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User's Manual

B Cells IgG Detection Dot-ELISA Kit

REF **DEIA-JY24044**

Σ **5×96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

The B cell ELISPOT assay determines the number of individual antibody secreting cells (ASC) in single cell suspensions and can identify ex vivo antigen-specific memory B cells. The cell suspensions used may originate from blood (PBMC) or secondary lymphoid organs.

Reagents And Materials Provided

1. Coating antibody* 1 vial 4 °C
2. Biotinylated detection antibody* 1 vial 4 °C
3. Streptavidin-HRP conjugate* 1 vial ≤-20 °C**
4. Recombinant IL-2* 1 vial ≤ -20 °C
5. R848 (Resiquimod) 1 vial (0.5 ml) ≤-20 °C**
6. AEC coloring system:

6.1. I. AEC stock solution 1 vial (4 ml) ≤-20 °C**

6.2 II. Substrate buffer (10x) 1 vial (5 ml) 4 °C

7. Blocking stock solution (10x) 1 vial (10 ml) 4 °C
8. Dilution buffer R (10x) 1 vial (10 ml) 4 °C
9. Tween-20 1 vial (5 ml) RT**

RT Room temperature (temperature between 20 °C and 26 °C)

* Lyophilized

** Store protected from light

Materials Required But Not Supplied

1. 96-well PVDF membrane-bottomed plates: Millipore cat. no. MSIP S4510 is recommended.
2. Adhesive cover slips.
3. Tubes and containers/plates to prepare solutions.
4. Tissue culture plates for preincubation (optional).
5. Sterile distilled water and demineralized water.
6. 70% ethanol.
7. Antigen of interest for coating*.
8. PBS pH 7.4 (home-made). For washing purposes only. Ingredients: $Na_2HPO_4 \cdot 2H_2O$, KH_2PO_4, NaCl and distilled water.
9. Sterile and pyrogen-free liquid PBS pH 7.4 (PBS-I): Thermo Fisher Scientific cat. no. 10010 is recommended. (Do not use PBS tablets. The filler in the tablets interferes with the coating process.)
10. Cell culture medium: RPMI-1640 supplemented with 2 mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml

streptomycin and 10% fetal calf serum.• Laminar flow hood (for sterile conditions), fume hood (for AEC substrate).

11. Pipetting devices.
12. For washing: squirt (wash or squeeze) bottle without sprout.
13. CO_2 incubator (37 °C, 100% humidity, 5% CO_2).
14. A reflected light microscope or an automated ELISPOT reader for spot counting.* Alternatively, antigen-specific immunoglobulin secreting B cells can also be analyzed by using the coating antibody provided with the kit in combination with a biotinylated antigen of interest as detection instead of the provided detection antibody. This procedure prevents the search for a PVDF membrane-binding antigen, and reduces the amount of antigen required.

Storage

Coating and detection antibodies

The vials with lyophilized coating and biotinylated detection antibody can be safely stored at 4 °C until the expiry date (indicated on the vials). After reconstitution, the antibodies are stable at 4 °C for at least 12 months when kept sterile. However, it is recommended that the reconstituted antibody solutions be divided into small aliquots for single use. These aliquots should be stored at ≤-20 °C (stable for at least two years).

IL-2

The vial with lyophilized recombinant IL-2 can be safely stored at ≤-20 °C until the expiry date (indicated on the vial). After reconstitution, the solution should be stored in small aliquots at ≤ -70 °C for single use (stable for at least one year).

R848

The vial with R848 can be safely stored at ≤-20 °C until the expiry date (indicated on the vial). It is strongly recommended to divide the solution into small aliquots for single use. These aliquots should be stored at ≤-20 C protected from light (stable for at least one year).

Conjugate

The vial with lyophilized streptavidin-HRP conjugate is stable until the expiry date (indicated on the vial) when stored protected from light at ≤-20 °C. After reconstitution, the reagent is stable at 4 °C for at least 2 months when kept sterile and protected from light. However, it is strongly recommended that the solution be divided into small aliquots for single use. These aliquots should be stored protected from light at ≤-20 °C (stable for at least one year).

AEC

The AEC stock solution should be stored at ≤-20 °C and is stable until the expiry date (indicated on the vial)*. Tightly close the vial after use. It is recommended that the solution be divided into small aliquots for single use in polypropylene vials. These aliquots should be stored ≤-20 °C protected from light at (stable for at least one year).

* Avoid exposure to light and air: tightly close the vial after use. Avoid contact with polystyrene pipettes and vials.

Substrate buffer

The substrate buffer is stable until the expiry date (indicated on the vial) when stored at 4 °C. Tightly close the vial after use.

Blocking and Dilution buffer

The vials with Blocking stock solution and Dilution buffer R can be safely stored at 4 °C until the expiry date (indicated on the vial). After opening, these solutions are stable for at least 6 months when kept sterile.

Tween-20

Tween-20 can safely be stored at RT (protected from light) and is stable until the expiry date (indicated on the vial).

Reagent Preparation

Note: Prepare reagents under sterile conditions (e.g. laminar flow hood).

PBS (for wash buffer)

5. 4 mM $Na_2HPO_4 \cdot 2H_2O$; 1.3 mM KH2PO4; 150 mM NaCl in distilled water (adjust to pH 7.4 and filter sterilize [0.2 µm] or autoclave).

For 1 ELISPOT plate: prepare 1 L PBS.

Wash buffer

PBS containing 0.05% Tween-20.

For 1 ELISPOT plate: add 0.5 ml of Tween-20 to 1 L PBS and mix gently but thoroughly.

Blocking buffer (1x)

Dilute Blocking stock solution (10x) in PBS-I.

For 1 ELISPOT plate: mix 2 ml Blocking stock solution (10x) gently but thoroughly with 18 ml PBS-I.

Dilution buffer (1x)

Dilute Dilution buffer R (10x) in PBS-I.

For 1 ELISPOT plate: mix 2 ml Dilution buffer R (10x) gently but thoroughly with 18 ml PBS-I.

Coating antibody (for analyzing total number of immunoglobulin secreting B cells)

Reconstitute the lyophilized antibody by injecting an appropriate volume (indicated on the vial) of sterile distilled water into the vial. Mix the solution gently for approximately 15 sec and allow it to stand at RT for 5 min. Avoid vigorous shaking.

For 1 ELISPOT plate: 50 µl is mixed gently but thoroughly with 5 ml PBS-I.

Antigen of interest (for analyzing number of antigen-specific immunoglobulin secreting B cells)

For coating, use 0.5-15 µg/ml antigen in PBS-I. (When using the antigen as detection, use 0.01-1 µg/ml biotinylated antigen in dilution buffer (1x).)

First, determine the optimal concentration.

Detection antibody

Reconstitute the lyophilized antibody by injecting an appropriate volume (indicated on the vial) of sterile distilled water into the vial. Mix the solution gently for approximately 15 sec and allow it to stand at RT for 5 min. Avoid vigorous shaking.

For 1 ELISPOT plate: 100 µl is mixed gently but thoroughly with 10 ml dilution buffer (1x).

Conjugate

Reconstitute the lyophilized contents by injecting an appropriate volume (indicated on the vial) of sterile distilled water into the vial. Mix the solution gently for approximately 15 sec and allow it to stand at RT for 5 min. Avoid vigorous shaking.

For 1 ELISPOT plate: 100 µl is mixed gently but thoroughly with 10 ml dilution buffer (1x).

Coloring system

The AEC coloring system consists of two items: a concentrated AEC stock solution* and a concentrated substrate buffer.

For 1 ELISPOT plate: mix 1 ml Substrate buffer (10x) thoroughly with 4.2 ml 70% ethanol and 4.8 ml demineralized water to reach a final concentration of substrate buffer (1x) in 30% ethanol. Add 660 µl AEC stock solution (toxic: use a fume hood) and mix thoroughly.

After mixing the solution should be clear.

This AEC solution should be used within 30 min after preparation.

* AEC stock solution must not come into contact with polystyrene pipettes and vials.

Reagents for in vitro activation of IgG memory B cells

Reconstitute the lyophilized IL-2 by injecting the appropriate volume (indicated on the vial) of sterile distilled water into the vial. Mix the solution gently for approximately 15 sec and allow it to stand at RT for 5 min. Avoid vigorous shaking.

The vial with R848 is thawed at RT for 10 min and then gently mixed.

Preincubation: combine both reagents in culture medium at a final concentration of 1/100 for IL-2 and 1/200 for R848.

The recommended preincubation time is 2-5 days at a recommended cell concentration of 2-5x10^6 cells/ml; see www.ucytech.com/cell-sample-preparation-b-cell-ELISPOT.

Assay Procedure

Before starting an ELISPOT experiment, an appropriate assay setup must be chosen.

Coating ELISPOT well	Cell stimuli during preincubation	Cells/ ELISPOT well	Detection ELISPOT well	Reveals
Provided coating antibody	IL-2+R848	$2x10^3$-$1x10^5$	Provided detection antibody	Total number of immunoglobulin secreting cells. Functionality of the cells and whether the assay works well.
Antigen of interest*	IL-2+R848	$1-3x10^5$	Provided detection antibody	Number of antigen-specific immunoglobulin secreting cells.
Provided coating antibody*	IL-2+R848	$1-3x10^5$	Biotinylated antigen of interest	Number of antigen-specific immunoglobulin secreting cells
PBS only (background response)	IL-2+R848	$1-3x10^5$	Provided detection antibody	False positive results.
Provided coating antibody	no preincubation step (no stimuli)	$2x10^3$-$1x10^5$	Provided detection antibody	Total number of spontaneously immunoglobulin secreting cells.**
Antigen of interest*	no preincubation step (no stimuli)	$1-3x10^5$	Provided detection antibody	Number of spontaneously antigen-specific immunoglobulin secreting cells.**
Provided coating antibody*	no preincubation step (no stimuli)	$1-3x10^5$	Biotinylated antigen of interest	Number of spontaneously antigen-specific immunoglobulin secreting cells.**
PBS only (background response)	no preincubation step (no stimuli)	$1-3x10^5$	Provided detection antibody	False positive results.
Provided coating antibody	none	none	Provided detection antibody	False positive results due to reagents or cell culture media.

Notes:

1. Controls should be tested for each sample on the ELISPOT plate.
2. All assay controls should follow the same procedure and incubation times as the cells tested on the

ELISPOT plate.
3. The above mentioned cell concentrations are guidelines. It is recommended to test the samples in triplicate and in serial dilutions in the ELISPOT procedure.
4. No more than 3x10**5** cells/well should be added in the ELISPOT plate. Higher concentration of cells will cause multiple cell layers, resulting in poor spot formation.
5. The volume of the cell preparations in the 96-well ELISPOT plate is 100 µl/well.

Note: All solutions should be at RT prior to use. Steps 1 till 11 should be performed under sterile conditions. In addition, estimate the time needed to prepare the (stimulated) cell suspensions, which should be ready for step 9, and plan accordingly.

1. Prewet the PVDF membrane of each well of the ELISPOT plate with 25 µl of 70% ethanol. Incubate for 1 min at RT.
2. Aspirate or firmly shake-out the ethanol. Immediately thereafter wells are rinsed twice with 200 µl PBS-I/well. The plate is subsequently emptied and tapped on tissue paper.
3. Add 50 µl of diluted coating antibody solution or 50 µl of a specific antigen into each well of the ELISPOT plate.
4. Cover the plate with a lid and incubate overnight at 4 °C.
5. Remove coating antibody solution and rinse each well 3x with 200 µl PBS-I. The plate is subsequently emptied with a firm shake-out action.
6. Add 200 µl blocking buffer (1x) into each well.
7. Cover the plate with a lid and incubate for at least 1 h at RT. During this incubation step start preparing the cell sample suspensions*.
8. If the cell suspensions are ready, remove blocking buffer from wells with a firm shake-out action (do not wash the wells).
9. Bring the cell suspensions into the wells of the ELISPOT plate. Add 100 µl/well.
10. Cover ELISPOT plate with lid and incubate at 37 °C, 5% CO2 and 100% humidity. The incubation time on the ELISPOT plate after a preincubation step can vary from 5-6 h for mouse spleen cells to 16-24 h for human and Old World monkey PBMC. The mentioned incubation times are guidelines. When other cell types are used, other incubation times should be considered.
11. Remove the bulk of cells with a firm shake-out action and rinse each well 2x with 200 µl PBS-I. The plate is subsequently emptied.
12. Wash the plate 5x with 250 µl wash buffer/well.*
13. Add 100 µl of diluted detection antibody into each well.
14. Seal the plate with an adhesive cover slip and incubate 2 h at RT (or overnight at 4 °C).
15. Empty plate. Remove and discard the underdrain from the bottom of the plate and wash both sides of the PVDF membrane 5x with wash buffer.
16. Add 100 µl diluted conjugate into each well.
17. Seal the plate with an adhesive cover slip and incubate 1 h at RT protected from light.
18. Empty plate and wash both sides of the PVDF membrane 5x with wash buffer.
19. Add 100 µl freshly prepared AEC solution into each well.
20. Cover plate with lid and incubate for 30 min at RT protected from light.
21. Stop the reaction by emptying the plate and thoroughly rinse both sides of the PVDF membrane with demineralized water.
22. Air-dry the plate at RT (protected from light).

23. Count spots by using a reflected light microscope or an ELISPOT reader. Note: Store the plate at RT at a dry place protected from light to prevent bleaching of spots.

Precautions

This kit is designed for research use only and not for use in diagnostic or therapeutic procedures.