Chapter 6
scFv Expression, Purification and Identification

6.1 Materials

pCYN2 B10 scFv plasmid was obtained as a gift sample from Fox Chase Cancer Center, Pennsylvania, USA. *E.coli* DH5α and BL21 (DE3) cultures were obtained as a gift from department of Microbiology and Biotechnology, The Maharaja Sayajirao University of Baroda, Vadodara, India. Agar powder and Luria broth (LB) were purchased from H.B. Chemicals, Vadodara, India. QuantiPro™ BCA protein assay kit, Bovine Serum Albumin, Isopropyl β-D-1-thiogalactopyranoside (IPTG), Protease Inhibitor Cocktail, Agarose, Ethidium Bromide, Bromophenol Blue, Calcium Chloride, Magnesium Chloride, Sodium Dodecyl Sulphate (SDS), Glucose, EDTA, Tris Chloride, Sodium Hydroxide, Potassium Acetate, Sodium Bicarbonate, Sodium Carbonate, Glycerol and Glacial Acetic Acid were of molecular biology grade and was purchased from Sigma-Aldrich, St-Louis, USA. Digestion enzyme Xho I and digestion buffer 'V1' was obtained from Fermentas International Inc., Canada. HIS-Select™ Spin Column was purchased from Sigma-Aldrich, St-Louis, USA. Cellulose dialysis tubing (Molecular weight cut of 12-14000 Da) were purchased from HiMedia Lab., Mumbai, India. Ethanol, Methanol, Hydrochloric acid (HCl) etc. and all other HPLC or molecular biology grade solvents were purchased from Merck, Germany. 96 well plates were purchased from Ghanshyam Trading Co., Vadodara, India.

6.2 pDNA Transformation Method

Calcium chloride method was used for bacterial transformations as the treatment of bacterial cells with chilled solution of calcium chloride and subsequent heat shock could cause transfection with bacetriophage-λ DNA (1). Same method was used by researchers to transform bacteria with plasmid DNA and *E.coli* chromosomal DNA (2). Chilling of bacterial cells in the presence of Ca^{2+} (divalent cations) helps cell membrane become permeable to plasmid DNA and treatment induces a transitory state of competence in the recipient bacteria, during which they are able to take up pDNAs derived from a variety of sources. In present research similar method of transformation was utilized for the transfection of pCYN2 B10 scFv in *E.coli* cells for pDNA isolation and protein expression. In this experiment, pCYN2 B10 scFv was transformed into *E.coli* strain DH5α for identification and into *E.coli* strain BL21 (DE3) for protein expression.

Protocol:

$CaCl_2$ method for transformation of pCYN2 B10 scFv into *E.coli* strains {DH5α and BL21 (DE3)}

1. Freshly sterilized Luria broth (LB) medium (5-10 mL) was inoculated with *E.coli* strains and incubated overnight on shaker incubator at 37°C.
2. Newly grown bacterial culture was used to inoculate 50 mL of sterilized LB and incubated on shaker incubator for 3-4 hrs to grow upto mid log phase.
3. After 3-4 hrs, mid log phase grown culture was centrifuged at 4000 rpm for 4 min at 4°C. Resultant pellet was treated with 1 mL of chilled 0.1 M $MgCl_2$, kept aside for 10 min and again centrifuged at 4000 rpm for 4 min at 4°C. Supernatant was discarded and pellet was collected.
4. Thereafter, 1 mL of chilled 0.1 M $CaCl_2$ was added to it and allowed to react for 10 min. Suspension was centrifuged at 4000 rpm, 4°C for 4 min and the culture was maintained in 100 µl of 0.1 M $CaCl_2$ in ice bath for 45 min.
5. pDNA was added to above cells product and resultant composition was incubated in ice bath for 45 min.
6. $CaCl_2$ treated pDNA was then subjected to heat shock at 42°C for 90 sec. and preserved in ice for 2 min.
7. Thereafter, 1 mL of LB was added and incubated for 1 hr at 30°C. Suspension was centrifuged and pellet was suspended in 100 µl of LB, mixed well and the content was streaked on antibiotic containing LB-agar medium (to allow identification of plasmid-containing colonies) with the help of sterile streaking loop.
8. Plates were incubated overnight at 37°C and single colony from the streaked plate was picked up and streaked again on the antibiotic-containing LB-agar medium so as to get the purified form pDNA of transformed cells. This single colony was further grown in liquid media to collect pDNA.

6.3 Plasmid preparation using alkaline lysis method for isolation of pDNA

6.3.1 Preparation of reagents

Following reagents were prepared for the isolation of pDNA (3).

Table 6. 1 Composition of different alkaline Lysis solutions

Reagent	Composition
Alkaline Lysis I	50 mM glucose + 25 mM Tris.Cl (pH = 8.0) + 10mM EDTA (pH = 8.0)
Alkaline Lysis II	0.2 N NaOH + 1% SDS (Prepare just before use)
Alkaline Lysis III	5M Potassium Acetate (60 mL) + Glacial Acetic Acid (11.5 mL) + Water (28.5 mL)

Table 6. 2 Preparation of stock solutions

Reagent	Method
Preparation of 1 M Glucose	9.01 gm of glucose was weighed accurately and dissolved in 40 mL water, the final volume was made to 50 mL.
Preparation of 1M Tris Cl	6.055 gm of Tris base was dissolved in 40 mL of water, pH was adjusted to the desired value using concentrated HCl and final volume was made upto 50 mL using deionized water. (E.g. for pH 2.1 mL of concentrated HCl was required for 50 mL).
Preparation of 0.5 M Di-Sodium Ethylene Diaminetetraacetate (EDTA) (pH 8.0)	9.34 gm of EDTA· $2H_2O$ was suspended in 40 mL of water and stirred vigorously on magnetic stirrer. pH of the solution was adjusted to 8.0 using sodium hydroxide to solubilize EDTA. It was then dispensed into aliquots and sterilized by autoclaving.
Preparation of 5 M Potassium Acetate	Potassium acetate (4.908 gm) was dissolved in 40 mL of milli Q water and final volume was made upto 50 mL. It was then sterilized by Autoclaving. The resulting solution was 3 M with respect to potassium and 5M with respect to acetate.
Preparation of RNAse	Pancreatic RNAse at a concentration of 10 mg/mL was added in 0.01 M potassium acetate (pH 5.2). This mixture was heated to 100°C for 15 min and allowed to cool slowly to room temperature (RT). pH of the solution was adjusted using 1M Tris Cl (pH 7.4) and dispensed into aliquots. Aliquots were stored at -20°C.
Preparation of Ampicillin	The stock solution of ampicillin (100 mg/mL in autoclaved water) was prepared and sterilized by filtration using 0.22 µ

antibiotic solution	membrane filter. Stock solution was then stored at -20° C. Amount of ampicillin required was transferred in a way to give final concentration of 100 µg/mL depending upon the volume of LB medium and LB-agar medium.

6.3.1.1 Preparation of Luria Broth (LB) medium

2 gm of LB was added in 100 mL deionized water and sterilized by autoclaving. After cooling to RT, calculated quantity of Ampicillin was added aseptically and stored at RT.

6.3.1.2 Preparation of LB agar plates

2 gm of LB and 3 gm of nutrient agar was added in 80 mL of deionized water, final volume was made to 100 mL and LB medium was sterilized by autoclaving. Mixture was then cooled to around 50-55 °C and 100 µl of Ampicillin (Stock 100 mg/mL) was added to it aseptically. Required volume of medium was transferred to sterile petri plates aseptically and allowed to solidify. Plates were then sealed with parafilm and stored at 4 ºC until used.

6.3.2 Alkaline lysis method for isolation of plasmid DNA (pDNA)

In 1979, Brinboim and Doly developed the alkaline lysis method of plasmid isolation. Alkaline lysis is a method used in molecular biology, to isolate plasmid DNA or other cell components such as proteins by breaking the cells. Bacteria containing the plasmid of interest is first grown, and then allowed to lyse with an alkaline lysis buffer consisting of a detergent sodium dodecyl sulfate (SDS) and a strong base sodium hydroxide (NaOH). SDS denatures bacterial proteins, and NaOH denatures chromosomal and plasmid DNA. The detergent cleaves the phospholipid bilayer of membrane and the alkali denatures the proteins which are involved in maintaining the structure of the cell membrane. The mixture is neutralized with potassium acetate, causing the covalently closed plasmid DNA to reanneal rapidly. Most of the chromosomal DNA and bacterial proteins precipitate as does the SDS, which forms a complex with potassium and are removed by centrifugation. The reannealed plasmid DNA from the supernatant can be recovered by precipitation using isopropanol or ethanol.

Protocol:

1. 50 mL LB medium was inoculated with pDNA containing *E.coli* strain and incubated overnight at 37 ºC.
2. Overnight grown culture was centrifuged for 5 min at 6000 rpm and pellet obtained was washed with double distilled autoclaved water.
3. The mixture was centrifuged again for 10 min at 6000 rpm and obtained pellets was suspended in 1.8 mL of Lysis I solution, mixed properly and incubated for 3 min on ice.
4. After the incubation period, 4.0 mL of freshly prepared Lysis II solution was added, mixed gently and allowed to stand for 1-2 min. Tube was closed tightly and the content was mixed by rapidly inverting the tube five times. (Note: Make sure that the entire surface of tubes comes in contact with solution II. **Do not VORTEX**)
5. Thereafter, 2.0 mL of Lysis III solution was added and vortexed in inverted position for 10 seconds to disperse solution III throughout the viscous bacterial lysate and incubated for 15-20 min in ice.
6. Above mixture was subjected to centrifugation for 10 min at 15000 rpm, supernatant was collected in a fresh tube, discarding cellular debris.
7. To the supernatant, equal volume of phenol: chloroform (1:1) was added, vortexed and centrifuged at 15000 rpm for 10 min. Upper phase was removed and equal volume of chloroform was added, again vortexed and centrifuged for 5 min at 15000 rpm.
8. To the supernatant 1 mL of ice-cold 70% EtOH was added and allowed to precipitate pDNA. Resultant solution was centrifuged and pellet was air dried. 20 µl of double distilled water or TE was added to dissolve the pellet.
9. 2ul RNase (10mg/mL) was added to above mixture and incubated for 20 minutes at room temperature to remove RNA.
10. pDNA was further analyzed by 1 % gel electrophoresis using 2-5 µl of pDNA.

6.4 Restriction analysis of small-scale preparations of plasmid DNA

Restriction enzymes that can be used to determine pCYN2 B10 scFv leading to different size fragments as shown in **Table 6.3**. Restriction endonucleases recognize short DNA sequences and cleave double-stranded DNA at specific sites within or adjacent to the recognition sequence into discrete fragments. In this study a number of independently transformed bacterial colonies were picked up and grown in small scale

cultures. pDNA was isolated, purified as explained previously and obtained pDNA was digested by restriction enzyme (RE) Xho I RE. Identification of digested plasmid was carried out on agarose gel (1 %) electrophoresis (2). Composition used for digestion study is given in Table 6.4. System was incubated at 37°C in water bath for 3 h and after incubation total amount of the digested pDNA was analysed on 1 % agarose gel electrophoresis in comparison with the λDNA ladder. Map of pCYN2 B10 scFv shown in **Figure 6.1**.

Table 6. 3 Fragment size and REs for pCYN2 B10 scFv

REs	Fragment (size)
Nco I + Xho I	Vector- (3.2 kb pCYN2) + Insert (0.850 kb scFv)
Nco I, Xho I Single digest	4.05 kb linearized plasmid

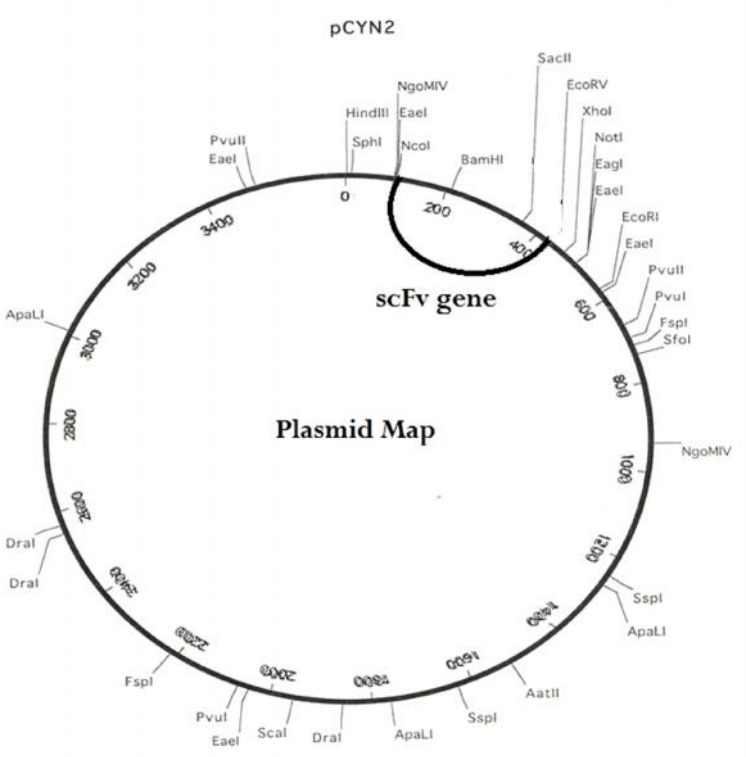

Figure 6. 1 pCYN2 B10 scFv

Table 6. 4 Composition for digestion studies

Component	Quantity (µl)
pDNA	2.0
RE Xho I	0.5
Digestion Buffer	2.0
BSA	2.0
Double Distilled Water	17.5
Total system	**20.0**

6.5 Agarose Gel Electrophoresis (AGE)

Agarose gel electrophoresis is a most commonly used method to separate DNA or RNA molecules according to their sizes.

Principle: Negatively charged nucleic acid molecules move through an agarose matrix when an electric field is applied. Shorter molecules move and migrate faster than longer ones. Thus, the process allows the separation of large and small fragments of pDNA. Molecular weight marker which is a mixture of known molecular weight DNAs helps to estimate size of DNA fragment (3). For effective separation of DNA of different sizes the agarose with different concentration given in **Table 6.5.**

Table 6. 5 Concentration of agarose in gel for effective separation of linear pDNA

Amount of Agarose in Gel (%)	Effective range of resolution of linear DNA fragment (kb)
0.5	1-30
0.7	0.8-12
1.0	0.5-10
1.2	0.4-7
1.5	0.2-3

6.5.1 Preparation of reagents

Composition of reagents used in AGE is given in **Table 6.6.**

Table 6. 6 Reagents for AGE

Buffer	Concentrated Stock Solution per Liter	Working Solution
50 x Tris- Acetate (TAE) – electrophoresis buffer	**50x stock solution:** 242 gm Tris Base, 57.1 mL glacial acetic acid, 37.2 g EDTA (pH = 8) in 1000 mL water	**1x working reagent** : 40 mM Tris acetate ; 2 mM EDTA
Ethidium bromide solution	**1000x stock:** 50 mg in 100 mL water	5 µg/mL
6x Buffer -gel loading buffer	0.25% (w/v) bromophenol blue + 0.25% (w/v) xylene cyanol + 30% (v/v) glycerol was prepared in autoclaved deionized water. Stored at 4◦C	

6.5.2 AGE Protocol

1. A clean, dry agarose gel cast was sealed to form a mold in gel loading tray and a comb was placed in the notches to form complete wells after solidification of poured agarose gel.
2. 1X TAE (electrophoresis buffer) was prepared and weighed quantity of agarose was dissolved in required volume of 1X TAE to form 1 % of agarose gel.
3. Neck of the flask was loosely packed with cotton plug and the slurry was heated in a microwave oven to dissolve agarose grains. Agarose gel was preserved at RT for further use.
4. Every time solid mass of agarose gel was melted in microwave oven, amount required to fill gel cast so as to form a gel thickness of around 3-5 mm was measured, ethidium bromide (from stock solution of 10 mg/mL in water to a final concentration of 0.5 µg/mL and mix thoroughly) was added to it and molten mass was then poured into gel cast.
5. After complete solidification of gel (35-40 minutes at RT), the comb was removed carefully and the gel was placed in the electrophoresis tank.
6. Electrophoresis tank was filled with the TAE buffer to cover the gel. A small quantity of pDNA (2-5µl) was mixed with the equal amount of gel loading buffer on parafilm using pipette and the mixture was slowly loaded into wells of the gel.
7. The electrophoresis unit was then closed with the lid and electrical voltage 100-150 was applied so that the DNA will migrate toward the anode (red lead).

(Note: If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis) and within few minutes, the bromophenol blue should start migrating from the wells into the body of the gel).

8. The gel was run until the bromophenol blue migrated the appropriate distance through the gel. (**Note:** During electrophoresis the ethidium bromide migrates towards the cathode in the direction opposite to that of DNA). Once the marker color was seen near the end of the gel (may be around 5 mm before the end of gel), the electric current was turned off and lead was removed.
9. The gel was taken from the electrophoresis tank, examined Gel Doc™ XR system (Bio-RAD, CA, USA) and picture was captured using gel documentation system (3).

6.6 Protein expression

Protein expression is a subcomponent of gene expression and refers to the way in which proteins are synthesized, modified and regulated in living organisms. Consisting of the stages after DNA has been translated into poly peptide chains, which gets folded into proteins. Protein expression is very common phenomenon used in the biomedical research to measure the presence and abundance of one or more proteins in a particular cell or tissue. Schematic representation of protein synthesis shown in **Figure 6.2.**

It is usually done in living host systems such as *E.coli* which is one of the most widely used bacterial host system for the production of heterologous proteins. Express usable amounts of protein, requirement of very short time to generate an overexpressing strain, cheap to grow, better characterization of its genetics and physiology are the advantages of bacterial host system hence, *E.coli* is a choice of living host for expression of commercially important proteins. Proteins are normally expressed in their soluble form. However, many times, they are also present in the form of insoluble recombinant proteins and are called as inclusion bodies (3).

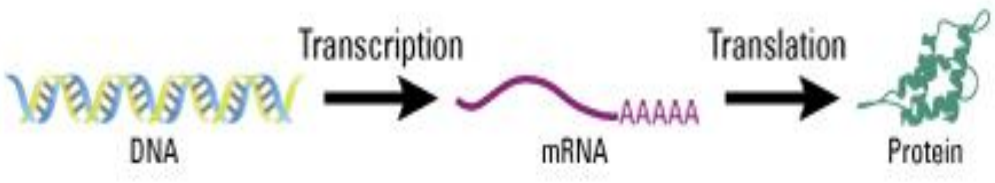

Figure 6. 2 Schematic representation of protein synthesis

Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Chemical formula: $C_9H_{18}O_5S$, Mol Wt.: 238.3) is a molecular mimic of allolactose (a lactose metabolite) that triggers the transcription of *lac operon*. Advantage of IPTG as inducer is that the presence of Sulphur (S) atom creates a chemical bond which is non-hydrolyzable by the cell, preventing the cell from "eating up" or degrading the inductant; therefore the IPTG concentration remains constant. IPTG induces the transcription of the gene coding for β-galactosidase, an enzyme that promotes lactose utilization, by binding and inhibiting the Lac 1 repressor. In cloning experiments, the *lacZ* gene is replaced with the gene of interest and IPTG is then used to induce gene expression. Many regulatory elements of the *lac* operon are used in inducible recombinant protein systems; IPTG is an effective inducer in the concentration range of 100 μM to 1.5 mM.

Protease Inhibitors: Crude cell extracts contain a number of endogenous enzymes, such as proteases and phosphatases, which are capable of degrading the proteins present in the extract. The best way to improve the yield of intact proteins is to add protease inhibitors for enzymes those are known to be present in the cell lysate. Thus**,** Protease inhibitors are the substances used to prevent degradation of protein and is added after induction and before cell lysis to prevent protein fragmentation. In this experiment protease inhibitor cocktail was used, which is a mixture of protease inhibitors with a broad specificity for the inhibition of serine, cysteine, and thermolysin like proteases and aminopeptidases. One mL of cocktail solution is recommended for the inhibition of endogenous enzymes found in 100 mL lysate from 20 g (wet weight) of *E coli* cells (4).

6.6.1 Preparation of reagents

6.6.1.1 Preparation of separating (resolving) and stacking gel

Stacking and separating gels were prepared as per the compositions given in the **Table 6.7.** All the ingredients required for the preparation of stacking and separating gel were mixed properly. Plates were assembled for the preparation of SDS PAGE. Just prior to pouring into the plates, APS and TEMED was added into stacking and separating gel mixtures, vortexed and poured into the plates. Plates filled with the respective gels were kept aside for the polymerization of gel to form a firm gel.

Table 6. 7 Composition of separating gel and stacking gel

Chemicals	12 % Separating Gel Mix	5 % Stacking Gel Mix
30% Acrylamide-bisacrylamide Solution	6 mL	1.3 mL
Distilled water	3 mL	5.1 mL
2.5X Tris-SDS Buffer (pH 8.8)	6 mL	---
5X Tris-SDS Buffer (pH 6.8)	---	1.6 mL
10% APS Solution	125 µl	75 µl
TEMED	7.5 µl	15 µl

6.6.1.2 Preparation of various solutions, buffers and reagents

Various solutions, buffers and reagents which are useful for the protein expression and purification were prepared as per the composition given in the **Table 6.8** and used during the experiments.

Table 6. 8 Compositions of buffers and reagents for protein expression & purification

Name of Solution	Procedure
10 % SDS	10% stock was prepared in de-ionized water and stored at RT
IPTG 100 mM Solution	0.238 gm of IPTG was dissolved in 10 mL milli Q water and sterilized by 0.22 µm disposable filter unit in a sterile tube under aseptic condition. Dispensed in 1 mL eppendorf and stored at -20ºC
1M Imidazole	Accurately weighed 0.681 gm of imidazole was dissolved in 10 mL of deionized water.
1M Sodium Chloride	5.85 gm sodium chloride was dissolved in 100 mL of deionised water
200 mM Tris. HCl	2.422 gm of Tris base was dissolved in 100 mL of deionised water
Tris Glycine Electrophoresis Buffer/ (5x Tank Buffer)	6.04 gm of Tris base (25mM) and 28.8 gm of glycine (192mM) was dissolved in 1800 mL of de-ionized water. 20 mL of SDS solution (10% w/v) was added and final volume

	was adjusted to 2000 mL with de-ionized water.
2x SDS-PAGE sample loading buffer	Tris Cl (pH 6.8) – 2.5 mL, β-mercaptroethanol – 0.2 mL (0.2% final), SDS (electrophoresis grade) – 0.4 g (4 % final), Bromophenol blue – 0.1 mg (0.001 % final), Glycerol – 2.0 mL (20% final) were dissolved in deionized water and volume was made upto 10 mL. Stored in 1 mL aliquots at −70°C
Preparation of 30% Acrylamide solution	A stock solution containing 29% w/v acrylamide and 1% w/v N, N' methylene bisacrylamide was prepared in deionized warm water (to assist the dissolution of bisacrylamide) and stored at 2-8ºC.
10% Ammonium per Sulphate (APS)	1.0 gm of APS was transferred to 8 mL of de-ionized water and the final volume was made to 10 mL.
1.5 M Tris Cl, pH 8.8	18.15 gm of Tris Cl was added in 80 mL of water, pH was adjusted to 8.8 by adding HCl and the volume was made to 100 mL with de-ionized water.
1.5 M Tris Cl, pH 6.8	6.05 gm of Tris Cl was added in 80 mL of water, pH was adjusted to 6.8 by adding HCL and final volume was made to 100 mL with de-ionized water.
Staining solution (Coomassie Brilliant Blue)	0.025 gm of Coomassie Brilliant Blue R-250 was dissolved in 100 mL of the methanol: acetic acid: water mixture (40:10:50) by stirring on a magnetic stirrer for about 3 hours and solution was filtered through whatman filter to remove any particulate matter.
Destaining Solution I	Methanol: Acetic acid: Double Distilled Water (50: 10: 40 v/v)
Destaining Solution II	Methanol: Acetic acid: Double Distilled Water (7: 9: 84 v/v)
Equilibration Buffer for purification	50 mM sodium phosphate with 0.3 M sodium chloride, pH 8.0
Wash Buffer for purification	50 mM sodium phosphate with 0.3 M sodium chloride and 5 mM imidazole, pH 8.0
Elution Buffer for	50 mM sodium phosphate with 0.3 M sodium chloride and

purification	250 mM imidazole, pH 8.0
Periplasmic extraction buffer	30 mM Tris-HCl pH 8, 20 % sucrose, 1mM EDTA
Osmotic shock buffer	5mM MgSO4
DNase stock	5 µg/mL stock solution prepared in deionised water
RNase stock	1 mg/mL stock solution prepared in deionised water
Saline solution	0.9 % sodium chloride in deionised water

6.6.2 Transformation of pCYN2 B10 scFv in *E.coli* BL21 (DE3)

Transformation of pCYN2 B10 scFv in *E.coli* BL21 DE3 was carried out using $CaCl_2$ method as per the protocol given in the **Section 6.2.1.**

6.6.3 Protocol for Induction, Expression and Extraction of scFv protein from BL21 (DE3) *E.coli*

1. 3 % LB/2% glucose/ 100µg/mL ampicillin inoculated with a single colony from a freshly transformed BL21 (DE3) plate and incubated at 30° C in shaking incubator; 250 rpm overnight.
2. Overnight culture was diluted 1/100 in 3 % LB / 0.1% glucose/ 100 µg/mL ampicillin. (For each liter of broth inoculate with 10 mL of the overnight culture).
3. This was incubated at 37ºC, 250 rpm until the OD 600 is 0.8- 1.0. Do not exceed 1.0. (It takes approx. 2-2.5 h if started with room temp broth).
4. IPTG was added to a final concentration of 0.5mM and Incubated at 30° C, 250 rpm for 5 hr. (**Note:** This timing can be altered depending on the protein, some are fine for 5 hr at 30 degrees, and some are better ON at 15 degrees).
5. Cultures were placed on ice and swirl to cool. Everything was kept at 4° C from this point.
6. Bacteria pelleted by centrifugation at 6000 rpm for 20 min at 4° C.
7. Pellet was resuspended in (20 mL/L of culture) periplasmic extraction buffer. Protease inhibitor were included in both the periplasmic and osmotic shock buffers and were added at the time of use. This was kept on ice for 20 mins prior to centrifugation.
8. Periplasmic extraction was centrifuged at 13000 rpm, 30 mins at 4 degrees and supernatant was preserved on ice.

9. Bacterial pellet was resuspended in (10 mL/L of culture) and kept on ice for 20 min, centrifuged at 13000 rpm and supernatant was preserved.
10. Periplasmic/osmotic shock extractions were combined and centrifuged at 18000 rpm for 30 min. Supernatant was dialyzed against PBS, O/N at 4 degrees.
11. Dialyzed samples were purified by using His Select Spin Columns.

6.6.4 Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS PAGE)

Principle: To separate different protein molecules of different shapes and sizes, they first have to be denatured so that the proteins no longer have any secondary, tertiary or quaternary structure. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone. SDS denatures all the proteins to their respective primary structure. SDS confers a negative charge to the polypeptide in proportion to its length.

Electrophoresis is used to separate complex mixtures of proteins (e.g., from cells, subcellular fractions, column fractions, or immunoprecipitates) to investigate subunit compositions, and to verify homogeneity of protein samples. Almost all analytical electrophoresis of protein is carried out using poly acrylamide gels under conditions that ensure dissociation of the proteins into their individual poly peptide sub-units and that minimize aggregation. Polyacrylamide gels are composed of chains of polymerized acrylamide that are cross linked by a bifunctional agent such as N, N' – methylene bisacrylamide. The effective range of separation of SDS-PAGE depends on the concentration of polyacrylamide used to cast the gel and on the amount of cross linking. Polymerization of acrylamide in the absence of cross linking agents generates viscous solutions having no practical value in the preparation of gels. Cross linking between acrylamide and bisacrylamide add rigidity and tensile strength to the gel and form pores through which the SDS-polypeptide complex passes. The size of these pores decreases as the bisacylamide: acrylamide ratio increases, reaching a minimum when the ratio is approximately 1:20. Most SDS- polyacrylamide gels are cast with a molar ratio of bisacrylamide: acrylamide of 1:29 which has been shown empirically to be capable of resolving poly peptide that differ in size by as little as 3%. Thus, sieving properties of the gels are determined by the size of the pores which is a function of the absolute concentration of acylamide and bisacrylamide used to cast the gel. SDS PAGE flow chart shown in **Figure 6.3.**

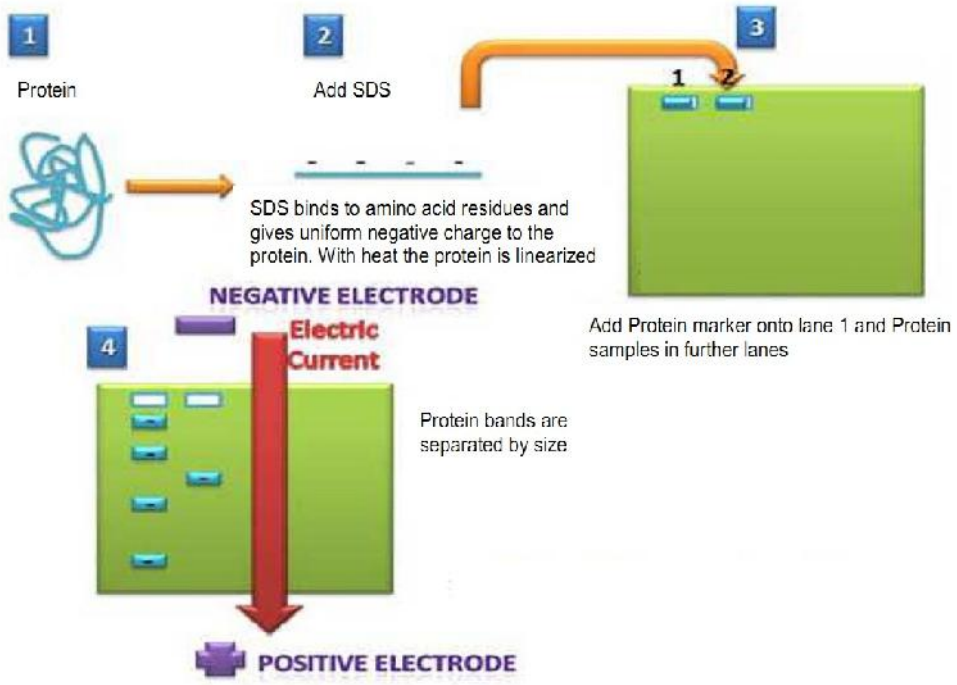

Figure 6. 3 SDS PAGE Flowchart.

Most commonly, the stronger anionic detergent SDS in combination with a reducing agent and heat is used to dissociate the proteins before they are loaded onto the gel. The denatured poly-peptides bind to SDS and become negatively charged. Because the amount of SDS bound is always proportional to the molecular weight of poly peptide and is independent of its sequence, SDS-poly-peptide complex migrate through poly acrylamide gels in accordance with the size of poly peptide. In polyacrylamide gel electrophoresis, proteins migrate in response to an electrical field through pores in the gel matrix; pore size decreases with higher acrylamide concentrations. The combination of gel pore size and protein charge, size, and shape determines the migration rate of the protein. At saturation, approximately 1.4 g of SDS is bound per gram of poly peptide. By using marker of known molecular weight protein, it is therefore possible to estimate the molecular weight of the polypeptide chain (3).

In most cases, SDS-PAGE is carried out with discontinuous buffer system in which the buffer in the reservoir is of a different pH and ionic strength than the buffer used to cast gel. The SDS-poly peptide complexes in the sample that is applied to the gel are swept along by a moving boundary created when electric current is passed between electrodes. After migrating through a stacking gel of high porosity, the

complexes are deposited in a low porosity zone of the resolving gel. The ability of discontinuous buffer systems to concentrate the entire sample into a very small volume greatly increases the resolution of SDS-polyacrylamide gels.

Discontinuous buffer system that is most widely used was originally devised by Ornstein (1964). The sample and stacking gel is made up of Tris.Cl buffer (pH 6.8), whereas resolving gel contains Tris. Cl (pH 8.8). Reservoir buffer contain Tris-glycine of pH 8.3, which is used to fill upper and lower chamber of the SDS PAGE unit. All components of the system contain 0.1% SDS. The chloride ions in the sample and stacking gel forms the leading edge of the moving boundary, and the trailing edge is composed of glycine molecules. Between leading and trailing edges of boundary is a zone of lower conductivity and steeper voltage of gradient which sweeps the poly peptides from the sample and deposits them on the surface of the resolving gel. The higher pH of the resolving gel favours the ionization of glycine, and the resulting glycine ions migrate through the stacked poly peptides and travel through the resolving gel immediately behind chloride ions. Freed from the resolving boundary, the SDS-poly peptide complexes move through the resolving gel in a zone of uniform voltage and pH and are separated according to size by sieving across the resolving gel.

6.6.4.1 Protocol for SDS-PAGE

1. Electrophoresis unit was assembled such that the glass plates are clamped to the unit along with the spacers placed in-between them at two vertical edges.
2. 1% agarose was prepared by dissolving 0.05 g of agarose in 5 mL of distilled water. Solution was heated to dissolve the agarose and a thin horizontal layer at the lower edge of the plates was added to seal the assembly. This was allowed to solidify by cooling it for 5-10 minutes.
3. 12% separating gel was prepared by adding the components as per mentioned in **Table 6.8** and gel was poured in-between the plates and allowed to solidify for an hour. Immediately after the gel was poured, distilled water was added to level the gel.
4. After an hour water was poured off by inverting the casting assembly.
5. 5% stacking gel was prepared by adding the components as per mentioned in **Table 6.8.** After addition of TEMED all the components were mixed gently by swirling the beaker. Stacking gel was poured on top of the separating gel and immediately the comb was placed avoiding air bubbles and allowed to solidify for 30 minutes.

(**Note:** Acrylamide is a potential neurotoxin and should be treated with great care. Always wear a face mask and use gloves.)

6. 1X Tris-Glycine-SDS gel running buffer was poured in the unit such that the buffer connects the two electrodes, and hence completes the flow of current. Comb was removed from the stacking gel carefully.
7. Sample Preparation: 4-6 tubes were taken for different protein samples and 1 tube for protein marker and labelled respectively. 20 µl of each sample was taken in the respective tube and 5 µl of 2X sample loading buffer was added to it. 3 µl of protein marker was taken in the respective tube and 4 µl of 5X sample loading buffer and 13 µl of Phosphate buffered saline was added. Tubes containing protein samples were boiled at 100^{o}C in a boiling water bath. (**Note:** Do not boil the tube containing Protein Marker.)
8. 20 µl of the samples were loaded immediately after the heat treatment in the wells created by the comb in the stacking gel.
9. Power cords were connected to the electrophoretic power supply according to the conventions: Red-Anode and Black- Cathode. Electrophoresis was carried out at 100 volts and 10 mA until dye front reaches 0.5 cm above the sealing gel.
10. The gel was carefully removed from in-between the plates using spatula into the plastic tray containing distilled water and washed for 1 minute. Water was discarded and staining destaining procedure was performed on resultant gel.
11. The orientation of the gel was marked by cutting a corner from the bottom of the gel that was close to the leftmost well. (**Note:** do not cut the corner from gels that are to be used for western blotting).

6.6.4.2 Protocol for staining and destaining of gel

1. After removing water, 50 mL of staining solution was added in the tray containing gel, till the bands become visible. Sometimes the gel may have to be kept overnight in the staining solution for visualization of the bands.
2. Gel was removed from the staining solution and the gel was washed by rinsing with distilled water till a considerable amount of stain leaches out from the gel. Distilled water was changed for 3-4 times.
3. 50 mL destaining solution was added to above gel and destaining was carried out with constant moderate shaking.
4. Destaining was continued till clear, distinct bands were observed.

5. Gel was removed from the destaining solution. Gel image was taken by using Gel Doc™ system (Bio-Rad USA) using SDS PAGE Protocol.

6.6.5 Protein Purification using HIS-Select™ spin column

The HIS-Select Spin Column is an immobilized metal-ion affinity chromatography (IMAC) product that allows rapid purification of small-scale crude cell extracts containing histidine tagged proteins. The HIS-Select Spin Column contains 20 mm spherical silica particles (100 nm pore size) with a hydrophilic layer. The silica is derivatized with a proprietary quadridentate chelate charged with nickel. HIS-Select Spin Columns are selective for recombinant proteins with histidine tags and exhibit very low non-specific binding of other proteins. The selectivity can be modulated with the inclusion of imidazole during chromatography. The binding capacity of a HIS-Select Spin Column is >500 µg per column as determined with an ~30 kDa histidine tagged protein recovered under high levels of protein expression. Protein purification was carried out using HIS-Select™ spin column as per manufacturer's protocol (5). The equilibration and wash buffer were supplemented with 1–10 mM imidazole and 0.15–0.5 M sodium chloride to reduce non-specific protein binding. Due to the unique selectivity of the chelate, 5 mM imidazole in the wash buffer is sufficient to obtain high purity samples (5).

6.6.5.1 Protocol for protein purification

Protein purification was performed as per manufacturer's protocol (5).

1. 500 µl of equilibration buffer was added to the spin columns and were closed with the help of collection tube lid.
2. Tubes were subjected to centrifugation at 2,000 rpm at room temperature for ~2 minutes. (**Note:** HIS-Select Spin Columns may also be used with an appropriate vacuum manifold in place of the centrifugation step).
3. Spin columns were removed from collection tube.
4. Collection tubes were emptied and spin column was placed back in the same collection tube.
5. The prepared cell extract was loaded on the column 500 µl of extract at one time (The column capacity up to 600 µl of extract at one time) and centrifuged as given in step 3.

6. Spin columns were removed from collection tubes and the flow-through was saved for later analysis.
7. Using a new collection tube, unbound protein was washed from the spin column using 500 µl of wash buffer with centrifugation. Collection tubes were emptied and samples were collected.
8. Washing step was repeated three times with 500 µl of wash buffer.
9. Using new collection tube, the targeted protein was eluted using up to 500 µl of elution buffer by using centrifugation as given in the step 3.
10. Samples were collected and analyzed for protein using BCA protein assay kit, QuantiPro BCA Reagent and SDS-PAGE.

6.7 Quantitative Protein Estimation

Protein Assay based on bicinchoninic acid (BCA) is a most sensitive and detergent-compatible method for the colorimetric detection and quantitation of total protein. This method is a combination of well-known biuret reaction, the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium and the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) with reagent containing bicinchoninic acid. The purple coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm (6).

6.7.1 Preparation of BSA Standards

Powder in the standard vial tapped to the bottom and 5 mg BSA dissolved in the 1 mL of water containing 0.05-0.1 % sodium azide. Preparation of diluted BSA standards given in **Table 6.9.**

Table 6. 9 Preparation of diluted BSA standards

Volume of the BSA Solution	Volume of Diluent (µL)	Final BSA Concentration (µg/mL)	Sample Name
300 µL of Stock	1200	1000	A
375 µL of A	125	750	B
250 µL of A	250	500	C
125 µL of A	375	250	D
75 µL of A	425	150	E
50 µL of A	450	100	F
25 µL of A	475	50	G
12.5 µL of A	487.5	25	H
6.25 µL of A	493.75	12.5	I

6.7.2 Preparation of BSA Working Reagent (BWR)

BWR prepared by mixing 50 parts of Reagent A (Contains sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartarate in 0.1 M sodium hydroxide) with 1 part of Reagent B (Copper Sulphate Solution). When Reagent A added initially to Reagent B turbidity was observed that quickly disappears upon mixing to yield a clear green BWR. Each test tube sample to be done require 2.0 mL of the BWR while the microwell plate samples require only 200 µL.

6.7.3 Assay Protocol

1. 0.2 mL of each standard and unknown sample was pipetted into appropriately labeled test tube and 0.2 mL diluent was used for blank tubes.
2. 2 mL BWR was added into each tube and mixed well.
3. Tubes were incubated at 60°C for 30 min as per mentioned in enhanced protocol (Assay Range 6.25 to 1000 µg/mL) or 30°C for 60 min as per normal protocol (Assay Range 25 to 500 µg/mL)
4. After incubation all tubes were cooled at room temperature and absorbance was measured at 562 nm (A-562) of each tube vs. water reference.
5. Average A-562 reading of the blanks subtracted from the Average A-562 reading of the standard and unknown samples.

6. Standard curve was prepared by plotting the average blank corrected A-562 reading for each BSA standard vs. its concentration in µg/mL and by using this standard curve, protein concentration for each unknown sample was determined.

6.9 Results and Discussion

6.9.1 Transformation and pDNA isolation

Transformation of pDNA was successfully carried out in *E.coli* DH5α (for determination of pDNA) and BL 21(DE3) (for protein expression). When transformed cells in *E.coli* DH5α and BL 21(DE3) subjected to single colony purification, easily identifiable single colonies of pDNA transformed cells were observed on antibiotic containing LB-Agar medium plates shown in **Figure 6.4** and **Figure 6.5** respectively.

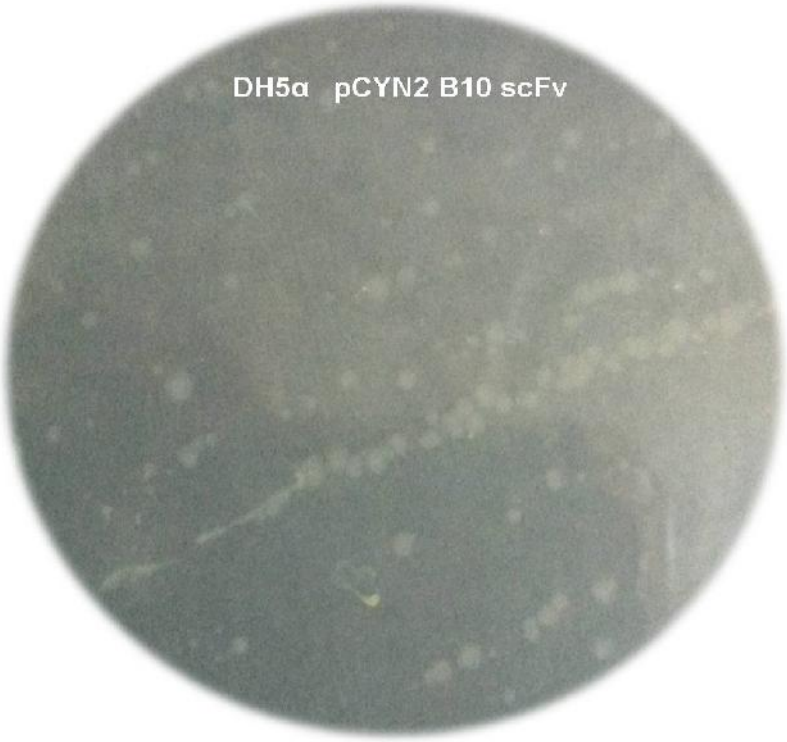

Figure 6. 4 Transformed cells in *E.coli* DH5α

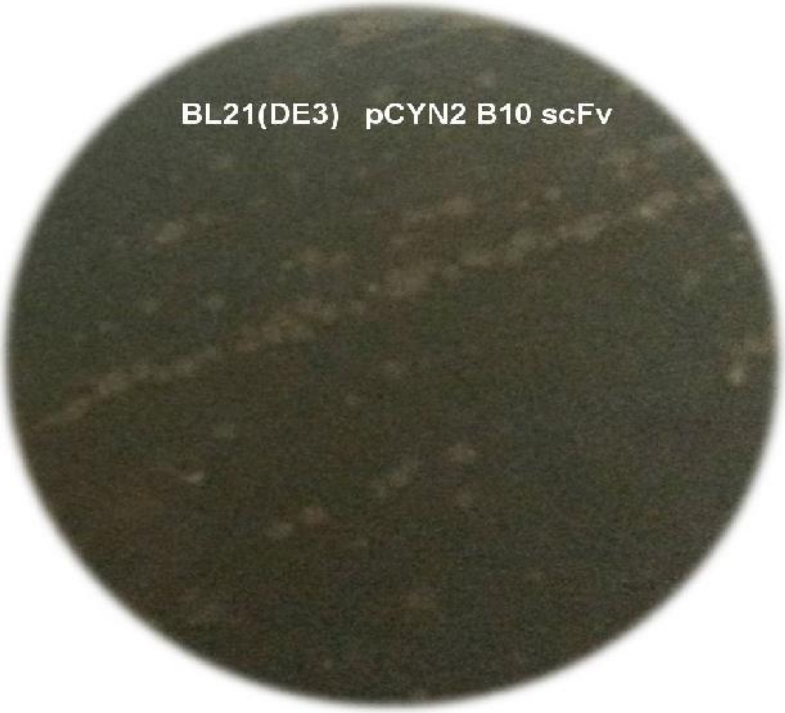

Figure 6. 5 Transformed cells in *E.coli* BL21 (DE3)

pDNA was successfully transformed and carefully isolated from the transformed cells using alkaline lysis method. Purified pDNA was further confirmed by the restriction enzyme digestion studies. After single digestion studies, a single band of 4.05 kilo base pairs was observed on the agarose gel indicating that the DNA was cut using Xho I and digested the pCYN2 B10 scFv leading to linear fragment of the said pDNA. **Figure 6.6** shows the results of Xho I single digestion of pDNA in comparison to molecular marker. The relative distance travelled by linearized plasmid DNA after digestion studies was compared with standard DNA ladder and results of the AGE confirmed that the size of linearized plasmid DNA under study was around 4.0 kilo base pair.

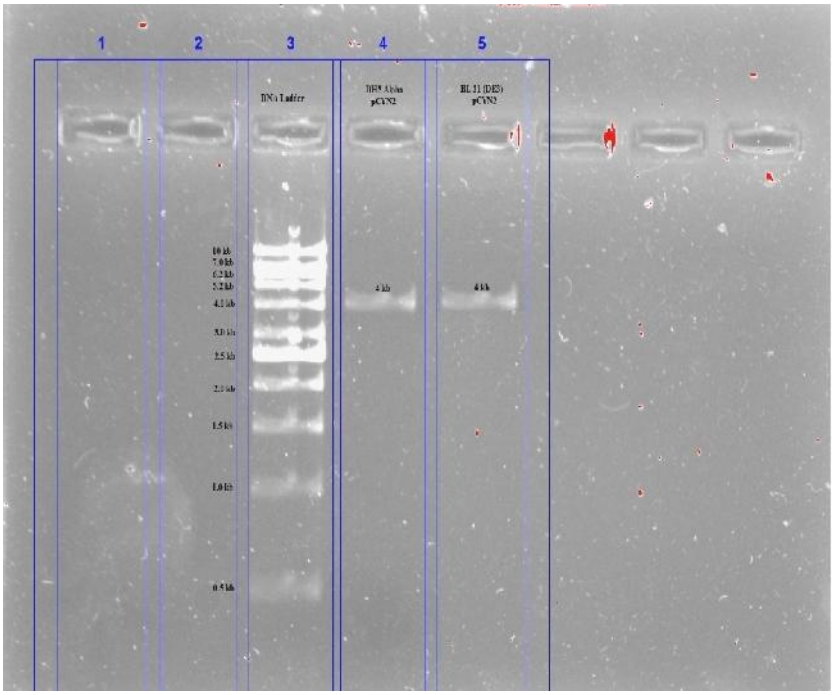

Figure 6. 6 pDNA (pCYN2 B10 scFv) by Xho I digestion

Lane 3 **–** Marker ladder, Lane 4 **–** pDNA in DH5α, Lane 5 **–** pDNA in BL 21 (DE3)

6.9.2 Protein Expression and Purification

Transformation of pDNA was successfully carried out in *E.coli* BL21 (DE3) (for protein expression). Transformed cells were subjected to single colony purification and easily identifiable single colonies of scFv pDNA transformed cells were observed on antibiotic containing LB-Agar medium. pDNA was carefully isolated from the transformed cells using alkaline lysis method. After alkaline lysis experiment, pDNA obtained was further used for protein expression studies. Protein expression study was successfully carried out in *E.coli* BL21 (DE3) strain. Expression of scFv was clearly observed after induction of scFv using IPTG shown in **Figure 6.7.** Induction was carried out at 30°C. The culture was then pelleted down and processed further for SDS-PAGE analysis. Results of the SDS PAGE studies clearly indicated induction of scFv in the cell lysate under the optimized conditions. Lane 4 and 6 clearly shows highly expressed band of scFv at 25 kDa (when compared to protein marker (PM). A band of 25 kDa was observed between the PM band of 20 and 29 kDa. However, no induction of expression was seen in uninduced scFv (Lane 3 and 5). While Lane 2 clearly indicated the purified form of scFv protein. Purification of histidine tagged protein was

done by using HIS-Select Spin Columns. This indicated that IPTG was helpful to induce the expression of 25 kDa scFv protein.

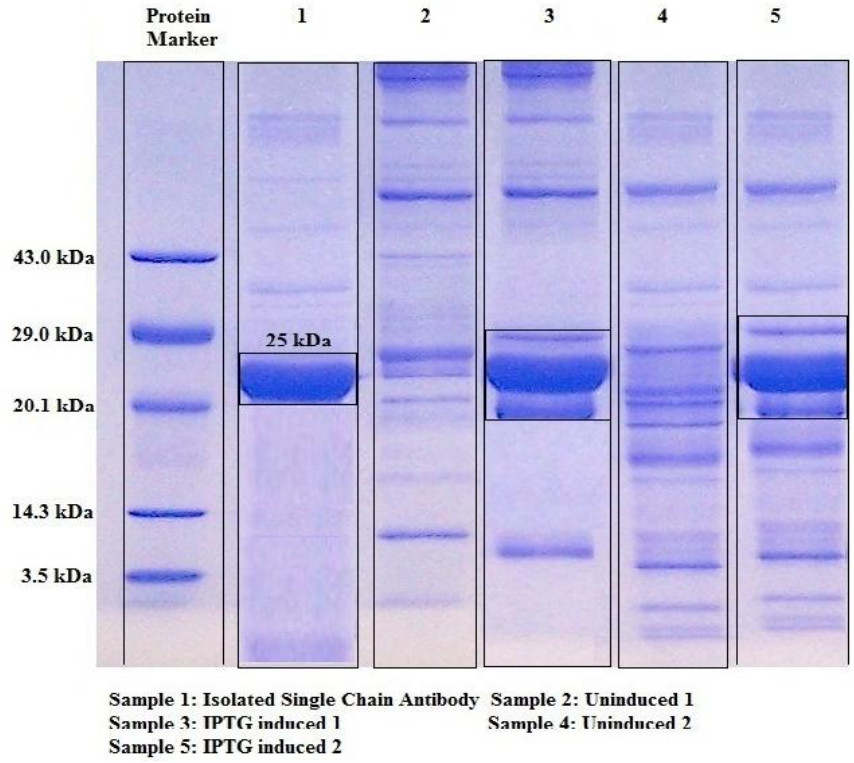

Figure 6. 7 Protein expression in cell lysate and purified scFv band on SDS PAGE Lane 1- Protein Marker Lane 2 –Purified scFv Lane 3 and 5- scFv uninduced (without IPTG); Lane 4 and 6 –scFv induced (with IPTG)

6.9.3 Quantification of Protein

Purified scFv was quantified by using BCA protein assay kit and the yield of the protein was found to be 1311.5 µg/mL isolated from 1000 mL bacterial culture calculated from the BCA standard curve that is already mentioned in **Section 3.7**.

6.9.4 Colour developent during BCA protein estimation

The color difference between protein samples before and after incubation during BCA protein estimation test shown in **Figure 6.8** and **Figure 6.9**.

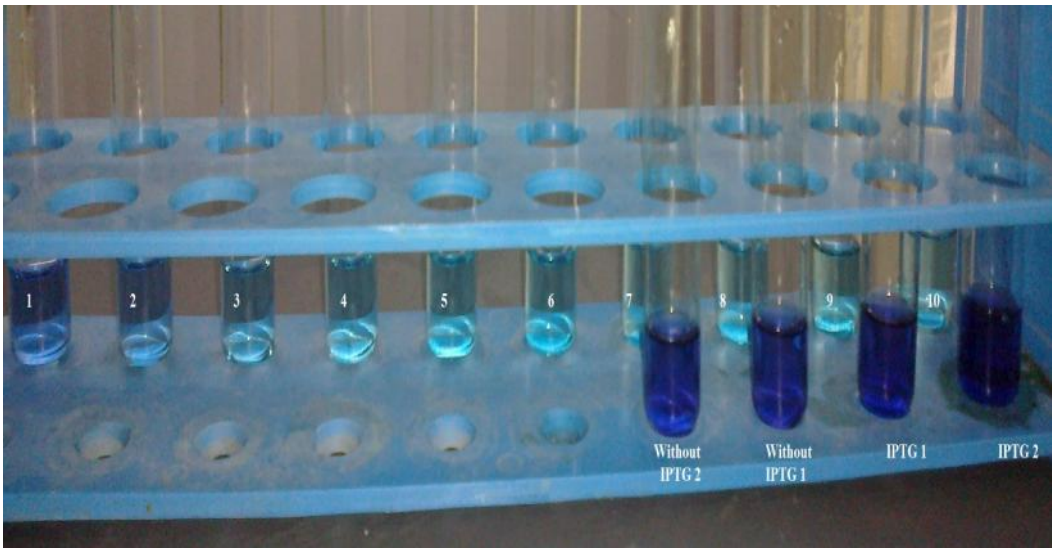

Figure 6. 8 Color of protein samples before incubation during BCA protein estimation test

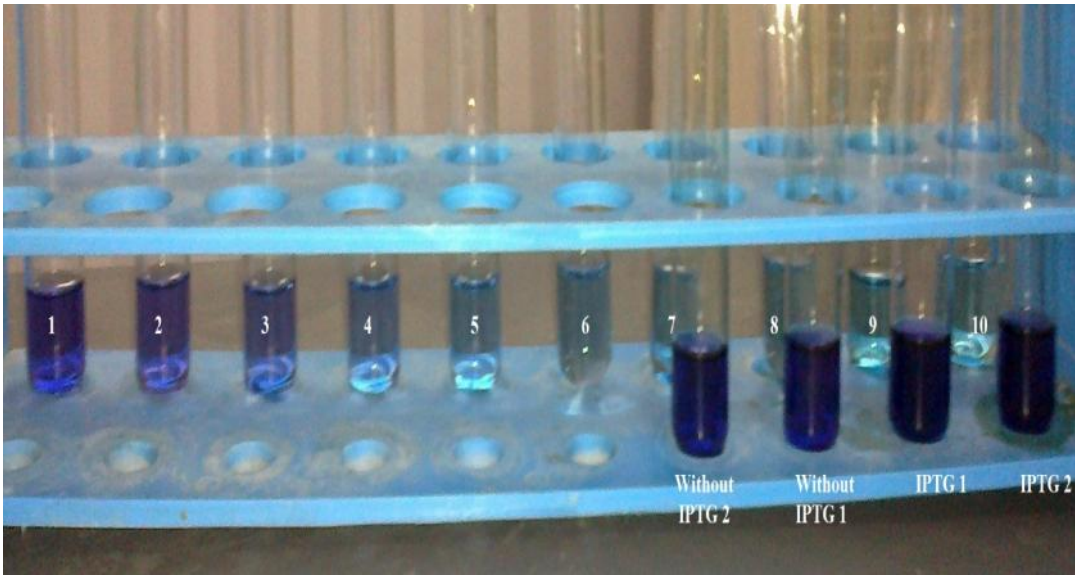

Figure 6. 9 Color of protein samples after incubation during BCA protein estimation test

6.10 References

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