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A.1. **Aim**

The detection and quantification of antibodies (Ab) by a sandwich Enzyme Linked ImmunoSorbent Assay. This assay can measure Ab titers from 0.78 ng/ml to 100 ng/ml from serum, supernatant or plasma, depending on which coating is used.

B.1. Equipment and instruments

- ELISA plates (Greiner microlon #655092)
- 50 ml tubes: Greiner Bio-one #22761
- Pipettes: 2 ml: ALP #PN2E1
  5 ml: ALP #PN5E25
  10 ml: ALP #PN10E25
  25 ml: ALP #PN25E1
- Deep well 96-well plates 1ml (NUNC, #278605)
- Plate washer (Bio-Tek instruments, ELx405)
- Plate reader (BIORAD microplate reader, model 680)

B.2. Materials

- Coating antigen : AMA-1, MSP-1, DICO 1,2,3 or Goat anti Hu, Rb, Ra or Mo.
- Coating Buffer : PBS (Gibco #10010-031)
- Blocking Buffer : PBS + 0,05% v/v Tween-20 + 3% w/v BSA
  Tween-20: Sigma # P1379
  BSA: Sigma #A9647
- Wash Buffer : PBS + 0,05% v/v Tween-20
- Dilution Buffer : PBS + 0,05% v/v Tween-20 + 0,5% w/v BSA
- Standard e.g. pool of final bleed of animals immunized with AMA-1, MSP-1, DICO 1,2,3 or total Hu, Rb, Rt or Mo IgG.
- Anti Hu, Rb, Ra or Mo IgG or IgM conjugated to Alkaline Phosphatase
- DEA buffer
  Diethanolamine: Sigma #D83303
  $MgCl_2 * 6H_2O$: Sigma #M0250
  To make 500 ml: 0,15% w/v $MgCl_2 * 6H_2O$ in 500 ml MQ (e.g. 0,75 g) + 492 µl Diethanolamine pH 9,8
- Substrate PNPP (Para Nitro Phenyl Phosphate hexahydrate) (Fluka cat# 71768)

C.1. Procedure

1. Coat ELISA plates with the desired coating, see schedule below for concentrations.
   Make dilutions in Coating Buffer and add 100 µl per well.
2. Incubate overnight (O/N) at 4°C covered with a plastic seal or lid.
3. Remove coating from plates by inverting the plates with a vigorous wrist action.
4. Block with 200 µl/well of Blocking Buffer.
5. Incubate 1 hour at Room Temperature.
6. Wash with plate washer program 9 (each well aspirated, filled with wash buffer and the wash buffer is aspirated out again. This cycle is performed five times).
7. Prepare samples and standards:
   Dilute pre immune samples 10 - 100 times in Dilution buffer as starting dilution and final bleed samples 1000 – 10000 times.
   Dilute the standard and make a curve starting at an IgG concentration of approximately 50 ng/ml.
   Example on how to make predilutions, check the end of the protocol
8. Add 100 µl of Dilution Buffer to all wells.

9. Add 100 µl of each pre diluted sample or standard IgG sample to the first wells of a column and make a 3-fold serial dilution over 8 wells using 50 µl. The standard IgG sample is serial diluted in 2-fold over 7 wells, using the last well as a blanc.
10. Incubate 1 hour at RT.
11. Wash with plate washer program 9.
12. Add 100 µl conjugate (Alkaline Phosphatase) in the right dilution (see schedule below).
13. Incubate 1 hour at RT.
14. Wash with plate washer program 9.
15. Add 100 µl of 1 mg/ml PNPP in DEA buffer.
16. Incubate 30 minutes at RT.
17. Read OD at 405 nm on a Microplate Reader.

D.1. Schematic:

Action	Material 1	Supplier	Concentration	Dilute in	Incubate
Coating	AMA-1, MSP-1 Dico 1, 2 or 3	BPRC	1 µg/ml	Coating buffer =	O/N 4 C
	Sh anti Rb IgG	Sigma R-3631	1 : 4000	PBS	
	Go anti Mo IgG	Thermo S. 31160	1 : 8000		
	Go anti Ra IgG	Pierce 31220	1 : 2000		
	Go anti Rb IgG	Thermo S. 31210	1 : 8000		
	Go anti Hu IgG	Thermo S. 31130	1 : 8000		
	Go Anti Hu IgM	Sigma I-2386	5 ug/mL		
Blocking	PBS-Tween-BSA	Merck 8.22181.0500 Sigma A-9647	0,05% Tween 3% BSA	PBS	1 hr RT
IgG Standard	Human IgG	Sigma I-4506	100ng/mL – 2 fold	Dilution buffer, after reconstitution in 150mM NaCl	
	Mouse IgG	Sigma I-5381	100ng/mL – 2 fold		
	Rabbit IgG	Sigma I-5006	100ng/mL – 2 fold		
Samples	Pre immune sera Immune sera		Start 10 – 100x Start 1000 – 10000x	Dilution buffer	
Conjugate	Go anti Rb IgG-AP	Pierce 31340	1:1250	Dilution buffer	1 hr RT
	Go anti Ra IgG-AP	Pierce 31350	1:1250		
	Go anti Mo IgG-AP		1:1250		
	Go anti Hu IgG-AP	Pierce 31310	1:1250		
	Go anti Hu IgM-AP	Sigma A-2189	1: 10000		
Substrate	PnPP	Fluka 71768	1 mg/ml	DEA buffer	30 min RT

D.2. Example on how to make pre-dilutions:

If the first dilution in the well should be 50ng/mL followed by 2 fold dilution series over seven wells, then the pre-dilution needs to be twice more concentrated, so 100ng/mL. After adding 50ul dilution buffer to each well, add 50ul of the pre-dilution (100ng/ml) to the top wells. Which dilutes it to 50ng/ml. Then 50ul is taken out to be resuspended in the next well, etc, to make the dilution series.