IP and ELISpot 应用与原理

主讲人: 林安婷
时　间: 2014/04/14

- 抗体在生物研究技术中的应用
- 免疫沉淀 (IP)
- IP 原理/实验流程/疑难排除
- 免疫分析 – 酶联免疫斑点分析法 (ELISpot)
- ELISpot 原理/实验流程/疑难排除
- ELISpot 与 ELISA 比较

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抗体在生物研究技术中的应用

- Western Blot (WB) 西方墨点法
- Immunohistochemistry /Immunocytochemistry (IHC/ICC) 免疫染色
- Immunofluorescence (IF) 免疫荧光
- Flow cytometry (FCM) 流式细胞术
- Immunoassay 免疫分析
- Immunoprecipitation (IP) 免疫沉淀

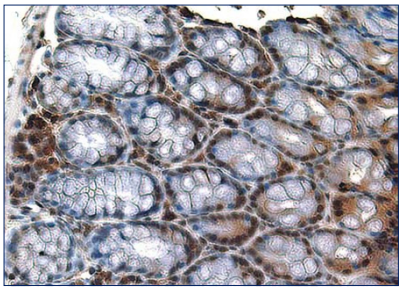
Western Blot 西方墨点法

- Specific recognition of very small amounts of protein in cell/tissue lysates.
- 细胞/组织样本中蛋白的特异性识别
- 蛋白经电泳分离后转膜

Immunohistochemistry / Immunocytochemistry 免疫染色

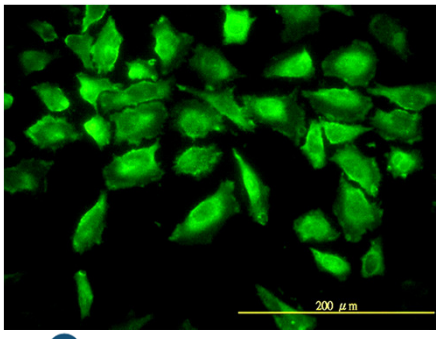
- Detection of the presence and the location of proteins *in situ* within tissues/cells
- 检测组织/细胞中蛋白的表达和定位

- IHC是针对固定的组织样本
- ICC是针对细胞样本



Immunoflourescence 免疫荧光

- Visualization specific protein or antigen in cells or tissue sections.
- 定位细胞或组织中的特异性蛋白
- 用荧光显微镜观察直标的荧光一抗或荧光二抗



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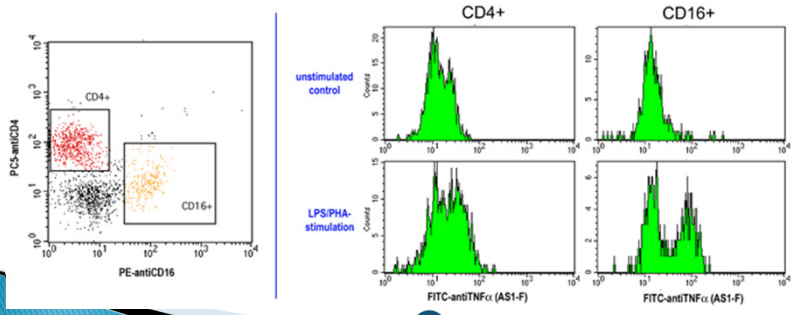
Flow cytometry 流式细胞术

- Identification of particular cell subsets and surface proteins that are regulated upon cell activities (e.g. differentiation, activation, or apoptosis)
- 识别特定的细胞亚群及表面抗原
- 通过荧光标记的抗体识别特定的细胞群，用流式细胞仪进行后续分析



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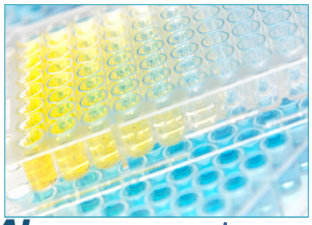
Immunoassay 免疫分析

- Measurement of the presence or concentration of a macromolecule in a solution
- 通过抗体或免疫球蛋白检测溶液中的大分子
- Variety of different labels to allow for detection of antibodies and antigens
  - Enzyme-linked immunosorbent assay (ELISA)
  - Radioimmunoassay (RIA)



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Immunoprecipitation 免疫沉淀

- Purification of a single antigen from a complex mixture using a specific antibody attached to a beaded support
- 用带珠子的特异性抗体纯化抗原
- Beads
  - resin, agarose, magnetic beads
  - 树脂珠,琼脂糖珠, 磁珠

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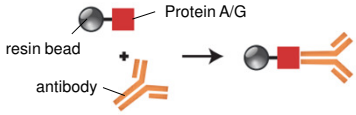
免疫沉淀的种类

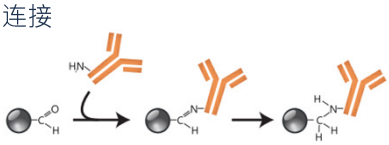
- Individual protein immunoprecipitation (IP)
  - Antigen isolation
- Protein complex immunoprecipitation (Co-IP)
  - Protein-protein interaction 蛋白复合体
- Chromatin immunoprecipitation (ChIP)
  - Protein-DNA interaction
- RNA immunoprecipitation (RIP)
  - Protein-RNA interaction

Immobilization of antibody

- :Indirect (via protein A/G)
  - 抗体和珠子通过Protein A/G 连接



- Direct (Abs are directly conjugated or crosslinked to beads)
  - 直接连接



Choose the correct protein A/G beads

- Protein A/G 珠子的选择

	Species	Subclass	Protein G	Protein A
Monoclonal	Human	IgG1	++++	++++
		IgG2	++++	++++
		IgG3	++++	---
		IgG4	++++	++++
	Mouse	IgG1	++++	+
		IgG2a	++++	++++
		IgG2b	+++	+++
		IgG3	+++	++
	Rat	IgG1	+	---
		IgG2a	++++	---
		IgG2b	++	---
		IgG2c	++	+
Polyclonal	Rabbit		+++	++++
	Cow		++++	++
	Horse		++++	++
	Goat		++	---
	Guinea Pig		++	++++
	Sheep		++	+/-
	Pig		+++	+++
	Rat		++	+/-
	Mouse		++	++
	Chicken		+	---
	Human IgG		++++	++++
	Human IgM		+	---
	Human IgE		+	---
	Human IgA		+	---

--- (weak or no binding) → ++++ (Strong binding)

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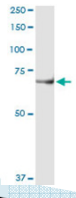
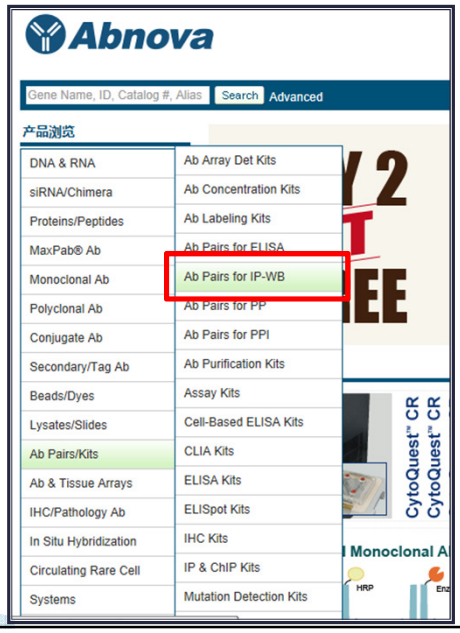
免疫沉淀(IP)抗体的选择

- Ab for WB
- Ab for IP (validated)
  - Abnova有超过2,500种
- Abnova Ab Pairs for IP-WB
  - 超过2,000组抗体对

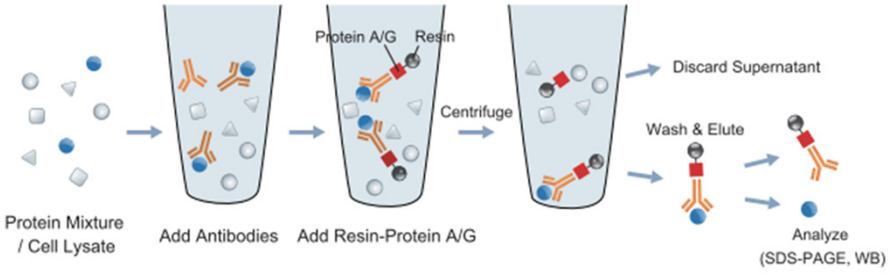
Antibody pair set content:
1. Antibody pair for IP: mouse monoclonal anti-ACO1 (300 ug)
2. Antibody pair for WB: rabbit polyclonal anti-ACO1 (50 ul)

  - 高特异性
  - 减小非特异性反应





IP实验流程



- Preparation of lysates 样本的准备
- Preclearing the lysates 样本的预洗
- Immunoprecipitation 免疫沉淀
- Wash/Elution/Analysis 洗涤/洗脱/分析

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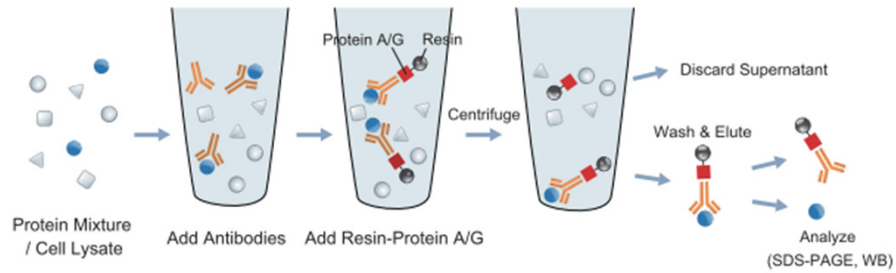
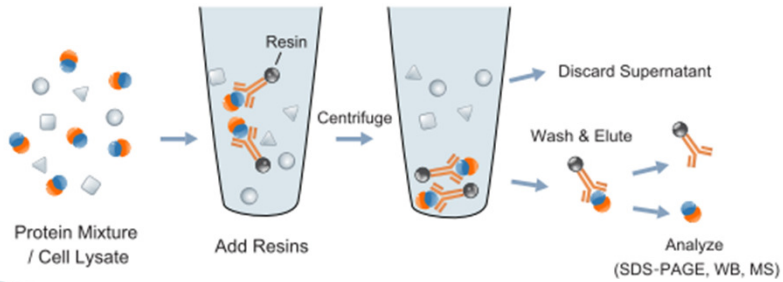
IP



Co-IP



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Preparation of lysates 样本的准备

- Minimizing denaturation of Ab binding sites 减少变质
- Lysis buffer (Harlow and Lane, 1999) 裂解液
  - Salts: 0–1 M
    Detergent: non-ionic: 0.1–2%
    ionic: 0.01–0.5%
  - Divalent cations: 0–10 mM
  - EDTA: 0–5 mM
  - pH: 6 – 9
- Protease inhibitor 蛋白抑制剂

Protein Mixture
/ Cell Lysate

Cell lysate	Tissue
Wash cells with ice cold PBS.	Dissect tissue on ice.
Lyse cells in ice cold lysis buffer	Homogenize tissue in ice cold lysis buffer
Pass the lysed suspension 5–10 times through a needle attached to a 1 ml syringe to fragment DNA	
Centrifuge and collect supernatant	

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Preclearing the lysates 样本的预洗

- Preventing non-specific binding 封闭以减小非特异性反应
  - Blocking the resin beads 封闭
    - BSA
  - Preclearing the lysates 预洗
    - Irrelevant Ab of the same species and isotype as the IP Ab
    - Normal serum

Preclearing the lysates
Incubate irrelevant Ab or serum with IP Ab at 4°C for 1 hr with gentle agitation
Add protein A/G resins* to the lysate
Incubate for 30 min at 4°C with gentle agitation
Spin in at 4°C for 10 min
Collect the supernatant

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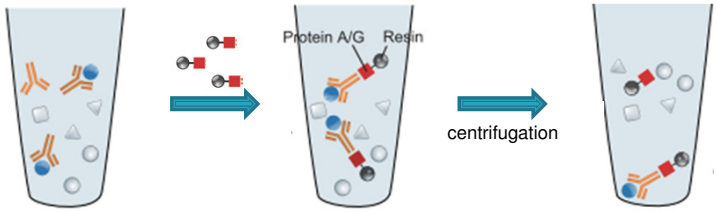
Immunoprecipitation 免疫沉淀反应



Incubation with Ab	Capture with Protein A/G resins	Precipitation
Incubate lysate with Ab for 1-12hr at 4°C with gentle agitation	Add protein A/G resins and incubate for 1-3 hours at 4°C with gentle agitation	Centrifuge for 30 sec at 4°C to precipitate Ag-Ab-protein A/G-resin complexes Discard supernatant

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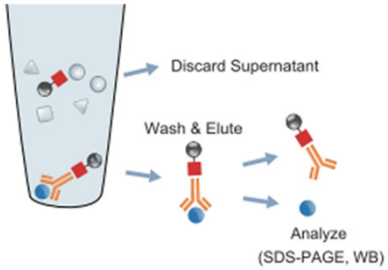
Wash/Elution/Analysis 洗涤/洗脱/分析



- Washing buffer
  - Salts, detergents
- Elution buffer
  - SDS sample buffer
    - 2% SDS, DTT/MeEtOH
  - Glycine buffer
    - 0.1-0.2 M Glycine, pH 2-3

Wash	Elution	Analysis
Wash pellet 5 times with lysis/washing buffer (on ice)	Resuspend pellet with elution buffer Vortex & centrifuge	Take the supernatant Run SDS-PAGE/WB

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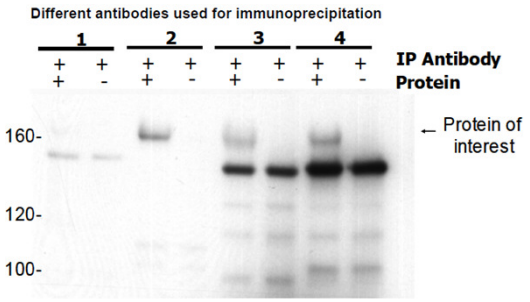
IP 疑难排除

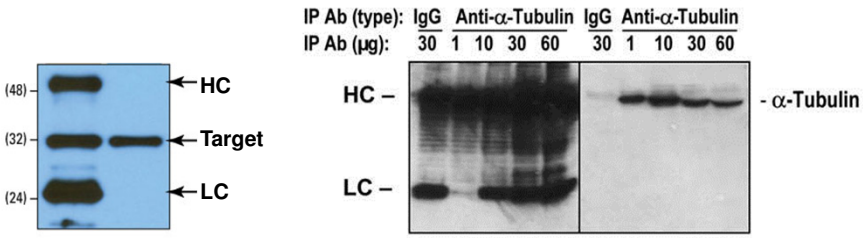
- High background
- High amount of antibody eluting
- No eluted target protein detected

High background 高背景



Possible Cause	Solution
Antigen degrading during IP	Use fresh protease inhibitors Keep sample cold at all time
Incomplete washing	Wash well by inverting
Non-specific binding to beads/Abs	Pre-block beads/Pre-clear the lysate Use less Ab Reduce the amount of sample loaded

High amount of Ab eluting



Possible Cause	Solution
Too much Ab eluting with the target protein	Reduce the amount of Ab Use 1° Ab from different species for WB Crosslink the Ab to the beads Elute with a gentle glycine buffer gradient

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No eluted target protein detected

Possible Cause	Solution
No/Low expression of target protein in sample used	If expression is low, increase the amount of lysate used
Insufficient capture Ab for target protein	Increase the concentration of Ab
Target protein has not eluted from the beads	Use the correct elution buffer with appropriate strength and pH
Ab has not bound to beads	Use the correct beads for the Ab isotype used
Incorrect lysis buffer used	Check if the Ab detects denatured or native protein Use the correct lysis buffer

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Enzyme-linked Immunospot Assay (ELISpot) 酶联免疫斑点法

- Detection of a single cell that secrets a protein of interest 适合检测分泌特定蛋白的细胞(外泌性蛋白)
  - cytokine, effector protein, receptor, surface marker, antibody
- The most sensitive cellular assays available
  - among 100,000 to 1,000,000 cells in the culture
  - Spot forming cells (SFC)

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Study Fields of ELISpot 研究领域

- monitor immune responses in both humans and animals.
  - T cell Function (T细胞功能)
  - Autoimmune (自体免疫)
  - Tumorigenisis (癌症肿瘤)
  - Vaccine and Drug development and analysis (疫苗与药物开发)
  - Infectious Disease (传染病)
  - Viral Infection (病毒性疾病)
  - Organ transplant (移植研究)

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ELISpot Kit 组分

Contents	ELISpot Kits
Target Ab	cytokine, effector protein, receptor, surface marker, antibody
Other Components	**Pre-coated PVDF plate(s)** **Detection Ab** **Enzyme conjugate** **Ready-to-use substrate buffer**

Humam: CD178; Granzyme B; IFN-gamma; IL-1beta; IL-2; IL-2sRa; IL-4; IL-5; IL-6; IL-10; IL-12; IL-13; IL-17A; IL-17A/F; IL-17F; Perforin; TNF-alpha; GM-CSF; M-CSF,…
Murine: IFN-gamma; IL-2
Rat: IFN-gamma; TNF-alpha

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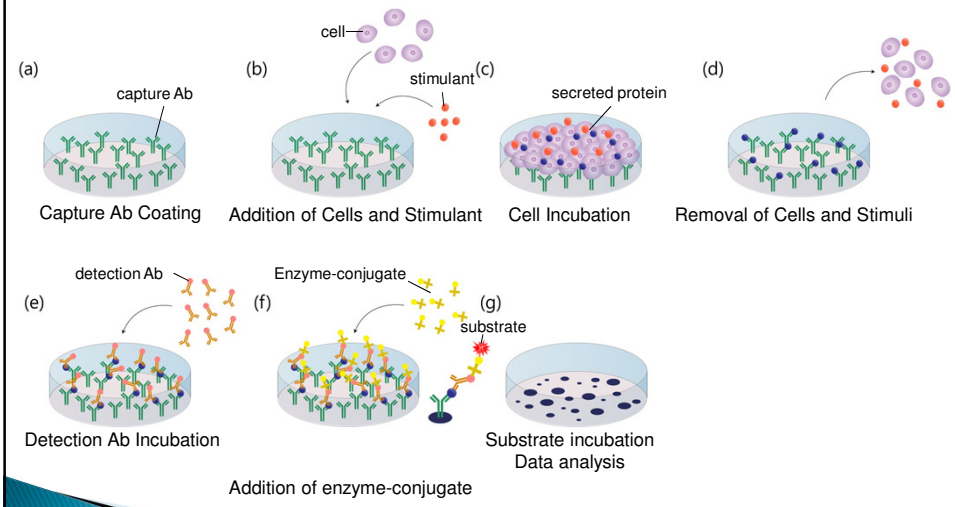
ELISpot 实验流程



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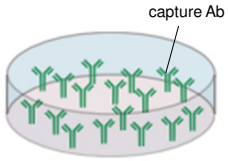
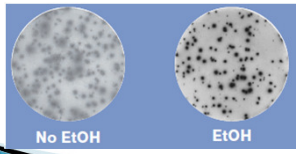
Capture Ab Coating 抗体包被

ELISpot plate with 96 wells



Pretreatment with EtOH	Immobilization of Capture Ab
Pretreat the PVDF plate with EtOH to obtain optimal spot quality Wash with sterile water	Immobilize target-specific capture Abs on the membrane of the well



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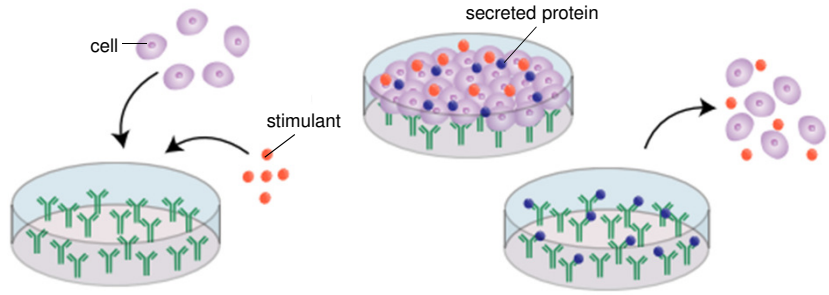
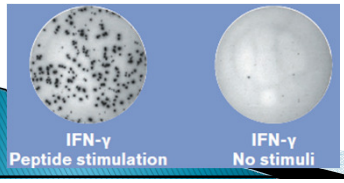
Cell incubation 细胞培养



Incubation with cells +/- stimulation	Removal of cells and stimulant
Add cells in the presence or absence of specific stimulus and incubate for a relevant time period to allow secreted protein induction and secretion	Wash off cells and stimulant Secreted protein bound by Capture Ab



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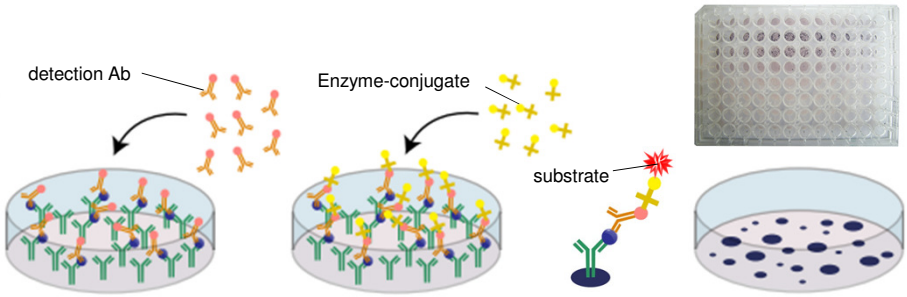
Detection & Analysis 检测&分析



Detection Ab incubation	Addition of Enzyme-conjugate	Substrate Incubation & Analysis
Add enzyme-conjugated or biotinylated detection Ab and incubate	Add streptavidin-enzyme conjugate If using biotinylated detection Ab	Add substrate (colored spot formed) Count spots and determine the number of responding cell

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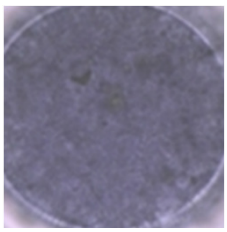
ELISpot 疑难排除

- High background
- No spots/very few spots
- Confluent spots
- Blank areas
- Poorly defined spots

High background



Possible Cause	Solution
Poor washing	Wash carefully
Too many cells secreting protein of interest	Reduce the number of cells per well Make series dilutions of cells
Plate not dried properly	Dry the plate longer before reading
Over developed plate	Reduce developing time

No spots/very few spots

Possible Cause	Solution
Not enough cells secreting protein of interest	Increase the number of cells
Ensure the cells are stimulated correctly	Use a positive stimulation control
Cells not incubated for long enough or may take time to respond to stimulant	Increase the cell incubation time or pre-treat cells with stimulant
Not enough primary or secondary Ab	Increase Ab concentration

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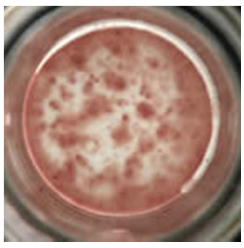
Confluent spots



Possible Cause	Solution
Poor coating, too much antibody	Improve capture Ab coating, less Ab
Prolonged cell culture	Reduce cell incubation time
Cells over-stimulated	Reduce the amount of stimulant

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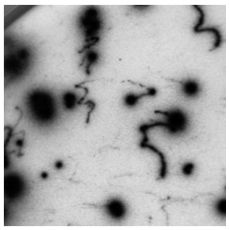
Blank areas

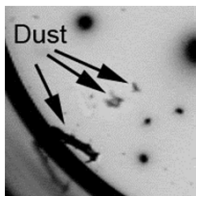
Possible Cause	Solution
foam formation during washing	Wash carefully
Cells unevenly distributed	Mix the cells gently to have a good homologous cell suspension before pipetting out into the wells
Damage from washing	Wash gently

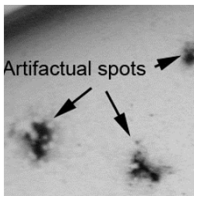
Poorly defined spots –stripy spots



Possible Cause	Solution
The movement of plates during cell incubation	Avoid moving of plates during incubation

Poorly defined spots
-small, dark, irregular spots





Possible Cause	Solution
Due to static eletricity, dust particles attached	Clean the underside of the well with 70% ethanol and compressed air
Cell wall fragments containing receptor-bound cytokines that stick to the coating Abs in the well.	These small artifactual spots are ignored by standard ELISpot readers.

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ELISpot 与 ELISA 比较

	ELISpot	ELISA
Measurement Format	Single Cell	Bulk Supernatant
T cell Functional Study	✓	✗
Sensitivity	200X – 400X more sensitive	–
Resolution	≠ Per cell source distinguishable	≒ Net source non-distinguishable
Dual Detection	✓	✗
Mail plate for analyses	✓	✗
Storage time/Sample Reusability	Weeks – months/✓	Hours/✗

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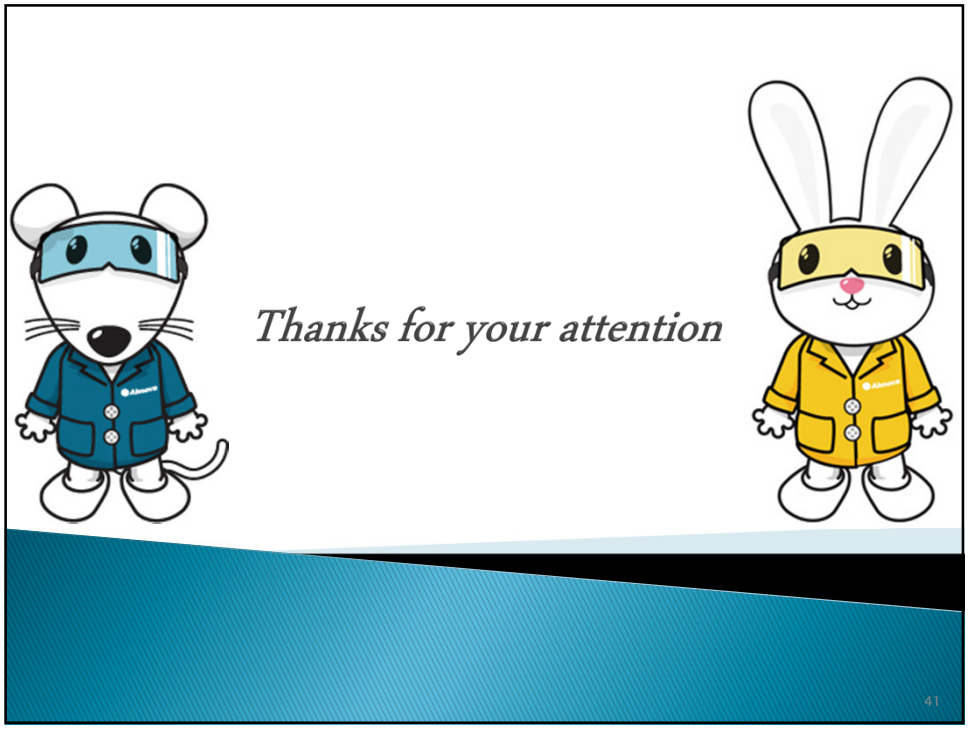

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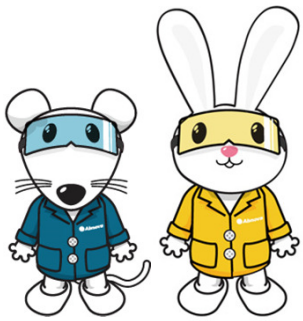
  - 多株抗体 (27,600+)
  - 蛋白质 (25,700+)



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