January 2024

PROTOCOL

Ferret Igκ/Igλ/IgG Three-Color FluoroSpot Assay

CONTENTS:

Ferret Basic Kit:

- Diluent B (2 bottles)
- Diluent C
- Plates: 96-well, high-protein-binding, PVDF filter plates
- Protocol

Ferret Igκ/Igλ/IgG Primary Kit:

- Anti-ferret Igκ Primary Ab
- Anti-ferret Igλ Primary Ab
- Anti-ferret IgG Primary Ab

Ferret Igκ/Igλ/IgG Secondary Kit:

- Anti-ferret Igκ (Biotin) Secondary Ab
- Anti-ferret Igλ (FITC) Secondary Ab
- Anti-ferret IgG ($Hapten_1$) Secondary Ab

Ferret Igκ/Igλ/IgG Tertiary Kit:

- SA-CTL-Red™
- Anti-FITC Alexa Fluor® 488
- Anti-$Hapten_1$ CTL-Yellow™

CRITICAL REAGENTS NOT INCLUDED:

- Coating antigen
- Low-binding 0.1µm syringe filters

STIMULATE B CELLS

- Cells need to already possess antibody-secreting capability directly ex vivo prior to isolation.

COAT THE ELISPOT PLATE

- Prepare coating anitgen solution in PBS (coating conditions should be optimized for each test antigen to achieve maximal assay sensitivity) 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 antigen coating solution.
- Seal plate in parafilm or place in a humid chamber and incubate overnight at 4°C.

FluoroSpot Protocol

CELL HARVEST AND PLATING

- Isolate cells and adjust to desired concentration using the assay medium.
- **Washing and blocking:** Decant the antigen 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 16-18 hours at 37°C. Do not stack plates. Avoid shaking plates by carefully opening and closing incubator door. Do not touch plates during assay incubation.

DETECTION

- **Prepare wash solutions:** Prepare Wash Buffers: PBS, distilled water and PBS-T (see Solutions).
- **Detection:** Prepare Anti-ferret Igκ/Igλ/IgG Primary 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-ferret Igκ/Igλ/IgG Primary Solution. Incubate for a minimum of 1 hour in the dark at room temperature.

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 • Margarete-Steiff-Str. 22 71277 Rutesheim, Germany
+49 (0) 228 8544 78-0 Phone • +49 (0) 228 8544 78-22 Fax
ctl-europe@immunospot.eu • www.immunospot.eu

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DETECTION CONTINUED

- Prepare Anti-ferret Igκ/Igλ/IgG Secondary 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 Anti-ferret Igκ/Igλ/IgG Secondary Solution. Incubate for a minimum of 1 hour 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 30 minutes in the dark at room temperature.
- Decant and wash plate twice with 150µl/well of distilled water. Optimal results can be obtained by removing the underdrain and flipping the plate face down, and filtering distilled water "back to front" through the plate using a vacuum manifold (Millipore #MSVMHTS00).
- 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.

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 (see Technical Tips) by adding fresh L-glutamine to a final concentration of 2mM. The 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-ferret Igκ, Anti-ferret Igλ and Anti-ferret IgG Primary Abs in Diluent B. For one plate, add 5µl of Anti-ferret Igκ, add 5µl of Anti-ferret Igλ and add 5ul of Anti-ferret IgG Primary Abs to 10ml of Diluent B and filter through a low-protein binding 0.1µm filter (Millipore #SLVV033RS or SLVVM33RS) (see Technical Tips).
- Secondary Solution: Dilute Anti-ferret Igκ, Anti-ferret Igλ and Anti-ferret IgG Secondary Abs in Diluent B and filter. For one plate, add 5µl of Anti-ferret Igκ (Biotin), add 5µl of Anti-ferret Igλ (FITC) and add 5µl of Anti-ferret IgG ($Hapten_1$) Secondary Abs to 10ml of Diluent B and filter through a low-protein binding 0.1µm filter.
- Tertiary Solution: Dilute SA-CTL-Red™, Anti-FITC Alexa Fluor® 488 and Anti-$Hapten_1$ CTL-Yellow™ in Diluent C. For one plate, add 25µl of SA-CTL-Red™ (visualizes Igκ), add 10ul of Anti-FITC Alexa Fluor® 488 (visualizes Igλ) and add 25µl of Anti-$Hapten_1$ CTL-Yellow™ (visualizes IgG) to 10ml of Diluent C and filter through a low-protein binding 0.1µm filter.
- 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 and proper excitation, Igκ spots will fluoresce red, Igλ spots will fluoresce green and IgG spots will fluoresce yellow.
- 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.
- 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.
- It is recommend to use a low-protein binding 0.1µm filter for filtering reagents after diluting in the diluents provided. However, if it is not possible to filter, high speed centrifugation of the antibody prior to transferring required volume into the diluents provided, is an alternative.
- 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. 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 however; fluorescent plates should be stored in the dark and scanned as soon as possible (within one week for measuring optimal signal).
- 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.
- 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.

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 human PBMC is available at ***www.immunospot.com.***

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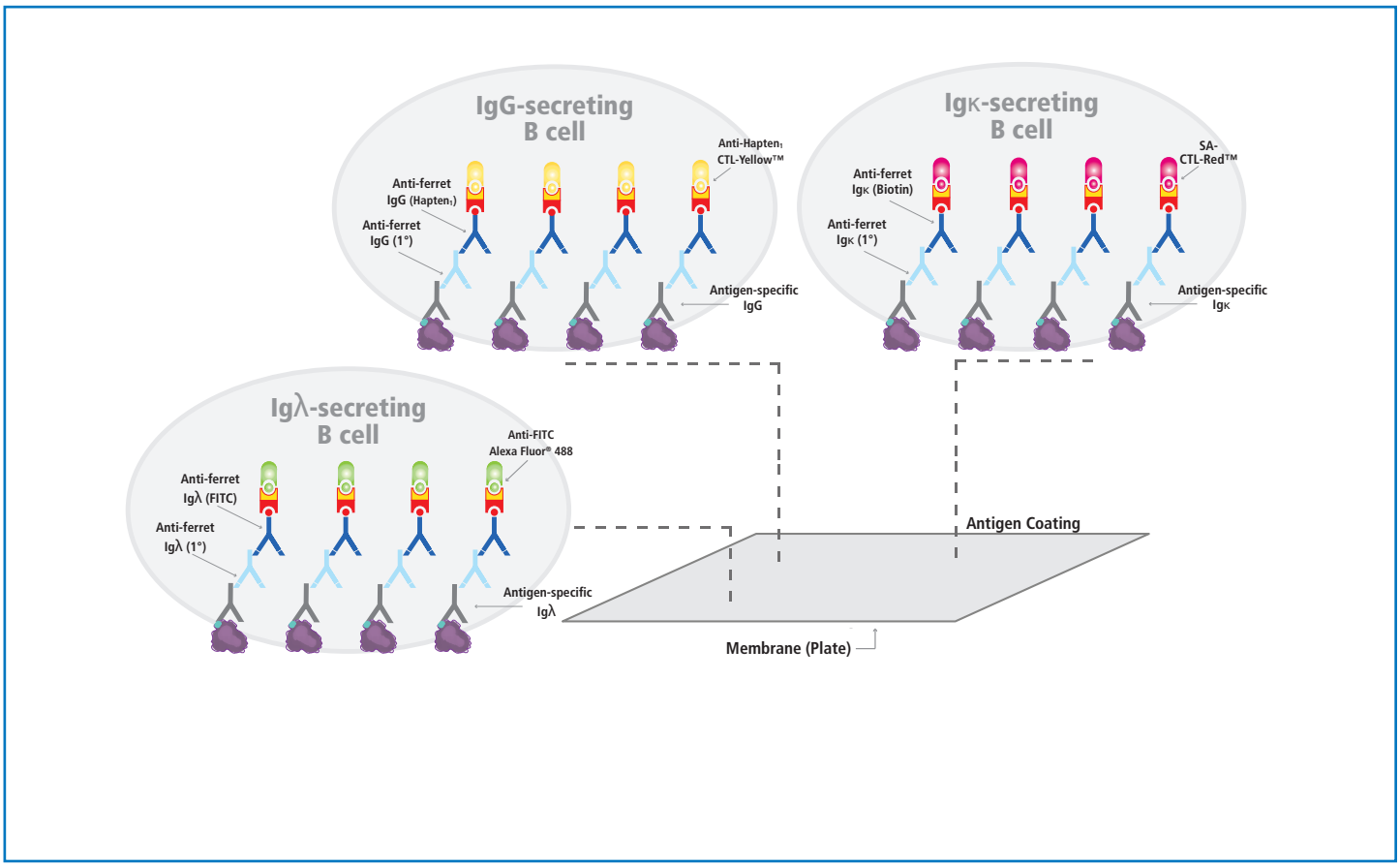
B cell ImmunoSpot® Assay

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. This system enables measurement of B cells secreting Igκ, Igλ and IgG antibodies directly ex vivo.

Principle of the Antigen-Specific Test

The principle for detection of antigen-specific Igκ, Igλ and IgG ASC, is illustrated in the figure below. The membrane is initially coated with the antigen itself and only ASC producing antibody with sufficient binding strength for the plate-bound antigen will generate a secretory footprint (shown in grey). The antigen-specific Igκ secretory footprints are detected by adding adding an Anti-ferret Igκ Primary Ab (shown in light blue), followed by a biotinylated Anti-ferret Igκ Secondary Ab (seen in dark blue) followed by the addition of Streptavidin conjugated to CTL-Red™ (SA-CTL-Red™); which when properly excited appear as red fluorescent "spots". The antigen-specific Igλ secretory footprints are detected by adding adding an Anti-ferret Igλ Primary Ab (shown in light blue), followed by a FITC-conjugated Anti-ferret Igλ Secondary Ab (seen in dark blue) followed by the addition of Anti-FITC Alexa Fluor®488; which when properly excited appear as green fluorescent "spots". The antigen-specific IgG secretory footprints are detected by adding adding an Anti-ferret IgG Primary Ab (shown in light blue), followed by a Hapten$_1$-conjugated Anti-ferret IgG Secondary Ab (seen in dark blue) followed by the addition of Anti-Hapten$_1$ CTL-Yellow™; which when properly excited appear as yellow fluorescent "spots".



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