PROTOCOL

Murine IgG2b / IgG3 Double-Color FluoroSpot Assay

CONTENTS:

Murine Ig Capture Kit:

- *CTL-Test™ B Medium*
- *B-Poly-S™*
- *Murine Ig Capture Ab*
- *Diluent A*
- *Diluent B*
- *Diluent C*
- *Plates: 96-well, low auto-fluorescent, high-protein-binding PVDF filter plates*
- *Adhesive plate sealing sheet*
- *Protocol*

Murine IgG2b Red Detection Kit:

- *Anti-murine IgG2b (Biotin) Ab*
- *Strep-CTL-Red™*

Murine IgG3 Green Detection Kit:

- *Anti-murine IgG3 (FITC) Ab*
- *Anti-FITC Alexa Fluor® 488*

YouTube

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 4 days.[3]

DAY -1: Coat the ELISPOT Plate

- Prepare *Capture Solution* containing the *Murine Ig Capture Ab* and prepare 70% ethanol. (see Solutions)
- Remove plate underdrain, pipette 15µl of 70% ethanol in each well and incubate for less than one minute. Add 150µl of PBS, decant, and wash with 150µl of PBS two more times.[4]
- Replace underdrain and immediately (before plate dries) pipette 80µl/well of the *Capture Solution* into the PVDF plate provided.[5]
- Seal plate in parafilm and incubate at 4°C overnight.

ELISPOT PROTOCOL

DAY 0: Cell Harvest and Plating

- **For *in vitro* ISC/ASC:** Harvest prestimulated cells and wash cells three times with *CTL-Test™ B.*[6]
- **For *in vivo* ISC/ASC:** Isolate splenocytes from spleen.
- **For both:** Adjust cells to desired concentration in *CTL-Test™ B.*[7]
- **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)[8]
- Plate cell dilution series for *in vivo/vitro* ISC/ASC.[9]
- Incubate for 4-24 hours at 37°C.[10]

ASC Detection

- Prepare Buffer Solutions: PBS, distilled water, and Tween-PBS. (see Wash Buffers)
- Prepare *Anti-murine IgG2b/IgG3 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 IgG2b/IgG3 Detection Solution.*
- Incubate at 4°C for a minimum of two hours wrapped in parafilm.[11]
- 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.
- Wash plate two times with distilled water, 200µl/well each time.
- Rinse membrane with tap water, decant and repeat three times.[12]
- Remove protective underdrain and rinse back of plate with tap water.[13]
- Let plate dry completely.[14]
- 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.) **Note:** Fluorescent signals must be read with compatible light source(s) and filter sets. The optimized settings differ depending on the model of instrument used. Please consult with Technical Support for assistance at +1 216-791-5084.[15]

See other side for Solutions and Technical Tips.

SOLUTIONS

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

- 70% Ethanol (not included): Dilute 190-200 proof ethanol. For 10ml, add 7ml of ethanol to 3ml of distilled water.
- *CTL-Test™ 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 Antibody* in *Diluent A.* For one plate, add 170µl of the *Murine Ig Capture Ab* to 8.5ml *Diluent A.*
- *Anti-murine IgG2b/IgG3 Detection Solution:* Dilute the *Anti-murine IgG2b (Biotin)* and *Anti-murine IgG3 (FITC) Detection Antibodies* in *Diluent B.* For one plate, add 25µl of the *Anti-murine IgG2b (Biotin)* and 25µl *Anti-murine IgG3 (FITC) Detection Ab* to 10ml of *Diluent B.*
- *Tertiary Solution:* Dilute *Strep-CTL-Red™* and *Anti-FITC Alexa Fluor® 488,* in *Diluent C* and filter. For one plate, add 50µl of *Strep-CTL-Red™* and 25µl of *Anti-FITC Alexa Fluor® 488* to 10ml of *Diluent C* and filter through a 0.22µm filter.

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 Kit 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 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, CTL recommends 9% for optimal results.

4 Activation of membrane with ethanol is instantaneous and can be seen visually as a graying of the membrane. Ethanol should be washed off as quickly as possible following activation.

5 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.

6 High background can result from antibody carryover from the prestimulation culture. To avoid it, wash the cells thoroughly with medium prior to the experiment.

7 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, CTL recommends 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.

8 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.

9 Resuspend cells carefully before pipetting by gently flicking the tubes with the 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.

10 Avoid shaking plates by carefully opening and closing incubator door. Do not touch plates during incubation to avoid disturbing the cells.

11 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.

12 The last two washes can be filtered through the plate using a 96-well vacuum manifold system.

13 When underdrain and gloves are wet, the underdrain may be slippery and difficult to remove. Wipe gloves and underdrain with paper towel before removing.

14 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.

15 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 and offers a trial version of ImmunoSpot® Software with the purchase of any kit. Email kitscanningservices@immunospot.com for more info. **Note:** Fluorescent signals must be read with compatible light source(s) and filter sets. The optimized settings differ depending on the model of instrument used. Please consult with CTL Analyzer Technical Support for assistance at +1 216-791-5084.

ADDITIONAL TIPS:

- Deviations from specified temperatures, timing requirements, number of washing steps, and specified reagent preparation volumes may alter the performance of the assay.
- Plates may 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.

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

C.T.L.

Cellular Technology Limited • 20521 Chagrin Blvd. • Shaker Hts., OH 44122-5350 USA
+1 216-791-5084 Phone • +1 888-791-4005 Toll Free US • +1 216-751-1373 Fax
kits@immunospot.com • www.immunospot.com

CTL-Europe GmbH • Hans-Boeckler-Str. 19-29, 53225 • Bonn, Germany
+49 (0) 228 8544 78-0 Phone • +49 (0) 228 8544 78-22 Fax
ctl-europe@immunospot.eu • www.immunospot.eu

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 IgG2b and IgG3 antibodies either *ex vivo,* or after *in vitro* prestimulation.

Principle of the Test

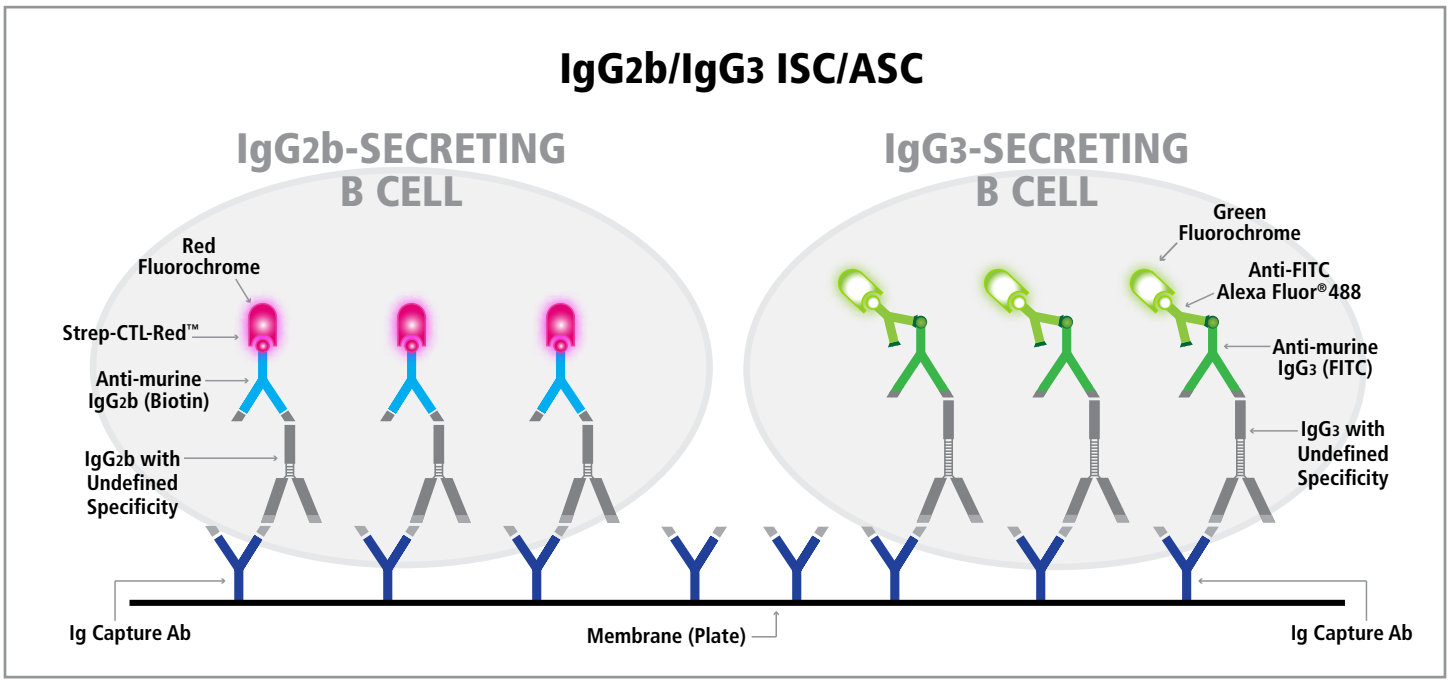
The principle for detection of all B cells that secrete IgG2b or IgG3 (ISC) is illustrated in the figure below. The membrane is coated with an *Anti-murine Ig Capture Antibody* shown in dark blue. As the B cells secrete antibodies (shown in gray), these antibodies are captured directly by the membrane-bound *Anti-murine Ig Capture Antibody*, irrespective of their class, subclass or antigen-specificity. Plate-bound IgG2b is detected by adding *Biotinylated Anti-murine IgG2b Antibody* followed by the addition of *Streptavidin-CTL-Red™*, or other Streptavidin-"color label," which when properly excited appears as red fluorescent "spots"— when using *CTL-Red™* label, or another color as per the label used. The plate-bound IgG3 is then detected by adding *FITC-labeled Anti-murine IgG3 Antibody* followed by the addition of *Anti-FITC Alexa Fluor® 488*. When properly excited, the frequency of IgG3-secreting cells appear as green fluorescent "spots."

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.

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



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Cellular Technology Limited • 20521 Chagrin Blvd. • Shaker Hts., OH 44122-5350 USA
+1 216-791-5084 Phone • +1 888-791-4005 Toll Free US • +1 216-751-1373 Fax
kits@immunospot.com • www.immunospot.com

CTL-Europe GmbH • Hans-Boeckler-Str. 19-29, 53225 • Bonn, Germany
+49 (0) 228 8544 78-0 Phone • +49 (0) 228 8544 78-22 Fax
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