



小鼠肿瘤坏死因子 α ELISpot 试剂盒 (ALP)

货号 : No. EK50349A

备注: 请在使用产品前仔细阅读本使用说明书 (以随货打印版为准)。不同批次试剂不可混用

预期用途: 本试剂盒用于定量测定细胞释放小鼠肿瘤坏死因子 α 的频率。

本产品仅供科研使用, 不能用于治疗 and 诊断。

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检测原理

酶联免疫斑点 (ELISPOT) 检测采用 ELISA 夹心法。PVDF 膜微孔板预包被了小鼠 TNF- α 特异性单克隆抗体，可以捕获由活化细胞分泌的小鼠 TNF- α 。在移除细胞并洗涤后，加入生物素标记的靶向 TNF- α 的二抗，形成“抗体-抗原-抗体”夹心复合物。随后加入链霉亲和素-碱性磷酸酶 (ALP) 结合物，进而实现生物素介导的结合。最后用底物显色，终止后在板孔底部膜表面形成不溶性斑点，每个斑点代表一个单独的 TNF- α 分泌细胞。斑点可以用 ELISPOT 分析仪进行自动计数或者用显微镜进行手动计数。

试剂盒组分和保存条件

请在有效期内使用试剂盒。

组分	数量	准备说明	储存
预包板	96T:1 板 10*96T:10 板	从真空包装中取出预包板后，应尽快使用。	真空储存于 2-8℃ ,直至保质期。
检测抗体	96T:1 支 50 μL 10*96T:1 支 500 μL	使用前需用含 0.5% 胎牛血清 (PBS-0.5% FBS)的 PBS 将检测抗体按1:350 进行稀释。 根据需要现用现配。	原液可在 2-8℃下保持稳定，直至保质期。 配制的工作液在工作日内用完并丢弃。根据需要现用现配。
Streptavidin-ALP	96T:1 支 90 μL 10*96T:1 支 900 μL	使用前需用含 0.5% 胎牛血清 (PBS-0.5% FBS)的 PBS 将 Streptavidin-ALP 按 1:170 进行稀释。	
BCIP/NBT-plus 稀释液	96T:1 瓶 15mL 10*96T:1 瓶 150mL	BCIP/NBT-plus 溶液 A 与稀释液按 1:250 的体积比进行混合, 随后加入与溶液 A 等体积的溶液 B 并轻轻混匀。溶液 A：稀释液: 溶液 B 的体积比为 1：250：1。例如：用移液管将 40 μL 溶液 A 滴入 10 mL 稀释液中，充分混合，然后加入 40 μL 溶液 B 并轻轻混合。 根据需要现用现配。	
BCIP/NBT-plus 溶液 A	96T:1 瓶 60 μL 10*96T:1 瓶 600 μL		
BCIP/NBT-plus 溶液 B	96T:1 瓶 60 μL 10*96T:1 瓶 600 μL		

检测流程

1. 预包板处理 (无菌操作)

- 从密封包装中取出预包板，用无菌PBS洗涤4次 (200 μl /孔)。
- 用含有10%胎牛血清(FBS)的无菌RPMI-1640培养基(200 μl /孔)对预包板进行处理，室温孵育至少30 min。

2. 细胞孵育 (无菌操作)

- 弃掉孔内液体，每孔先加入刺激物，然后加入100 μl 的细胞悬液。细胞和刺激物的浓度应根据实际情况进行调整，各组设置建议如下：
 - a. 阳性对照孔: 对于多克隆刺激剂，如LPS (0.1-0.5 $\mu\text{g}/\text{ml}$)，建议采用比实验组更少的细胞数，如 1×10^5 cells/孔。
 - b. 阴性对照孔: 细胞数要与实验孔中的细胞数保持一致，不加刺激物。
 - c. 背景对照孔: 不加细胞，不加刺激物，加入培养基。
 - d. 实验组：建议铺板细胞总数 $1.5-2.5 \times 10^5$ cells/孔，根据实验需求加入实验者自己的刺激物 (用培养基进行刺激物的配制)。

注意 建议在检测样本时设置复孔。

- 加样完成后，盖好板盖，置于37°C，5% CO₂培养箱中孵育18-48 h。在此期间不要移动板子，盖好的板子建议用铝箔纸包裹，避免蒸发。

3. 斑点检测

注意：使用含有0.5% FBS的PBS (PBS-0.5% FBS) 稀释检测抗体和Streptavidin-ALP。PBS应用0.2 μm 滤膜进行过滤。在洗涤和孵育缓冲液中避免使用吐温或其他去污剂。

- 弃掉孔内液体，每孔加入 200 μl 4°C预冷的去离子水，4 度冰箱放置 10 分钟低渗裂解细胞。
- 弃掉孔内液体，每孔加入200 μl 的PBS，清洗5次。

注意：PBS浸泡 1 min, 保证清洗干净，下述所有洗涤步骤要求相同。
- 用含有0.5% FBS的PBS (PBS-0.5% FBS) 将检测抗体按1:370进行稀释，100 μl /孔加入板中，室温孵育2 h。

注意 稀释后的检测抗体应用0.2 μm 的滤膜过滤，以降低非特异性斑点的产生。
- 弃掉孔内液体，每孔加入200 μl 的PBS，清洗5次。
- 用含有0.5% FBS的PBS (PBS-0.5% FBS) 将Streptavidin-ALP按1:170进行稀释，100 μl /孔加入板中，室温孵育1 h。
- 弃掉孔内液体，每孔加入200 μl 的PBS，清洗5次。
- 用0.45 μm 滤膜过滤现配的BCIP/NBT-plus底物溶液，每孔加入100 μl ，室温避光孵育 5-30 min，根据斑点生成情况选择终止显色时间。

注意 显色时间太长可能导致背景颜色变深。
- 终止显色时用大量自来水/去离子水冲洗，建议揭开板底座，冲洗膜的底部。
- 将膜板放置在室温阴凉处，待膜板完全干燥后再进行读数。可使用斑点分析仪或显微镜进行斑点计数及分析。

注意：请勿在高于37°C的温度下干燥膜板，否则可能导致膜破裂。
- 室温避光存放膜板，以便日后进行重新分析。

常见问题及解决办法

问题描述	可能原因分析	改进建议
PVDF 膜背景深，着色重	*洗板不彻底 *膜是湿的，未干透	*提高洗板次数，保证每次浸泡 1 min *PVDF 膜板完全干透后再读板
阴性对照孔出现斑点	*培养基、血清或 DMSO 中的内毒素或其他污染物激活细胞，导致额外的非特异性斑点产生 *细胞被污染 *细胞活率低	*使用无内毒素的试剂 *在板处理和细胞孵育过程中注意严格无菌操作 *优化细胞分离提取流程，确保细胞的活性和功能性（建议细胞活率> 90%）
斑点形状不规则或成团	*细胞破碎 *细胞聚集	*优化细胞分离提取流程，确保细胞活性 *细胞悬液充分混匀，确保呈单细胞状态
阳性对照孔斑点数少或者斑点模糊	* 酶活力下降 *显色试剂温度低，出现氧化现象 *刺激物效价低	* 提高酶浓度 *显色试剂使用前平衡至室温，勿使用已出现氧化沉淀的试剂 *提高刺激物浓度
斑点位置不均匀，存在边缘效应	*铺板后震动细胞板，造成细胞位移 *加入刺激物时力度过大，冲散细胞悬液 *板子堆放在培养箱中	* 在铺板完成后，尽量不要晃动细胞板，轻缓平移放入培养箱 * 采取先加刺激物后加细胞的方式 *将每块板单独放在架子上，使热量均匀分布到每个孔
读板时即使对参数进行了调整 ,但孔中斑点密集，无法被全部计算在内	*斑点融合在一起，无法区分单个斑点	*减少加入孔中的细胞数量



Mouse TNF- α ELISpot Kit (ALP)

Cat. No. EK50349A

Note: Please read this instruction manual carefully before using the product (the printed version with the product shall prevail). Different batches of reagents cannot be mixed.

Intended Use: The kit applies for the detection of mouse TNF- α at the cellular level by counting TNF- α -secreting cells.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.
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PRINCIPLE OF THE ASSAY

The enzyme-linked immunospot (ELISPOT) assay employs a sandwich ELISA-based methodology. A 96-well polyvinylidene difluoride (PVDF) membrane plate, pre-coated with mouse TNF- α -specific monoclonal antibodies, captures mouse TNF- α secreted by activated cells. Following cell removal and washing, biotin-conjugated secondary antibodies targeting TNF- α are added to form antibody-antigen-antibody "sandwich" complexes. Subsequent incubation with streptavidin-alkaline phosphatase (ALP) conjugates enables biotin-mediated binding. Addition of chromogenic substrate culminates in the formation of insoluble spots at the membrane interface, with each spot representing an individual TNF- α -secreting cell. Spot quantification is achieved by automated ELISPOT analysis or manual counting using a microscope.

KIT CONTENTS AND STORAGE

Please use the kit before the expiration date.

Components	Amount	Preparation instructions	storage
Pre-coated microplate	1plate (96 tests)	Once removed from vacuum bag, the plate should be used as soon as possible.	Vacuum storage can store at 2-8℃ until expiration date.
Detection Antibody	1 vial	Dilute the detection antibody 1:370 in PBS containing 0.5% fetal calf serum (PBS-0.5% FBS) before use. Dilute fresh as needed.	Primary liquid are stable at 2 - 8℃ until expiration date. To be reconstituted, the working fluid is used within the working day and discard. So dilute fresh as needed.
Streptavidin-ALP	1 vial	Dilute the Streptavidin-ALP 1:170 in PBS-0.5% FBS.	
BCIP/NBT-plus Dilution	1 bottle	BCIP/NBT-plus Solution A and the Dilution should be mixed together in a 1:250 volume, followed by the addition of Solution B in the same volume as Solution A and mixed gently. The volume ratio of Solution A: Dilution: Solution B is 1:250:1. For example: pipette 40 μL of Solution A into 10 mL of the Dilution buffer, mix thoroughly, then add 40 μL of Solution B and mix gently. Dilute fresh as needed.	
BCIP/NBT-plus Solution A	1 bottle		
BCIP/NBT-plus Solution B	1 bottle		

ASSAY PROCEDURE

1. Plate Set-up (sterile conditions)

- Remove the plate from the sealed package and wash 4 times with sterile PBS (200 µl/well).
- Condition the plate with sterile RPMI-1640 culture media (200 µl/well) containing 10% fetal calf serum (FBS) as used for the cell suspensions, Incubate for at least 30 minutes at room temperature.

2. Incubation of cells in plate (sterile conditions)

- Empty the plate, then add stimulus followed by the 100 µl cell suspensions to each well. The concentration of cell and stimulus should be adjusted according to the actual situation. The recommended group settings are as follows.
 - a. Positive control: Fewer cells such as 1×10^5 cells/well are needed for polyclonal activators, e.g., LPS (0.1-0.5 µg/ml).
 - b. Negative control: The cell concentration is kept consistent with the experimental wells in the absence of stimulus.
 - c. Background control: No cells, no positive stimulus, add to the medium.
 - d. Experimental group: The recommended cell number per well is $1.5-2.5 \times 10^5$, and add your own stimulus as required by the experiment (prepare the stimulus using culture medium).

Note: It is recommended to set duplicate wells for samples to be tested.

- Put the plate in a 37°C and 5% CO₂ incubator for 18-48 hours. Do not move the plate during this period and it is recommended to wrap the plate in aluminum foil to avoid evaporation.

3. Detection of spots

Note: Use PBS containing 0.5% FBS (PBS-0.5% FBS) for dilution of the detection antibody and Streptavidin-ALP. The PBS should be filtered (0.2 µm) for optimal results. Avoid the inclusion of Tween or other detergents in the washing and incubation buffers.

- Remove the cells and medium by emptying the wells, add 200 µl of 4°C pre-cooled deionized water to each well, and place in a 4°C refrigerator for 10 minutes to perform hypotonic lysis of the cells.
- Remove the cells by emptying the plate and wash 5 times with PBS, 200 µl/well.

Note: Soak in the washing solution for 1 minute to ensure thorough cleaning. The same requirement applies to all subsequent washing steps.

- Dilute the detection antibody 1:370 in PBS-0.5% FBS. Add 100 µl/well and incubate for 2 hours at room temperature.

Note: Diluted detection antibody can be filtered (0.2 µm) to reduce the risk of unspecific background.

- Empty the plate and wash 5 times with PBS, 200 µl/well.
- Dilute the Streptavidin-ALP 1:170 in PBS-0.5% FBS and add 100 µl/well. Incubate for 1 hour at room temperature.

- Empty the plate and wash 5 times with PBS, 200 µl/well.
- Filter the ready-to-use substrate solution (BCIP/NBT-plus) through a 0.45 µm filter and add 100 µl/well. Develop until distinct spots emerge in positive wells (usually 5-30 minutes).

Note: Too long the color development time will cause the background color in the experimental well to become darker.

- Stop color development by washing extensively in tap water. If desirable, remove the underdrain (the soft plastic under the plate) and rinse the underside of the membrane.
- Leave the plate to dry. Plates should be completely dry before analysis. Inspect and count spots in an ELISpot

reader or in a microscope.

Note: Do not dry the microplate at a temperature higher than 37°C; this may cause cracking of the membrane filters.

- For the option to re-analyze later, store plate in the dark at room temperature.

TROUBLESHOOTING GUIDE

Problem	Possible reason	Solution
Dark background of the PVDF membrane	*Plate is insufficiently washed *The membrane is wet	*Increase the washing times to ensure soaking time *Microplates should be analyzed until the PVDF membranes are completely dry
Spots appear in the negative control wells	*Endotoxins or other contaminants in the medium, serum, or DMSO may activate immune cells *The cells are contaminated *Cell viability is low	*Use endotoxin-free reagents *Pay attention to aseptic procedures in the step of Plate Set-up and Incubation of cells in plate *Ensure high viability (recommended viability > 90%) as well as functionality of the cell sample
Spots are irregular in shape and severely clumped	*Cell disruption *Cell aggregated	*Optimize the cell separation process to ensure cell activity *The cell suspension is thoroughly mixed to ensure a unicellular state
Positive Control spots were low and fuzzy	* Enzyme activity is decreased *The temperature of the Substrate reagent is low and oxidized * The titer of the stimulus is low	* Increase enzyme concentration *Balance the color reagent to room temperature before use, do not use reagents that have oxidized precipitation * Increase the concentration of stimulus
The spots are unevenly positioned and have an edge effect	*Shake the cell plate after plating, causing cell displacement *Add the stimulus too vigorously to flush the cell suspension *Stack the plates in the incubator	*After plating, try not to shake the plate, and gently pan it into the incubator *Use the method of adding the stimulus first and then the cells *Place each plate individually on the shelf to allow an even distribution of heat to each microwell
Not all spots are counted in the crowded well despite algorithm and parameter adjustments	*Spots are confluent. Single spots cannot be discriminated	*Decrease the number of cells added per well