SP002-EN.01

Mouse Interferon-γ (IFN-γ) ELISPOT Kit

Catalog Number：RAS-SP002

Pack Size: 96 tests

IMPORTANT: Please carefully read this manual before performing your experiment.

For Research Use Only. Not For Use In Diagnostic Or Therapeutic Procedure

INTENDED USE

Mouse Interferon-γ (IFN-γ) ELISPOT Kit is used to detect IFN-γ at the cellular level. It is for research use only.

PRINCIPLE OF THE ASSAY

This assay kit is used to measure the levels of mouse Interferon-γ (IFN-γ) by employing a standard sandwich-ELISA format. The polyvinylidene difluoride (PVDF)-backed micro plate in the kit has been pre-coated with Anti-IFN-γ Antibody. IFN-γ secreted by cells binds to Anti-IFN-γ Antibody fixed on the microplate. Then add the Biotin-Anti-IFN-γ Antibody to the plate and form Antibody-antigen-biotinylated antibody complex. Next add Streptavidin-HRP to the plate, incubate and wash the wells. At last, load the substrate into the wells and red brown spots will form at the bottom of the plate hole after termination. Each individual spot representing an individual IFN-γ secreting cell. The spots can be counted with an automated ELISpot reader system or manually using a stereomicroscope.

MATERIALS PROVIDED

Table1. Materials provided

Catalog	Components	Size (96 tests)	Format	Storage	
				Unopened	Opened
RSP002-C01	Pre-coated Anti-IFN-γ Antibody Microplate	1 plate	Solid	2-8°C	2-8°C
RSP002-C02	Positive Stimulus	60 μg	Power	2-8°C, avoid light	-70°C, avoid light
RSP002-C03	Biotin-Anti-IFN-γ Antibody	50 μL	Liquid	2-8°C	2-8°C
RSP002-C04	Streptavidin-HRP	50 μL	Liquid	2-8°C	2-8°C
RSP002-C05	Washing Buffer (10×)	50 mL	Liquid	2-8°C	2-8°C
RSP002-C06	Dilution Buffer (1×)	50 mL	Liquid	2-8°C	2-8°C
RSP002-C07	AEC Dilution	25 mL	Liquid	2-8°C	2-8°C
RSP002-C08	AEC Solution A (20×)	0.8 mL	Liquid	2-8°C, avoid light	2-8°C, avoid light
RSP002-C09	AEC Solution B (20×)	0.8 mL	Liquid	2-8°C	2-8°C
RSP002-C10	AEC Solution C (20×)	0.8 mL	Liquid	2-8°C, avoid light	2-8°C, avoid light

SRORAGE

1. The unopened kit is stored at 2°C to 8°C and its expiration date can be found on the kit label.

2. This kit is validated for single use only. Results obtained using previously opened or reconstituted reagents may not be reliable.

Note: *Do not use reagents past their expiration date.*

REAGENTS/EQUIPMENT NEEDED BUT NOT SUPPLIED

Clean bench;

CO_2 incubator;

ELISpot reader or microscope;

Single or multi-channel micropipettes and pipette tips;

Sterile RPMI-1640 culture media;

REAGENT PREPARATION

Bring all reagents and samples to room temperature (20℃-25℃) before use. If crystals have formed in buffer solution, place the sample until the crystals have completely dissolved.

ASSAY PROCEDURE

1. Working Solution Preparation

1.1 Positive stimulus:

According to Table 2, prepare the provided lyophilized product into a storage solution with sterile water, dissolve at room temperature for 15 to 30 min, and mix by gently pipetting, this step must be performed in a sterile environment.

Table 2. Preparation method

ID	Components	Size (96 T)	Storage solution concentration.	Reconstituted water Vol.
RSP002-C02	Positive Stimulus	60 μg	600 μg/mL	100 μL

1.2 Sterile culture media:

RPMI-1640 medium with 10% fetal bovine serum can be used, and it is recommended to add dual antibodies.

1.3 Preparation of 1×Washing Buffer:

Dilute 50 mL 10×Washing Buffer with ultrapure water/deionized water to 500 mL.

1.4 Preparation of Biotin-Anti-IFN-γ Antibody working fluid:

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Dilute Biotin-Anti-IFN-γ Antibody at 1:2000 with 1×Dilution Buffer. Please prepare it for one-time use only.

1.5 Preparation of Streptavidin-HRP working fluid:

Dilute Streptavidin-HRP at 1:2000 with 1×Dilution Buffer. The prepared working fluid should avoid light. Please prepare it for one-time use only.

1.6 Preparation of 1×AEC Solution: Please prepare it for one-time use only. The proportion of preparation is shown in Table 3.

Table 3. Preparation method

Preparation Vol	AEC Dilution	AEC Solution A (20×)	AEC Solution B (20×)	AEC Solution C (20×)
1 mL	0.85 mL	50 µL	50 µL	50 µL
5 mL	4.25 mL	250 µL	250 µL	250 µL
10 mL	8.25 mL	500 µL	500 µL	500 µL

2. Pre-coated Microplate treatment (Aseptic procedure)

Add 200 µL Sterile RPMI-1640 culture media to each well, incubate at room temperature for 30 min, then remove the remaining solution.

3. Add Stimulus and cell suspensions (Aseptic procedure)

Add 50 µL stimulus to each well, then add 50 µL cell suspensions,

a. Positive control: The cell concentration can be 1×10^5 cells/well and the final intracellular concentration of positive stimulus is 6.0 µg/mL (Dilute 600 µg/mL of positive stimulus to 12 µg/mL with basic medium).

b. Negative control: The cell concentration can be 1×10^5 cells/well without positive stimulus.

c. Background control: No cells, no positive stimulus, add to the medium.

d. Experimental: The concentration of cell and stimulus should be adjusted according to the actual situation.

Note: *It is recommended to set three doable holes for samples to be tested.*

4. Incubation (Aseptic procedure)

Seal the plate with microplate sealing film and incubate at 37°C and 5% CO_2 incubator for 20-48 hour.

Note: *The covered plate is recommended to be covered with aluminum foil, and the cultivation time can be adjusted according to the actual situation.*

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5. Washing

Remove the remaining solution by aspiration, add 250 µL of 1×Washing Buffer to each well, then soak for 60 s, remove any remain8ing 1×Washing Buffer: by aspirating or decanting, invert the plate and blot it against paper towels. Repeat the wash step above for six times.

Note: *Remove the remaining solution by aspiration before washing the plate in this step, add 200 µL cold deionized water to each well, and the hypotonic lytic cells can be placed at 2-8℃ for 5-10 minutes to help the washing effect.*

6. Add Biotin-Anti-IFN-γ Antibody

For all wells, add 100 µL Biotin- Anti-IFN-γ Antibody (dilute at 1:2000) working solution. Please prepare it for one-time use only. Seal the plate with microplate sealing film and incubate at 37℃ for 1 h.

7. Washing

Repeat step 5.

8. Add Streptavidin-HRP

For all wells, add 100 µL Streptavidin-HRP (dilute at 1:2000) working solution. Please prepare it for one-time use only, avoid light. Seal the plate with microplate sealing film and incubate at 37℃ for 1 h.

9. Washing

Repeat step 5.

Note: *After washing for the fifth time remove the bottom plate, the bottom surface and bottom plate of the membranes can be washed with deionized water, blot it against clean paper towels and prevent damage to the membranes, the bottom plate can be closed, and then washing for the Sixth time to ensure that the experimental background is clean.*

10. Substrate Reaction

Add 100 µL AEC Substrate Solution to each well. Incubate at room temperature for 5-30 min, avoid light.

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

11. Termination

Remove the remaining solution by aspiration, Open plate base and rinse the microplate with deionized water for 3-5 times. Place the plate in the shade at room temperature, dry naturally, and close the base.

12. Calculation of results

The microplate can be analyzed by counting spots using either a microscope or a specialized automated ELISpot

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reader.

PRECISION

Inter/Intra-assay precision was evaluated by stimulating 1×10^5 cells/well Mouse Spleen Cells with 6.0 μg/mL ConA at 37℃ and 5% CO2 incubator for 20 h and repeated for six wells.

Well	Number of Spots Counted		
	Batch-1	Batch-2	Batch-3
1	275	291	276
2	306	311	285
3	325	276	284
4	330	296	289
5	344	271	270
6	315	298	279
Intra-assay-Mean	316	291	281
Intra-assay-SD	23.845	14.816	6.892
Intra-assay-CV%	7.5%	5.1%	2.5%
Inter-assay-Mean	296		
Inter-assay-SD	21.907		
Inter-assay-CV%	7.4%		

PRECAUTIONS

1. This kit is for research use only and is not for use in diagnostic or therapeutic applications.
2. Please strictly follow the instructions for operation. Individual experimental steps are aseptic operation. PVDF membrane is attached to the bottom of the plate, and the gun head should not touch the bottom when adding liquid, so as not to damage the PVDF membrane.
3. Do not mix or substitute reagents with those from other kits or other lot number kits.
4. Bring all reagents and samples to room temperature before use and ensure the crystals have completely dissolved.
5. The kit is stored at 2°C to 8°C, do not use the kit that has expired, and try to use the kit once opened.

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TROUBLESHOOTING GUIDE

Problem	Cause	Solution
Dark background color of the filter membrane	* Plate is insufficiently washed * The membrane is wet	* Increase the washing times to ensure soaking time * Microplates cannot be analyzed accurately until the PVDF filter membranes are completely dry
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
Spots vary in size and shade	* When this phenomenon occurs in positive controls, the cell status is uneven and the survival rate is low	* Optimize the cell separation process to ensure cell activity * Strengthen cell washing to remove dead cells
	* When the positive control is normal, the degree of specificity of the stimulus is low	* Change stimulus
The number of spots in the wells is lower	* When the positive control is normal, there are stimulus-specific problems and the level of normal cell response is low	* Change the concentration of the stimulus * Increase the number of cells added per well
	* When the positive control spots were small, the number of surviving cells added was too small.	* Activity count was performed to improve cell activity * Increase the number of cells added per well
The number of spots in the wells is high	* Too many cells were added to the well	* Decrease the number of cells added per well

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