# ELISpot Flex: Bovine IgG (ALP)

Product Code: 3150-2A

**CONTENTS:**

**Vial 1 (green top)**
Capture mAb: MT134 (800 µl)
Concentration: 0.5 mg/ml

**Vial 2 (yellow top)**
Detection mAb: MT391, biotin (40 µl)
Concentration: 0.5 mg/ml

**Vial 3 (white top)**
Streptavidin-ALP (50 µl)

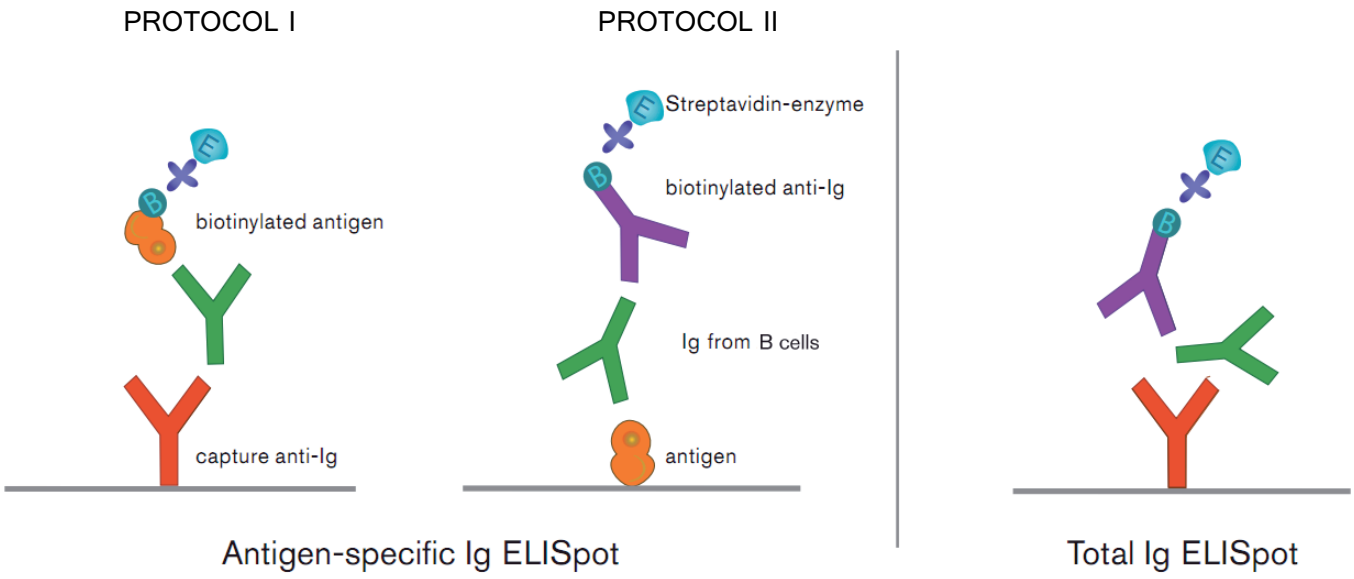
**Vial 4 (black top)**
Polyclonal activator: R848 (100 µl)
Concentration: 1 mg/ml

Antibodies are supplied in sterile filtered (0.2 µm) PBS with 0.02% sodium azide. Streptavidin-ALP is supplied in 0.1 M Tris buffer with 0.002% Kathon CG. R848 is supplied in sterile filtered (0.2 µm) PBS containing 1% DMSO. Vials have been overfilled to ensure recovery of the specified amount.

**STORAGE:**

Shipped at ambient temperature. On arrival antibodies and Streptavidin-ALP should be stored refrigerated at 4-8°C. R848 should be stored at -20°C. The expiry date indicates how long unopened products, stored according to instructions, are recommended for use.

# Schematic illustration of the different protocols



# Kit description

**Please read through before starting the assay**

This kit contains reagents for detection and enumeration of B cells secreting IgG antibodies. B cells secreting antigen-specific IgG as well as B cells secreting IgG irrespective of antigen specificity (total IgG) can be measured.

**Detection of antigen-specific B cells**

Detection of antigen-specific IgG-secreting B cells, visualised as spots, can be made using either of two protocols (Protocol I or II). The choice of protocol will depend on several factors such as the available amount and molecular nature of the antigen. The use of biotinylated antigen (Protocol I) may be more sensitive and result in spots of higher quality. In addition, the antigen is used in smaller quantities and is not present during cultivation.

**Protocol I:** Please note that antigen must be biotinylated prior to starting the experiment. IgG from all secreting B cells are captured by anti-IgG antibodies coated on the ELISpot plate. The secretion of specific IgG is then visualised by the addition of biotinylated antigen followed by Streptavidin-enzyme and substrate.

**Protocol II:** In the conventional way of performing B cell ELISpot, the antigen is coated on the ELISpot plate and antigen-specific IgG secreted by the B cells bind to the immobilised antigen. Spots are detected after addition of biotinylated anti-IgG followed by Streptavidin-enzyme and substrate.

**Detection of total IgG-producing B cells**

Both protocols allow determination of the total number of IgG-secreting B cells, used as a positive control or for establishing the proportion of antigen-specific B cells.

# Hints and comments

**Please read through before starting the assay**

These suggestions are based on the detection of immune responses using PBMC. If using B-cell clones, mixtures of separated cell fractions etc., other protocols may have to be considered.

**Plates**

We recommend the use of PVDF-based membrane plates. Maximal antibody binding capacity of these plates is obtained by a brief treatment with ethanol. It is essential that the membrane is not allowed to dry after the treatment. If this occurs the treatment step (A2-A3) needs to be repeated before adding the coating antibody.

**Plate washing**

Washing of plates can be done using a multi-channel micropipette. In washing steps not requiring sterile conditions (C1-C5), a regular ELISA plate washer can also be used, provided that the washing head is adapted to the ELISpot plates. Avoid getting liquid on the underside of the membrane as this may cause leakage due to capillary drainage. If using MAIPSWU plates, always remove the plate from the plate tray before manually emptying the plate.

**Cells**

Both fresh and cryopreserved cells may be used with good results. However it is recommended that the latter are rested for at least one hour to allow removal of cell debris before addition to the plate. As a guideline, 100,000-500,000 cells per well may be used to assess antigen-specific responses. For detection of total IgG, less cells (e.g. 25,000-50,000 cells per well) may be used to avoid confluent spot formation. It is recommended to set up samples in triplicates or duplicates.

***In vivo* activated B cells**

B cells that have been activated *in vivo*, for instance as a result of vaccination, may be analysed directly in the ELISpot wells without prior stimulation. The time-point for detection of cells secreting antigen-specific antibodies after immunization needs to be established by the user.

**Memory B cells - *in vitro* activation**

Memory B cells may require polyclonal stimulation before secreting detectable amounts of antibody. Pre-stimulation can be made with R848 at 1 µg/ml in tubes for 48-72 hours. After pre-stimulation, wash the cells to ensure removal of any secreted antibodies. Resuspend the cells in medium and add the cell suspension to the ELISpot plate.

**Serum**
The assay requires use of serum-free cell culture medium, e.g. AIM-V, since IgG present in bovine serum will interfere with the assay.

**Buffers**
PBS for washing and dilution should be filtered (0.2 µm) for optimal results. Although possible to use, we do not recommend the inclusion of Tween or other detergents in the washing and incubation buffers.

**Substrate development**
Develop until distinct spots are visible in positive wells (usually 10-30 minutes). A general darkening of the membrane may occur but disappears after drying.

**Biotinylation of antigen**
If biotinylated antigen will be used for detection, (Protocol I), this should be labeled before starting the ELISpot experiment. A suitable reagent for biotinylation is e.g. Sulfo-NHS-LC-Biotin (Pierce product code 21335). Further information on biotin and biotin conjugation may be found at www.piercenet.com

-The protein antigen to be biotinylated with Sulfo-NHS-LC-Biotin should be in a suitable amine-free buffer (e.g. PBS, pH 7.4). If solution contains sodium azide, this should first be removed e.g. by dialysis. For small volumes (< 200 µl) a MINI-dialysis unit Slide-A-Lyser (10.000 MWCO, Pierce) can be used. The same dialysis unit can be used for removal of free biotin (see below). Conjugation is normally performed using a 20- to 50-fold molar excess of biotin to protein where the higher ratio is for protein at low concentration.

-The Sulfo-NHS-LC-Biotin should be dissolved in pure $H_2O$ and immediately added to the antigen solution. After incubation for 30 minutes at room temperature, free biotin may be removed by dialysis against PBS or other buffer.

The concentration of the biotinylated antigen in the assay is typically 0.01-1 µg/ml but depends on the size of the antigen and the degree of biotinylation and should be established separately.

# Protocol I

**(using biotinylated antigen)**

## A Preparation of ELISpot plate (sterile conditions)

1. Dilute the coating mAb MT134 to 10 µg/ml in sterile PBS, pH 7.4.
2. PVDF plates need to be treated (activated) with ethanol before coating. PVDF plates from Millipore, type MAIPSWU should be treated with 50 µl 70% ethanol/well for maximum 2 minutes. PVDF plates, type MSIP should be treated with 15 µl 35% ethanol/well for maximum 1 minute. After ethanol treatment, immediately proceed to plate washing.
3. Wash plate 5 times with sterile water, 200 µl/well.
4. Add 100 µl/well of the antibody solution and incubate overnight at 4-8°C.

## B Incubation of cells in plate (sterile conditions)

**See Hints and Comments section for details on cell stimulation.**

1. Wash plate 5 times with sterile PBS, 200 µl/well, to remove excess antibody.
2. For analysis of *in vivo* activated B cells, add the cell suspension to the ELISpot plate. For analysis of memory B cells, the cells can be pre-activated separately in tubes prior to addition to the plates.
3. Put the plate in a 37°C humidified incubator with 5% $CO_2$ and incubate for 16-24 hours. Do not move the plate during this time and take measures to avoid evaporation (e.g. by wrapping the plate in aluminium foil).

## C Detection of spots

1. To remove the cells, empty the plate and wash 5 times with PBS, 200 µl/well.

2a. **Antigen-specific IgG**
Dilute biotinylated antigen to a suitable concentration (0.01-1 µg/ml) in PBS. Add 100 µl/well and incubate for 2 hours at room temperature.

2b. **Total IgG**
Dilute the detection mAb MT391-biotin to 0.1 µg/ml in PBS. Add 100 µl/well and incubate for 2 hours at room temperature.

3. Wash plate as above (step C1).
4. Dilute the Streptavidin-ALP (1:1000) in PBS and add 100 µl/well. Incubate for 1 hour at room temperature.
5. Wash plate as above (step C1).
6. Add 100 µl/well of substrate solution (e.g. BCIP/NBT) and develop until distinct spots emerge.
7. Stop colour development by washing extensively in tap water. If desirable, remove the plate from the plate tray or the underdrain and rinse the underside of the membrane.
8. Leave the plate to dry. Inspect and count spots in an ELISpot reader or in a dissection microscope.
9. Store plate in the dark at room temperature.

# Protocol II

**(using coated antigen)**

## A Preparation of ELISpot plate (sterile conditions)

1a. **Antigen-specific IgG**
Dilute the antigen to suitable concentration (1-50 µg/ml in sterile PBS, pH 7.4).

1b. **Total IgG**
Dilute the coating mAb MT134 to 10 µg/ml in sterile PBS, pH 7.4

2. PVDF plates need to be treated (activated) with ethanol before coating. PVDF plates from Millipore, type MAIPSWU should be treated with 50 µl 70% ethanol/well for maximum 2 minutes. PVDF plates, type MSIP should be treated with 15 µl 35% ethanol/well for maximum 1 minute. After ethanol treatment, immediately proceed to plate washing.
3. Wash plate 5 times with sterile water, 200 µl/well.
4. Add 100 µl/well of the antigen or antibody solution and incubate overnight at 4-8°C.

## B Incubation of cells in plate (sterile conditions)

**See Hints and Comments section for details on cell stimulation.**

1. Wash plate 5 times with sterile PBS, 200 µl/well, to remove excess antibody
2. For analysis of *in vivo* activated B cells, add the cell suspension to the ELISpot plate. For analysis of memory B cells, the cells can be pre-activated separately in tubes prior to addition to the plates.
3. Put the plate in a 37°C humidified incubator with 5% $CO_2$ and incubate for 16-24 hours. Do not move the plate during this time and take measures to avoid evaporation (e.g. by wrapping the plate in aluminium foil).

## C Detection of spots

1. To remove the cells, empty the plate and wash 5 times with PBS, 200 µl/well.
2. Dilute the detection mAb MT391-biotin to 0.1 µg/ml in PBS. Add 100 µl/well and incubate for 2 hours at room temperature.
3. Wash plate as above (step C1).
4. Dilute the Streptavidin-ALP (1:1000) in PBS and add 100 µl/well. Incubate for 1 hour at room temperature.
5. Wash plate as above (step C1).
6. Add 100 µl/well of substrate solution (e.g. BCIP/NBT) and develop until distinct spots emerge.
7. Stop colour development by washing extensively in tap water. If desirable, remove the plate from the plate tray or the underdrain and rinse the underside of the membrane.
8. Leave the plate to dry. Inspect and count spots in an ELISpot reader or in a dissection microscope.
9. Store plate in the dark at room temperature.

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CERTIFIED ISO 9001 Quality management systems

CERTIFIED ISO 13485 Medical devices Quality management systems

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