

***Green and Red Fluorescent
Proteins:
Cloning, Gene Expression
and Protein Purification***

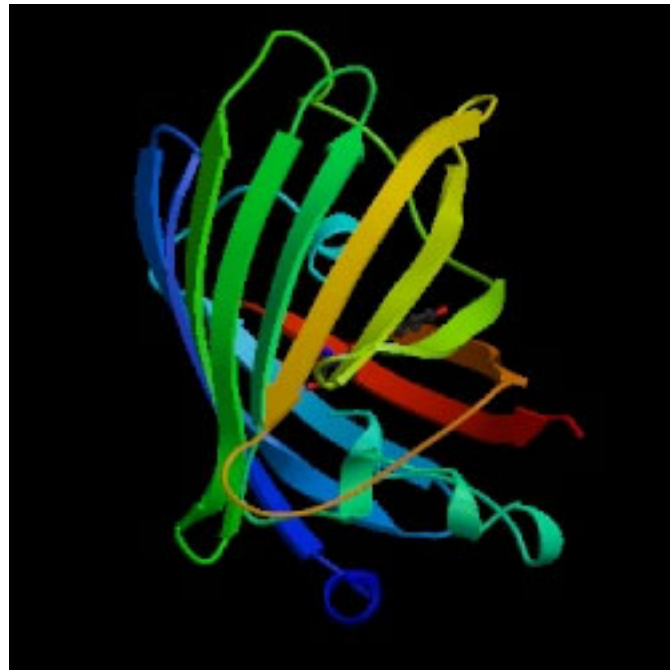
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BCH 467

General Biochem Lab



Fall, 2004

BCH 467, Fall 2004

Lab #1

Restriction Map of DNA

Introduction: What color is your DNA?

You will be assigned to investigate either unknown DNA sample A or B. The DNA contains a gene encoding either a red fluorescent protein (RFP) or a green fluorescent protein (GFP). In the first experiment, the DNA will be cut and the resulting fragments separated by agarose gel electrophoresis. From the sizes of the fragments, you should be able to piece together a “map” of the DNA. Subsequently you will determine the sequence of a portion of the DNA. By comparing the DNA sequence to the amino acid sequences of the red and green fluorescent proteins, you should be able to identify the gene. In the third experiment, you will use a diagnostic PCR test to identify your gene. Combining all of these results should lead to a detailed description of the DNA.

Background information on these types of procedures is covered in Chapters 11 and 12 of the textbook (Ninfa and Ballou, 1998, *Fundamental Laboratory Approaches for Biochemistry and Biotechnology*, pp. 277-299 and pp. 313-323).

A general introduction to fluorescent proteins is given in the article: Matz, M. V., Lukyanov, K. A., and Lukyanov S. A., 2002, “Family of the green fluorescent protein: journey to the end of the rainbow”, *BioEssays* 24, 953-959. More details on the biochemistry of fluorescent proteins can be found in the review: Zimmer, M., 2002, “Green fluorescent protein (GFP): applications, structure, and related photophysical behavior” *Chem. Rev.* 102, 759-781.

Cover: structure of GFP (PDB structure file 1EMB)

Restriction Map of DNA

In the laboratory, DNA is commonly contained in plasmids, which are small, circular pieces of DNA that can replicate independently of the chromosome. DNA is often manipulated using restriction enzymes, which cut the DNA at specific recognition sites. The sizes of DNA fragments can be determined using agarose gel electrophoresis with visualization of the DNA by ethidium bromide staining. In this experiment, you will use these tools to determine how many sites your plasmid contains for three restriction enzymes, and how far apart the sites are on the DNA.

MATERIALS

1. L20 pipettor, sterile tips, sterile 1.5 ml eppendorf tubes
eppendorf tube rack, float
2. Plasmid A or B
(50 nanograms/microliter in 1X digestion buffer)
You will investigate either plasmid A or plasmid B. Use the same type of plasmid unknown for all of the experiments in the DNA section.
3. 1X digestion buffer
(10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 100 mM NaCl, 1 mM 2-mercaptoethanol)
4. Restriction endonucleases BamHI, PstI, and ScaI
(2 units/microliter in 1X digestion buffer, on ice)
(one unit is the enzyme activity that completely cleaves 1 microgram λ DNA in 1 hour at 37 °C)
Sequences of restriction sites:
BamHI: GGATCC
PstI: CTGCAG
ScaI: AGTACT
5. gel loading solution
(30% glycerol, 10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.025% bromophenol blue)
6. DNA size markers (1 kb ladder, New England Biolabs)
(50 nanograms/microliter in 0.006% xylene cyanol FF, 0.006% bromophenol blue, 0.06% orange G, 2.5% Ficoll 400, 10 mM Tris-HCl, pH 7.9, 10 mM EDTA)
7. 37 °C water bath
8. 0.5X TBE
(44.5 mM Tris base, 44.5 mM boric acid, 1.0 mM EDTA)
9. molten agarose
(1% agarose in 0.5X TBE, boiled and then cooled to ~55 °C)
10. horizontal gel electrophoresis apparatus and power supply
11. staining solution
0.5 micrograms/ml ethidium bromide in 0.5X TBE
12. UV light box, Polaroid camera and film

PROCEDURE

1. Set up restriction digests of the plasmid DNA:

Prepare three single digestions and three double digestions with the restriction enzymes BamHI, PstI, and ScaI, and one sample with no enzyme added. Each digestion should contain 250 ng of plasmid and 10 units of each enzyme in the digestion in 1X digestion buffer with a total volume of 20 μ l. Use sterile eppendorf tubes and sterile pipet tips to avoid contaminating the solutions with nucleases, and use a fresh tip each time an aliquot is taken from the stocks.

Example of digestion setup:

	1 BamHI	2 PstI	3 ScaI	4 BamHI & PstI	5 BamHI & ScaI	6 PstI & ScaI	7 uncut
Digestion buffer	10 μ l	10 μ l	10 μ l	5 μ l	5 μ l	5 μ l	15 μ l
Plasmid (50 ng/ μ l)	5 μ l	5 μ l	5 μ l	5 μ l	5 μ l	5 μ l	5 μ l
BamHI (2 U/ μ l)	5 μ l			5 μ l	5 μ l		
PstI (2 U/ μ l)		5 μ l		5 μ l		5 μ l	
ScaI (2 U/ μ l)			5 μ l		5 μ l	5 μ l	

2. Incubate digestion tubes in the 37 °C water bath for at least 30 minutes.

3. Pour an agarose gel:

Insert the gel casting tray into the casting fixture. Slowly pour 50 ml of molten agarose into the gel tray. Insert the comb into the slots in the casting tray. Allow the gel to harden undisturbed at room temperature.

4. Set up the gel electrophoresis apparatus:

Add 300 ml 0.5X TBE to the main gel box. When the gel is solidified (fully opaque), transfer the casting tray to the main gel box. Remove the comb.

5. Load and run the samples:

When the incubation is done add 5 μ l of loading buffer to each sample.

Load 20 μ l of each sample in a separate lane on the gel.

Load 10 μ l of the size markers in the remaining lane on the gel.

Record the order of the samples in the lanes.

Place the cover on the gel box, connect the electrodes, and set the power supply to 100 volts.

Electrophorese at 100 volts for 1 hour or until the blue dye is approximately 2/3 of the way down the gel. The size marker contains three dyes: xylene cyanol FF migrates at approximately 4 kb, bromophenol blue at approximately 300 bp and orange G at approximately 50 bp.

6. Stain and photograph the gel:

When the electrophoresis is finished, bring the gel to the TA, who will transfer the gel to the ethidium bromide solution.

Incubate the gel in the ethidium bromide solution for approximately 15 minutes.

The TA will then rinse the gel briefly in 0.5X TBE and place the gel on the UV transilluminator.

The TA will photograph the gel using the Polaroid camera.

WEAR GLOVES DURING THIS STEP.

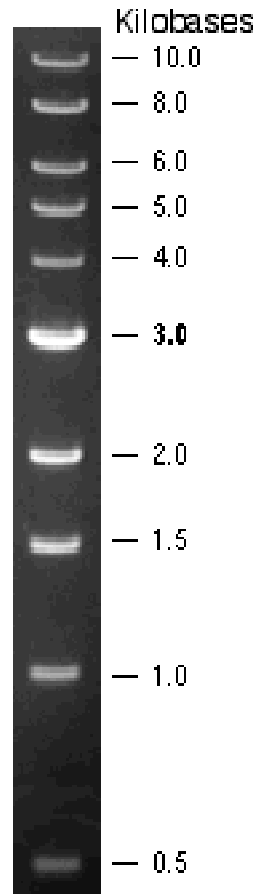
DO NOT LOOK DIRECTLY AT THE LIGHT BOX WITHOUT THE COVER.

ANALYSIS:

On the photograph of the gel, measure the migration distance from the well to the band for the size markers and plasmid fragments.

For the size markers, plot $\log(\text{size in kilobase pairs})$ versus migration distance or use a semilog plot. Fit a straight line in the linear region of the size markers, and use this to calculate the sizes of the plasmid fragments.

DNA size markers
(1 kb ladder, New England Biolabs)



Using the fragment sizes determined from the analysis of the gel photograph, determine the restriction map of your plasmid. Show the distances between the restriction sites of the three enzymes.

The plasmid can be thought of as having two parts: the vector and the insert. A vector is a plasmid that has been engineered to be able to “carry” pieces of DNA. Different genes can be inserted into restriction sites on the vector. The vectors used for the fluorescent protein genes are called pRSETB and pQE30. The pRSETB vector contains 1 ScaI site, and the pQE30 vector contains 2 ScaI sites. The fluorescent protein genes are in the inserts, which do not contain ScaI sites.

Before the next lab period, you should determine whether your vector is pQE30 or pRSETB.

DNA Exercise, Lab #1

Beads on a String

A plasmid can be thought of as a circle of DNA with restriction sites marking specific locations on the string. In this exercise, you solve a puzzle using logic similar to that needed for making a restriction map.

The DNA is represented by a string and the restriction sites are marked by beads of different colors. Imagine that the string is cut at the beads and figure out the distances in the units delineated by the knots. Do not count the beads as part of the length. Please do not actually cut the string.

On the worksheet, record the lengths of the pieces for each of the single and double cuts.

Exchange worksheets with your lab partner.

Draw a map of your partner's string using the information from the lengths of string. Check your map using your lab partner's string.

Please note that flipping over or rotating the map yields an equivalent plasmid.

λ Phage Map

The λ phage packages its DNA as a linear fragment that is 48.5 kb in length. Digests of λ DNA are often used as size markers in agarose gel electrophoresis.

The sizes of the fragments that would be obtained upon single and double digestion with several different restriction enzymes are given. Use this information to construct a restriction map of the λ DNA. The NarI site is indicated on the map for you.

Note that the sizes of the fragments have been rounded to the nearest 0.1 kB. Because of this, the map will not be very precise, and the fragments may not add up exactly.

Example

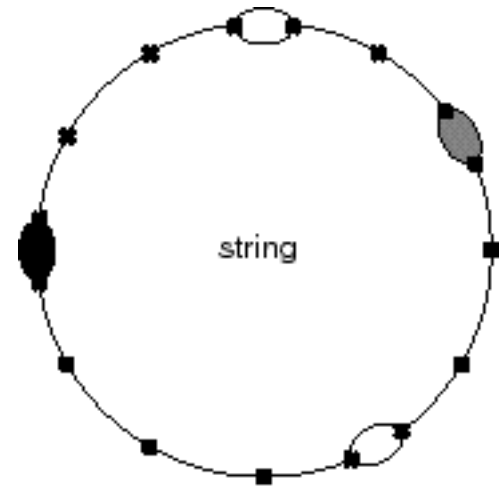
The single digests tell you how many of each site there are. In this example, white cuts twice and so yields two fragments, and the gray and black only cut once yielding one linear fragment.










The sum of the fragments in each digest yields the total length. For this plasmid, the total length is 12 units. Draw a circle to represent the plasmid and divide it into twelve units.

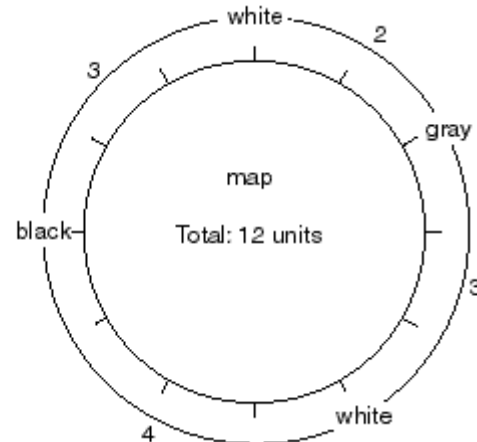
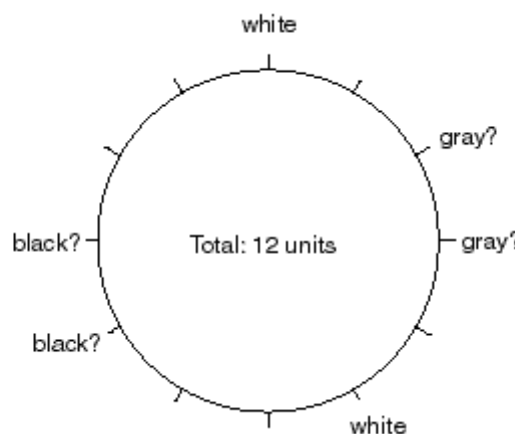
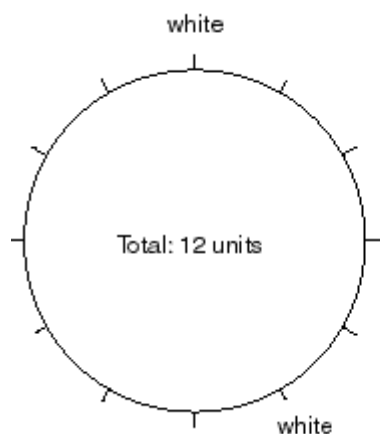
From the single digests you know there are two white sites five units apart, but you don't know where the gray and black sites are relative to the white.

From the double digests of the white and gray, you can't tell that the 5 unit white fragment is cut into pieces with lengths of 2 and 3, but there is still an ambiguity as to where these sites are. Similarly the white and black digest tells you that the 7 unit fragment is cut into pieces of 3 and 4 units, but the black site could be in one of two places.

Using the information from the gray and black digest resolves the ambiguity, since the correct combination yields gray and black sites that are 5 and 7 units apart.



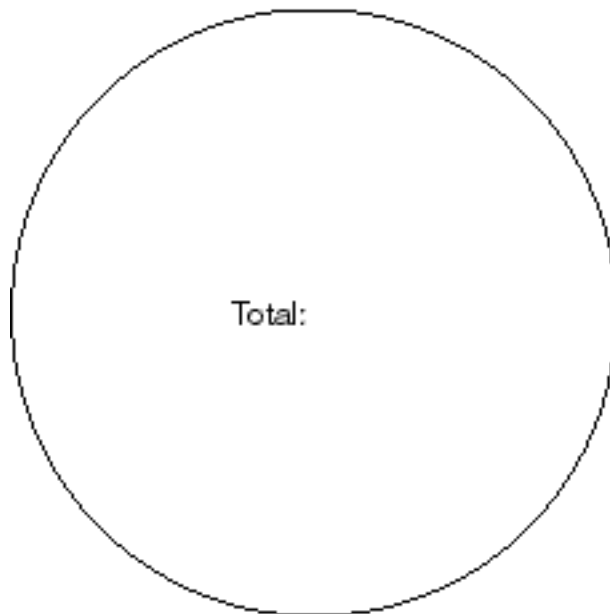
	lengths if cut
	<u>5, 7</u>
	<u>12</u>
	<u>12</u>
 & 	<u>2, 3, 7</u>
 & 	<u>3, 4, 5</u>
 & 	<u>5, 7</u>



Worksheet

Type of Cut	Bead Color	Length if Cut
Single Bead Color		
Single Bead Color		
Single Bead Color		
Double Bead Color		
Double Bead Color		
Double Bead Color		

Map:

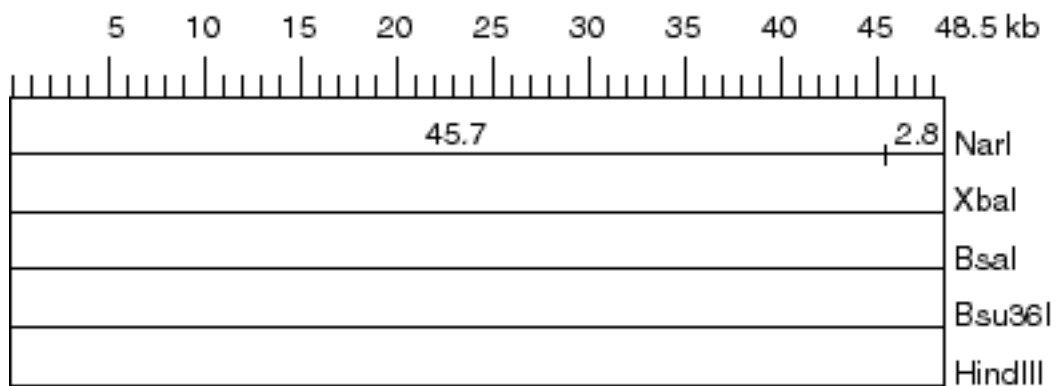


λ Phage Map

Digests:

NarI	XbaI	BsaI	Bsu36I	NarI-XbaI
45.7	24.5	31.3	26.7	24.5
2.8	24.0	11.4	14.2	21.2
		5.8	7.6	2.8
NarI-BsaI	XbaI-BsaI	NarI-Bsu36I	XbaI-Bsu36I	BsaI-Bsu36I
31.3	18.2			
11.4	13.1	26.7	24.5	15.3
3.0	11.4	11.4	14.2	11.4
2.8	5.8	7.6	7.6	8.4
		2.8	2.2	7.6
				5.8
HindIII	NarI-HindIII	XbaI-HindIII	BsaI-HindIII	Bsu36I-HindIII
23.1				
9.4	23.1	23.1	11.7	23.1
6.7	9.4	9.4	11.4	6.8
4.4	6.7	6.7	9.4	6.7
2.3	2.8	4.4	5.3	4.4
2.0	2.3	2.3	4.4	2.6
0.6	2.0	1.4	2.3	2.0
	1.5	0.6	2.0	1.6
	0.6	0.6	1.4	0.8
			0.6	0.6

Fragment sizes:

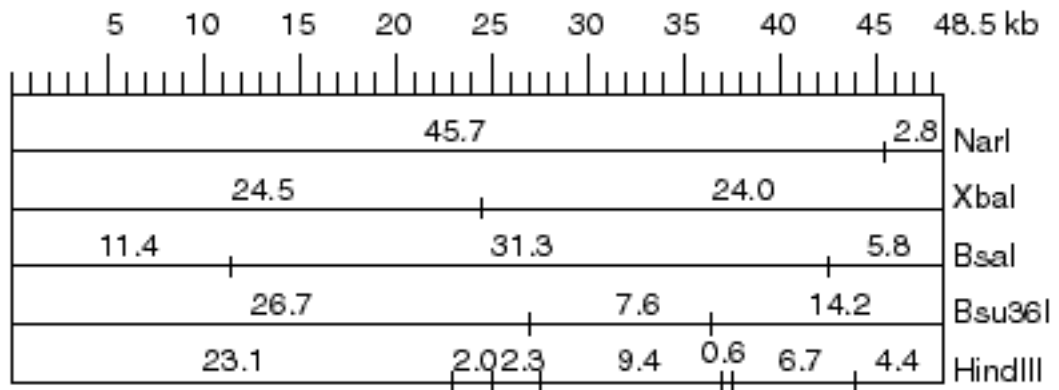


Map:



λ Phage Map - Key

Fragment sizes:



Map:



Lab Report, Lab #1:

Prepare a lab report including the sections: Title, Abstract, Introduction, Materials and Methods, Results, Discussion and References. This lab report is worth 100 points.

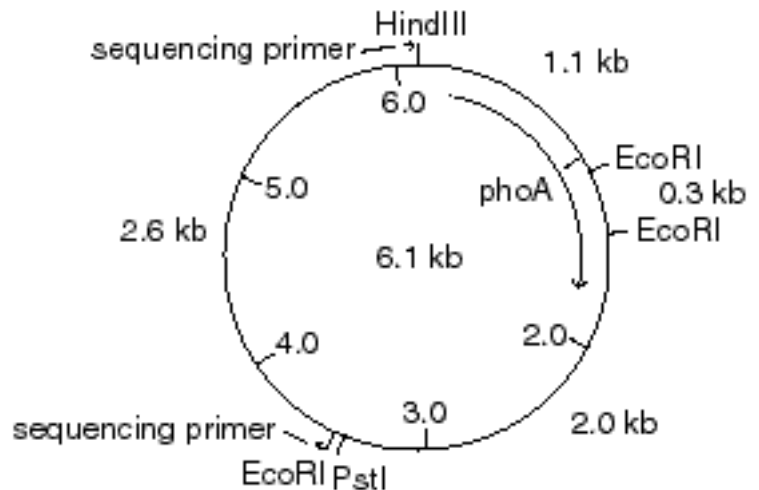
Present the evidence that you have for the identification of your plasmid as either plasmid A (pRSETB) or plasmid B (pQE30).

Your results should include a plot of log (size in kilobase pairs) vs. migration distance (cm) for the DNA size markers. Measure the migration distance for your plasmid fragments, and determine their size (kb) by using the plot as a standard curve.

Prepare a detailed map of your plasmid. Mark the locations of the restriction sites for all three restriction enzymes used, and the distances between them. Use the example below as a guide. At this point, you do not need to mark the location of the sequencing primer and gene insert. That will follow later.

Example of a plasmid map

This plasmid consists of the pUC19 vector with an insert containing the *phoA* gene from *E. coli*. This gene encodes the alkaline phosphatase protein. A scale is marked on the inside of the plasmid, with the HindIII site arbitrarily designated as 0. The locations of restriction sites for HindIII, EcoRI, and PstI are marked on the outside, as well as the distances between the sites.



BCH 467, Fall 2004

Lab #2

DNA Sequencing

Genes can be identified in a piece of DNA by determination of the nucleotide sequence. DNA is most commonly sequenced using the dideoxy method with fluorescently labeled chain termination nucleotides. The sequencing reactions are run on a polyacrylamide gel and the fluorescence of the fragments is used to identify the bases in a semi-automated procedure. In this experiment, you will determine the DNA sequence for the portion of your plasmid that contains the beginning of the fluorescent protein gene. After adding a sequencing primer to your DNA sample you will take a “field trip” to the ASU sequencing facility for a demonstration of these procedures. Sequencing results will be returned the following week.

MATERIALS

1. L20 pipettor, sterile tips, sterile 1.5 ml eppendorf tube
eppendorf tube rack
2. Plasmid A or B
(0.25 microgram/microliter in 10 mM Tris-HCl, pH 8.0)
Use the same plasmid DNA unknown (either A or B) as in Lab #1.
3. Sequencing primers for pRSETB and pQE30
10 nanograms/microliter in 10 mM Tris-HCl, pH 8.0)
From the mapping results, you should know whether the vector in your plasmid is pRSETB or pQE30. Based on this information, choose the appropriate sequencing primer for your plasmid.

PROCEDURE

1. Set up a sample for sequencing:
Prepare a sequencing sample containing 250 ng plasmid and 20 ng primer in a total volume of 6 μ l.
Use sterile eppendorf tubes and sterile pipet tips to avoid contaminating the solutions with nucleases, and use a fresh tip each time an aliquot is taken from the stocks.

Example of sequencing sample preparation:

Plasmid DNA (0.063 μ g/ μ l)	4 μ l
Primer (10 ng/ μ l)	2 μ l

Label the tube with the number assigned to you by your TA and record your number.

2. Take the samples to the ASU DNA sequencing facility:
When everyone is ready, bring the sequencing samples to Room 538 of the Life Sciences Building E-Wing, where Scott Bingham will explain DNA sequencing techniques.
Leave the samples at sequencing lab.

ANALYSIS: Determine whether your sequence codes for the GFP or RFP fusion protein.

Amino acid sequence of GFP fusion protein (native protein numbering in parenthesis)

```
MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDPPEAFMFSKGEELFTGVVP      50 (13)
ILVELDGDVNGHKFSVSGEGEGDATYGKLTLLKFICTTGKLPVPWPPTLVTT      100 (63)
LTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVK      150 (113)
FEGDTLVNRIELKGIIDFKEDGNILGHKLEYNNSHNVYIMADKQKNGIKV      200 (163)
NFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNE      250 (213)
KRDHMLVLEFVTAAGITHGMDELYK                                    275 (238)
```

Amino acid sequence of RFP fusion protein (native protein numbering in parenthesis)

```
MRGSHHHHHHGSRSKNIKEFMRFKVRMEGTVNGHEFEIEGEGEGRPYE      50 (39)
GHNTVKLKVTKGGPLPFAWDILSPQFQYGSKVYVKHPADIPDYKKLSFPE      100 (89)
GFKWERVMNFEDGGVVTVTQDSSLQDGCFIYKVKFIGVNFPSDGPVMQKK      150 (139)
TMGWEASTERLYPRDGV LKGEIHKALKLKDGGHYLVEFKSIYMAKKPVQL      200 (189)
PGYYYVDSKLDITSHNEDYTIVEQYERTEGRHHLFL                    236 (225)
```

Genetic Code

GGG	G	Gly	TGG	W	Trp
GGA	G	Gly	TGA	*	End
GGT	G	Gly	TGT	C	Cys
GGC	G	Gly	TGC	C	Cys
GAG	E	Glu	TAG	*	End
GAA	E	Glu	TAA	*	End
GAT	D	Asp	TAT	Y	Tyr
GAC	D	Asp	TAC	Y	Tyr
GTG	V	Val	TTG	L	Leu
GTA	V	Val	TTA	L	Leu
GTT	V	Val	TTT	F	Phe
GTC	V	Val	TTC	F	Phe
GCG	A	Ala	TCG	S	Ser
GCA	A	Ala	TCA	S	Ser
GCT	A	Ala	TCT	S	Ser
GCC	A	Ala	TCC	S	Ser
AGG	R	Arg	CGG	R	Arg
AGA	R	Arg	CGA	R	Arg
AGT	S	Ser	CGT	R	Arg
AGC	S	Ser	CGC	R	Arg
AAG	K	Lys	CAG	Q	Gln
AAA	K	Lys	CAA	Q	Gln
AAT	N	Asn	CAT	H	His
AAC	N	Asn	CAC	H	His
ATG	M	Met	CTG	L	Leu
ATA	I	Ile	CTA	L	Leu
ATT	I	Ile	CTT	L	Leu
ATC	I	Ile	CTC	L	Leu
ACG	T	Thr	CCG	P	Pro
ACA	T	Thr	CCA	P	Pro
ACT	T	Thr	CCT	P	Pro
ACC	T	Thr	CCC	P	Pro

Worksheet for analysis of DNA

features	Start of fusion protein									
DNA										
protein	Met	Arg	Gly	Ser	His					

features										
DNA										
protein										

features										
DNA										
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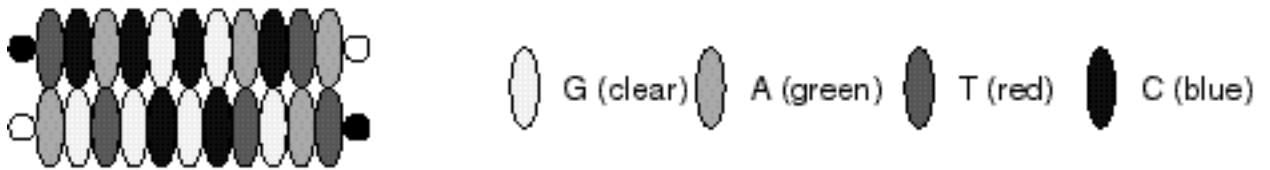
features										
DNA										
protein										

Exercise, Lab #2

In order to translate a DNA sequence into a protein sequence, you need to know where to start, since double-stranded DNA has six reading frames. Sometimes it's necessary to translate all six frames to see which one matches the information you have about a protein. In this exercise you decode the six frames of a short segment of DNA. The double-stranded DNA fragment is represented by pairs of beads that are color-coded to identify the base. Remember that the genetic code assumes that the DNA is written in the 5' to 3' direction. The polarity of ends of the DNA is marked by smaller beads. Only one of the frames will spell a common three letter English word when translated using the genetic code and the one-letter amino acid abbreviation. Write the sequence of the word that makes sense in both the one letter amino acid abbreviation and using the full names of the amino acid residues.

Bead Code		Genetic Code					
5'	black	GGG	G	Gly	TGG	W	Trp
3'	white	GGA	G	Gly	TGA	*	End
G	clear	GGT	G	Gly	TGT	C	Cys
A	green	GGC	G	Gly	TGC	C	Cys
T	red	GAG	E	Glu	TAG	*	End
C	blue	GAA	E	Glu	TAA	*	End
		GAT	D	Asp	TAT	Y	Tyr
		GAC	D	Asp	TAC	Y	Tyr
		GTG	V	Val	TTG	L	Leu
		GTA	V	Val	TTA	L	Leu
		GTT	V	Val	TTT	F	Phe
		GTC	V	Val	TTC	F	Phe
		GCG	A	Ala	TCG	S	Ser
		GCA	A	Ala	TCA	S	Ser
		GCT	A	Ala	TCT	S	Ser
		GCC	A	Ala	TCC	S	Ser
		AGG	R	Arg	CGG	R	Arg
		AGA	R	Arg	CGA	R	Arg
		AGT	S	Ser	CGT	R	Arg
		AGC	S	Ser	CGC	R	Arg
		AAG	K	Lys	CAG	Q	Gln
		AAA	K	Lys	CAA	Q	Gln
		AAT	N	Asn	CAT	H	His
		AAC	N	Asn	CAC	H	His
		ATG	M	Met	CTG	L	Leu
		ATA	I	Ile	CTA	L	Leu
		ATT	I	Ile	CTT	L	Leu
		ATC	I	Ile	CTC	L	Leu
		ACG	T	Thr	CCG	P	Pro
		ACA	T	Thr	CCA	P	Pro
		ACT	T	Thr	CCT	P	Pro
		ACC	T	Thr	CCC	P	Pro

Example



Color Translation:

black 5'	red T	blue C	green A	blue C	clear G	blue C	clear G	green A	blue C	red T	green A	white 3'
white 3'	green A	clear G	red T	clear G	blue C	clear G	blue C	red T	clear G	green A	red T	black 5'

Genetic Code Translation of Six Reading Frames:
(Strands must be written in the 5' to 3' direction.)

Strand 1 Frame 1		TCA S	CGC R	GAC D	TA
Strand 1 Frame 2	T	CAC H	GCG A	ACT T	A
Strand 1 Frame 3	TC	ACG T	CGA R	CTA L	
Strand 2 Frame 1		TAG *	TCG S	CGT R	GA
Strand 2 Frame 2	T	AGT A	CGC R	GTG V	A
Strand 2 Frame 3	TA	GTC V	GCG A	TGA *	

*HAT: histidine-alanine-threonine

Worksheet

Color Translation:

Genetic Code Translation of Six Reading Frames:
(Strands must be written in the 5' to 3' direction.)

Strand 1 Frame 1					
Strand 1 Frame 2					
Strand 1 Frame 3					
Strand 2 Frame 1					
Strand 2 Frame 2					
Strand 2 Frame 3					

Exercise #2 Decoding - Key

CLEAR	GREEN	RED	CLEAR	BLUE	CLEAR	RED	GREEN	RED	G A T G C G T A T	DAY
GREEN	BLUE	BLUE	BLUE	CLEAR	BLUE	RED	GREEN	BLUE	A C C C G C T A C	TRY
RED	CLEAR	CLEAR	BLUE	GREEN	BLUE	RED	GREEN	RED	T G G C A C T A T	WHY
RED	BLUE	BLUE	GREEN	GREEN	CLEAR	RED	GREEN	BLUE	T C C A A G T A C	SKY
BLUE	GREEN	RED	CLEAR	GREEN	CLEAR	GREEN	GREEN	RED	C A T G A G A A T	HEN
GREEN	CLEAR	CLEAR	CLEAR	GREEN	GREEN	CLEAR	GREEN	RED	A G G G A A G A T	RED
RED	CLEAR	CLEAR	CLEAR	GREEN	GREEN	GREEN	BLUE	CLEAR	T G G G A A A C G	WET
RED	GREEN	BLUE	CLEAR	GREEN	CLEAR	RED	BLUE	BLUE	T A C G A G T C C	YES
RED	CLEAR	RED	CLEAR	BLUE	CLEAR	GREEN	CLEAR	GREEN	T G T G C G A G A	CAR
BLUE	CLEAR	BLUE	CLEAR	BLUE	CLEAR	RED	GREEN	RED	C G C G C G T A T	RAY
RED	BLUE	CLEAR	GREEN	RED	BLUE	GREEN	BLUE	RED	T C G A T C A C T	SIT
BLUE	BLUE	CLEAR	GREEN	RED	BLUE	CLEAR	GREEN	GREEN	C C G A T C G A A	PIE

BCH 467, Fall 2004

Lab #3

PCR

PCR is a relatively quick and efficient way to amplify small amounts of DNA. Since the amplification relies on the hybridization of primers that are complementary to the DNA, this technique can also be used to identify the presence of particular genes in a DNA sample. In this experiment you will use primers specific for the gene encoding the green fluorescent protein and primers specific for the gene encoding the red fluorescent protein. Only one of these sets of primers should result in amplification of your DNA.

MATERIALS

1. L20 and L200 pipettors, sterile tips, PCR tubes
eppendorf tube rack
2. Plasmid A or B
(10 nanograms/microliter in 10 mM Tris-HCl, pH 8.0)
Use the same plasmid DNA unknown (either A or B) as in DNA Experiments I-1 and I-2.
3. PCR primers
(40 nanograms/microliter in water)
Primer GF: GAACTTTTCACTGGAGTTGTC
Primer GR: CCCAGCAGCTGTTACAAACTC
Primer RF: GTTATCAAGGAGTTCATGAGG
Primer RR: CTCGGTTCTTTCATACTGCTC
4. 2X PCR buffer
(20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM MgCl₂, 0.002% gelatin, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.4 mM TTP, 0.06 units/microliter Taq DNA polymerase, inert red dye)
5. sterile H₂O
6. thermocycler
7. 0.5X TBE
(44.5 mM Tris base, 44.5 mM boric acid, 1.0 mM EDTA)
8. molten agarose
(1% agarose in 0.5X TBE, boiled and then cooled to ~55 °C)
9. horizontal gel electrophoresis apparatus and power supply
10. DNA size markers (1 kb ladder, New England Biolabs)
(50 nanograms/microliter in 0.006% xylene cyanol FF, 0.006% bromophenol blue, 0.06% orange G, 2.5% Ficoll 400, 10 mM Tris-HCl, pH 7.9, 10 mM EDTA)
11. staining solution
0.5 micrograms/ml ethidium bromide in 0.5X TBE
12. UV light box, Polaroid camera and film

PROCEDURE

1. Set up PCR samples:

Prepare one reaction with the GFP primer set (GF and GR) and one reaction with the RFP primer set (RF and RR). Each reaction solution should contain 50 ng of the plasmid and 200 ng of each primer in a 1X PCR buffer with a total volume of 50 μ l in a PCR tube. The PCR buffer contains buffer, nucleotide mix, Taq polymerase and dye. Use sterile pipet tips to avoid contaminating the solutions with nucleases, and use a fresh tip each time an aliquot is taken from the stocks.

Example of PCR sample preparation:

	1 Green primers	2 Red primers
Sterile water	10 μ l	10 μ l
Plasmid (10 ng/ μ l)	5 μ l	5 μ l
Primer GF (40 ng/ μ l)	5 μ l	
Primer GR (40 ng/ μ l)	5 μ l	
Primer RF (40 ng/ μ l)		5 μ l
Primer RR (40 ng/ μ l)		5 μ l
PCR buffer (2X)	25 μ l	25 μ l

2. Run the PCR cycles:

Place the tubes in the heating block of the thermal cycler.

All the samples in the section must be started at the same time.

The TA will start the thermal cycler.

Run the thermocycler with the following temperatures and times:

Initial denaturation: 94 °C 5 min

20 cycles of

Denaturation: 94 °C 30 sec

Annealing: 55 °C 30 sec

Extension: 72 °C 30 sec

Final extension: 72 °C 5 min

The entire PCR program will take approximately 1 hour.

3. Pour an agarose gel:

Insert the gel casting tray into the casting fixture. Slowly pour 50 ml of molten agarose into the gel tray. Insert the comb into the slots in the casting tray. Allow the gel to harden undisturbed at room temperature.

4. Set up the gel electrophoresis apparatus:

Add 300 ml 0.5X TBE to the main gel box. When the gel is solidified (fully opaque), transfer the casting tray to the main gel box. Remove the comb.

5. Load and run the samples:
 - Turn off the power supply.
 - Load 20 μl of each sample in a separate lane on the gel.
 - Load 10 μl of the size markers in a separate lane.
 - Record the order of the samples in the lanes.
 - Place the cover on the gel box, connect the electrodes, and set the power supply to 100 volts.
 - Electrophorese at 100 volts for 1 hour or until the orange dye of the size markers is approximately 2/3 of the way down the gel. The size marker contains three dyes: xylene cyanol FF migrates at approximately 4 kb, bromophenol blue at approximately 300 bp and orange G at approximately 50 bp.

6. Stain and photograph the gel:
 - When the electrophoresis is finished, bring the gel to the TA, who will transfer the gel to the ethidium bromide solution.
 - Incubate the gel in the ethidium bromide solution for approximately 15 minutes.
 - The TA will then rinse the gel briefly in 0.5X TBE and place the gel on the UV transilluminator.
 - The TA will photograph the gel using the Polaroid camera.
 - WEAR GLOVES DURING THIS STEP.**
 - DO NOT LOOK DIRECTLY AT THE LIGHT BOX WITHOUT THE COVER.**

ANALYSIS:

Identify your gene based on which set of primers resulted in a PCR product for your plasmid.

Determine the size of the PCR product as in Experiment #1:

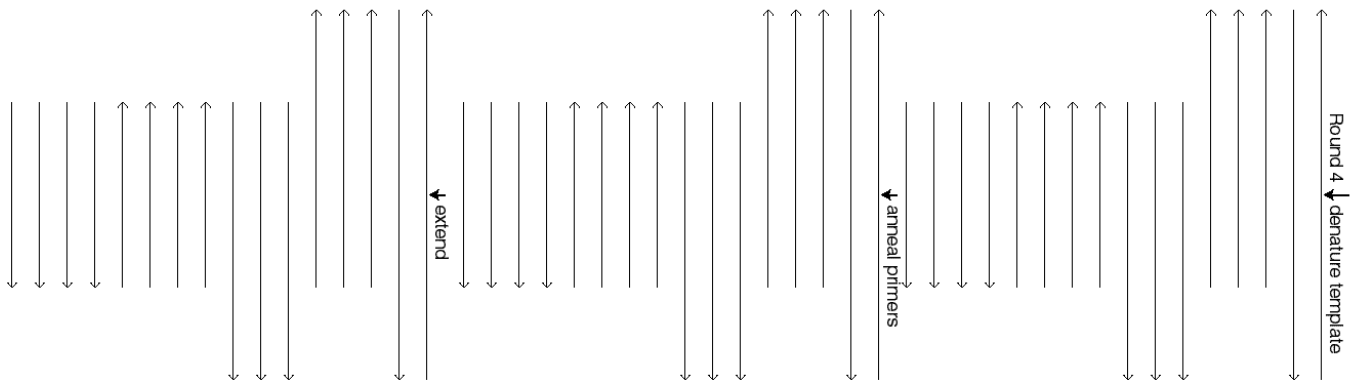
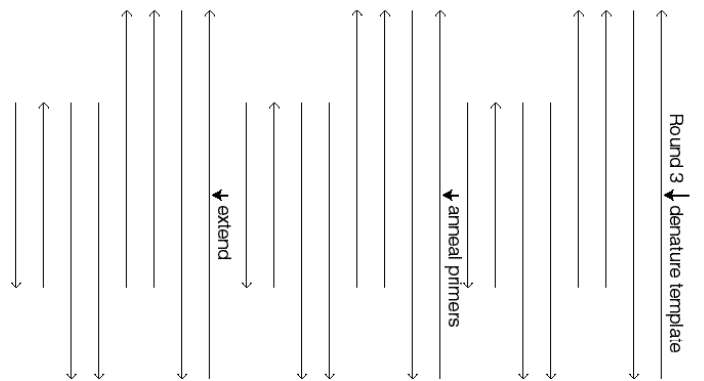
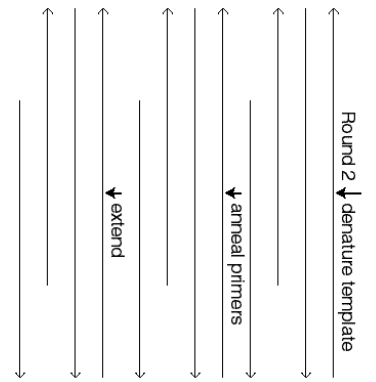
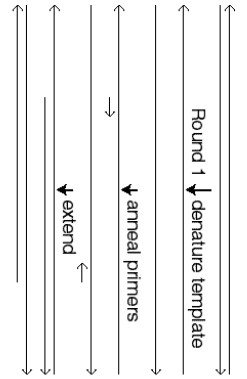
- Measure the migration distance for the size markers and PCR product on the gel photograph.
- For the size markers, plot $\log(\text{size in kilobase pairs})$ versus migration distance or use a semilog plot.
- Fit a straight line in the linear region of the size markers.
- Using the parameters from the fit, calculate the size of the PCR product.

The melting temperature of an oligonucleotide is the temperature at which 50% of the oligonucleotides are annealed to a perfectly matched complementary template. Calculate the melting temperature (T_m) of the PCR primers using the formula:

$$T_m = 4\text{ }^\circ\text{C} \times (\text{total number of G and C}) + 2\text{ }^\circ\text{C} \times (\text{total number of A and T})$$

Exercise, Lab #3

The first round of PCR and the templates for three subsequent rounds are shown. Draw the primers and extension products for the 2nd, 3rd and 4th rounds of PCR. How many double stranded fragments are present at the end of each round?



Lab Report II

(Labs #2 and #3, 200 points)

Prepare a lab report including the sections: Title, Abstract, Introduction, Materials and Methods, Results, Discussion and References. This lab report is worth 200 points.

Present the evidence that you have for the identification of the fluorescent protein gene in your plasmid. Compare the sequencing and PCR methods for identifying the gene.

Summarize your results with a detailed map of your plasmid. Use the example below (pUC19 with PhoA insert) as guidance. Mark the locations of the restriction sites and the distances between them. Show the approximate locations of the fluorescent protein gene, the PCR primers, and the sequencing primer.

Report the DNA sequence and protein sequence for at least the first 110 amino acid residues of the fluorescent protein gene. Note where the following are in your sequence:

- The beginning of the gene encoding the fluorescent fusion protein
- The 6X His tag region near the amino terminus (containing 6 sequential histidine codons)
- One of the restriction sites that you mapped
- One of the PCR primers

Were there any places where the translated DNA sequence did not match the protein sequence? Was the DNA sequence reliable in these regions? How often could the nucleotide sequence not be determined (marked as “N”)? What limits how far the sequence can be read?

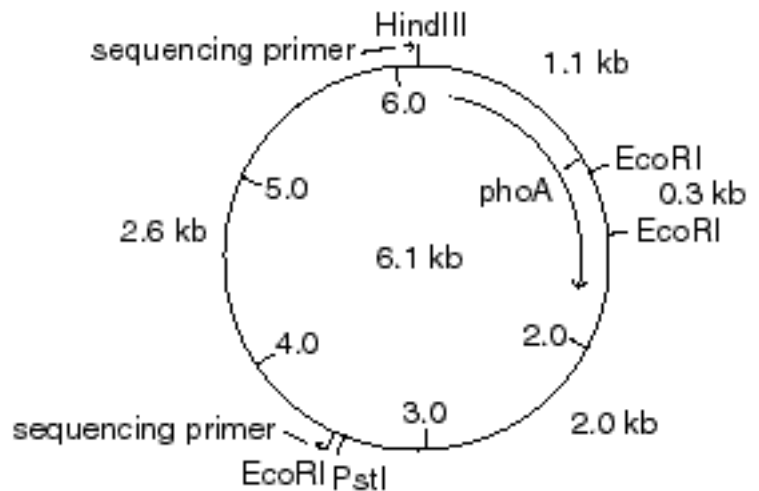
One base pair has a molecular weight of approximately 660 Da. Convert the size of the plasmid DNA in base pairs to the molecular weight. How does this compare to the size of a small protein such as green fluorescent protein (27,000 Da)? Explain why you used an agarose gel rather than a polyacrylamide gel to separate the DNA restriction fragments.

The annealing temperature during PCR is usually set to 4 to 10 °C below the melting temperature of the primers. Was this true for the PCR that you carried out? Predict what would happen if the annealing temperature were much higher and much lower than this.

In the next set of labs, you will insert the DNA into the common laboratory bacterium *Escherichia coli*. Make a prediction as to what color the bacterial colonies will be.

Example of map and sequence

This plasmid consists of the pUC19 vector with an insert containing the *phoA* gene from *E. coli*. This gene encodes the alkaline phosphatase protein. A scale is marked on the inside of the plasmid, with the HindIII site arbitrarily designated as 0. The locations of restriction sites for HindIII, EcoRI, and PstI are marked on the outside, as well as the distances between the sites. The locations of the *phoA* gene and two sequencing primers are also marked.



Below is a part of the sequence of this plasmid. The beginning of the *phoA* gene is translated into the corresponding amino acid sequence, marked with the one-letter code. The locations of the sequencing primer and the HindIII site are also indicated.

```

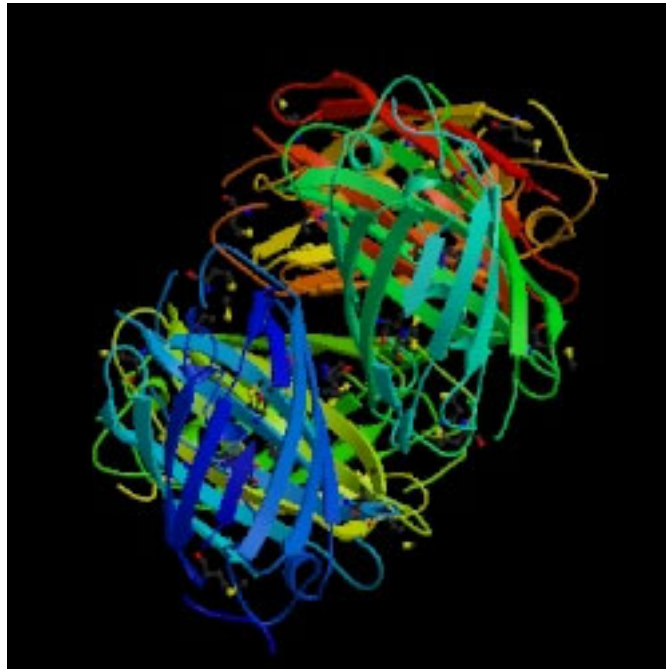
Sequencing primer
----->
GTGAGCGGATAACAATTTACACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTTGG      60
AGATTATCGTCACTGCAATGCTTCGCAATATGGCGCAAATGACCAACAGCGGTTGATTG      120
ATCAGGTAGAGGGGGCGCTGTACGAGGTAAAGCCCGATGCCAGCATTCCTGACGACGATA      180
CGGAGCTGCTGCGCGATTACGTAAAGAAGTTATTGAAGCATCCTCGTCAGTAAAAAGTTA      240

                                Start of phoA gene
ATCTTTTCAACAGCTGTCATAAAGATGTCACGGCCGAGACTTATAGTCGCTTTGTTTTTA      300
      M S R P R L I V A L F L      12
TTTTTTAATGTATTTGTACATGGAGAAAATAAAGTGAAACAAAGCACTATTGCACTGGCA      360
F F N V F V H G E N K V K Q S T I A L A      32
CTCTTACCGTTACTGTTTACCCCTGTGACAAAAGCCCGGACACCAGAAATGCCTGTTCTG      420
L L P L L F T P V T K A R T P E M P V L      52
GAAAACCGGGCTGCTCAGGGCGATATTACTGCACCCGGCGGTGCTCGCCGTTTAACGGGT      480
E N R A A Q G D I T A P G G A R R L T G      72
GATCAGACTGCCGCTCTGCGTGATTCTCTTAGCGATAAACCTGCAAAAAATATTATTTTG      540
D Q T A A L R D S L S D K P A K N I I L      92
CTGATTGGCGATGGGATGGGGGACTCGGAAATTACTGCCGCACGTAATTATGCCGAAGGT      600
L I G D G M G D S E I T A A R N Y A E G      112
GCGGGCGGCTTTTTTAAAGGTATAGATGCCTTACCGCTTACCGGGCAATACACTCACTAT      660
A G G F F K G I D A L P L T G Q Y T H Y      132
  
```

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Lab #4

DNA Ligation



Cover: structure of RFP tetramer (PDB structure file 1G7K)

What color are your bacteria?

In the previous labs, you should have identified the gene on your plasmid as coding for either GFP or RFP. In order to confirm the identification of the gene, you will insert the DNA into the common laboratory bacterium *Escherichia coli*. The bacteria should “express” the gene under certain growth conditions. The fluorescent proteins will be identified by measuring the absorption and fluorescence spectra of cell extracts.

The effect of altering the plasmid on the bacterial expression will also be investigated. Plasmids A and B each contain two EcoRI sites. The smaller EcoRI fragment contains most or all of the fluorescent protein gene, and the larger fragment contains the ampicillin resistance gene of the vector. The plasmids will be cut at the EcoRI sites. The fragments generated by this digestion will then be joined using a DNA ligase enzyme.



DNA LIGATION

DNA fragments generated by restriction endonucleases can be joined together by a ligation reaction and then taken up by bacteria in a process known as transformation. In the ligation reaction, DNA ligase catalyzes the formation of a phosphodiester bond between adjacent 5' phosphate and 3' hydroxyl groups. Although *E. coli* is not naturally transformable, the bacteria can become “competent” for DNA uptake after treatment with calcium chloride. This week (experiment #4), you will cut your plasmid with the restriction enzyme EcoRI, purify the digest using a spin column and then ligate the product overnight. Next week (experiment #5), you will transform “uncut”, “cut”, and “cut and ligated” plasmid DNA into *E. coli*.

MATERIALS

1. L20 and L200 pipettors, sterile tips, sterile 1.5 ml eppendorf tubes
eppendorf tube rack, float
Use sterile eppendorf tubes and sterile pipet tips to avoid contaminating the solutions with nucleases and to avoid contamination with other microorganisms.
2. Ice
3. Plasmid A or B
(80 nanograms/microliter in 1X digestion buffer)
Use the same plasmid DNA unknown (either A or B) as in the Part I DNA labs.
4. 1X digestion buffer
(50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol (DTT))
5. Restriction endonuclease EcoRI
(2 units/microliter in 1X EcoRI digestion buffer, on ice)
6. DNA purification spin columns (QIAquick PCR purification Kit)
7. 37° C water bath
8. 65° C water bath
9. 2X ligation buffer
(1X = 50 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA, pH 7.5)
10. T4 DNA ligase
(400 units/microliter in 2X ligation buffer, where one unit is defined as the amount of enzyme required to give 50% ligation of Hind III fragments of λ DNA (5' DNA termini concentration of 0.12 µM) in 20 µl of 1X T4 DNA ligation reaction buffer in 30 minutes at 16 °C)

PROCEDURE

1. Digestion

Set up two tubes with plasmid DNA. One tube will have the uncut sample with no enzyme added and the other tube will be for EcoRI digestion. The digest should contain 800 ng DNA and 20 units of enzyme in a total volume of 40 μ l of 1X EcoRI digestion buffer.

Example of digestion setup:

	Digestion 1	Digestion 2
Plasmid (80 ng/ μ l) in 1X digestion buffer	5 μ l	10 μ l
1X digestion buffer	15 μ l	20 μ l
EcoRI (2 U/ μ l)		10 μ l

Incubate at 37 °C for 30 minutes.

Incubate at 65 °C for 20 minutes to inactivate the EcoRI enzyme.

Place on ice briefly to cool, and then centrifuge the eppendorf tube for approximately 30 seconds in the microcentrifuge to collect all the liquid in the bottom of the tube.

2. DNA purification (Qiagen)

Purify the two digest reactions using the spin columns provided, and the manufacturer's protocol (Qiagen QIAquick PCR purification kit).

3. Ligation

Set up a second set of tubes and transfer 200 ng of DNA from the digestion tubes. One of the second set of tubes will contain the uncut sample, one will contain a cut sample and one will be ligated. The ligation should contain 200 ng EcoRI-digested DNA and 40 unit ligase in a total volume of 20 μ l 1X ligation buffer. The final concentration of DNA in each of the tubes should be 10 ng/ μ l.

Example of ligation setup:

	Ligation 1	Ligation 2	Ligation 3
Plasmid (20 ng/ μ l) from digestion	10 μ l digestion 1	10 μ l digestion 2	10 μ l digestion 2
2X ligation buffer	10 μ l	10 μ l	5 μ l
Ligase			5 μ l

Incubate at 4° overnight.

DNA Exercise Lab #4: Cut and Paste

In DNA manipulation, it is often necessary to transfer a fragment of DNA from one plasmid vector to a different plasmid, or otherwise rearrange DNA fragments, in a procedure called subcloning. For this exercise, you want to subclone a green or a red gene from a purple plasmid into a blue plasmid.

Obtain a set of four "*E. coli*" cells.

Do a "plasmid isolation" from the "*E. coli*." The copy number, or number of plasmids per cell, for the plasmids in this exercise is one. These are very low copy number plasmids. A high copy number plasmid has hundreds of copies per cell.

Do a "restriction digest" at the black and white sites of the plasmids, leaving "sticky ends" on each fragment. The large blue and purple fragments represent vectors and the small blue and red or green fragments represent inserts.

Mix the fragments together and give them to your lab partner. Have your lab partner “ligate” the fragments together into the four possible combinations of vector and insert. The black sticky ends should ligate only with other black sticky ends and the white sticky ends should ligate with white sticky ends. Each ligation product should have one vector and one insert

Your lab partner should then perform a “transformation” to insert the recombinant plasmids back into the *E. coli* cells (one plasmid per cell) and return the cells to you.

To determine which cell contains the red or green gene in the blue plasmid, perform another “plasmid isolation”.

Using the following information, determine the characteristics of *E. coli* with each of the four possible recombination products:

The large blue fragment contains a gene for tetracycline resistance.

The purple fragment contains a gene for kanamycin resistance.

Complete blue plasmids allow the *E. coli* to produce β -galactosidase, which turns *E. coli* blue when plated on a medium containing X-gal.

Worksheet:

Plasmid construction	<i>E. coli</i> Colony Phenotype			
	Tetracycline resistant	Kanamycin resistant	Turns blue when plated on x-gal	Remains white when plated on x-gal
Purple and red or green				
Blue and blue				
Purple and blue				
Blue and red or green				

How you could pick the *E. coli* that contains the desired construction (the red or green gene in the blue plasmid)?

What step could you include after the digestion and before the ligation in order to increase the chances of getting cells with the desired construction?

Please return the plasmids to their original state (blue with blue and purple with red or green) before returning the *E. coli* to your TA.

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Lab #5

Transformation and Protein Expression in Liquid Culture

The ligation reactions you prepared last week were incubated at 4° for one week (overnight would usually be sufficient). This week, you will transform *E. coli* cells with the uncut, cut, and cut and ligated plasmids. You will then spread the transformed cells on Petri dishes to allow them to grow into colonies.

The nutrient medium contained in the agar plates also contains the antibiotic ampicillin. Hence, only cells that express the ampicillin resistance gene (Amp^r) will be able to grow. Amp^r codes for β -lactamase, an enzyme that degrades ampicillin. If the ampicillin is not degraded, it will prevent bacterial cell wall biosynthesis, hence cells without Amp^r will not be able to multiply. Cells that grow into colonies should contain the intact plasmid (pRSetB or pQE30).

You will also inoculate liquid medium with a starter culture. The starter culture was prepared by the TA on the previous day. The TA inoculated 5 ml LB broth with one freshly transformed *E. coli* colony. The *E. coli* cells are strain JM109(DE3) and contain the plasmid pRSetB with the *gfp* insert. pRSetB belongs to the pET family of vectors, where the expression of insert DNA is controlled by the T7 promoter, a phage promoter. The T7 RNA polymerase is very specific for T7 promoters and it does not recognize DNA from other sources. The T7 RNA polymerase is about 5 times faster than *E. coli* RNA polymerase, so genes controlled by T7 promoters can be overexpressed. Overexpression only works if the T7 RNA polymerase is supplied to cells. This is accomplished by using a strain that is lysogenic for a fragment of the phage DE3, which contains (among other things) the gene coding for T7 RNA polymerase. Expression of this polymerase is induced by the addition of lactose or IPTG to the medium. These inducers bind and inactivate a repressor protein. We will use IPTG (isopropyl beta-D-thiogalactopyranoside) as an inducer, a non-metabolizable analog.

Materials and Supplies

1. Competent *E. coli* cells
(strain JM109-DE3 or XL1-blue in 0.1 M $CaCl_2$)
2. LB broth
(1% tryptone, 0.5% yeast extract, 0.05% NaCl)
3. 37° shaker-incubator
4. Petri plates with LB agar medium
(1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, 1% lactose, 100 μ g/ml ampicillin)
5. sterile colony spreaders (disposable)
6. 37° incubator
7. Erlenmeyer flasks containing 100 ml sterile LB broth (3 per section).

8. IPTG (dry powder).
9. Ampicillin stock solution, 100 mg/ml. Stored frozen (-20 deg), thawed at the beginning of lab.
10. Shaker-incubator, flask clamps.
11. Absorbance spectrophotometer.
12. Disposable plastic cuvettes, 1 ml volume.
13. Microcentrifuge
14. Falcon culture tubes, sterile (36 per section).
15. Squirt bottle containing 70% ethanol.

Discard biological waste (anything with *E. coli*) in an appropriate container.

A. Expression of GFP in liquid culture

This experiment will take approximately four hours. Work in groups of four students. Get started right away, then use the lag time to do the transformation (B) below.

1. Add a total of 20 mg ampicillin to 50 ml LB broth, supplied in a 250-ml Erlenmeyer flask.
2. Using sterile technique, pour the 5-ml starter culture into the 250-ml Erlenmeyer flask.
3. Set the absorbance spectrophotometer to $\lambda = 600$ nm, and set a cuvette containing LB broth in both the sample and reference compartment. Autozero. Remove the sample compartment cuvette, and replace with a cuvette containing the inoculated medium. Read the optical density at 600 nm. This will be your zero timepoint. The optical density is proportional to cell density.
4. Set the Erlenmeyer flask in the shaker and shake at 37° C.
5. Collect an OD reading at 600 nm every 30 min. Plot a growth curve directly into your notebook as you go along.
6. After 2 hours of shaking, add 25 mg IPTG to the culture. Carefully weigh out the IPTG on the analytical balance wearing gloves and using new weigh paper to avoid contamination.
7. Take your last OD reading about 2 hours after induction by IPTG. Cell growth should have slowed down due to expression of GFP protein.
8. Transfer 1 ml culture into each of two eppendorf tubes, and spin in the microcentrifuge for one minute. Discard the supernatant (biological waste). In the dark, shine UV light at the pellets (wearing UV-goggles). If GFP has been expressed by the cells, they should glow green.
9. Label the eppendorfs with your group's names and store at -20° until later. We will load a small amount on a protein gel in a few weeks.

B. Transformation

Set up three sterile tubes on ice and add 45 μ l competent *E. coli* cells. Add 50 ng of plasmid DNA that has been (1) not cut, (2) digested with EcoRI and (3) digested with EcoRI and ligated.

Example of transformation setup:

	Transformation 1 uncut	Transformation 2 cut	Transformation 3 cut and ligated
E. coli	45 μ l	45 μ l	45 μ l
Plasmid DNA	5 μ l ligation 1	5 μ l ligation 2	5 μ l ligation 3

Mix the *E. coli* and DNA gently.

Incubate on ice for 5 minutes.

Place in 37° C water bath for 30 seconds.

Incubate on ice for 2 minutes.

Add 200 μ l LB broth.

Incubate at 37 °C with shaking for 1.5 hours.

Spread 50 μ l of the cells on a Petri plate until the liquid is absorbed.

Leave in 37 °C incubator.

Plates will be incubated overnight at 37 °C, then incubated at room temperature for four days, and then stored at 4 °C until next week.

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Lab #6

Protein Expression on Solid Media

Colonies should have grown on the transformation plates from the previous week. Each colony arises from a single cell. The large colonies are ampicillin resistant. Smaller “satellite” colonies that do not contain a plasmid may also be visible. Colonies with fluorescent protein genes should be colored due to the expression of the protein. The absorption and fluorescence spectra of the green and red fluorescent proteins can be measured after lysing the bacterial cells with detergent.

MATERIALS

1. L1000 pipettor, tips, 1.5 ml eppendorf tubes
2. Plates from DNA Experiment Lab #4
3. Colony spreaders
4. Cell lysis buffer
(20 mM Tris-HCl, pH 7.5, nonionic detergent)
5. DNase I solution
(deoxyribonuclease I, 2 mg/ml)
6. 1 ml clear-sided cuvetts
7. spectrophotometer
8. fluorimeter
9. UV light box

Discard biological waste (anything with *E. coli*) in an appropriate container.

PROCEDURE

1. Record the number and color of the colonies on each plate from last week.
Place the plates under the cover on the UV light box. Record which colonies are fluorescent.
2. Resuspend the cells.
Add 1 ml of the lysis buffer to the surface of the plate from the “uncut” plasmid transformation. Resuspend the colonies with a colony spreader.
Tilt the plate slightly and pick up as much liquid as possible, then transfer to a 1.5 ml eppendorf tube.
3. Prepare a crude cell extract.
Vortex the tube for approximately 1 minute to disperse the bacterial cells.
Add 10 μ l DNase solution.
Incubate at room temperature for 15 minutes.
Centrifuge 5 minutes, 13,000 rpm, in microcentrifuge.
Transfer 0.5 ml of the supernatant to a 1 ml clear-sided cuvet.

Add 0.5 ml lysis buffer.

(Note: If the student's experiment was not successful and a petri dish with colonies provided by the TA is used, dilute the supernatant 10-fold before proceeding).

4. Measure the absorption spectrum:
 - a. Set the wavelength to 700 nm.
 - b. Set cuvettes containing blank solution (lysis buffer) into the reference and sample cell.
 - c. Autozero.
 - d. Collect baseline.
 - e. Replace the blank solution in the sample cell with your crude cell extract.
 - f. Collect an absorbance scan from 700 nm to 300 nm.

Determine the wavelength of the absorption peaks.

5. Measure the fluorescence spectrum.

For the green cells, scan the emission spectrum from 475 nm to 575 nm using 455 nm excitation.
For the red cells, scan the emission spectrum from 550 nm to 650 nm using 530 nm excitation.
Determine the wavelength of the fluorescence emission peak.

DNA Exercise Lab #6: Changing Color

Several types of mutations have been shown to shift the absorption and emission wavelengths of fluorescent proteins. Mutations are often introduced using an oligonucleotide in a PCR-based procedure. The mismatched bases should be in the middle of the oligonucleotide used as the PCR primer so that it is held down by base pairing on either side. Often a restriction site change that makes a silent mutation (that is, a mutation that does not change the amino acid sequence) is included to provide a method for screening the mutagenesis products.

Design oligonucleotides for the following mutations that change the color of the fluorescent protein. The mutations are designated by the wild type amino acid residue, the amino acid residue number, and the altered amino acid residue, such that the mutation threonine to tyrosine at residue 203 is called T203Y. For each mutation, include a silent mutation that introduces one of the restriction sites in the table on the following page.

Green to Yellow: T203Y

Mutation: threonine to tyrosine at residue 203

For example, in this oligonucleotide, an Eco47III (AGCGCT) restriction site could be introduced.

Original sequence:

↓Residue 203

DNA	CAT	TAC	CTG	TCC	ACA	CAA	TCT	GCC	CTT	AGC
protein	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	Ser

Modified sequence:

DNA										
protein										

Green to Blue: Y66H

Original sequence:

↓Residue 66

DNA	ACT	ACT	CTG	ACT	TAT	GGT	GTT	CAA	TGC	TTT
protein	Thr	Thr	Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe

Modified sequence:

DNA										
protein										

Red to Green: K70M

Original sequence:

↓Residue 70

DNA	CAG	TAT	GGA	AGC	AAG	GTA	TAT	GTC	AAG	CAC
protein	Gln	Tyr	Gly	Ser	Lys	Val	Tyr	Val	Lys	His

Modified sequence:

DNA										
protein										

Restriction Enzyme	Recognition Site
ApaLI	GTGCAC
BsrGI	TGTACA
Eco47III	AGCGCT
HindIII	AAGCTT
SnaBI	TACGTA

GGG	G	Gly	TGG	W	Trp
GGA	G	Gly	TGA	*	End
GGT	G	Gly	TGT	C	Cys
GGC	G	Gly	TGC	C	Cys
GAG	E	Glu	TAG	*	End
GAA	E	Glu	TAA	*	End
GAT	D	Asp	TAT	Y	Tyr
GAC	D	Asp	TAC	Y	Tyr
GTG	V	Val	TTG	L	Leu
GTA	V	Val	TTA	L	Leu
GTT	V	Val	TTT	F	Phe
GTC	V	Val	TTC	F	Phe
GCG	A	Ala	TCG	S	Ser
GCA	A	Ala	TCA	S	Ser
GCT	A	Ala	TCT	S	Ser
GCC	A	Ala	TCC	S	Ser
AGG	R	Arg	CGG	R	Arg
AGA	R	Arg	CGA	R	Arg
AGT	S	Ser	CGT	R	Arg
AGC	S	Ser	CGC	R	Arg
AAG	K	Lys	CAG	Q	Gln
AAA	K	Lys	CAA	Q	Gln
AAT	N	Asn	CAT	H	His
AAC	N	Asn	CAC	H	His
ATG	M	Met	CTG	L	Leu
ATA	I	Ile	CTA	L	Leu
ATT	I	Ile	CTT	L	Leu
ATC	I	Ile	CTC	L	Leu
ACG	T	Thr	CCG	P	Pro
ACA	T	Thr	CCA	P	Pro
ACT	T	Thr	CCT	P	Pro
ACC	T	Thr	CCC	P	Pro

Lab Report III (Labs #4, #5 and #6)

Calculate the transformation efficiencies for the “uncut”, “cut” and “cut and ligated” plasmids:

$$\text{Transformation efficiency} = (\text{number of colonies})/(\mu\text{g DNA})$$

To do this, the amount of DNA that resulted in the number of colonies on each plate needs to be calculated. Remember that not all of the transformation mixture was plated. If no colonies were observed on a plate, calculate what the efficiency would be if there had been one colony. The efficiency of this transformation must be less than this.

The “uncut” plasmid is mostly supercoiled, the “cut” plasmid is linear, and the “cut and ligated” plasmid is relaxed circular DNA. How can you explain different transformation efficiencies for each type of DNA? What other factors besides the transformation efficiency could affect the number of colonies on the “cut and ligated” sample?

Using the molecular weight of the plasmid determined previously, convert the amount of DNA that you used in the transformation of the “uncut” plasmid to the number of moles of DNA. Using Avogadro’s number, convert the number of moles of DNA to the number of molecules of DNA in the transformation. Assuming that each colony arose from one molecule of DNA, what percentage of the DNA molecules were successfully transformed? What might limit the number of colonies obtained from the transformation?

Provide an explanation for any changes in the color of the colonies on the plates for the transformations with “cut” plasmid DNA and “cut and ligated” plasmid DNA compared to “uncut” plasmid DNA. Draw the three possible ligation products and indicate what color each would be. Explain how the concentration of DNA in the ligation could affect the ratio of the colors observed for the colonies.

Different color variants have been made by altering the amino acid sequences of GFP and RFP. Several of these are listed below. From your results on the absorption and emission spectra, determine which strain of GFP or RFP you have. The peaks will vary slightly depending on the conditions, so find the one that is closest to your observations. Does your gene sequence support this assignment?

<u>Variant</u>	<u>absorption maximum</u>	<u>emission maximum</u>
Wild type GFP	395 nm, 470 nm	504 nm
Y66H	384 nm	448 nm
Y66W	436 nm	485 nm
F64L, S65T	488 nm	507 nm
S65G, S72A, K79R, T203Y	514 nm	527 nm
Wild type RFP	558 nm	583 nm
K70M	480 nm	499 nm
K83M	564 nm	602 nm

Plot a growth curve for the liquid-culture GFP expression experiment. Did your growth curve level off after IPTG induction? Do you believe that the IPTG induction worked? Why or why not?

Lab #7

Protein purification via Ni-affinity chromatography

During the next three lab periods, you will purify green fluorescent protein (GFP) from bacterial cell paste. This protein has been overexpressed the pET expression system that we used previously in the lab: the *E. coli* strain used is JM109(DE3), and the expression plasmid carrying the gene coding for GFP is pRSetB. Cells were grown by the TA in four one-liter batches in a shaker/incubator until the optical density (OD600) reached 1.0, then induced with IPTG and allowed to grow for another four hours. The cells were then harvested by centrifugation. This procedure is much like lab #5 (protein expression in liquid culture), though larger in scale. The weight of the wet cell paste was determined (between 10 and 20 g depending on batch), and cells were frozen at -80°.

This week, you will be given frozen *E. coli* cell paste. The paste will be suspended in cell suspension buffer and sonicated to burst the cells open. The batch will then be divided between the student groups. You will clarify the lysate by centrifugation, then purify the protein by Ni-affinity chromatography. The protein will be bound to the resin, the impurities will be washed off with low imidazole buffer, and GFP will be eluted with high imidazole buffer. Fractions containing purified GFP protein will be dialyzed overnight to remove imidazole.

Materials:

- 1) EGFP Cell Pellet (1 per section)
- 2) Lysis Buffer (50mM HEPES pH 7.9, 300mM NaCl, 3mM BME, 10% Glycerol, 0.1mM PMSF). Add PMSF immediately before use, along with a small spec of DNase and 100µl 1M MgCl₂.
- 3) Branson 450 Sonifier
- 4) Beckman J2-21 Centrifuge
- 5) Beckman JA-20 Rotor
- 6) 50 ml Polypropylene Centrifuge tubes
- 7) 1 Glass pipet and bulb
- 8) 1 syringe column (3 ml) with glass wool
- 9) 2-way stopcock, and yellow stopper
- 10) Column stand and clamp
- 11) 4mls of Ni-NTA superflow slurry
- 12) 40 glass test tubes and test tube rack
- 13) Column wash Buffer (50mM HEPES, 7.9, 300mM NaCl)
- 14) Low imidazole buffer (20mM Imidazole, 50mM HEPES, 7.9, 300mM NaCl)
- 15) High imidazole buffer (100mM Imidazole, 50mM HEPES, 7.9, 300mM NaCl)
- 16) 20 x 1ml cuvettes, disposable, UV-transparent (Methacrylate, from Fisher)
- 17) Dialysis tubing, molecular weight cut off of 12,000-14,000Da
- 18) 0.2M Acetic acid

Procedure:

- 1) A frozen cell pellet on ice will be provided to your section. The pellet is thoroughly broken into pieces by mechanical means, then resuspended in 100 ml Lysis Buffer. This process is demonstrated by the TA. Follow your TA to PSD327 for sonication of the cell suspension. The

TA will demonstrate sonication of the entire suspension for 5 minutes. Sonication will be carried out for 5 minutes using a large sonicator tip and an amplitude of 7 (about 60% power), alternating 1 minute on, 1 minute off. The cell suspension should be kept on ice for the entire duration.

- 1) After breaking open the cells, clarification of the extract from cellular debris is done by centrifugation. Your TA will demonstrate this for you as well. The cell suspension will be split in two aliquots (50 ml each) and transferred into 50 ml polypropylene centrifuge tubes. The tubes will be balanced against each other to be of equal weight. They will be centrifuged for 45 minutes at 14,000 rpm in a high-speed centrifuge. After the centrifugation is complete, each student pair will obtain 17 ml supernatant.
- 2) Obtain a 3ml syringe from your TA. This syringe body will be used as a column. Stuff the bottom with glass wool while wearing gloves. Obtain a 2-way stopcock, and a yellow stopper. Set up your column using a stand and a clamp. Obtain test tubes and a test tube stand.
- 3) Pour the column: Obtain 4 ml of the Ni-NTA slurry (50%) and pour it into the column while the stopcock is closed. Allow the resin to settle for a few minutes. Open the stopcock and allow most of the excess liquid to drain into a beaker. Do not allow the resin to dry out. Final resin bed volume should be about 2ml. There should be a 2 ml reservoir above the bed.
- 4) Wash the column with 10 ml **water**. Gently apply the water to the top of the column by addition of 1 ml at a time with a pipette. Do not perturb the resin bed. Allow most but not all of the water to drain into a beaker.
- 5) Equilibrate the column with 10 ml **Column wash buffer**. Carry out this step as in step 4).
- 6) Load 17ml of cell extract and start collecting 1-ml fractions **immediately**. To do so, first set up a series of test tubes in a row using a test tube holder. Number them sequentially with a felt-tip pen. Mark them to indicate the height of 1 ml solution. Now gently apply the clarified cell extract to the top of the column while collecting the eluent with tube #1. When 1 ml has been collected, replace tube #1 with tube #2, etc. Allow all of the extract to partition into the column resin while taking care not to dry out the stationary phase.
- 7) Wash the column with 15 ml of **Column wash buffer**. Continue to collect fractions sequentially.
- 8) Wash the column with 10 ml of **Low imidazole buffer**. Note the fraction collected when applying the low imidazole buffer.
- 9) Elute the protein with 10 ml of **High imidazole buffer**. Again, note the fraction number when applying the high imidazole buffer.

- 10) Measure the absorbance at 280 nm (A_{280}) for every 2nd fraction. Use column wash buffer as a blank. The absorbance at 280 nm gives a measure of total protein content in that fraction. Imidazole will also absorb at that wavelength. Plot fraction number against A_{280} directly in your notebook. The resulting graph is called a chromatogram. This graph should be included in your report.
- 11) Pool fractions of the second peak: These are the fractions that eluted after application of the high-imidazole buffer. They should contain GFP and look green to the eye. Take an absorbance scan of this GFP pool from 600 nm to 240 nm. Use column wash buffer as a blank.
- 12) Start Dialysis: Wear gloves. Obtain Dialysis tubing from your TA. Cut off an appropriate length of tubing. The tubing holds about 3.3 ml/cm. 15 cm should be sufficient to hold the protein pool and to tie knots at the ends. Soak the tubing in water for about 10 minutes before use. Tie off one end of the tubing, then transfer the protein pool into the tubing using a pipette. Tie off the other end and use an eppendorf to label your sample. Place into the 2 liter Column wash buffer (dialysis buffer) designated for your section.
- 13) Clean up your column: Wash the column with 15 ml water, then 15 ml 0.2 M acetic acid, and last another 25 ml water. Recycle the resin into a designated container.

Lab #8

Cleavage of poly-histidine tag, followed by anion exchange chromatography

This week, you will continue to purify your GFP sample. During dialysis, the imidazole buffer has been removed. In addition, the protein was dialyzed against buffer containing no sodium chloride. This will be important to get efficient binding to the DEAE resin. You will proceed to remove the poly-histidine tag by digesting the protein with α -chymotrypsin. This protease cleaves after aromatic residues if the site is accessible. Since the polyhistidine tag contains a phenylalanine right before the start of the mature protein sequence, and since the tag is disordered (not part of the eleven-stranded β -barrel), chymotrypsin will cleave the protein at this site. It will also degrade the tag into short peptides.

After protease digestion, you will further purify GFP via anion exchange chromatography. GFP is retained strongly by the resin due to charge-interactions, whereas the peptides and other protein impurities elute more rapidly.

Materials:

- 1) 2 L Dialysis buffer (20mM HEPES, 1mM EDTA, pH=7.9)
- 2) Alpha-chymotrypsin stock solution (1mg/ml)
- 3) 100 mM PMSF stock solution
- 4) DEAE Cellulose, 70% slurry
- 5) 1.5 cm ID x 10 cm glass column, stop cock, yellow stopper
- 6) Glass Test tubes x 25
- 7) Test tube rack
- 8) Low-salt buffer (20mM HEPES, 50mM NaCl, pH=7.9)
- 9) High-salt buffer (20mM HEPES, 300mM NaCl, pH=7.9)
- 10) 15 x 1.5 ml cuvettes (UV-transparent)
- 11) P1000 Pipettes and tips

Procedure:

- 1) Retrieve your protein sample from the dialysis bath. Your samples were removed from dialysis in Column Wash Buffer and dialyzed against 20mM HEPES, pH 7.9, 1 mM EDTA in order to prepare them for this week. Remove a 1-ml sample of this His-tagged EGFP for both SDS-PAGE analysis (Lab # 9) and for the optical properties lab (Lab #10). Label your eppendorf tube with your group name/section. Store at 4°C.
- 2) Start the 6His-tag digest: Add 0.5 ml of alpha-chymotrypsin to your protein sample. Mix gently and allow the reaction to proceed at room temperature for 1 hour. Take your EGFP sample to your TA to have the reaction stopped. This will be done by addition of 50 μ l of 100mM PMSF (phenylmethanesulfonylfluoride), a potent and toxic protease inhibitor. Wear gloves during the following steps, to protect your hands from the toxicity of PMSF.
- 3) Set up the column: Obtain a column, stopcock, and yellow stopper from your TA. Set up the column using a stand and clamp. Obtain test tubes and a test tube stand as well.

- 4) Obtain the DEAE cellulose from your TA. This resin is a 70% slurry. Take 25 to 30 ml slurry and pour it into the column with the stopcock in the closed position. Allow the resin to settle for a few minutes. This should result in a final bed volume of around 8 ml. Open the stopcock and allow most of the excess liquid to drain into a beaker. Do not let the resin dry out. There should be about a 10ml reservoir above the bed.
- 5) Equilibrate the column in **20mM HEPES pH=7.9**. Use about 3 bed volumes.
- 6) Load the digested protein onto the column, and start collecting 2-ml fractions **immediately**. (Number the test tubes sequentially in the test tube holder. Measure out 2 ml of water and mark the test tubes to that approximate volume.)
- 7) Wash column with 2 bed volumes of **20mM HEPES, 7.9**.
- 8) Wash the Column with 2 bed volumes of **low-salt buffer (20mM HEPES, 50mM NaCl, pH=7.9)**.
- 9) Elute the protein with 1 bed volume of high-salt buffer (**20mM HEPES, 300mM NaCl, pH=7.9**).
- 10) Collect A_{280} measurements of every other fraction. Plot the absorbance versus fraction number in your notebook. Mark the addition of the low-salt buffer and the addition of the high-salt buffer in your chromatogram.
- 11) Compare the chromatogram with the fractions that look green. Did GFP elute under high-salt conditions? Pool peak fractions containing GFP. Take an absorbance scan of your pooled fractions from 600 nm to 240 nm. Use 20 mM HEPES, 300mM NaCl, pH=7.9 as a blank.
- 12) Label a 15 ml falcon tube with your group name/section, and store your protein at 4°C for use in subsequent experiments.

Lab #9

BCA and Bradford Protein Assays

This week, you will determine the protein concentration in your EGFP pools, before and after cleavage of the 6His-tag (post-Nickel column and post-DEAE column). You will use chromogenic protein assays that are based on dye binding (Bradford) or copper binding (BCA) to the protein. You will assay each of your protein pools in triplicate, then determine the average and standard deviation for each pool and for each assay.

In both assays, standards and samples must be prepared at the same time, and the absorbance must be read at the same time. Work in pairs, **DO NOT SPLIT UP THE WORK AND TRY TO RUN BOTH ASSAYS SIMULTANEOUSLY!**

You will compare the results obtained from each assay with each other, and with the protein concentration obtained via absorbance at 280 nm (Lab #10).

BRADFORD ASSAY

Bradford Materials:

- 1) Bradford dye reagent
- 2) BSA (bovine serum albumin) stock solution (10 mg/ml)
- 3) 4-ml cuvettes
- 4) P1000 pipets and tips
- 5) P200 pipets and tips
- 6) Eppendorf tubes
- 7) Disposable glass culture tubes
- 8) Deionized Water

Bradford Procedure:

- 1) Prepare a series of BSA standard solutions that range from 0 mg/ml to 2 mg/ml. To do so, label a series of eppendorfs with the following concentrations: 0.0, 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 mg/ml BSA. Notice that this will include two eppendorfs with no (0.0) BSA. Now determine the dilution of the BSA stock necessary to obtain the final BSA concentration, and fill in the dilution table below.

Bradford dilution table:

Final concentration in eppendorf (mg/ml)	BSA stock to add (μ l)	Water to add (μ l)	Final Volume (μ l)
0.0			1000
0.0			“
0.2			“
0.4			“
0.6			“
0.8			“
1.0			“
1.2			“
1.4			”
1.6			“
1.8			“
2.0			“

- 2) For the standard curve, label a series of glass culture tubes in the same manner as the eppendorfs. For the unknown protein solutions, label an additional six glass test tubes with the following labels:

plus Tag#1	minus Tag#1
plus Tag#2	minus Tag#2
plus Tag #3	minus Tag#3

- 3) Transfer 60 μ l each of the standard solutions from the respective eppendorf into the correctly labeled glass test tube. Use clean pipette tips, and wear gloves to avoid contamination of your sample with skin proteins (again, your series of standards will include two test tubes with no BSA).
- 4) Transfer 60 μ l each of the protein pools (plus 6His-tag and minus 6His-tag) into the correctly labeled glass test tubes. These are your “unknowns”. Each pool will be assayed in triplicate.
- 5) Add 3 ml of Bradford Reagent directly to each glass test tube. Add the reagent in series, starting with the standards (from low to high BSA), then immediately afterwards to the six sample test tubes.
- 6) Gently vortex each tube, again performing the task in the same sequence and without taking breaks.
- 7) Allow the test tubes to stand at room temperature for 5 minutes.
- 8) Read the absorbance of each tube at 595 nm. Use plastic disposable cuvettes (the dye will stain them). Autozero the spectrophotometer at 595 nm, using the 0.0 mg/ml BSA standards in both the sample and reference cells. Read and record the absorbance of each standard and sample, assaying the tubes in the same sequence as before. Do not take any breaks during this procedure.
- 9) Sketch your standard curve directly in your notebook (you will prepare a more accurate plot for your report). Plot mg/ml along the x-axis and A_{595} along the y-axis. Is the curve linear?

Include the absorbance of your samples. Can you estimate the concentration of your EGFP pools directly off the standard curve?

BCA ASSAY

BCA Materials:

- 1) BCA solution A: Pre-mixed Solution
- 2) BCA solution B: 4% copper sulfate·5 H₂O
- 3) Working reagent: Mix 50 volumes of BCA solution A with 1 volume of BCA solution B
- 4) BSA stock solution (10 mg/ml)
- 5) 4-ml cuvettes
- 6) P1000 pipets and tips
- 7) P200 pipets and tips
- 8) Water bath set to 37°C (or heat block if the glass tubes fit).

BCA Procedure:

This procedure is similar to the Bradford assay.

- 1) Prepare a set of BSA standards in the range of 0.0 mg/ml to 2.0mg/ml. You may use the same standard solutions (prepared in eppendorfs) that you used for the Bradford assay.
- 2) Add 100 µl protein sample (either standard or unknowns) to a series of labeled glass test tubes.
- 3) Add 3 ml of BCA working reagent to each glass test tube.
- 4) Mix well by gently vortexing.
- 5) Incubate the test tubes at 37°C for 30 minutes.
- 6) Cool to room temperature and read the absorbance at 560 nm against an appropriate blank (the 0.0 mg/ml BSA standard).

Lab Report # IV:

Include a short description of the biochemical properties of GFP (1–2 paragraphs) in the Introduction section. This is in addition to the usual discussion of the theory behind the experimental techniques.

Make sure to include the chromatograms for both the Ni-column and DEAE column purification steps in your report.

Lab #10

Polyacrylamide gel electrophoresis (SDS-PAGE)

In this lab, you will determine the molecular weight of your purified protein using denaturing gel electrophoresis. In addition, you will examine the crude cell extract and two stages of protein purification to determine whether the purification procedure was effective, and whether removal of the 6HisTag via chymotrypsin digest was efficient. From the gel, you will estimate the purity of your final protein pool.

Materials:

- 1) Gel casting frame, electrode assembly, power supply
- 2) Glass plates, spacers, loading comb
- 3) P1000 Pipettes and tips
- 4) P200 Pipettes and tips
- 5) P2 Pipette and tips
- 6) 15-ml Falcon tubes x 2
- 7) Heat block

Stock Solutions:

- 8) 30% acrylamide/bis solution:

acrylamide	29.2 g
bis	0.8 g
water	100 ml

filtered and stored in the dark at 4 °C.

- 9) 1.5 M Tris-HCl (pH 8.8)

Tris base	18.15 g
water	50 ml

pH adjusted to 8.8 with 1 N HCl (about 24.3 ml)
volume adjusted to 100 ml with water.

- 10) 1.0 M Tris (pH 6.8)

Tris base	6 g
water	40 ml

pH adjusted to 6.8 with 1 N HCl
volume adjusted to 50 ml with water.

- 11) 10% (w/v) SDS

SDS 10 g
volume adjusted to 100 ml with water

12) 10% (w/v) ammonium persulfate (APS)

Ammonium persulfate 1 g
adjust volume to 100 ml with water (shelf life 1 week)

13) TEMED

14) Protein MW Markers: Biorad Low-MW range.

Diluted 1 : 20 in Reducing Laemmli Sample Buffer.
Heated for 5 min at 95 °C.

15) 2X Reducing Laemmli Sample Buffer

62.5 mM Tris-HCl pH 6.8
25% glycerol
2% SDS
0.01% bromphenol blue
5% beta-mercaptoethanol (BME)

16) 1X Running Buffer (Tris/Glycine/SDS buffer)

Tris base	12 g	(25 mM Tris)
glycine	57.6 g	(250 mM glycine)
SDS	4 g	(0.1% SDS)
water	4 liter	(pH 8.3)

(no need to adjust the pH)

17) Coumassie Stain (traditional)

Coumassie Brilliant Blue R-250
1 g in 500 ml of 50% methanol/10% acetic acid
(filtered through Whatman No. 1 filter paper)

Coumassie Stain (Biosafe)

Purchased from Biorad. No dilution necessary. Water-based.
(can be discarded in the sink)

16) Fixing and Destaining Solution

50% v/v methanol
10% v/v acetic acid

Procedure:

- 1) Assemble the glass plates and spacers in the casting frame and test for water leaks. **Wear gloves at all stages of gel handling. Acrylamide is a potent neurotoxin.**
- 2) Pour the resolving gel: We will run a 15% acrylamide gel. Add the components in order from top to bottom to a glass beaker. When adding the TEMED, insert the pipette tip below the solution, and then mix by inversion. Gently swirl solution before pouring into the gel mold. Fill to 2 cm below the top of the shorter glass plate. Gently overlay the solution with water using a pasteur pipette. You will not see the polyacrylamide-water interface clearly until the gel has polymerized.

Resolving gel:

Component	Volume (ml)
H ₂ O	1.1
30% acrylamide mix	2.5
1.5M Tris (pH 8.8)	1.3
10% SDS	0.05
10% ammonium persulfate	0.05
TEMED	0.002

- 3) Allow the resolving gel to polymerize for 30 minutes.
- 4) Prepare samples for loading onto the gel: Label eppendorf tubes with the sample name. Obtain a Molecular weight marker aliquot.

Sample Preparation:

Molecular Weight Markers

The stock solution provided to you is ready for loading.
When ready, load 5 µl onto the gel.

Frozen cell pellet (from Lab #5)

- a. Add 50 µl Reducing Laemmli Sample Buffer to the cell pellet.
- b. Heat at 100 °C for 10 min with intermittent vigorous vortexing.
- c. Spin in eppendorf centrifuge for 1 min.
- d. When ready, load 5 µl supernatant onto the gel.

Dialyzed 6His-tagged EGFP, post-Nickel column (saved from Lab #8)

- a. Dilute sample: Add to eppendorf: 2 µl sample, 5 µl water, 7 µl loading buffer.
- b. Concentrated sample: Add to eppendorf: 7 µl sample, 7 µl loading buffer.

- c. Heat at 100 °C for 10 min with intermittent vigorous vortexing.
- d. Spin in eppendorf centrifuge for 1 min.
- e. Load 12 µl onto the gel.

Mature EGFP, post-DEAE column (6His-tag cleaved)

- a. Dilute sample: Add to eppendorf: 2 µl sample, 5 µl water, 7 µl loading buffer.
- b. Concentrated sample: Add to eppendorf: 7 µl sample, 7 µl loading buffer.
- c. Heat at 100 °C for 10 min with intermittent vigorous vortexing.
- d. Spin in eppendorf centrifuge for 1 min.
- e. Load 12 µl onto the gel.

- 5) Pour the stacking gel: The Stacking gel will be a 5% acrylamide gel. Add the components in order to a beaker. Swirl gently after addition of TEMED. Allow the water to drain from the separating gel by tilting the gel assembly, and catching the water with a KimWipe. Pour the stacking gel solution into the mold on top of the separating gel. Fill to the top.

Stacking gel:

Component	Volume (ml)
H ₂ O	1.4
30% acrylamide mix	0.33
1.0M Tris (pH 6.8)	0.25
10% SDS	0.02
10% ammonium persulfate	0.02
TEMED	0.002

- 6) Gently insert the comb, minimizing the trapping of air bubbles. Air will inhibit polymerization. Allow the polymerization reaction to proceed for 30 minutes.
- 7) Remove the gel assembly from the casting frame and insert into the electrode assembly. Keep the comb in place.
- 8) Pour running buffer in the buffer dam. Carefully remove the comb without perturbing the wells formed by the teeth of the comb. Convince yourself that there are 10 sample wells.
- 9) Load the samples onto the gel according to the gel loading table below. To do so, use a 20-µl pipette, fill the tip with the required sample volume, then carefully apply the sample above the intended well by resting the pipette tip on the lower glass plate. You will not be able to insert the pipette tip directly into the well. Release the sample slowly, and it will drop down into the well by gravity (due to the glycerol). Record any mistakes or spillage into an adjacent well.

Gel Loading Table:

Lane #	Sample Name	Volume (µl)
1	(leave empty)	----
2	pellet	5

3	(leave empty)	----
4	6His-tagged EGFP (concentrated)	10
5	6His-tagged EGFP (dilute)	10
6	MW markers	5
7	Mature EGFP (concentrated)	10
8	Mature EGFP (dilute)	10
9	MW markers	5
10	(leave empty)	----

- 10) Pour the rest of the running buffer into the outer chamber of the electrode assembly. Apply power at 70 V and run the samples through the stacking gel. When the dye front reaches the resolving gel, turn up the voltage to 120 V. Watch to make sure the dye front does not run off the gel. When the dye front is near the bottom of the gel (about 1 cm from the bottom), turn off the power.
- 11) Remove the gel sandwich, disassemble the glass plates and carefully remove the stacking gel with a spatula and discard. Place the resolving gel in a small tray containing water. Change the water twice, each time rinsing the gel for about 5 min.
- 12) Immerse the gel in the Coomassie dye staining solution until it is uniformly blue. This may take several hours, hence the gels will be stained overnight.
- 13) The following day, your TA will rinse the gel in water, then transfer it into the destaining solution (water if Biosafe dye was used). The Coomassie dye will be soaked out of the gel, and only protein bands will remain blue.
- 14) The TA will wash the gels in water (3 changes, 5 min each), then soak them in 20% ethanol/10% glycerol for one hour. The gel will be photocopied or scanned, then soaked for another hour in fresh 20% ethanol/10% glycerol. The alcohol will shrink the gel and keep it from tearing during the drying procedure.
- 15) The TA will mount the gels between two sheets of cellophane, allow to dry overnight, and return them to you during lecture on the following Monday. The photocopied or scanned images will be available during lecture as well.
- 16) You are required to paste either the image of the gel, or the dried gel itself, directly into your report.

Lab #11

Optical Properties and Extent of Chromophore Formation in EGFP

In this lab, you will characterize the optical properties of your purified protein pool. You will carry out absorbance and fluorescence spectrophotometry on EGFP with and without the 6His-tag. You will then determine the total protein concentration in your two protein pools via UV absorbance, and use this number to calculate the purification yield (total mg protein obtained at the end of purification).

You will then determine the total chromophore concentration. To do so, you will denature the protein under basic conditions (NaOH), then measure the absorbance of the chromophore anion in the unfolded protein. You will use the chromophore extinction coefficient to calculate total amount of chromophore present. Last, you will determine the molar ratio of chromophore to protein, and calculate the extent (percent) chromophore maturation in your purified EGFP pools (\pm 6His-tag).

Materials:

- 1) 1.5 ml UV-transparent cuvettes (UV-cutoff 220 nm)
- 2) EGFP protein pools from your purification: + 6His tag and – 6His tag, estimated concentration 0.5 to 1.0 mg/ml
- 3) Eppendorfs
- 4) 4-sided fluorimeter cuvettes

Stock solutions:

- | | | |
|----|--------------------|-----------------------------------|
| 1) | Base: | 0.2 M NaOH |
| 2) | HEPES/NaCl Buffer: | 50 mM HEPES pH 7.9
300 mM NaCl |
| 3) | 8 M urea buffer: | 8.0 M urea
50 mM HEPES pH 7.9 |

Procedure:

- 1) Obtain your EGFP samples from your TA.: post-Nickel column (+ 6His tag) and post-DEAE (- 6His tag).
- 2) Transfer 200 μ l of each protein into a labeled eppendorf. Dilute 5-fold by addition of 800 μ l HEPES buffer.
- 3) Collect an absorbance scan on each protein from 550 nm to 250 nm. To do so, autozero buffer against buffer, and collect a baseline. Then place the protein in the sample chamber, collect the scan and print.

- 4) Note total absorbance at 280 nm and determine the absorbance maxima of the chromophore in the visible region of the spectrum. There will be two peaks, a major peak due to the chromophore anion, and a minor peak or “shoulder” due to the neutral (protonated) form of the chromophore. These two species exist in equilibrium with each other in the protein’s interior.
- 5) Calculate the protein concentrations for the two protein pools. First, use Beer’s Law to determine the protein concentration in the cuvette. The extinction coefficient is a semi-empirical extinction calculated from the amino acid composition of the protein (web page: <http://au.expasy.org/cgi-bin/protparam>). Calculate both molar concentration (mmol/ml) and weight concentration (mg/ml).

$$\text{EGFP + 6HisTag:} \quad \epsilon_{280} = 21,050 \text{ M}^{-1} \text{ cm}^{-1} \quad (\text{MW} = 31,079 \text{ g/mol})$$

$$\text{EGFP - 6HisTag :} \quad \epsilon_{280} = 19,770 \text{ M}^{-1} \text{ cm}^{-1} \quad (\text{MW} = 26,894 \text{ g/mol})$$

Then determine the concentration in the original protein pools. To do so, you must take the dilution factor used for your absorbance measurements into account.

- 6) Determine the total volume of your purified protein pools, then calculate the total protein yield (in mg) for each pool.
- 7) Transfer 100 μl of each protein pool into a clean eppendorf, and add 900 μl 0.2 N NaOH. At high pH (>11), base-denaturation of the protein will occur, and the chromophore will be present in the anionic form only.
- 8) Collect absorbance scans of the base-denatured samples from 550 nm to 250 nm. Blank against 0.2 N NaOH.
- 9) Determine total absorbance at 448 nm. This is the absorbance maximum of the chromophore anion when the protein is denatured (unfolded, essentially unstructured). The chromophore is now solvent-exposed instead of buried in the protein’s interior, hence the absorbance maximum is shifted.
- 10) From the absorbance, calculate the molar concentration of the chromophore in the cuvette, using Beer’s Law and the following extinction coefficient:

$$\epsilon_{448} = 37,000 \text{ M}^{-1} \text{ cm}^{-1}$$
- 11) Now calculate the molar chromophore concentrations in the original protein pools, taking the dilution factor into account.
- 12) Obtain the percent chromophore in your samples by dividing the chromophore molarity by the protein molarity, and multiplying by 100%. Has the chromophore formed completely in your protein pools?
- 13) Perform a fluorescence emission scan on your purified EGFP pools. To do so, transfer 1.5 ml HEPES/NaCl buffer into a 4-sided fluorimeter cuvette, then add 50 μl protein to the cuvette. Set the excitation wavelength to 455 nm, and scan the emission intensity from 475 to 575 nm. Does the presence of the 6HisTag alter the optical properties of your protein?

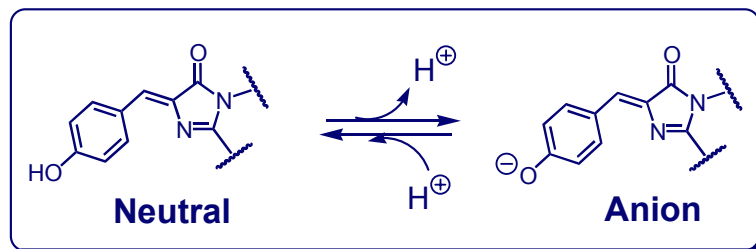
- 14) Chemically denature EGFP (– 6HisTag): Add 50 μl protein to 450 μl 8 M urea solution in an eppendorf. Heat for 5 min at 95 $^{\circ}\text{C}$ (heat block). Does the protein pool remain green? Note any visible changes in your notebook. Do you believe the protein is still fluorescent? Wear gloves when working with urea!
- 15) Collect an absorbance scan of the urea-denatured protein. Blank against urea buffer. Do you believe the chromophore is still present? Why or why not? If so, what is the absorbance maximum of the chromophore in 8 M urea? How does it compare to the base-denatured protein? How does it compare to the absorbance of the protein in the native state?

Lab #12

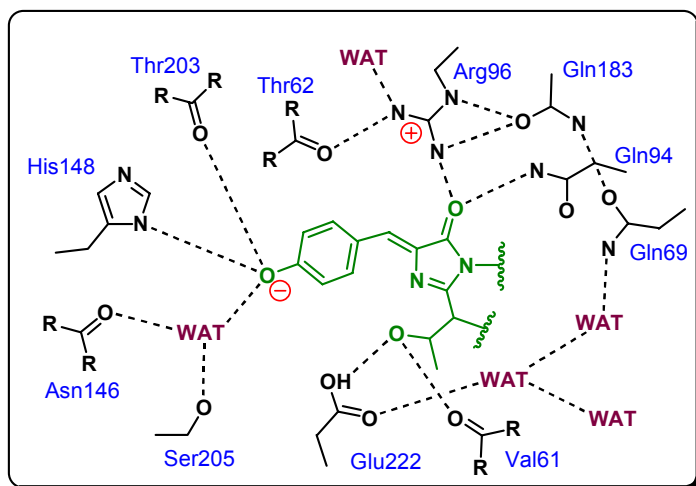
pH Titration of EGFP: Determination of Chromophore pK_a

This week, you will further characterize your final, purified EGFP pool (post-DEAE). In the mature, native protein, the chromophore is buried in the interior of the β -barrel. Hence, its optical properties are modulated by the immediate protein environment, and differ from the properties of the chromophore when exposed to aqueous solvent.

The chromophore is known to have two charge states. In native EGFP, the neutral form absorbs at 400 nm, and the anion absorbs at 489 nm. One form can be converted into the other by a change in solution pH. When the protein is denatured in 8 M urea, the chromophore pK_a has been shown to be 8.1,



whereas in the native protein, the pK_a is shifted significantly. In the protein's interior, the interactions of the chromophore with amino acid side chains will modulate the chromophore's titration behavior. Shown below is a schematic representation of the immediate protein environment around the chromophore in the center of the beta-barrel. Hydrogen bonds are shown as dashed lines. This information has been obtained from protein crystal structures.



From this schematic, can you guess whether the chromophore pK_a is shifted up or down from 8.1? Which charge state of the chromophore is preferentially stabilized by the protein? To answer this question, consider any protein side chain charges positioned close to the chromophore.

In this lab, you will determine the pK_a of native EGFP by carrying out a pH titration. You will measure absorbance spectra of the protein in buffers with increasing pH, and observe the conversion of the 400-nm peak to the 489-nm peak. You will then use the total absorbance at 489 nm as a function of pH to determine the chromophore pK_a in the native protein.

Materials:

- 1) 1-ml UV-transparent cuvettes
- 2) purified EGFP pool (6His-tag cleaved)
- 3) P1000 pipets and tips
- 4) P200 pipets and tips
- 5) Eppendorf tubes
- 6) Buffers in the range of pH 5 to pH 7: All buffers are 75mM Buffer, 140mM NaCl.
 - a. Acetate Buffer pH 4.6
 - b. Acetate Buffer pH 5.0
 - c. Acetate Buffer pH 5.3
 - d. PIPES Buffer pH 5.8
 - e. PIPES Buffer pH 6.0
 - f. PIPES Buffer pH 6.5
 - g. HEPES Buffer pH 7.0
 - h. HEPES Buffer pH 8.0

Instrumentation:

Dual-wavelength UV-VIS absorbance spectrophotometer

Procedure:

- 1) Obtain your EGFP sample of mature EGFP (minus 6His-tag) from the TA.
- 2) Label a series of eppendorfs with the pH of the respective buffers. Transfer 200 μ l of EGFP into each eppendorf, then add 800 μ l of the appropriate buffer to each eppendorf. Gently mix each tube by pipetting up and down. Do not introduce air bubbles since this leads to protein denaturation.
- 3) Transfer HEPES buffer to two UV-transparent cuvettes, place them into the sample and blank compartments of the spectrophotometer, autozero at 550 nm, and collect a baseline scan from 550 to 250 nm. Now scan each EGFP solution between 550 and 250 nm, using the same baseline correction (no need to collect a new baseline for each sample, though you may autozero at 550 nm before starting each scan).
- 4) Determine the total absorbance at 489 nm for each scan.
- 5) In your notebook, sketch a plot of pH vs A_{489} (pH along the x-axis).

- 6) Hand-fit a sigmoidal curve to your plot, and estimate the pK_a of the chromophore in EGFP.
- 7) Calculate ΔpK_a for the chromophore (denatured-protein pK_a minus native-protein pK_a). Is the pK_a shifted up or down in the native protein? Using the schematic above, can you explain your observations? Was your prediction correct?

Lab Report V:

This lab report will include SDS-PAGE (lab #10), Optical Properties and Percent Chromophore (Lab #11), and Chromophore pK_a (Lab #12). Include the following in your report:

SDS-PAGE

The purpose of this lab was to determine the molecular weight of your purified protein, to determine whether the purification procedure was effective, and whether removal of the 6HisTag was efficient. You will estimate the homogeneity of your final protein pool.

1. Include an image of your gel in the report.
2. Plot relative mobility versus log (molecular mass) in kilodaltons. Use one of your standard lanes to do this analysis.
3. From this plot, determine the MW of EGFP, before and after 6His-Tag cleavage (post-nickel column and post-DEAE column).
4. Was all of the 6His-tag removed by the digest? If not, estimate percent cleavage.
5. Roughly (visually) estimate the percent EGFP in your crude cell extract (pellet, lane 2).
6. Roughly (visually) estimate the homogeneity of EGFP before 6His-Tag removal. For example, do you think the protein is 30% pure, 80% pure, or 95% pure? How about in the final purified protein pool?
7. Based on your observations, do you believe the purification procedure worked well? What kinds of improvements would you suggest?

OPTICAL PROPERTIES, PERCENT CHROMOPHORE

The purpose of this lab was to characterize the optical properties of purified EGFP, to calculate the purification yield (total mg protein obtained at the end of purification) from 280-absorbance, and to calculate the extent (percent) chromophore maturation in your purified EGFP pools (\pm 6His-tag).

Answer all questions given in the protocol for Lab # 11.

Under 5), you are asked to calculate protein concentrations for the two protein pools from their absorbance at 280 nm. How do these concentrations compare to the protein concentrations determined via the Bradford and BCA assays (Lab #8)?

CHROMOPHORE pK_a

Answer all questions given in the protocol for Lab #12.

