**AMEER TAHA**

**LABORATORY REPORT**

**FOR**

**GREEN FLOURESCENT PROTEIN**

**BIO 230**

**PROFESSOR NONTERAH**

**4/18/2021**

**ABSTRACT**

The objective of the experiment was to attempt transformation of GFP in *E. coli* cells using pGLO plasmid. Protein transformation is a genetic engineering technique which can be utilized to express green fluorescent protein (GFP) in *E. coli* cells. The pGLO plasmid was genetically engineered to contain 4 sections including the replication origin, an ampicillin resistant component, the green fluorescent component which was isolated from a new species of starfish, and the fluorescent triggering component which is the arabinose sugar.

Four bacterial streaked plates were observed under UV light which includes the -DNA LB plate, -DNA LB/amp plate, +DNA LB/amp plate, and the +DNA LB/amp/ara plate. The –DNA LB plate which contains only the nutrient agar and the *E. coli* bacterial cells was observed to have an ample growth of bacteria but the spread do not fluoresce when subjected under UV light. The –DNA LB/amp plate which contains nutrient agar streaked with *E. coli* showed no growth due to the presence of ampicillin which is an antibacterial drug. The +DNA LB/amp plate was observed to have individual colonies that survived in an ampicillin treated plate. This ampicillin resistance is due to the ampicillin resistant component of the plasmid caught by the *E. coli* cells that survived. Finally, the +DNA LB/amp/ara plate contains individual colonies that fluoresce under the UV light due to the presence of arabinose sugar which is a triggering factor for the cells to fluoresce. Upon calculation, the efficiency for the protein transformation was determined to be 1152.5 cells/µg of DNA.

**BACKGROUND**

The green fluorescent protein (GFP) is a type of protein that was found originally in fluorescent jellyfish (Shimomura, 2009) but for this experiment the GPF in the engineered plasmid was isolated from the new species of starfish. This single protein which has become the most important tool in contemporary bioscience has undergone a very laborious characterization (Sanders and Jackson, 2009). Bacterial transformation is a process that enables cells to catch available DNAs from the environment and upon successful transformation process; the cells will have the property of the DNA that was bonded to it.

The microorganism cells used in this experiment to express the GFP were from *E. coli* cells. The pGLO DNA introduced to the *E. coli* cells was genetically engineered to contain four components that dictate the properties of the plasmid. This plasmid has the origin replication component, an ampicillin resistant component which would prevent the cells that bonded with the plasmid from being killed in the presence of the ampicillin drug due to its ampicillin resistance property, a GPF component which is the component that is responsible for glowing green under the UV light, and the trigger switch which is the arabinose sugar component which signals the GFP to produce a green light under the UV light (Diaz-Montealegre and Mills, N.D).

The experiment was carefully designed to give students the chance to observe and understand the protein transformation process of *E. coli* using pGLO plasmid DNA. This enables the students to learn how to prepare bacterial cultures using aseptic practices. The students will observe if the transformation successfully happened based on the growth of *E. coli* cells in different controlled conditions such as normal agar plate, an agar plate which contains ampicillin, and an agar plate which contains arabinose sugar. The transformed proteins will then undergo purification using chromatography and characterization using electrophoresis. Electrophoresis allows visualization of the phenotypic properties of the protein and with the SDS-PAGE extension kit the single protein band that is responsible for the specific fluorescent trait can be identified. In addition, the students will also be learning important laboratory techniques.

**MATERIALS AND METHODS**

The apparatus, materials, and equipment used to conduct this experiment are the following:

UV lamp, incubator, freezer, water bath, freezer, centrifuge, electrophoresis set-up, spectrophotometer, micro test tube, markers, sterile transfer pipette, ice bath, sterile loop, petri dish.

The reagents used to conduct this experiment are the following:

Calcium chloride or CaCl2 (which served as the transformation solution), pure *Escherichia coli* plate, pGLO plasmid DNA, nutrient broth, nutrient agar, TE solution, lysozyme, liquid equilibrium buffer for chromatography column, and buffers.

PROTEIN TRANSFORMATION PROCEDURE

Two micro test tubes were secured and labelled as +DNA and –DNA. Then a 250 µL of calcium chloride solution were transferred to each tube. This will make the membrane of the *E. coli* cells to loosen up which makes it able to accept the plasmid DNA upon addition of the plasmid. The micro tubes were placed in a beaker containing ice. One colony of *E. coli* was carefully transferred into the two micro test tubes.

pGLO plasmid DNA was scooped and inoculated into the micro test tube labelled +DNA. The micro test tube that was labelled –DNA does not contain the pGLO plasmid DNA and this served as the blank sample. The tubes were returned to the beaker with ice and were incubated for about 10 minutes. It was made sure that new inoculating loops were used in every inoculation process. While the micro test tubes were incubating in the ice, 4 petri dishes were secured and labelled as -DNA LB, -DNA LB/amp, +DNA LB/amp, and +DNA LB/amp/ara. The micro test tubes that were prepared previously were heat shocked for 50 seconds at 42 ºC and were returned immediately to the ice bath and was incubated for additional 2 minutes. The tubes were removed in the ice bath and put into the bench at room temperature. About 250 µL of LB nutrient broth to each of the tubes and were incubated at room temperature for 10 minutes. About 100 µL of transforms and controls were transferred into each of the appropriate agar plates and were evenly spread. The plates were stacked and incubated at 37 ºC for 24 hours.

BACTERIAL CULTURE PREPARATION

The plates were removed from the incubator after the 24 hours incubation period and were observed using UV light. On the +DNA LB/amp/ara plate, green colonies were observed. On the +DNA LB/amp plate, white colonies were identified. Two culture tubes identified as (+) and (-) were prepared. A scoop of green colony was transferred in the (+) tube and white colony to (-) tube. The test tubes were vigorously shaken and were incubated for 24 – 48 hours at 32 ºC.

PURIFICATION

The culture tubes were taken from the incubator and were observed under UV light. The difference in color from both tubes was noted. A volume of 2 mL of culture from the (+) tube was pipetted into a micro test tube labelled (+) and was centrifuged for a period of 5 minutes. The supernatant was poured and the residue was observed under UV light. About 250 µL of TE solution was transferred into the residue and completely mixed. A drop of lysozyme was added into the solution. The tube was again observed under UV light and then put into the freezer.

A chromatography column was prepared by letting 2 aliquots of 1 mL liquid buffer solution to completely drain from the column. The micro test tube was removed from the freezer and thawed. The tube was centrifuged at maximum speed for a period of 10 minutes, and immediately observed under UV. 250 µL of the supernatant was transferred into a new micro test tube labelled as (+). About 250 mL of binding buffer was transferred into the tube and was then refrigerated.

This part will be the chromatography process. Three collection tubes were labelled as 1, 2, and 3. The refrigerated supernatant containing a volume of 250 µL was loaded into the column by pipetting it gently to the side of the column. While dripping, the column was observed under UV. The effluent was collected in test tube 1. The procedure was repeated for test tube 2 now using wash buffer. The procedure was then repeated for test tube 3 now using TE buffer. The tubes were then put into freezer.

BRADFORD PROTEIN ASSAY AND ELECTROPHORESIS

A BSA standard with initial concentration of 1000 µg/mL was diluted into 750, 500, 375, 250, and 125 µg/mL. The BSA standards were subjected to spectrophotometer analysis and read the absorbance at 595 nm wavelength. The data generated were used to prepare a standard calibration curve which was used to calculate for the concentration of the unknown solution.

Samples labelled as “pre-purification GFP” and “post-purification GFP” were thawed and centrifuged. 2 aliquots of 10 µL pre-purification GFP sample were transferred into micro test tubes and labelled as “native” and “heat treated”. Two aliquots of 25 µL post-purification GFP sample were transferred into micro test tubes labelled also as “native” and “heat treated”. Each tubes were added an equal volume of Laemmli sample buffer and were allowed to equilibrate for 5 minutes at room temperature. The tubes labelled “heat treated” were heated at 95ºC for 5 minutes on a water bath and then cooled at room temperature afterwards.

The electrophoresis gel box was loaded with samples in the following order: 10 µL Kaleidoscope standard, 10 µL heated pre-purification, 10 µL heated post-purification, 20 µL heated pre-purification, 20 µL heated post-purification, 20 µL Kaleidoscope standard, 10 µL native pre-purification, 10 µL native post-purification, 20 µL native pre-purification, 20 µL native post-purification. After about 30 minutes of electrophoresis, the gel was removed from the frame and strained and gently rinsed with deionized water for three times. The gel was transferred into a clear plastic wrap and was observed under UV light to examine the lines 6-10. Photos of the product were taken to document. About 40 mL of Coomassie Blue stain was added into the tube, covered it and shaken it throughout the day after the gel has been inserted back into a strainer. After 24 hours, the gel was stained overnight. The migration distances of any visible bands were measured starting from the bottom of the well.

**RESULTS**

Table 1. Summary of observations from the plates with and without pGLO plasmid

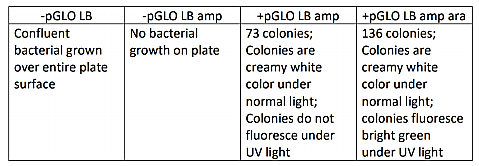


Figure 1. Photo of the plates with and without pGLO plasmid observed under UV light

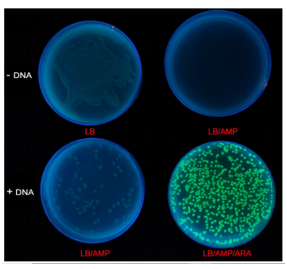


Table 1 summarizes the observation from the plates labelled with –DNA LB, -DNA LB/amp, +DNA LB/amp, and +DNA LB/amp/ara. The –DNA LB plate which contains no pGLO plasmid showed a spread growth of the *E. coli* bacteria. The -DNA LB/amp which also contains no pGLO plasmid but treated with ampicillin showed no growth of the *E. coli* bacteria. The +DNA LB/amp plate which was treated with ampicillin and where the pGLO plasmid was introduced contains a number of 73 individual colonies. Finally, the +DNA LB/amp/ara which contains ampicillin, arabinose sugar, and pGLO plasmid has 136 individually glowing colonies of *E. coli*  cells.

TRANSFORMATION EFFICIENCY CALCULATION

The total number of colonies in the +DNA LB/amp/ara plate is 136 colonies.

The concentration of DNA is 0.06 microgram/ microliter.

The volume of pGLO used was 10 microliters.

**Total amount of DNA (microgram/ microliter) : 0.06 µg / µl \* 10 µl = 0.6 µg of pGLO**

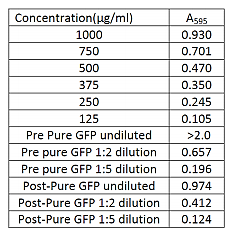
To find the volume of DNA spread on the plate, we add the 10 µl to the 500 µl of the nutrient broth and transformation solution: 10 + 500= 510 µl.

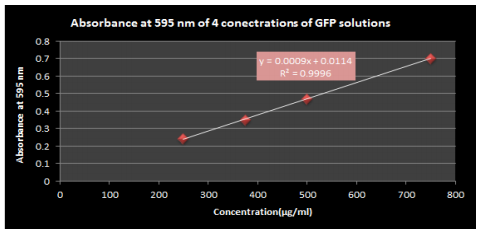
**Fraction of DNA used** = (Volume spread on LB/amp/Ara plate)/ (Total Volume on test tube)

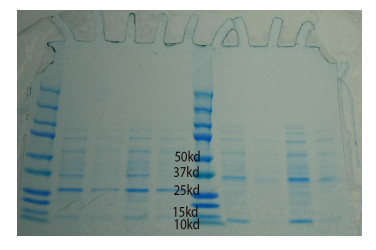
Using 100 µl to put on each plate: 100/510 = **0.19608.**

To calculate how much DNA we spread on the plate: 0.6 µg \* 0.19608 = 0.118 µg of pGLO spread on plate.

Since the number of colonies present on the plate were 136 colonies. Transformation Efficiency: (total number of cells growing on agar plate / amount of DNA on agar plate). **Transformation efficiency = 136 colonies / 0.118 µg = 1152.5 cells/µg**

Table 2. Data used for standard calibration curve for Bradford protein assay

Figure 2. Standard calibration graph plotted using Microsoft Excel

Figure 3. Gel electrophoresis result

**1**

**2**

**3**

**4**

**5**

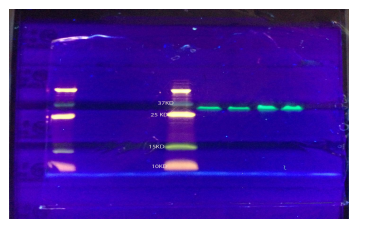
**6**

**7**

**8**

**9**

**10**

Figure 4. Pre-stained gel (UV light) showing native GFP bands

**1**

**2**

**3**

**4**

**5**

**6**

**7**

**8**

**9**

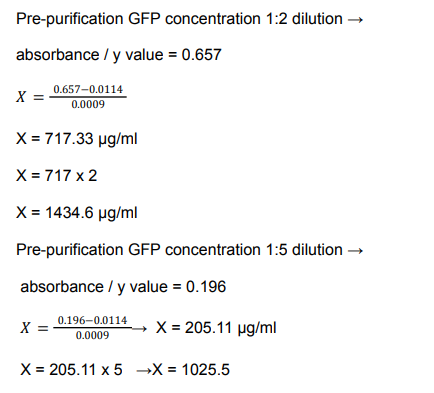
**10**

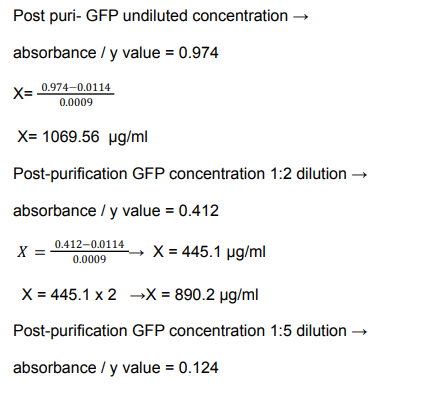
**LEGEND:**

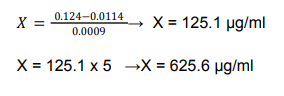
(1)10 µL Kaleidoscope standard, (2) 10 µL heated pre-purification, (3) 10 µL heated post-purification, (4) 20 µL heated pre-purification, (5) 20 µL heated post-purification, (6) 20 µL Kaleidoscope standard, (7) 10 µL native pre-purification, (8) 10 µL native post-purification, (9) 20 µL native pre-purification, and (10) 20 µL native post-purification

PRE AND POST PURE GFP CONCENTRATION CALCULATIONS

The equation of the line from the standard calibration curve y = 0.0009x + 0.0114 was used to calculate for the concentrations of the pre and post pure GFP given their absorbance indicated in Table 2.

x = Protein concentration and y = absorbance at 595 nm.





**DISCUSSIONS**

The first part of this experiment was intended to observe the transformation of GFP to *E. coli* proteins upon the introduction of the pGLO plasmid. Upon confirmation that GFP was expressed to *E. coli* cells, protein purification was attempted and upon completion of the purification processes the concentration and the molecular weight was determined using Bradford protein assay and gel electrophoresis, respectively.

Table 1 of the results section summarizes the data and observation collected for the four plates of *E. coli* cells under different plate conditions. For the –DNA LB plate, a spread of the bacteria abundantly grew on the surface of the plate was observed which is expected because the media supports the growth of the *E. coli* bacteria. Upon addition of the antibacterial drug ampicillin to the plate labelled –DNA LB/amp, all the bacterial cells were killed due to the denaturation of *E. coli* protein by the ampicillin, hence, no growth was observed in this plate. For the +DNA plates, an ample amount of pGLO plasmid was introduced into the bacterial cells. To observe if there were *E. coli* cells that caught the pGLO plasmid, the condition of the agar plates were prepared accordingly. pGLO plasmid contains an antibacterial, specifically ampicillin, resistant component which would allow the cells to thrive even in the presence of ampicillin. In the +DNA LB/amp plate, there were several individual *E. coli* colonies that grew on the plates. Therefore, these cells have caught the pGLO plasmid which is responsible to the antibacterial property. The plate containing +DNA LB/amp/ara was also able to grow *E. coli* colonies and for this plate the colonies fluoresce under the influence of the UV light. This is due to the presence of the trigger switch (arabinose sugar) that allows transformed *E. coli* proteins to glow. Hence, from this portion of the experiment, transformation of the *E. coli* protein using green fluorescence protein from the pGLO plasmid is a success. From this, the transformation efficiency or the capacity of *E. coli* cells to absorb foreign DNA such as the DNA from the pGLO plasmid was calculated and was found to be 1152.5 cells/ µg.

The second step for this experiment is the purification of the transformed protein (fluorescent) which grew on +DNA LB/amp/ara plate. During the purification step, Hydrophobic Interaction Chromatography (HIC) separates the protein components based on their hydrophobic property. This would be a good separation technique because GFP is strongly hydrophobic which can be easily separated from other proteins. High levels of salt prevent binding of molecules to hydrophobic molecules, which is why the column was cleansed first with a buffer with a high salt content. This would facilitate the washing of all hydrophobic proteins collected in the collection tube. The subsequent washings were made with a buffer with decreasing salt contents. The last wash was performed using a zero-salt buffer Tris-EDTA, which makes the stored GFP in the third collection tube easier to wash. On this portion of the HIC, we now have the purified GFP as evidenced by the fluorescing substance under UV light.

Bradford protein assay allowed us to calculate the protein concentration based on its absorbance measured using spectrophotometry. For this to become possible, a series of dilution of protein standards were prepared to create a calibration curve which produced a linear equation y = 0.0009x + 0.0114. Various protein solutions were prepared and have absorbances (and concentrations) which include the pre-pure GFP (1:2) 0.657 (717.33 µg/ml), pre-pure GFP (1:5) 0.196 (205.11 µg/ml), post-pure GFP undiluted 0.974 (1069.56 µg/ml), post-pure GFP (1:2) 0.412 (225.11 µg/ml), post-pure GFP (1:5) and 0.124 (125.11 µg/ml). It can be noted in the calculated concentrations that the undiluted purified protein has a lower concentration compared to the undiluted un-purified protein. This is because purification processes separated all other proteins present and only retain the protein of interest which is the GFP. The best dilution to use for other analysis is the 1:2 dilution because as more diluted the sample is the greater the error in analysis might be encountered.

Electrophoresis was used to separate proteins based on their molecular weight. Proteins that are lighter are able to migrate faster on the gel and the heavier ones migrate slower. The proteins that did not go through purification process contains more bands such as those seen in figure 3 on samples 2, 4, 7, and 9 compared to the purified protein such as the sample numbers 3, 5, 8, and 10. One good property of GFP is that is a robust protein which is partially denatured. Even after denaturation, the partially denatured protein is still visible under UV light and gel electrophoresis can help in the UV visualization process (Bird et. al, 2015). Figure 4 shows, however, that the partially denatured proteins did not show any fluorescence activity which means that all proteins were totally denatured including the protein responsible for the fluorescence activity. Denaturation process destroys the protein’s secondary and tertiary structure. On the other hand, the pre and post purification proteins that were not heated showed fluorescence under the influence of UV light and as shown in figure 4 the protein for samples 7,8, 9, and 10 that fluoresce lie on the same band. The weight of the protein can be measured in relation to the weights of the standard ladder as also seen in figure 4. Based on the given weight of the standard, the weight of the protein can be estimated to fall between 25 KD and 37 KD – approximately 33 KD.

**CONCLUSION**

This experiment was a success in general since the students were able to observe the expression of GFP (isolated from the new species of starfish) in the *E. coli* cells as evidenced by the 136 individual colonies that grew on +DNA LB/amp/ara plate which fluoresce under UV light. Using Bradford protein assay and the plotted standard calibration curve with equation of the line y = 0.0009x – 0.0114, the concentrations for un-purified and purified proteins (diluted and undiluted) were determined. The molecular weight of the GFP protein was determined with the help of gel electrophoresis and UV light. The fluorescent protein band was estimated to have a molecular weight of about 33 KD. Even though the processes made were tedious, the established experimental design was able to help achieve all positive results from this experiment.

**REFERENCES**

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