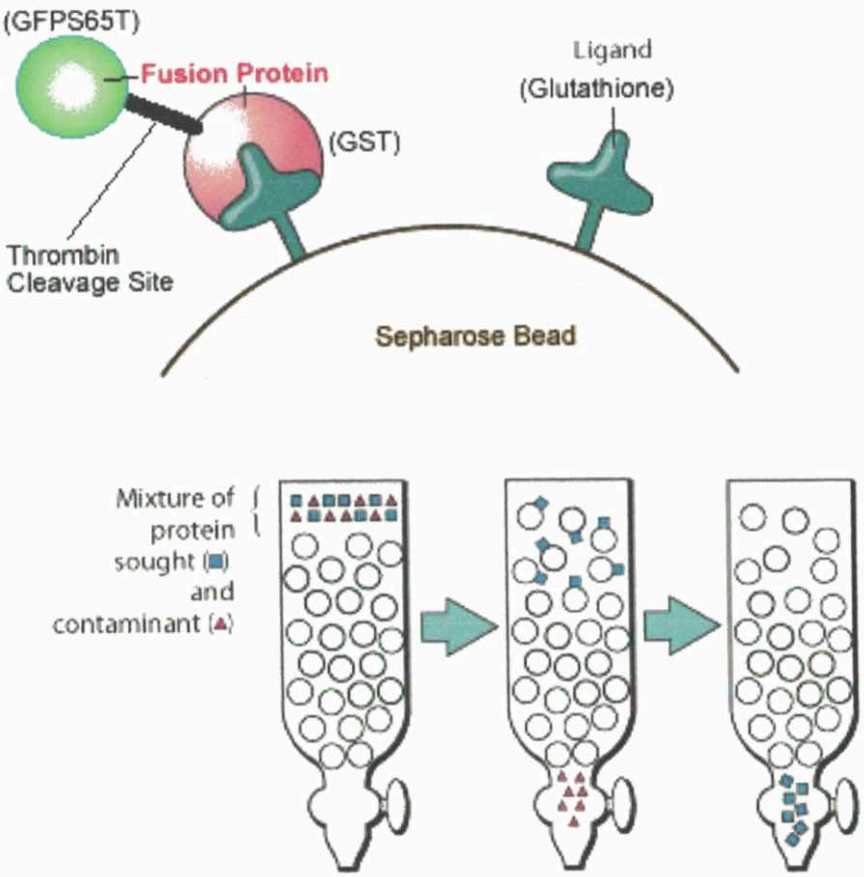
BIOC 2000 – Practical Report



A cartoon of affinity chromatography by Adrian English.

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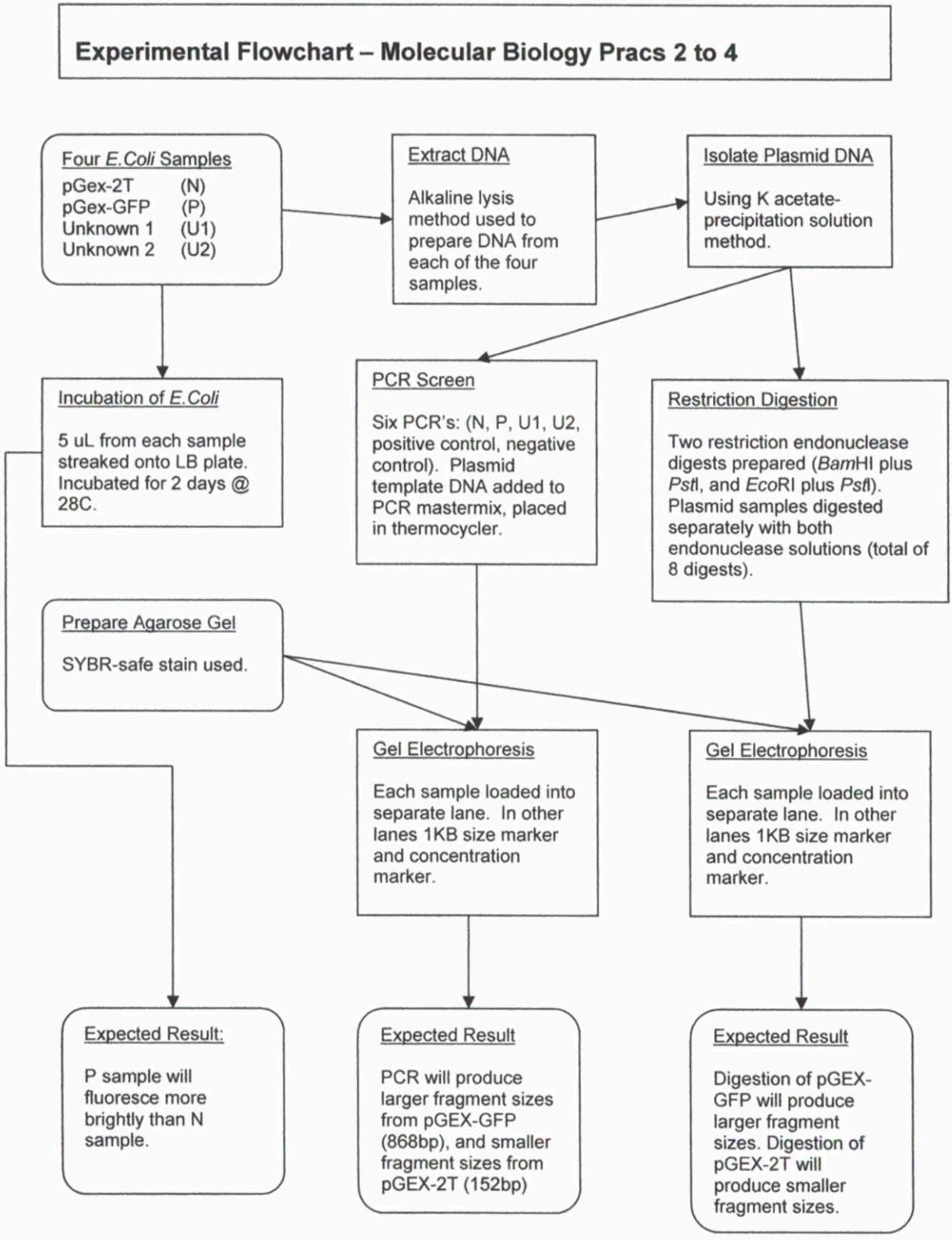
Abstract

Part one of this experimental series is concerned with production of Green Fluorescent Protein (GFP) in an *E.Coli* bacterial model. The protocols employed focus on isolation and detection of the GFP plasmid in unknown bacterial samples; necessary to confirm plasmid uptake and selection of appropriately transformed subjects. PCR and restriction digestion techniques are used in combination with electrophoretic detection methods. Transformed subjects were correctly identified and used to produce GFP for treatment in part two of the series. Part two of the experimental series is concerned with purification and quantification of GFP using affinity chromatography and spectrophotometric techniques. Highly purified GFP was successfully recovered and subsequent quantitation protocols produced generally agreeable concentration measures.

Part One Molecular Biology – Practicals 2 to 4



Introduction and Experimental Rationale

Green Fluorescent Protein (GFP) is a protein that emits a green fluorescent light (508nm) when excited by light at 395nm wavelength. GFP is encoded by a specific gene (first isolated from the jellyfish *Aequorea victoria)* that can be fused to genes that encode proteins of interest (Lodish p382). When proteins of interest are produced, the recombinant DNA also produces GFP, causing the protein of interest to display a green fluorescent colour. Colourisation aids identification and observation of the target. GFP has many possible research applications and its manipulation is thus an important technique in molecular biology.

The purpose of the following series of experiments is to confirm the existence of GFP in several *E.coli* samples, and to isolate plasmid DNA from samples so that the presence of a particular plasmid (pGEX-GFP) can be verified. Methods for DNA extraction, restriction digestion, DNA amplification (through PCR), and gel electrophoresis have been used to achieve these ends.

The second set of experiments in concerned with purification and quantitation of GFP using affinity chromatography. Protein purification is covered in detail in part two, however it is important to mention at this stage that production of GFP also includes production of an associated fusion protein partner bound to (the N-terminus of) the GFP. The fusion protein is called glutathione-S-transferase (GST) and has a binding affinity for a substrate called glutathione. This binding affinity allows efficient purification in an affinity chromatography column, explained further in part two – Protein Chemistry.

The alkaline lysis method of DNA extraction has been utilised because it allows the separation of chromosomal and plasmid DNA. This method employs detergents to break down the cell wall and uses a strong base to denature DNA. When denatured strands of chromosomal DNA re-anneal, the nucleotides are not necessarily complimentary because the strands become mixed, and as such there is a low instance of specific base pairing. Due to the circular architecture of plasmid DNA, specific base pairing can be achieved when re-annealing occurs. The differences in re-annealing result in different solubilities that can be exploited for the purpose of isolation. Alternative methods, such as glass beads or sonication, may be necessary for some organisms with tough cell walls (eg yeasts) but alone do not cause denaturing of DNA (necessary for plasmid DNA isolation).

Producing visible colonies of *E.Coli* bacteria is achieved by placing streaks from the sample liquid onto a growth medium and incubating. In this experiment, Lysogeny Broth (LB) is used. Alternatively, agar or super optimal broth (SOB) can be used as a growth medium. SOB has been shown to achieve higher plasmid transformation efficiency (Hanahan 1983). However, transformation efficiency is not crucial to this experiment and use of LB is adequate.

P-GEX, the vector plasmid used in this series of experiments, contains an ampicillin resistance gene that confers resistance to the antibiotic ampicillin. The LB contains ampicillin and eliminates bacteria from the colony that do not possess a pGEX plasmid. The LB plates also contain IPTG, a reagent used as a molecular analog of a lactose metabolite. This reagent triggers the transcription of the *lac* operon which is present in the pGEX plasmid and is the primary means of inducing expression of the GFP-GST fusion protein. IPTG can not be metabolized by *E.coli*, which ensures a relatively stable concentration and rate of expression of the *lac* controlled fusion protein gene (Hansen 1998)

The pGEX-GFP plasmid contains essentially the same architecture as the pGEX-2T plasmid, however an additional gene is present to encode for the GST-GFP fusion protein. GFPS65T indicates that a mutant variety of GFP has been used that substitutes Serine for Threonine at position 65 in the amino acid sequence of the protein (done to improve stability of fluorescence).

Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis techniques are used to determine which plasmid is present in each of the two unknown samples being tested.

Restriction enzymes (from the type II family) recognise a specific nucleotide sequence, and then cut the nucleotide's phosphodiester bonds at that same recognition site, yielding separated DNA fragments. The placement of enzyme recognition sites in relation to the location of the GFPS65T gene will produce different size fragments in digested p-GEX depending upon the presence of the GFPS65T gene. A *BamH*I and *Pst*I restriction enzyme digest will produce larger fragment sizes in pGEX-GFPS56T than in pGEX-2T.

PCR uses specific primer nucleotides that bind to particular regions of plasmid DNA that show base pair complimentarity. The polymerase binds to the 3' end of the primer sequence and extends the primer using nucleotides that match the corresponding plasmid DNA. Forward and reverse primers ensure that after the first few PCR cycles, a specific fragment of DNA has been copied from the plasmid. The target sequence then undergoes exponential amplification to yield a large quantity of the DNA fragment of interest.

Comparison of amplified fragments is performed using gel electrophoresis whereby the negatively charges DNA fragments are pulled through an agarose gel matrix using an electrical current. Distance travelled through the matrix is a function of DNA fragment size, and can be observed to infer DNA fragment size from the tested samples.

The forward primer (5'pGEX) is complimentary to the sequence 38-61 base pairs upstream of the *BamH*I recognition site, and has a primer annealing temperature (Tm) of 76°C. The reverse primer (3'pGEX) is complimentary to the sequence 52-75 base pairs upstream of the *BamH*I recognition site, and also has a primer annealing temperature (Tm) of 76°C. This combination of primers should amplify an 868bp fragment from the pGEX-GFPS65T plasmid, and a 152bp fragment from the pGEX-2T plasmid (BIOC2000 Prac manual).

94°C – denature
65°C – primer anneal.
76°C – polymerase extension

The PCR reaction consists of several steps whereby the reaction mix is heated and cooled to effect enzymatic action of the polymerase. Initially the reaction mix is heated to 94°C to denature the DNA strands. The high temperature increases the kinetic vibration of DNA molecules to the point whereby it overcomes the weak hydrogen bonding forces keeping the complimentary DNA strands together. The mix is then cooled to 72°C, approximately the primer annealing temperature (Tm) of 76°C, allowing forward and reverse primers to bind to complimentary plasmid DNA. The mix is then cooled to a temperature that allows the *Taq* polymerase to extend the primer sequences, thereby copying the relevant plasmid DNA. PCR cycles are repeated until the process is complete, at which time the mix is cooled to 4°C to pause any further reaction.

PCR is very sensitive to any possible DNA fragment contamination requiring the use of a template free negative control to protect against this type of error going undetected. The PCR machine is also initially preheated for five minutes at 94°C to lyse or denature any contaminants. DNA is added last to the reagent mix and the then maintained on ice prior to undergoing PCR to prevent premature reaction.

The aim of the DNA mini-prep protocol is to isolate plasmid DNA from samples of *E.Coli*. The experimental hypothesis purports that a single stranded chromosomal DNA will precipitate in high salt, whereas super coiled plasmid DNA remains in solution when neutralised. The difference in solubility of the two DNA types allows separation and isolation of plasmid DNA. The aim of fluorescence testing is to detect the presence of E.Coli bacteria that possess the GFP insert. The experimental hypothesis purports that *E.Coli* colonies grown on an LB plate will exhibit a green fluorescent colour if they possess the GFP gene and produce GFP. The aim of the gel electrophoresis experiment is to determine if plasmid DNA isolated from unknown *E.Coli* samples are either the pGEX-2T or the pGEX-GFP plasmid. The experimental hypothesis purports that plasmids of the same nucleotide sequence will display identically sized fragments on an electrophoresis gel when digested with the same restriction endonucleases. Similarly sized plasmids will also create similar banding patterns on an electrophoresis gel. Figure 1 below summarises the samples and expected outcomes.

Sample	Plasmid	Expected Outcome	Purpose
P	pGEX-GFPS65T	Larger fragment / High fluorescence	pGEX-GFP reference
N	pGEX-2T	Smaller fragment / Low or no fluorescence	pGEX-2T reference
U1	Unknown 1	Unknown	Determine plasmid
U2	Unknown 2	Unknown	Determine plasmid
Positive Control	pSUPERBRIGHT	Super high fluorescence	Positive Control

Figure 1: Samples used in part one of the experimental series.

Method

Figure 2 lists the experimental methods used (in chronological order) and where to find detailed method explanations in the BIOC 2000 practical manual. Figure 3 shows all the components in the Polymerase Chain Reaction for each sample. Figure 4 shows the stock reagent volumes used for the restriction endonuclease digests.

Method	BIOC2000 Prac Manual Page Number
Plasmid DNA Miniprep – Alkaline Lysis Method	31
Fluorescence Testing – Culturing using LB plate	33
PCR Screen	34
Restriction Endonuclease Digestion	35
Agarose Gel Electrophoresis	38-39

Figure 2: List of experimental methods used and location of detailed instructions in prac manual.

Component	N	P	U1	U2	TF-Neg Cntrl
Template DNA	2µl	2µl	2µl	2µl	0µl
Forward Primer	0.5µl	0.5µl	0.5µl	0.5µl	0.5µl
Reverse Primer	0.5µl	0.5µl	0.5µl	0.5µl	0.5µl
Reaction Buffer	2µl	2µl	2µl	2µl	2µl
Taq polymerase	0.8µl	0.8µl	0.8µl	0.8µl	0.8µl
dNTPs	0.4µl	0.4µl	0.4µl	0.4µl	0.4µl
Water	13.8µl	13.8µl	13.8µl	13.8µl	15.8µl
Total Volume	20µl	20µl	20µl	20µl	20µl

Figure 3: PCR components and quantities.

***BamH*I + *Pst*I Digests**			
Component	Final Concentration	1 Reaction (20 µl)	Master Mix (4.5 reactions; 67.5 µl)
10x *BamH*I digestion buffer	1 x	2.0 µl	9 µl
100 mg/ml BSA	1 mg/ml	0.2 µl	0.9 µl
*BamH*I 20 U/µl	10 U / 20 µl	0.5 µl	2.25 µl
*Pst*I 20 U/µl	10 U / 20 µl	0.5 µl	2.25 µl
Plasmid template	100 ng / µl	5.0 µl	-
Sterile H2O	-	11.8 µl	53.1 µl
Final Volume	-	20.0 µl	67.5 µl

***EcoR*I + *Pst*I Digests**			
Component	Final Concentration	1 Reaction (20 µl)	Master Mix (4.5 reactions; 67.5 µl)
10x *EcoR*I digestion buffer	1 x	2.0 µl	9 µl
100 mg/ml BSA	1 mg/ml	0.2 µl	0.9 µl
*EcoR*I 20 U/µl	10 U / 20 µl	0.5 µl	2.25 µl
*Pst*I 20 U/µl	10 U / 20 µl	0.5 µl	2.25 µl
Plasmid template	100 ng / µl	5.0 µl	-
Sterile H2O	-	11.8 µl	53.1 µl
Final Volume	-	20.0 µl	67.5 µl

Figure 4: Restriction endonuclease digest reagent volumes.

Results

Plasmid DNA Miniprep: Each sample (P, N, U1, U2) underwent the alkaline lysis protocol, producing a bacterial lysate mixture that was precipitated using the K acetate precipitation solution. After the K acetate pptn solution was added and the mixture was spun, each sample separated into three distinct layers. The bottom layer appeared milky white, and the top two layers appeared liquid with a distinct separation. The top layer, containing the plasmid DNA, was removed and underwent further centrifuging which produced a small white pellet that was barely visible on the bottom side of the tube. The appearance of the white pellet indicated that DNA was precipitated from the solution (confirmed by further testing explained below).

Fluorescence Testing: After incubation, the plate was observed under blue light (Figure 5). A reference diagram (Figure 6) has been provided to show the streak locations for each of the samples before incubation. Colonies were scored for brightness on a scale of 0 to 5 (Figure 7).

Figure 5: Incubated LB plate observed under blue light.

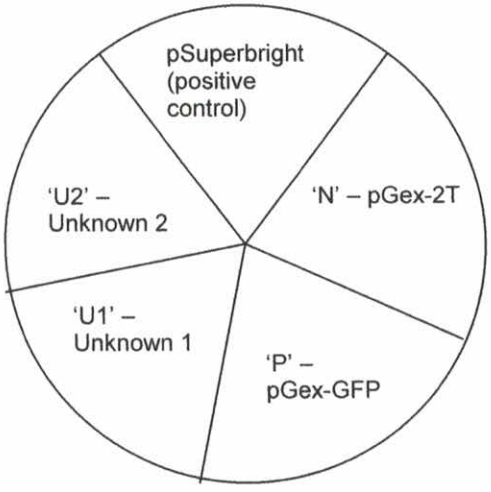


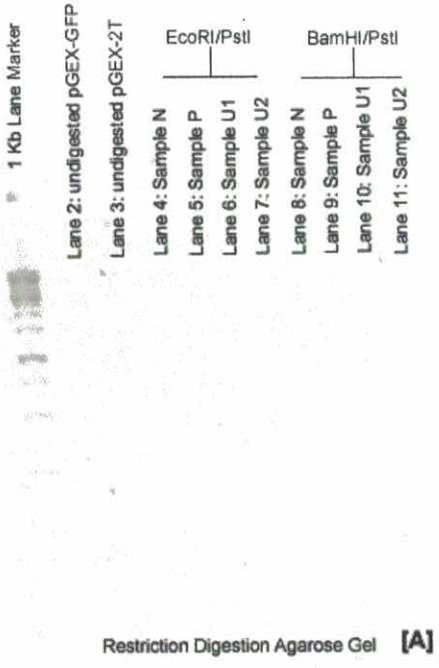
Figure 6: Diagram showing streak locations for each sample.

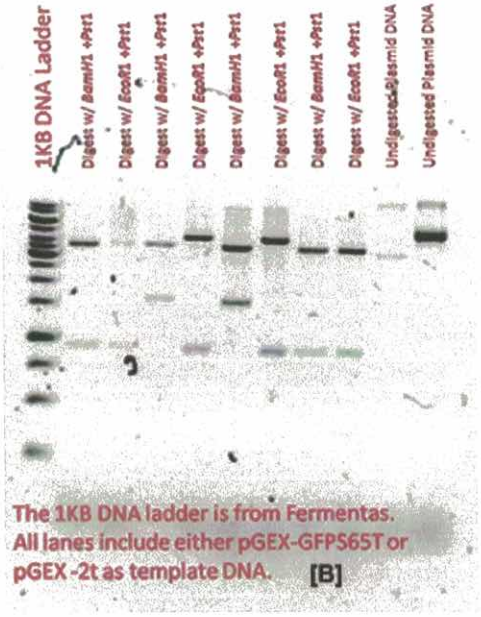
Fluorescence testing showed three distinct levels of brightness. The pSuperbright positive control displayed the highest level of brightness, samples P and U1 showed a medium level of brightness, and samples N and U2 showed a low level of brightness (summarised in Figure 7 below).

Clone Number	PCR Result (Product size indication)	Fluorescence (Brightness on a scale of 0 – 5)	Restriction Fragment Sizes (Product size indication)	Conclusion
N – pGEX-2T	~150bp	1	971bp and 3977bp	pGEX-2T
P – pGEX-GFP	~900bp	3	961bp and 4703bp	pGEX-GFP
U1 – Unknown 1	~900bp	3	961bp and 4703bp	pGEX-GFP
U2 – Unknown 2	~150bp	1	971bp and 3977bp	pGEX-2T

Figure 7: Collated results from PCR, Restriction digest, and fluorescence analysis.

Gel electrophoresis was performed using a 1% agarose gel, run at 100V for 1 hour. Photos of the agarose gels from PCR and restriction digestion products (with lanes samples marked) are shown below in Figure 8. The restriction digestion gel result was not as expected and indicated experimental error because there was not a visible quantity of DNA in any of the lanes. A photo of the expected result (a successful gel electrophoresis of restriction digestion products) is shown.





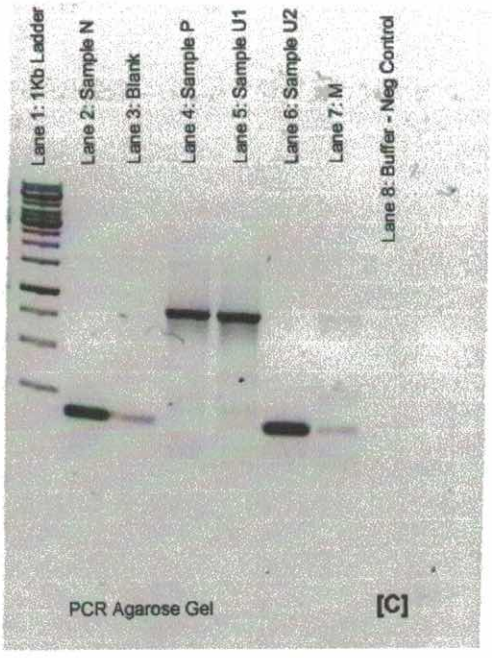


Figure 8: Agarose gel electrophoresis results. [A] - The restriction digestion gel showing an unexpected result (no DNA bands). [B] – An ideal restriction digestion gel showing the expected result. [C] – The PCR gel with lanes marked.

Discussion

When DNA from U1 was cut with PstI and BamHI, two bands were produced of approx 1000bp and 4700bp in size indicating U1 contained the pGEX-GFP plasmid. When the PCR gel was analysed, it showed U1 produced a 900bp band, confirming it contained the pGEX-GFP plasmid. Fluorescence testing also indicated that sample U1 contained the pGEX-GFP plasmid as that colony matched the fluorescence intensity of the P sample. The sample U2 contained the pGEX-2T plasmid.

The p-GEX plasmid has several restriction enzyme recognition sites, mapped below in Figure 9. The restriction enzyme sites, and the corresponding number of base pairs from *lac* 1 (the binding site) is indicated. The thrombin cleavage site describes the amino residues that are used to link the GST and GFPS65T proteins together. Thrombin cleaves (cuts) the amino acid sequence at this location and allows the GFPS65T to be separated from its fusion protein partner (GST). The thrombin cleavage site is located just before the BamHI cleavage site (931bp).

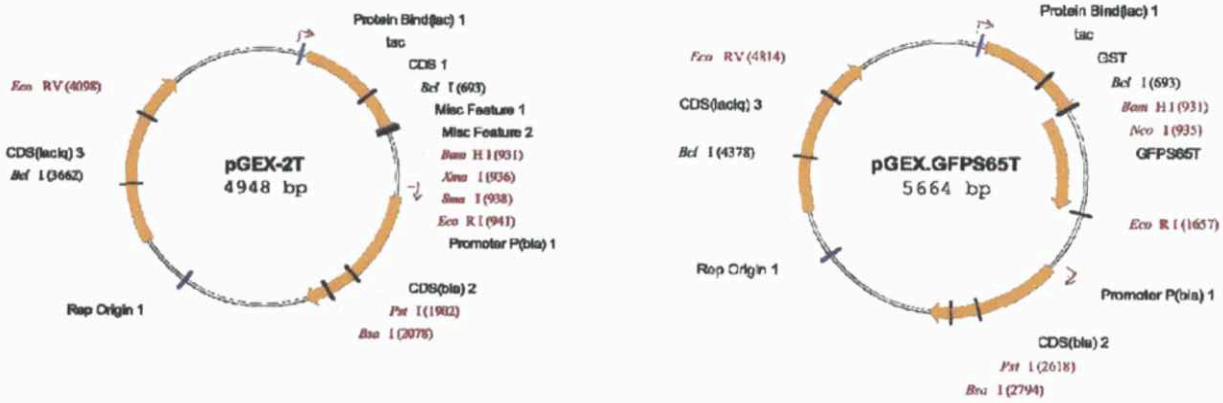


Figure 9: Plasmid diagrams for pGEX-2T and pGEX-GFPS65T (BIOC2000 prac manual).

The restriction digest agarose gel failed to produce a meaningful result. This may have been due to several factors. Pipetting error may have resulted in unsuccessful DNA recovery causing an absence of DNA in the gel lanes. During preparation of the restriction digest gel, there was an error in measuring apparatus that may have resulted in a higher concentration of agarose (above 5%) being added to the gel mixture. Perhaps this resulted in a more dense gel that did not allow migration of DNA (although this is unlikely because the reference ladder functioned correctly). There may have been some contamination (RNA, bacterial DNA) that may have caused the restriction enzymes to fail. Lastly, there may have been too little DNA recovered to create a visible band.

Despite the failure of the restriction digest gel to produce a meaningful result, the PCR gel and the fluorescence testing did produce a consistent conclusion. The use of more than one detection method created some redundancy in this experiment however this redundancy allowed the experimental series to recover from the restriction digest gel error and still draw a solid conclusion; that the unknown sample 1 (U1) possessed the GST-GFPS65T plasmid.

The experimental hypothesis for plasmid DNA isolation was confirmed, by appearance of a pellet (that was able to suspended in TE buffer) and by further analysis of DNA using electrophoretic methods. The experimental hypothesis for fluorescence testing was also confirmed, showing successful incubation of all colonies (including the positive control). The differences in fluorescence were substantial enough to easily differentiate the unknown samples. Overall, the results from each experiment (except the failed restriction digestion gel) agreed with each other.

Aside from the restriction digestion gel, the other experiments performed well. In terms of experimental design, three methods of plasmid detection may not be the best use of resources when two methods could suffice. In this particular experiment, the GST-GFPS65T plasmid produced a physical difference in *E.Coli* colonies that was observable with the naked eye. Not all expressed proteins produce physical manifestations that are directly observable. If the experiment concerned a protein that had no observable manifestation then using two electrophoretic methods for detection (PCR and restriction digestion) would be necessary, and plating of sample cells may be redundant.

Plasmid identification is necessary to ensure that the correct colony is selected for growth and protein expression. The protein expressed by the correctly identified colony is purified and quantified in part two – protein chemistry. The RED DOT clone used (sample U1) would be the best clone to use for expression of GFP. The table below in Figure 10 summarised class results for the various unknown clones.

Class of *E.Coli* Transformant	**PCR Result**	**Fluorescence**	**Insert Type**
Desired vector ("P")	pGEX-GFP	Yes	GST-GFPS65T
Vector without insert ("N")	pGEX-2T	No	GST
Blue Dot	pGEX-GFP	Yes	GST-GFPS65T
Red Dot	pGEX-GFP	Yes	GST-GFPS65T
White Dot	pGEX-2T	No	GST
Green Dot	pGEX-2T	No	GST

Figure 10: Summarised class results for all unknown clone samples.

Bioinformatics Analysis

Figure 11 summarises molecular weight and isoelectric point data for GST, GFPS65T, and the fusion protein GST-GFPS65T (expasy). Isoelectric point is important because it signifies the pH at which the protein molecule has no net charge. Electrophoretic methods rely on molecular charge and users of this protocol must ensure that the pH of buffer solution is not near the isoelectric point of the target protein, otherwise there will be no migration through the gel.

Protein	Entry Name	Primary accn #	Molecular Weight (Da)	Isoelectric Point (pI)
GST	GST26_SCHJA	P08515	25,499	6.09
GFP	GFP	P42212	26,886	5.67
GFPS65T	-	-	26900.35	5.67
GST-GFPS65T	-	-	52525.14	5.84

Figure 11: Molecular weight and isoelectric point data for selected proteins.

Figure 12 below shows three hydropathy plots for GST, GFPS65T, and GST-GFPS65T. Hydrophobic residues have a positive hydrophobicity value of over 1. Hydrophilic (polar/charged) residues have a negative hydrophobicity value. Analysing the plots below suggest that GST is completely soluble in water (entirely hydrophilic), whereas the GFPS65T does have a single hydrophobic region suggesting lower solubility. The fusion protein plot shows a combination of properties shown in the individual GST and GFPS65T plots – a single region of hydrophobicity amongst hydrophilic residues. Figure 13 and 14 on the following page show screen shots of GST and GFP records from the Uni Prot Blast database.

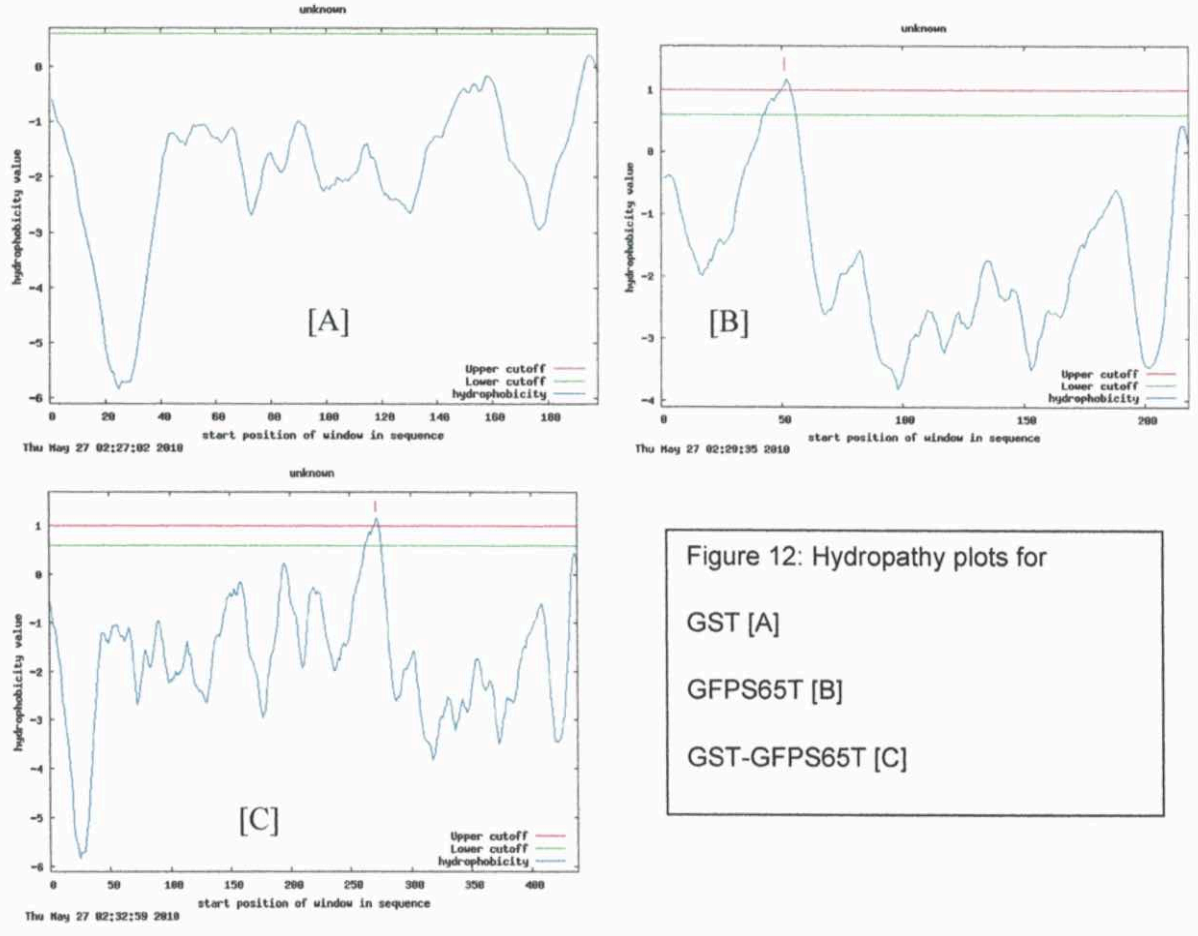


Figure 12: Hydropathy plots for

GST [A]

GFPS65T [B]

GST-GFPS65T [C]

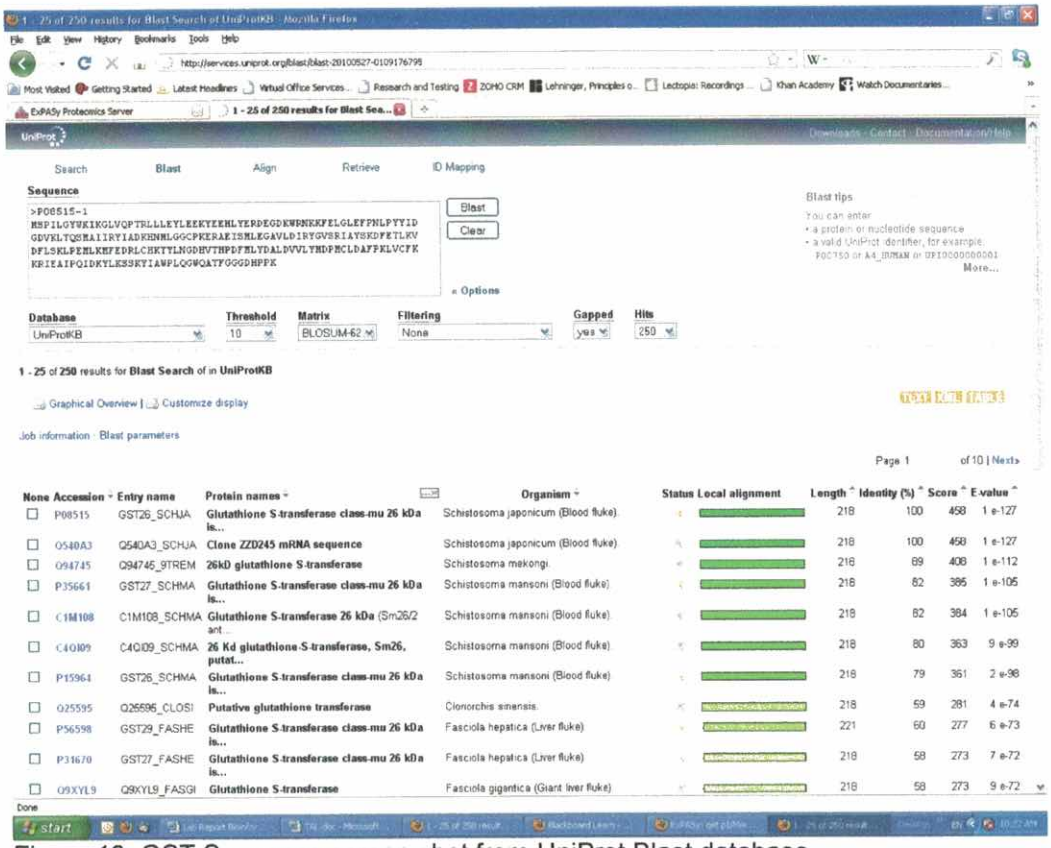


Figure 13: GST Sequence screen shot from UniProt Blast database.

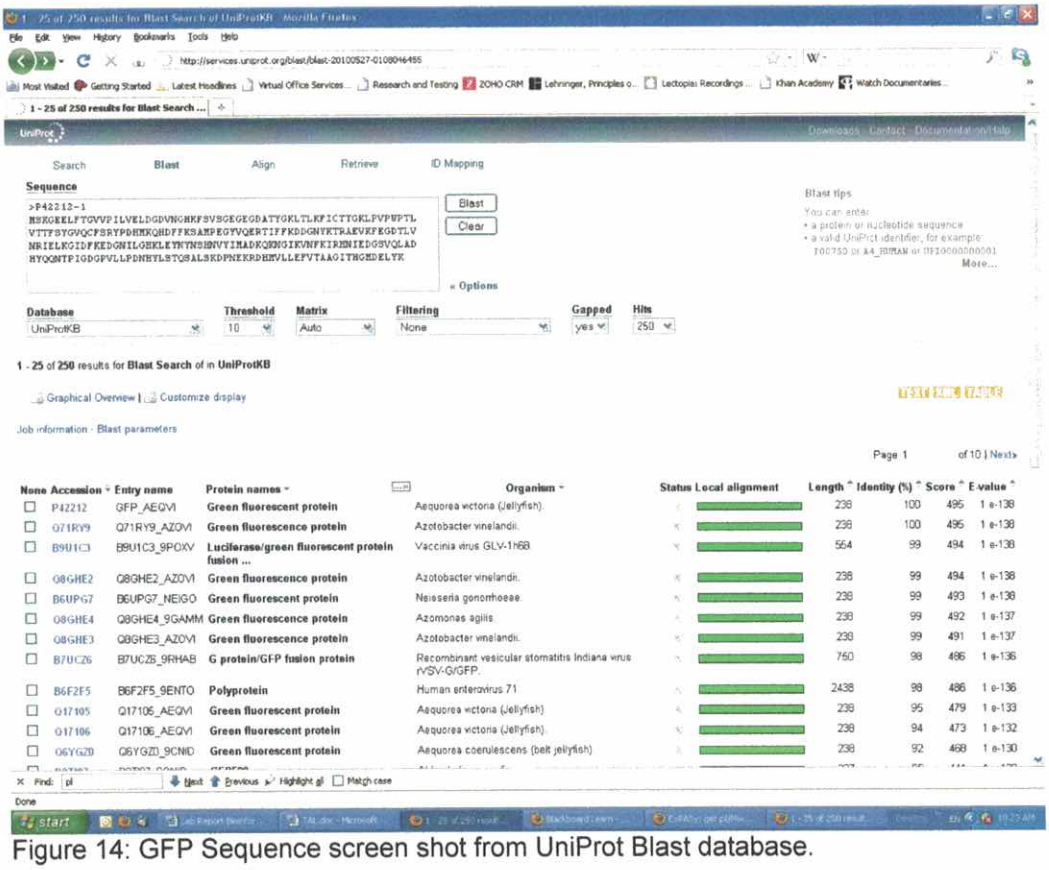
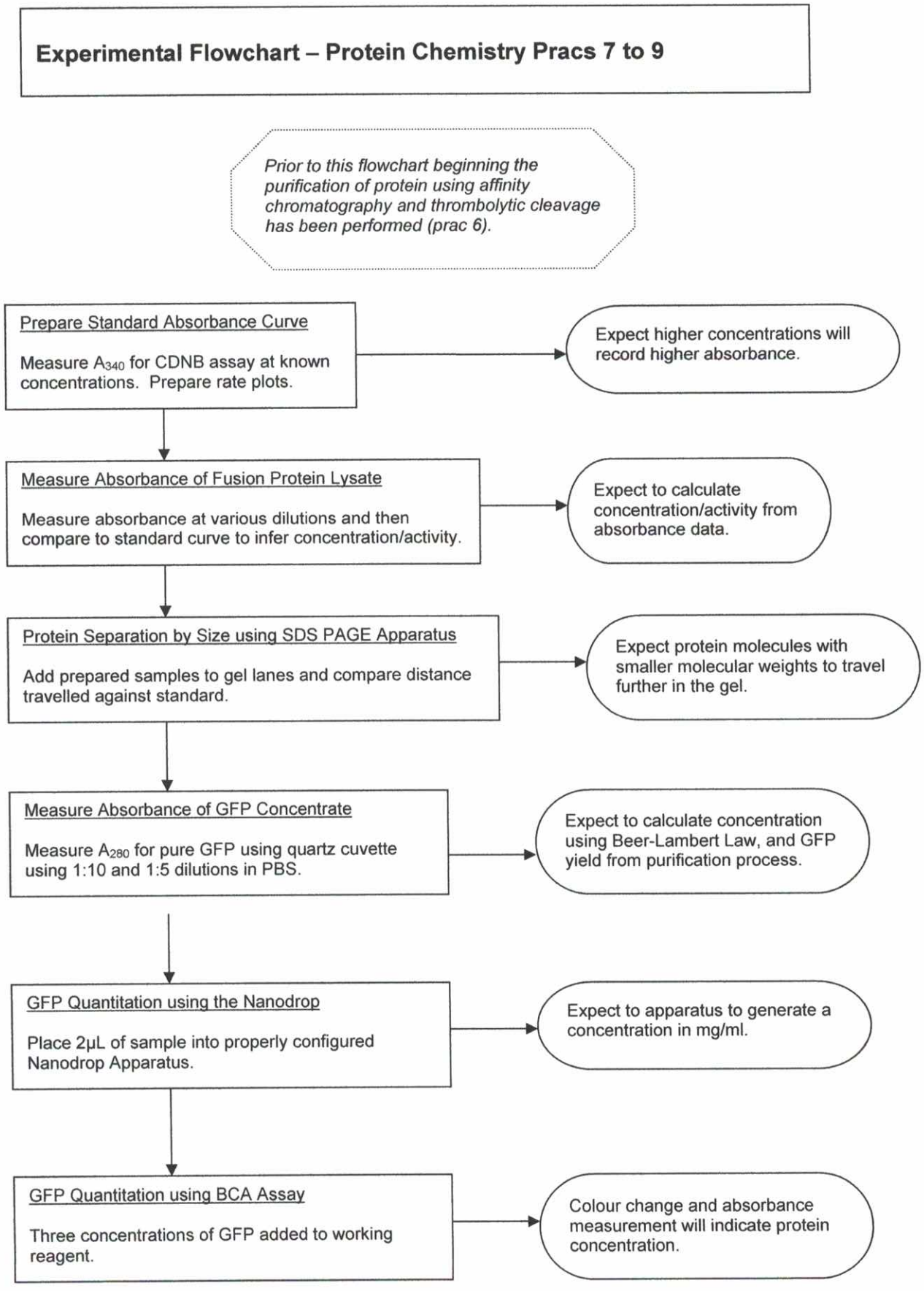


Figure 14: GFP Sequence screen shot from UniProt Blast database.

Part Two - Protein Chemistry (Practicals 7 to 9)



Introduction and Experimental Rationale

Part two of this experimental series picks up where the molecular biology series ends, and is broadly concerned with quantitation and purification of the GFP fusion protein. Protein purification is achieved using affinity chromatography rather than relying on exploitation of GFP's native properties to isolate it (such as size and charge). Affinity chromatography is faster and produces higher protein yields than more traditional methods as it relies on the bonding of the GST part of the fusion protein to a specific substrate (BIOC2000 Prac book). The substrate, glutathione, is covalently linked to sepharose (a type of resin) and is produced in the form of tiny beads. The beads are placed in a column that allows crude protein mixture to pass through. As the crude mixture passes, the GST ligand binds to the resin bound glutathione, immobilising the fusion protein inside the column. The specific binding behaviour of GST to its substrate allows isolation of the target protein.

The GFP fusion protein could have alternatively been produced with a histidine tag instead of GST. His-tagged proteins have an affinity for divalent cations (such as Co^{2+}) (BIOC2000 Prac Book). Use of alternative tag and substrate chemistry is possible however the concept is exactly the same – that a specific binding affinity will allow a target protein to be captured in the affinity chromatography column.

Once the crude protein mixture has passed through the column, a reagent is added to cleave the bond between the GST part of the fusion protein and the GFP. In this instance, thrombin is used to cleave the bond, releasing just the GFP to pass through the column for collection.

Several measurement techniques are used for quantitation including spectrophotometry (measuring absorbance of light), SDS-PAGE, Nanodrop, and BCA Assay. Measuring absorbance of light (at a particular wavelength) over time firstly requires the preparation of a standard curve. The standard absorbance curve measures absorbance for known concentrations over time, and is the reference that will be used for comparison against samples of unknown concentration. The measurement of absorbance has a kinetic nature, because reactions in the sample will change the absorbance of light over time (as new chemical species are produced). To prepare the standard curve, a synthetic substrate for GST is used instead of glutathione (CDNB). The resulting conjugate absorbs light at 340nm and is not easily affected by other chemical species or contaminants, allowing measurement of concentration using crude cell lysate as well as purified protein.

Protein separation by size is achieved using sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). This is an electrophoretic technique that relies on detergents (SDS) to denature and negatively charge protein 'strands' (covalently linked amino acid residues at the primary protein structure level). The strands are then able to travel through a gel matrix, pulled toward a positively charged terminal. Smaller sized protein strands travel further through the gel than larger sized molecules during the same time interval, allowing separation of proteins by size. A banding pattern is produced in the gel, and when compared against a reference ladder, the apparent molecular weight can be estimated.

The Nanodrop is a specialised apparatus that places a small droplet of sample (2 µl) between two arms. When the two arms clamp together a beam of light is shone through the droplet and absorbance is measure. The Nanodrop is based upon spectrophotometry principles but is linked to a computer allowing the apparatus to calculate a direct concentration measure, rather than just an absorbance reading. Results from the Nanodrop are compared against concentration measures generated by measuring absorbance using a traditional spectrophotometry apparatus and applying the Beer-Lambert law.

The last quantitation method used is a Bicinchoninic Acid Assay (or BCA Assay) – a type of enzyme assay that produces a colour change according to the level of protein concentration. The BCA assay relies on chemical reactions between the peptide bonds in proteins to modify a copper ion that then reacts with bicinchoninic acid. The reaction forms a product that exhibits a purple colour and an absorbance of light at 562nm wavelength (Smith 1985). The aims and experimental hypothesis for the series of experiments in part two is described below.

AIM: To prepare a standard absorbance curve using a CDNB assay at known concentrations.
HYPOTHESIS: Absorbance of light at 340nm will increase over time for any given concentration. Higher concentrations will result in more light absorbance.

AIM: To measure absorbance of the GST-GFP fusion protein in crude cell lysate.
HYPOTHESIS: A sample with an unknown concentration of GST-GFP will produce a specific absorbance of light at 340nm. This absorbance measure can be compared to a standard curve to infer concentration.

AIM: To measure molecular weight of GFP.
HYPOTHESIS: Proteins with smaller molecular weights will travel further in an electrophoretic gel apparatus than larger molecular weights, due to opposite charge attraction (negatively charged protein is attracted to the positively charged terminal inside the gel apparatus).

AIM: To calculate concentration and yield by measuring absorbance of purified GFP protein.
HYPOTHESIS: Absorbance of light at 280nm is related to concentration of GFP protein by the Beer-Lambert law.

AIM: To determine GFP concentration in a purified protein sample using a BCA assay.
HYPOTHESIS: Protein concentration will determine the extent of a chemical reaction, the product of which produces and observable colour change. The degree of colour change and the resulting absorbance measure will indicate protein concentration.

Method

Figure 15 lists the experimental methods used (in chronological order) and where to find detailed method explanations in the BIOC 2000 practical manual. The concentrations of GST-GFP used in the preparation of the standard curve are shown below in figure 16.

Method	BIOC2000 Prac Manual Page Number
CDNB Assay and Absorbance Measurement of Lysate	63-65
SDS-PAGE	70-71
Absorbance Measurement of GFP Concentrate	72
Protein Quantitation using the Nanodrop	73
Protein Quantitation using BCA Assay	73-76

Figure 15: List of experimental methods used and location of detailed instructions in prac manual.

GST-GFP (µg)	Volumes (µl)	
	GST-GFP stock (0.1 µg/µl)	H_2O to 40 µl
0	0	40
0.5	5	35
1.0	10	30
2.0	20	20
3.0	30	10
4.0	40	0

Figure 16: GST-GFP standards used to construct a standard absorbance curve.

Results

Figure 17 is a table showing absorbance readings recorded at 340nm wavelength for known concentrations of GST-GFP for experiment one, construction of the standard curve.

	GST-GFP Concentration (µg) – Standard Curve					
Time (hh:mm:ss)	**0.0**	**0.5**	**1.0**	**~~2.0~~**	**3.0**	**4.0**
0:00:00	0	0	0	~~0~~	0	0
0:00:30	0.003	0.009	0.018	~~0.13~~	0.082	0.085
0:01:00	0.007	0.022	0.041	~~0.261~~	0.172	0.176
0:01:30	0.011	0.036	0.06	~~0.386~~	0.265	0.265
0:02:00	0.015	0.047	0.081	~~0.502~~	0.354	0.351
0:02:30	0.019	0.06	0.103	~~0.616~~	0.438	0.43
0:03:00	0.023	0.073	0.122	~~0.723~~	0.513	0.511
0:03:30	0.026	0.085	0.142	~~0.815~~	0.586	0.589
0:04:00	0.03	0.097	0.162	~~0.915~~	0.656	0.665
0:04:30	0.034	0.11	0.182	~~1.013~~	0.726	0.738
0:05:00	0.038	0.123	0.202	~~1.103~~	0.796	0.805

Figure 17: GST-GFP Absorbance over time at 340nm wavelength.

Figure 18 (below) is a standard curve rate plot of rate (A_{340} / min) versus GST-GFP concentration. During construction of the standard curve, the absorbance readings for the 2.0 µg standard were deemed to be inaccurate. The data points were outliers as they showed higher absorbance than the 4.0 µg standard. The data for the 2.0 µg standard was omitted from the line of best fit calculation. The equation for the line of best fit is: Absorbance = 0.0382 x GST-GFP concentration (in µg).

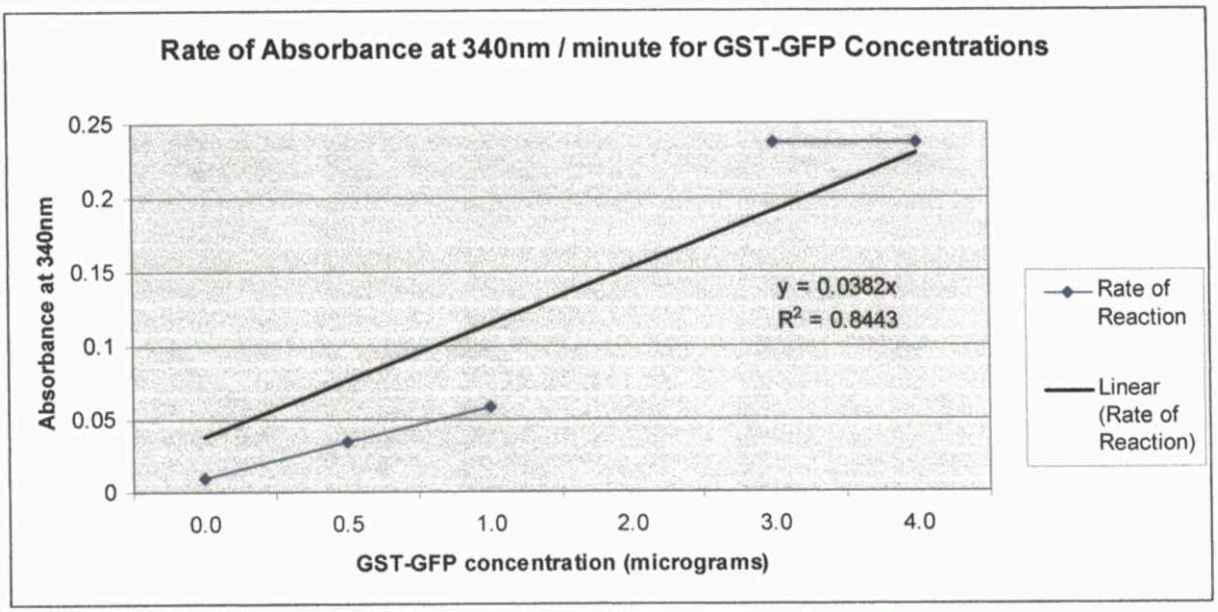


Figure 18: A rate plot of rate (A_{340} / min) versus GST-GFP concentration used as the standard curve.

In experiment two, A_{340} was measured for induced lysate samples diluted with H_2O (1:8, 1:16, and 1:32) over a five minute period (as with the standard curve preparation). The concentration of GST-GFP in the lysate for the 1:16 dilution was calculated to be 1.41 µg/µl, and for the 1:32 dilution is was calculated to be 1.15 µg/µl. The average concentration of GST-GFP in the lysate sample using spectrophotometric analysis was 1.28µg/µl. SDS-PAGE produced the following banding patterns show below in Figure 19.

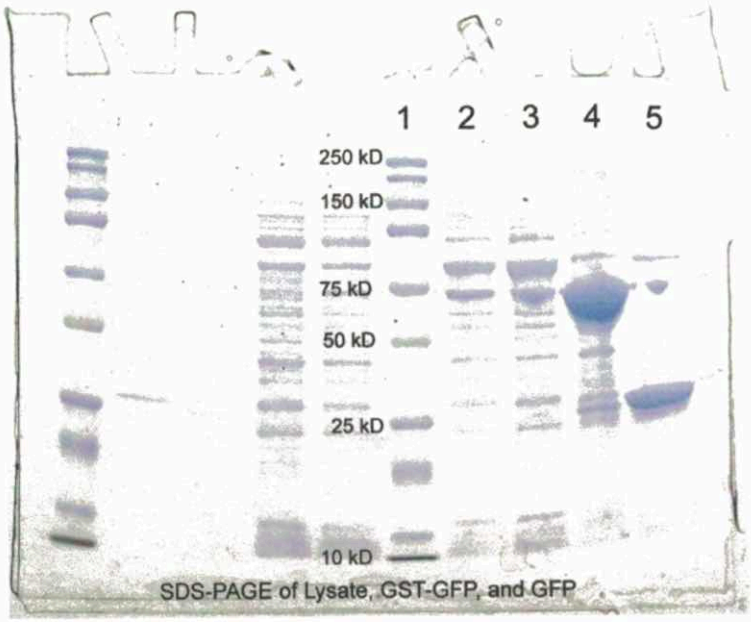


Figure 19: Photograph of SDS-PAGE result.
Lane 1 – Marker
Lane 2 – Induced lysate
Lane 3 – Flow through
Lane 4 – Resin bound fusion protein
Lane 5 – Purified GFP

Spectrophotometric measurement of GFP concentrate diluted 1:5 at A_{280} produced an absorbance reading of 0.550. A GFP concentration of 50 µg/ml produces an A_{280} of 0.1. Using the Beer-Lambert law and the absorbance reading, the purified GFP sample was calculated to have a specific concentration of 1375 µg/ml, or 1.375 mg/ml. Activity was calculated at 0.408 units / µg.

The Nanodrop apparatus was also used to measure concentration of purified GFP and produced a reading of 2.4 mg/ml, with a molecular weight reading of 26.98 kDa.

The BCA Assay (shown below in Figure 20) was performed with standards added in rows A1 to C9 (triplicate). Diluted GFP was added to rows A10 to C12 (triplicate) with dilution factors indicated. Spectrophotometric analysis at A_{550} indicated that column 10 had an absorbance of 1.067 (which most closely corresponded to the standard in column 3), column 11 had an absorbance of 0.43 (approximately corresponding to standard in column 6), and column 12 had an absorbance of 0.328 (approximately corresponding to standard in column 6). Figure 21 (below) summarises protein quantitation data.

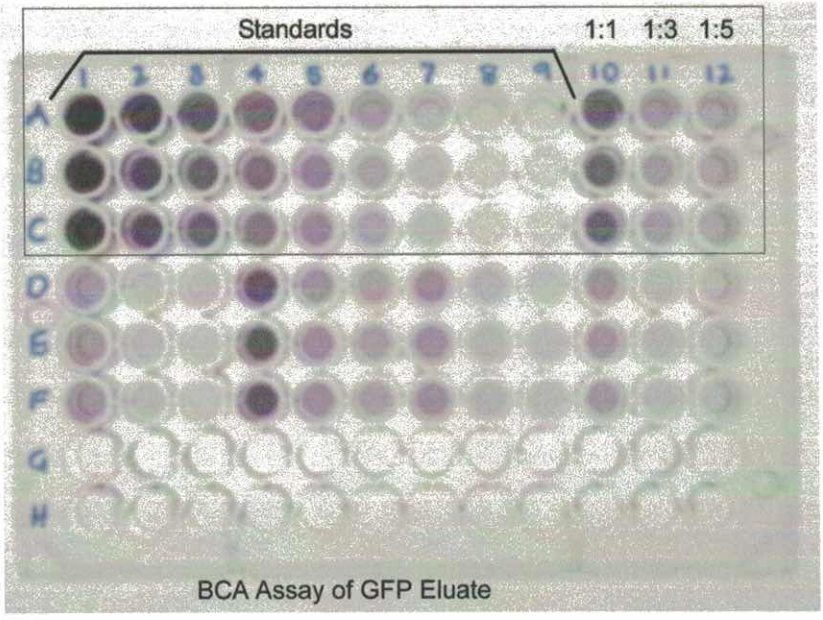


Figure 20: BCA Assay of GFP eluate at 1:1 dilution (column 10), 1:3 dilution (column 11), and 1:5 dilution (column 12). Rows A to C were used to perform the assay in triplicate.

Sample Vol (ml)	Assay Method	GFP Protein in sample (mg/ml)	Total in sample (mg)	% Recovery of GFP compared to original lysate
0.00175	CDNB	0.00128	0.00000224	100
0.20	Spectrophotometer	1.375	0.275	-
0.0015	Nanodrop	2.4	0.0036	-
0.075	BCA	1.68	0.126	-

Figure 21: Summarised protein quantitation results.

Discussion

Overall the protein quantitation experiments provided inconsistent data, although results from the Nanodrop, BCA, and Spectrophotometer experiments generated concentration measures within the range of 2 mg/ml +/- 0.7 mg/ml of GFP protein. While inconsistent, the concentration results are within a reasonable range.

Generation of the standard curve was problematic, and produced absorbance data that was inconsistent with expected results. Absorbance was expected to increase with concentration; however the 2.0 µg standard generated absorbance readings that were significantly higher than the 4.0 µg standard). The 3.0 µg and 4.0 µg standards generated very similar absorbance readings which was also unexpected.

The corresponding rate of absorbance plot produced an inconsistent curve that was overlayed with a line of best fit (R^2 value of 0.84). Pipetting error may explain erroneous absorbance data. It is unlikely that contaminants influenced absorbance as the CDNB assay was used with cell lysate, specifically because it has been determined to work in the presence of contaminants. The spectrophotometer was not suspected of faulty operation, although this can only be confirmed by using an alternative apparatus and repeating protocols. CDNB assay results were impacted by the problems encountered constructing the standard curve. To correct standard curve errors the procedure could be repeated or a relevant curve sourced from the literature.

SDS-PAGE produced an expected result showing a consistent relationship between protein size and distance travelled in the gel (smaller molecules travelling further). The resin bound fusion protein sample showed a high concentration at about the 75kDa band.

The purified GFP sample showed a high concentration at about the 25kDa band – which is consistent with the theoretical molecular weight of GFP. As the resin bound protein molecule is larger than the cleaved protein, it was expected to travel a smaller distance in the gel.

The induced lysate sample showed feint banding at the 75kDa level which was expected, and reflected the presence of resin bound GFP prior to purification. The induced lysate sample did not show any banding at the 25kDa level which was also expected and indicated that there was not yet any free GFP.

The flow through sample showed feint banding at both the 25kDa and 75kDa levels indicating trace leftover amounts of resin bound and free GFP. The flow through sample illustrates that while affinity chromatography is highly efficient it does not recover 100% of the target protein.

Spectrophotometric analysis of purified GFP produced results consistent with the other methods used, and was successfully executed without any major difficulties. The initial dilution of 1:10 produced an absorbance reading that was below the ideal working range of the spectrophotometer apparatus. This was easily corrected by using a 1:5 dilution. The Beer-Lambert law was applied to the absorbance readings to generate concentration. This law assumes a pure protein. The banding pattern of the purified GFP sample in the SDS-PAGE experiment (lane 5) produced feint bands other than the major GFP band at 25kDa. This suggests that there were impurities contained within the purified GFP sample – which may have had an influence upon the accuracy of this result. Calculation of total yield of GFP from the purification process was not possible due to the errors encountered generating the standard curve.

Quantitation of protein using SDS-PAGE proved to be subjective (using dye strength and reference bands), and as such concentration measures using this approach were not included in the result.

The Nanodrop apparatus was highly automated and produced a direct concentration measure from a very small sample size. The Nanodrop produced a quality measure as part of its output that indicated that there were contaminants present in the purified sample (purity of approximately 95%). This indication agreed with the SDS-PAGE banding pattern in lane 5 (purified GFP) showing feint bands in addition to the expected GFP band at 25kDa. The molecular weight output from the Nanodrop was in agreement with the theoretical molecular weight. Computerised processing of absorbance data reduced the risk that human calculation error could have influenced the concentration result, and as such the concentration measure from the Nanodrop provided an important benchmark against the other methods used.

The BCA Assay produced a result that was acceptable when compared against results from the other methods used. The assay did not appear to be impaired by any contaminants.

Calculation of overall percentage yield was hampered by the initial problems encountered with the standard curve and CDNB assay. It is expected that affinity chromatography would increase percentage yield, to what extent was not able to be determined with high confidence but is estimated to produce an approximate 10 fold increase in purity.

In conclusion, the experimental series in part two confirmed that affinity chromatography using GST and glutathione substrate does produce a highly purified protein sample (estimated at approximately 95% purity). The effectiveness of thrombin to cleave GFP from it's GST tag was also confirmed by the SDS-PAGE result.

The experimental series generated a sufficient amount of data to specify GFP concentration within a reasonable tolerance, although the individual results from the different protocols were not exactly the same. The data generally confirmed experimental hypothesis, other than the standard curve experiment which was primarily due to experimental error.

Experimental Roundup

The experimental series as a whole demonstrates core biochemical methods used to produce and purify a protein of interest. Using the methods and theoretical background of this experimental series, it should be possible to produce a range of proteins in high concentrations. Multiple detection and quantitation techniques were employed. This provided some redundancy that allowed recovery from individual experimental errors, and a cross check between protocols aimed at producing a similar result. With refinement and mastery of technique, the experimental series could be simplified, and some of the overlapping protocols removed to produce a result in less time (and with less expense). However maintaining some level of redundancy as a cross check of experimental results is valuable and worth an investment of additional time.

In part one, the experimental aim to isolate plasmid DNA was successfully achieved, as was the aim to detect the presence of *E.Coli* with GFP plasmids using plating and fluorescence testing. The restriction enzyme gel was not successfully achieved; however the success of the PCR gel did allow the aim of determining plasmid type from unknown samples to be successfully confirmed.

In part two, the experimental aim to prepare a standard absorbance curve was not completely successful, although a line of best fit was generated from available results with a reasonable R^2 value. Generation of a standard curve did appear to be a simple protocol, but may be more sensitive than originally presumed.

The experimental aim to measure absorbance of GST-GFP in crude cell lysate was successful; however the calculations needed to produce a concentration measure were influenced by the (in)accuracy of the standard curve. This dependency and the complexity of various concentration calculations provided a larger opportunity for experimental error to occur than with other protocols used. The Nanodrop provided an important reference to this protocol and was an invaluable piece of apparatus.

The aim to calculate concentration from a purified GFP sample was successful. Using a known relationship between absorbance and concentration improved confidence in the result. The aim to quantify GFP using SDS-PAGE proved to be too subjective; however it provided valuable data on the presence of protein products (and their molecular weights). The aim to deduce concentration using BCA assay was also successful but was not an overly insightful protocol.

Further purification protocols, such as ION exchange or SEC could be employed as part of the series. However the effort may not be worth the additional gain in purity, as affinity chromatography is highly efficient. The factors influencing the level of protein expression in *E.Coli* would be an interesting topic of further study. Methods from this series could be employed in the production of protein and measurement of protein purity from colonies exposed to various expression factors.

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