Lab Summary Notes (6.29.25 ~ 8.3.25)

Lab #1: Pipetting Practice & Recombinant Plasmid Cloning (6.29~7.3)

Main Objectives:

- Learn proper use of motor pipettes and micro pipettes.
- Transfer accurate liquid volumes and measure pipetting accuracy.
- Understand transformation of recombinant plasmid DNA into E. coli.
- Observe colony formation after heat shock and antibiotic selection.

Procedure

<table>
<tr><td rowspan="3">Accuracy Test for Pipetting (Lab 1-1)</td><td>Materials
1. Micropipettes (P10, P20, P200, P1000)
2. Pipette (5 mL), pipette tips
3. Microcentrifuge tubes
4. Flask (250 mL), water, digital balance
5. 1% methylene blue</td></tr>
<tr><td>Steps:
1. Prepare a 250 mL Erlenmeyer flask and fill it with water. Also, prepare one 15 mL culture tube per person. Remove the cap from each culture tube.
2. Place the culture tube on a digital scale and tare to zero.
3. Prepare a pipette tip and open the packaging just enough to expose the tip. Do not remove the entire wrapper.
4. Attach the pipette tip firmly to the end of the motor pipette. Ensure it is securely connected.
5. Carefully remove the pipette tip from its wrapper, making sure it does not touch anything.
6. Hold the flask with one hand and insert the pipette tip into the water with the other. At eye level, aspirate the target volume of water accurately.
7. Remove the pipette tip from the flask and place the flask back on the table.
8. Hold the culture tube and insert the pipette tip inside. Dispense the water by pressing the release button on the motor pipette.
9. Measure the weight of the culture tube to calculate the mass of the transferred water.</td></tr>
<tr><td>Purpose:</td></tr>
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- This exercise verifies the accuracy and consistency of motor pipette handling by comparing the expected and actual mass of water dispensed. It trains precision in aspirating and dispensing liquid volumes for future experimental use.

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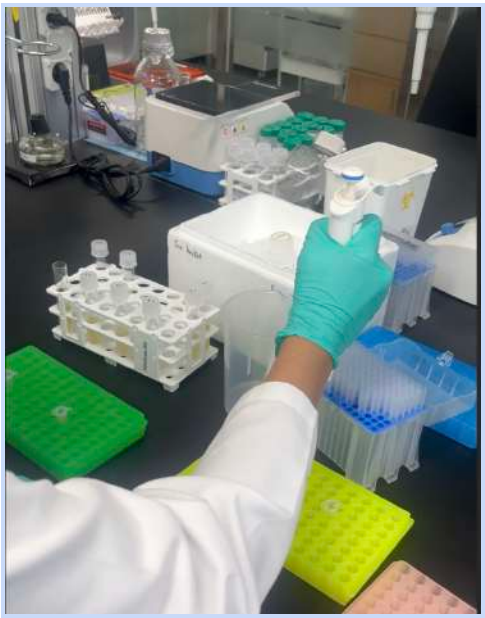
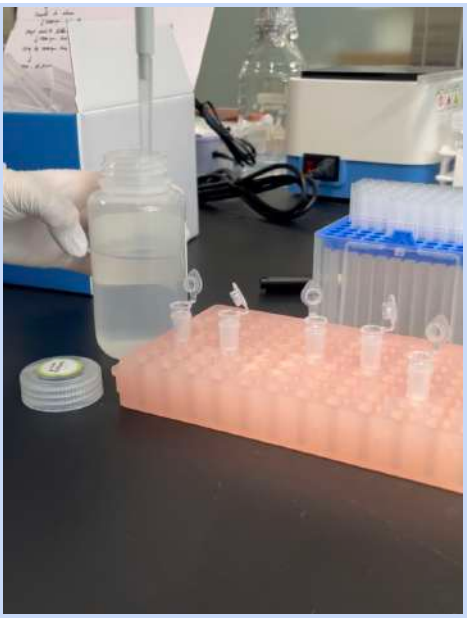
<tr><td></td><td>   </td></tr>
<tr><td rowspan="2">Transformation of Recombinant Plasmid DNA

(Lab 1-2)</td><td>

Material:

1. 37°C shaking incubator
2. 42°C water bath
3. Ice and an ice bucket
4. 1.5 mL microcentrifuge tube
5. Sterile spreader
6. LB media and LB agar plates
7. Competent E. coli cells
8. Plasmid DNA containing the NUSA gene (NUSA_pET28a)

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Steps:

1. Place the 1.5 mL tube containing the competent cells on ice.
2. Bring the provided Kanamycin-containing agar plate to room temperature.
3. Add 5 µL of plasmid DNA to the competent cells. Mix by gently tapping the bottom of the tube with your finger.
   <u>Do not pipette at this stage!</u>
4. Keep the mixture of competent cells and plasmid on ice for 20 minutes.
5. Submerge two-thirds of the tube in a 42°C water bath for 1 minute and 30 seconds to apply heat shock.
6. Return the tube to ice for another 2–3 minutes.
7. Add 1 mL of LB media to the tube and incubate in a 37°C shaking incubator for 1 hour.
8. Centrifuge for 30 seconds. Carefully discard the supernatant, leaving behind the cell pellet and a small amount of media.
9. Transfer the competent cell/plasmid mixture onto the LB agar plate and

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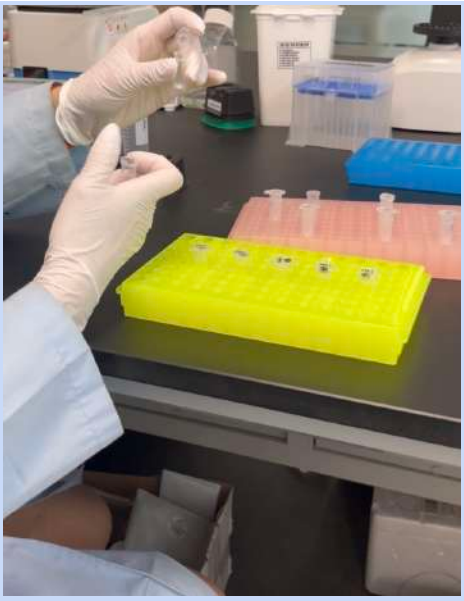
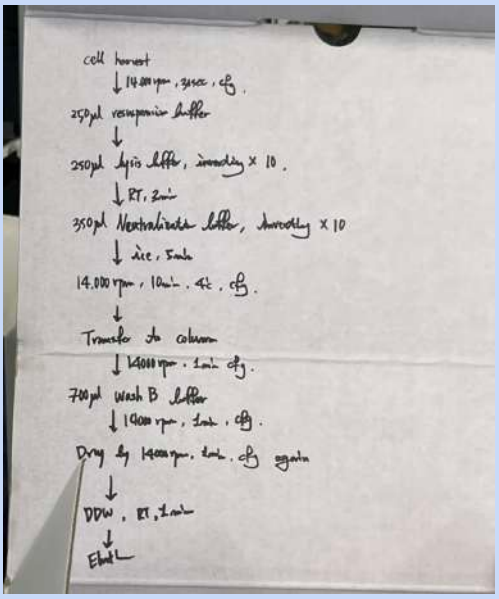
<tr><td rowspan="3"></td><td>spread evenly using a sterile spreader.
10. Observe colony growth the next day and store the plate in the refrigerator.</td></tr>
<tr><td>Purpose:
- In experiments using microorganisms as hosts, DNA is the most fundamental material, so securing a large quantity of DNA is crucial. To achieve this, a method called transformation is used, where foreign DNA is introduced into host cells. This involves making bacterial cells receptive to plasmid DNA by applying physical or chemical stimuli. Once the plasmid enters the cell, the transformed cells are cultured. This method not only helps obtain large amounts of DNA but is also used for various lab applications involving recombinant plasmids, such as library construction.</td></tr>
<tr><td> 
 
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<tr><td rowspan="2">Extraction and Purification of Plasmid DNA

(Lab 1-3)</td><td>Material:
1. Plasmid purification kit (Enzynomics)
2. Centrifuge</td></tr>
<tr><td>Steps:
1. Cell Resuspension
a. Harvest the bacterial cell pellet.
b. Add 250 µl of Buffer S1 and resuspend the pellet thoroughly by pipetting.
2. Cell Lysis
a. Add 250 µl of Buffer S2 and mix gently by inverting the tube.
b. Do not vortex.
3. Neutralization
a. Add 350 µl of Buffer S3 and invert the tube to mix.
b. Centrifuge at 13,000 rpm for 10 minutes to pellet cell debris and chromosomal DNA.
4. Supernatant Transfer</td></tr>
</table>

	a. Carefully transfer the clear supernatant to a new spin column. b. Centrifuge at 13,000 rpm for 1 minute to bind plasmid DNA to the membrane. 5. Washing Step 1 a. Add 700 µl of Buffer AW to the spin column. b. Centrifuge at 13,000 rpm for 1 minute and discard the flow-through. 6. Washing Step 2 (Optional) a. (Optional) Add 500 µl of Buffer PW for an additional wash. b. Centrifuge at 13,000 rpm for 1 minute and discard the flow-through. 7. Dry Spin a. Centrifuge once more at 13,000 rpm for 1 minute to remove residual ethanol. 8. Elution a. Place the spin column in a clean 1.5 mL tube. b. Add 50 µl of Buffer EB to the center of the membrane. c. Centrifuge at 13,000 rpm for 1 minute to elute the purified plasmid DNA.
	Purpose: - Extract and purify plasmid DNA from E. coli that was transformed with the cloned gene in the previous session.
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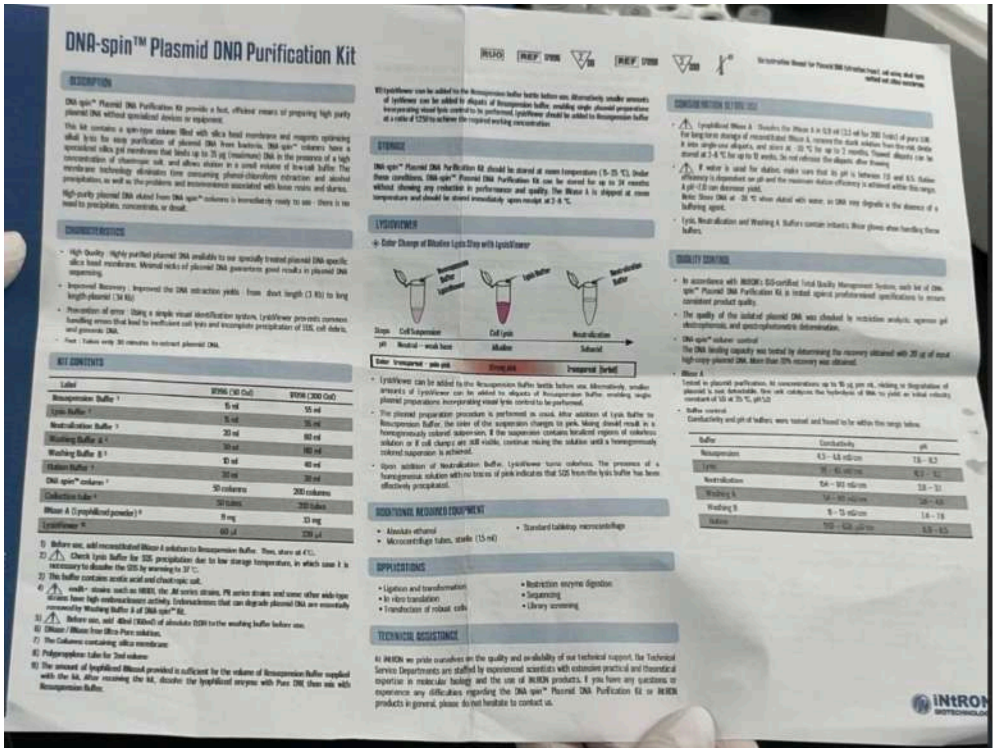
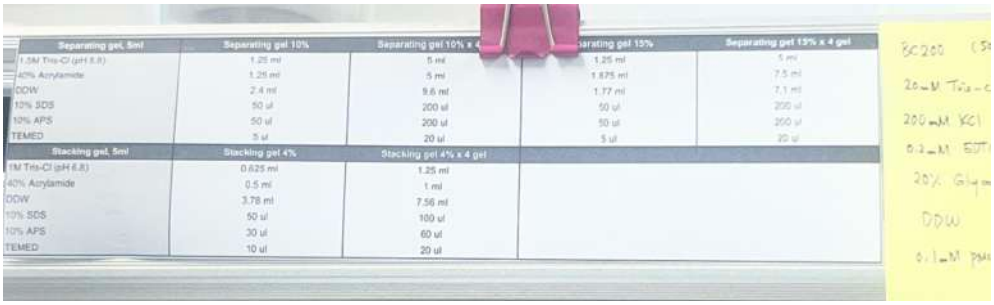

DNA-spin™ Plasmid DNA Purification Kit
DESCRIPTION
CHARACTERISTICS
KIT CONTENTS
STORAGE
LYSISVIEWER
ADDITIONAL REQUIRED EQUIPMENT
APPLICATIONS
TECHNICAL ASSISTANCE
CONSIDERATION BEFORE USE
QUALITY CONTROL
iNtRON

Lab #2: Protein Expression & Identification (7.13 ~ 7.20)

Procedure

<table>
<tr><td rowspan="4">Protein Expression (Lab 2-1)</td><td>Materials
1. 37°C shaking incubator
2. LB media in 5 mL tubes and 50 mL flasks
3. 1M IPTG
4. Spectrophotometer</td></tr>
<tr><td>Steps:
1. Inoculate a single colony into 5 mL of LB medium containing 50 μg/mL kanamycin and incubate for 12 hours.
2. Transfer 1–2% of the pre-cultured medium into 50 mL of fresh LB liquid medium.
3. Measure the optical density (O.D.) at 1-hour intervals.
4. When O.D. reaches between 0.5 and 0.7, add IPTG to a final concentration of 0.5 mM.
5. After 4 hours, measure O.D. again and harvest the cells using a centrifuge.
6. Discard the remaining LB supernatant and freeze the pellet for storage.</td></tr>
<tr><td>Purpose:
- Induce protein expression in previously transformed expression E. coli by culturing the cells and adding IPTG as a chemical inducer.</td></tr>
<tr><td> 
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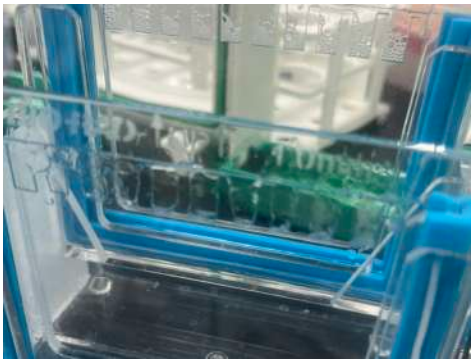
SDS-PAGE gel **(Lab 2-2)**	Material: 1. Protein sample (10 μL) 2. 2× Laemmli sample buffer ○ Tris-HCl (pH 6.8): Stabilizes the pH of the protein sample ○ Sodium Dodecyl Sulfate (SDS): Denatures proteins and gives them a negative charge, allowing separation by size ○ Glycerol: Increases sample density for easier gel loading ○ Bromophenol Blue: A tracking dye to monitor sample migration ○ β-mercaptoethanol or DTT: Reduces disulfide bonds to fully unfold proteins 3. SDS-PAGE gel kit 4. SDS running buffer (Tris-Cl, glycine, SDS) 5. Staining solution, destaining solution

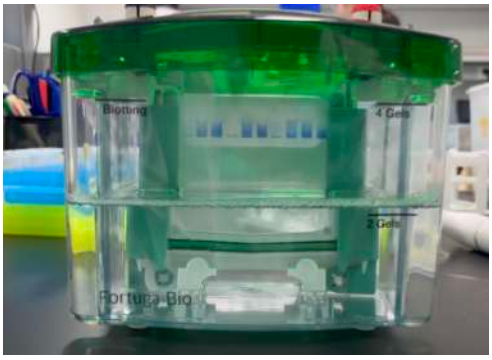
Steps:

1. Prepare SDS-PAGE Gel as written in the kit instruction
2. Prepare Protein Sample
    a. Mix the protein sample with Laemmli sample buffer.
3. Denature Protein
    a. Heat the mixed sample at 95°C for 5 minutes using a heating block to unfold protein structure.
4. Load and Run Gel
    a. Load the sample into the gel wells and run the gel at 180V for 1 hour.
5. Staining
    a. Remove the gel and incubate in a staining solution for 15 minutes.
6. Destaining
    a. If staining is successful, incubate in a destaining solution for 30 minutes to reduce background.
7. Analyze
    a. Check the protein bands and take a photo for documentation.

Purpose:

- Use SDS-PAGE to measure protein bands by molecular weight and determine the size and amount of the protein.





Lab #3: BCA + Affinity Assay (7.27 ~ 8.3)

Main Objectives:

- Produce functional MHC/peptide complexes by expressing, refolding, and quantifying proteins, then analyzing the structural or binding characteristics of the resulting complexes.

Procedure:

BCA Assay (Lab 3-1)	Materials 1. BSA (Bovine Serum Albumin) 2. BCA assay kit 3. 96well plate
	Steps: 1. Preparation of Standards and Working Solutions a. Prepare diluted BSA (bovine serum albumin) standards using the same diluent as the test samples. b. Use the standard preparation table as a reference. c. Each 1 mL of BSA stock is sufficient to prepare a full dilution series. 2. Prepare BCA Working Reagent (WR) a. Mix 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1 ratio). b. Calculate the total volume of WR needed using this formula: (# of standards + unknowns) × (# of replicates) × (volume per well) 3. Microplate Procedure (Sample:WR = 1:8)

<table>
<tr><td></td><td>
a. Pipette 25 µL of each standard or unknown sample into individual wells of a 96-well microplate.
i. Working concentration range: 20–2000 µg/mL
b. Add 200 µL of prepared WR to each well.
c. Mix thoroughly on a plate shaker for 30 seconds.
d. Cover the plate and incubate at 37°C for 30 minutes.
e. Bring the plate to room temperature, then measure absorbance at 562 nm using a plate reader.
4. Plotting the standard curve
a. X-axis: Protein concentration (µg/µL)
b. Y-axis: Absorbance at 562 nm (OD_{562})
c. Apply linear regression to determine the concentration of unknown samples based on the standard curve (the slope)
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Purpose:
- To quantitatively analyze the protein content of unknown samples using the BCA method.
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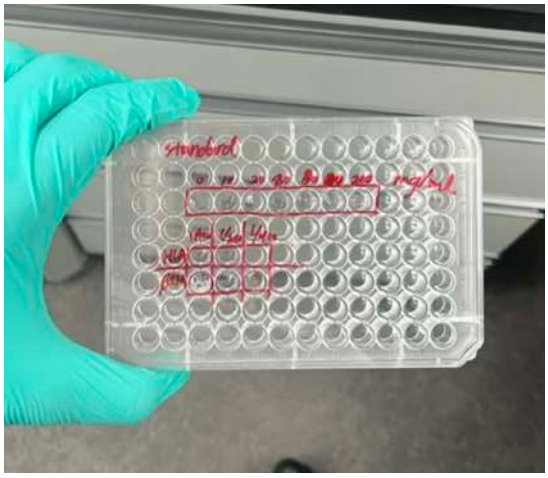

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<tr><td>Refolding of MHC/peptide complexes</td><td>
Material:
1. Refolding buffer (250 ml)
○ 100 mM Tris-HCl (3.925 g)
○ pH 8.0 400 mM L-arginine-HCL (21 g)
○ 2 mM NaEDTA (1 ml stock solution [0.5M])
○ 0.5 mM oxid. glutathione (0.077 g)
○ 5 mM red. glutathione (0.385 g)
2. Guanidine solution (100 ml)
○ 3M guanidine-HCL (28.7 g)
○ pH 4.2 10 mM sodium acetate (0.082 g)
○ keep at RT 10 mM NaEDTA (2 ml stock: 0.5 M)
3. Protease inhibitors (stock solutions):
○ PMSF, 100 mM (174.2 mg in 10 ml 2-propanol, stored at RT)
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(Lab 3-2)	○ Pepstatin, 2 mg/ml (5 mg in 2.5 ml DMSO) ○ Leupeptin, 2 mg/ml (5 mg in 2.5 ml dH2O)
	Steps: 1. Calculate the quantities of HC and b2m needed for refolding: a. HC: 18.6 mg (3 x 6.2 mg) (final concentration approx. 3 mM) b. b2m: 13.2 mg (3 x 4.4 mg) (final concentration approx. 6 mM) 2. Thaw recombinant protein stocks (in 8 M urea) and add 6.2mg HC to each of 3 a. Eppendorf tubes (3 x 4.4 mg for b2m). One sample of HC each and b2m is used immediately for refolding, and the others are stored at -20°C until use. 3. Cool 200ml refolding buffer in a 250-300 ml flask to 10°C (on ice) 4. Add protease inhibitors to refolding buffer: a. 2 ml PMSF (stock 100 mM) b. 100 ml pepstatin (stock 2 mg/ml) c. 100 ml leupeptin (stock 2 mg/ml) 5. Dissolve 12 mg of MHC-restricted peptide in the refolding buffer (if solubility is a problem. 6. Just before injection, dilute the HC and b2m samples 1:2 with guanidine solution (add max. 500 ml per sample) 7. Injection and dilution of HC and b2m into the refolding solution: a. inject the b2m-in-guanidine solution i. Use a #27 gauge needle and a 1 ml syringe, and inject near the vigorously rotating stir bar to obtain fast and efficient dilution. 2m refolds relatively easily and remains stable even in the absence of HC. b. Inject the HC-in-guanidine solution i. Follow the same procedure as described above for 2m. HC alone is very unstable and precipitates rapidly if not stabilized in a trimeric HC/ 2m/peptide complex. Therefore, the order of dilutions/injections (first peptide and 2m, then HC) is quite important. The consecutive injection of small aliquots -instead of the single application of the whole amount of protein- increases the yield of refolded MHC . 8. Place refolding at 10°C for 8 hours (constantly and slowly stirring the solution) a. We place the solution in a closed box in a cold room. 9. Take the second aliquot of HC and b2m, make “in-guanidine-solutions”, and inject into the refolding buffer as described above. 10. Incubate another 6-12 hrs at 10°C (constantly and slowly stirring the solution) 11. Take the last aliquots of HC and b2m, make “in-guanidine-solutions” and inject into the refolding buffer as described above. 12. Incubate for an additional 24 hrs at 10°C (constantly and slowly stirring the solution)
	Purpose:

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- Use SDS-PAGE to measure protein bands by molecular weight and determine the size and amount of the protein.

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<tr><td rowspan="3">Comparative analysis of the complexes formed

(Lab 3-3)</td><td>

Material:

1. Nickel coated 96 well plate
2. Complex protein solutions
3. Micro plate reader

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Steps:

1. Equilibrate the wells to be analyzed on a nickel-coated plate with a refolding buffer for approximately 5 minutes.
2. After removing the refolding buffer, various complex solutions and blank samples are placed into the wells.
3. Incubate at RT for 2 hours to overnight.
4. After removing the solution, wash with a buffer about 5 times.
5. After filling 100 ul of buffer, measure fluorescence using a micro plate reader.

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Purpose:

- To check if MHC/peptide complexes successfully formed by measuring how much fluorescence signal they produce when bound to a nickel-coated plate.

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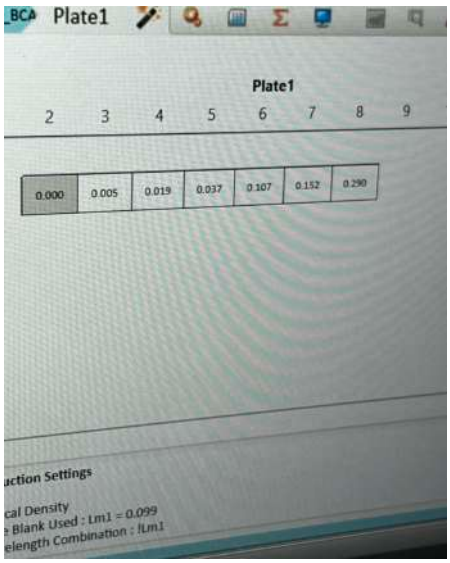
<tr><td></td><td> 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Recorded by Aiden Ha