Human IgA ELISpot Antibody Pair Kit

For detection and enumeration of cells secreting human immunoglobulin A

Catalog #100-2272 1 Kit

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Product Description

Human Immunoglobulin A (IgA) ELISpot Antibody Pair Kit is intended for those who want the flexibility of setting up their own ELISpot assay. This kit includes capture and detection antibodies, streptavidin-alkaline phosphatase (SA-ALP) conjugate, and polyclonal B cell stimuli. It is designed for the detection and enumeration of cells secreting human IgA in a suspension of peripheral blood mononuclear cells (PBMCs). ELISpot is commonly used for evaluating immune responses, such as determining the frequency and isotype of circulating antigen-specific memory B cells or antibody-secreting cells (ASCs). Soluble IgA is secreted by IgA+ ASCs and plays an important role in neutralizing invading pathogens, particularly at mucosal surfaces. ELISpot is highly sensitive and can detect secreted analytes at a single-cell level. This assay is used in research of infectious diseases and vaccines.

This assay can be used for quantifying antigen-specific IgA-secreting cells and/or total IgA-secreting cells, depending on how the assay is performed. The assay is based on a modified version of the sandwich ELISA method, in which cells are added to ELISpot plates pre-coated with either capture antibodies specific for human IgA (**Protocol 1**) or an antigen of choice (**Protocol 2**). Secreted IgA is then captured throughout the culture period before the cells are washed away. Captured IgA is detected by the addition of a biotinylated antigen or detection antibody, followed by SA-ALP which binds to the biotinylated antigen or antibody. Addition of the chromogenic enzyme substrate, 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP)/nitroblue tetrazolium (NBT), results in the formation of colored spots. The number, size, and intensity of these spots are directly proportional to the frequency of antigen-specific or total IgA-secreting cells in the sample and the amount of immunoglobulin secreted.

NOTE: This kit includes sufficient reagents for 4 x 96-well ELISpot plates. BCIP/NBT substrate (e.g. Catalog #200-1016) and ELISpot plates (e.g. Millipore Sigma Catalog #MSIPS4W10/MAIPSWU10) are required for use with this kit and are available for purchase separately. For detecting antigen-specific immune responses, an antigen of choice is also required.

This kit is supplied in two boxes (Catalog #200-1012/200-1013).

Product Information

All components listed below are stable until expiry date (EXP) on label. Components may be shipped at room temperature (15 - 25°C) but should be stored as indicated upon receipt.

COMPONENT NAME	SIZE	STORAGE	DESCRIPTION
Anti-human IgA mAb (MT20), biotin	100 µL	Store at 2 - 8°C.	Biotinylated anti-human IgA antibody (0.5 mg/mL); detection antibody
Anti-human IgA mAb (MT57), biotin	100 µL	Store at 2 - 8°C.	Biotinylated anti-human IgA antibody (0.5 mg/mL); detection antibody
Anti-human IgA mAb (MT57), unconjugated	1200 µL	Store at 2 - 8°C.	Anti-human IgA antibody (0.5 mg/mL); capture antibody
R848, Activator	100 µL	Store at -20°C.	R848 (1 mg/mL); positive control stimulus for IgA secretion
Recombinant human IL-2, lyophilized	1 µg	Store at -20°C.	Lyophilized recombinant human IL-2; positive control stimulus for IgA secretion
Streptavidin-ALP for ELISpot	50 µL	Store at 2 - 8°C.	SA-ALP conjugate

Materials Required but Not Included

- Absorbent paper
- Aluminum foil (optional)
- BCIP/NBT Substrate (e.g. Catalog #200-1016)
- Biohazard safety cabinet certified for Level II handling of biological materials
- ELISpot plates with PVDF membrane (e.g. Millipore Sigma Catalog #MSIPS4W10/MAIPSWU10)

- ELISpot reader (e.g. Mabtech IRIS™ 2/ASTOR™ 2) or dissection microscope
- Ethanol solution, 35% or 70%
- Incubator, 37°C
- Multi-channel pipettor (e.g. Catalog #38110) with appropriate pipette tips or automatic plate washer adapted for ELISpot plates for non-sterile steps of washing
- Phosphate-buffered saline (PBS) containing 0.5% fetal bovine serum (FBS; PBS + 0.5% FBS)
- PBS, sterile and filtered (0.2 µm), OR Dulbecco's phosphate-buffered saline (D-PBS; Without Ca++ and Mg++), pH 7.4 (Catalog #37350)
- Pipettor (e.g. Catalog #38059/38060) with appropriate pipette tips (e.g. Catalog #38032/38033)
- Reagent reservoir (e.g. Catalog #38080)
- Sterile water
- Syringe and syringe filter (0.45 µm and 0.2 µm, e.g. Catalog #200-0583)
- Timer
- Tubes for preparing dilutions (e.g. Catalog #100-0092/200-0521)

Preparation of Reagents and Materials

A. Recombinant Human IL-2, Lyophilized

Centrifuge vial of Recombinant human IL-2, lyophilized before opening. Reconstitute in 1 mL sterile PBS (final concentration of 1 µg/mL) by pipetting the solution down the sides of the vial. Let it sit for 15 minutes, then vortex.

NOTE: After reconstitution, use immediately, or aliquot and store at -20 to -80°C. Avoid repeated freeze-thaw cycles.

B. Samples and Cell Culture Medium

Fresh or cryopreserved PBMCs may be used in the assay. If using cryopreserved cells, it is recommended to incubate cells in culture medium at 37°C for ≥ 1 hour to return the cells to their resting state before beginning the experiment. For optimal results, remove any cell debris by allowing it to settle and carefully transfer the cell suspension to a new tube or filter the cell suspension through a cell strainer (e.g. Catalog #27215).

NOTE: For ELISpot assay, the suggested seeding density for detecting antigen-specific responses is 1 - 5 x 10^5 cells/well. The suggested seeding density for detecting total IgA-secreting cells is ~5 - 7.5 x 10^4 cells/well. Cell density may be reduced to prevent confluent spot formation. Duplicate or triplicate wells for all samples are recommended.

CELL CULTURE MEDIUM

Complete medium (cell culture medium + serum) should be screened for low background staining. FBS is recommended over human serum which may contain antibodies that can interfere with the assay. Alternatively, serum-free medium may be used.

C. Stimulation

IN VITRO ACTIVATED B CELLS

Memory B cells may require polyclonal stimulation in order to differentiate into IgA+ ASCs. It is suggested to culture PBMCs at ~1 x 10^6 cells/mL in complete medium with the stimuli included in this kit in an appropriate tissue culture-treated plate (e.g. Catalog #38017) or in a tube (e.g. Catalog #38057). Stimulate PBMCs with 1 µg/mL R848, Activator and 10 ng/mL Recombinant human IL-2, lyophilized (see Preparation of Reagents and Materials, section A) to induce IgA secretion by activated B cells. Incubate cells at 37°C, 5% CO_2 for 48 - 72 hours. It is recommended to stimulate at least twice as many cells as needed for the ELISpot assay to account for any cell loss during this process.

Cells should then be collected and thoroughly washed with fresh complete medium to remove the secreted antibodies prior to adding to the ELISpot plate.

IN VIVO ACTIVATED B CELLS

B cells that have been activated into ASCs in vivo (e.g. following vaccination) may be able to be analyzed by ELISpot assay directly, without the stimulation step. Cells secreting antigen-specific antibodies are typically detected in the circulation, 6 - 9 days after vaccination.

D. Assay Controls

It is recommended to also include negative controls (e.g. unstimulated cells or naive donor) in your assay to determine the number of cells spontaneously producing the immunoglobulin of interest. Wells with medium only may also be used to determine the presence of any background staining.

E. Capture Antibody

Dilute Anti-human IgA mAb (MT57), unconjugated 1 in 33.3 in sterile PBS (final concentration of 15 µg/mL).

Example: For one plate, add 300 µL Anti-human IgA mAb (MT57), unconjugated to 10 mL sterile PBS.

F. Detection Antibody

- For detecting **antigen-specific IgA-secreting cells**, use Anti-human IgA mAb (MT57), biotin and follow **Protocol 2** in Direction for Use.
- For detecting **total IgA-secreting cells**, use Anti-human IgA mAb (MT20), biotin. Both **Protocols 1 and 2** in Direction for Use could be followed.

Dilute your Detection Antibody of choice 1 in 500 in PBS + 0.5% FBS (final concentration of 1 µg/mL). Diluted Detection Antibody may be filtered using a 0.2 µm syringe filter to reduce the risk of non-specific background.

Example: For one plate, add 20 µL of your Detection Antibody of choice to 10 mL PBS + 0.5% FBS.

G. SA-ALP

Dilute Streptavidin-ALP for ELISpot 1 in 1000 in PBS + 0.5% FBS.

Example: For one plate, add 10 µL Streptavidin-ALP for ELISpot to 10 mL PBS + 0.5% FBS.

H. BCIP/NBT Substrate Solution

For instructions on how to prepare this substrate solution, refer to the Product Information Sheet (Document #10000033749), available at www.stemcell.com, or contact us to request a copy.

Directions for Use

The following instructions are for detection of immunoglobulin-secreting cells using PBMCs. If using other cell types (e.g. B cell clones or isolated cells), adjust accordingly.

Please read the entire protocol before proceeding. **Sterile technique should be used for steps 1 - 8 of Protocols 1 and 2.**

NOTE: In all washing steps, each well must be washed thoroughly and blotted dry by tapping the plate upside down on absorbent paper to remove any remaining liquid before adding the next reagent. Once the PVDF membrane is wetted, ensure it does not dry out during the assay.

NOTE: Cross-contamination of reagents may invalidate assay results. Permanently labeled, dedicated, multi-channel pipettor reservoirs for reagents are recommended.

- For detecting **antigen-specific IgA-secreting cells**, follow **Protocol 1** if your antigen of choice is biotinylated or follow **Protocol 2** if your antigen of choice is to be used for coating. **Protocol 1** may be preferred due to the smaller quantity of antigen required and potential for higher sensitivity and spot quality.
- For detecting **total IgA-secreting cells**, both protocols follow identical steps but have been included for ease of use when running in parallel to antigen-specific detection.

NOTE: Total IgA-secreting cells may be used as a positive control or for determining the proportion of antigen-specific IgA-secreting cells.

PROTOCOL 1

1. Prepare samples as described in Preparation of Reagents and Materials, sections B and C.
2. Prepare fresh ethanol solution (35% or 70%) using > 99% ethanol and sterile water. Treat the PVDF membrane of your ELISpot plate with the fresh ethanol solution as follows:
   - For MSIPS4W10 plates: add 20 µL/well of 35% ethanol solution. Do not let sit for > 1 minute.
   - For MAIPSWU10 plates: add 50 µL/well of 70% ethanol solution. Do not let sit for > 2 minutes.

   NOTE: If the membrane dries out during this treatment, repeat the process.
3. Wash each well five times with 200 µL sterile water.
4. Add 100 µL/well of diluted Capture Antibody (see Preparation of Reagents and Materials, section E). Cover plate to prevent evaporation and incubate overnight at 2 - 8°C.
5. Wash each well five times with 200 µL sterile PBS.
6. Add 200 µL/well of complete medium (see Preparation of Reagents and Materials, section B) to condition the plate and incubate at room temperature (15 - 25 °C) for at least 30 minutes.
7. Remove the medium and add 100 µL/well of the cell suspension.
8. Incubate at 37°C and 5% CO_2 for 16 - 24 hours. Cover the plate (e.g. by wrapping the plate in aluminum foil) to avoid evaporation.

   NOTE: Do not stack or move plate during incubation.
9. Remove cells and wash each well five times with 200 µL PBS and blot dry.

10. Add biotinylated antigen or antibody of choice as follows and incubate at room temperature for 2 hours.
    - For detecting **antigen-specific IgA-secreting cells**, add 100 µL/well of biotinylated antigen of choice diluted in PBS + 0.5% FBS. Use 0.01 - 1 µg/mL of biotinylated antigen. Titrate the concentration for optimal performance.
    - For detecting **total IgA-secreting cells**, add 100 µL/well of diluted Detection Antibody (Anti-human IgA mAb (MT20), biotin, see Preparation of Reagents and Materials, section F).
11. Wash each well five times with 200 µL PBS and blot dry.
12. Add 100 µL/well of diluted SA-ALP (see Preparation of Reagents and Materials, section G) and incubate at room temperature for 1 hour.
13. Wash each well five times with 200 µL PBS and blot dry.
14. Add 100 µL/well BCIP/NBT Substrate Solution (see Preparation of Reagents and Materials, section H). Develop until distinct spots emerge (approximately 5 - 30 minutes).

    NOTE: Darkening of the membrane may occur during development, but it disappears after drying.
15. Wash the plate thoroughly with tap water to stop color development. If needed, remove the underdrain (i.e. soft plastic under the plate) and rinse the bottom of the membrane.
16. Leave the plate to dry overnight at room temperature.
17. Using an ELISpot reader (recommended) or dissection microscope, inspect and count spots. Each spot represents the footprint of an individual cell.

    NOTE: Measurement of antigen-specific IgA-secreting cells and total IgA-secreting cells are often performed in parallel in order to determine the ratio of antigen-specific cells. Alternatively, when quantifying total IgA-secreting cells following stimulation, this can be compared to the number of cells spontaneously secreting IgA in your unstimulated control.
18. Store the plate at room temperature in the dark.

PROTOCOL 2

1. Prepare samples as described in Preparation of Reagents and Materials, sections B and C.
2. Prepare fresh ethanol solution (35% or 70%) using > 99% ethanol and sterile water. Treat the PVDF membrane of your ELISpot plate with the fresh ethanol solution as follows:
    - For MSIPS4W10 plates: add 20 µL/well of 35% ethanol solution. Do not let sit for > 1 minute.
    - For MAIPSWU10 plates: add 50 µL/well of 70% ethanol solution. Do not let sit for > 2 minutes.

    NOTE: If the membrane dries out during this treatment, repeat the process.
3. Wash each well five times with 200 µL sterile water.
4. Add capture antigen or antibody of choice as follows. Cover plate to prevent evaporation and incubate overnight at 2 - 8°C.
    - For detecting **antigen-specific IgA-secreting cells**, add 100 µL/well of antigen of choice diluted in sterile PBS. Use 1 - 50 µg/mL of antigen for coating. Titrate the concentration for optimal performance.
    - For detecting **total IgA-secreting cells**, add 100 µL/well of diluted Capture Antibody (see Preparation of Reagents and Materials, section E).
5. Wash each well five times with 200 µL sterile PBS.
6. Add 200 µL/well of complete medium (see Preparation of Reagents and Materials, section B) to condition the plate and incubate at room temperature (15 - 25 °C) for at least 30 minutes.
7. Remove the medium and add 100 µL/well of the cell suspension.
8. Incubate at 37°C and 5% CO_2 for 16 - 24 hours. Cover the plate (e.g. by wrapping the plate in aluminum foil) to avoid evaporation.

    NOTE: Do not stack or move plate during incubation.
9. Remove cells and wash each well five times with 200 µL PBS and blot dry.
10. For detecting antigen-specific IgA-secreting cells, add 100 µL/well Anti-human IgA mAb (MT57), biotin. For detecting total IgA-secreting cells, add 100 µL/well Anti-human IgA mAb (MT20), biotin (see Preparation of Reagents and Materials, section F). Incubate at room temperature for 2 hours.
11. Wash each well five times with 200 µL PBS and blot dry.
12. Add 100 µL/well of diluted SA-ALP (see Preparation of Reagents and Materials, section G) and incubate at room temperature for 1 hour.
13. Wash each well five times with 200 µL PBS and blot dry.
14. Add 100 µL/well BCIP/NBT Substrate Solution (see Preparation of Reagents and Materials, section H). Develop until distinct spots emerge (approximately 5 - 30 minutes).

NOTE: Darkening of the membrane may occur during development, but it disappears after drying.

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18. Store the plate at room temperature in the dark.

Troubleshooting

PROBLEM	POSSIBLE CAUSE	RECOMMENDED ACTION
Blank wells	ELISA substrate or incorrect ELISpot substrate used	Ensure BCIP/NBT ELISpot substrate is used.
	Reagents not added in correct sequence or missed from some wells	Follow the sequence of incubation and wash steps as outlined in Directions for Use. Wells may be able to be recovered by repeating Step 9 onward in the Directions for Use.
Too few spots	Low seeding density	Increase seeding density. Follow the suggested seeding density outlined in Preparation of Reagents and Materials, section B.
	Low/no cell viability	Ensure cell viability is ≥ 90% before beginning the assay. Include a positive control to confirm cells are viable and functional.
Too many spots	High seeding density	Reduce seeding density. Follow the suggested seeding density outlined in Preparation of Reagents and Materials, section B.
	Stimulation time too long	Incubation time may need to be optimized depending on the stimulus used. Follow recommended incubation time outlined in Directions for Use.
High spot count in negative control wells	Cells not rested	Ensure cryopreserved cells are rested for ≥ 1 hour before use.
	Non-specific activation	Ensure cell culture medium does not contain any reagents that may stimulate the cells non-specifically.
	Contamination	Start assay with new cells and ensure sterile technique is used.
Spots very faint	Incorrect storage conditions or expired reagents	Ensure reagents are stored at the recommended storage conditions and have not passed their expiry dates.
	Incubation time too short	Follow the directions for the multiple incubation steps outlined in Directions for Use. Incubation time may need to be optimized depending on the stimulus used.
	Incorrect dilution of Detection Antibody or SA-ALP	Follow the directions for dilution of Detection Antibody and SA-ALP outlined in Preparation of Reagents and Materials.
	Spots underdeveloped	Ensure BCIP/NBT Substrate Solution is brought to room temperature before use. The substrate should be left on until distinct spots are visible by eye in the positive control wells (approximately 5 - 30 minutes).
Spots unevenly distributed or smeary	Cells unevenly distributed	It is recommended to first add the stimulus or medium to the wells, then the cell suspension. Ensure the plate is kept in a horizontal position during incubation.
	Plate moved during incubation	Ensure the plate is not disturbed during the incubation period.
	Presence of cell clumps	A cell strainer may be used to remove aggregates from the cell suspension before use.
	PVDF membrane dried out	Work efficiently to ensure that PVDF membrane does not dry out between steps.

PROBLEM	POSSIBLE CAUSE	RECOMMENDED ACTION
	Uneven dispersion of reagents	Gently tap the plate upside down on an absorbent tissue to remove any leftover wash buffer. After the addition of diluted antibody, ensure the solution covers the bottom of the well. Avoid bubble formation.
Poor replicates	Unequal volumes in wells	Ensure that pipettes function properly.
	Unequal cell numbers between wells	Ensure cell suspensions are thoroughly mixed before adding to each well.
	Carry-over between wells	Ensure pipette tips are changed between wells.
Dark membrane	Inappropriate serum selection	Use FBS or serum-free medium. FBS should be screened to ensure low background staining. Avoid using human serum as this may contain heterophilic antibodies or secreted analytes that can interfere with the assay.
	Presence of detergents	Avoid including Tween or other detergents in washing and incubation buffers.
	Presence of dimethyl sulfoxide (DMSO)	Ensure final DMSO concentration (if used) is < 0.5%.
	Presence of previously secreted analyte	Wash cells thoroughly and resuspend in fresh culture medium just before plating.
	Insufficient washing	Ensure that each well is washed five times with 200 µL PBS. Ensure that multi-channel pipettor fills and empties reproducibly without touching the reagents on the plate. Blot dry wells before proceeding to the next step. Increase cycles of washes and soaking time between washes.
	Overdevelopment	BCIP/NBT Substrate Solution should be left on until distinct spots are visible by eye in the positive control wells (approximately 5 - 30 minutes). Monitor the wells closely during this time.
	PVDF membrane still wet	Ensure the membrane is completely dry before imaging.
Assay artifacts	Presence of cell debris	Ensure cryopreserved cells are rested before use, debris is removed, and cell viability is ≥ 90%.
	Precipitate present in assay reagents or buffers	Ensure BCIP/NBT Substrate Solution is filtered (0.45 µm) before use. PBS and diluted Detection Antibody may also be filtered (0.2 µm) for optimal results.
	Membrane damaged by pipette tips	Ensure pipette tips do not touch the bottom of the wells.
	False positives	Check for false positives by plating wells with medium only (no cells).

Related Products

For a complete list of ELISpot kits, ELISA kits, and related products from STEMCELL Technologies, visit www.stemcell.com, or contact us at techsupport@stemcell.com.