酵素純化與分析

Enzyme Purification and Analysis

國立台灣大學 微生物與生化所 莊榮輝
Professor RH Juang, Institute of Microbiology and Biochemistry

酵素純化與分析

B2

- 酵素純化方法 Enzyme purification methods
- 酵素分析方法 Enzyme analysis methods
- 問題集 Problems

酵素分析方法 Enzyme analysis methods

1. 蛋白質定量法 Protein determination
2. 酵素活性測定法 Enzyme activity assay
3. 電泳檢定法 Electrophoresis
4. 分子量決定法 Molecular weight determination
5. 蛋白質構造與組成分析
   Protein structure and composition analysis
6. 免疫學工具的利用 Immunochemical tools
7. 蛋白質科技 Protein technology

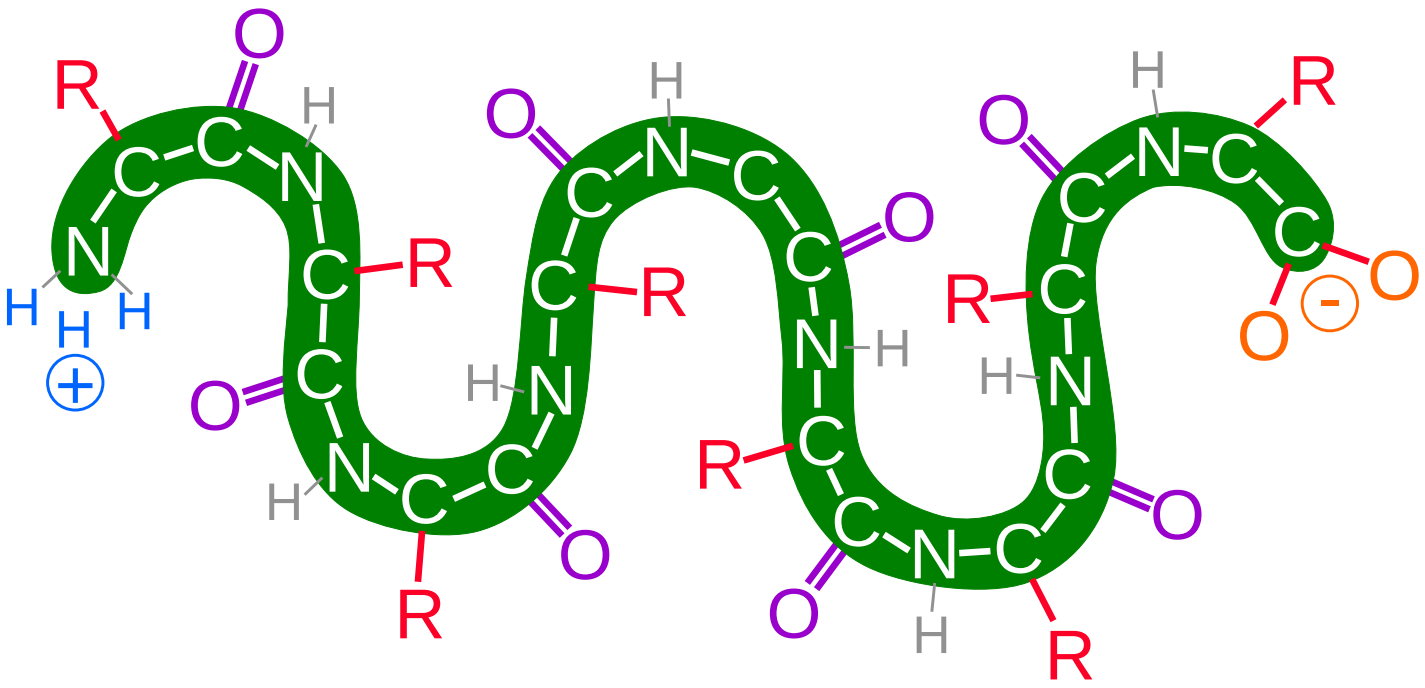
1 蛋白質定量法 Protein determination methods

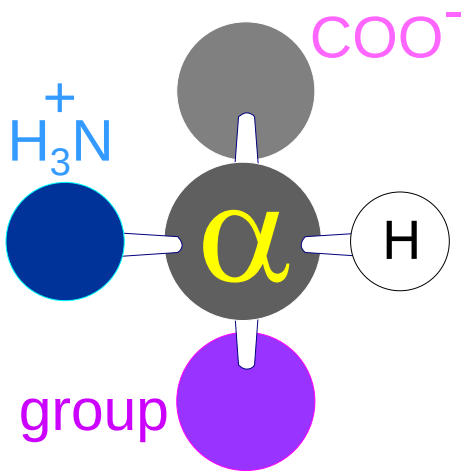
- 1.1 Biuret method
- 1.2 Lowry method
- 1.3 UV absorbance
- 1.4 Coomassie Blue (dye binding) method
- 1.5 Other methods

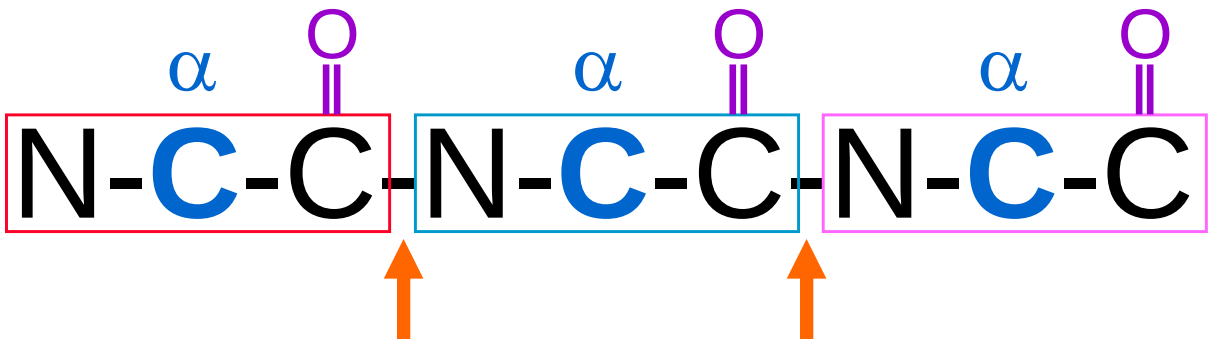
蛋白質構造的骨架 Backbone of protein molecule

Constant

Variable

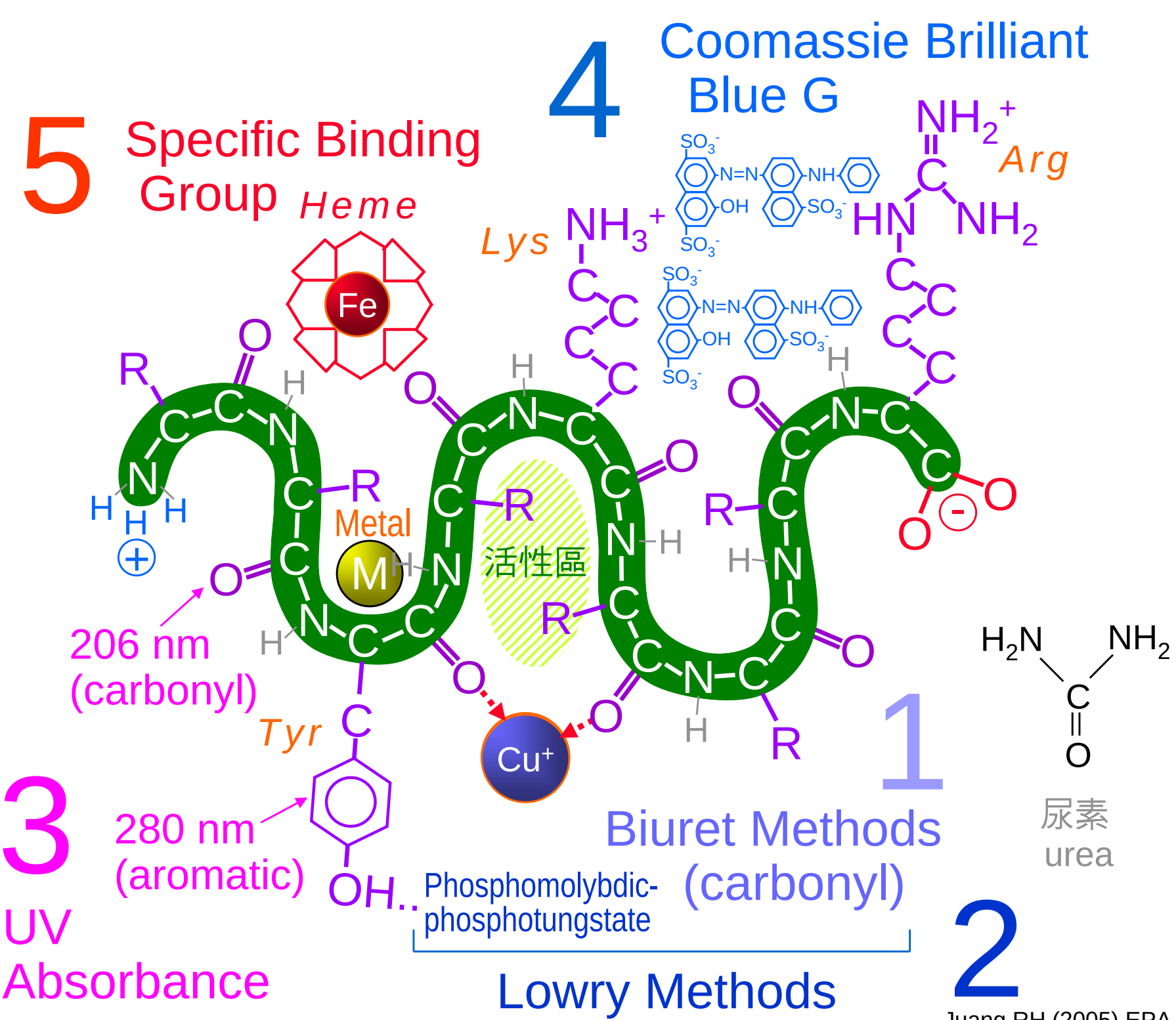






Juang RH (2005) EPA

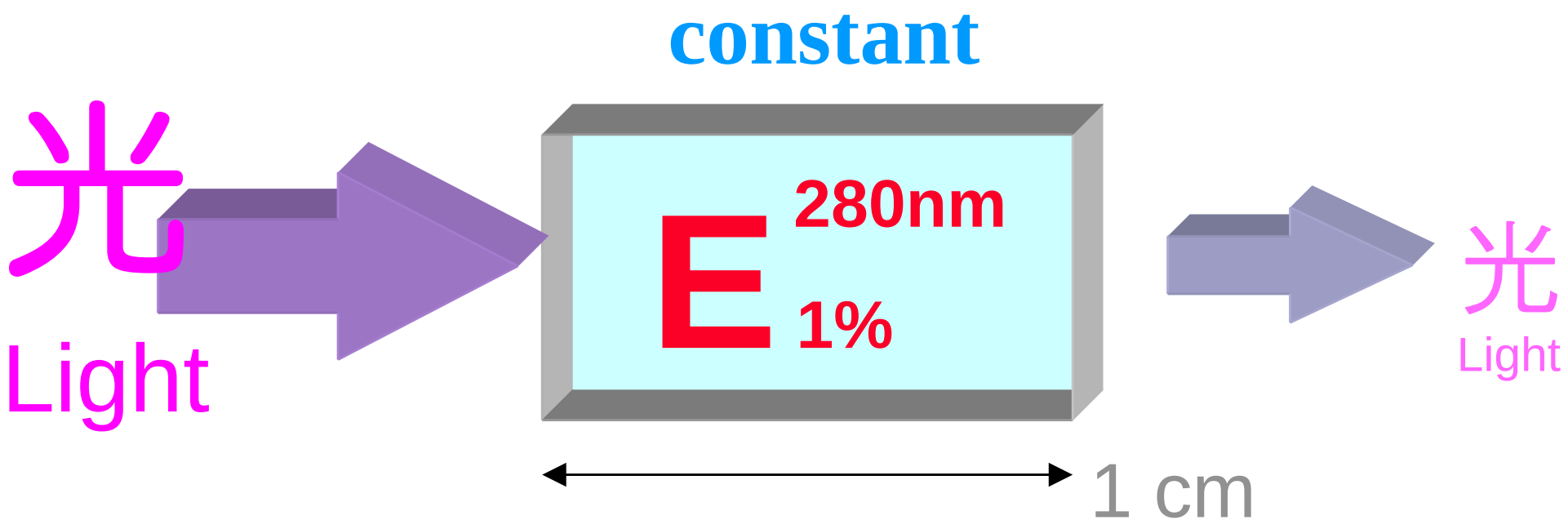
各種蛋白質定量法原理



Juang RH (2005) EPA

分子消光係數 Molar extinction coefficient

- A constant indicating the capacity of light absorbance for a molecule



吸光值 $A = E \times b \times c$

1 *10* *0.1%*

蛋白質消光係數 UV absorbance by proteins

- 280 nm - Aromatic Groups (Side chain)

  1 mg/mL 溶液→ 吸光度 (280 nm) = **1** 約值

- 192 nm - Carbonyl Groups (Backbone)

  1 mg/mL 溶液→ 吸光度 (192 nm) = **60**

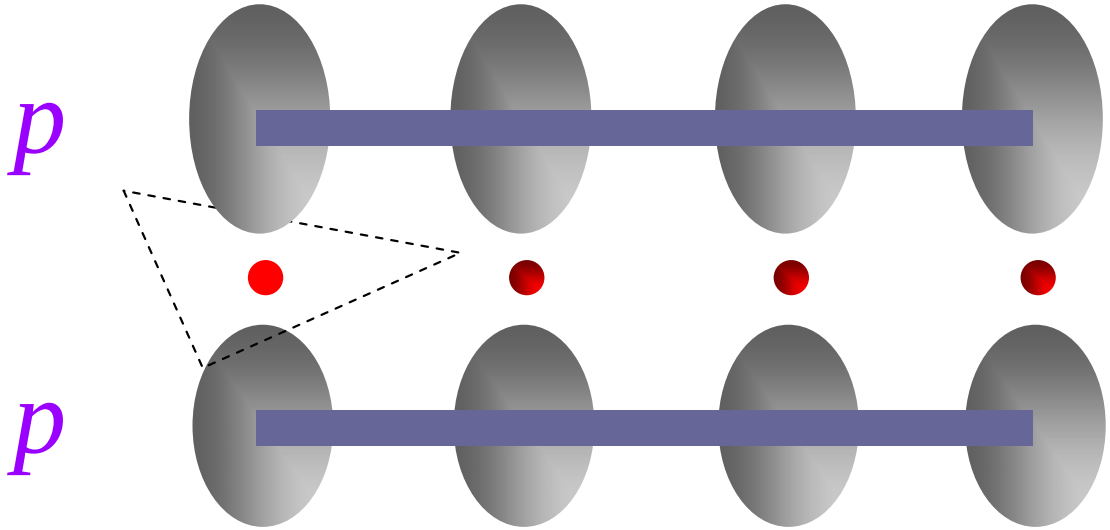
  (206 nm) = **29**

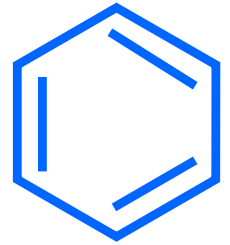
200 nm UV light is interfered heavily by O_2

共軛雙鍵 Conjugated double bonds

sp^2 sp^2 sp^2 sp^2

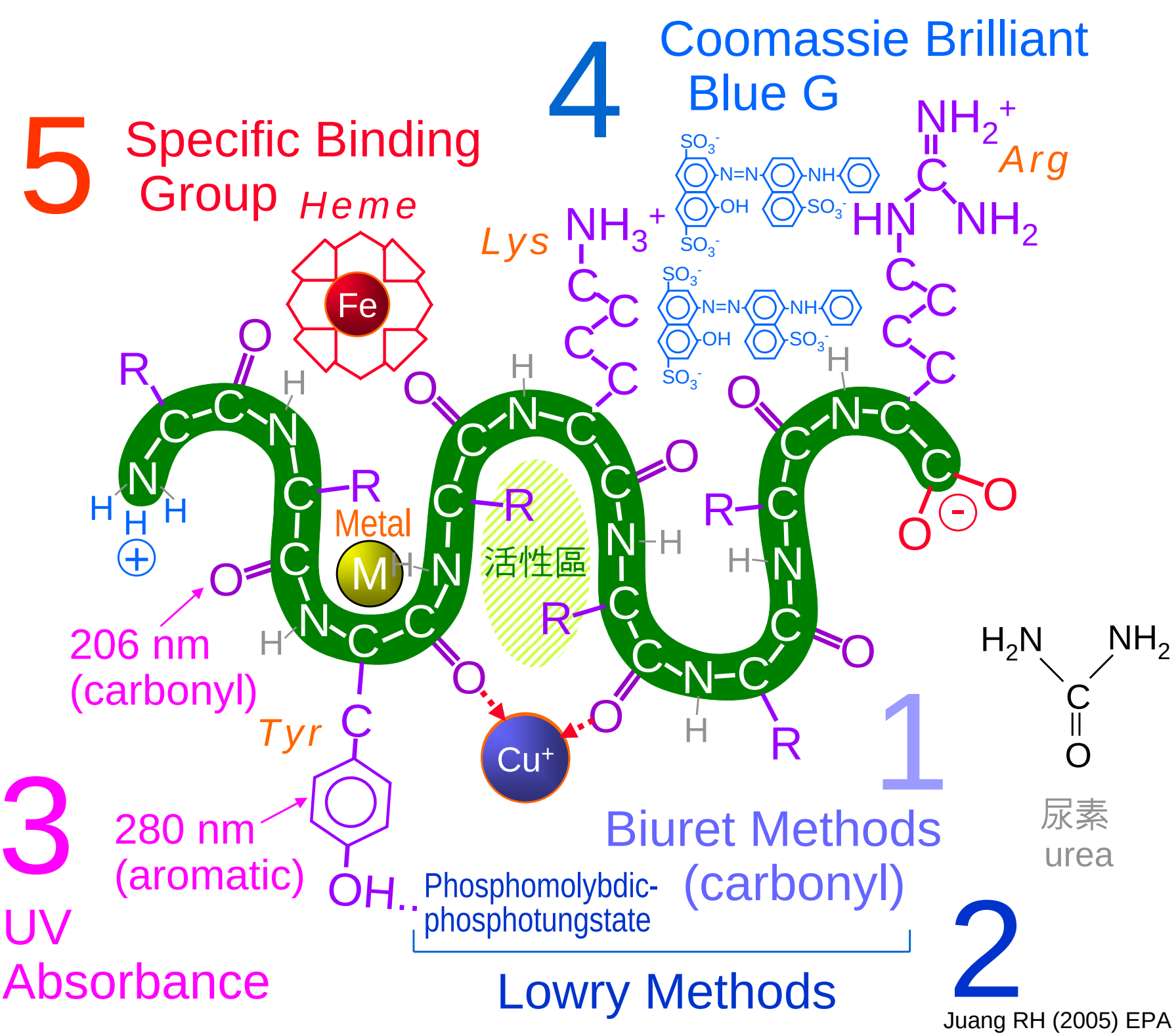
—C=C—C=C—





p 電子共振 resonance

各種蛋白質定量法原理

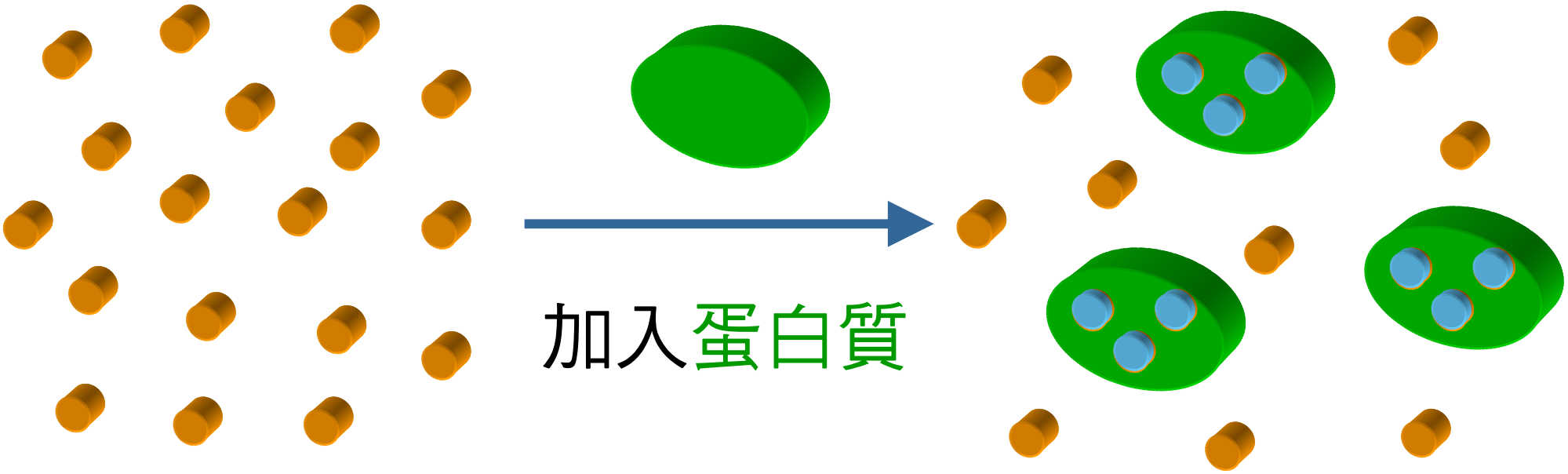


Juang RH (2005) EPA

Bradford Method

Coomassie Brilliant Blue G-250

470 nm　　CBG is an *indicator*　　595 nm

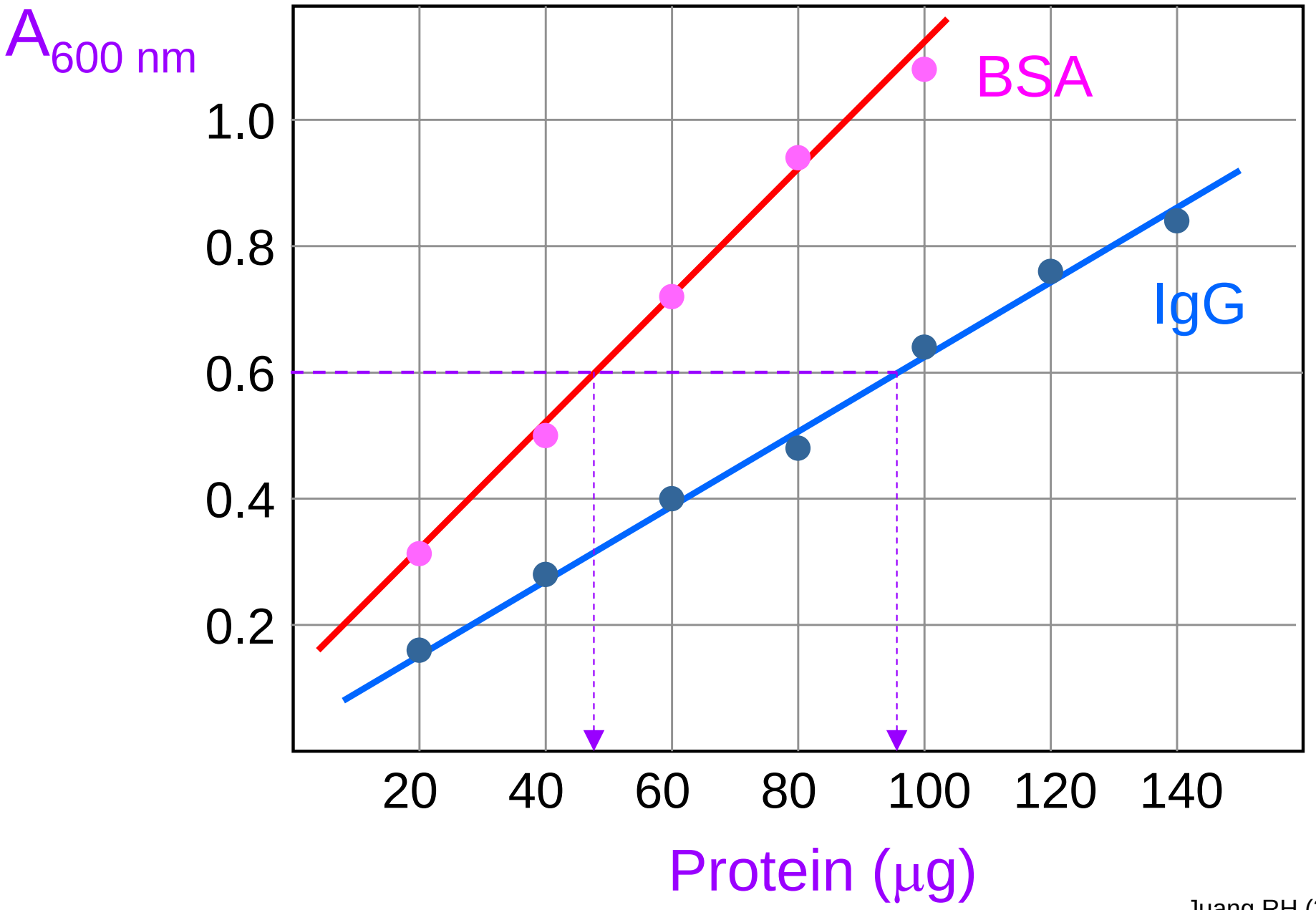


酸性環境下呈茶色
Brown (acidic)

與蛋白質結合變藍色
Blue (pH ↑)

Juang RH (2005) EPA

不同蛋白質的定量差異 Deviation of standards



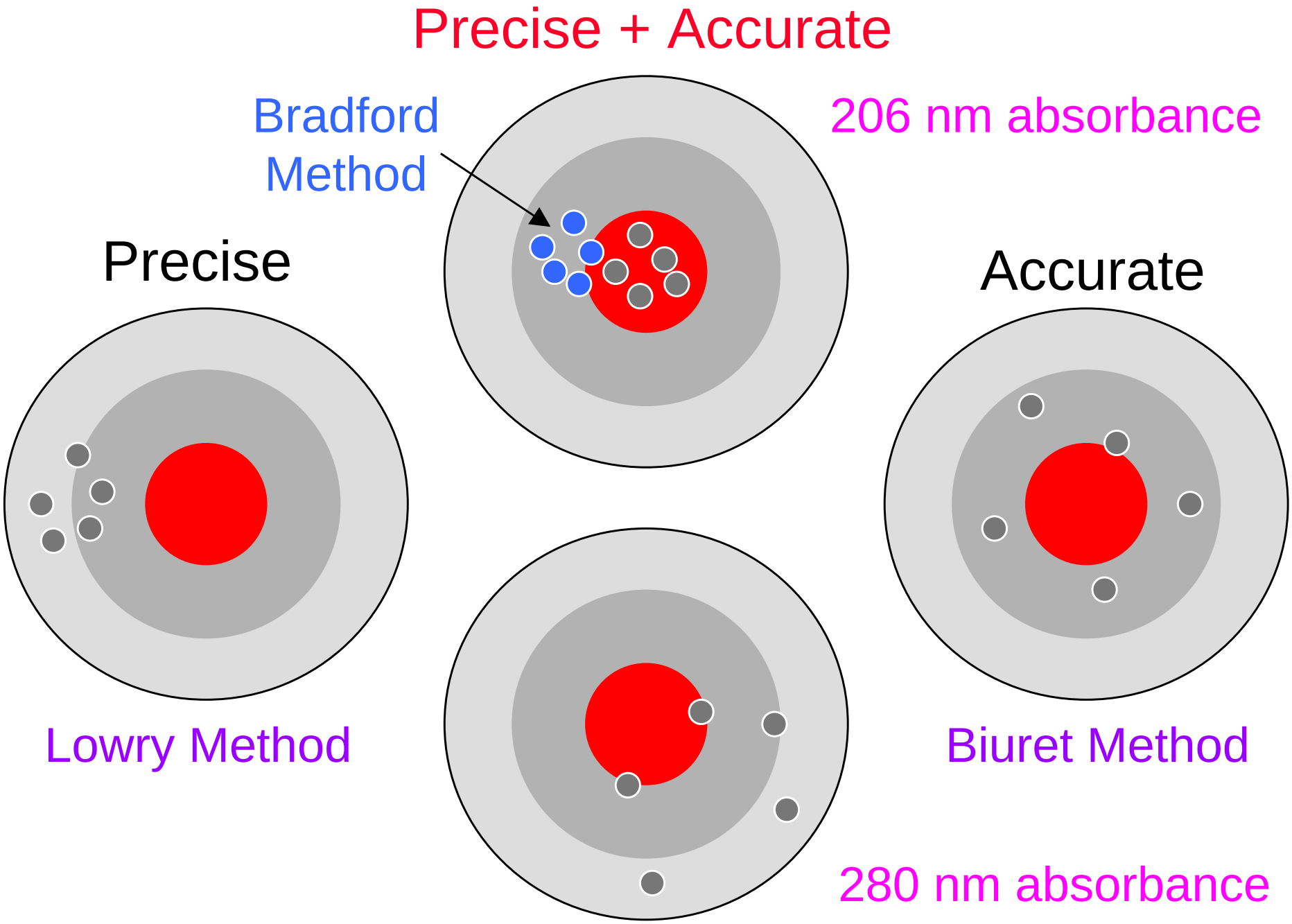
Juang RH (2005) EPA

各種蛋白質定量法的比較

Methods	Precision	Accuracy	Remarks
Biuret	0.05 - 5 mg	High	Rapid, Corrosive, Interference
Lowry	0.05 - 0.5 mg	Medium	Slow, Interference
Absorbance 280 nm	0.05 - 2 mg	Low	Sample recoverable, Interference
Absorbance 205 nm	0.01 - 0.05 mg	High	Sample recoverable, O_2 interference
Bradford Dye-binding	0.01 - 0.05 mg	M - H	Rapid, Interference, Color staining

Comparison of the protein determination methods

Juang RH (2005) EPA



Juang RH (2005) EPA

2 酵素活性測定法 Enzyme activity assay methods

- 2.1 催化反應 Catalytic reaction

  活性測定時要注意一些基本原則

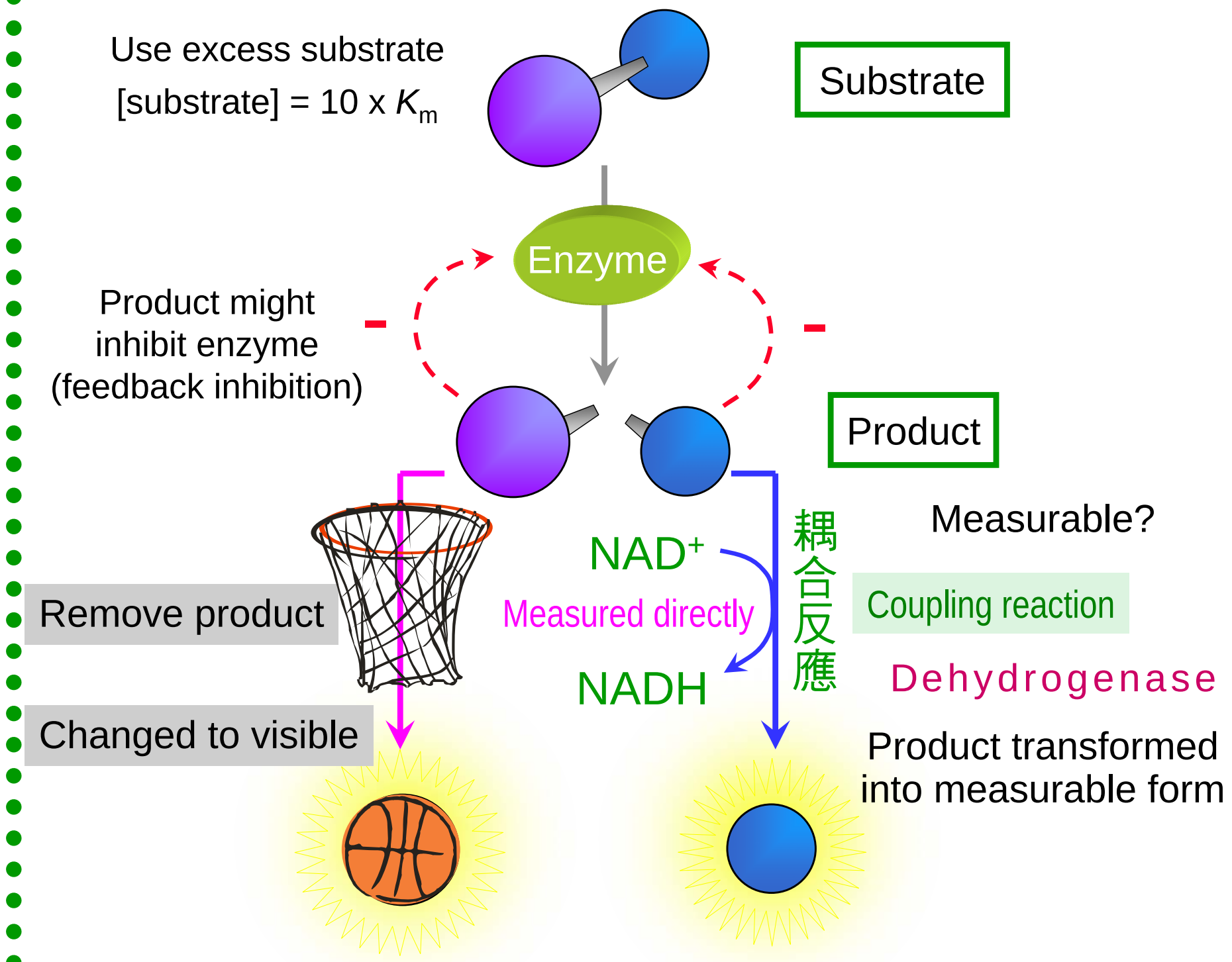
- 2.2 酵素活性分析 Enzyme assay methods

  反應速率 = 生成物 (P) / 時間 (t)

- 2.3 維持酵素活性 Maintain activity

  很多酵素在細胞外容易失去活性

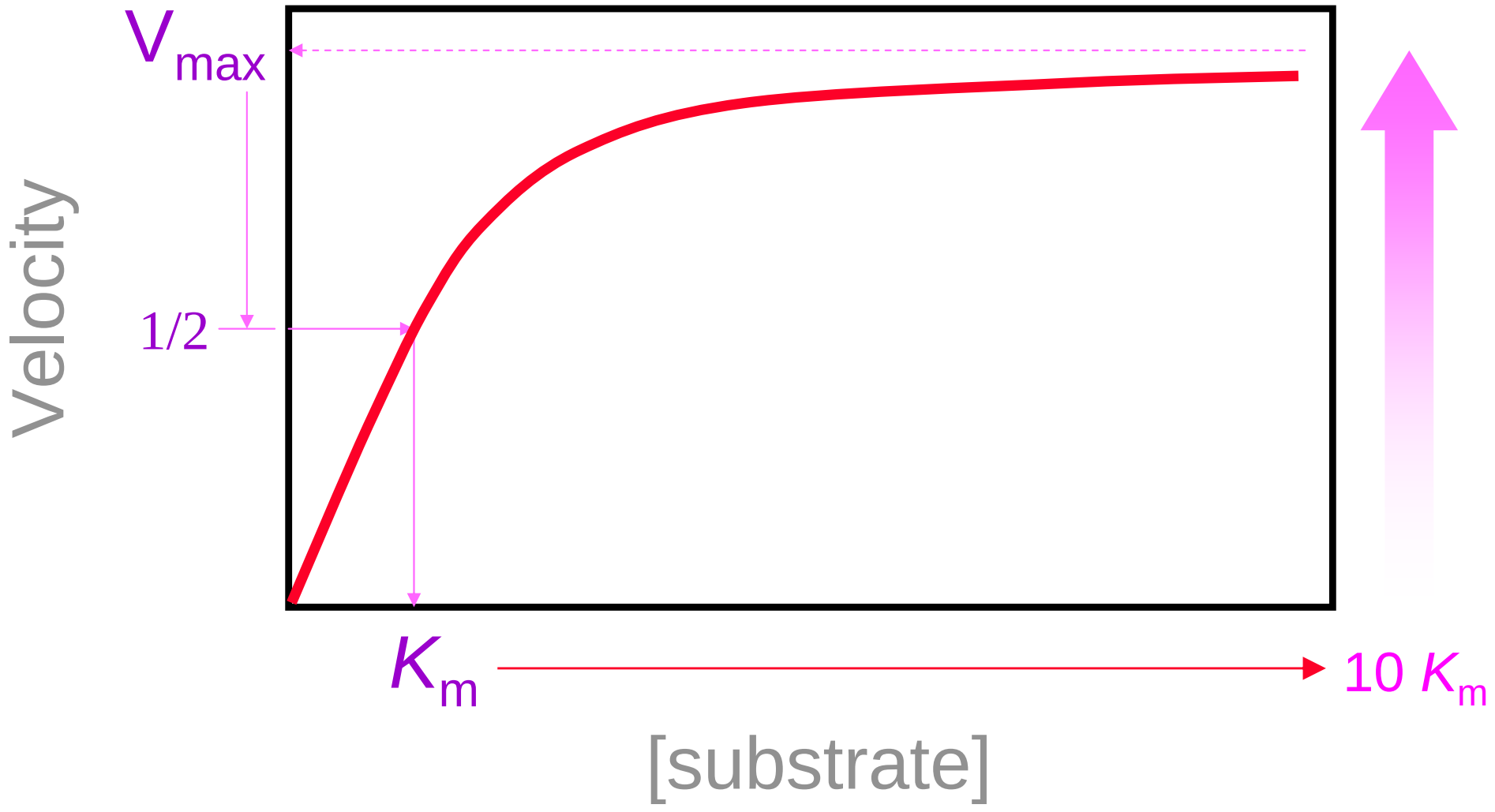
酵素反應及偵測方法



Enzyme catalysis and detection methods

Juang RH (2005) EPA

基質量使用十倍 K_m　[Substrate] = 10 K_m

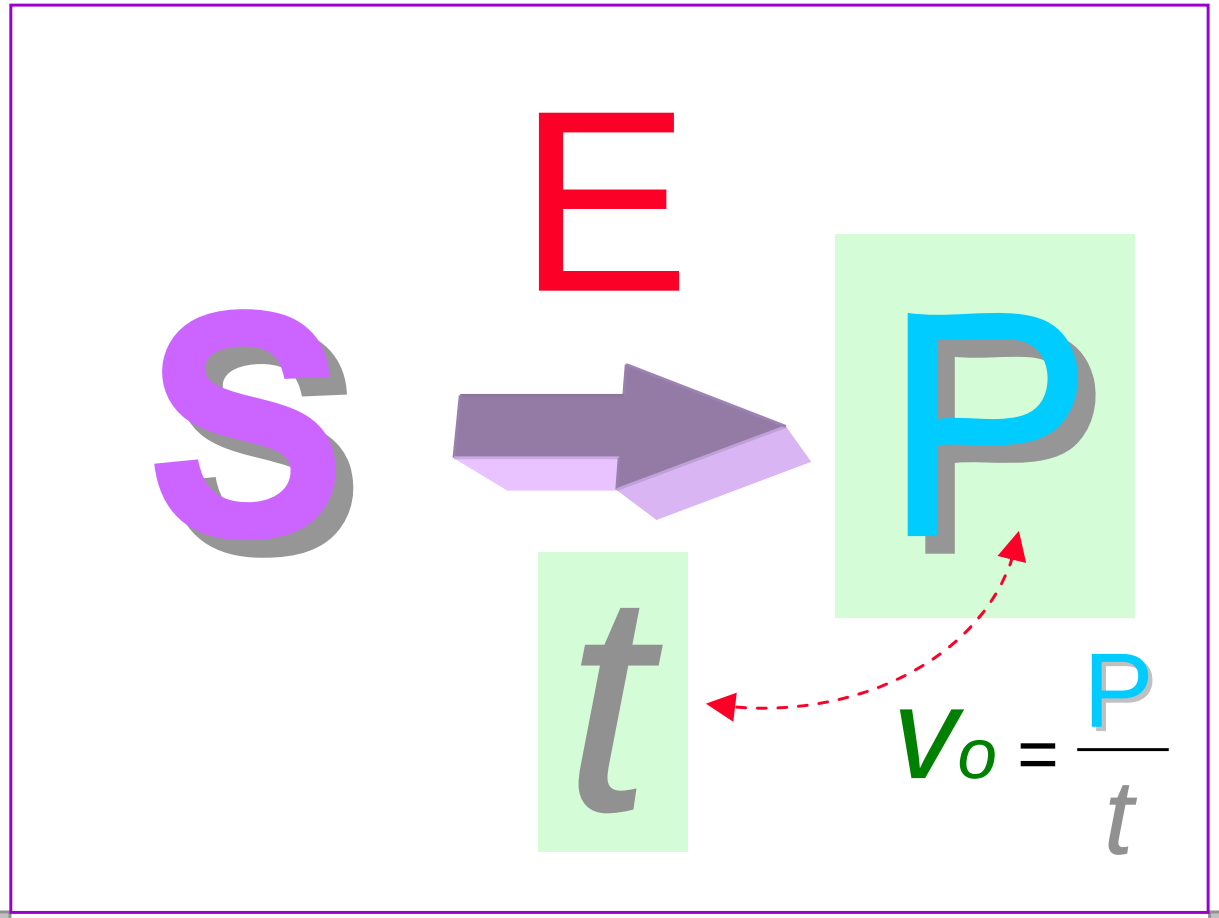


酵素活性測定 Determine enzyme activity (SEPt)

Optimized enzyme concentration

Substrate excess

10 x K_m



Product measurable

pH

酸鹼度

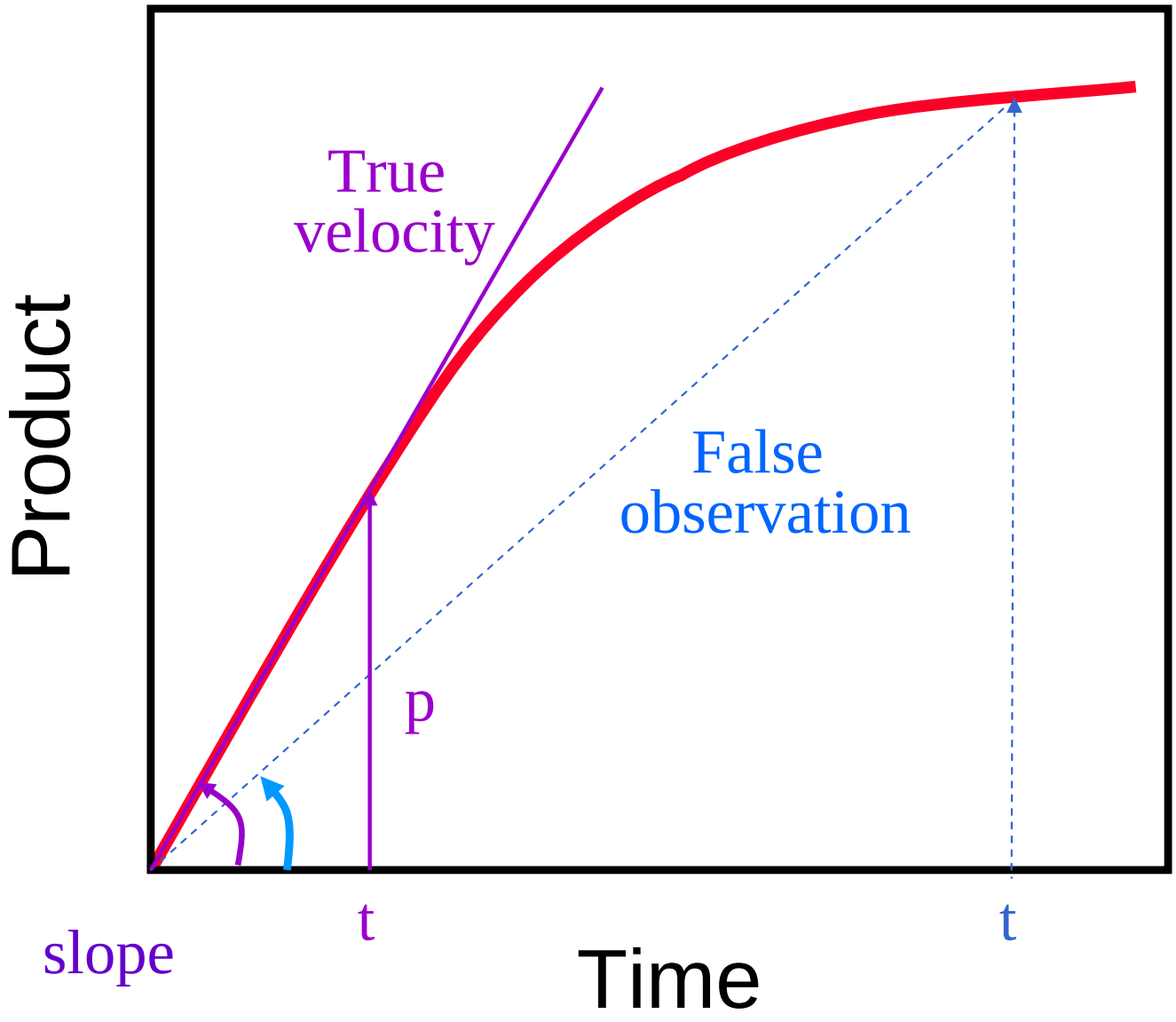
Proper reaction time

Temperature

溫 度

Juang RH (2005) EPA

反應速率需成線性 True velocity of the reaction



$$v_o = \frac{p}{t}$$

Juang RH (2005) EPA

2.2 酵素活性分析 Enzyme assay methods

- 2.2.1 酵素活性測定方法 Assay methods
  在一定時間內測得生成物的產量
- 2.2.2 中止酵素反應方法 Stop the reaction
  中止酵素的方法不得破壞生成物或干擾測定
- 2.2.3 連續測定法 Continuous measuring
  連續測定可不用刻意中止酵素反應
- 2.2.4 澱粉磷解脢活性分析 Assay for L-SP
  以生化方法可以偵測到澱粉磷解脢的活性

2.2.1 酵素活性測定方法 Assay methods

- a. 直接測定生成物 Measuring product directly

  酒精去氫酶 (alcohol dehydrogenase, deHase)

  $Alcohol + NAD^+ \rightarrow Acetaldehyde + NADH + H^+$

- b. 耦合反應法 Coupling reaction

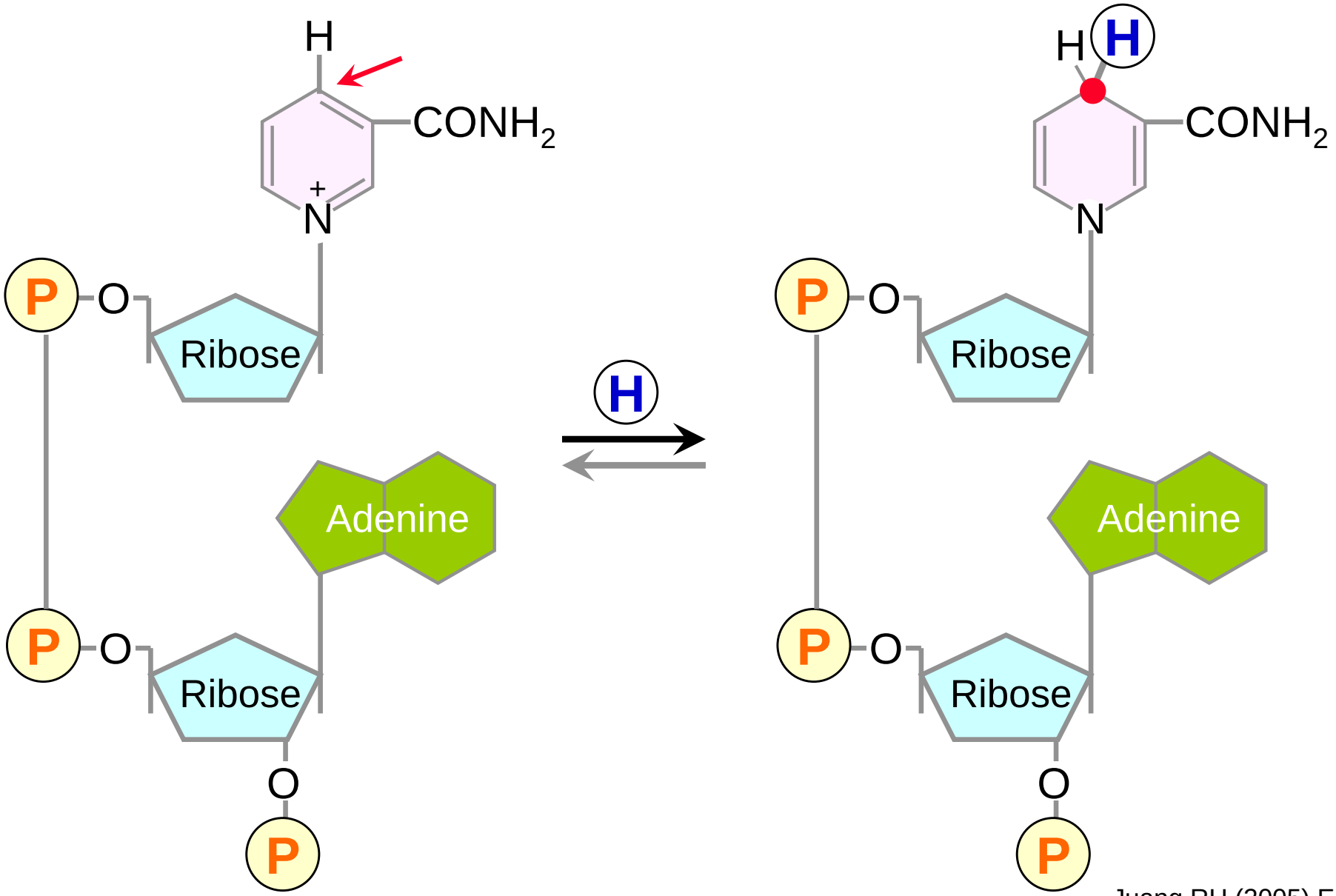
  S → P → Q 可耦合到去氫酶反應 (coupled to deHase)

- c. 化學測定法 Chemical method
- d. 放射線測定法 Tracer method
- e. 測壓法 Manometry (for gaseous product)
- f. 電極 Electrode (for pH or O_2 change)
- g. HPLC 檢定法 Your last choice

輔脢 NADH 作用機制 Action of coenzyme NADH

NADP+ Oxidized form

NADPH Reduced form



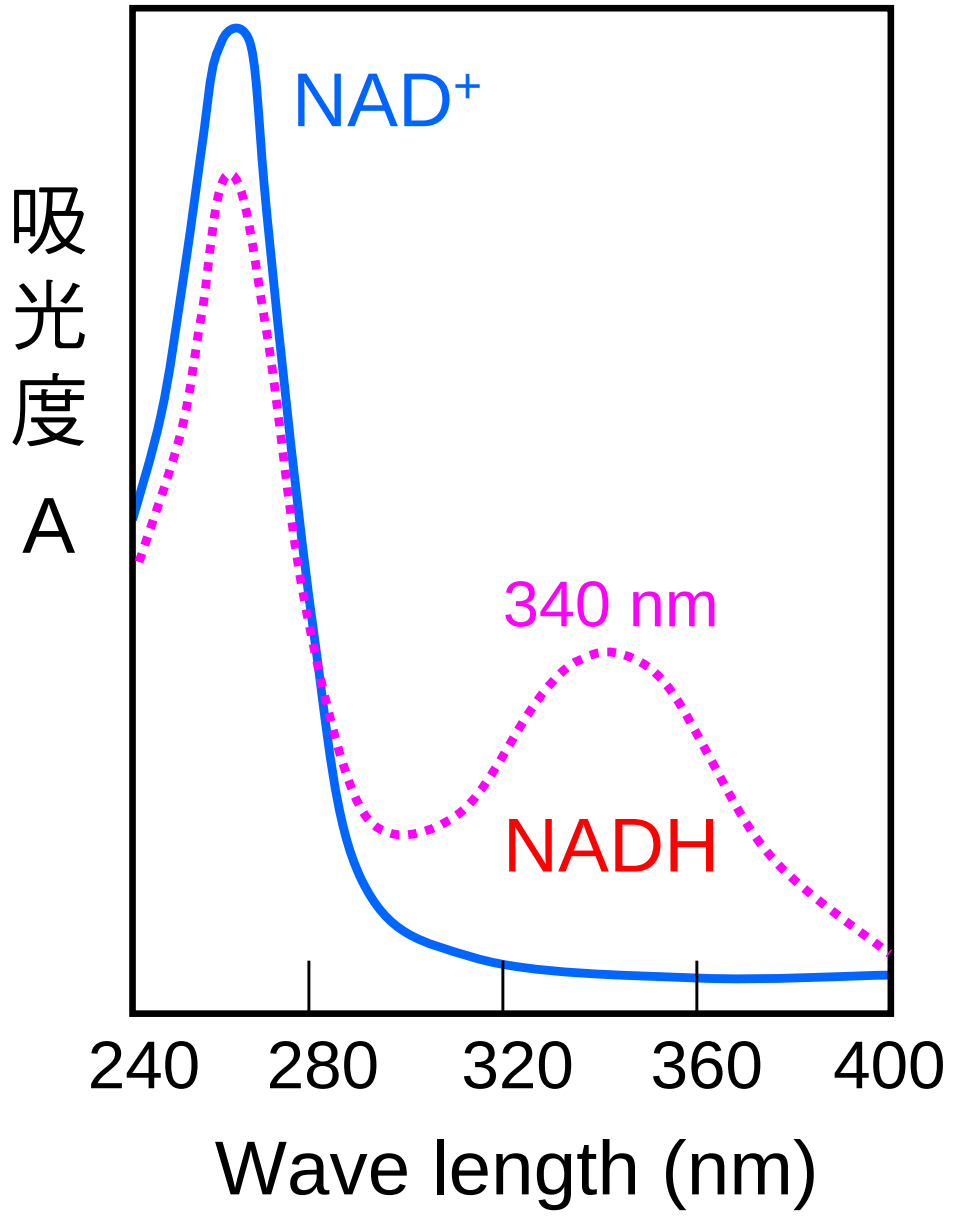
Adapted from Alberts et al (2002) Molecular Biology of the Cell (4e) p.86

Juang RH (2005) EPA

Coenzyme NADH

NAD$^+$/NADH 的轉換可以
耦合 340 nm 吸光度變化

$NAD^+ \rightleftharpoons NADH$ 340 nm



Dehydrogenase (去氫酶)

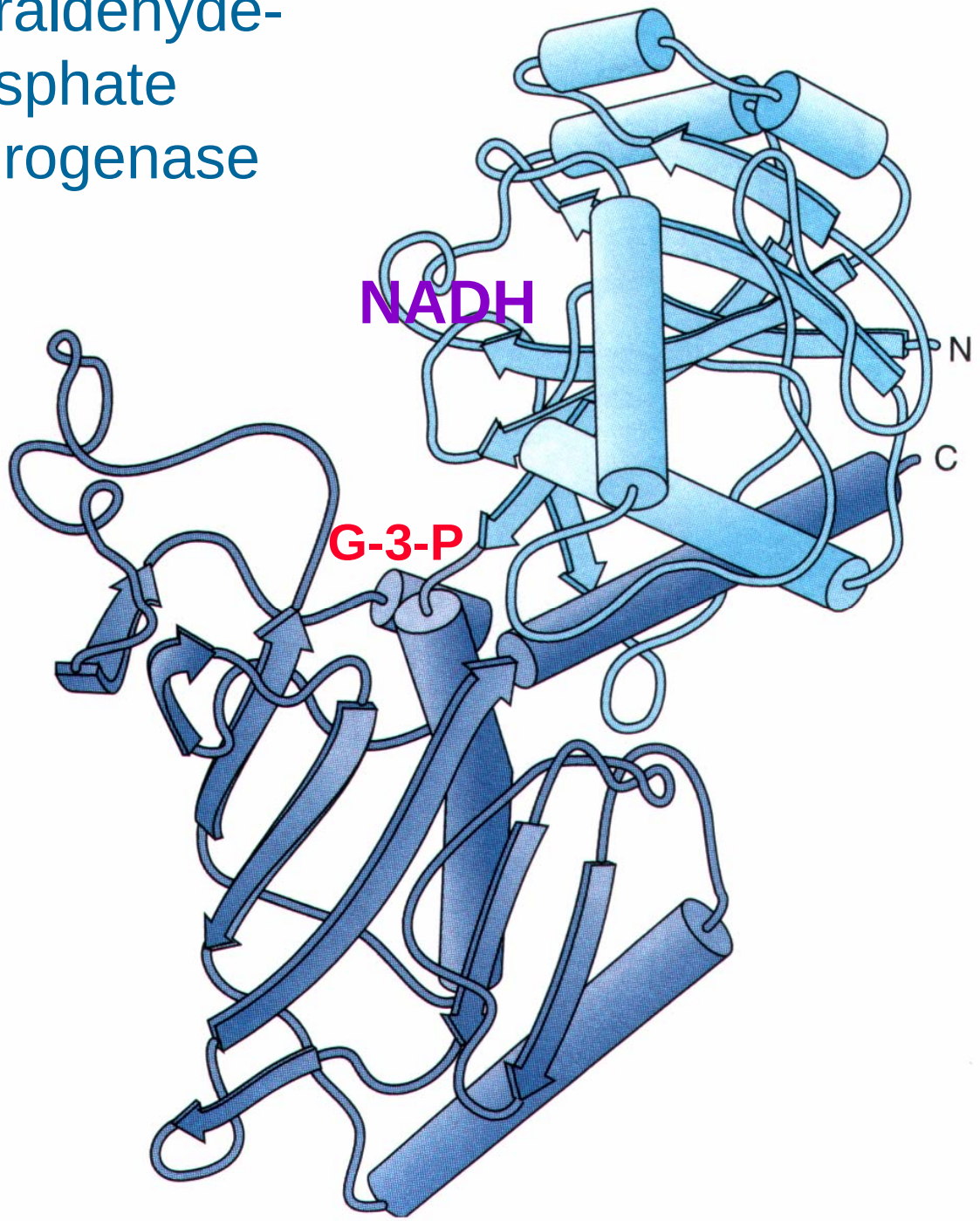
Glyceraldehyde-3-P deHase

- Dehydrogenases use NADH or NADPH as coenzyme
- Have similar NAD$^+$ Binding domain (Convergent evolution)

Juang RH (2005) EPA

一個典型的去氫酶

Glyceraldehyde-3-phosphate dehydrogenase



NADH Binding Domain (conserved)

substrate Binding Domain (variable)

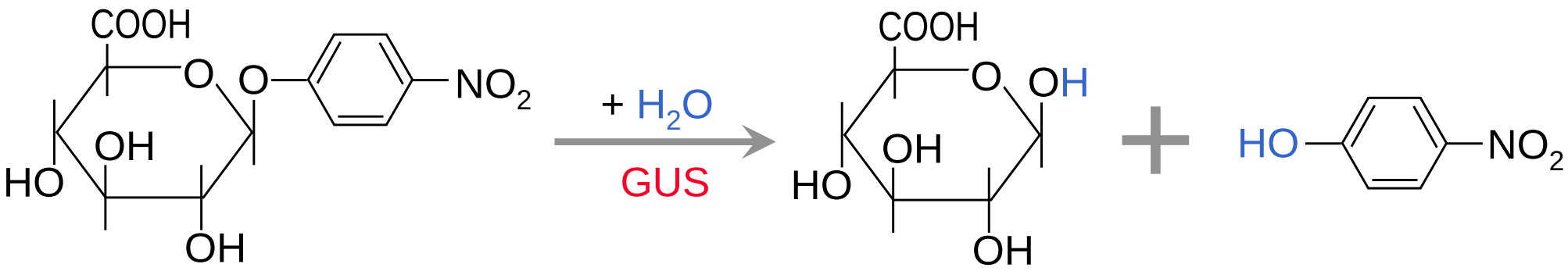
A typical dehydrogenase molecule contains two domains

Kleinsmith & Kish (1995) Principles of Cell and Molecular Biology (2e) p.25

GUS activity assay - using synthetic substrate

Substrate (colorless)

Products (yellow)



p-Nitrophenyl β-D-glucuronide (*p*NPG)

β-D-Glucuronic acid

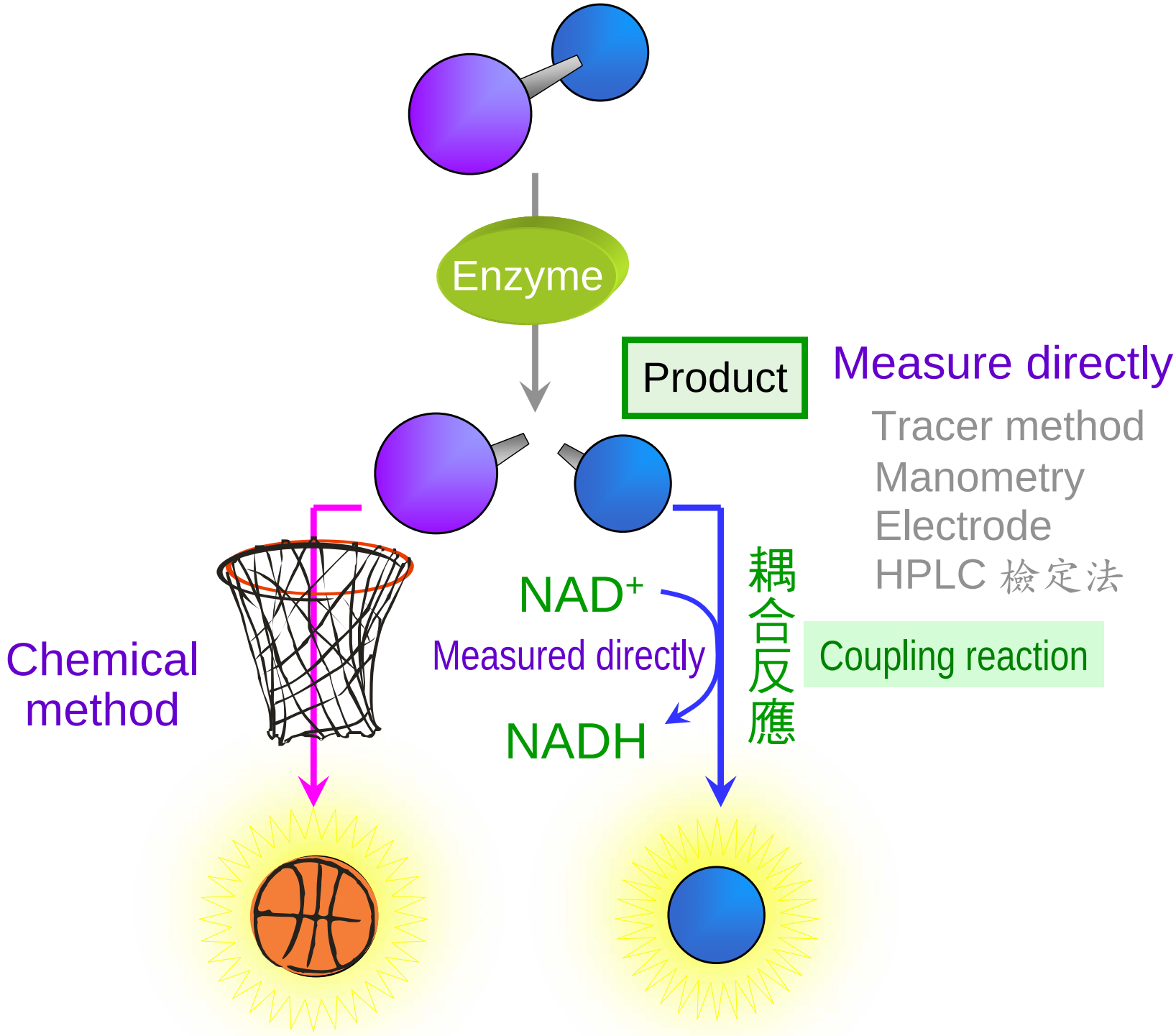
p-Nitrophenol (yellow)

415 nm

GUS (glucuronidase) is extensively used for a reporter in cloning.

Juang RH (2005) EPA

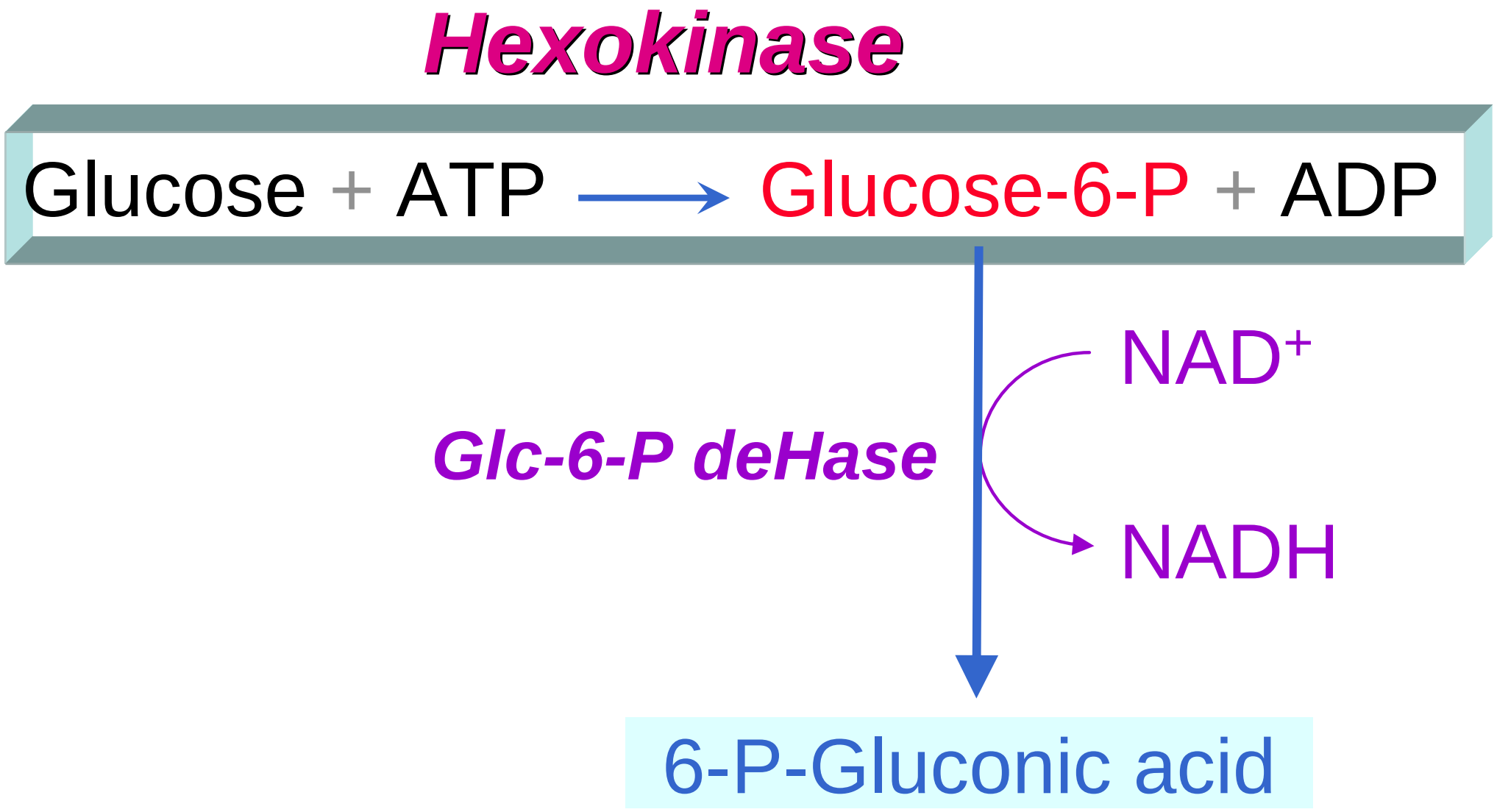
酵素偵測方法



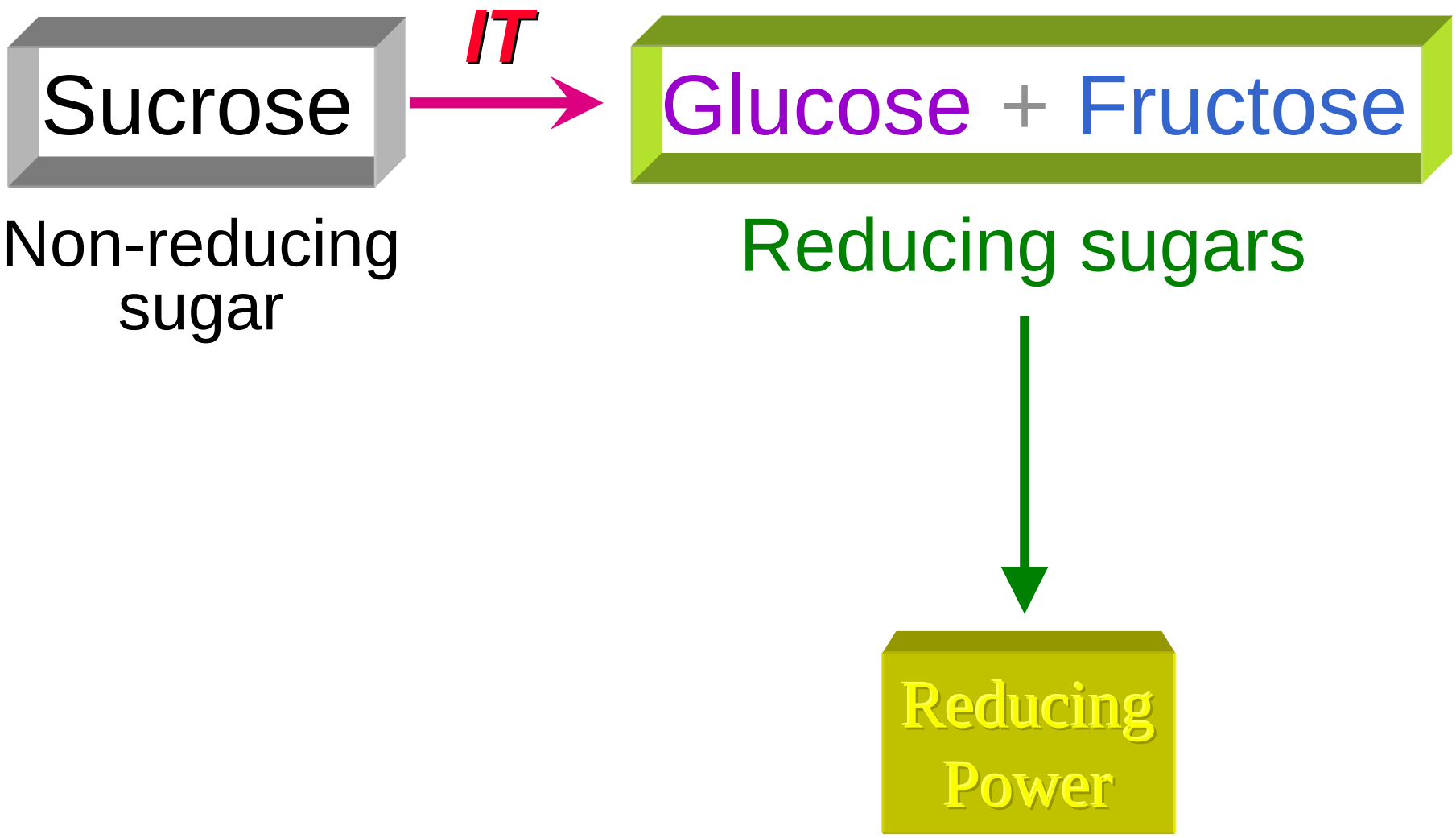
Enzyme detection methods

Juang RH (2005) EPA

Coupled to *dehydrogenase* (NAD^+-NADH)

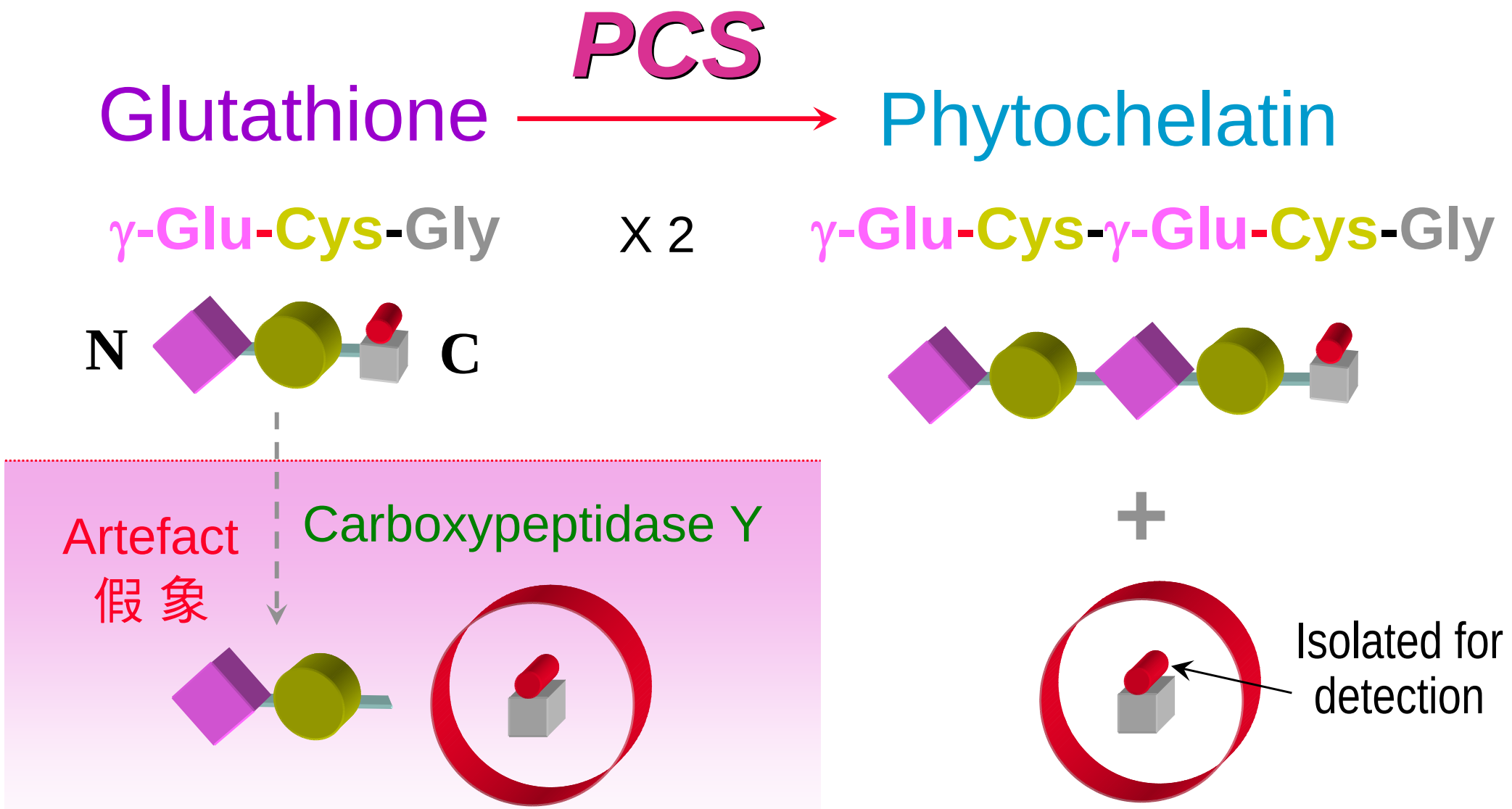


轉化腌 *Invertase (IT)*



Juang RH (2005) EPA

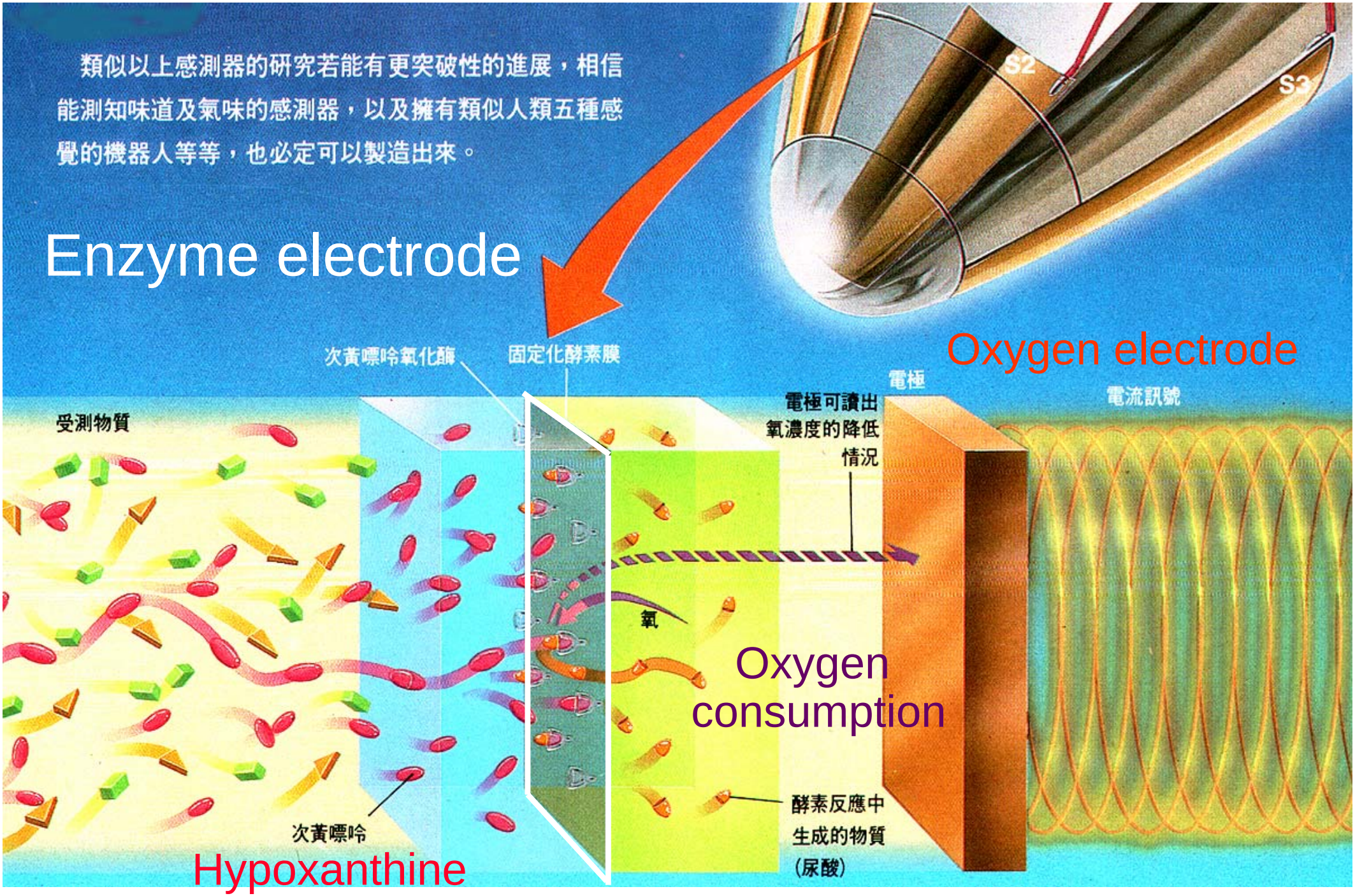
放射線測定法 Using radioactive tracer



It is safer to measure phytochelatin directly by HPLC

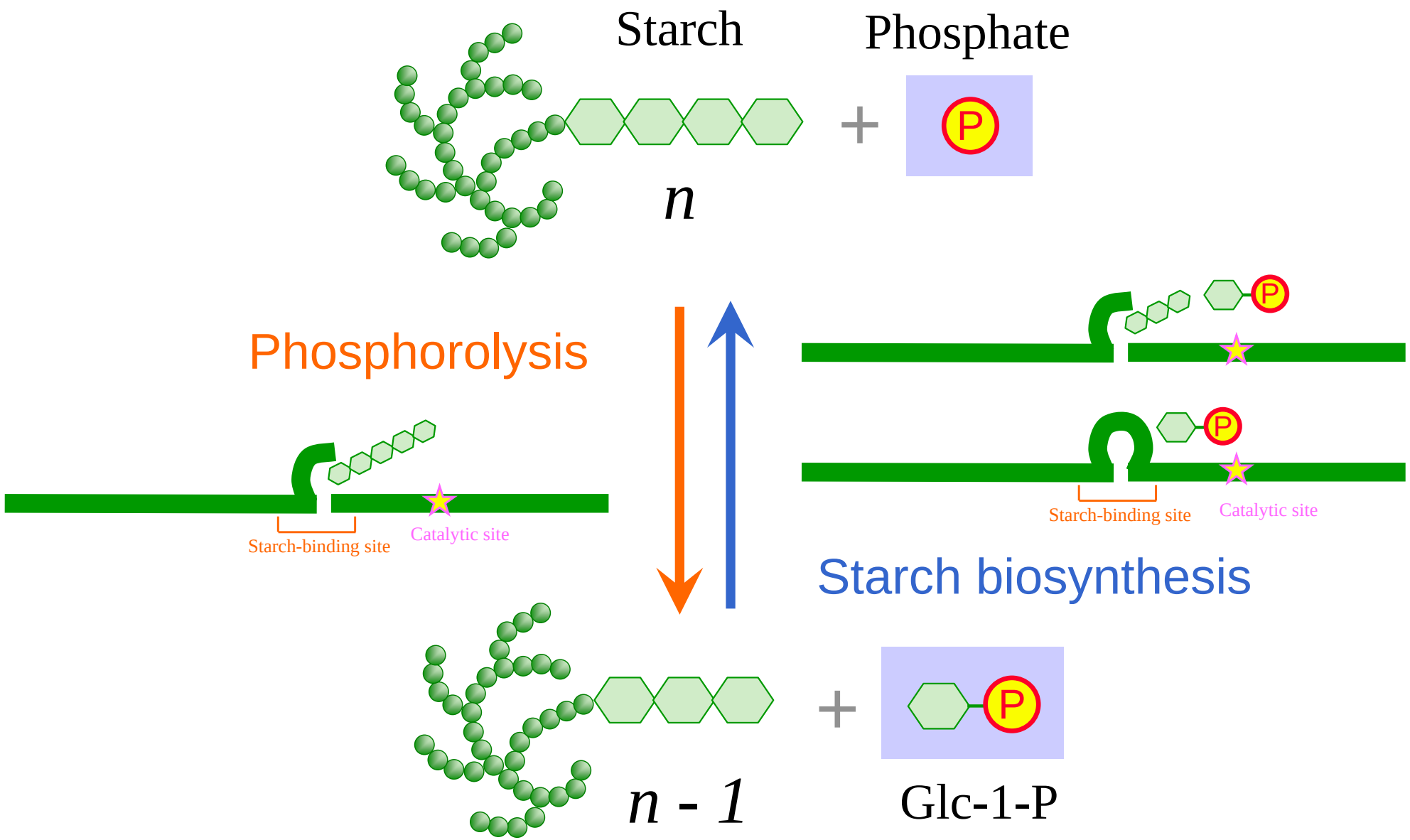
Juang RH (2005) EPA

A sensor to monitor the quality of meat



牛頓 (1985) no. 23 p.108

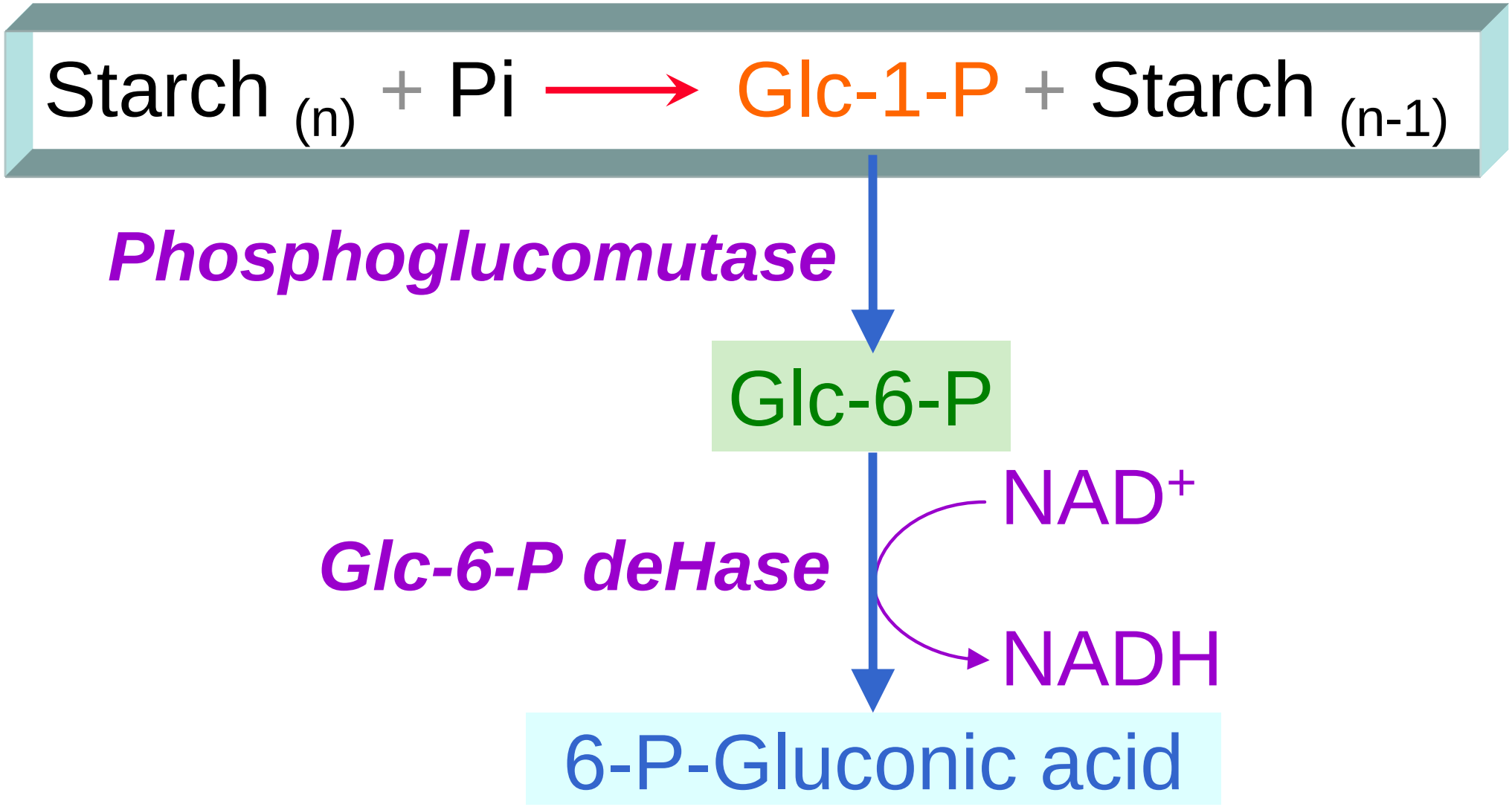
2.2.4 澱粉磷解脢活性分析 Assay for L-SP



Juang RH (2005) EPA

Coupled to *dehydrogenase*

Starch phosphorylase (phosphorolysis)

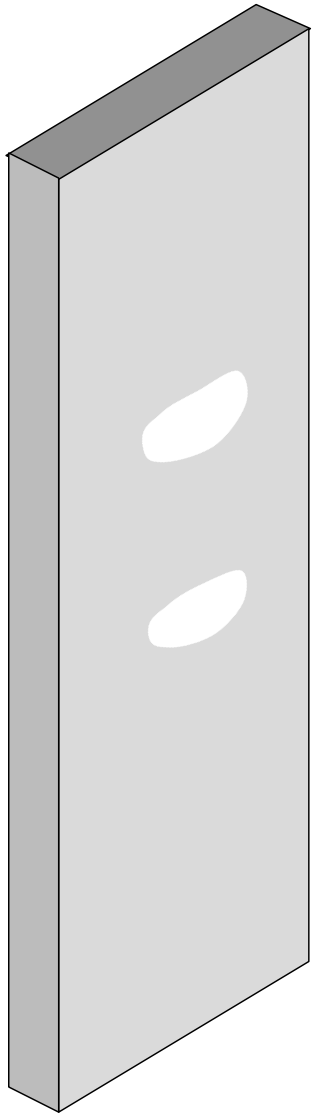


Juang RH (2005) EPA

澱粉磷解酶活性分析及干擾

A

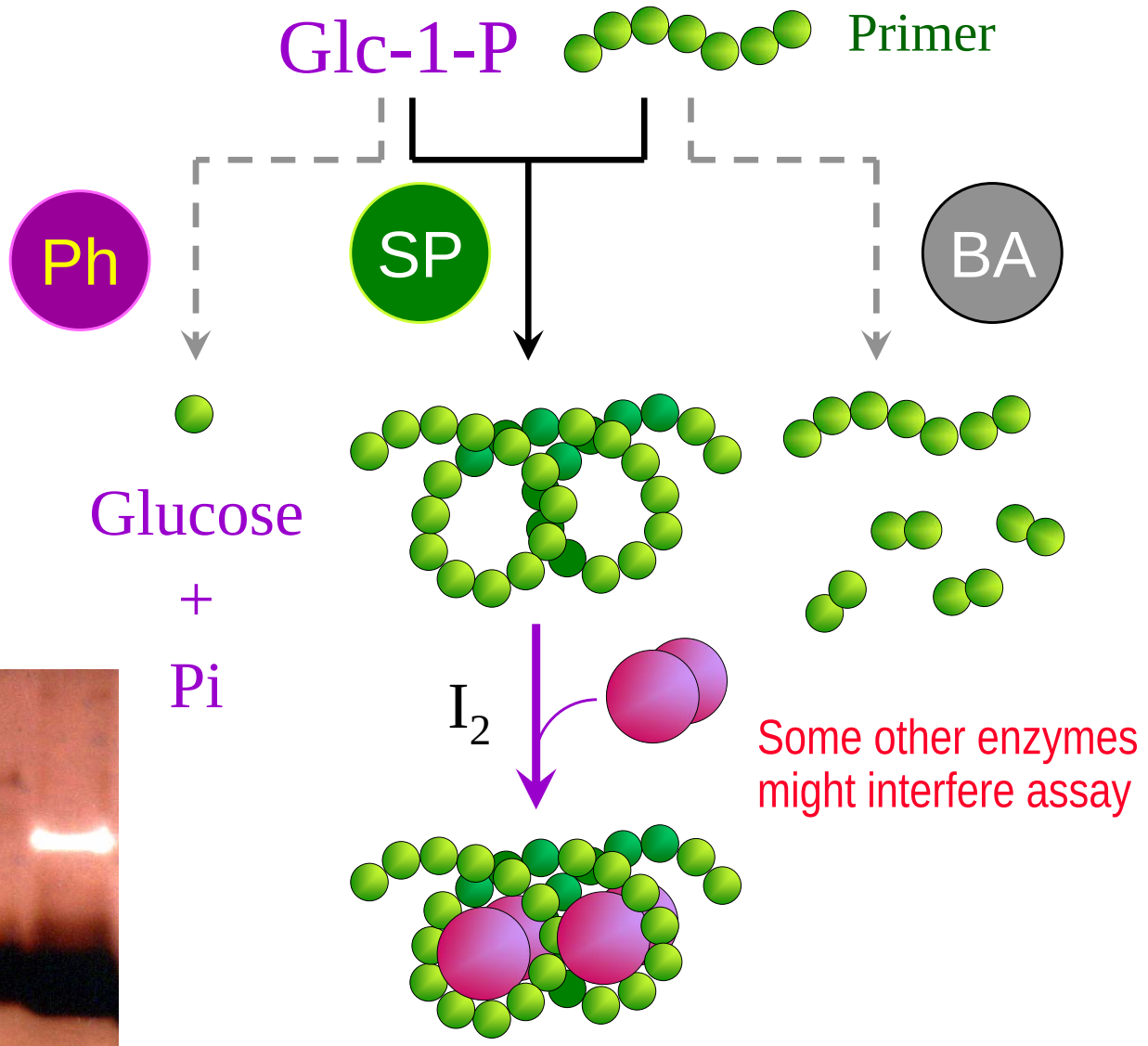
Activity staining



Native PAGE

B

Activity assay and interference



Direct observation of the color product

Some other enzymes might lead to false (+) or (-) results

Juang RH (2005) EPA

2.2.2 中止酵素反應方法 Stop enzyme reaction

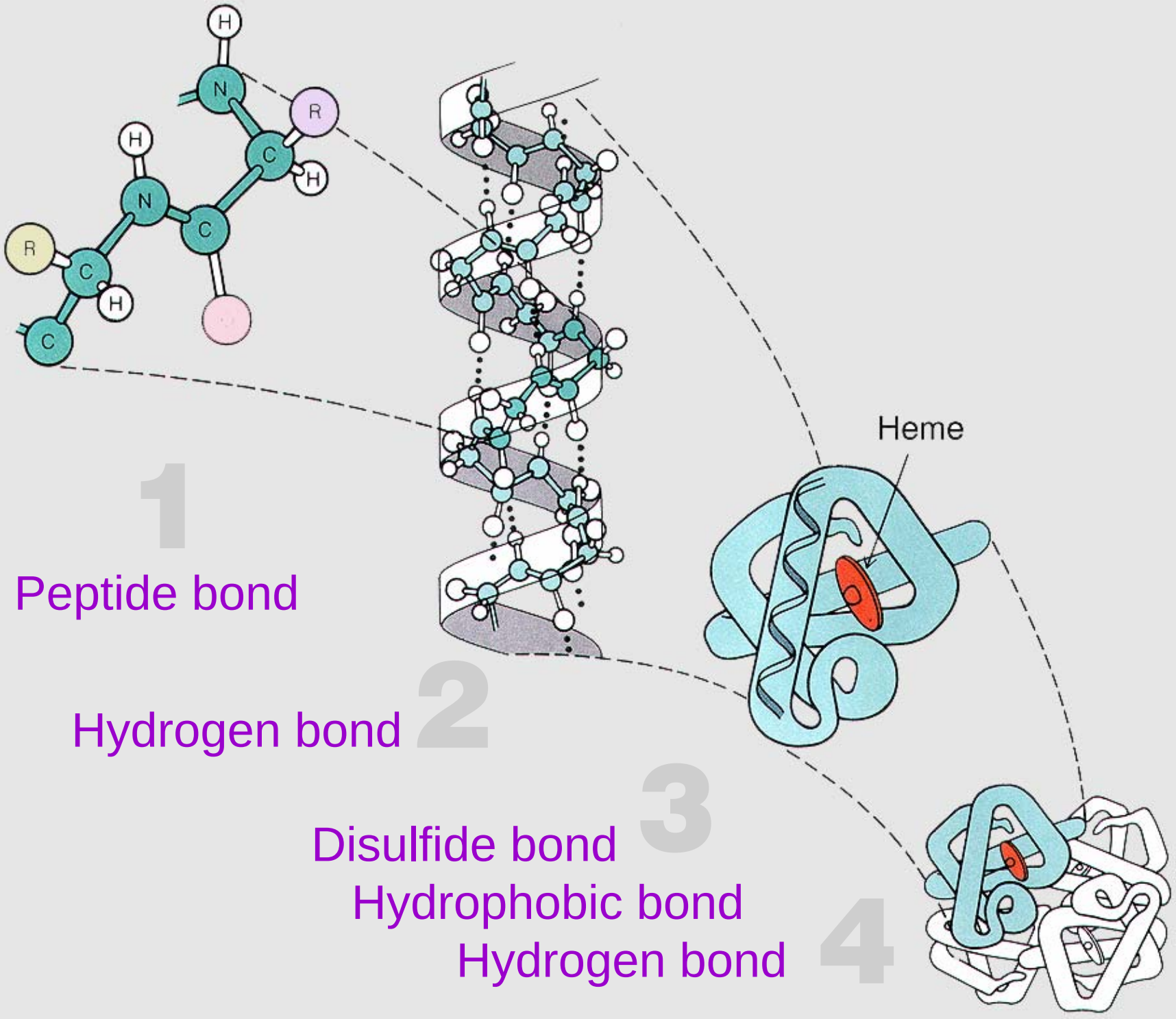
- How to denature the enzyme effectively ?

Change *p*H	TCA	Chemical
Rapidly heating	Boiling	Physical
Add denaturant	SDS	Chemical
Add metal chelator	EDTA	Chemical
Add enzyme inhibitor	PMSF	Chemical
Add non-radioactive substrate (pulse-chase)		Physical

- The product should not be destroyed
- No interference to the detection method

Juang RH (2005) EPA

蛋白質的四級構造

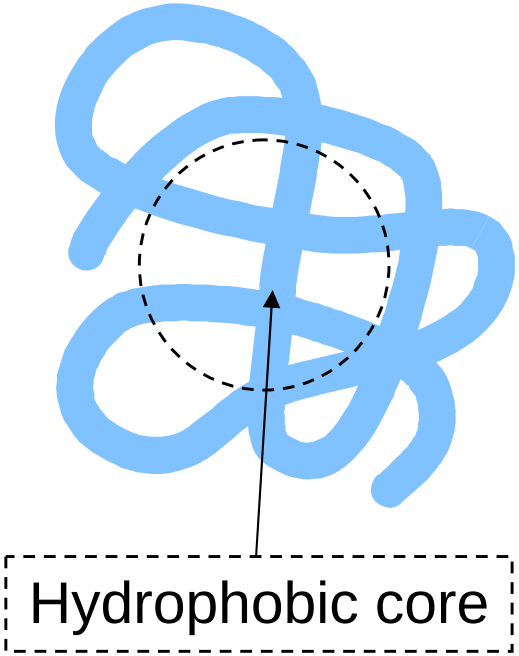


Mathews & van Holde (1990) Biochemistry (1e) p.200, Fig. 6.25

Four levers of protein structure

SDS 在蛋白質表面均勻敷上一層負電

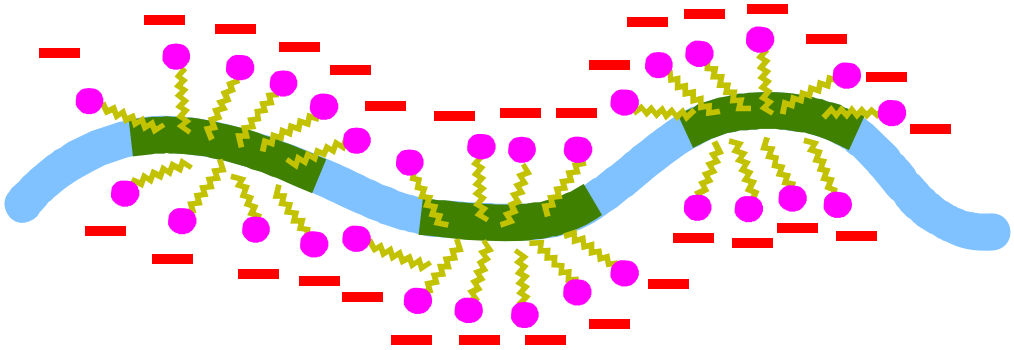
Native protein



SDS

boiling

Protein is denatured to linear form



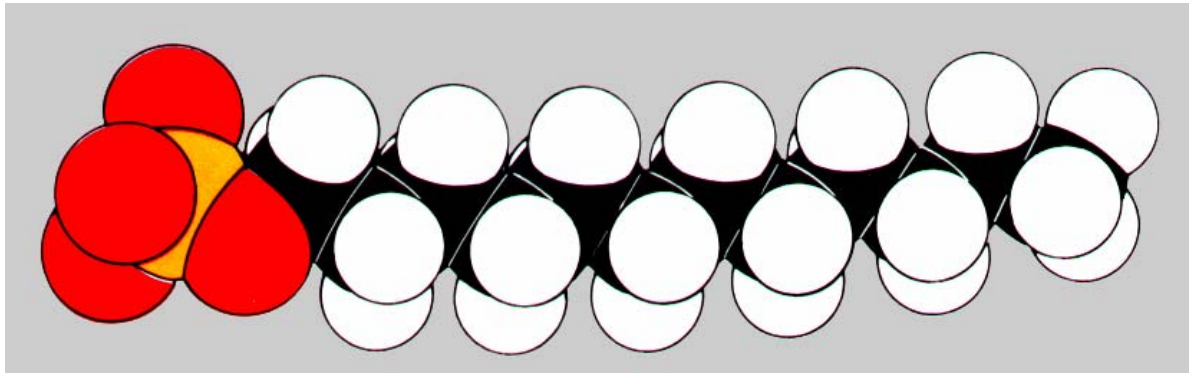
Its surface covered with negatively charged SDS uniformly

+ Mercaptoethanol: to break the disulfide bonds

Juang RH (2005) EPA

常見的變性劑

Urea	$NH_2\text{-}C(=O)\text{-}NH_2$
Guanidine HCl	$NH_2(=NH_2^+)\text{-}NH_2$
Mercaptoethanol	$HOCH_2CH_2SH$
SDS	$SO_4^-\text{-}(CH_2)_{11}CH_3$



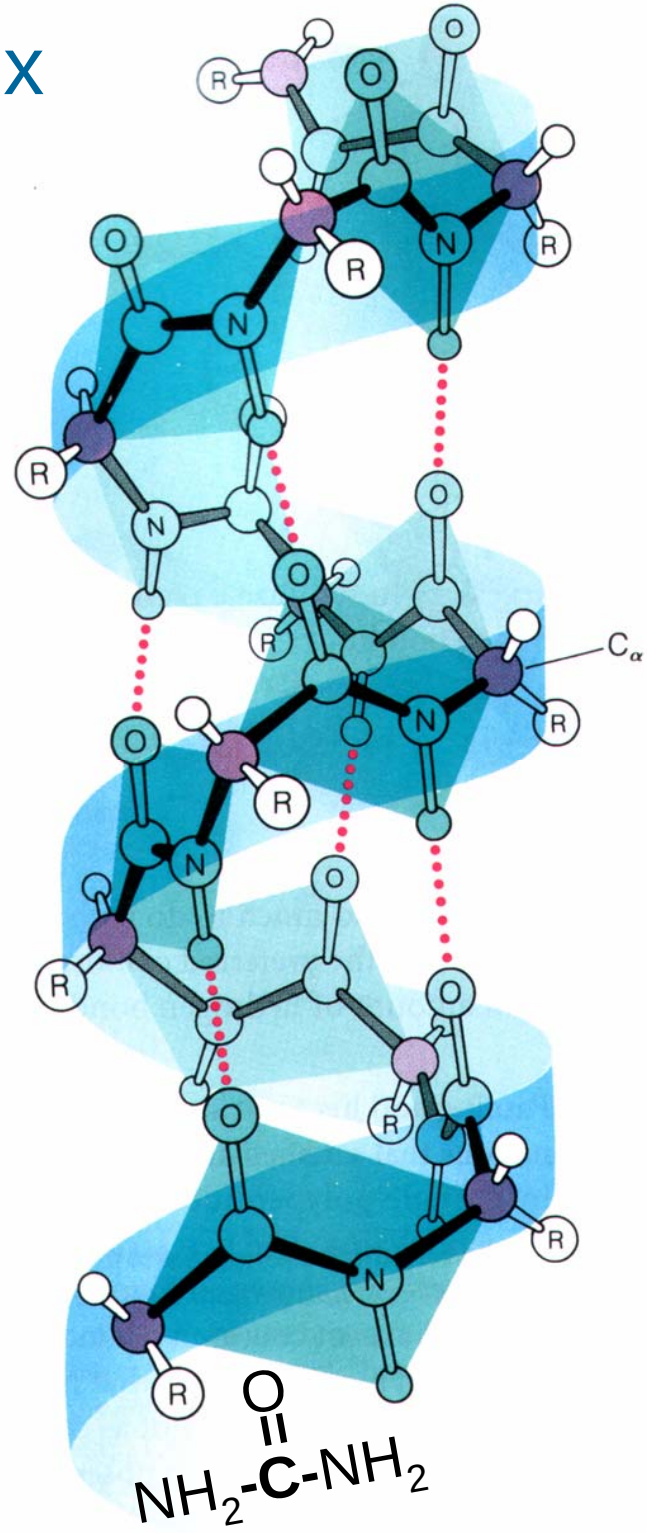
Common denaturants for proteins

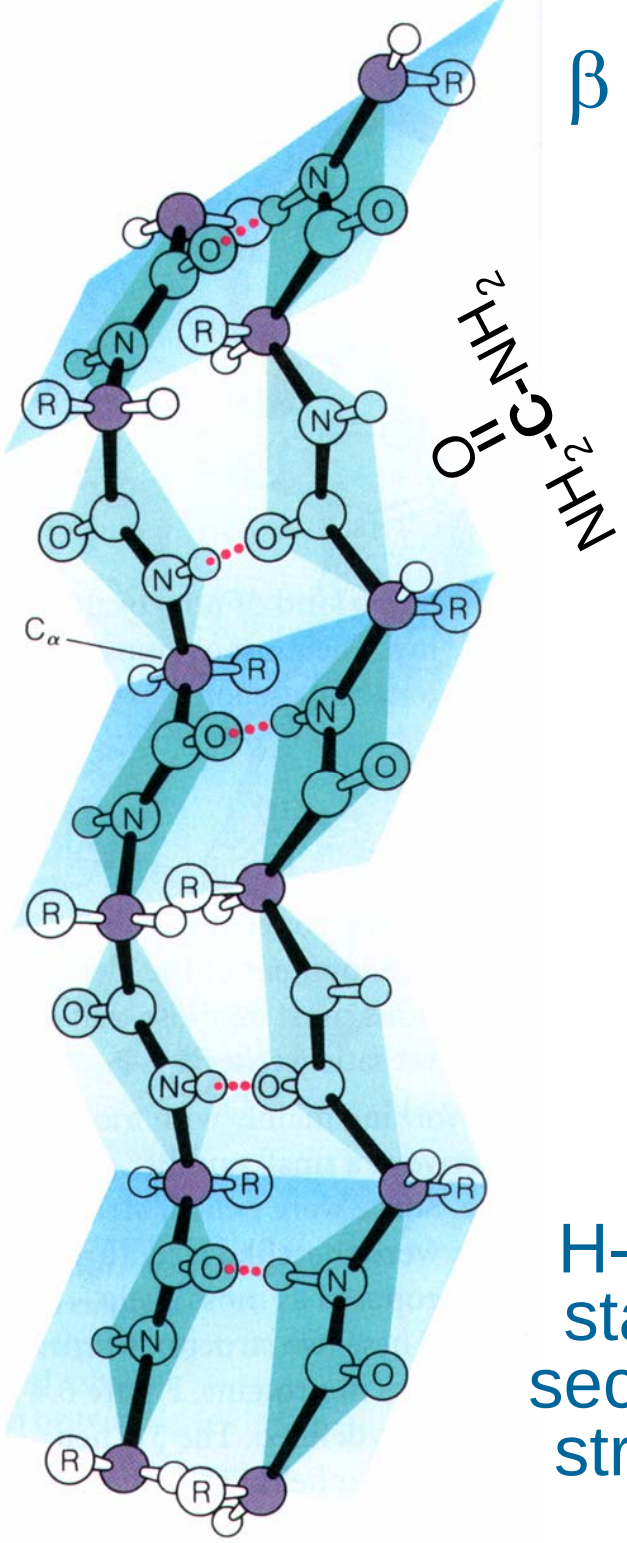
Juang RH (2005) EPA

蛋白質二級構造

α helix

β sheet

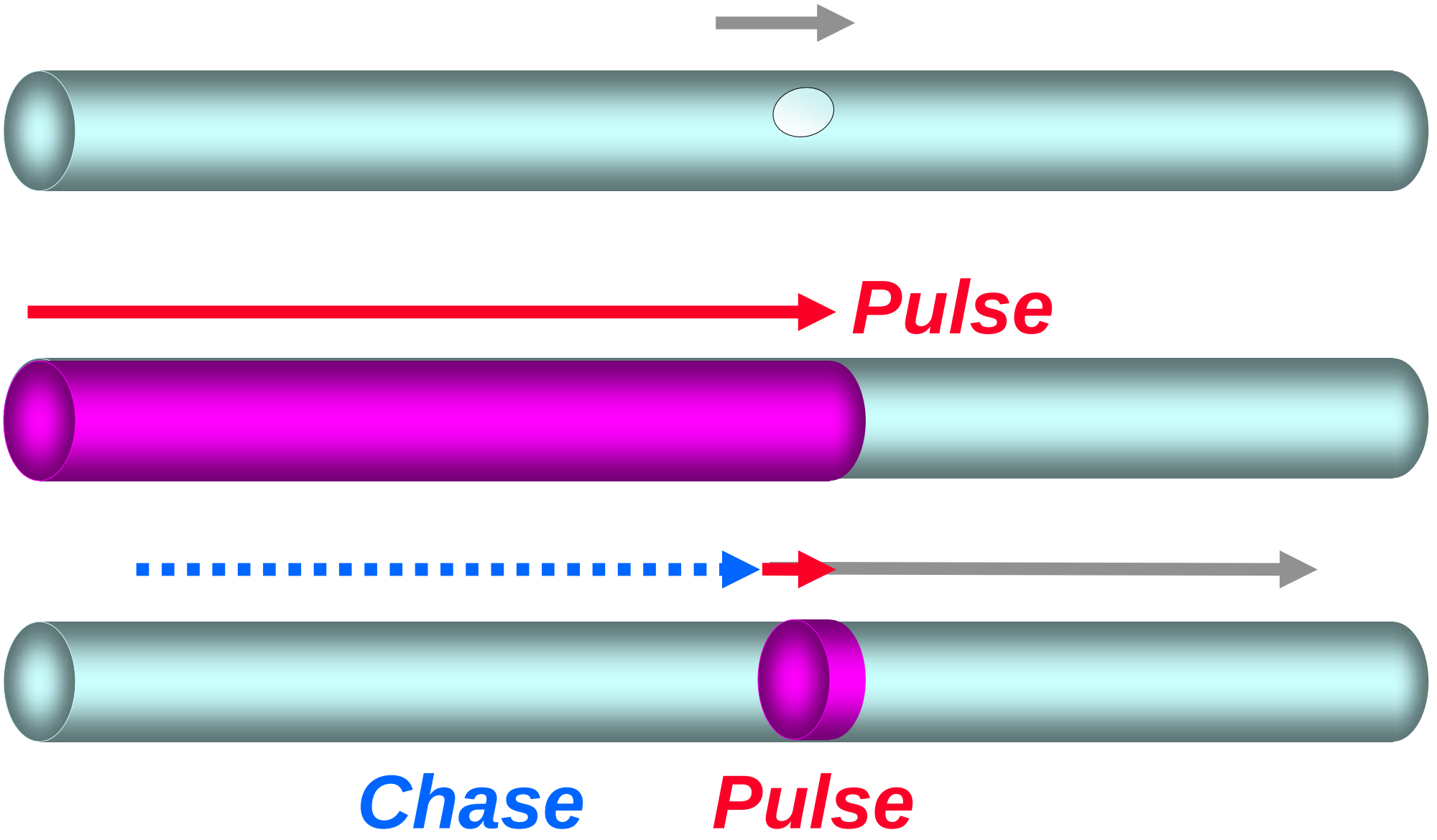




Mathews et al (2000) Biochemistry (3e) p.164

H-bonds stabilize secondary structure

Pulse-chase

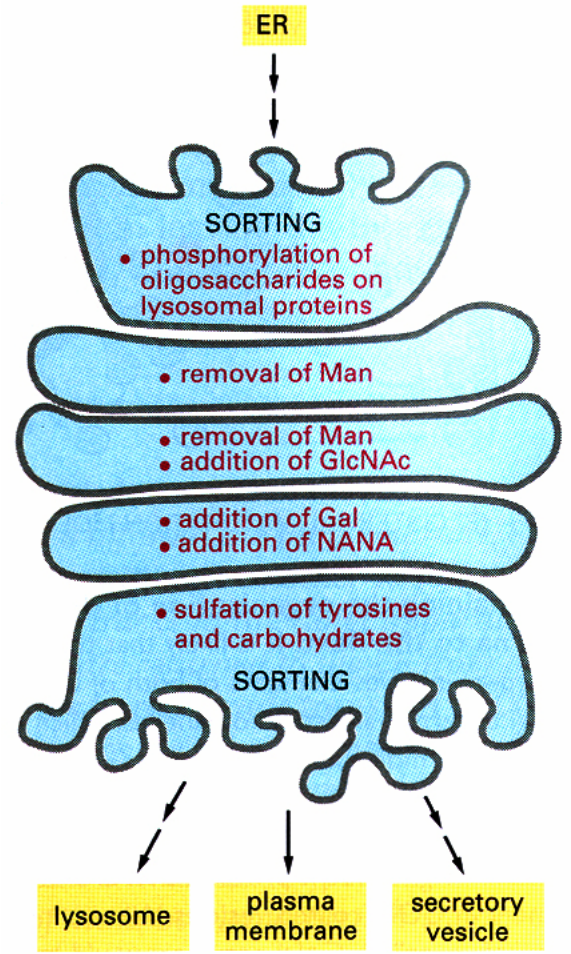


To measure the flow speed of water in a glass tube

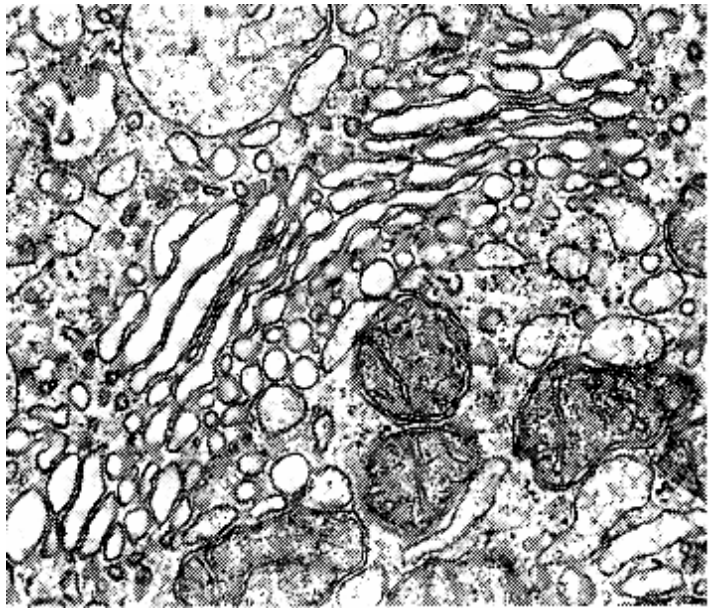
Juang RH (2005) EPA

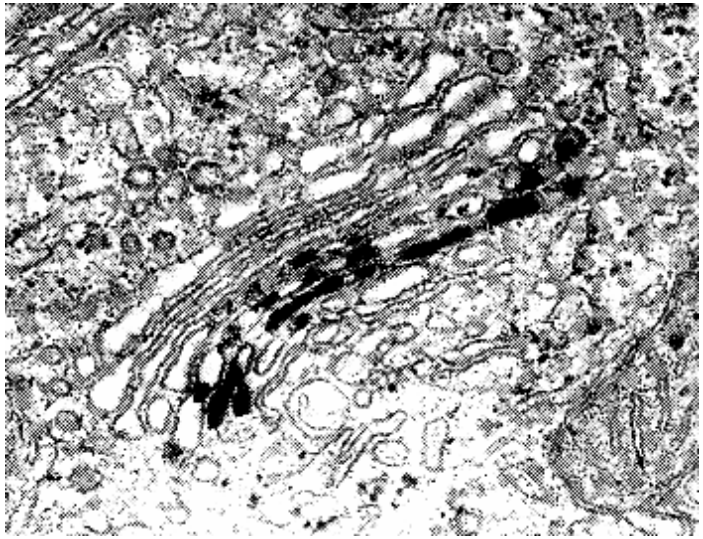
高爾基氏體的蛋白質運送 Golgi transportation

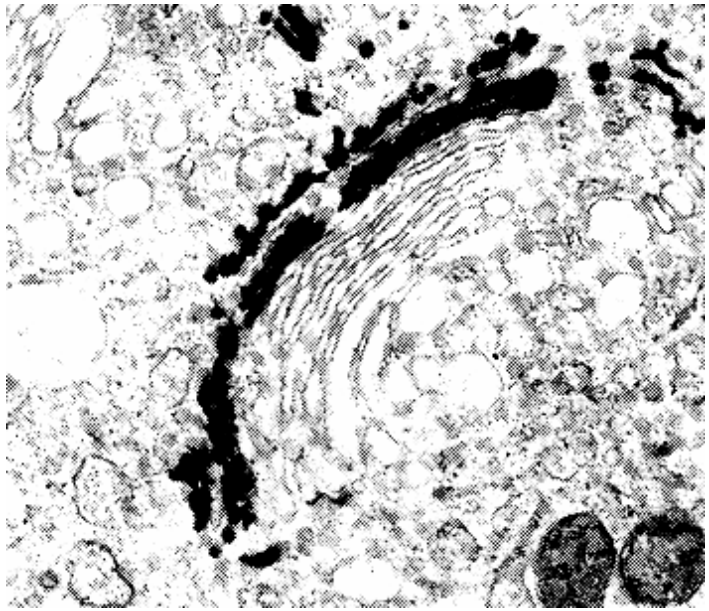
Inner side

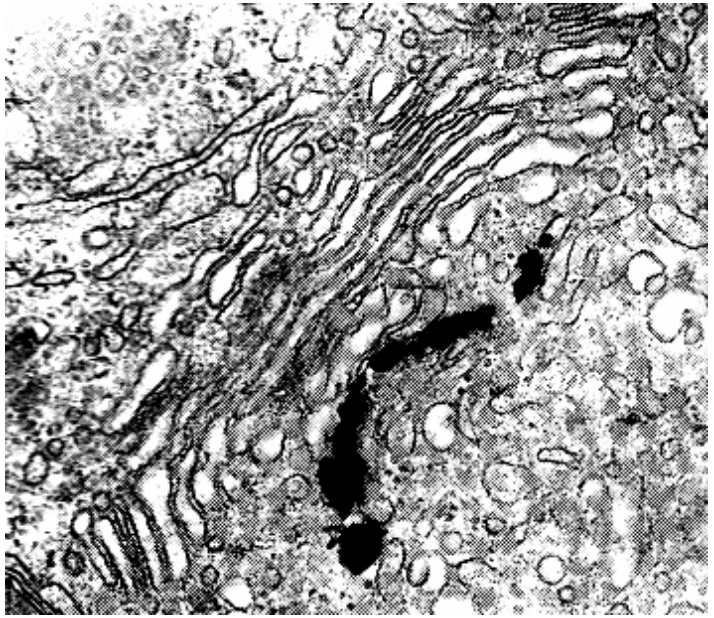


Outer side









Alberts et al (2002) Molecular Biology of the Cell (4e) p.736-737

2.3 維持酵素活性 Maintain enzyme activity

- 2.3.1 緩衝液 Buffer

  可維持穩定的酸鹼度及離子濃度以保酵素活性

- 2.3.2 試劑的保存 Reagents

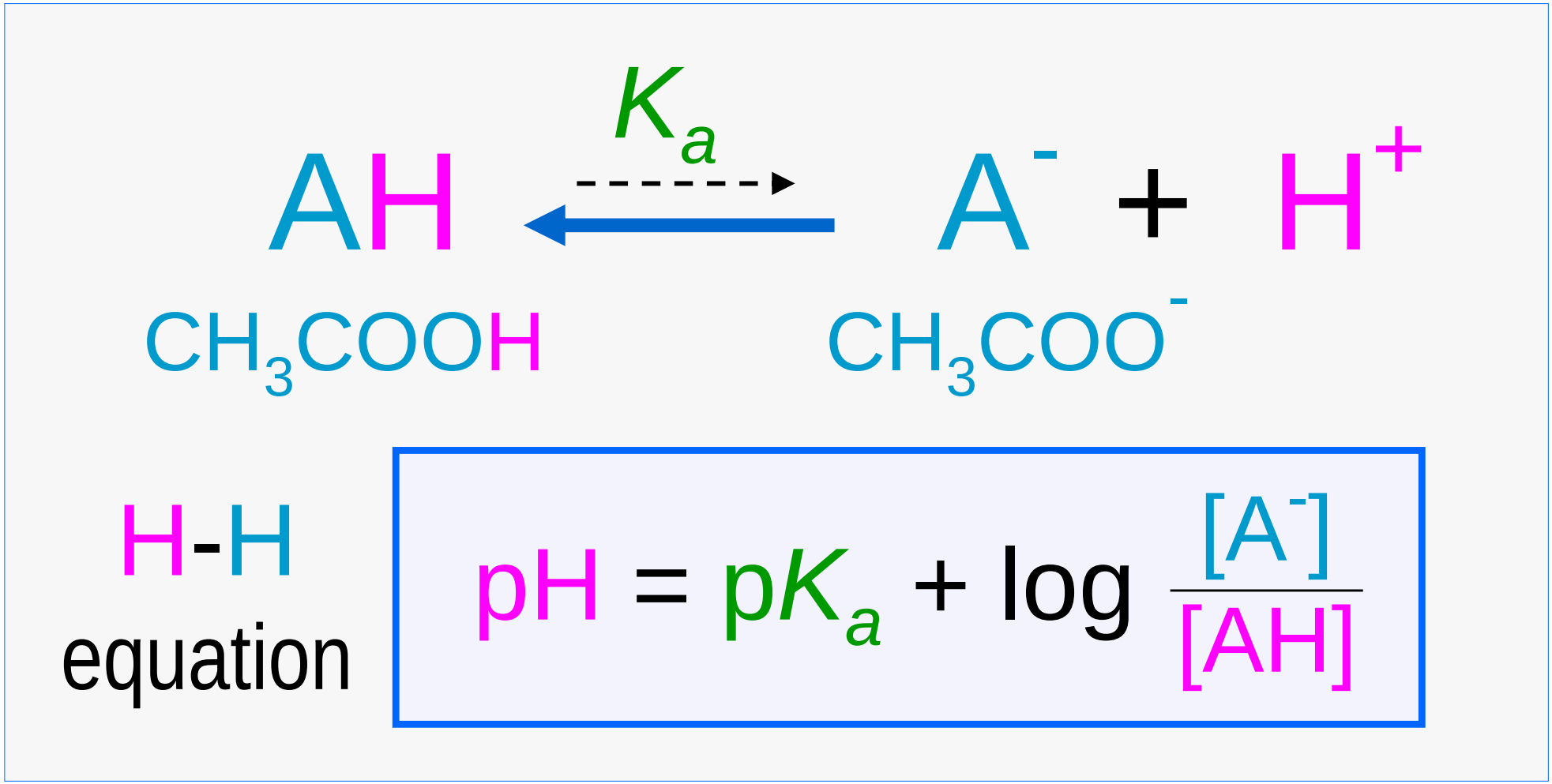
  試劑要依指示保存在適當的地方

- 2.3.3 酵素活性之維持 Maintain the activity

  注意酵素失活的原因有助保持其最高活性

- 2.3.4 酵素活性單位 Enzyme activity unit

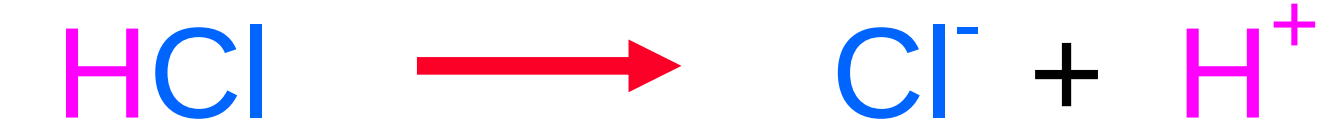
弱酸鹼可作為緩衝分子 Buffer is weak acid/base



H-H equation

$$pH = pK_a + \log \frac{[A^-]}{[AH]}$$

Strong acid



Juang RH (2005) EPA

如何推導公式

$$K_a = \frac{[A^-]\,[H^+]}{[AH]} \qquad \frac{\text{dissociated}}{\text{associated}}$$

(1) K_a 的定義

$$\log K_a = \log [H^+] \frac{[A^-]}{[AH]}$$

(2) 兩邊取 log

$$\log K_a = \log [H^+] + \log \frac{[A^-]}{[AH]}$$

分解右邊 log

$$-\log [H^+] = -\log K_a + \log \frac{[A^-]}{[AH]}$$

(3) 移項

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{[A^-]}{[AH]}$$

(4) 定義 -log 為 p

Henderson-Hasselbalch equation

How the pK_a of a buffer contribute to its buffering effect

How to derive H-H equation from K_a

Juang RH (2005) EPA

弱酸如何作為緩衝液分子

$AH \rightleftharpoons A^- + H^+$

example Acetic acid

$CH_3COOH = CH_3COO^- + H^+$

◎ K_a is the dissociation constant

$$K_a = \frac{[A^-][H^+]}{[AH]} = \frac{1}{10^5}$$

pK_a of acetic acid ($pK_a = 5$)

◎ K_a is derived to get H-H equation

一、兩邊取 log

二、移項取出 $[H^+]$

三、定義 p 為 -log (pH = -log$[H^+]$)

H-H equation

$$pH = pK_a + \log \frac{[A^-]}{[AH]}$$

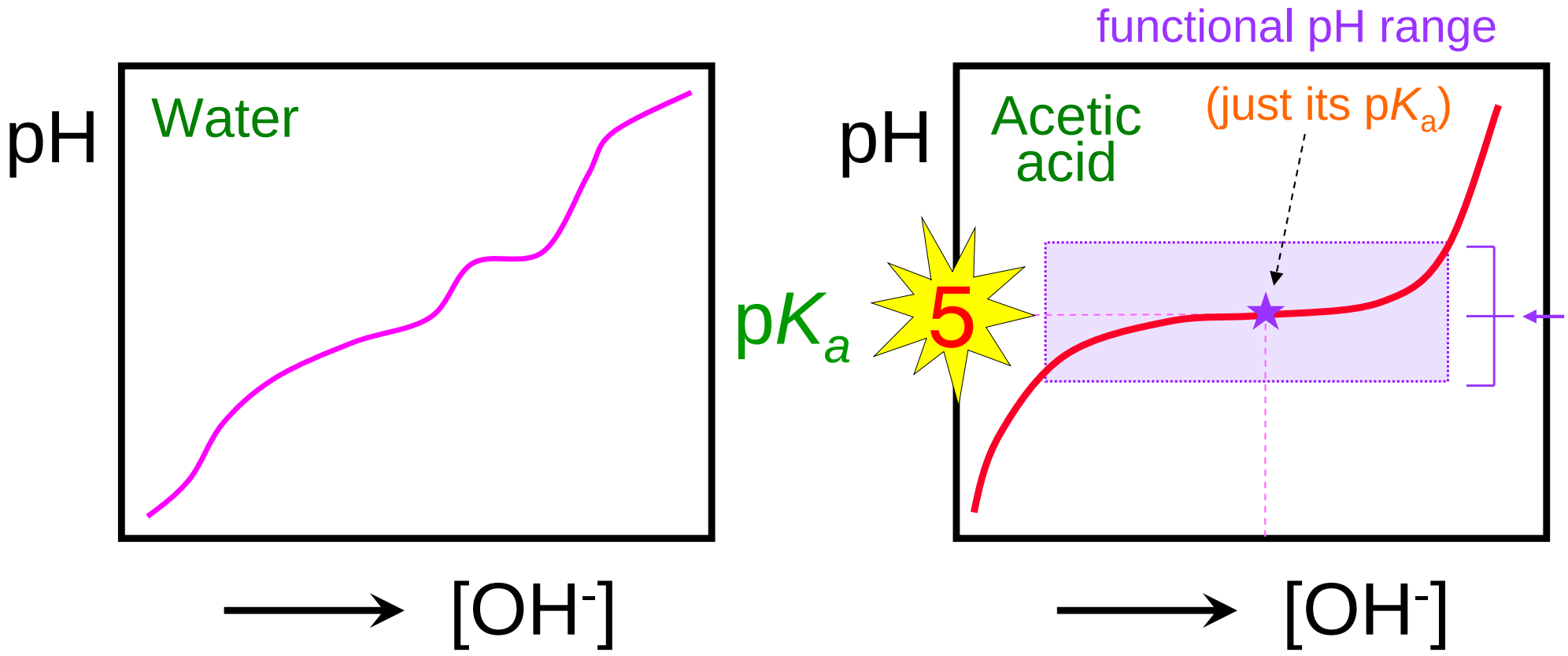
5 5

dissociated / associated

pH = constant pK_a ? ….. when $[A^-] = [AH]$, log 1 = 0

Why weak acid could serve as a buffer ?

弱酸在其 pK_a 上下有緩衝作用



H-H equation

$$pH = pK_a + \log \frac{[A^-]}{[AH]}$$

6 5

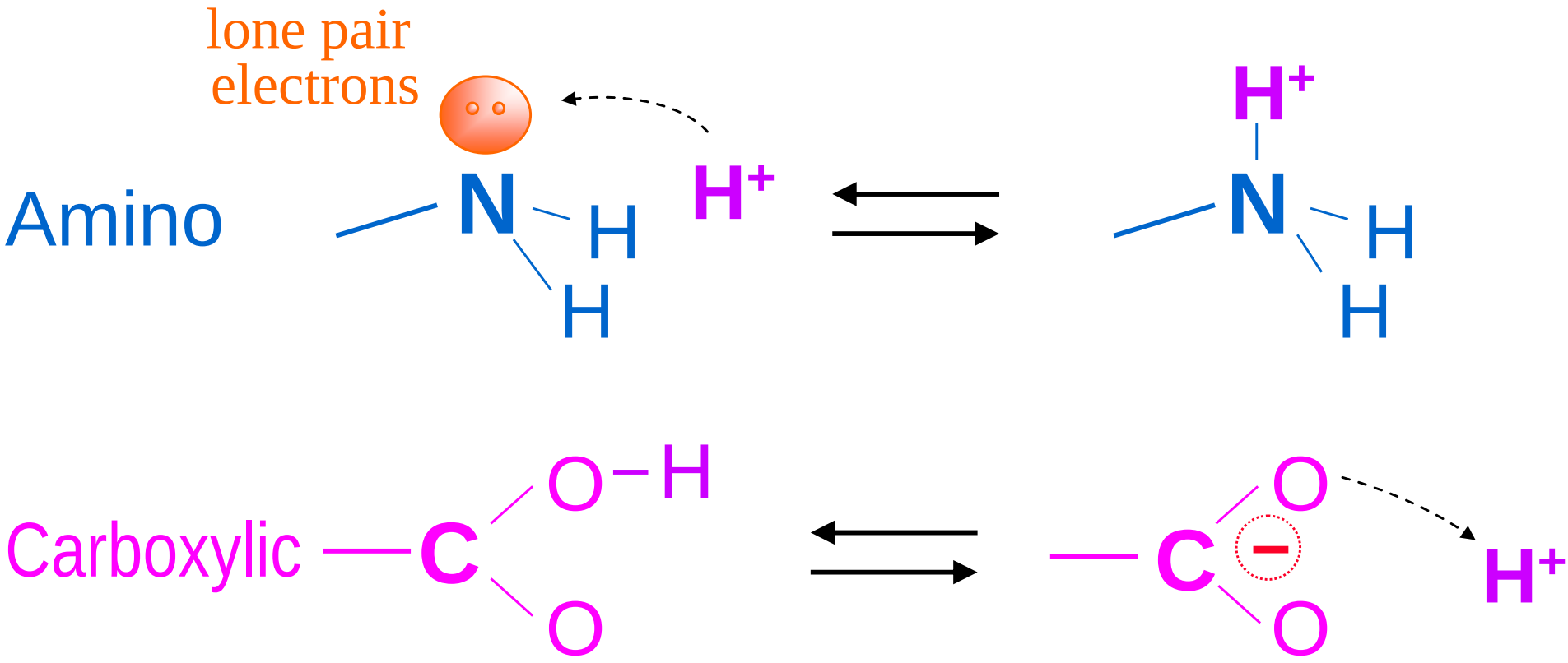
dissociated / associated

Acetic acid has highest buffer effect at its pK_a (pH 5)

Juang RH (2005) EPA

質子可以吸著或脫離一基團

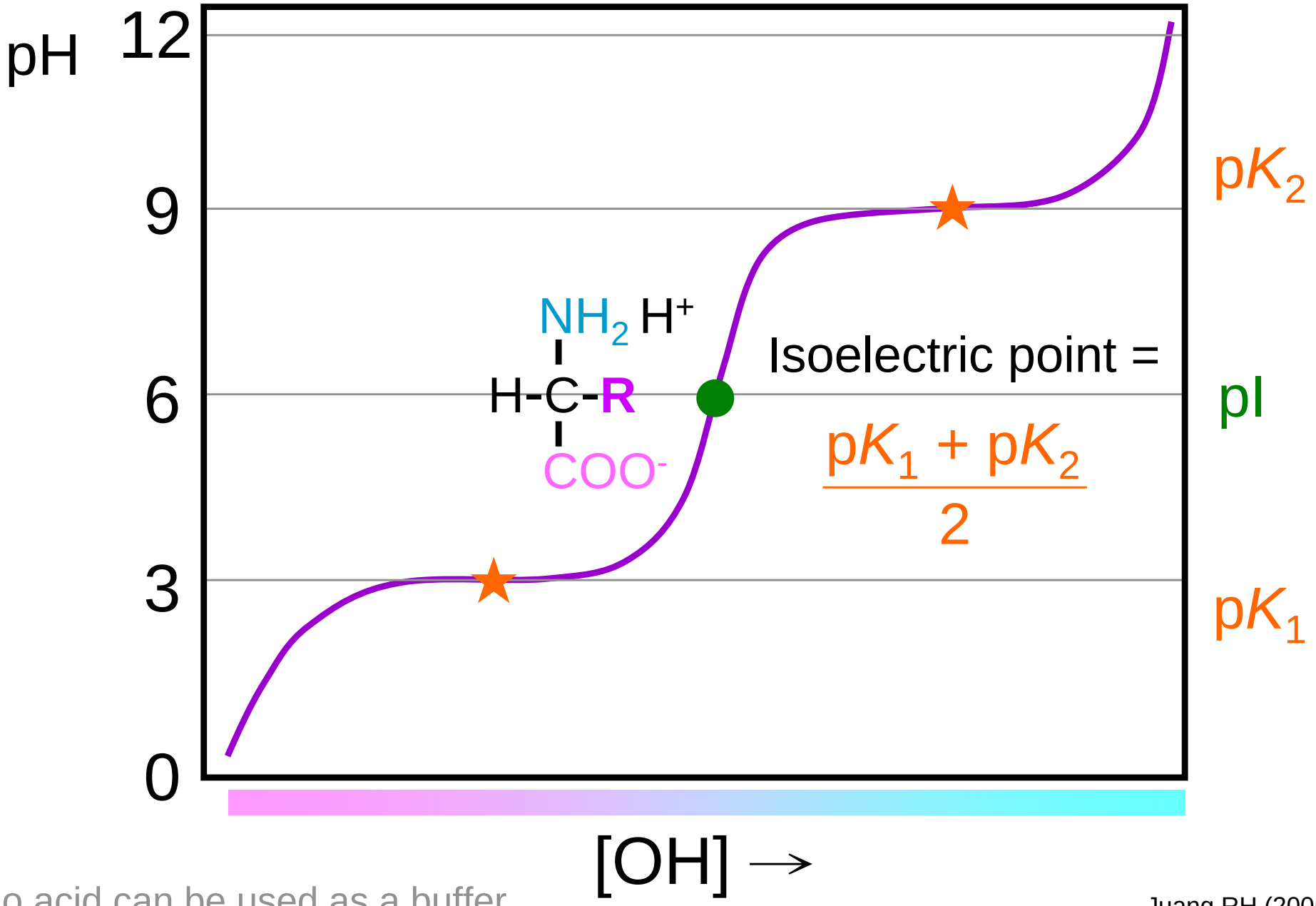
Proton： The smallest and most abundant particle in the living cell controlling the pH and the charge property of a molecule



Ampholyte: A molecule contains both positively and negatively charged groups

Proton can enter or leave a functional group relatively freely

胺基酸的緩衝作用範圍 Amino acid as a buffer



Amino acid can be used as a buffer

Juang RH (2005) EPA

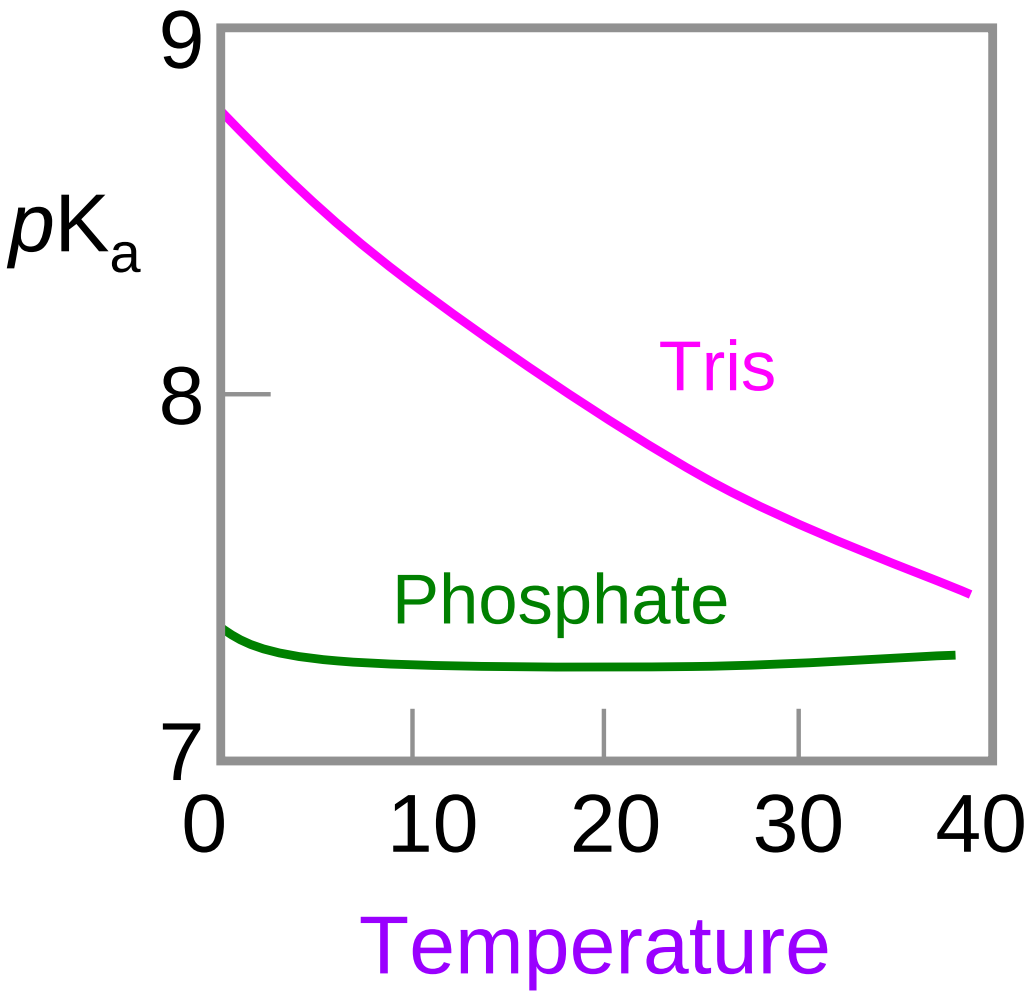
各種常用緩衝液及其使用範圍 Common buffers

Buffer	pH	Remarks
Formate	3.0 - 4.5	Volatile, could be removed by lyophilization
Citrate	3.0 - 6.2	Bind with divalent metal ions
Acetate	3.7 - 5.5	Volatile, could be removed by lyophilization
Phosphate	5.8 - 8.0	Precipitated with Ca; crystallized at low temperature
HEPES	6.5 - 8.5	Low toxicity, used in cell culture
Tris	7.1 - 8.9	pH effected by temperature; special pH electrode required
Borate	9.1 - 9.0	
Carbonate	9.7 - 10.7	Bind with divalent metal ions
Universal	2 - 12	Contains several buffers at various pH ranges

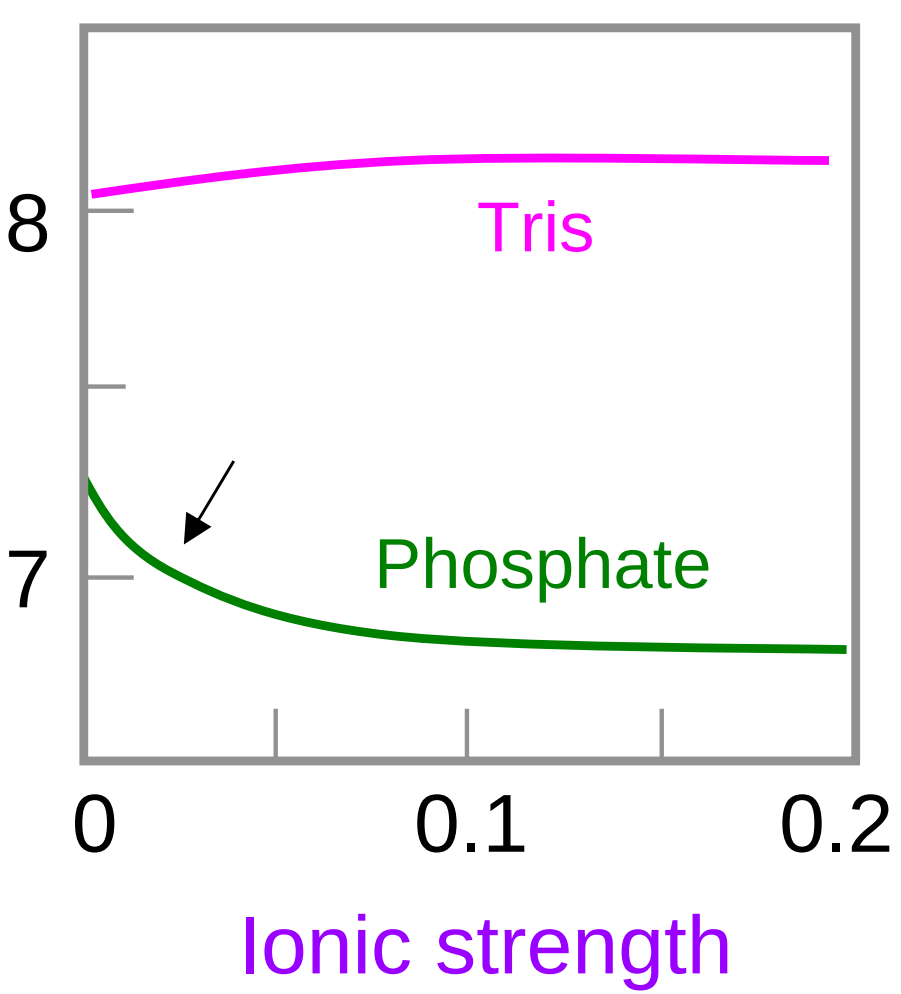
Juang RH (2005) EPA

緩衝液使用注意 Notice for two common buffers

Tris is affected by temperature



Diluted phosphate buffer raises its pH



Juang RH (2005) EPA

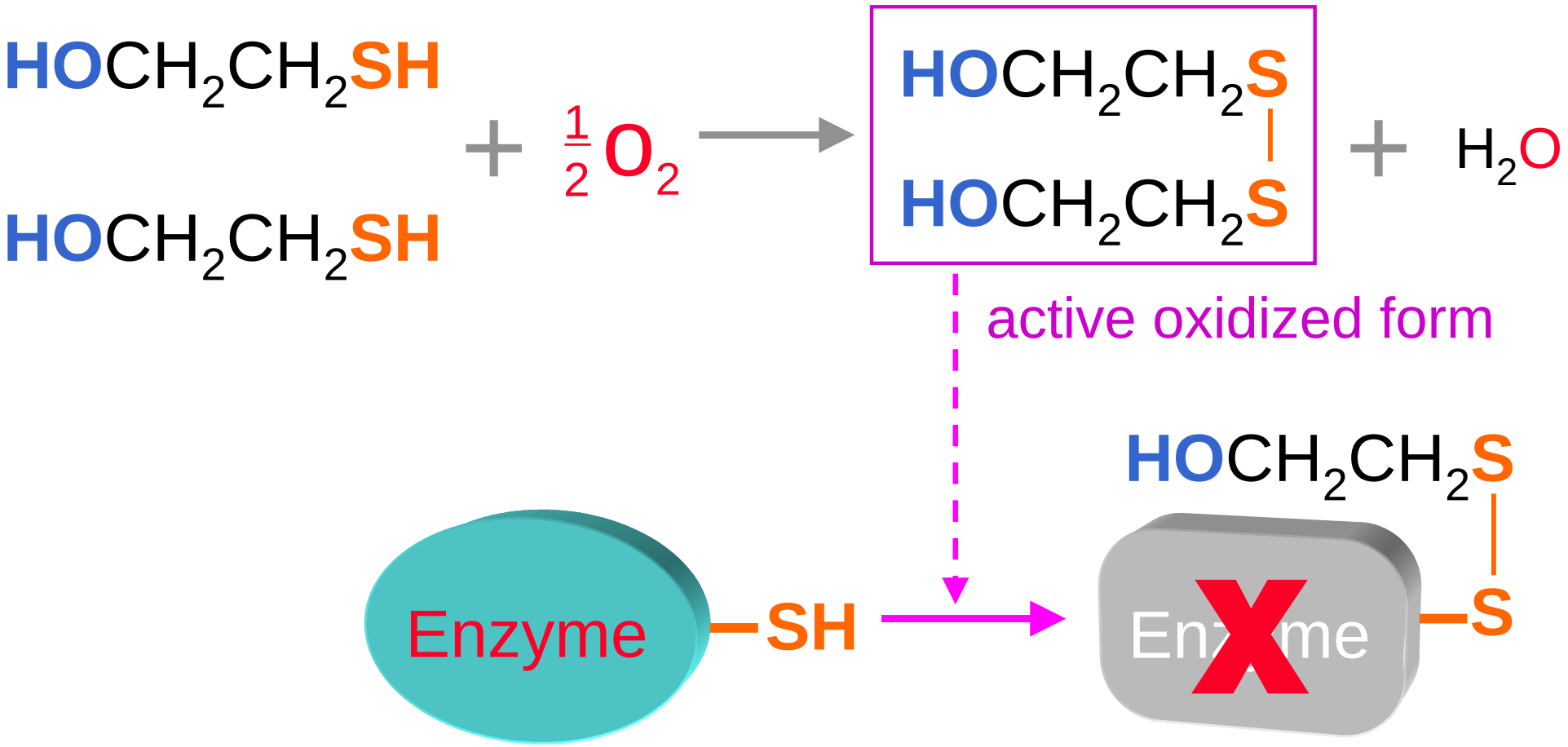
緩衝液常用的添加物 Some common additives

Additives	Action	Concentration
NaN_3 (sodium azide)	Antimicrobials	0.01%
EDTA	Remove metal ions	0.1 - 1 mM
β-Mercaptoethanol	Antioxidant	1 - 10 mM
Dithiothreitol (DTT or DTE)	Antioxidant	1 - 5 mM
BSA (bovine serum albumin)	Stabilizer	0.1 - 10 mg/mL
Tween-20, Triton X-100	Surfactant	0.5 - 0.05%
Glycerol, glucose	Antifreeze	50%
PMSF, TPCK, TLCK, benzamidine etc.	Proteinase inhibitor	Trace amount

Beta-*mercaptoethanol*

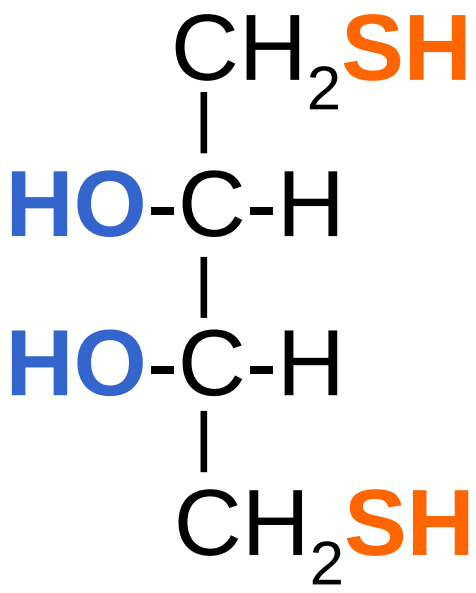
α β

HOCH_2CH_2**SH** **HO**CH_2CH_3

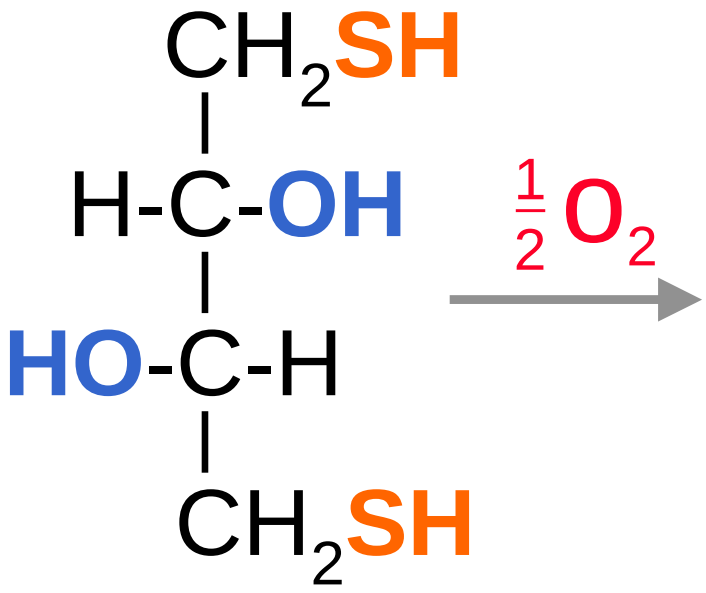


Juang RH (2005) EPA

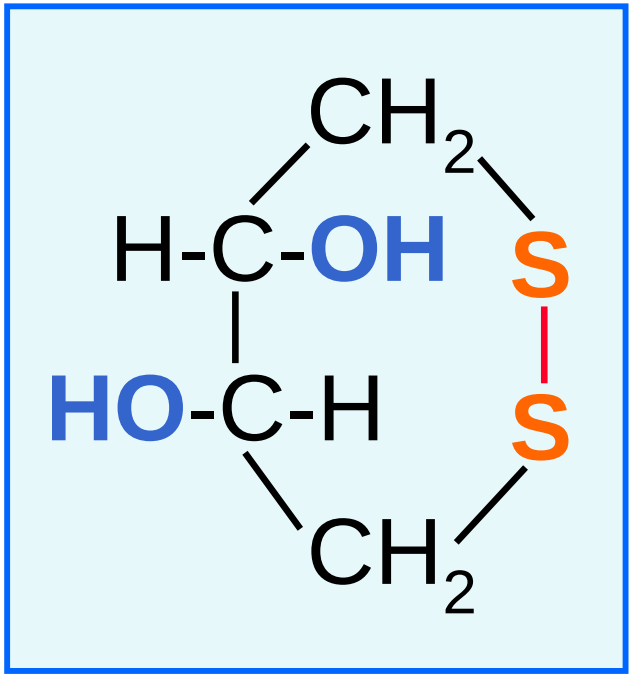
Dithiothreitol (DTT)



Dithioerythritol
(DTE)



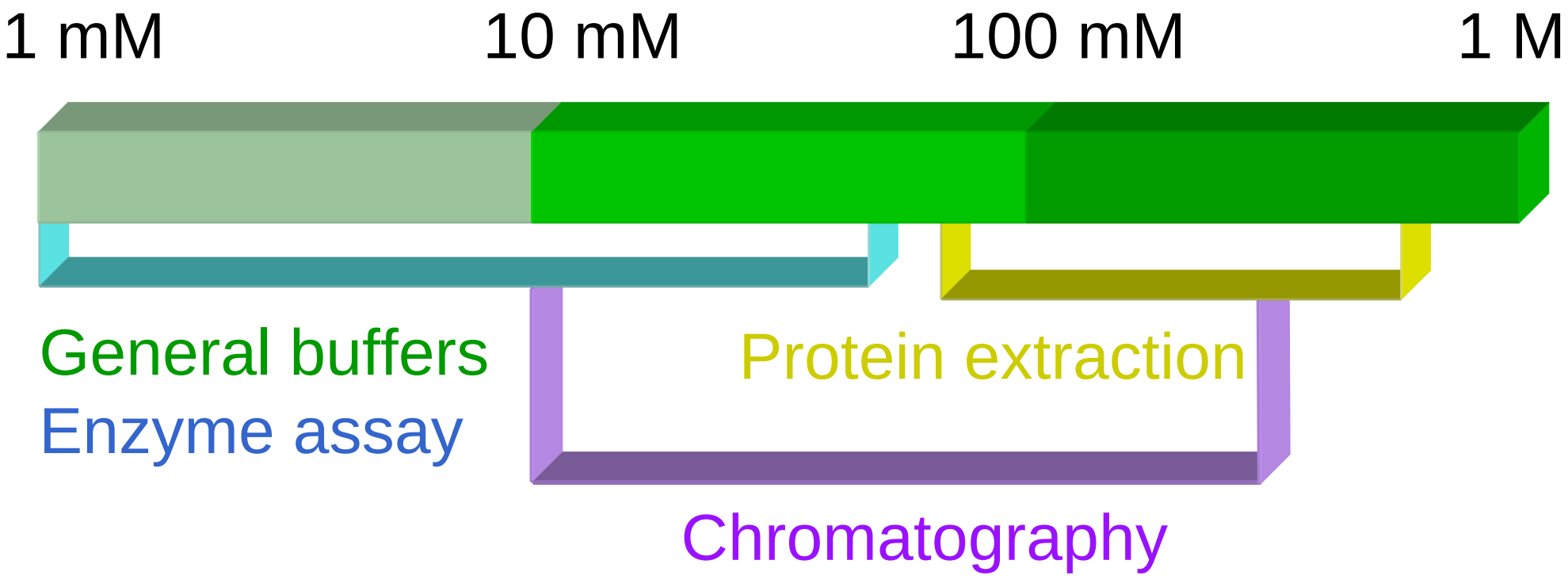
Dithiothreitol
(DTT)



Stable cyclic
oxidized form

Juang RH (2005) EPA

緩衝液使用濃度範圍 Concentration ranges

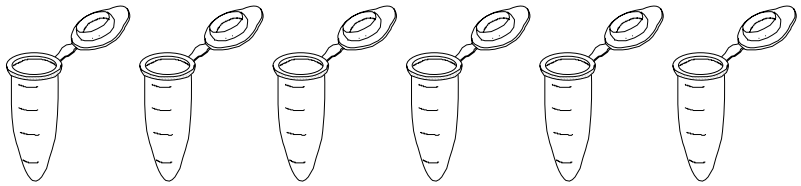


Juang RH (2004) ECX

2.3 維持酵素活性 Maintain enzyme activity

- 2.3.1 緩衝液 Buffer
  可維持穩定的酸鹼度及離子濃度以保酵素活性
- 2.3.2 試劑的保存 Reagents
  試劑要依指示保存在適當的地方
- 2.3.3 酵素活性之維持 Maintain the activity
  注意酵素失活的原因有助保持其最高活性
- 2.3.4 酵素活性單位 Enzyme activity unit

試劑的保存注意事項



a. Avoid humidity

Open the bottle of a frozen reagent only when its temperature has been brought back to the room temperature

b. Stored in frozen state

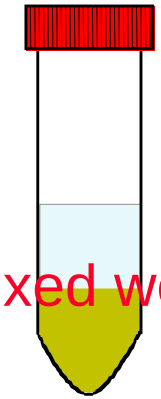
(1) Frequently used reagents should be frozen in aliquot

(2) Avoid repeatedly freezing-thawing

(3) Certain enzymes are very sensitive to freezing L-SP

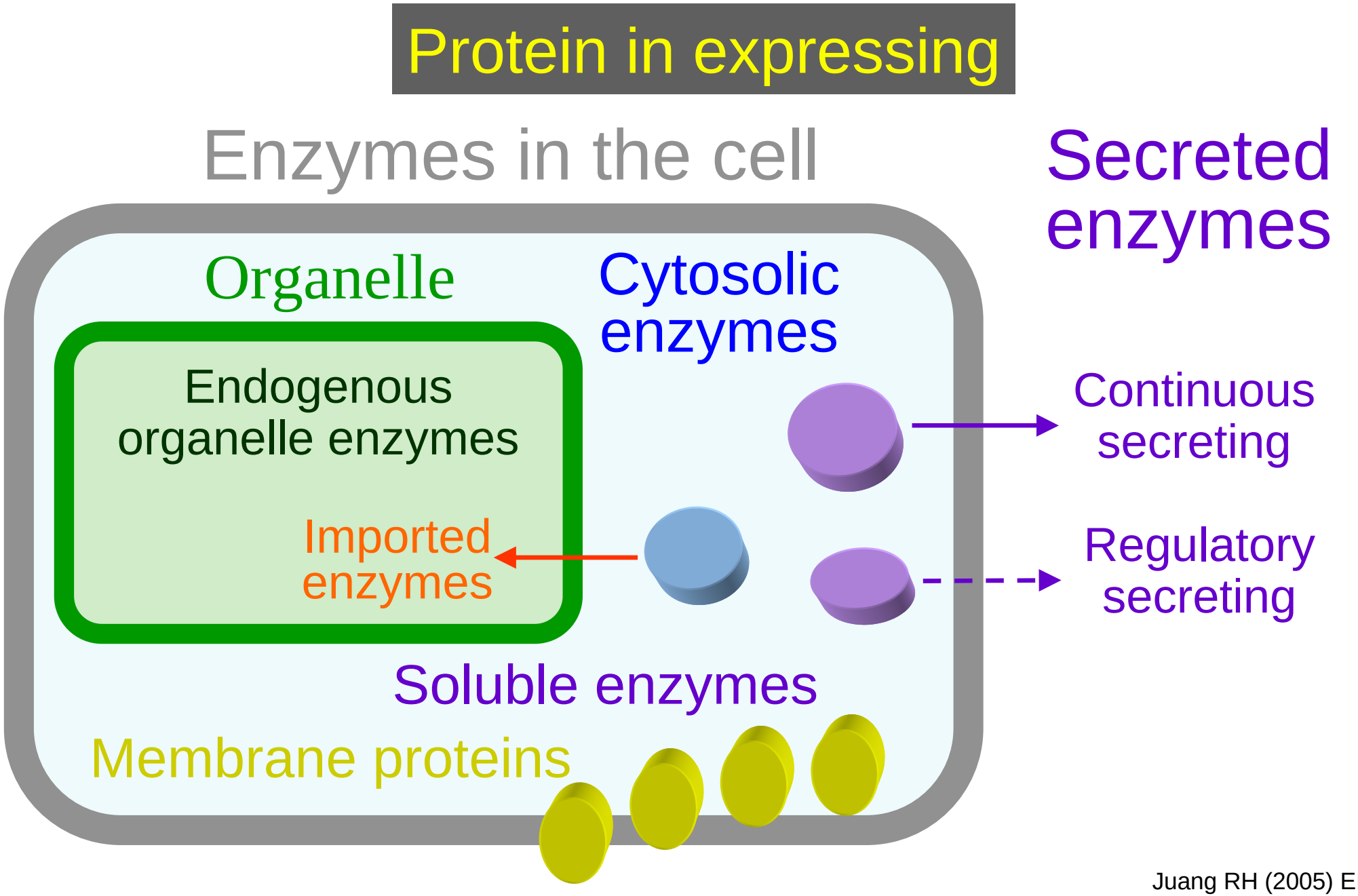
c. Frozen in glycerol

Protein stored in -20℃ in 50% glycerol



d. Avoid light and microbe

細胞內外酵素分佈 Cellular distribution of enzymes



Juang RH (2005) EPA

各種酵素的安定性都不同 Enzyme stability

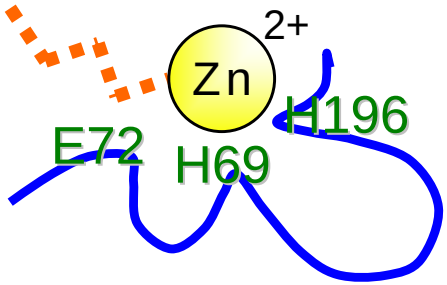
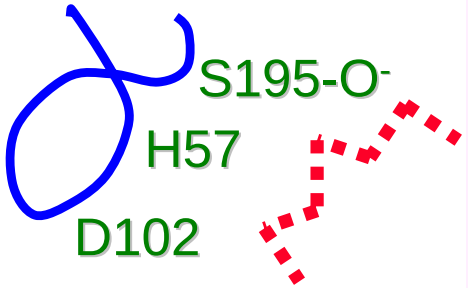
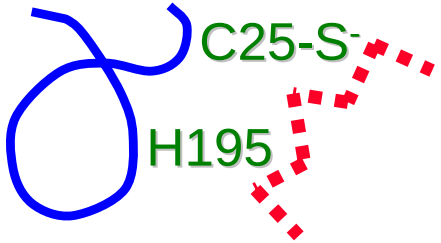
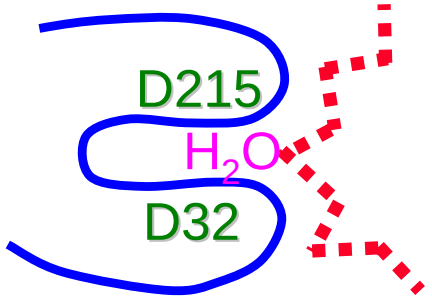
Reasons enzyme lost its activity:

Protein *Denatured*	Physical/chemical denaturation
Active site destroyed	Chemical reaction
Protease proteolysis	Inactivated by degradation
Enzyme *Inhibitor*	Natural or synthetic inhibitors

Physical denaturation	Heating, freezing, foaming, adsorbing
Chemical denaturation	Extreme pH, oxidation, heavy metals

Juang RH (2005) EPA

蛋白胨的專一性及其抑制劑 Protease families

Family	Example	Mechanism	Specificity	Inhibitor
Metal Protease	Carboxy-peptidase A		Non-polar	EDTA EGTA
Serine Protease	Chymotrypsin Trypsin		Aromatic Basic	DFP TLCK TPCK
Cysteine Protease	Papain		Non-specific	PCMB Leupeptin
Aspartyl Protease	Pepsin Renin		Non-specific	Pepstatin

Juang RH (2005) EPA

3 電泳檢定法 Electrophoresis

- 3.1 電泳原理 Basic principles

  帶有電荷的分子都可在電場中泳動

- 3.2 聚丙烯醯胺膠体電泳 PAGE

  是蛋白質最常用的電泳介質

- 3.3 其它相關技術 Related techniques

  另有一些與電泳相關的檢定技術

3.1 電泳原理 Basic principles for electrophoresis

- 3.1.1 蛋白質的泳動率 Protein mobility

  泳動率與蛋白質所帶的電荷及大小有關

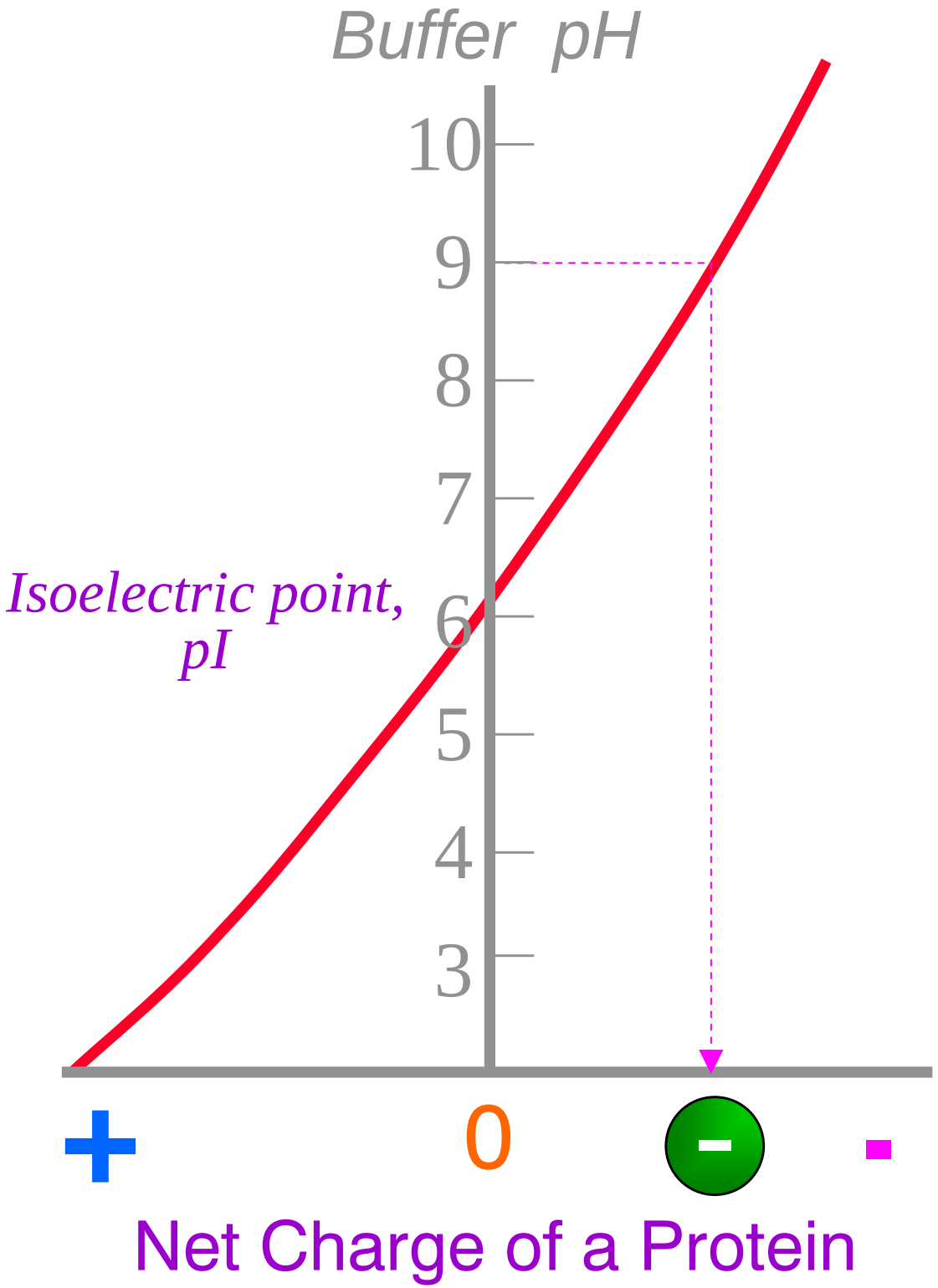
- 3.1.2 電泳的種類 Types of electrophoresis

  電泳的種類與方式很多，但原理是不變的

- 3.1.3 電泳設備及系統選擇 Available systems

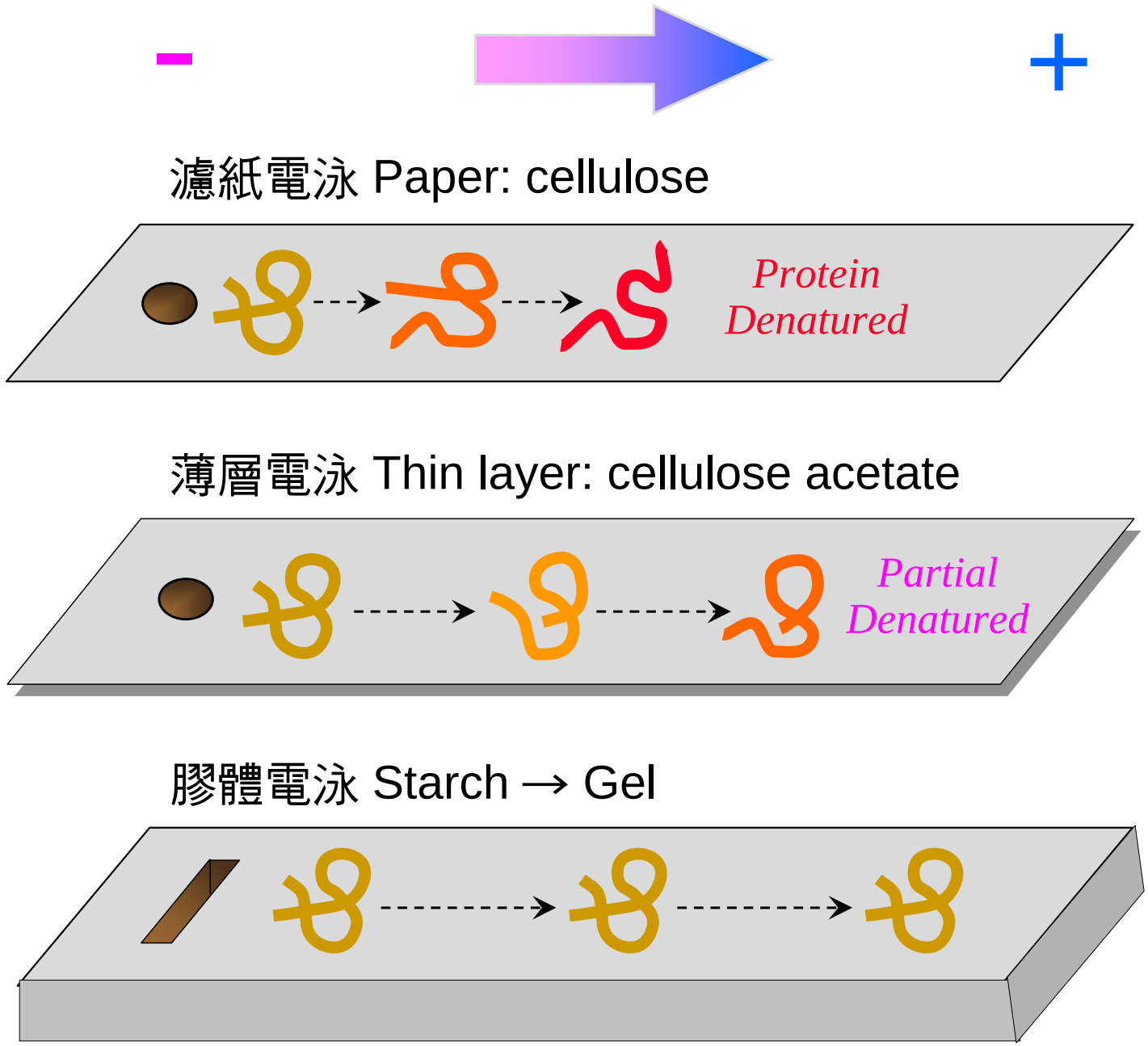
  要選擇正確的系統及適用的設備

環境影響分子的帶電性質



Juang RH (2005) EPA

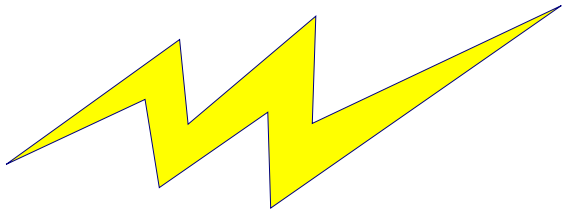
電泳形式的演進



Historical evolution of electrophoresis design

Juang RH (2005) EPA

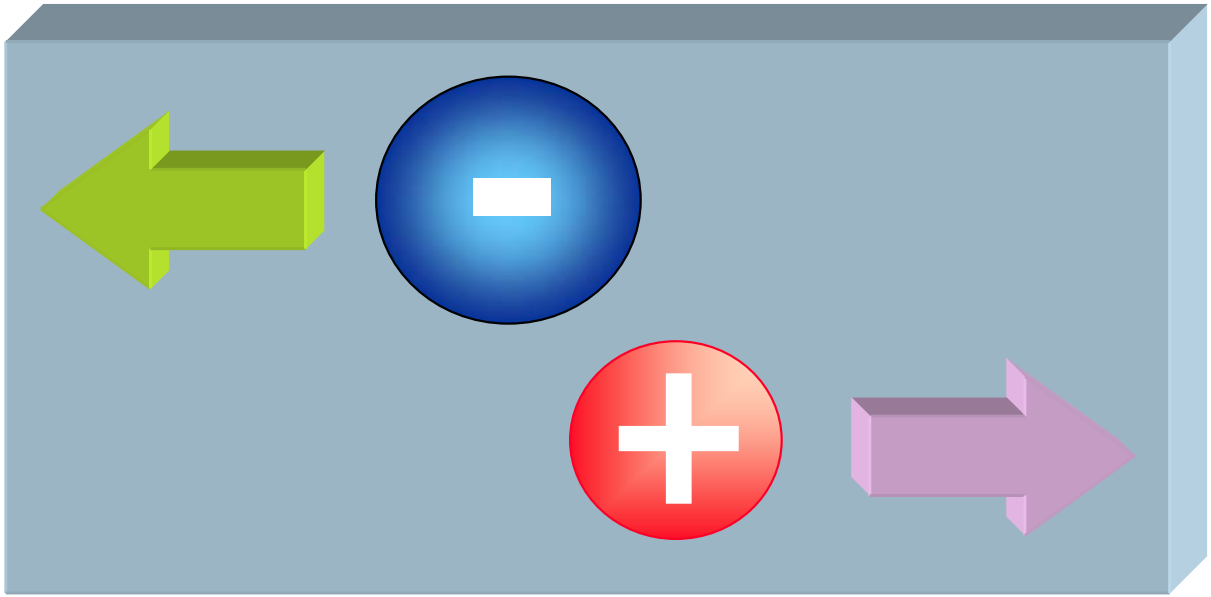
影響泳動率的因素 Factors affecting mobility



外加電流電壓　　Input *current, voltage*

ANODE

+



CATHODE

-

Friction　　***Charge***

分子量 分子形狀
Molecular weight, shape

分子的等電點
Isoelectric point

Juang RH (2005) EPA

3.2 聚丙烯醯胺膠體電泳 PAGE

- 3.2.1 PAGE 種類 PAGE categories

  依蛋白質樣本是否變性可分成兩大類

- 3.2.2 PAGE 膠体組成 Gel composition

  是單体小分子的聚合反應

- 3.2.3 PAGE 系統解剖 System anatomy

  說明膠体的構成及電泳的分離機制

- 3.2.4 結果不佳時 Trouble shooting

  舉出電泳操作時可能出現的問題

膠體的聚合反應 Gel polymerization

Ammonium persulfate (free radical initiator)

$(O_4S\text{-}SO_4)^{2-} \longrightarrow 2\ SO_4^-$

Free radical producer

Acrylamide (monomer)

Basic unit of the gel polymer

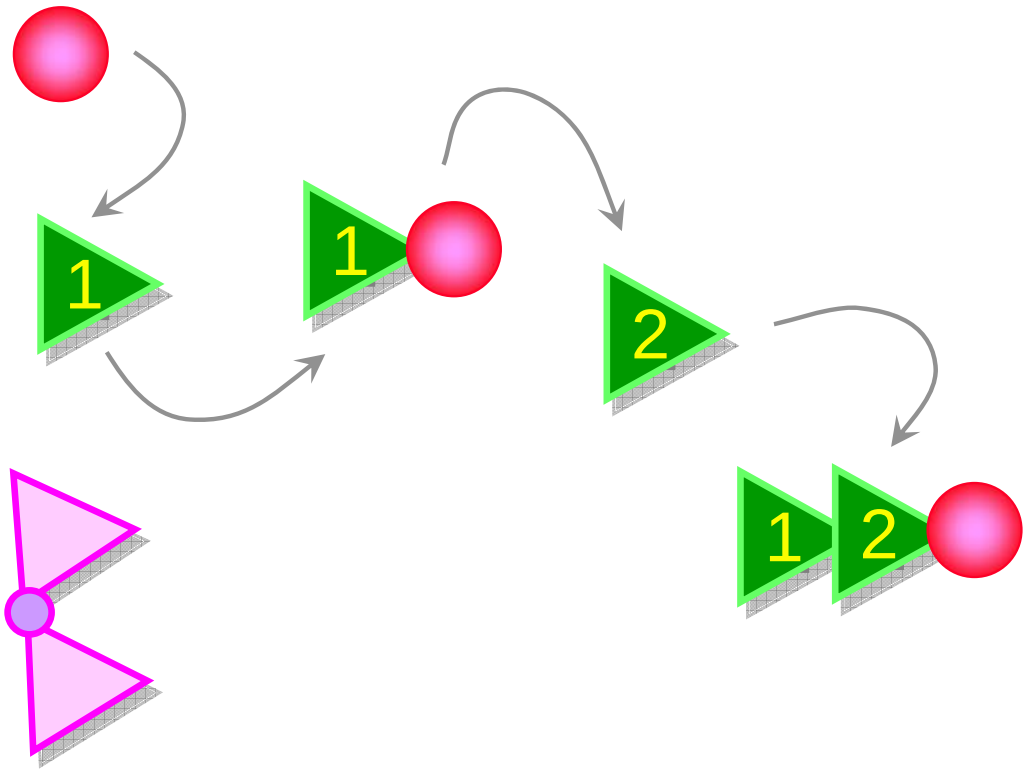
Bis(acrylamide) (bridge)

Cross-linking the gel

TEMED (catalyst)

Help the transfer of radical

SDS (Sodium dodecyl sulfate)



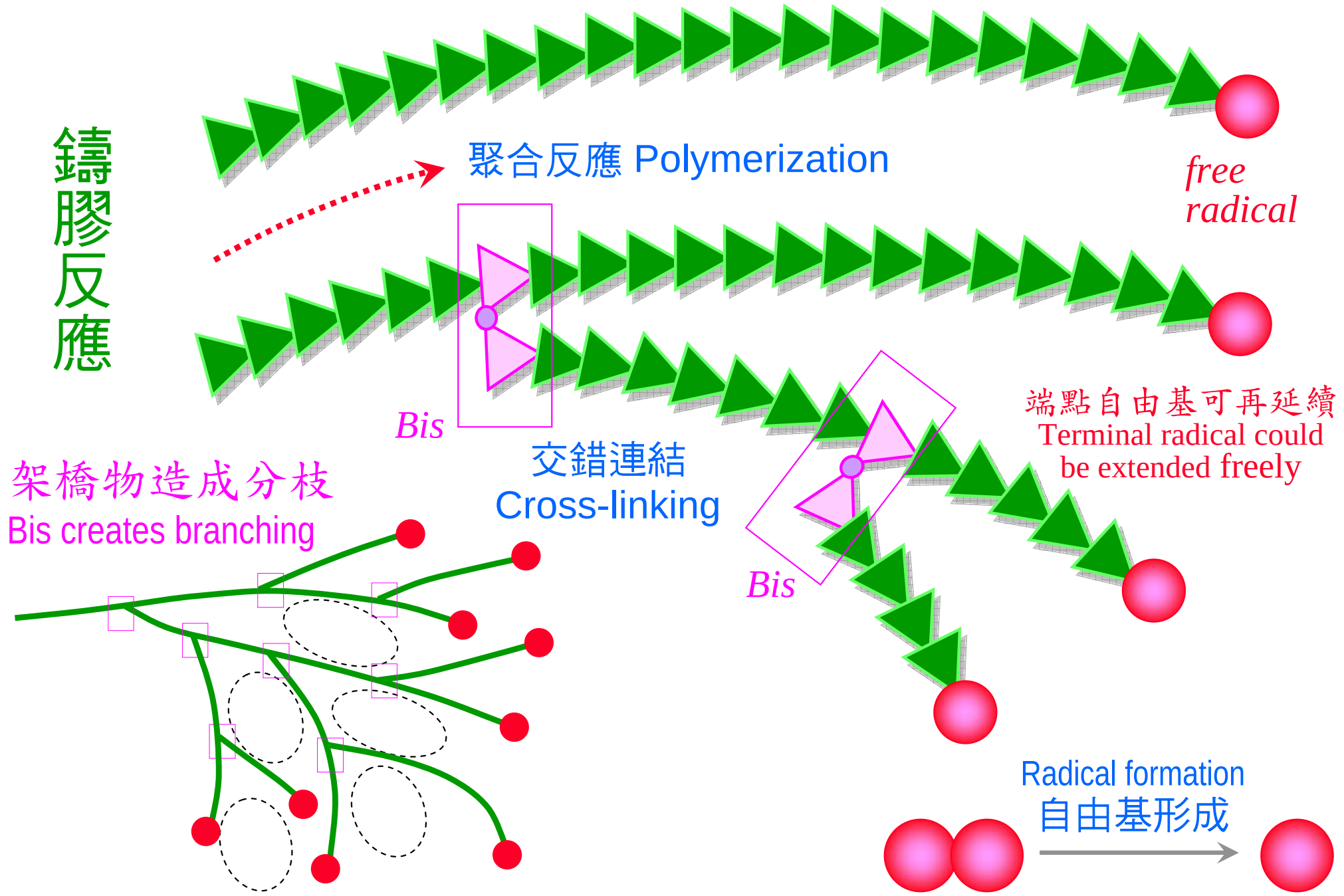
$CH_2=CH\text{-}CO\text{-}NH_2$

Acrylamide is toxic

Heating starch might produce it

Juang RH (2005) EPA

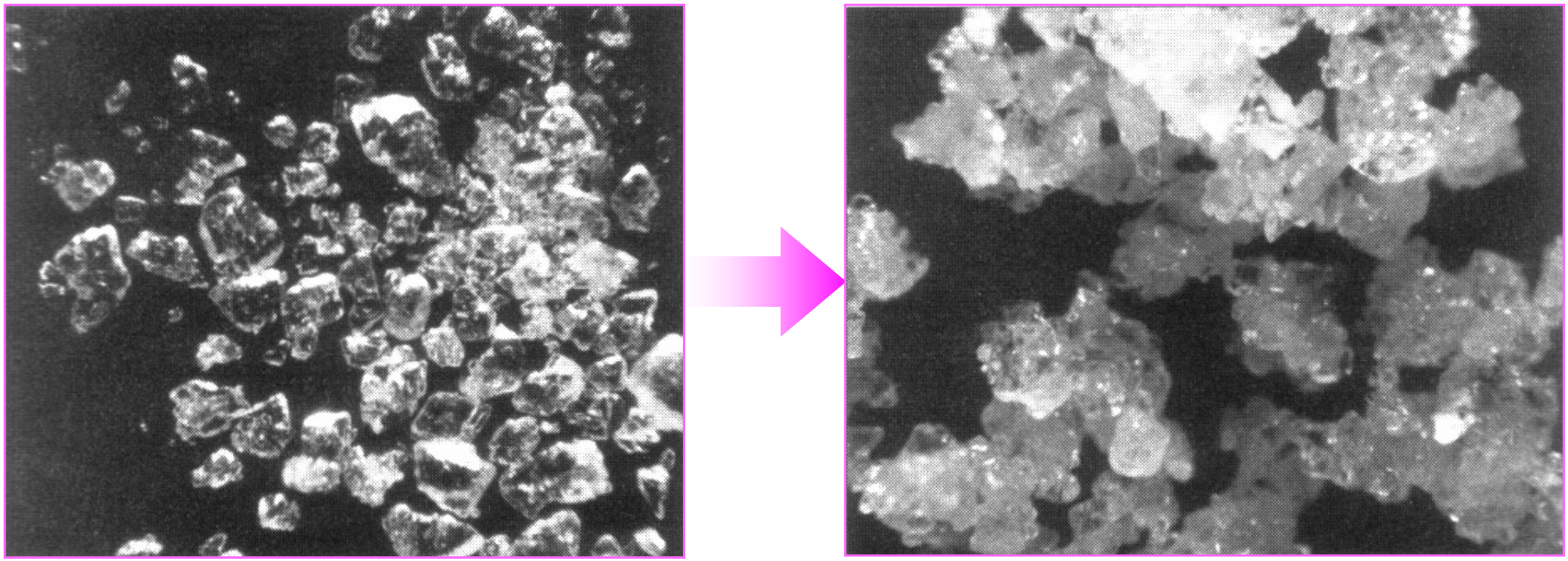
單體聚合反應 Polymerization from the monomer



Sample protein migrates in the space created by polymerization

Juang RH (2005) EPA

凝膠反應注意 A problem in gel polymerization

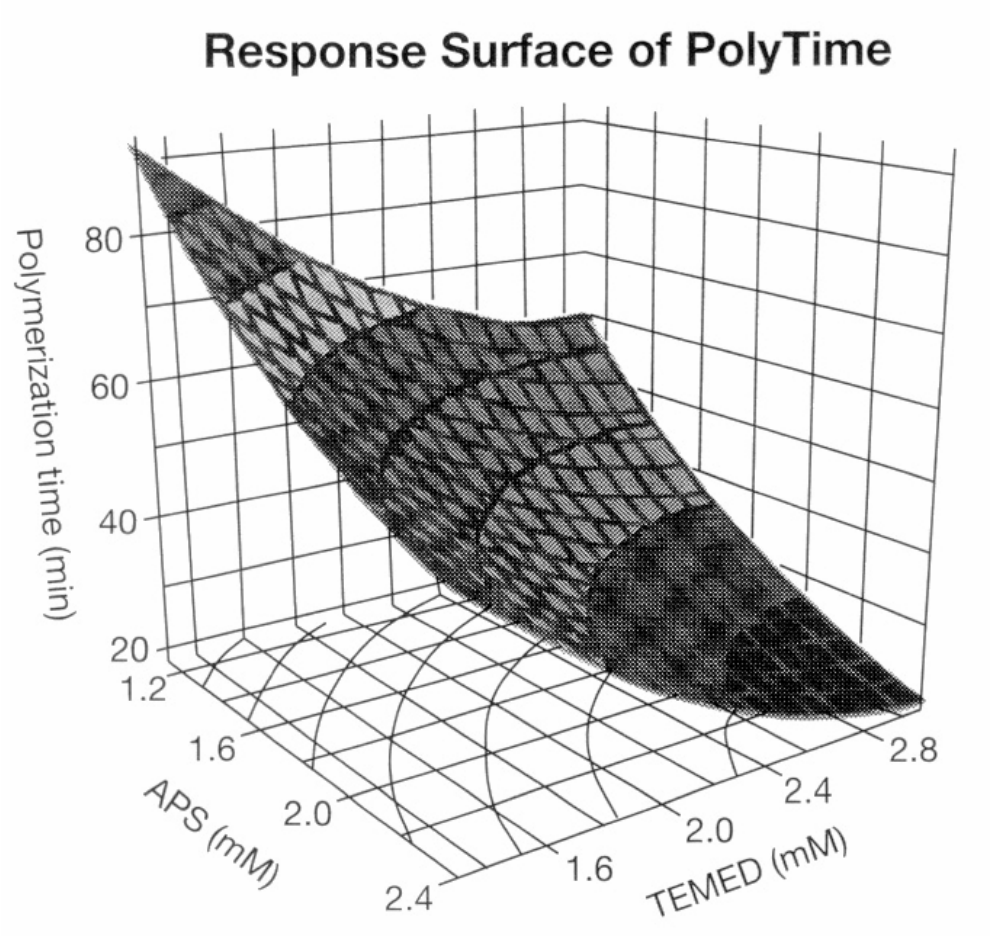


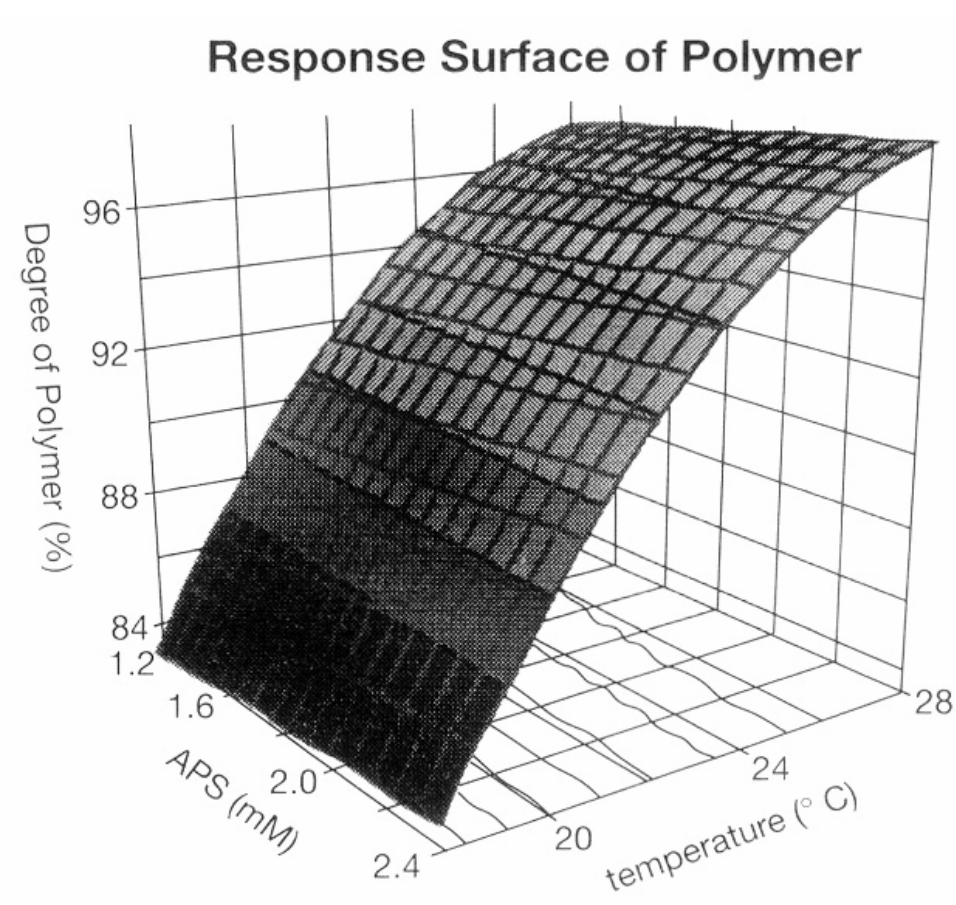
- APS 很容易因潮解而失去效用

APS is highly hygroscopic, and therefore loses its function

Pharmacia: Acrylamide Gel Casting Handbook p.11

凝膠反應條件 Examine polymerization conditions





- 凝膠時間與 APS 或 TEMED 量成反比
  Polymerization time is reduced when APS or TEMED increases

- 凝膠程度 (%) 與溫度成正比但與 APS 濃度無關
  Percentage of polymerization is proportional to the temperature, but not related to APS concentration

Pharmacia: Acrylamide Gel Casting Handbook p.12, 13

電泳膠體系統組成 Composition of PAGE system

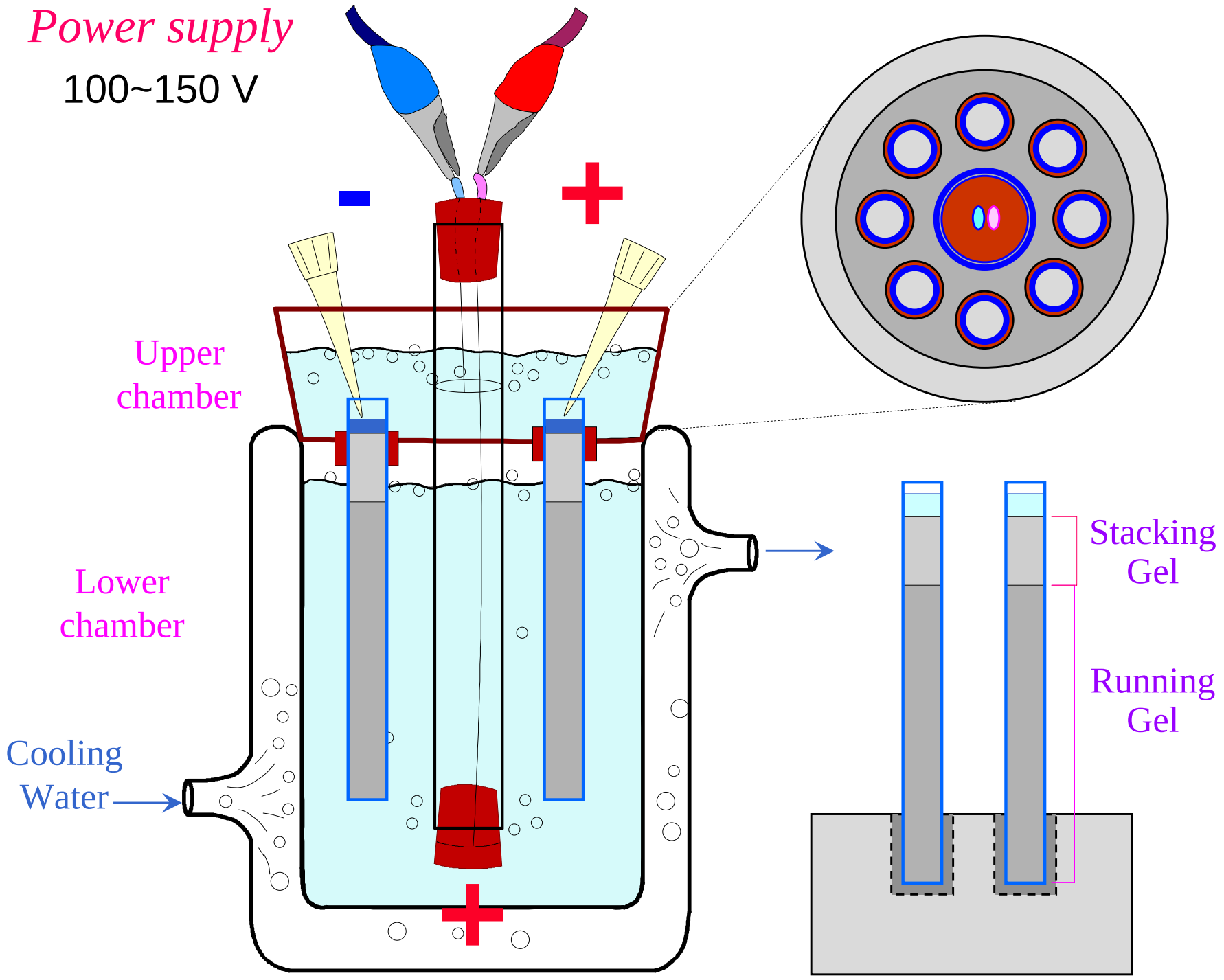
	PAGE system		Buffer	pH	Gel %
1	Cathode buffer		Tris-glycine	8.3	-
2	Sample		Tris-glycine	8.3	-
3	Gel	Stacking	Tris-HCl	6.9	5%
4		Running	Tris-HCl	8.3	7.5~20%
5	Anode buffer		Tris-glycine	8.3	-

- 膠體不連續性有焦聚樣本的作用

The gel discontinuity results in the stacking effect for sample molecules

Juang RH (2005) EPA

直立式電泳裝置



The vertical rod gel is the prototype of modern electrophoresis Juang RH (2005) EPA

焦集膠體主要角色 Key molecules in stacking gel

Glycine: Negative charged

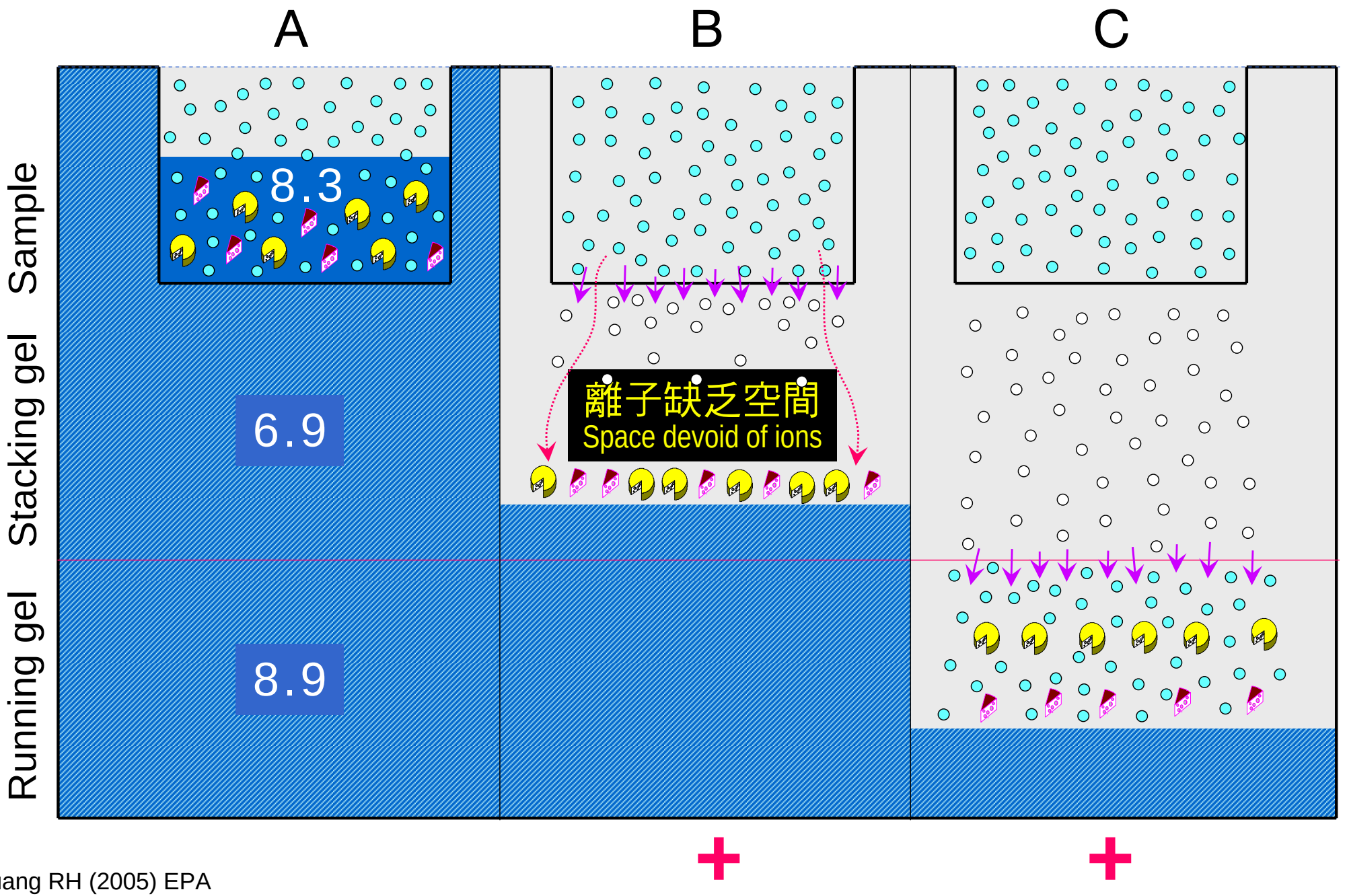
No net charge

Chloride ion:

Proteins: Small molecule

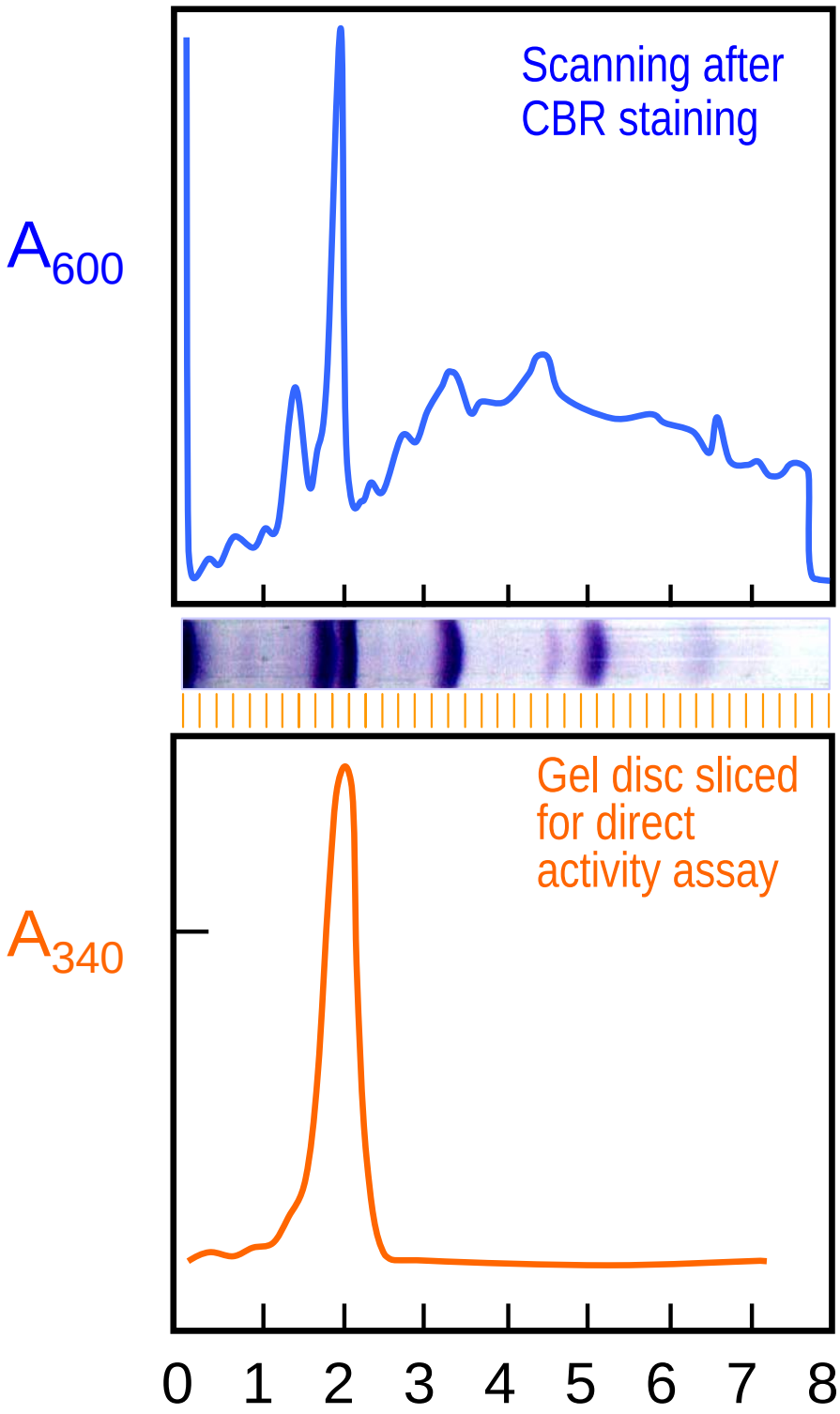
Large molecule

焦集膠體的作用原理 How stacking effect works



Juang RH (2005) EPA

電泳膠體中酵素活性測定

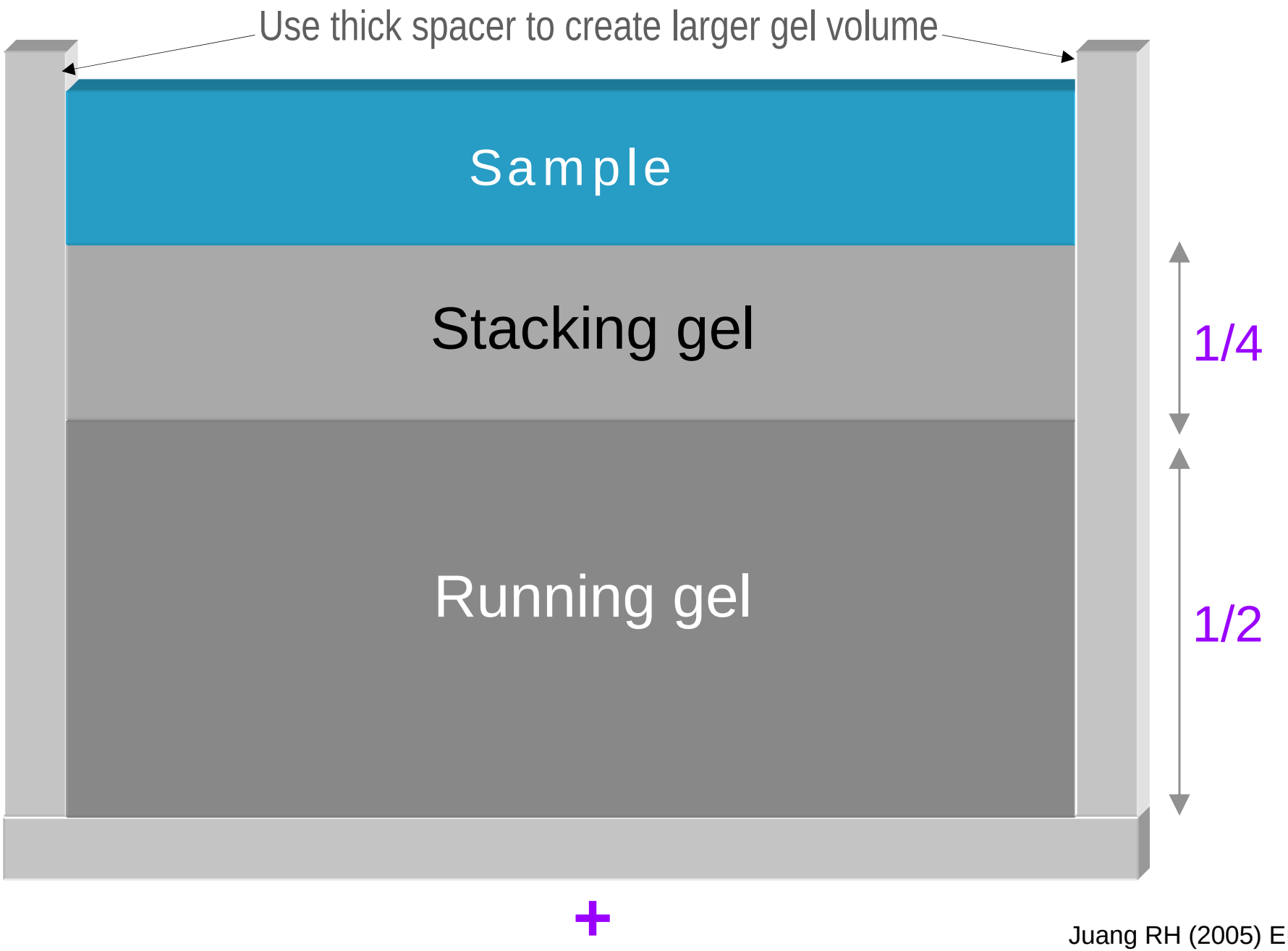


Native PAGE
Non-denaturing PAGE

SDS-PAGE

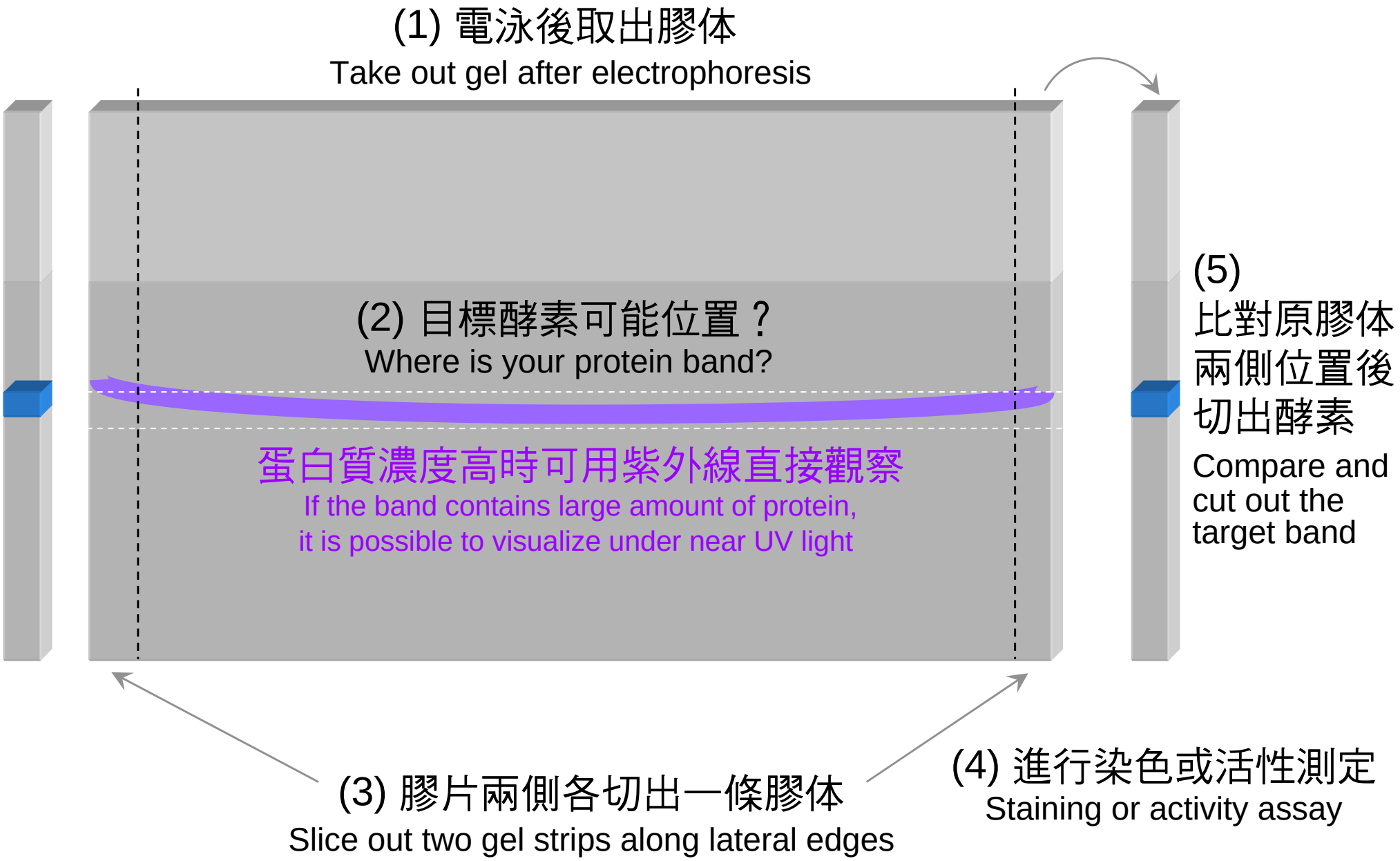
Juang RH (2005) EPA

製備式電泳膠片 Preparative gel



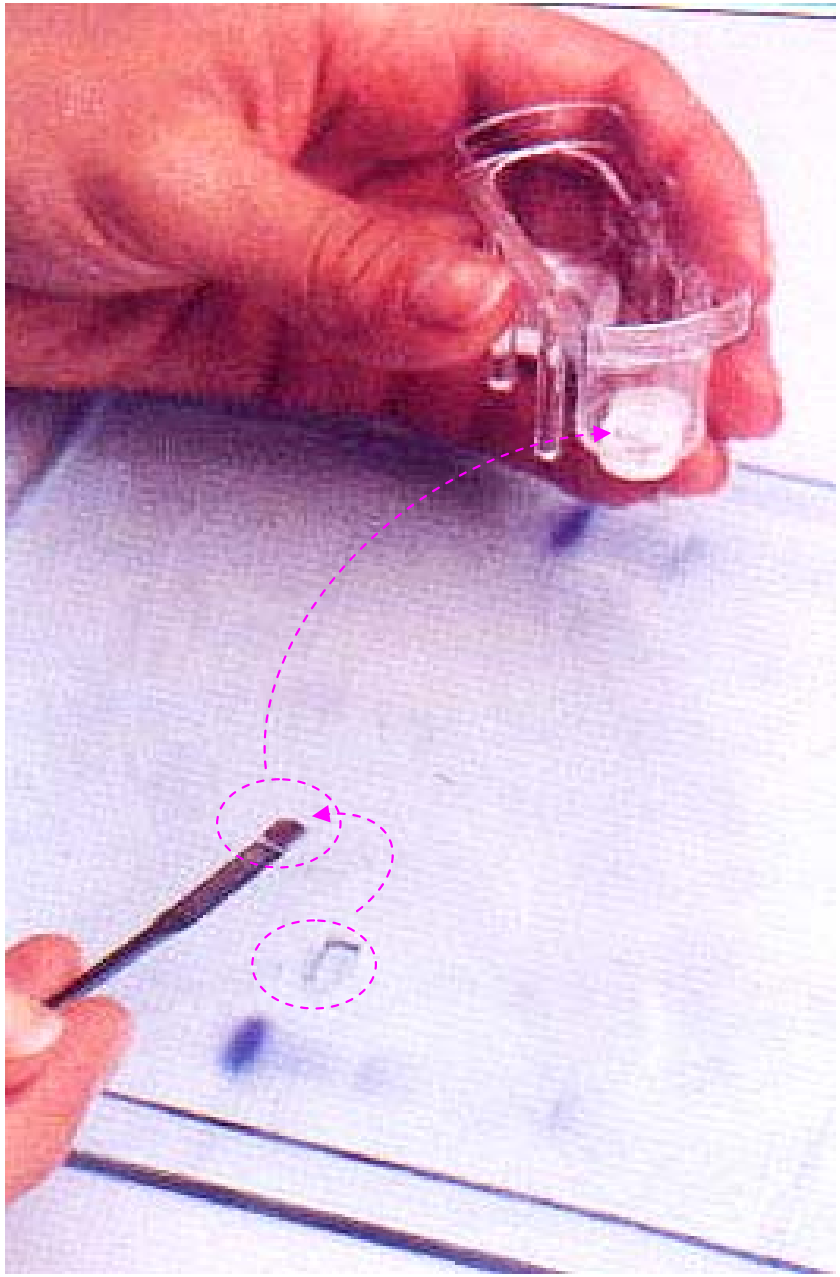
Juang RH (2005) EPA

製備式電泳操作 Detect protein band on the gel



Juang RH (2005) EPA

電泳膠體蛋白質溶離 Protein eluted from the gel

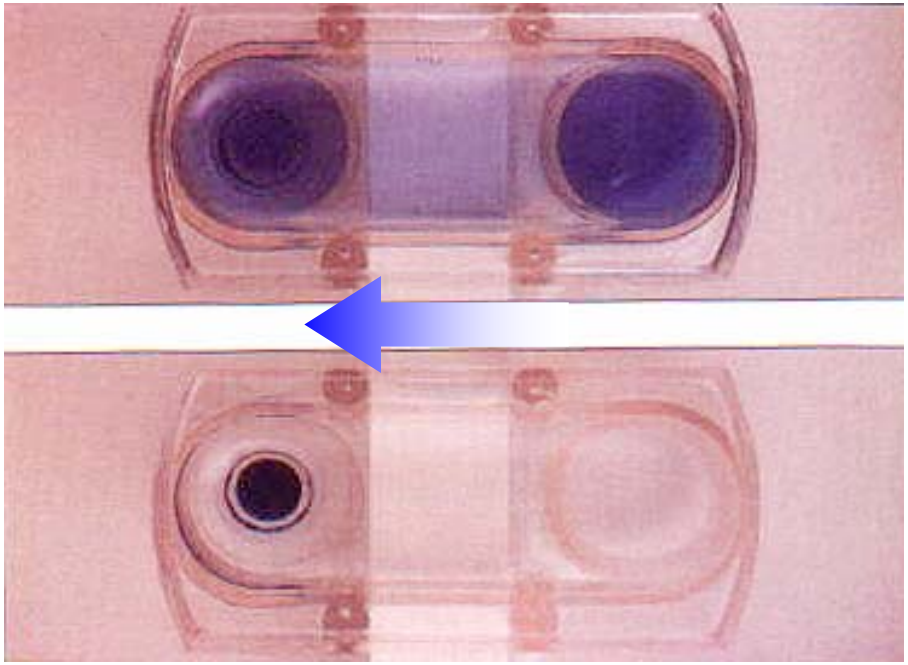


ISCO: Little Blue Tank Concentrator

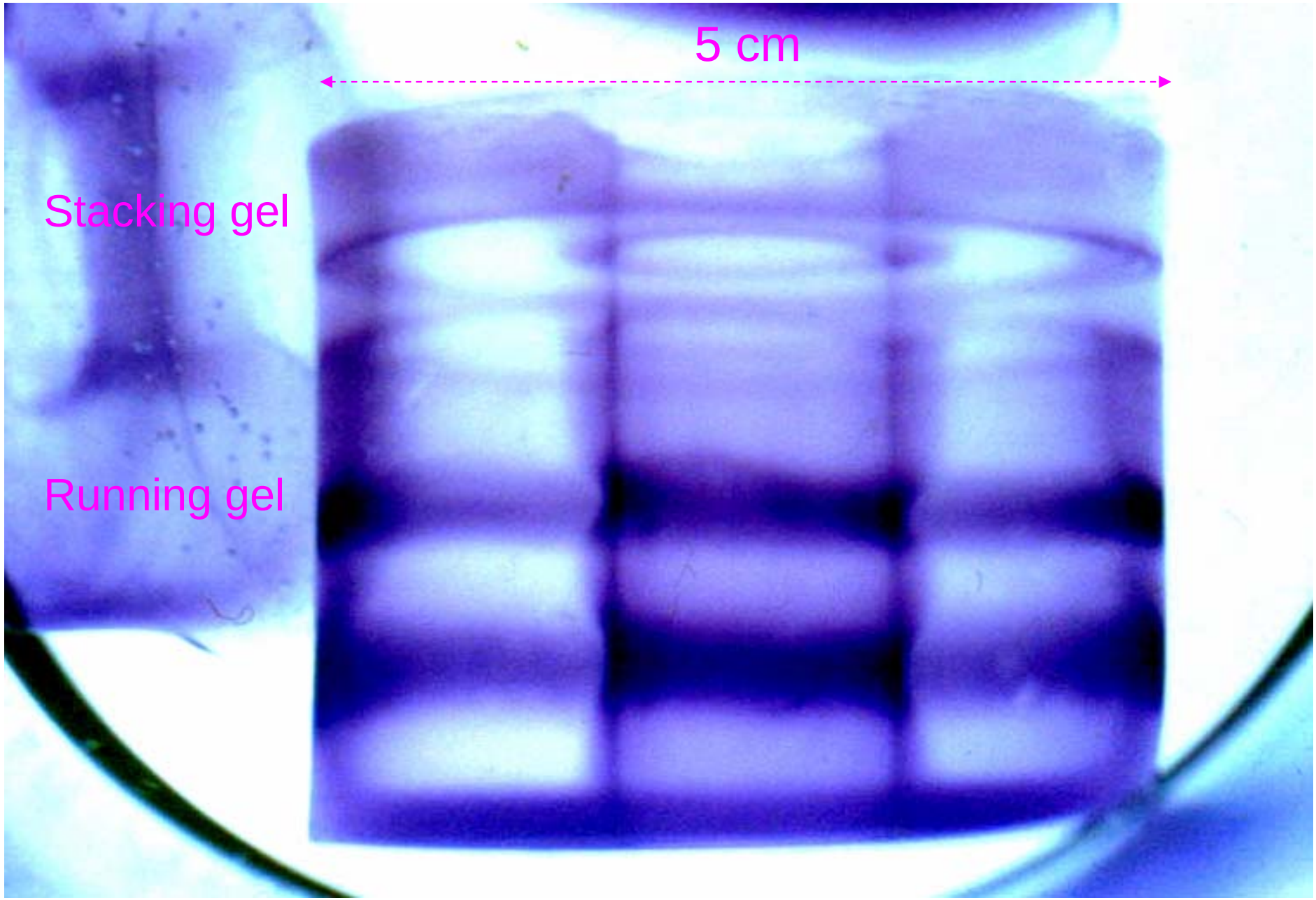
- 直接挖出膠體進行溶離

Cut out and eluted

Little Blue Tank



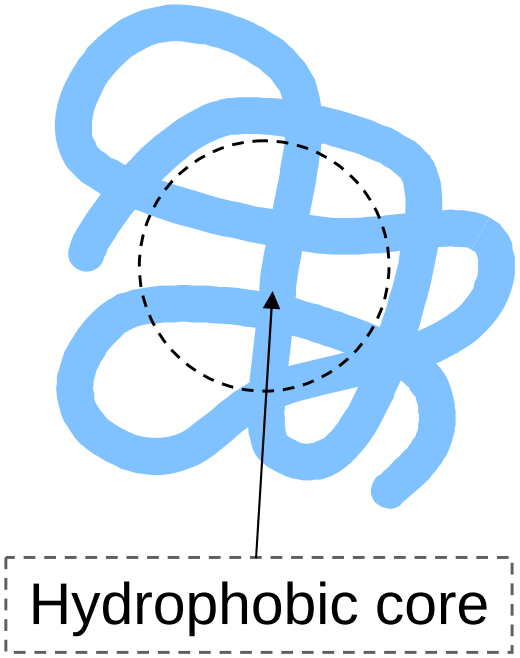
巨無霸的製備式電泳 Jumbo size preparative gel



Juang RH (2005) EPA

SDS 在蛋白質表面均勻敷上一層負電

Native protein

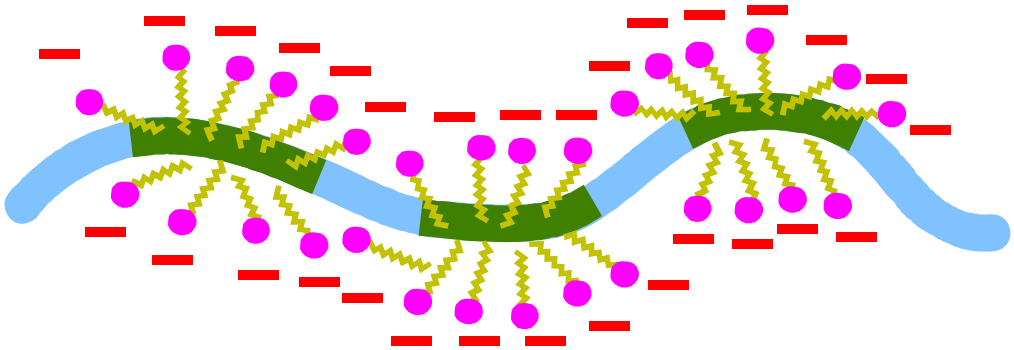


0.1%

SDS

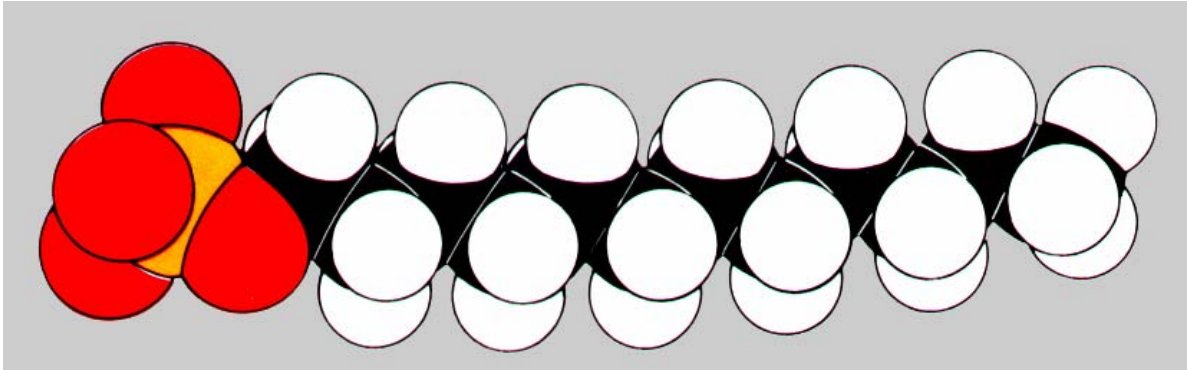
boiling

Protein is denatured to linear form



Its surface covered with negatively charged SDS uniformly

Polar head

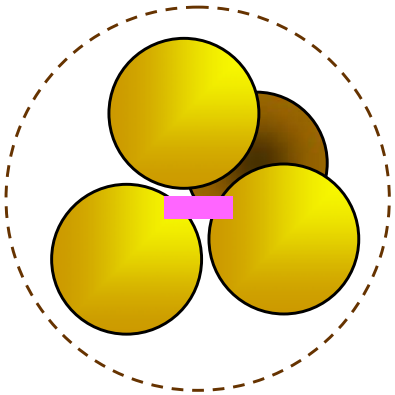


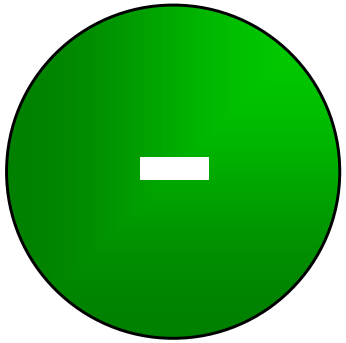
Non-polar tail

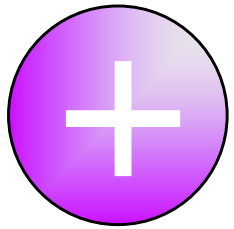
Juang RH (2005) EPA

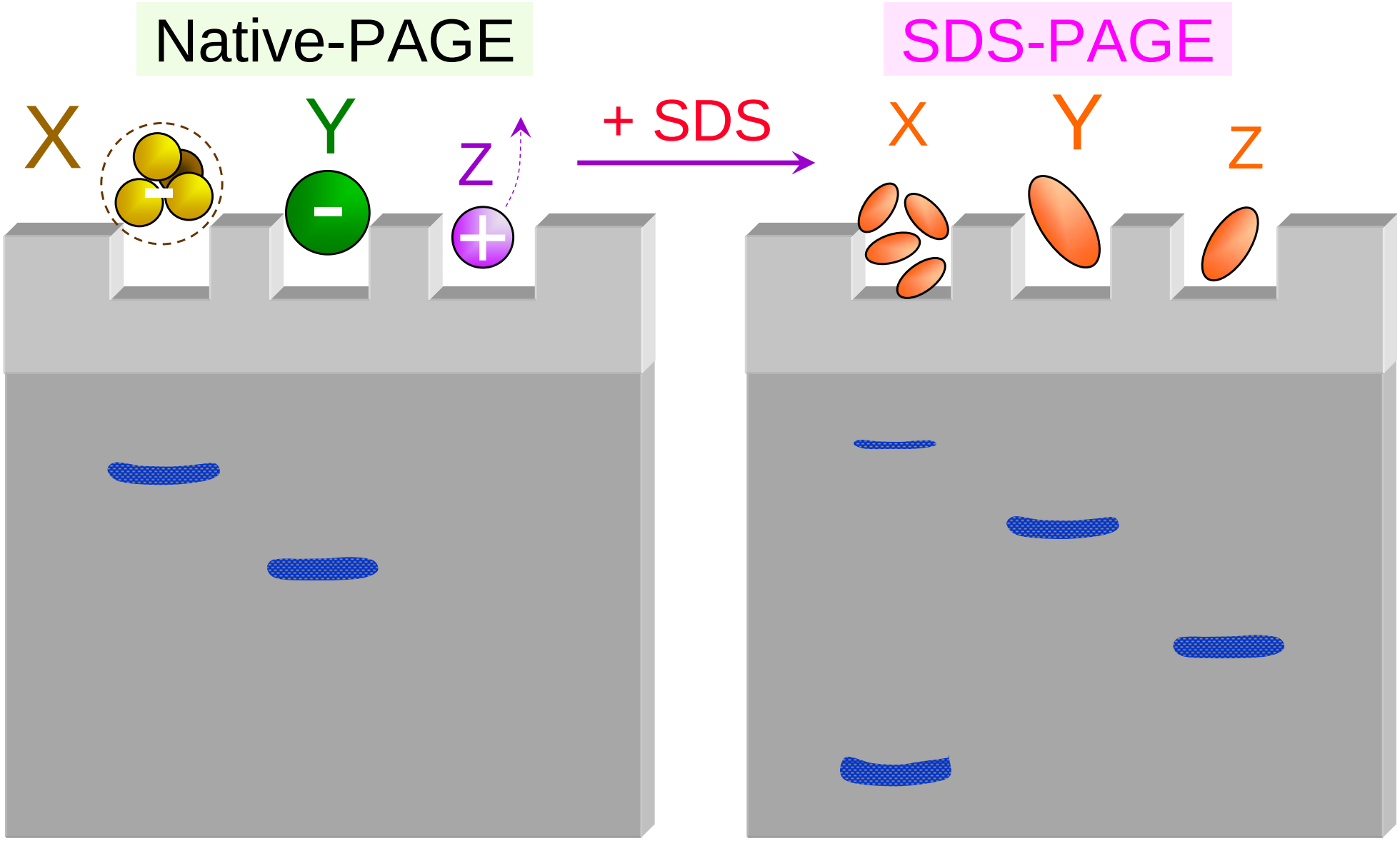
三種不同性質蛋白質的電泳比較 An example

Protein	Quaternary Structure	Molecular Weight	pI	Mobility	
				Native PAGE	SDS-PAGE
X	Tetramer	(40,000)x4	5.8	Slow	Fast
Y	Monomer	88,000	5.2	Fast	Slow
Z	Monomer	60,000	9.3	Upward	Medium

X 

Y 

Z 



+

分子量 及 淨電荷密度

均影響泳動率

molecular weight and net charge density

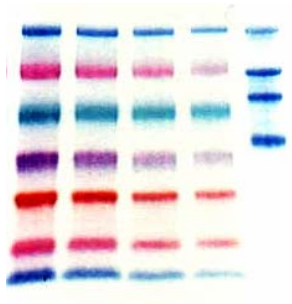
+

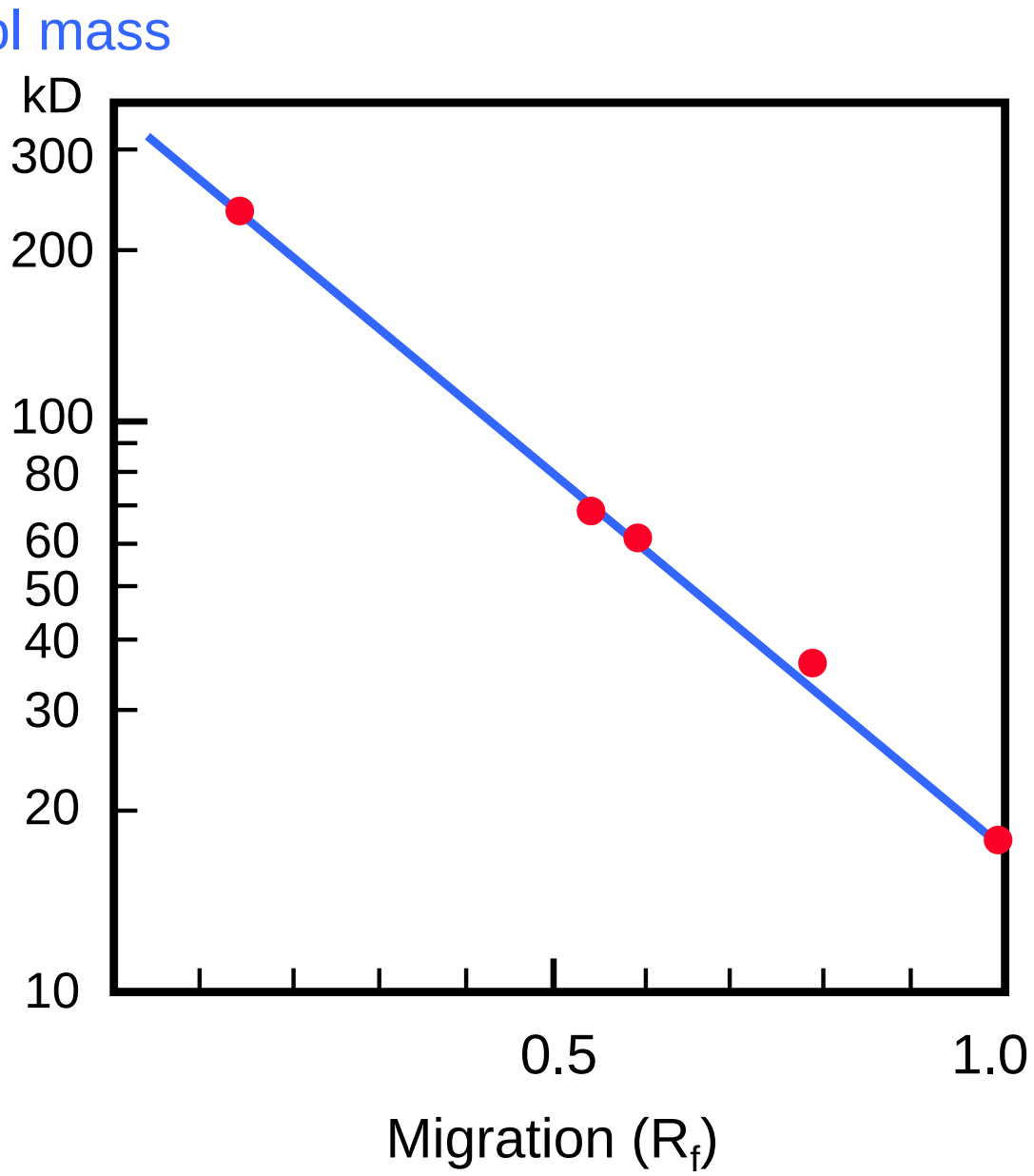
只有 分子量 影響泳動率

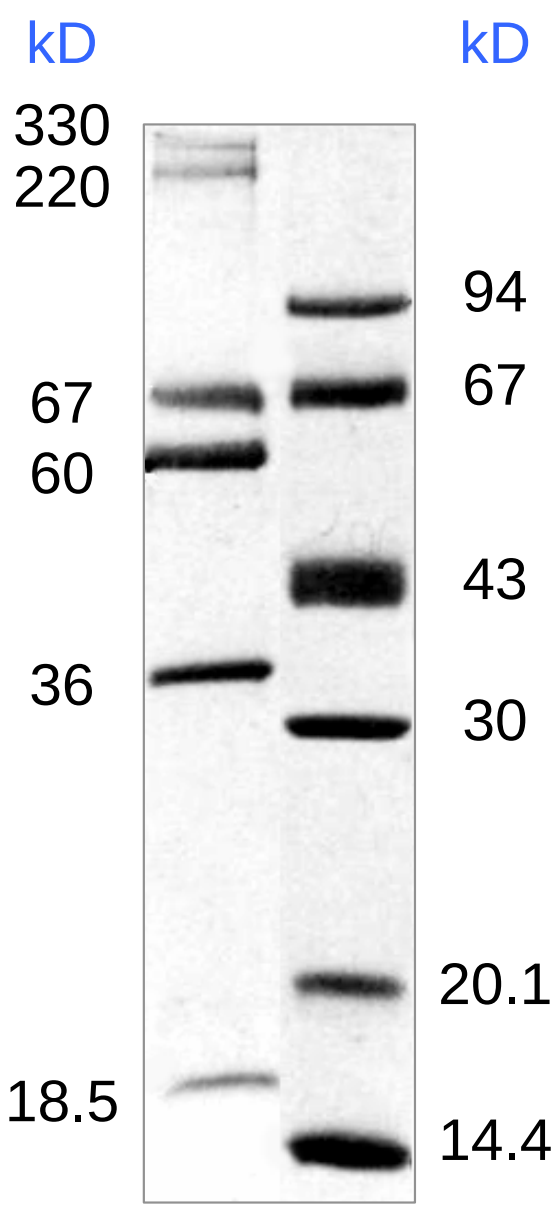
only molecular weight

Juang RH (2005) EPA

測定單元體分子量 SDS-PAGE for subunit MW

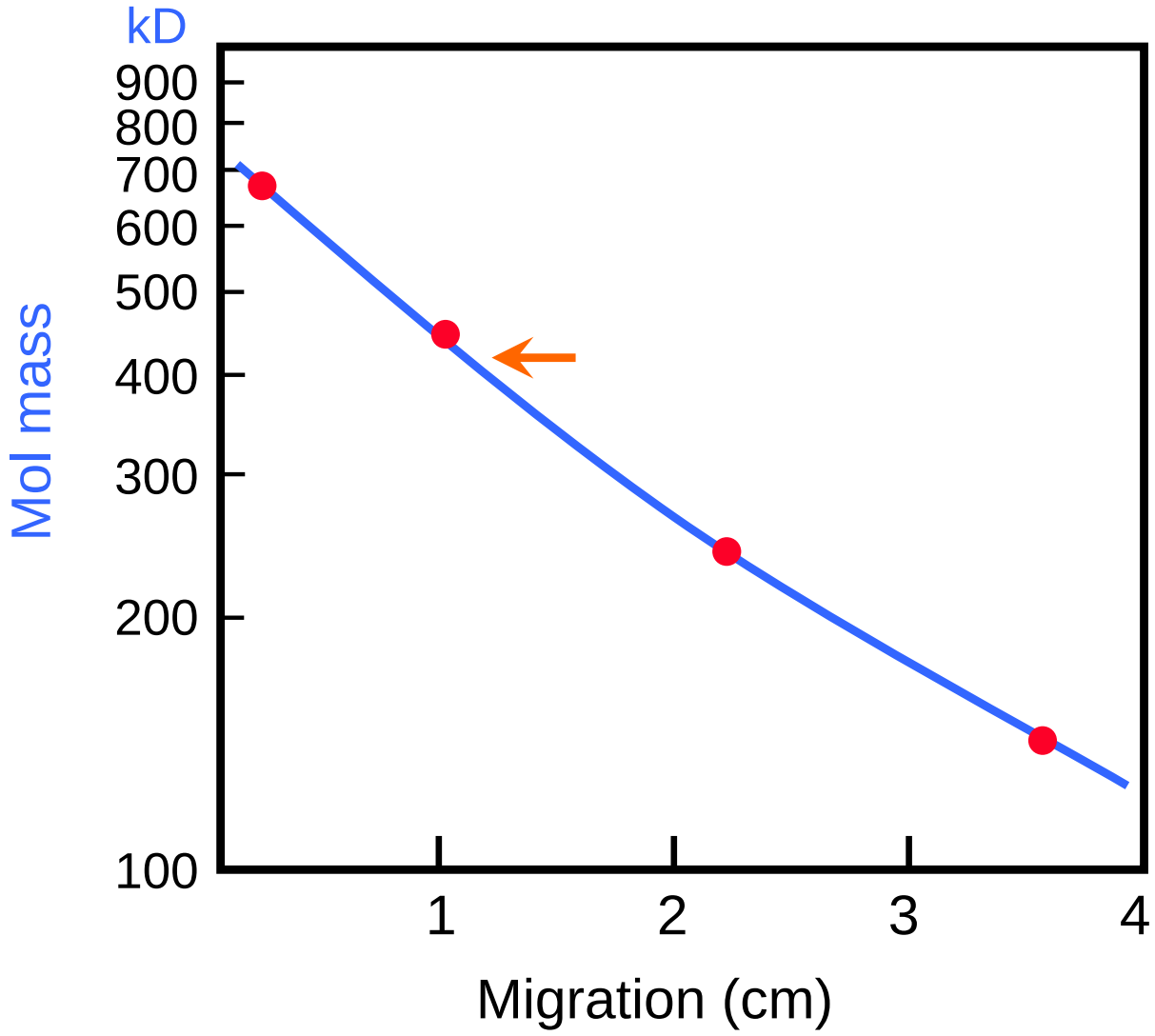


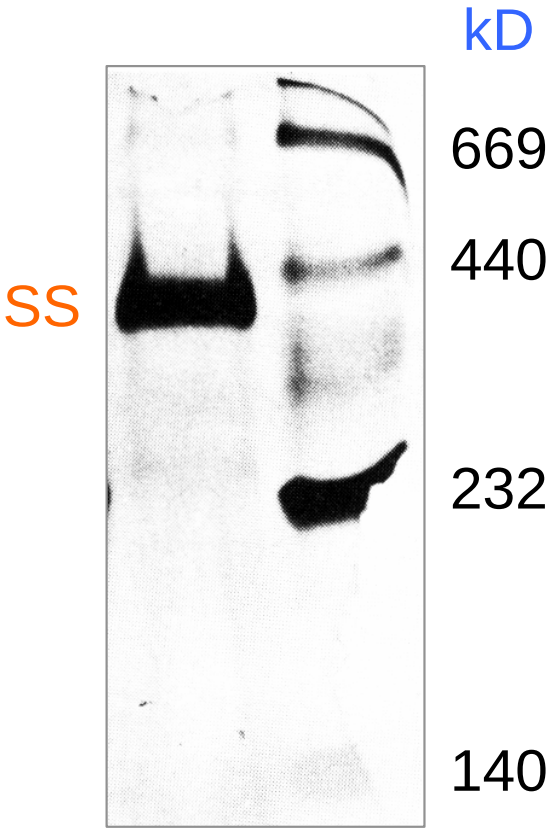




Pharmacia: Molecular Markers for electrophoresis

原態分子量測定 Disc-PAGE for native MW





- 不能以 disc-PAGE 為唯一實驗依據

Don’t take disc-PAGE MW determination as your only evidence

Juang RH (2005) EPA

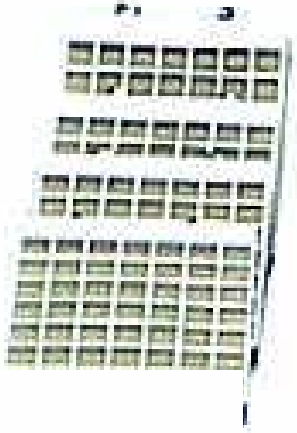
電泳系統的選擇 Choose your systems

電源供應器 Power Supply	● 100 - 250 V	● 100 - 500 mA	
電泳槽 Electrophoretic Unit	Vertical vs Horizontal	Rod vs Slab	Regular vs Mini gel

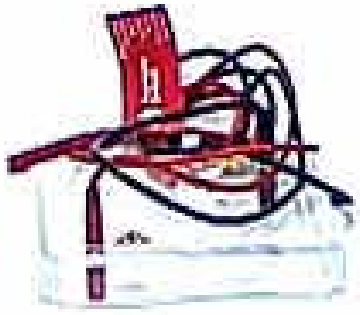
電泳系統選擇 System Choice	■ Gel casting	■ Materials	■ Samples
Vertical	Rod of slab	Acrylamide	Protein
Horizontal	Slab	Agarose	NA Isozyme
Vertical	Vertical slab	Mixed type	DNA sequencing

電泳槽及相關設備 Instruments and equipments

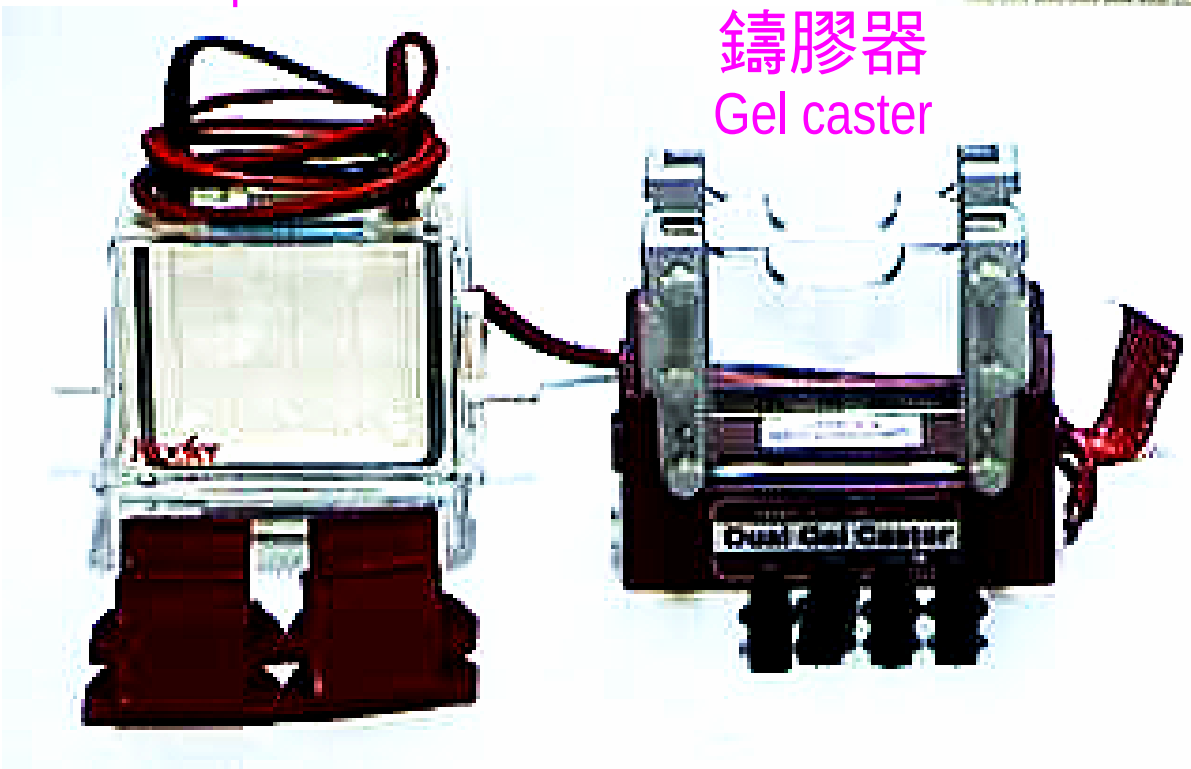
轉印三明治
Transfer sandwich



轉印槽
Gel transfer



電泳槽
Electrophoresis unit



鑄膠器
Gel caster

供電器 Power supply

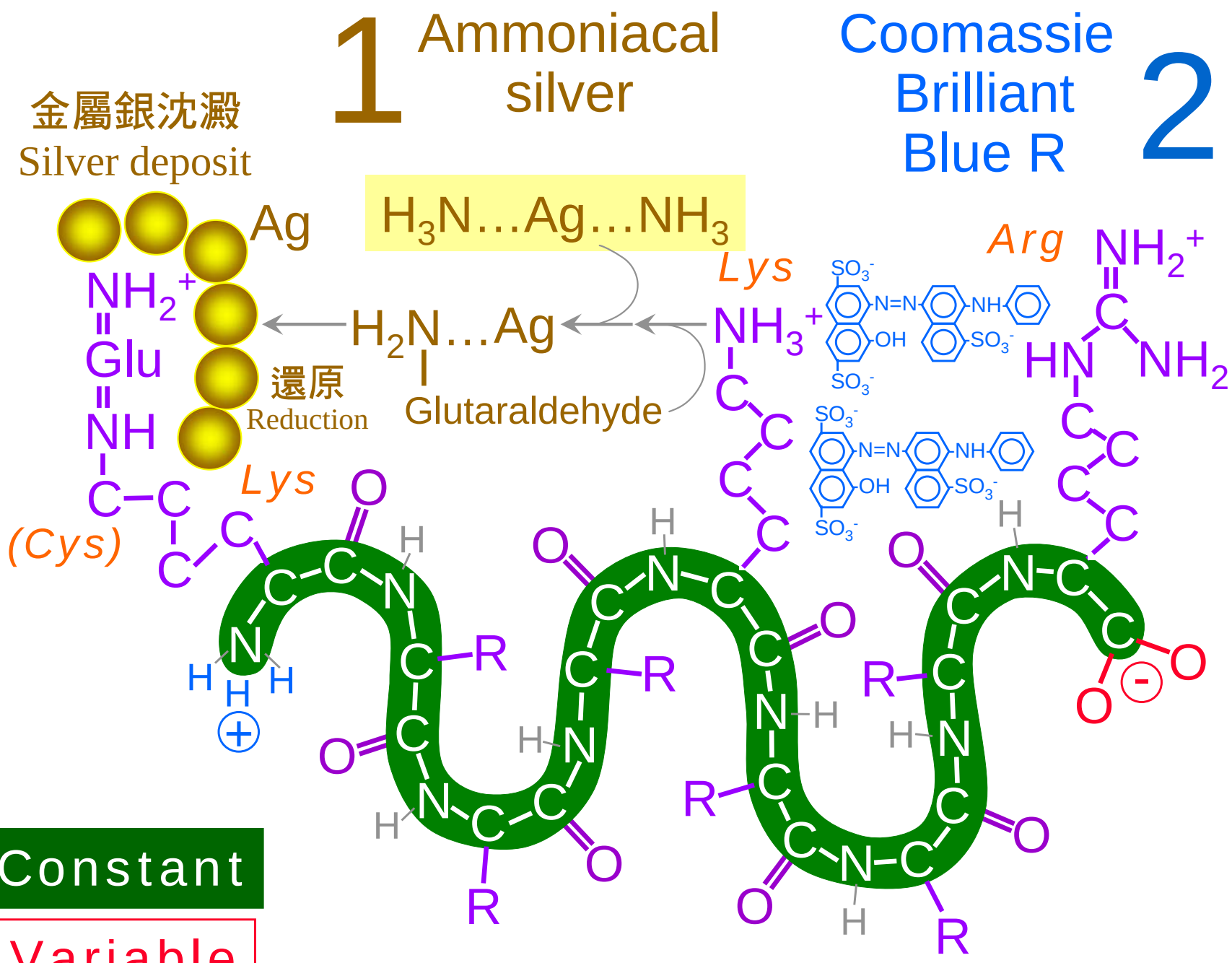
Juang RH (2005) EPA

3.3 其他相關技術 Other related techniques

- 3.3.1 染色及乾燥 Gel staining & drying
  電泳膠片可以用各種方法染出蛋白質色帶
- 3.3.2 等電焦集法 Isoelectric focusing
  依蛋白質分子的等電點來分離
- 3.3.3 二次元電泳 2D electrophoresis
  解析力極高，可分析成份複雜的樣本
- 3.3.4 蛋白質轉印法 Protein transfer
  電泳後可再把蛋白質色帶轉印到薄膜上

各種蛋白質染色法機制

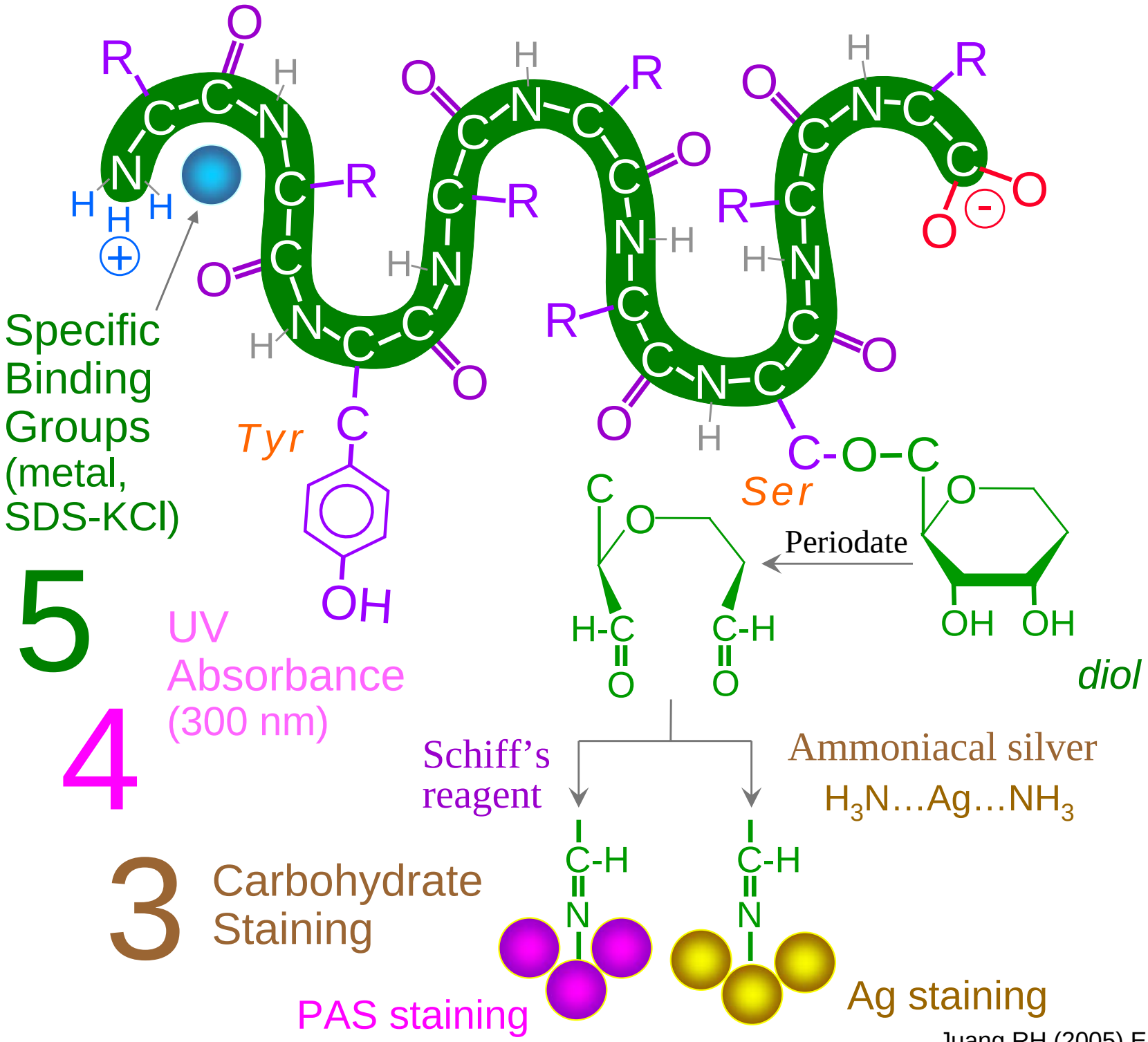
OHC-C-C-C-CHO



Gel staining methods and their mechanism

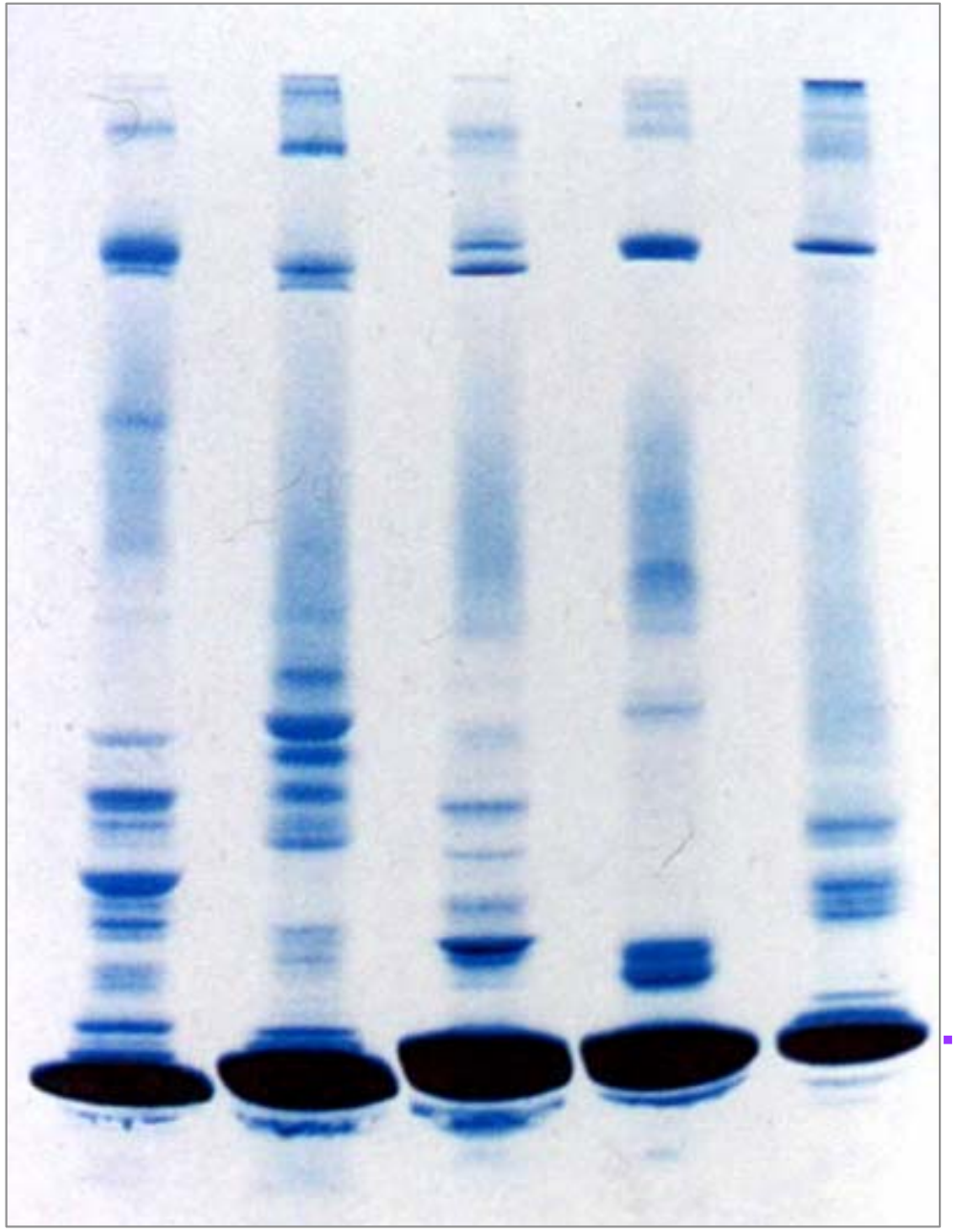
Juang RH (2005) EPA

各種蛋白質染色法機制

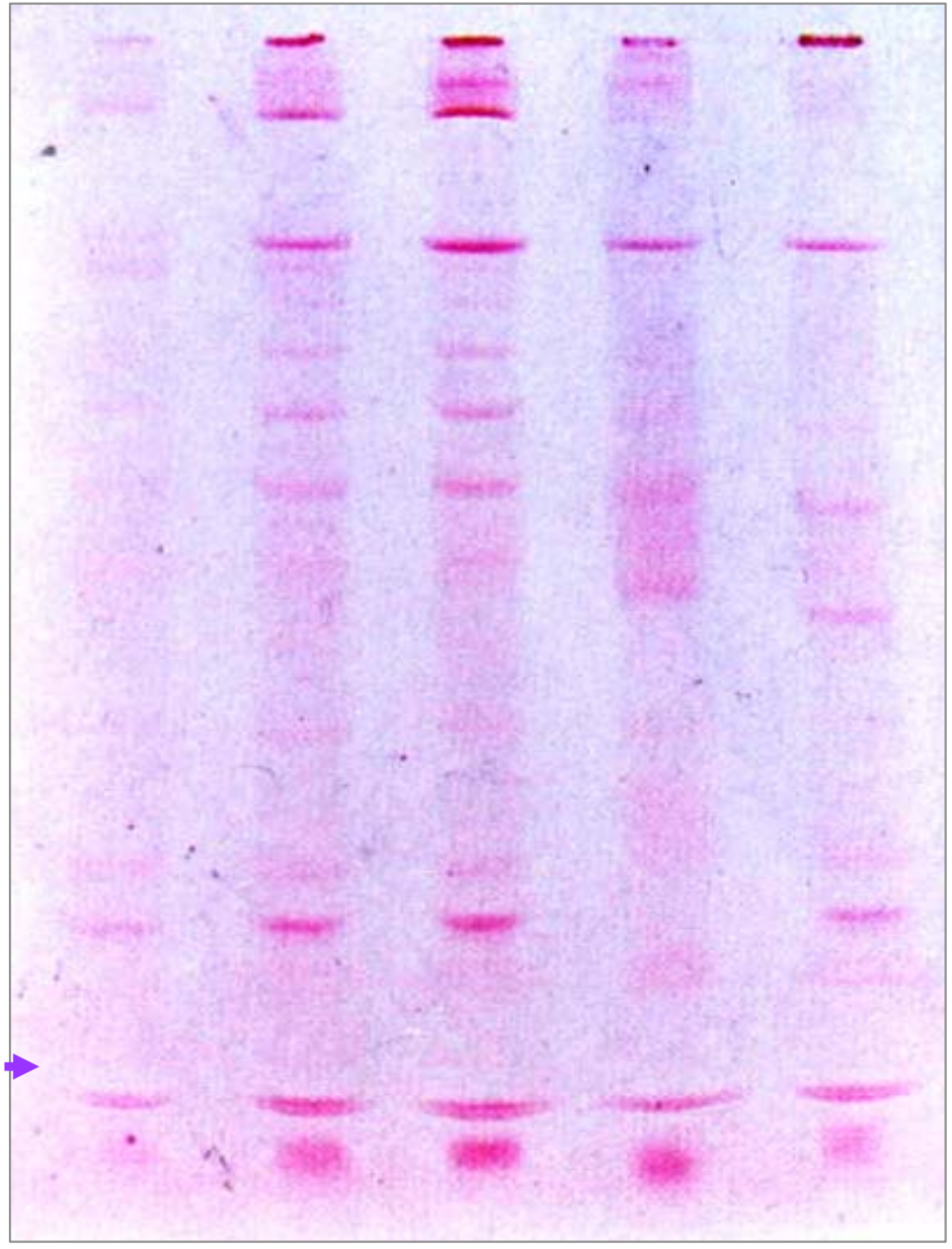


Juang RH (2005) EPA

血清電泳染色結果 Serum stained by CBR / PAS

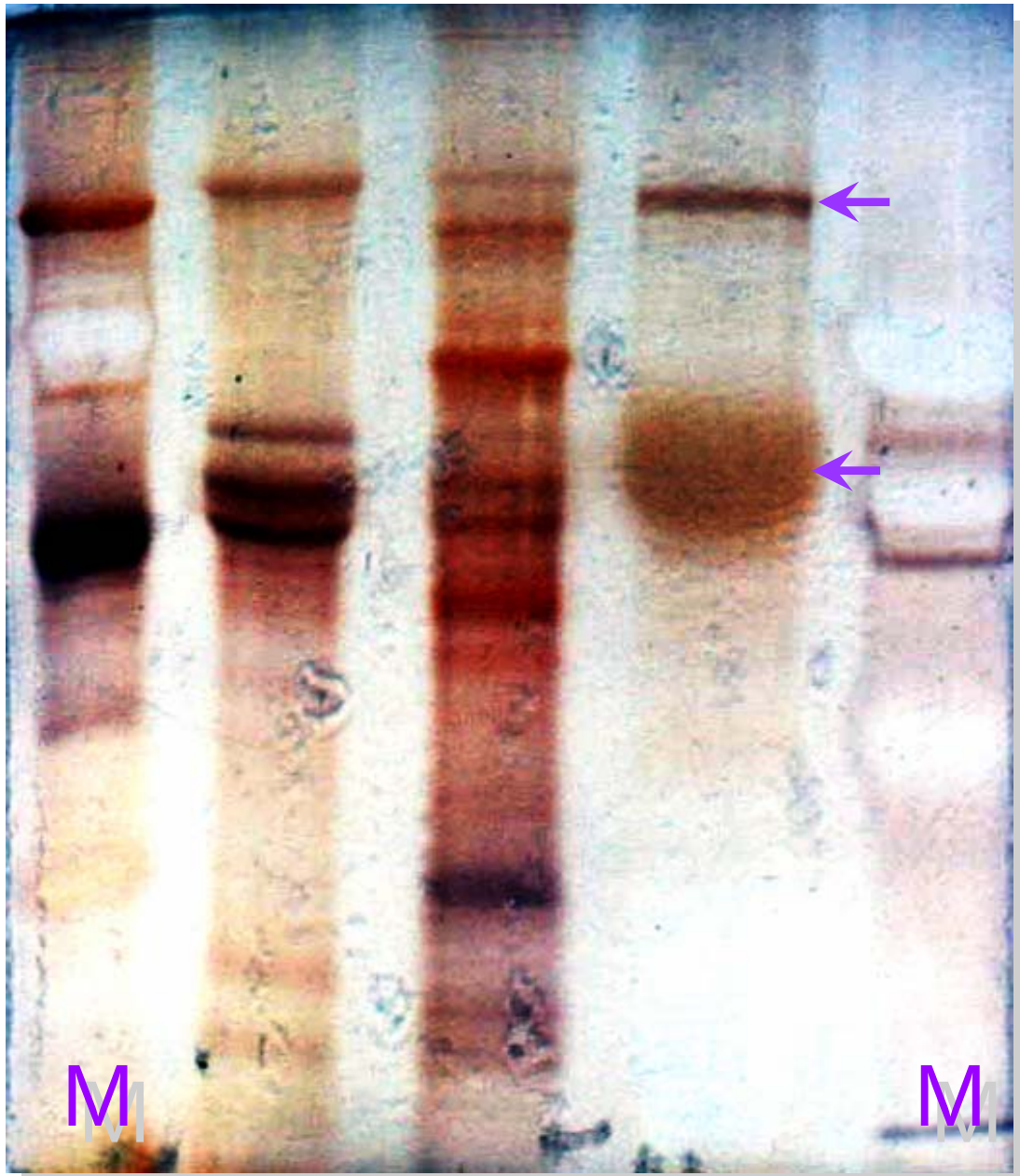


Pharmacia: Electrophoresis



Albumin can't be stained with PAS

SP 醣染色結果 Glycoprotein stained with $AgNO_3$

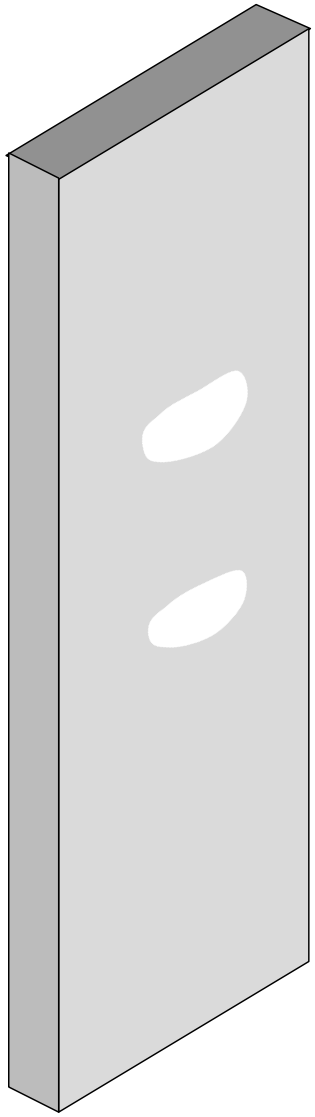


Juang RH (2005) EPA

澱粉磷解酶活性分析及干擾

A

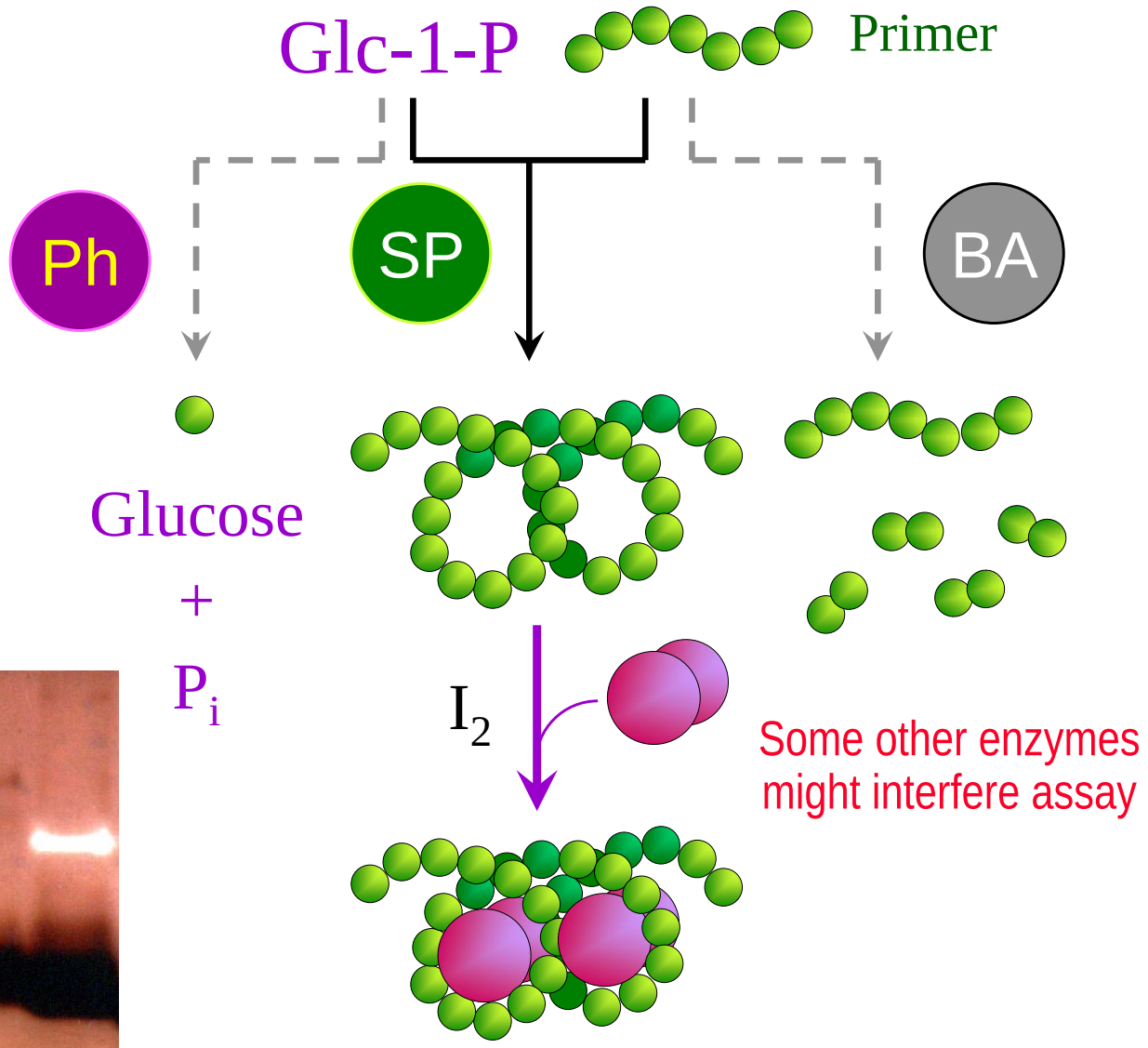
Activity staining



Native PAGE

B

Activity assay and interference



Direct observation of the color product

Not every enzyme can be stained by its activity on the gel

Juang RH (2005) EPA

膠片呈色法比較 Comparison of staining methods

Coomassie Brilliant Blue R-250 Staining	Protein	Med sensitivity ●●	Simple, rapid
Ammoniacal Silver Staining	Protein	High sensitivity ●●●	Complex steps
Periodic Acid - Schiff's Reagent	Carbohydrate	Low sensitivity ●	Complex steps
UV Absorbance (300 nm)	Protein or nucleic acid	Low sensitivity ●	Illuminate gel directly
Autoradiography	Radioactive labeled molecule	High sensitivity ●●●	Radioactive hazard
KCl Precipitation	Protein coated with SDS	Low sensitivity ●	Simple, rapid
Activity Staining	Enzyme reaction (insoluble product)	High sensitivity ●●●	Variable

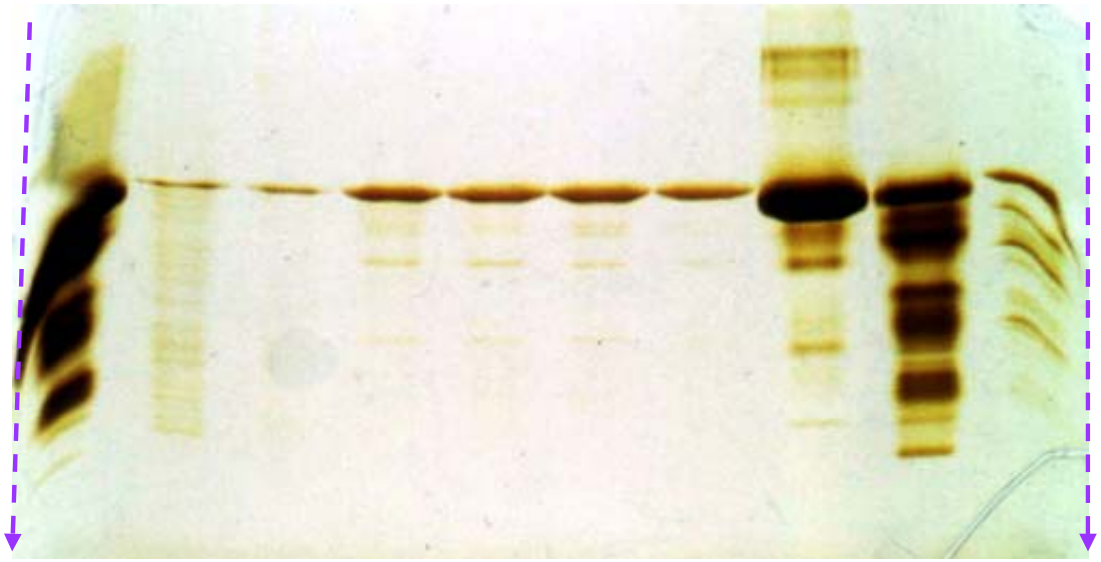
Juang RH (2005) EPA

電泳結果不佳十大原因 Trouble shooting (Top 10)

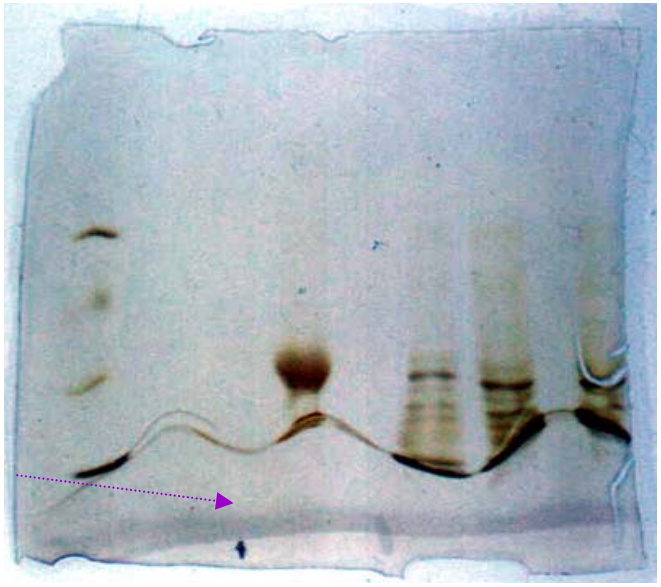
Can't polymerize	Wrong APS concentration or inactivated APS
Band twisting	Bad gel casting or gel trapped bubbles
Band diffusing, tailing	Samples have high-salt or extreme pH
Some lanes failed	Sample wells cleaned (tooth-brushing) ?
Partly polymerized	APS not dissolved completely?
Dye front line leaned	Uneven temperature or leaking electrode buffer
Gel with vertical lines	Impurity in gel or reagents
No stacking effect	Check *p*H of C solution (stacking gel)
Wrong mobility	Forgot SDS in gel, buffer or sample?
Gel became sticky	Forgot Bis in gel solution?

Juang RH (2005) EPA

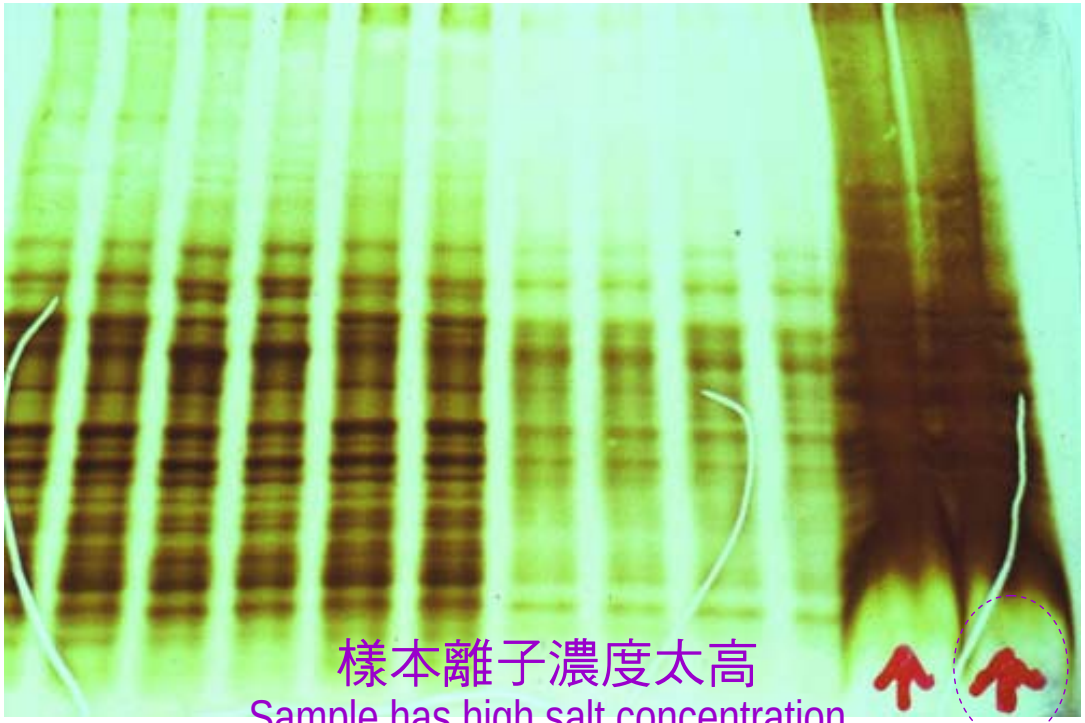
各種電泳染色的問題 Some gel problems



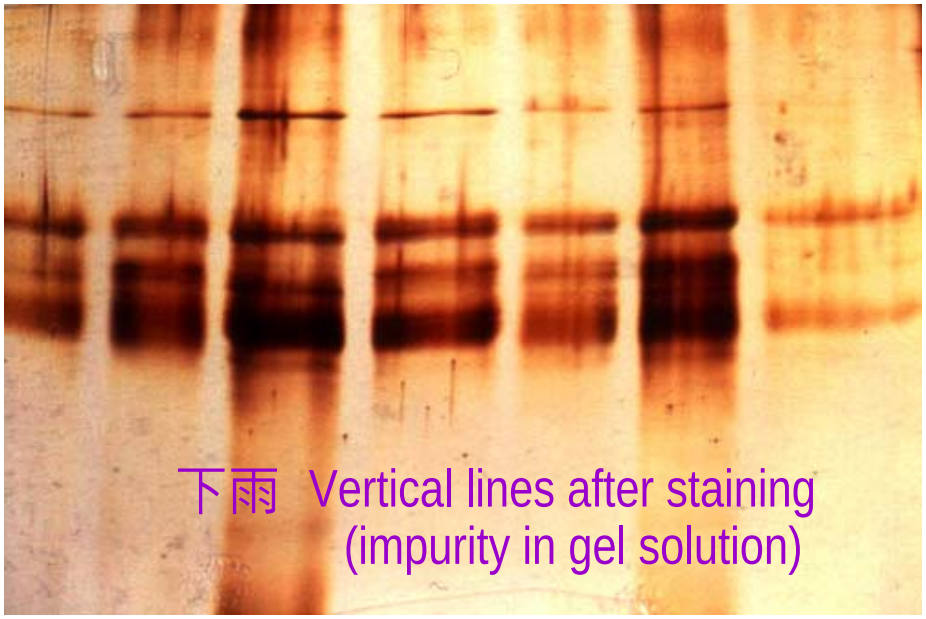
暗流
Leaking buffer conducting the electric current



凝膠不均勻
Gel is unevenly polymerized



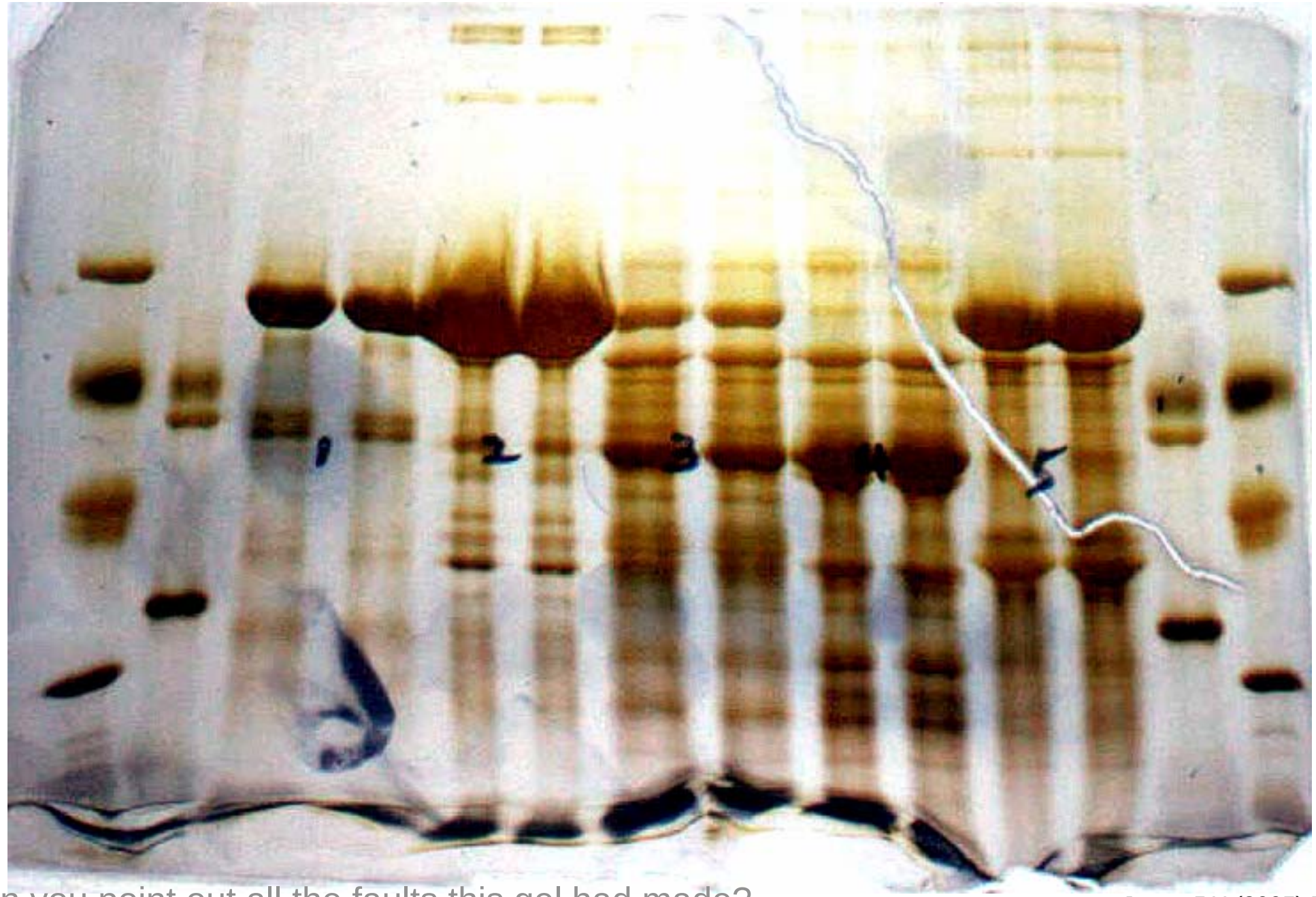
樣本離子濃度太高
Sample has high salt concentration



下雨 Vertical lines after staining (impurity in gel solution)

Juang RH (2005) EPA

各種電泳染色的問題 A malformed gel



Can you point out all the faults this gel had made?

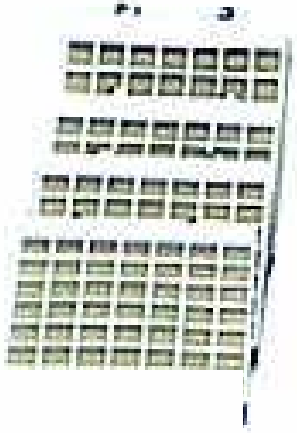
Juang RH (2005) EPA

3.3 其他相關技術 Other related techniques

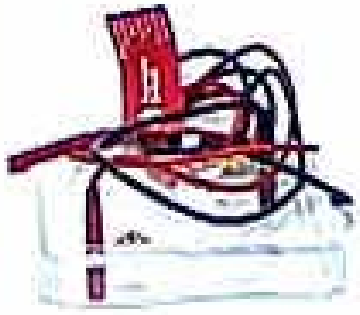
- 3.3.1 染色及乾燥 Gel staining & drying
  電泳膠片可以用各種方法染出蛋白質色帶
- 3.3.2 等電焦集法 Isoelectric focusing
  依蛋白質分子的等電點來分離
- 3.3.3 二次元電泳 2D electrophoresis
  解析力極高，可分析成份複雜的樣本
- 3.3.4 蛋白質轉印法 Protein transfer
  電泳後可再把蛋白質色帶轉印到薄膜上

電泳槽及相關設備 Instruments and equipments

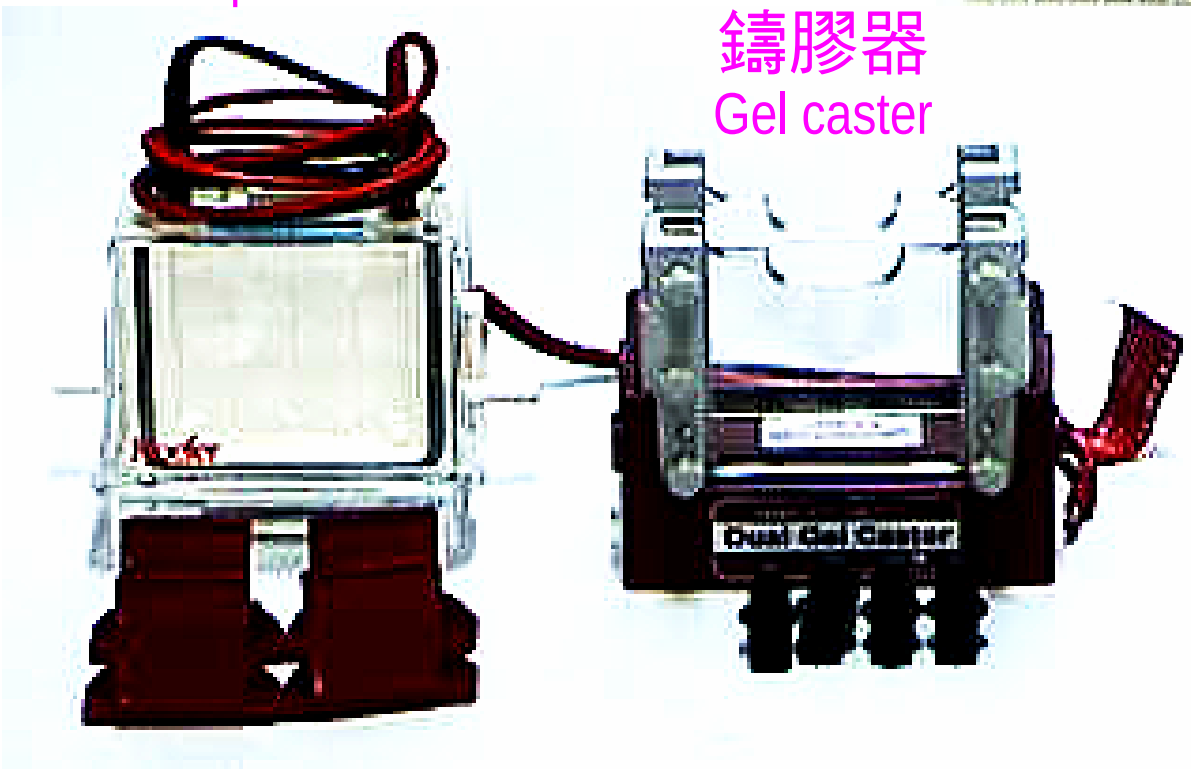
轉印三明治
Transfer sandwich



轉印槽
Gel transfer



電泳槽
Electrophoresis unit



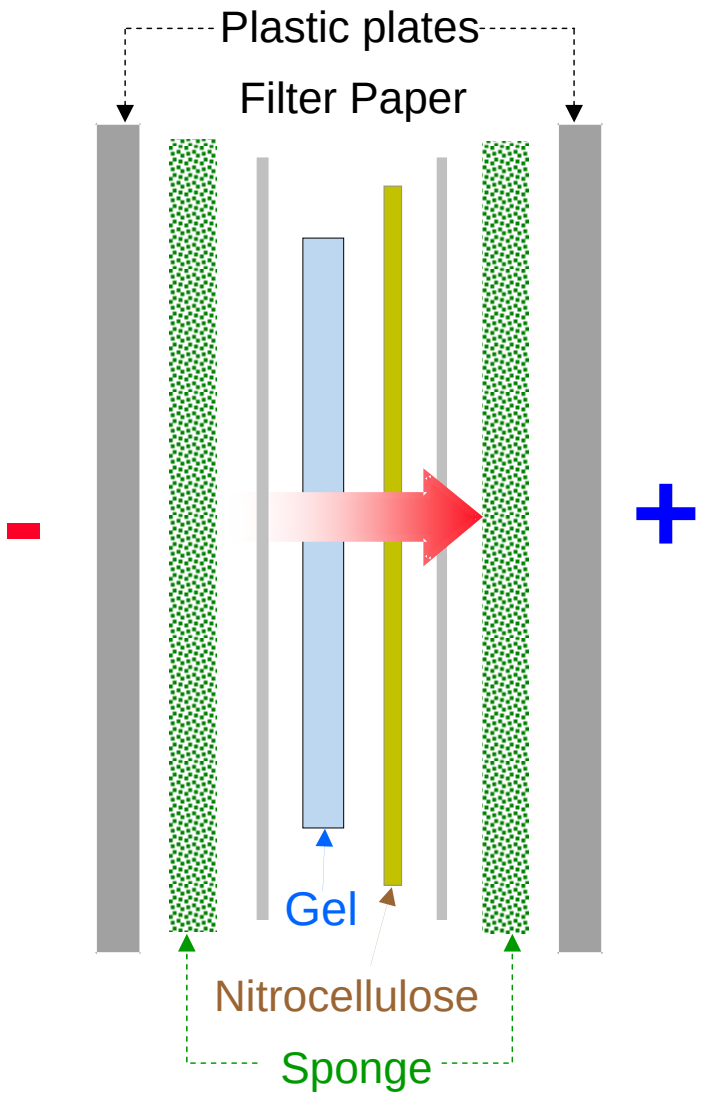
鑄膠器
Gel caster

供電器 Power supply

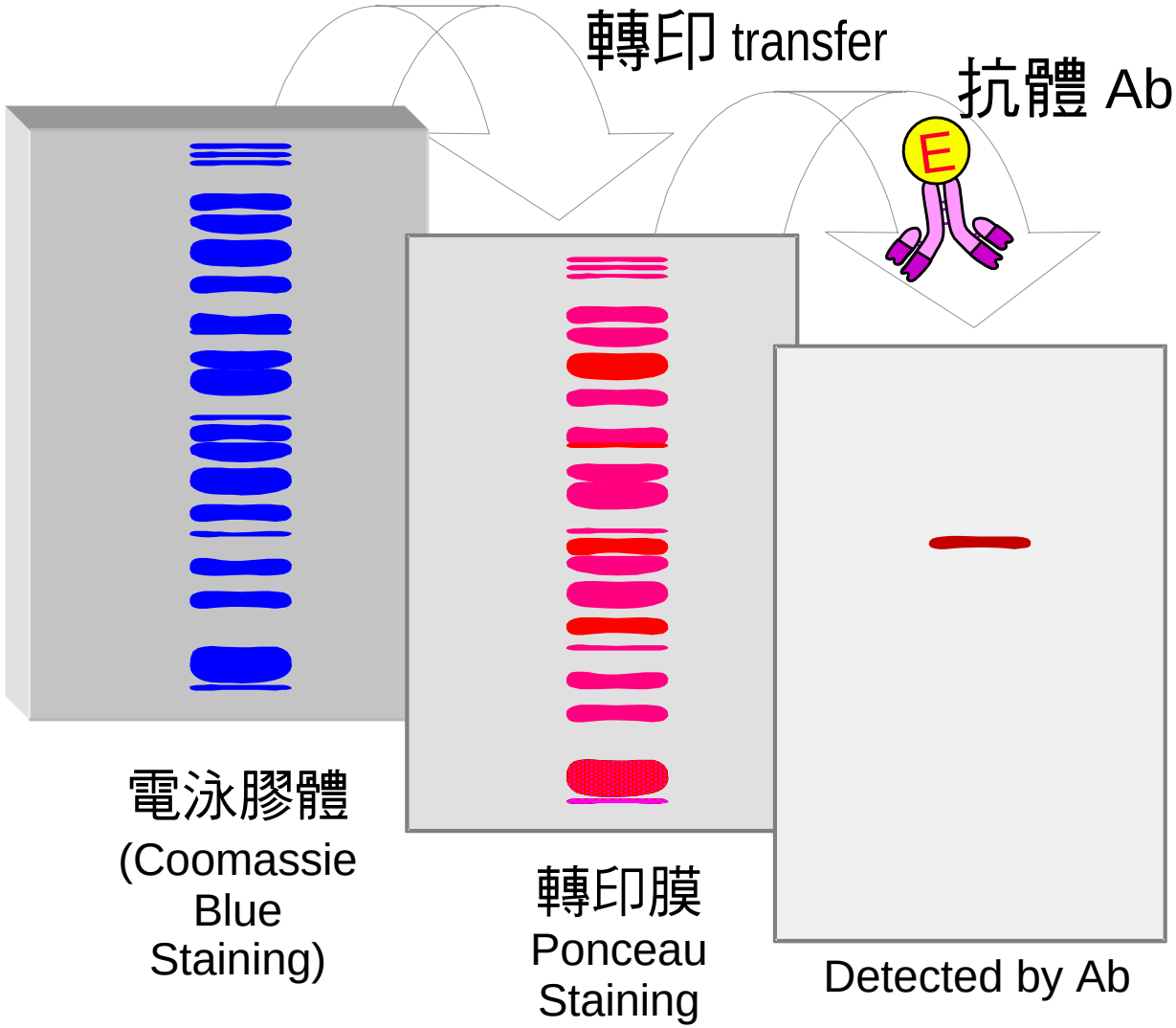
Juang RH (2005) EPA

轉印及免疫染色 Protein transfer and staining

A 轉印三明治
Transfer sandwich

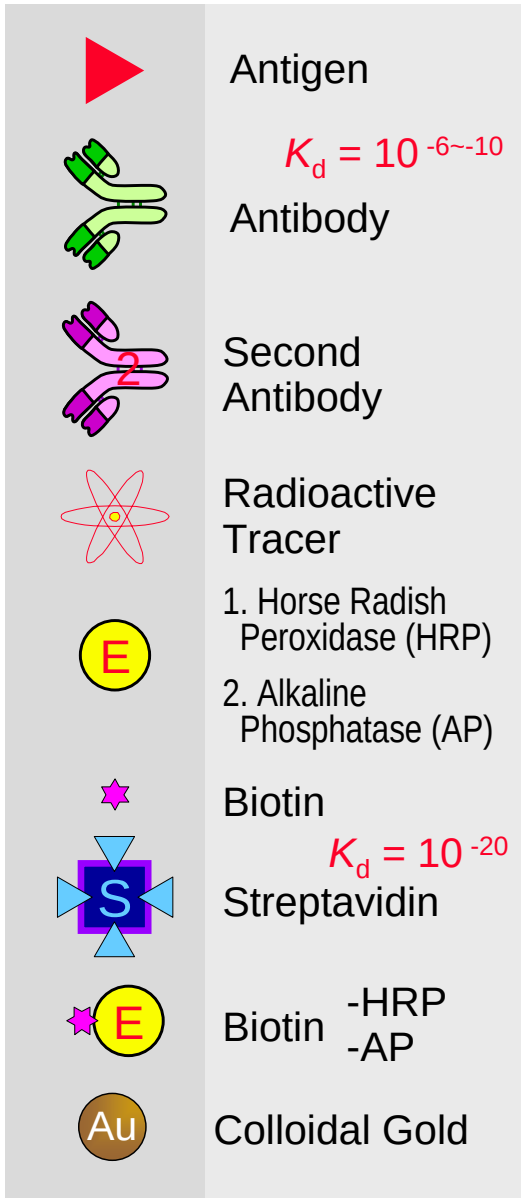


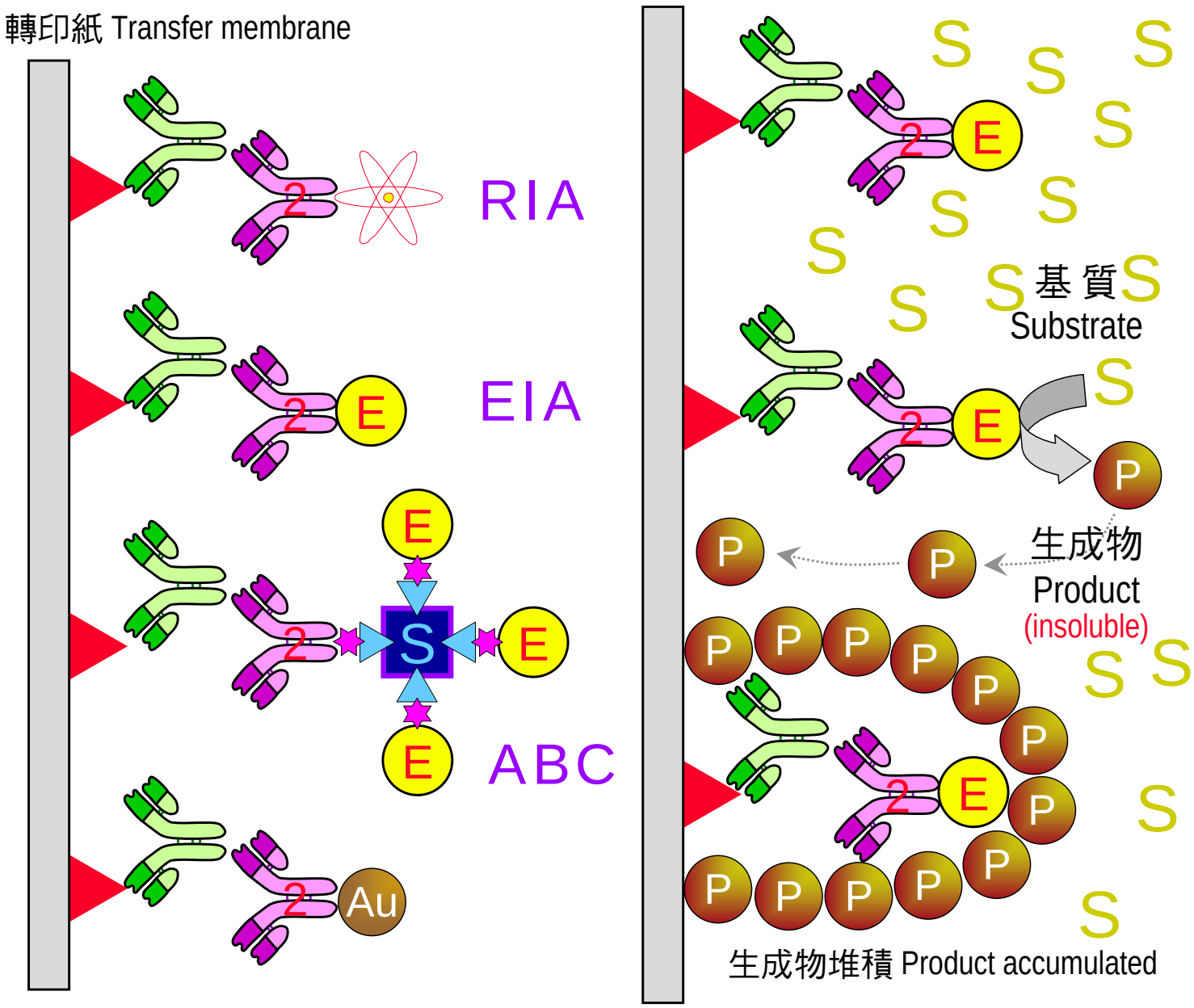
B 免疫染色流程及結果
Immunostaining procedure and result



Juang RH (2005) EPA

免疫轉印種類與呈色機制 Immunoassays



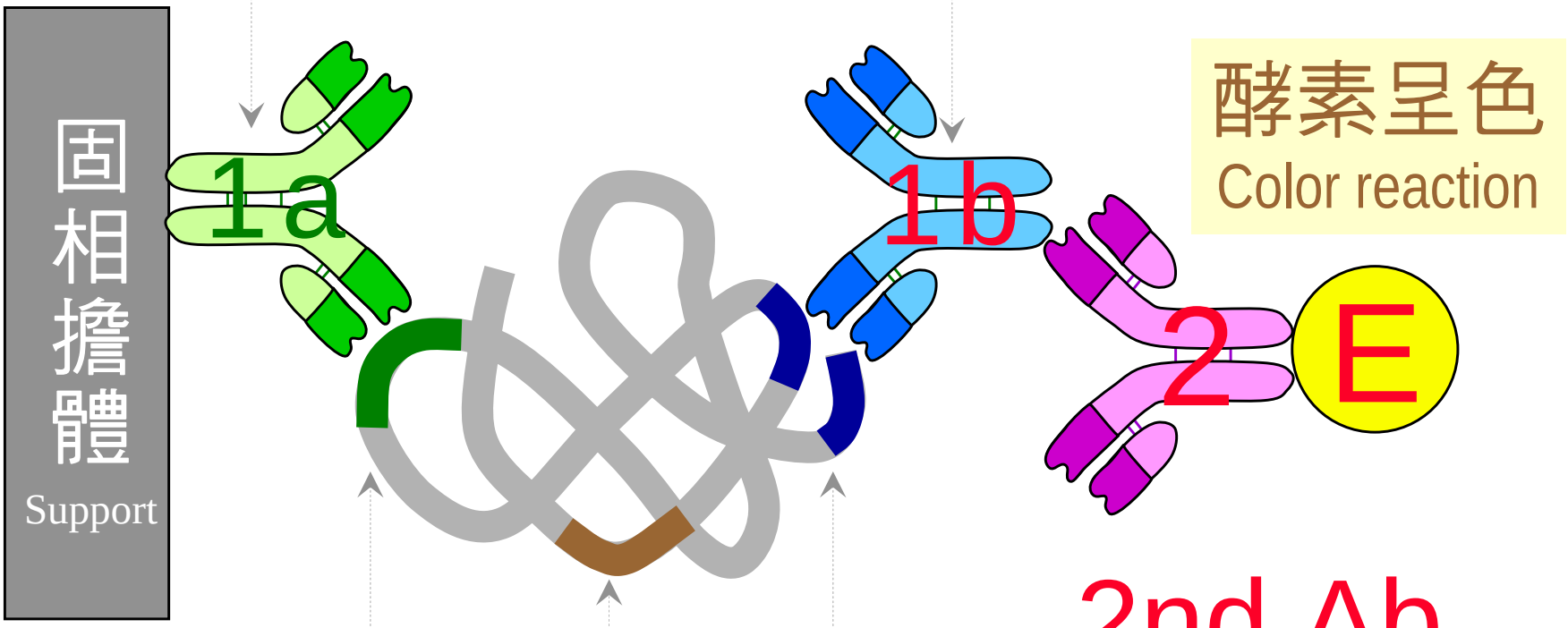


Juang RH (2005) EPA

三明治免疫分析法 Sandwich ELISA method

使用不同動物來源的兩種抗體

Use two Ab from different animal sources



抗原要有多個抗原決定基

Ag contains at least two epitopes

Juang RH (2005) EPA

標誌酵素及呈色劑

Peroxidase

Horse radish peroxidase (HRP) 山葵過氧化酶

Substrate: DAB (brown) 4CN (blue)

Sensitivity: 500 pg

Phosphatase

Alkaline phosphatase (AP) 鹼性磷酸酶

Substrate: BCIP + NBT (blue)

Sensitivity: 100 pg

Substrate: AMPPD (Chemiluminescenct)

Sensitivity: 10 pg (very sensitive)

Enzymes used in 2nd Ab-enzyme conjugates

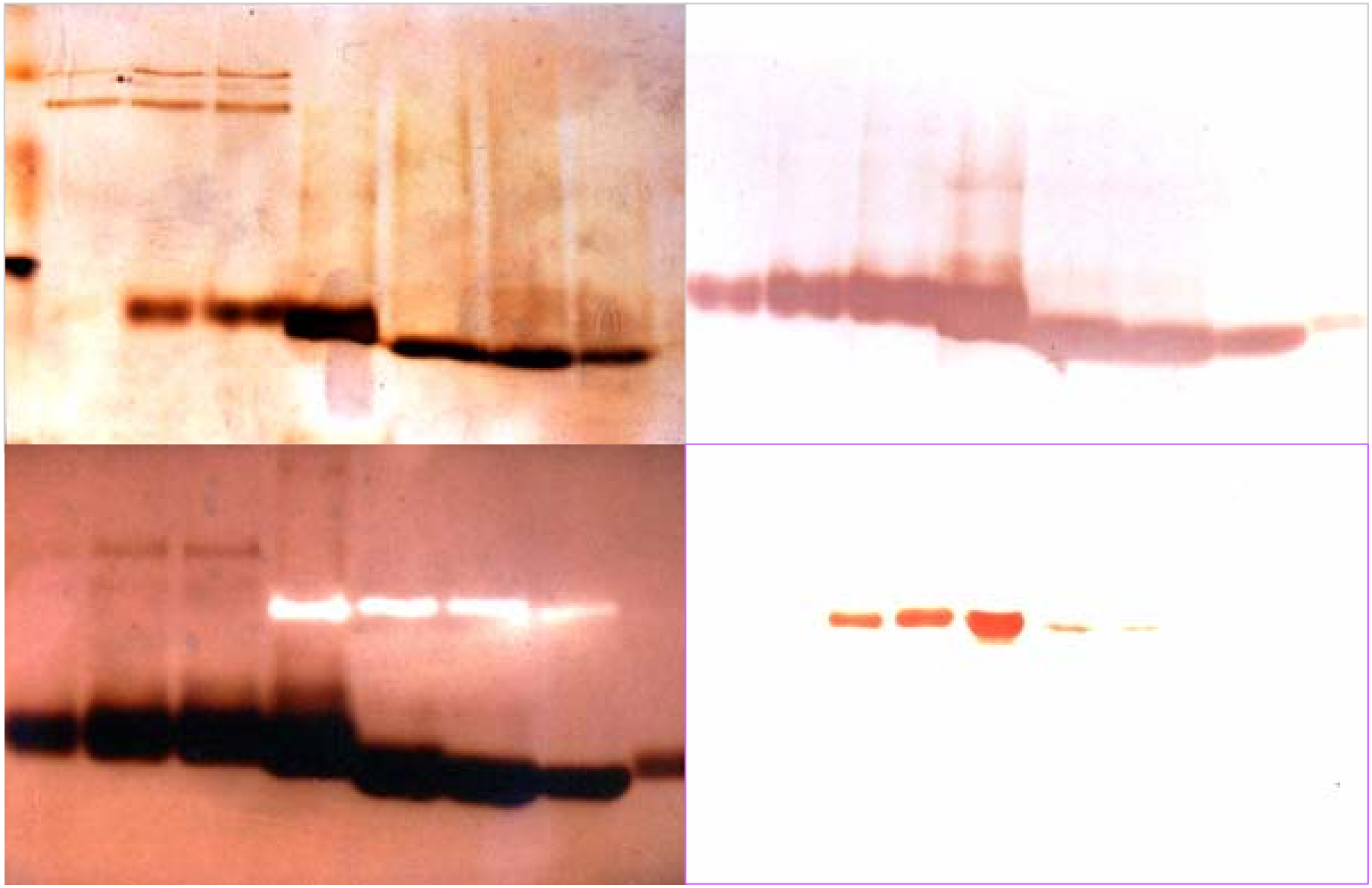
澱粉磷解酶染色方法比較 Staining L-SP

Gel filtration fractions

Immunostaining

Silver staining

Activity staining



Disc-PAGE

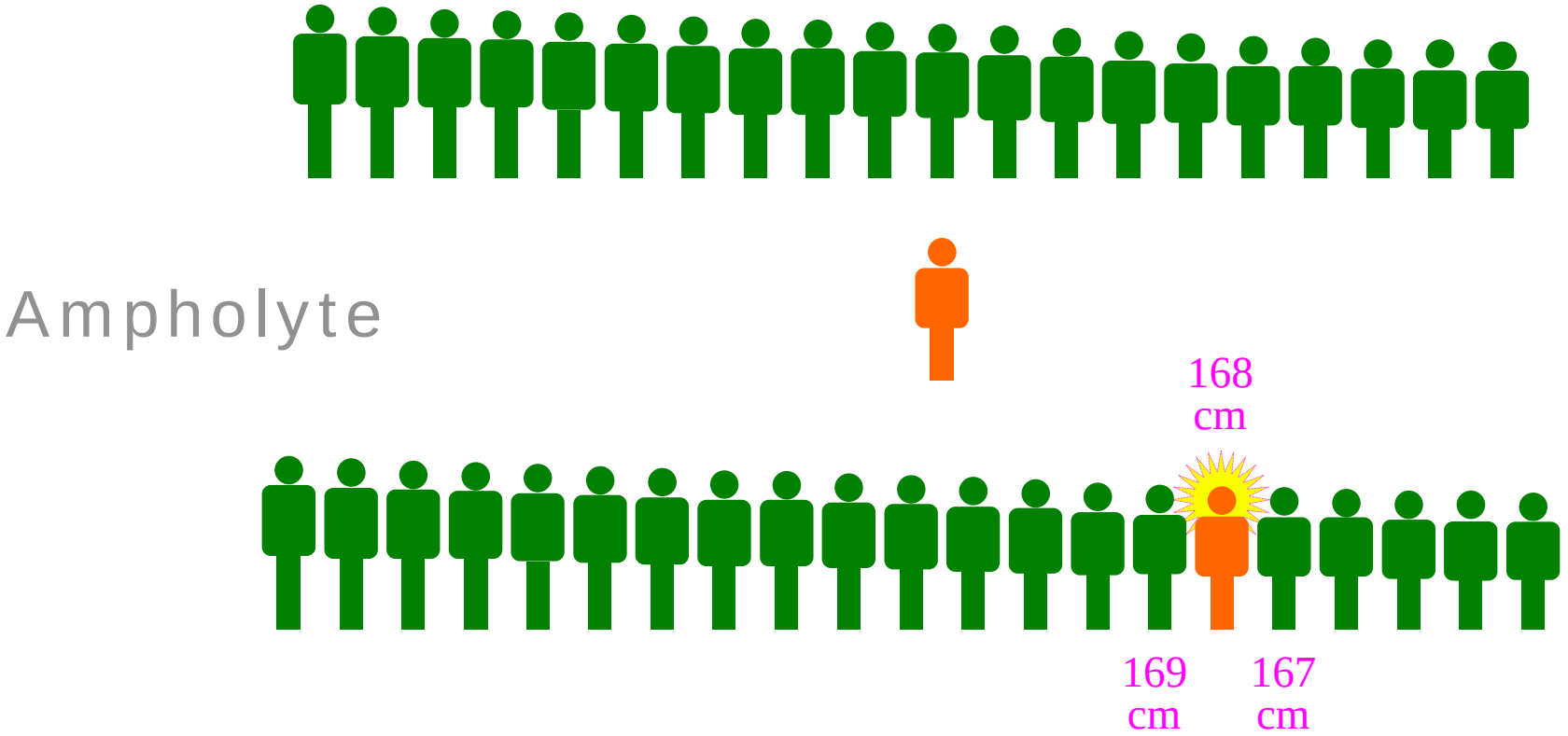
SDS-PAGE

Juang RH (2005) EPA

3.3.2 等電焦集原理 Principle for isoelectric focusing

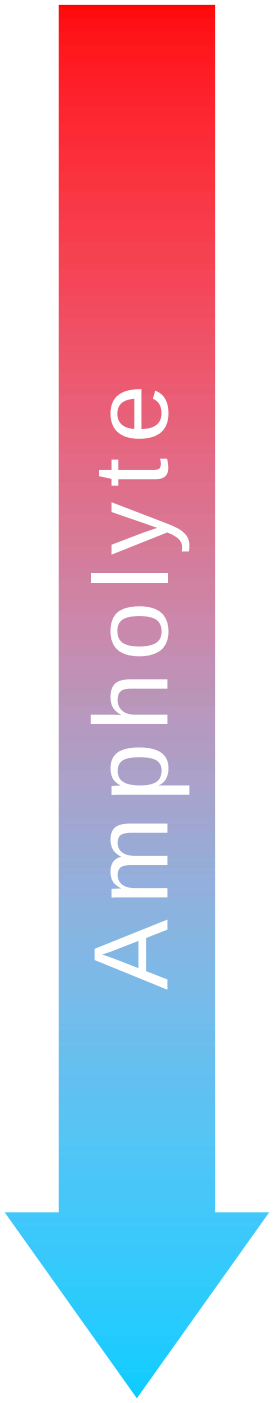
樣本分子 在一已知 pH 梯度 中焦集

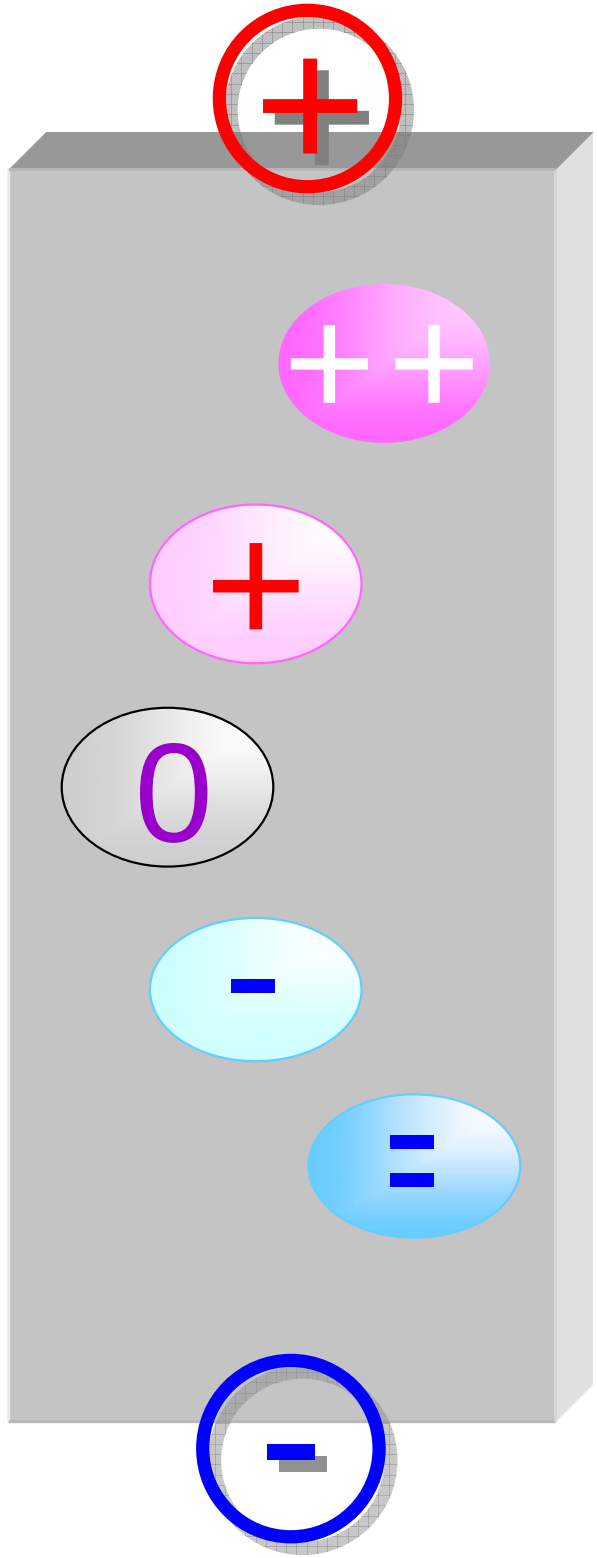
Sample molecules are focused in a preformed pH gradient



Juang RH (2005) EPA

等電焦集法的焦集方式





在低 pH 處帶正電被排斥

At lower pH gel, sample is positively charged and repelled

焦集在等電點

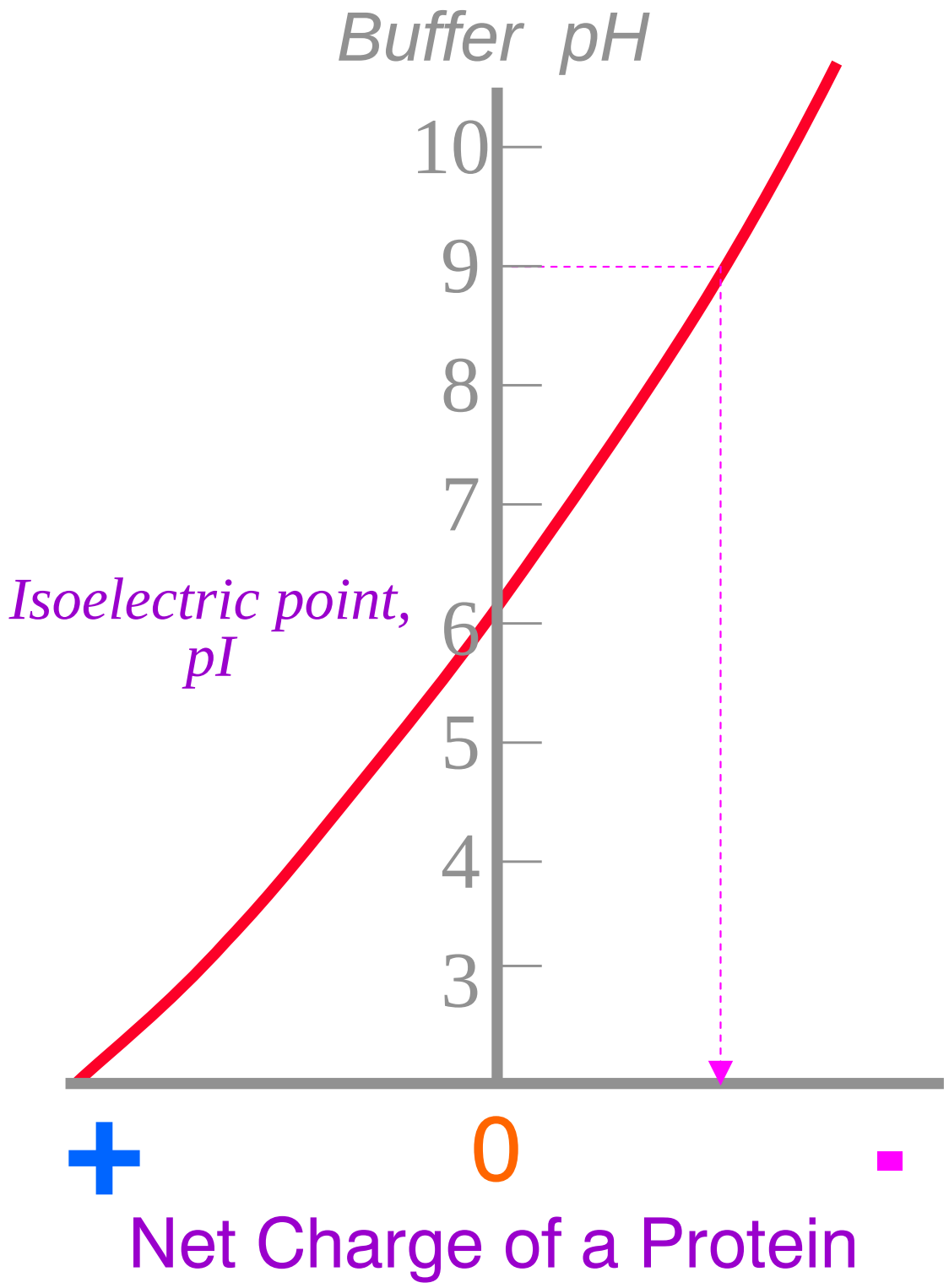
Focused at its pI

在高 pH 處帶負電亦被排斥

At higher pH, sample is negatively charged and also repelled to move upward

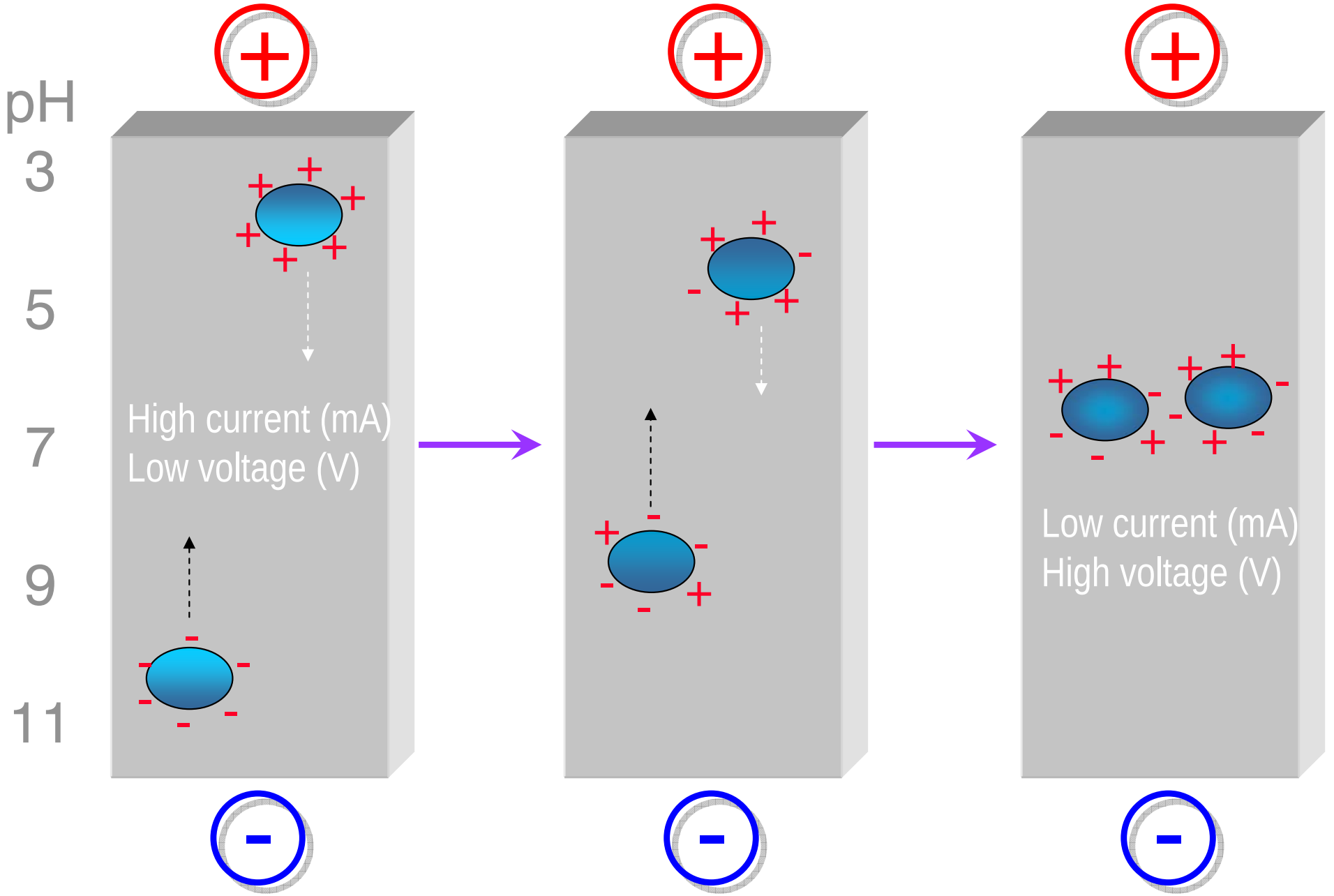
Juang RH (2005) EPA

環境影響分子的帶電性質



Juang RH (2005) EPA

等電焦集的運作機制 Action mechanism of IEF



Adapted from Alberts et al (2002) Molecular Biology of the Cell (4e) p.487

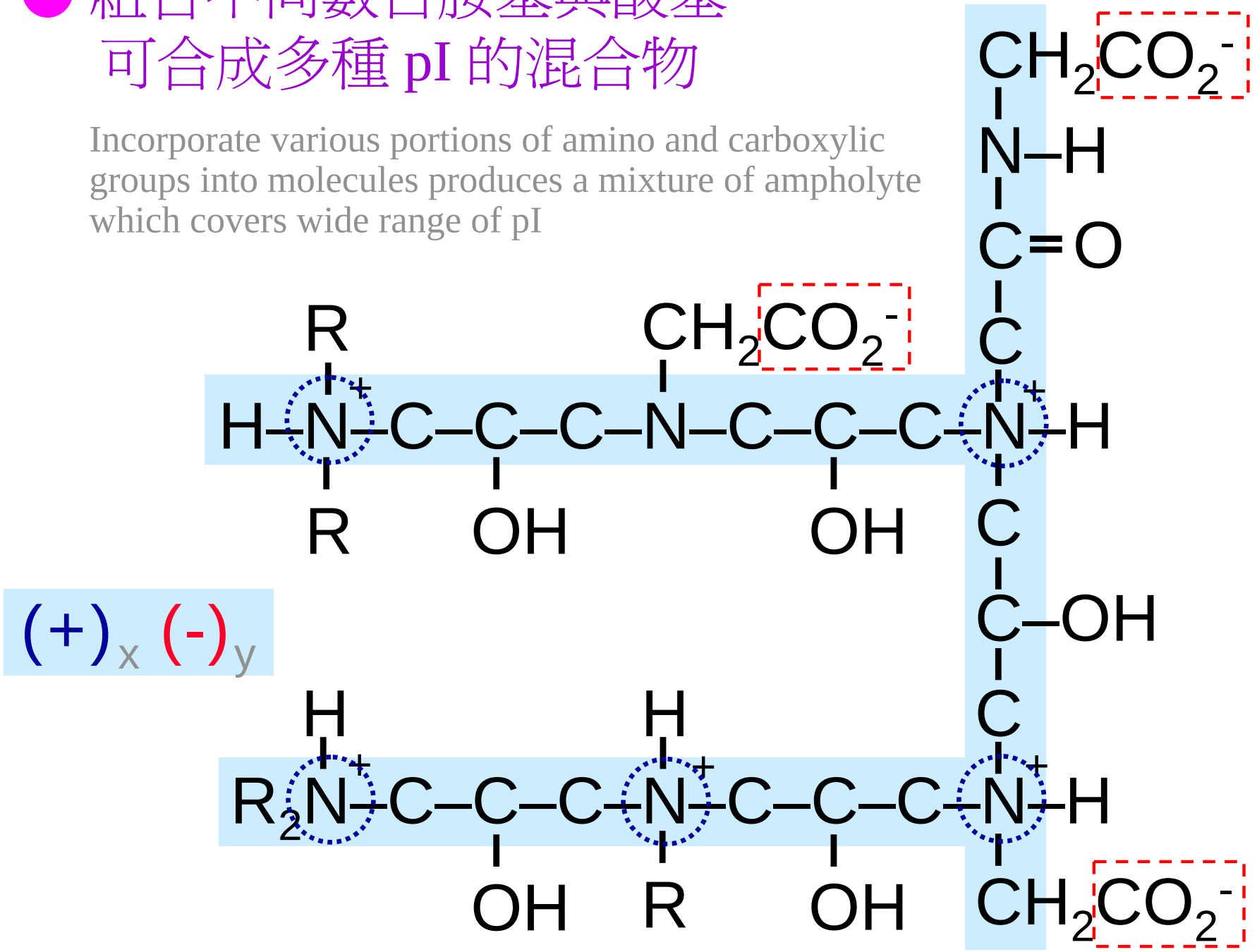
Sample can be applied at any place on the gel

Juang RH (2005) EPA

等電焦集法 雙性離子構造

● 組合不同數目胺基與酸基
可合成多種 pI 的混合物

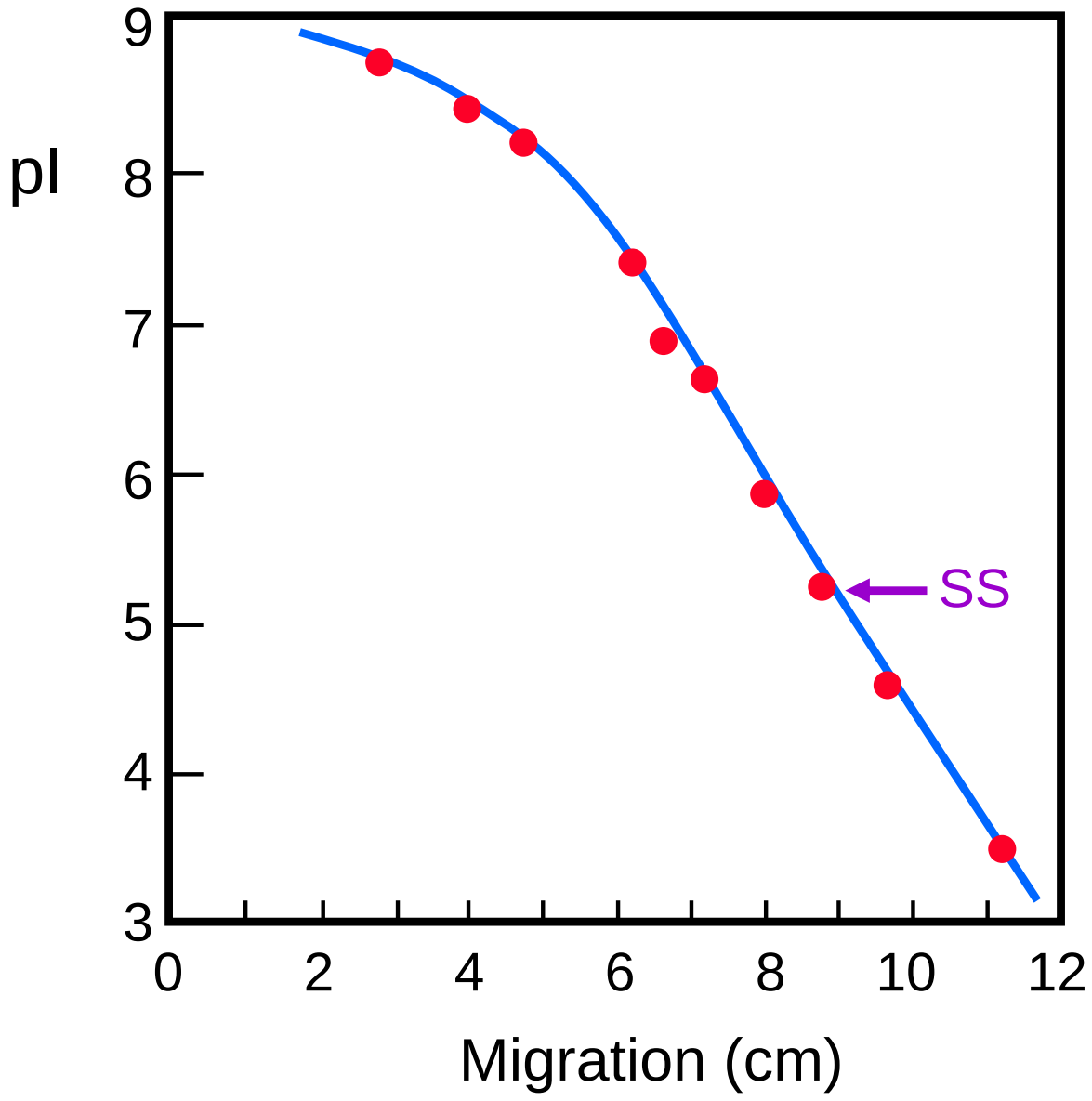
Incorporate various portions of amino and carboxylic groups into molecules produces a mixture of ampholyte which covers wide range of pI



The mixture of ampholytes are synthesized by combinatory chemistry

Juang RH (2005) EPA

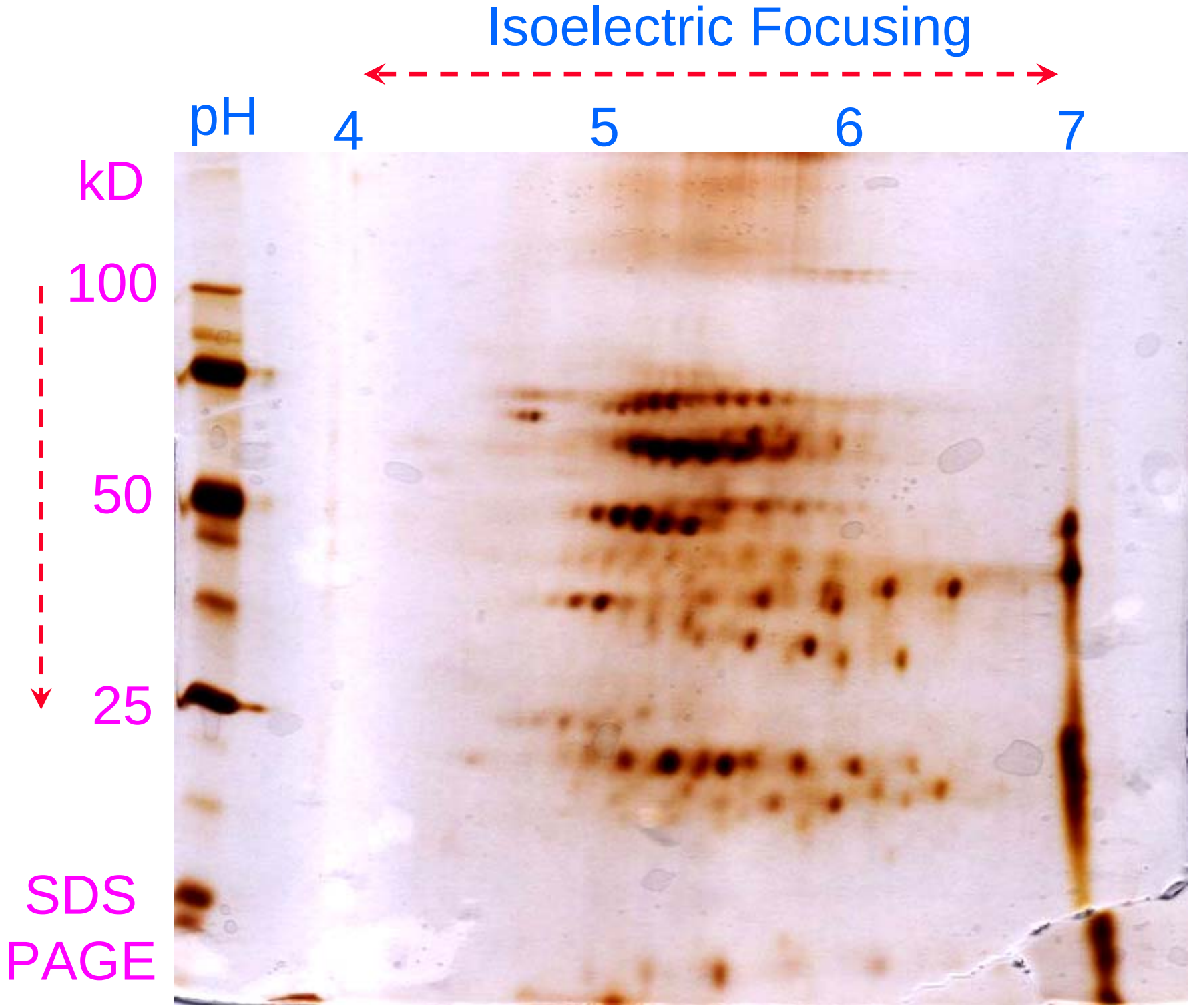
等電點標準校正線 Standard curve for IEF



Standard curve is established by proteins with known pI

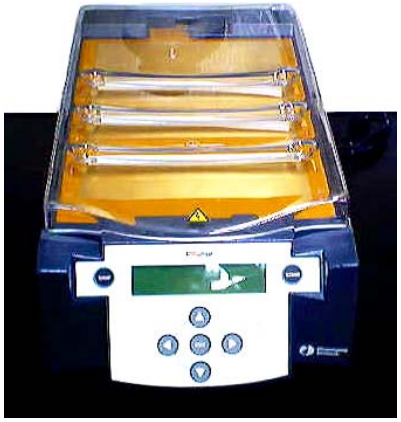
Juang RH (2005) EPA

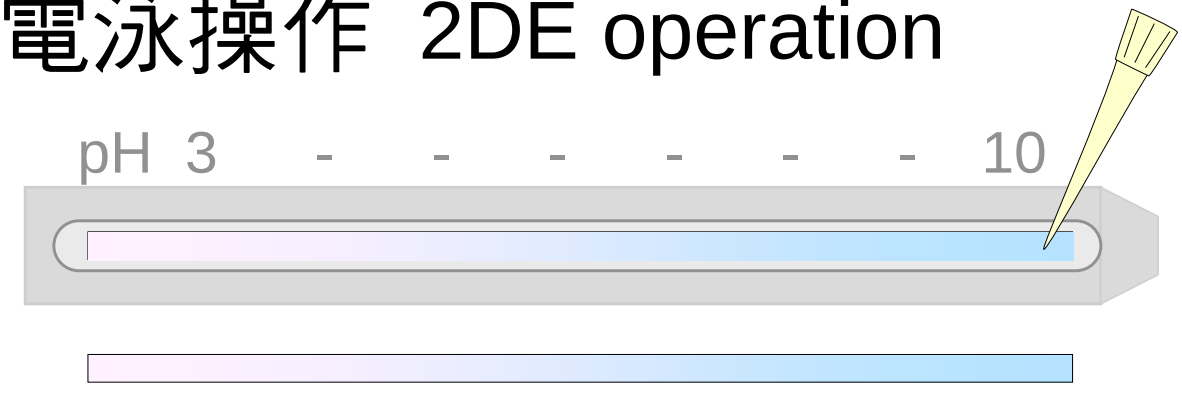
二次元電泳

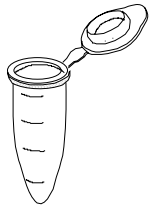

Isoelectric Focusing
pH
4
5
6
7
kD
100
50
25
SDS
PAGE
Juang RH (2005) EPA

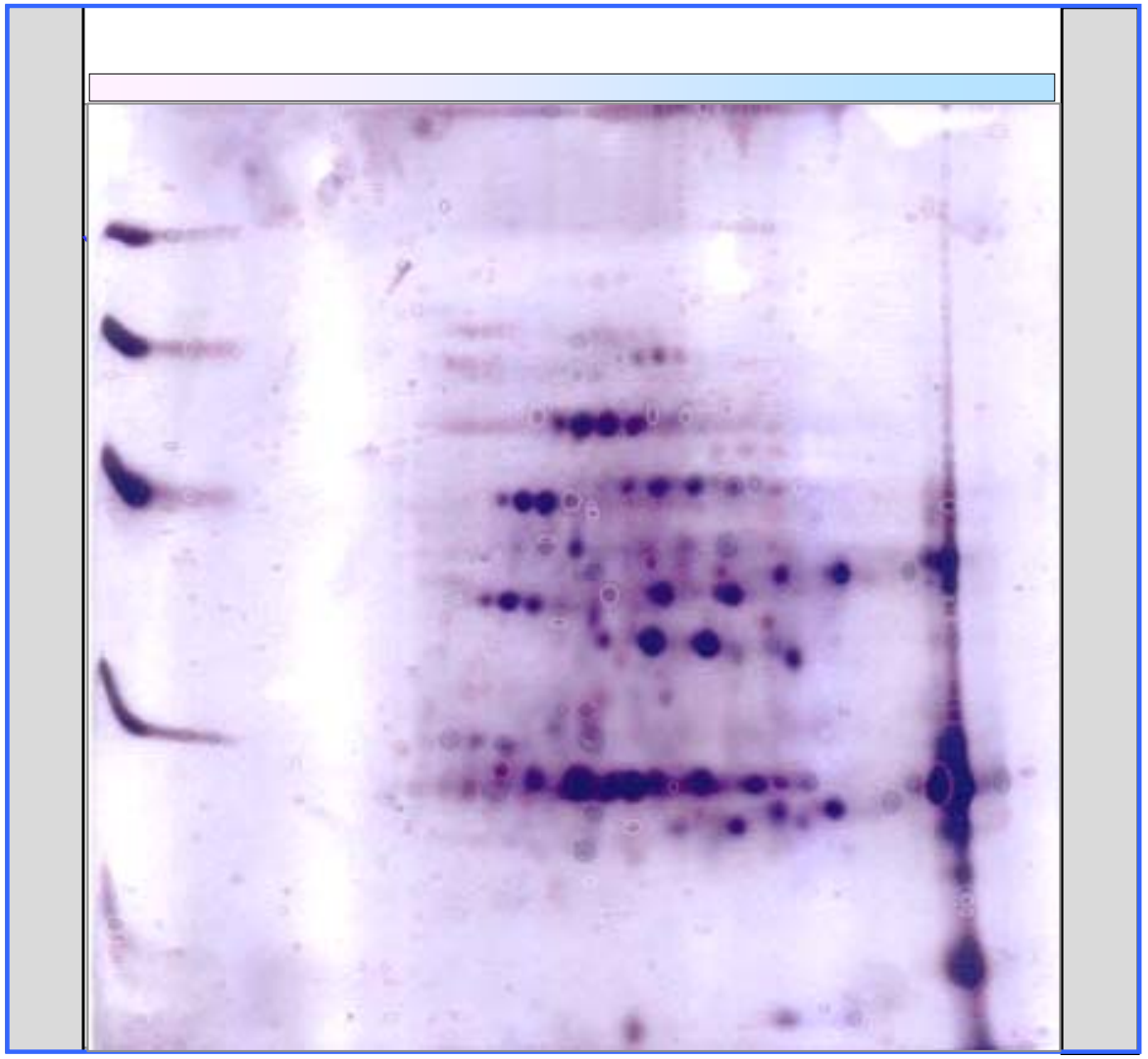
二次元電泳操作 2DE operation

(1) IEF
等電焦集電泳









(2)
SDS-PAGE
分離膠體

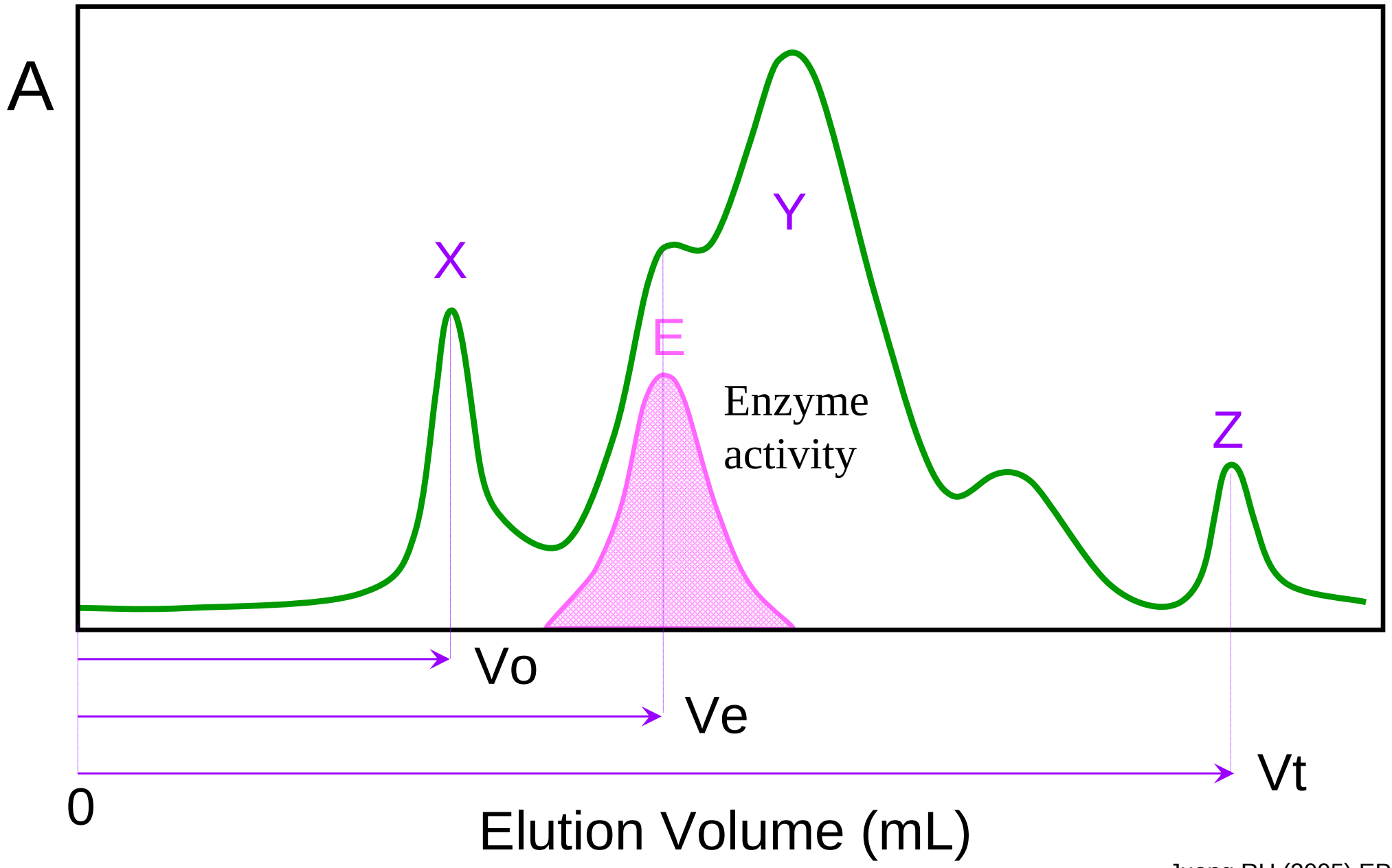
(3)
Staining
染色脫色

Juang RH (2005) EPA

4 分子量決定法 Molecular weight determination

- 4.1 膠体過濾法 Gel filtration
  依蛋白質分子量的大小測定
- 4.2 梯度電泳法 Gradient PAGE
  可佐證分子量的測定
- 4.3 其它分子量測定方法 Other methods
  超高速離心法 Ultracentrifugation
  由胺基酸序列計算 Deduced for amino acid sequence
  質譜儀分析 Mass spectrometry

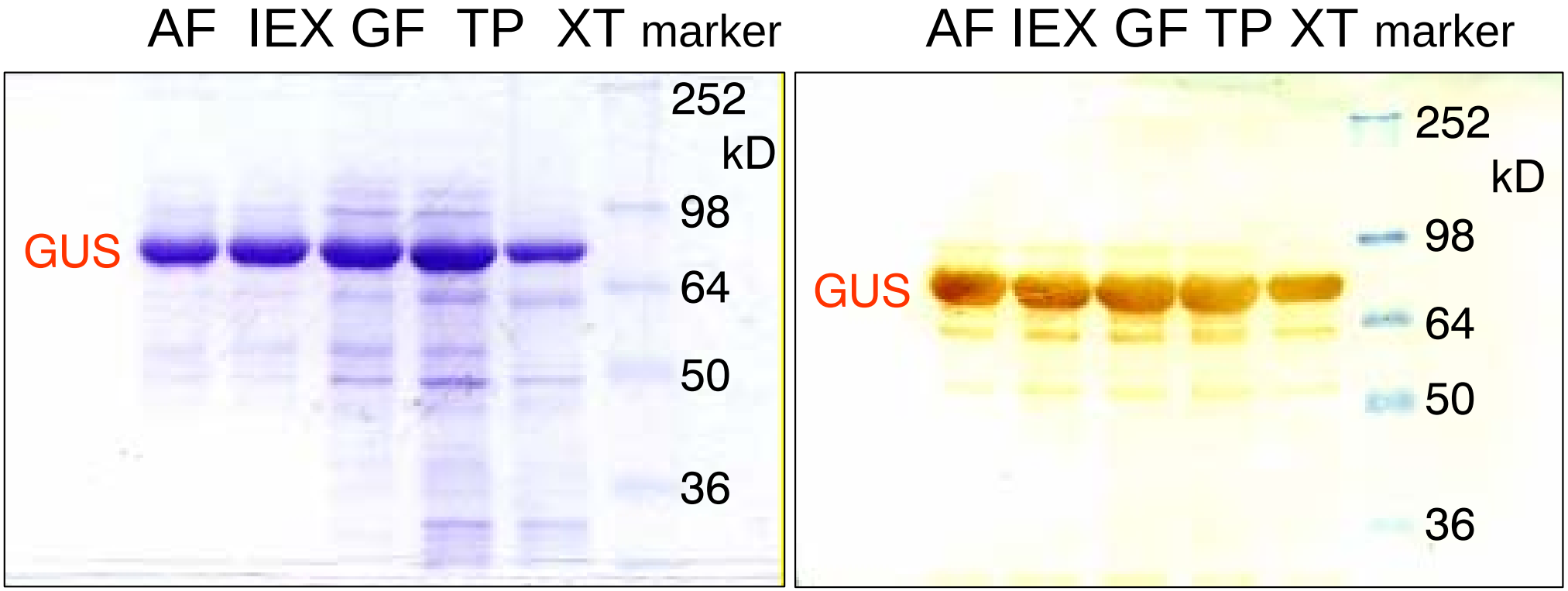
膠体過濾法依原態分子量分離 Native MW



Juang RH (2005) EPA

以電泳及抗體檢定表現蛋白質 GUS

← 純化步驟順序 Purification steps



10% SDS-PAGE

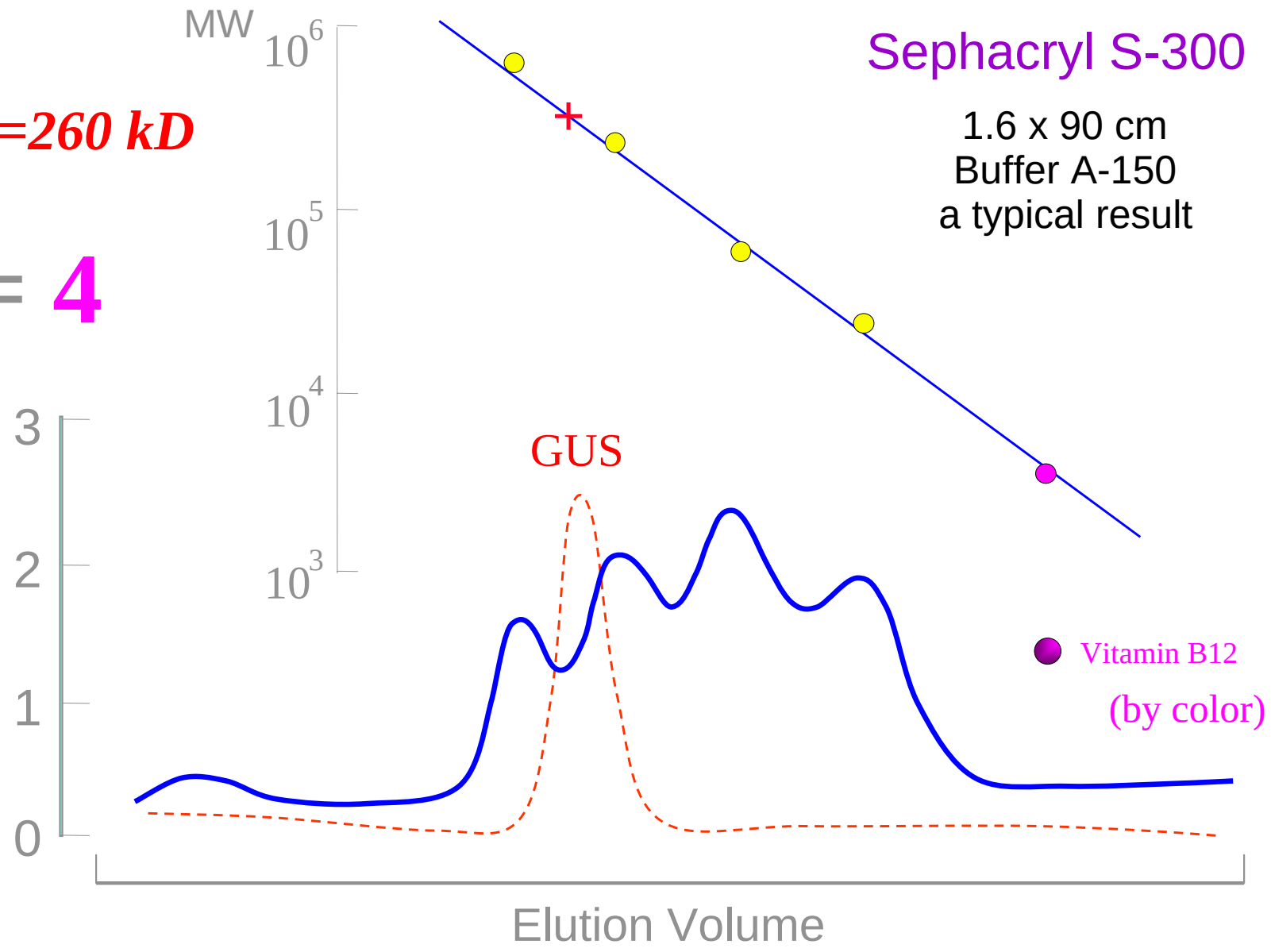
Western Transfer

MW = 70 kD

Expressed protein GUS is detected by SDS-PAGE and immunostaining

Juang RH (2005) EPA

以膠體過濾法求得 GUS 原態分子量



MW=260 kD

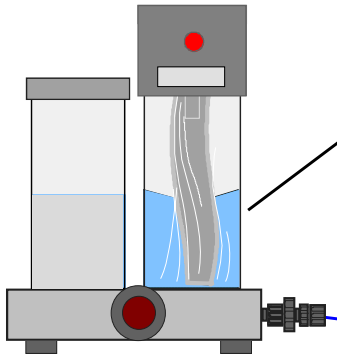
$$\frac{260\ kD}{70\ kD} = 4$$

Native molecular weight of GUS is determined by gel filtration

Juang RH (2005) EPA

梯度電泳片的製備 Prepare the gradient gel

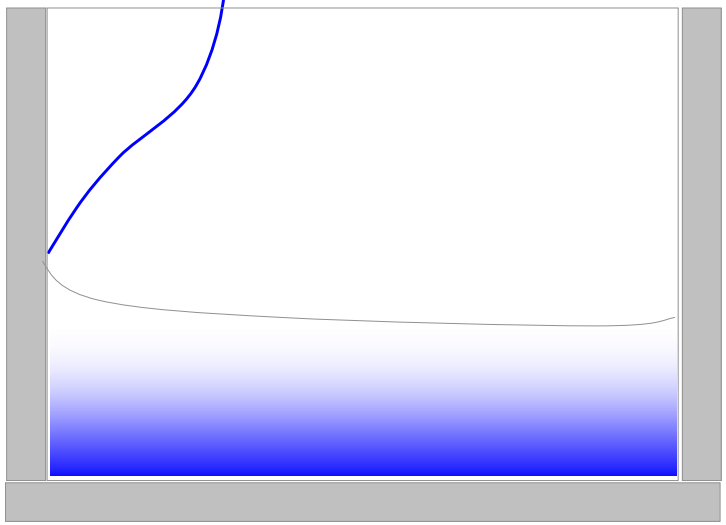
Gradient mixer



若在高限溶液中
加入少量染劑
Blue dye added in the upper limiting solution

5% 20%

Upper-limiting solution



由下方開始
注入梯度溶液
Start the gradient from the bottom

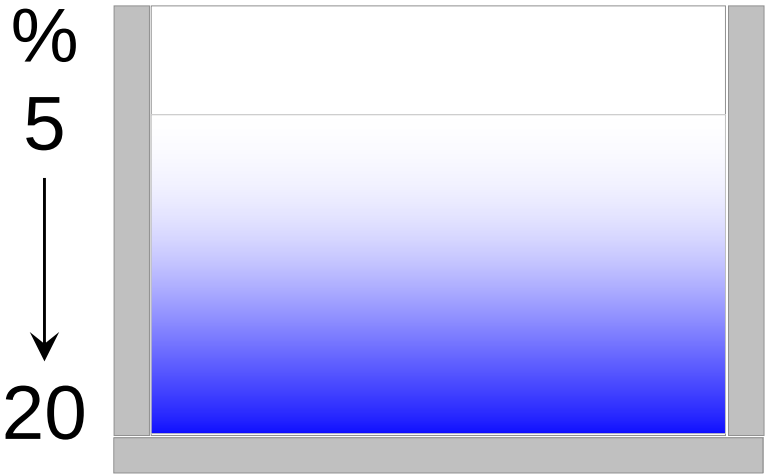
Determine MW by native-PAGE

(1) Sample protein pI < 8.0

(2) Use gradient gel

(3) Longer running time

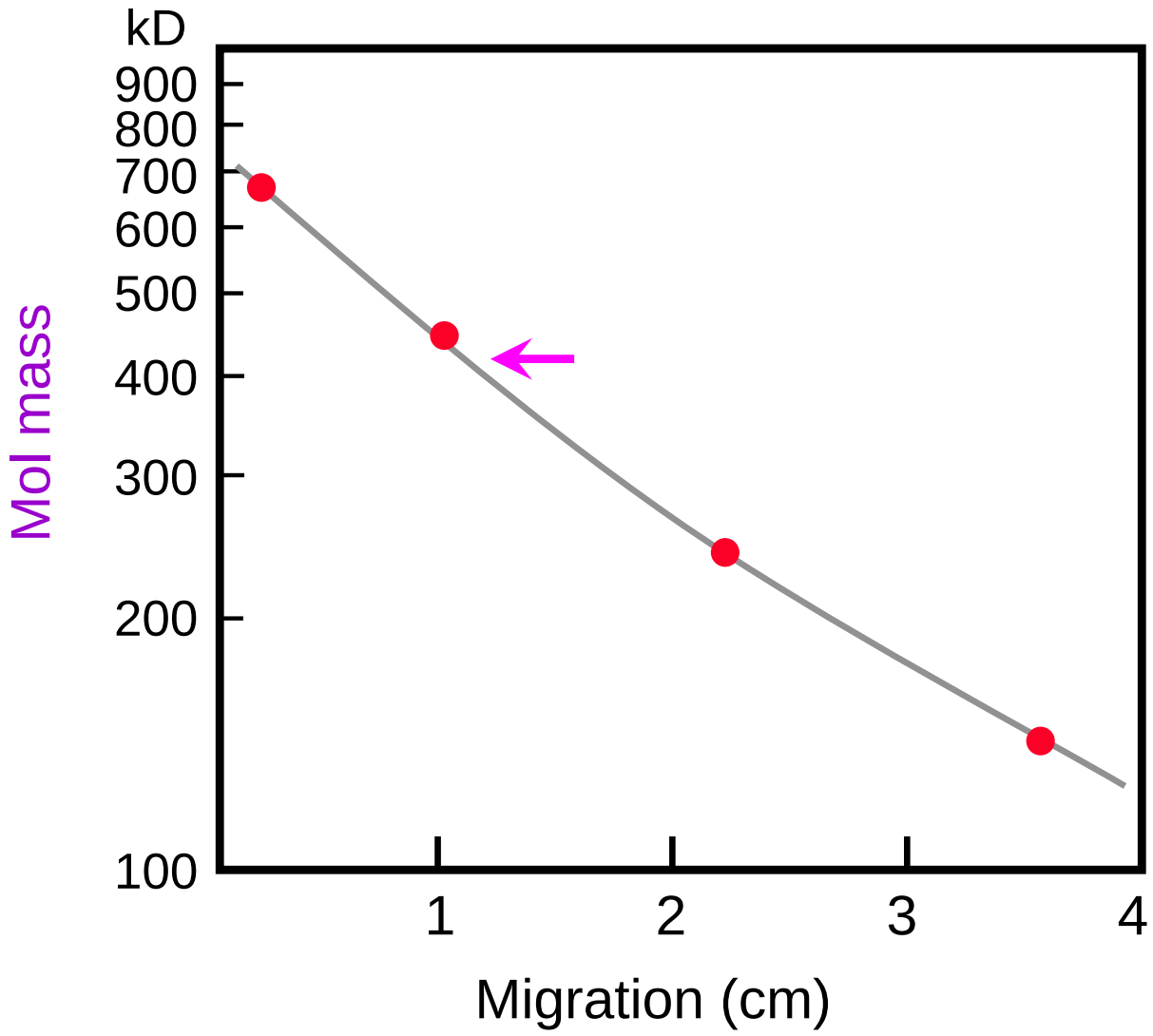
可觀察所拉梯度是否均勻
The blue color shows how the gradient formed in the gel

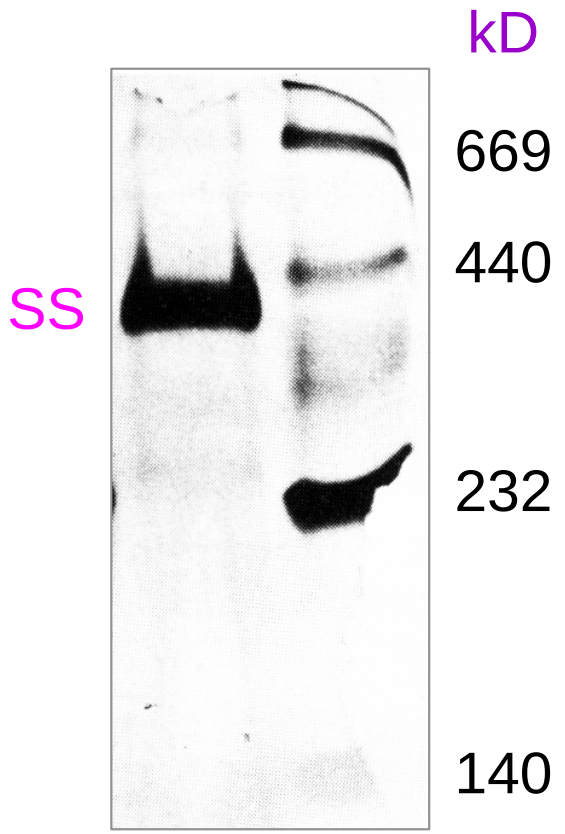


You can use commercial pre-cast gradient gel (various gel percentage)

Juang RH (2005) EPA

原態分子量 Gradient PAGE for MW determination



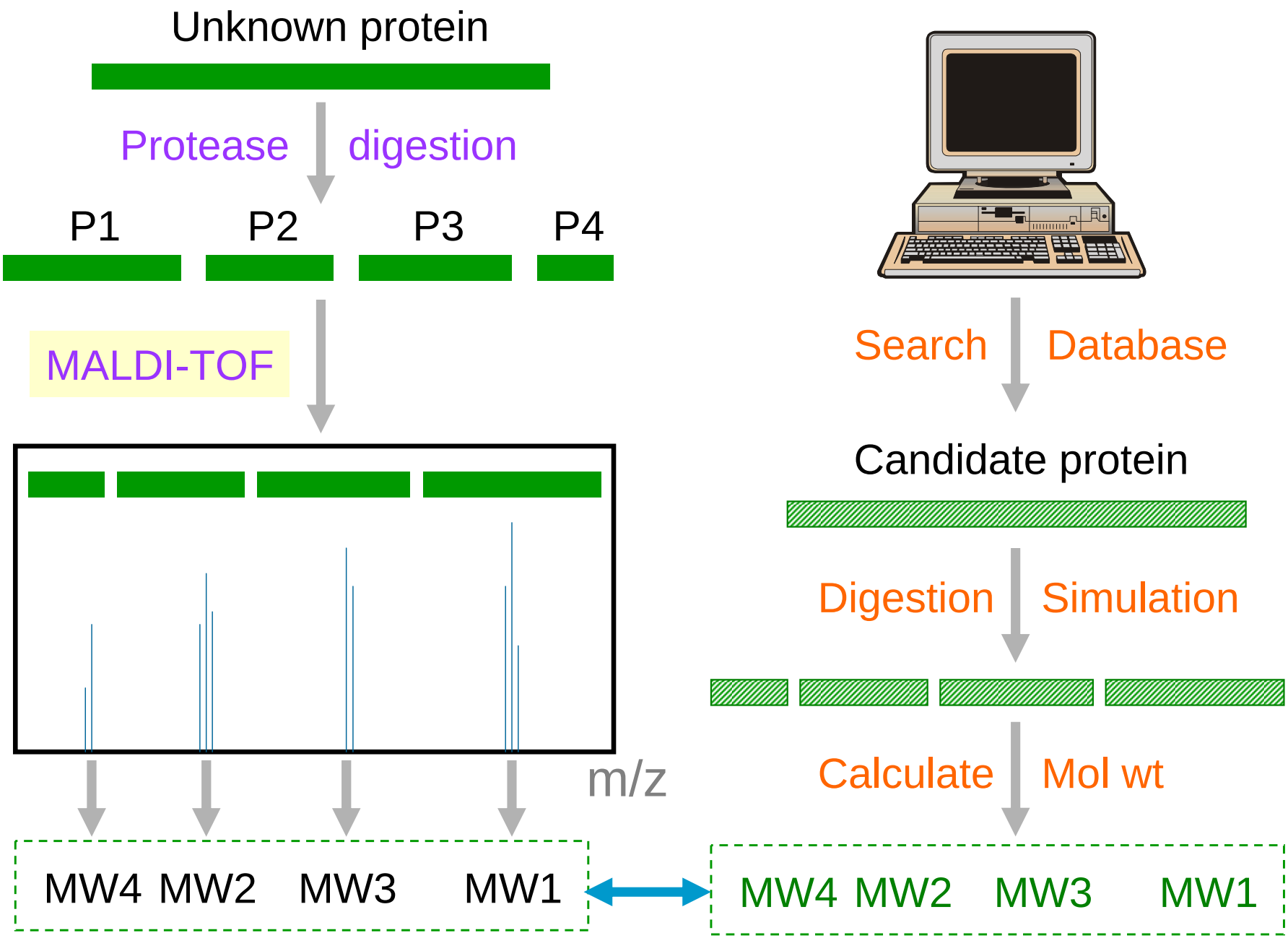


- 不能以 disc-PAGE 為唯一分子量證據

You can't take disc-PAGE as your only evidence for MW determination

Juang RH (2005) EPA

質譜儀可檢定蛋白質身分

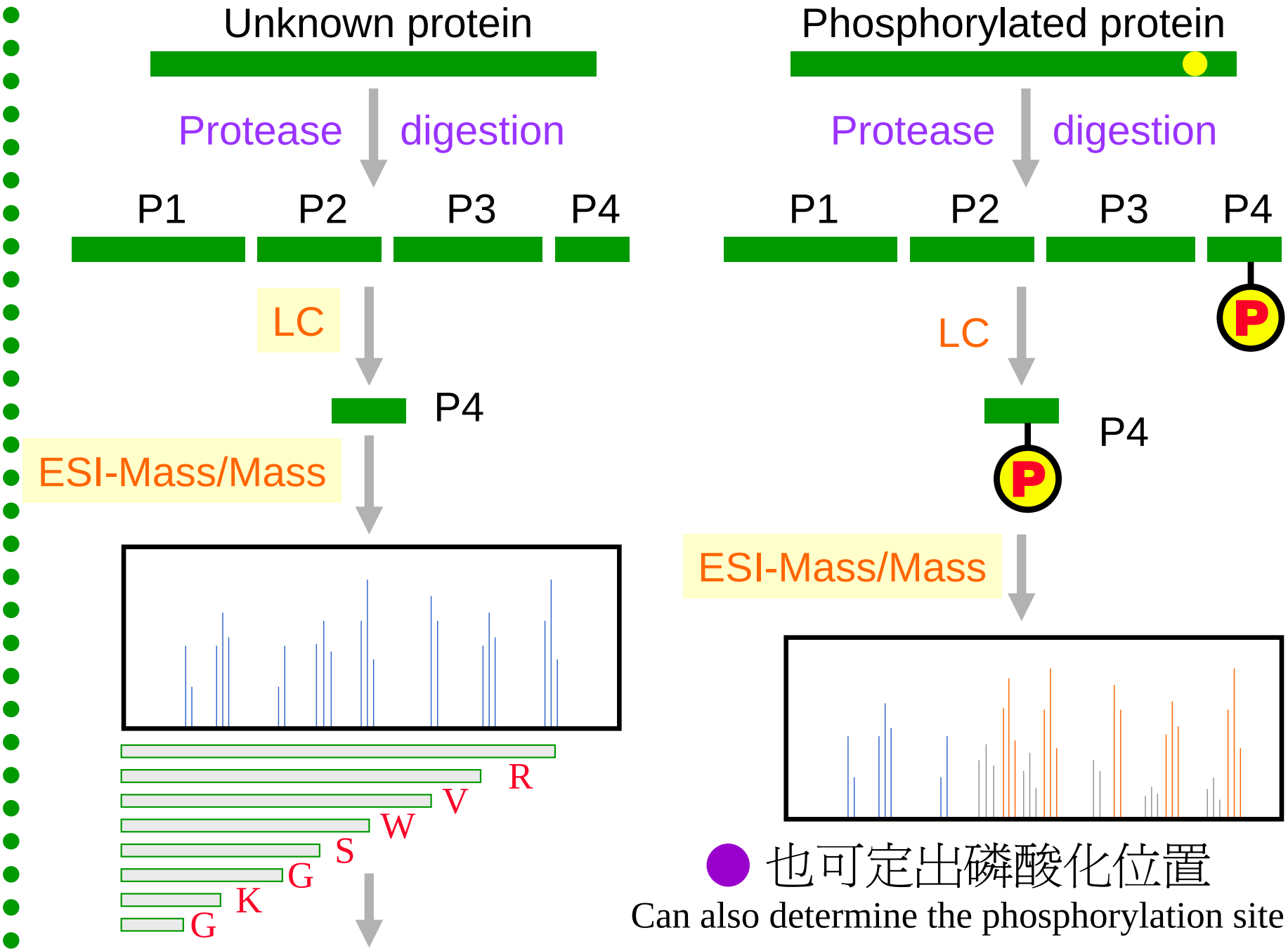


● 比對各片段分子量可確定該蛋白質身分

Tryptic fragments identified by MALDI-TOF could identify an unknown protein

Juang RH (2005) EPA

以質譜儀進行蛋白質序列分析



● 也可定出磷酸化位置

Can also determine the phosphorylation site

● 質譜儀可直接定序

Determine the amino acid sequence by LC/MS/MS

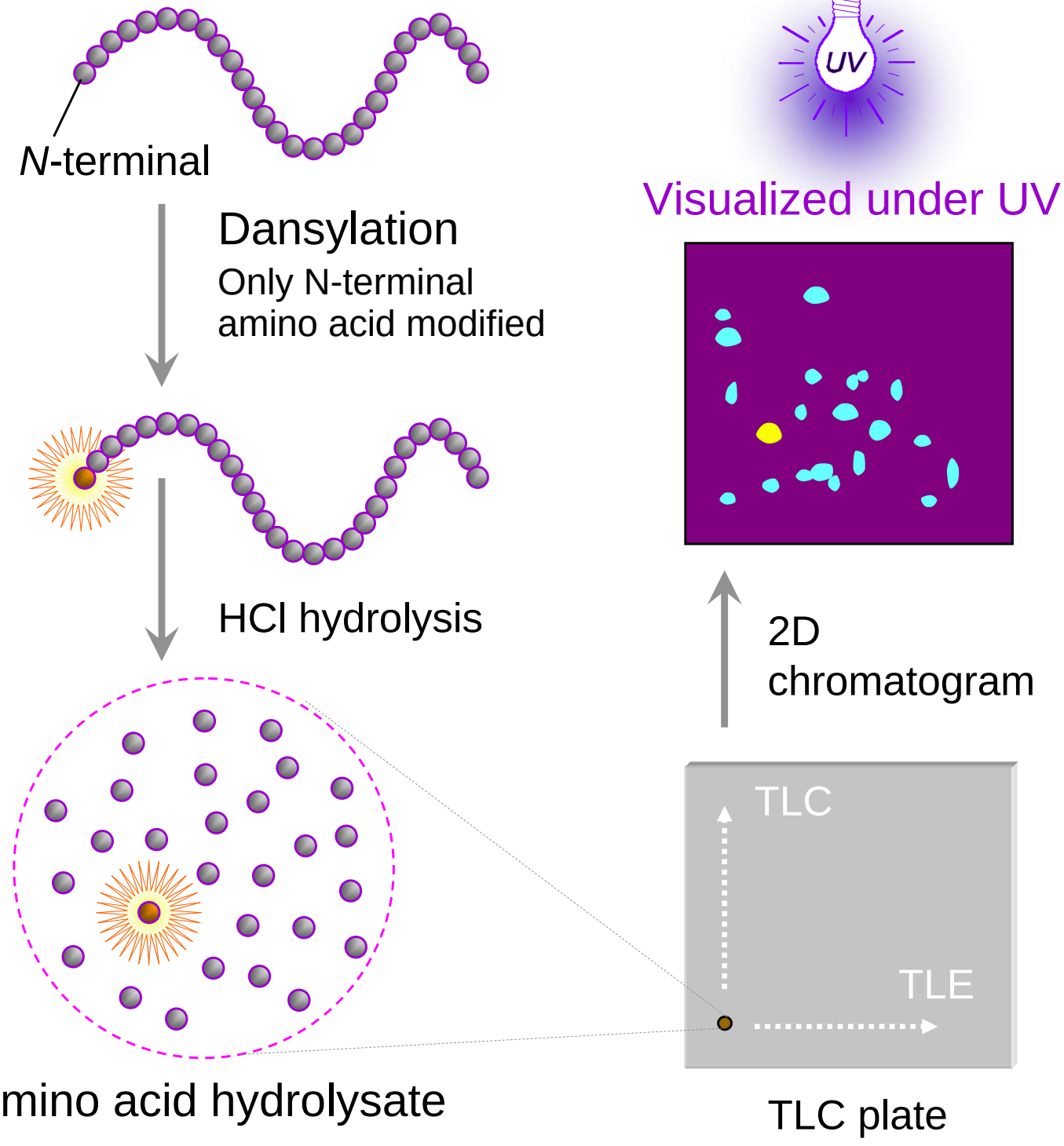
Juang RH (2005) ECX

5 蛋白質構造與組成分析 Protein structure analysis

- 5.1 N-端或 C-端胺基酸 Terminal determination
  通常都直接定序但 C-端較爲困難
- 5.2 胺基酸組成分析 Amino acid composition
- 5.3 胺基酸定序法 Amino acid sequence
  - 5.3.1 From cDNA sequence
  - 5.3.2 Edman degradation or ESI/MS/MS
- 5.4 胜肽圖譜 Peptide mapping
- 5.5 其它相關方法 Other methods

Some old techniques become very useful in modern proteomic study

决定N端胺基酸



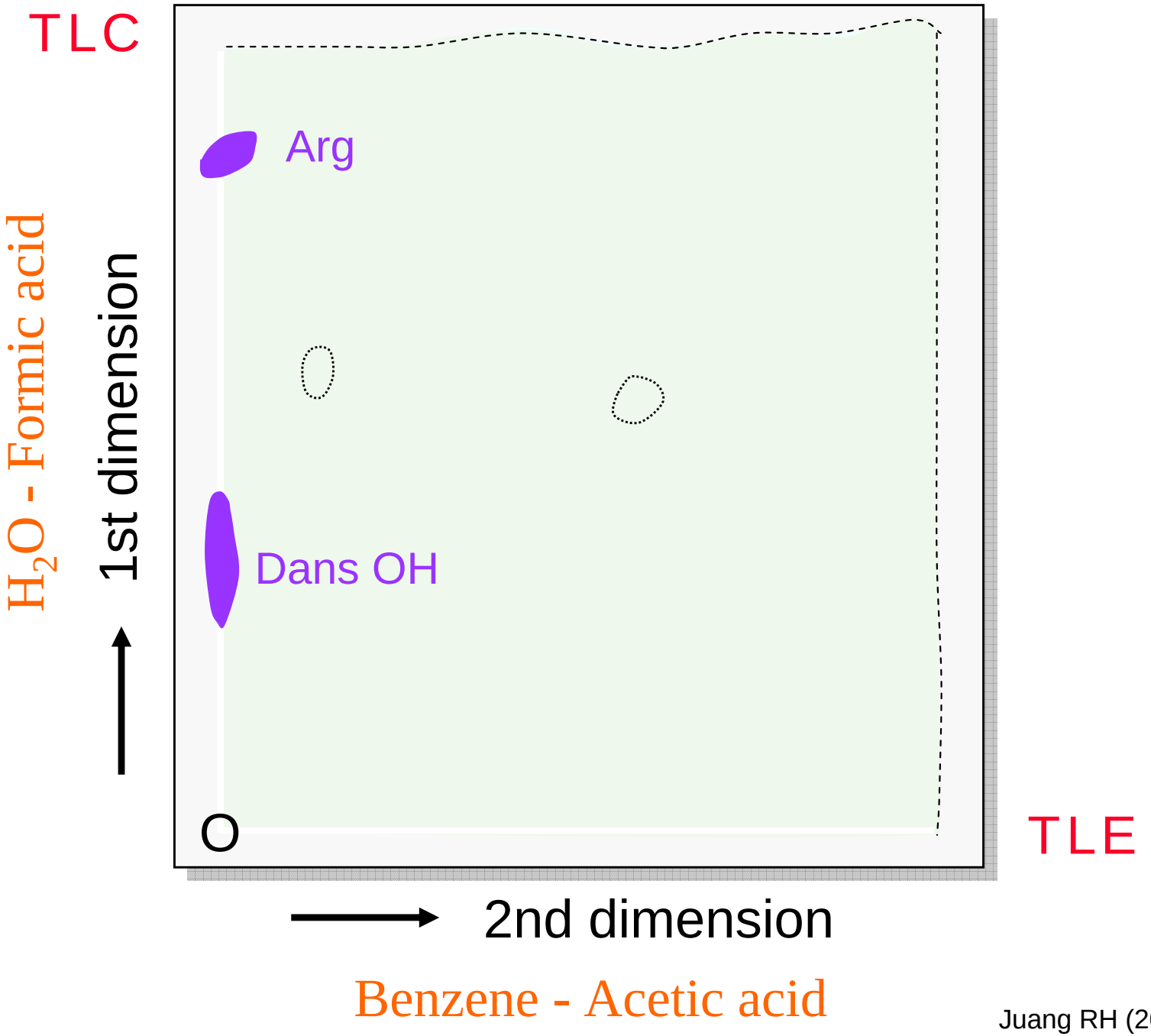
Determine the N-terminal amino acid by dansylation

Juang RH (2005) EPA

以薄層層析法鑑定胺基酸

二次元薄層層析電泳可分離並檢定二十種胺基酸

20 amino acids are separated and identified on 2D TLC/TLE



Juang RH (2005) EPA

蛋白質酸性水解 Total acid hydrolysis of protein

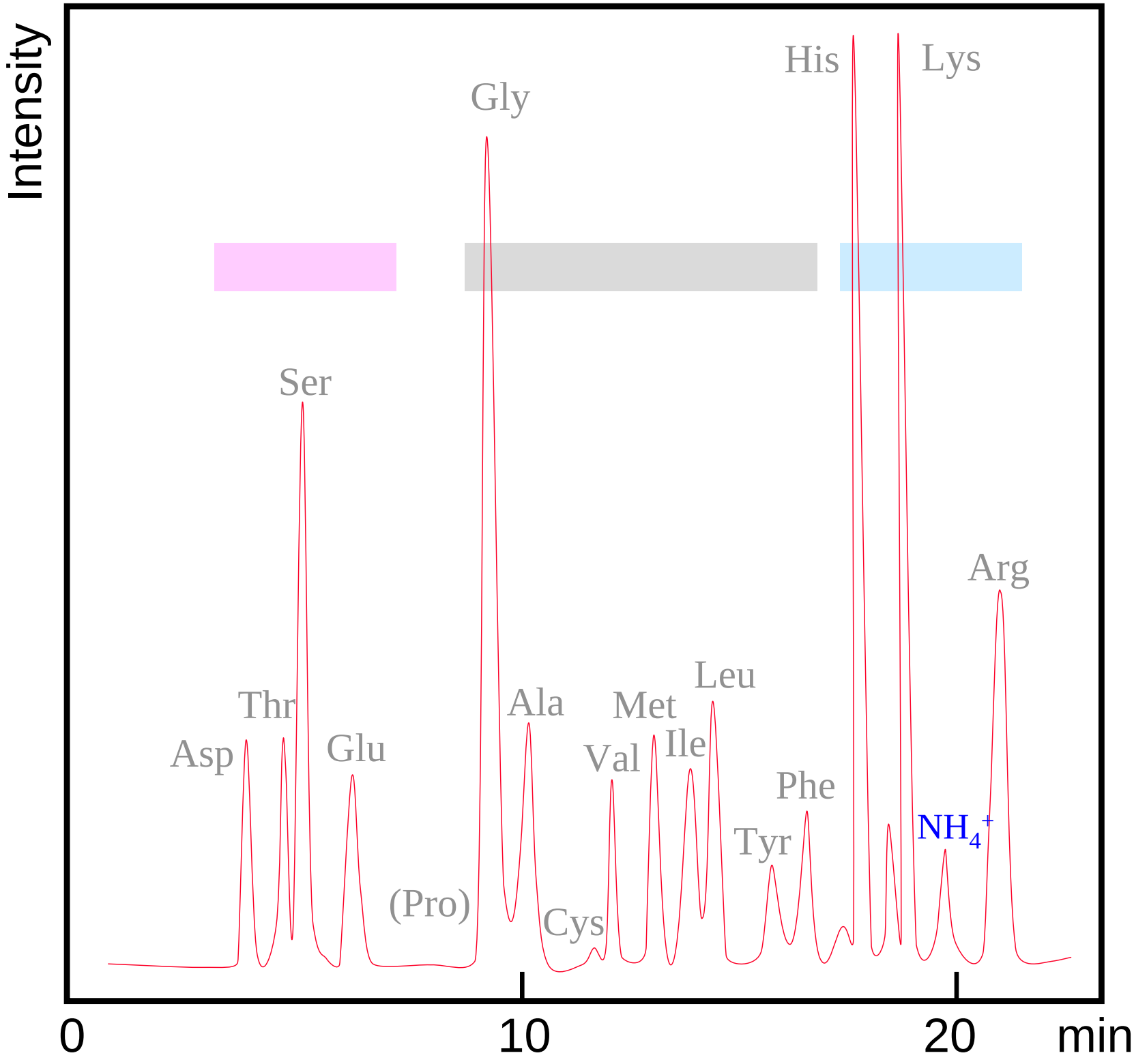
- Reagent: 6 N HCl or 4 N methanesulfonic acid
- Condition: 110C, 24 hours, under vacuum
- Detection: by HPLC (ion exchange) next slide
- Notice: Some amino acids are destroyed (Trp)

Cys-Cys broken to Cys

Gln & Asn are acidified to Glu & Asp

(Glu + Gln → Glx; Asp + Asn → Asx)

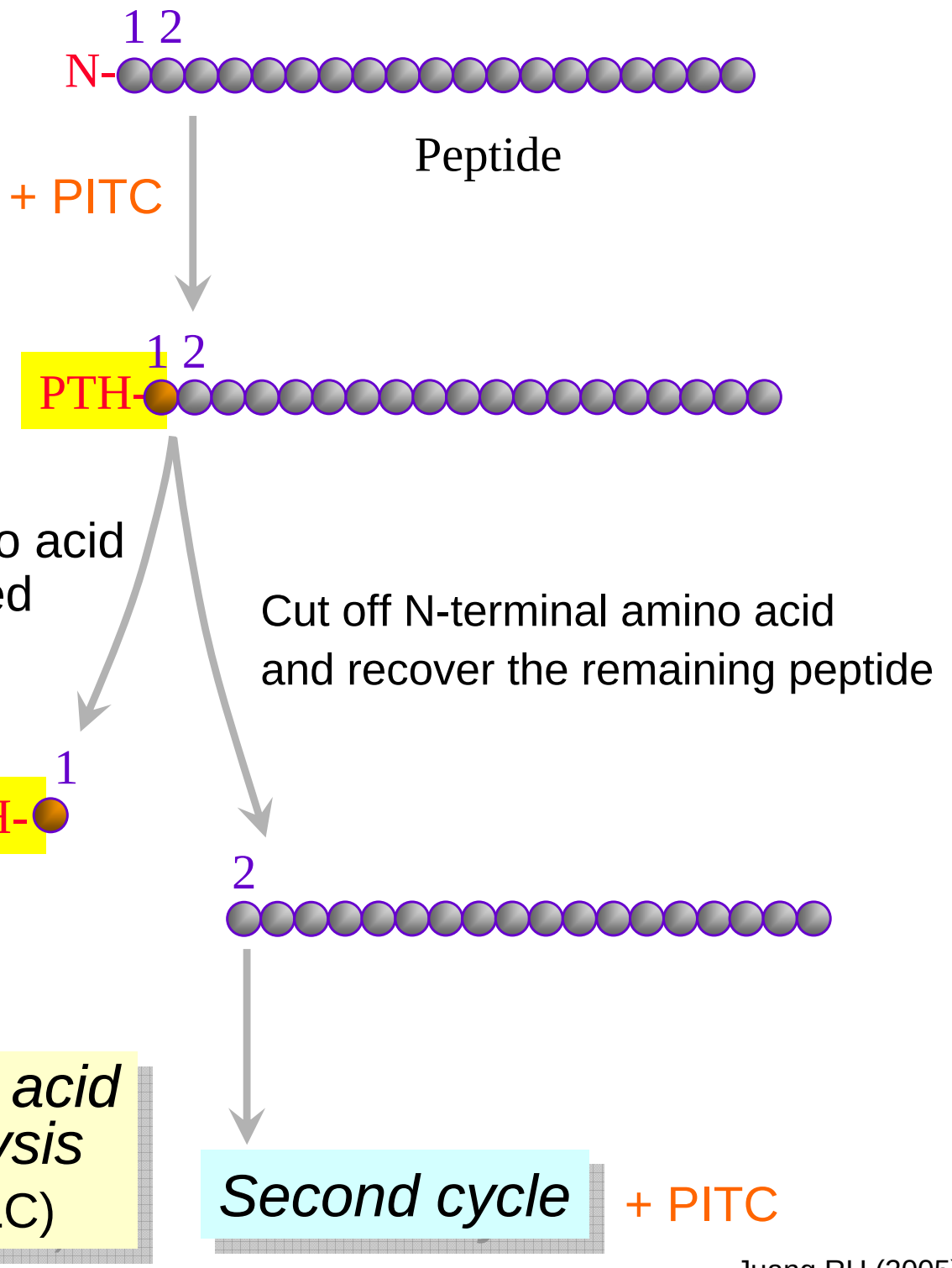
以液相層析分離鑑定胺基酸



Juang RH (2005) EPA

決定胺基酸序列

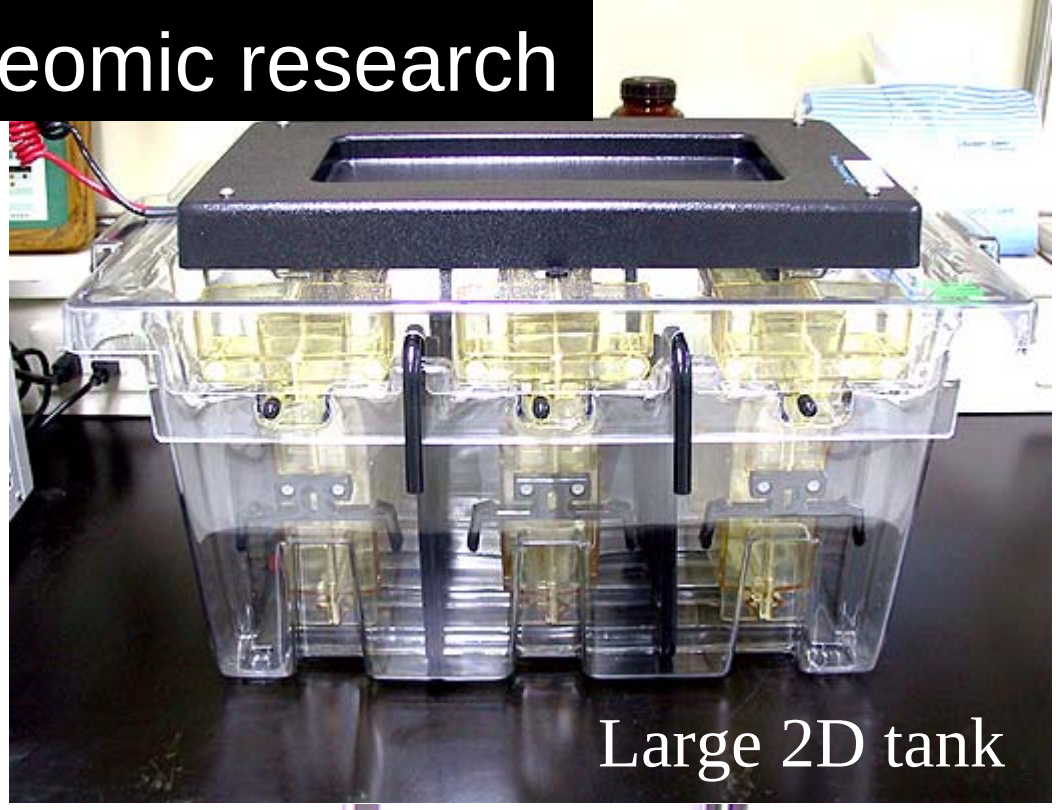
Edman Degradation
for amino acid sequencing



Juang RH (2005) EPA

Tools for proteomic research

LC/MS/MS



Large 2D tank

Amino acid sequence analyzer

Juang RH (2005) EPA

5.4 胜肽圖譜 Peptide mapping

5.4.1 蛋白質專一性水解 Specific proteolysis

- 專一性內切酶 Specific *endo*-peptidase

  Trypsin, Chymotrypsin, *Sa* protease

- 化學反應法 Chemical method

  CNBr

5.4.2 胜肽群檢定方法 Identify peptides

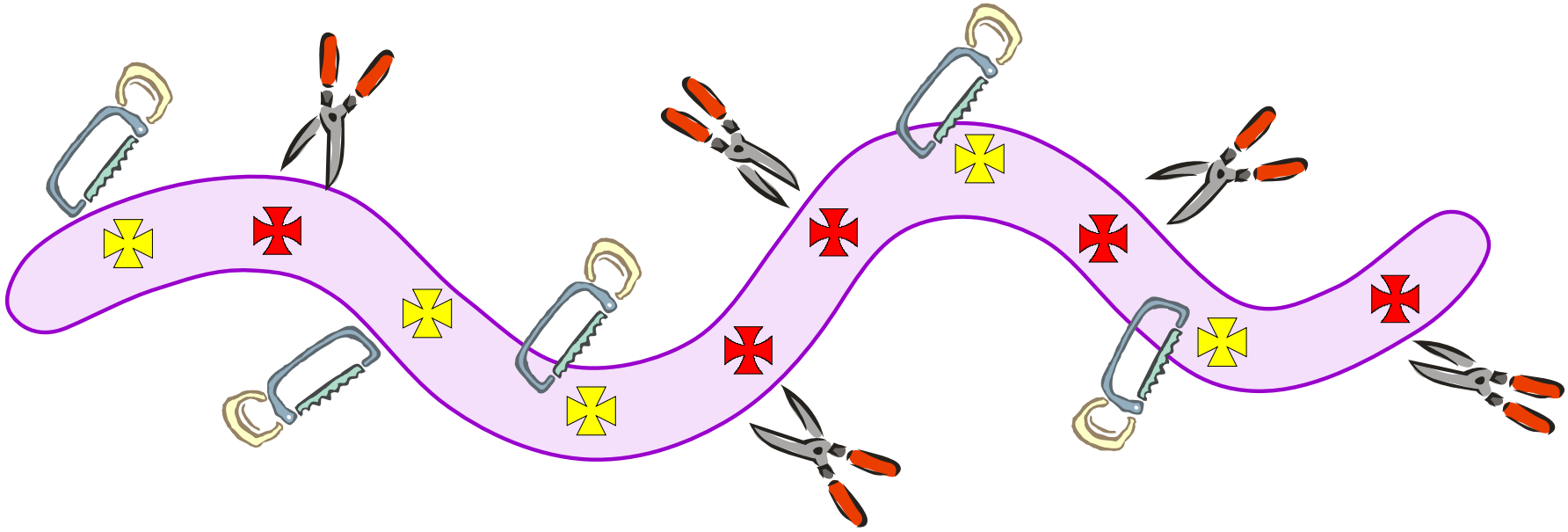
TLE/TLC HPLC SDS-PAGE

Peptide mapping has become a very important tool in proteomic study

蛋白質的專一性水解 Specific proteolysis

使用專一性蛋白酶
Use specific endo-protease

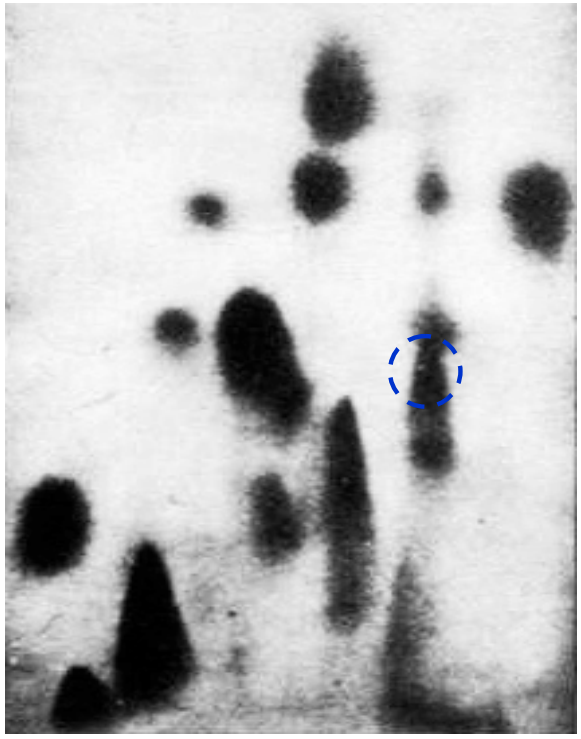
變性蛋白質
Protein denatured



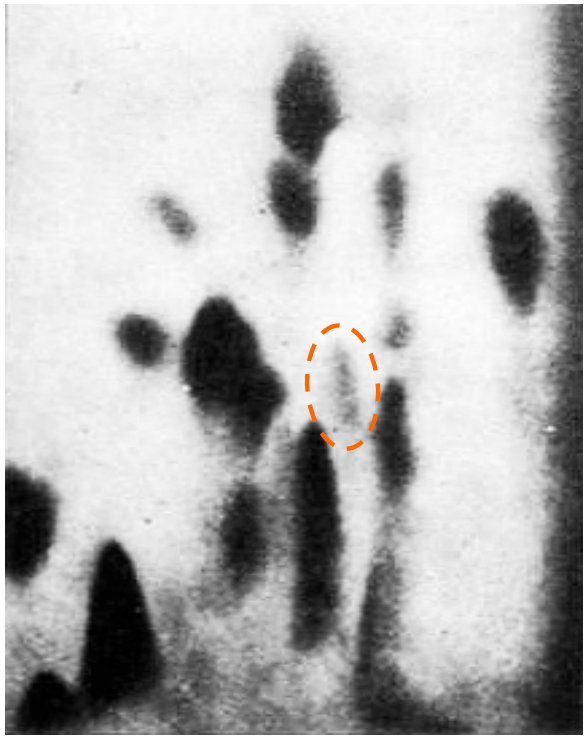
Specific protease can generate a set of fixed peptides for every protein

以雙向層析電泳鑑定胜肽

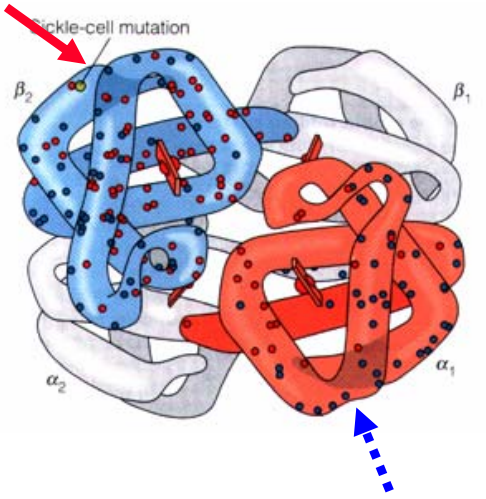
Hemoglobin A



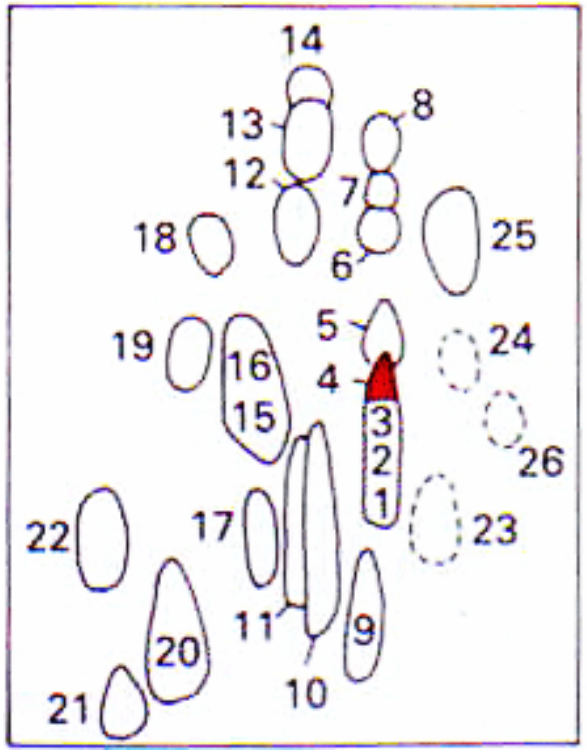
Hemoglobin S

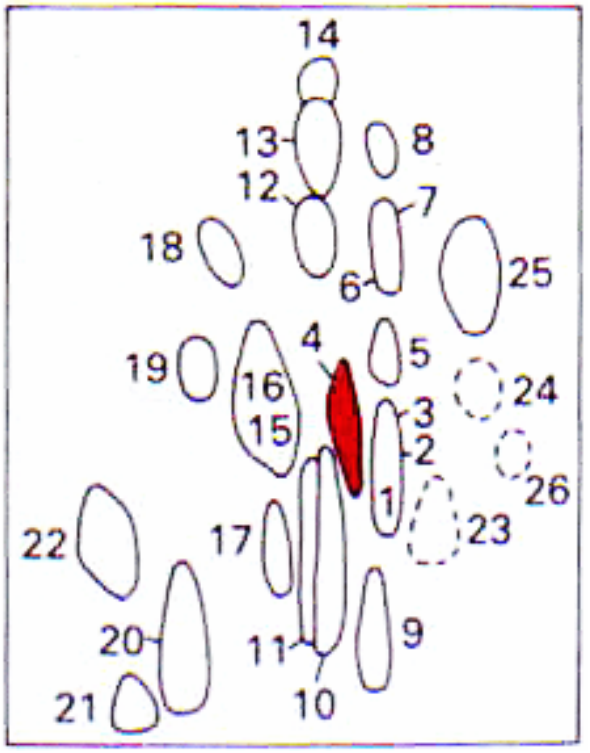


鐮型血球
Sickle cell



血紅蛋白四號片段

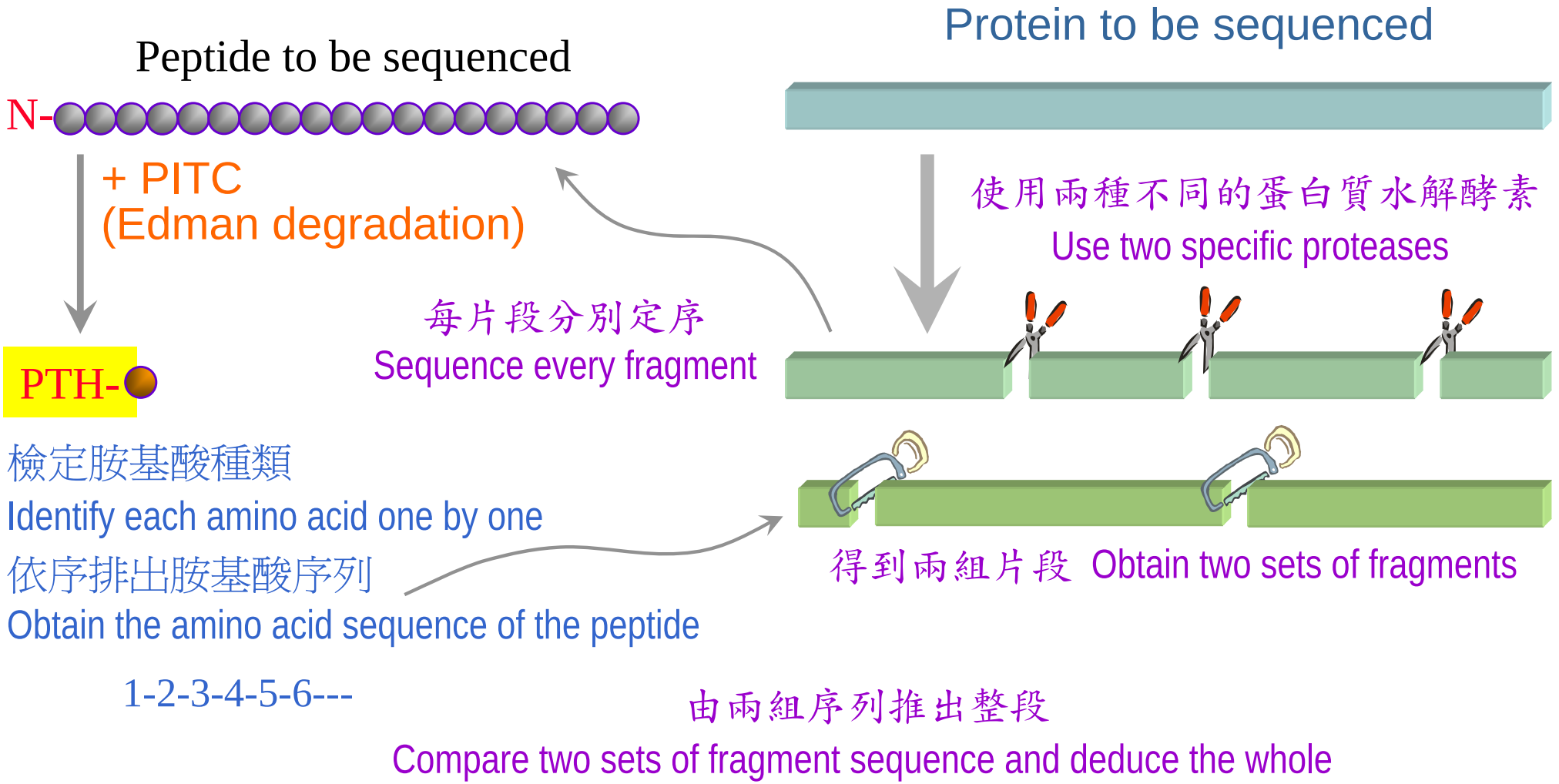




色析 TLC

電泳 TLE

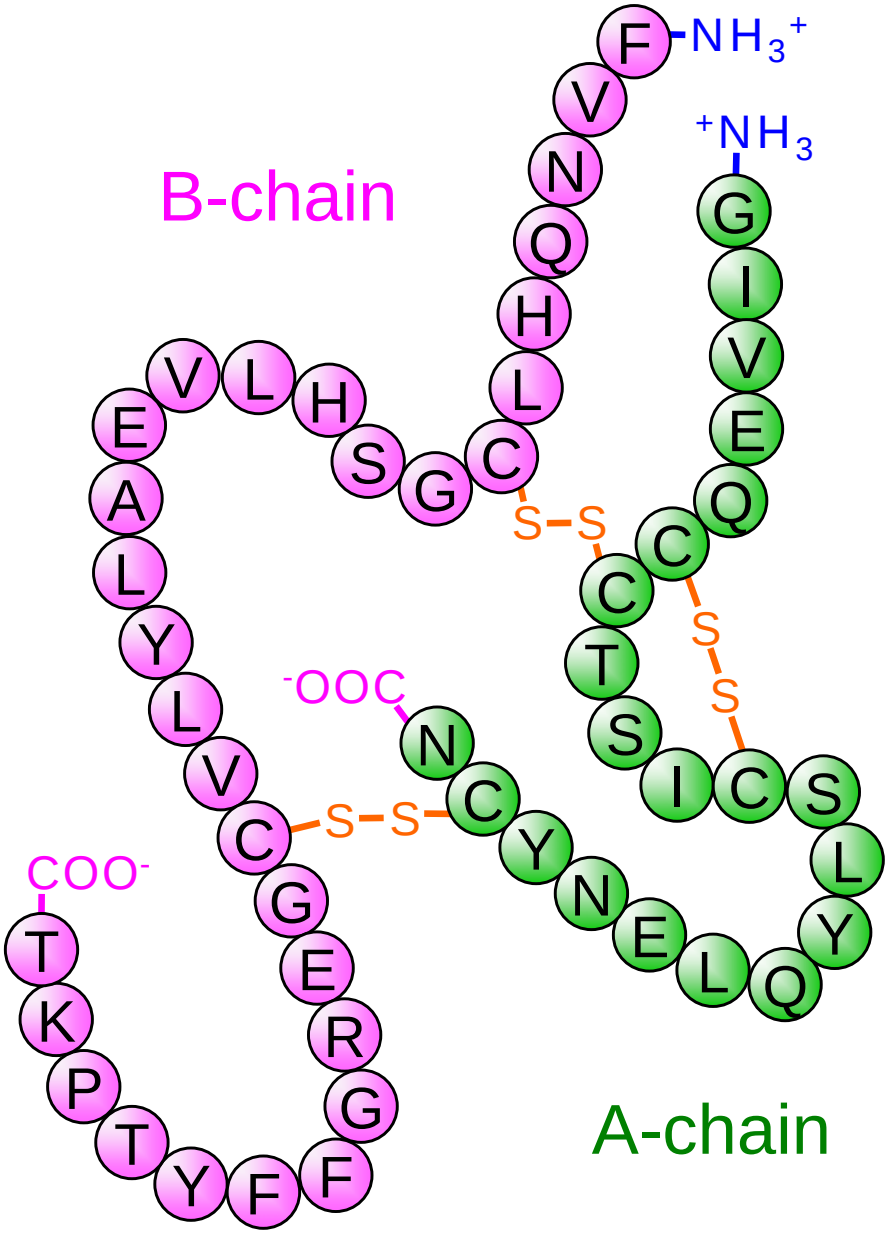
以傳統胺基酸定序法決定蛋白質序列



The traditional protein sequencing method using Edman degradation

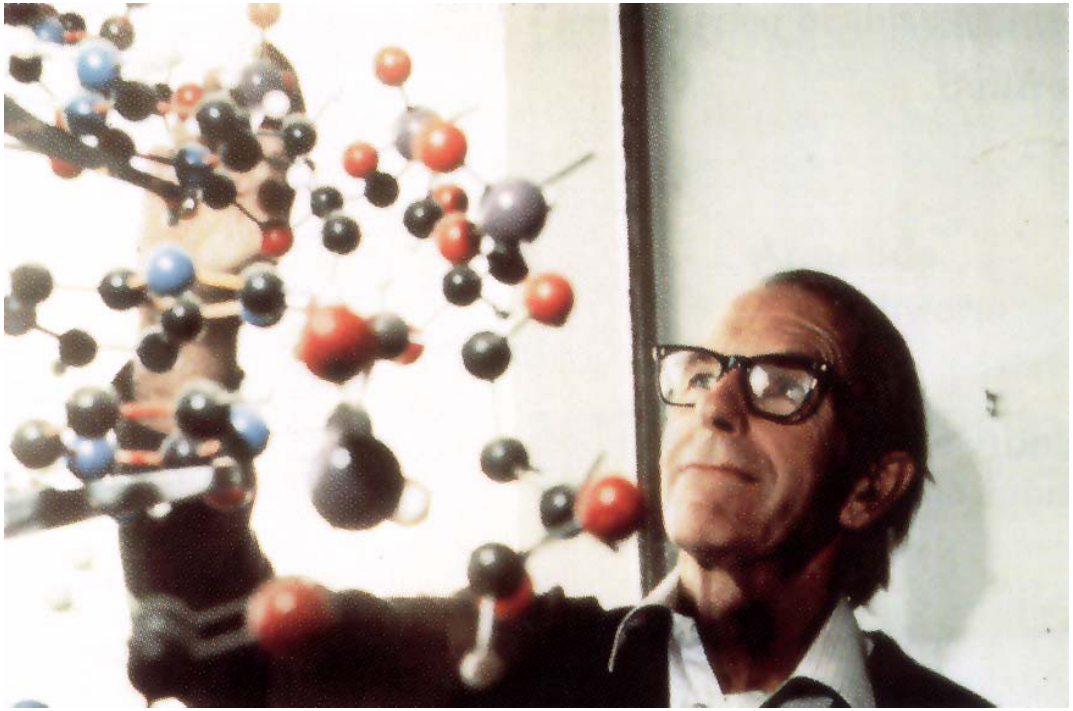
Juang RH (2005) EPA

以傳統胺基酸定序法決定蛋白質序列



F. Sanger (1958, Cambridge U)

Insulin 胰島素 (A, B chains)



Nelson & Cox (2000) Principles of Biochemistry (3e) p.142

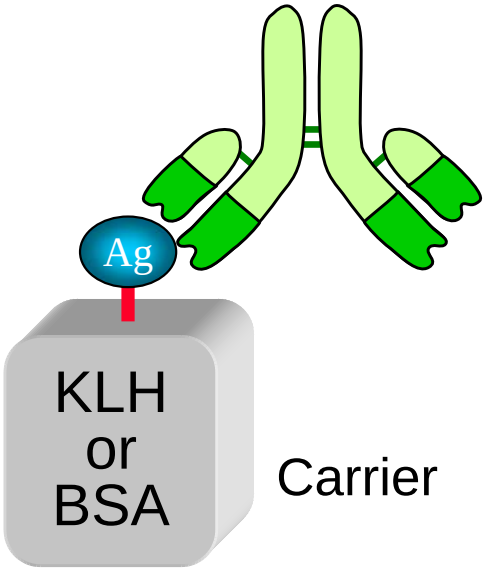
6 免疫學工具的利用 Immunological tools

- 6.1 抗原製備 Antigen preparation
- 6.2 免疫流程 Immunization protocol
- 6.3 抗體製備 Antibody preparation
- 6.4 抗體的應用 Antibody application

Juang RH (2005) EPA

抗原的種類 Antigen origin

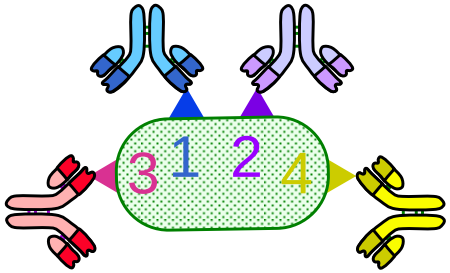
- 巨分子抗原 Macromolecules
  - Protein, polysaccharide, nucleic acid
- 小分子抗原 Small molecules
  - Conjugated to carrier before immunization
- 半抗原 (hapten) aflatoxin, citrinin
  - Carrier is required
- 人工合成胜肽 Synthetic peptides
  - Carrier is required
  - Produce monospecific Ab



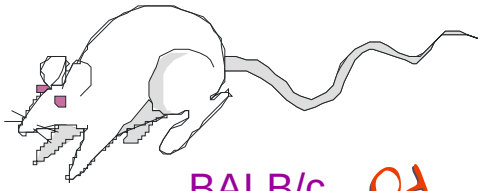
KLH: keyhole limped haemocyanin (貝類血青素蛋白); BSA: bovine serum albumin

基礎免疫學 Essential immunology

- 免疫系統： 先天及後天免疫系統
  Immune systems (innate and adaptive)
- 免疫反應： 遭遇→動員→掃蕩→休止
  Immune response (four stages)
- 抗体分子： 有兩個專一性抗原結合區
  Antibody molecule (two specific binding sites)
- 單株抗体： 只對其專一性抗原基作用
  Monoclonal antibody (very specific reagent)



小白鼠免疫流程



BALB/c

加佐劑製成乳劑
+ adjuvant → emulsion

Antigen (50 μg/mouse)
Emulsified in 0.5 mL
Freund's Complete Adjuvant

At least three booster shots,
same dose in 0.5 mL
Freund's Incomplete Adjuvant

Booster shots might be reduced
if TiterMax is use as adjuvant

0
2
4
6
8
10
12
14
wk

Trial Bleeding → Titer Determination

全採血

Total Bleeding, < 1 mL

Pristane (0.5 mL)

NS-1 Cell (10^6 cells)

Ascites Fluids

腹水 X mL (X = 1-10)

The immunization protocol for antiserum or ascites production

Juang RH (2005) EPA

免疫球蛋白純化流程

Ascites or serum (X mL)

↓ spin down cells (discard)

↓ + 2X mL PBS

↓ ammonium sulfate (AS) fractionation 0~40% sat.

↓ spin down pellet

Pellet

↓ resuspended in 40% AS

↓ spin down pellet

Pellet

↓ dissovled in X mL PBS

↓ dialysis in PBS three changes

↓ spin down precipitate

Supernatant

↓ + glycerol (equal volume)

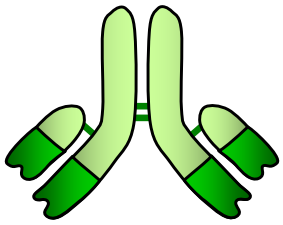
IgG (stored in freezer)

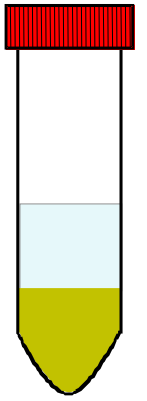
沉澱 Precipitation

清洗 Washing

透析 Dialysis

保存 Stock





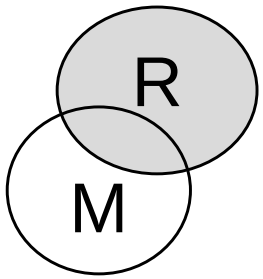
Juang RH (2005) EPA

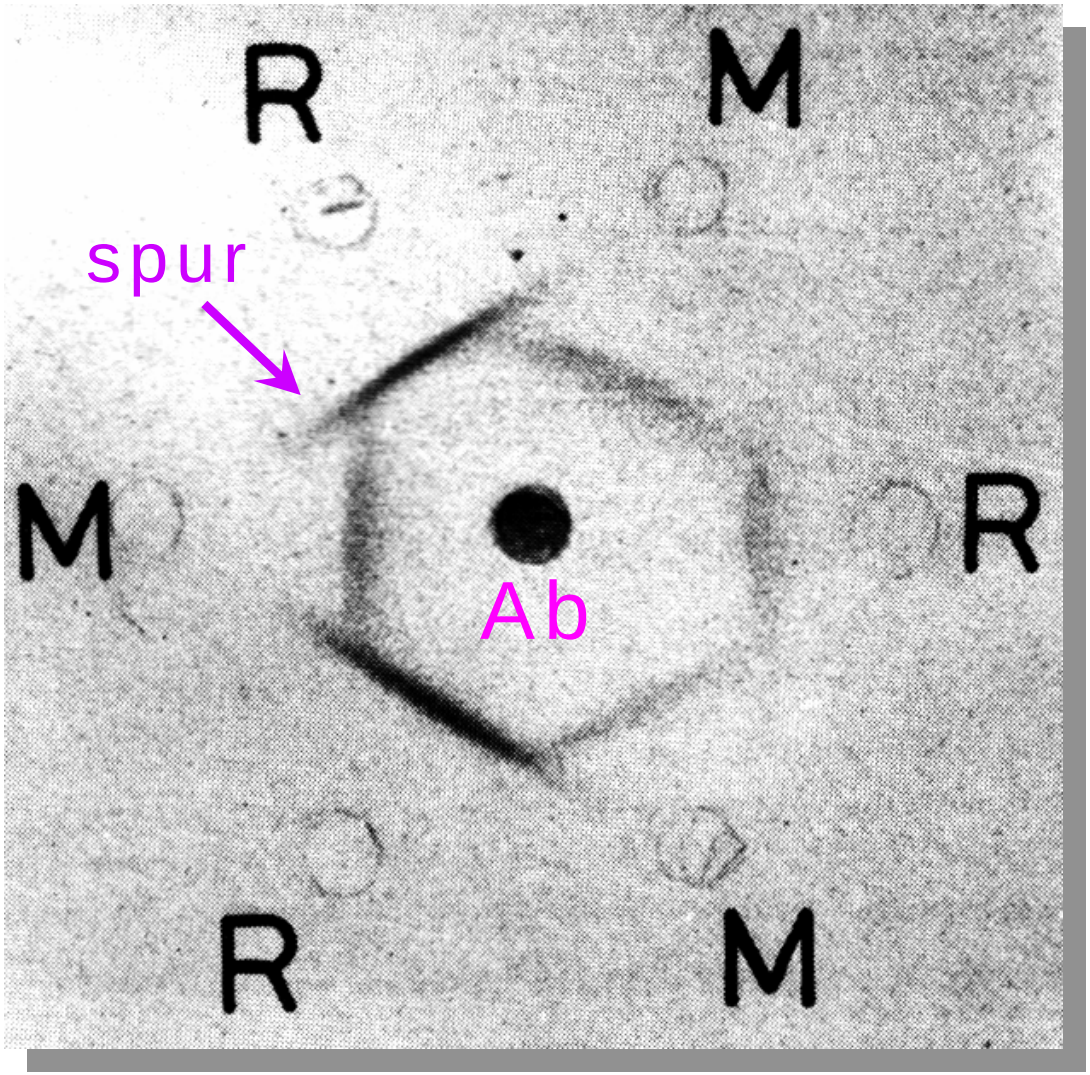
6.4 抗體的應用 Applications of Ab

- **轉印及免疫染色法** Western blot & immunostaining
  - 應用最廣 最有效率
- **免疫沉澱法** Immunoprecipitation (pull-down)
  - 另一種檢定專一性抗原的方法
- **親和層析法** Affinity chromatography
  - 最快速有效的純化方法
- **雙向免疫擴散法** Double diffusion
  - 古老但仍有其特色及應用
- **酵素免疫分析法** Enzyme immunoassay
  - 可分析大量樣本 (ELISA)
- **抗體晶片** Antibody chip
  - 專一快速地同時進行多種分析

雙向免疫擴散法

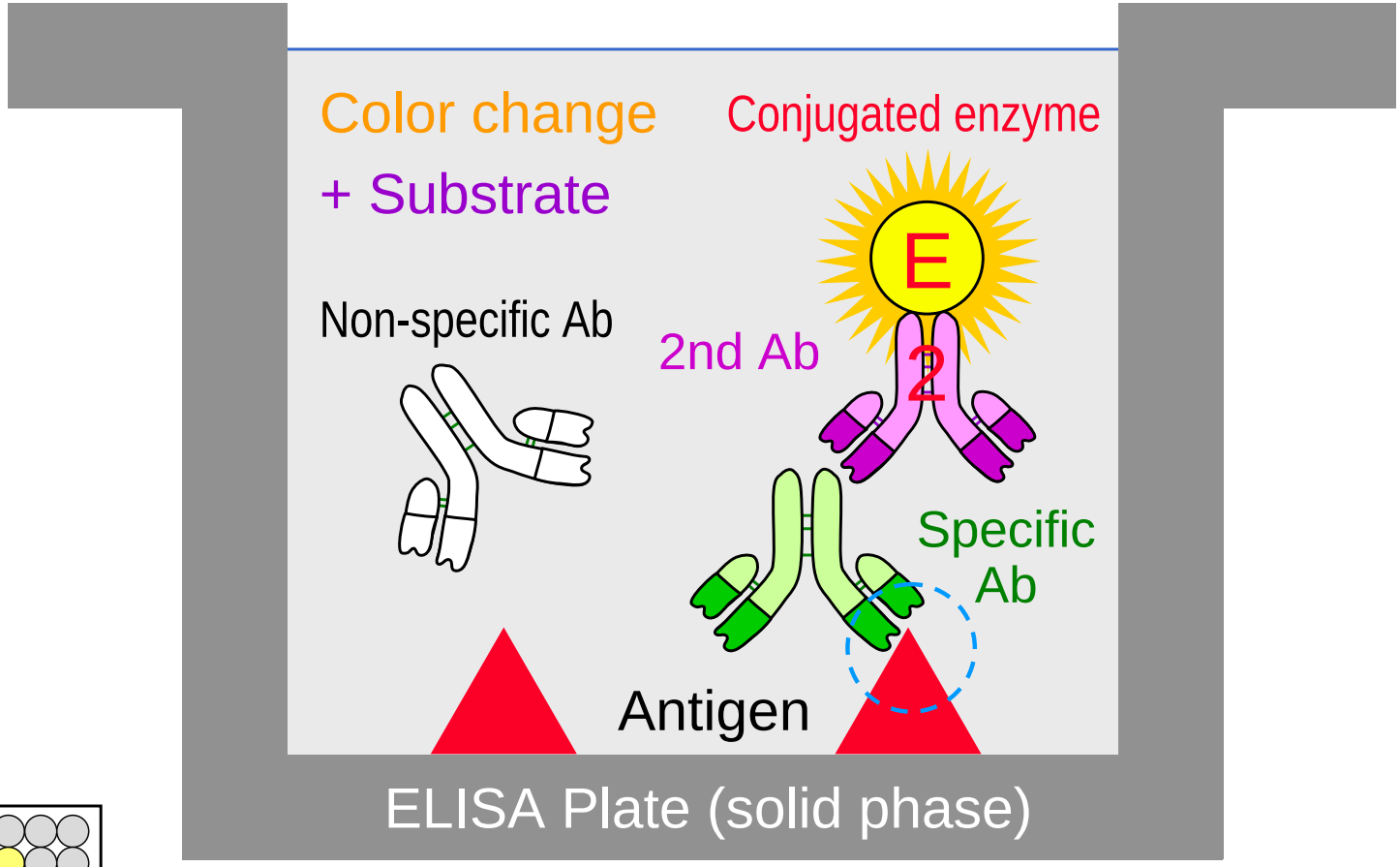
Outer wells: Rice (R) and maize (M) sucrose synthase (Ag)
Central well: Antiserum against rice sucrose synthase (Ab)

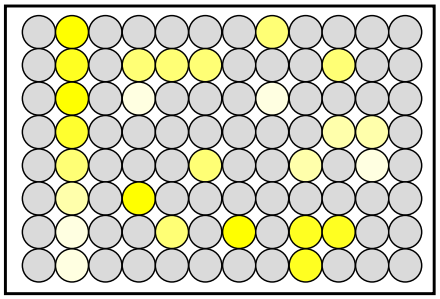




由沉澱線交叉情形得以推測抗原分子間的關係
The crossing-over of the precipitin lines reveals the structural relationship between the antigen molecules

酵素免疫分析法 ELISA

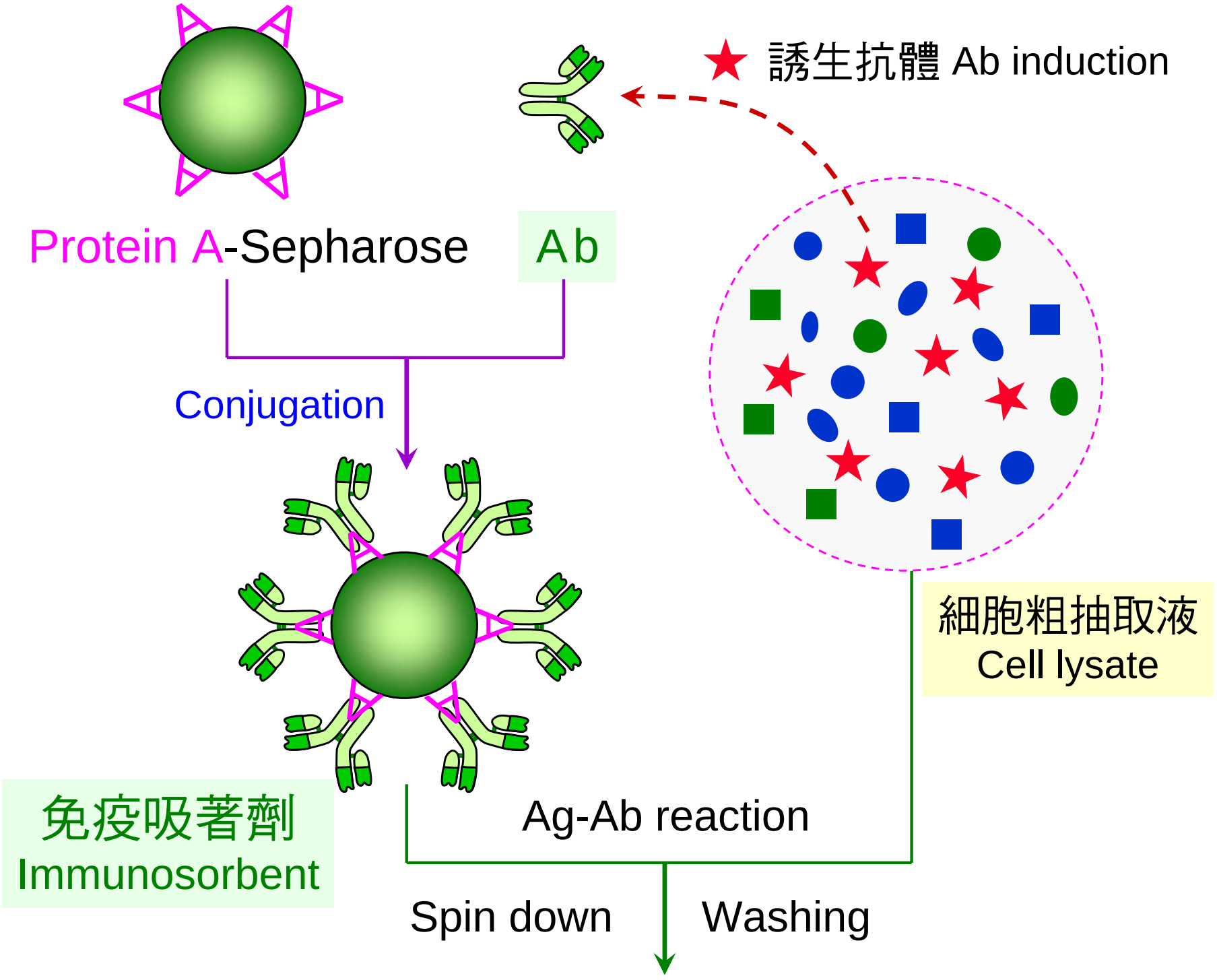




To detect the Ab in the sample

Juang RH (2005) EPA

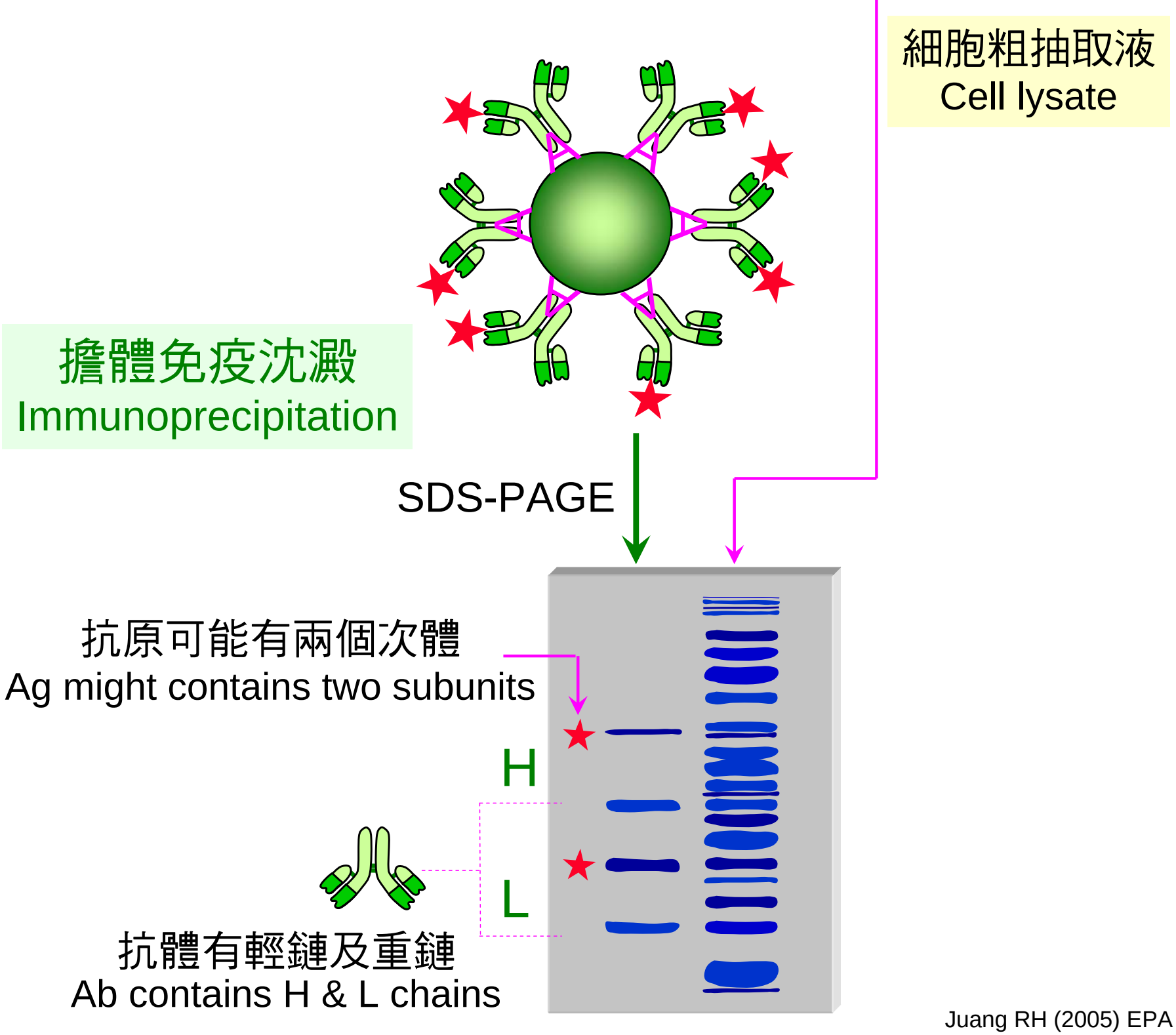
擔體免疫沈澱的原理及應用



Solid support-based immunoprecipitaion

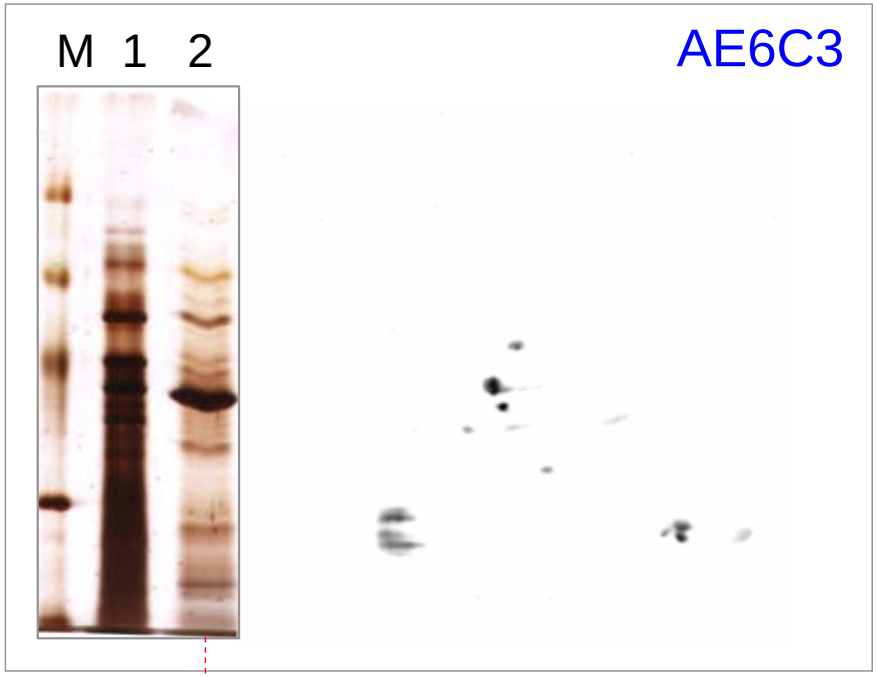
Juang RH (2005) EPA

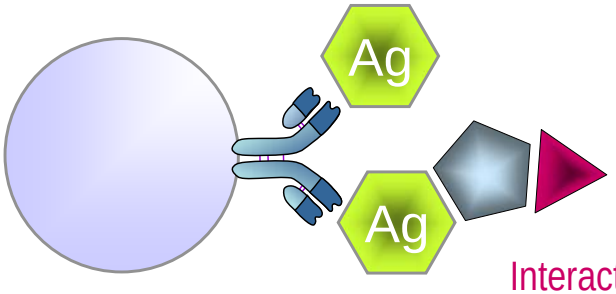
擔體免疫沈澱的原理及應用

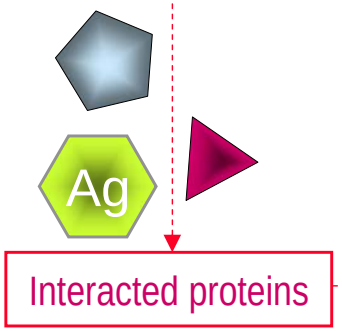


Juang RH (2005) EPA

抗體免疫沈澱與蛋白質交互作用







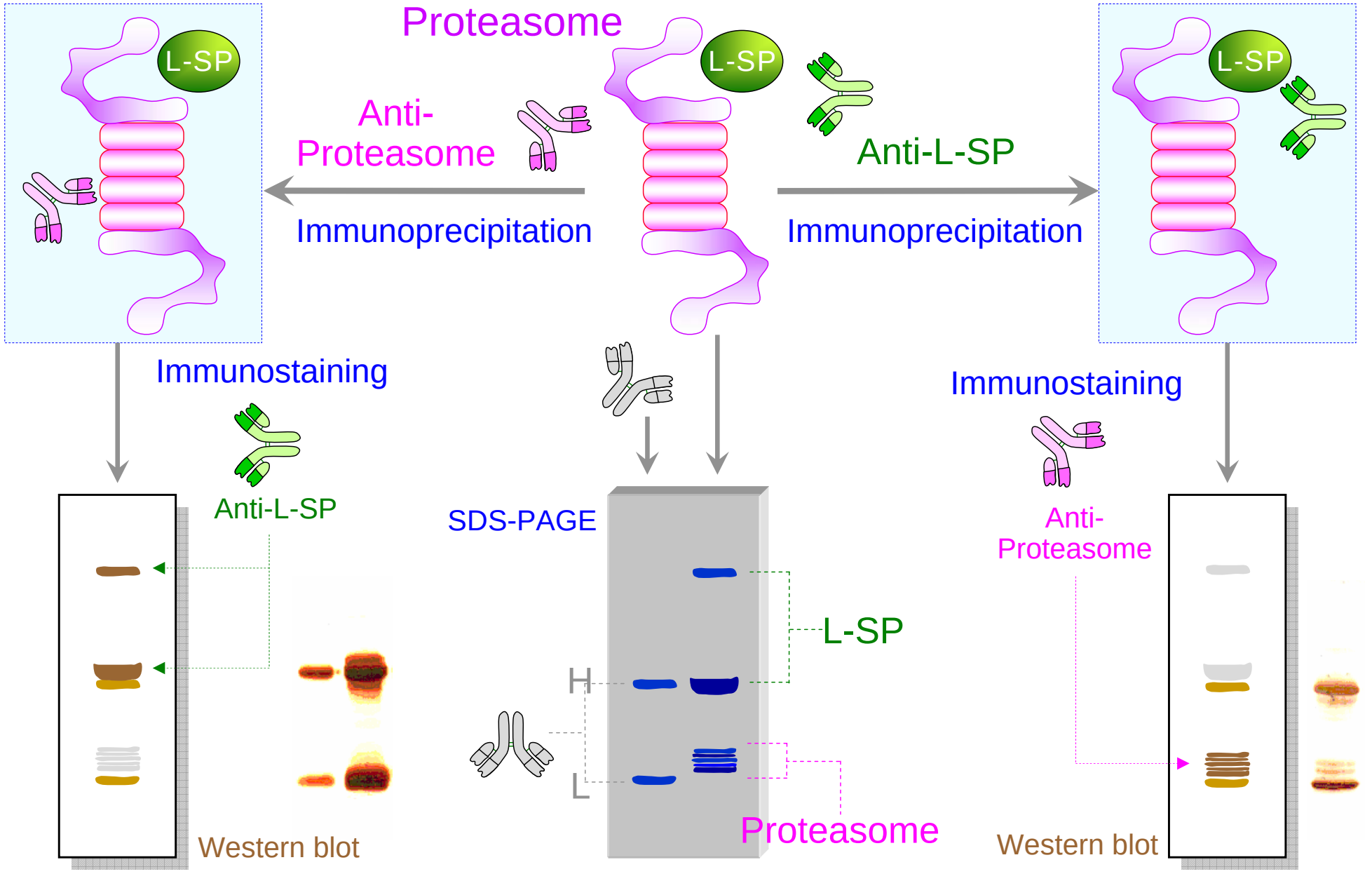
LC/MS/MS

Protein ID	Match peptide
Adenosylhomocysteinase	IVLTIIR DSAAVFAWK HSLPDGLMR LVGVSEETTTGVK
Histone H4 (wheat)	IFLENVIR IDGLIYEETR TVRAMDVVYALKR
Fructose bisphophate aldolase	VTPEVIAEYTVR IGPNEPSQLAIDLNAQGLAR
Triosephosphate isomerase	TNVSPEVAESTR VIACVGETLEQR
NAD-dependent malate dehydrogenase	DDLFNINAGIVK
Histone H3	ASAPATGGVK
Putative lipase	DQVLEEVRR

Pull down proteins interacted with Ag

Juang RH (2005) EPA

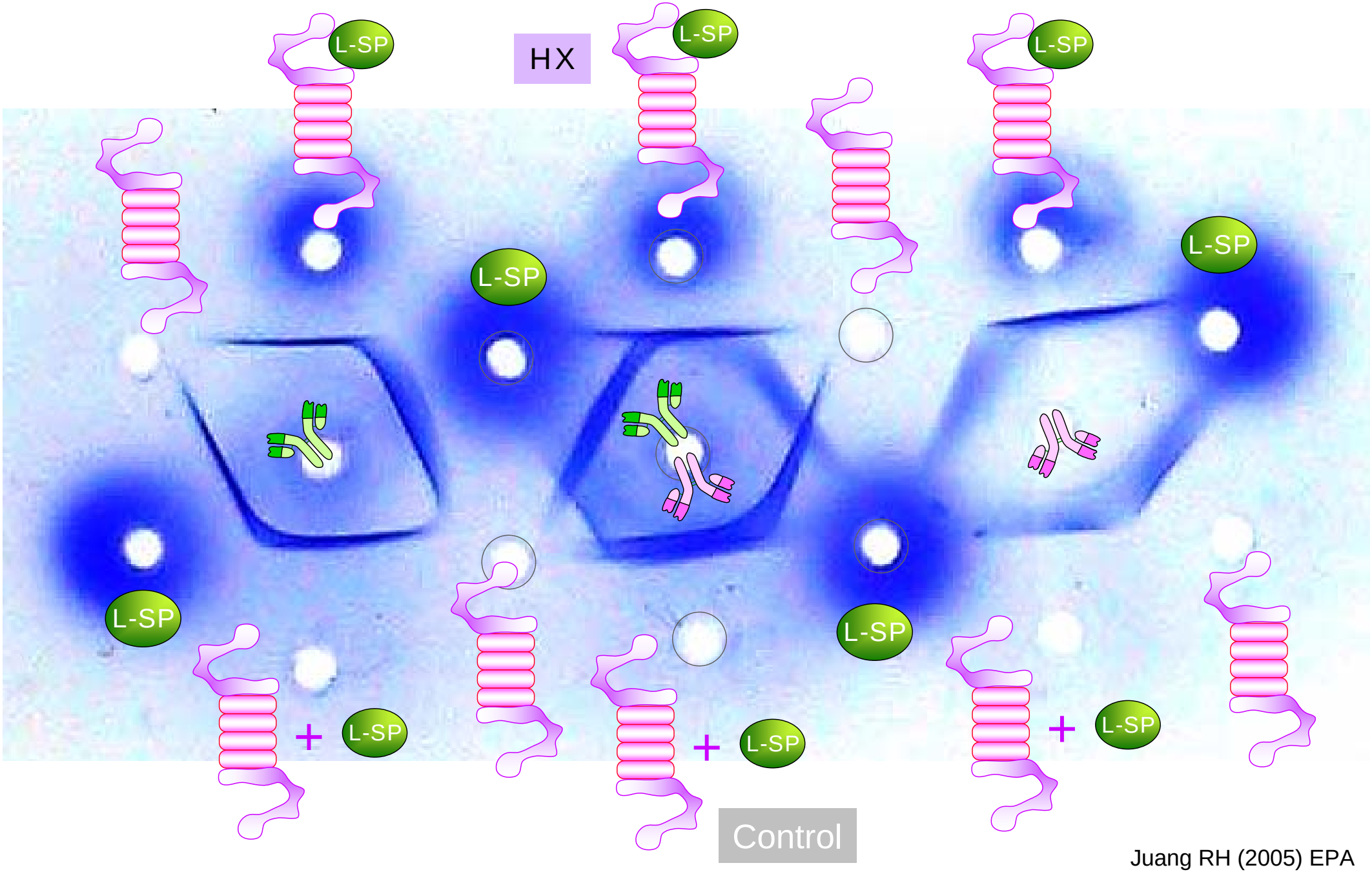
免疫沈澱證明分子間結合 Protein interactions



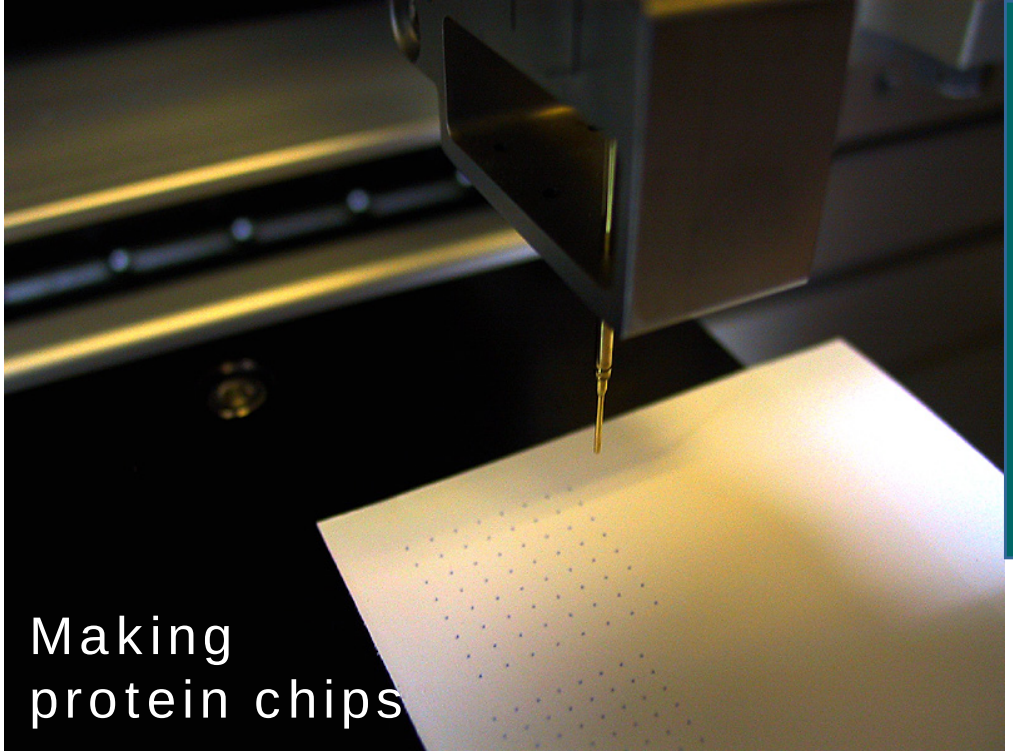
Immunoprecipitation is useful in detecting the interaction between two proteins

Juang RH (2005) EPA

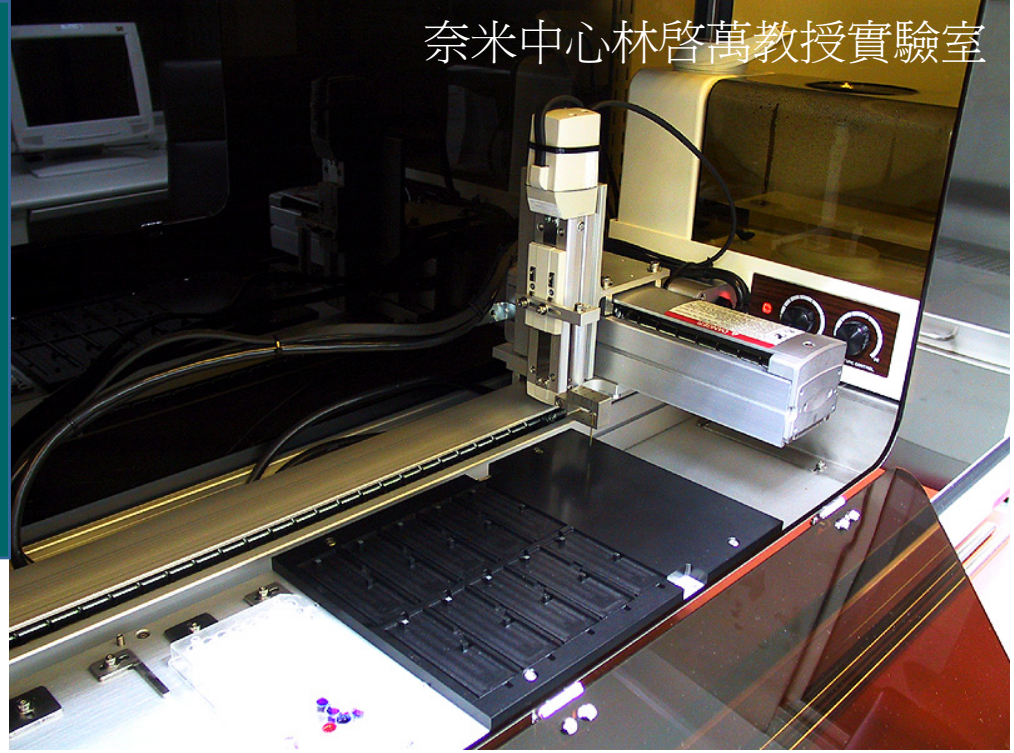
雙向免疫擴散 Double diffusion works



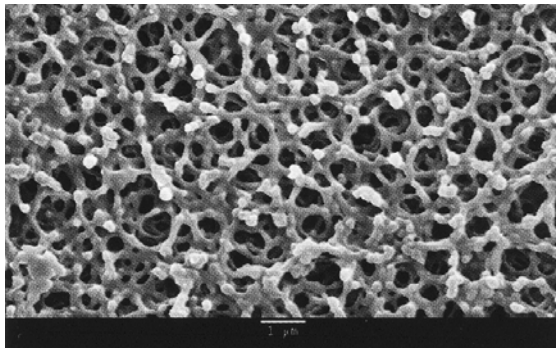
Juang RH (2005) EPA



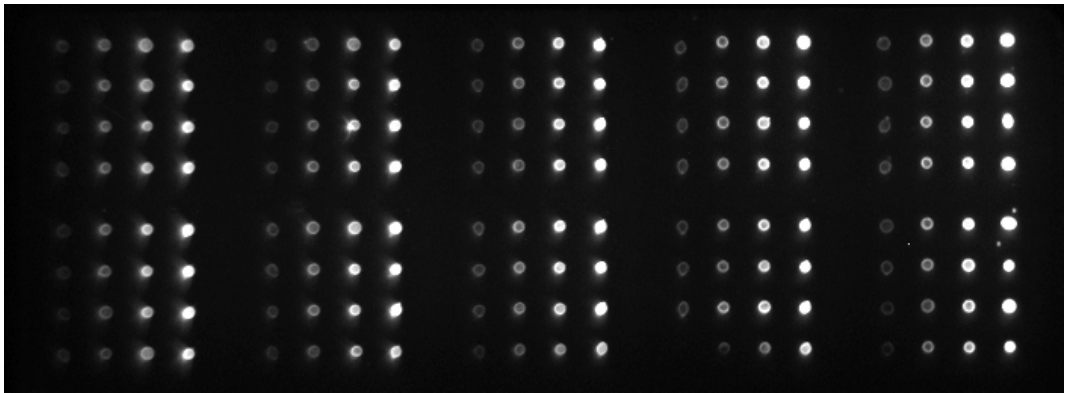
蛋白質晶片試製

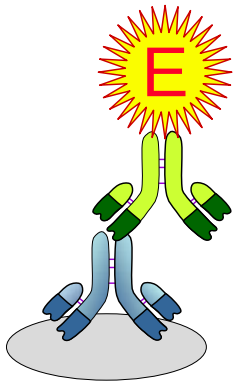


Nitrocellulose

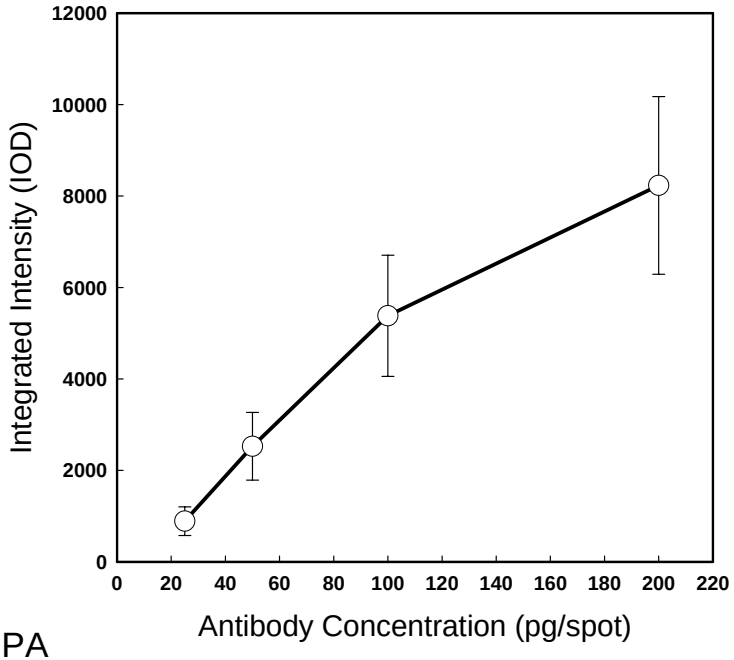


Schleicher & Schuell

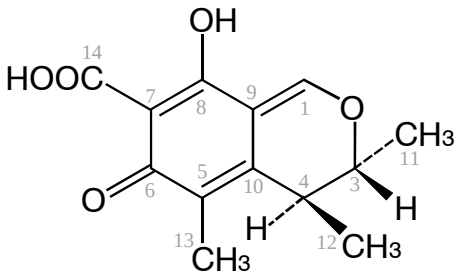




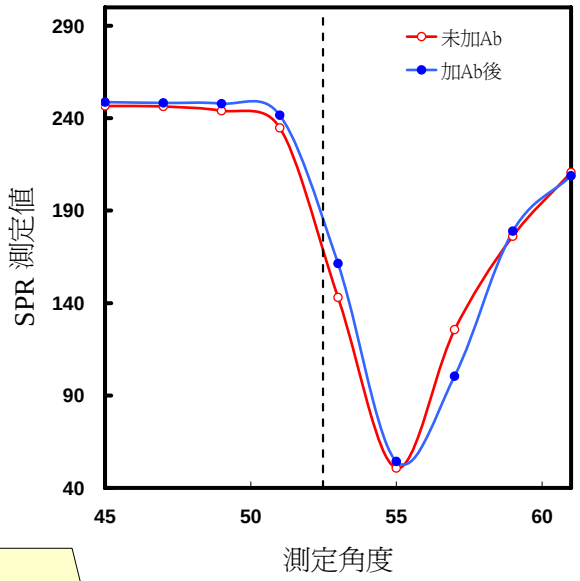
Juang RH (2005) EPA

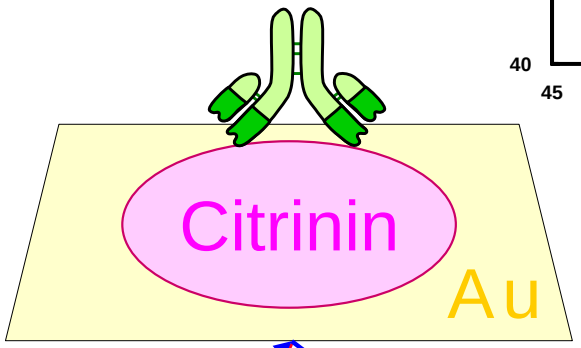


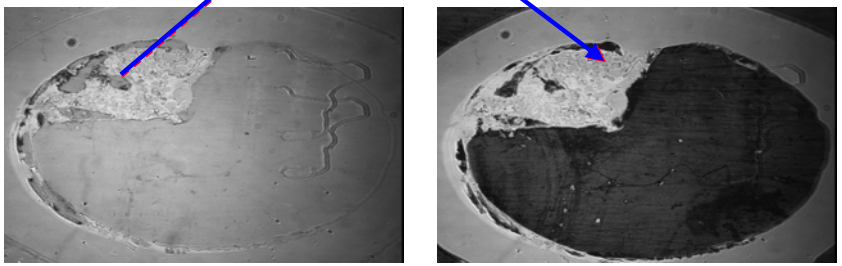
表面電漿共振 (SPR) 可檢測兩分子間的結合



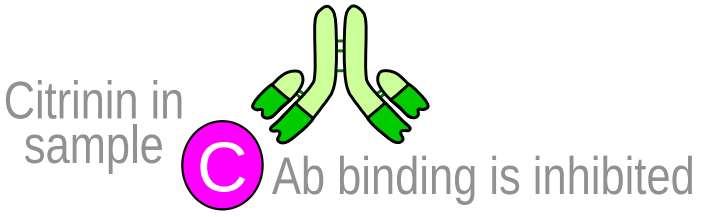
抗體抗原結合對 SPR 的影響

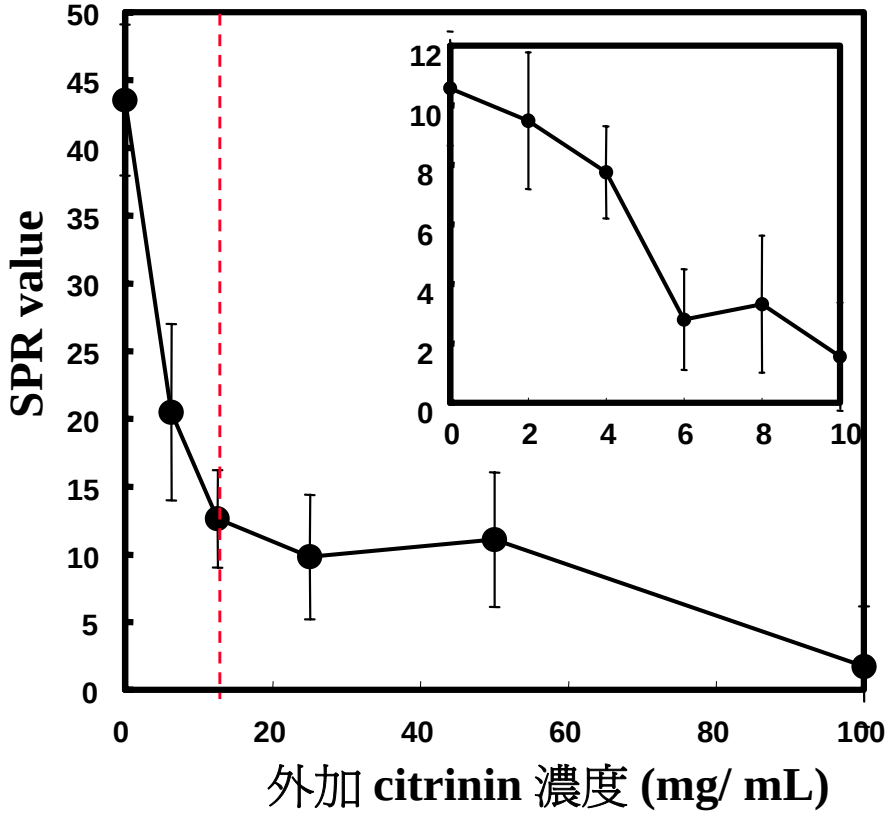






(台大醫工所林啓萬教授)





Juang RH (2005) EPA

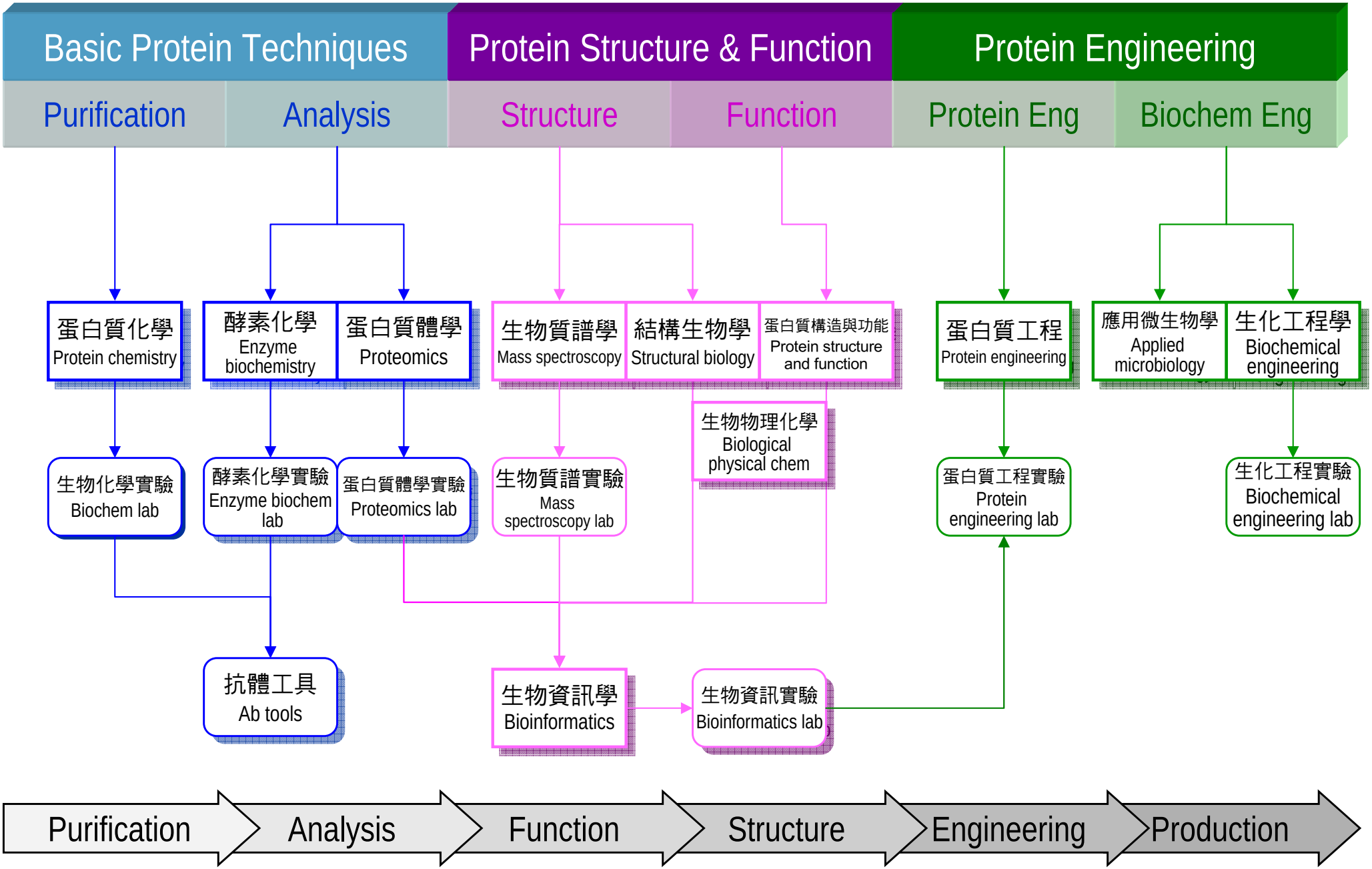
Surface plasmon resonance (SPR) detects the binding of two molecules directly

7 蛋白質科技 Protein technology

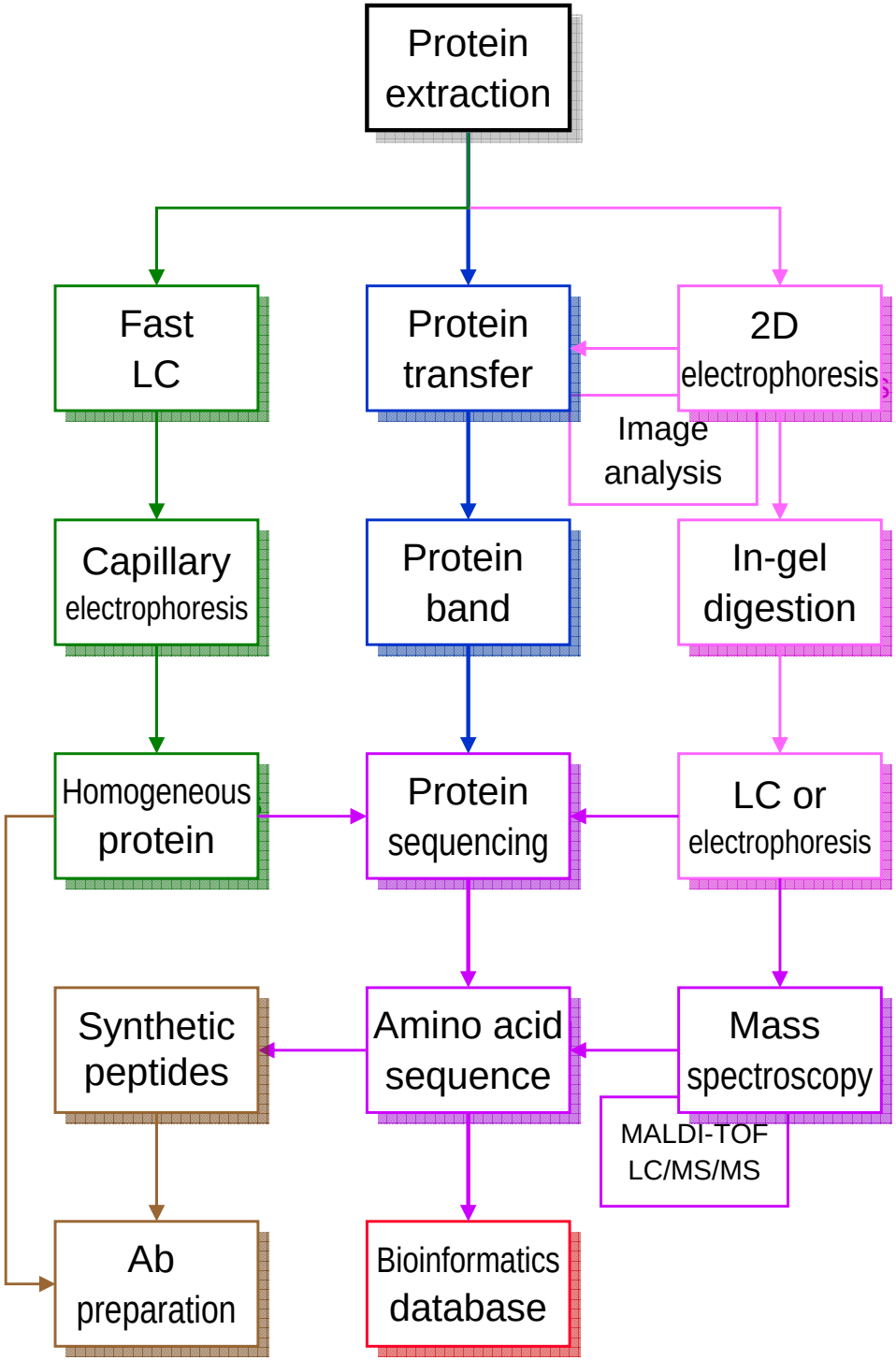
- 7.1 蛋白質科技範疇 An overview
- 7.2 蛋白質微量分析及檢定 Microanalysis
- 7.3 蛋白質體研究 Proteome research

蛋白質科技
Protein Technology

蛋白質科技相關課程與階段 Related courses



蛋白質的微量分離及檢定



蛋白質科技
Protein Technology

蛋白質純化分析新貌

A new look for EPA

1 電泳及轉印

Electrophoresis and transfer

2 二次元電泳

2D electrophoresis

3 膠體內水解

In-gel digestion

4 微量分離純化

Micropurification and analysis

微量分析系統

Microanalysis

抗體製備

Ab preparation

生物資訊學

Bioinformatics

Juang RH (2005) EPA

Genome

基因表現不一定完全反映到蛋白質

Gene expression is not totally reflected in protein level

由基因體較難預測蛋白質的修飾及調控

It is difficult to predict the protein modification and regulation from genomic level

也無法預測蛋白質間的交互作用

Nor can you predict the protein interactions

Proteome

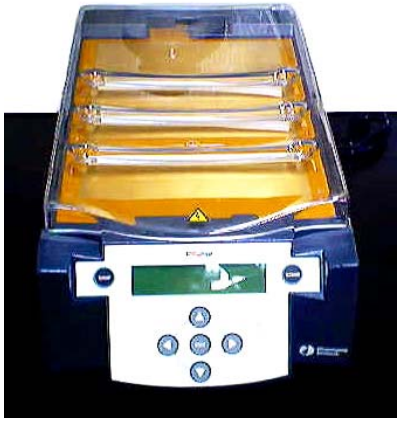
Proteome is much complex than its genome

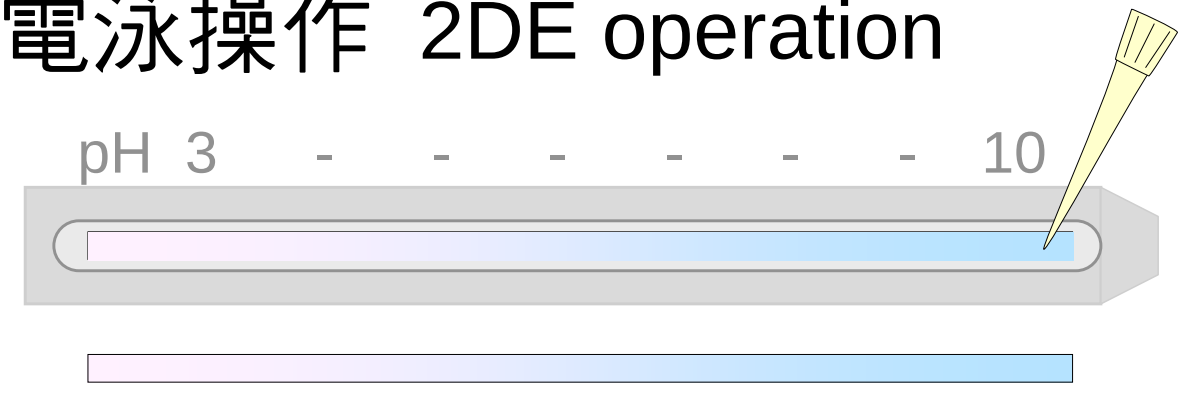
Juang RH (2005) EPA

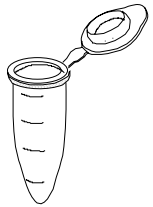
二次元電泳操作 2DE operation

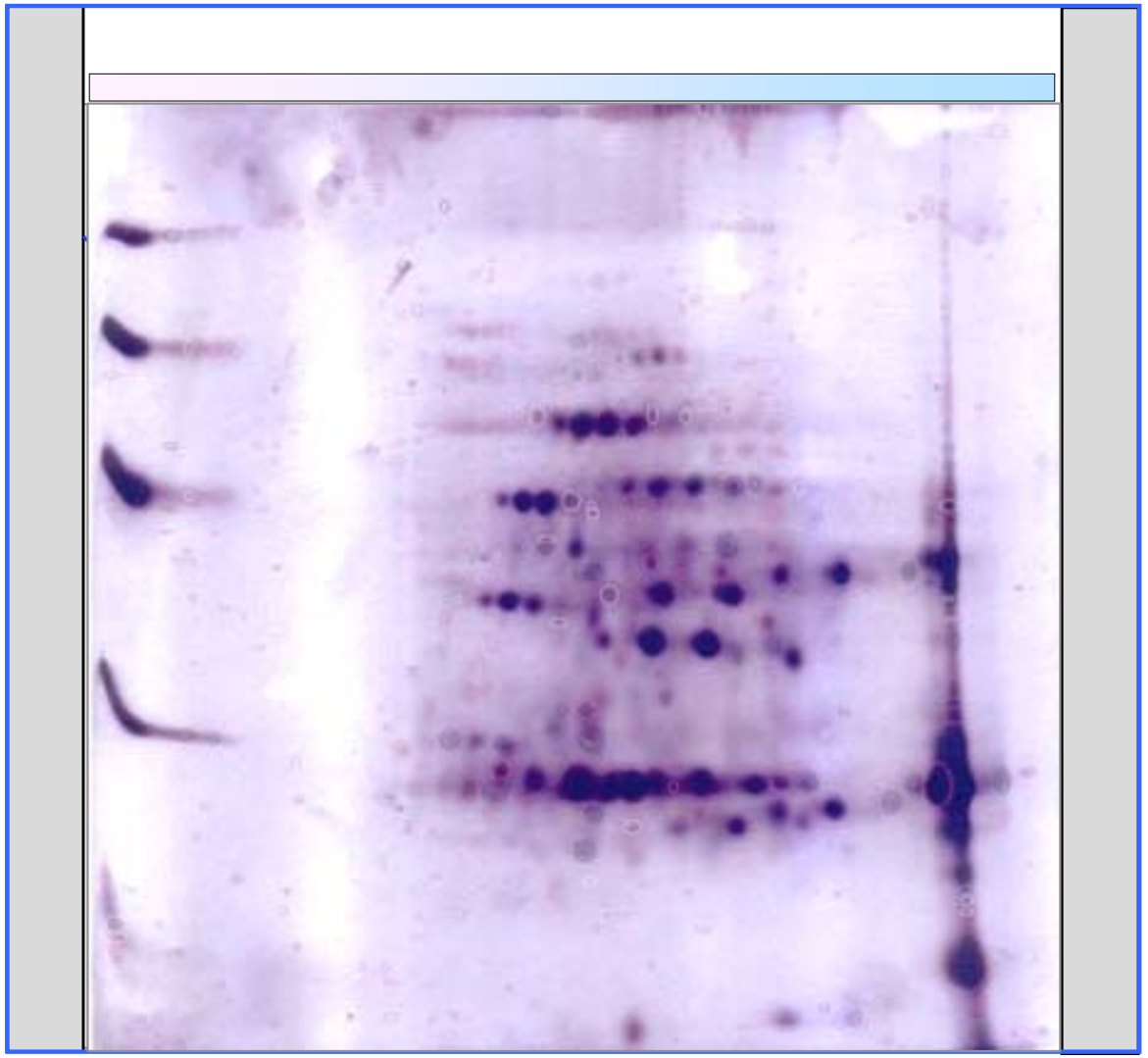
Juang RH (2005) EPA

(1) IEF
等電焦集電泳





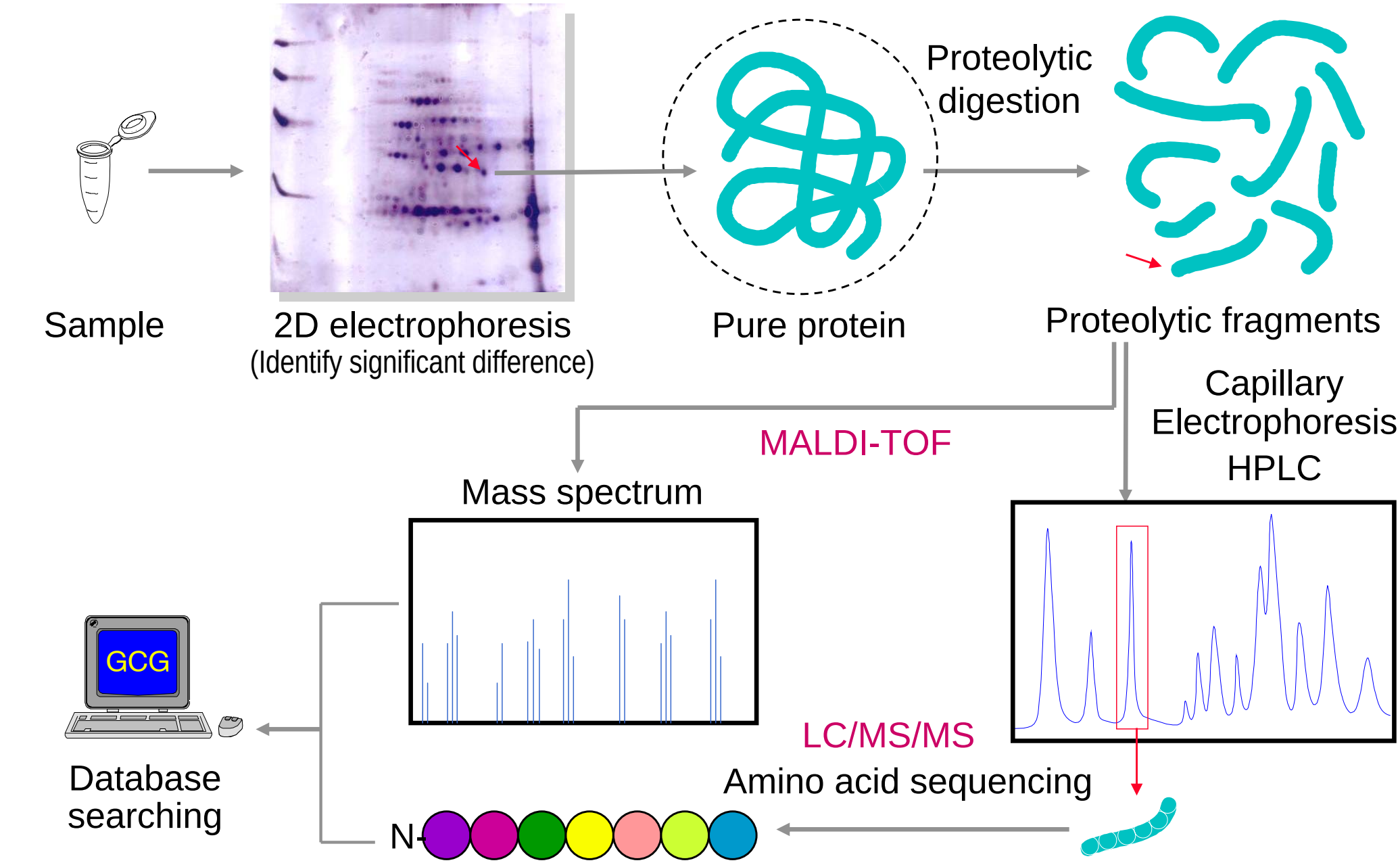




(2)
SDS-PAGE
分離膠體

(3)
Staining
染色脫色

蛋白質體可綜觀蛋白質的消長與身分



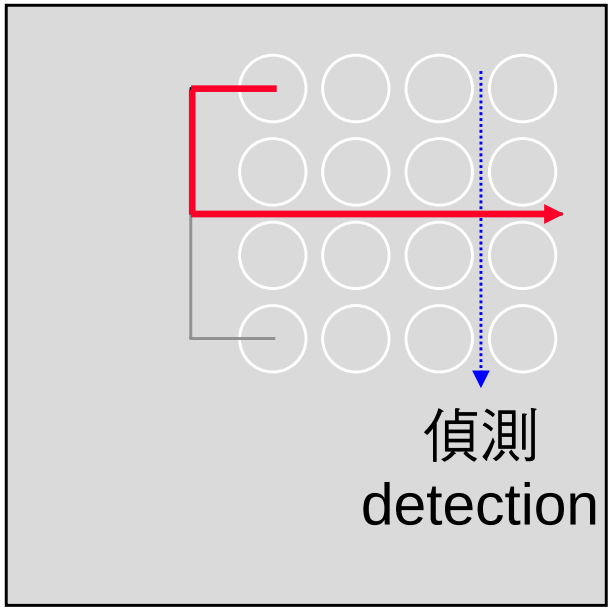
2D tool provides insight from comparing proteomic difference

Juang RH (2005) Proteomics

微流體平台 Microfluidics, Lab-on-a-chip

Agilent 所有蛋白質純化與活性分析均予微小化

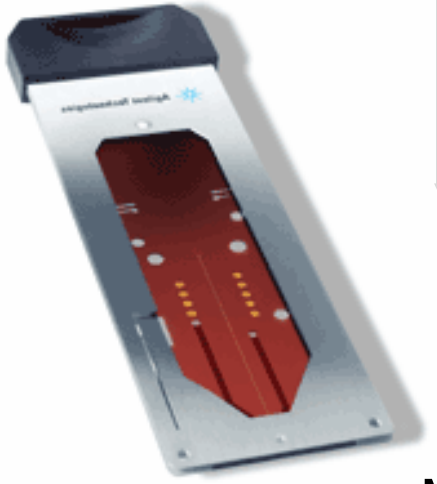
Agilent 2100 bioanalyzer

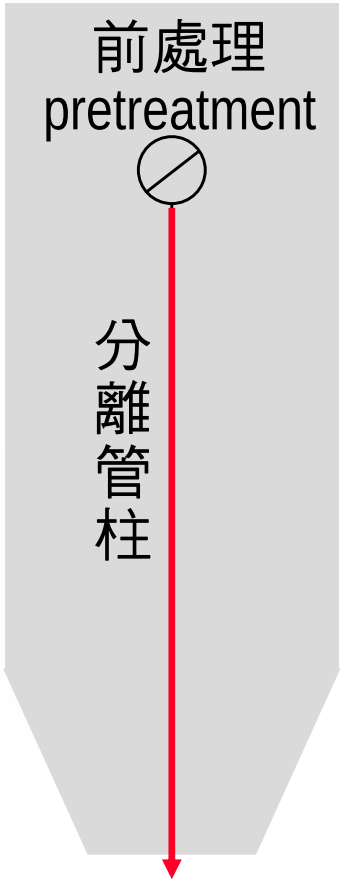


毛細管電泳
Capillary electrophoresis

http://www.chem.agilent.com/Scripts/Phome.asp

Agilent HPLC-Chip/MS





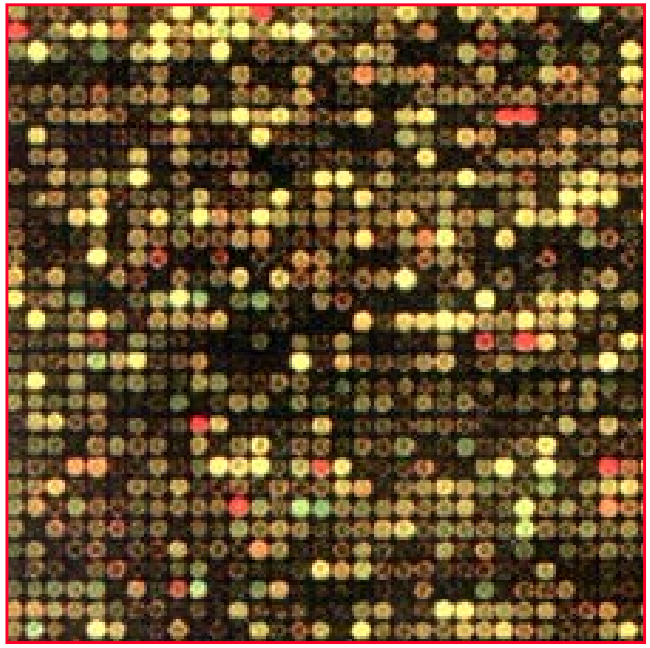
質譜儀分析
Mass analysis

Minimize protein purification and analysis in one chip

Juang RH (2005) EPA

現代蛋白質科技特點 Modern protein technology

- 高產能 High-through put
- 快速 High-speed
- 微量 Micro-scaled



Juang RH (2005) EPA