Protocol_504_Antibody Isotype ELISA on mouse serum/intestinal wash/fragment culture

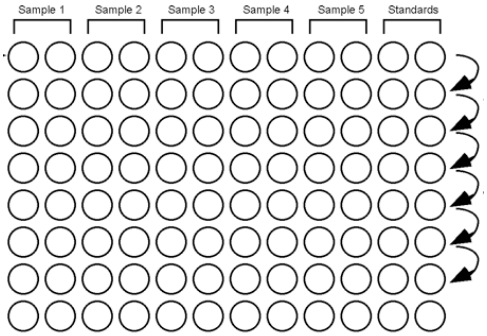
Reagents

- ELISA plates (NUNC)
- Coating buffer: 0.1 M NaHCO3 (3.18 g Na2CO3 + 5.88 g NaHCO3 made up to 1000 ml with water).
- Blocking buffer: 2% w/v BSA in PBS (10 g BSA made up to 500ml with PBS, filter-sterilised; store at 4°C, and only open in the laminar flow hood).
- Washing buffer: 0.05% Tween 20 in PBS (5 ml Tween 20 in a 15 ml tube; add about 8ml water and mix well, then add it all to 10 liters of PBS)
- Diluent: 0.5% BSA in PBS, sterile filtered, store at 4°C.
- Substrate buffer: 0.1 M sodium phosphate monobasic NaH2PO4, pH 4 (13.8 g NaH2PO4.H2O made up to 1000ml). Per 10 ml (needed for 1 plate), add 1 mg ABTS (Boehringer) and 5 µl H2O2 just before use and mix.
- Antibodies: see table at the end of the protocol

Method

1. Coat the ELISA plates with 100 ∫l/well of the coating antibody (see table). Wrap plates and incubate at 4°C overnight. This can also be over the weekend, but in this case make sure that there is a lid over the top plate, otherwise it will dry out.
2. In the morning, flick off the coating solution into the sink, wash plates 4x by immersing in washing buffer and flicking the buffer from the plate into the sink. Blot the plates by tapping onto paper towelling.
3. Add 100 µl/well of Blocking buffer. Incubate at room temperature for 1-2 hours.
4. During the blocking stage you can mark the plates with the samples that they will contain, and prepare your samples. Please note: Samples are prepared in columns A1-A10, standard is prepared in Columns A11-A12.
    - 4a. **Serum samples**: On a separate plate, fill the top wells (A1-A10) with 159.5 µl of diluent. Add 110 µl of diluent to all other wells (B1-H10). Add 5.5 µl of each serum sample into the top row (initial dilution of serum sample = 1/30). Perform a **1/3** titration down the plate by transferring 55 µl, as depicted in the following diagram.
    - 4b. **Intestinal wash samples**: Add 110 µl diluent the top wells (A1-A10). Add 55 µl of diluent to all other wells (B1-H1). Add 55 µl of each intestinal wash sample into the top row (initial dilution of intestinal wash = 1/3). Perform a **1/2** titration down the plate by transferring 55 µl, as depicted in the following diagram.
    - 4c. **Fragment culture (Fc) concentrate:** Add 60 µl diluent to the top wells (A1-A10) and add 60 µl diluent to all other wells. Add 30 µl of sample to the top row (initial dilution of Fc = 1/3). Perform a **1/3** titration down the plate by transferring 30 µl, as depicted in the following diagram.
    - 4d. **Standard:** Add 159.5 µl of diluent to the top well (A11-A12) and 110 µl to all other wells. Pre-dilute the standard as indicated below. Add 5.5 µl of the pre-diluted standard into the top row. Perform a **1/3** titration down the plate by transferring 55 µl, as depicted in the following diagram.

NB: Please note that for serum and intestinal samples, you can use a multichannel to dilute both the samples and the standard at the same time, since 55 µl is transferred each time. However, for fragment cultures, the standard has to be diluted separately.



Transfer 55 µl (30 for Fc) between rows with 12-channel pipette

Discard 55µl (30 for Fc) from the 7th row leaving the 8th rov

5. Flick off the blocking buffer into the sink. Do NOT wash the plate. Blot the plates by tapping onto paper towelling.

6. Transfer the diluted samples to the ELISA plate using a 12-channel pipette, starting with row 8 (most dilute) and ending with row 1 (most concentrated).
   6a. Serum samples: Transfer 100 µl of the titration to the ELISA plate.
   6b. Intestinal wash: Transfer 50 µl of the titration to the ELISA plate.
   6c. Fragment culture**:** Transfer 50 µl of the titration to the ELISA plate.
   6d. Standard: Always transfer the same volume as the samples.

7. Incubate 90 min at RT.
8. Wash the plates 4x.
9. Add 100 µl of secondary antibody (see table) and incubate for 30-60 minutes at room temperature.
10. Wash 4x. Add 100 µl of substrate buffer and wait 30-60 min for the colour reaction to be optimal, then read at OD405nm in a 96 well plate reader.

NOTE 1: These dilutions series have worked well in the past. However, your samples may be different. If you expect your samples to be out of range, please do a test-run first, to determine the optimal dilutions to use.
NOTE 2: The standard is always diluted in the same way; This dilution series has already been optimized and will be the same in every assay.
NOTE 3: Please make sure that the template you use for analysis is the correct one!!!

Immunoglobulin isotype	Coating antibody[1]	Standard[2]	Secondary antibody[3]
IgM	Goat anti-mouse IgM 1020-01 Southern	Purified mouse myeloma IgM 02-6800 Zymed	Anti-mouse IgM μ chain specific; peroxidase conjugated A8786 Sigma
IgG_1	Goat anti-mouse IgG1 1070-01 Southern	Purified mouse myeloma IgG1 02-6100 Zymed	Anti-mouse IgG γ chain specific; peroxidase conjugated A3673 Sigma
IgG_{2a}	Goat anti-mouse IgG2a 1080-01 Southern	Purified mouse myeloma IgG2a 02-6200 Zymed	
IgG_{2b}	Goat anti-mouse IgG2b 1090-01 Southern	Purified mouse myeloma IgG2b 02-6300 Zymed	
IgG_3	Goat anti-mouse IgG3 1100-01 Southern	Purified mouse myeloma IgG3 553486 Pharmingen (stock 0.5 mg/ml)	
IgA	Goat anti-mouse IgA 1040-01 Southern (tube 41 unlabelled)	Purified mouse IgA 553476 PharMingen (stock 0.5 mg/ml)	Anti-mouse IgA α chain specific; peroxidase conjugated A4789 Sigma

[1] Use a dilution of 1:1000 for all coating antibodies

[2] Dilute all standards except IgG3 and IgA as follows: 6μl standard added to 94μl diluent – put 5.5μl of this dilution into the standard top wells (step 4 of the method). For IgG3 and IgA add 12μl to 88μl of diluent – again add 5.5μl of this into the top well. By doing this the top well contains a final concentration of 2μg/ml of antibody.

[3] Use a dilution of 1:1000 for all secondary antibodies