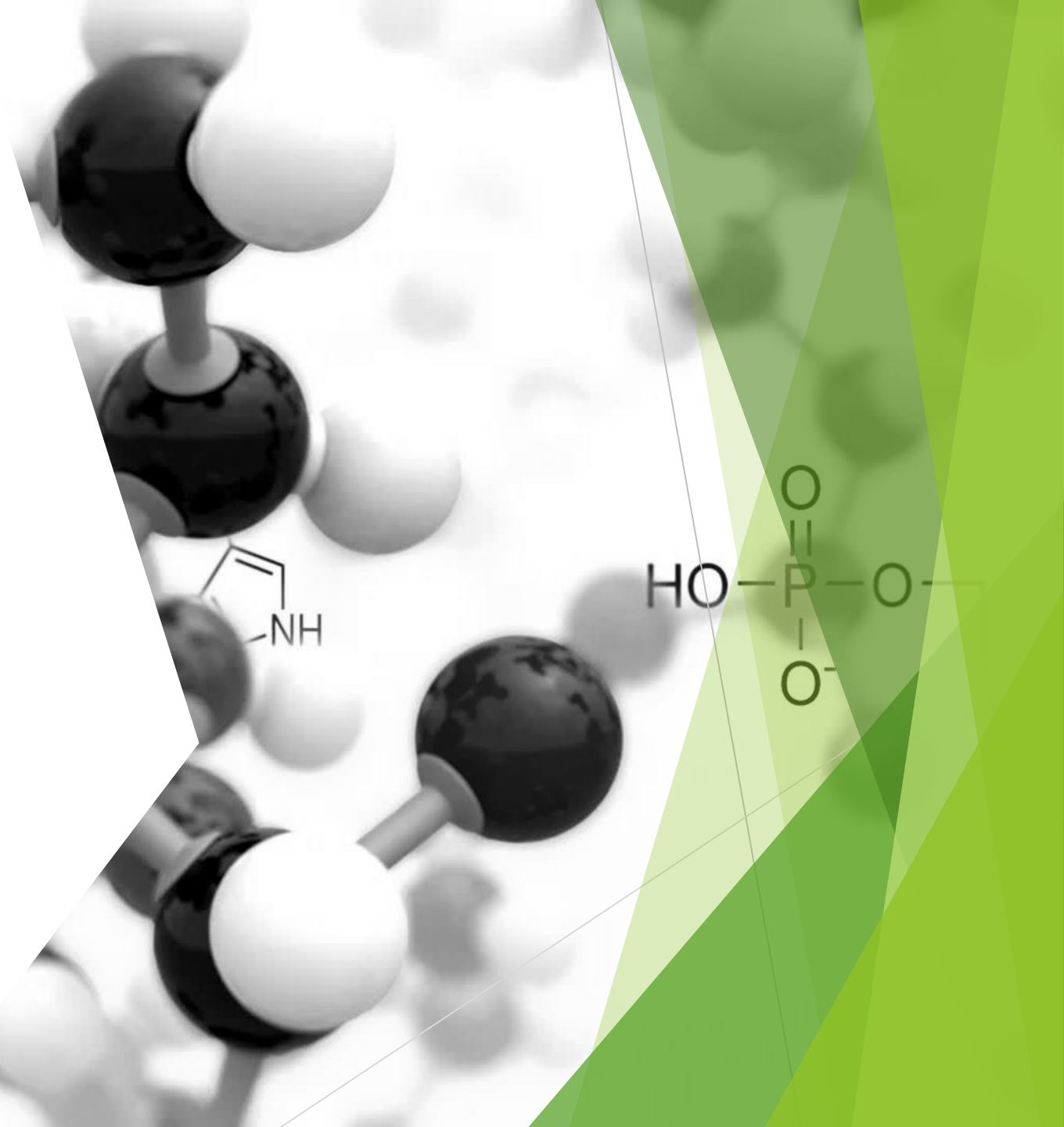
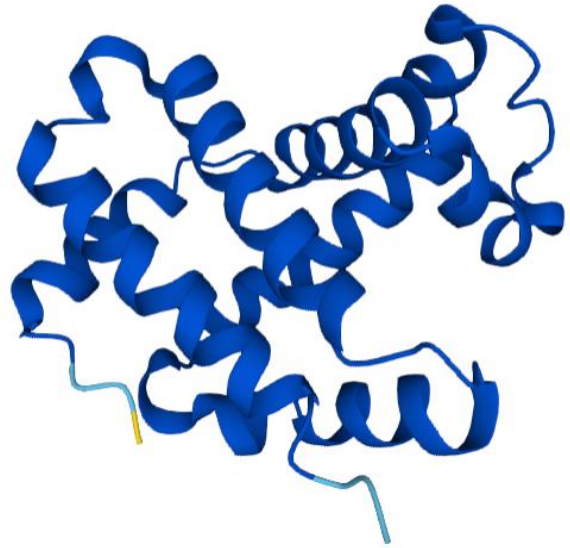
# Assessing Mutations In Beta-Globin Gene For Improved Protein Folding



# Background



- Thalassemia is a disease that is caused by mutations in the hemoglobin protein.
  - Symptoms include:
    - Fatigue, weakness, pale skin, slow growth and bone deformities (especially in infants)
  - Types include:
    - Alpha-Thalassemia
      - Caused by deletions of amino acids
    - Beta-Thalassemia
      - Caused by mutations of amino acids
      - $\beta^0$ vs. $\beta^+$ (no change in protein production vs. decrease in protein production)

```
        10         20         30         40         50         60         70         80         90         100        110
MVHLTPEEKS AVTALWGKVN VDEVGGEALG RLLVVYPWTQ RFFESFGDLS TPDAVMGNPK VKAHGKKVLG AFSDGLAHLD NLKGTFATLS ELHCDKLHVD PENFRLLGNV

       120        130        140
LVCVLAHHFG KEFTPPVQAA YQKVVAGVAN ALAHKYH
```

- **Goal:** Identifying mutations in the beta-globin gene that either decrease or increase protein production.
- **Hypothesis:** Depending on amino acid properties:
  - Protein production will increase (more bends in protein)
  - Protein production will decrease (protein folds incorrectly)
  - Protein production will cease (mutations were too drastic)

Images: 3D structure and amino acid sequence of beta-Globin, as per UniProt

# Methods
# Preparation, Transformation & Inoculation

- Preparation
  - Plasmid pet15B, provided by GenScript
  - Plasmid dissolved and vortexed
- Transformation
  - 2 μL of PDNA (plasmid DNA) transformed into 50 μL of DH5-Alpha E.coli Competent Cells
  - 25 μL of PDNA + E.coli solution plated onto 2 Ampicillin Luria Broth plates (each).
- Inoculation
  - In a tube, 3 μL of Ampicillin was added (100 μg/mL) into 3 mL of Luria Broth.
  - Two colonies from each plate would be scooped out by a heated loop and inoculated into a separate tube.
  - The tubes would be labeled: $C1_1$, $C1_2$, $C2_1$, $C2_2$ (where C1 is clone 1 and C2 is clone 2).

# Methods:

# Plasmid Miniprep and Gel Electrophoresis

### Plasmid Miniprep

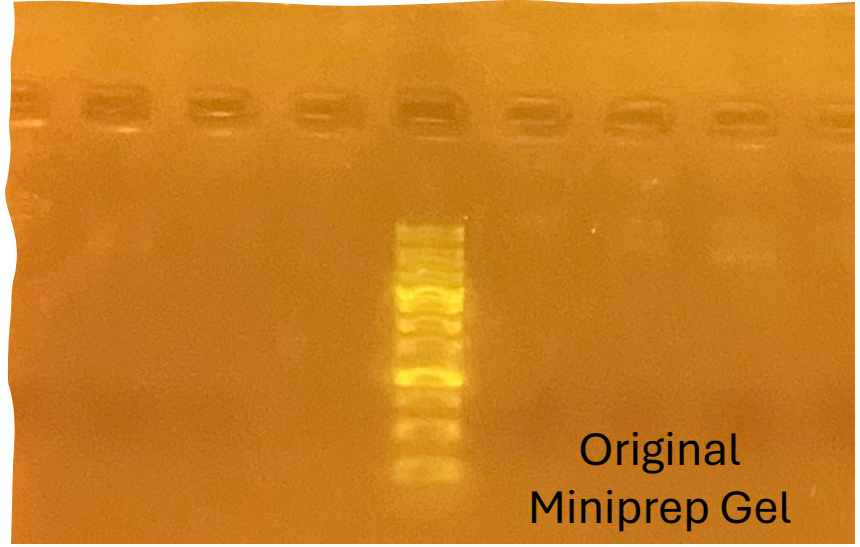
- Initial Prep (Centrifuge)
- Buffer Protocol
  - RNAse Suspension Buffer (P1)
    - Eradicates RNA
  - Lysing Buffer (P2)
    - Breaks apart cells
  - Neutralization Buffer (N3)
    - Neutralizes pH
- Centrifuge/Wash Protocol
  - Zymo Spin Column
    - Binds to DNA, filters out debris
  - Wash 1 and Wash 2
    - Pushes debris through spin column
  - Elution Buffer
    - Dissolves spin column; leads to purified DNA

### Making the Gel

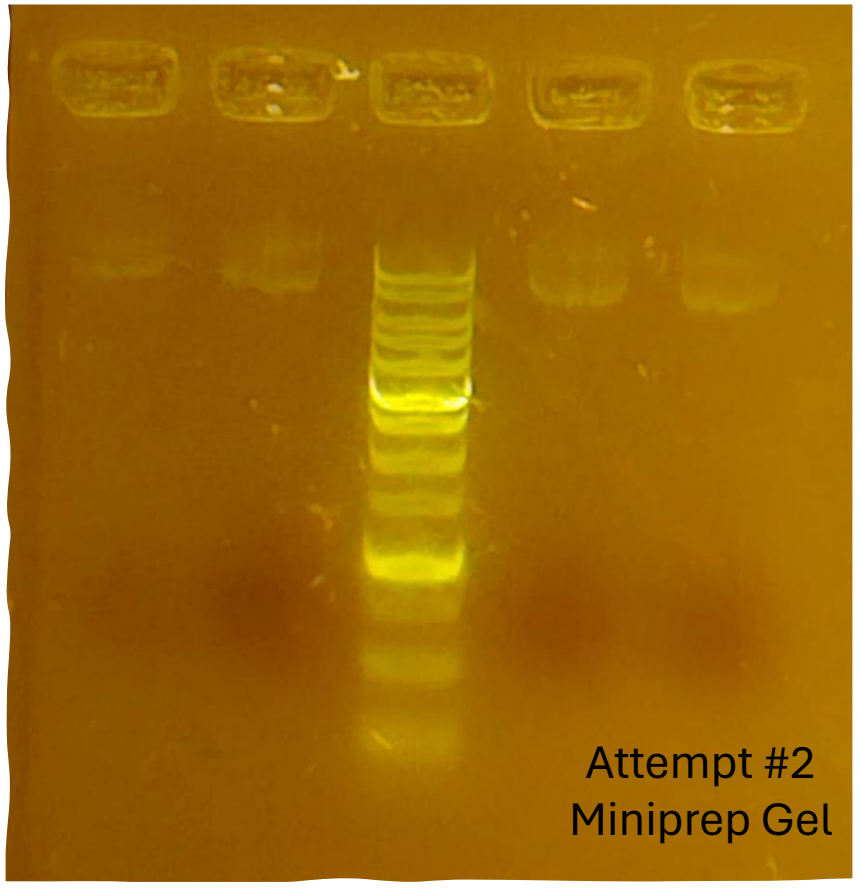
- Add 0.5 gm Agarose Gel
- Add 50 mL TAE Buffer
- Heat thrice for 30 seconds
- Add 5 µL SYBR Green (DNA staining dye; safer replacement of Ethidium Bromide)
- Add combs to create wells

### Running the Gel

- Add 1 uL of Loading dye
- Add 2 uL DNA
- Pipette into wells
- Turn machine on (150 V)



Original Miniprep Gel

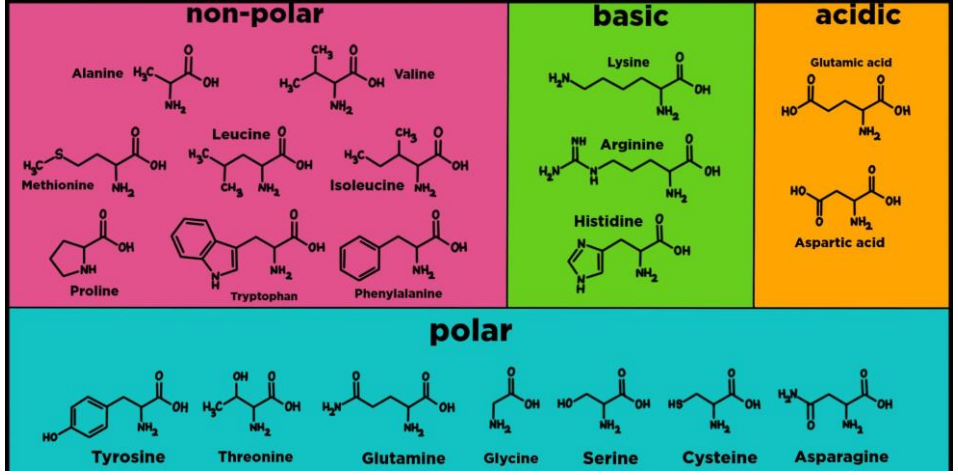


Attempt #2 Miniprep Gel

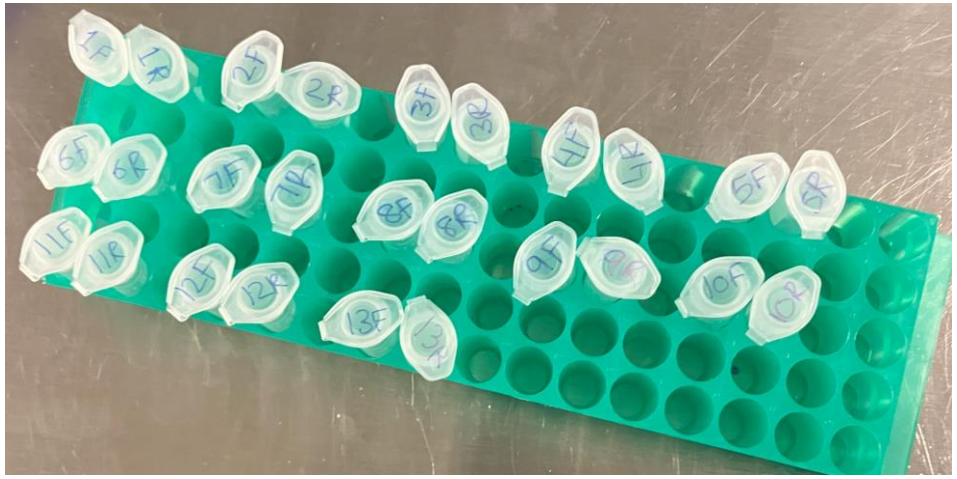
# Methods:
# Finding Mutations & Primers

- Due to the nature of Alpha-Thalassemia (deletion of an amino acid), further examination of the topic was eliminated. Deletion of an allele is harder to send for sequencing.
- Beta-Thalassemia, however, is caused by a *mutation* of an amino acid, specifically in the Beta chain of the Hemoglobin protein (also known as Beta-globin).
- To identify the most prevalent mutations, reviews from NIH, such as “The Molecular Basis of β-Thalassemia” were used.
- Table 2 from the review had a list of common Beta-globin mutations that were associated with the causation of Beta-Thalassemia.
- To narrow down the missense mutations left, I analyzed the properties of the amino acid before mutation, and after.

Images from top to bottom: Properties of amino acid chart, 26 GenScript primers for 13 mutations, Primers dissolved in 20 uL of sterilized water



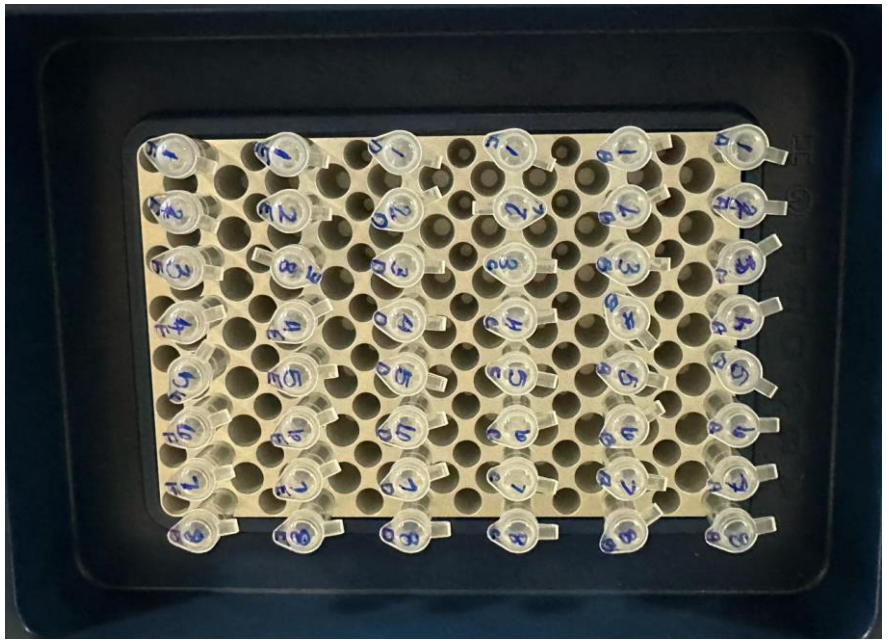




# Methods: Polymerase Chain Reaction

Goal: Denature plasmid DNA and amplify it by adding primers with mutations.

Images of Gradient PCR

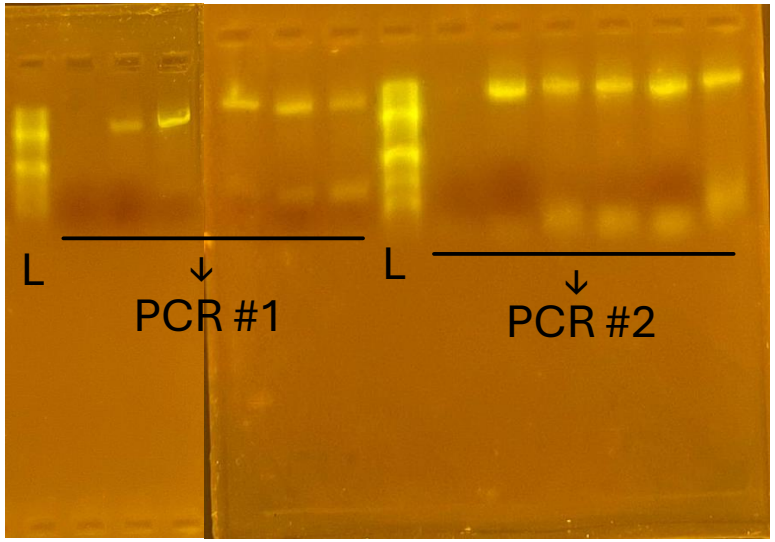
## First PCR

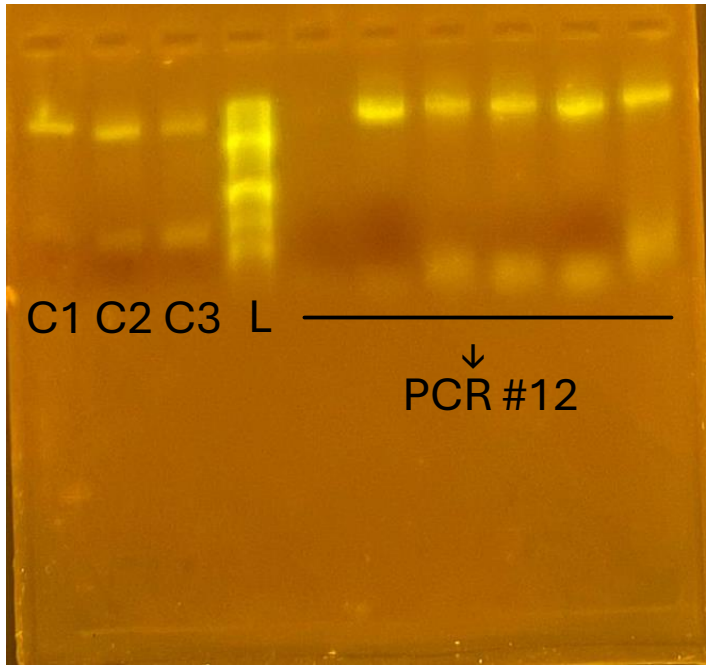
- Prepared PCR 14x Mix
  - 28 uL of sterilized water
  - 14 uL of C2 DNA
  - 182 uL Accuris High Fidelity Master Mix
- Pipette 14x Mix into 13 tubes (one for each mutation)
- Add 2 uL of dissolved FORWARD primer into respective tubes.
- Add 2 uL of dissolved REVERSE primer into respective tubes.
- Run PCR in thermal cycler at 63 deg C.

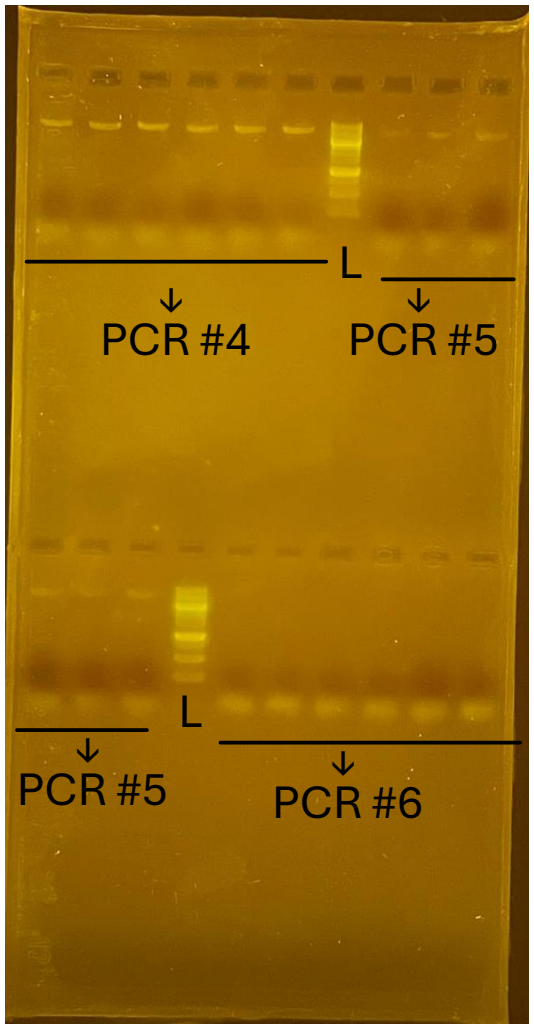
## Gradient PCR

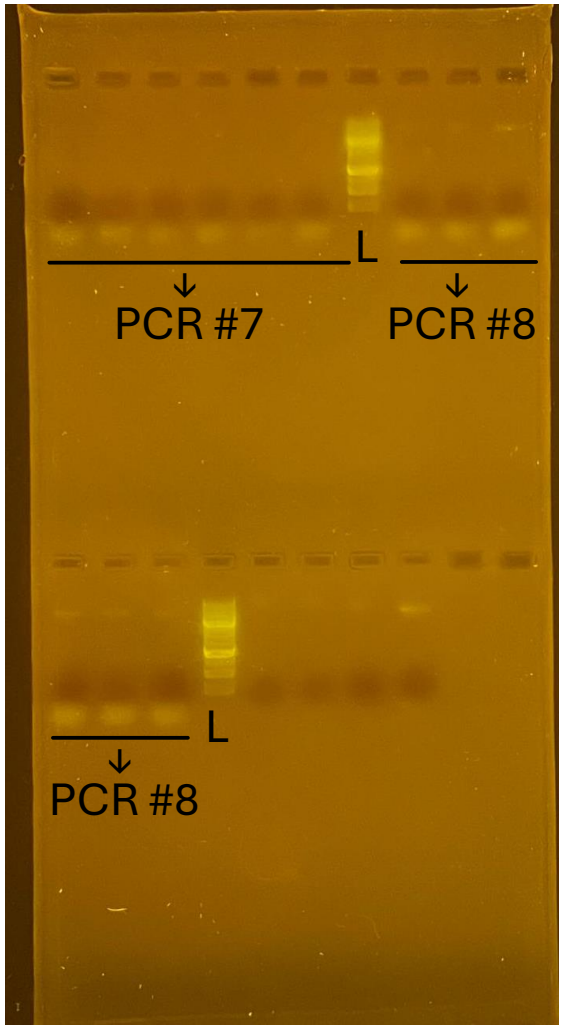
- Goal: Identify ideal annealing temperature for EACH primer.
- Experiment:
  - Each primer is made into 8 PCR solutions.
  - Each solution experiences different temperature.
  - Compare results by running a gel and identifying the strongest band.

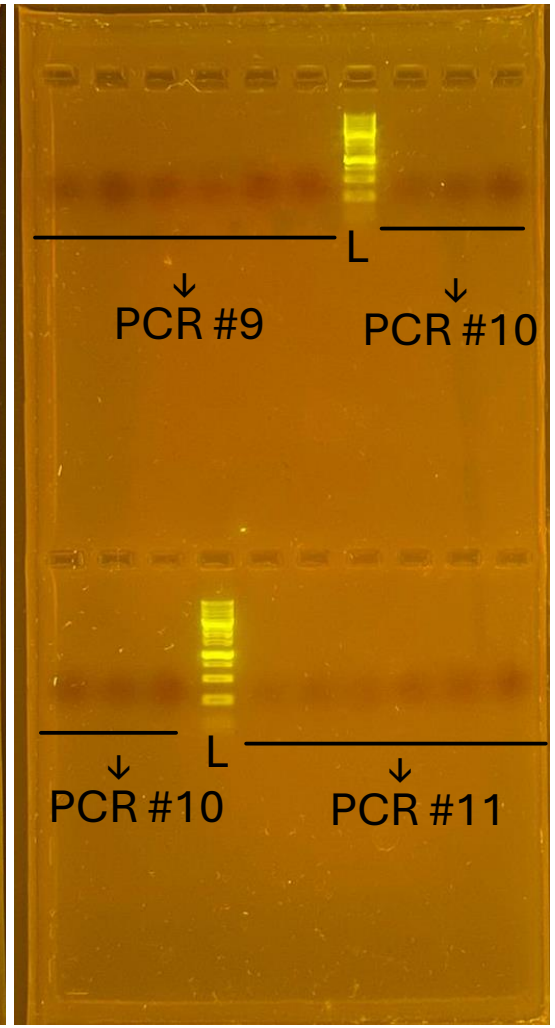
# Images of Gradient PCR Gels



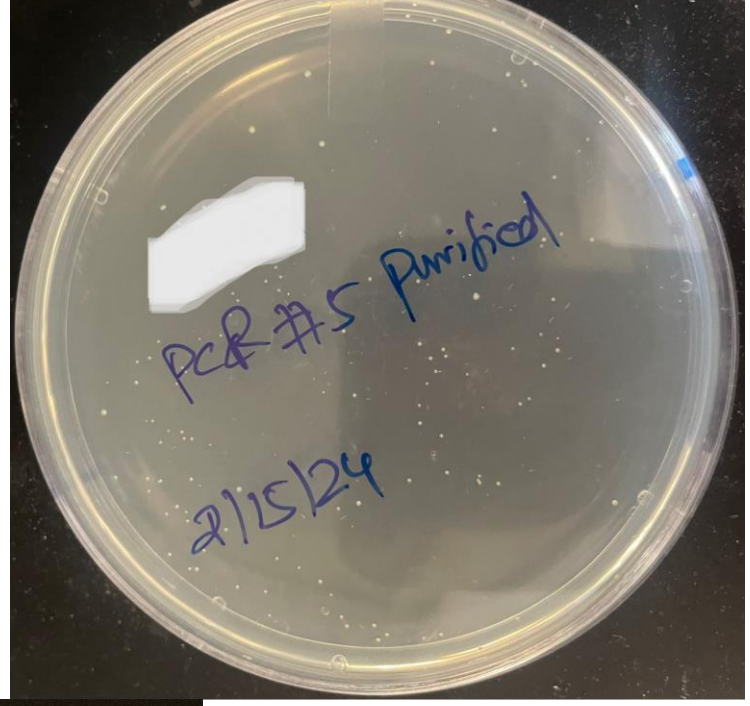
# Methods:

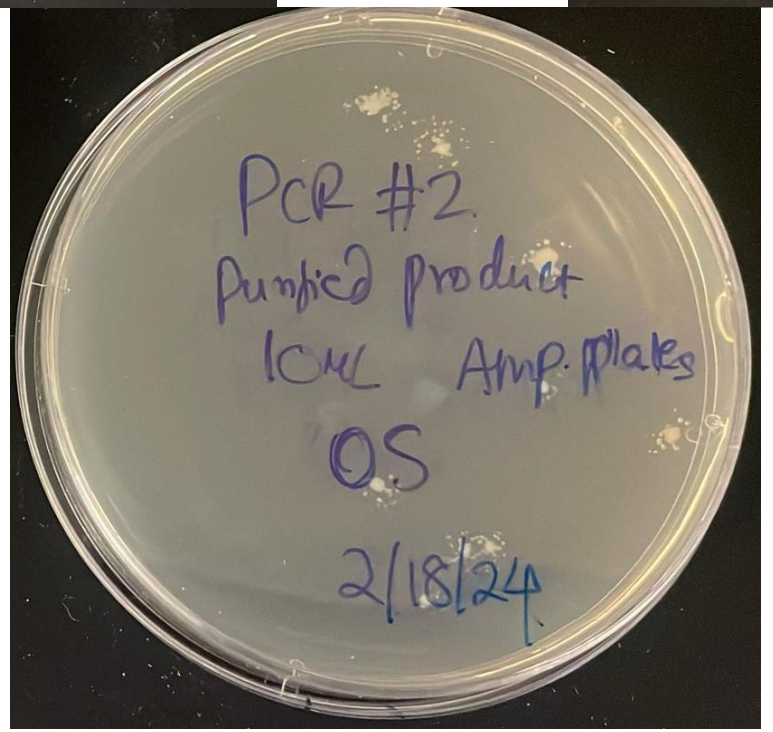
# Post-PCR (Miniprep, Inoculation, Transformation)

1) Perform miniprep on best PCR products (PCR #1, PCR#2, PCR#5 and PCR#8)
2) Transform into E.coli (as shown in images to the left)
3) Inoculate into Luria Broth
4) Perform another miniprep to isolate purified DNA

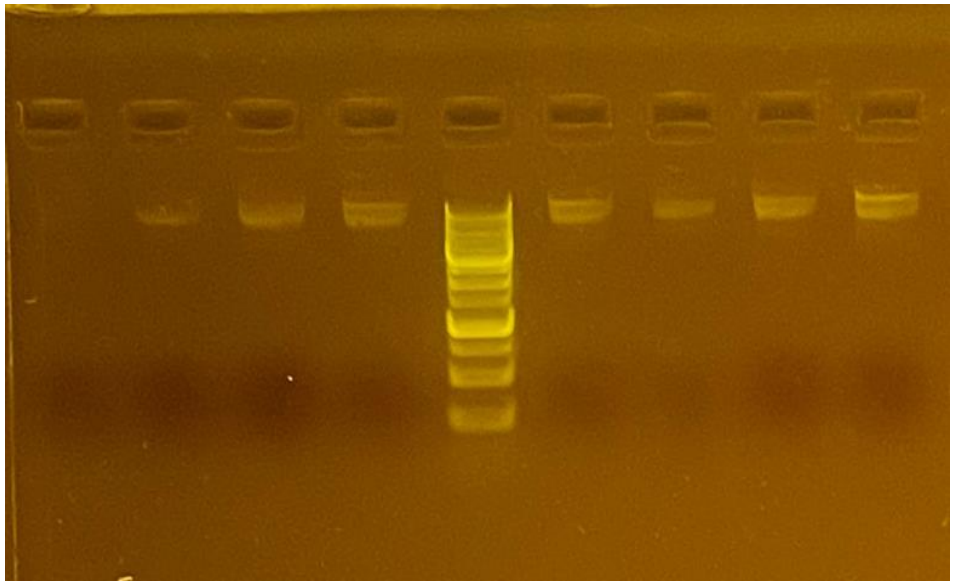
Goal:

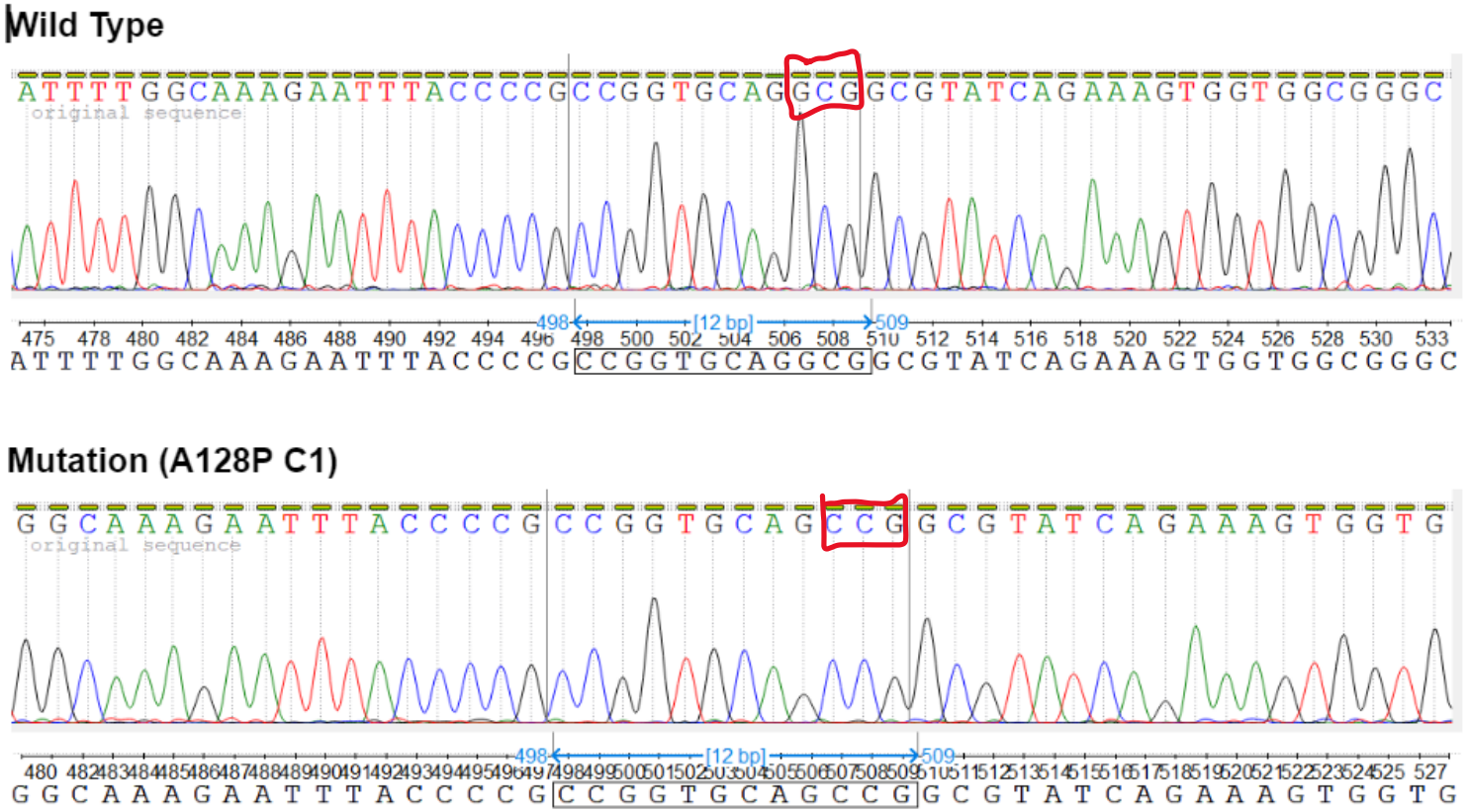
- Isolate PCR product DNA
- Let plasmid multiply in host body
- Isolate purified DNA again
- Prepare mutated DNA and WT DNA for sequencing (next step)

# Methods: Sequencing





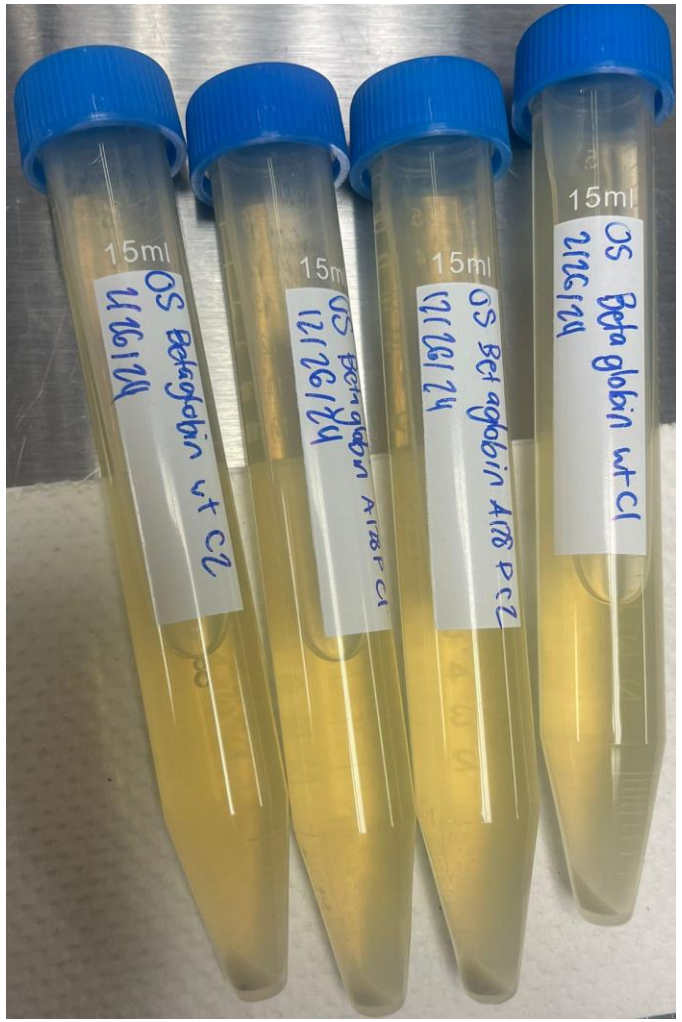

**Loading Order**

Lane 1 → Wild Type C1
Lane 2 →PCR #2 C1
Lane 3 → PCR#1 C1
Lane 4 → PCR #1 C2
Lane 5 → Ladder
Lane 6 → PCR #4 C1
Lane 7 → PCR #4 C1
Lane 8 → PCR #8 C1
Lane 9 → PCR #8 C2

**Purpose of sequencing:**

- To determine the nucleotide bases that make up the amino acids.
- Any deviations from the original Beta-Globin nucleotide sequence will help identify mutations.

# Methods: Recording OD and Protein Production in 30 mL Culture




Above: 30 mL Culture

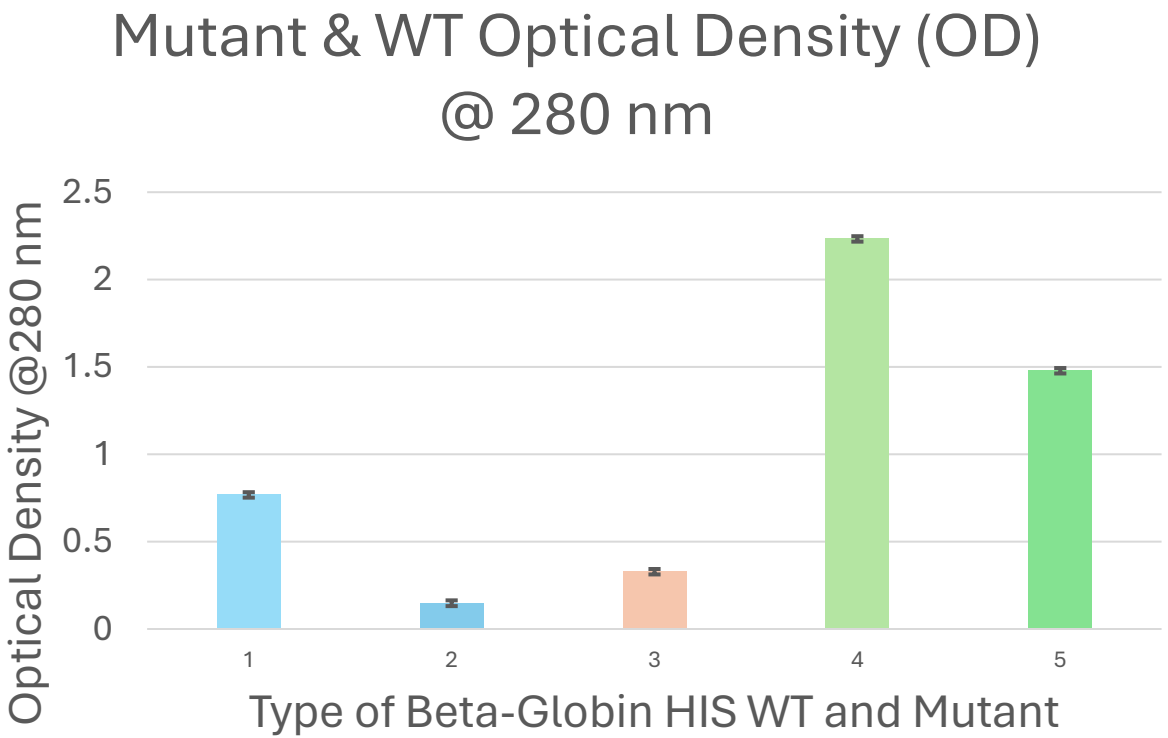

## Recording Optical Density (OD)

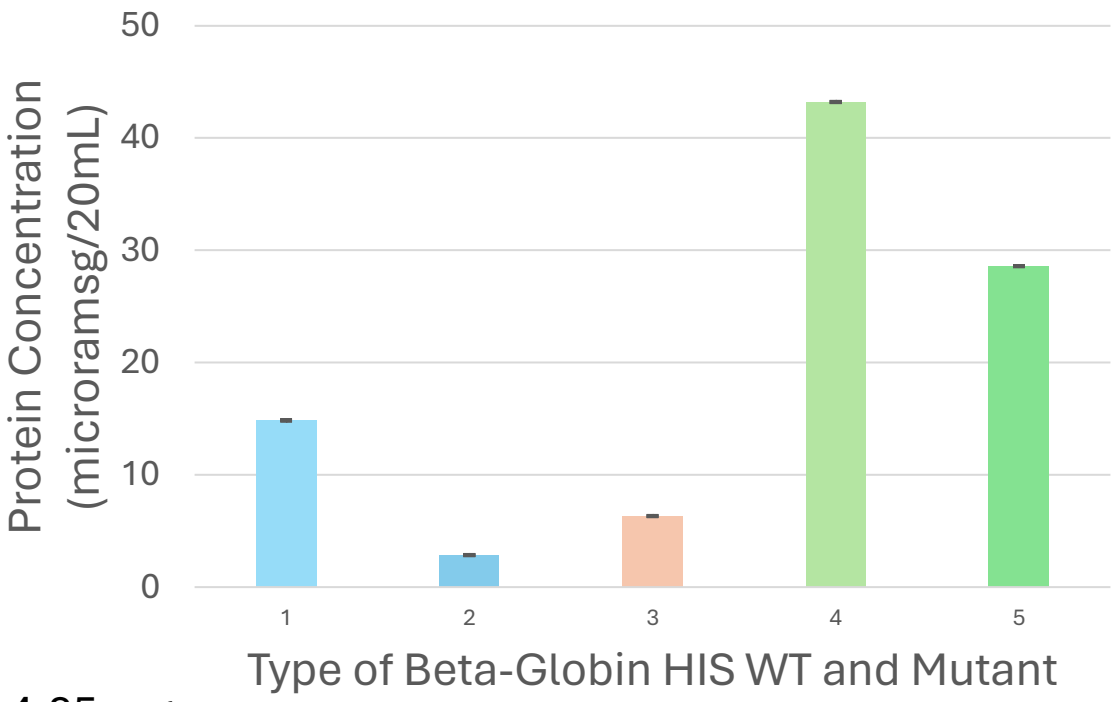
1) Based on results of sequencing, inoculate the mutated DNA into 30 mL of Nutrient Broth
2) Add IPTG to initiate protein production
3) Measure OD via a spectrophotometer and record every 2 hours.

## Recording Protein Production (in mg)

1) Add IPTG to initiate protein production
2) Add 2 mL of Proteaise Inhibitor Mix (100x) to culture.
3) In a microcentrifuge tube, add silica beads up to the line.
4) Pour as much of bacterial culture as possible into the microcentrifuge tube.
5) Place in MonoLyser and spin thrice at 5x speed and twice at 10x speed.
6) Repeat the above steps for all 30 mL of the culture.
7) Repeat steps 1 to 6 for all mutants and the WT.

# Data/Results




- Wild Type average protein production of approximately 14.85 mg.
- L32E had approximately 2.85 mg protein produced.
- L106P had approximately 6.33 mg protein produced.
- **Mutations L32E and L106P both showed decreased protein production in comparison to WT.**
- L110P had approximately 43.21 mg protein produced.
- A128P had approximately 28.6 mg protein produced.
- **Mutations L110P and A128P both showed increased protein production in comparison to WT.**

# Analysis

- L110P likely has the most protein production due to the change from a non-polar amino acid to a special case amino acid.
    - Proline, a special case amino acid, is unique due to its ring structure, which creates bends in the protein structure.
    - Additional bends or loops in the structure could be a reason for increased protein production.
- L32E likely has the least protein production due to the change from a non-polar amino acid to a polar amino acid.
    - Non-polar amino acids are hydrophobic and tend to exist in the core of a protein
    - Polar amino acids are hydrophilic and create the external structure of the protein.
    - Removing an amino acid from the core of the protein may lead to instability, and therefore decreased protein production.

# Future Work

- Identifying epitopes using mutations that show *increase* in protein production.
- Use an assay kit to evaluate different aspects of epitope, including pH, presence of different components, weight, etc.