Diaclone ELISpot development

General Protocol

Note: This protocol is given as a general procedure to assist when using Diaclone Capture and Detection antibodies for ELISpot testing. Optimal dilutions of all reagents, samples and controls as well as the incubation times should be determined by each laboratory for every application.

For research use only

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Reagents and Materials required but not supplied

- Detection Solutions (e.g. Streptavidin AP and BCIP/NBT)
- Ethanol
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS)
- Cell stimulation reagents (e.g. PMA, Ionomycin)
- CO_2 incubator
- Tween 20
- Phosphate Buffered Saline (PBS)
- Coating Buffer (e.g. 1X Phosphate Buffered Saline (PBS))
- Wash Buffer (e.g. 0.1% PBS-T Solution)
- Blocking Solution (e.g. 2% dried skimmed milk in 1xPBS or cell culture medium)
- Dilution Buffer (e.g. 1% BSA PBS Solution)
- Miscellaneous laboratory plastic and/or glass, if possible sterile
- 5 ml and 10 ml graduated pipettes
- Adjustable volume single and multi-channel micropipettes with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Glass-distilled or deionised water
- 96 well PVDF bottomed plates (we recommended Millipore plates catalogue # MSIPN4510, MSIPS4510 and M8IPS4510)

***Note**: if the above recommended plates are used please take the following points into consideration during the assay. Peel of the bottom of the plate after step 16 below and wash both sides of the membrane during step 17. During step 18 be careful to support the plate as the bottom has been removed. Then finally during step 19 ensure both sides of the membrane are washed.*

ELISpot Plate Coating and Blocking

1. Add 25µl of 35% ethanol to every well
2. Incubate plate at room temperature (RT) for 30 seconds
3. Empty the wells by flicking the plate over a sink & gently tapping on absorbent paper. Thoroughly wash the plate 3x with 100µl of 1xPBS
4. Add 100µl of diluted **capture antibody** (as per insert using coating buffer) to every well
5. Cover the plate and incubate at 4°C overnight
6. and wash the plate once with 100µl of 1X PBS per well
7. Add 100µl of **Blocking solution** to every well
8. Cover the plate and incubate at RT for a minimum of 2 hours

ELISpot Assay Procedure

9. Empty the wells as per step 3 and wash the plate once with 100µl of 1X PBS per well
10. Add 100µl of **sample, positive and negative controls** cell suspension to appropriate wells providing the required of number cells and concentration of stimulant
11. Cover the plate and incubate for the appropriate time at 37°C in a CO_2 incubator. Take care not to agitate the plate during this incubation

***Note**: The most appropriate incubation time for each experiment must be empirically determined as this can vary dramatically dependant on the specific activation conditions, cell type and analyte of interest.*

12. Empty the wells as per step 3 and add 100µl of wash buffer to every well and allow to soak for 10mins at 4°C
13. Empty the wells as per step 3 and wash the plate 3x with 100µl wash buffer
14. Add 100µl of diluted **Detection Antibody** (as per insert using dilution buffer) to every well and incubate for a minimum of 1 hour at RT
15. Empty the wells as per step 3 and wash the plate 3x with 100µl wash buffer
16. Add 100µl of diluted **Streptavidin-HRP** (e.g. 1: 5000 working dilution) to every well cover the plate and incubate for a minimum of 1 hour at RT
17. Empty the wells as per step 3 and wash the plate 3x with wash buffer, once washed ensure all remaining buffer has been removed
18. Add 100µl of BCIP/NBT buffer to all wells and incubate for 10mins

***Note**: It is very important to monitor spot formation during this incubation period to determine the optimum incubation time (this can be either more or less than the recommended 10 mins). Ensure the assay is not over developed leading to high background levels.*

19. To stop the development reaction wash the wells 3 times with distilled water ensuring every well is full during each wash.
20. Remove any remaining water following step 3 above and allow the plate to dry.
21. Once dry the spots in each well can be counted either manually using a microscope or automated using a detection system.