EFFECT OF FREEZE AND THAW CYCLE AND INCUBATION PERIOD ON THE SOLUBILISATION OF INCLUSION BODY PROTEIN

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Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering

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Dedicated to my family and my friends.

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ABSTRACT

Overexpression of recombinant protein in bacteria result in the formation of inactive protein. These inactive proteins associate forming insoluble protein aggregates which is referring to inclusion bodies (IBs). Generally, IBs are pure and the aggregated protein inside it has native-like secondary structure which is a bioactive protein. To recover the insoluble and active protein is a major problem encountered. Solubilisation does play a crucial role by unfolded the protein and thus help it to refold properly so that functional bioactive protein can be recovered. Example of mild solubilisation method using low concentration of urea and combine with freeze and thaw method has been proven to increase the efficiency of the recovering of bioactive protein. For freeze and thaw process there are factors that affect the overall process which are freezing incubation period and number of process cycle. Incubation period affect the process by determining the amount of stress needed to be applied so that unfolding process can occur. Number of cycle does affect the protein stability in terms of the occurrence of protein degradation probability. Thus the objective of this research is to investigate effect of freeze and thaw cycle an incubation period on the solubilisation of IBs. For incubation period the experiment was conducted between 1 to 4 days whereas for freeze and thaw cycle experiment conducted between cycle 1 to 4. Moreover, the performance of these two parameters were analysed using native-polyacrylamide gel electrophoresis (n-PAGE) to determine the functional for enhance green fluorescent protein (EGFP) amount and Bradford assay to determine the total protein amount. In this study, incubation period did affected the performance of solubilisation rate in which the IBs being solubilised and then proceed for refolding process and has been proven achieved active form of EGFP. For number of process cycle, it did not affect the solubilisation rate on determining the amount of functional EGFP recovered.

Keywords : Inclusion bodies, solubilisation, freeze and thaw, incubation period, number of cycle

ABSTRAK

Ekspresi protein rekombinan di bakteria menyebabkan terjadinya inaktif protein. Inaktif protein ini akan bergumpal dan membentuk gumpalan protein yang tidak larut, di mana dikenalpasti sebagai badan inklusi (IBs). Secara general, IBs adalah asli dan akumulasi protein di dalamnya mengandungi struktur sekunder yang dikenali sebagai natif protein di mana ia adalah aktif protein. Salah satu masalah yang dihadapi ialah apabila untuk mendapatkan semula inaktif protein yang tidak larut. Demikian ini, cara untuk mengatasi masalah tersebut adalah melalui kaedah penglarutan. Penglarutan memainkan peranan penting di mana ia membuka ikatan protein yang bergumbal dan membentuk ikatan yang sempurna untuk menghasilkan perotein yang berfungsi. Penglarutan sederhana dengan menggunakan kaedah urea yang berkepekatan rendah dan digabung dengan kaedah pembekuan dan pencairan dibuktikan dapat meningkatkan kadar effisien bagi mendapatkan protein yang aktif. Terdapat faktor yang mempengaruhi proses pembekuan dan pencairan iaitu kadar pembekuan masa dan bilangan kitaran proses. Kadar pembekuan masa mempengaruhi proses tersebut dengan menentukan daya yang diperlukan untuk membuka ikatan protein terakumulasi. Bilangan kitaran proses menentukan kestabilan protein dari aspek kebarangkalian degradasi protein. Objektif kajian ini adalah untuk mengkaji kesan kadar pembekuan masa dan bilangan kitaran proses terhadap penglarutan badan inklusi. Bagi kadar pembekuan, eksperimen dikaji antara julat 1 hari hingga 4 hari manakala bagi faktor bilangan kitaran proses, ekperiman dikaji pada kitaran 1 hingga 4. Dua faktor ini akan memberi impak terhadap prestasi kadar penglarutan. Dua factor ini akan dianalisis melalui natif polyacrylamide gel elektroforesis (n-PAGE) untuk menentukan bilangan EGFP yang berfungsi dan Bradford assay untuk menentukan jumlah protein. Justeru, melalui dua faktor yang mempengaruhi kaedah tersebut terbukti dapat menghasilkan protein yang berfungsi. Melalui kajian ini, kadar pembekuan masa mempengaruhi kadar penglarutan dimana IBs dilarutkan dan diteruskan untuk proses pengikatan semula dan dibuktikan bahawa EGFP aktif dapat dihasilkan. Bilangan kitaran proses mempengaruhi kadar penglarutan dengan menentukan bilangan EGFP yang berfungsi.

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LIST OF SYMBOLS

%	percentage
°C	Degree Celcius
Avg	Average
COV	Coefficient of Variation
g	gram
hr	hour
min	minute
mL	Milli Litre
Std	Standard Deviation
(v/v)	volume/volume
μg	Micro gram
μL	Micro litre

LIST OF ABBREVIATIONS

APS	Ammonium persulphate
BSA	Bovine Serum Albumin
CBB	Coomassie Brilliant Blue
EDTA	Ethylenediaminetetraacetic acid
E. coli	*Escherichia coli*
EGFP	Enhanced Green Flourescent Protein
HCl	Hydrochloric acid
IBs	Inclusion bodies
Inc.P	Incubation period
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
LB	Luria Bertani
N.of.cy	Number of cycle
n-PAGE	Native Polyacrylamide Gel Electrophoresis
OD	Optical Density
r-hGH	Human growth hormone
rpm	Rotation per minute
TEMED	Tetramethyl ethyldiamine

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

IBs are an aggregated protein that has been found in cytoplasm or periplasm of expression host which occur during high level of expression. Besides that, it is also a pure and insoluble protein. IBs form when the high concentration of polypeptide chain emerging from ribosome and thus lead to formation of partially folded or misfolded protein that occur in cytoplasm (Ventura, 2005). These intermediate proteins have the surface exposed to hydrophobic patches which will bring the protein to assemble together and form the IBs. Protein will functioning very well, if native secondary structure is maintained. When this aggregated protein is not properly folded, the native structure is disrupted. The IBs have its own advantage and disadvantage. The IBs become a nuisance factor for biotechnology and pharmaceutical industries. Abnormal protein aggregation can cause more than 20 different diseases in human being (Stefani and Dobson, 2008). Heterologous protein overexpression in *Escherichia coli (E.coli)* lead to protein accumulating in dense water insoluble aggregates. One of the example is expression of EGFP in *E.coli* contain only small amount of soluble protein whereas most of the protein is in insoluble particles (Tsumoto *et al*., 2003). EGFP has a very useful application in order to monitor folding on protein over expression. Over expression can be easily measured using fluorescent spectrometry (Tsumoto *et al*., 2003).

1.2 Motivation

The disadvantages of IBs have been monopolied by the recent studies. Nevertheless, this IBs can be view as a positive side in large scope. First of all it can be considered an advantage for basic research as for protein production. It is also play crucial role in biomedicine field and use as an alternative method to produce low cost proteins. In biomedicine field it can be used as naturally immobilized enzymes or as nanomaterials based on its specification as a pure recombinant protein (Garcia-Fruitos *et al*., 2009). IBs are very useful in biocatalysis process and provide innovative stage in industrial catalysis market (Roessl *et al*., 2010). Besides that, by understanding protein aggregation that occur in inclusion body we can discover strategies to control this process. IBs will be used as model to study insoluble protein deposits that lead to some complex human disease (Ramon *et al.,* 2014).

IBs as a source of almost pure protein (Ramon *et al.,* 2014). In order to obtain the native folded and active protein, solubilisation and refolding are the most crucial steps (Burgess, 2009). The effectiveness of solubilisation process will affect the refolding efficiency. Mild solubilisation is one of the method for recovery of bioactive proteins. Mild solubilisation method can preserve the existing native-like secondary structure during refolding and allow for higher recovery of bioactive form (Singh *et al.,* 2014*)*. This is because it will help the protein to fold properly by preventing the hydrophobic interactions and inhibit the molecules aggregation during refolding.

1.3 Problem Statement

Freeze and thaw method combined with low concentration of urea has been extensively studied to increase the efficiency of the solubilisation process (Strambini and Gabellieri, 1996). Freeze and thaw affect the protein stability in two different categories which are physical and chemical degradation. For physical degradation, freezing is a condition in which physical stress is applied by the formation of ice crystal hence applied several stresses for denaturing IBs. In terms of chemical degradation, freezing affect the environment of the buffer solutes which will result the change in buffer solution pH. Thus , it is very important to study the factors that affect the freeze and thaw method. The factors are pH buffer, rate of freezing and thawing, number of cycle and incubation period. pH change will affect the performance of the process(Cao *et al*.,2003) because it does affect the instability of the protein. Number of cycle does affect the protein stability in terms of the occurrence of protein degradation probability and thus indirectly determine the recovery efficiency of the functional protein. Incubation period affect the process by applying different amount of stress needed for certain protein to undergone denaturing. The amount of stress may irreversibly denature complex macromolecular structure, and could alter the protein stability. If too much of stress applied, the protein will degraded, if less of stress applied than the required stress needed, the protein will not be denatured. From this two condition the recovery of bioactive protein cannot be achieve because it does directly affect the solubilisation process, where the unfolding and refolding of the protein could not be established.

1. 4 Objective

The objective of this research is :

1) To investigate the effects of freezing incubation period and number of freeze and thaw cycle on solubilisation of enhanced green fluorescent protein inclusion bodies(EGFP IBs).

1.5 Scopes of Study

The following are the scopes of this research:

1. Producing EGFP IBs from recombinant *E. coli* cells

2. Study the two factors that affect freeze and thaw process
   - Incubation period (1 – 4 days)
   - Number of cycle (1 - 4 cycles)

3. Analyse the solubilisation performance using protein analyses as below:

   a. Native-polyacrylamide gel electrophoresis (n-PAGE) to determine functional EGFP amount.
   b. Bradford assay to determine total protein amount

CHAPTER 2

LITERATURE REVIEW

2.1 Enhanced Green Fluorescent Protein

2.1.1 Discovery of Enhanced Green Fluorescent Protein

The jellyfish *Aequorea victoria* is bioluminescent where it does produce light with the aid of chemical reactions that provide energy for photon emission and emits green light (Davenport and Nicol, 1955). Shimumora had completed the study of the active component bioluminescent and identified it as a protein named aequorin. Surprisingly, the light emission of purified aequorin peaked in the blue part of visible spectrum and supposedly this light emission should be distinctly green. Hence Shimomura and his colleagues isolated yet another protein discovering strong green fluorescence. Therefore, EGFP was discovered as a companion protein to aeqorin the famous chemiluminescent protein from *Aequorea* jellyfish that are slightly greenish in sunlight and greenish fluorescence in the ultraviolet of Mineralite (Shimomura *et al*., 1962). It contain high resolution of crystal structure that emitting internal fluorophore efficiently. The illustration of *Aequoreo victoria* jellyfish can be seen in Figure 2.1.

Figure 2.1: *Aequoreo victoria* jellyfish

2.1.2 Properties of Enhanced Green Fluorescent Protein

The highly fluorescent properties of the protein originally come from process of energy transfer of $Ca^{2+.}$ Ca^{2+} (blue light) is an activated photoprotein and also responsible to produce energy during oxidation. This highly fluorescent contain chromophore which specifically known as p-hydroxybenzylideneimidazolinone (Cody *et al*., 1993). It is formed from residues 65–67, which are Ser-Tyr-Gly in the native protein comprise of modified 238 amino acids within the polypeptide (Prasher *et al*., 1994). The diagram of chromophore structure is as shown in the Figure 2.2.

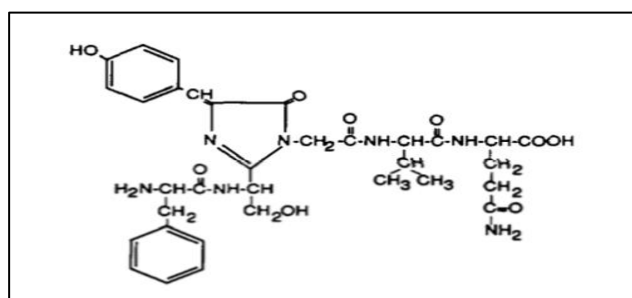

Figure 2.2: Fluorescent chromophore form by amino acid in the primary structure (adapted from Prasher *et al*.,1994).

EGFP has crystal structure which is an 11-stranded β-barrel threaded by an α-helix running up the axis of the cylinder (Yang *et al*., 1996). The chromophore is attached to the α-helix and buried at the centre of the cylinder known as (β-can). The illustration of the structure can be seen as in the Figure 2.3.

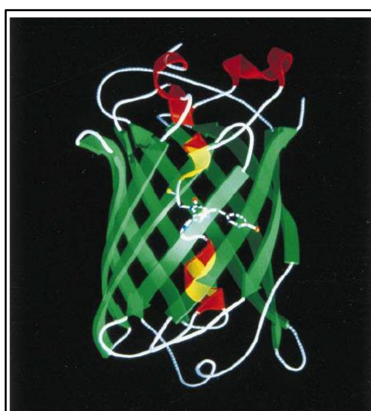

Figure 2.3: α-helix shape structure containing chromophore (adapted from Prasher *et al*.,1994)

2.1.3 Characteristic of Enhanced Green Fluorescent Protein

The fluorescent of EGFP can be measured by fluorescent spectrometry in order to monitor the protein folding (Tsumoto *et al* ., 2003).Excitation spectrum of EGFP fluorescent has a dominant maximum at 400nm whereas the emission spectrum has a sharp maximum about 505 nm (Tsien *et al*., 1998). Figure 2.3 showing the fluorescence excitation and emission spectra of native EGFP from *Aequoreo victoria*. There are a few factors that affect the fluorescence of EGFP, which are temperature, pH and also the availability of the oxygen. First and foremost, EGFP requires an oxygen to become fluorescent. However when it reach maturation, it does not require oxygen to become fluorescent. Other than that, EGFP when exposed to high temperature which is to be specific at 78°C, 50 % of fluorescent will be lost (Ward *et at*., 1982). pH affect the fluorescent by base and acidic treatment for example addition of guanidine hydrochloric acid will lead to the loss of fluorescence. This phenomena can be treat by applying neutralization process in which the denaturant guanidinine hydrochloric acid will be removed. Thus, the fluorescence of the protein can be attained to its original form. EGFP can be produced in both active and inactive IBs in *E.coli* expression system depending on the growth condition. Previous study has shown that expression of EGFP in *E.coli* contain only small amount of soluble protein whereas most of the protein is in insoluble particles (Tsumoto *et al*., 2003)

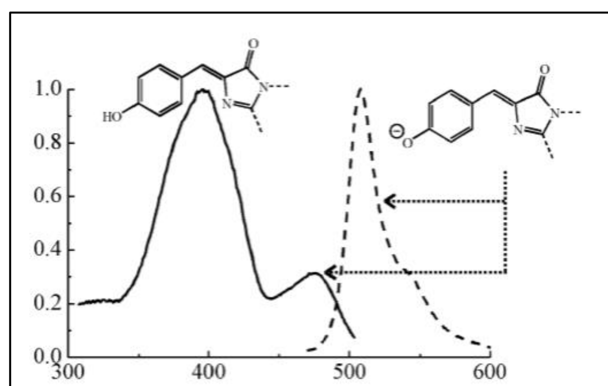

Figure 2.4: Fluorescence excitation(Tsien *et al* ., 1998)

2.1.4 Application of Enhanced Green Fluorescent Protein

The speciality of EGFP that has two photon adsorption, and a unique property which form chromophore of three amino acids within its primary structure make it applicable to be use as data storage, diagnostic photochemical application, fusion tag, and gene marker. The most common use of EGFP is to monitor the location, movement and chemical reactions involving proteins expressed. As a reporter gene, EGFP indicate the level of gene expression in cell by measuring the intensity of the fluorescence (Chalfie *et al*., 1994). In fusion tag application, EGFP visualize dynamic cellular event by monitoring protein localization. To be precise in biology field, when EGFP was fused with cellular protein, made it possible to directly study biology of protein due to its ability to fold. Thus, this mechanism make it possible to reveal wealth data, including information steady state distribution and dynamic history. Chalfie et al. (1994) reported EGFP used as an intrinsic intracellular reporter in *E. coli* and *Caenorhabditis elegans*. EGFP can be used as a transcriptional probe for monitoring non-product information such as pH, oxygen, temperature, and nutrient availability in bioprocess technology March *et al.,* (2003).

The most widely used application of EGFP is fluorescence resonance energy transfer (FRET). FRET is a quantum-mechanical phenomenon which transfers energy from an excited donor fluorophore to another acceptor fluorophore, within 10-100 Å. Protein interactions can be investigated by using this method in vivo and in vitro since the efficiency of FRET is determined from the distance between fluorophores. During this application, EGFP acts as intracellular molecular sensor (Sakamoto et al., 2014). March et al. (2003) found that EGFP can be used as a transcriptional probe for monitoring non-product information such as pH, oxygen, temperature, and nutrient availability in bioprocess technology.

2.2 Inclusion Bodies Protein

2.2.1 Characteristic of Inclusion Bodies

Over expression of recombinant protein in bacteria result in the formation of inactive protein. Inactive protein associate forming IBs. IBs composed from network of partially folded protein inside in which the properly folded protein is trapped inside. IBs contain variable amount of natively folded protein or partially folded protein(Gonzalez *et al*., 2008). It can be found in cell like bacteria, yeast, and mammals (Tyedmers *et al*., 2010). It also contains pure and active protein and the protein inside it have extensive native-like secondary structure. IBs have characteristic as dense, large and apparently spherical or cylindrical particle that contained 80 to 95 % of heterologous expressed protein (Wang, 2009). Small heat shock proteins (IbpA and IbPA), chaperones (DnaK system), phospholipids from membrane and nucleic acid and other background proteins are found in IBs (Jurgen *et al*., 2010). Besides that, IBs has structure of cross-β which is similar to the structure that found in amyloid fibers. The illustration of amyloid structure can be seen as shown in Figure 2.5.

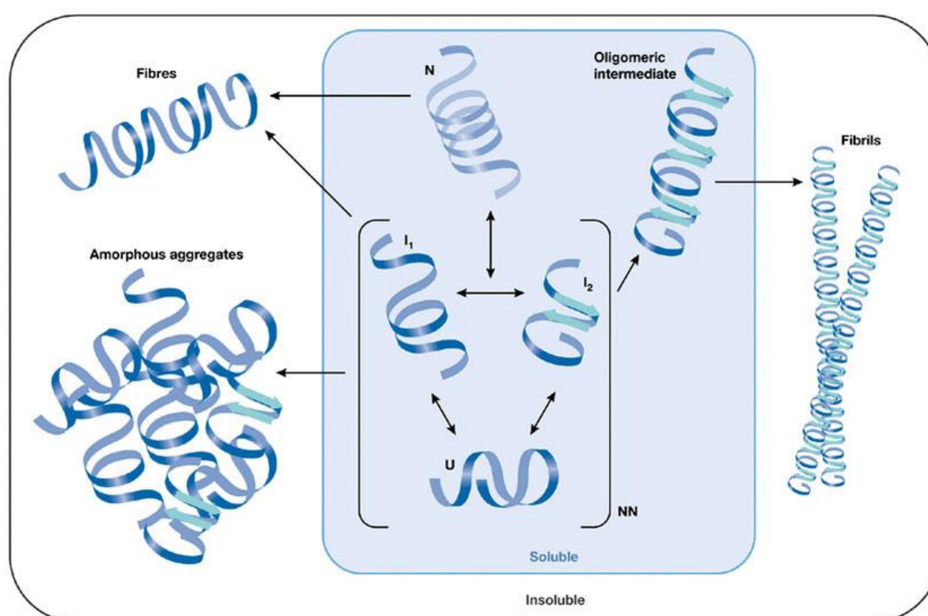

Figure 2.5: Amyloid fiber structure

2.2.2 Formation of Inclusion Bodies

First of all, formation of IBs can be categorized under physical induced and chemical induced. For physically induced, IBs are formed by result from unbalanced equilibrium between aggregated and soluble protein of *E. coli* (Villaverde *et al* .,2003). Aggregation in here is described as specific intermolecular interaction among single type of protein molecule (Speed *et al*., 1996). Intermolecular interaction will lead to accumulation of partially folded or misfolded expressed protein. The major condition that lead to the interaction is due to non-covalent hydrophobic bond or ionic interaction between the molecule. For chemically induced IBs are formed due to the result of disulphide bond formation and non-disulphide crosslink. Many of the chemical reaction can directly crosslink the protein and thus change the hydrophobicity of the protein. Example for disulphide bond formation when free cys residues in protein can be oxidized resulting disulphide linkage such as bFGF (Shahrokh *et al* ., 1994). Table 2.1 below showing the mechanism for IBs formation.

Table 2.1: Mechanism for IBs formation.(adapted from Wang, 2015)

Mechanism of formation	Type
Physical aggregation	Folding and unfolding intermediates (De Yong *et al*., 1993)
	Nucleation and growth of protein aggregates (Krishnamurthy and Manning, 2002)
	Reversibility and specificity of physical aggregation(Fink, 1998)
	Thermodynamics of protein aggregation (Patro and Pryzybycien, 1996)
Chemical induced aggregation	Disulfide bond formation
	Non-disulfide crosslinking pathways

For folding and unfolding intermediate protein, hydrophobicity of the protein play the crucial role. The concept of aggregation of protein begin with the presence of patches of hydrophobic groups that act as initiator. It does determined the correct folding of polypeptide chain into functional protein. Folding and unfolding intermediate protein is not stable and poorly populated and it composed of patches of contiguous hydrophobics

group that create the aggregation. In contrast for completely folded or unfolded protein do not aggregated easily. This is due to hydrophobic side chains are out of contact with water and it is scattered from each other (Uversky *et al* ., 1999).

For the second mechanism which is nucleation and growth of protein. There are two proposed models describing the formation of inclusion bodies as a consequence of the self assembly of non- native monomers into growing polymers of higher sizes. The model can be described by the schematic diagram below as shown in Figure 2.6 and there is diagram showing the illustration of the mechanism as shown in Figure 2.7.

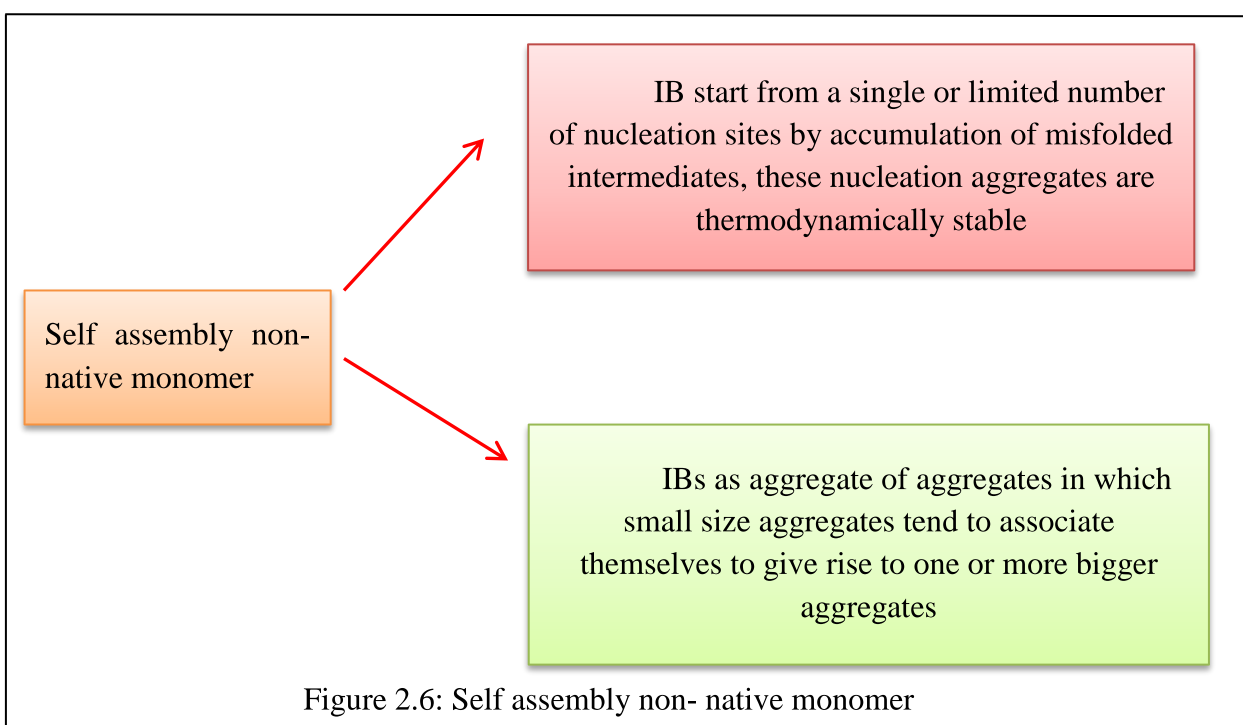

Figure 2.6: Self assembly non- native monomer

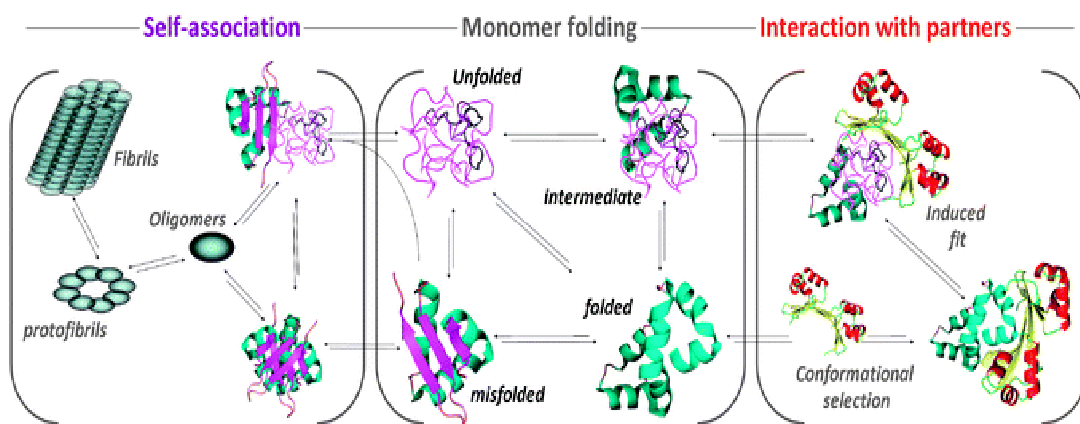

Figure 2.7: Diagram illustrating the mechanism formation of IBs

Next for reversibility of protein aggregation, it does depend on stage of aggregation. Mainly there are 2 stages of aggregation. During initial stage aggregation, formation of soluble aggregates may be reversible however during second stage formation of insoluble aggregates is irreversible. For example thermally induced protein aggregation is often irreversible, such as thrombin (Boctor and Mehta, 1992), recombinant pGH (Charman *et al*., 1993), recombinant human megakaryocyte growth and development factor(rhMGDF) (Narhi *et al*., 1999). Physical aggregation is a result of strong and non-specific protein-protein interactions (Durbin and Feher, 1996) . For example BSA aggregates easily because of the formation of incorrect intermolecular salt bridges (Giancola *et al*., 1997). Yet the highlighted issue here is that aggregation may occur by specific interaction of certain conformations of protein intermediates rather than by nonspecific interactions (Shin *et al*., 2002). This statement is supported from the evidence of the fibril growth of a sequence of *E. coli*. The protein was specific, as each peptide could be nucleated by fibrils of the same peptide but not by fibrils of closely related sequences (Jarrett and Lansbury, 1993).

For thermodynamic mechanism, free energy change associated with the protein aggregation process. When there is protein aggregation, it will led to increase in overall free energy of the system. This is due to the lost of certain number of monomer conformational and translational state.. Protein with low native energies tend to have a higher energetic barrier for aggregation (Istrail *et al* ., 1999). This condition will not help the aggregation process to take place.

Next for chemical induced aggregation, disulfide bond is form when free cys residue in protein undergone oxidation. This process will next initiate thion disulfide exchanges which will result in protein aggregation such as bFGF and β-galactosidase. Nevertheless, without free cys disulfide-bonded protein can still undergone aggregation through disulfide exchange β-elimination. This case is valid for lyophilized insulin during storage (Costantino *et al*., 1994). usually disulfide formation will cause of protein precipitation in solid state, but not always in liquid state. Covalent dimers may form in the protein from non-disulfide crosslinking bond. Insulin has been proven to form transamidated dimers.

2.2.3 Formation of Inclusion Bodies in *E.coli*

In bacterial cell, aggregation fall under category of self assembly type. Specifically, in *E.coli* the formation of IBs begin at the cytoplasm of the cell. At the cytoplasm , the protein are simultaneously synthesized on multiple location. Various transitional folding states of the target protein are formed, however some of the protein intermediates are failed to fold into native conformation. The failed protein will be degrade by the cells, while the others aggregate into smaller proto-aggregates (Schrodel and De Marco, 2005). Then the targeted recombinant protein is incorporated into proto-aggregates due to cross molecular stereoscopic interaction, hydrophobicity ,and exceeding the solubility limit. Protein precursors start to aggregate and then are glued together into single IBs. This IBs grow in the cell as a sphere until it reaches the cell wall then prolonged into cylinder shape. When the cell divide IBs stay at one side of the cell and grow further whereas the other cell remains empty. Illustration of this process can be seen from Figure 2.8.

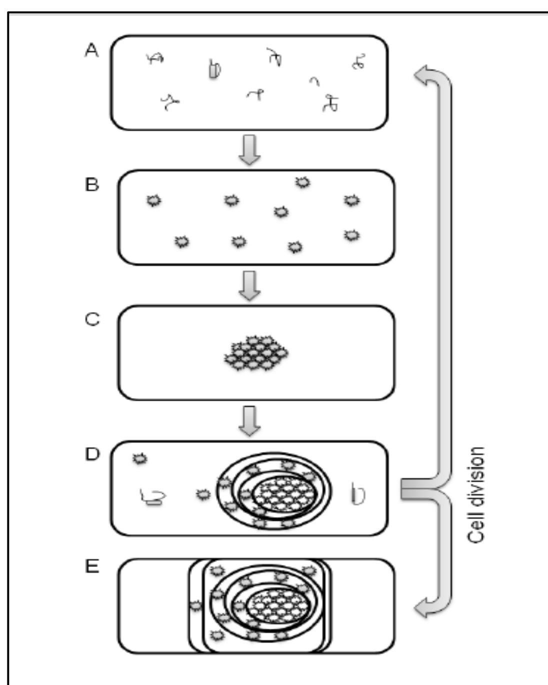

Figure 2.8: IBs formation in *E.coli* cell
(adapted from Petenel and Komel, 2011)

In order to have a better understanding of the whole concept, there is a must to understand the details process that occurring during the folding step. As mentioned above, during folding step some of the protein are failed to fold. Hence various

transitional folding intermediates protein are present in the cell with properly folded protein. Cells' quality control machinery maintains kinetic equilibrium between soluble and aggregated forms of the protein. Soluble fraction is composed of single protein molecules same like soluble aggregates. The properly folded proteins are also trapped inside the soluble aggregates. Soluble aggregates are further aggregated into insoluble aggregates known as IBs.

2.2.4 Protein Recovery From Inclusion Bodies

In industry and for research purpose , recombinant proteins are widely used and the need for their low-cost production is increasing. *E.coli* is one of the best known and most often used host organisms for economical protein production. As mention in previous section during over expression, protein aggregates which is IBs are formed. Formation of IBs remain as a big hurdle, as it is considered as deposits of inactive protein (Burgess, 2009). Fortunately, there is a lot of inisiative to produce the soluble protein in bacteria by altering the production process(Sorensen and Mortensen, 2005) co-expression of chaperones (De Marco *et al.*, 2005), by altering the target protein (pointmutations, fusion proteins) (Rinas *et al.,* 1992). In order to obtain pure bioactive protein, there are some procedure that must be established. The overall process for recovering the bioactive protein is illustrated in Figure 2.9. The procedure are isolation of purified inclusion bodies from *E.coli* cells, solubilisation of IBs, refolding of solubilised protein and purification. Based on the previous study there are two methods involve for purification of IB which are traditional method and novel method. For the traditional method there are four steps involve as mention above. Solubilisation and refolding are the most crucial step in order to obtain higher recovery of bioactive protein.

Isolation is the first step and it is a method in which the IBs are isolate from the bacterial cell and cell debris by using low speed of centrifugation. After centrifugation of the cell lysate and removal of supernatant, the IBs were contaminated by the cell envelope and the membrane cellular debris. During the isolation procedure, purification step is also including in this step. Purification in this step is by washing the IB pellet by using detergent containing buffer such as Triton X-100 which will be helping in removing the contaminants. Hence pure IB is obtained. The choice for cell disruption create a big impact on the protein quality (Ventura *et al.,* 2006). For this isolation

process, there are several lysis method involve. The list of the method for isolation can be seen in Table 2.2.

Table 2.2: List of lysis method (Rodriguez- Camona *et al*., 2010)

Lysis method	**Method uses**
Non mechanical	• Lysozyme • Non ionic detergent
Mechanical	• Homogenizer • French press • Sonication
Combined	• Sonication + homogenizer • Sonication + lysozyme • French press + lysozyme

The second step is solubilisation. In this step the aggregated protein are solubilised or denatured by used denaturant reagents (Fischer *et al.,* 1992). During this step the need to choose the solubilisation method is very important because it does affect the efficiency of bioactive protein produce. Basically there is two type of solubilisation which is conventional and mild solubilisation. This step is the most crucial because its determine the efficiency of the refolding rate.

Refolding is a process in which the solubilised protein are refolded back by removal of solubilisation agent. Higher refolding rate can be achieved by using refolding buffer (Fischer *et al.,* 1992). However there is some circumstance for this process which is the aggregation of the targeted protein and most of the folding method involve in used of certain additives to inhibit aggregation. Thus it is shown that to improve the yield of refolding rate common additives are used in refolding buffer . Table 2.3 showing the type of common additives used as refolding buffer.

Table 2.3: Common additives used in refolding buffer (Singh *et al.,* 2015)

Category additive	**Example of additive under certain category**
Chaotropes	Urea, guanidine hydrocholride
Amino acid	Glycine, Arginine, proline
Sugars and polyhydric alcohol	Sucrose, polyethylene glycol, sorbitol, glycerol
Others	Sulfobetaines, substituted pyridines and pyrolles, acid substituted aminocyclohexanes

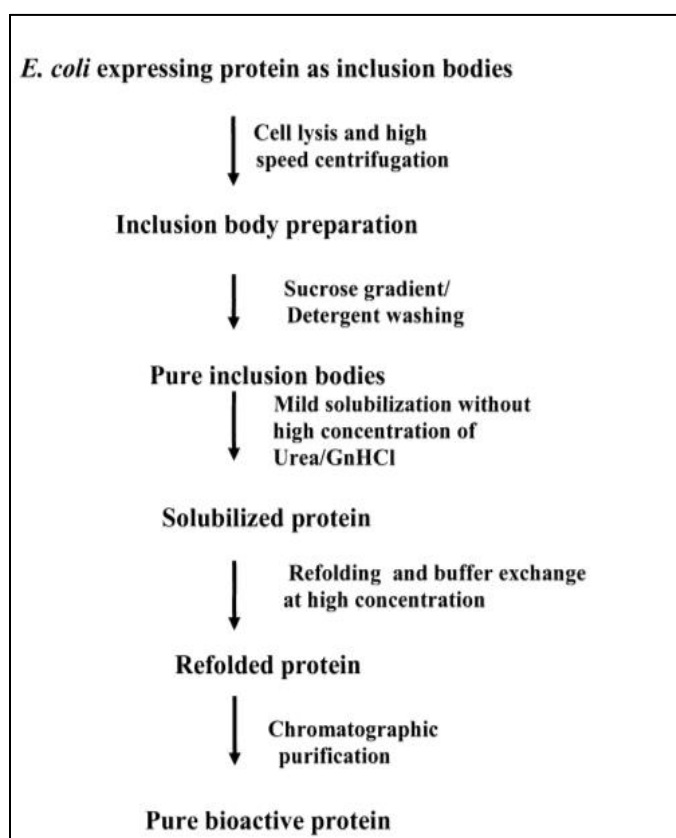

Figure 2.9: Summary of step to recover bioactive protein

2.2.5 Advantages and Disadvantages of Inclusion Bodies

IBs have been greatly used in Biomedicine field as naturally immobilized enzymes or as nanomaterials based on its specification as a pure recombinant protein (Garcia-Fruitos *et al*., 2009). Besides that, it is very useful in biocatalysis process and provide innovative stage in industrial catalysis market (Roessl *et al*.,2010). As immobilized enzyme, IBs have an advantage of being easily separated and recycled than their soluble counterparts, thus it is more preferred choice of biocatalysis. Moreover, IBs can use to discover strategies to control aggregation process by understanding the aggregation that occur in IBs. However, there are some disadvantages of IBs which the protein aggregation become a major obstacle in a rapid commercialization of potential drug candidates. This is because the protein instability due to the aggregation become a major problem in protein drug development. Other than that, protein aggregation will lead to more than 20 different degenerative diseases in human being (Stefani and Dobson, 2008).

2.3 Solubilisation of Inclusion Body Protein

2.3.1 Conventional Solubilisation

As mention in previous part, there are two types of solubilisation which are the conventional solubilisation and the mild solubilisation. Conventional solubilisation uses high concentration of chaotrophic reagents like urea and guanidine hydrochloride (Monera *et al*., 1994). It also uses an additional reducing agent such as beta-mercaptoethanol, dithiothreitol or cysteine in order to prevent inter disulphide formation. Other than that uses of EDTA (chelatin agent) will help to prevent metal catalysed air oxidation of cyteines. Solubilisation using high concentration of chaotropes will cause a complete disruption of protein structure. This will lead to aggregation of protein molecule during refolding process (Singh *et al.,* 2015). In conclusion this method will result in low recovery of refolded protein. This is because there is kinetic competition between the rate of aggregation and the rate of refolding. Based on the kinetic law, protein aggregation is a high rate of reaction whereas refolding is the first order reaction (Singh and Panda, 2005). Logically, this statement proved that the rate of aggregation is more than the rate of folding at high initial concentration of protein (Singh and Panda, 2005). Thus this process required dilution of

solubilised protein in order to increase the efficiency of refolding rate. This conventional method will also generate random coil structure during refolding process. Hydrophobic amino acid patches are exposed and will lead to loss of secondary structure (Qi *et al*., 2005). In conclusion , one of the way to reduce protein aggregation is by having refolding process in which the intermediates are beyond the aggregated structure. Mild solubilisation help to solubilise IB without generating the random coil configuration,by preventing the hydrophobic interaction during initial stage of refolding.

2.3.2 Mild Solubilisation

One of the example of mild solubilisation is in which the IBs are solubilised under low concentration of urea (Upadhay *et al.,* 2014). This method is very efficient because it does reduce the protein aggregation by making sure that the refolding process of partially folded protein beyond the aggregated structure. Mainly aggregation occur by non-native hydrophobic interaction between folding intermediates in which hydrophobic patches are exposed. Hence, mild solubilisation will help the process by not generating random coil and improve the recovery by prevention of hydrophobic interactions during the initial stage of refolding (Khan *et al*., 1998). Furthermore, it is a method which the protein is solubilized under mild alkaline pH 8 and low urea concentration. To be precise in low concentration of urea and mild alkaline pH the protein could not be denatured completely (Clark, 2001). The purpose urea is for physical separation of the molecules by disrupting the hydrophobic interactions of protein molecules (Patra *et al.,* 2000). Hence there is freeze and thaw method combined with low concentration of urea has been extensively studied to increase the efficiency of the solubilisation process (Strambini and Gabellieri, 1996). Figure 2.10 shows the illustration between conventional and mild solubilisation method.

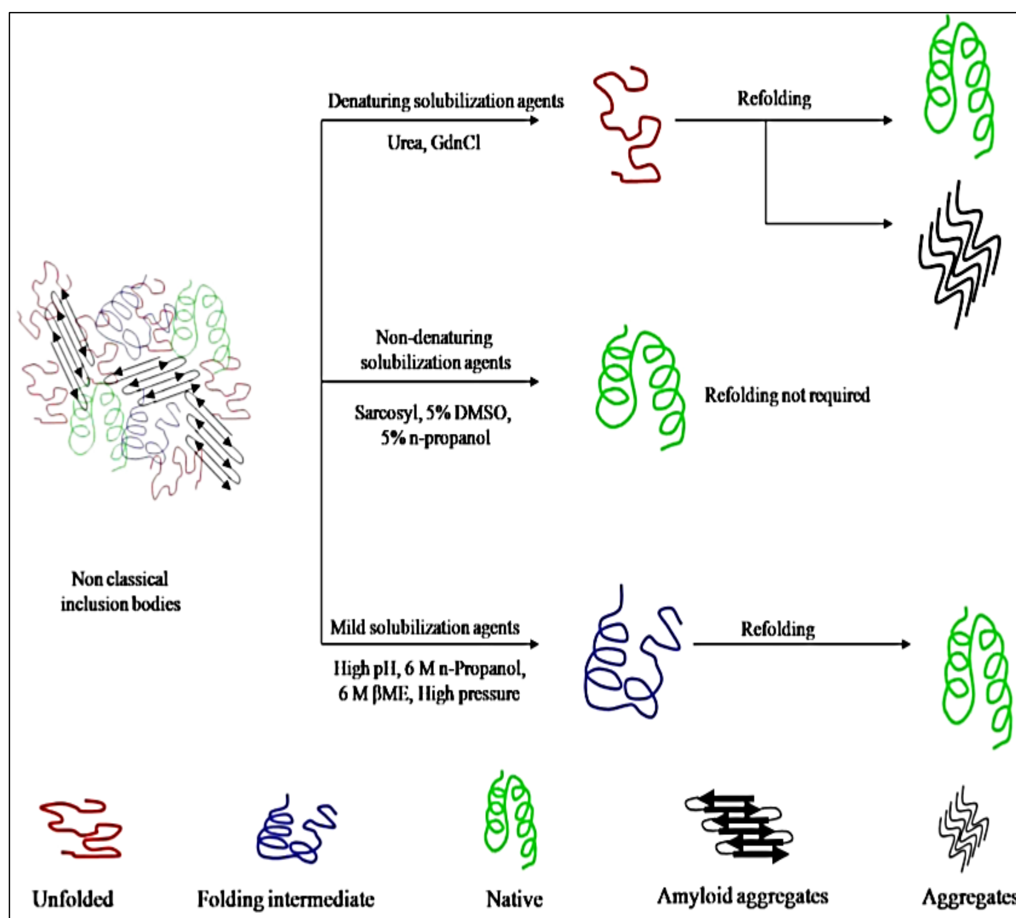

Figure 2.10: Refolding process in mild and harsh solubilisation (Singh *et al.*, 2015)

2.3.3 Method for Mild Solubilisation

For more efficient solubilisation, several mild solubilisation methods have been studied in order to keep the protein in partially folded form to prevent aggregation during refolding. There are 6 methods involve which are using high pH buffer, high hydrostatic pressure, detergents, organic solvent, low concentration of urea and non-denaturing solubilisation agent. For the first method by using high pH buffer which is higher than 12 and combine with 2 M urea has been proven success to solubilised IBs. High pH buffer does not completely unfold the native like protein structure, it does preserve it in solubilised state (Khan RH *et al.*, 1998). Pure r-hGH IBs were solubilized at different pH in 100 mM Tris buffer ranging from pH 3 to 13. The percent solubilisation of human growth hormone was monitored. When increasing pH from 6 to 12.5, solubilisation rate of r-hGH from IBs was observed increasing as well. Further addition of urea in 100 mM Tris buffer at pH 12.5 did not further increase solubilisation of r-hGH from the IBs. In 100 mM Tris buffer at pH 12.5 containing 2 M urea, a maximum

of 6 mg ml^{-1} of r-hGH were solubilised from the IBs. Use of 2 M urea did not unfold the protein completely and preserved the native-like secondary structure. Use of high pH will result in better solubilization as it was distant from the isoelectric point of human growth hormone which is 4.9. Combination of alkaline pH and 2 M urea destabilized both the ionic and hydrophobic interactions which are the major cause of protein aggregation in IBs of r-hGH. High hydrostatic pressure help solubilising process by disrupting intermolecular interactions during high pressure range of 2-4 kbar. Removal of applied pressure will lead to refolding process (John *et al.,* 1999). By applying 2.4 kbar pressure at -9°C and refolding at 0.4 kbar at 20°C, has been proven improve refolding of recombinant endostatin (Chura-Chambi *et al*., 2013). For EGFP IBs, demonstrated that incubation at pressure levels higher than 1 kbar disrupt the refolding of EGFP to the native state, likely by intefering the connections involved in the protein's native structure. Thus, high yields of biologically active EGFP were only obtained by applying of 2.4 kbar for 30 min to dissociate the aggregates with an incubation at lower pressure levels of 0.35 to 0.70 kbar, will help EGFP refolding, for 16 h (Malavasi *et al.,* 2011) Solubilisation with detergent like N-Lauroylsarcosine and Lauroyl-L-glutamate and combined with 2 M urea has been proved to increase the yield of bioactive protein (Kudou *et al*., 2011). By using organic solvent based solubilisation like β-mercaptoethanol and n-propanol has been proven to increase the efficiency and inhibit aggregation during refolding (Singh *et al* .,1998). From previous researcher has been proven that Lauroyl-L-glutamate at concentration of 2% can caused denaturation of both BSA and interleukin-6 . When the detergent concentration was reduced to 0.1%, the native structure was restored, suggesting that the bound detergents have dissociated. n-PAGE showed that the mobility of BSA or interleukin-6 was identical when the protein sample load on the n-PAGE contained 0%, 0.1% and 0.2% Lauroyl-L-glutamate. The ability of Lauroyl-L-glutamate to solubilise IBs was tested using E. coli expressed interleukin-6 and microbial transglutaminase. From this result, IBs were readily solubilised by 2% Lauroyl-L-glutamate Solubilising inclusion bodies in non-denaturing buffers such as Tris-Cl, without help of any solubilising agent will enhance the process in such a way where the bioactive protein can be achieved without required any refolding process. For example the inclusion bodies of N-acetyl-d-glucosamine 2-epimerase was reported to be active after solubilised using Tris-HCl buffer at pH 7 (Lu and Lin , 2012). The amount of proteins solubilised increased with the pH of the solubilisation solution. The percentage of inclusion bodies being solubilised increased

from 12.8% to 86.7% when the solution pH was increased from 5.6 to 11.7. An abrupt increase in protein recovery was observed between pH 9.5 and pH 10.0. Other than that, more inclusion bodies could be solubilised under pH 13.0 Tris solution. However under pH 13.0 extensive hydrolysis of epimerase occurred which result in low recovery. Low concentration of urea has also been used in many cases, and it results into extraction of recombinant protein without refolding step. This method does not completely denatured the solubilised protein molecules (Clark, 2001).

2.4 Freeze and Thaw Method

2.4.1 Introduction

As mention in earlier part, low concentration of urea cannot denatured the protein completely. Freeze and thaw method is one of the mild solubilisation process (Pikal-Cleland *et al*., 2000). Freeze and thawing method combined with 2 M urea can solubilised many IBs expressed in E. *coli* (Qi *et al.,* 2015). Hence it is very crucial to understand the role of this method before applying it to any protein. First and foremost, the fundamental of the protein is that it must attain the stability of its structure in order to maintain their activity. Protein will loss the activity if aggregation occur. Freeze and thaw affect the protein stability in two different categories which are physical and chemical degradation. Mainly freezing is a condition in which physical stress is applied by the formation of ice crystal hence applied several stresses for denaturing process of the protein. Thawing is a condition in which the applied stress is removed, so that the protein will refold back properly. Freezing induced several complex physical and chemical changes, which by mean resulting denaturation of protein with possibility of irreversible aggregates. Different protein have the different resistance towards the stress applied. Different stress applied will lead to aggregation with different characteristic. The condition applied for this process is very important by considering about other parameter involve. The physical stress may completely denatured the protein and refolding ability can be achieved at higher efficiency (Cao *et al*., 2003). Freeze and thaw method has been proven successfully solubilise the homogenous suspension of EGFP in 20 mM potassium phosphate buffer at pH 8 containing 2 M urea at -20°C of freezing temperature (Xingmei Qi, 2005). From this method the yield of recovery of bioactive protein is high and can applied for maximizing the recovery of proteins from IBs expressed in *E.coli*.

2.4.2 Application

Effect of freezing and thawing can be applied on preservation of meat. Freezing has been a great preserving technique for meat and its can be last for long time. Other than that, meat can be preserved in a condition similar to that of normal state and can be kept satisfactory for six months. Fresh meat remains almost same food value and flavor after proper freezing. Freezing and thawing are complex processes that involve heat transfer process. It is also related to physical and chemical changes which can affect the quality of the meat products (Li *et al*., 2002). Other than that slow freezing method can applied for the treatment of infertility (Chen.C *et al*.,1986). Treatment of infertility in here does for cryopreservation of oocytes, by maintaining the viability of the cell.

2.5 Factors Affecting Freeze and Thaw Method

2.5.1 Buffer pH

Different type of buffer used can affect the pH value. Hence, when pH is changing it will have a significant impact on the stability of the protein (Maity e*t al.,* 2009). During freezing in sodium phosphate buffer, a significant decreased of pH was observed from 7.0 to 3.8 ((Pikal-Cleland *et al.,* 2000). This is because due to crystallization of disodium salt (Sarciaux *et al.,* 1999). pH shift during freezing of sodium phosphate buffer depend on the eutectic temperatures and the concentration of various salt components relative to their solubility. The monobasic salt is more soluble than dibasic because of the eutectic temperature of monobasic salt is -9.7°C which is higher than eutectic temperature of dibasic salt -0.5°C (Vandenberg and Rose, 1959).

2.5.2 Rate of Freezing and Thawing

The freezing and thawing rate during freeze-thaw process can affect the degree of freezing concentration, surface area of ice-liquid interface, and the duration of protein exposed to the stress (Kueltzo et al., 2008). There are two type of freezing involve which are slow and rapid freezing. During rapid freezing, it was proven that less protein degradation occur (Pikal ,1994). However, different concept was found by Cao et al (2003) that stated rapid cooling will cause higher degree of supercooling which in turns yield a large number of small ice crystal, which generated large surface area to proteins that will lead to surface denaturing of protein. Other than that, rapid freezing rate may cause protein partial unfolding and cause the structural changes after

adsorption ice to the protein surface (Ugwu and Shireesh, 2004). This effect can cause the protein to be aggregate.

Besides, in slow freezing rate (<1℃/min), much larger ice crystals were formed which created less surface area to proteins and resulted in decreasing protein surface denaturation (Cao et al., 2003). Thus only limited number of denaturing site exist and directly impact the protein aggregation which will be reduce.

Slow thawing rate will caused more protein damage than the fast thawing rate (Cao *et al.,* 2003). At slow thawing rate, recrystallization occurs more which it changes the ice solution interface and causes more protein damage. Hence rapid thawing is more recommended.

2.5.3 Number of Cycle

Number of cycle does affect the protein stability in terms of probability degradation of protein occurence and thus indirectly determine the recovery efficiency of the functional protein. Repeated cycle of freezing and thawing processes will cause protein degradation and loss of activity (Patel *et al.,* 2011). It also can damaged cell envelope (Franks, 1981) and disrupted the integrity of membrane (Lee et al., 1985). Protein solubility decreased when the freeze-thaw cycles increased (Benjakul and Bauer, 2000). For better recovery of intracellular protein it is recommended that the cell disruption using freeze and thaw process was limited to three cycles (Johnson and Hecht, 1994).

CHAPTER 3

METHODOLOGY

3.1 Introduction

In this chapter, functional EGFP was recovered by using mild solubilisation method under freeze and thaw process. There are several procedures involve in order to recover the pure bioactive protein as shown in Figure 3.1.

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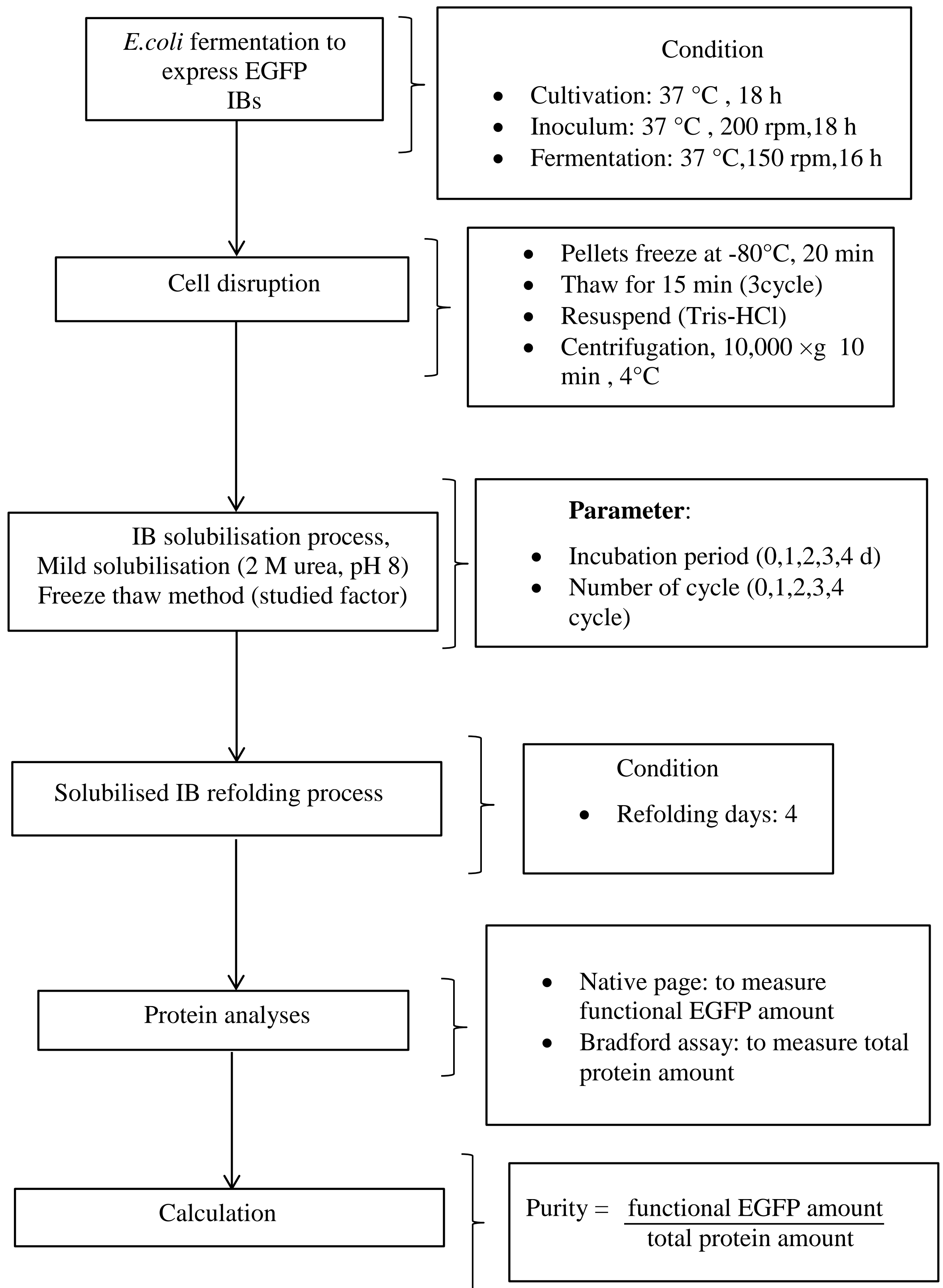

Figure 3.1: Experiment flow chart

3.2 Materials

The chemicals that were used in this study are LB broth, LB agar, ampicillin, IPTG, for fermentation of *E. coli* cell for expression of EGFP. For detergent washing, chemicals used are EDTA, Triton X and urea. For the analysis method, the chemicals needed are Tris-base, HCl, glycine, APS, acrylamide- bis solution 40% (37:5:1), butanol, TEMED, bromophenol blue, CBB, BSA, ethanol, ortho-phosphoric acid

3.3 Experimental Methods

3.3.1 Cultivation of recombinant Enhanced Green Fluorescent Protein

E. coli strain BL21(DE3) harboring the plasmid pRSETEGFP was used for expression of EGFP. It was cultured on LB agar and were put in an incubator at temperature of 37 °C for 18 h. *E. coli* cells were growth by producing colony. For inoculum preparation, ampicillin at final concentration of 100 µg/ml was added in 50ml LB broth. A single cell was cultured in the 50 ml LB broth in a shaker incubator at 37 °C and 200 rpm for 18 h. 5% (v/v) of inoculum was transferred into 200ml LB broth in empty flask by following the ratio of 0.2 of medium to empty flask.Then, the cells were grown at 37 °C and 150 rpm with OD reached the range 0.8 to 1.0, a final concentration of 1mM IPTG was added to induce EGFP IBs expression for another 16 h (Qi *et al.*, 2005).

3.3.2 Harvesting and cell washing

The cells were harvested at 8000 ×g at 4°C for 10 min (Johnson and Hecht, 1994). Supernatant was discharged, the cell pellets were washed with 20mM Tris-HCl buffer pH 8 at 10% (w/v). Cell suspension were centrifuged at 8000 ×g at 4°C for 10 min. The clarified supernatant were discharged and the cell pellets were kept.

3.3.3 Freeze-thaw method

The cell pellets were frozen at -80°C for 20 min. Then the pellets were thawed for 15 min in water. This step was repeated for another 2 times. Next, the pellets were resuspended with 20 mM Tris-HCl buffer pH 8.0 at 10% (w/v). Cell lysate were centrifuged at 10,000 ×g for 10 min at 4°C . Supernatant consist of soluble protein was discharged and the pellets were kept.

3.3.4 Detergent washing

Cell pellets were washed with detergent solution (20 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 1% Triton X, 1 M urea, pH 8.0) to eliminate cell debris, (Rodríguez *et al*., 2010) . The pellets were preceding for centrifugation at 10,000 ×g at 4°C for 10 min, supernatant were discharged. This 2 steps were repeated for another 2 times .The pellets were washed with 20 mM Tris-HCl buffer pH 8.0 at 10% (w/v) to remove detergent. Lastly, the centrifugation were carried out again by using the same condition which is at 10,000 ×g at 4°C for 10 min. Supernatant were discharged and the cells pellet were kept in -20°C.

3.3.5 Urea solubilisation with freeze and thaw process

The cell pellets were resuspended with 20mM Tris HCl, 2M urea, pH 8 at 10% (w/v). Cell suspension was distributed into several 1.5mL microcentrifuge tube which every tube was consist of 1 mL of cell suspension. Freeze and thaw method was carried out under 2 different parameter. First parameter is freezing incubation period (1, 2, 3, and 4 days). Second condition is number of freeze and thaw cycle (1, 2, 3, 4 and 5 cycles. After the freeze and thaw process, the cell suspension was centrifuged at 10,000 ×g at 4°C for 10 min. Lastly, the supernatant were collected and its volume was recorded for the protein analyses which are n-PAGE for determining functional EGFP amount and Bradford assay for determining total protein amount.

3.4 Analytical Method

3.4.1 Preparation for n-PAGE

Gel- based imaging method was conducted to quantify the amount of EGFP (Chew *et al.,* 2009). Protein samples were undergone process of electrophoresis and separated by native polyacrylamide gel by using an Omnipage mini vertical system (Cleaver scientific). Resolving gel was prepared (Table 3.3) and was polymerised in gel cassette. Butanol was added on top of the layer in order to make the layer smooth and to reduce air bubble. After the gel has been polymerised, the butanol was washed away with distilled water. After that, the stacking gel (Table 3.3) was prepared and loaded on top of the resolving gel. Comb was inserted into the gel cassette and the stacking gel was let to polymerise. Figure 3.4.1 shows the set-up of the analyses method. As shown from the picture, the sample and dye were loaded in the stacking gel.

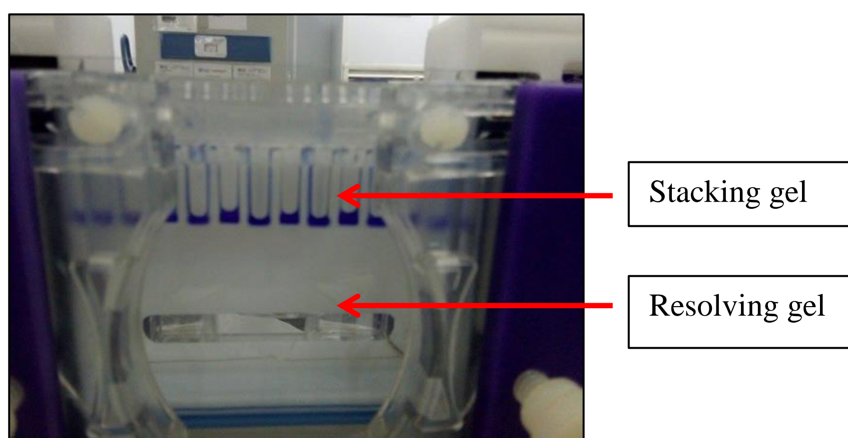

Figure 3.4.1: Analysis process for **n-PAGE**

Table 3.3: Formulation for preparation of stacking gel and resolving gel

	15%(w/v) resolving gel	**4%(w/v) stacking gel**
Acrylamide mix 40%(w/v) acrylamide and 1.07%(w/v) bisacrylamide (37.5 : 1)	3750 µL	400 µL
Distilled water (autoclaved)	3750 µL	2600 µL
10% (w/v) APS	62.58 µL	26.72 µL
TEMED	10.12 µL	5.6 µL
4x native lower buffer (1.5 M Tris-HCl, pH 8.8)	2500 µL	
4x native upper buffer (0.5 M Tris-HCl, pH 6.8		1000 µL

After polymerisation, protein sample was mixed with 6×sample buffer [0.35M Tris-HCl (pH 6.8), 0.3% (v/v) glycerol, and 0.05% (w/v) bromophenol blue] and the electrophoresis was carried out under a constant current of 25 mA for one gel for 90 min. After electrophoresis, fluorescent image of EGFP on the gel was captured by a using bio-imaging system(Alpha-Innotec) at an ultraviolet (UV) wavelength of 365 nm and an exposure time of 1 s. The camera settings were adjusted (aperture at 2.80 mm, zoom at 70 mm, and focus at 0.7 mm) and transIllumination UV was chosen as standard lighting. After making sure that the setting of the camera is proper, then the fluorescent image was captured. The EGFP fluorescence intensity on the gel was quantified using AlphaEase FC software by drawing boxes around the fluorescent image. The amount of functional EGFP was determined from standard curve equation, $y = 53{,}994{,}732.43x - 22{,}762{,}035.96$ (intensity versus the amount of pure EGFP).

3.4.2 Preparation for Bradford assay

For standard BSA preparation, 2 to 10 µg, 10 to 20 μL of dH_2O were prepared and pipetted into a 96-well plate. Bradford reagent [0.05% (w/v) CBB G-250, 23.75% (w/v) ethanol, and 42.5% (w/v) ortho-phosphoric acid] with volume of 200 μL was added into the microplate. The volume of sample added is 20 μL . Absorbance at wavelength of 595 nm was measured by using a microplate reader (Infinite M200 Pro) .Figure 3.4.2 showing the illustration of amount sample and BSA added. Triplicate measurements were taken in order to get an average absorbance value. After measuring the BSA absorbance, BSA curve yield an equation, y = 0.0926x. From the sample absorbance and the BSA curve quation, the total protein amount in 20 μL was obtained by taking the value of absorbance sample divide by the gradient of the curve.

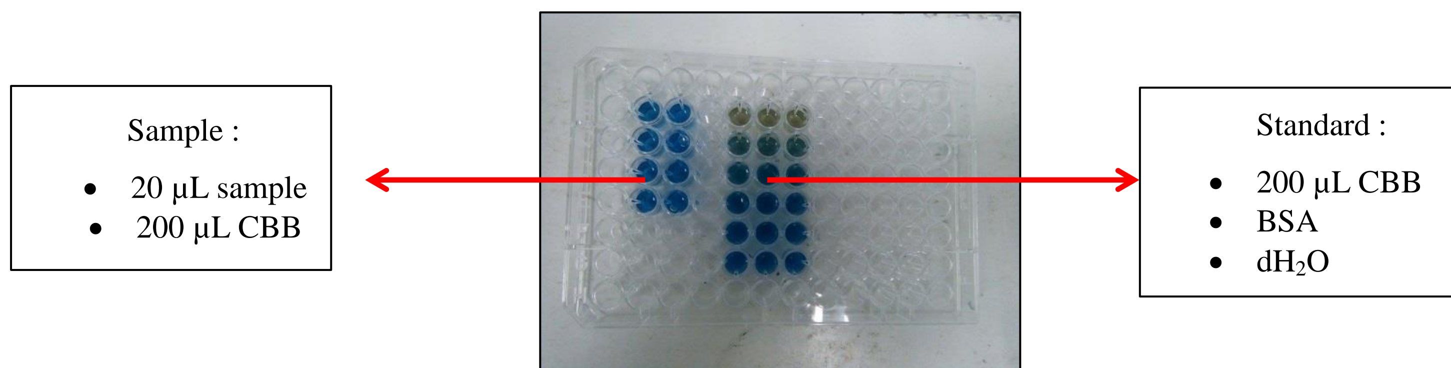

Figure 3.4.2: Amount of sample and BSA added

3.5 Calculation

$$\text{Purity} = \frac{Functional\ EGFP\ amount\ in\ supernatant}{total\ protein\ \ amount\ in\ supernantant} \text{ x } 100\%$$

CHAPTER 4

RESULT AND DISCUSSION

4.1 Introduction

This chapter is mainly about the results and discussion on effect of freeze and thaw cycle and freezing incubation period for solubilisation of inclusion body protein for recombinant EGFP recovery.

4.2 Effect of Incubation Period

Sample of EGFP protein was examined under different freezing incubation period during freezing and thawing process. First it is very important to consider how does the incubation period relates to the freezing. Incubation period is the time taken for the protein to undergone freezing process. It gives the big impact on the amount of stress applied on the protein for denaturing process. Hence, it will affects the functional protein recovery. Figure 4.1 shows the yield of recovered functional EGFP amount under different freezing incubation period. For the period ranging from 0 to 1 day, the graph shows a very large significant increase of amount of functional EGFP. When the incubation period is further increased, the graph showing a gradual increase in the trend. Even though, the trend is increasing, the percentage of increment for every incubation period does not vary too much. Between incubation period 0 day to 1 day the percentage of increment is 20%, for incubation period 1 day to 2 days is 4.76%, for incubation period 2 days to 3 days is 2.44%, for incubation period 3 days to 4 days is 4.65%.This is because when freezing occur, protein can undergone denaturation by the formation of ice crystal (Bhatnagar *et al.,* 2007). Formation of ice crystal is the physical stress which

is the driving force in denaturing the protein. The physical stress may completely denatured the protein and refolding ability can be achieved at higher efficiency (Cao *et al.*, 2003). For incubation period ranging from 1 to 4 days, the functional amount EGFP is ranging from 40 to $43\mu g$. From the graph it can be concluded that increasing the period will result in more functional protein being solubilised and refolded properl.By analysing the graph, the gradually increased in trend, which by mean that after the first day incubation period ,the amount of functional protein desired already achieved the saturation limit. It might be because of the functional protein has been degraded when incubation period increasing.

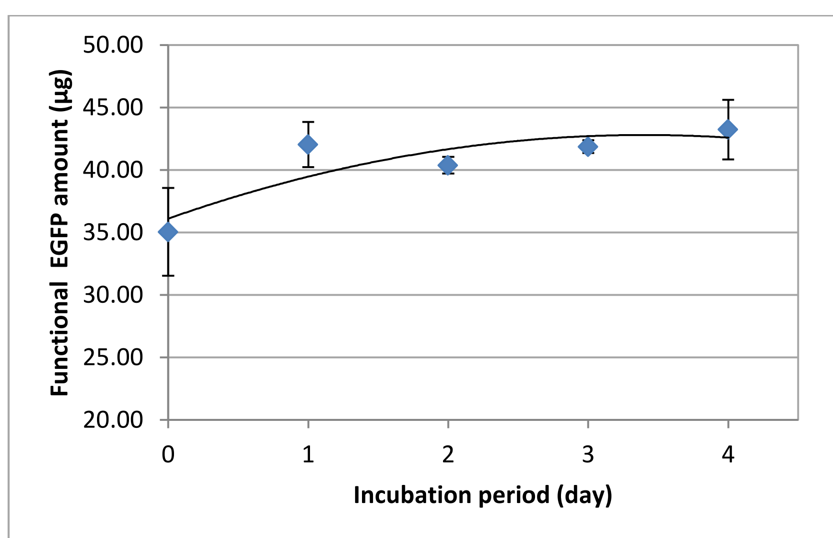

Figure 4.1: The recovered functional EGFP amount under different incubation period

After undergone several incubation period due to the stress applied on the protein which it cannot resist towards it which then lead to degradation. Thus it contain more un-functional protein amount rather than functional protein amount. Besides that, when more un-functional protein was obtained it contributed to the increasing in the total protein amount. From figure 4.3, it can be concluded that, as incubation period increasing the total protein amount will increased as well due to the increased in un-functional EGFP amount and *E.coli* cell protein.This shown that the incubation period for one day is already sufficient to recover the functional EGFP protein. By increasing the day of incubation, there will be not so much effect on recovery of bioactive protein.

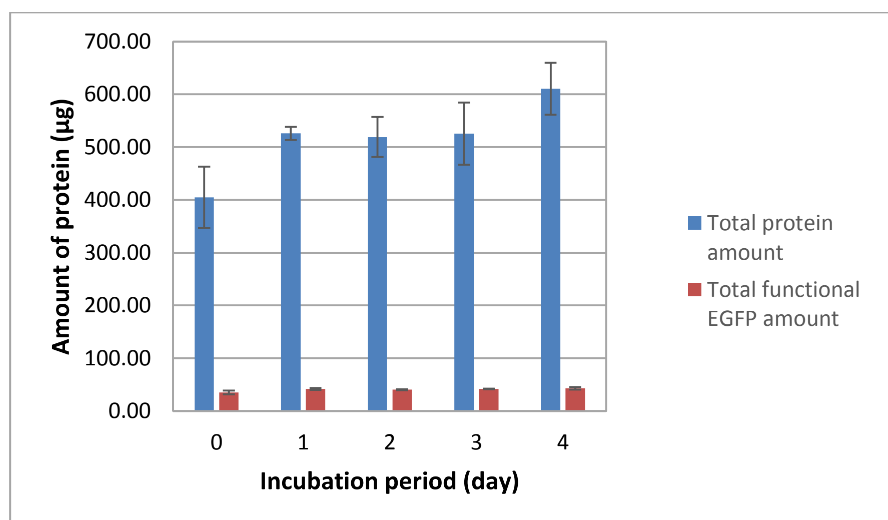

Figure 4.3: Comparison between total amount protein and total functional EGFP amount

In order for the protein to carry out biological activity,it must maintained native secondary and higher order structures (Cleland *et al*., 1993). So there is a need to understand what is the important criteria that contribute to the protein stability in order for it to function. Basically for protein instability can be separated into 2 categories which are physical instability and chemical instability. Chemical instabilities involve processes that make or break covalent bonds, generating new chemical entities. Chemical instabilities is related to chemical degradation. Example of chemical degradation process are oxidation, disulphide exchange, condensation reaction, and proteolysis. Physical instability refers to any process whereby the protein changes its physical state without any change in the chemical composition. Example of protein instability process are denaturation, surface adsorption, aggregation and precipitation.

Denaturation causes the loss of the globular or three-dimensional structure that most proteins adopt. This globular structure is referred to as the native state. Consequently, upon unfolding or denaturation, the protein changes its physical state, but the chemical composition remains the same. Denaturation can involve the loss of secondary or tertiary structure or both.The amount of the stress applied is related to denaturation process which it disrupt the physical properties of the protein by altering the shape of the structure. Example of denaturation in drug protein are often accompanied by covalent and non-covalent aggregates that not only can destroy the activity of the drug, but also cause adverse side effects(Carpenter and Chang, 1996).

Other than that, increasing in stress will not only help in solubilising the functional EGFP, it also at the same time solubilising the undesired protein. Figure 4.2, shows the trend of the purity is decreasing dramatically from day 0 to 4. For day 0 the purity obtained is 8% whereas for period day 4 the purity decrease to 7.2%. Purity is defined as the functional EGFP amount over the total protein amount. Some part of total protein maybe non-functional EGFP. There is possibility the freezing condition degraded the EGFP, hence affect the refolding process. For freezing period from day 1 to 4,solubilisation majorly occur on undesired protein but it did not the remaining EGFP-IBs. This is because when freezing occur ice crystal are formed, the buffer salt and protein are concentrated. This condition is subjected to cryoconcentration in which proteins experience high concentration environment. Cryoconcentration affects the protein structure solution through change in pH and ionic strength (Singh et al., 2011). When the protein in high concentration during freezing, aggregation will take place (Wang and Hanson, 1988). Aggregation affects mass balance of protein solutions and decreases the concentration of targeted protein (Patel et al., 2011). So this is why the amount of the targeted protein being solubilised is less than undesired protein.

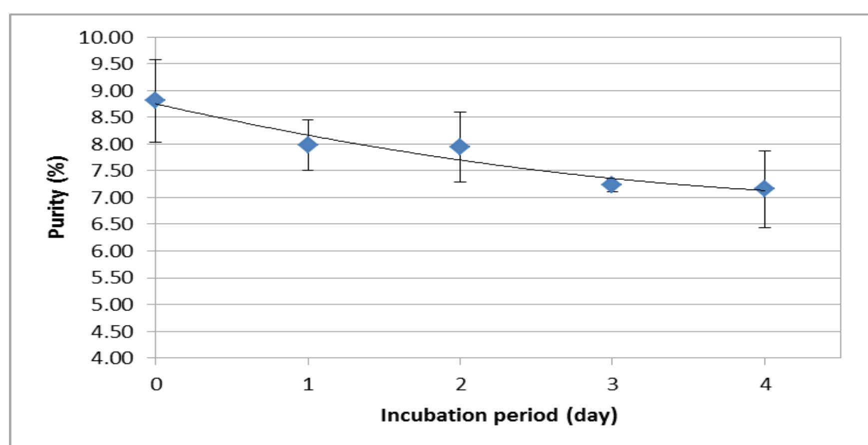

Figure 4.2: Purity of functional EGFP amount under different incubation period

During the freezing process, the amount of the stress applied to the protein affect the stability of the protein. Protein are marginally stable, in frozen state it is readily denature by various stress. Increasing in total amount of protein is contributed by the main factor in freezing which are low temperature and high destabilizing salt concentration (Brandts *et al.,* 1970). This two factor will lead to the damage of the protein in terms of damaging the cell membrane. Other reason that attribute to the

increasing in the total number of protein is during the cell isolation process. Cell isolation is a method in which the IBs are isolate from the bacterial cell and cell debris cellular by using low speed of centrifugation. After this step, the cell pellet is washed by detergent in order to remove the cell debris. The most important concept in here is that during cell isolation it might be not all the cellular components is getting rid. Some one of the cell remain as a whole cell, and this cell together with the IBs pellet were proceed for the mild solubilisation. Then for the mild solubilisation, both IBs pellet and the cell were solubilised. The IBs pellet was unfold and refold into the bioactive form and the cell were dissolve in the urea. Hence the total number of protein is increasing because it is governed by the amount of the cell that dissolved in the urea. The purpose of this study is to study the effect freezing incubation period on the solubilisation of EGFP IBs Freezing incubation period did affect the solubilisation of EGFP IBs by applying stress that are capable for denaturing during solubilisation process then proceed for refolding process in obtaining functional EGFP. Hence, it is proven that to use 0 day incubation period is sufficient for the solubilisation, because the number of functional amount protein is quite similar from period 0 day to 4 day.

4.3 Effect of Number of Cycle Process

To study the solubilisation capacity and the recovery of functional EGFP, sample of EGFP protein was examined under different number of cycle of freezing and thawing process. Qi *et al.,* (2015) reported that single freeze and thaw method under 2 M of urea could solubilised different type of IBs aggregates expressed in *E. coli*. Number of cycle does affect the protein stability in terms of probability degradation of protein occurence and thus indirectly determine the recovery efficiency of the functional protein. Figure 4.4 shows the yield of recovered functional amount of EGFP in different number of cycle process. From the figure, it was found that from zero cycle to 3 cycles of freezing and thawing processes, the amount of functional EGFP remained same. However, when more cycle were applied the amount of functional EGFP was increased from 45 to 50 μg. There was a slight increase in the functional EGFP as the number of process cycle increase. It might be because of EGFP can solubilise but cannot refold back properly. This situation is might be because of the cell disruption that occur during solubilisation. During solubilisation chemical denaturation occur in which the unfolding

of the protein by addition of chaotropes like urea. Actually, urea appears to delayed the hydrophobic collapse associated with formation of the globular native state (Stumpe and Grubmuller, 2009). When freezing occur, the urea will be in highly concentrated so the high concentration of urea will cause a complete disruption of protein structure. Unlike excluded solutes, chaotropes appear to bind to proteins, reducing their chemical potential. When the unfolded state has a much larger surface area than the native state ,the chemical potential of the unfolded state is lowered . When it falls below that of the native state, the protein unfolds. It has been reported that the addition of high concentrations of urea can alter the pKa of amino acid side chains by 0.3 to 0.5 units (Marti,2005). When the alteration of pKa occur it will lead to aggregation. So during that solubilisation process, there was kinetic competition between the rate of aggregation and the rate of refolding. Based on the kinetic law, protein aggregation is a high rate of reaction whereas refolding is the first order reaction (Singh and Panda, 2005). Logically, this statement proved that the rate of aggregation is more than the rate of folding at high initial concentration of protein (Singh and Panda, 2005) This will lead to aggregation of protein molecule during refolding process (Singh *et al.,* 2015). It is shown that the solubilisation does occurring by unfolding the protein but the refolding step cannot take place due to aggregation that occur.

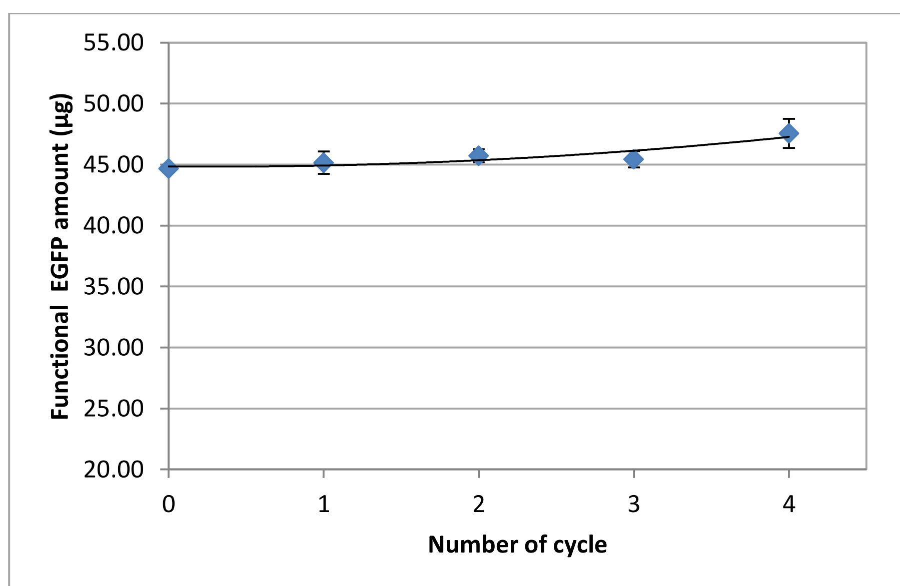

Figure 4.4: The recovered functional EGFP amount under different number of cycle

However, from zero cycle to fifth cycle the purity achieved showed a slight decrease in the trend of the graph from figure 4.5. The highest value of the purity achieved was during cycle 1 which is 17%. This has been proven that by increasing the number of process cycle will result in low purity of EGFP functional amount. It might be because EGFP failed to refold properly do to the degradation. During repeated freezing and thawing, there was repeated melting and reformation of ice crystal which will lead to the cell damage. Repeated cycle of freezing and thawing processes will cause protein degradation and loss of activity (Patel *et al.,* 2011). It also can damaged cell envelope (Franks, 1981) and disrupted the integrity of membrane (Lee et al., 1985). *E. coli* cell wall structure consist of an inner membrane , outer membrane and periplasmic space between the membrane which also contains one to two layers of peptidoglycan (Sekiguchi and Yamamoto, 2012). EGFP was located at the cytoplasm and can be exported to bacterial perisplam (Dammeyer and Tinnefeld, 2012), but EGFP was found inactive in the perisplasmic space (Feilmeier et al., 2000). Peptidoglycan is the main stress-bearing component which found on the bacterial cell wall (Vollmer and Seligman, 2010). Repeated stress applied might be destroying the protective peptidoglycan layer, causing disruption effect. In addition, freezing and thawing of *E. coli* cells without help of cryoprotective agent, such as glycerol, will decreases their viability (Calcott and MacLeod, 1975). Ability of the *E.coli* cell to survive is proportional to the number of freeze-thaw cycles that cells experience (Packer *et al.,* 1965). So it is proven here that EGFP IBs undergone degradation because of the repeated stress that has been applied on it which was beyond the limitation. So it recommendable that for better recovery of intracellular protein the cell disruption using freeze and thaw process was limited to three cycles (Johnson and Hecht, 1994).

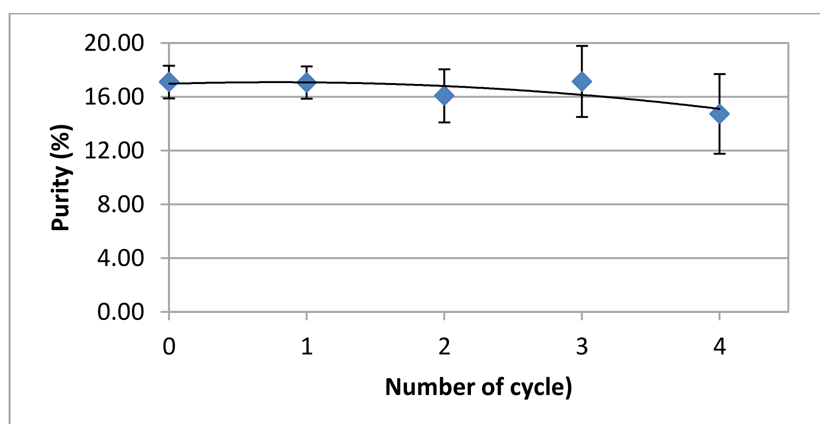

Figure 4.5: Purity of functional EGFP under different number of cycle

Comparison between mild solubilisation and conventional solubilisation

Mild solubilisation method can be classified as an effective method because it can reduce the protein aggregation by making sure that the refolding process of partially folded protein beyond the aggregated structure. Mainly aggregation occur by non-native hydrophobic interaction between folding intermediates in which hydrophobic patches are exposed. Hence, mild solubilisation will help the process by not generating random coil and improve the recovery by prevention of hydrophobic interactions during the initial stage of refolding (Khan *et al*., 1998). Furthermore, it is a method which the protein is solubilized under mild alkaline pH 8 and low urea concentration. One of the example of mild solubilisation process is the recovery of human growth hormone from IBs . Pure r-hGH IBs were solubilised at different pHs in 100 mM Tris buffer ranging between pH 3 to13 and percent solubilisation of r-hGH was studied. Solubilisation of r-hGH from IBs was observed by increasing the pH from 6 to 12.5. The efficiency of solubilisation was further increases by incorporating 2M urea in 100 mM Tris buffer at pH 12.5 .Further addition of urea in 100 mM Tris buffer at pH 12.5 did not further increase solubilisation of r-hGH from the IBs. In 100 mM Tris buffer at pH 12.5 containing 2M urea, a maximum of 6mgml^{-1} of r-hGH were solubilised from the inclusion bodies. 2M urea did not unfold the protein completely and preserved the native-like secondary structure.

Conventional solubilisation uses high concentration of chaotrophic reagents like urea and guanidine hydrochloride (Monera *et al*., 1994). Tsumoto *et al.,* 2003 studied the solubilisation performance of GFP expressed in *E.coli* using high non –denaturing concentration of guanidine hydrochloride. From the studied decreasing the concentration of guanidine hydrochloride from 0.5 M to 2 M will result in more EGFP being solubilised which shown that the fluorescence can be observed. Increasing the concentration to 6 M has been proven could not solubilised EGFP and it cause denaturation to occur so no any fluorescence being observed

From this comparison it is proven that the mild solubilisation result in more recovery of protein. However, low concentration of urea and mild alkaline pH the protein could not be denatured completely (Clark, 2001). Thus by adding the method freeze and thaw process the efiiciency of the process will result in better recovery.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In conclusion, the effects of incubation period and number of process cycle affect the performance of freeze-thaw processes. Incubation period affect the solubilisation performance of EGFP by determining the amount of stress needed to be applied during the denaturing process. If too much of stress applied on EGFP, it can lead to the aggregation which EGFP actually can unfold but failed to refold back decause due to its structure that has been disrupted by the stress. If the amount of stress is sufficient enough, it will help denaturation which unfold the protein and refolding process can be achieved in order to achieve functional EGFP. Number of cycle does affect the solubilisation performance by affecting EGFP stability in terms of probability degradation occurrence. If the process cycle was repeated for many times, the denaturation of EGFP occur but after that the structure of *E.coli* cells has been degraded due to the cell stability that cannot resist towards the stress. So the refolding of EGFP cannot occur. If the cycle was limited to certain times the functional EGFP can be obtained. In this study period one was considered as the most effective as high purity (8%) and high yield (42 μg) of EGFP recovered from *E. coli* cells was achieved. The highest purity of recovered EGFP was achieved by number of process cycle 1 which was around 17%. The yield of recovered EGFP was around 45 μg .

5.2 Recommendation

It is recommendable that incubation period should be limited to certain limit, because the EGFP has its own stability towards the stress applied. Other than that, the number of cycle should also be limited and cannot across certain range that might cause degradation occur. Besides that, for the cell disruption method it is recommendable to choose mechanical method in order to make sure all the cell debris has been removed before proceed for solubilisation. If the cell debris did not remove completely it affect the solubilisation which some of the cell remain as a whole cell, and this cell together with the IB pellet were proceed for the mild solubilisation. Furthermore, it is very crucial to choose the right additive for refolding buffer and it is recommendable to choose additive under category of sugars and polyhydric alcohol because this type of additives can prevent aggregation during refolding.

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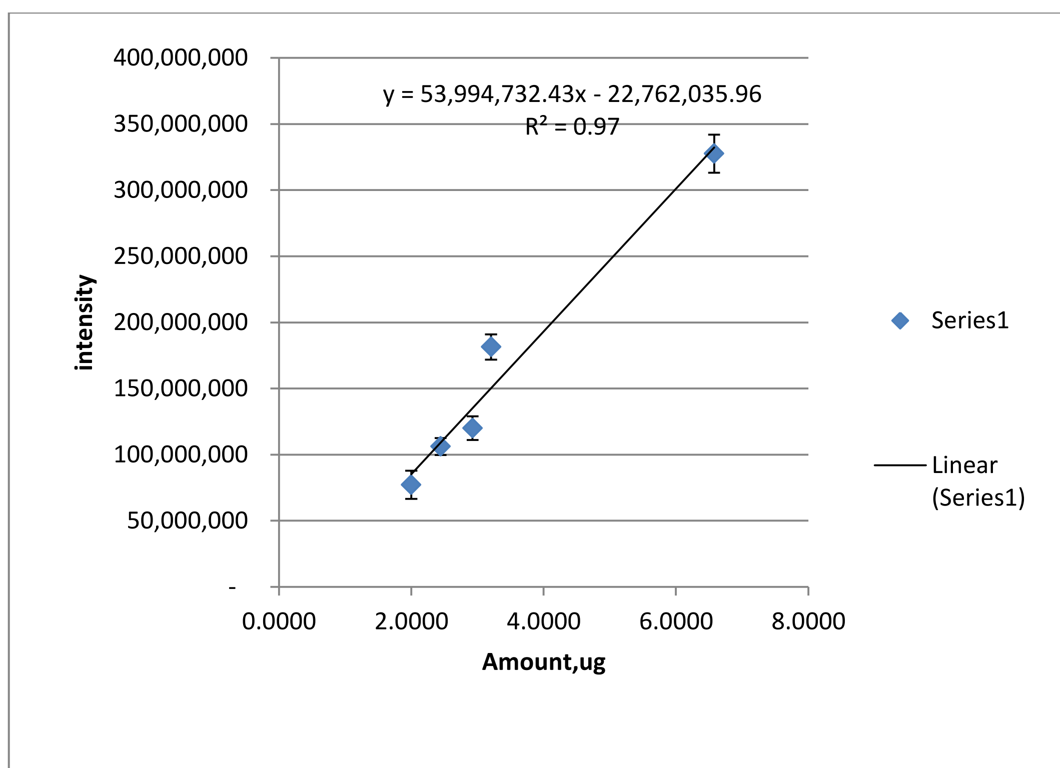
CHAPTER 7 APPENDIX

Appendix A: Data for standard curve

Table A1 shows the intensity value at different amount of EGFP

	Intensity						
Amount (µg)	Run 1		Run 2		Avg	Std	Cov
6.57	344108726.25	326073275.25	305320402.25	334621113.75	327530879.38	14322415.42	4.37
3.20	182486762.25	167129025.50	181676049.75	194102814.50	181348663.00	9569076.80	5.28
2.92	114492050.00	128286086.50	108113180.00	129199131.00	120022611.88	9012702.12	7.51
2.45	111852125.00	100810842.25	98623704.25	113323219.50	106152472.75	6502326.82	6.13
1.99	88422850.75	84128978.25	75910659.50	60303117.50	77191401.50	10736967.22	13.91

Figure A1 shows the standard curve of intensity versus amount of pure EGFP



Appendix B: Data for effect of incubation period

a) Intensity Gel 1, Run 1

Inc. P (d)	IMAGE 1		IMAGE 2		IMAGE 3		IMAGE 4		IMAGE 5	
	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)
0	3610080	3419384	3875173	3745927	3879847	3760377	4182303	4057151	3676391	3548041
1	5018185	4158281	8614037	7569005	5508951	4694231	5487151	4519738	5611299	4690154
2	6187365	5283060	9835476	7931730	5800183	4886839	5065363	4037189	7851336	6787892
3	6909063	5402826	10990738	10508856	9152959	7515326	8754931	7084665	8013415	6725007
4	7813137	6195057	12123793	10811381	6961513	5233352	7151931	5431446	9591260	7862215

Inc. P (d)	GEL 2							
	Intensity 1	Intensity 2	Intensity 3	Intensity 4	Intensity 5	Avg	STD	COV
0	N.D	181,179	136,338	219,791	171,705	177,253	34301.18	19.35
1	918,473	870,340	746,817	842,565	991,194	873,878	90677.01	10.38
2	1,038,390	947,654	874,392	1,018,502	1,228,553	1,021,498	132532.26	12.97
3	1,317,201	1,273,471	1,333,548	1,480,916	1,412,819	1,363,591	82711.52	6.07
4	1,650,141	1,597,585	1,712,476	1,819,660	1,859,317	1,727,836	110630.77	6.40

b) Intensity Gel 2 Run 1

Inc. P (d)	IMAGE 1		IMAGE 2		IMAGE 3		IMAGE 4		IMAGE 5	
	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)
0	3745311	3627467	5735930	5554751	4693187	4556849	3745295	3525504	3604887	3433182
1	5153664	4235191	5098561	4228221	5046128	4299311	5318843	4476278	5002284	4011090
2	4599868	3561478	4520384	3572730	4796298	3921906	4968217	3949715	4765256	3536703
3	7586044	6268843	7396262	6122791	7212587	5879039	7654726	6173810	7051913	5639094
4	9526729	7876588	8031039	6433454	8291073	6578597	9426207	7606547	8766442	6907125

Inc. P (d)	GEL 1							
	Intensity 1	Intensity 2	Intensity 3	Intensity 4	Intensity 5	Avg	STD	COV
0	N.D	129,246	119,470	125,152	128,350	125,555	4420.6009	3.5208
1	859,904	1,045,032	814,720	967,413	921,145	921,643	90227.7926	9.7898
2	904,305	N.D	913,344	1,028,174	1,063,444	977,317	80472.7520	8.2340
3	1,506,237	N.D	1,637,633	1,670,266	1,288,408	1,525,636	173315.3293	11.3602
4	1,618,080	1,312,412	1,728,161	1,720,485	1,729,045	1,621,637	179086.2369	11.0435

c) Intensity Gel 3, Run 1

Inc. P (d)	**IMAGE 1**		**IMAGE 2**		**IMAGE 3**		**IMAGE 4**		**IMAGE 5**	
	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)
0	5107408	5098871	5240324	5159508	4763324	4552872	4556651	4441708	5327777	5300618
1	6600330	5961332	6068438	5490045	6812449	6304434	6795902	6243245	7369776	7143575
2	8104879	6544008	8112143	6460304	8589359	6497420	8635900	7613129	9418325	8000435
3	8081397	6131702	8248162	6213229	8570291	6400748	7698726	6285605	7543576	5701660
4	1115777	8336688	10968081	8153917	10016685	7402036	1068924	8824820	1327523	1085656

Incubation period (d)	GEL 3							
	Intensity 1	Intensity 2	Intensity 3	Intensity 4	Intensity 5	Avg	STD	COV
0	n.d	n.d	210,452	114,943	n.d	162,698	67535.06	41.51
1	638,998	578,393	508,015	552,657	n.d	569,516	54690.49	9.60
2	1,560,871	1,651,839	n.d	1,022,771	1,417,890	1,413,343	277616.05	19.64
3	1,949,695	2,034,933	2,169,543	1,413,121	1,841,916	1,881,842	288143.28	15.31
4	2,821,086	2,814,164	2,614,649	1,864,427	2,418,669	2,506,599	395376.29	15.77

d) Intensity Gel 1, Run 2

Inc. P (d)	IMAGE 1		IMAGE 2		IMAGE 3		IMAGE 4		IMAGE 5	
	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)
0	4201438	3927324	4499430	4174922	4895760	4548192	3906317	3639879	4522934	4412755
1	6001657	4875497	6636668	5412776	6399234	5047018	6522230	5202339	6223429	4952446
2	6015985	4538682	7977230	6450460	7250002	5544577	7724121	6027815	7789347	5909392
3	7218495	5422083	8118315	6142349	8561012	6507323	8371856	6307183	8645707	6452959
4	1348754	8195657	1418441	9013213	12798303	7727293	15131352	9939990	12665497	7665157

Inc. P (d)	GEL 1							
	Intensity 1	Intensity 2	Intensity 3	Intensity 4	Intensity 5	Avg	STD	COV
0	274,114	324,508	347,568	266,438	N.D	303,157	33985.23	11.21
1	1,126,160	1,223,892	1,352,216	1,319,891	1,270,983	1,258,628	79270.27	6.30
2	1,477,303	1,526,770	1,705,425	1,696,306	1,879,955	1,657,152	143422.19	8.65
3	1,796,412	1,975,966	2,053,689	2,064,673	2,192,748	2,016,698	130267.84	6.46
4	5,291,892	5,171,204	5,071,010	5,191,362	5,000,340	5,145,162	100810.96	1.96

e) Intensity Gel 2, Run 2

Inc. P (d)	IMAGE 1		IMAGE 2		IMAGE 3		IMAGE 4		IMAGE 5	
	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)
0	6067179	6041860	5886473	5773086	6462978	6391448	6475310	6309389	5789955	5683232
1	11582943	10539922	12168458	11048906	1235841	11145007	10046714	8196478	12795145	1151128
2	9921878	8156294	11035047	9189747	1131649	9470275	12186475	11044931	10007378	8023939
3	10749381	8687163	13301593	10991939	1466142	12352535	14892195	12258931	13278511	1061870
4	12644474	9129417	16154021	12095545	1706010	12738553	16093137	11801667	15292751	1113164

Inc. P (d)	GEL 2							
	Intensity 1	Intensity 2	Intensity 3	Intensity 4	Intensity 5	Avg	STD	COV
0	N.D	113,387	71,530	165,921	106,723	114,390	38953.87	34.05
1	1,043,021	1,119,552	1,213,403	1,850,236	1,283,860	1,302,014	319804.07	24.56
2	1,765,584	1,845,300	1,846,223	1,141,544	1,983,439	1,716,418	330778.45	19.27
3	2,062,218	2,309,654	2,308,887	2,633,264	2,659,809	2,394,766	251165.54	10.49
4	3,515,057	4,058,476	4,321,547	4,291,470	4,160,587	4,069,427	327346.81	8.04

f) Intensity Gel 3, Run 2

Inc. P (d)	**IMAGE 1**		**IMAGE 2**		**IMAGE 3**		**IMAGE 4**		**IMAGE 5**	
	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)
0	4148846	4034978	4319124	4218293	4978629	4774081	4643891	4517032	4343523	4218694
1	6171656	4391891	9493953	7494268	8363202	6460195	8288891	6502528	6997288	4907564
2	7795406	6002572	6957014	4847645	6861082	4943798	6307021	4419956	9119592	7018120
3	9660802	7031185	10448649	7771693	1034529	7664814	9116591	6544451	9328544	6757942
4	1345703	9397480	13711317	9789964	1233142	8469949	1255054	8649171	11730631	7769503

Inc. P (d	GEL 3							
	Intensity 1	Intensity 2	Intensity 3	Intensity 4	Intensity 5	Avg	STD	COV
0	113,868	100,831	N.D	126,859	124,829	116,597	11959.50	10.26
1	1,779,765	1,999,685	1,903,007	1,786,363	2,089,724	1,911,709	134745.76	7.05
2	1,792,834	2,109,369	1,917,284	1,887,065	2,101,472	1,961,605	139105.34	7.09
3	2,629,617	2,676,956	2,680,476	2,572,140	2,570,602	2,625,958	53728.62	2.04
4	4,059,557	3,921,353	3,861,471	3,901,376	3,961,128	3,940,977	75401.96	1.91

g) Intensity

Inc.P (d)	RUN 1					
	Gel 1	Gel 2	Gel 3	Avg	STD	COV
0	125,555	177,253	162,698	55,168	26659.06	17.18
1	921,643	873,878	569,516	788,345	191010.99	24.23
2	977,317	1,021,498	1,413,343	1,137,386	240004.45	21.10
3	1,525,636	1,363,591	1,881,842	1,590,356	265117.80	16.67
4	1,621,637	1,727,836	2,506,599	1,952,024	483202.65	24.75
Inc.P (d)	RUN 2					
	Gel 1	Gel 2	Gel 3	Avg	STD	COV
0	303,157	114,390	116,597	178,048	108353.18	60.85
1	1,258,628	1,302,014	1,911,709	1,490,784	365176.58	24.49
2	1,657,152	1,716,418	1,961,605	1,778,392	161410.80	9.07
3	2,016,698	2,394,766	2,625,958	2,345,807	307566.83	13.11
4	5,145,162	4,069,427	3,940,977	4,385,189	661282.10	15.08

Avg Run1	Avg Run2	Avg	STD	COV
155,168	178,048	166,608	71676.28	43.02
788,345	1,490,784	1,139,565	464716.65	40.78
1,137,386	1,778,392	1,457,889	395889.75	27.15
1,590,356	2,345,807	1,968,082	486996.79	24.74
1,952,024	4,385,189	3,168,606	1429824.78	45.124

h) Amount 10 uL

Inc.P (d)	RUN 1					
	Amount Gel 1	Amount Gel 2	Amount Gel 3	Avg	STD	COV
0	0.4238	0.4248	0.4245	0.4244	0.0004	0.1163
1	0.4386	0.4377	0.4321	0.4361	0.0035	0.8110
2	0.4396	0.4404	0.4477	0.4426	0.0044	1.0042
3	0.4498	0.4468	0.4564	0.4510	0.0049	1.0886
4	0.4515	0.4535	0.4679	0.4577	0.0089	1.9551
Inc.P (d)	RUN 2					
	Amount Gel 1	Amount Gel 2	Amount Gel 3	Avg	STD	COV
0	0.4271	0.4236	0.4237	0.4248	0.0020	0.0047
1	0.4448	0.4456	0.4569	0.4491	0.0067	0.0150
2	0.4522	0.4533	0.4578	0.4544	0.0029	0.0065
3	0.4589	0.4659	0.4701	0.4650	0.0056	0.0122
4	0.5168	0.4969	0.4945	0.5027	0.0122	0.0243

Avg Run1	Avg Run2	Avg	STD	COV
0.4244	0.4248	0.4246	0.0013	0.3126
0.4361	0.4491	0.4427	0.0086	1.9442
0.4426	0.4544	0.4486	0.0073	1.6345
0.4510	0.4650	0.4580	0.0090	1.9692
0.4577	0.5027	0.4802	0.0265	5.5140

i) Concentration

Inc.P	RUN 1					
(d)	Conc Gel 1	Conc Gel 2	Conc Gel 3	AVG	STD	COV
0	0.0423	0.042	0.0424	0.0424	4.9373	0.1163
1	0.0438	0.0437	0.0432	0.0436	0.0003	0.8110
2	0.0439	0.0440	0.0447	0.0442	0.0004	1.0042
3	0.0449	0.0446	0.0456	0.0451	0.0005	1.0886
4	0.0451	0.0453	0.0467	0.0457	0.0008	1.9551
Inc.P	RUN 2					
(d)	Conc Gel 1	Conc Gel 2	Conc Gel 3	AVG	STD	COV
0	0.0427	0.0423	0.0423	0.0424	0.0002	0.4723
1	0.0444	0.0445	0.0456	0.0449	0.0006	1.5057
2	0.0452	0.0453	0.0457	0.0454	0.0002	0.6577
3	0.0458	0.0465	0.0470	0.0465	0.0005	1.2249
4	0.0516	0.0496	0.0494	0.0502	0.0012	2.4359

Avg Run1	Avg Run2	Avg	STD	COV
0.0424	0.0424	0.0425	0.0001	0.3126
0.0436	0.0449	0.0443	0.0009	1.9442
0.0442	0.0454	0.0449	0.0007	1.6345
0.0451	0.0465	0.0458	0.0009	1.9692
0.0457	0.0502	0.0480	0.0026	5.5140

j) Amount sample

Inc.P	RUN 1						
(d	volume (uL)	Gel 1	Gel 2	Gel 3	AVG	STD	COV
0	750	31.7914	31.8632	31.8430	31.8326	0.0370	0.1163
1	1000	43.8629	43.7745	43.2108	43.6161	0.3538	0.8111
2	900	39.5694	39.6431	40.2962	39.8363	0.4000	1.0042
3	750	n.d	n.d	n.d	n.d	n.d	n.d
4	900	40.6434	40.8204	42.1185	41.1941	0.8054	1.9552
Inc.P	RUN 1						
(d)	volume (uL)	Gel 1	Gel 2	Gel 3	AVG	STD	COV
0	900	38.4457	38.1311	38.1348	38.2372	0.1806	0.4723
1	900	40.0383	40.1107	41.1269	40.4253	0.6087	1.5057
2	900	40.7026	40.8014	41.2101	40.9047	0.2690	0.6577
3	900	41.3019	41.9321	42.3175	41.8505	0.5127	1.2250
4	900	46.5165	44.7235	44.5094	45.2498	1.1022	2.4359

Avg Run1	Avg Run2	Avg	STD	COV
31.8326	38.2372	35.0349	3.5099	10.0183
43.6161	40.4253	42.0207	1.8035	4.2919
39.8363	40.9047	40.3705	0.6599	1.6346
#DIV/0!	41.8505	41.8505	0.5127	1.2250
41.1941	45.2498	43.2220	2.3833	5.5140

k) Absorbance Sample

Inc.P (d)	RUN 1								
	Absorbance Measure 1	Absorbance -blank Measure 1	Absorbance Measure 2	Absorbance -blank Measure 2	Absorbance Measure 3	Absorbance -blank Measure 3	Avg	Std	COV
0	1.0704	0.7378	1.0519	0.7193	1.2186	0.886	0.7810	0.0913	11.6990
1	1.2137	0.8811	1.3148	0.9822	1.186	0.8534	0.9056	0.0677	7.4866
2	1.1901	0.8575	1.3533	1.0207	1.2832	0.9506	0.9429	0.0818	8.6824
3	1.4044	1.0718	1.375	1.0424	1.3817	1.0491	1.0544	0.0154	1.4613
4	1.4363	1.1037	1.6074	1.2748	1.3642	1.0316	1.1367	0.1249	10.9891

Avg Run1	Avg Run2	Avg	STD	COV
0.7810	0.8359	0.8084	0.0388	4.8016
0.9056	1.0256	0.9655	0.0848	8.7901
0.9429	0.9503	0.9466	0.0052	0.5502
1.0544	1.0723	1.0633	0.0126	1.1902
1.1367	1.1128	1.1247	0.0168	1.5004

l) Total amount of protein in 20 uL

Inc.P (d)	RUN 1					
	Total GFP amount	Total GFP amount	Total GFP amount	Avg	STD	COV
0	8.8571	8.6350	10.6362	9.3761	1.0969	11.6990
1	10.5774	11.7911	10.2448	10.8711	0.8138	7.4866
2	10.2941	12.2533	11.4117	11.3197	0.9828	8.6824
3	12.8667	12.5138	12.5942	12.6582	0.1849	1.4613
4	13.2496	15.3037	12.3841	13.6458	1.4995	10.9891

Inc.P (d)	RUN 2					
	Total GFP amount	Total GFP amount	Total GFP amount	Avg	STD	COV
0	9.8583	9.6506	10.5966	10.0352	0.4971	4.9542
1	13.1236	12.0276	11.7851	12.3121	0.7131	5.7925
2	10.5966	10.8295	12.7983	11.4081	1.2095	10.6023
3	12.8595	12.6818	13.0780	12.8731	0.1984	1.5414
4	14.1260	12.4393	13.5126	13.3593	0.8537	6.3904

Avg Run1	Avg Run2	Avg	STD	COV
9.3761	10.0352	9.7056	0.8428	8.6845
10.8711	12.3121	11.5916	1.0446	9.0122
11.3197	11.4081	11.3639	0.9868	8.6842
12.6582	12.8731	12.7657	0.2080	1.6298
13.6458	13.3593	13.5026	1.1025	8.1655

m) Concentration (ug/uL)

Inc.P (d)	RUN 1					
	Concentration	Concentration	Concentration	Avg	STD	COV
0	0.4428	0.4317	0.5318	0.4688	0.0548	11.6990
1	0.5288	0.5895	0.5122	0.5435	0.0406	7.4866
2	0.5147	0.6126	0.5705	0.5659	0.0491	8.6824
3	0.6433	0.6256	0.6297	0.6329	0.0092	1.4613
4	0.6624	0.7651	0.6192	0.6822	0.0749	10.9891
Inc.P (d))	RUN 2					
	Concentration	Concentration	Concentration	Avg	STD	COV
0	0.4929	0.4825	0.5298	0.5017	0.0248	4.9542
1	0.6561	0.6013	0.5892	0.6156	0.0356	5.7925
2	0.5298	0.5414	0.6399	0.5704	0.0604	10.6023
3	0.6429	0.6340	0.6539	0.6436	0.0099	1.5414
4	0.7063	0.6219	0.6756	0.6679	0.0426	6.3904

Avg Run1	Avg Run2	Avg	STD	COV
0.4688	0.5017	0.4852	0.0421	8.6845
0.5435	0.6156	0.5795	0.0522	9.0122
0.5659	0.5704	0.5681	0.0493	8.6842
0.6329	0.6436	0.6382	0.0104	1.6298
0.6822	0.6679	0.6751	0.0551	8.1655

n) Purity

Inc.P (d))	RUN 1					
	Gel 1	Gel 2	Gel 3	Avg	STD	COV
0	9.57	9.84	7.98	9.13	1.0033	10.9877
1	8.294	n.d	8.44	8.36	0.1003	1.1994
2	8.542	7.19	7.85	7.86	0.6762	8.6048
3	n.d	n.d	n.d	n.d	n.d	n.d
4	6.817	5.93	7.56	6.77	0.8162	12.0621
Inc.P (d))	RUN 2					
	Gel 1	Gel 2	Gel 3	Avg	STD	COV
0	8.67	8.78	8.00	8.4812	0.4230	4.988
1	n.d	7.41	7.75	7.5829	0.2433	3.209
2	8.54	8.37	7.16	8.0212	0.7541	9.403
3	7.14	7.35	7.19	7.2251	0.1093	1.514
4	7.32	7.99	7.32	7.5423	0.3873	5.135

Avg Run1	Avg Run2	Avg	STD	COV
9.13	8.4812	8.806	0.7753	8.80
8.36	7.5829	7.974	0.4762	5.97
7.86	8.0212	7.940	0.6467	8.15
n.d	7.2251	7.225	0.1093	1.51
6.77	7.5423	7.155	0.7118	9.95

Appendix C: Data for effect of number of cycle

a) Intensity Gel 1, Run 1

N.of .cy	IMAGE 1		IMAGE 2		IMAGE 3		IMAGE 4		IMAGE 5	
	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (backgroun d)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)
0	17625331	16646768	19412688	18496287	13734157	12840576	24436210	22906341	22525683	21425949
1	13576770	12663878	14069172	13070921	14101036	13345280	19164681	18446393	17093503	16118024
2	15563357	13837379	15331538	13599372	13037395	11608594	21217349	19179295	18241225	16403230
3	16709221	15404503	15314444	13996464	12345626	11137134	20349741	18792747	16851377	15293062
4	15043256	12721008	17529519	14684043	12732385	10470235	16562683	13475684	18514860	15664534

N.of . cy	GEL 1							
	Intensity 1	Intensity 2	Intensity 3	Intensity 4	Intensity 5	Avg	STD	COV
0	978,563	916,401	893,581	1,529,869	1,558,315	1,175,346	338200.8981	28.7745
1	912,892	998,251	755,756	718,288	975,479	872,133	127925.1069	14.6680
2	1,725,978	1,732,166	1,428,801	2,038,054	1,837,995	1,752,599	220651.5147	12.5899
3	1,304,718	1,317,980	1,208,492	1,556,994	1,558,315	1,389,300	159389.0977	11.4726
4	n.d	2,845,476	1,951,658	3,086,999	2,850,326	2,683,615	500823.0699	18.6622

b) Intensity Gel 2, Run 1

N.of cy.	IMAGE 1		IMAGE 2		IMAGE 3		IMAGE 4		IMAGE 5	
	IDV (sample)	IDV (background)	IDV (sample)	IDV] (background)	IDV (sample)	IDV (backgroun d)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)
0	23338778	21348237	23023684	20852830	23799929	21426568	23255021	21339820	27860234	25340738
1	14439361	13273402	23097130	21658634	22485803	21065557	21835387	20243913	19447315	17913577
2	15301399	13744569	21330929	19578116	21485521	19628440	21862408	19969265	19574686	17574047
3	16081306	14744278	21412598	19994022	19935618	18489181	19017150	17486971	18618651	17105801
4	14186051	3166955	20237337	19470529	13075683	11854376	14060515	12872210	18845663	17677474

N.of. cy	GEL 2							
	Intensity 1	Intensity 2	Intensity 3	Intensity 4	Intensity 5	Avg	STD	COV
0	1,990,541	2,170,854	2,373,361	1,915,201	2,519,496	2,193,891	253859.2813	11.5711
1	1,165,959	1,438,496	1,420,246	1,591,474	1,533,738	1,429,983	163340.439	11.4225
2	1,556,830	1,752,813	1,857,081	1,893,143	2,000,639	1,812,101	167940.4165	9.2677
3	1,337,028	1,418,576	1,446,437	1,530,179	1,512,850	1,449,014	77680.06355	5.3608
4	n.d	766,808	1,221,307	1,188,305	1,168,189	1,086,152	214019.2897	19.7043

c) Intensity Gel 3, Run 1

N.of. cy.	**IMAGE 1**		**IMAGE 2**		**IMAGE 3**		**IMAGE 4**		**IMAGE 5**	
	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)
0	21644743	19146158	23058679	20531013	23852384	21319925	21793464	19321078	22262153	20233419
1	22711871	20915329	22451850	20634926	24682881	22804381	22027822	20384741	21500588	19998554
2	23622220	21311889	23910035	21629339	25113435	22601246	20638048	18505714	18798606	16779910
3	22229316	20716791	25091439	23533647	21611950	20209918	20439866	18123640	18354220	17211516
4	21775063	17878516	21588378	17338092	25340752	20433578	18623360	14927492	21274654	18011403

N.of. cy	GEL 3							
	Intensity 1	Intensity 2	Intensity 3	Intensity 4	Intensity 5	Avg	STD	COV
0	2,498,585	2,527,666	2,532,459	2,472,386	2,028,734	2,411,966	215595.2495	8.9386
1	1,796,542	1,816,924	1,878,500	1,643,081	1,502,034	1,727,416	152910.0219	8.8520
2	2,310,331	2,280,696	2,512,189	2,132,334	2,018,696	2,250,849	187528.9097	8.3315
3	1,512,525	1,557,792	1,402,032	n.d	1,142,704	1,403,763	185929.1113	13.2450
4	3,896,547	4,250,286	4,907,174	3,695,868	3,263,251	4,002,625	618783.3174	15.4594

d) Intensity Gel 1, Run 2

N.of .cy	IMAGE 1		IMAGE 2		IMAGE 3		IMAGE 4		IMAGE 5	
	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)
0	23055831	19990590	24212763	21263373	21439170	18536182	31714094	27864034	24819399	21395073
1	23609889	21545953	22847721	20634918	17867104	15765971	26427359	23879361	21500107	18946768
2	24793898	23023815	22072184	20086184	17598354	15882892	24027032	21967258	24990741	22809047
3	21202206	19079390	21735069	19355268	18401246	16130780	18700089	16164303	22162669	19679865
4	19531724	17220669	14828212	12524512	14576060	12274535	13321578	11002126	15177570	12707063

N.of. cy	GEL 1							
	Intensity 1	Intensity 2	Intensity 3	Intensity 4	Intensity 5	Avg	STD	COV
0	3,065,241	2,949,390	2,902,988	3,850,060	3,424,326	3,238,401	356288.7768	11.0020
1	2,063,936	2,212,803	2,101,133	2,547,998	2,553,339	2,295,842	213763.8025	9.3109
2	1,770,083	1,986,000	1,715,462	2,059,774	2,181,694	1,942,603	175575.0742	9.0381
3	2,122,816	2,379,801	2,270,466	2,535,786	2,482,804	2,358,335	148749.6817	6.3074
4	2,311,055	2,303,700	2,301,525	2,319,452	2,470,507	2,341,248	64934.33757	2.7734

e) Intensity Gel 2, Run 2

N.of .cy	IMAGE 1		IMAGE 2		IMAGE 3		IMAGE 4		IMAGE 5	
	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)
0	17507684	16114489	20392687	18910647	18993351	17528182	15158390	13570407	19778414	18229602
1	18781131	17490129	18305596	16950041	18883815	17409051	17187413	15743973	15532434	14054619
2	19855144	18402821	22707330	21253096	17921631	16369264	19036632	17588398	19639340	17985464
3	15090151	13612180	20048811	18339173	17094422	15332633	14843171	13131745	19158160	17297799
4	14412998	11894819	19297540	16252195	20426901	17335992	18246446	15034809	26875841	21995185

N.of. cy	GEL 2							
	Intensity 1	Intensity 2	Intensity 3	Intensity 4	Intensity 5	Avg	STD	COV
0	1,393,195	1,482,040	1,465,169	1,587,983	1,548,812	1,495,440	75767.2599	5.0665
1	1,291,002	1,355,555	1,474,764	1,443,440	1,477,815	1,408,515	82196.7797	5.8357
2	1,452,323	1,454,234	1,552,367	1,448,234	1,653,876	1,512,207	90446.7219	5.9811
3	1,477,971	1,709,638	1,761,789	1,711,426	1,860,361	1,704,237	140502.7503	8.2443
4	2,518,179	3,045,345	3,090,909	3,211,637	n.d	2,966,518	307016.8290	10.3494

f) Intensity Gel 3, Run 2

N.of .cy	**IMAGE 1**		**IMAGE 2**		**IMAGE 3**		**IMAGE 4**		**IMAGE 5**	
	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)	IDV (sample)	IDV (background)
0	17545455	16257062	22926971	21774659	18495055	16991121	18053530	16576448	22668448	21124234
1	19198386	17275904	20940486	18968584	18957555	17012319	17364397	15401641	20706125	18606142
2	21230547	18951830	19496566	17257838	18522904	16211263	17960077	15674202	19057011	16950600
3	34304532	31635778	25625652	23559942	21815511	19868156	25458301	23237044	26274697	23631865
4	21621459	19149583	18121532	15583377	17348747	14652640	16730282	14159249	17470977	14753048

N.of. cy	GEL 3							
	Intensity 1	Intensity 2	Intensity 3	Intensity 4	Intensity 5	Avg	STD	COV
0	1,288,393	1,152,312	1,503,934	1,477,082	1,544,214	1,393,187	166669.5622	11.9631
1	1,922,482	1,971,902	1,945,236	1,962,756	2,099,983	1,980,472	69416.4902	3.5050
2	2,278,717	n.d	2,311,641	2,285,875	2,106,411	2,245,661	93903.8299	4.1815
3	2,668,754	2,065,710	1,947,355	2,221,257	2,642,832	2,309,182	331112.7241	14.3389
4	2,471,876	2,538,155	2,696,107	2,571,033	2,717,929	2,599,020	105141.1587	4.0454

g) Intensity

N.of. cy	RUN 1					
	Gel 1	Gel 2	Gel 3	Avg	STD	COV
0	1,175,346	2,193,891	2,411,966	1,927,067	660078.2667	34.2529
1	872,133	1,429,983	1,727,416	1,343,177	434198.8234	32.3262
2	1,752,599	1,812,101	2,250,849	1,938,516	272119.3991	14.0375
3	1,389,300	1,449,014	1,403,763	1,414,026	31151.7988	2.2030
4	2,683,615	1,086,152	4,002,625	2,590,797	1460450.2430	56.3706
N.of. cy	RUN 2					
	Gel 1	Gel 2	Gel 3	Avg	STD	COV
0	3,238,401	1,495,440	1,393,187	2,042,343	1037077.9530	50.7788
1	2,295,842	1,408,515	1,980,472	1,894,943	449803.8617	23.7370
2	1,942,603	1,512,207	2,245,661	1,900,157	368564.7844	19.3965
3	2,358,335	1,704,237	2,309,182	2,123,918	364284.1512	17.1515
4	2,341,248	2,966,518	2,599,020	2,635,595	314235.3463	11.9227

Avg Run1	Avg Run2	Avg	STD	COV
1,927,067	2,042,343	1,984,705	780051.3487	39.3
1,343,177	1,894,943	1,619,060	497669.0026	30.7
1,938,516	1,900,157	1,919,337	290511.6603	15.1
1,414,026	2,123,918	1,768,972	452386.3037	25.6
2,590,797	2,635,595	2,613,196	945127.2699	36.2

h) Amount 10uL

N.of. cy	RUN 1					
	Amount Gel 1	Amount Gel 2	Amount Gel 3	Avg	STD	COV
0	0.4433	0.4621	0.4662	0.4572	0.0122	2.6736
1	0.4377	0.4480	0.4535	0.4464	0.0080	1.8013
2	0.4540	0.4551	0.4632	0.4574	0.0050	1.1017
3	0.4472	0.4483	0.4475	0.4477	0.0005	0.1289
4	0.4712	0.4416	0.4956	0.4695	0.0270	5.7605
N.of. cy	RUN 2					
	Amount Gel 1	Amount Gel 2	Amount Gel 3	Avg	STD	COV
0	0.4815	0.4492	0.4473	0.4593	0.0192	0.0418
1	0.4640	0.4476	0.4582	0.4566	0.0083	0.0182
2	0.4575	0.4495	0.4631	0.4567	0.0068	0.0149
3	0.4652	0.4531	0.4643	0.4608	0.0067	0.0144
4	0.4649	0.4765	0.4696	0.4703	0.0058	0.0123

Avg Run1	Avg Run2	Avg	STD	COV
0.4572	0.4593	0.4583	0.0144	3.152
0.4464	0.4566	0.4515	0.0092	2.041
0.4574	0.4567	0.4571	0.0053	1.177
0.4477	0.4608	0.4543	0.0083	1.844
0.4695	0.4703	0.4700	0.0175	3.725

i) Concentration

N.of. cy	RUN 1					
	Conc Gel 1	Conc Gel 2	Conc Gel 3	Avg	STD	COV
0	0.0443	0.0462	0.0466	0.0457	0.0012	2.6735
1	0.0437	0.0448	0.0453	0.0446	0.0008	1.8012
2	0.0454	0.0455	0.0463	0.0457	0.0005	1.1016
3	0.0447	0.0448	0.0447	0.0447	0.0001	0.1288
4	0.0471	0.0441	0.0495	0.0469	0.0027	5.7605
N.of. cy	RUN 2					
	Conc Gel 1	Conc Gel 2	Conc Gel 3	Avg	STD	COV
0	0.0481	0.0449	0.0447	0.0459	0.0019	4.1810
1	0.0464	0.0447	0.0458	0.0456	0.0008	1.8242
2	0.0457	0.0449	0.0463	0.0456	0.0006	1.4944
3	0.0465	0.0453	0.0464	0.0460	0.0006	1.4638
4	0.0464	0.0476	0.0469	0.0470	0.0005	1.2372

AVG Run1	Avg Run2	Avg	STD	COV
0.0457	0.0459	0.0458	0.0014	3.1521
0.0446	0.0456	0.0452	0.0009	2.0412
0.0457	0.0456	0.0457	0.0005	1.1770
0.0447	0.0460	0.0454	0.0008	1.8441
0.0469	0.0470	0.0470	0.0017	3.7246

j) Amount

N. of. cy	RUN 1						
	volume (uL)	Gel 1	Gel 2	Gel 3	Avg	STD	COV
0	1000	44.3328	n.d	n.d	44.3328	n.d	n.d
1	1000	43.7712	44.8044	45.3553	44.6436	0.8042	1.8013
2	1000	45.4019	45.5121	46.3247	45.7462	0.5040	1.1017
3	1000	44.7291	44.8397	44.7558	44.7749	0.0577	0.1289
4	1000	47.1262	n.d	49.5690	48.3476	1.7274	3.5728
N. of. cy	RUN 2						
	volume (uL)	Gel 1	Gel 2	Gel 3	Avg	STD	COV
0	1000	n.d	44.9256	44.7363	44.8309	0.1339	0.2987
1	1000	46.4080	44.7646	45.8239	45.6655	0.8331	1.8242
2	1000	45.7538	44.9567	46.3151	45.6752	0.6826	1.4945
3	1000	46.5237	45.3123	46.4327	46.0896	0.6747	1.4638
4	1000	46.4921	47.6501	46.9695	47.0372	0.5820	1.2373

Avg Run1	Avg Run2	Avg	STD	COV
44.3328	44.8309	44.6649	0.3028	0.6779
44.6436	45.6655	45.1546	0.9217	2.0412
45.7462	45.6752	45.7107	0.5380	1.1770
44.7749	46.0896	45.4322	0.6747	1.4850
48.3476	47.0372	47.5614	1.1960	2.5146

k) Absorbance sample

N.of. cy	RUN 1								
	Absorbance Measure 1	Absorbance -blank Measure 1	Absorbance Measure 2	Absorbance -blank Measure 2	Absorbance Measure 3	Absorbance -blank Measure 3	Avg	Std	COV
0	1.0465	0.7628	0.9013	0.6176	0.7377	0.4540	0.6115	0.1545	25.2657
1	0.7068	0.4231	0.7552	0.4715	0.7930	0.5093	0.4680	0.0432	9.2332
2	0.8909	0.6072	0.8957	0.6120	0.8097	0.5260	0.5817	0.0483	8.3073
3	0.7144	0.4307	0.7444	0.4607	0.6936	0.4099	0.4338	0.0255	5.8876
4	1.0343	0.7506	1.0327	0.749	0.7750	0.4913	0.6636	0.1492	22.4894

N.of. cy	RUN 2								
	Absorbance Measure 1	Absorbance -blank Measure 1	Absorbance Measure 2	Absorbance -blank Measure 2	Absorbance Measure 3	Absorbance -blank Measure 3	Avg	Std	COV
0	0.7612	0.4775	0.8122	0.5285	0.7583	0.4746	0.4935	0.0303	6.1427
1	0.7930	0.5093	0.7913	0.5076	0.8217	0.5380	0.5183	0.0170	3.2957
2	0.8097	0.5260	0.7530	0.4693	0.7448	0.4611	0.4854	0.0353	7.2799
3	0.6936	0.4099	0.9331	0.6494	0.8233	0.5396	0.5329	0.1198	22.4944
4	0.7750	0.4913	0.9083	0.6246	0.7659	0.4822	0.5327	0.0797	14.9648

Avg Run1	Avg Run2	Avg	STD	COV
0.6115	0.4935	0.5525	0.0833	15.0934
0.4680	0.5183	0.4931	0.0355	7.2173
0.5817	0.4854	0.5336	0.0680	12.7568
0.4338	0.5329	0.4833	0.0701	14.5117
0.6636	0.5327	0.5981	0.0925	15.4779

l) Total amount of protein in 20 uL

N.of. cy	RUN 1					
	Total GFP amount	Total GFP amount	Total GFP amount	Avg	STD	COV
0	8.2375	6.6695	4.9028	6.6033	1.6683	25.2657
1	4.5691	5.0917	5.5000	5.0536	0.4666	9.2332
2	6.5572	6.6090	5.6803	6.2822	0.5218	8.3072
3	4.6511	4.9751	4.4265	4.6843	0.2757	5.8876
4	8.1058	8.0885	5.3056	7.1666	1.6117	22.4894

N.of. cy	RUN 2					
	Total GFP amount	Total GFP amount	Total GFP amount	Avg	STD	COV
0	5.1565	5.7073	5.1252	5.3297	0.3273	6.1427
1	5.5000	5.4816	5.8099	5.5971	0.1844	3.2957
2	5.6803	5.0680	4.9794	5.2426	0.3816	7.2799
3	4.4265	7.0129	5.8272	5.7555	1.2946	22.4944
4	5.3056	6.7451	5.2073	5.7526	0.8608	14.9648

Avg Run1	Avg Run2	Avg	STD	COV
6.6033	5.3297	5.9665	1.2817	21.4822
5.0536	5.5971	5.3254	0.4351	8.1708
6.2822	5.2426	5.7624	0.7010	12.1654
4.6843	5.7555	5.2199	1.0223	19.5854
7.1666	5.7526	6.4596	1.391	21.5360

m) Concentration

N.of .cy	RUN 1					
	Concentration	Concentration	Concentration	Avg	STD	COV
0	0.4118	0.3334	0.2451	0.3301	0.0834	25.2657
1	0.2284	0.2545	0.2750	0.2526	0.0233	9.2332
2	0.3278	0.3304	0.2840	0.3141	0.0260	8.3072
3	0.2325	0.2487	0.2213	0.2342	0.0137	5.8876
4	0.4052	0.4044	0.2652	0.3583	0.0805	22.4894
N.of .cy	RUN 2					
	Concentration	Concentration	Concentration	Avg	STD	COV
0	0.2578	0.2853	0.2562	0.2664	0.0163	6.1427
1	0.2750	0.2740	0.2904	0.2798	0.0092	3.2957
2	0.2840	0.253	0.2489	0.2621	0.0190	7.2799
3	0.2213	0.3506	0.2913	0.2877	0.0647	22.4944
4	0.2652	0.3372	0.2603	0.2876	0.0430	14.9648

Avg Run1	Avg Run2	Avg	STD	COV
0.3301	0.2664	0.2983	0.0640	21.4822
0.2526	0.2798	0.2662	0.0217	8.17084
0.3141	0.2621	0.2881	0.0350	12.1654
0.2342	0.2877	0.2609	0.0511	19.5854
0.3583	0.2876	0.3229	0.0695	21.5360

n) Amount sample

N.of cy	RUN 1						
	volume (uL)	Total protein amount	Total protein amount	Total protein amount	Avg	STD	COV
0	1000	n.d	n.d	245.1404	245.1404	n.d	n.d
1	1000	228.4557	254.5896	275.0000	252.6818	23.3307	9.2332
2	1000	327.8618	330.4536	284.0173	314.1109	26.0940	8.3073
3	1000	232.5594	248.7581	n.d	240.6587	11.4542	4.7595
4	1000	405.2916	404.4276	265.2808	358.3333	80.5870	22.4894

N.of .cy	RUN 2						
	volume (uL)	Total protein amount	Total protein amount	Total protein amount	Avg	STD	COV
0	1000	257.8294	285.3672	256.2635	266.4867	16.3697	6.1428
1	1000	275.0000	274.0821	290.4968	279.8596	9.2235	3.2957
2	1000	284.0173	253.4017	248.9741	262.1310	19.0829	7.2799
3	1000	n.d	350.6479	291.3607	321.0043	41.9224	13.0598
4	1000	265.2808	337.2570	260.3672	287.6350	43.0441	14.9648

Avg Run1	Avg Run2	Avg	STD	COV
266.4867	245.1404	257.9482	16.4524	6.3782
279.8596	252.6818	270.3084	14.3081	5.2933
262.1310	314.1109	285.8291	32.2726	11.2909
321.0043	240.6587	282.8564	50.3698	17.8076
266.4867	245.1404	257.9482	16.4524	6.3782

o) Purity

N.of. cy	RUN 1					
	Gel 1	Gel 2	Gel 3	Avg	STD	COV
0	18.0847	n.d	n.d	18.0847	n.d	n.d
1	19.1596	17.5987	16.4928	17.7504	1.3399	7.5483
2	13.8479	13.7726	16.3105	14.6437	1.4440	9.8611
3	19.2334	18.0254	17.9917	18.4168	0.7074	3.8408
4	11.6277	n.d	12.2566	11.9422	0.4447	3.7236
N.of. cy	RUN 2					
	Gel 1	Gel 2	Gel 3	Avg	STD	COV
0	n.d	15.7431	17.4571	16.6001	1.2120	7.3012
1	16.8756	16.3326	15.7743	16.3275	0.5507	3.3726
2	16.1095	17.7413	18.6024	17.4844	1.2661	7.2415
3	13.2679	n.d	n.d	13.2679	n.d	n.d
4	17.5256	14.1287	18.0397	16.5647	2.1252	12.8297

Avg Run1	Avg Run2	Avg	STD	COV
18.0847	16.6001	17.0950	1.2121	7.0902
17.7504	16.3275	17.0389	1.2028	7.0591
14.6437	17.4844	16.0640	1.9739	12.2876
18.4168	13.2679	17.1296	2.6384	15.4028
11.9422	16.5647	14.7157	2.9526	20.0645