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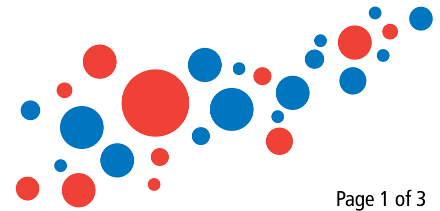
THE ELISPOT Source



PROTOCOL

Murine IgG2a Single-Color Enzymatic ELISPOT Assay

CONTENTS

- *CTL Test™ B Medium*
- *B-Poly-S™*
- *Murine Ig Capture Ab*
- *Anti-murine IgG2a (Biotin) Detection Ab*
- *Strep-AP*
- *S1 (Substrate component 1)*
- *S2 (Substrate component 2)*
- *S3 (Substrate component 2)*
- *Diluent A*
- *Diluent B*
- *Diluent C*
- *Diluent Blue*
- *Plates: 96-well, high-protein-binding, PVDF filter plates*
- *Adhesive plate sealing sheet*
- *Protocol*

Visit our YouTube channel for several helpful videos on working with ELISPOT and FluoroSpot assays: **www.youtube.com/user/ImmunoSpot.**

Protocols and technical resources available at **www.immunospot.com.**

PROCEDURE for polyclonal prestimulation of B cells to obtain *in vitro* ASC

DAY -4: Prestimulate B Cells[1] *(Indices refer to footnotes below.)*

- Prepare *CTL-Test™ B Culture Medium.*[2]
- Adjust cells (freshly-harvested splenocytes or thawed from cryopreserved splenocytes) in *CTL-Test™ B* to 4 million/ml.
- Add *B-Poly-S™* reagent to cells in *CTL-Test™ B* 1:1000 (e.g., 10μl *B-Poly-S™* to 10ml *CTL-Test™ B*).
- Culture cells in a 37°C humidified incubator 7-10% CO_2 for four days.[3]

DAY -1: Coat the ELISPOT Plate

- Prepare *Capture Solution* containing the *Murine Ig Capture Ab.* (see Solutions)
- Pipette 80μl/well of the *Capture Solution* into the PVDF plate provided.[4]
- Seal plate with parafilm and incubate at 4°C overnight.

ELISPOT PROTOCOL

DAY 0 / 1: Cell Harvest and Plating

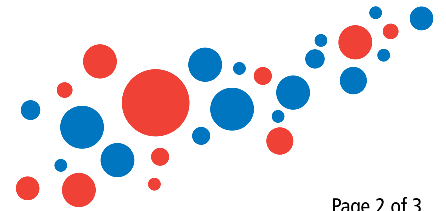
- **For *in vitro* ISC/ASC:** Harvest prestimulated cells and wash three times with *CTL-Test™ B.*[5]
- **For *in vivo* ISC/ASC:** Isolate splenocytes from spleen.
- **For both:** Adjust cells to desired concentration in *CTL-Test™ B.*[6]
- **Prepare the plate for the assay:** Decant the *Capture Solution* plated the previous day.
- Wash plate one time with 200μl/well of sterile PBS at room temperature. (see Solutions)[7]
- Plate cell dilution series for *in vivo/vitro* ISC/ASC.[8]
- Incubate for 4-24 hours at 37°C.[9]

ASC Detection

- Prepare Buffer Solutions: PBS, distilled water, and Tween-PBS. (see Wash Buffers)
- Prepare *Anti-murine IgG2a Detection Solution.* (see Solutions)
- Wash plate two times with PBS and two times with 0.05% Tween-PBS, 200μl/well each time.
- Add 80μl/well of *Anti-murine IgG2a Detection Solution.*
- Incubate at room temperature for a minimum of two hours wrapped in parafilm.[10]
- Prepare *Tertiary Solution.* (See Solutions)
- Wash plate three times with 0.05% Tween PBS, 200μl/well.
- Add 80μl/well of *Tertiary Solution.* Incubate at room temperature for one hour.
- During incubation, prepare *Blue Developer Solution.* (see Solutions)
- Wash plate two times with 0.05% Tween-PBS, 200μl/well, then two times with distilled water, 200μl/well.
- Add *Blue Developer Solution,* 80μl/well. Incubate at room temperature for 15-20 minutes.
- Stop reaction by gently rinsing the plate membrane with tap water, decant, and repeat rinsing three times.
- Remove protective underdrain and rinse back of plate with tap water. If using strip plates, there is no underdrain.[11]
- Let plate dry completely.[12]
- Scan and count plate. (CTL has scanning and analysis services available and offers a trial version of ImmunoSpot® Software with the purchase of any kit. Email kitscanningservices@immunospot.com for more info.)[13]

See other side for Solutions and Technical Tips.

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



SOLUTIONS

All solutions should be freshly-made prior to use. It is important to quick-spin vials before use to ensure content volumes.

- *CTL-Test™ B, B Cell Culture Medium*[2]: Prepare medium by adding 1% fresh L-glutamine. The amount of medium needed will depend on variables such as cell yield and number of samples tested but will be no less than 20ml for one full plate.
- *Capture Solution:* Dilute the *Murine Ig Capture Ab* in *Diluent A.* For one plate, add 170µl of the *Murine Ig Capture Ab* to 8.5ml *Diluent A.*
- *Anti-murine IgG2a Detection Solution:* Dilute the *Anti-murine IgG2a (Biotin) Detection Ab* in *Diluent B.* For one plate, add 25µl of the *IgG2a (Biotin) Detection Ab* to 10ml of *Diluent B.*
- *Tertiary Solution:* Dilute *Strep-AP* 1:1000 in *Diluent C.* For one plate, add 10µl *Strep-AP* to 10ml of *Diluent C*.
- *Blue Developer Solution:* Add the *Substrate Solutions* in sequential steps to 10ml of *Diluent Blue.*

  For one plate:
  Step 1 — Add 160µl of *S1* to 10ml of *Diluent Blue.* Mix well!
  Step 2 — Add 160µl of *S2*. Mix well!
  Step 2 — Add 92µl of *S3*. Mix well!

  It is recommended to make the Blue Developer Solution within ten minutes of use and keep it protected from direct light.

Wash Buffers (not included)

For each plate prepare:
- 0.05% Tween-PBS: 100µl Tween-20 in 200ml PBS
- PBS, sterile, 100ml
- Distilled water, 100ml

FOOTNOTES

1. Four-day prestimulation cultures are recommended for most *in vitro* ASC measurements. Adhere strictly to sterile conditions for prestimulation culture. The *B-Poly-S™* provided with this Assay System is sufficient for stimulation of 320 million splenocytes at 4 million cells/ml. Additional *B-Poly-S™* can be purchased separately, Cat. #CTL-mBPOLYS-200.
2. For reasons of standardization, we recommend the use of specialized serum-free media, such as CTL-Test B™, that has been specially developed for B cell ELISPOT work (Cat. # CTLTB-005). With a pretested fetal calf serum (FCS) available, R10 medium is also suitable: RPMI 1640 with 10% FCS, 1% L-glutamine, 1% Penicillin-Streptomycin, plus 1µM 2ME. With either medium, make sure it is reconstituted with fresh L-glutamine. L-glutamine is unstable at 4°C, therefore, keep stock frozen and resupplement medium with freshly-thawed L-glutamine after seven days. Prepare more culture medium than required for the prestimulation culture, as it will be needed for later washing steps and use in the ELISPOT assay. Store medium at 4°C protected from light. Before adding medium to cells, place into a CO_2 incubator with lid ajar to warm up and equilibrate the pH.
3. If a 24-well plate is used, plate 1ml of the cell suspension per well. For larger scale polyclonal stimulation cultures, traditional culture flasks can be used. Suggested conditions for culture flasks are approximately 3ml of 4 million/ml cell suspension per cm^2. For example, in a T-25 flask, we recommend 8ml of total cell suspension, for a T-75 flask, 25ml. While splenocytes can be cultured at a range of % CO_2, we recommend 9% for optimal results.
4. To maximize the use of each plate, an adhesive plate-sealing sheet has been included to cover unused wells for use in subsequent assays. Use your thumbs to firmly adhere the sheet to the plate and a razor blade to cut the sheet to expose only the necessary wells.
5. High background can result from antibody carryover from the prestimulation culture. To avoid it, wash the cells thoroughly with medium prior to the experiment.
6. After *in vitro* stimulation in certain Ig classes such as IgA, frequencies can be rather high, whereas *in vivo* ISC/ASC and *in vitro* ASC tend to occur in rather low frequencies. Therefore it is important to establish the frequency range and then cover it in serial dilution. For the detection of low-frequency Ig-producing cells we recommend concentrations of $1x10^6$, $5x10^5$, $2.5x10^5$, and $1.25x10^5$ cells per well. In the case of very rare Ig-secreting cells, magnetic bead-based enrichment of B cells is recommended (see Saletti et al., *Nature Protocols,* 2013, 8, 1073). For high-frequency Ig-secreting cells, continue dilution series, skip two dilutions and plate cells at 15,000, 10,000 and 5,000 cells per well. Keep the diluted cells in a CO_2 incubator with lid open until pipetting into the assay plate.
7. Decant plate containing the PBS just before adding the cells. While processing plates, the PVDF membrane at the bottom of the wells must remain wet.
8. Resuspend cells carefully before pipetting by gently flicking the tubes with lid closed, do not resuspend by pipetting as shear forces harm the cells. Plate the splenocytes in 100µl of media/well using large orifice tips. Once the plate is completed, gently tap the sides to permit even sedimentation of the cells and immediately place into a 37°C humidified incubator. Do not stack plates.
9. Avoid shaking plates by carefully opening and closing incubator door. Do not touch plates during incubation to avoid disturbing the cells.
10. From this point forward, the assay will require approximately 3.5 more hours to complete. If the cell preparation prior to incubation will result in a long day, it is acceptable to incubate the detection solution overnight at 4°C.
11. When underdrain and gloves are wet, the underdrain may be slippery and difficult to remove. Wipe gloves and underdrain with paper towel before removing.
12. To completely dry plate, place in running laminar flow hood for two hours, or on the bench top for 24 hours at a 45° angle on paper towels. Do not dry the ELISPOT assay plates at temperatures exceeding 37°C as this may cause the membrane to crack. Spots may not be readily visible while the membrane is still wet and the background may appear elevated. Scan and count plates only after membranes have completely dried.
13. Proper image analysis equipment is recommended to capture and document the wealth of information generated by B cell ELISPOT assays. If you do not have access to a qualified ImmunoSpot® Analyzer, CTL has scanning and analysis services available and offers a trial version of ImmunoSpot® Software with the purchase of any kit. Email kitscanningservices@immunospot.com for more info.

ADDITIONAL HINTS:

- Deviations from specified temperatures, timing requirements, number of washing steps, and specified reagent preparation volumes may alter the performance of the assay.
- Plates can be washed manually or with a suitable automated plate washer with adjusted pin length and flow rate so membranes and spots are not damaged (CTL recommends CTL 405LSR).
- To avoid damage to the PVDF membrane in the wells, do not touch the membrane with pipette tips or with the plate washer. The PVDF membrane is permeable and protected by an underdrain. Avoid direct contact between the well bottom and wet surfaces, including paper towels or any other materials that will absorb liquid.

See other side for Contents and Procedure.
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B Cell ELISPOT Assay

Circulating B cells in the lymphoblastoid/plasmablast state can only secrete antibodies 5-10 days after antigen encounter *in vivo*. Such *in vivo*-activated antibody-secreting cells can be directly plated and measured in B cell ELISPOT assays. Spontaneous immunoglobulin-secreting plasmablasts that have known antigen specificity are commonly referred to as *in vivo*-specific antibody-secreting cells (*in vivo* ASCs), whereas, immunoglobulin-secreting cells that have unknown or undefined antigen specificity after *in vivo* activation are called *in vivo* ISC. Resting B memory cells, however, need to be prestimulated *in vitro* with mitogen for 4-10 days before they start secreting antibody and become detectable in B cell ELISPOT assays. The *B-Poly-S*™ reagent provides optimal polyclonal stimulation of murine B cells. If the antigen specificity of *in vitro* stimulated B cells is known (when tested in antigen-specific B cell ELISPOT assays), one refers to the cells as *in vitro* ASCs. If the antigen specificity of such *in vitro* stimulated B cells is not known (when tested in antigen-specific B cell ELISPOT assays) one refers to the cells as *in vitro* ISCs. This system detects all B cells producing IgG2a antibodies either *ex vivo,* or after *in vitro* prestimulation.

Principle of the Test

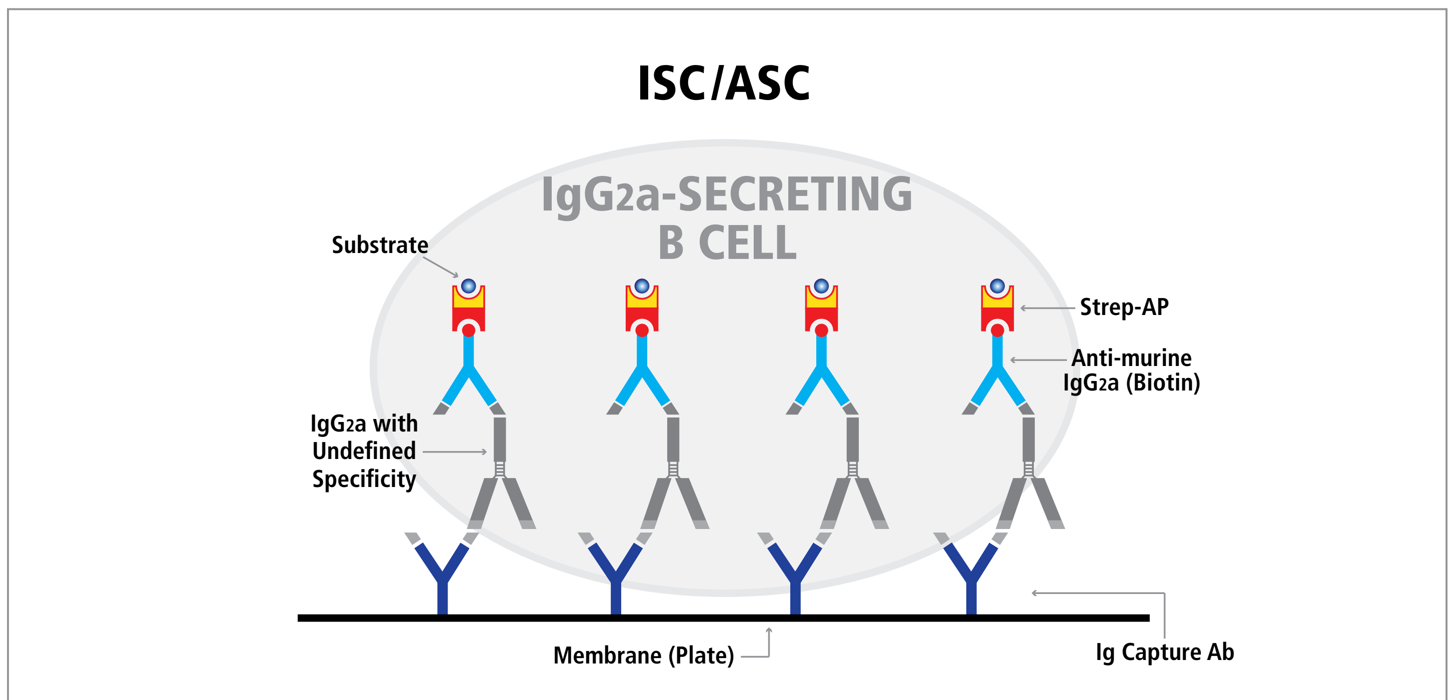
The principle for detection of all B cells that secrete IgG2a (ISC) is illustrated in the figure below. The membrane is coated with an *Anti-murine Ig Capture Antibody* shown in dark blue. As the B cells secrete antibodies (shown in gray), these antibodies are directly captured by the membrane-bound *Anti-murine Ig Capture Antibody*, irrespective of their class, subclass, or antigen-specificity. The plate-bound IgG2a is detected by adding *Biotin-labeled Anti-murine IgG2a Antibody* followed by the addition of *Streptavidin-Alkaline Phosphatase (Strep-AP)* and a substrate that consists of three subcomponents *(S1, S2, S3)*, which generates a blue precipitate or "spot."

Nomenclature for Ig Secreting B Cells

	SPONTANEOUSLY SECRETES* IG AT ISOLATION	SPONTANEOUSLY SECRETES† WITH POLYCLONAL STIMULATION
Undefined Ag Specificity	*In vivo* ISC	*In vitro* ISC
Defined Ag Specificity	*In vivo* ASC	*In vitro* ASC

*Plasmablast that underwent human stimulation less than 12 days ago
†Memory B cell that underwent polyclonal stimulation for a minimum of 4 days

For detection of *in vivo/vitro* ASC, the membrane is coated with the antigen itself, rather than the *Ig Capture Antibody*. Utilizing this method, only antigen-specific antibodies released by the *in vivo/vitro* ASC will bind to the membrane-bound antigen. These antigen-specific antibodies can then be detected using the same procedure as described for the *in vivo/vitro* ISC. Coating conditions need to be optimized for each antigen. CTL can assist you in optimizing coating conditions for various antigens.



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