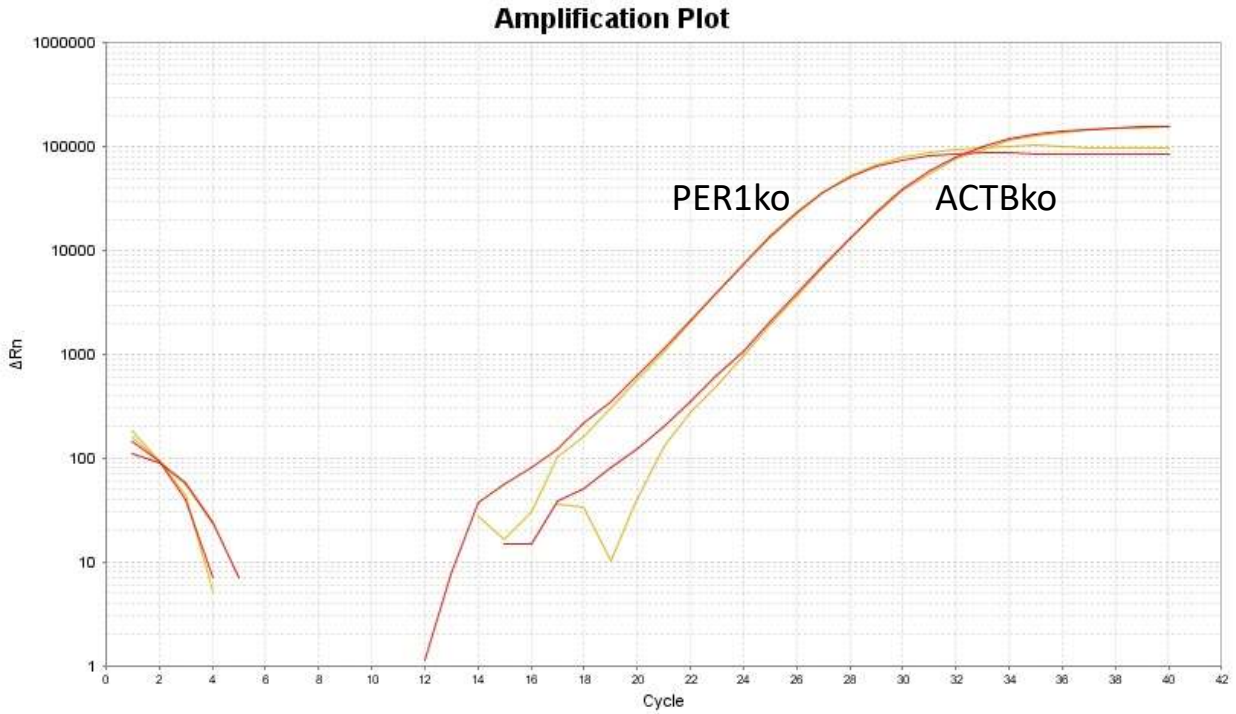
Selection Efficiency

A549 Cell testing

- Columns:
  - 1: 1kb ladder
  - 2: ΔA+ ΔB
  - 3: ΔA
  - 4: ΔB
  - 5: PER1ko
  - 6: NT

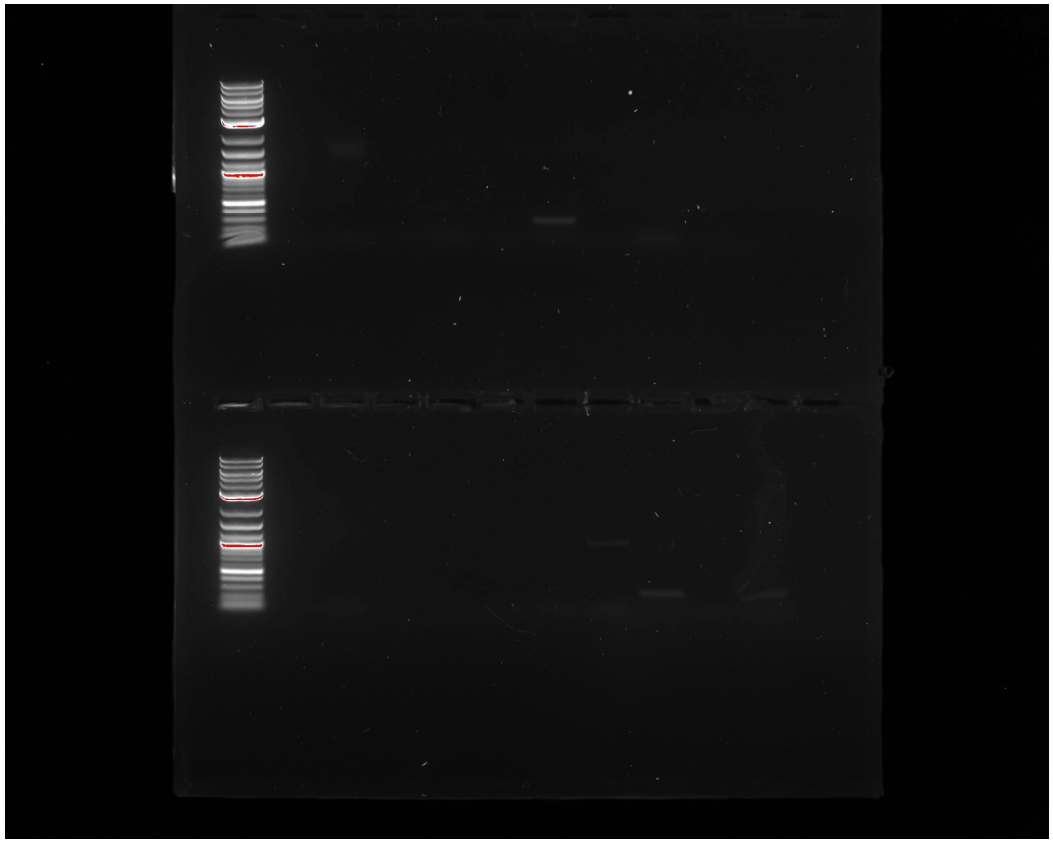


qPCR 293T Preliminary Tests



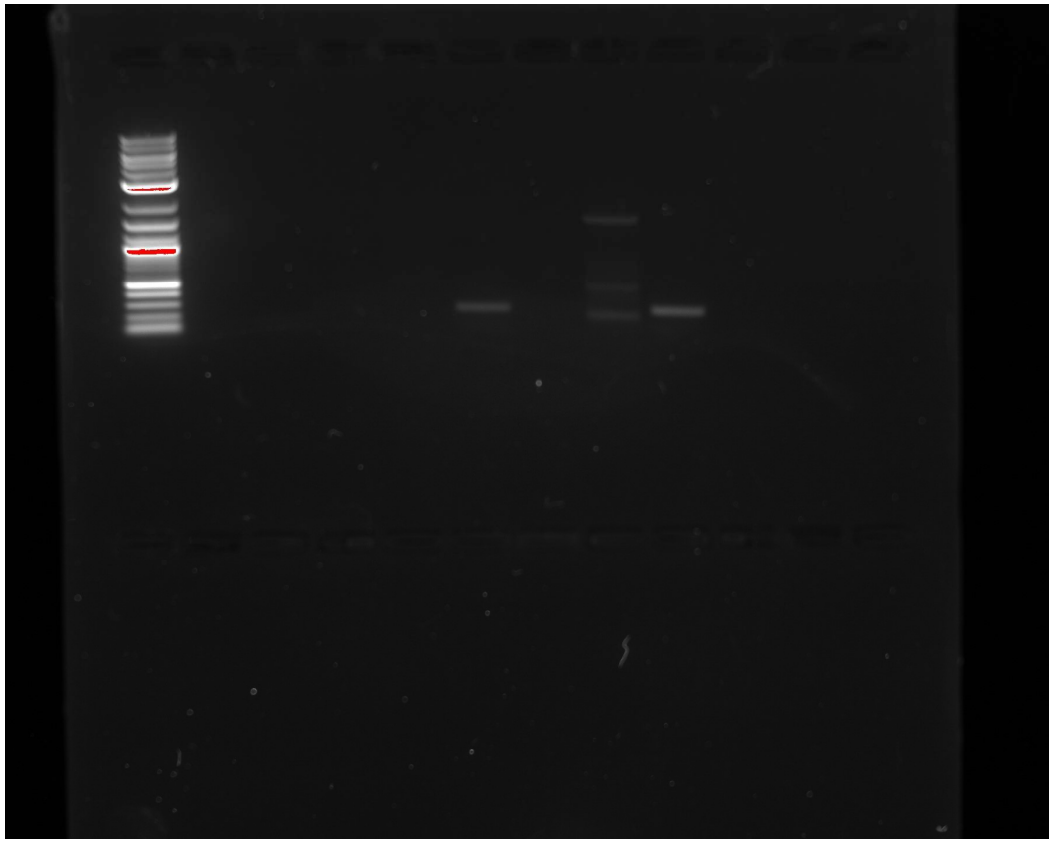
ΔB Testing

- Follow-up Tests – all enhancer B regions
- Every other column is genomic primer B, then NT control:
  - ΔA+ΔB
  - ΔB
  - B1
  - B1-B2
  - B1-B3
  - B2-B4
  - B3-B4
  - B4
  - NT Transfection



ΔA Testing

- Follow-up Tests – just ΔA+ΔB, ΔA, and NT



Future Directions

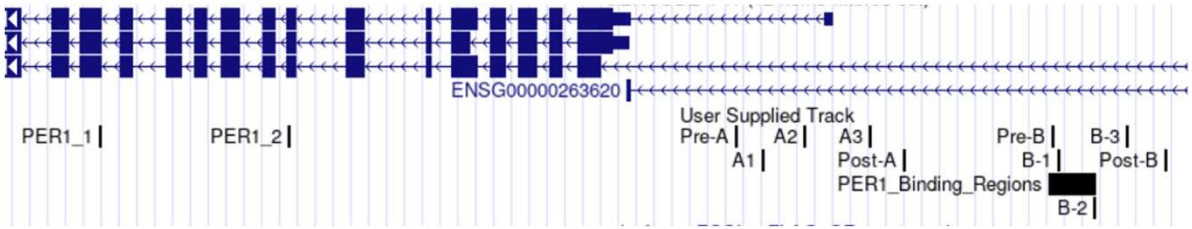
- Re-grow cells and try to run PCR without puro (assuming growth is sufficient)
  - For both ΔA and ΔB tests
- Try puro selection with more time (and maybe less puro)
- qPCR all genomic DNA
- Sanger Sequencing (should PCR/qPCR succeed)
- Grow and send colonies that failed at GeneWiz
  - *Region A3-A4 failed growth in original colonies

Thank you

It’s been a blast getting to know everyone in the lab, and I look forward to seeing you all in the future!

Project Design

- After speaking with Schuyler, we have thought that this entire project should be reduced in size
  - Option 1 - Delete enhancers with Hyper LB and Enhanced AS
    - Will allow me to test the orthologs against each other
    - I will not be testing as many pairs for cutting
    - 120 transfections
  - Option 2 – Use only one ortholog and cut out regions of the enhancer as well.
    - Will allow me to examine smaller regions and search for critical areas
    - 132 transfections
- On the next slides, I will show you the full potential project



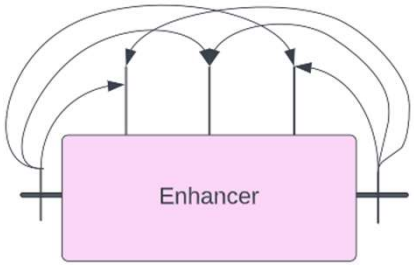
Experimental Design

GOAL FOR THIS WEEK: Proof of Concept, testing to confirm the precision and ensure that we can actually detect changes in the system.

- 3 Replicates for all below:
- Experiment 1 – Control Experiment Tests – Enhanced AS
  - Target Enhancers with ko (Together and each on their own)
  - Target PER1 with ko
  - Target Olfactory control with ko
  - Total: 5*3=15
  - Testing the “pre” and “post” enhancers on their own, Hyper LB and Enhanced AS
    - Total: 4*3 = 12
  - Vehicle Control
    - Total: 3

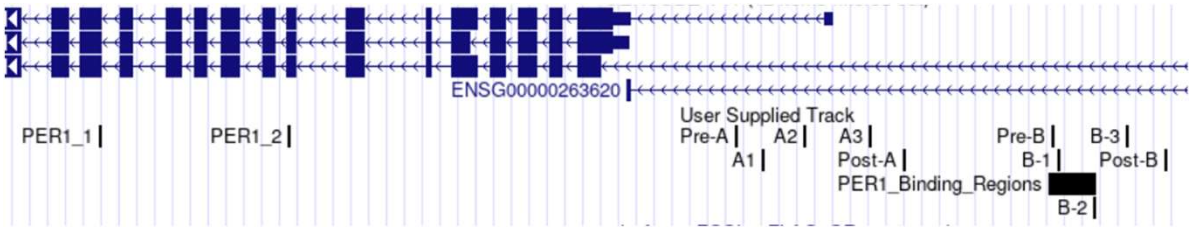
Final Total: 30 tests

- Tested with qPCR PER1 gene Expression
- May also Sanger Sequence the locus to confirm what fraction of cells are edited



Experimental Design

In simple terms, I have validated the controls, now I am testing the regions. But I still will include the controls again, for a total of 72 tests in experiment 3.

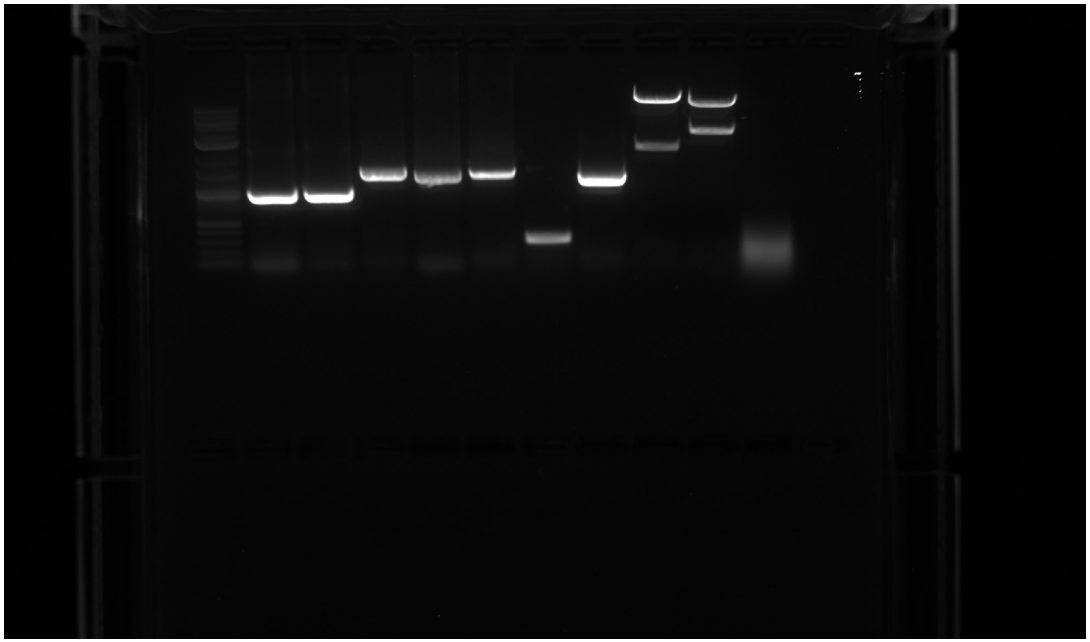


GOAL FOR NEXT WEEK: Cutting out subfeatures. Identify important enhancer regions.

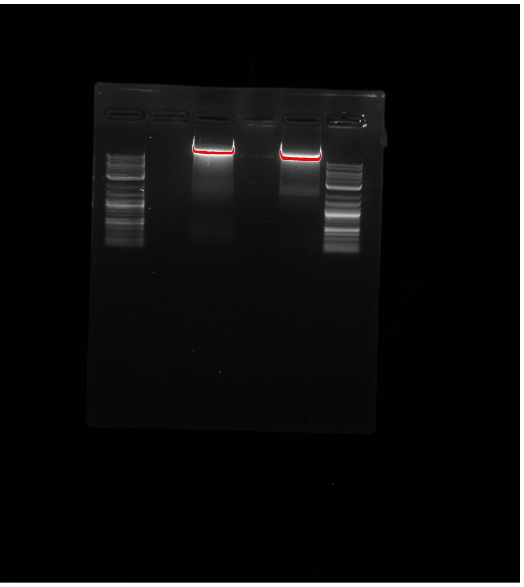
- 3 Replicates for all below:
- Experiment 3 – Secondary Experiment Tests
  - 6 regions (3 for each enhancer based on the highest efficacy between “pre” and “post”), additional controls, and Enhanced AS
  - Total: 6*3 = 18
  - Repeat Controls
    - Experiment 1without the anchor guide tests
    - Total: 30-12=24
  - Tested with qPCR PER1 gene Expression
  - May also Sanger Sequence the locus to confirm what fraction of cells are edited

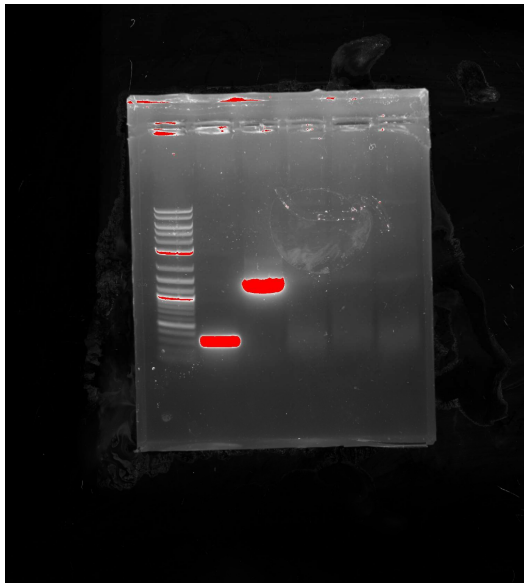
42 total tests for Experiment 3

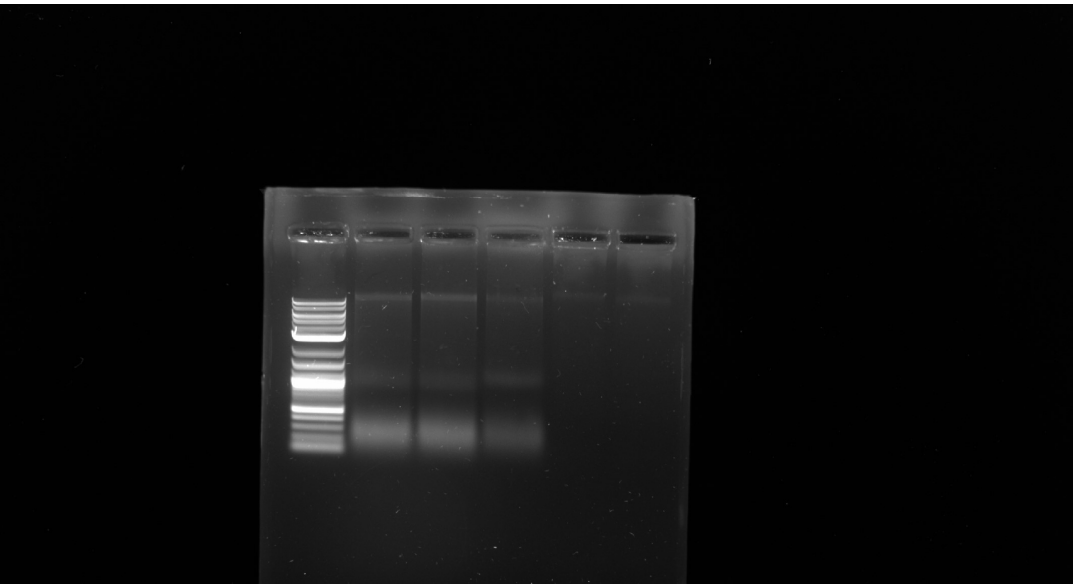
Gels



- From January 25, testing Gibson cloning using some of my first developed primers. Goals:
- 5' Make A908D mutation As dCas12a ultra and LB dCas12. The LB reaction did not work (last one). The other did seem to cut where the should.





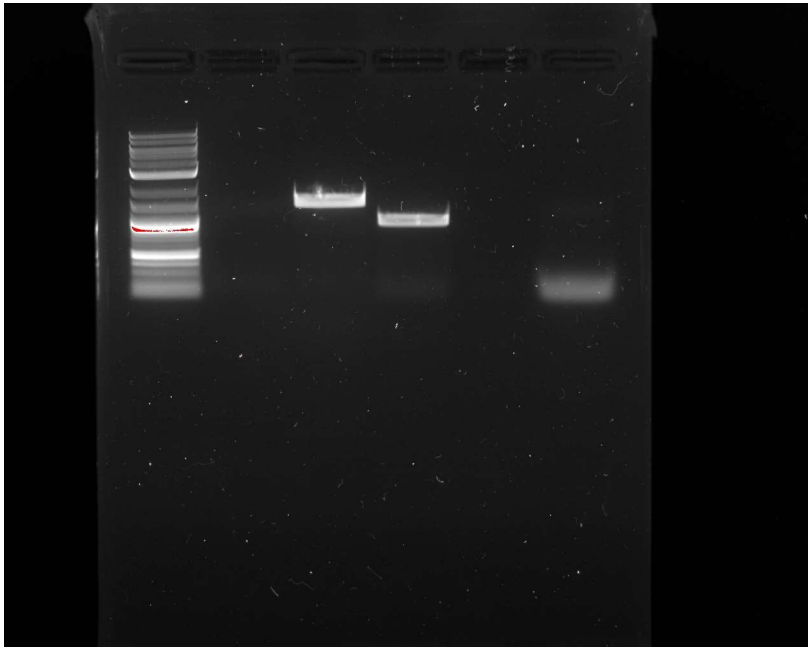


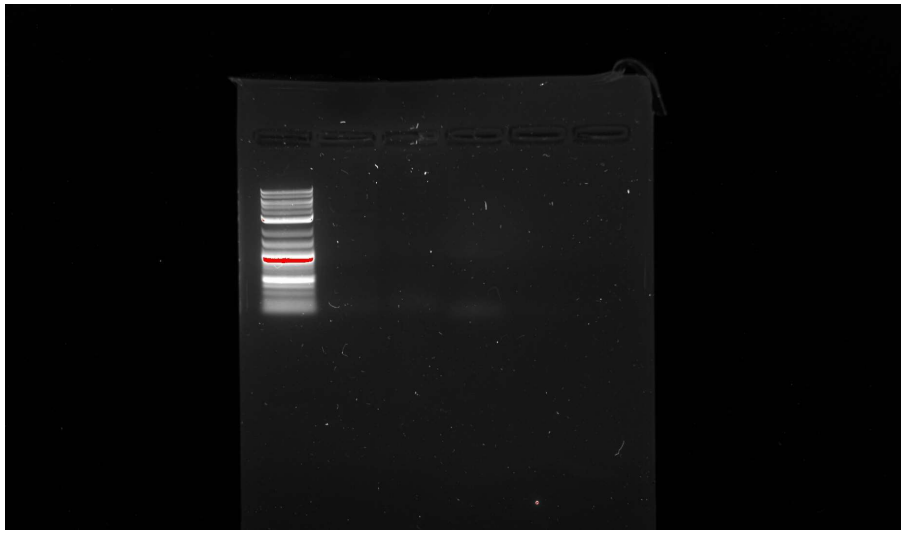
Gels

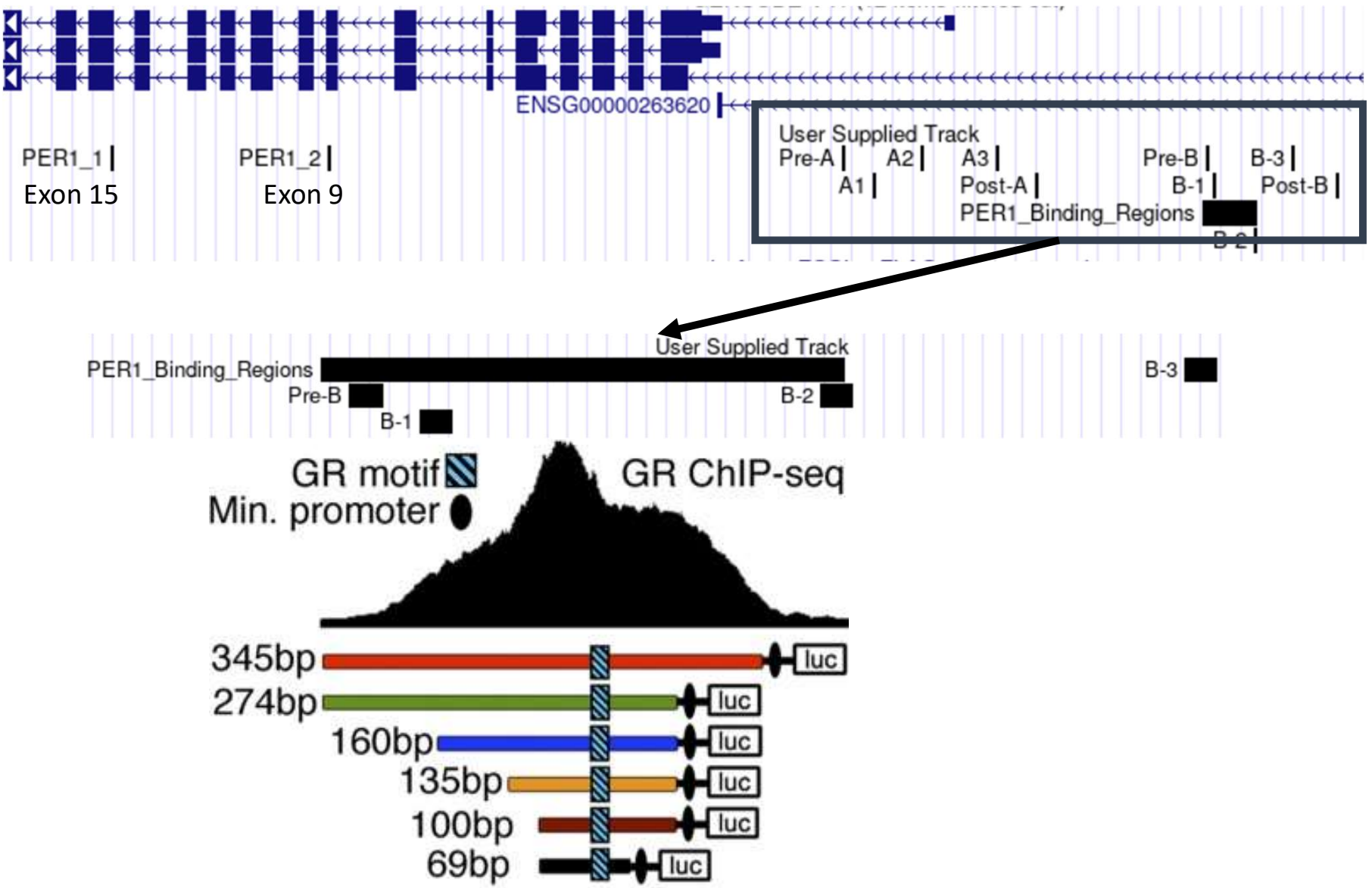
- From first week of February. I retried the LB reaction, and it failed, see bottom left.
- As such, I first retried it with a Hyper LB using different primers, top right, which worked, leading me to replate and try again with the original primers in hyper LB, which became the top left.

Gels

- We received the ordered primers in the last week of January, which I extracted from the plate and then tested the genomic primers with 293T genomic DNA. I tried it twice since the first time completely failed. One of both enhancer A and enhancer B genomic primers succeeded the second time. I later learned that the reason the control failed (see left last column) was that I used the wrong primers






ENSG00000263620
PER1_1
Exon 15
PER1_2
Exon 9
User Supplied Track
Pre-A
A1
A2
A3
Post-A
PER1_Binding_Regions
Pre-B
B-1
B-3
Post-B
B-2
User Supplied Track
PER1_Binding_Regions
Pre-B
B-1
B-2
B-3
GR motif
Min. promoter
GR ChIP-seq
345bp
274bp
160bp
135bp
100bp
69bp
luc

What I've done

- Designed gRNAs
- Transfection
- Send to genewiz
- Miniprep (grow liquid cultures)
- Create my own cultures from designed primers
- Tested in enhanced AS and hyper LB, but our hyper LB backbone kinda didn't work first time and decided to focus on AS
- Test expression with qPCR
- Didn't look great, retried two more times, decided that we only had two weeks left so focus on the experiment
- Designed and ran most parts of the experiment