

BIOCHEMISTRY EXPERIMENT MANUAL



吳繡賢 李惠春

Tzu Chi University 慈濟大學

Biochemistry Experiment Manual

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Licensing

A detailed breakdown of this resource's licensing can be found in [Back Matter/Detailed Licensing](#).

1: Gravimetric Calibration of Micropipettes

I. Introduction

To ensure the accuracy of the experiment, it is essential that the measuring devices used in the experiments be accurate and reliable. That is, it has to be calibrated at the beginning of the experiment. Micropipette is one of the most important measuring devices used in modern biochemistry laboratory. Today's experiment is the gravimetric calibration of volumetric pipette.

II. Materials and Methods

A. Materials and Equipment:

1. Deionized water
2. 50 ml beaker
3. Micropipette and tips
4. Electronic analytical balance

B. Procedure:

1. Select one of the Micropipettes (20, 200 or 1000 ml).
2. Bring some deionized water with a clean beaker (50-100 ml), allow it to reach room temperature (record the temperature).
3. Put a weighing dish on the electronic analytical balance and zero.
4. Pipette 10, 100 or 500 ml of water, respectively with 20, 200 and 1000 ml pipette and transfer the water content to the weighing dish. Record the weight.
5. Repeats step 3 and 4 for a total of 10 times.
6. Reset pipette to its max. volume setting after use.

Tips for Pipette Calibration

The process of pipette calibration can be very involved and complicated if the correct steps are not followed. Follow the few guidelines listed below to make the process a little less tedious.

1. Prior to performing any type of pipette calibration, clean and inspect the pipette, both internally and externally. A clogged nosecone can cause the pipette to deliver inconsistent volumes, even though the calibration on the pipette might be correct.
2. If any parts are worn or damaged, replace them prior to starting the calibration process. If a seal or O-ring is worn, it could cause the pipette to leak and deliver inconsistent volumes, but the calibration on the pipette could be fine.
3. Use the manufacturer's recommended pipette tips. Many times the pipette tip can be the reason why the pipette is not delivering the correct volumes.

When beginning the process of gravimetrically testing pipettes, the following conditions must be fulfilled:

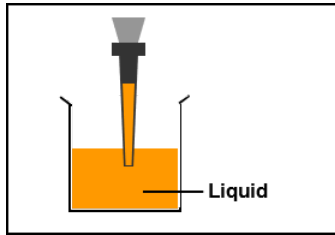
1. Evaporation protection for the weighing chamber. This can be done with the aid of moist blotting paper or an evaporation trap.
2. Testing area should be free of any drafts, direct sunlight or heat.
3. Distilled and degassed water should be used as a sample solution.
4. The pipette, the water, and the room must be maintained at a constant temperature of $20-25\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$.
5. When working with volumes $\leq 20\text{ ml}$ water should be removed from the weighing dish. When working with volumes $> 20\text{ ml}$ liquid should be added to the weighing dish.

Proper Pipetting Technique

Proper pipetting technique, in addition to a calibrated pipette will ensure that the correct volume is aspirated and dispensed.

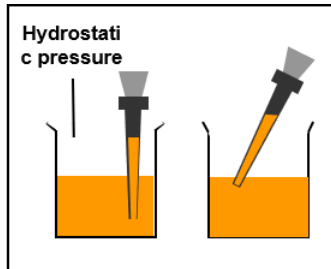
Correct Tip Immersion

Pipette tip is immersed only a few millimeters in solution and is held at a 90° angle from the solution.



Incorrect Tip Immersion

Increasing the angle of the pipette or the depth at which the tip is immersed can increase the hydrostatic pressure and therefore increase the volume aspirated. A 30° incline can increase the liquid column by 0.15%, resulting in improper volumes.



III. Results

1. Reorganize your data in the following table.

Room temperature on the day of the experiment: _____ $^\circ\text{C}$

Density of water at this temperature (unit): _____

** Please record to the next decimal place(請紀錄至小數點下一位)

		P1000	P200	P20
Pipette number				
Set Volume (ul)				
Measured weight (mg)	1			
	2			
	3			
	4			
	5			
	6			
	7			
	8			
	9			
	10			
Mean (mg)				
SD (mg)				
CV%				
Measured Vol. (ul)				
% Error				

2. Calculate the mean, standard deviation ($SD = \{[Sx^2 - (Sx)^2/n]/(n-1)\}^{1/2}$) and % coefficient of variation (% CV = [SD/mean] x 100).
3. Calculate the percent error by first converting the weight of water to volume: % Error = [(Experimental value - expected value) / Expected value] x 100.

IV. Question and discussion

1. 為什麼本實驗需要知道實驗時的水溫是多少？

如果水溫改變，會影響測量的「準確度」還是「精確度」？

Why it is important to know the temperature of water in the conversion of weight to volume? If the temperature changes, "accuracy" or "precision" of the measurement will be affected?

2. 請寫出下列名詞的定義及在本實驗中代表的意義: SD, CV%, and Error%。

Please define SD, CV%, and Error%, and explain their meanings in this exercise.

3. 請比較你和旁邊兩組的P1000數據，哪一組的P1000吸量管最不準確？請說明你的理由。

Which P1000 pipette, among yours and your neighbor groups', is the least reliable one?

Please describe your reasoning based on the values of mean, SD, or CV%.

4. 根據你得到的P1000、P200和P20三支吸量管的數據判斷，哪一支吸量管最不準確？
請說明你的理由。

Which pipette, among the ones you tested, is the least reliable one?

Please describe your reasoning based on the values of mean, SD, or CV%.

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CHAPTER OVERVIEW

2: Preparation of Ovalbumin and Lysozyme

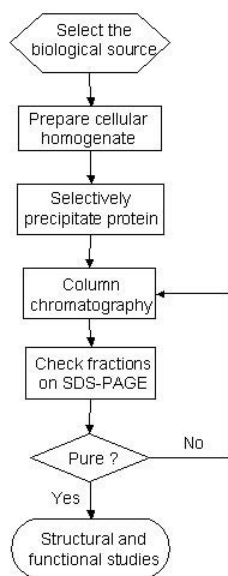
The most abundant protein in avian egg white is ovalbumin, occupying about 55% of the total proteins in newly deposited eggs. Ovalbumin is made of a single polypeptide with a molecular weight of 44,500 Da. It is thought to be a storage protein serving as an amino acid source for developing embryo. Lysozyme (M_r 14,600) is an enzyme also abundant in egg white as well as human tears that catalyzes the hydrolytic cleavage of polysaccharides in the protective cell walls of some families of bacteria. Lysozyme, because it can lyse (or degrade) bacterial cell walls, serves as a bactericidal agent.

In Exp 2-4, we will prepare these two proteins from chicken egg white applying the general strategy for protein purification. In Exp 5, we will carry out protein gel electrophoresis to check the purity of these two proteins.

Getting Started with Protein Purification

Protein purification is usually a multi-step process exploiting a wide range of biochemical and biophysical characteristics of the target protein, such as its source, relative concentration, solubility, charge, and hydrophobicity. The ideal purification strives to obtain the maximum recovery of the desired protein, with minimal loss of activity, combined with the maximum removal of other contaminating proteins.

A typical protocol for purification of a soluble cellular protein involves the disruption of the cellular membrane, followed by a differential centrifugation in a density gradient to isolate the protein from subcellular particles and high molecular weight aggregates. Further purification may utilize selective precipitation by addition of inorganic salts (salting out) or addition of miscible organic solvent to the solution containing the protein. Final purification will include a combination of techniques that separate based on molecular charge, molecular size, and affinity.



[2.1: Protein Fractionation](#)

[2.2: Protein Quantification](#)

[2.3: Gel Filtration Chromatography](#)

[2.4: Ion-exchange Chromatography](#)

Video of Experimental Procedures: [2.1](#) & [2.2](#)

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2.1: Protein Fractionation

I. Introduction

Fractional precipitation by salt is almost invariably used at an early stage of a protein purification procedure to obtain an initial purification and concentration. Further purification is then achieved by chromatographic methods, which have higher resolving power, but generally lower capacity.

When high concentrations of salt are present, proteins tend to aggregate and precipitate out of solution. This technique is referred to as “**salting out**”. Protein precipitates are aggregates of protein molecules large enough to be visible and to be collected by centrifugation. The distribution of hydrophilic and hydrophobic residues at the surface of a protein determines its solubility properties. When proteins are in solution, water molecules form a shell over hydrophobic patches on the protein. The water is ordered in the shell so as to maximize hydrogen bonds. This ordering is energetically unfavorable. At high salt concentrations, water is pulled from the hydrophobic patches to associate with salt ions. This forces hydrophobic patches on the surface of the protein to interact with each other, resulting in protein aggregation or precipitation.

Ammonium Sulfate Precipitation

Salting out by adding sufficient amounts of ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) is a convenient, nondenaturing way to concentrate a protein. Protein precipitation using $(\text{NH}_4)_2\text{SO}_4$ is achieved by dehydration in the microenvironment of the protein molecule. In solution, a large number of water molecules are bound to the sulfate ion (SO_4^{2-}), reducing the amount of water available to interact with the protein molecules. At a particular concentration of $(\text{NH}_4)_2\text{SO}_4$, an insufficient quantity of unbound water will remain to keep a given protein species in solution, resulting in the precipitation of that protein. A saturated $(\text{NH}_4)_2\text{SO}_4$ solution in water is ~ 4M with a density of 1.235 g/ml compared to a protein aggregate density in this solution of 1.29 g/ml. This difference makes it possible to pellet out the aggregates by centrifugation.

II. Materials and Methods

Materials:

1. Egg white 蛋白溶液
2. Saturated ammonium sulfate solution (75 g in 100 ml of water)
3. 1% NaCl

Procedure:

Preparation of crude egg albumin.

1. Add 0.5 ml saturated ammonium sulfate solution to 0.5 ml egg white drop by drop, vortex to mix between addition. ***If the salt is added too quickly, then high local concentrations will develop and proteins will be precipitated that would remain soluble at the target ammonium sulfate concentration. Such proteins may not readily re-dissolve, and reduce the effectiveness of fractionation.***

將0.5 ml的飽和硫酸銨溶液，逐滴加入0.5 ml的蛋白溶液中，每加入一滴硫酸銨溶液後，為避免局部硫酸銨濃度過高，請立刻混和均勻。[請注意操作蛋白質溶液時，應避免出現起泡的現象，泡泡的表面張力會將蛋白變性。]

2. The mixture is allowed to stand on ice for 30 min to allow protein precipitation taking place.

加完硫酸銨溶液後，靜置於冰上30分鐘，讓蛋白沉澱。

3. Centrifuge at 12000 rpm for 5 min. Remove the supernatant by pipette as much as possible.

以12000 rpm的離心力離心5分鐘，將上清液以微量吸管盡可能的吸乾，但是不要碰觸到蛋白沉澱。

4. Add 1 ml of 1% NaCl to resuspend the pellet (crude albumin), by vortex or pipetting. Remove any undissolved material by centrifuge. (12000 rpm, 5 min)

加入1 ml 1%NaCl，以微量吸管輕輕的反覆沖洗蛋白沉澱，使其重新溶解；如無法完全溶解，最後再利用12000 rpm的離心力離心5分鐘，將少許的不溶物移除。

III. Results:

1. Prepare 1 ml of 5% and 2.5% crude albumin each for Exp. 2.2

請準備1 ml 5%和2.5%粗白蛋白溶液，實驗2.2將會用到。

2. Transfer 400 μ l, 400 μ l, and 50 μ l of crude albumin to three separate eppendorf tubes. Label the tubes with group number and store at -20°C freezer for the chromatography separation (Exp. 2.2 and 2.3) and gel electrophoresis later.

將400 μ l, 400 μ l, 與50 μ l 的粗白蛋白溶液，分裝到3個1.5 ml微量離心管，請在管外標明你的組別號碼，保存到零下20度的冰箱，在接下來的實驗2.2、2.3、3將會用到。

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2.2: Protein Quantification

I. Introduction

Accurate determination of protein concentrations is fundamental for all quantitative measurements of biochemical interactions. A quick, useful estimation of protein concentration is to simply read the OD (optical density) at 280 nm of the protein solution using UV spectrophotometer. Protein absorb light at 280 nm due to the presence of tyrosine and tryptophan residues. However, many other substances (such as nucleic acids) also absorb at 280 nm resulting false estimation of protein concentration.

The Bradford Assay

The Bradford protein assay involves the addition of an acidic dye, Coomassie Brilliant Blue G-250, to the protein solution. The Coomassie blue dye binds to basic and aromatic amino acids resulting in a shift of the absorbance maximum from 465 nm (brownish) to 595 nm (blue). The amount of protein present in the sample is determined by performing a simple colorimetric reaction and comparing the results with those obtained from known amounts of protein.

II. Materials and Methods

Materials:

1. 5% and 2.5% crude albumin from Exp. 2A
2. 2 mg/ml bovine serum albumin (BSA) stock. 小牛血清白蛋白標準溶液
3. 1% NaCl solution.
4. Coomassie Brilliant Blue solution 染劑
5. Disposable cuvette and spectrophotometer 塑膠丟棄式比色管

Procedure:

1. Construct a standard curve. Prepare 5 dilutions from a 2 mg/ml protein stock solution of bovine serum albumin (BSA). Prepare 1 ml minimum

	Std 1	Std 2	Std 3	Std 4	Std 5
BSA Conc. (mg/ml)	0.02	0.04	0.08	0.120	0.2
2 mg/ml BSA stock (ml)					
Add 1% NaCl (ml)					

請利用1% NaCl將2 mg/ml小牛血清白蛋白標準溶液做適當稀釋，配製出0; 0.02; 0.04; 0.08; 0.12; 0.2 mg/ml等不同蛋白濃度的標準溶液，每個濃度至少要配製1 ml，請將稀釋的方法填在上表。

3. Set up and label test tubes, make duplicates.

在空試管外標明Blank及樣品名稱[五個不同蛋白濃度標準品、兩個未知樣品]，每個樣品請準備兩個重複，共需15支試管。

4. Add 2 ml of the dye reagent to all test tubes.

在空試管中加入2 ml染劑。

5. Add 0.1 ml protein samples to the labeled test tubes (a) standard BSA solutions prepared from step 1; (b) 2.5% and 5% crude albumin from Exp. 2A.

在試管中，分別加入0.1 ml蛋白溶液 [五個不同蛋白濃度標準品、兩個未知樣品: 實驗2A的5%和2.5%粗白蛋白溶液]。
Blank: 加入0.1 ml 1% NaCl。

6. Mix the content by gentle vortexing.

利用試管震盪器將溶液混和均勻。

7. Measure the absorbance at 595 nm with spectrophotometer.

利用光度計測量每一管595 nm的吸光度。

III. Results:

1. Construct the standard curve by plotting OD_{595} of the standards versus protein concentration of the known.

請利用五個蛋白標準溶液的濃度與吸光度讀值，劃出標準曲線。

2. Calculate the protein concentration of the unknowns (the original crude albumin) from the standard curve and the dilution factors if necessary.

參照標準曲線，計算出兩個未知樣品的蛋白濃度。

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2.3: Gel Filtration Chromatography

I. Introduction

Gel filtration chromatography is also known as size exclusion, molecular sieve, gel permeation chromatography. The primary objective of gel filtration is to achieve rapid separation of molecules based on size. The gel, which is in bead form for easy column packing, consists of an open, cross-linked three-dimensional molecular network of pores into which molecules of less than the pore size may penetrate. The pores within the beads are of such sizes that some are not accessible to large molecules, but smaller molecules can penetrate all pores. The extent to which a molecule enters the pores depends on its shape as well as molecular weight. Therefore, separation depends on the difference in the ability of various molecules to enter these pores. Proteins above the molecular weight exclusion limit cannot enter the pores. Consequently, they come through the column as a sharp peak in the void volume.

II. Materials and Methods

Materials:

1. Protein sample (crude albumin from Exp. 2A mixed with Vit. B12)

蛋白樣品[實驗2A的粗白蛋白加維他命B12]

2. Buffer: 50 mM Tris-HCl, pH 7.0, 50 mM NaCl

3. Column, test tubes and test tube rack

4. Pre-swollen Sephadex G-50 (fine particle), degassed.

除氣、預先吸水膨脹過的Sephadex G-50膠體過濾凝膠溶液

[請注意凝膠溶液只能以玻棒輕輕攪拌，**不可以**攪拌子與震盪器劇烈攪拌，會導致膠體顆粒碎裂，影響分離效果。]

5. Spectrophotometer and quartz cuvette (*handle with care!!*)

石英比色管-價錢昂貴請特別小心

Procedure:

1. Assemble column apparatus. Mark the 50 ml solution level on the column.

將管柱組裝好，利用量筒量取50 ml的水，倒入管柱中，在管柱外50 ml處做上記號，再將水倒掉。

2. With the valve closed, then transfer the G-50 slurry to the column along the column wall. **Be careful to avoid air bubbles trapped in the gel.**

將管柱底部的開關關閉，再將Sephadex G-50凝膠溶液沿著管柱邊緣，慢慢倒入管中，**不要讓氣泡在管柱內形成。**

3. Open the valve to allow buffer flowing by gravity. The G-50 beads will begin to settle, keep adding gel slurry and buffer till the settled beads reached the 50 ml mark on the column. **Be careful not to let the gel run dry.**

將管柱底部的開關打開，緩衝溶液隨即流出，G-50膠體顆粒會開始沉降在管柱底部，在膠體顆粒還沒有完全沉降前，繼續倒入剩下的G-50膠體溶液，直到沉降的膠體顆粒體積達到50 ml。請注意，絕對**不要讓管柱乾掉**，膠體的上方一定要有緩衝溶液蓋住。

4. After the column is packed, adjust the flow rate to 2 ml/min.

待管柱填充好，調整流速到2 ml/min。

5. With minimum solution above the gel matrix, add protein sample by gently layered on top of the gel matrix, to keep a **flat gel surface**.

蛋白樣品要加入管柱前，請先將管柱上的緩衝溶液放掉，待緩衝溶液的高度降到膠體表面時，請**馬上**將管柱的開關關閉。再將蛋白質樣品溶液，輕輕地加到膠體上方，絕對不要破壞膠體表面的平整，蛋白樣品加完後，請打開管柱底部開關。

6. After the sample sink below the gel matrix, add more buffer to elute the protein mixture.

待蛋白樣品全部進入膠體內，關上開關，慢慢地加入緩衝溶液蓋過膠體表面，同樣的，絕對不要破壞膠體表面的平整。

7. Begin collecting eluent immediately after loading the sample (2 ml/tube).

打開管柱底部開關，開始以每根試管2 ml收集流出液，同時並適當的補充管柱上的緩衝溶液，絕對**不要讓管柱乾掉**。

8. Measure the absorbance at 280 nm with a spectrophotometer and quartz cuvette (*handle with care!!*).

利用光度計測量每一根試管收集溶液的280 nm讀值，石英比色管-**價錢昂貴請特別小心**。

9. Store the fractions of the peak absorbance for gel electrophoresis, (Exp. 5).

將含有蛋白質吸收峰試管的樣品吸取1ml至微量離心管中，標示組別後交至講桌上，以便統一冷凍保存，留待實驗5電泳分析時使用。

III. Results:

1. Plot absorbance at 280 nm against tube number.

請依照試管的管數，與其280 nm吸光度讀值作圖。

2. Look up the molecular weight of ovalbumin, lysozyme, and Vit. B12, and predict the eluted order of these molecules.

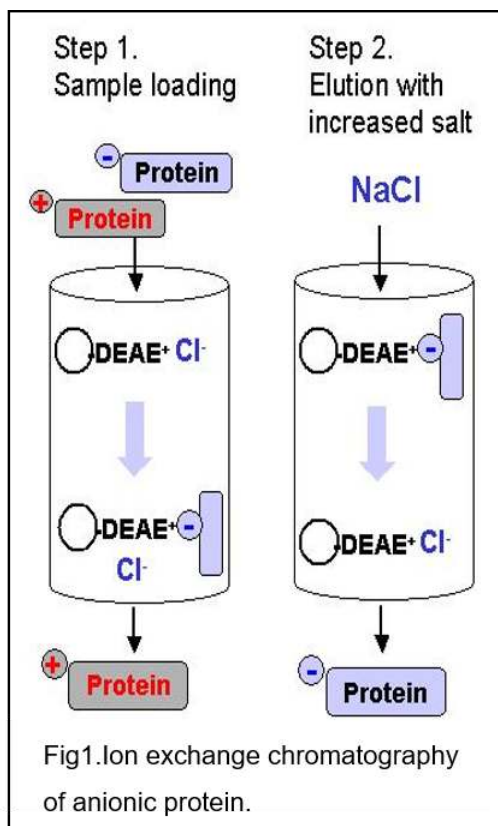
請找出雞卵蛋白、溶菌酶、維他命B12的分子量，並預測他們流出管柱的順序。

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2.4: Ion-exchange Chromatography

I. Introduction

Proteins contain charged groups on their surfaces, which enhance their interactions with solvent (water) and hence their solubility. At physiological pH, some of these charged groups are cationic (positively charged, e.g., lysine), whereas others are anionic (negatively charged, e.g., aspartate). Since proteins differ from each other in their amino acid sequence, the net charge possessed by a protein at physiological pH is determined by the relative numbers of these charged groups (i.e., negatively charged proteins possess more negatively charged groups than positively charged groups). This also underlies the different **isoelectric point (pI)** of proteins. *The pI of a protein is defined as the pH where this protein has an equal number of positive and negative charges.* The pI of most proteins is between pH 5 and pH 9. *When pH is above pI, the proteins have a negative charge; below pI their net charge is positive.*



Ion exchange chromatography separates proteins (1) on the basis of their charge type (cationic or anionic) and, (2) on the basis of relative charge strength (e.g. strongly anionic from weakly anionic). Separation of proteins by ion exchange chromatography requires differential binding of the proteins to the ion exchange matrix (e.g. a positively charged group such as DEAE, diethylamino ethyl). After; proteins are applied to an ion-exchange column, those proteins which have no affinity for the matrix are removed during a wash of the column. Then, the adsorbed proteins are removed in an elution step (Fig 1). Since different proteins generally **bind** to an ion exchange with differing affinities, it is usually possible to separate them by gradually increasing the salt concentration during the elution step. The act of binding would displace a counter-ion (such as Cl^- with a loading buffer containing 0.1 M chloride) from the ion exchanger. If a higher concentration of the counter-ion were applied (for example, 0.3 M Cl^-), a protein with a low net charge might be displaced. To remove another protein with a greater net charge, 0.5 M chloride may be required. By progressively raising the counter-ion concentration, separation of proteins can be achieved.

II. Materials and Methods

Materials:

1. Crude albumin from Exp. 2.1 蛋白樣品[實驗2.1的粗白蛋白]
2. Buffer: 50 mM Tris-HCl, pH 7.0, containing 0, 0.1M, 0.2M, 0.3M, 0.4M and 0.5M NaCl
3. Column, test tubes and test tube rack

DEAE-cellulose 離子交換凝膠 [請注意凝膠溶液只能以玻棒輕輕攪拌，**不可以**攪拌子與震盪器劇烈攪拌，會導致膠體顆粒碎裂，影響分離效果。]

4. Spectrophotometer and quartz cuvette (*handle with care !!*)

石英比色管-價錢昂貴請特別小心

Procedure:

1. Assemble column apparatus. Mark the 2 ml solution level on the column.

將管柱組裝好，利用量筒量取2 ml的水，倒入管柱中，在管柱外2 ml處做上記號，再將水倒掉。

2. With the valve closed, add 5 ml buffer to the column. Then transfer the DEAE cellulose slurry to the column along the column wall.

將管柱底部的開關關閉，先倒入2 ml的緩衝溶液，再將DEAE-cellulose 離子交換凝膠溶液沿著管柱邊緣，慢慢倒入管中，**不要讓氣泡在管柱內形成**。

3. Open the valve to allow buffer flowing by gravity. The beads will begin to settle, keep adding gel slurry and buffer till the settled beads reached the 5 ml mark on the column. Be careful to avoid air bubbles trapped in the gel and not to let the gel run dry.

將管柱底部的開關打開，緩衝溶液隨即流出，DEAE-cellulose膠體顆粒會開始沉降在管柱底部，在膠體顆粒還沒有完全沉降前，繼續倒入剩下的DEAE-cellulose膠體溶液，直到沉降的膠體顆粒體積達到2 ml。請注意，絕對**不要讓管柱乾掉**，膠體的上方一定要有緩衝溶液蓋住。

4. After the column is packed, adjust the flow rate to 2 ml/min.

待管柱填充好，調整流速到2 ml/min。

5. With minimum solution above the gel matrix, add 4 ml protein sample (10X dilution from Exp 2A) by gently layered on top of the gel matrix.

蛋白樣品要加入管柱前，請先將管柱上的緩衝溶液放掉，待緩衝溶液的高度降到膠體表面時，請**馬上**將管柱的開關關閉。再將4 ml蛋白質樣品溶液[實驗2A所準備的樣品，稀釋10倍後使用]，輕輕地加到膠體上方，絕對不要破壞膠體表面的平整，蛋白樣品加完後，請打開管柱底部開關。

6. After the sample completely enters the gel, the column is washed with 6 ml of the same buffer.

待蛋白樣品全部進入膠體內，關上開關，慢慢地加入緩衝溶液蓋過膠體表面，同樣的，絕對不要破壞膠體表面的平整。

7. Begin collecting fractions after sample loading, 2 ml/tube.

打開管柱底部開關，開始以每根試管2 ml收集流出液，同時並適當的補充管柱上的緩衝溶液，絕對**不要讓管柱乾掉**。

8. Then elute with a series of buffer containing increasing concentration of salt: 6 ml 0.1M NaCl, 6 ml of 0.2 M NaCl, 6 ml of 0.3 M NaCl, 6 ml of 0.4 M NaCl, and 6 ml of 0.5 M NaCl.

依次以不同的緩衝溶液沖提管柱 [逐步提高緩衝溶液內鹽的濃度]，6 ml 0.1M NaCl, 6 ml of 0.2 M NaCl, 6 ml of 0.3 M NaCl, 6 ml of 0.4 M NaCl, and 6 ml of 0.5 M NaCl.

9. Measure the absorbance at 280 nm with a spectrophotometer.

利用光度計測量每一根試管收集溶液的280 nm讀值，石英比色管-價錢昂貴請特別小心。

10. Store the fractions of peak absorbance for gel electrophoresis (Exp. 5)

將含有蛋白質吸收峰試管的樣品吸取1ml至微量離心管中，標示組別後交至講桌上，以便統一冷凍保存，留待實驗5電泳分析時使用。

III. Results:

1. Plot absorbance at 280 nm against tube number.

請依照試管的管數，與其280 nm吸光度讀值作圖。

2. Look up the pI of ovalbumin, lysozyme, and predict the eluted order of these molecules.

請找出雞卵蛋白、溶菌酶的等電點，並預測他們流出管柱的順序。

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3: Gel Electrophoresis of Protein

I. Introduction

Electrophoresis

Biomolecules often carry positive or negative electrical charges. When placed in an electric field, charged molecules move towards the electrode of opposite charge due to the phenomenon of electrostatic attraction. Electrophoresis is the separation of charged molecules in an applied electric field. The relative mobility of individual molecules depends on several factors the most important of which are net charge, charge/mass ratio, molecular shape and the temperature, porosity and viscosity of the matrix through which the molecule migrates.

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis in acrylamide gels is frequently referred to as PAGE, being an abbreviation for *polyacrylamide gel electrophoresis*. Cross-linked polyacrylamide gels are formed from the polymerization of acrylamide monomer in the presence of smaller amounts of *N,N'*-methylenebisacrylamide (normally referred to as 'bis'-acrylamide). Note that bis-acrylamide is essentially two acrylamide molecules linked by a methylene group, and is used as a cross-linking agent. Acrylamide monomer is polymerized in a head-to-tail fashion into long chains and occasionally a bis-acrylamide molecule is built into the growing chain, thus introducing a second site for chain extension. Proceeding in this way a cross-linked matrix of fairly well-defined structure is formed (Fig 5-1). The polymerization of acrylamide is initiated by the addition of ammonium persulphate (APS) and catalyzed by *N,N,N',N'*-tetramethylethylenediamine (TEMED).

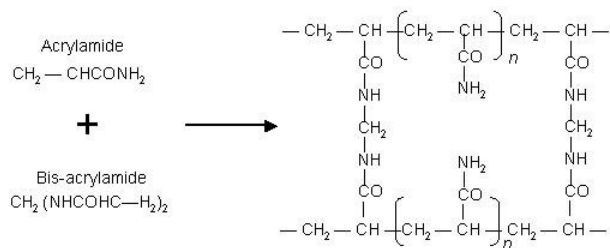


Fig 5-1. The formation of polyacrylamide gel from acrylamide and bis-acrylamide.

Acrylamide gels are defined in terms of the total percentage of acrylamide (T %) present, and the pore size in the gel can be varied by changing the concentrations of both acrylamide and bis-acrylamide. The low percentage gels (e.g. 4%) have large pore sizes and are often used in the stacking gel system of PAGE or to separate DNA. Gels of between 9% and 20% acrylamide are often used in SDS-PAGE to separate protein according to their size.

$$T \% = (a + b) \times 100 / \text{total volume (ml)}$$

a = weight of acrylamide in g

b = weight of bis-acrylamide in g

SDS-PAGE

Proteins can be dissociated into their constituent polypeptide chains by an anionic detergent, sodium dodecyl sulphate (SDS), after the reduction of any disulphide bonds. The SDS binds to the polypeptide chain producing a rod-shaped complex, the length of which is dependent upon the relative molecular mass of the protein. The large number of these strongly anionic detergent molecules bound by the protein (approximately one SDS per two amino acids) effectively masks the native charge of the protein and at a neutral pH results in a relatively constant charge to mass ratio for all proteins. As a result, the electrophoretic mobility of all protein-SDS complexes is approximately equal but the molecular sieving effect of polyacrylamide gel results in a relative mobility which is inversely related to the size of the complex. Under certain conditions, this inverse relationship can be demonstrated by a linear plot of the relative mobility of the protein against the logarithm of its relative molecular mass (Fig 5-2). It is necessary to use a series of known proteins in order to produce a calibration curve.

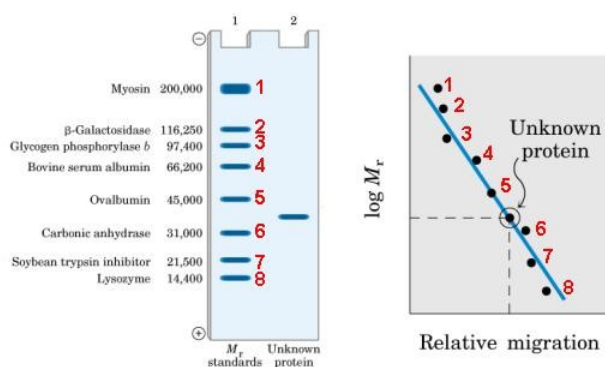


Fig 5-2. The electrophoretic mobility of a protein on an SDS-PAGE is related to its molecular weight. A plot of $\log M_r$ of the marker protein versus relative protein mobility is linear. Adopted from Lehninger, Principle of Biochemistry, 3rd ed.

Electrophoretic techniques

The equipment required for electrophoresis consists basically of two items, a power pack (power supply) and an electrophoresis unit. Electrophoresis units are available for running either vertical or horizontal gel systems. Vertical slab gel units of the type shown in Fig. 5-3 are commercially available and routinely used to separate protein in acrylamide gels. The gel is formed between two glass plates that are clamped together but held apart by plastic spacers. Gel dimensions are typically 12 cm x 14 cm, with a thickness of 0.5 to 1 mm. A plastic comb is placed in the gel solution and is removed after polymerization to provide loading wells for samples.

When the apparatus is assembled, the lower electrophoresis tank buffer surrounds the gel plates and affords some cooling of the gel plates. The power pack supplies a direct current between the electrodes in the electrophoresis unit. All electrophoresis is carried out in an appropriate buffer to maintain a constant state of ionization of the molecules being separated. Any variation in pH would alter the overall charge and hence the mobilities (rate of migration in the applied field) of the molecules being separated.

12.1 General principles

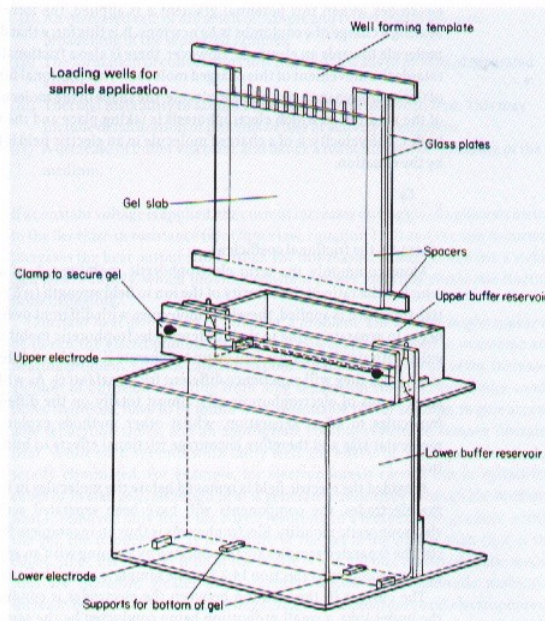


Fig. 12.1. A typical vertical gel apparatus, such as that used for separating proteins in a polyacrylamide gel. Note that, once the gel has set, the lower spacer is removed prior to the gel being run.

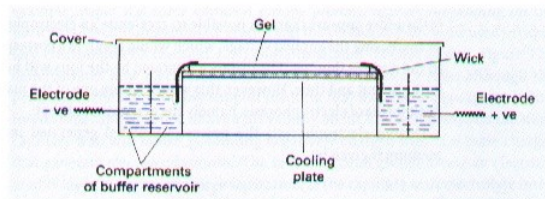


Fig. 12.2. A typical horizontal apparatus, such as that used for immunoelectrophoresis, isoelectric focusing and the electrophoresis of DNA and RNA in agarose gels.

Fig 5-3 A typical vertical gel apparatus used for separating proteins in a polyacrylamide gel.

Sample preparation

Samples to be run on SDS PAGE are firstly boiled for 5 min in sample buffer containing **b-mercaptoethanol** and SDS. The mercaptoethanol reduces any disulphide bridges present that are holding together the protein tertiary structure, and the SDS binds strongly to, and denatures, the protein. It is then heated at 100°C for 2-5 min in order to denature the protein and expose the total length of the polypeptide chain to the detergent. The sample buffer also contains a tracking dye, usually **bromophenol blue**, that allows the electrophoretic run to be monitored, and glycerol, which gives the sample solution density thus allowing the sample to settle easily to the bottom when injected into the loading well. Once the samples are all loaded, a current is passed through the gel and electrophoresis is performed on polyacrylamide gel and the bands subsequently visualized using an appropriate dye.

II. Materials and Methods

Stock solutions:

A. Acrylamide stock solution (30%):

29.2 g acrylamide

0.8 g bis-acrylamide

add d.H₂O to make 100 ml

B. 4X Running Gel Buffer: 1.5 M Tris-HCl, pH 8.8

C. 4X Stacking Gel Buffer: 0.5 M Tris-HCl, pH 6.8

D. Tank Buffer: 0.025 M Tris-HCl, 0.192 M glycine, 0.1 % SDS, pH 8.3

E. 5X Sample Buffer:

0.375 M Tris-HCl, 10% SDS, 50% glycerol, 12.5% b-mercaptoethanol, 0.02% bromophenol blue, pH 6.8

F. Staining solution:

0.1% Coomassie blue R-250, 10% acetic acid, 40% methanol.

G. Destaining solution: 10% acetic acid, 20% methanol.

Procedure:

i. Casting the running gel

1. **Assemble gel sandwich (Fig 5-4).** Add H₂O to test for leakage.

請依照圖5-4組裝電泳玻片，組裝好加入純水，看看會不會滲漏。

f. Repeat steps a–e for additional gels.

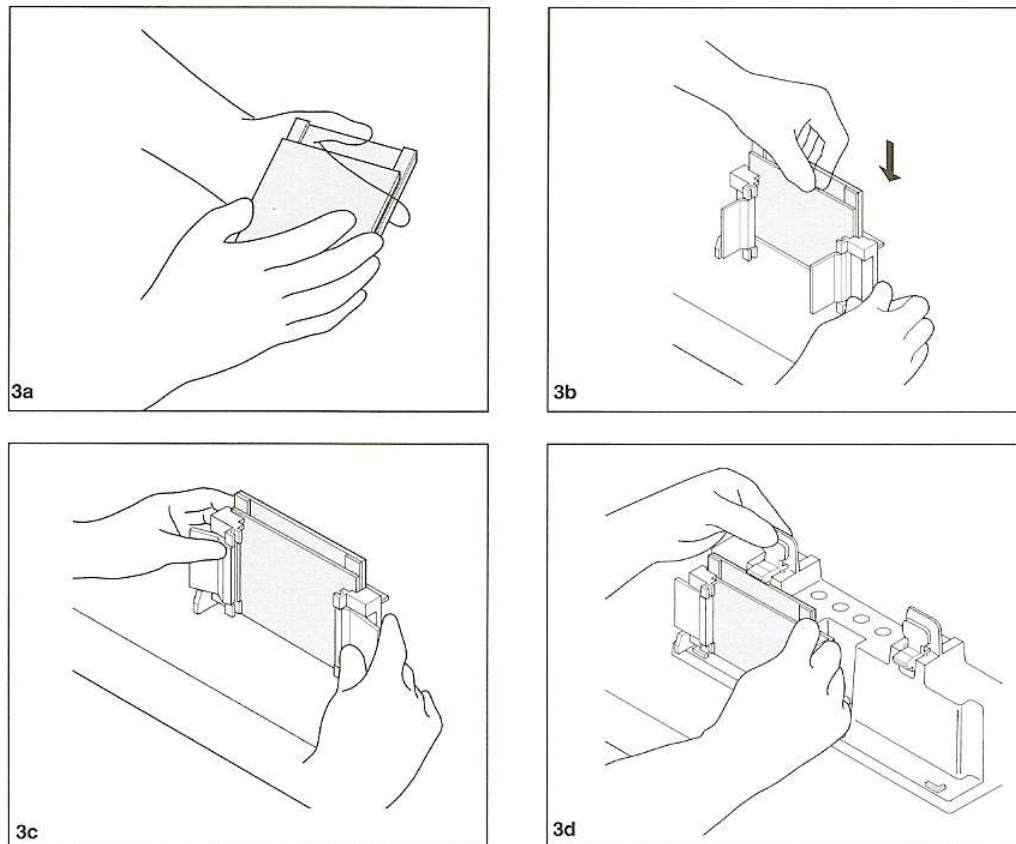


Fig. 3. Assembling the Mini-PROTEAN casting stand and frame.

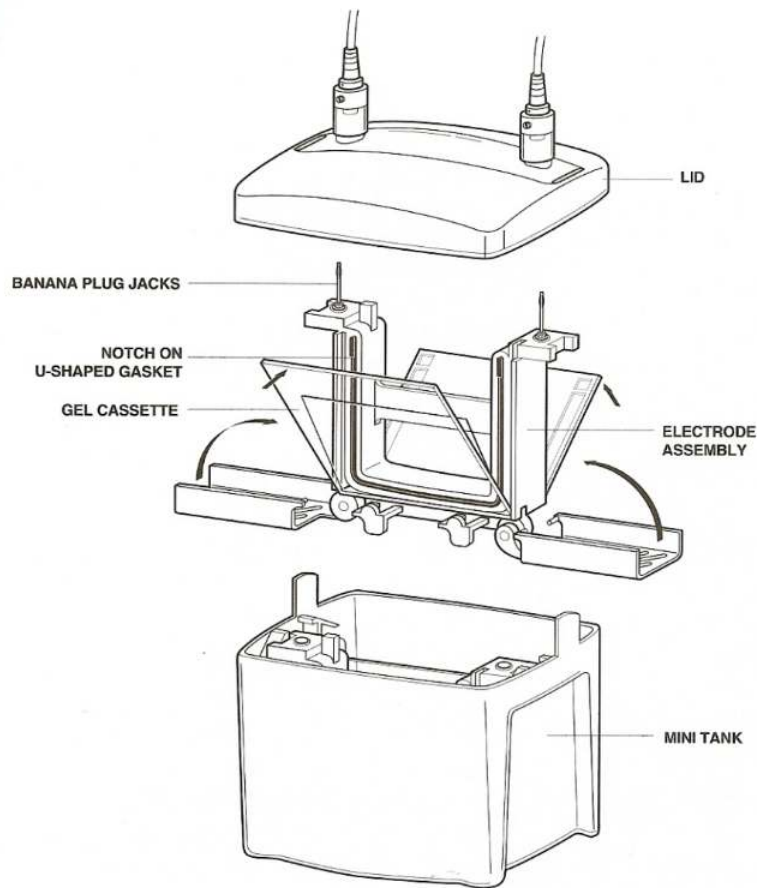


Fig. 1. Assembling the Mini-PROTEAN Tetra cell.

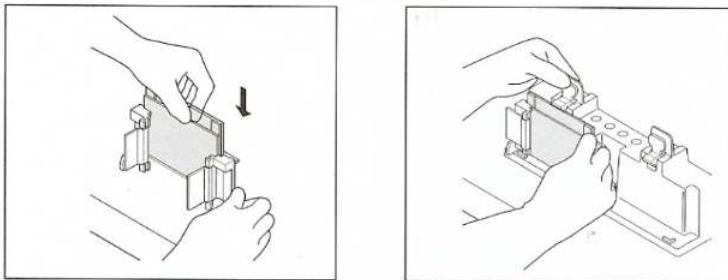


Fig. 2. Assembling the Mini-PROTEAN Tetra cell casting frame and casting stand.

Electrophoretic separation.

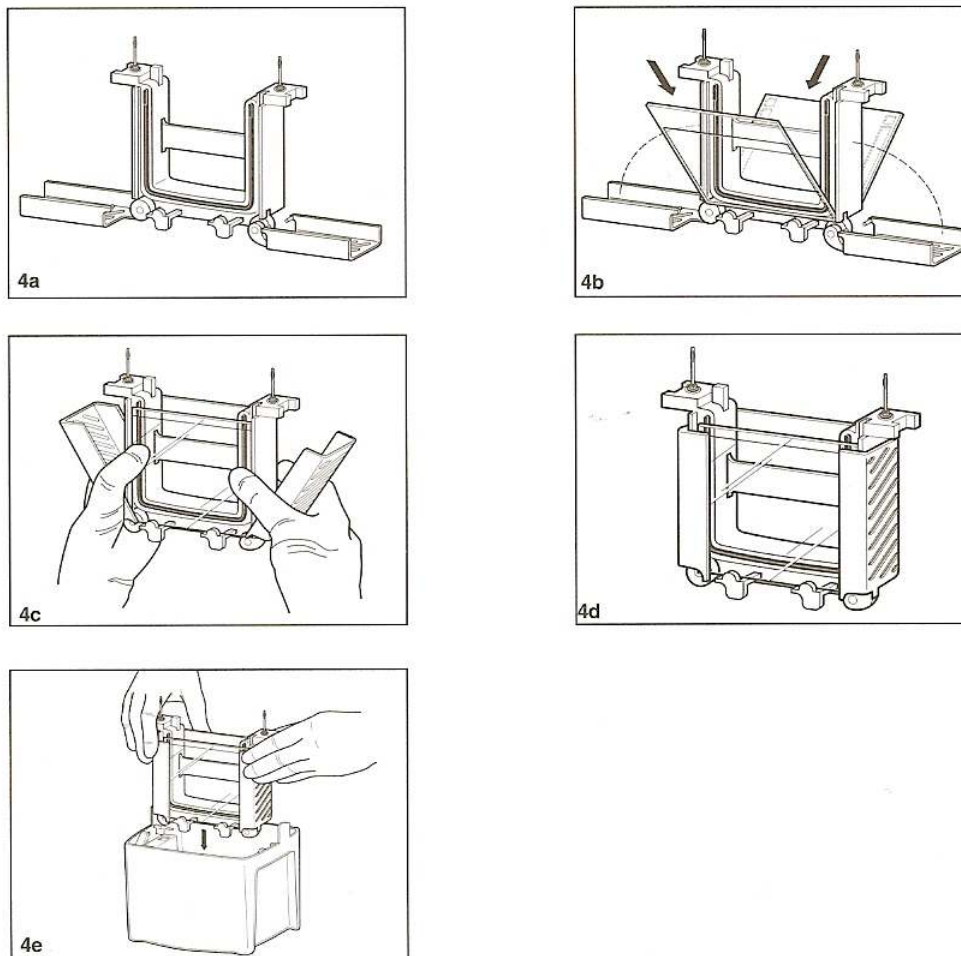


Fig. 4. Assembling the Mini-PROTEAN Tetra Cell Electrophoresis Module.

2. Combine solution in a flask or beaker according to Table 5-1. Acrylamide is a neurotoxin, gloves should be worn at all times. Add ammonium persulfate and TEMED last, and mix by swirling container gently.

依照下表5-1配製電泳凝膠溶液[running gel]，請注意丙烯醯胺[acrylamide]具有神經毒性，務必全程戴上手套操作實驗！過硫酸銨[ammonium persulfate]與TEMED最後再加入，輕輕晃動，將溶液混合均勻。

3. Carefully **introduce solution into gel sandwich** by pipetting along a spacer. This minimizes the possibility of air bubbles becoming trapped within the gel.

將電泳凝膠溶液慢慢地加入兩片電泳玻片間，不要讓氣泡產生。

4. On top of the running gel, gently **layer about 1-5 mm of water** to keep the gel surface flat.

為確保電泳凝膠凝固後表面的平整，在電泳凝膠溶液的上方，輕輕地加入1-5 mm高度的水，加完水後，應看到水與凝膠溶液間的介面形成一條平整的直線。

5. Allow gel to polymerize (15-30 min). When the gel has polymerized, a distinct interface will appear between the separating gel and the water, and the gel mold can be tilted to verify polymerization.

等待膠體聚合需15-30分鐘，膠體聚合好後，水與凝膠溶液間的介面會因折射度的改變，而變得更清楚。

Table 5-1 Recipes for acrylamide gel

	Running Gel (6 ml) 12.5% acrylamide	Stacking Gel (2ml) 4% acrylamide
Acrylamide monomer solution (30%)	$\underline{x_r}$ ml	$\underline{x_s}$ ml
4X Gel Buffer	1.5 ml (pH 8.8)	0.5 ml (pH 6.8)
10% SDS	60 ul	20 ul
d. H ₂ O	$\underline{y_r}$ ml $y_r = 6 - (1.5 + 0.06) - x_r$	$\underline{y_s}$ ml $y_s = 2 - (0.5 + 0.02) - x_s$
10% Ammonium Persulfate (APS)	30 ul	10 ul
TEMED	5 ul	3 ul

ii. Cast stacking gel

1. Pour off water covering the running gel.

倒掉覆蓋在凝膠上的水層。

2. Prepare the **stacking gel solution** as before and pipet it onto separating gel until solution reaches top of front plate.

依照上表5-1配製電泳凝膠溶液[stacking gel]，**請注意丙烯醯胺[acrylamide]具有神經毒性，務必全程戴上手套操作實驗！**過硫酸銨[ammonium persulfate]與TEMED最後再加入，輕輕晃動，將溶液混合均勻。將電泳凝膠溶液慢慢地加入兩片電泳玻片間，直到前面玻片的頂端。

3. **Carefully insert comb** into gel sandwich. Tilting the comb at a slight angle is helpful for insertion without trapping air bubbles.

將電泳梳插入兩片電泳玻片間，請避免產生氣泡

4. **Allow stacking gel to polymerize** (about 30 min).

等待膠體聚合需30分鐘

5. After stacking gel has polymerized, remove comb carefully, making sure not to tear the well ears. It is useful to rinse wells with electrophoresis buffer prior to loading in order to remove unpolymerized acrylamide and any contaminants.

膠體聚合好後，將電泳梳輕輕拔出，不要破壞樣品槽間的凝膠，可以用電泳槽的緩衝溶液清洗還沒有完全聚合的聚丙烯胺及其他的污染物。

iii. Assemble Electrophoresis Unit and Run a Gel

1. Assemble the electrophoresis unit, fill upper and lower chamber with electrophoresis buffer.

將組裝好電泳玻片及配件置入壓克力電泳槽內，在內側與外側倒入緩衝溶液。外側的緩衝溶液要蓋過玻片下的白金線，內側的緩衝溶液要高過內部的電泳玻片。

iv. Preparing and Loading samples

1. Combine protein sample 20 ml and 5x sample buffer 5 ml in an Eppendorf tube.

將蛋白樣品20 ml與5x sample buffer 5 ml混和均勻放入微量離心管。

2. Heat at 100°C for 5 min.

100°C加熱5分鐘。

3. Spin down protein solution for 1 second in microfuge.

在微量離心機離心1秒。

4. Introduce 10 ml sample solution into well using a pipetman.

利用微量吸管慢慢加入 10 ml 樣品，避免汙染到旁邊的樣品槽。

5. Include molecular weight standards in one or both outside wells.

在最旁邊的樣品槽，加入蛋白質分子量標準品。

6. Connect to the power pack. Use constant voltage of 200 V for 1 hours or till the dye front reaches the bottom of the gel.

將電泳槽蓋子蓋上、電源線連接好[正、負電極要接對]，將電源供應器設定在固定電壓200V 跑1小時，或是讓藍色的染料跑到玻片底部。

v. Staining and Destaining the gel

1. Stain the gel for ~30 min with shaking and then destain the gel for overnight.

在振盪器上膠片可以染30分鐘，退染所需時間較長，可以讓其退染一個晚上。

2. Scan the gel for record the next day.

隔天將膠片掃描存檔。

III. Results:

I. Identify the protein bands on the gel and measure the distance it traveled in the running gel. Plot the log [MW of the markers] against the mobility for standard curve and determine their linear relationship.

II. Identify the protein in the unknown sample and estimate their molecular weight.

Video of Experimental Procedures:

- 蛋白質電泳 Part I - 鑄膠與製備樣品篇 (<https://youtu.be/s8onQ6aZQMU>, 5:27)
- 蛋白質電泳 part II - 製備集膠與加入樣品 (<https://youtu.be/xpphS2LkSNY>, 3:44)
- 蛋白質電泳 part III - 結果解讀與報告 (<https://youtu.be/GeqWGybHeQw>, 5:19)

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4: Kinetic Characteristics of Acid Phosphatase

I. Introduction

Phosphatases are enzymes that catalyze the hydrolysis of phosphate monoesters with consequent release of inorganic phosphate and are widely distributed in nature.

The seeds of plants are a particularly rich source of typical acid phosphatases. The amount of phosphatase activity in seeds usually increases sharply upon germination and then falls as the seedling develops. The precise physiological function of this phosphatase is not known, but it is probably employed in releasing phosphate from organically bound storage forms, such as inositol hexaphosphate (phytic acid), for use in the metabolism of the germinating seedling.

In this experiment, acid phosphatase from wheat germ is used to develop skills in assaying and studying general kinetic properties of enzymes. The assay to be used takes advantage of the broad specificity of the phosphatase by using the artificial substrate, *p*-nitro-phenyl phosphate (pNPP). The degree of hydrolysis of the substrate is determined by photometric measurement of the *p*-nitrophenol liberated in the reaction. In alkaline solution the *p*-nitrophenolate ion absorbs light strongly in the region of 405 nm.

Since you are able to use a spectrophotometer and standard cells with a 1.0 cm light path, you can use the extinction coefficient for *p*-nitrophenol in 0.02M NaOH ($\epsilon = 18.8 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 405 nm) to calculate the mmoles of product formed from your absorbance readings.

II. Materials and Methods

A. Materials:

1. 0.06 M Disodium *p*-nitrophenyl phosphate (pH 4.8), (pNPP, keep cold)
2. 0.5 M Sodium hydroxide
3. 0.05 U/ml Acid phosphatase in 0.1% bovine serum albumin.
4. 0.025 M Sodium phosphate (pH 4.8)
5. 0.05 M Citrate buffer (pH 4.8)

B. Equipment:

1. Test tubes
2. Pipette and tips
3. Water bath
4. Test tube rack
5. Spectrophotometer

C. Procedure:

A. Determination of the Michaelis Constant:

Because the assay for phosphatase is a fixed time assay, the stock enzyme solution is diluted to yield a constant rate of product formation throughout the assay period. This is particularly important in this experiment because phosphatase is subject to product inhibition.

1. Prepare a series of tubes containing 50 ml of 3, 6, 12, 30 and 60 mM pNPP.
2. Add 200 ml of 0.05 M citrate buffer (pH 4.8) to each tube to yield a final volume of 250 ml.
3. Incubate the substrate tubes and the tube of enzyme for 5 minutes in the 37°C water bath.
4. At time intervals add 0.25 ml of enzyme to substrate tube after 30 sec to another. Mix and immediately remove to water bath.
5. After 10 min add 0.5 ml of 0.5 M sodium hydroxide and 2 ml H₂O to each tube. This stops the enzyme-catalyzed reaction and makes the pH alkaline for measurement of *p*-nitrophenol.

6. Measure the absorbance at 405 nm of these tubes against the corresponding “Zero-time” tube as a blank.

B. Inhibition by Inorganic Phosphate:

1. Repeat the experiment for determination of the Michaelis constant, with the modification that 0.05 ml of 0.025 M sodium phosphate (pH 4.8) is added to each tube and the volume of buffer added to each tube is adjusted so that the final volume (before adding enzyme) is 0.25 ml. (Note: This experiment must be done with the same dilution of acid phosphatase and on the same day as the determination of the Michaelis constant. why?).

方法:

1. 準備並標記實驗用玻璃試管(雙重複): 共21支。

Blank + I(S only:5*2) + (S+I:5*2)

2. 分別將50ml各濃度pNPP (3, 6, 12, 30, 60 mM) 加到組別I及II。

3. 組別I:加200ml 0.05M citrate buffer ;

組別II:加150ml 0.05M citrate buffer 和50ml 0.025M Na₃PO₄ (inhibitor), 此時總體積皆為250ml。

4. 將組別I, II的試管以及酵素放到37°C水浴槽靜置5分鐘, 使溫度皆達37°C。

※步驟5和6皆在水浴槽旁操作。

5. 在T=0 min時, 加250ml酵素到試管 → 震盪 → 放回37°C水浴槽。間隔30秒加250ml酵素至下一管, 重覆步驟5至所有試管皆加入酵素。

6. 在T=10 min時, 加500ml 0.5M NaOH 到試管終止反應 → 震盪 → 放回試管架。間隔30秒重覆步驟6至所有試管皆終止反應。

7. 將所有試管帶回座位, 每管加入2ml d.H₂O → 震盪使混合均勻。

8. Blank: 250ml citrate buffer + 250ml 酵素 + 500ml 0.5M NaOH + 2ml d.H₂O = 3ml (total)。

9. 讀OD₄₀₅ nm。

III. Results

1. Calculate the m moles of p-nitrophenol liberated by the enzyme at each substrate concentration and time in the presence and absence of inhibitor.

利用Lambert-Beer's Law: $A/\epsilon = [pNP]_{3ml\ solution}$; $\epsilon = 18.8 \times 10^3 M^{-1}cm^{-1}$, 分別計算兩組在各個受質濃度時pNP之莫耳數。

2. Calculate the reaction velocity (m moles of p-nitrophenol formed/ml of reaction mixture/10 min) at each substrate concentration.

分別計算兩組在各個受質濃度時pNP的V_o值。

3. Prepare a Lineweaver-Burk plot of your data, express pNPP concentration in moles/liter. Determine the V_{max} and the Michaelis constant for pNPP, expressing each in appropriate units.

分別將V_o與受質濃度取倒數, 作Lineweaver-Burk圖 (S only及S+I 兩條線畫在同一個圖), 由直線關係式分別計算出在 S only及S+I時的V_{max}及K_m值。

4. Which type of inhibition do you observe?

依據你的作圖結果, 判別sodium phosphate為哪一類型的抑制劑?並說明此抑制劑的特徵。

Video of Experimental Procedures:<https://youtu.be/J4ixSqWid2Y> (8:25)

Thumbnail Acid Phosphatase, but Astrojan [Wikimedia CC-BY 4.0](#)

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5: Glucose Tolerance Test and Determination of Blood Glucose

Introduction

Glucose is the major energy source for the body processes. The energy that is necessary for much of the molecular synthesis and metabolic processes of the cells is provided by the metabolism of glucose. Therefore, the level of circulating glucose is of vital interest. Fasting blood glucose level above or below the normal range usually indicate disease. Blood glucose determinations are, therefore used for diagnosis and follow-up of abnormalities of carbohydrate metabolism.

Oral Glucose Tolerance Test (OGTT)

OGTT is a test including series of measurement of blood glucose before and after glucose administration. These values are useful to evaluate individuals and establish values for normal and disease state. The recommended procedures are:

1. A fasting blood sample should be collected after 10-16 hours fast.
2. A glucose dose of 50g is prepared in flavored water (such as lemon juice) at a concentration no greater than 25g/dl, and should be drunk in about 5 minutes.
3. Blood samples should be collected at 30 minutes intervals for 2 hours.

Determination of glucose

Blood glucose concentration is measured using a modified glucose oxidase method. The oxidation of sample glucose is catalyzed by glucose oxidase to form hydrogen peroxide and gluconate. The hydrogenperoxide is then determined by the peroxidase oxidative coupling of N,N-diethylaniline (N,N-DEA) with 4-amino-antipyrine (4-AAP).

Glucose oxidase



Peroxidase



Purple dye (detect at 553 nm)

II. Materials and Methods :

A. Materials :

1. Phosphate buffer (50 mM) (can be stored at room temperature for certain period of time)

Weigh 6.8 gm KH_2PO_4 , bring up with 900 ml of distilled H_2O (dH_2O), adjust pH to 7.0 with approximately 53 ml, 1M NaOH or 6 ml 10 M NaOH. Bring up to 1 liter with dH_2O .

2. Combined reagent (100 ml): make fresh each day and store refrigerated until use.

Glucose oxidase 0.01963 g (50,000 U / 0.37 g solid)

Peroxidase (Horseradish) 0.00333 g (50,000 U / 0.264 g solid)

Aminoantipyrine 0.062 g

Sodium azide 0.1 g

N,N-Diethylaniline 50 ml

Transfer the above reagents to a 100 ml volumetric flask and dilute to volume with phosphate buffer.

3. Glucose standard solution :

Transfer 1 g of glucose into a 100 ml volumetric flask and diluted to volume with aqueous 0.2% benzoic acid solution. From this stock solution, prepare 50, 100, 150, 200, 300 mg / dl with 0.2% benzoic acid. Store refrigerated.

B. Equipment

1. Spectrophotometer

2. Cuvettes

3. Borosilicate glass tubes

C. Procedure:

1. Pipet 20ml of blood sample (plasma or serum), or standards, or 0.2% benzoic acid to 10 ml test tubes.

吸取20ml之血液檢體 (血漿或血清), 標準品, 及0.2%苯甲酸 到10ml之測試管中。

2. Add 5 ml combined reagent to each tube. Invert with parafilm to mix and record the time.

每根測試管中加入5ml之combined reagent. 以石蠟膜封住管口倒置混合並紀錄時間

3. Let stand at room temperature for 30 minutes, Read the absorbance against the combined reagent blank at 553 nm.

於室溫靜置30分鐘, 以combined reagent當作空白對照值, 記錄波長553 nm下的吸光值

IV. Reference :

1. Kabasakalian, P., Kalliney, S. and Westcett, A. (1974) Enzymatic blood glucose determination by colorimetry of N,N-Diethylaniline-4-Aminoantipyrine. *Clin. Chem.* 20 , 606-607.

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Video of Experimental Procedures : <https://youtu.be/TUTixCmDknU>

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6: Quantitative Determination of Serum

Introduction

A. Cholesterol has been implicated in the development of atherosclerosis and heart disease which are the leading causes of death in many countries. Since High-density lipoproteins (HDLs) and Low-density lipoproteins (LDLs) are the cholesterol-rich lipoprotein fractions, a high or low of cholesterol in the blood reflect a high or low level of these lipoproteins in our body. Measurement of blood cholesterol is important in the diagnosis of hyperlipoproteinemia and identification of the risk for cardiovascular disease.

B. Principle: The assay is based on the enzyme driven reaction that quantifies both cholesterol esters and free cholesterol. Cholesterol esters are hydrolyzed via cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase into the ketone cholest-4-en-3-one plus hydrogen peroxide. The hydrogen peroxide is then detected with a highly specific colorimetric probe. Horseradish peroxidase catalyzes the reaction between the probe and hydrogen peroxide, which bind in a 1:1 ratio (Figure 1). Samples are compared to a known concentration of cholesterol standard in a 96-well microtiter plate format.

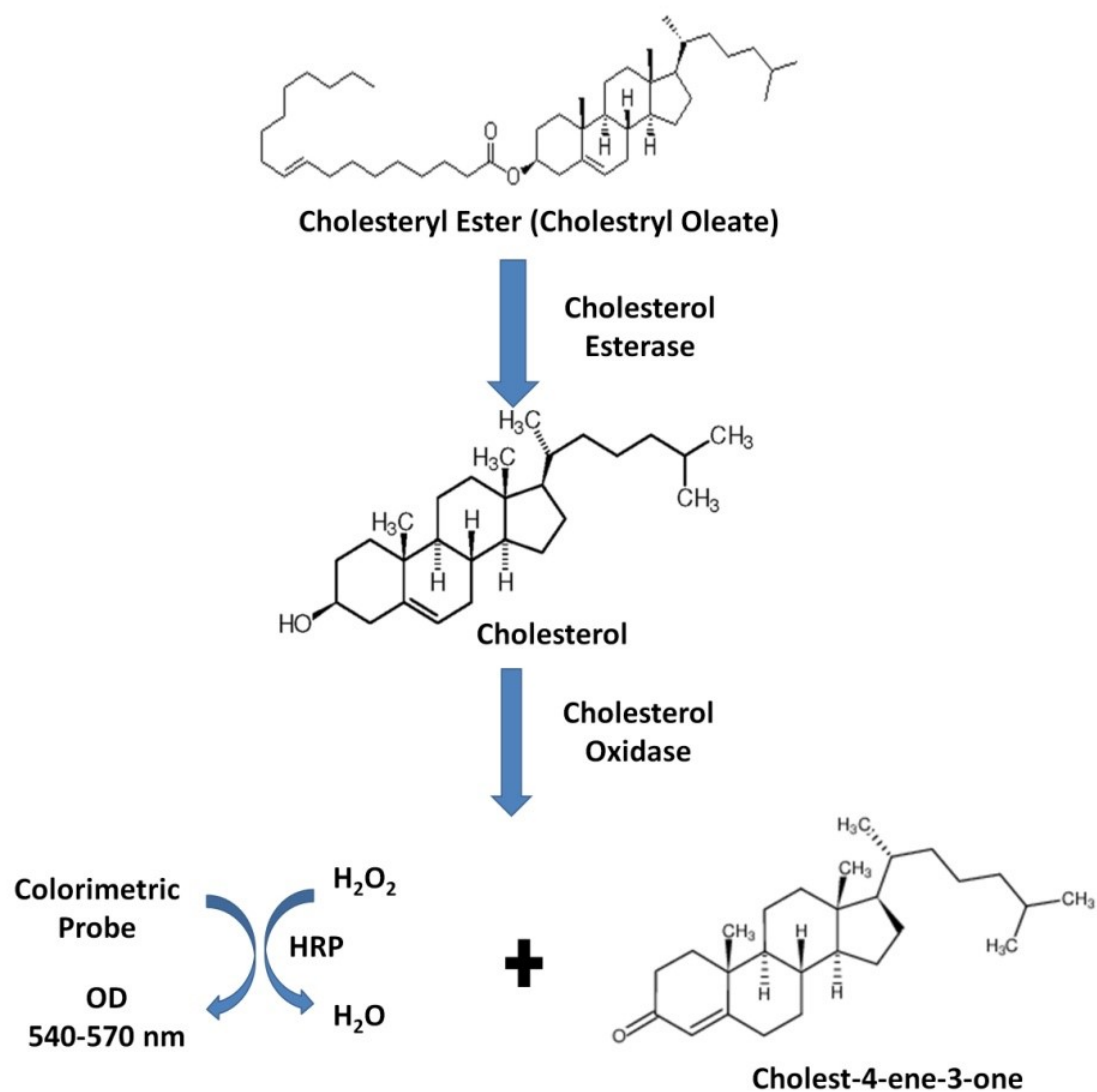


Figure1. Colorimetric Cholesterol Assay Principle

II. Materials and Methods:

A. Materials:

1. Assay Diluent (1X) prepared from Cell Biolab's "Total Cholesterol Assay Kit".
2. Cholesterol Standard: 250 μM cholesterol solution in 1X Assay diluent.
3. 50X Colorimetric Probe prepared from Cell Biolab's "Total Cholesterol Assay Kit".
4. Horseradish peroxidase (HRP).
5. Cholesterol Esterase
6. Cholesterol Oxidase

B. Equipment:

1. 96-well microtiter plates
2. Spectrophotometric microplate reader capable of reading excitation in the 540-570 nm absorbance range.

C. Procedure:

1. Preparation of Cholesterol Standard Curve

Use this 250 μM solution to prepare a series of the cholesterol standards according to Table below.

Tube	Final cholesterol Conc. (μM)	250 μM cholesterol standard (μl)	Assay diluent (μl)
1	0	0	50
2	50	10	40
3	100	20	30
4	200	40	10

(配製標準樣品四管，如表0、50、100、200 μM ，各一管)

2. Preparation of Samples

Serum samples must be diluted at 1:50 (add 20 μl of serum to 980 μl of 1X Assay Diluent) and 1:100 (add 10 μl of serum to 990 μl of 1X Assay Diluent) with Assay Diluent. This will provide values within the range of the standard curve.

(準備未知濃度血清樣品，利用1X assay diluent分別將樣品稀釋50倍和100倍，各一管)

3. Preparation of the Reaction Reagents

Cholesterol oxidase	16 μl
Horseradish peroxidase	16 μl
Colorimetric Probe	16 μl
Cholesterol esterase	3.2 μl
1X Assay Diluent	1000 μl

For best results, place the Reaction Reagent on ice and use within 30 minutes of preparation. Do not store the Cholesterol Reaction Reagent solution.

(依表格配製反應試劑。此試劑最好在30分鐘內使用完畢。)

※請注意是二組共同配製一管共用，且配置前請將藥品先離心!

4. Add 50 μL of the diluted cholesterol standards or samples to a 96-well microtiter plate.

(將標準樣品和未知樣品分別取50 μl 加入96孔盤，四組共用一盤，如下圖)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std1		Std1		Std1		Std1					
B	Std2		Std2		Std2		Std2					
C	Std3		Std3		Std3		Std3					
D	Std4		Std4		Std4		Std4					
E	Un1		Un1		Un1		Un1					
F	Un1		Un1		Un1		Un1					
G	Un2		Un2		Un2		Un2					
H	Un2		Un2		Un2		Un2					
	組別一		組別二		組別三		組別四					

5. Add 50 μL of the prepared Cholesterol Reaction Reagent to each well and mix the well contents thoroughly.

(在每個含有樣品的well加入50 μl 的反應試劑)

6. Cover the plate wells to protect the reaction from light. Incubate the plate for 45 minutes at 37°C.(用鋁箔紙包覆96孔盤達避光效果，再將此96孔盤放到暖房或37°C烘箱45分鐘)

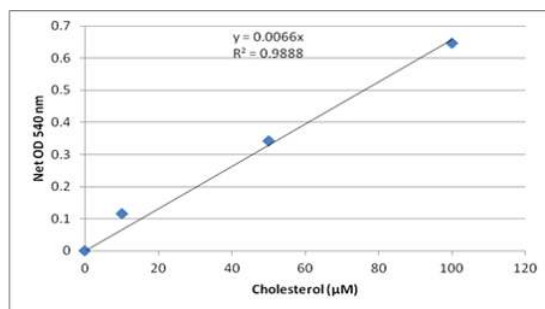
7. IMMEDIATELY read the plate with a spectrophotometric microplate reader in the 540 nm.

(45分鐘後立即使用微量光譜儀讀取波長540nm的吸光值)

8. Plot the Cholesterol Standard Curve.

(畫出標準曲線，並算出回歸曲線公式)

9. Calculate the concentration of cholesterol within samples by comparing the sample absorbance values to the cholesterol standard curve.



(利用公式算出未知樣品濃度)

10. For the conversion of results from μM to mg/dl , divide the cholesterol concentration(μM) by 25.9.

(將算出的 μM 的濃度除以25.9換算成 mg/dl 的濃度，50 μM =1.93 mg/dl)

III. References

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6. Cell Biolabs, Inc.-Total Cholesterol Assay Kit (Colorimetric), Catalog Number : STA-384

Video of Experimental Procedures : https://youtu.be/oR4w8wLS_VE

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CHAPTER OVERVIEW

7: Recombinant DNA

I. Introduction:

In the past fifteen years, we have witnessed a complete revolution in molecular genetics and biochemistry. This revolution has not been the result of a single new development in instrumentation or a theoretical breakthrough, but rather the application of a variety of techniques collectively referred to as genetic engineering or recombinant DNA technology. This new technology involves the *in vitro* modification and recombination of genetic material from different organisms to create new gene combinations; the product is often described as recombinant DNA. These techniques are applicable both to prokaryotes and eukaryotes.

Understanding the structure, function, and regulation of genes and their products is essential to an appreciation of biological systems. This also involves understanding the organization of an organism's genome. Before the advent of recombinant DNA techniques, the understanding of gene regulation was hampered by the complexity of the average eukaryotic genome, which may contain up to 109 bp in approximately 50,000 genes. Investigators need to be able to isolate and study a gene in a purified form to analyze its structure and function. Molecular cloning of DNA provides a mechanism for isolating a single discrete segment of DNA from a population of genes, purifying this segment to homogeneity, and amplifying the DNA segment to produce enough pure material for chemical, genetic, and biological analysis.

A typical cloning experiment requires: (1) the DNA of interest (target DNA); (2) a cloning vector; (3) restriction endonucleases; (4) DNA ligase; and (5) a prokaryotic cell to serve as the biological host.

1. Target DNA: Almost any gene or DNA sequence can be cloned. The purpose for doing so range from basic research on gene structure, function, and regulation; to industrial applications such as over-expression of a biological molecule. DNA can be isolated from virtually any source: plant, animals, bacteria, fungi, etc.

2. Cloning Vectors: Bacterial plasmids are small (3-15 kb), closed circular, extrachromosomal pieces of DNA that replicate autonomously and can easily be placed into and extracted from their bacterial host. Since their initial discovery, certain plasmids have been highly modified in order to make them ideal for molecular cloning purposes. Unnecessary sequences have been deleted, polylinkers or multiple cloning sites have been inserted, their origin of replication has been modified to allow them to replicate to extremely high numbers within the bacteria cell (>400 copies/cell in some cases), and easily identifiable genetic markers (such as antibiotic resistance or the *LacZ* gene) have been inserted making identification of bacterial colonies containing recombinant plasmids easy and rapid. There are a large number of plasmids (referred to often as cloning vectors) commercially available for virtually any purpose.

3. Restriction Endonucleases: Restriction enzymes or restriction endonucleases cut (or digest) DNA in a site-specific manner. They were originally identified in bacteria where they protect the cell from infection by bacteriophages by destroying any DNA it recognizes as foreign (based on differences in methylation). Each type of restriction enzyme cleaves at its own unique sequence which is almost always a palindromic sequence from four to six base pairs in length. Some enzymes cleave the DNA in the exact center of their recognition sequence leaving even or blunt ends on the cut DNA but most enzymes cleave off-center and leave complementary single-stranded ends on the pieces of DNA. These are often referred to as "sticky ends" and are easier to ligate together than blunt-ended DNA. A large number of restriction enzymes (>150) are commercially available in purified form.

4. DNA Ligase: This enzyme catalyzes the formation of phosphodiester bonds between adjacent nucleotides (assuming one nucleotide has a free 3 hydroxyl group, and one has a free 5 phosphate). This means it can join two pieces of DNA together as one. DNA isolated from different sources that have been digested with a restriction endonuclease(s) can be ligated together provided they have complementary sticky ends or both have blunt ends.

5. Biological Host: Once the sequence of interest has been cut with restriction enzyme(s) and ligated into a cloning vector (which was digested with a compatible enzyme(s) it is placed into an *Escherichia coli* host strain for propagation. One method for introducing foreign genetic material (recombinant DNA molecules) into bacterial cells is called transformation. There are a number of *E. coli* strains that can be used for this purpose. Almost all contain DNA repair, methylation, restriction, and recombination deficient genotypes. This limits the ability of the bacteria to reject or mutate the foreign DNA. Once the bacteria are transformed,

they are spread onto an agar plate. Each individual bacterium multiplies on the agar plate to form a colony. All the bacteria within a colony are genetically identical and are referred to as clones.

Once the recombinant molecules have been introduced into bacteria they must be screened to make sure they carry the correct recombinant molecule. The three most common methods of screening clones are antibiotic selection, insertional inactivation, and restriction enzyme digestion/agarose gel electrophoresis. Antibiotic selection requires a cloning vector carrying an antibiotic resistance gene. When bacteria are plated on an agar plate containing the appropriate antibiotic, only those bacteria that are transformed will form colonies. This method of selection is almost performed immediately after transformation. Insertional inactivation requires a plasmid carrying an additional antibiotic resistance or the *LacZ* gene. When the sequence of interest is cloned into the plasmid poly-cloning site, it interrupts this additional gene (either *LacZ* or antibiotic resistance). This causes inactivation of the gene. In the case of *LacZ*, bacteria containing non-recombinant plasmids (functional *LacZ*) will turn blue on an agar plate containing the chromogenic substrate X-gal. If *LacZ* is interrupted by the insertion of foreign DNA (recombinant molecule), the colonies will be unable to metabolize X-gal and will remain white in color. Using these two methods (antibiotic selection and insertional inactivation), the very first agar plate can separate transformed from non-transformed bacteria and distinguish bacteria colonies containing recombinant from bacteria clones containing non-recombinant DNA molecules. Once appropriate clones are identified, they are grown in larger quantity and their plasmid DNA is extracted. The plasmid DNA (or plasmid mini-prep) is then digested with restriction enzymes and the DNA fragments are resolved on an agarose gel. If the recombinant molecule is correct, the pattern and the size of the DNA fragments should be as predicted.

One recently developed technique which bypasses many of the traditional lengthy or cumbersome protocols involved in isolating the target DNA is the polymerase chain reaction, commonly referred to as PCR. This new technique was devised by Kary Mullis of Cetus Corp. in the mid 1980's, and has since become common place in many laboratories. Using this method, a single target DNA sequence in any region of a genome (fly, mouse, human, etc.) can be rapidly amplified to thousands or millions of copies, thereby rendering it plentiful enough for sequencing or cloning. In brief, the PCR technique involves first the separation (denaturation) of the two strands of a double helical DNA by heating the DNA to a high temperature. Each of the two separated strands is then annealed at a lower temperature with one or two specific primers designed to hybridize to a sequence flanking the target region to be amplified. Polymerization reaction then extend inward from the two primers to create two new double stranded DNA molecules, each containing one newly-synthesized strand and one template strand. These reactions are catalyzed by a thermostable DNA polymerase (Taq polymerase). The DNA molecules are subsequently denatured again, hybridized again with the two flanking primers, and extended again by the Taq polymerase. This cycle of strand separation, primer annealing and polymerization can be carried out for many cycles, thereby exponentially amplifying the target DNA template. For instance, a unique sequence run through 30 cycles of PCR will be amplified 109 times (2^Xn ; where X =number of double stranded templates before cycle 1, and n =number of cycle).

II. Experimental Design:

In this section, we are going to experience some of the basic recombinant DNA techniques. These include: (1) to prepare the plasmid DNA, pUC19/tRNA^{2f}Met (K12), from the *E. coli* cells (TG-1 strain) using the alkali lysis method and quantitate the plasmid DNA by spectrophotometry. (2) to characterize the plasmid DNA by restriction enzyme digestion and analyze the digested DNA fragments on agarose gel by gel electrophoresis. (3) to amplify the *E. coli* tRNA^{2f}Met gene from the plasmid DNA pUC19/tRNA^{2f}Met(K12) by the PCR technology and analyze the amplicone (220 bp) on agarose gel by gel electrophoresis.

The pUC19/tRNA^{2f}Met(K12) plasmid contains the *E. coli* initiator tRNA gene fragment (220 bp). This gene fragment is amplified by PCR from the *E. coli* K12 strain and cloned into the Sma I site of the pUC19 vector. The sequence of the tRNA^{2f}Met gene is shown in the Figure 1. Two PCR primers will be used in our experiment. Their sequences are:

L primer: 5-TAC GTC CGT CTC GGT ACA CCA A--3 (22mer)

R primer: 5-CAA ATC CCA CTA CGA AGG CCG A--3 (22mer)

M13mp18 vector seq

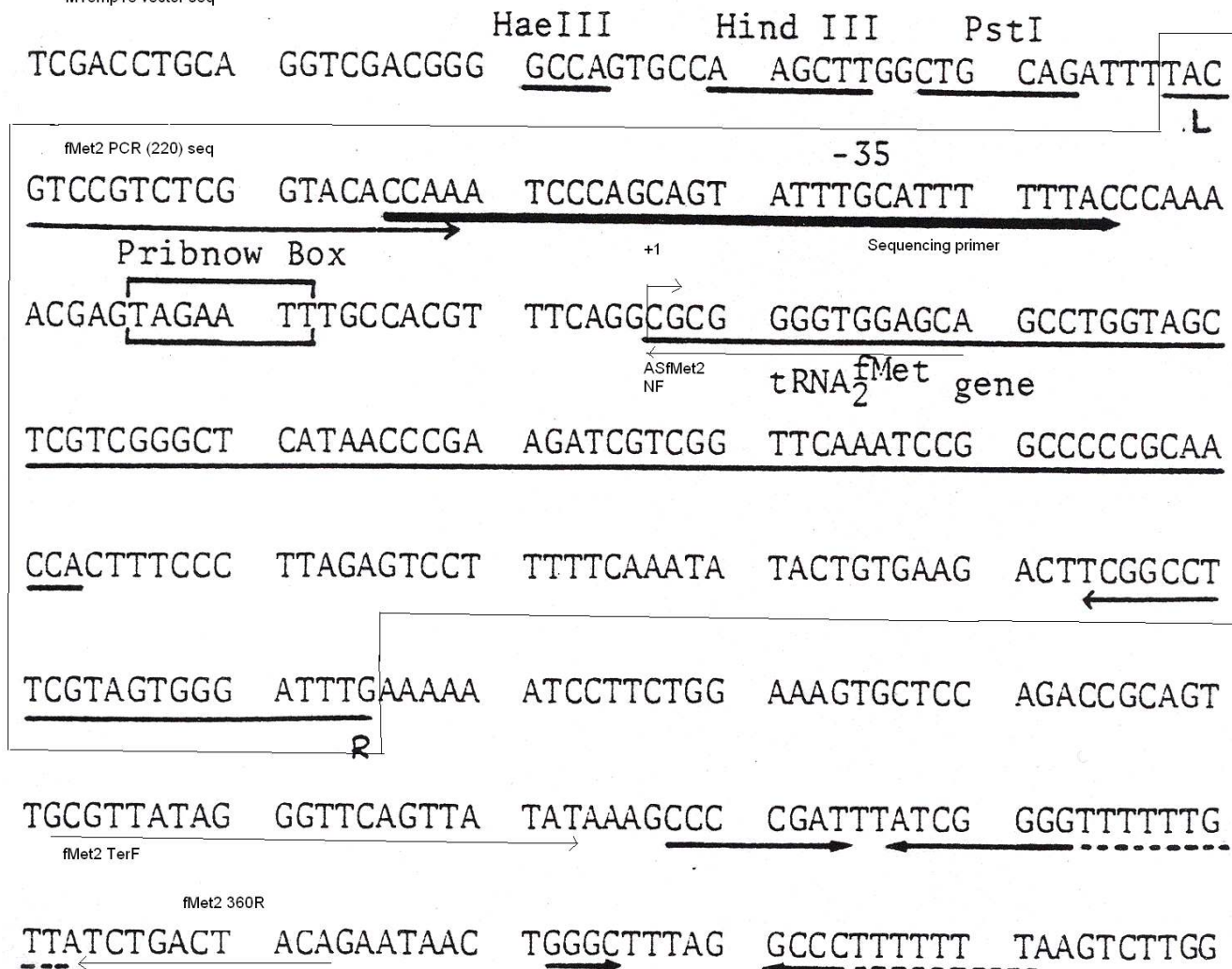


Fig. Sequences of E.coli tRNA^{fMet2} gene and its flanking sequences cloned into M13mp18 vector. Box area shows the sequences of fMet2 PCR product (220 bp). The gene fragment was PCR amplified by L/R primers and cloned into the SmaI site of pUC19 vector. The flanking sequences are replaced by the pUC19 vector sequences in pUC19/fMet2 clone. The locations of other primers are also indicated.

III. References

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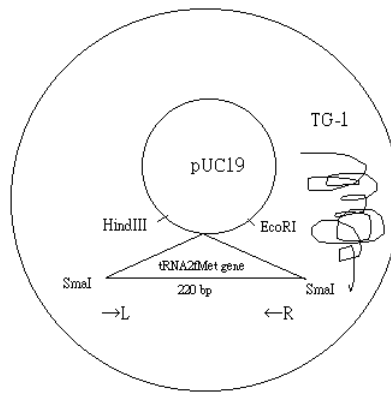


Fig. The scheme of pUC19/tMet2 clone. Strain for plasmid preparation is TG-1/pUC19/tRNA^{fMet}₂. The genotype of TG-1 is: K12, Δ (*lac-Pro*) *supE thi hsdD5/F' traD36 proA⁺ B⁺ LacI^q lacZ* Δ M15

[7.1: Plasmid DNA Preparation and Nucleic Acid Quantitation](#)

[7.2: Restriction Enzyme Digestion of Plasmid DNA and Agarose Gel Electrophoresis](#)

[7.3: Gene Amplification by Polymerase Chain Reaction \(PCR\)](#)

Video of Experimental Procedures : [7.1](#)、[7.2](#)、[7.3](#)

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7.1: Plasmid DNA Preparation and Nucleic Acid Quantitation

I. Introduction:

DNA can be isolated by different methods, depending on whether the DNA is chromosomal or extrachromosomal (plasmid or bacteriophage). Plasmids are found in a variety of bacterial species; they are double-stranded, closed circular DNA molecules that often contain genes that confer certain selective advantages to the bacterial host in specific environments. For small plasmids, 3-15 kb, the alkaline lysis technique exploits the covalently closed circular or supercoiled property of plasmid DNA molecules. When double-strand DNA is exposed to high pH, the two strands of the helix dissociate into two single strands. The process of denaturation is reversible and under the appropriate conditions the two complementary strands of the double helix will reassociate, or reanneal, to the double stranded form. However, the reassociation of strands is a time dependent process, and when a denatured solution of DNA is chilled or neutralized rapidly, the complementary strands are unable to reassociate and remain in the single-stranded form. When a supercoiled DNA is denatured, the two single-stranded circular DNA molecules remain intertwined. Because these two strands are in proximity to each other due to supercoiling, the rate of reassociation is much faster than the reassociation of complementary non-supercoiled DNA strands. Therefore, denatured supercoiled DNA can rapidly reanneal to form a double-stranded molecule. As a result of this property, a mixture of chromosomal and plasmid DNA can be denatured with alkali, then returned rapidly to a neutral pH, and the chromosomal DNA will remain predominantly in the single-stranded form. A selective precipitation can then be used to separate the single-stranded chromosomal DNA from the double-stranded plasmid DNA.

Another property that makes supercoiled DNA different from linear DNA is its lessened ability to bind intercalating dyes such as ethidium bromide. These are molecules that insert between the stacked purine/pyrimidine base pairs of double-stranded (ds) DNA. As each molecule intercalates, the overall buoyant density of the DNA molecules decreases. Supercoiled DNA will bind and accumulate dye molecules until the DNA can no longer be twisted without nicking one of the strands to relieve the tension. In contrast, linear DNA is free to rotate about its axis and relieve the distortion induced by the binding of an intercalating agent. Consequently, the linear molecule can bind more dye than the supercoiled DNA molecule. As a result, the density decrease caused by the binding of the dye to linear DNA will be greater than that caused by the binding of the dye to the supercoiled DNA. Utilizing this principle, a mixture of supercoiled and linear DNA saturated with EtBr can be separated by equilibrium centrifugation in a CsCl density gradient.

When a solution of CsCl is subjected to extremely high centrifugal force, the molecules will actually begin to separate within the solution. The cesium molecules will begin to migrate away from the axis of rotation and will form a linear density gradient within the centrifuge tube. In this case, the solution will be spun at approximately 60,000 rpm which is approximately 300,000xg ($RCF = 11.17r(N/1000)^2$ where r =radius in cm. and N =rpm). The DNA/EtBr complexes will migrate within this gradient to a point where they remain in equilibrium. Less dense molecules (linear DNA/EtBr) will not migrate as far as more dense molecules (supercoiled DNA/EtBr) and thus form two separate and distinct bands. RNA and any contaminating proteins will appear as a precipitate.

During this session, an *E. coli* plasmid, pUC19/tRNA^{2fMet}(K12), will be isolated using the alkaline lysis method and purified by the QIAGEN resin-tip technique. This plasmid DNA will then be used as the PCR template DNA. The tRNA^{2fMet} gene fragment from the plasmid, pUC19/tRNA^{2fMet}(K12), will be amplified using the PCR technique.

II. Materials and Methods:

A. Each group will receive the following materials:

1. Buffer S1	5. Buffer W2
2. Buffer S2	6. Buffer E
3. Buffer S3	7. TE buffer
4. Buffer W1	

B. Common materials and equipment:

1. Spectrophotometer	7. Parafilm
2. Quartz cuvettes: 0.5 ml volume, 0.2 ml is enough for measurement	8. RQ H ₂ O
3. Bench-top centrifuge	9. TE buffer
4. PM spin column	10. Ink marker
5. Eppendorf tube rack	11. Labeling tapes
6. Eppendorf tube	

Buffer	Composition	Storage
Buffer S1: (Resuspension buffer)	100 g/ml RNase A, 50 mM Tris/HCl, 10 mM EDTA, pH 8.0	4C
Buffer S2: (Lysis buffer)	200 mM NaOH, 1% SDS	RT
Buffer S3: (Neutralization buffer)	4 M guanidine hydrochloride, 0.5 M potassium acetate, pH 4.2	4C
Buffer W1: (Equilibration buffer)	5 M guanidine hydrochloride, 20 mM Tris-HCl, pH 6.6	RT
Buffer W2: (Wash buffer)	20 mM NaCl, 2 mM Tris-HCl, pH 7.5	RT
Buffer E: (Elution buffer)	10 mM NaCl, 2 mM Tris-HCl, pH 8.5	RT
TE:	10 mM Tris/HCl, 1 mM EDTA, pH 8.0	RT

C. Procedure:

1. In the day before the experiment, inoculate a 3 ml LB medium supplemented with 50 mg/ml ampicillin with the isolated colonies of *E. coli* containing the plasmid, pUC19/tRNA2fMet(K12). Grow at 37C overnight with shaking.

在實驗前一天，在LB培養皿上，挑選帶有質體pUC19/tRNA2fMet(K12)的大腸桿菌單一菌落，接種到一試管，裡面有內含50 mg/ml ampicillin抗生素 3 ml LB培養基。

2. Transfer two 1.5 ml of the culture to two eppendorf tubes and spin at 12,000 rpm for 1 minutes. (To balance your two tubes in the opposite positions.)

將3 ml菌液分裝到2個1.5 ml微量離心管內，以每分鐘12000轉速離心1分鐘，離心前，請確認此兩個微量離心管的體積一樣的，在離心機轉子內要對稱放置。

3. Remove supernatant by pipetting. Resuspend the bacterial pellet in 0.2 ml of buffer S1.

吸取出上清液，務必吸取乾淨，不要碰觸到離心下來的菌體沉澱。將菌體沉澱重新懸浮於 0.2 ml buffer S1，不能看到有小塊狀不溶的沉澱懸浮，要完全均質化。

4. Add 0.2 ml of buffer S2, mix gently by inverting the tube 10 times, and incubate at room temperature for 2 minutes.

加入0.2 ml buffer S2，請輕輕的上下顛倒混勻離心管10次，不可以劇烈震盪，至於室溫下2分鐘。

5. Add 0.3 ml of buffer S3, mix immediately but gently by inverting the tube 10 times.

加入 0.3 ml buffer S3，請輕輕的上下顛倒混勻10次。

6. Centrifuge for 5 minutes at 12,000 rpm in a mini centrifuge. Transfer supernatant promptly to a clean tube.

以每分鐘12000轉速離心5分鐘，將上清液轉移到另外一個新的微量離心管，不要碰觸到白色沉澱。

Binding

7. Place PM spin column into a new Eppendorf tube. Apply the supernatant (from step 6) to the PM spin column by pipetting. Do not take the white precipitates.

將PM spin column放置入一個新的eppendorf離心管中。將第6步驟的上清液吸取到PM spin column內，不要吸到白色的沉澱。

8. Centrifuge for 1 minute at 12,000 rpm in a mini centrifuge. Discard the flow-through. Place the PM spin column back to the same Eppendorf tube.

以每分鐘12000轉速離心1分鐘，倒掉濾液後，將PM spin column 放回原來的Eppendorf tube。

Wash

9. Add 0.4 ml buffer W1 into the PM spin column. Centrifuge for 0.5 minute at 12,000 rpm in a mini centrifuge. Discard the flow-through. Place the PM spin column back to the same Eppendorf tube.

在PM spin column內加入0.4ml buffer W1，以每分鐘12000轉速離心0.5分鐘，倒掉濾液後，將PM spin column 放回原來的Eppendorf tube。

10. Add 0.6 ml buffer W2 into the PM spin column. Centrifuge for 0.5 minute at 12,000 rpm in a mini centrifuge. Discard the flow-through. Place the PM spin column back to the same Eppendorf tube.

在PM spin column內加入0.6ml buffer W2，以每分鐘12000轉速離心0.5分鐘，倒掉濾液後，將PM spin column 放回原來的Eppendorf tube。

11. Centrifuge for 2 minute at 12,000 rpm in a mini centrifuge to remove residue Buffer W2.

以每分鐘12000轉速離心2分鐘，將殘留在PM spin column上的Buffer W2徹底移除。

Elution

12. Place the PM spin column into a NEW Eppendorf tube. Then, add 0.2 ml Buffer E into the PM spin column. Centrifuge for 2 minute at 12,000 rpm to elute your plasmid DNA samples.

將PM spin column放入一個新的Eppendorf tube內。加入0.2 ml Buffer E，以每分鐘12000轉速離心2分鐘，將plasmid DNA 流洗出管柱。

Quantitate plasmid DNA as follows:

For quantitating nucleic acids, absorbance readings are taken at wavelengths of 260 and 280 nm. The A₂₆₀ allows a calculation of the nucleic acid concentration in the sample. An A₂₆₀ of 1.0 corresponds to approximately 50 g/ml of double-strand DNA, or 40 g/ml of single-strand DNA or RNA. The ratio of A₂₆₀/A₂₈₀ provides an estimate of the purity. Homogeneous preparations of DNA and RNA have value of 1.8 and 2.0 respectively. Values significantly lower than this indicate contamination with protein and/or phenol.

Operation Procedure:

- Zero the spectrophotometer in the nucleic acid mode between 220 and 300 nm with 200 ml of TE buffer as the blank. Use 0.5 ml quartz cuvettes.
- Obtain your prepared plasmid DNA. Measure the absorbance after diluting 50 ul in 150 ul of TE buffer and record the values.
- Calculate the concentration of the DNA solution A₂₆₀ dilution factor (4) 50 g/ml = DNA concentration in mg/ml.
- Calculate the A₂₆₀/A₂₈₀.
- Label your undiluted plasmid DNA sample with your group number, and the DNA concentration in g/ml. Store your plasmid DNA preparation in a -20°C freezer.

Questions:

- Why the pH and NaCl concentration in S1, S2, S3, W1, W2, E buffers are important for plasmid purification?
- Why the buffer S2 can disrupt the bacterial cells?

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7.2: Restriction Enzyme Digestion of Plasmid DNA and Agarose Gel Electrophoresis

I. Introduction:

During this procedure, the plasmid DNA will be cut or digested with the restriction enzymes Eco RI and Hind III I to generate the tRNA gene fragment. These enzyme sites are located within the poly-cloning site of pUC19 plasmid by the synthetic primers.

II. Materials and Methods:

A. Materials:

1. Eco RI: 5 u/ul	14. 100 ml flask
2. HindIII: 5 u/ul	15. 100 ml cylinder
3. 10X R.E. buffer (H)	16. UV box/camera
4. 6x agarose gel dye	17. 37 ⁰ C dry bath
5. pUC19/tRNA ₂ ^{fMet} : Eco RI/Hind III cut	18. Gel electrophoresis apparatus
6.H ₂ O	19. Centrifuge
7. Ice	20. Balance
8. 0.5 L 0.5x TBE	21. Microwave oven
9. Agarose	22.Marker DNA (A) : 100 bp markers
10. Eppendorf tube	23. Marker DNA (B) : pUC19/tRNA ₂ fMet , Eco RI / Hind III digested
11. Yellow tip (box)	24. Backup plasmid DNA
12. Blue tip (box)	25. Spatula
13. Ink markers	26. Weighing paper

10 X TBE running buffer: Per liter

0.89 M Tris-borate 108 g

0.89 M Boric acid 55 g

0.02 M EDTA (pH 8.0) 7.4 g (or 40 ml of 0.5 M EDTA, pH 8.0)

B. Procedure:

1. Set- up the following reactions:

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
	Marker A	Marker B	Intact	Digested (your own plasmid DNA)	Digested (Backup plasmid DNA (0.5 µg) from TA)
H ₂ O			3.5	1.5	10.5
10xbuffer (H)			1.5	1.5	1.5
plasmid DNA			10	10	1
Eco RI (5 U/ul)			0	1	1
Hind III (5 U/ul)			0	1	1
Total vol.(ul)	15	15	15	15	15

2. Incubate the sample tube 3, 4 and 5 in a 37°C dry bath for one hour to insure complete digestion.

將試管3,4,5置入預設為37°C的乾浴加熱器1小時，靜待酵素反應完成。請利用這段時間，依照下列步驟，配製電泳用洋菜膠片。

3. Prepare a 1.5% agarose gel:

a. Weigh out 0.75 g agarose.

b. Add 50 ml of 0.5 X TBE buffer.

c. Microwave 2-3 min to completely dissolve, allow to cool to about 50~60°C for a few minutes.

微波加熱，要確認洋菜膠顆粒要完全溶解，不可以有微小的顆粒懸浮，冷卻數分鐘後將溶液到50-60°C。

d. Add 2.5 l of 10 mg/ml EtBr to into the gel, mix well. [EtBr is toxic!]

加入2.5 ml 10 mg/ml EtBr到膠體溶液。EtBr劇毒，請小心。

e. Prepare gel tray, adjust comb.

f. Pour gel. Let gel to solidify for 30-60 min.

靜待膠體凝固，凝固後，膠體會變成不透明。

g. Remove comb, put gel tray into electrophoresis tank and add 0.5 x TBE buffer to tank. Make sure add enough buffer to cover the gel.

小心的垂直拔出電泳梳，然後將膠片至於電泳槽中，倒入0.5X TBE buffer到電泳槽中，要蓋過膠片。

h. Load samples into gel wells as following:

	lane 1	lane 2	lane 3	lane 4	lane 5
	Marker A	Marker B	intact	digested	Digested (from TA)
DNA (μl)	15	15	15	15	15
6x agarose gel dye (ul)			3	3	3

5. Load the above samples in the corresponding gel lanes. Marker A and B already contain gel dye, and add them into agarose gel well directly.

Marker A與B是有顏色的，不需再加入6x agarose gel dye，可以直接加入電泳膠片的樣品槽內。

6. Electrophoresis will be carried out for 30-40 min at 100 volts. Wear gloves when handling gel and be very careful because EtBr is a potent mutagen.

7. Place the gel on a UV light box, turn on the UV light and take a photograph of the gel for your lab report.

Questions:

1. On an agarose gel, why bromophenol blue and xylencyanol dyes migrate toward the same direction as plasmid DNA? Why EtBr can bind DNA and migrate toward the opposite direction?

2. Establish a DNA ladder standard curve and calculate your insert and vector DNA fragment size.

(<https://www.youtube.com/watch?v=Bemr91UkUAA> or <https://www.youtube.com/watch?v=z8Hz2WNnGY4>)

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7.3: Gene Amplification by Polymerase Chain Reaction (PCR)

I. Introduction:

In this experiment, we are going to amplify *E. coli* tRNA^{fMet}(k12) gene by PCR technique. Two oligo DNA, NEWF2R and NEWF2L, are the primers. Both primers are 22 nucleotides long. Template DNA is pUC19/tRNA^{fMet} prepared in the experiment 9. The PCR product is a 220 bp fragment. Their corresponding sequences are shown in the introduction section.

II. Materials and Methods:

A. Materials:

1. <i>Taq</i> DNA polymerase: in 0.2 ml PCR tube	9. Marker B: pUC19/tRNA ^{fMet} : Eco RI /HindIII digested (with gel loading dye)
2. 10x <i>Taq</i> polymerase buffer	10. 0.5x TBE buffer: 0.5 L
3. dNTPs mix	11. Thermocycler
4. Oligo primers	12. Gel electrophoresis apparatus
5. Template DNA	13. Microwave oven
6. RQ H ₂ O	14. Balance
7. 6x agarose gel dye	15. Ink marker
8. Marker A : 100 bp markers	

B. Procedure:

1. Set up PCR conditions.
2. Add into a 0.2 ml PCR tube the following reagents in the correct order:

a. PCR protocol:

Tube#	1	2	3
Component	reaction	Positive control	Negative control
1. double distilled water	18 ul	18	19
2.10 X PCR buffer	2.5 ul	2.5 ul	2.5 ul
3. dNTPs mix (2.5 mM/each)	2 ul	2	2
4. Primer L& Primer R mixture (100ng/ul)	1 ul	1	1
5. <i>Taq</i> DNA polymerase (5U/ul)	0.5 ul	0.5	0.5
6. Template (pUC19/tRNA ^{fMet}) (1ng=E9 copies)	1 ul (template from your own preparation)	1 (template from TA)	0
Total vol. (l)	25 ul	25	25

b. PCR conditions:

	Temperature	Time (min:sec)	Cycle
Template denaturation	95	3:00	1
Template denaturation	95	0:30	25
Primer annealing	55	0:30	
Enzyme extension	72	0:30	
Final extension	72	3:00	1

3. Start the PCR reaction.

4. While PCR is running, prepare a 1.5% agar.

5. When PCR reaction is finished, mix with 5 ml of 6X agarose gel dye mix, load into a 1.5% agarose gel. A marker DNA (pUC19tRNA^{fMet} cut by Eco RI and HindIII) with dye is loaded on the gel side by side. Gel electrophoresis is set up at 100 volts for 30-45 min.

6. Marker A and B already contain gel dye, and add them into agarose gel well directly.

Marker A與B是有顏色的，不需再加入6x agarose gel dye，可以直接加入電泳膠片的樣品槽內。

Gel setting:

	lane 1	lane 2	lane 3	lane 4	lane 5
	Marker A (100 bp ladders)	Marker B (pUC19/fMet2 E/H cut)	Reaction	Positive control	Negative control
DNA (ml)	20 (with dyes)	20 (with dyes)	25	25	25
6x agarose gel dye (ml)	0	0	5	5	5
Total vol. (ul)	20	20	30	30	30

7. Photograph of the gel results.

Note:

1. PCR caculation:

The average weight of a base pair (bp) is 650 Daltons.

The size of tRNA^{fMet} gene is 220 bp.

The size of pUC19 plasmid is 2686 bp.

Template used in this PCR reaction is

$$1 \text{ ng DNA} / ((220+2686)*650) = 5.3 \times 10^{-16} \text{ mole}$$

$$5.3 \times 10^{-16} \text{ (mole)} \times 6.02 \times 10^{23} = 3.2 \times 10^8 \text{ (template molecule number)}$$

$$N_t = N_o \times 2^{\text{cycle\#}}$$

No = initial template input

Nt = final product output (amplicon)

IV. Questions:

1. Based on the OD260 reading of your own plasmid DNA preparation, how many copies of template you added into your PCR reaction? If the PCR efficiency is 100%, how many copies of PCR products can be obtained after 25 cycles?
2. 1 mg of *E. coli* genomic DNA equal to how many molecules? How about human cells?

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8: Ligation of a restriction enzyme digested plasmid DNA

Introduction:

DNA ligase of *E. coli* is a polypeptide of molecular weight 75,000. The comparable T4-induced enzyme is somewhat smaller (63,000 to 68,000). Both enzymes catalyze the synthesis of phosphodiester bonds between adjacent 5'-phosphoryl and 3'-hydroxyl groups in nicked duplex DNA, coupled to the cleavage of the pyrophosphate bond of DPN (*E. coli*) or ATP (T4). Phosphodiester bond synthesis catalyzed by both enzymes occurs in a series of these discrete steps and involves the participation of two covalent intermediates. A steady state kinetic analysis of the reaction-catalyzed *E. coli* ligase supports this mechanism, and further demonstrates that enzyme-adenylate and DNA-adenylate are kinetically significant intermediates on the direct path of phosphodiester bond synthesis.

In this experiment, the plasmid DNA pGFPuv (map as shown below) was first cut by NcoI, which disrupt the GFPuv gene. As contrast to the ccc form of plasmid DNA, the linearized plasmid has zero or low transformation efficiency. However, when the linerized plasmid is resealed by T4 DNA ligase, its transformation efficiency will be increased dramatically. The condensed plasmid will have higher transformation efficiency.

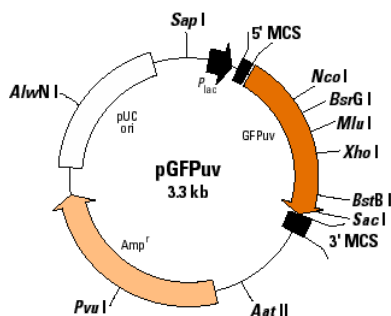


Table. Ligation reaction set up

	1	2
	Ligation (ul)	Negative control (no ligase added) (ul)
Insert DNA (2-10 ng or in a ratio of 10x or more to vector), for self ligation, add ddH ₂ O	1	1
Cut vector (25 ng/ul)	0.5	0.5
2x rapid ligation buffer	2.5	2.5
T4 DNA ligase (20 U/ul)	1	0
H ₂ O	0	1
Total volume (ul)	5	5

Mix well and spin briefly

Incubate at room temperature for 5 min to 1 hr.

Use 2.5ul for transformation,

Note:

1. Insert DNA can be a purified gene fragment from gel, or a DNA fragment in low melting gel slice (melt the gel slice at 60C for 2 min before use).

2. For PCR products with a 3'-A , use the TA cloning vector pEZT-T (linearized); for PCR products with blunt ends, use a vector with blunt ends such as pEZT-blunt (CIP treated); for gene fragments with sticky ends, use vector with compatible ends.

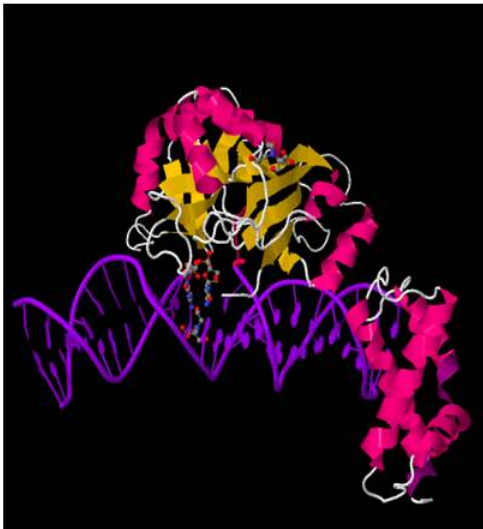
3. Transformation efficiency is the number of transformed cells (transformants) generated by 1 µg of supercoiled plasmid DNA in a transformation reaction. A known quantity of plasmid DNA (1 ng) is used as the control.

Transformation efficiency (transformants/µg) is calculated as follows:

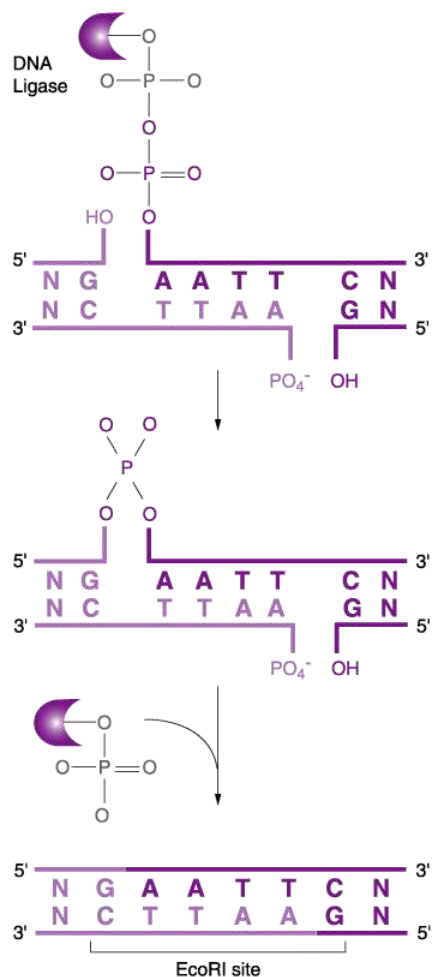
of colonies on plate/ng of DNA plated X 1000 ng/µg

4. 2xrapid ligation buffer: 60 mM Tris-HCl, pH 7.8, 20 mM MgCl₂, 20 mM DTT, 2 mM ATP, 10% PEG 8000.

Fig. Structure of T4 RNA Ligase 2 with Nicked 5'-Adenylated nucleic acid duplex containing a 3'-deoxyribonucleotide at the nick



<http://www.pdb.org/pdb/explore/jmol.do?structureId=2HVR&bioNumber=1>



Note: The substrate of ligase is the nicked ds DNA. Two intermediates are present: ligase-adenylate and DNA-adenylate.

Ligation of nicks in DNA and RNA – Which T4 ligase should you choose?

Substrate Black = DNA Green = RNA	Rate of Ligation at 37°C (mol nicks joined/mol protein/min)		
	T4 DNA Ligase	T4 RNA Ligase 1	T4 RNA Ligase 2
5'—3' 3'—5'	-	+	+++
5'—3' 3'—5'	-	-	+++
5'—3' 3'—5'	-	-	+++
5'—3' 3'—5'	+++	+	+++
5'—3' 3'—5'	-	-	-
5'—3' 3'—5'	-	-	-
5'—3' 3'—5'	-	-	-
5'—3' 3'—5'	+++	-	-

+++ rate is greater than 10

++ rate is 1 - 10

+ rate is .1 - 1

- rate is less than 0.1

(Courtesy of Dr. Richard Bowater, University of East Anglia, Norwich, UK. Original graph appears in Bullard D. and Bowater R. (2006) Biochem J., 398, 135-144.)

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9: Transformation of ligated plasmid DNA

Introduction:

Transformation is the idea of transferring DNA from one organism to another. This is generally done by breaking down the membrane of the cell, allowing DNA to move into the cell. Then the cell membrane is reconstructed and the new DNA is now part of the cell. In this experiment, pGFPuv (ligated circular and unligated linear, and a 1ng control plasmid (pGFPuv) used for measuring transformation efficiency) will be used for transformation.

Transformation & Storage Solution (2X TSS) enables researchers to take advantage of the simple system described by Chung *et al.*¹ for the preparation, long-term storage and transformation of competent *E. coli*. Early log-phase cells are suspended in 1X TSS: a solution containing polyethylene glycol, dimethyl sulfoxide, and divalent cations in a bacterial growth medium. The transforming DNA is added to the TSS-treated cells, the mixture is incubated on ice, and the transformed cells are plated on selective media. Transformation efficiencies of E6-E8 colonies/ug of DNA are routinely obtained without lengthy host cell preparation or heat shock (1, 2). For example, *E. coli* strains DH1, DH5, HB101, JM109, LE392, MM294, SCS-1, and XL-1 gave transformation efficiencies of 1.5-6 x E7 colonies/ug of DNA.

Precise transformation efficiencies will depend on both the host strain and the nature and quality of the transforming DNA. For added convenience, TSS-treated competent cells can be prepared in advance and stored at -70C for later use with little or no loss in transformation efficiency. For example, TSS-treated competent *E. coli* JM109 cells showed no significant loss of transformation efficiency after 1-18 weeks at -70C. However, the transformation efficiency of TSS-treated *E. coli* K802 competent cells was reduced several-fold after similar treatment. Fresh TSS-treated competent cells should be used when the total number of recombinants, the ligation or transformation efficiency or the percentage of correct recombinants is expected to be low.

References :

1. Chung, C.T. *et al.*, (1989) *Proc. Natl. Acad. Sci. USA* 86, 2172.
2. Groth, D. *et al.*, (1979) *Anal. Biochem.* 240, 302.

One-Step Preparation of Competent *E. coli* Cells by TSS

1. Grow cells in 3 ml LB at 37C to early exponential phase (OD₆₀₀=0.3-0.4).(ex. make a 1/50 dilution (60 ul) of a overnight culture into a fresh LB medium and grow for about 1 hr)
2. Make (1ml aliquots cells in autoclaved 1.5ml tubes)*3.
3. Pellet cells by centrifugation at 1000 xg for 10 min. at 4°C (or 12,000 rpm for 1 min. in a mini-centrifuge).
4. Resuspend cells of each tube in 1/20 their original volume (50ul) in ice-cold TSS solution.
5. For storage, freeze immediately in liquid nitrogen (or dry ice in ethanol) and store at -70 °C.
6. Add 2.5 ul of ligated, unligated plasmid DNA and 1ul of control plasmid (see the following Table) to the competent cells (corresponding to 100 pg-10 ng of transforming DNA)
7. Mix the cells and DNA and place on ice for 10 min, then at room temperature for 10 min, and then again on ice for 10 min.
8. Add 0.5 ml of LB broth and incubate the tubes at 37°C for 1hr.
9. A brief spin and remove 450 ul of the supernatant.
10. Resuspend the cell ppt and plate the cells on the appropriate selective media.

Table. Transformation set up

	1	2	3
Transformation	ligation	Negative control (no ligase)	uncut pGFPuv control (1 ng/ul)
DNA	2.5 ul	2.5 ul	2.5 ul
Competent cells	50 ul	50 ul	50 ul



Figure. Fluorescent phenotype of a petri-dish with ampicillin on a UV lamp. DH5a/pGFPuv clone (Left) and a fluorescent negative control clone (Right).

Table. Preparation of transformation and storage solution (TSS)

LB	For 100 mL LB, add the following:
10% PEG 8000	10 g PEG 8000
20 mM glucose	1 ml of 2M glucose
5% DMSO	5 mL DMSO
10 mM MgCl ₂	1 ml of 1M MgCl ₂
10 mM MgSO ₄	1 ml of 1M MgSO ₄

Note: Final pH should be 6.5

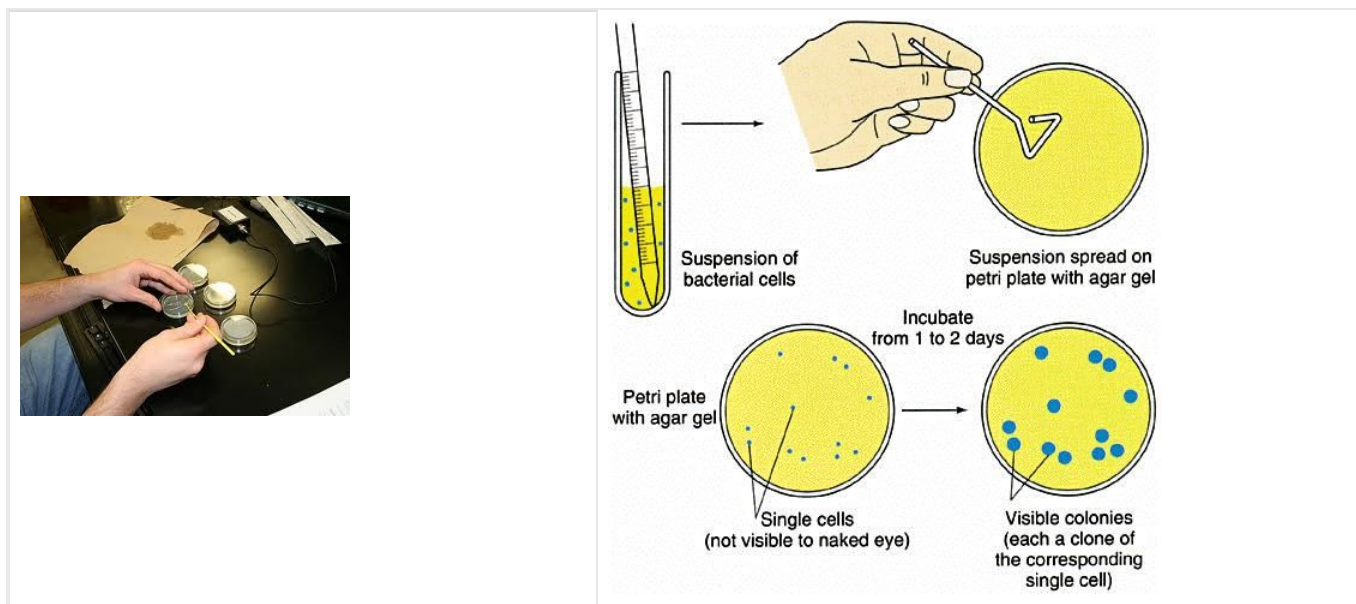


Fig. Scheme for the transformation

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10: Green Fluorescence Protein (GFP) Purification

Green Fluorescence Protein (GFP) Purification

– Hydrophobic Interaction Chromatography –

For the past years, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has become one of the most widely studied and exploited proteins in biochemistry and cell biology. Its amazing ability to generate a highly visible, efficiently emitting internal fluorophore is both intrinsically fascinating and tremendously valuable. High-resolution crystal structures of GFP offer unprecedented opportunities to understand and manipulate the relation between protein structure and spectroscopic function. GFP has become well established as a marker of gene expression and protein targeting in intact cells and organisms. Mutagenesis and engineering of GFP into chimeric proteins are opening new vistas in physiological indicators, biosensors, and photochemical memories.

Solutions:

TE buffer 10 mM Tris, 1 mM EDTA (pH 8.0)

Equilibration buffer TE plus 2.0 M $(\text{NH}_4)_2\text{SO}_4$ (pH 8.0)

Binding buffer TE plus 4.0 M $(\text{NH}_4)_2\text{SO}_4$ (pH 8.0)

Wash buffer TE plus 1.3 M $(\text{NH}_4)_2\text{SO}_4$ (pH 8.0)

Growing Cell Cultures (Pre-lab preparation)

1. Remove the transformation plates from the incubator and examine using the UV light. Identify several green colonies that are not touching other colonies on the LB/amp plate. Identify several white colonies on the LB/amp plate.
2. Obtain two culture tubes containing the growth media LB/amp/ara. Label one "+" and one "-". Using a sterile loop, lightly touch the loop to a green colony and immerse it in the "+" tube. Using a new sterile loop, repeat for a white colony and immerse it in the "-" tube (it is very important to pick only a single colony). Spin the loop between your index finger and thumb to disperse the entire colony.
3. Cap the tubes and place them in the shaking incubator or on the shaking platform and culture overnight at 32 °C or 2 days at room temperature. Or cap the tubes and shake vigorously by hand. Place in the incubator horizontally at 32 °C for 24–48 hours. Remove and shake by hand periodically when possible.

Bacterial Concentration

1. Remove "+" liquid cultures from the shaker and observe with the UV light. Note any color differences between the two cultures. Label 2 microtubes with your group number. Transfer 1.5 ml of "+" liquid culture into each microtube. Spin the microtubes for 1 minute at 12000 rpm.
2. Pour out the supernatant and observe the pellet under UV light.
3. Add 100 µl of TE buffer to each tube. Resuspend the pellet thoroughly by rapidly pipetting up and down several times. Combine the samples into one tube (~ 200µl)
4. Add 20 µl of lysozyme to the resuspended bacterial pellet to initiate enzymatic digestion of the bacterial cell wall. Mix the contents gently by flicking the tube. Incubate at room temperature for 20 minutes. Observe the tube under the UV light.
5. Place the microtube in the -20°C freezer for 20 min. The freezing causes the bacteria to rupture completely.
6. Remove the microtube from the freezer and thaw using hand warmth. Place the tube in the centrifuge and pellet the insoluble bacterial debris by spinning for 5 minutes at 12000rpm.
7. After the 5 minute spin, immediately remove your tube from the centrifuge. Examine the tube with the UV light.
8. Transfer the supernatant into a new microtube labeled "+". This is the sample for HIC chromatography.

Bacterial Lysis

1. Remove the microtube from the freezer and thaw using hand warmth. Add 200 μ l (same volume) Binding buffer to mix with the samples.

Hydrophobic Interaction Chromatography (HIC)

1. Add 1 ml HIC matrix suspension to the empty column. Allow all of the liquid buffer to drain from the column (~3–5minutes).
2. Prepare the column by adding 1 ml of Equilibration buffer to the top of the column. Drain the buffer to the 1 ml mark on the column.
3. Label collection tubes 1, 2, 3, ... and place the tubes in a rack. Place the column in collection tube 1. When the last of the buffer has reached the surface of the HIC matrix proceed to the next step below.
4. Carefully and gently load HIC sample onto the top of the column. Hold the pipette tip against the side of the column wall, just above the upper surface of the matrix and let the supernatant drip down the side of the column wall. Examine the column using a UV light. Note your observations. After it stops dripping transfer the column to collection tube 2.
5. Add 1 ml of Wash buffer and let the entire volume flow into the column. Examine the column using the UV light. Note your observations. After the column stops dripping, transfer it to tube 3.
6. Add 500 μ l of TE Buffer and let the entire volume flow into the column. Examine the column using the UV light and note your observations.
7. Repeat step 6 two more times. Examine all collection tubes and note any differences in color between the tubes.
8. Transfer 500 μ l of the greenest sample into two microtubes each (labeled with your group number). These are the samples for gel electrophoresis next time.

HIC matrix regeneration

1. HIC matrix material is very expensive and we need to regenerate it so we can use it again. To regenerate the column after this experiment, add about 2-3 column volumes of 1.3 M Wash Buffer. Follow with 3-5 column volume of distilled water. Now column is ready for reused immediately. For long term storage, the matrix is stored in 10% Ethanol.

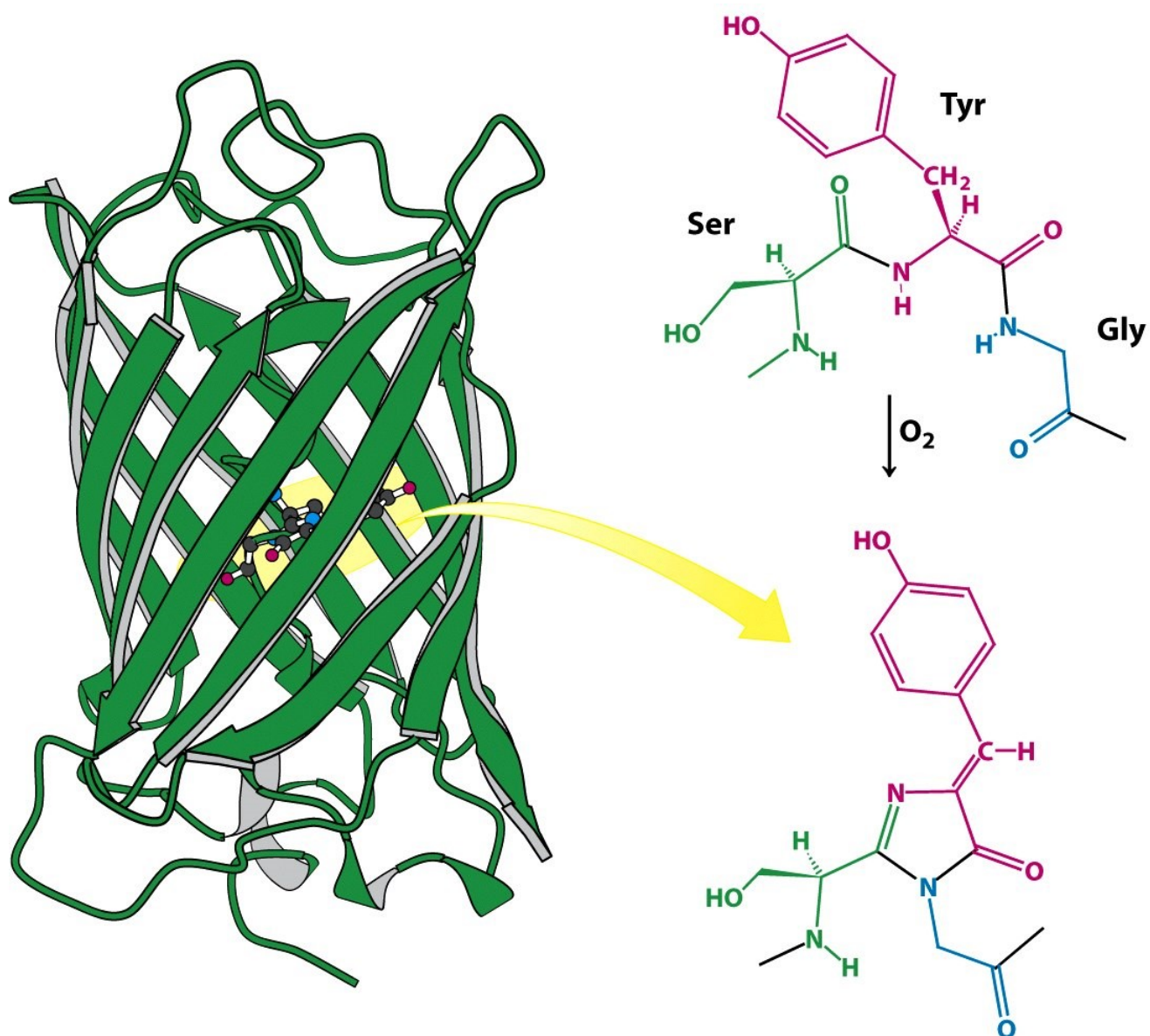


Figure 2-68a
Biochemistry, Sixth Edition
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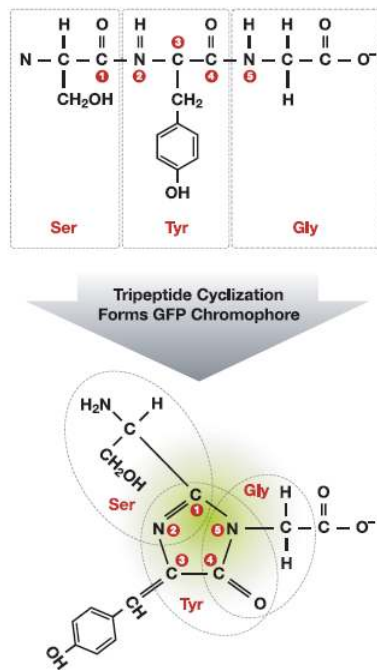


Figure 1. (Left). The tertiary structure of GFP is barrel-like, consisting of 11 beta sheets depicted as the ribbons and an internal chromophore of 3 adjacent amino acids, depicted as ball-and-stick. (Right) Cyclization of the tripeptide Ser-Tyr-Gly. The active chromophore of GFP is comprised of three adjacent amino acids in the primary polypeptide chain. The three amino acids are enzymatically converted to an active cyclic chromophore *in vivo*.

Laboratory write-up

1. What color was the bacterial pellet after centrifuging the intact cells? After centrifuging the lysed cells? What color were the supernatants? What does this tell you?
2. List your predictions and observations for the sample and what happens to the sample when the following buffers are added to the HIC column.

Collection tube No.	Prediction	Observations under UV (Column and collection tube)
Tube 1 Sample in Binding Buffer		
Tube 2 Sample in Wash Buffer		
Tube 3 Sample in Elution Buffer		

3. Using the data table above, compare how your predictions matched up with your observations for each buffer.
 - a. Binding buffer
 - b. Wash buffer
 - c. Elution buffer
4. Based on your results, explain the roles or functions of these buffers.
 - a. Equilibration buffer
 - b. Binding buffer

- c. Wash buffer
 - d. TE (Elution) buffer
5. Were you successful in isolating and purifying GFP from the cloned bacterial cells? Identify the evidence you have to support your answer.

Video of Experimental Procedures:



thumbnail Erin Rod [Wikipedia CC-BY-SA 4.0](#)

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