ELISA的原理及衍伸應用及Western Blot的常見問答集

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亞諾法生技

什麼是ELISA?

- Enzyme-linked immunosorbent assy 酵素免疫分析法
- 利用固體承載物，以及抗體抗原專一性，對於樣品中的目標物進行檢測
- 利用最後呈色的量級進行定量

ELISA types 分類及原理

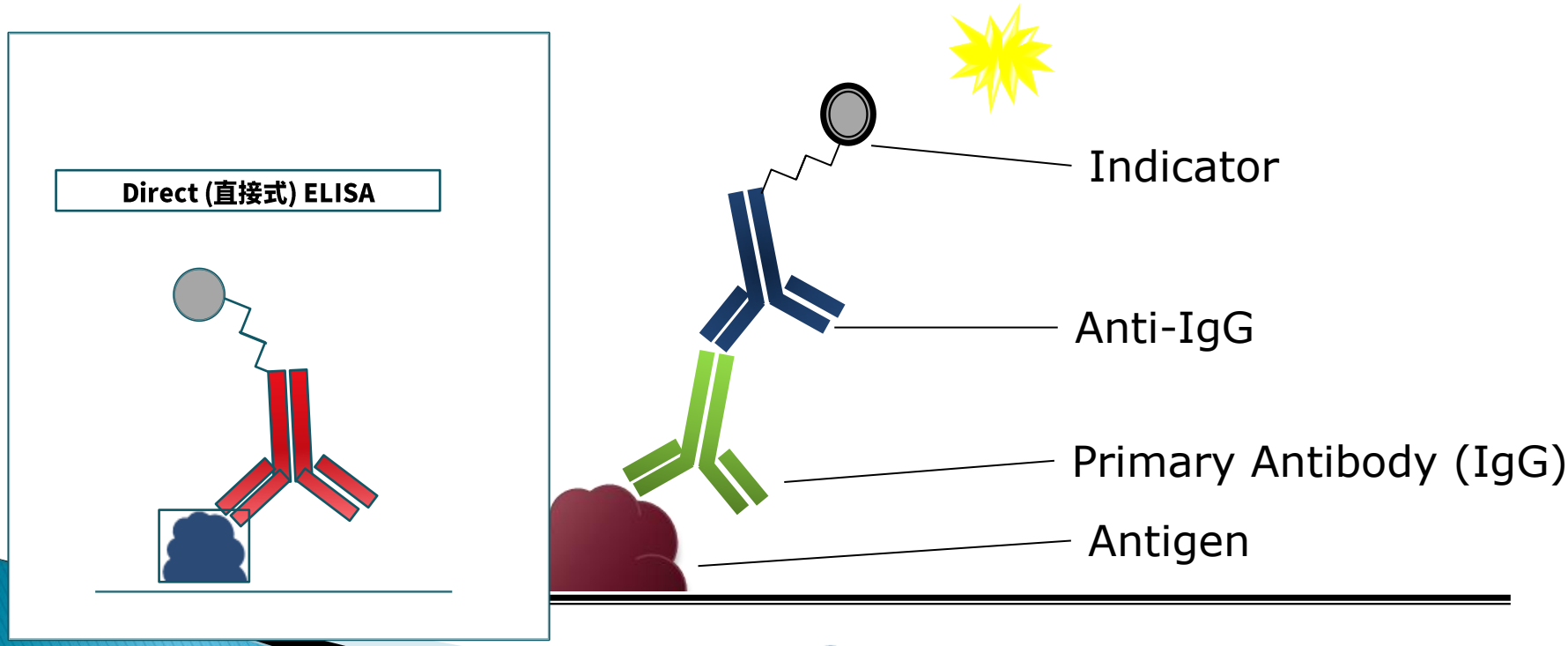
- ELISA Assay procedure 內容物與檢測介紹
- Data analysis 數據分析
- ELISA application ELISA 之應用
- ELISA trouble shooting 疑難排除
- Western Blot trouble shooting WB 疑難排除

ELISA types (ELISA 之分類)

- Indirect (間接式) ELISA
- Direct (直接式) ELISA
- Sandwich (雙抗夾心) ELISA
- Competitive (競爭性) ELISA

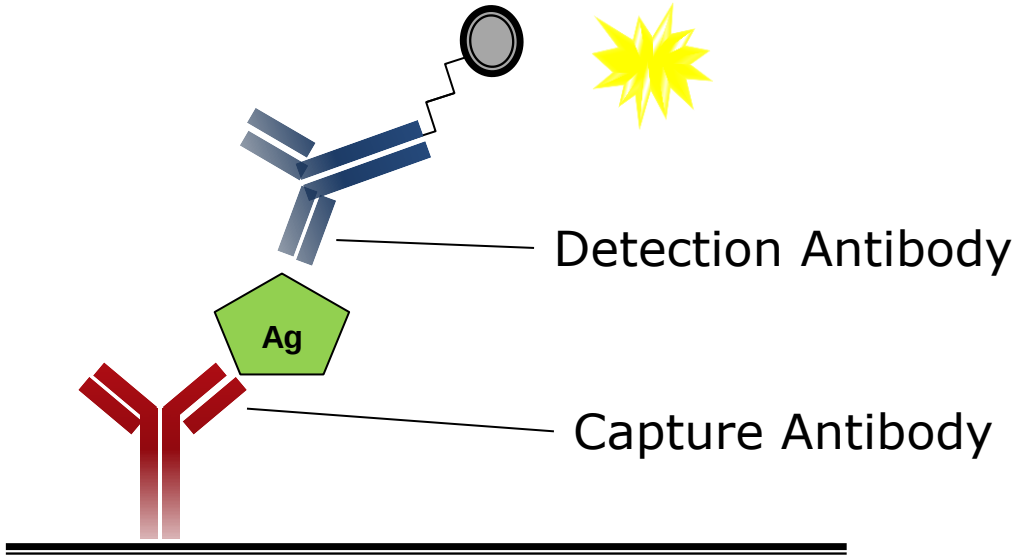
Indirect (間接式) ELISA

- Antigen: immobilized on the plate
- Primary Antibody (一抗): IgG
- Secondary Antibody (二抗): Anti-IgG
- Indicator enzyme: HRP (Horse Radish Peroxidase)
- Chromogen: TMB



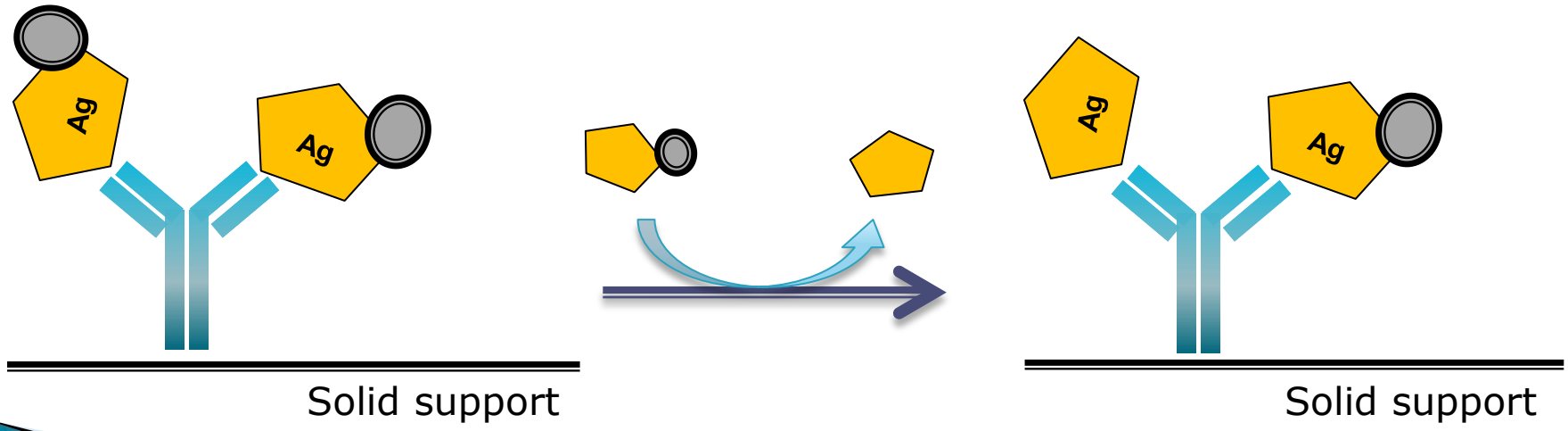
Sandwich (雙抗夾心) ELISA

- **Antibody Pair**
  - Recognize different Epitopes (抗原表位)：不適用分子量較小抗原表位少之標的偵測物。
  - Capture Antibody (用於捕獲抗原): 常用單株抗體
  - Detection Antibody (用於偵測被捕獲之抗原): enzyme-labeled
- HRP (Indicator)
- TMB (Chromogen)



Competitive (競爭型) ELISA

- Capture Antibody/Antigen (捕獲)
- Competitor Required (競爭者):
  - antigen vs. Indicator-labeled antigen
- Color development: HRP & TMB



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ELISA 內容物介紹

- Antibody/antigen coating (96-well) plate
- Standards/calibrators (標準樣本)
- Diluent (稀釋液)
- Capture & detection antibody
- Chromogen (顯色劑)
- Stop solution (終止液)

Sample Pretreatment (樣本前處理)

- 細胞培養液上清液, 血清

  以離心去除非水溶性顆粒.

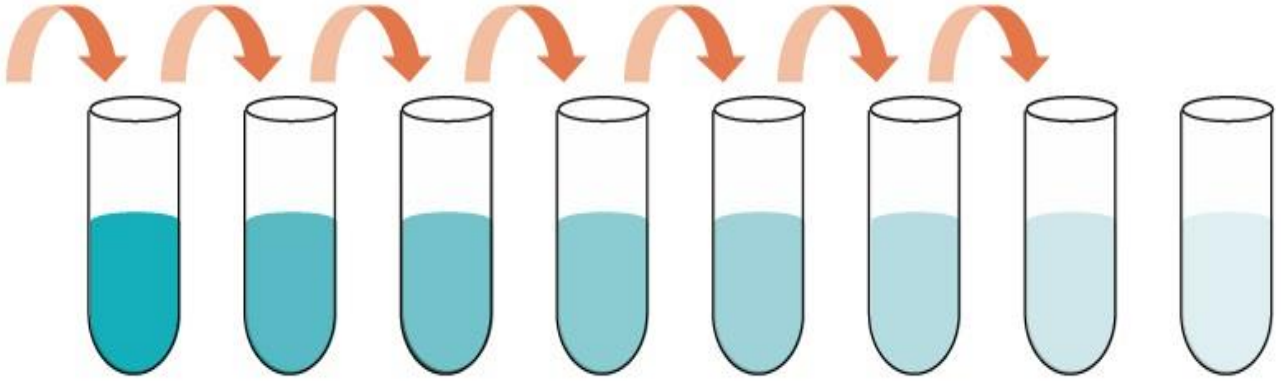
- 血漿

  加入 citrate, EDTA 或 heparin 作為抗凝劑, 再離心並收集上清液.

- 檢測前需再經過稀釋
- 樣本經前處理後應盡速檢測, 或分裝後保存於-20°C.

Standard Preparation (標準樣本)

- 以稀釋緩衝液將標準樣本進行系列稀釋



ELISA常規步驟 (雙抗夾心)

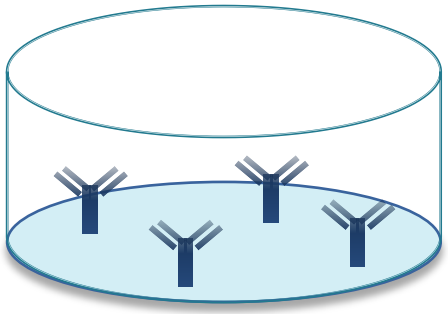
樣本前處理

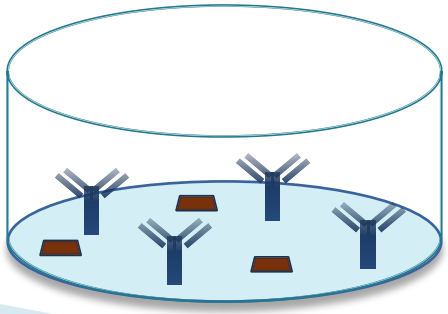
標準樣本
系列稀釋

包被

清洗

封閉





清洗

加入樣本/
標準品

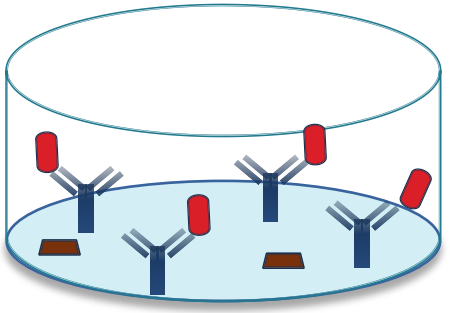
清洗

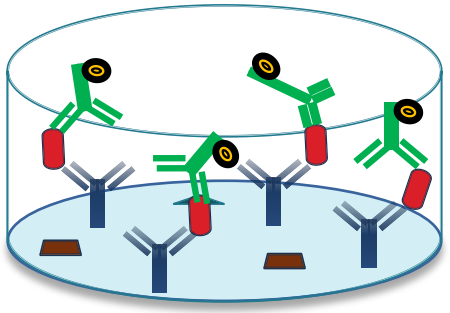
加入檢測抗體

清洗

呈色

吸光度測定





Blocking (封閉)

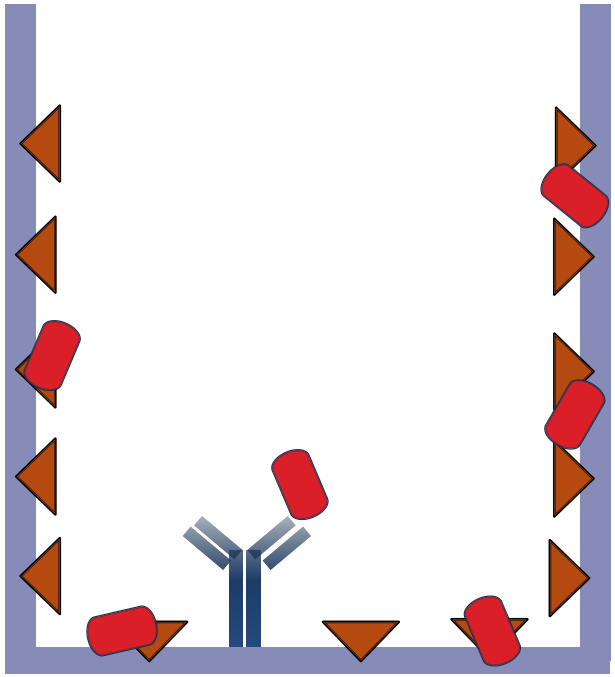
- 封閉非專一性蛋白質結合位點
- Blocking Reagent:

  1% BSA (Immunoassay Grade)

  10% FBS

  5% skim milk in PBS

  5% serum



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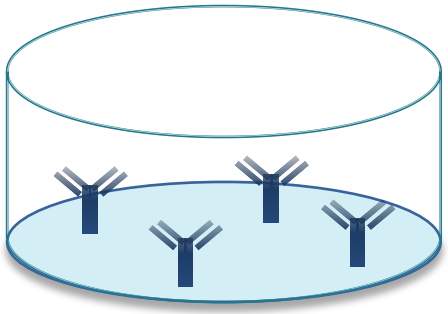
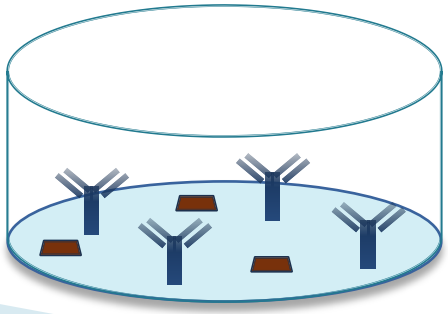




清洗

加入樣本/
標準品

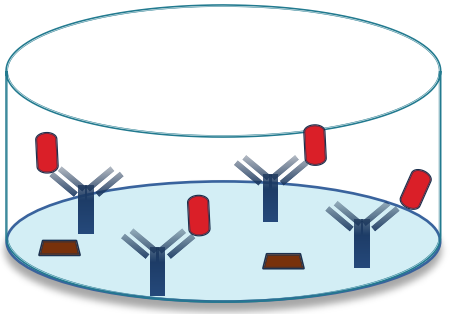
清洗

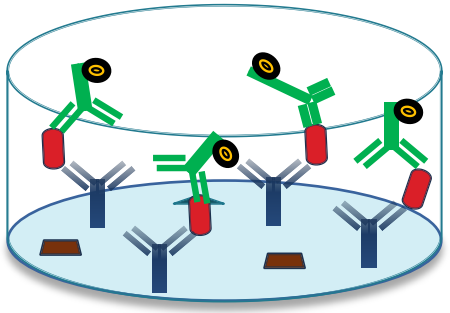
加入檢測抗體

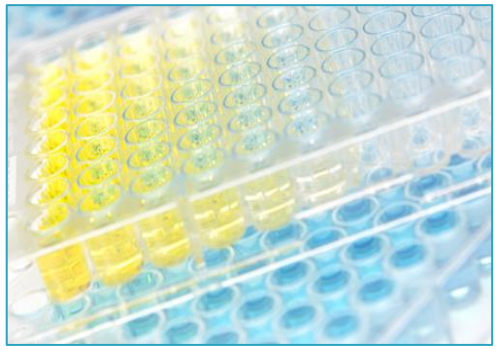
清洗

呈色

吸光度測定







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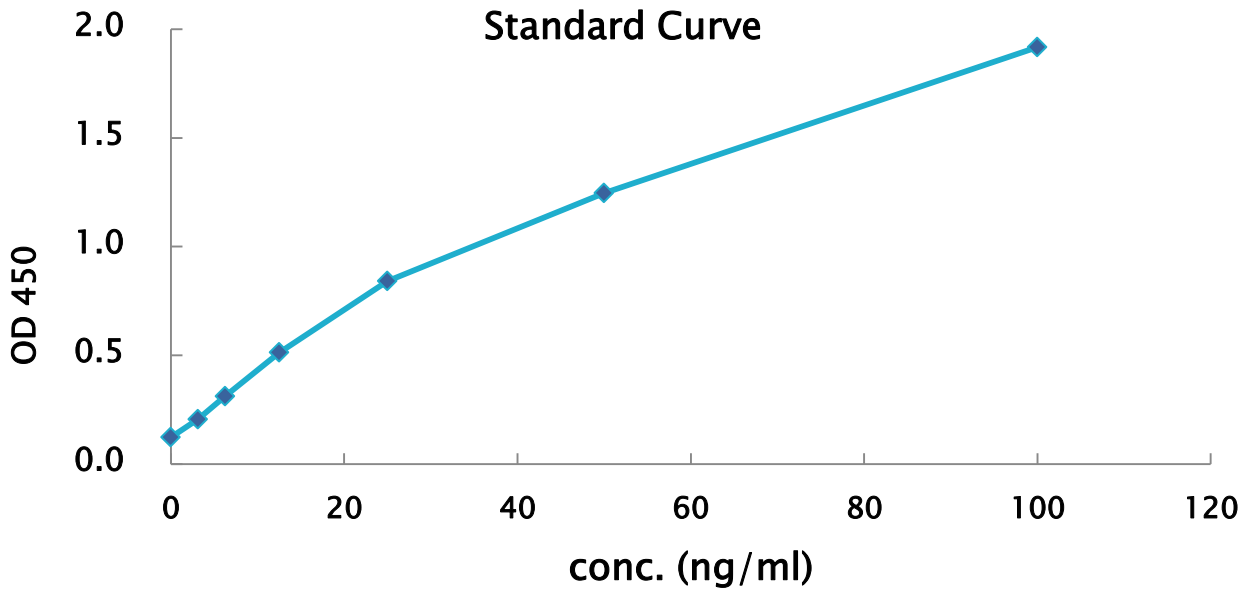
Data Analysis

- Standard curve (標准曲線)
- Precision (精確度)
- Accuracy (准確度)
- Sensitivity (靈敏度)
- Specificity (特異性)

Standard Curve (標准曲線)

	LipoDox conc. (ng/ml)						
Con.	0	3.13	6.25	12.5	25	50	100
OD1	0.12	0.22	0.34	0.48	0.85	1.32	1.99
OD2	0.12	0.19	0.29	0.55	0.83	1.17	1.84
AVG	0.12	0.21	0.31	0.51	0.84	1.25	1.92
CV	1%	10%	11%	9%	2%	9%	6%

變異係數
(coefficient of variation)
Sample CV < 15%
Blank CV < 20%



Precision (精確度)

- Intra-assay (內部檢測) Precision (Precision within an assay)
- Inter-assay (批間檢測) Precision (Precision between assays)

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	4	5	6
n	20	20	20	60	60	60
Mean (nmol/L)	6.9	18.2	40.2	10.9	25.7	67.2
Standard deviation	0.3	1.3	2.5	0.7	1.4	3.8
CV (%)	4.3	7.1	6.2	6.4	5.4	5.7

Less than 10%

Accuracy 准確度(Recovery Studies)

- 樣本成分會影響吸亮度之測定
- Recovery: 80-120%

Sample	Average % Recovery	Range
Cell culture media	97	85 - 104%
Serum	98	93 - 103%
EDTA plasma	100	94 - 105%
Heaprin plasma	98	92 - 104%
Citrate plasma	101	92 - 106%

Sensitivity (靈敏度)

- Limit of Detection (檢測限)

  樣品中所含的目標蛋白可被檢測出之最低量或濃度.

Specificity (專一性)

Metanephrine (甲基腎上腺素) ELISA Kit (KA1890)

	Substance	Cross Reactivity (%)
		Metanephrine
Analytical Specificity (Cross Reactivity)	Derivatized Metanephrine	100
	Derivatized Normetanephrine	0.15
	Derivatized 3-methoxytyramine	< 0.001
	Adrenaline	3.3
	Noradrenaline	< 0.001
	Dopamine	< 0.001
	Vanillic mandelic acid, L-Dopa, Homovanillic acid, L-Tyrosin, Tyramin	< 0.001

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ELISA 之應用

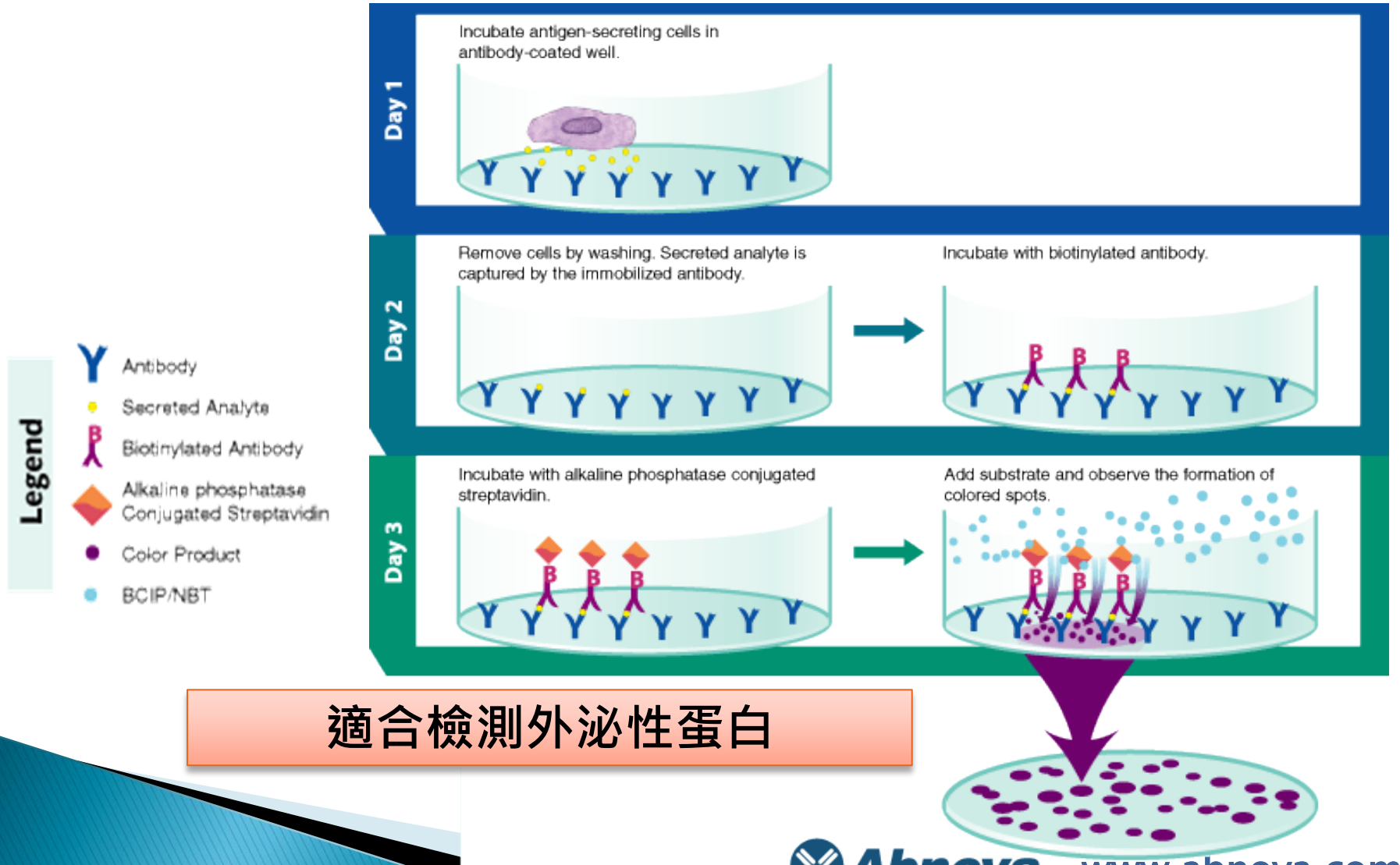
Application

- ELISPOT
- Cell-Based ELISA Kit

Related product in Abnova

- Ab Pair for ELISA (1000+)
- ELISA Kit (2000+)
- Cell-Based ELISA Kit (500+)

ELISPOT (Enzyme-linked Immunospot Assay)（酶聯免疫斑點法）



適合檢測外泌性蛋白

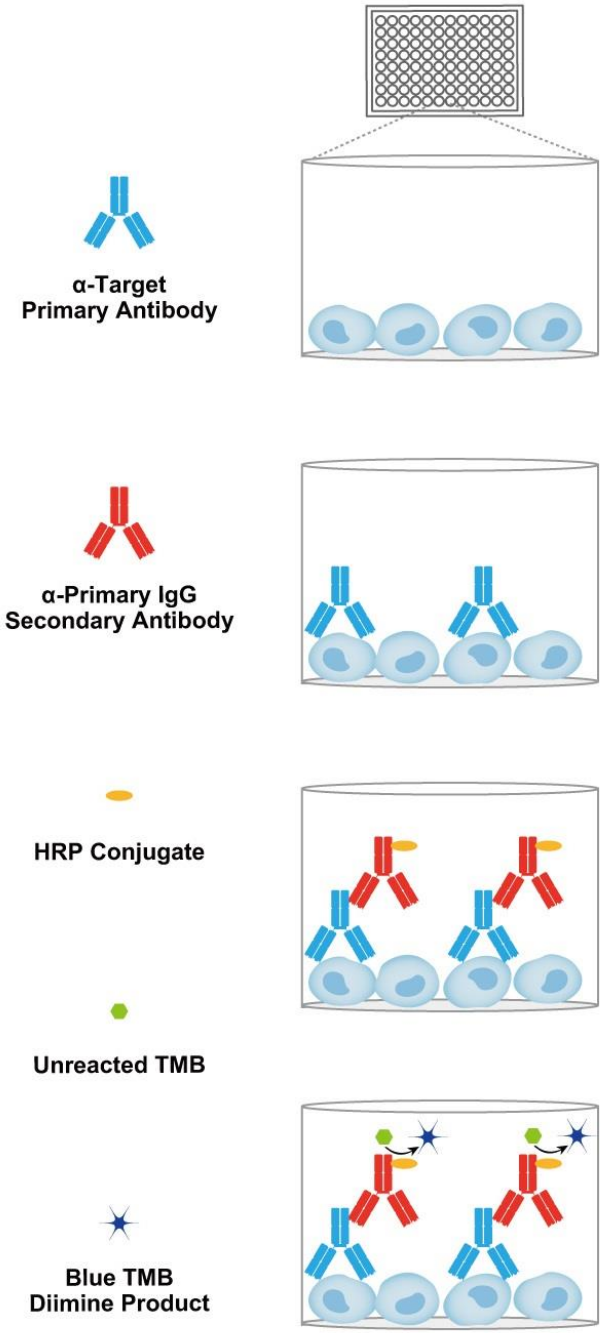
Cell-Based ELISA

將細胞接種到各孔的底部上。將細胞固定和封閉。

加入一抗和胞內之專一性抗原結合。

加入二抗。

HRP 酶將加入之 TMB 氧化。顏色由藍變黃，加入酸將反應終止，再測量吸光值 (450nm)。



Advantages of Cell-based ELISA Kit

	Cell-Based ELISA	Western Blot
樣本類型	活細胞即可 (Suspension cells, loosely attached cells, attached cells)	細胞裂解液
樣本前處理	不須額外處理	須將細胞裂解
檢測過程	快速	較費時 (Gel electrophoresis, Transfer)
檢測限	ng/mL	mg/mL
應用	Qualitative/phosphory-lation detection	Qualitative/phosphoryl-ation detection

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Weak or no signal 無信號

Possible Cause	Solution
The protein amount is added into well insufficiently.	Ensure extract contains enough amount of proteins.
Incubation time and temperature is incorrect.	Ensure the incubation time and temperature described in the protocol are correctly followed.
Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation

High background 高背景

Possible Cause	Solution
Poor blocking	Try a different blocking agent.
Contaminated wash buffer	Make fresh wash buffer
Insufficient washing	Increase number of washes Increase time of soaking between in wash
Incubation time and temperature is incorrect.	Ensure the incubation time and temperature described in the protocol are correctly followed.
Primary and secondary antibodies	Try decreasing the antibody concentration and/or the incubation period.

Anomalous signal 讀值異常

Possible Cause	Solution
Reagents not fresh or not at the correct pH	Use freshly prepared solutions and reagents.
Reagent added in incorrect order, or incorrectly prepared	Review protocol and check the concentration of reagents.
Bubbles in the wells	Make sure you remove air bubbles within wells.
Uneven temperature around work surface	Avoid incubating plate in areas where environmental conditions vary, and use plate sealer
Filth at the bottom of 96-well plate	Make sure the surface is clean before adding the reagents into wells

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- **Western Blot trouble shooting 疑難排除**

什麼是Western Blot?

- 利用膠體電泳法分離樣品中的蛋白質、轉漬到膜上後，使用對目標蛋白具專一性的抗體進行蛋白質偵測。

Western Blot 起源?

- 史丹佛大學的George Stark團隊提出並發表於1979年.
  - They used passive transfer of the proteins, then 125-I-labeled Protein A for detection.
- 瑞士Harry Towbin團隊1979年發表接近於當代流程的方法
  - developed the method of electrophoretic transfer of proteins to membranes, their procedure also used secondary antibodies for detection.
- 西雅圖W. Neal Burnette
  - gave the technique the name “Western blotting” in 1979

Western Blot Procedure

SDS-PAGE

(SDS-polyacrylamide gel electrophoresis)

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Transfer

PVDF
Nitrocellulose

|

Blocking

|

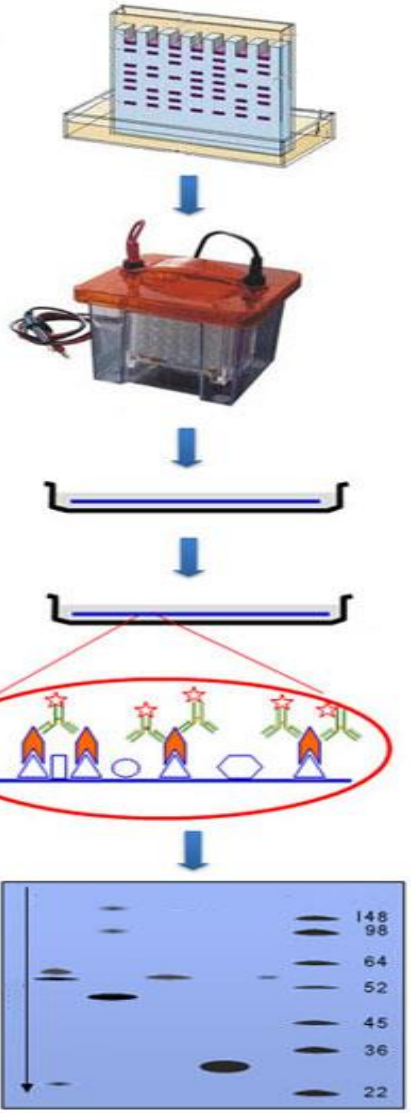
Primary and Secondary
Detection Reagents

|

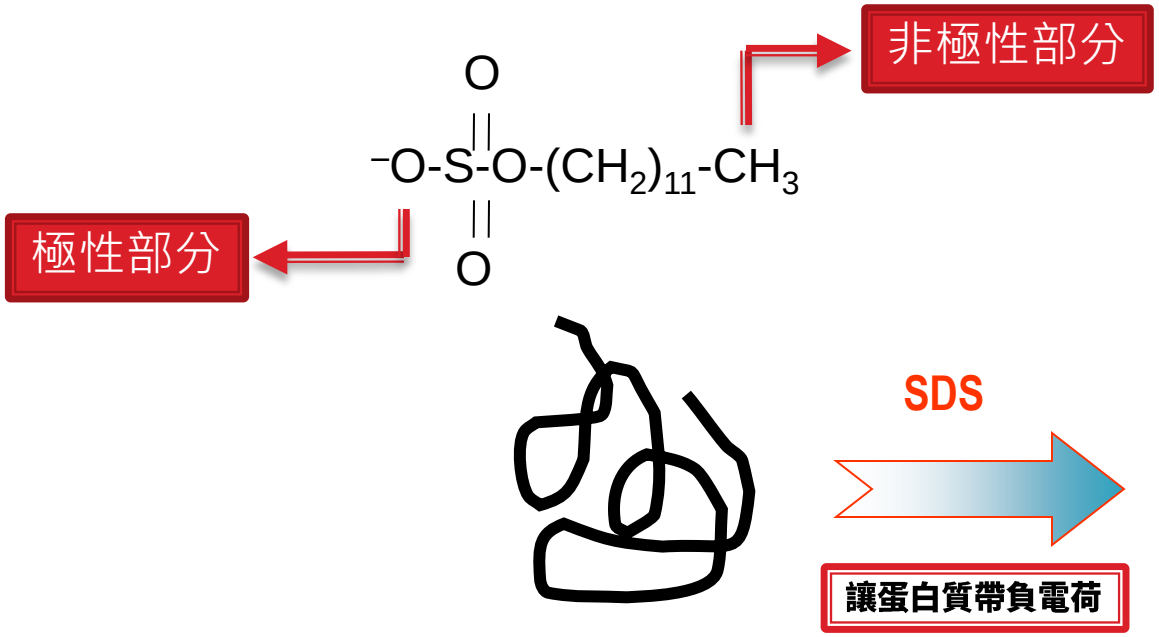
Enzyme Substrates

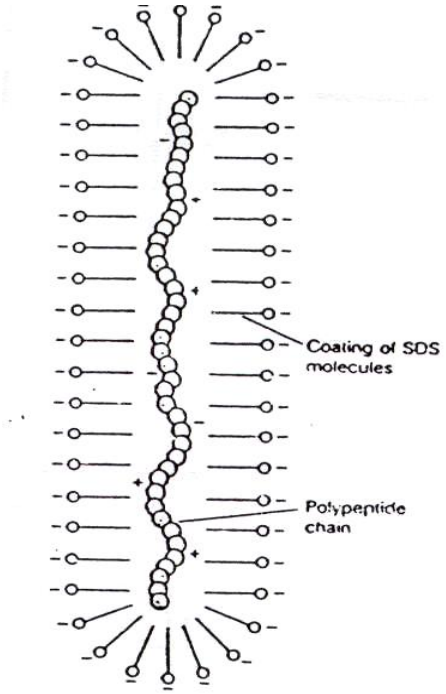
|

Film



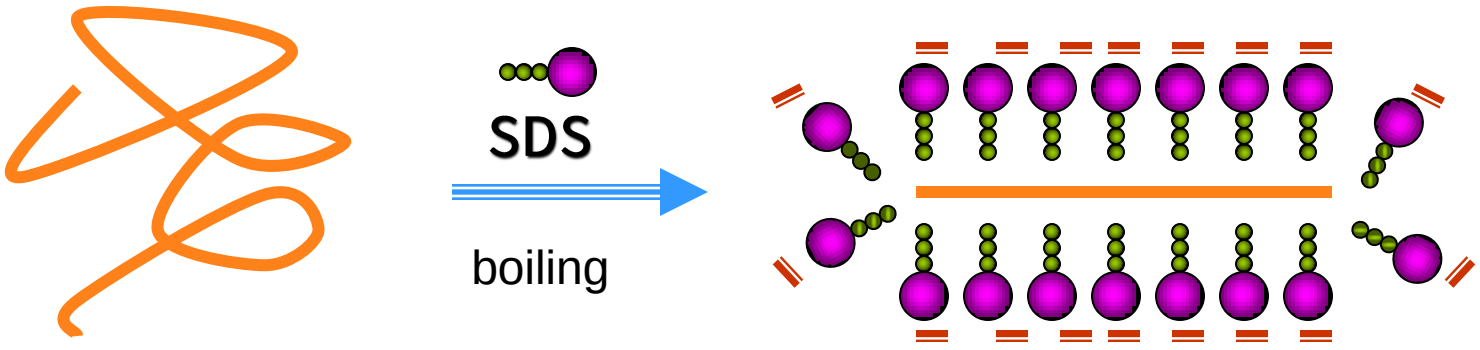
Sodium Dodecyl Sulphate (SDS)





・SDS-PAGE 系統中，樣本分子的泳動率，
僅取決於其分子量，而與原來分子所帶的電荷無關

Sodium Dodecyl Sulphate (SDS)



SDS 分子上的非極性區 (綠色尾巴)，會與蛋白質上的非極性區結合，並且使蛋白質變性，成為一線狀長條分子，上面佈滿 SDS 分子。SDS 分子的極性區 (紫色圓點)，則露在外面，以增加親水性。

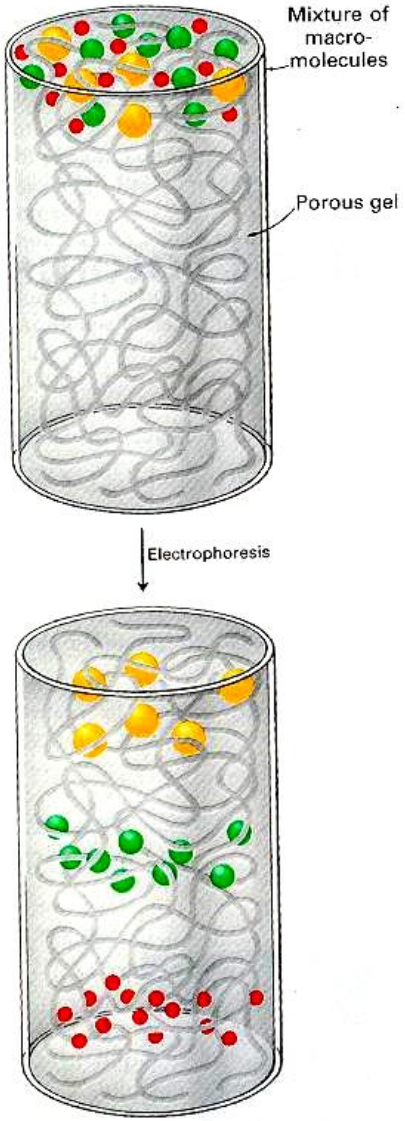
SDS-PAGE 系統中，樣本分子的泳動率，僅取決於其分子量，而與原來分子所帶的電荷無關。

Electrophoresis (電泳)

帶電分子在電場作用下，向著與其電性相反的電極移動，稱為電泳

$$\text{泳動率 } (\mathbf{v}) \sim \frac{\text{外加電壓 } (\mathbf{E}) \times \text{分子淨電荷 } (\mathbf{q})}{\text{分子泳動時與介質間之摩擦力 } (\mathbf{f})}$$

PAGE



利用一個分子篩來分離大小不同的蛋白質

Blocking (封閉)

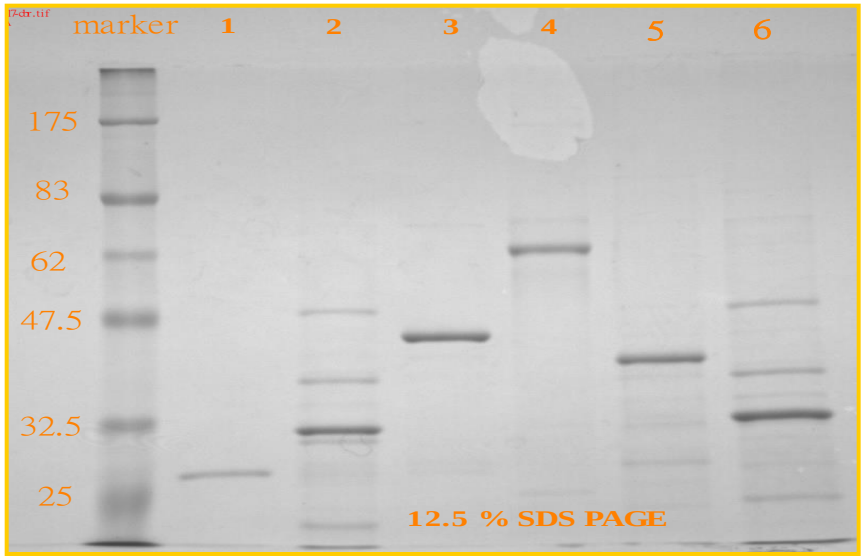
- 封閉膜上非專一性蛋白質結合位點
- 減少抗體非專一性結合，進而減低背景干擾
- Blocking Reagent:

  1% BSA (Immunoassay Grade)

  10% FBS

  5% skim milk in PBS

  5% serum



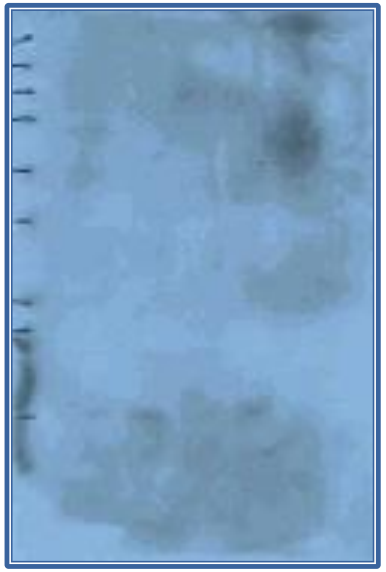
1. Western blotting：0.5ug /lane （12/18）

	Marker	1	2	3	4	5	6
Ag		GST	nv84	419	mouse serum	n98	nv8
1st Ab		Rat anti-GST(3000 X)					
2nd Ab		HRP-Goat-anti-Rat IgG(10000x)					

WB Troubleshooting

- Weak or no signal 無信號
- High background 高背景
- Multiple bands 多帶現象
- Speckled or Dotted Appearance 出現不均勻的斑點
- White bands on a black blot 出現白色條帶
- Smile effect of the bands 條帶“微笑”效應
- Bands Smeared, Bands not Sharp 條帶不銳利清晰

- **Weak or No Signal 無信號**

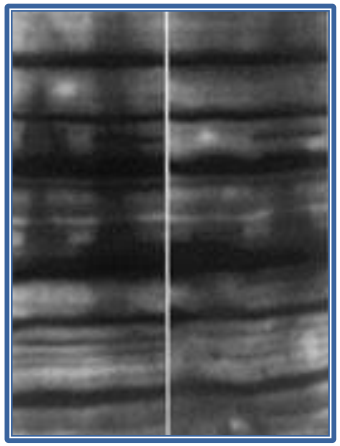


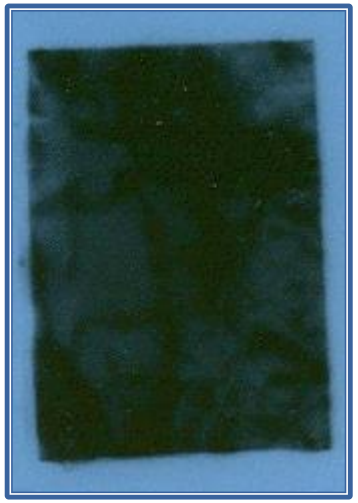
Troubleshooting

- **Weak or No Signal 無信號**

Possible Cause	Solution
Insufficient antigen	Load at least 20-30 µg protein per lane; Use protease inhibitors.
The 1st Ab and the 2nd Ab are not compatible	Use 2nd Ab that was raised against the species in which the 1st Ab was raised.
2nd Ab was inhibited by sodium azide	Do not use sodium azide together with HRP-conjugated antibodies.
Detection system	Check that the detection reagents are being stored correctly and used as recommended.

- High Background 高背景



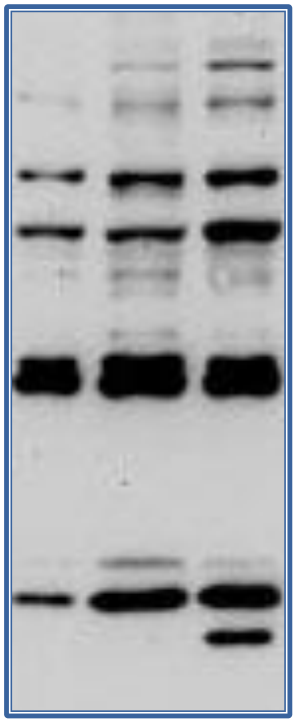


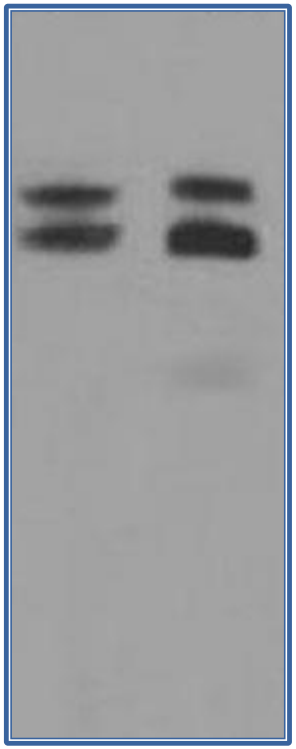
Troubleshooting

- **High Background 高背景**

Possible Cause	Solution
Poor blocking	Try a different blocking agent (5% non-fat dry milk, 3% BSA, or normal serum).
Insufficient washing	Washing should be thorough at each step.
Choice of membrane	Nitrocellulose membrane is considered to give less background than PVDF.

- Multiple Bands 多帶現象



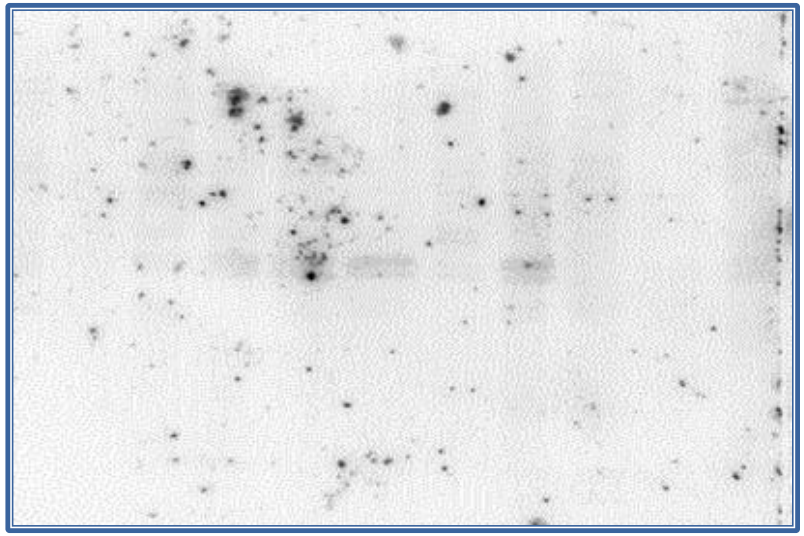


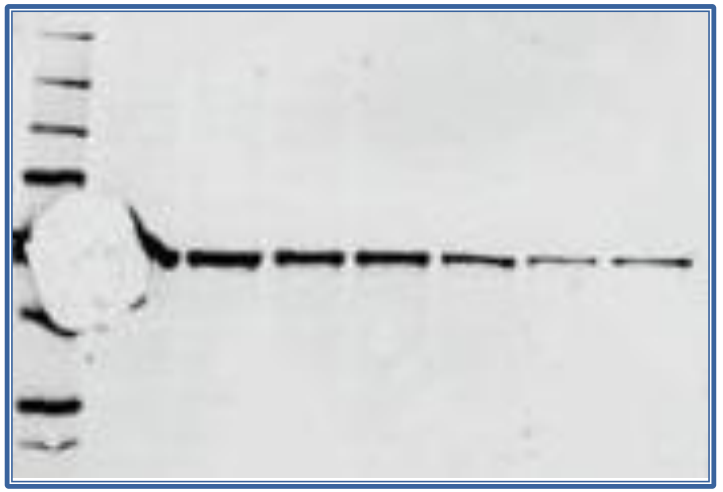
Troubleshooting

- **Multiple Bands 多帶現象**

Possible Cause	Solution
Post-translation modification (acetylation, methylation, phosphorylation, glycosylation)	Examine the literature and use an agent to de-phosphorylate, de-glycosylate.
SDS caused nonspecific binding	Wash blots after transfer. Do not use SDS during immunoassay procedure.
Primary and secondary antibodies	Try decreasing the antibody concentration and/or the incubation period.

- Speckled or Dotted Appearance 不均勻的斑點





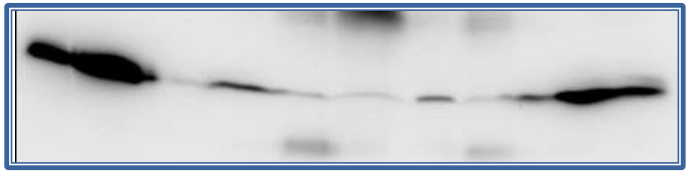
Troubleshooting

- **Speckled or Dotted Appearance 不均勻的斑點**

Possible Cause	Solution
Black Spots	Filter or change a new blocking reagent to avoid the antibodies are binding to blocking reagent.
White Spots	Make sure you remove air bubbles when preparing the gel for transfer.

- **Other question**

Smile effect of the bands



Bands Smeared

Troubleshooting

Problem	Possible Cause	Solution
Smile effect of the bands	Migration is too fast or too hot	Slow down the migration or run the gel in the cold room or on ice.
Bands Smeared, Bands not Sharp	Bands smeared due to hot gel, high voltage	Decrease voltage, and prepare new running buffer.

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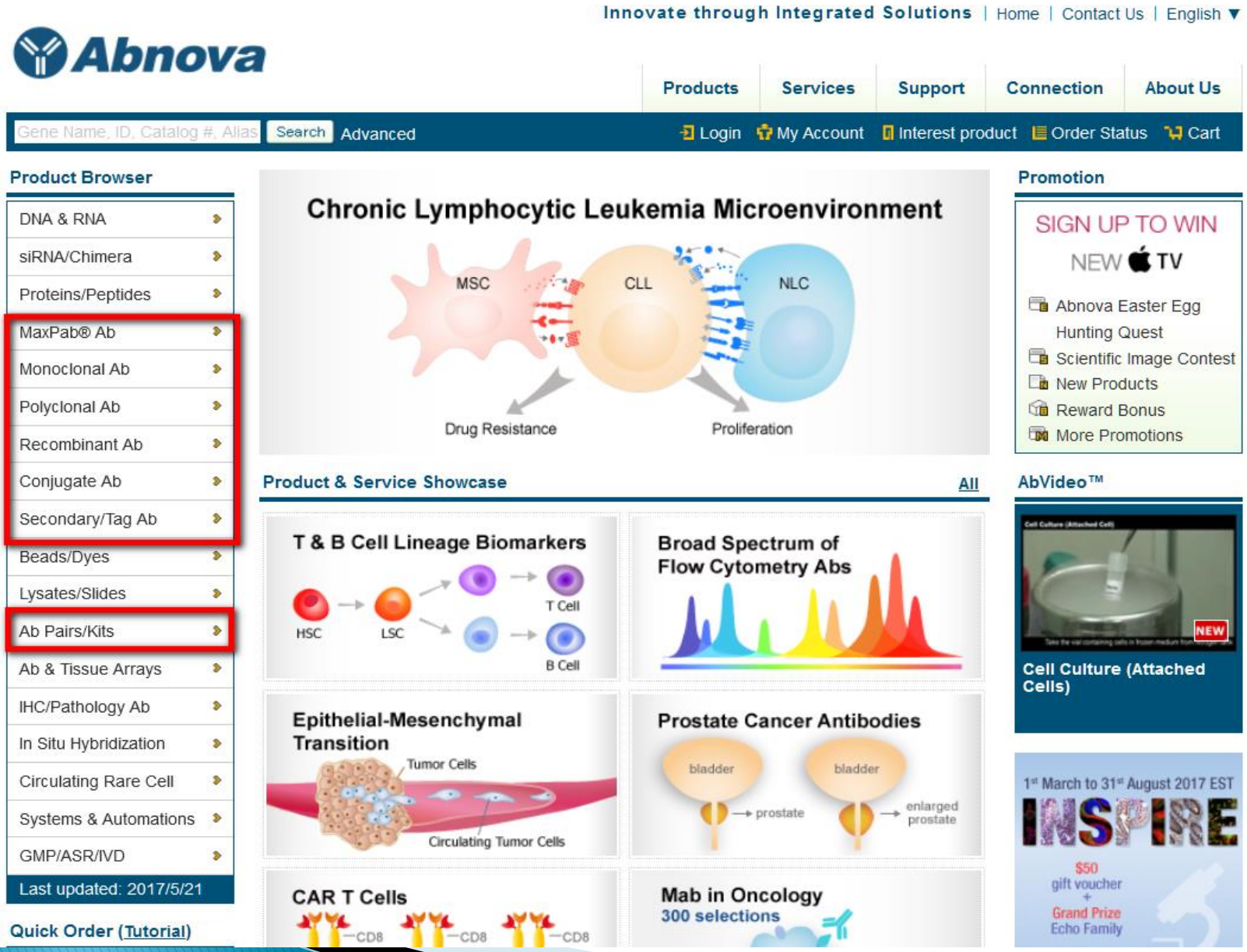

Thanks for your attention