7 電泳檢定法 Electrophoresis

7.1 電泳原理 Basic principles

帶有電荷的分子都可在電場中泳動

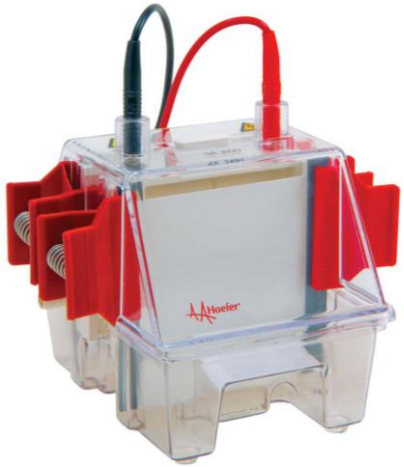
7.2 聚丙烯醯胺膠体電泳 PAGE

是檢測蛋白質最常用的電泳形式

錄影
Ana 7a

7.3 其它相關技術 Related techniques

一些與電泳相關的技術：染色、轉印、IEF、2DE



錄影
Ana 7b

7.1 電泳原理 Basic principles for electrophoresis

7.1.1 蛋白質的泳動率 Protein mobility

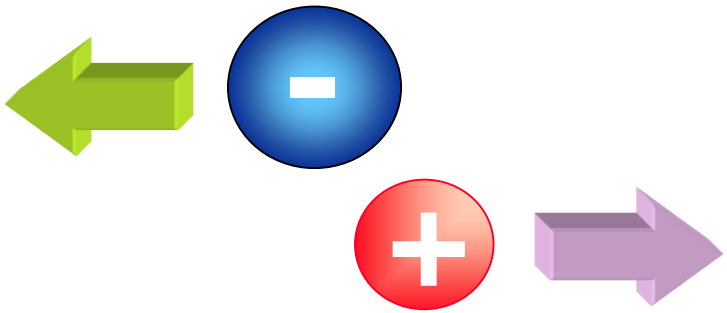
泳動率與蛋白質所帶的電荷及大小有關

7.1.2 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

7.1.3 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



蛋白質分子的帶電性質

環境影響分子的帶電性質

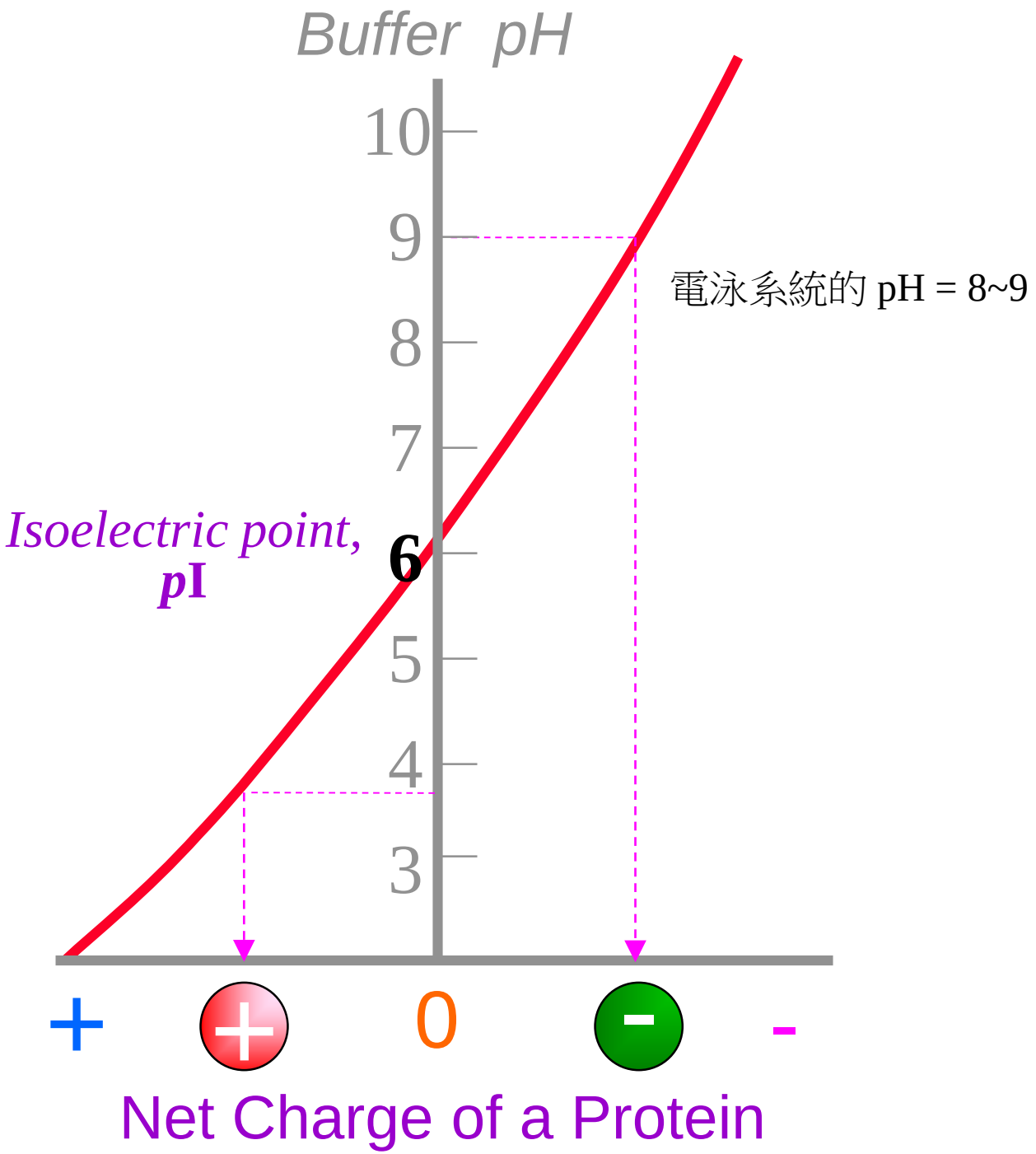
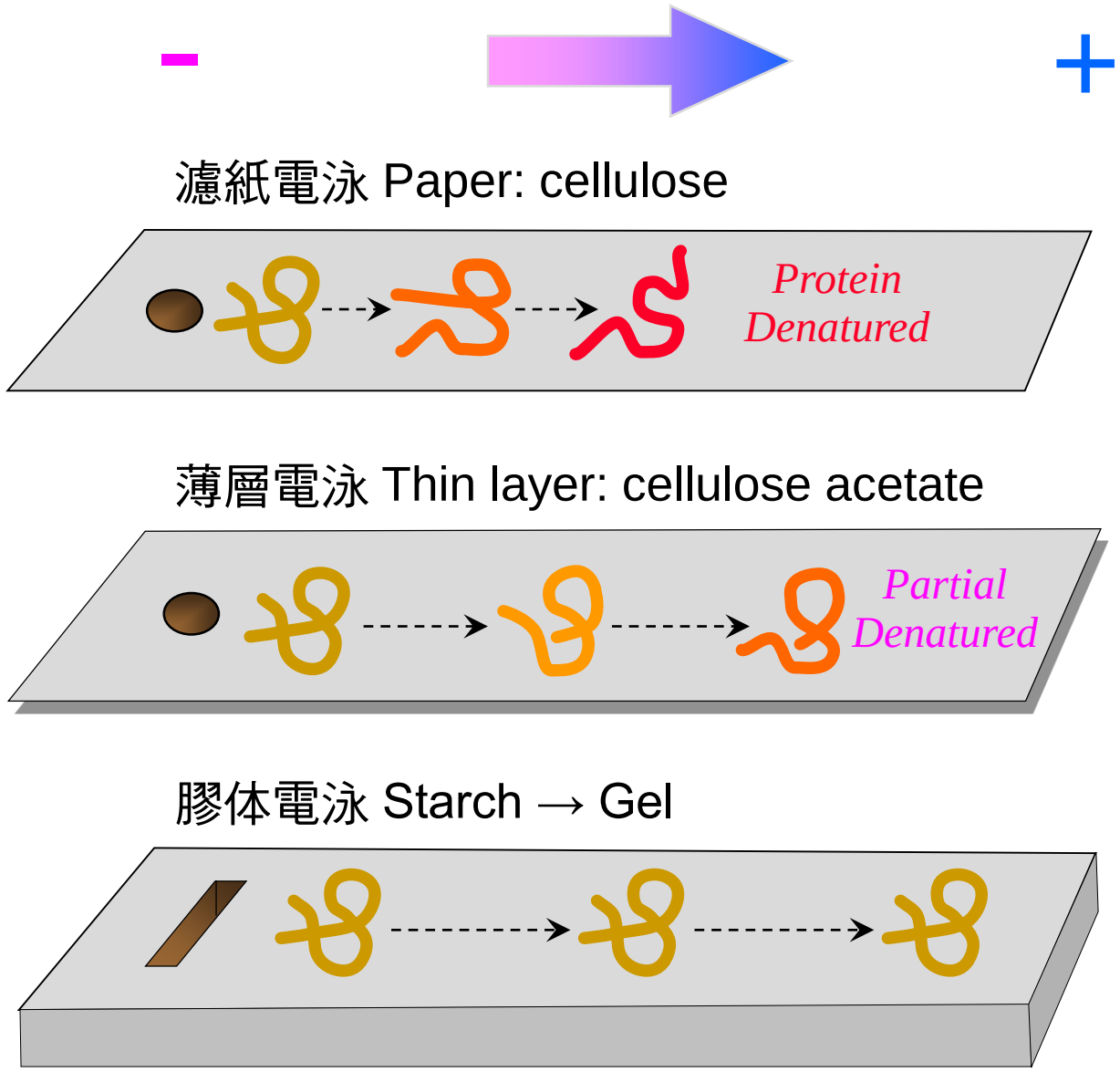


圖 7.1

電泳形式的演進

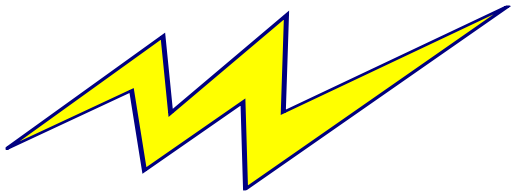


Historical evolution of electrophoresis design

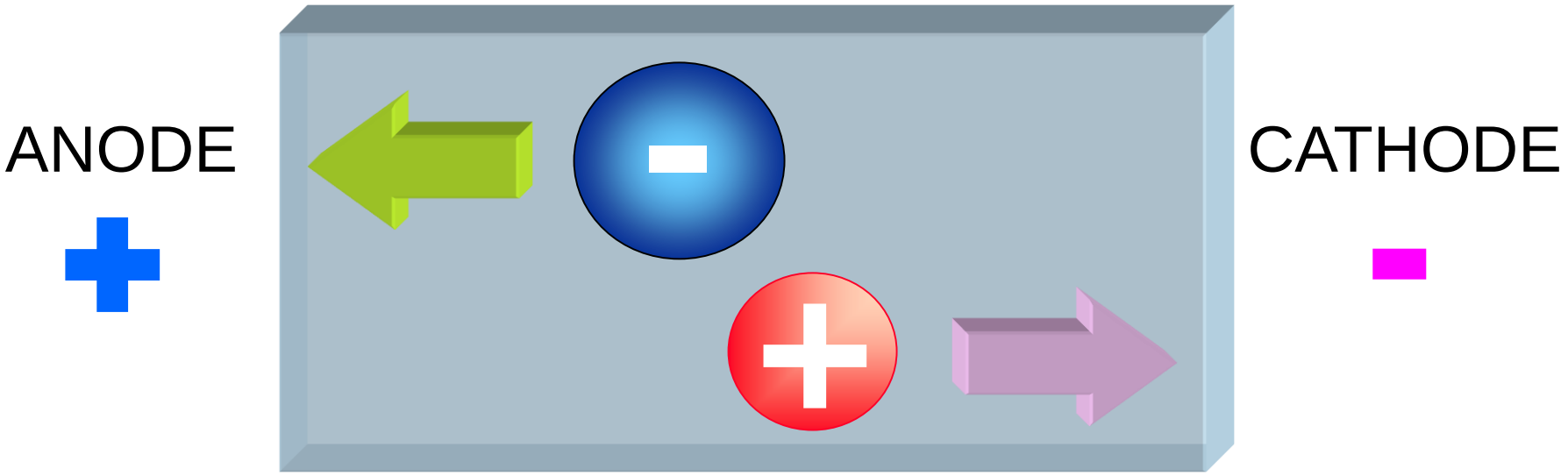
圖 7.2

影響泳動率的因素 Factors affecting mobility

影響整個系統



外加電流電壓 Input *current, voltage*



Friction ***Charge***

分子量 分子形狀
Molecular weight, shape

分子的等電點
Isoelectric point

影響個別分子

7.2 聚丙烯醯胺膠体電泳 PAGE

7.2.1 PAGE 種類 PAGE categories

依蛋白質樣本是否變性可分成兩大類

7.2.2 PAGE 膠体組成 Gel composition

是單体小分子的聚合反應

7.2.3 PAGE 系統解剖 System anatomy

說明膠体的構成及電泳的分離機制

7.2.4 結果不佳時 Trouble shooting

舉出電泳操作時可能出現的問題

膠体的聚合反應 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

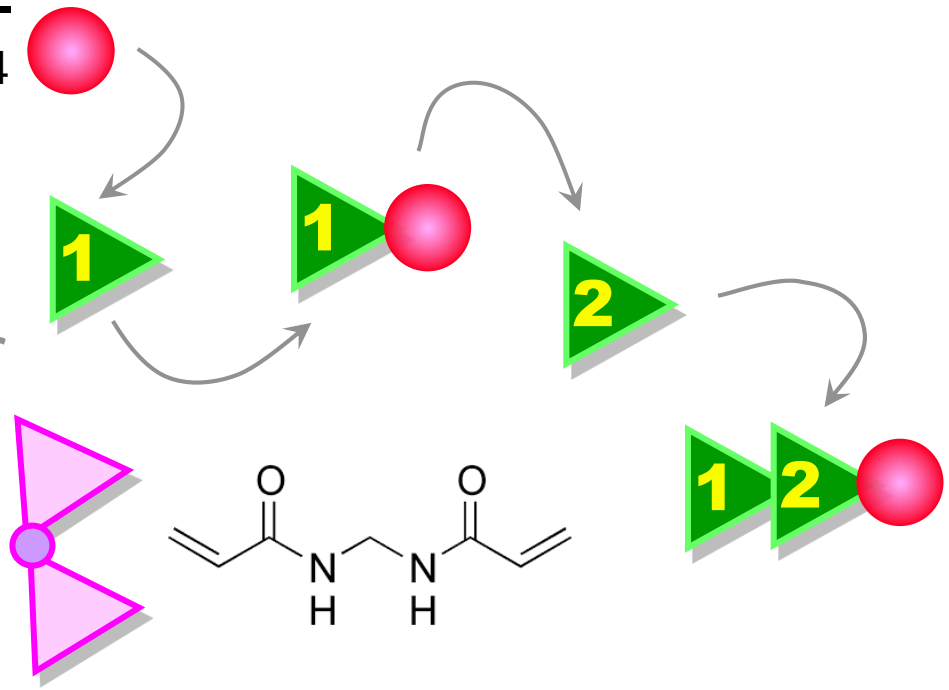
Bis(acrylamide) (bridge)

Cross-linking the gel

TEMED (catalyst)

Help the transfer of radical

SDS (Sodium dodecyl sulfate)

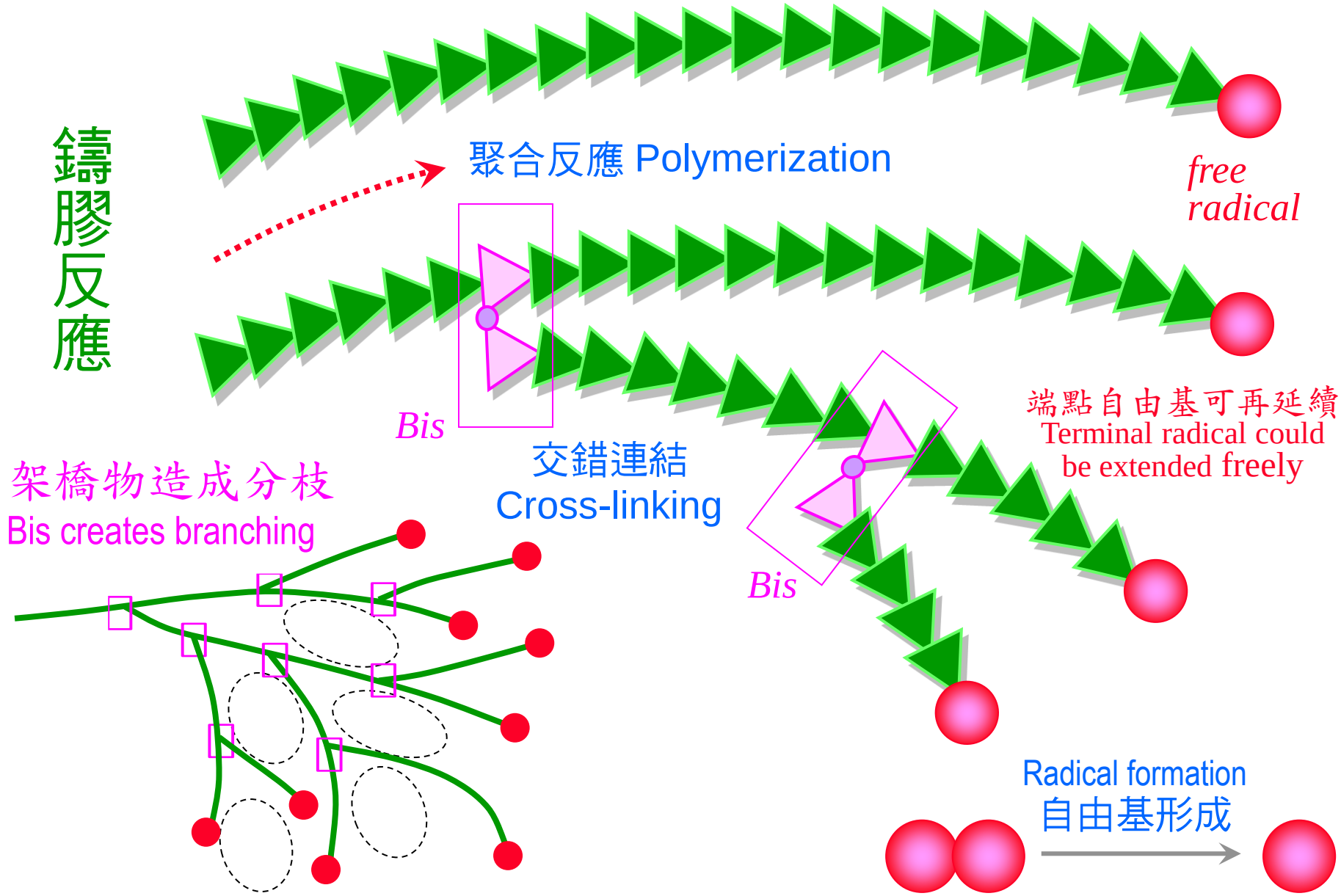


$CH_2{=}CH\text{-}CO\text{-}NH_2$

Acrylamide is toxic

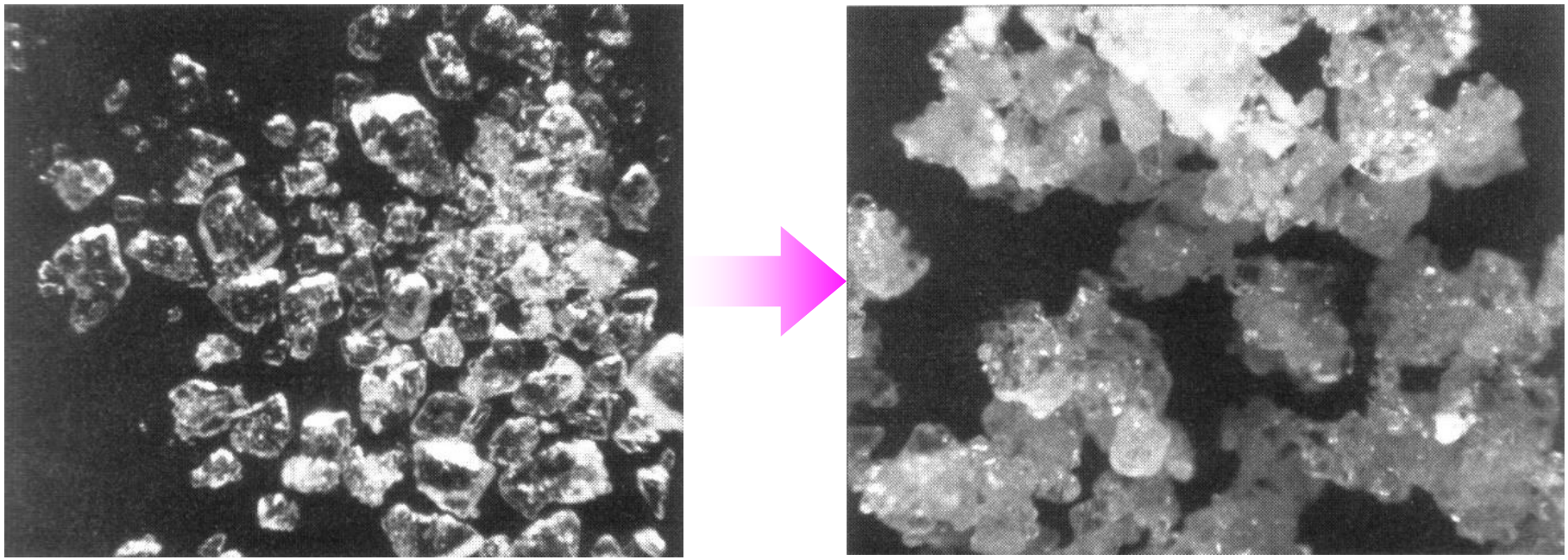
Heating starch might produce it

單体聚合反應 Polymerization from the monomer



Sample protein migrates in the space created by polymerization

凝膠反應注意 A problem in gel polymerization

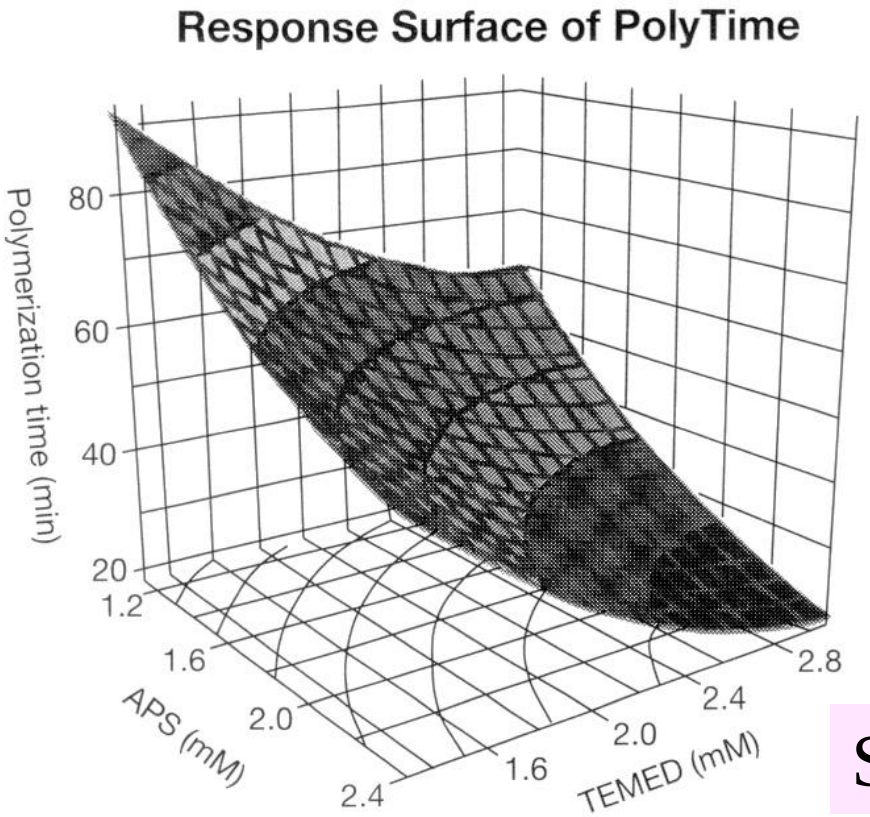


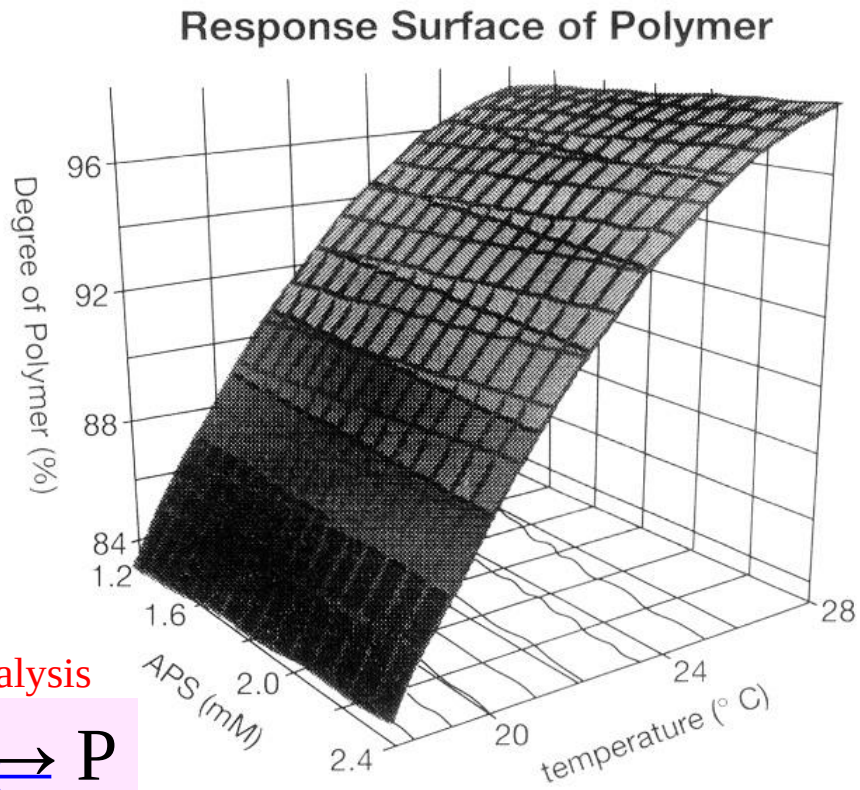
- APS 很容易因潮解而失去效用

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





catalysis

$S \rightleftharpoons P$

equilibrium

- 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 百思不解

Percentage of polymerization is proportional to the temperature, but not related to APS concentration

Pharmacia: Acrylamide Gel Casting Handbook p.12, 13

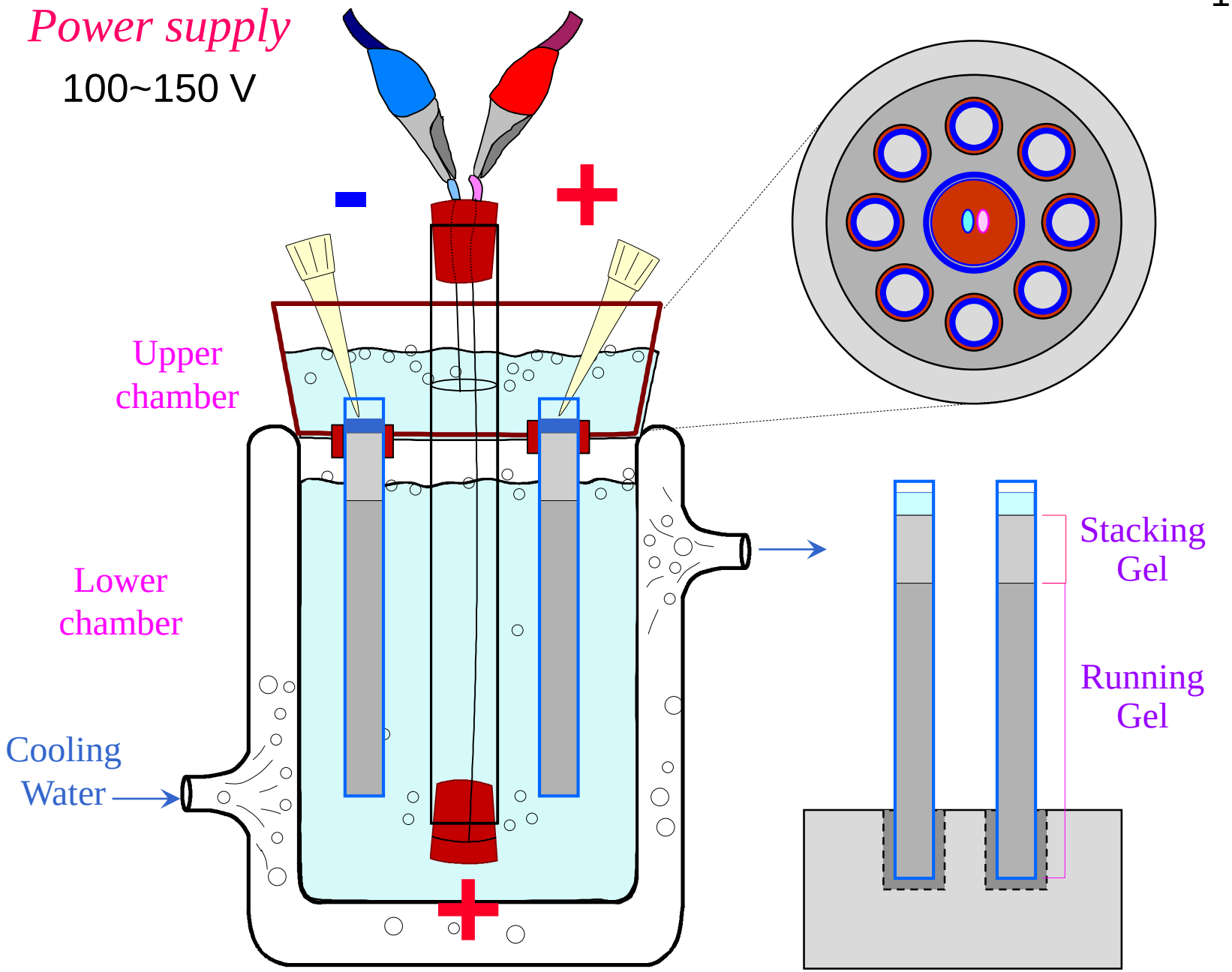
電泳膠体系統組成 Composition of PAGE system

	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	-

- 膠体不連續性有焦聚樣本的作用

The gel discontinuity results in the stacking effect for sample molecules

直立式電泳裝置



The vertical rod gel is the prototype of modern electrophoresis

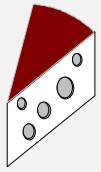
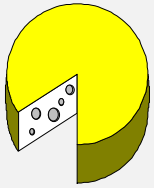
圖 7.3

焦集膠体主要角色 Key molecules in stacking gel

Glycine: Negative charged

No net charge

Chloride ion:

Proteins:  Small molecule  Large molecule

焦集膠体的作用原理 How stacking effect works

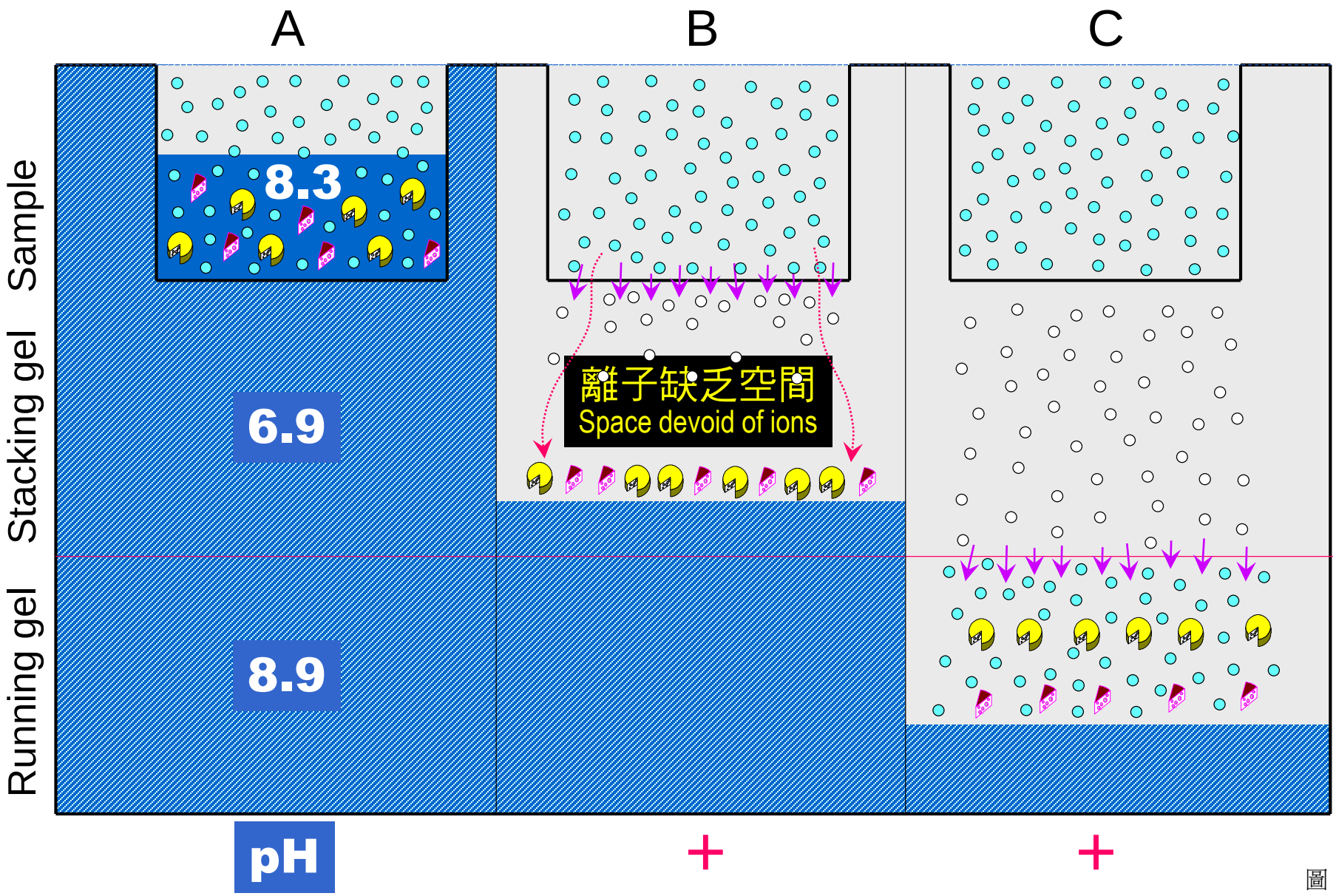
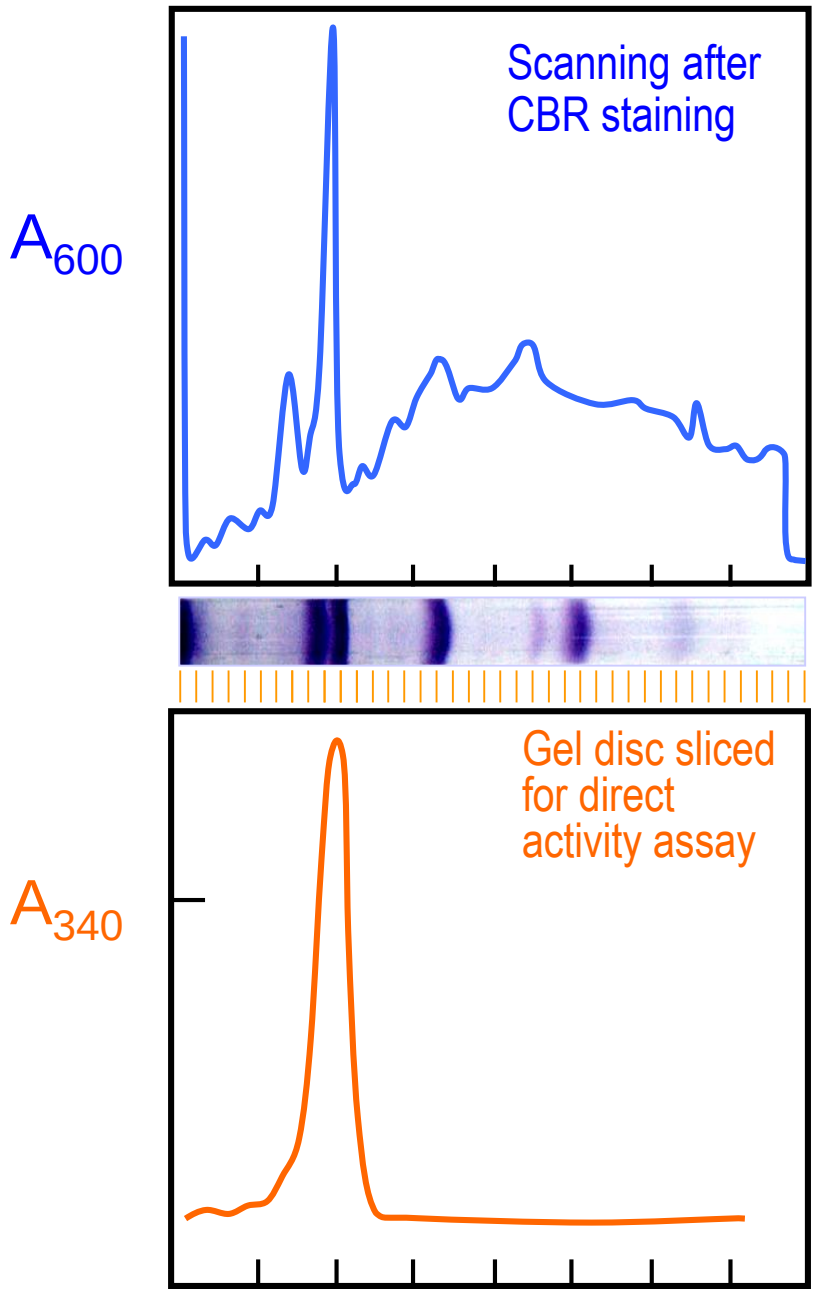


圖 7.4

電泳膠体中酵素活性測定

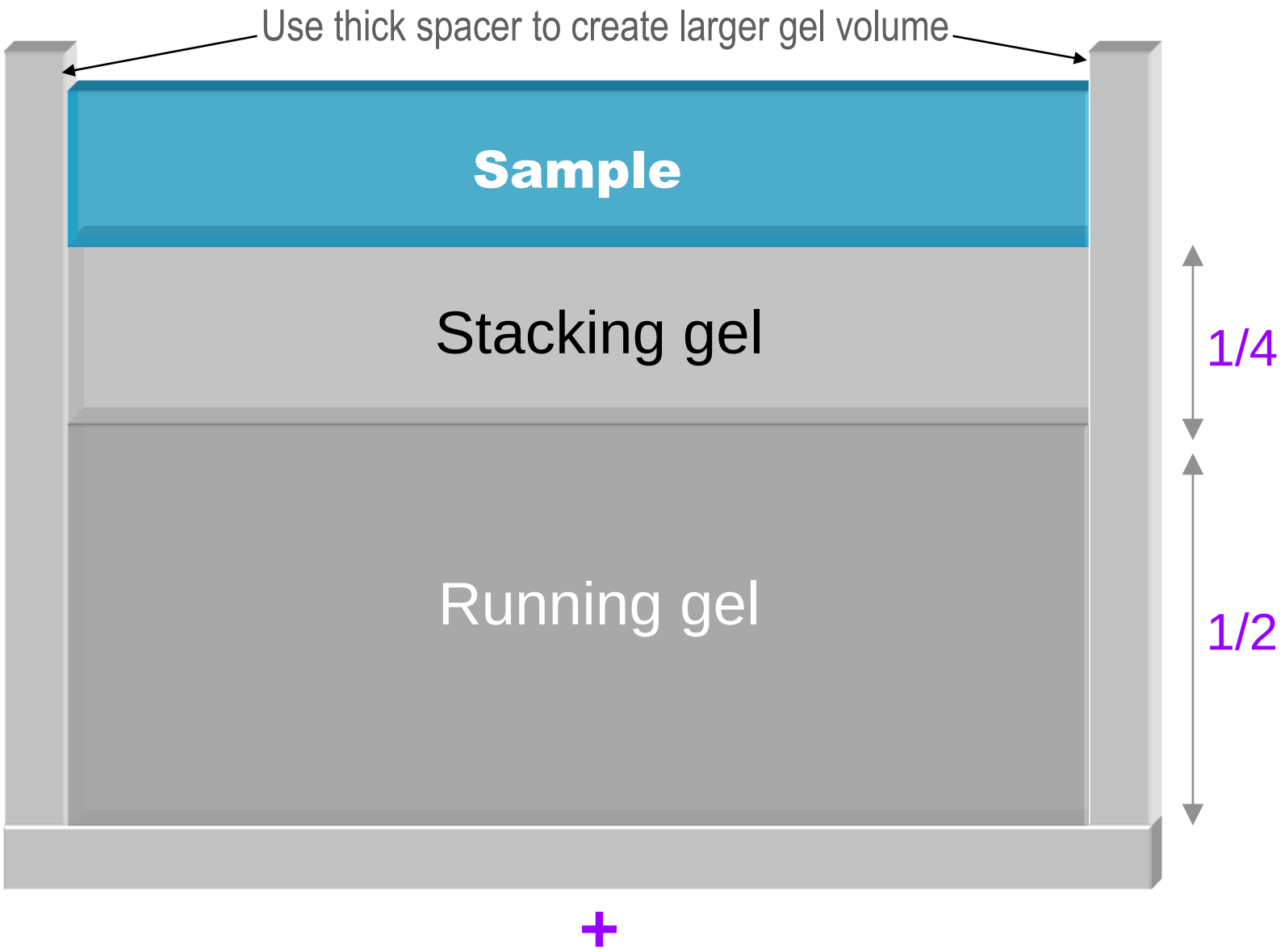


Native PAGE
Non-denaturing PAGE

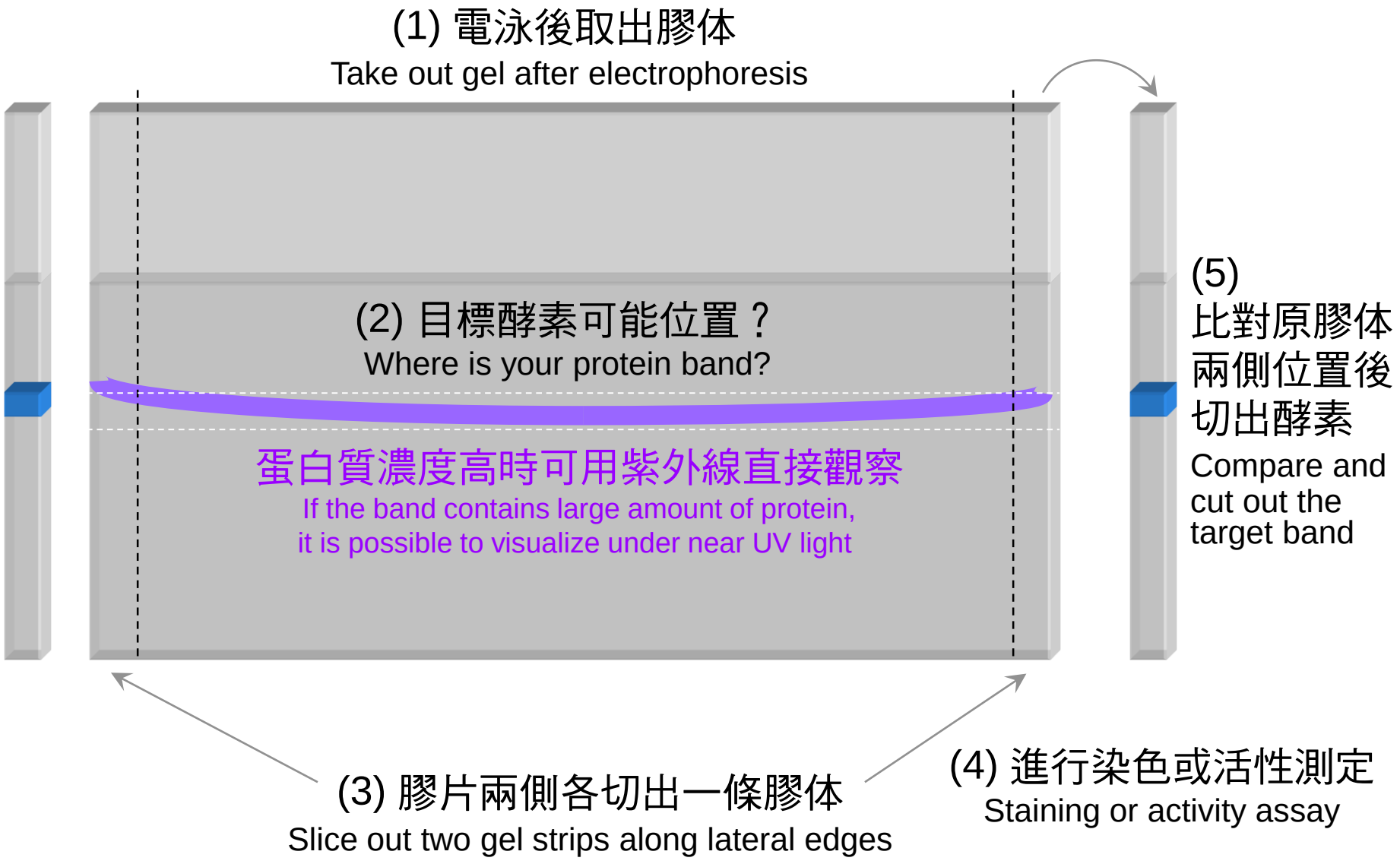
~~SDS-PAGE~~

(但有少數酵素在 SDS 下還有活性)

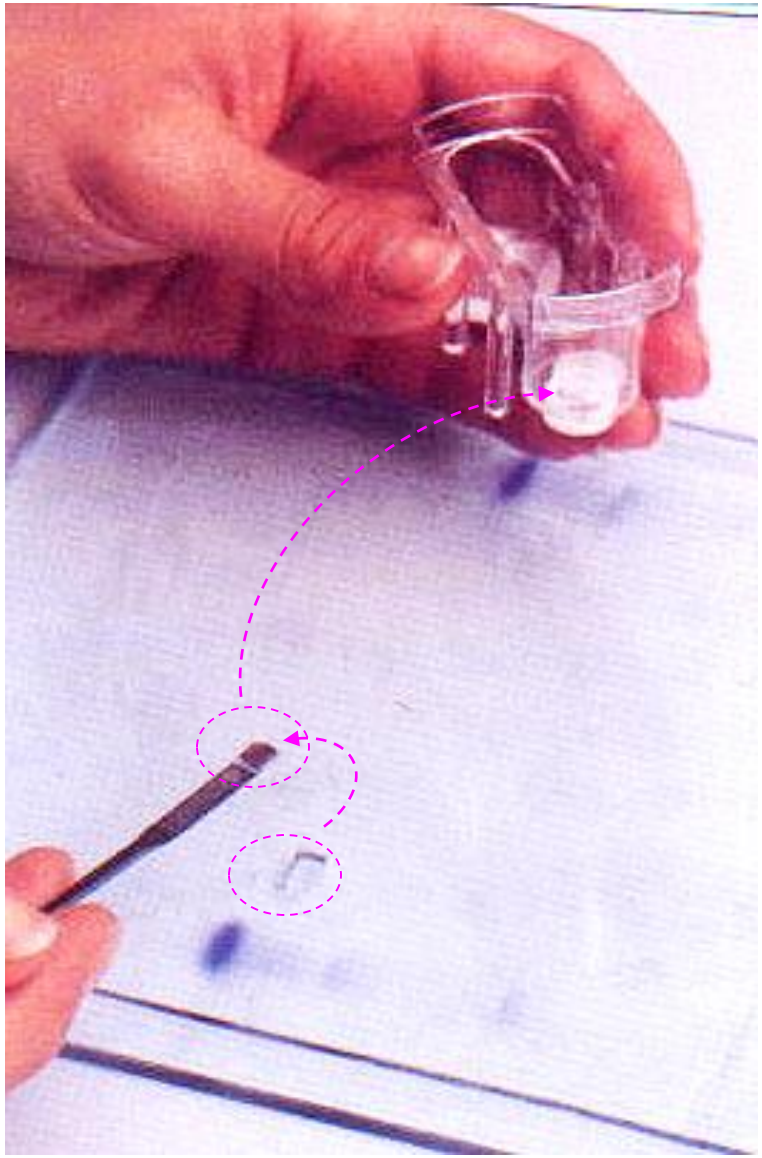
製備式電泳膠片 Preparative gel



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



電泳膠体蛋白質溶離 Protein eluted from the gel

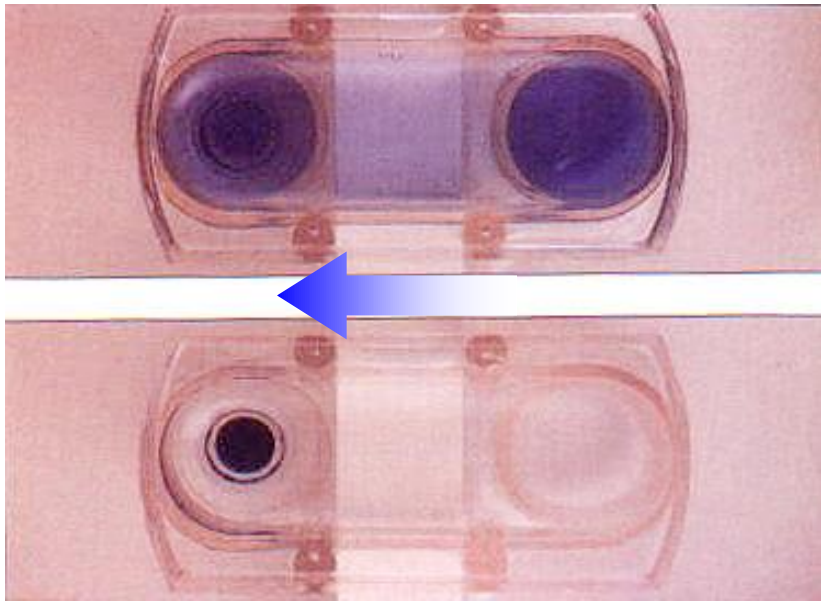


ISCO: Little Blue Tank Concentrator

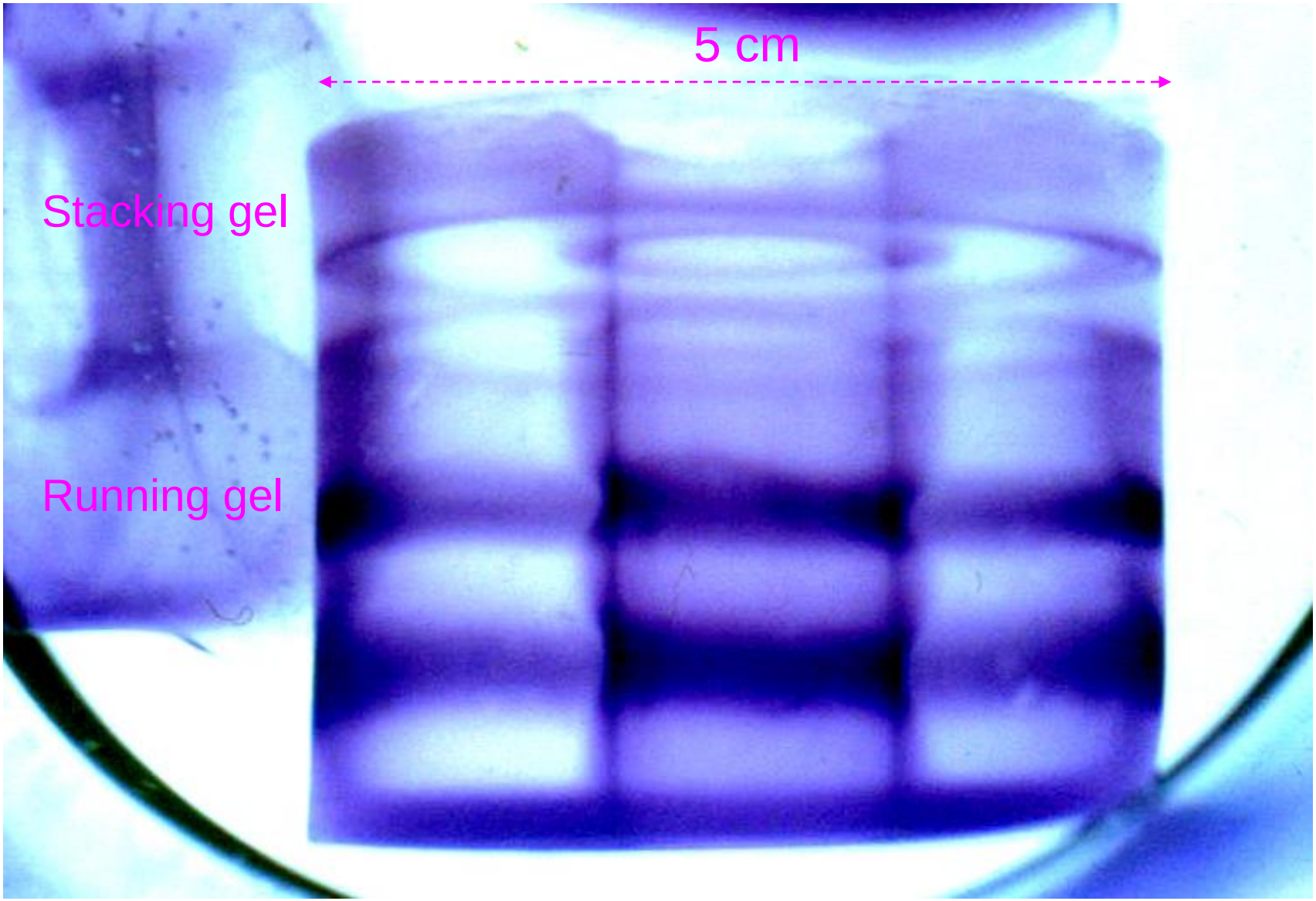
- 直接挖出膠体進行溶離

Cut out and eluted

Little Blue Tank

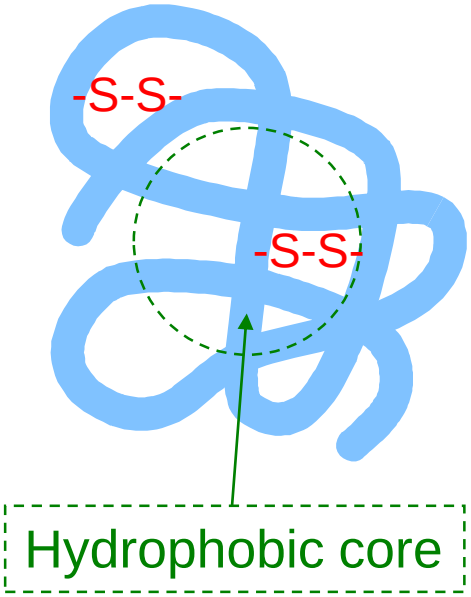


巨無霸的製備式電泳 Jumbo size preparative gel

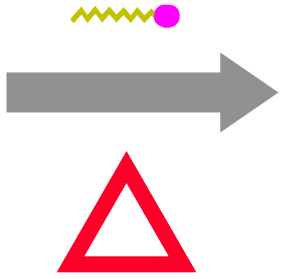


SDS 在蛋白質表面均勻敷上一層負電

Native protein (in water)

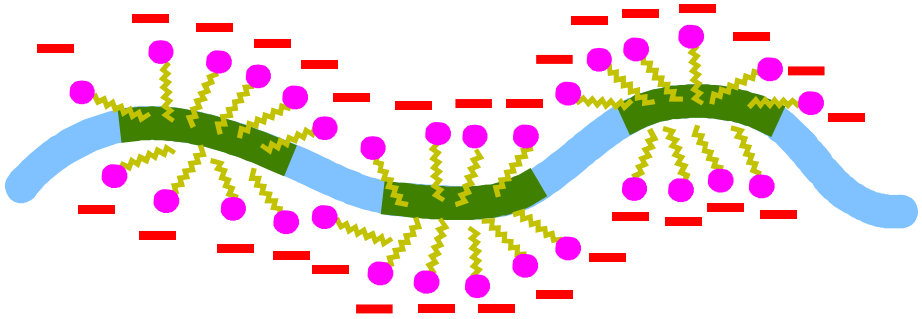


SDS



boiling

Protein is denatured to linear form

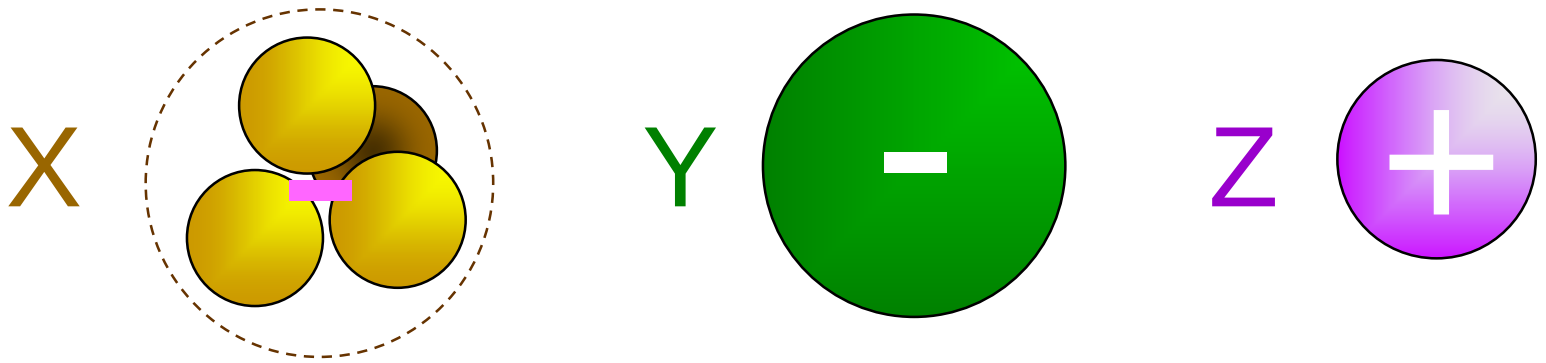


Its surface covered with negatively charged SDS regularly

+ SDS: block the non-polar amino acid residues
+ β-Mercaptoethanol: break the disulfide bonds (-S-S-)
Boiling: destroy the H-bonds and denature protein

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



影響泳動率的因素 Molecular weight & charge

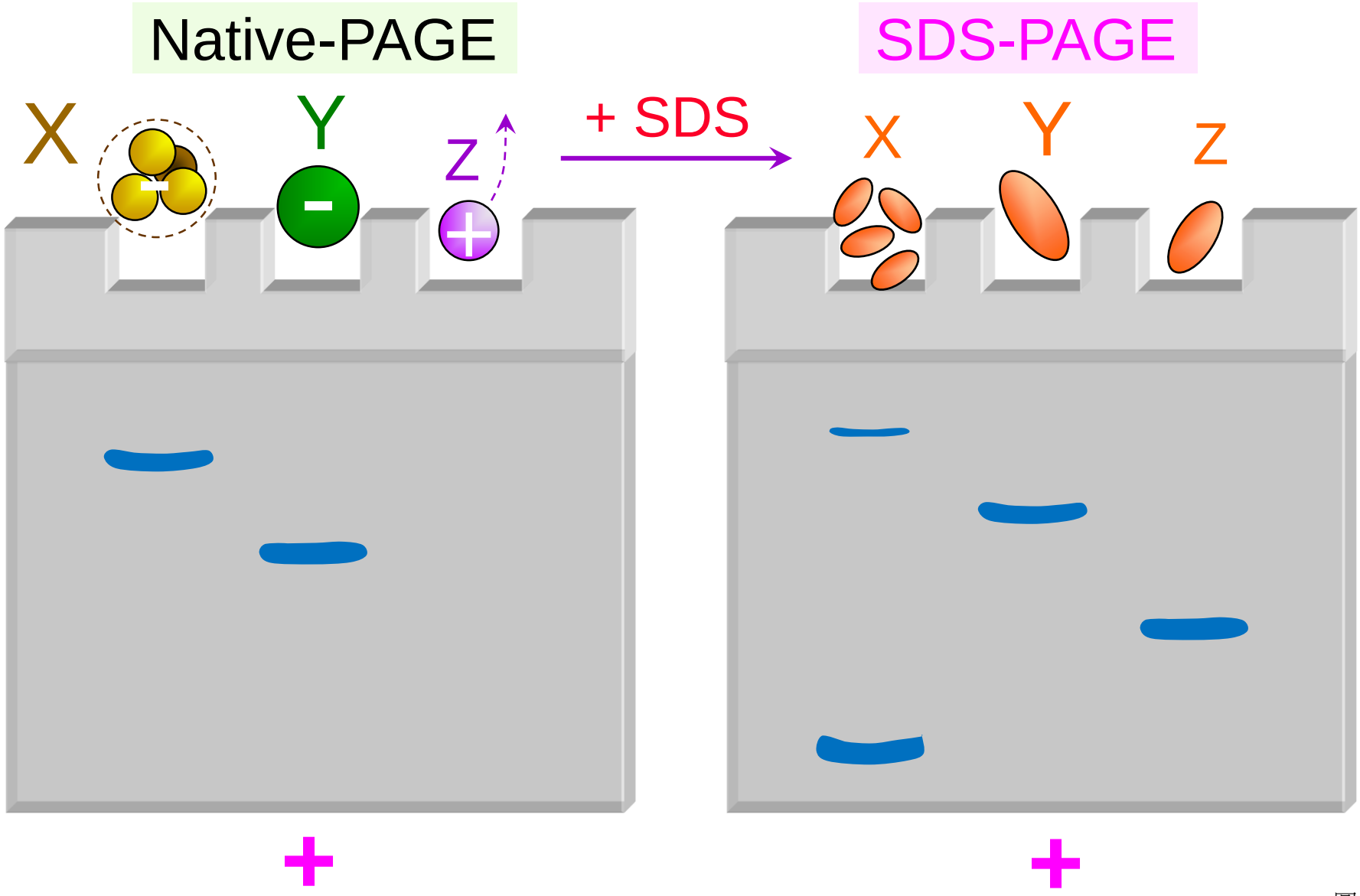
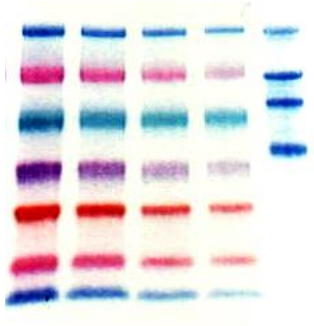
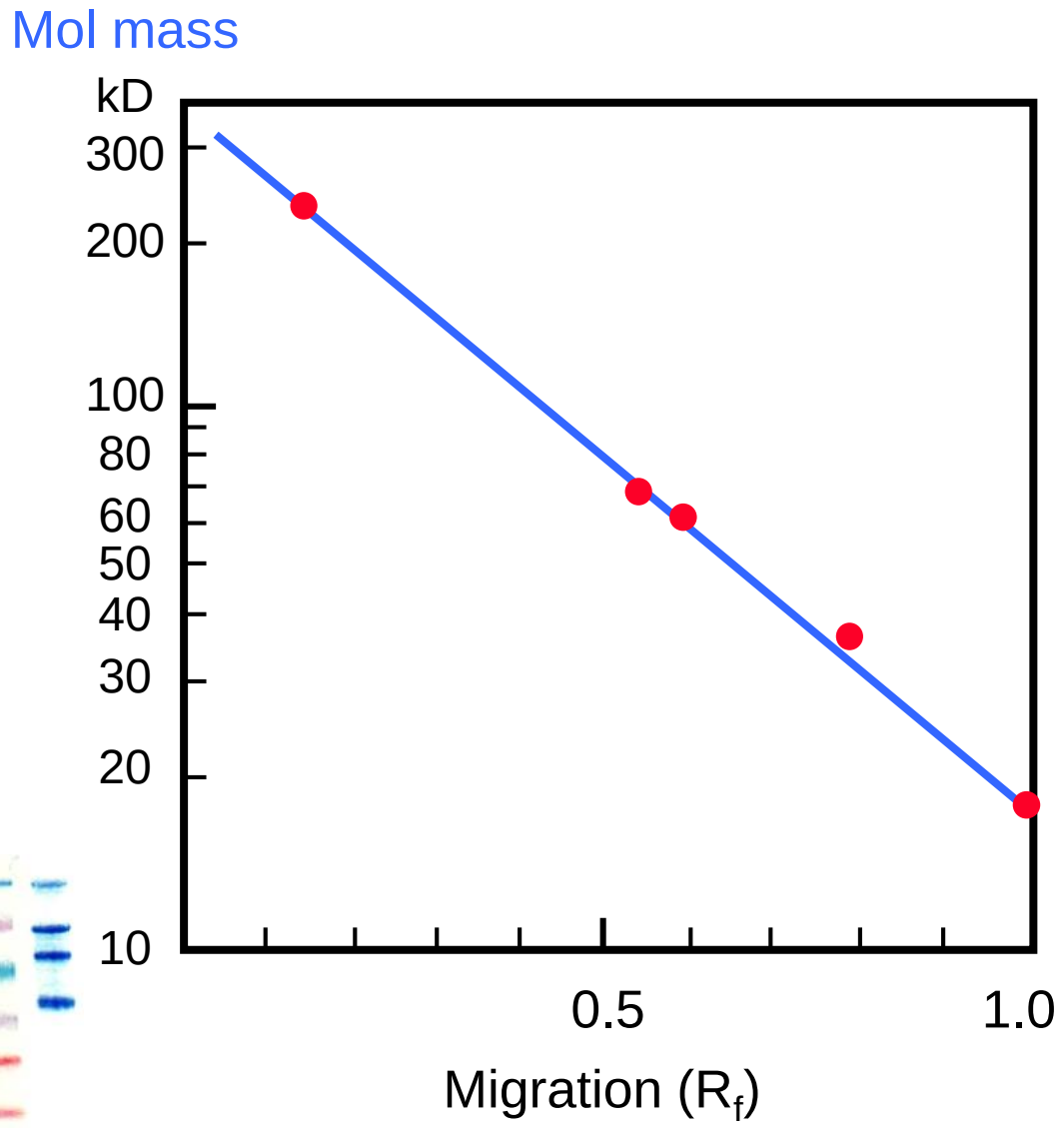
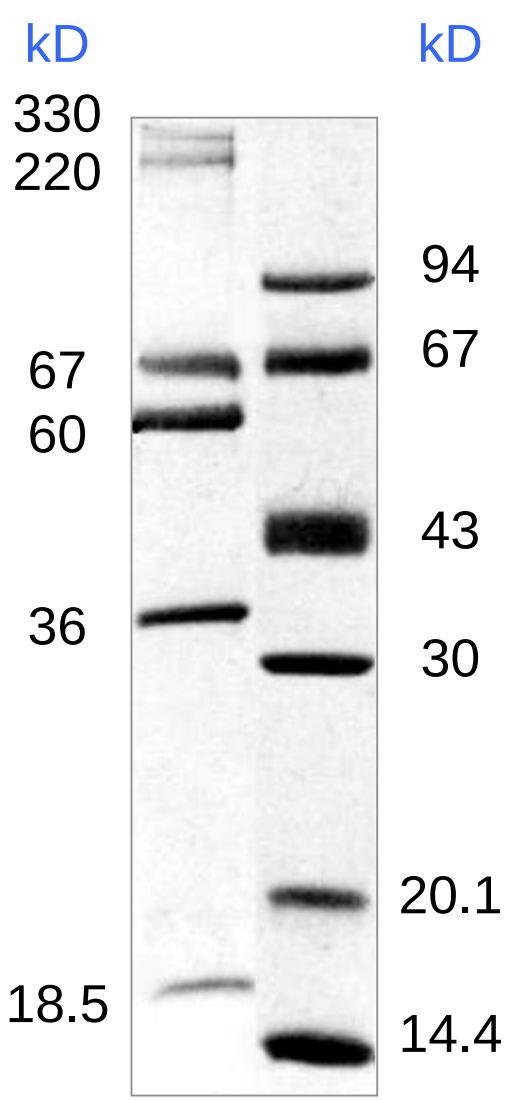


圖 7.5

測定単元体分子量 SDS-PAGE for subunit MW

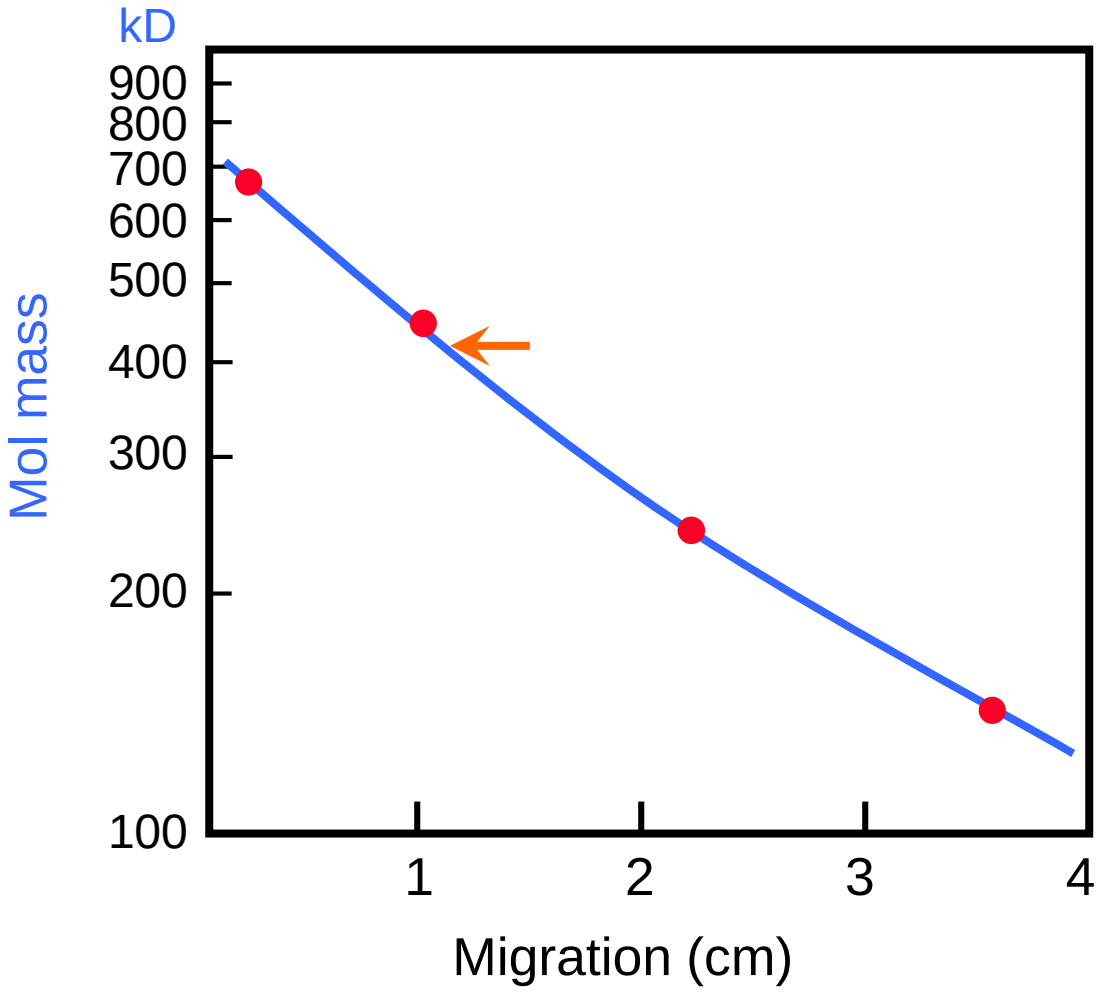


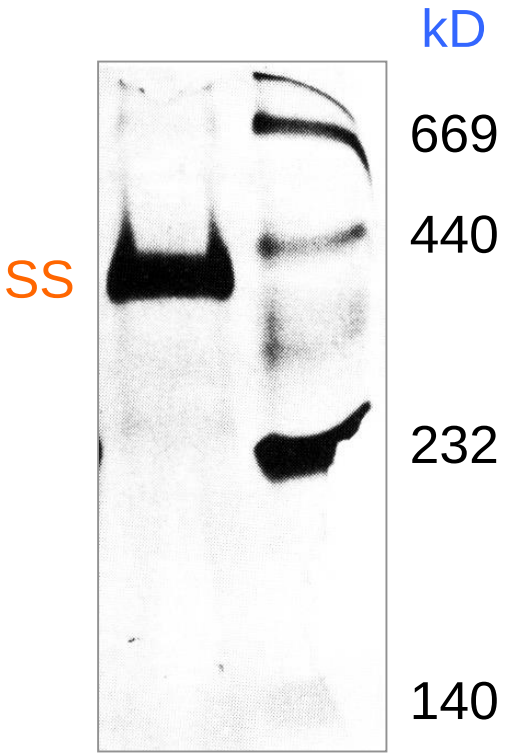




Pharmacia: Molecular Markers for electrophoresis

原態分子量測定 Disc-PAGE for native MW





- 不能以 disc-PAGE 做為唯一的實驗依據

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

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 or slab	Acrylamide	Protein
Horizontal	Slab	Agarose	NA Isozyme
Vertical	Vertical slab	Mixed type	DNA sequencing

(1) 蛋白質樣本最常用的選擇畫有底線

(2) 蛋白質最常用：迷你垂直式 acrylamide slab 以 100 V 進行電泳

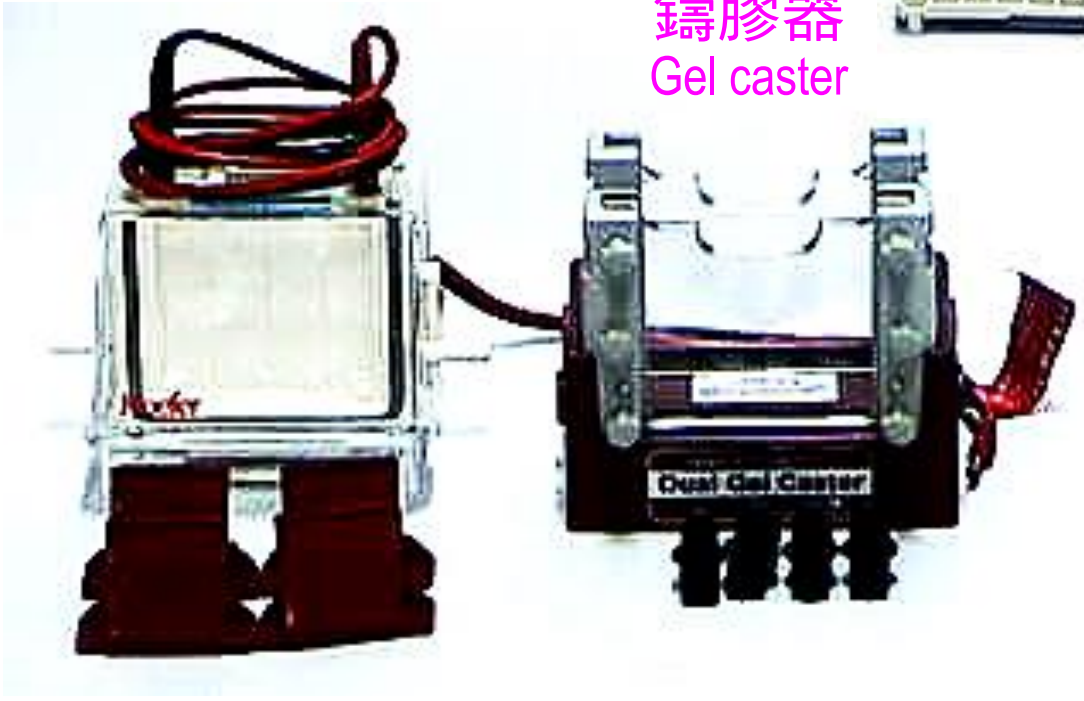
電泳槽及相關設備 Instruments and equipment

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

7.3 其他相關技術 Other related techniques

7.3.1 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

7.3.2 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

7.3.3 等電焦集法 Isoelectric focusing

依蛋白質分子的等電點來分離

7.3.4 二次元電泳 2D electrophoresis

解析力極高，可分析成份複雜的樣本

各種蛋白質染色法機制

1 Ammoniacal Silver

金屬銀沈澱
Silver deposit

binding acidic amino acids

$H_3N…Ag…NH_3$

$H_2N…Ag$

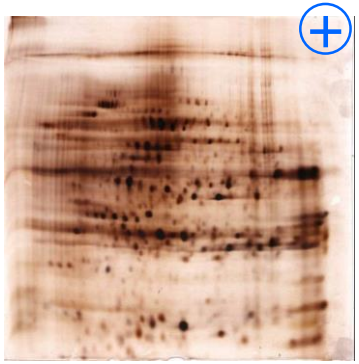
Glutaraldehyde
OHC-C-C-C-CHO

還原
Reduction

2

Coomassie Brilliant Blue R

or non-polar amino acids



Gel staining methods and mechanism

Constant

Variable

各種蛋白質染色法機制

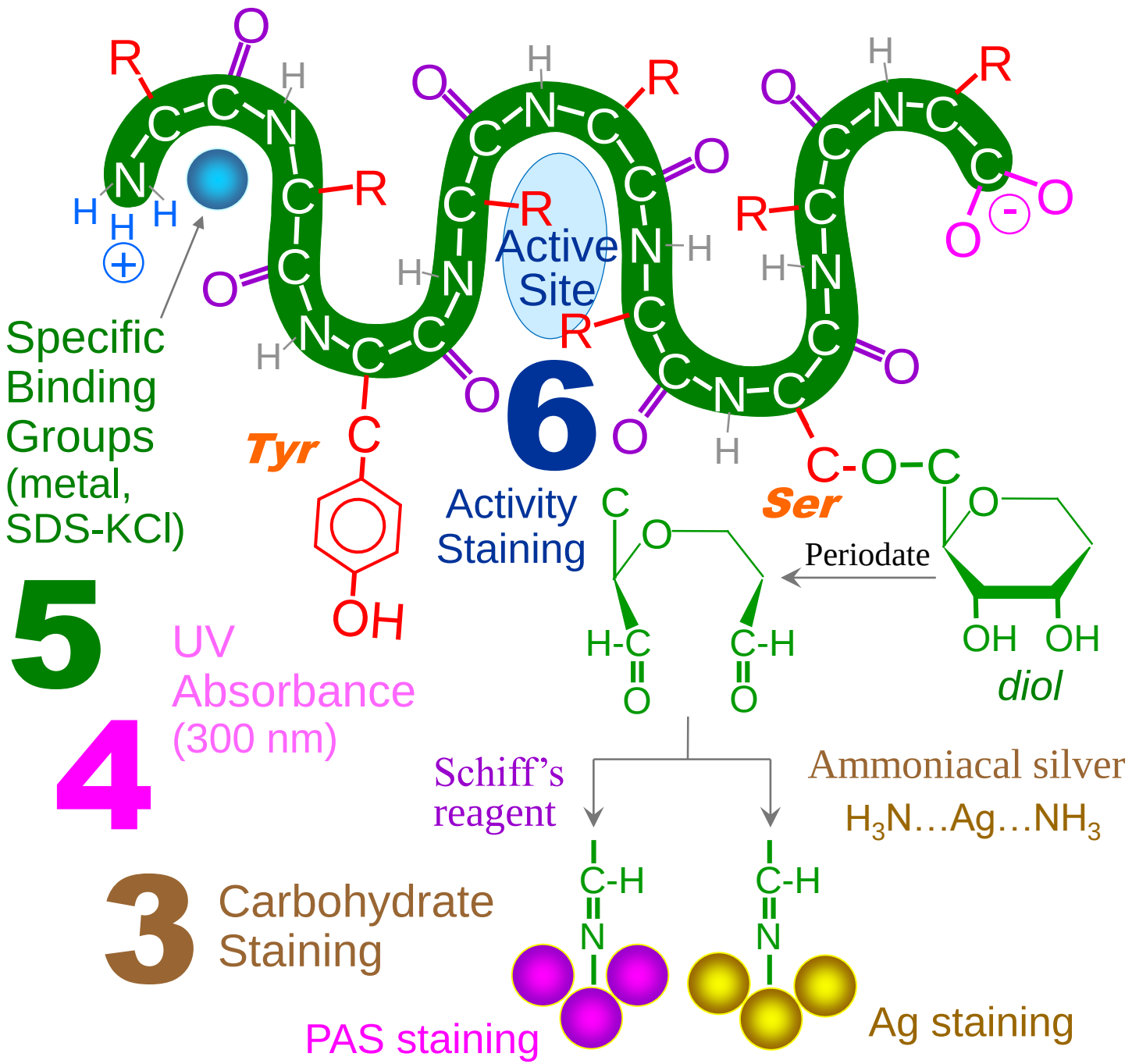
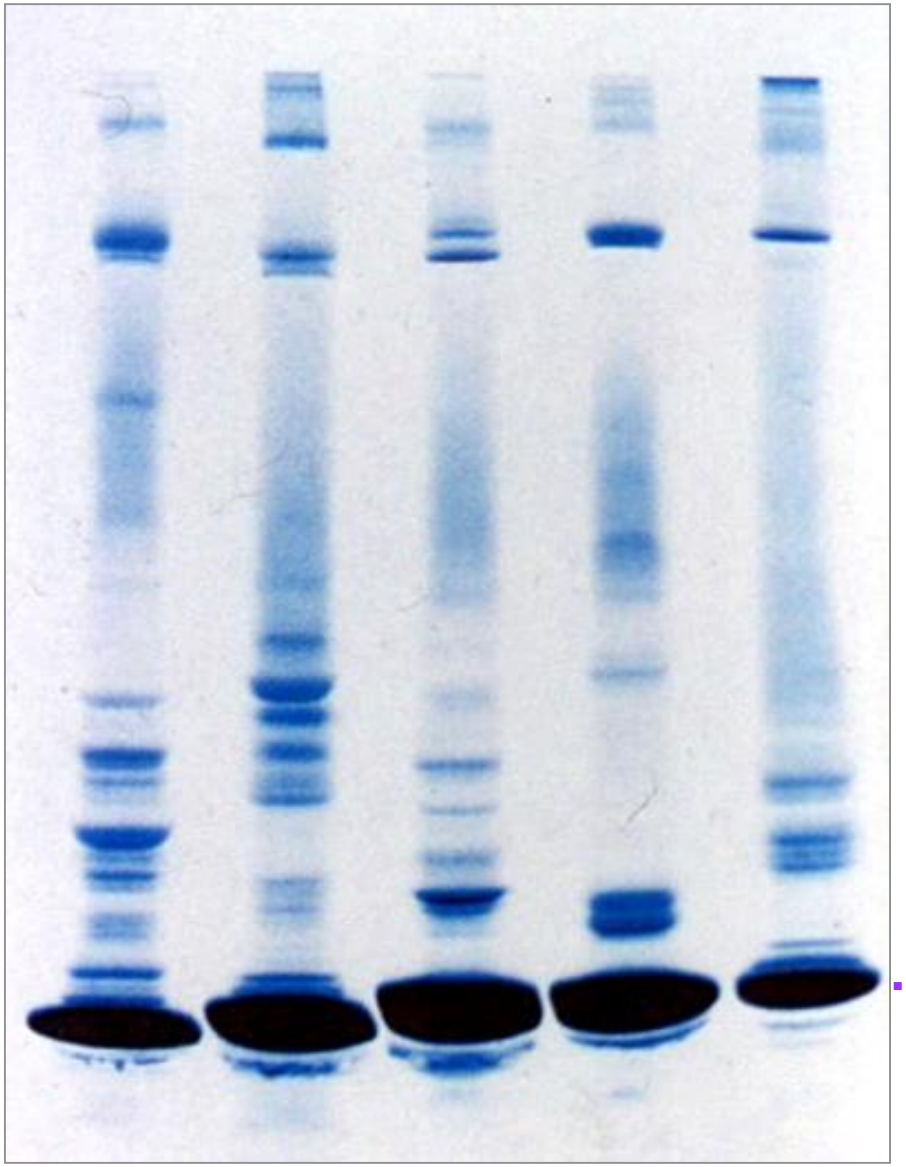
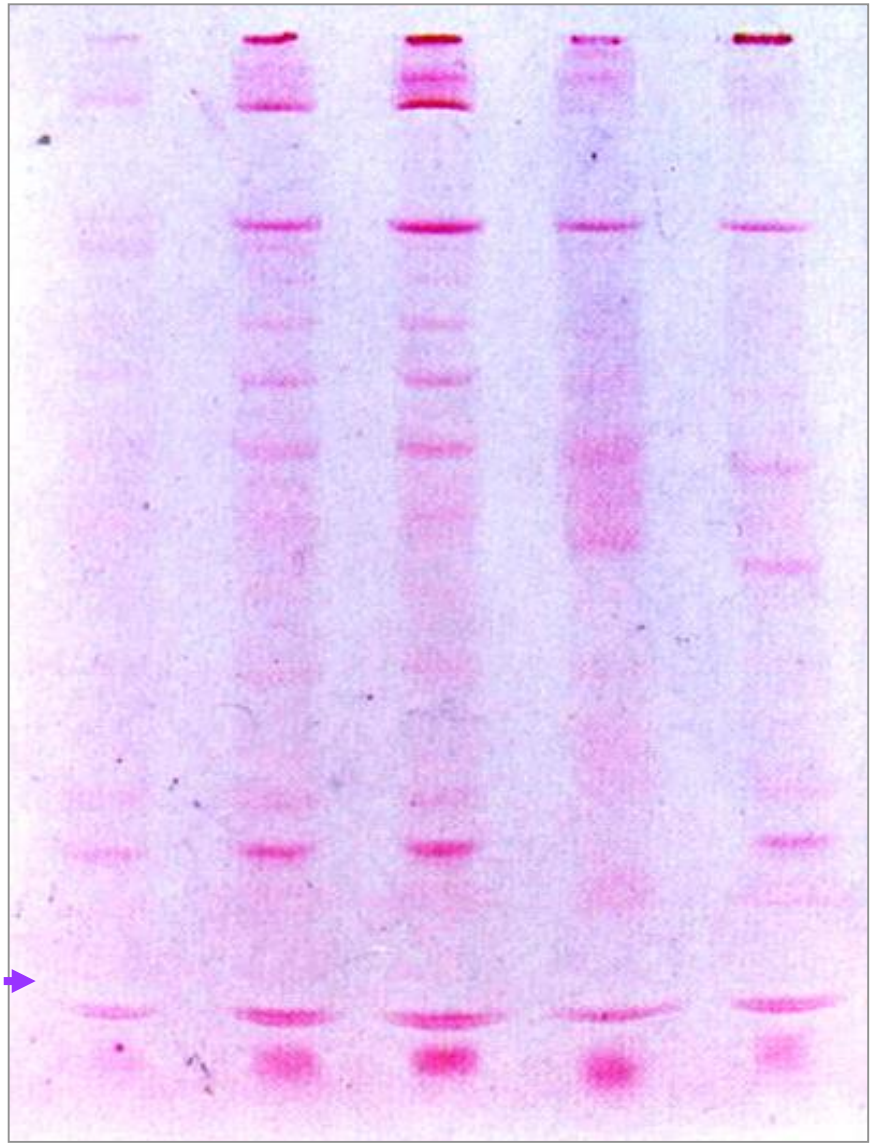


圖 7.6

血清電泳染色結果 Serum stained by CBR / PAS

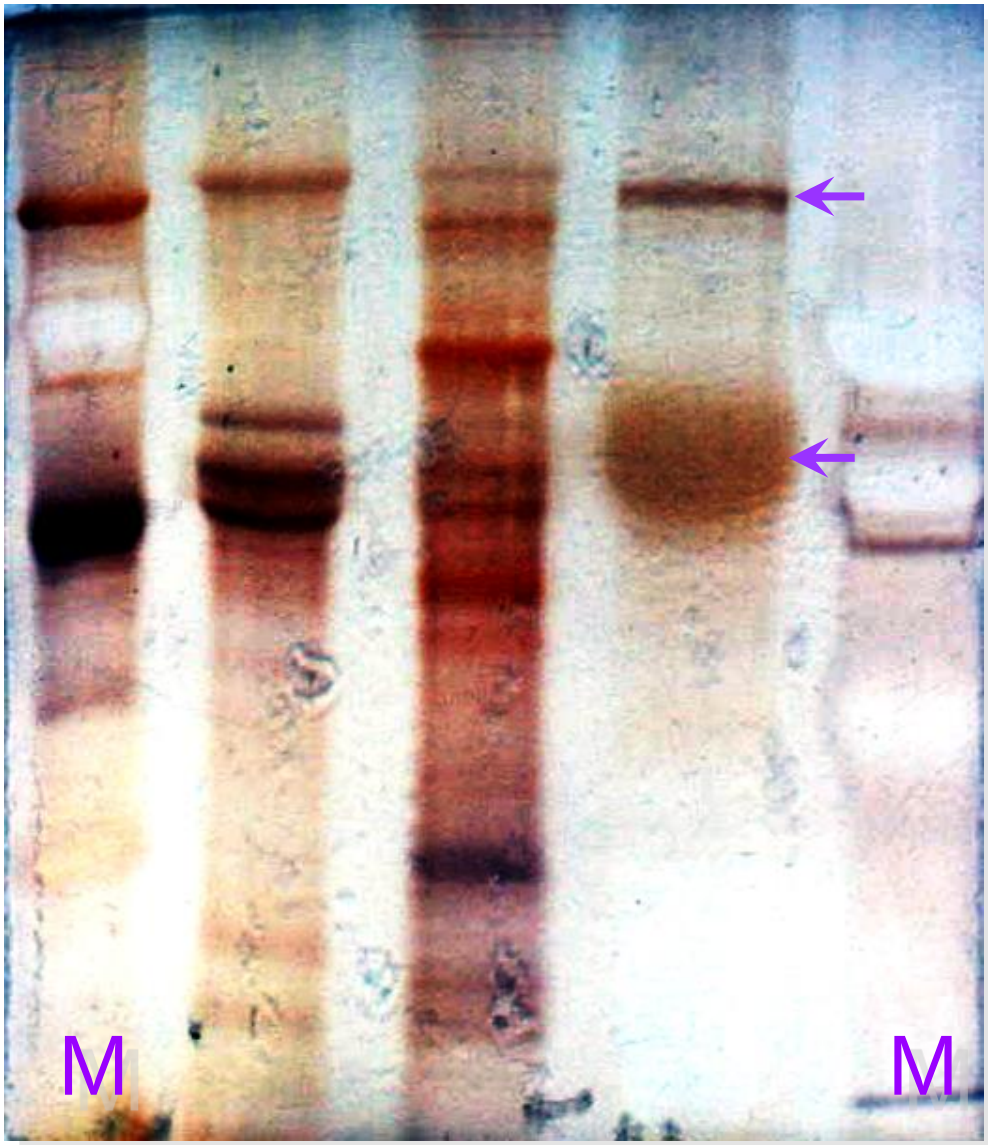




Pharmacia: Electrophoresis

Albumin can't be stained with PAS

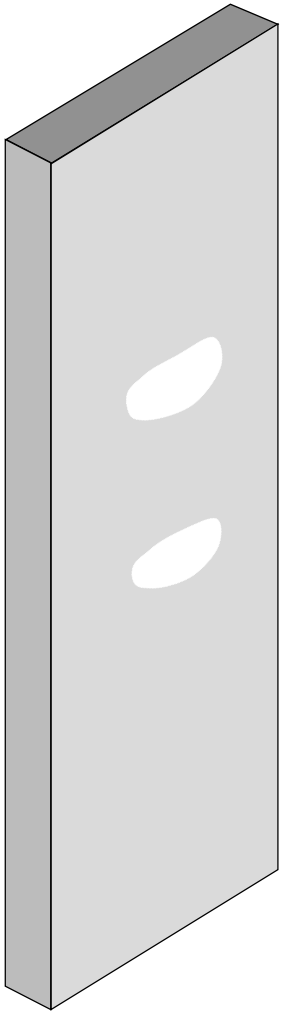
SP 醣染色結果 Glycoprotein stained with $AgNO_3$



Assay by starch synthesis

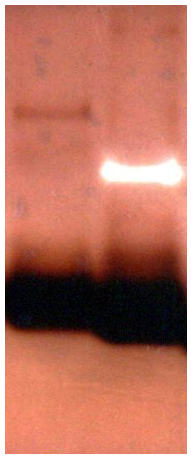
A

Activity staining



BA

SP



Native PAGE

B

Activity assay and interference

Direct observation of the color product

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

圖 6.3

■ 膠片呈色法比較 Comparison of staining methods

Methods	Targets	Sensitivity	Remarks
Coomassie Brilliant Blue R-250 Staining	Protein	Med sensitivity ●●	Simple, rapid (最常用)
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 (3.1)
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 ●●(●)	Depended on enzyme assay

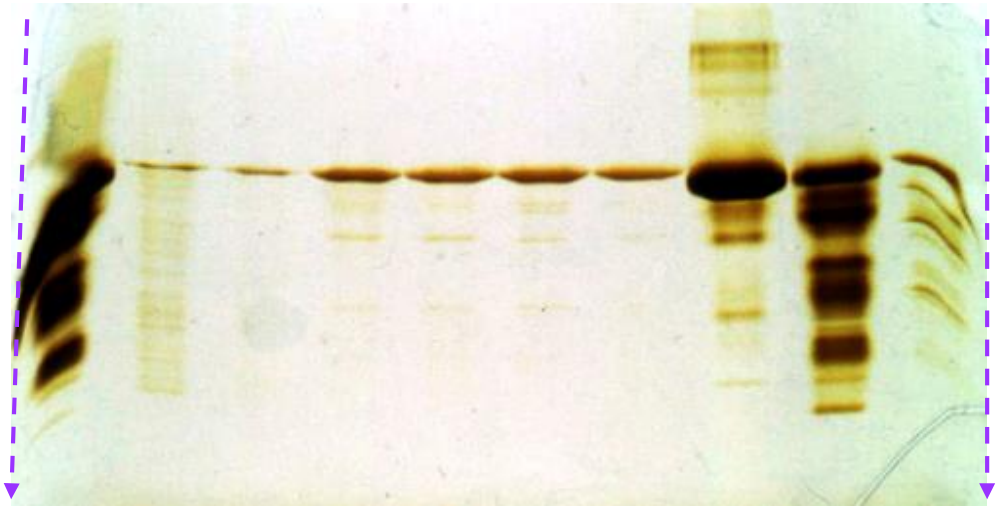
電泳結果不佳十大原因 Trouble shooting (Top 10)

膠片情況	可能原因
色帶扭曲	鑄膠不良或有氣泡陷入 (或樣本成分影響)
膠体無法凝結	APS 濃度太低或已經失效 (室溫太低)
色帶擴散、拖尾	樣本中的鹽濃度太高或極端 pH
只有部分樣本跑不好	樣本槽是否清潔乾淨？ (充分刷牙)
凝膠不完全	APS 是否溶解完全？ (凝膠可放過夜)
染料前線歪斜	室內溫度是否均勻？緩衝液有洩漏？
膠片出現許多直線條	膠体或試劑成分含有雜質 (或有小氣泡)
似乎沒有焦集作用	各種試劑 pH 是否正確？(尤其是 C 溶液)
蛋白質樣本泳動率不對	膠体、緩衝液、樣本溶液中忘了 SDS？
膠体沒有凝結變成黏液	膠体溶液中忘了加 Bis？ (無法架橋)

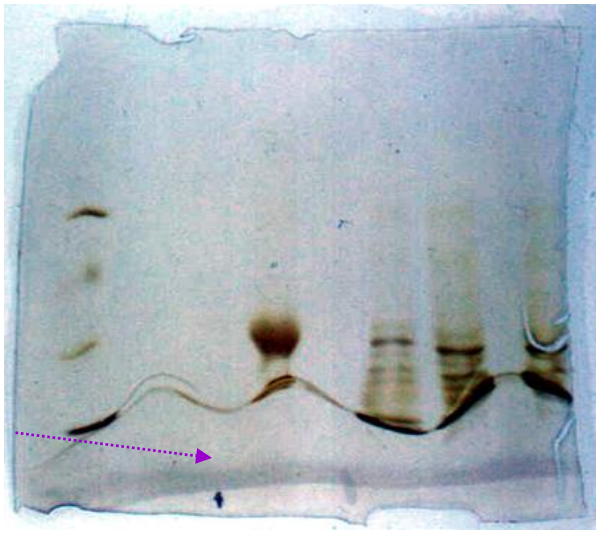
電泳結果不佳十大原因 Trouble shooting (Top 10)

What's wrong?	Possible causes
Band twisting	Bad gel casting or gel trapped bubbles
Can't polymerize	Wrong APS concentration or inactivated APS
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?

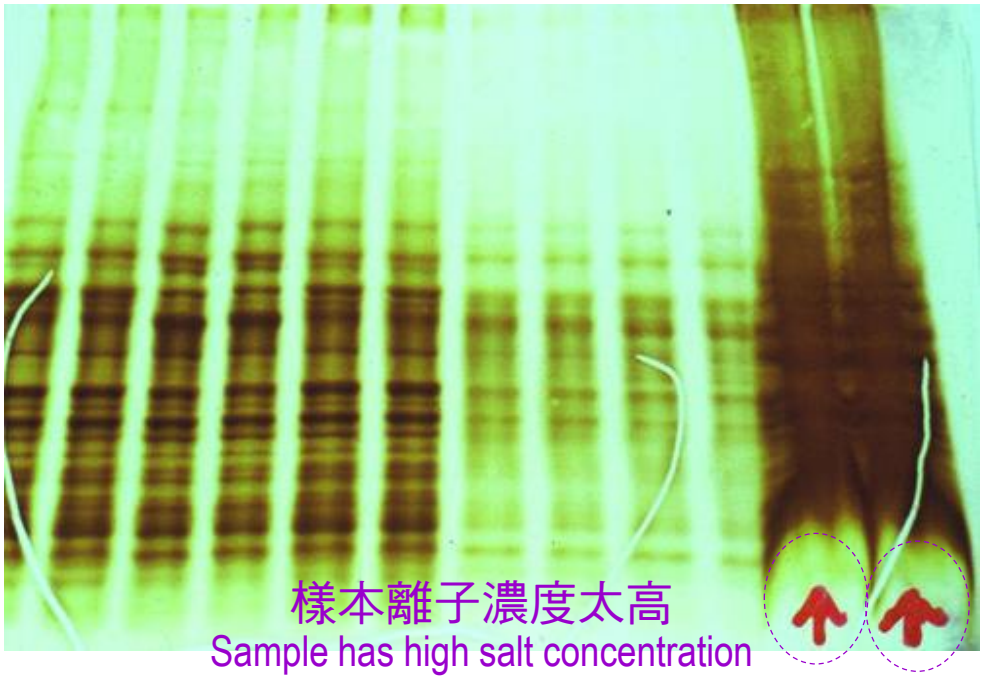
各種電泳染色的問題 Some gel problems



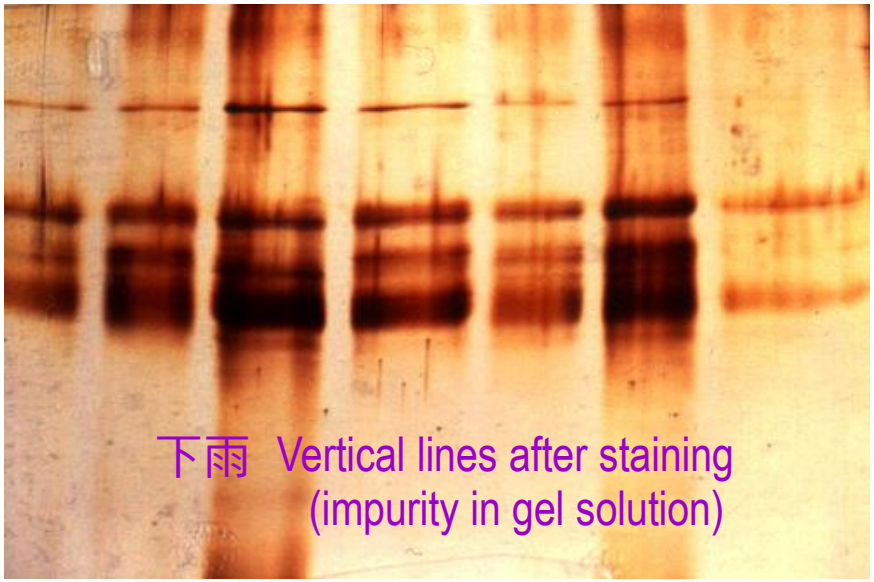
暗流
Leaking buffer conducting the electric current
(膠體與 spacer 之間有空隙)



凝膠不均勻
Gel is unevenly polymerized

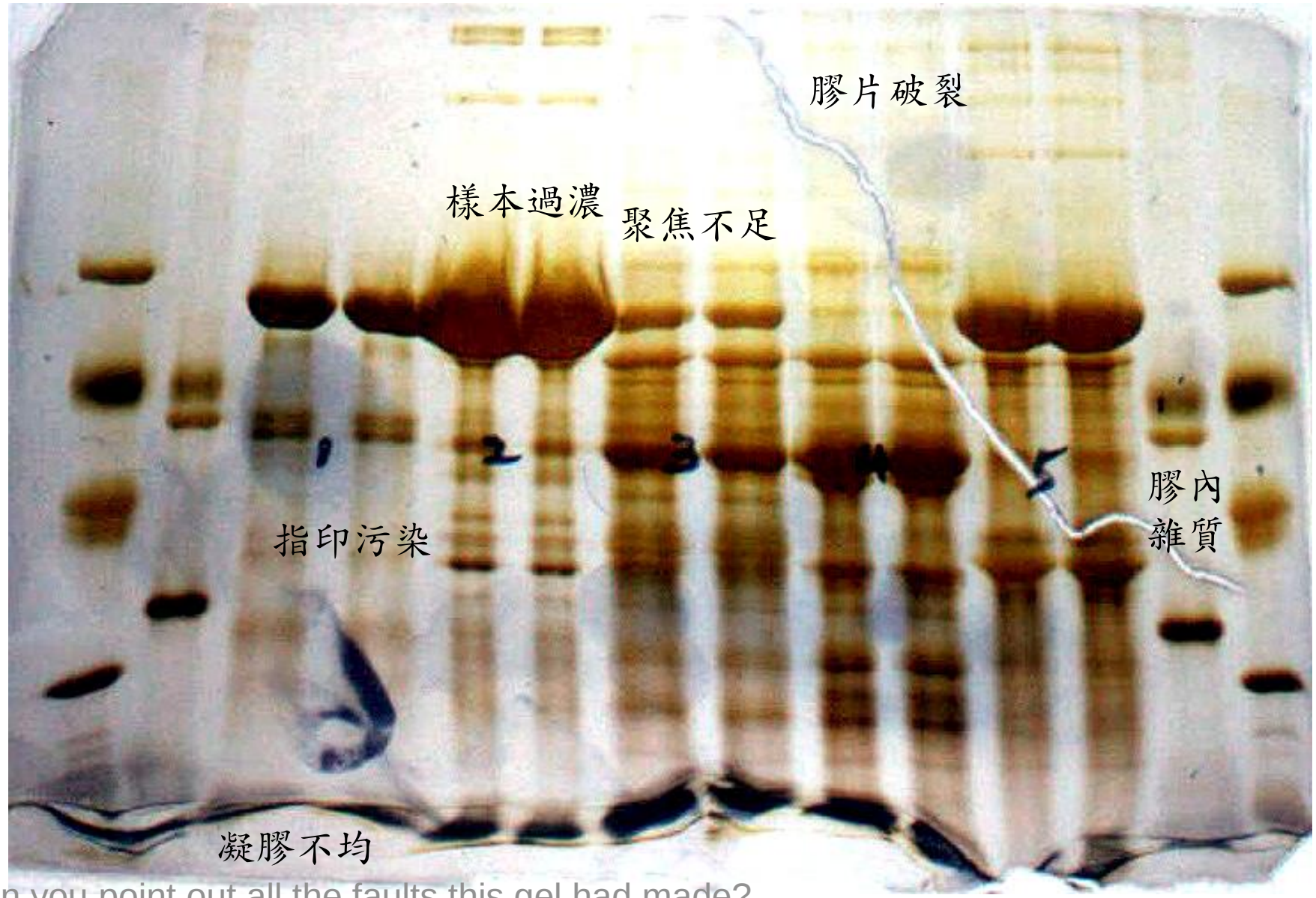


樣本離子濃度太高
Sample has high salt concentration



下雨 Vertical lines after staining (impurity in gel solution)

各種電泳染色的問題 A malformed gel

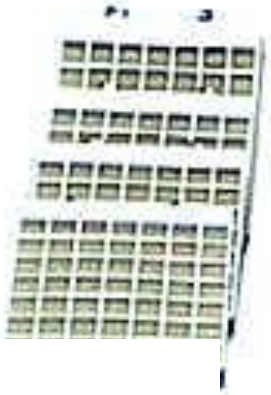


Can you point out all the faults this gel had made?

電泳槽及轉印設備 Electrophoresis and transfer

Western Blotting

轉印三明治
Transfer sandwich



轉印槽
Gel transfer

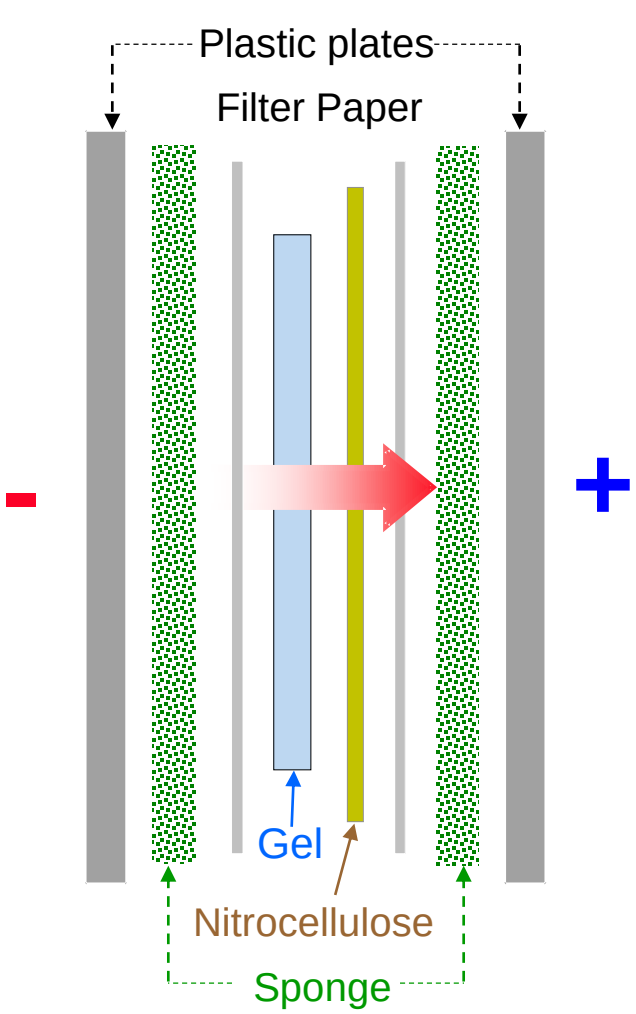
電泳槽
Electrophoresis unit

鑄膠器
Gel caster

供電器 Power supply

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

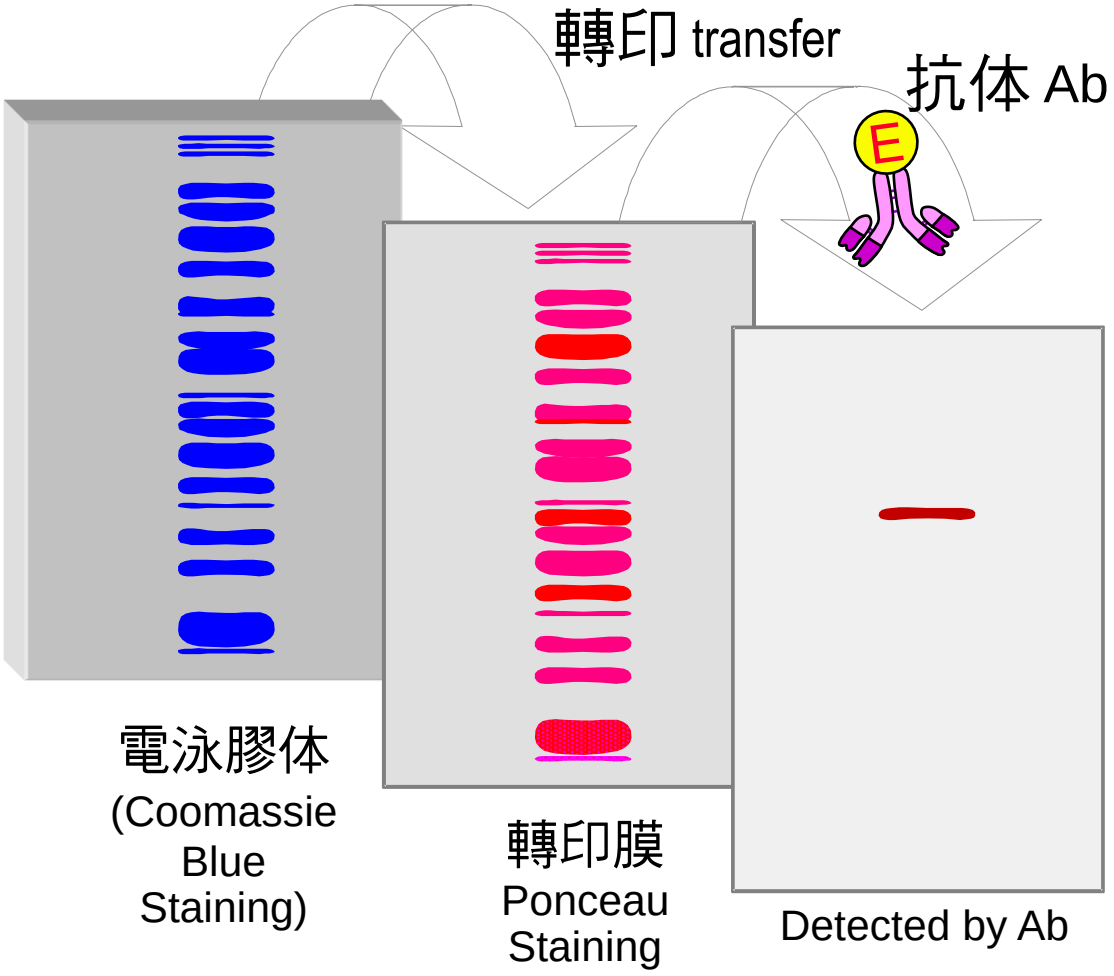
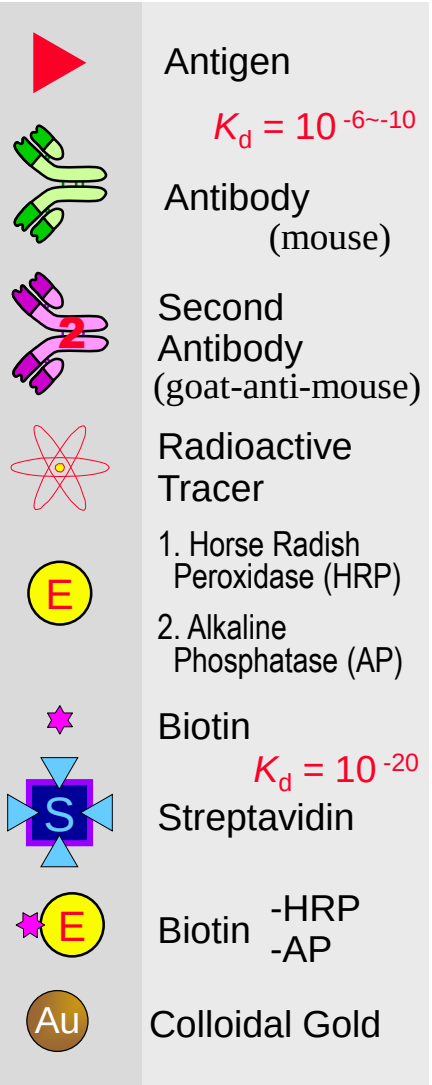
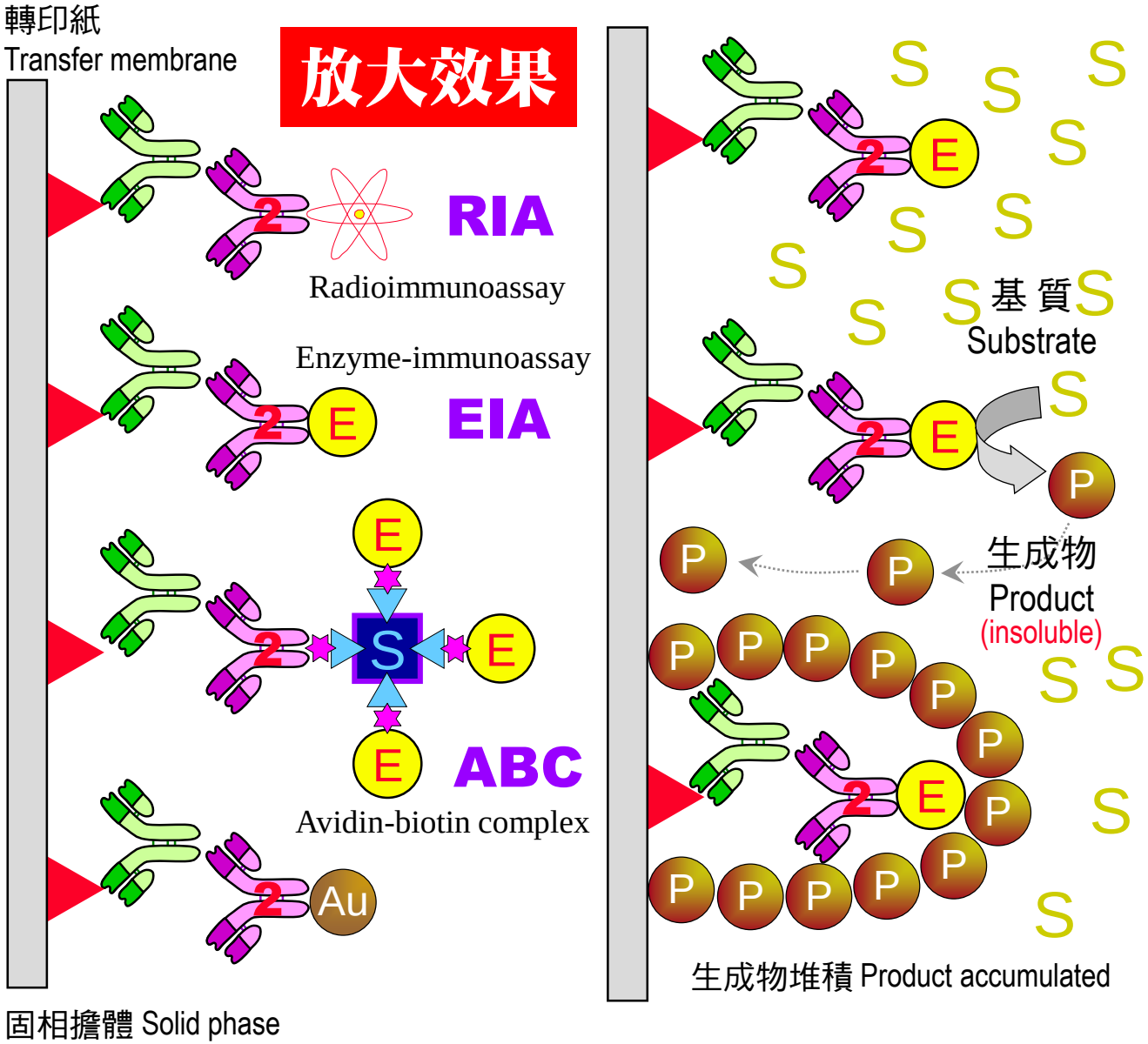


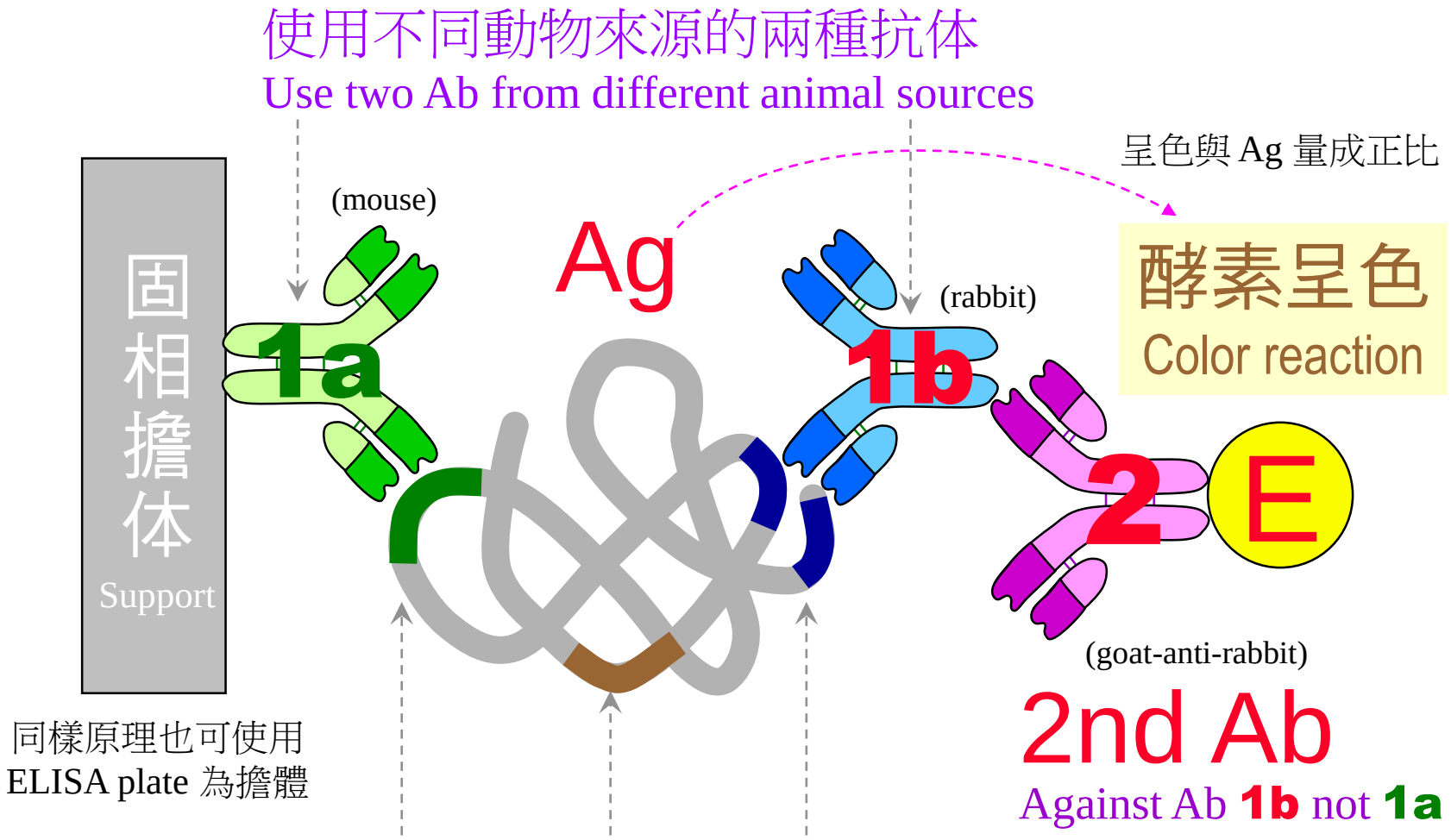
圖 7.7

免疫轉印種類與呈色機制 Immunoassays





三明治免疫分析法 Sandwich ELISA method



固相擔體 Solid phase:
ELISA plate, Western blot,
Affinity gel, Enzyme immobilization

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)

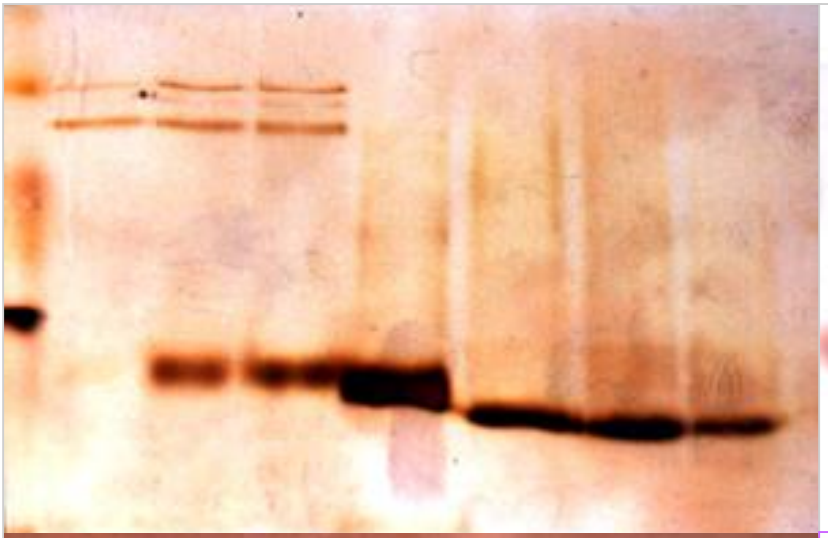
澱粉磷解酶染色方法比較 Staining L-SP

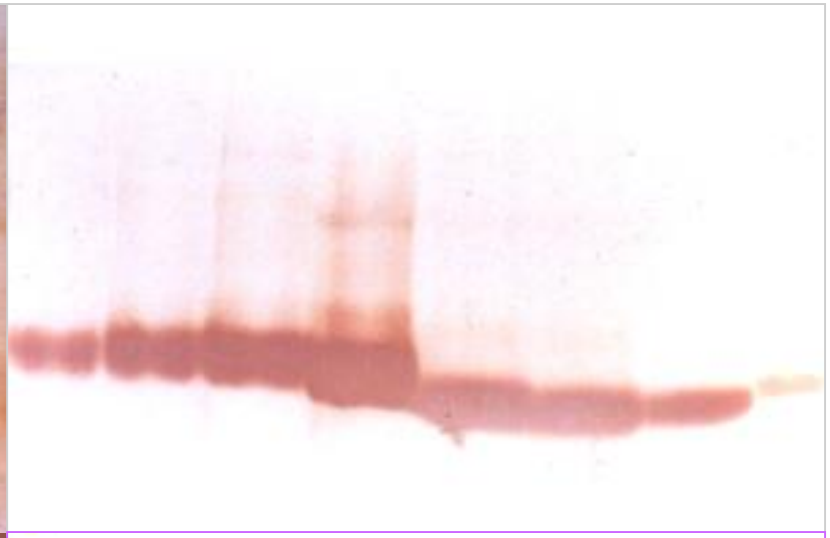
Gel filtration fractions

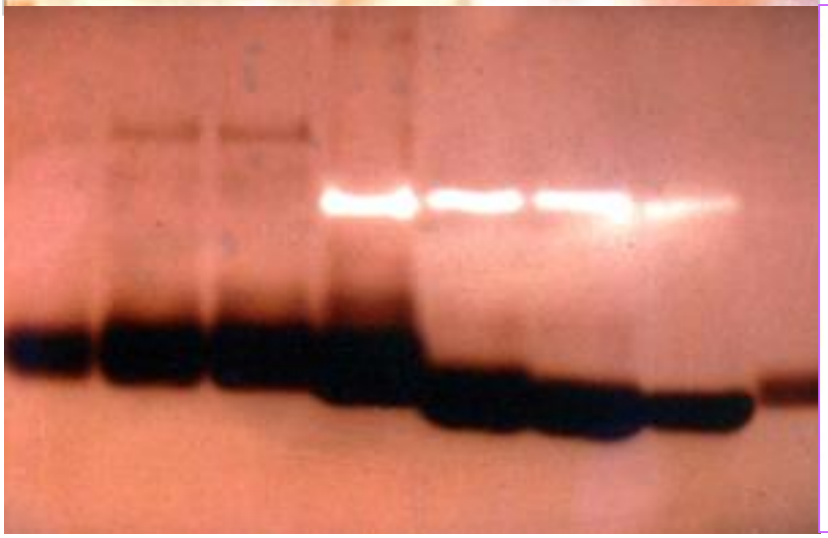
Immunostaining

Silver staining

Activity staining







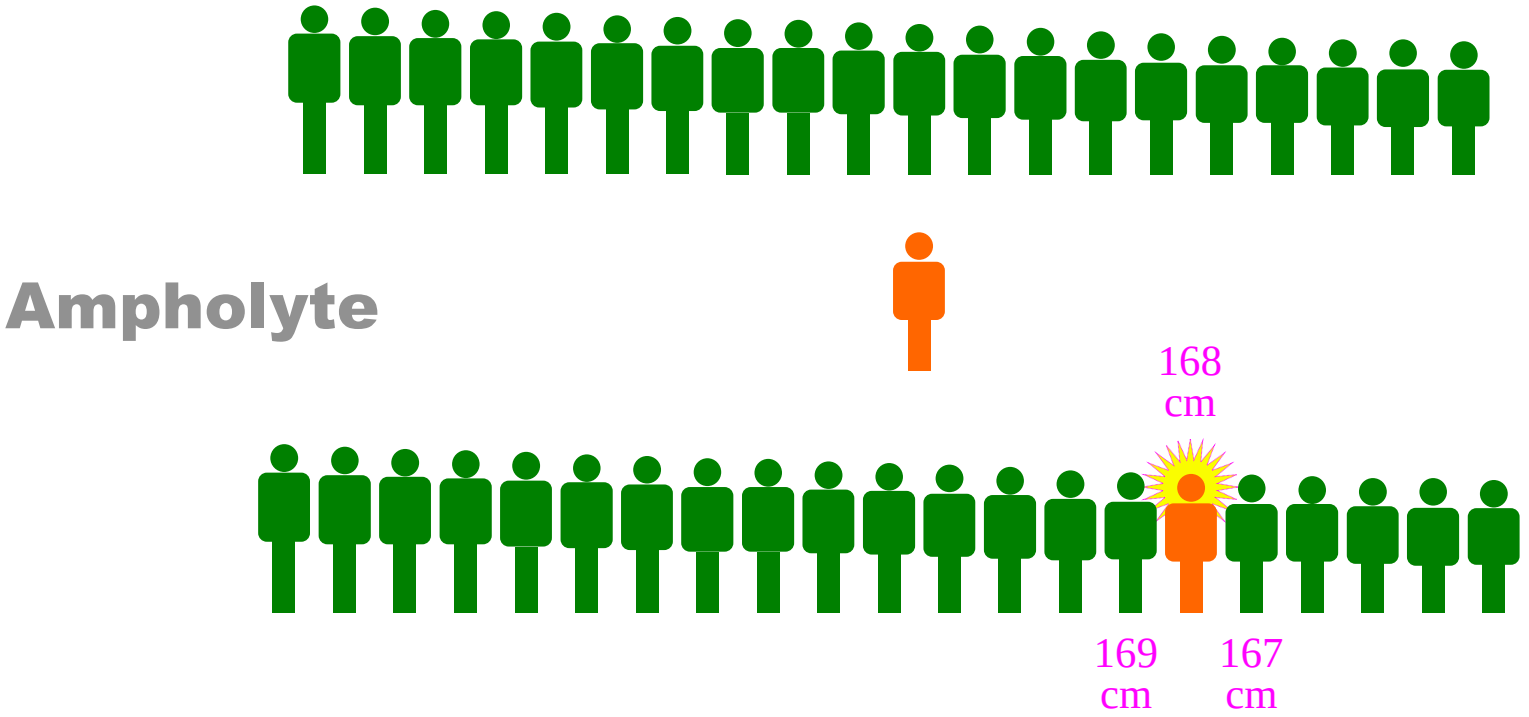
Disc-PAGE

SDS-PAGE

7.3.3 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



(1) 與 Chromatofocusing (2.3.5) 的原理一樣

(2) 都是用 Ampholyte 形成 pH 的連續梯度

環境影響分子的帶電性質

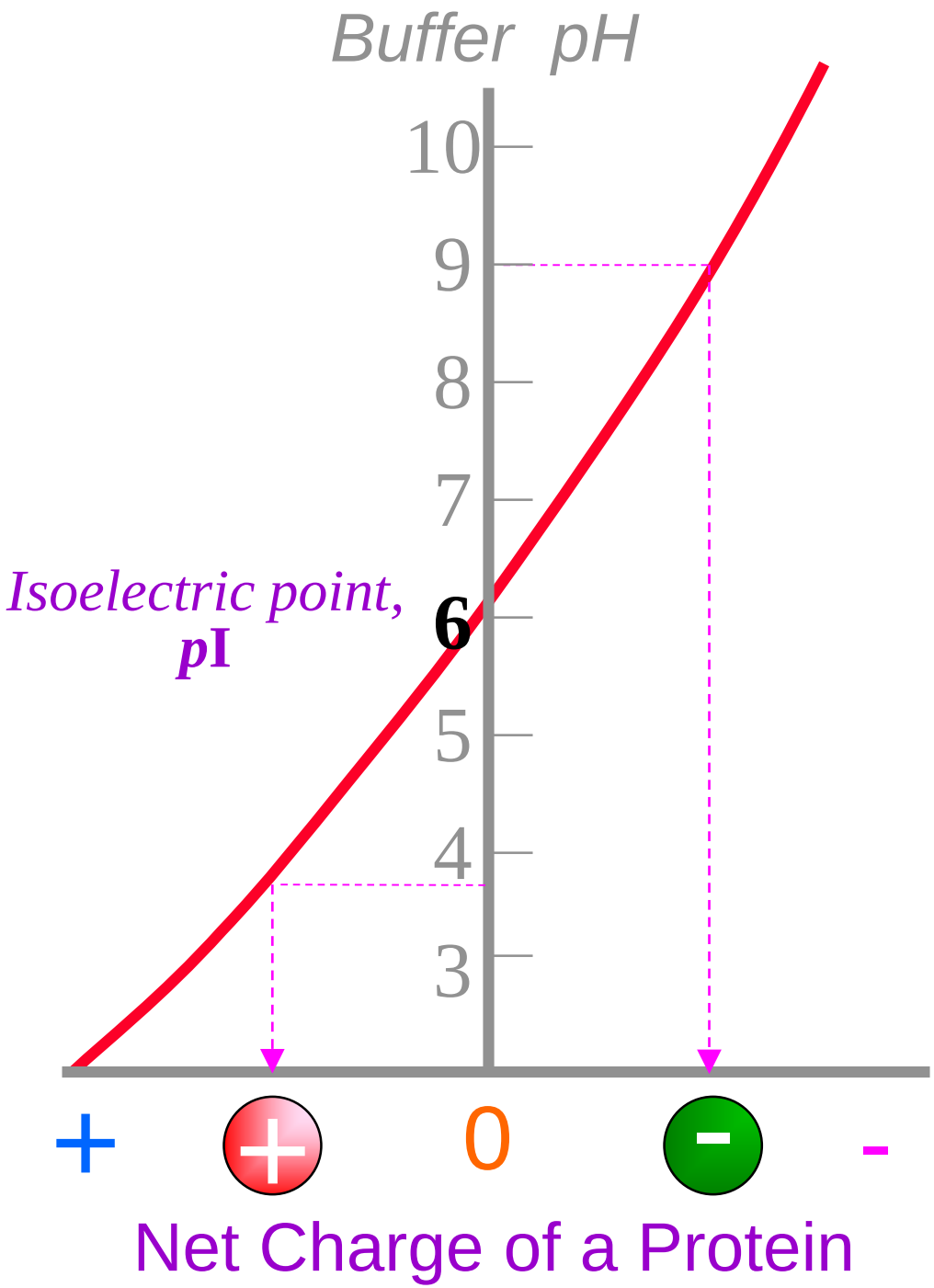
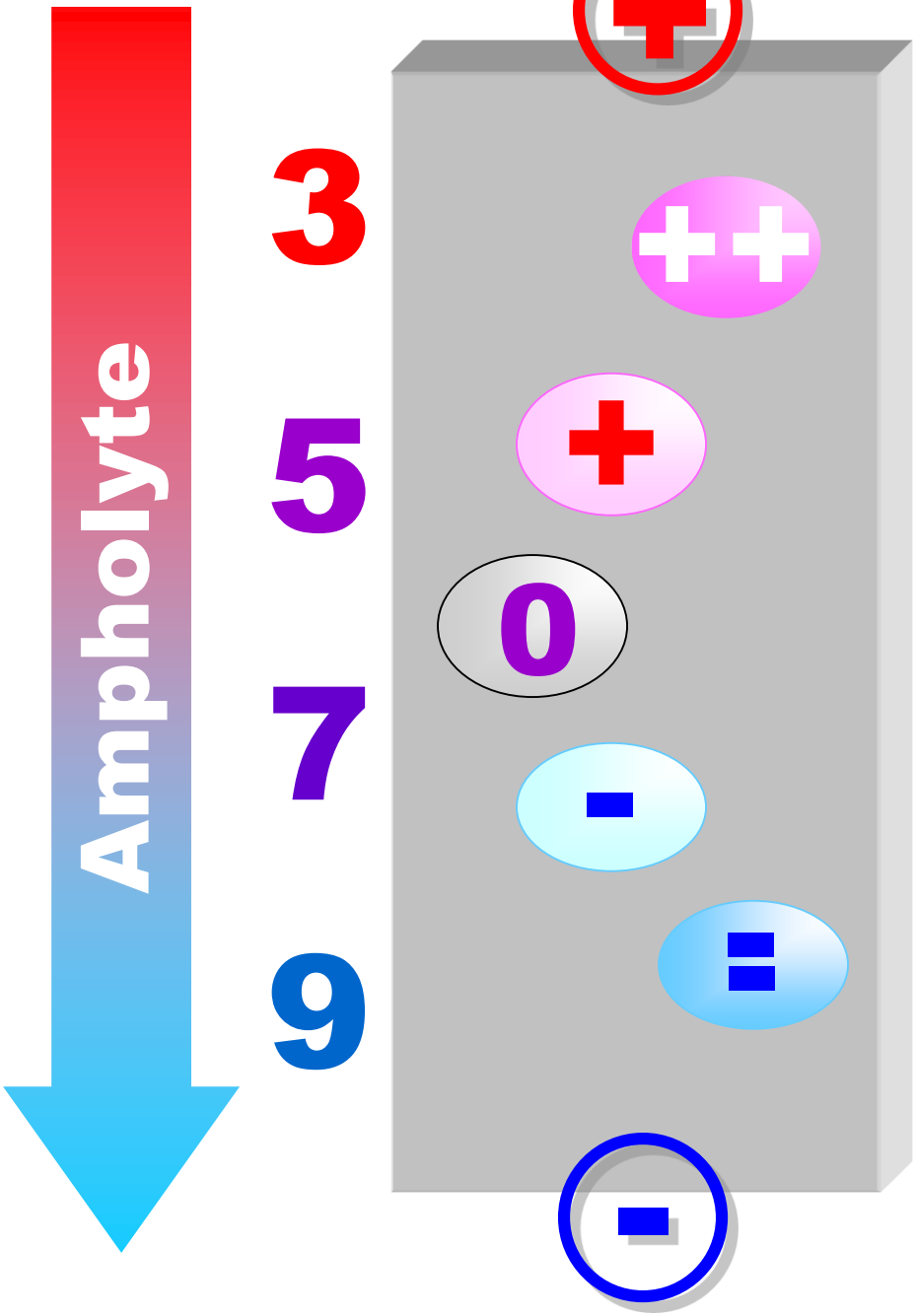


圖 7.1

等電焦集法的焦集方式



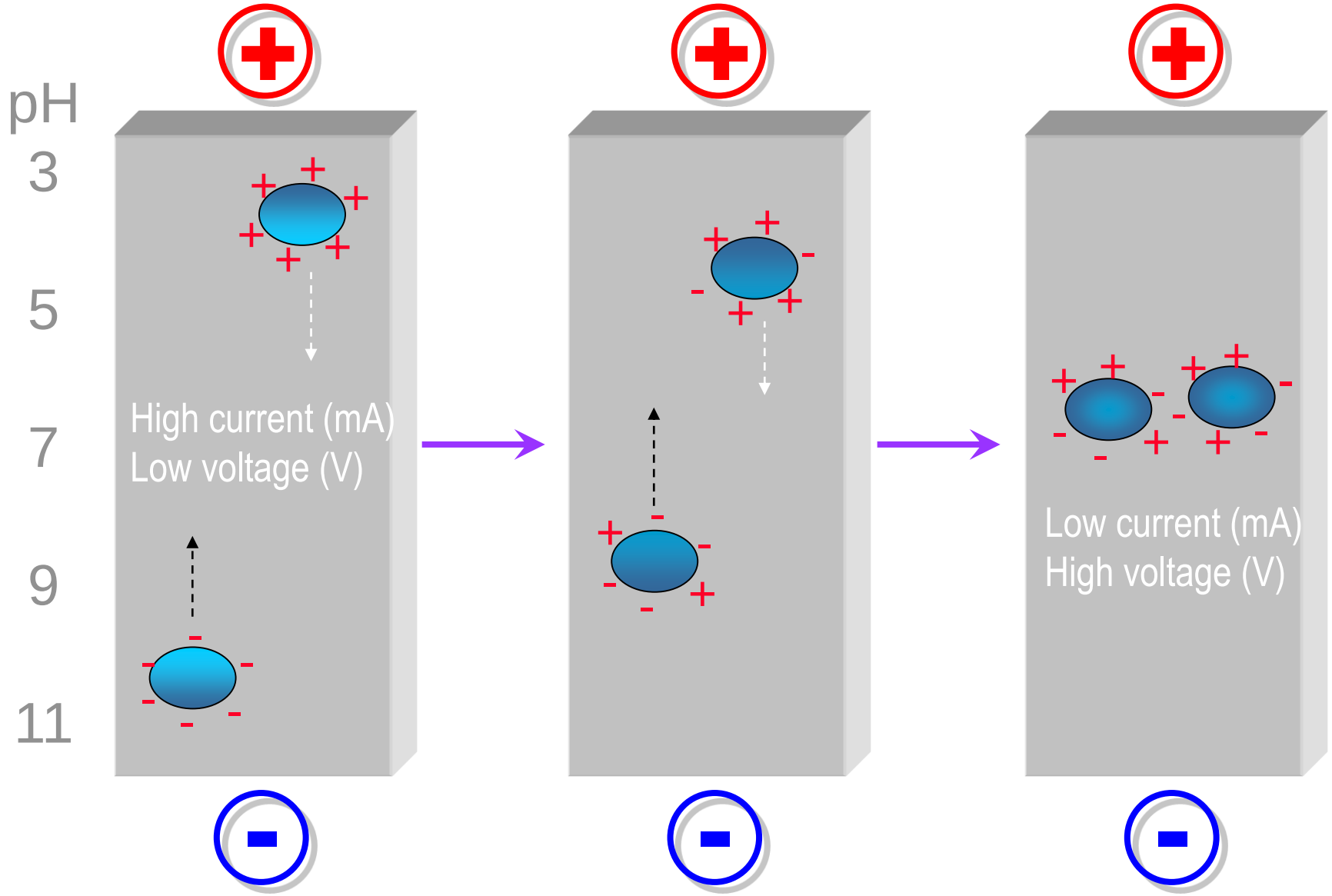
在低 pH 處帶正電被排斥
At lower pH gel, sample is positively charged and repelled

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

圖 7.8

等電焦集的運作機制 Action mechanism of IEF

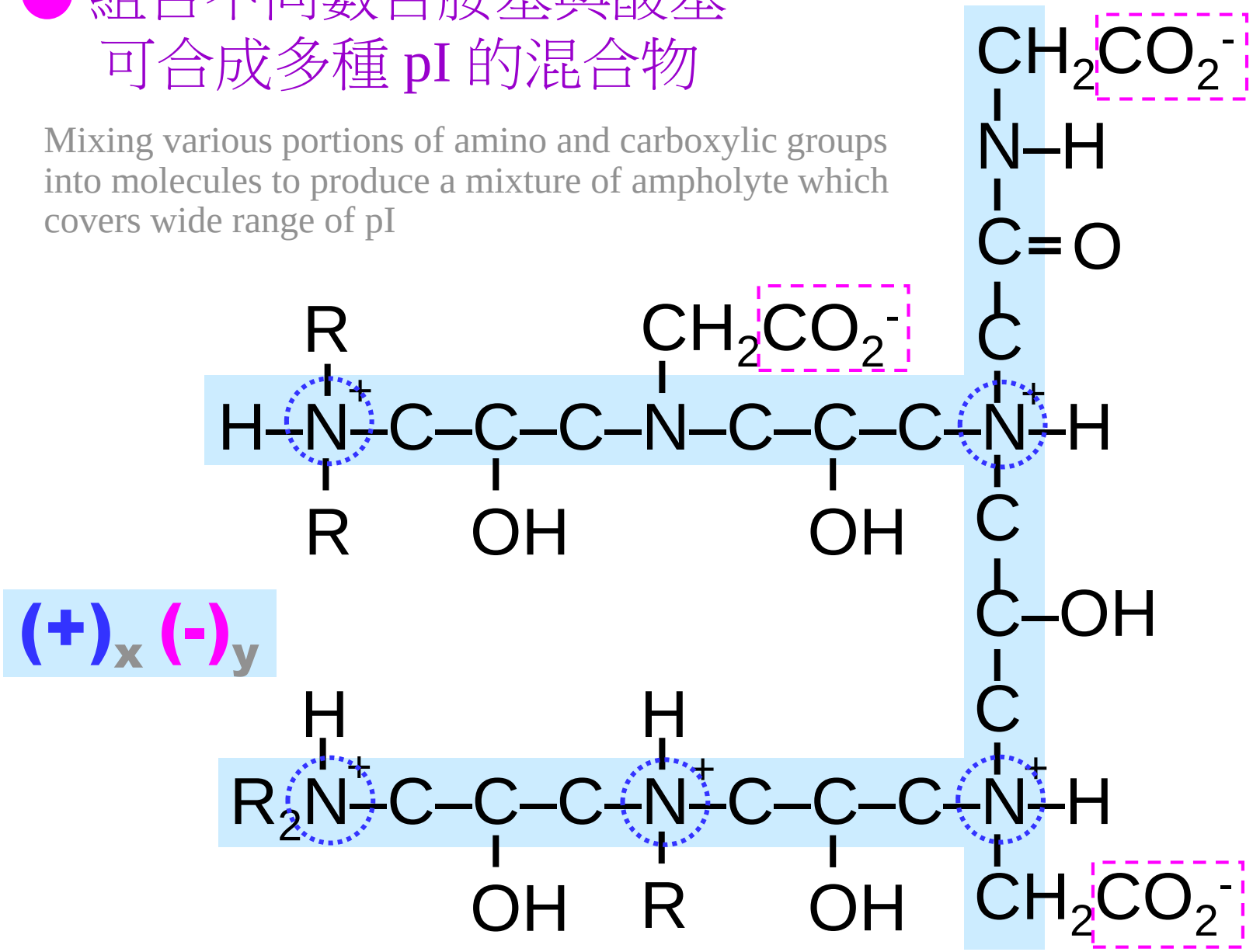


Sample can be applied at any place on the gel

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

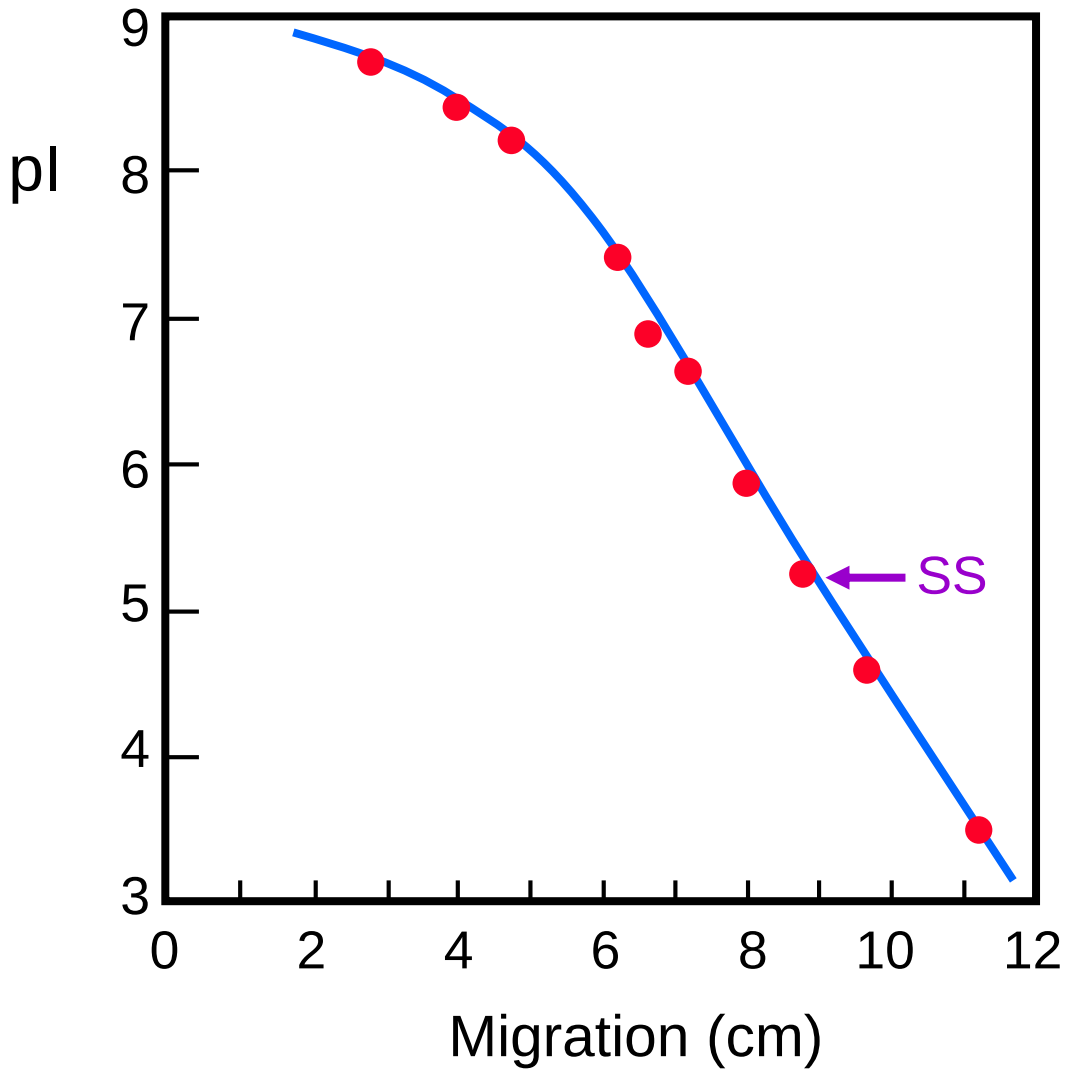
組合不同數目胺基與酸基可合成多種 pI 的混合物

Mixing various portions of amino and carboxylic groups into molecules to produce a mixture of ampholyte which covers wide range of pI



The mixture of ampholytes are synthesized by combinatory chemistry

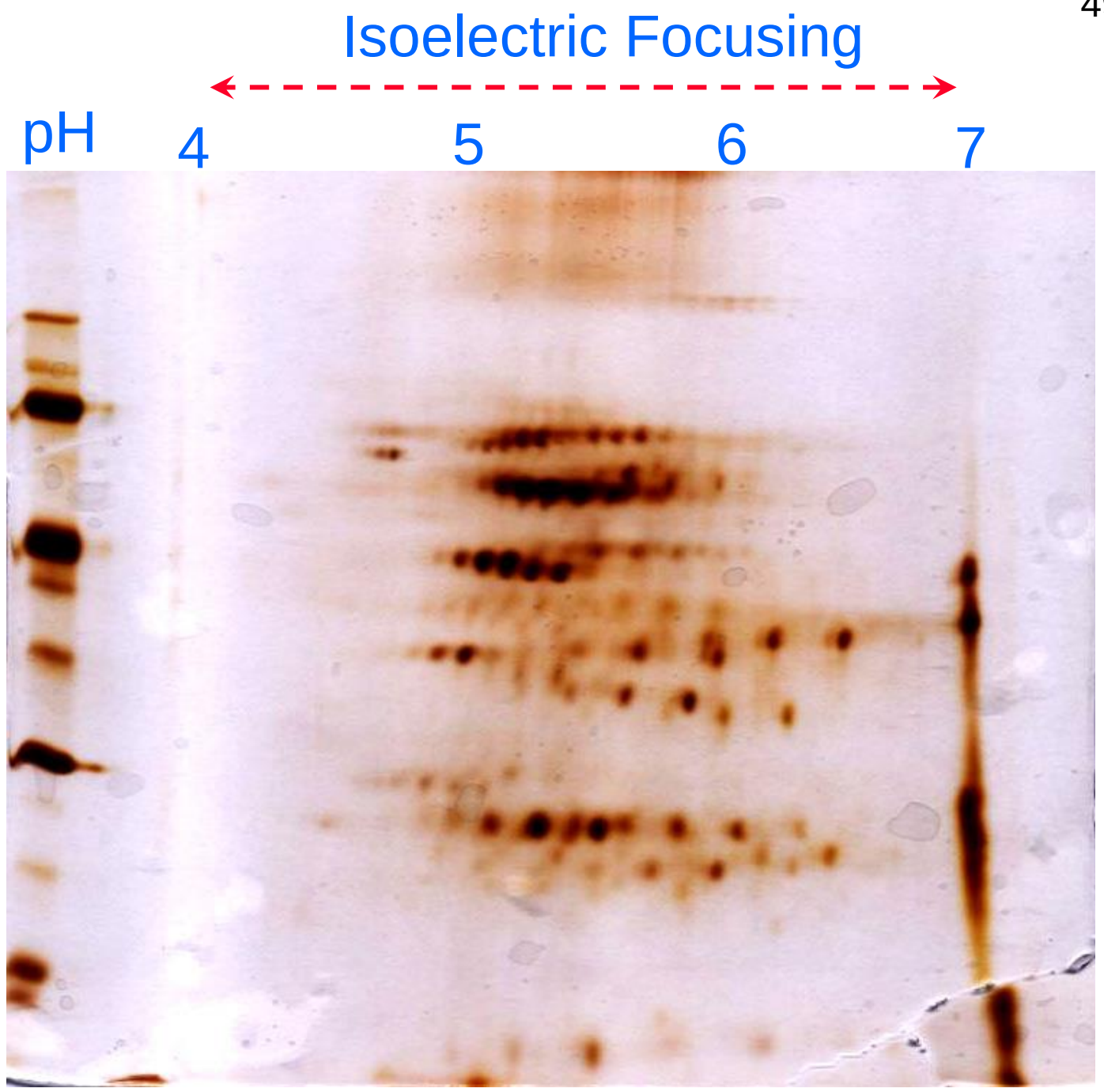
等電點標準校正線 Standard curve for IEF



Calibration line is established by proteins with known pI

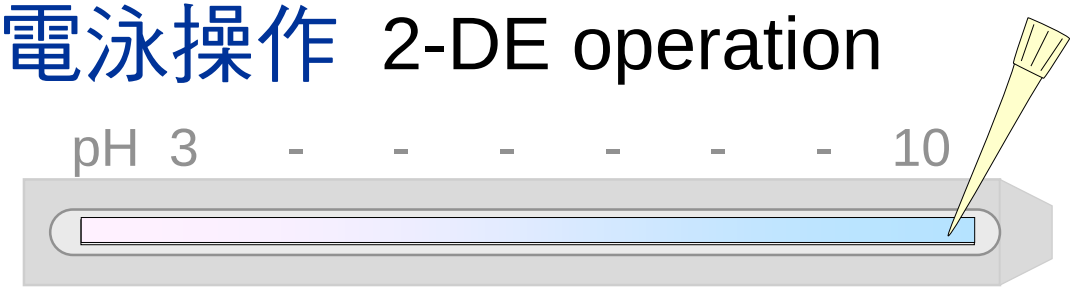
二次元電泳

2-DE

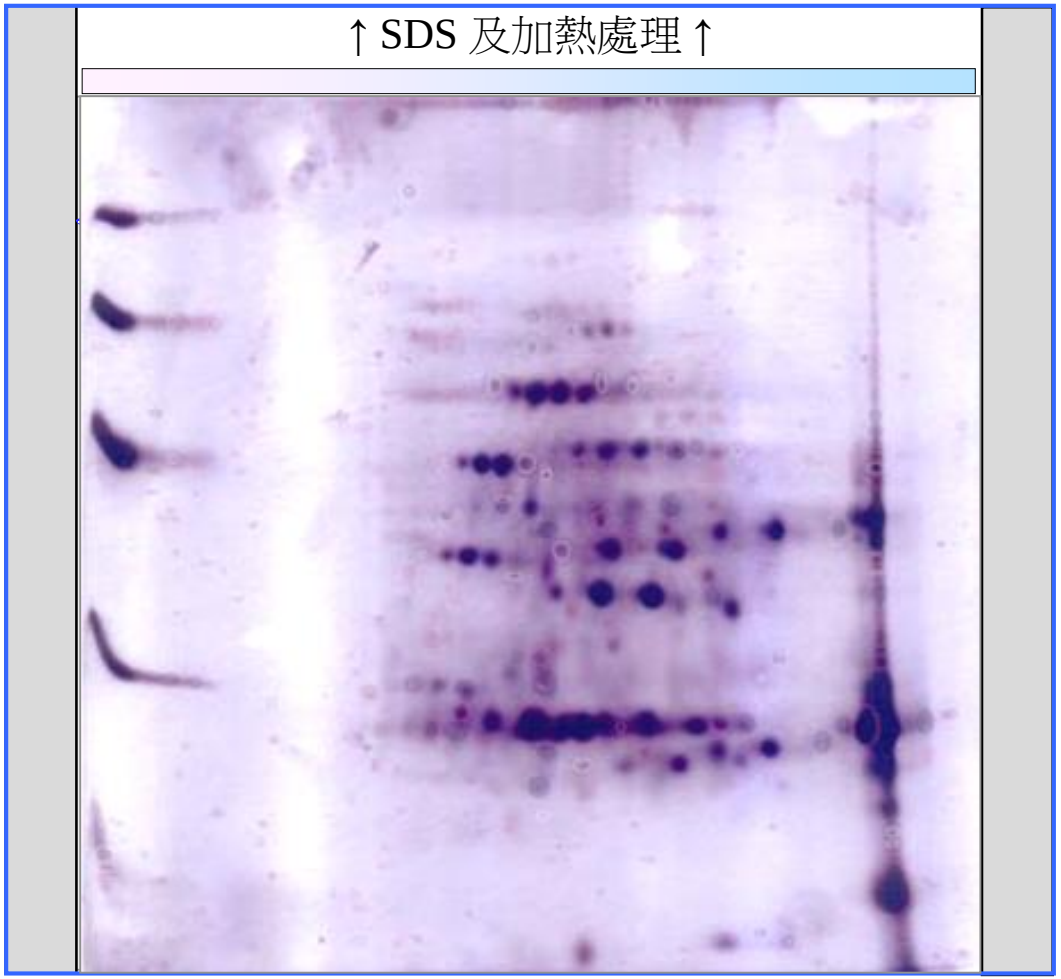


二次元電泳操作 2-DE operation

(1) IEF
等電焦集電泳



↑注意 pH 梯度的產生↑

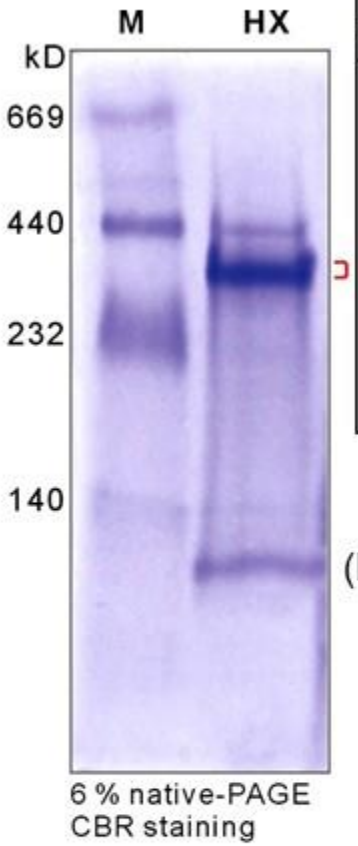


(2)
SDS-PAGE
分離膠体

(3)
Staining
染色脫色

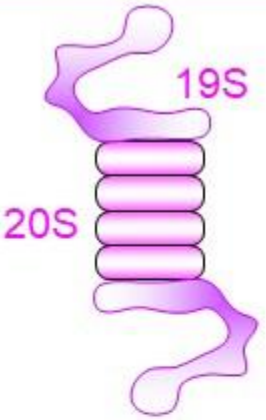
圖 7.9

HX is a high-molecular-weight form of L-SP



6 % native-PAGE
CBR staining

no.	Full protein name	Matched peptide	Sequence coverage (%)	Match score	Species	Accession number
1	20S proteasome α-subunit	8	29	333	*Lycopersicon esculentum*	CAA74725
	20S proteasome α-subunit	6	19	252	*Glycine max*	AAC28135
	20S proteasome α-subunit	6	21	145	*Arabidopsis thaliana*	CAA74025
	20S proteasome α2-subunit	6	16	236	*Arabidopsis thaliana*	AAG48830
	starch phosphorylase	4	5	228	*Ipomoea batatas*	1802404A
	20S proteasome α1-subunit	7	21	217	*Nicotiana tabacum*	CAB39975
	20S proteasome α1-subunit	4	13	174	*Oryza sativa*	XP_470540
	20S proteasome α5-subunit	6	17	190	*Glycine max*	AAF70292
	20S proteasome α3-subunit	5	17	184	*Euphorbia esula*	AAF34770
	20S proteasome α3-subunit	5	15	159	*Petunia x hybrida*	AAC35982

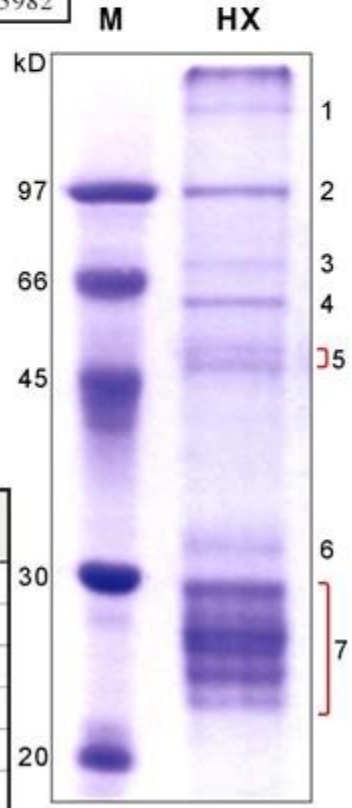


(L-SP)

HX

Native-PAGE → Band 1 → LC/MS/MS

SDS-PAGE → Band 1~7 → LC/MS/MS



12.5 % SDS-PAGE
CBR Staining

no.	Full protein name	Matched peptide	Sequence coverage (%)	Match score	Species	Accession number
1	starch phosphorylase	10	14	220	*Ipomoea batatas*	1802404A
2	starch phosphorylase	86	51	1727	*Ipomoea batatas*	T10947
3	starch phosphorylase	18	19	428	*Ipomoea batatas*	1802404A
4	chaperonin 60	19	25	451	*Cucurbita*	CAA50218
5	starch phosphorylase	30	28	454	*Ipomoea batatas*	1802404A
6	20S proteasome α6-subunit	36	29	269	*Nicotiana benthamiana*	AAN07899
7	20S proteasome α-subunit	13	28	466	*Lycopersicon esculentum*	CAA74725

Blue-native 2-D PAGE and immunostaining for L-SP