virax biolabs

# ELISpot Human IL-8 Kit

**UNCOATED PLATES**

## Instructions for use

**Catalogue Numbers:**

| | |
|---|---|
| **2126-01** | Uncoated 96-well plate – 1 plate |
| **2126-05** | Uncoated 96-well plates – 5 plates |

**Note:** This protocol (version 1.1) is given as a general procedure to assist when using **Virax Biolabs** Capture and Detection antibodies for ELISpot testing. Optimal dilutions of all reagents, samples and controls as well as the incubation times should be determined by each laboratory for every application.

**For research use only**

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## INTENDED USE

The Human IL-8 (CXCL8) Enzyme-Linked ImmunoSpot (ELISpot) assay is a highly sensitive method used to detect and quantify individual cells that secrete specific cytokines or other soluble molecules. This assay is utilized in immunology to monitor cellular immune responses at the single-cell level and reliably detects and measures human IL-8 (CXCL8) secretion by stimulated effector cells.

## INTRODUCTION

### Description

Interleukin-8 (IL-8 / CXCL8) is a pro-inflammatory chemokine that plays a key role in the recruitment and activation of immune cells, particularly neutrophils. It is secreted by multiple cell types, including monocytes, macrophages, epithelial cells, and endothelial cells, in response to inflammatory stimuli or microbial products. IL-8 is best known for directing neutrophil chemotaxis to sites of infection or tissue injury and enhancing their activation and degranulation. In addition to its role in innate immunity, IL-8 can also act on subsets of T cells, particularly memory and activated T cells that express CXCR1 or CXCR2 promoting their migration into inflamed tissues and contributing to the shaping of local immune responses. Due to its role in immune cell recruitment, inflammation, and tissue remodelling, IL-8 is widely used as a biomarker in studies of infection, inflammation, and tumour immunology.

**This kit is for research use only and is not to be used in diagnostic procedures.**

## STORAGE

Product is shipped at ambient temperature. Store product contents; antibodies, enzyme conjugate, substrate and plates at 2-8°C upon receipt. Expiry of the kit and reagents is stated on the box label. Required storage conditions should be maintained to ensure the stated expiry of the kit components.
Return reagents immediately to 2-8°C after use. Handle reagents under sterile conditions. Any contamination of the reagents or wrong storage conditions will invalidate the shelf life of the product.

## CONTENTS

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| **Plates** | 96 well uncoated ELISpot plate(s) / strips |
| **Capture mAb** | IL-8 capture antibody |
| **Detection mAb** | Biotinylated IL-8 antibody |
| **Enzyme conjugate** | Streptavidin-ALP (Alkaline Phosphatase) |
| **Substrate** | BCIP/NBT Ready-to-use solution |
| **Blocking agent** | Bovine Serum Albumin (BSA) |

## REAGENTS AND MATERIALS REQUIRED BUT NOT SUPPLIED

- Various sterile laboratory consumables
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, (FCS))
- Cell stimulation reagents (e.g. PMA, Ionomycin, peptide pool, CD3, CD28)
- $CO_2$ incubator
- Phosphate Buffered Saline (PBS) / Dulbecco's phosphate buffer saline (DPBS)

## REAGENTS PREPARATION

### a) Blocking Buffer

Cell culture medium containing 10% FCS or serum-free medium containing 10% BSA. Use same cell culture medium as used for the cell suspension.

### b) Dilution Buffer

Add 1g of BSA into 100mL of sterile PBS/DPBS to prepare 1% BSA solution. To ensure buffer sterility, it is recommended to filter 1% BSA solution through a 0.2µm filter.

### c) Capture Antibody

⚠ **Prepare this reagent immediately prior to use.**

Dilute the capture mAb to 15µg/mL in sterile PBS and mix well.

### d) Detection Antibody

⚠ **Prepare this reagent immediately prior to use.**

For one plate, dilute 100µL of antibody into 10mL of Dilution Buffer and mix well. It is recommended to filter the detection antibody through a 0.2µm. Any unused stock antibody can be kept at -20°C for long term. It is recommended to aliquot the reagent prior to storage.

### e) Streptavidin-Alkaline Phosphatase (ALP) conjugate

⚠ **Prepare this reagent immediately prior to use.**

For one plate, dilute 10µL of Streptavidin-ALP conjugate into 10mL of Dilution Buffer and mix well. It is recommended to filter the streptavidin-ALP conjugate through a 0.2µm filter to reduce the nonspecific background.

### f) BCIP/NBT

Normal appearance of this ready-to-use reagent is clear to pale yellow. Crystalline precipitate can occur in the product sometimes. In this case, filter the reagent through a 0.2µm filter. Discard if solution is turbid or purple.

## ELISPOT PROTOCOL

### a) Plate Activation and Blocking

⚠ **Work under sterile conditions.**

1. Remove plate from its sealed package.
2. To activate the plate, add 15µL 35% ethanol (EtOH) per well and incubate for maximum 1 minute.
   Note: MSIPS4W10 (white plates), MSIPS4510 (clear plates) and M8IPS4510 (strip plates) all require this activation step.
3. Wash the plate 5 times with sterile PBS, 200µL per well.

4. Add diluted capture antibody solution, 100µL per well.
5. Incubate overnight at 2-8°C.
6. Following the overnight incubation; wash plate 5 times with 200µL per well sterile PBS to remove the excess capture antibody.
7. Add 100µL per well of blocking buffer.
8. Cover the plate and incubate at room temperature for at least 30 minutes.

Do not tap the plate. Empty the wells by flicking.

## b) Cell Incubation

⚠ **Work under sterile conditions.**

At this step, either frozen or freshly isolated cells can be used. While working with frozen samples, it is suggested that to leave thawed cells at 37°C for at least one hour in order to remove cell debris.

1. Remove blocking buffer.
2. Pipette 50µL of stimulant and 50µL of cell suspension per well. Preferably stimulant and cell suspension can be mixed prior to plating. Cell suspension should not be added first.
3. Place the plate in a $CO_2$ and temperature controlled humidified incubator (37°C and 5% $CO_2$) with lid on.
4. Incubate the plate for desire length of time depending on the stimulant and cell type i.e. 18-48 hours.

At this step, plate can also be wrapped using aluminium foil to prevent evaporation and allow equal heat distribution. Do not stack plates in order to maintain continuous and equal flow. Do not move plate during incubation to avoid cytokine "trailing" or streaks and ensure accurate spot formation.

## c) Detection Antibody

1. Remove cells and wash the plate 5 times with 200µL of PBS, per well.
2. Pipette 100µL per well of detection antibody to every well.
3. Cover the plate and incubate at room temperature for 1 hour 30 minutes.
4. Empty the wells and wash the plate 5 times with 200µL of PBS, per well.
5. Pipette 100µL of diluted streptavidin-ALP conjugate to every well.
6. Cover the plate and incubate at room temperature for 1 hour.
7. Empty the wells and wash the plate 5 times with 200µL of PBS, per well.
8. Add 100µl of ready-to-use BCIP/NBT substrate per well and incubate in the dark.
9. Observe coloured spot development until distinct spots form.
10. To stop the spot development, wash the wells thoroughly with running water.
11. Tap dry the plate on lint free absorbent paper.

12. Air-dry the plate at room temperature then read the wells in an ELISpot reader.

⚠ **Make sure wells are completely dry before spot counting.**
**Wet membrane increases background noise and interferes with spot detection and clarity.**

Store plate in the dark at room temperature.

## USEFUL INFORMATION

### a) Plate Handling and Washing

ELISpot membrane is delicate. Handle the plate with extra care during pipetting and washing as the PVDF membrane may easily be damaged, i.e. puncture of the membrane.

It is also important not to allow membrane to dry for the duration of the assay.

Automated plate washers or squirt bottles with a wide spout can be used for the washing steps that do not require sterile conditions. However, vigorous dispensing for both manual and automated washing should be avoided to prevent damaging the PVDF membrane and dislodging antibodies from the well surface as this could lead to inaccurate results and poor reproducibility.

Wash efficiency can be improved by allowing wash buffer to sit in the wells for a short time, i.e. 1 minute.

Following the detection antibody incubation (Section c, step 4), removing the underdrain and washing both sides of the membrane can help to reduce any background signal.

### b) Buffer

To ensure sterile conditions during washing and blocking steps, use sterilized PBS or filter through a 0.2µm filter to avoid background.

### c) Serum

FCS is recommended to use for conditioning the wells. Prepare 10% FCS in cell culture medium for cell blocking. However, some batches of FCS may non-specifically activate the cells and can therefore be the cause of non-specific background. Test different batches of FCS to assess for low background reactivity and high antigen-specific responses before use.

Non-human serum (i.e. primate or rodent serum) can be used as alternative for FCS.

### d) Sample

Fresh or frozen PMBCs can be used. When working with frozen cells, it is recommended to rest the cells at 37°C for at least an hour to remove the cell debris.

It is recommended to use between 150,000 and 250,000 viable cells per well for the assay. However, cell number should be optimised while using polyclonal activators as higher cell concentration can lead to confluent spot formation and overlapping spots.

### e) Stimuli

Samples can be stimulated using the antigen of interest, i.e. infected cells, vaccine, virus, and peptide pool. If the cell type and/or cytokine/protein of interest requires longer incubation, then a pre-incubation step can be added, however, prolonged cell incubation can lead to excessive cytokine/protein secretion. This will cause the larger spots to start merging and become indistinguishable. Therefore, incubation time and the amount of stimulant should be optimized depending on the cell type and antigen of interest.

### f) Assay Controls

For positive control wells, anti-CD3/CD28 antibodies, phytohemagglutinin (PHA), and concanavalin A (ConA) are the commonly used T cell activators. Negative control wells should contain only unstimulated cells and cell culture medium. However, if anti-CD28 is used as a stimulant in the other wells, it should also be added to the negative control to help identify if the response is solely due to the stimulant, i.e. peptide pool.

### g) Detection Antibody and Streptavidin-ALP Conjugate

It is recommended to filter the detection antibody and the conjugate through a 0.2µm filter to reduce background noise.

### h) Spot Development

It is recommended to allow 5-30 minutes to develop spots. However, it is crucial to optimise spot development time as overdevelopment leads to increased background signal. Wash wells extensively under running water to stop spot development.

virax biolabs

BioCity Glasgow,
Bo'Ness Road, Newhouse,
Lanarkshire ML1 5UH
United Kingdom

www.viraxbiolabs.com
support@viraxbiolabs.com