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# Rat IL-4 ELISPOT Kit with sterile PVDF Plates

**Catalog No:** CKR034A
CKR034S

**Size:** 2 x 96 tests
5 x 96 tests

### Intended use

The cytokine ELISPOT assay is designed to enumerate cytokine secreting cells in single cell suspensions of lymphoid tissue, CNS tissue, bone marrow or preparations of peripheral blood mononuclear cells (PBMC). The assay has the advantage of detecting only activated/memory T-cells and the cytokine release can be detected at the single cell level, allowing direct determination of T-cell frequencies. The high sensitivity and easy performance, allowing a direct enumeration of peptide-reactive T-cells without prior *in vitro* expansion, makes the ELISPOT assay eminently well suited to monitor T-cell responses. The higher sensitivity if ELISPOT in comparison to that of ELISA or intracellular staining is due to the plate-bound antibodies directly capturing the cytokine released by the cell before it is diluted in the supernatant, trapped by high-affinity receptors or degraded by proteases. The sensitivity of the assay lends itself to measurement of very low frequencies of cytokine-secreting cells (1/300,000).

### Brief description of the ELISPOT assay

Cells are incubated in the wells of the ELISPOT plate precoated with a high-affinity monoclonal antibody to which the cytokine, produced during incubation, will bind. Subsequently, cells are washed away. Areas in which the cytokine has been trapped are detected with a combination of biotinylated anti-cytokine detection antibodies and Streptavidin-horseradish peroxidase (Streptavidin-HRP). The last step in the assay is the addition of AEC (3-amino-9-ethylcarbazole) yielding a red zone ("spot"). This zone reveals the site of cytokine secretion.

### Contents of the kit

| Part# | Reagents | Quantity | Storage conditions |
|---|---|---|---|
| CKR034S-A | Coating antibody, lyophilized | 1 vial | 4°C |
| CKR034S-B | Biotinylated detection antibody, lyophilized | 1 vial | 4°C |
| CKR034S-C | Streptavidin-HRP conjugate, lyophilized | 1 vial | -20°C |
| CKR034S-D | AEC stock solution | 1 vial (4 ml) | -20°C in the dark |
| CKR034S-E | AEC Substrate buffer capsules | 5 capsules | Room temperature |
| CKR034S-F | Blocking stock solution (10x) | 1 vial (10 ml) | 4°C |
| CKR034S-G | Dilution buffer R (10x) | 1 vial (10 ml) | 4°C |
| CKR034S-H | Tween-20 | 1 vial (5 ml) | Room temperature |
| CKR034S-P | 96-well PVDF membrane plate with lid | 5 | Room temperature |
| CKR034S-Z | Adhesive cover slip | 5 | Room temperature |

**Hazard Information:**

**AEC (3-amino-9-ethylcarbazole) is toxic** and tumorigenic and should be handled in a chemical fume hood with extreme care. Avoid contact with skin and eyes by wearing protective clothing, gloves and eye/face protection. In case of contact with skin, wash intensely with water and soap. Upon ingestion or contact with eyes, rinse immediately with plenty of water for at least 15 minutes and seek medical help.**

** LD50 oral; rat and mouse: 150 mg/kg, AEC contents in 5 plate format vials is 20 mg AEC/vial.

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**Additional reagents/materials required for assay performance:**

- Sterile distilled water
- 70% ethanol.
- Phosphate buffered saline (PBS): home-made, filter-sterilized or autoclave. For washing purposes only.
- Wash buffer: 1 x PBS containing 0.05% Tween-20 (PBST).
- Sterile and pyrogen free PBS (PBS-I)
- Pipetting devices.
- $CO_2$-incubator (37°C, 100% humidity).
- Culture medium
- Cell stimuli
- Squirt (wash or squeeze) bottle with wide spout for washing, see Addendum**
- Tissue culture plates or tubes for pre-stimulation (optional).
- A dissecting microscope or an immunospot image analyzer for spot counting.

**Storage of reagents**

- The vials with lyophilized coating antibodies and biotinylated detection antibodies can be safely stored at 4°C up to the kit expiration date. After reconstitution, the reagents are stable for a minimum of 6 months at 4°C when kept sterile. However, it is strongly recommended to divide the reconstituted antibody preparations into small aliquots for single use. These aliquots should be stored frozen (at ≤-20°C). Under these conditions, the reagents are stable for at least one year.

- The vial with lyophilized Streptavidin-HRP conjugate should be stored at ≤-20°C until the expiry date. After reconstitution, the reagents are stable for a minimum of 2 months at 4°C when kept sterile. However, it is strongly recommended to divide the reconstituted conjugate into small aliquots for single use. These aliquots should be stored at ≤-20°C. Under these conditions, the conjugate is stable for a minimum of one year.

- The reconstituted Streptavidin-HRP rapidly loses activity when kept at room temperature.

- The AEC stock solution should be protected from light and should be stored at ≤ -20°C until the expiry date. It is recommended to divide the solution into small aliquots for single use in polypropylene vials. These aliquots should be stored at ≤ -20°C protected from light.

- The substrate buffer capsules are stable until the expiry date when stored at room temperature, in a moisture-free environment.

- Blocking stock solution (10x) and Dilution buffer (10x) should be stored at 4°C until the expiry date. After opening these solutions are stable for a minimum of 6 months when kept sterile.

- Tween-20 can best be stored at room temperature until the expiry date.

## Preparation of Reagents

**Important:** Prepare reagents under aseptic conditions (e.g. Laminar Flow Hood).

### 1. CKR034S-A: Coating Antibody

Reconstitute the lyophilized contents of the vial by injecting **250 µl** of sterile distilled water into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 5 minutes at room temperature.
For one ELISPOT plate, 50 µl of the solution is pipetted out of the vial and thoroughly mixed with 5 ml PBS-I.

### 2. Blocking Stock Solution (1x)

Dilute Blocking stock solution R (10x) in PBS-I.
For one ELISPOT plate, 2 ml is thoroughly mixed with 18 ml PBS-I.

### 3. Dilution Buffer (1x)

Dilute dilution buffer (10x) in PBS-I.
For one ELISPOT plate, 2 ml is thoroughly mixed with 18 ml PBS-I.

### 4. CKR034S-B: Biotinylated detection antibodies

Reconstitute the lyophilized contents by injecting **500 µl** sterile distilled water into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 5 minutes at room temperature.
For one ELISPOT plate, 100 µl is thoroughly mixed with 10 ml Dilution buffer R (1x).

### 5. CKR034S-C: Streptavidin-HRP conjugate

Reconstitute the lyophilized contents by injecting **500 µl** of sterile distilled water into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 5 minutes at room temperature.
For one ELISPOT plate, 100 µl is thoroughly mixed with 10 ml Dilution buffer R (1x).

### 6. AEC coloring system

The AEC coloring system consists of two items: a concentrated AEC stock solution and a substrate buffer capsule.
To prepare AEC substrate solution, dissolve the contents of one capsule in 57 ml water. After complete dissolution, 43 ml 70% ethanol is added to reach a final concentration of 30% ethanol. 10 ml of this solution is thoroughly mixed with 660 µl AEC stock solution (toxic, use a fume hood). After mixing, the solution should be clear. This amount is sufficient for one ELISPOT plate and should be used within 30 minutes after preparation.
**Do not bring AEC stock solution into contact with polystyrene pipettes and vials.**

### 7. PBS Wash Buffer

5.4 mM $Na_2HPO_4$-H2O, 1.3 mM $KH_2PO_4$, 150 mM NaCl, pH 7.4 sterile
For one ELISPOT plate, prepare 1 liter PBS, add 0.5 ml Tween-20, mix thoroughly.

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## ELISPOT method

Be sure to read the 'Preparation of reagents' and 'Directions for washing' before carrying out the assay.

***Note: Use ELISPOT plates and reagents under aseptic conditions (e.g. Laminar Flow Hood) for steps 1 to 6.***

1. Prewet the PVDF-membranes by adding 25 µl of 70% ethanol to each well. Incubate for 1 minute at room temperature.

2. Aspirate or firmly shake out the ethanol. Immediately thereafter rinse wells 2x with PBS-I. Subsequently empty plates and tap on tissue paper.

3. Add 50 µl of properly diluted coating antibodies to each well. Cover the plate with a lid and incubate overnight at 4°C.

4. Decant solution from wells. Wash wells 3x with 200 µl PBS-I/well. Add 200 µl Blocking Buffer R (1x) to each well. The plate is covered with a lid and incubated for at least 1 hour at room temperature. During this incubation start preparing cell samples.

5. Decant solution from wells. Do not wash the wells. Dilute the cells in Culture medium containing an appropriate stimulus (polyclonal stimulus or antigen). Add cells to the wells of the ELISPOT plate. Add 100 µl/well.

   Triplicates of 3 x $10^6$ cells/ml are often used to assess antigen-specific responses. For polyclonal stimuli, the cell number may have to be reduced to $\pm 10^4$ cells/ml. No more than 3 x $10^5$ cells/well should be suspended in the ELISPOT plate.

6. Cover the ELISPOT plate with a lid and incubate at 37°C, 5% $CO_2$, and 100% humidity. The incubation time can vary from 72 to 24 hours. Specific activation conditions will vary, depending on cell type, cytokine of interest, kinetics of cytokine release and whether a pre-incubation step was included in the procedure .

   *Please note: Do not disturb the plate during incubation. After step 7, aseptic conditions are no longer needed.*

7. Remove the bulk of cells with a firm shake out action and wash 2x with PBS-I at 200 µl/well. Thereafter wells are washed 5x with 250 µl Wash Buffer/well .

8. Discard Wash buffer and add 100 µl of properly diluted biotinylated detection antibodies to each well. Seal the plate with an adhesive cover slip and incubate 2 hours at room temperature or overnight at 4°C.

   *From this step on, it is critical to wash/rinse both sides of the PVDF membrane*

9. Decant solution from wells. Remove and discard the underdrain from the bottom of the plate and wash both sides of the PVDF membrane 5x with Wash Buffer. Add 100 µl of properly diluted Streptavidin-HRP conjugate solution into each well. Seal the plate with an adhesive cover slip and incubate 1 hour at room temperature.

10. Decant solution from wells. Wash both sides of the membrane 5x with Wash buffer.

11. Add **freshly prepared** AEC substrate solution, 100 µl/well. Cover the plate with a lid and incubate for 30 minutes at room temperature in the dark.

12. Stop color development by thoroughly rinsing both sides of the membrane with demineralized water.

13. Air dry plate at room temperature and count spots by use of a dissecting microscope or an immunospot image analyzer.

    To prevent fading of spots, store the plate at a dry place in the dark.

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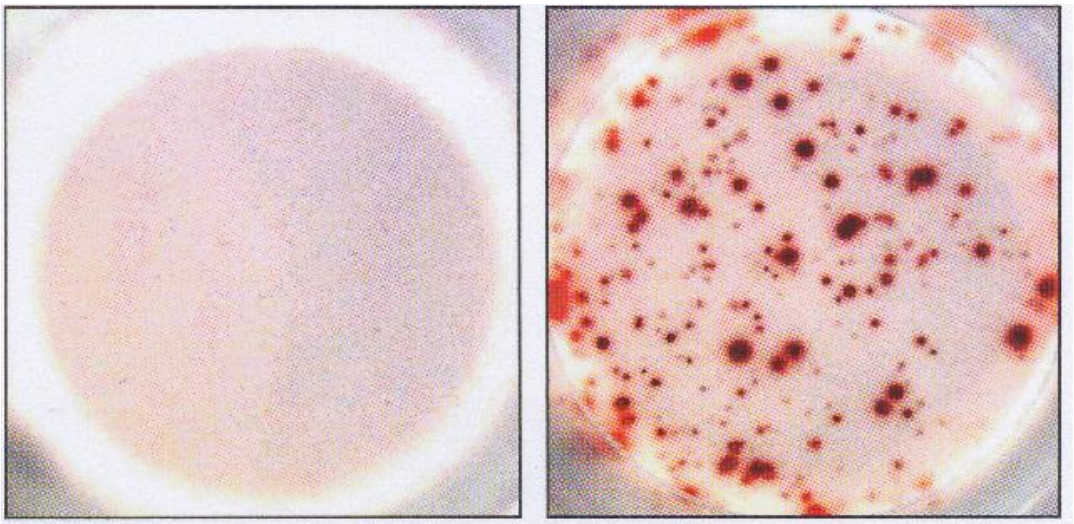
**Typical Data:**



Example of IL-4 specific spots produced by rat (BN) splenocytes. Preincubation: 42h, incubation ELISPOT: 20h. Left: no stimulus, $2x10^5$ cells. Right: Concanavalin A, $2x10^5$ cells.

**NOT FOR HUMAN USE. FOR RESEARCH ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE.**

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