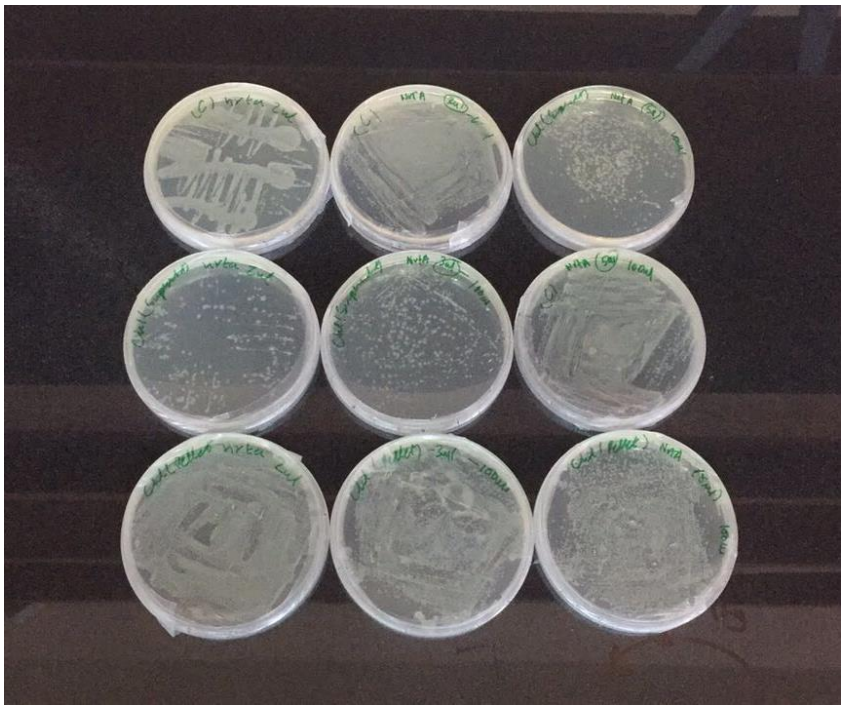
Collaboration with Team Taipei

The plasmid sent by the team containing NrtA gene was successfully transformed, lysed and the SDS-PAGE was performed later. Following are the step by step processes that we followed to help the team in confirming whether the protein is properly synthesized in the cell by running the protein on gel i.e. 45k Da.

1. Transformation:

The plasmid was transformed in DH5-alpha cells and proper colonies were grown on plates after 18-24hrs.



Fresh broth for cultures were prepared, single colony was inoculated in a falcon tube and incubated in the shaker incubator for 20hrs at 37 degree centigrade.

2. SDS-Page Preparation

12% of separating gel was prepared for the protein of 45kDa.

Protocol of Separating Gel

Components	Volume
H_2o	1.67 ml

Acrylamide mix (30%)	2 ml
Separating gel buffer (1.5M Tris pH 8.8)	1.25 ml
20% SDS	25ul
TEMED	2.5ul
10% Ammonium per sulfate	50ul
Total	5 ml

Protocol of Stacking Gel

Components	**Volume**
H_2o	1.702 ml
Acrylamide mix (30%)	0.5 ml
Stacking gel buffer (1.5M Tris pH 8.8)	750 ml
20% SDS	15ul
TEMED	3ul
10% Ammonium per sulfate	30ul
Total	3 ml

Sample Loading Buffer 4X (10 ml):

Components	**Quantity**
Glycerol	5 g
SDS	1 g
EDTA	0.0372 g
Tris-HCL (0.5 M pH 6.8)	3.153 g

Above mentioned three components were dissolved in 0.5 M Tris-HCL, the pH was adjusted to 6.8 and the final volume to 10ml. Bromophenol blue dye was added to give deep blue color to the solution. Aliquots were prepared and stored at -20° C.

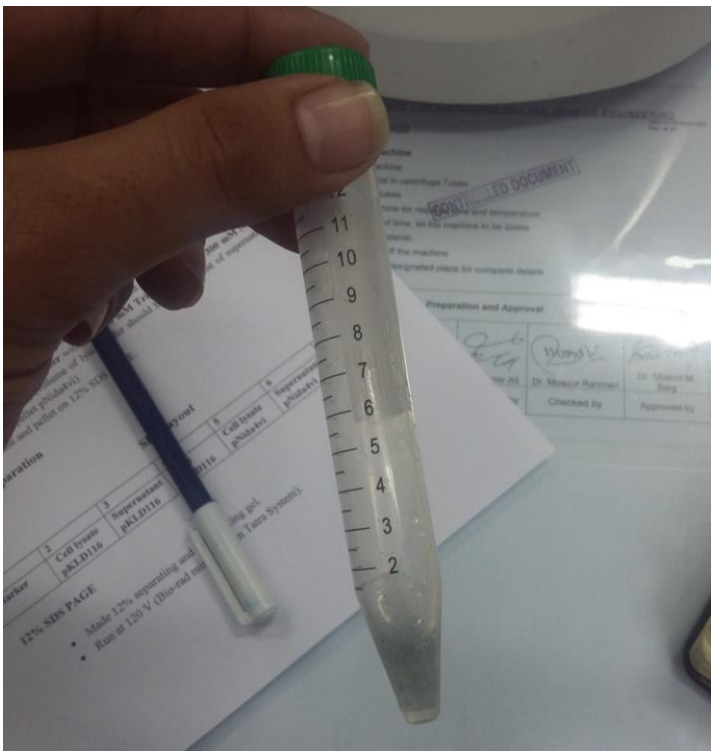
Running Buffer 1X (1 L):

Components	**Quantity**
Glycine	14.4 g
Tris base	3 g
SDS	1 g
Adjust volume to 1 L with distilled water.	

While the separating gel was left to be polymerized, culture containing bugs were centrifuged for 20 minutes to get separated form media. Following that, supernatant was discarded and the pellet was dissolved in resuspension buffer

During polymerization of the stacking gel, cell lysis was performed with the help of glass beads.

Cell Lysis Protocol



i. Re-suspend DH5- alpha pellet cells (from 25 ml culture) in 2.5 ml pre-chilled lysis buffer with urea (10 mM Tris HCl, pH 8.0, 300 mM NaCl, 2mM β-ME, 8 M Urea) and vortex. Also, re-suspend NrtA bugs pellet (from 25 ml culture) in 2.5 ml pre-chilled lysis buffer with urea (10 mM Tris HCl, pH 8.0, 300 mM NaCl, 2mM β-ME, 8 M Urea) by vortex.

ii. In separate falcon tube, take 0.5 ml glass beads (2-15 ml falcon x 0.5 ml), wash glass beads with ethanol then with distilled water. Lastly, washing with 1 ml re-suspension buffer (glass bead should by 1/3rd of cell culture) is done.

iii. Add 1.5 ml from 15 ml culture of dissolved pellet of DH5- alpha and the bugs of NsrtA in the washed glass beads (15 ml falcon) separately.

iv. Add lysozyme (0.12 mg/15ml cells). (Dissolve 2.25 mg lysozyme in 100 µl of re-suspension buffer. Then add 50 µl of it in each falcon tube)

v. Vortex for 30 secs and incubate on ice for 30secs (repeat this step for 25 times).

vi. After 25 cycles, leave these falcon tubes on ice for further 20 minutes to settle down the beads.

vii. Take out the supernatant from these beads (cell lysate DH5-alpha/ cell lysate NrtA).

viii. Centrifuge supernatant at 8000 - 12,000 rpm for 20 minutes at 4°C.

ix. Take out supernatant (supernatant DH5- alpha/ supernatant NrtA).

x. Re-suspend the pellets of both cells (DH5-alpha/ NrtA containing cells) in lysis buffer with urea (10 mM Tris HCl, pH 8.0, 300 mM NaCl, 2mM bMe, 8 M Urea). The volume of lysis buffer should be equal to that of supernatant.

Protein sample preparation

Components	Quantity
Dye	200 ul
SDS	200 ul
Beta mercaptoethanol	20 ul
Take 10 ul from the mixture and add 40 ul protein sample in it.	

Protein dye and the sample were mixed and incubated for 10 mins at 95° C. The gel was run at 170 volts for 40 minutes.

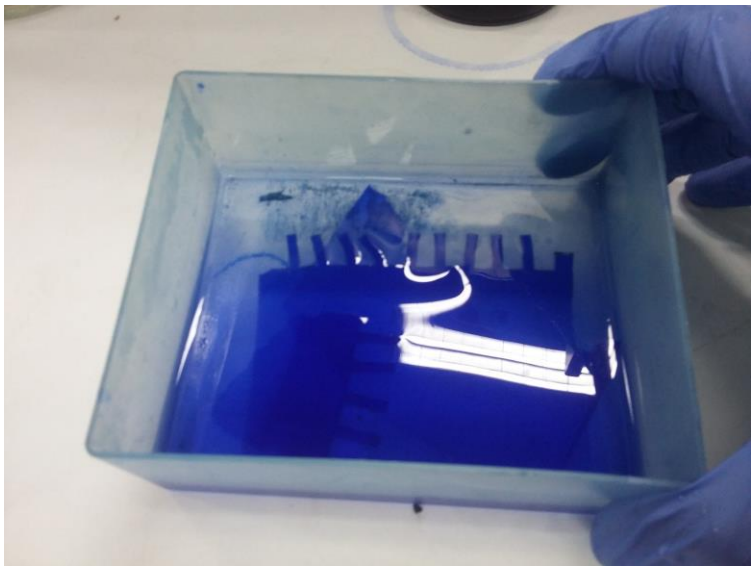
Staining Solution

Components	Quantity
Acetic Acid	50 ml
Isopropanol	125 ml
Coomassaie B. Blue R-250 (0.025%)	0.125 g
Distilled water	325 ml
Total Volume	500 ml

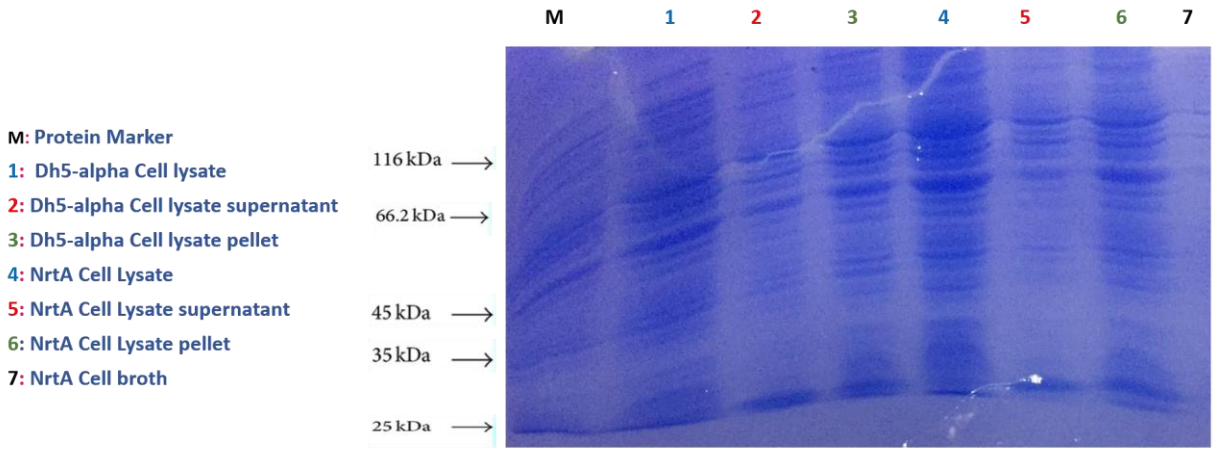
After overnight staining, the gel was de-stained in the de-staining solution.

De-Staining Solution:

Components	Quantity
Acetic Acid	50 ml
Distilled water	450 ml
Total Volume	500 ml



Gel Visualization



Without purifying NrtA protein from the NrtA containing cells, it cannot be easily concluded that NrtA protein is specifically being produced in the cell.

It is suggested by iGEM Peshawar team to attach a tag to your specific protein for exact identification.