Contents, kit for 4 plates:

Vial 1 (green top)
Monoclonal antibody 107 (600 μl)
Concentration: 1 mg/ml

Vial 2 (purple top)
Biotinylated monoclonal antibodies 107/182/101 (50 μl)
Concentration: 1 mg/ml

Vial 3 (white top)
Streptavidin-Alkaline Phosphatase (50 μl)

Activators
Anti-CD40 monoclonal antibody S2C6 (80 μl)
Concentration: 1 mg/ml

Lyophilized recombinant human IL-4 (1 μg)

Storage:
Shipped at ambient temperature. On arrival antibodies and conjugate should be stored at 4-8 °C. Human IL-4 should be stored frozen at -20 °C. The expiry date indicates how long unopened products, stored according to instructions, are recommended for use. Antibodies are supplied in sterile filtered PBS with 0.02% sodium azide, except mAb S2C6 that is supplied without azide. Streptavidin-ALP is supplied in 0.1 M Tris buffer with 0.002% Kathon CG. Vials have been overfilled to ensure recovery of stated quantity.
A **Preparation of ELISpot plate (sterile conditions)**
1. Dilute the coating antibody (107) to 15 μg/ml in sterile PBS, pH 7.4.
2. Remove the ELISpot plate from the package and if using a PVDF plate, pre-wet the membrane by adding ethanol. PVDF-plates from Millipore Corp., MAIPSWU, should be treated with 50 μl 70% ethanol per well for 2 minutes. PVDF-plates, type MSIP, should be treated with 15 μl 35% ethanol per well for maximum 1 minute.
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)**
1. Remove excess antibody and wash plate 5 times with sterile PBS, 200 μl/well.
2. Add 200 μl/well of medium containing 10% of the same serum as used for the cell suspensions. Incubate for at least 30 minutes at room temperature.
3. Remove the medium and add the cell suspension. The included anti-CD40 mAb and recombinant IL-4 can be used to induce IgE secretion (see Hints and comments).
4. Put the plate in a 37°C humidified incubator with 5% CO₂ 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. Remove the cells by emptying the plate and wash 5 times with PBS, 200 μl/well.
2. Dilute the detection antibodies (107/182/101-biotin) to 1 μg/ml in PBS containing 0.5% fetal calf serum (PBS-0.5% FCS). Add 100 μl/well and incubate for 2 hours at room temperature.
3. Wash as above (step C1).
4. Dilute the Streptavidin-ALP (1:1000) in PBS-0.5% FCS and add 100 μl/well. Incubate for 1 hour at room temperature.
5. Wash 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 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.
Hints and comments

These suggestions are based on the detection of immune responses using PBMC. If using cell clones, separated cell fractions etc., the protocol may have to be modified. The assay can be used for study of in vivo-induced IgE-secreting cells as well as for study of IgE secretion induced by anti-CD40 mAb and IL-4. Please note that stimulation with anti-CD40/IL-4 induces switching to IgE and IgG1 and results with stimulated cells may therefore not be representative of the situation in vivo.

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 (A2-3) needs to be repeated before adding the coating antibody.

Plate washing
Always remove the plate MAIPSWU from the plate tray before manually emptying the plate. 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 plate leakage.

Cells
IgE secretion can be induced by pre-activation of cells with anti-CD40 mAb and IL-4. Stimulate cells with a mixture of mAb S2C6 (1 μg/ml) and IL-4 (10 ng/ml) in tubes for approx. 5 days. After pre-activation, wash the cells to remove secreted antibodies. Freshly prepared and cryopreserved cells may be used. It is recommended that the latter are rested for at least one hour to allow for the removal of cell debris. Triplicates with 100,000-300,000 cells/well are recommended but the cell number may need to be modified depending on the expected spot frequencies. Other incubation times have to be established by the user. * Reconstitute IL-4 with 1 ml PBS to obtain 1 μg/ml. Leave for 15 min and then vortex. Use directly or store in aliquots at -20°C.

Serum
The serum should be selected to support cell culture and give low background staining. We recommend the use of fetal calf serum. Alternatively serum-free medium evaluated for cell culture can be used. Human serum is not recommended as it may contain heterophilic antibodies or intrinsic analyte which may interfere with the assay.

Detection antibody
To reduce unspecific background it is recommended to filter (0.2 μm) the working dilution of detection mAb.

Assay controls
The number of cells responding to stimulation is often compared to the number of cells spontaneously producing IgE, determined by incubating the same number of cells in the absence of stimuli. Polyclonal activators, such as anti-CD40 mAb and IL-4, can be used as control stimuli for assessment of cell viability and functionality of the ELISpot assay. See above.

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.
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