LAB SCHEDULE

|  |  |
| --- | --- |
| DAY | WORK |
| **Day 1** | * PREPRATORY DAY.   + A. – To prepare the agar plates and liquid state media containing chloramphenicol and   Gentamicin antibiotics.   * + B. – To resuspend pVDL9.3, pbad33-GM plasmids, and gene block in TE buffer.   + C. – To create plasmid construct from Pbad33-Gm and gene block insert.   + D. – To create chemically competent cells. |
| **Day 2** | * CELL TRANSFORMATION.   + To dual transform bacterial cell with transformed plasmid pbad33-Gm and pVDL9.3. |
| **Day 3** | * Cell inoculation into liquid media   + **A.-** Check for fluorescence on agar plate #1/2.   + **B.-**Inoculating a Liquid Bacterial Culture from monoclonal colony on agar plate #1/2.   + **C.-** Prepare appropriate concentrations of inducers from stock. |
| **Day 4** | * CELL INDUCTION DAY   + **A.** -To create 25 homogenous LB broth cell cultures.   + **B.** -To find out at what levels of IPTG, L-arabinose secretion of fusion protein is maximum.   + **C.** Prepare samples for ELISA test.   + **D.** Create a glycerol stock of dual transformed shuffle cells (50% v/v). |
| **Day 5** | * ELISA DAY   + To run ELISA test on the cell lysate supernatant collected and treated yesterday. |

Use of Ssp dnaB derived mini-intein as a fusion partner for production of recombinant human insulin peptide leveraging Hemolysin transport system(Type I secretion system) in Escherichia coli.  
Ganesh Kishore  
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Abstract  
   
To develop a system for localized insulin production within cells—to be potentially housed in a hollow fiber bioreactor—we are engineering a fusion protein with the structure: insulin – N-terminal cleaving intein – HlyA. The N terminal cleaving mini intein is derived from Ssb DnaB (Mathys et al., 1998). This fusion protein will be secreted by type I secretion system which has been previously used for export of Shiga-like toxin IIeB subunit Salmonella Typhimurium aroA, where there were no quantifiable protein present in the cytoplasm and the secreted protein showed binding to its respectable receptor. (tzschaschel et al., 1996)  
The fusion protein would be cloned into plasmid Pbad33-Gm with expression induced by L-arabinose Induced.  
The associated secretion machinery—HlyB and HlyD—will be expressed under the control of the pVDL9.3 vector, which are under induction of lactose/IPTG by lac promoter.  
TolC, the third component of the T1SS complex, is natively expressed in the Shuffle *E. coli* strain used for this system.  
Cleavage of the insulin fusion protein will be triggered by change of pH (6.5-7), activating the N-terminal cleaving intein, thereby releasing free insulin from the fusion construct.

*Proposal:  
If we could direct even 10% of global protein production into insulin and could purify 90% of the produced insulin, we could produce 0.5 g of insulin in a 500 ml Hollow fiber Bioreactor at OD600 = 6(OD can go as high as 100/200 in optimized systems), induced for 6 hours a day can produce insulin for ~360 patients in a single day.(\*\*The whole system aka bioreactor + purification system could be housed in dimensions of a computer! \*\*)  
\*\*\*In E. coli SHuffle cells at OD600 = 1, grown at 22–25°C in rich media, the global production of 300-amino-acid proteins is ~37.3 mg/L/hour (1.13 μmol/L/hour). Insulin peptide production is predicted to range from 0.373–9.325 mg/L/hour and 0.0113–0.2825 μmol/L/hour (11.3–282.5 nmol/L/hour), corresponding to 1–25% of the global protein synthesis rate. \*\*\*  
  
Note: For Phase I and II experimental validation, the gene constructs will include fluorescent markers and TEV excision tags. These elements will be removed in the final therapeutic construct, provided the early-stage results are successful.*

*Biosafety Note: Experiments using non-pathogenic E. coli Shuffle strains and standard molecular techniques align with BSL-1 guidelines. I will strictly adhere to protocols including proper material handling, autoclaving waste, PPE use (gloves, lab coats), and workspace hygiene to ensure safety and compliance.*

*\*\*Additional notes to self -   
add loxP/Cre system verification cut- and run all the parameters again  
design 2d model of the bioreactor- Blue print*

Lab Checklist

# Chemicals & Reagents

☐ Liquid LB medidia/powder.

☐ Distilled water

☐ Antibiotics- 4mg Gentamicin (10 μg/mL sol), 10 mg Chloramphenicol (25 μg/mL sol)

☐ Inducers -IPTG, Arabinose powders.

☐ High-fidelity T4 DNA ligase, HindIII, XbaI

☐ Detergents- CHAPS or Triton X-100

☐ TE Buffer

☐ Insulin ELISA kit.

# DNA & Vectors

☐ Native pVDL9.3 vector (Promoter: LacI-Plac, low copy, 7400 bp)

☐ Native pBAD33-Gm vector (Promoter: araBAD, low copy, 5500 bp)

☐ Gene block insert for pBAD33-Gm

# Machines and Equipment

☐ Autoclave  
☐Centrifuge  
☐ Water Bath  
☐ Vortex Mixer  
☐ Shaking Incubator   
☐ Bunsen Burner  
☐ Autoclaved toothpicks/ inoculation loop  
☐ Eppendorf tubes  
☐ fluorescence microplate reader (Plate reader), Black Flat bottom 96 deep well plate.  
☐ Spectrophotometer  
☐ Refrigerator

☐ Petri dishes (90 mm) X5

☐ 15 ml conical tubes X3

☐ Autoclavable 500 mL bottles

☐ 5/10ml glass pipette, micropipettes (P10, P20, P200, and P1000) and tips

☐ Microcentrifuge tubes

☐ Autoclave tape

**DAY 1: Preparatory Day**🡪 Plating & Vector Creation.  
  
**AIM** –   
A. – To prepare the agar plates and liquid state media containing chloramphenicol and   
Gentamicin antibiotics.  
B. – To resuspend pVDL9.3, pbad33-Gm Plasmids   
C. – To create plasmid construct from Pbad33-Gm and gene block insert.  
D. – To create chemically/electrocompetent cells  
  
MATERIALS REQUIRED –   
**A. Agar plate preparation (Add gene protocol)**Two 500ml bottles(#1 and #2)For 400 mL of LB:  
4 g NaCl, 4 g Tryptone, 2 g Yeast Extract and dH2O to 400 mL, 10 mg chloramphenicol (25 µg/mL), 4mg Gentamicin (10 µg/mL), pipette, autoclave tape.  
For 5 agar plates from Petri dish (90 mm ~20ml LB), 1.2g agar powder. **B. Plasmid and gene block resuspension (NovoPro protocol, IDT protocol)**pVDL9.3, pbad33 plasmids, gene block, Centrifuge, vortex, gene block, etc. **C. Plasmid transformation (NovoPro protocol)**PCR thermal cycler, Heat blocks or Water bath at 42°C and 80°C, Water bath at 42°C, Incubator at 37°C**,** Eppendorf tubes, Native Pbad33-Gm vector, HindIII, XbaI, Ligase Buffer.  
  
**D.** **Preparing electrocompetent cells (Addgene protocol)**  
E. coli cells (SHUFFLE strain),LB (Luria-Bertani) broth and agar plates(without antibiotics), Calcium chloride (CaCl2),Ice, Sterile microcentrifuge tubes, Centrifuge, Incubator set to 37 °C, Pipettes and tips, Sterile distilled water.

METHODOLOGY -  
**A. Agar plate preparation**I. to make 400 mL of LB, weigh out the following into glass bottle #1:  
4 g NaCl,  
4 g Tryptone,  
2 g Yeast Extract,  
 dH2O to 400 mL,  
  
II. Cover the opening of the bottle #1 with its cap or aluminum foil (not air-tight) and tape the bottle with autoclave tape. 🡪 Place the gel mix in the autoclave and run on 121 ℃ under 20 psi for at least 30 min.🡪 tape will darken during the autoclave process indicating process is complete.  
  
III. Prepare a water bath at 60 ℃ with sufficient water to submerge ~75% of the bottle #1 containing molten gel mixture  
  
IV. Once cooled, transfer 100 ml of LB solution into another bottle #2 and add 1.2 g Agar to bottle #2 🡪 swirl to form a medium/agar colloid.  
  
V. Add 3/4th of the prepared antibiotics (3 mg gentamycin, 7.5 mg chloramphenicol) to the Bottle#1.  
  
VI. Autoclave Bottle #2 and repeat step III, 🡪 add 1/4th of the prepared antibiotics (1 mg gentamycin, 2.5 mg chloramphenicol to the Bottle#2.   
  
VII. Pour 20ml content of Bottle #2 into one 90 mm plates 🡪 store at 4°C  
  
VIII. Add 2 mg IPTG and 80 mg L-arabinose to remaining 80 ml and pour it into 4 90 mm plate.  
(Final result🡪

|  |  |  |  |
| --- | --- | --- | --- |
| item | Contents | Volume | Quantity |
| LB liquid | Antibiotics | 300 ml | X1 |
| Agar plate 1-4 | Antibiotics + inducers (0.1mM IPTG,0.1% L-arabinose) | 20 ml | X4 |
| Agar plate 5 | Antibiotics | 20 ml | X1 |

\* Plates 1-4: LB + Chloramphenicol (25 μg/mL) + Gentamicin (10 μg/mL) - Used for selecting double transformants. Plates 1 and 2 will be plated, plate 3 will serve as a control, and plate 4 as a backup. \*  
\*\*Plate 5: LB + Chloramphenicol (25 μg/mL) + Gentamicin (10 μg/mL) - For colony PCR in case plate 1 and 2 fail to grow colonies). \*\*

\*\*Even though we probably will not use all liquid LB, it would be good practice to make more than we need in case contamination occurs in liquid cultures on day 3/etc.\*\*  
**B. Resuspending the gene block & Plasmids (IDT protocol)**  
1. *Centrifuge:* at ≥3,000×g for 3-5 seconds.

2. *Resuspend:* According to the Table

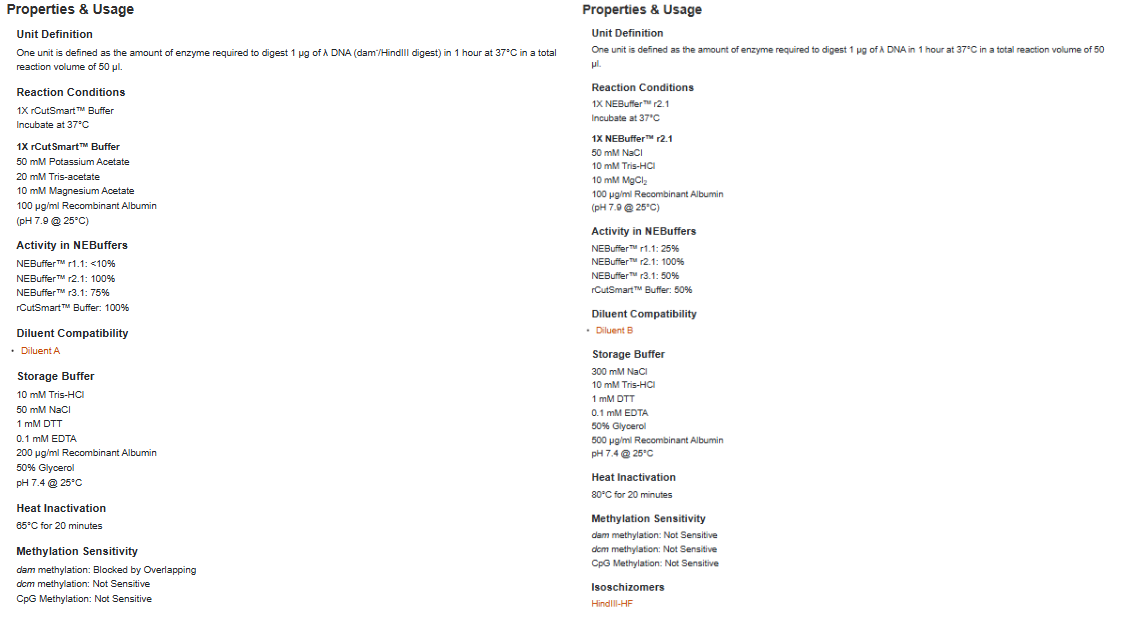
|  |  |  |  |
| --- | --- | --- | --- |
| Final Stock Concentration | Resuspension volume of TE buffer (μL) for IDT gene fragments/plasmids. Synthesis Scale | | |
|  | 250 ng | 500 ng | 1000 ng |
| 10 ng/µL | 25 | 50 | 100 |
| 20 ng/µL | Not recommended | 25 | 50 |
| 50 ng/µL | Not recommended | 10 | 20 |

\*50 ng/µL stock concentration will be done/preferred\*  
\*\*To get 1ng of DNA for next step, 20 µL of 50 ng/µL stock will lead to 1 µg of DNA\*\*  
\*\*\*We will do this same step for plasmid suspension\*\*\*  
\*\*\*\*pbad33-Gm and gene block would be stored at -20 ℃ after plasmid transformation\*\*\*\*

4. Briefly vortex the tube and then do a quick spin to concentrate the liquid at the bottom. Speed is less than 5000×g.

5.Store the plasmid/gene block at -20 ℃.  
  
 **C. pbad33-Gm plasmid Transform Protocol (NEB protocol, IDT protocol) \*gene block🡪pbad33-Gm\***I. Linearize the vector by combining in an Eppendorf tube-

|  |  |
| --- | --- |
| REAGENT | AMOUNT |
| Plasmid DNA | 1 µg (20 µL from 50 ng/µL stock) |
| NEBuffer™ r2.1(100% activity for both) | 5 µl (1X) |
| Xbal | 10 units is sufficient, generally 1µl is used |
| HindIII | 10 units is sufficient, generally 1µl is used |
| Total Reaction Volume (++distilled water till) | 50 µl |
| Incubation Time | 1 hour |
| Incubation Temperature | 37°C |

\*\*Do the same for the gene block to reveal sticky ends\*\*  
\*\*1 µg (1000 ng) DNA in 50 µl, thus 1 µl of solution contains 20 ng of DNA. \*\*  
  
 Xbal HindIII  
**  
\***Since we cannot heat inactivate HindIII without risking damage to DNA, we will do a purification by PCR/DNA Purification Kit for pbad33 plasmid backbone and do a gel extraction for the gene block insert. **\*  
Ligation protocol (NEB protocol)**I. Set up the following reaction in a microcentrifuge tube on ice.

|  |  |
| --- | --- |
| Component | Amount Used in 20 μl Reaction |
| T4 DNA Ligase Buffer (10X) \* | 2 μl |
| Vector DNA (5,5 kb) | 50 ng (2.5 µl of plasmid RE solution) |
| Insert DNA (1.6 kb) | 45 ng (2.25 µl of insert RE solution) |
| Nuclease-free water | To bring volume up to 20 μl |
| T4 DNA Ligase | 1 μl |

\* The T4 DNA Ligase Buffer should be thawed and resuspended at room temperature.  
\*\* T4 DNA Ligase should be added last  
\*\*\* ligation using a molar ratio of 1:3 vector to insert

II. Gently mix the reaction by pipetting up and down and microfuge briefly.

III. For cohesive (sticky) ends, incubate at 16°C overnight or **room temperature for 10 minutes** (we will incubate for 10 minutes in room temperature).

IV. Heat inactivate at 65°C for 10 minutes.

V. Chill on ice and transform 1-5 μl of the reaction into 50 μl competent cells.

**D. Prep Chemically competent cells (Add gene protocol)**  
I. inoculate a single colony of E. coli into 5-10 ml of LB broth 🡪Incubate the culture at 37 °C with shaking until it reaches an optical density (OD600) of about 0.4–0.6. (3-4 hours)

Step 2: Chilling Cells on Ice

II. Transfer the culture to a sterile centrifuge tube 🡪 Place the tube on ice for 10-15 minutes to cool down the cells (10-15 minutes).  
  
III. Centrifuge the culture at 4,000 x g for 10 minutes at 4 °C 🡪 Carefully decant and discard the supernatant.

IV. Gently resuspend the cell pellet in an equal volume of cold, sterile 100 mM calcium chloride (CaCl2) solution 🡪 Incubate on ice for 30 minutes, gently inverting the tube every 5-10 minutes.

V. Centrifuge the cells again at 4,000 x g for 10 minutes at 4 °C Carefully remove the supernatant, leaving the cell pellet Resuspend the pellet in a smaller volume (usually 100-200 μl) of cold 100 mM CaCl2 solution.

VI. Divide the competent cells into small aliquots (usually 20-100 μl) and store them in sterile microcentrifuge tubes.

VII. Flash-freeze the aliquots in liquid nitrogen and store them at -80 °C until needed (the next day)

**DAY 2: CELL TRANSFORMATION**  
  
AIM – To transform bacterial cell with transformed plasmid pbad33-Gm and pVDL9.3.  
  
MATERIALS REQ –  
Shaking incubator at 37 °C, Stationary incubator at 37 °C, Water bath at 42 °C, Ice bucket filled with ice, Microcentrifuge tubes, Sterile spreading device  
2. LB agar plate (with appropriate antibiotic) as,

|  |  |  |
| --- | --- | --- |
| Agar plates | antibiotic | inducer |
| Plate 1/2 | + | **+(0.1mM IPTG, O.1% L arabinose)** |
| Plate 5 | + | - |

SOC media, Competent cells, transformed pbad33-Gm DNA, Native pVDL9.3.  
  
  
METHODOLOGY-

**1 Double transformation into E. coli (iGem protocol).**\*\*it’s for BL21(DE3) but should work nevertheless. \*

I. Prepare dilutions of the plasmids with a concentration of 2 ng/μL

II. Switch on the water bath and set temperature at 42 °C. Also turn on the heat/shaking-block and set up to 37 °C.

III. Load a bucket with ice from the ice machine

IV. Take the bacterial cells and SOC (Super optimal broth with catabolite repression) out of the -80 °C freezer. Transfer the cells directly to ice. Do not touch the bottom of the tube that contains the cells.

V. Thaw the cells on ice for ~5 minutes

VI. Add 1 μL of each plasmid into 20 μL bacteria. Mix well. Make sure you work near the Bunsen burner flame.

VII. Leave the cells on ice for 5 minutes

VIII. Heat shock the cells for 30 seconds at 42°C

IX. Return the cells directly to ice for 2 minutes

X. Add 80 μL of SOC solution to the bacteria.

XI. Incubate for 60 minutes at 37 °C and 300 rpm.

XII. Plate cells onto the agar plate in the 37 °C incubator. Place plate upside down.

*Note:  
1–5 μL DNA (1 pg–100 ng) per 50 μL competent cells (do not exceed 5 μL DNA)*  
  
  
**DAY 3: Cell inoculation into liquid media**  
AIM-  
**A.-** Check for fluorescence on agar plate #5  
**B.-**Inoculating a Liquid Bacterial Culture from monoclonal colony on agar plate#1  
**C.-** Preparation of appropriate amounts of inducers to be added

MATERIALS REQUIRED -  
**A.** **fluorescence Check-** Agar plate #5, UV light source  
  
**B.** **Inoculation of Liquid Bacterial Culture-** liquid LB media, 15 ml conical tubed, autoclaved toothpicks/inoculation loop,  
  
**C.** **Preparation of appropriate amounts of inducers to be added** - Arabinose powder, IPTG powder.

METHODOLOGY -  
**A. fluorescence Check-**  
I. In a dimly lit/dark room allow the UV rays from UV source to hit agar plate #5.  
  
II. If fluorescence occurs, it means that our cells are transformed and functioning as intended.

**B. Inoculation of Liquid Bacterial Culture-**

I. Transfer 10 ml of LB media to 15 ml conical tube.

II. Using a sterile pipette tip or toothpick, select a single colony from your LB agar plate.

III. Drop the tip or toothpick into the liquid LB and swirl.

IV. Loosely cover the culture with sterile aluminum foil or a cap that is not air tight.

V. Incubate bacterial culture at 37°C for 12-**18 hrs** in a shaking incubator.  
\* Do the same for an additional tube\*  
\*\*Do the same for an 3rd tube but this time no colonies (only toothpick-Control group)  
🡪growth occurs in 3rd means that sample was contaminated\*\*

**C. Preparation of appropriate amounts of inducers to be added-  
For Arabinose (w/v)-**

|  |  |  |
| --- | --- | --- |
| Arabinose (%w/v) | Grams per 0.5 mL | Milligrams |
| 0.01 % | 0.00005 g | 0.05 mg |
| 0.05 % | 0.00025 g | 0.25 mg |
| 0.1 % | 0.0005 g | 0.50 mg |
| 0.5 % | 0.0025 g | 2.50 mg |
| 1 % | 0.005 g | 5.00 mg |

**For IPTG** **(1mM)**Molecular weight of IPTG is 238.3 g/mol.

|  |  |  |
| --- | --- | --- |
| Desired Concentration (mM) | Moles in 0.5 mL | Mass of IPTG (mg) |
| 0.01 mM | 5 × 10⁻⁹ mol | 0.0012 mg |
| 0.02 mM | 1 × 10⁻⁸ mol | 0.0024 mg |
| 0.04 mM | 2 × 10⁻⁸ mol | 0.0048 mg |
| 0.1 mM | 5 × 10⁻⁸ mol | 0.0119 mg |
| 0.6 mM | 3 × 10⁻⁷ mol | 0.0715 mg |
| 1.0 mM | 5 × 10⁻⁷ mol | 0.1192 mg |

DAY 4: CELL INDUCTION DAY  
AIM-   
**A.** -To create 25 homogenous LB broth cell cultures.  
**B.** -To find out at what levels of IPTG, L-arabinose secretion of fusion protein is maximum.  
**C.** Prepare samples for ELISA test   
**D.** Create an glycerol stock of dual transformed shuffle cells(50% v/v)..  
  
MATERIALS REQ –  
**A. Creation of 25 homogenous LB broth cell cultures.**  
Liquid bacterial culture of day 2, IPTG, Eppendorf tubes.  
  
**B. Plate reader fluorescence test**Plate reader, Flat-bottom Black 96 well plate, appropriate amounts of IPTG and L-arabinose, Multichannel pipette, GFP protein at 1.0 mg/mL (1.0 μg/μL).  
  
**C. Preparing the sample for ELISA test**CHAPS or Triton X-100, Tubes, beakers, and cylinders.

METHODOLOGY -   
**A. Creation of 25 homogenous LB broth cell cultures.**I. Vortex the liquid culture prepared the previous day, Check and note down its OD (should be ~4 after 18 hours incubation ).  
II. Pipette 0.25 ml of liquid culture of it into 25 samples each as shown in the plate reader configuration below.  
III. Pipette 0.25 ml of LB Broth media into the 25 samples each.  
IV. Add the inducers according to the matrix shown below.  
  
**B - Plate reader fluorescence tests**  
WE Create an **A-B Expression Matrix** to determine at what concertation of inducers leads to maximum fluorescence in the supernatant the cell culture.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| IPTG/ ARABINOSE (W/V) | IPTG  0.1mM | IPTG  0.2mM | IPTG  0.4mM | IPTG  0.6 mM | IPTG  1 mM |
| 0.01% ARABINOSE | UD1 | UD2 | UD3 | UD4 | UD5 |
| 0.05%  ARABINOSE | UD6 | UD7 | UD8 | UD9 | UD10 |
| 0.1%  ARABINOSE | UD11 | UD12 | UD13 | UD14 | UD15 |
| 0.5%  ARABINOSE | UD16 | UD17 | UD18 | UD19 | UD20 |
| 1%  ARABINOSE | UD21 | UD22 | UD23 | UD23 | UD25 |

The 25 samples will be run through the plate reader at the following configuration and parameters-

|  |
| --- |
| NC – Negative control (0.5 ml LB broth) BK - Blank sample (○) SD – Standard (Known Concentrations of sfgfp) UD- Unknown sample (undetermined) λ excitation 🡪488 λ emission 🡪510 Bandwidth 🡪 5–10 nm (5 preferred) Gain 🡪 Auto Read Type🡪 Top read. |

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| \ | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | UD1 | UD2 | UD3 | UD4 | UD5 | ○ | SD1 | SD5 | ○ | ○ | ○ | ○ |
| B | UD6 | UD7 | UD8 | UD9 | UD10 | NC | SD2 | SD6 | ○ | ○ | ○ | NC |
| C | UD11 | UD12 | UD13 | UD14 | UD15 | ○ | SD3 | SD7 | ○ | ○ | ○ | ○ |
| D | UD16 | UD17 | UD18 | UD19 | UD20 | ○ | SD4 | SD8 | ○ | ○ | ○ | ○ |
| E | UD21 | UD22 | UD23 | UD24 | UD25 | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| F | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| G | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| H | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |

|  |  |
| --- | --- |
| Standard | Concentration 1.0 mg/mL (1.0 μg/μL) soln to be added |
| SD1 | 0.5 μL |
| SD2 | 1.0 μL |
| SD3 | 1.5 μL |
| SD4 | 2.0 μL |
| SD5 | 2.5 μL |
| SD6 | 3.0 μL |
| SD7 | 3.5 μL |
| SD8 | 4.0 μL |

\*Distilled water will be added till the volume is 0.5 ml for each\*  
\*\* Base and Increments are based on predicted protein produced for cells in 0.25 mL at OD₆₀₀ = 0.4 at 10 minute intervals accounting for 10% of their global protein synthesis\*\*  
\*\*\*The Plate reader will be used 60 minutes after induction has been done\*\*\*   
  
After getting the results for which Concentration is fluorescence maximum,  
we will continue to do another plate reading but this time with supernatant of the culture. -

For example, if there was maximum fluorescence in UD12,   
we will test the supernatants again of-  
UD1, UD2, UD6, UD7, UD11, UD12 Supernatant to know at what concentration of IPTG is secretion of fusion protein by Type I secretion system saturated.  
  
So we know that

**C. Preparing samples for ELISA test \***  
I. Lyse cells using lysing buffer → resuspend pellet in lysis buffer → incubate on ice (10–30 min) → vortex occasionally.

II. Centrifuge lysate at spin at 10,000–15,000 g, 10–15 min, 4°C → collect supernatant (contains fusion protein.  
  
IV. incubate for 16-24 hrs at room temperature and pH 6.5–7 to allow the intein cleavage.  
  
V. Mix the following (NEB protocol)

|  |  |
| --- | --- |
| REAGENT | AMOUNT |
| Supernatant | 45 µl |
| 1 Protease Reaction Buffer (10X) | 5 µl (10X) |
| TEV Protease | 1µl |
| Total Reaction Volume (++distilled water till) | 50 µl |
| Incubation Time | 1 hour |
| Incubation Temperature | 30°C |

or at 4°C overnight.  
\*\*just before Elisa day\*\*  
  
**D. Create a glycerol stock of dual transformed shuffle cells (50% v/v).**I. Add 500 μL of the overnight culture to 500 μL of 50% glycerol in a 2 mL screw top tube or cryovial and gently mix.

II. Freeze the glycerol stock tube at -80°C.

**DAY 5: Elisa DAY.**  
AIM - To run ELISA test on the cell lysate supernatant collected and treated yesterday.  
  
MATERIALS REQUIRED -  
<according to Agilent ELISA kit>  
Insulin ELISA kit (stored at 2°C to 8°C) by Thermofisher/Agilent/others, CHAPS or Triton X-100, Tubes, beakers and cylinders for reagent preparation, Redistilled water, Magnetic stirrer, Vortex mixer, Microplate reader with 450 nm filter, Microplate shaker (700–900 cycles per minute, orbital movement),Microplate washing device with overflow function (recommended but not required).  
  
METHODOLOGY (**Agilent protocol**)-   
I. Prepare enzyme conjugate 1X solution and wash buffer 1X solution.

II. Prepare sufficient microplate wells to accommodate calibrators, controls, and samples in duplicate.

III. Pipette 25 μL each of calibrators, controls and samples into appropriate wells.

IV. Add 100 μL of enzyme conjugate 1X solution to each well.

V. Incubate on a plate shaker (700-900 rpm) for 1 hour at room temperature (18–25°C).

VI. Wash 6 times with 700 μL wash buffer 1X solution per well using an automatic plate  
washer with overflow-wash function, after final wash, invert and tap the plate firmly  
against absorbent paper. Do not include soak step in washing procedure.

Or manually,  
Discard the reaction volume by inverting the microplate over a sink. Add 350 μL wash  
buffer 1X solution to each well. Discard the wash solution, tap firmly several times  
against absorbent paper to remove excess liquid. Repeat 5 times. Avoid prolonged  
soaking during washing.

VII. Add 200 μL Substrate TMB into each well.

VIII. Incubate on the bench for 15 minutes at room temperature (18–25°C).

IX. Add 50 μL Stop Solution to each well.  
Place plate on a shaker for approximately 5 seconds to ensure mixing.

X. Read optical density (OD) at 450 nm and calculate results accordingly.  
Read within 30 minutes.

Note to the Reader:  
I. If the Shuffle strain cannot allocate at least 10% of global protein production to fusion proteins or produces dysfunctional insulin, I would switch to the BLDE3 strain with the CyDisCo system implemented and other necessary adjustments made.  
  
II. I am assuming that TolC, which is the final effector for export of fusion protein is naturally present in the levels necessary to export fusion protein at 10% of global synthesis rate. But if results in plate reader show that export of fusion protein does not increase with increase with HlyB, HlyD (meaning TolC becomes a limiting factor), I would -  
Replace the native tolC RBS and promoter with stronger versions to improve export efficiency.