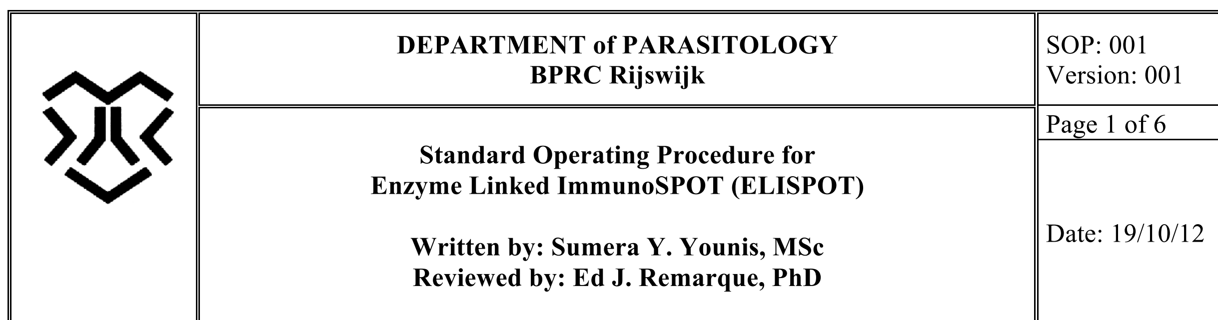

	DEPARTMENT of PARASITOLOGY BPRC Rijswijk	SOP: 001 Version: 001
	Standard Operating Procedure for Enzyme Linked ImmunoSPOT (ELISPOT) Written by: Sumera Y. Younis, MSc Reviewed by: Ed J. Remarque, PhD	Page 1 of 6 Date: 19/10/12

Index **Page**

A.1.	**Aim**	**2**
A.2.	**Brief description of the assay**	**2**
B.1.	**Equipment and instruments**	**2**
B.2.	**Materials**	**2**
C.1.	**Procedure**	**3**
	Day 0: Coating of ELIspot Membrane Plates	**3**
	Day 0: Preparation splenocytes suspension	**3**
	Day 0: Stimulating cells	**3**
	Day 1: Blocking of ELIspot Membrane Plates	**4**
	Day 1: Harvest supernatant and wash prestimulated cells	**4**
	Day 1: Incubation of prestimulated cells on ELIspot Membrane Plates	**4**
	Day 2: Detection and development of spots	**4**

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A.1. Aim

The determination of interferon-gamma (IFN-γ) and Interleukine-5 (IL-5) secreting splenocytes following specific antigenic stimulation by an Enzyme Linked ImmunoSPOT.

A.2. Brief description of the assay

Mouse splenocytes are stimulated for 24 hours with specific antigen(s) prior to their transfer to a PVDF-membrane plate that is coated with IFNγ or IL-5 specific capturing antibodies (so-called indirect ELISPOT). After another overnight incubation cells are removed and the captured cytokine is stained with IFNγ or IL-5 specific detector antibody-conjugate and AEC as a substrate (generating red spots). The number of spots observed corresponds to with the number of cytokine-secreting cells in the original suspension.

B.1. Equipment and instruments

- Plates: 96well F-bottom Greiner Bio-one # 655180
  96well V-bottom Greiner Bio-one # 651180
- 50 mL tubes: Greiner Bio-one # 22761
- 15 mL tubes: Greiner Bio-one # 188271
- Cell strainers (70 μm): BD Falcon # 352350
- Pipettes: 2 mL: ALP # PN2E1
  5 mL: ALP # PN5E25
  10 mL: ALP # PN10E25
  25 mL: ALP # PN25E1
- PVDF membrane plates: Millipore # MSIPS4510
- Cryo's: Greiner Bio-one # 126263
- Nalgene Filter Unit 500 mL: VWR # 734-5082
- Cell counter: Casy Counter – Cell counter and Analyser system, model TT
- ELISPOT reader: Aelvis
- Incubator: New Brunswick Scientific, model: Innova co-170

B.2. Materials

- Foetal Calf Serum: Gibco # 10270.
  Heat inactivated and filtered using Nalgene filter unit.
- RPMI 1640: Gibco # 52400-025
- Culture medium (CM): RPMI 1640 supplemented with 1% Glutamax + 1% Penicillin/Streptomycin + 10% filtered heat inactivated FCS
  Glutamax: Gibco # 35050
  Penicillin-Streptomycin: Gibco # 15140-122
- Freezing mix: FCS + 20% DMSO
  Dimethylsulfoxide (DMSO): Sigma # D2640
- PMA/Iono (positive control): mix of phorbol-myristate-acid and ionomycin.
  PMA: Sigma # P1585 at 50 μg/mL in stock
  Ionomycine: Sigma # IO-634 at 1 mg/mL in stock.

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Final concentration PMA 50 ng/mL; ionomycin 1 µg/mL
- Antigens: AMA1, HBsAg and Ag85A
- Casyton: ready-to-use isotonic dilution for cell cultures especially developed for use with CASY Cell Counter + Analyzer Systems.
- ELISPOT kits: U-CyTech # CT317-PR5 (IFNγ) and # CT321-PR5 (IL-5)
  Containing:
  - Coating cytokines (lyophilized)
    - Reconstitute in sterile MilliQ and use 100x in PBS
  - Biotinylated detector antibodies (lyophilized)
    - Reconstitute in sterile MilliQ and use 100x in Dilution Buffer R
  - Streptavidin-HRP conjugate (lyophilized)
    - Reconstitute in sterile MilliQ and use 100x in Dilution Buffer R
  - AEC coloring system: AEC stock solution and Substrate buffer capsules
    - Content of 1 substrate capsule dissolved in 100 mL 30% EtOH (substrate mix)
    - 3,33 mL AEC stock solution added to substrate mix
    - AEC solution filtered over a 45 µm filter
  - Blocking stock solution R (10x)
    - Prepare by diluting to 1x in PBS
  - Dilution Buffer R (10x)
    - Prepare by diluting to 1x in PBS
  - Tween-20
- Coating Buffer: PBS Gibco # 10010-015
- Wash Buffer: PBS + 0,05% v/v Tween-20
  Tween-20: Sigma # P1379

C.1. Procedure

Day 0: Coating of ELIspot Membrane Plates (Sterile condition)

1. Dilute the Coating antibody 100x in sterile PBS.
2. Prewet membrane plate(s) with 100 µL/well of filtered 70% EtOH and incubate for 10 min at room temperature (RT).
3. Remove EtOH from plates by inverting the plates with a vigorous wrist action.
4. Wash plates twice with 200 µL/well PBS.
   Leave PBS to soak for 3 min between washes.
5. Coat plates with 100 µL/well 100x diluted coating cytokine.
6. Incubate plates o/n at 4 °C

Day 0: Preparation splenocytes suspension

1. Spleens are mashed by passing them through a 70 µm cell strainer and taken up in 25 mL RPMI
2. Centrifuge cells for 8 min at 1400 rpm, normal brake
3. Discard supernatant and wash cells again with 25 mL RPMI
4. Centrifuge cells for 8 min at 1400 rpm, normal brake
5. Discard supernatant and take up cell pellet in 5 mL CM

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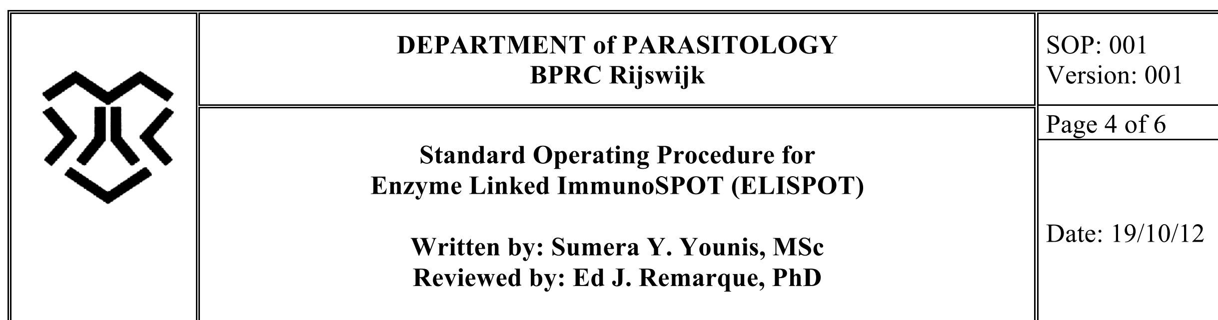

6. Add 10 µL cell suspension to 10 mL Casyton solution and use for cell counting with Casy Counter prog 6 (3 counts in every 200 µL)
   * Note: for non-Casy counter users: an counting chamber such as Bürker-Türk could be used to calculate number of cells in a cell suspension. Different counting chambers may have other specifications; important variables in these counting chambers are the depth of a chamber and the number of smallest squares per cm^2. When counting chambers with different specifications are used, different algorithms have to be followed for the correct calculation of the number of cells per mL. It is important to check the specifications of the counting chamber in use and follow the calculation instructions that go with individual counting chambers.
7. Calculate amount of cells

Day 0: Stimulating cells

1. Dilute splenocytes suspension to a 2x higher concentration. So since we need 5 $x10^6$ cells/mL we will prepare 10 $x10^6$ cells/mL in CM.
2. Prepare 2x concentrated stimuli in CM.
   AMA1, HBsAg and Ag85A at 20 µg/mL [final: 10 µg/mL in assay]
   PMA/Iono at 2 µg/mL [final: 1 µg/mL in assay]
3. Add antigen stimulus in triplicate and PMA/Iono stimulate in duplicate to 96well F-bottom plates. Add 50 µl/well splenocytes to same 96well F-bottom plates
   Final volume: 100 µL/well, according to the predefined stimulation scheme, as follows:
   A. 50 µl/well 2x concentrated stimulus (using culture medium as negative control in triplicate)
   B. 50 µl/well 2x PBMC suspension [final: 5 $x10^5$ cells/well in assay]
4. Incubate overnight (for 20-24 h) at 37 °C in 5% CO_2.

Day 1: Blocking of ELIspot Membrane Plates (Sterile condition)

1. Discard coating and wash membrane plates 5 times with sterile PBS 200 µL/well.
2. Block plates by adding 200 µL/well Blocking solution
3. Incubate for 1 h at 37 °C

Day 1: Harvest supernatant and wash pre-stimulated cells (Sterile condition)

1. Pre-warm CM in a water bath at 37°C.
2. Resuspend stimulated splenocytes in the F-bottom plates and transfer to new 96well V-bottom plates.
3. Centrifuge for 5 min at 500*x* g at RT
4. Harvest supernatant into new 96well F-bottom plates and store at -80°C for measurement of secreted cytokines at later time point (pooled triplicates - duplicates).
5. Wash cell pellets in V-bottom plates with 100 µL pre-warmed CM.

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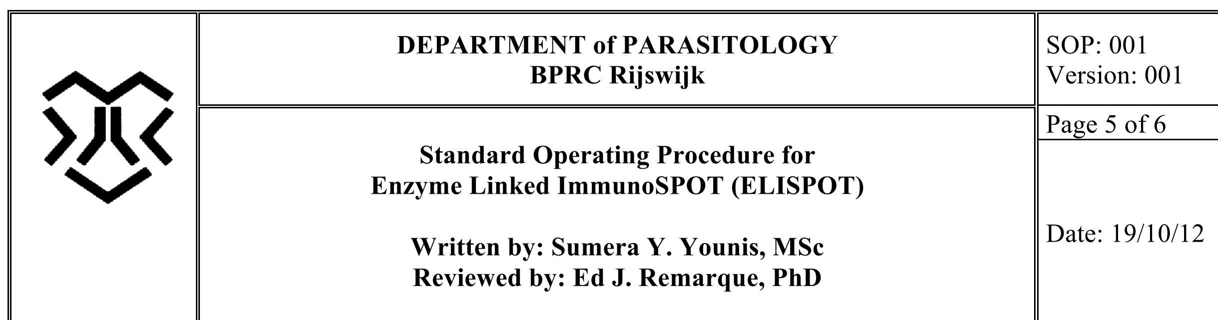

6. Centrifuge for 5 minutes at 500*x* g at RT
7. Discard supernatants and resuspend cells in 100 µL pre-warmed CM.

Day 1: Incubation of pre-stimulated cells on ELIspot Membrane Plates (Sterile condition)

1. Remove the Blocking solution and transfer 100 µL pre-stimulated cell suspension to membrane plates.
2. Incubate at 37°C humidified incubator with 5% CO_2 for 20-24 h.
   Do not move the plate during incubation and take measures to avoid evaporation (e.g by wrapping the plates in plastic foil or put the plates in a humidified container).

Day 2: Detection and development of spots

1. Flick off the bulk of cells and wash the membrane plates 5 times with 200 µL/well PBS.
2. Dilute the detection antibody 100x in Dilution buffer and add 100 µL/well.
3. Incubate for 1 h at 37 °C
4. Discard detection antibody and wash **both sides** of the membrane plates 5 times with PBS-T, 200 µL/well using the wash comb, gravity and PBS-T container
   Flick of PBS-T and remove the plastic cover carefully from the bottom of the plates; the PVDF membrane is exposed now on both sides. Rinse the exposed bottom-side of the membranes thoroughly with PBS-T using the wash comb, gravity and PBS-T container.
   So both sides of the membrane are washed.
5. Make sure plastic cover from the bottom is rinsed thoroughly and carefully re-adjust the plastic covers on the bottom of the plates.
6. Dilute the streptavidin-HRP 100x in Dilution buffer and add 100 µL/well.
7. Incubate for 1 h at 37 °C
8. Wash 3 times with 200 µL/well PBS-T using the wash comb, gravity and PBS-T container as described in step 4.
9. Make sure plastic cover from the bottom is rinsed thoroughly and carefully re-adjust the plastic cover on the bottom.
10. Add 100 µL/well freshly prepared AEC solution
11. Incubate plates for develop 25±5 min in the dark until distinct spots emerge.
12. Colour development stopped by washing both sides of membrane extensively with tap water.
13. Tap membrane plates dry on tissue paper and let them dry at RT in dark
14. When membranes are completely dry (at least 24 h or longer), count the spots using the AELVIS
    - Visually check the counting results.
    - Calculate frequency as spots per million (spm) of splenocytes.

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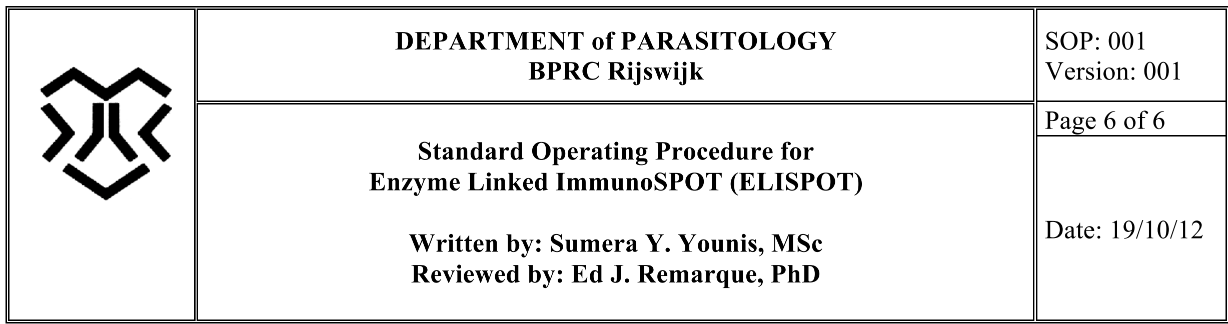

* Note: AELVIS settings: Eli.Expert ROI = 72% – Bright = 95% – MinSize = 8 – MaxSize = 5000 – MinInt = 10 – MinCirc = 50 – Invert = off – Slope = XL – Develop = B – Separate = 10 – Poll = on – Overdev = on.

15. Store plates in the dark at RT.

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