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INDIRECT ELISA

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All steps are carried out at room temperature unless stated otherwise.
Recipes for all solutions (highlighted) in **bold** are included at the end of the protocol.

1. Antigen coating:

a. Dilute purified antigens to a final concentration of 3.0 μg/ml in **antigen-coating buffer** and add 50 μl of diluted antigen to each well of a 96-well ELISA plate.
b. Carefully cover the plate with adhesive plastic and incubate at 4°C overnight.

2. Blocking:

a. Empty the wells of **antigen-coating buffer** and wash 3 times with 200 μl **PBST buffer** for 5 minutes each time.
b. Add 200 μl **blocking buffer** per well to block residual protein-binding sites. Cover the plate with adhesive plastic and incubate for 1–2 h at 37°C.

3. Antibody incubation:

a. Dilute your primary antibody of choice with **blocking buffer** in a series e.g. 1:500, 1:1000, 1:2000, 1:4000 and so on, empty the wells of **blocking buffer** and then add 100 μl of each dilution per well. Repeat in duplicate, or triplicate, for accuracy. Cover the plate with adhesive plastic and incubate for 1 h at 37°C.
b. Empty the wells and wash 3 times with 200 μl **PBST buffer** for 5 minutes each time.
c. Dilute the HRP-conjugated secondary antibody with **blocking buffer** at an optimal concentration (a dilution factor within 1:10,000-1:100,000 is recommended) and add 100 μl of secondary antibody solution to each well. Cover the plate with adhesive plastic and incubate for 1 h at 37°C.
d. Empty the wells and wash 3 times with 200 μl **PBST buffer** for 5 minutes each time.

4. Signal detection:

a. Add 100 μl TMB substrate (mix equal volumes of **TMB buffer A** and **buffer B**) to each well with a multichannel pipette. Color development should peak after 15 minutes, at which time it should be stopped by adding 100 μl of 2 M H_2SO_4 per well. Read absorbance at 450 nm.

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Buffers Needed

Antigen coating-buffer	For 1000 ml	PBST buffer	For 1000 ml
100 mM $NaHCO_3$	8.4 g	10 mM Na_2HPO_4	1.42 g
Adjust pH to 9.6		1.8 mM NaH_2PO_4	0.22 g
Add ddH_2O to 1000 ml		140 mM NaCl	8.19 g
		0.2 % Tween 20	2 ml
		Adjust pH to 7.4	
		Add ddH_2O to 1000 ml	

Blocking buffer	For 100 ml
5% non-fat dry milk	5 g
Add PBST buffer to 100 ml	

TMB buffer A	For 500 ml	TMB buffer B	For 500 ml
$NaAc•3H_2O$	13.6 g	TMB (first dissolved in 3 ml DMSO)	0.15 g
Citric acid	1.6 g	EDTA-2Na	0.2 g
30% H_2O_2	0.3 ml	Citric acid	0.95 g
Add ddH_2O to 500 ml		Glycerol	50 ml
		Add ddH_2O to 500 ml	

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