September 2021

PROTOCOL

Mouse IgG1/IgG2a Double-Color ELISPOT Assay

CONTENTS:

STIMULATE B CELLS *(OPTIONAL)*

- Prepare assay medium (see Technical Tips for recommended assay medium).
- Adjust cells (freshly-isolated from blood or thawed cryopreserved splenocytes) to 1-4 x10^6/ml in assay medium.
- Add 10µl Mouse B-Poly-S™ reagent per 10ml of assay medium.
- Culture cells in a 37°C humidified incubator 5-10% CO_2 for 3-5 days.

COAT THE ELISPOT PLATE

- Prepare Capture Solution containing the Anti-mouse Igκ/λ Capture Ab and prepare 70% EtOH (see Solutions).
- **Pre-wetting:** Remove underdrain from the PVDF plate and set aside. Pipette 15µl of 70% EtOH into each well and then immediately add 180µl/well of PBS. Decant and wash again with 180µl/well of PBS.
  Note: Activation of the membrane with EtOH is instantaneous and can be seen visually as a graying of the membrane. It is important to be sure that the EtOH solution has wicked across the entire membrane of all wells before adding the first PBS wash.
- **Coating:** Decant PBS from plate, blot excess on paper towel and replace the underdrain. Do not press directly on the bottom of the underdrain. Instead, line up the underdrain, and put pressure on the plate frame to spread force evenly across the entire plate. Quickly pipette 80µl/well of the Capture Solution or Antigen Solution.
- Seal plate in parafilm or place in a humid chamber and incubate overnight at 4°C.

ELISPOT Protocol

DAY 1: CELL HARVEST AND PLATING

- **For in vitro ASC:** Harvest prestimulated splenocytes and wash cells twice with assay medium and adjust cells to desired concentration.
- **For in vivo ASC:** Isolate cells and adjust to desired concentration using the assay medium.
- **Washing and blocking:** Decant the coating solution plated the previous day.
- Add 150µl/well of sterile PBS to remove excess unbound antibody or antigen.
- Decant PBS, blot excess on paper towel and add 150µl/well of assay medium.
- Incubate for a minimum of 1 hour at room temperature.
- Decant plate and add 100µl/well of assay medium.
- Add 100µl/well of assay medium containing cells at the desired concentration using large orifice tips. Once completed, gently tap the sides of the plate to distribute cells and immediately place into a 37°C humidified incubator, 5-10% CO_2.
- Incubate for 4-8 hours at 37°C (see Technical Tips). Do not stack plates. Avoid shaking plates by carefully opening and closing incubator door. Do not touch plates during assay incubation.

For laboratory research use only. Not for use in diagnostic or therapeutic procedures.

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DAY 2: DETECTION

- **Prepare wash solutions:** Prepare Wash Buffers: PBS, distilled water and PBS-T (see Solutions).
- **Detection:** Prepare Anti-mouse IgG1/IgG2a Detection Solution (see Solutions).
- After the completion of assay incubation, decant assay medium containing cells and wash plate twice with 150µl/well of PBS.
- Wash plate twice with 150µl/well of PBS-T. Decant final wash and blot excess on paper towel without allowing the membrane to dry.
- Add 80µl/well of Anti-mouse IgG1/IgG2a Detection Solution. Incubate for 2 hours in the dark at room temperature.
- Prepare Tertiary Solution (See Solutions).
- Wash plate twice with 150µl/well of PBS-T. Decant final wash and blot excess on paper towel without allowing the membrane to dry.
- Add 80µl/well of Tertiary Solution. Incubate for 1 hour in the dark at room temperature.
- Prepare CTL-TrueBlue Substrate Solution (see Solutions).
- Decant and wash plate twice with 150µl/well of distilled water. Decant final wash and blot excess on paper towel without allowing the membrane to dry.
- Add 80µl/well of CTL-TrueBlue Substrate Solution. Incubate for 10 minutes in the dark at room temperature.
- Wash plate twice with 150µl/well of distilled water. Decant final wash and blot excess on paper towel.
- Add 80µl/well of CTL-TrueRed Substrate Solution. Incubate for 7 minutes in the dark at room temperature.
- Decant plate and remove underdrain. Wash both sides of the plate gently using tap water. Decant final wash and blot excess on paper towel.
- Dry plate in a running laminar flow hood or overnight face down on paper towel in the dark.
- Scan and count plate. CTL has scanning and analysis services available. Email kitscanningservices@immunospot.com to inquire. Please consult with Technical Support for assistance at +1-216-791-5084.

SOLUTIONS

- All solutions should be freshly-made prior to use. It is important to quick-spin vials contained in the kit before use to ensure content volumes.
- 70% EtOH: Dilute 190-200 proof EtOH. For 10ml, add 7ml of EtOH to 3ml of distilled water.
- Assay medium: Prepare assay medium by adding fresh L-glutamine to a final concentration of 2mM. Amount of medium needed will depend on variables such as cell yield and number of samples tested, but will not be less than 35ml for one full plate; warm to 37°C before using.
- Capture Solution: Dilute Anti-mouse Igκ and Anti-mouse Igλ Capture Ab in Diluent A. For one plate, add 85µl of Anti-mouse Igκ and 85µl of Anti-mouse Igλ Capture Ab to 8.3ml of Diluent A.
- Detection Solution: Dilute Anti-mouse IgG1 and Anti-mouse IgG2a Detection Ab in Diluent B and filter. For one plate, add 10µl of Anti-mouse IgG1 (FITC) Detection Ab and 10µl of Anti-mouse IgG2a (Biotin) to 10ml of Diluent B and filter through a low-protein binding 0.1µm filter (Millipore #SLVV033RS).
- Tertiary Solution: Dilute FITC-HRP and SA-AP in Diluent C. For one plate, add 10µl of FITC-HRP and 10µl SA-AP to 10ml of Diluent C and filter through a low-protein binding 0.1µm filter.
- Substrate Solution: Dilute substrate components by adding reagents in order. It is recommended to make the Substrate Solution within 10 minutes of use and protect from direct light.

  CTL-TrueBlue - For one plate: Step 1 – Aliquot 10ml of Diluent Blue. Step 2 – Add 160µl of S1 and mix well. Step 3 – Add 160µl of S2 and mix well. Step 4 – Add 92µl of S3 and mix well.

  CTL-TrueRed - For one plate: Step 1 – Aliquot 10ml of Diluent Red. Step 2 – Add 180µl of R1 and mix well. Step 3 – Add 160µl of R2 and mix well.

- Wash Buffers **(not included)**: For each plate prepare 200ml distilled water, 200ml PBS and 200ml of PBS-T (100µl Tween-20 into 200ml PBS = 0.05% v/v).

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TECHNICAL TIPS

- Upon successful completion of the assay IgG1 spots will appear red and IgG2a spots will appear blue.
- A suitable assay medium for usage in B cell ImmunoSpot® is RPMI 1640 with pre-tested 10% FCS, 2mM L-glutamine, 100U/ml Penicillin, 100µg/ml Streptomycin, 8mM HEPES and 50µM 2-mercaptoethanol. Even brief exposure to a mitogenic serum can cause high background while other sera can have suppressive effects.
- Deviations from specified temperatures, timing requirements, number of washing steps and specified reagent preparation volumes may alter the performance of the assay. Longer incubation times beyond 8 hours may increase assay sensitivity, but also can increase background membrane staining. Longer substrate incubation times will result in darker spots. Lighter spots have larger differences between the RGB values making it easier to separate colors during analysis. Shorter substrate incubation times are encouraged for double-color enzymatic assays.
- It is highly recommended to aliquot and freeze the B-Poly-S™ or B-Poly-SE™ reagent in single-use aliquots to avoid repeated freeze/thaw cycles.
- The coating concentration of antigens used for ImmunoSpot® assays needs to be optimized by the end user. Only pre-wet and coat one to two plates at a time to avoid drying of the membrane before addition of coating solution.
- It is recommended to prepare the next reagent solution prior to decanting the plate to avoid drying of the membrane between detection steps.
- For antigen-specific assays, an initial cell concentration of 1-5 x 10^5 cells/well may be required. A serial titration of cells is recommended in order to determine the optimal cell concentration. However, a lower initial cell concentration (1-5 x 10^4 cells/well) may be more appropriate for total Ig assays and incubation times beyond 8 hours. CTL recommends using the CTL-LDC™ Kit for accurate live/dead cell counts.
- The PVDF membrane is permeable and protected by an underdrain. Do not push the underdrain too far up on the plate after the pre-wetting step, as this may result in leaking of the membrane. Instead, pressing down on the plate frame or the lid itself is preferred. It is OK if the underdrain falls off after the cell incubation, just avoid direct contact of the membrane with your hands, wet surfaces or absorbent materials. To prevent damage to the PVDF membrane in the bottom of the well, avoid touching with pipette tips or the plate washer.
- The PVDF membrane must remain wet during the detection steps. After blotting the plate on paper towel to remove excess liquid, immediately add the next detection solution to avoid the membrane from drying.
- Scan and count plates only after they have completely dried. ELISPOT plates are stable and can be scanned weeks to months after assay completion without any loss of signal when stored away from direct light.
- Optimal removal of fibers and debris is achieved by removing the underdrain, flipping the plate face down and filtering distilled water "back to front" through the plate using a vacuum manifold (Millipore #MSVMHTSOO).
- Plates may be washed manually or using a suitable plate washer with adjusted pin length and flow rate so membranes and spots are not damaged (CTL recommends the CTL 405LSR).
- Data analysis: The CTL ImmunoSpot® Analyzers along with the ImmunoSpot® Software have advanced features that permit automated, objective recognition of spots, gating and counting. An ELISPOT data management tool, SpotMap®, is also available to facilitate high-throughput ELISPOT work.

The CTL team can assist with data analysis and troubleshooting, along with customizing ImmunoSpot® assays to suit your needs. Please contact us at ***kits@immunospot.com***
Visit our YouTube channel for several helpful videos on working with ELISPOT assays and PBMC: ***www.youtube.com/user/ImmunoSpot.***
The CTL Thawing Protocol for cryopreserved mouse splenocytes is available at ***www.immunospot.com.***

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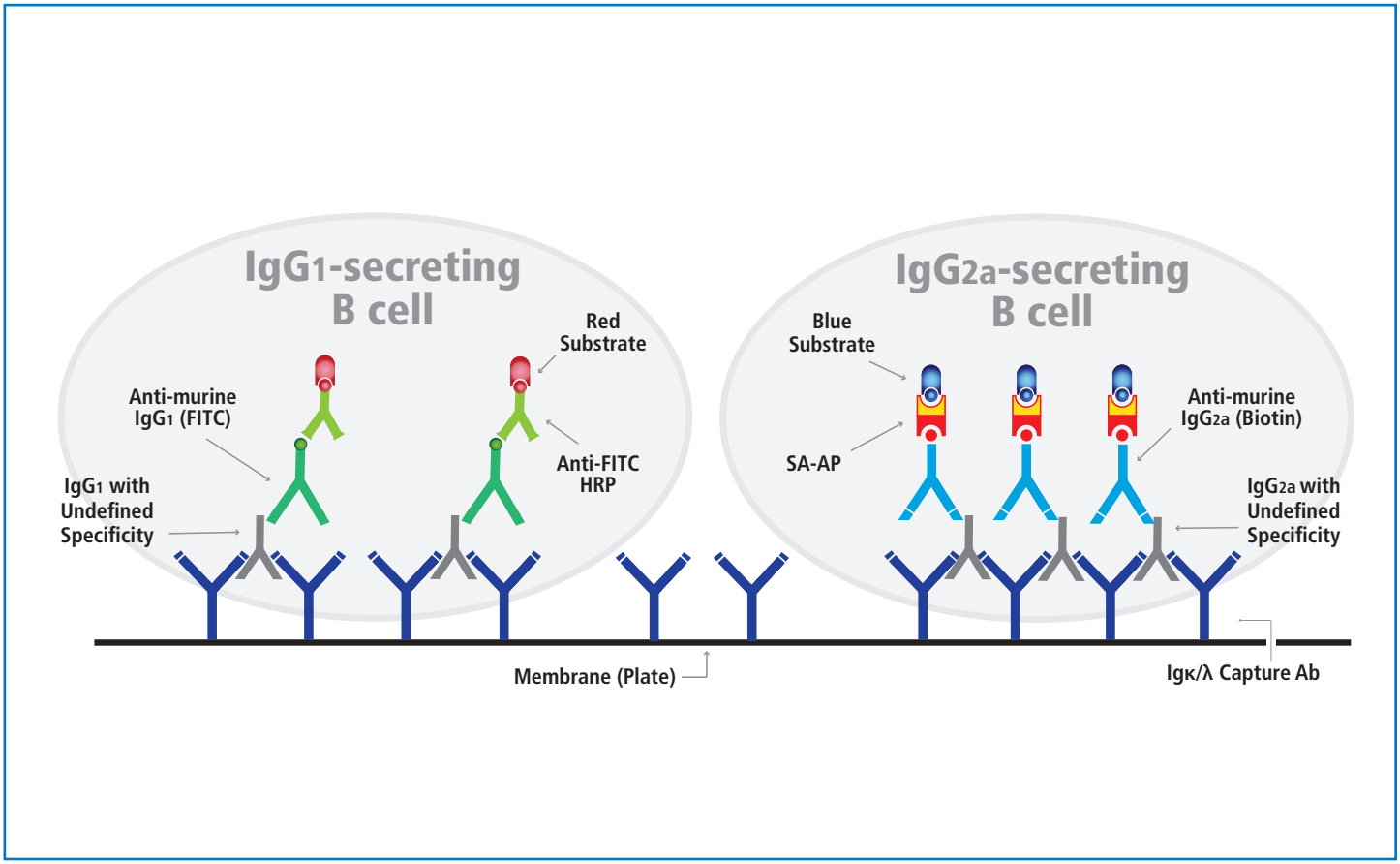
B cell ImmunoSpot® Assay

When provided with the appropriate stimuli in vivo, B cells acquire a lymphoblastoid/plasmablast phenotype and begin to secrete their unique B cell receptor as immunoglobulin (Ig); more commonly referred to as antibody. Such Ig-secreting plasmablasts, also known as antibody-secreting cells (ASC), can be directly plated and measured in B cell ImmunoSpot® assays. Resting memory B cells, however, require prior stimulation in vitro to promote their differentiation into Ig-secreting plasmablasts to enable detection in B cell ImmunoSpot® assays. This system enables measurement of B cells secreting IgG1 or IgG2a antibodies either directly ex vivo, or after in vitro prestimulation.

Principle of the Test

The principle for detection of all B cells that secrete IgG1 or IgG2a is illustrated in the figure below. The membrane is coated with Anti-mouse Igκ/λ Capture Ab (shown in dark blue). As the B cells secrete antibody (shown in gray), these antibodies are directly captured irrespective of their class, subclass or antigen-specificity. The plate-bound IgG1 is detected by adding FITC-labeled Anti-mouse IgG1 Ab, followed by the addition of Anti-FITC Horseradish Peroxidase (FITC-HRP) and a substrate that consist of two sub-components (R1 and R2); which generates a red precipitate or "spot". Plate-bound IgG2a is detected by adding a biotinylated Anti-mouse IgG2a Ab, followed by the addition of Streptavidin-Alkaline Phosphatase (SA-AP) and a substrate that consists of three subcomponents (S1, S2 and S3); which generates a blue precipitate or "spot".

For detection of antigen-specific IgG1 or IgG2a ASC, the membrane needs to be coated with the antigen itself, rather than the Anti-mouse Igκ/λ Capture Ab. Utilizing this method, only antibodies with sufficient binding strength will remain bound to the antigen-coated membrane. These antigen-specific antibodies can then be detected using the same procedure as described above. Coating conditions should be optimized for each test antigen to achieve maximal assay sensitivity. CTL can assist in optimizing coating conditions for various antigens.



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