SOP: SP039.2
Modified: 7/14/22 KE

Indirect ELISA Assay

Materials and Reagents (for a colorimetric ELISA):

1. 96-well microplate, clear, flat bottom, non-sterile (Immulon 4HBX, Fisher Scientific Cat# 14-245-53)
2. Multichannel pipet
3. 200 µL pipet tips
    - *use filter tips if using/handling biological fluids*
4. TBST and TBS (Note 1)
    - *These buffers will be used as wash buffers and as "standard diluents" for diluting reagents*
5. Blocking solution (3% BSA in TBST)
6. Sigma PNPP developer tablets [4-Nitrophenyl phosphate disodium salt hexahydrate] (Sigma Cat# N9389-50TAB)
7. Pierce™ Diethanolamine Substrate Buffer (5X) (ThermoFisher/Fisher Scientific, Cat# PI34064)
8. Primary antibody being tested
9. Antigen testing against
10. Secondary antibody
11. Stopping solution (2N NaOH)
    - In *normality*, NOT *molarity*
    - *Optional – if wanting to do a single read instead of a kinetic read*
12. Multichannel pipet with 200 µL pipet tips (***use filter tips if handling biological/biohazardous fluids***)
13. Plate sealers (clear, sterile OR non-sterile, NOT PCR sealers)
14. 0.22 µm sterile filter units (PES membrane), 1 L
15. Microplate reader
    - ***Plate reader should be able to read absorbance***
16. Titer plate shaker (Lab-Line Instruments, Inc., Model #4625)
    - ***ALL shaking steps should be performed at 200 rpm (speed "2" on titer plate shaker)***

Protocol:

1. _____ Coat ("plate") a 96-well microplate with appropriate ***target*** antigen or sample (serum, CFP, WCL, etc.) at 100 µL *per well* (indicated as 100 µL/well) *in* **coating buffer** (Notes 2 -4).

2. _____ Cover microplate with plate sealer and incubate ELISA microplate overnight at 4°C, gently rocking (Notes 5 & 6).

3. _____ Decant plate contents (Note 7).

4. _____ Wash plate thrice with 200 µL/well of **TBST** wash buffer (Note 8).

5. _____ Block ELISA plate with 300 µL/well of blocking solution (in **TBST**) and ***incubate*** for 1 hr, shaking at room temperature (Notes 9 & 10).

6. _____ Decant plate contents (*directly into sink*, blocking solution is ***not*** biohazardous).

7. _____ Wash plate thrice with 200 µL/well of **TBST** wash buffer (Note 8).

8. _____ ***Dilute*** primary antibody in TBST and plate at 100 µL/well. ***Incubate*** for 1 hr, shaking at room temperature (Note 11).

9. _____ Decant plate contents (Note 7).

10. _____ Wash plate thrice with 200 µL/well of **TBST** wash buffer (Note 8).

11. _____ ***Dilute*** secondary antibody in **TBS** and plate at 100 µL/well. ***Incubate*** for 30-45 min, shaking at room temperature (Notes 12 & 13).

12. _____ Decant plate contents (*directly into sink*, secondary antibody is ***not*** biohazardous).

13. _____ Wash plate thrice with 200 µL/well of **TBS** wash buffer (Note 14).

14. _____ ***Prepare*** AP substrate developer and plate at 100 µL/well (Note 15 & 16).

15. _____ Inspect microplate for bubbles before reading.

16. _____ ***Place*** microplate in microplate reader immediately after addition of the developing solution. Make sure microplate reader is set to room temperature (~ 25°C).

17. _____ Read microplate at **405 nm** (absorbance) using a kinetics read (15, 30, 45 min intervals), shaking continuously. See Note 17 for setup of kinetics Gen 5 protocol on microplate reader.

18. _____ When finished, collect developer from microplate and wash plate once with 200 µL/well of water. Collect initial developer and wash as ***hazardous waste*** (Note 15).

Notes:

1. Make sure to choose diluent/wash buffer carefully. TBST is generally used with an alkaline phosphatase (AP) substrate/developer. This is due to the fact that the phosphate in PBST can inhibit/hinder development with an AP enzyme (see Reference 1). Below are the Dobos laboratory recipes for TBST and TBS.

   TBST Recipe (for 1 L):
   - 1.21 g tris base
   - 8.77 g sodium chloride (NaCl)
   - 2.5 mL 20% Tween® 80

   Dissolve ingredients one at a time before adding the subsequent ingredient. Mix ingredients well. QS to 1 L with ultrapure (Milli-Q) water and ***pH to 7.4****. Store at 4°C.*

   TBS Recipe (for 4 L):
   - 4.85 g tris base
   - 34.66 g sodium chloride (NaCl)

   Dissolve ingredients one at a time before adding the subsequent ingredient. Mix ingredients well. QS to 4 L with ultrapure (Milli-Q) water and ***pH to 7.45****. Store at room temperature.*

2. There are different coating buffer recipes. However, a sodium bicarbonate/sodium carbonate coating buffer is one of the more common to use (see Reference 2).

   Coating Buffer Recipe (for 1 L):
   - 1.5 g anhydrous Na_2CO_3 (sodium carbonate)
   - 2.93 g anhydrous $NaHCO_3$ (sodium bicarbonate)

   Dissolve ingredients one at a time before adding the subsequent ingredient. Mix ingredients well. QS to 1 L with ultrapure (Milli-Q) water and ***pH to 9.60****. 0.22 µm filter and store at room temperature. Coating buffer should be stored no more than 1 month at room temperature. Make sure to check for growth/floaties with each use.*

3. A concentration of 1 µg/100 µL of ***purified*** antigen is typical for coating/plating. More crude samples (culture filtrate protein (CFP) and whole cell lysate (WCL)) are generally coated/plated at a concentration of 5 µg/100 µL. The concentration of antigen can increase or decrease depending on individual assays.

4. Be sure to include a positive control, negative controls, and a blank. The positive controls are generally serum from immunizations, another LOT of MAB supernatant, another LOT of PAB serum and depend on assay, but are known to give a positive ELISA result. The negative controls (recommended to use multiple) are generally a result of taking out a "piece" of the ELISA. Negative controls allow for the identification of any non-specific binding interactions between reagents and can create a baseline for negativity. Table 1 below illustrates some negative controls that can be utilized for an **INDIRECT ELISA** (other ELISA types may include more reagents, and thus, more negative controls). A blank control, PBST or TBST only, should be added as well (see Table 1 below).

Table 1: Indirect ELISA Negative Controls

Negative Control	Coating Buffer (CB)	Antigen	Blocking Solution	Primary Antibody	Secondary Antibody (AP or HRP)
NO Antigen	Y	N (CB only)	Y	Y	Y
NO Primary Antibody	Y	Y	Y	N (Diluent)	Y
NO Secondary	Y	Y	Y	Y	N (Diluent)
NO Substrate	Y	Y	Y	Y	Y
Block Only	Y	N (CB only)	Y	N (Diluent)	Y
PBST or TBST Only	Y	N (CB only)	N (Diluent)	N (Diluent)	N (Diluent)

**ALL negative controls receive substrate/developer*
*** Y = reagent added, N = no reagent added, substituted with coating buffer OR diluent (indicated in table)*

5. Some people choose not to rock microplate for coating. The idea behind rocking the microplate is to create an even coating of the wells. *Too vigorous* of rocking can lead to coating the sides of the wells.
6. The old version of the protocol (SP039.1) gave more incubation options: 37°C for 1 hour *or* 25°C for 4 hours. However, optimization of these incubation times would need to be performed before sticking to an incubation parameter other than the 4°C, overnight.
7. ***If plate contents contain biological/biohazardous waste, the liquid must be handled and disposed of properly as biohazardous waste (cannot be decanted into sink).*** *However, if plate contents do NOT contain any biological/biohazardous waste, then the liquid can be decanted directly into sink*. Make sure to check wells are void of residual liquid (can tap microplate a couple more times). Make sure to tap on *dry spot* of towel after each wash as to not contaminate microplate with backsplash of liquid on towel.
8. Washes should be performed ***quickly***. Wash buffer should be added using a multichannel pipet (with 200 µL capacity), gently tapped, then decanted in sink (3-4 shakes). ***IF washes are performed after an incubation with biological fluids/biohazardous waste, the washes must be handled and disposed of properly as biohazardous waste (cannot be decanted into sink)***. The microplate should be tapped 3-4 times on a towel for liquid removal. Make sure to check wells are void of residual liquid (can tap microplate a couple more times). Make sure to tap on *dry spot* of towel after each wash as to not contaminate microplate with backsplash of liquid on towel.
9. The most consistent % blocking solution is typically a 3% BSA solution. A 1% BSA blocking solution can be used but may lead to insufficient blocking.
10. Tween detergent is used to aid in non-specific interactions/binding of reagents (see Reference 3). Tween® 20 can be used instead of Tween® 80.
11. Dilute the primary antibody to the proper dilution. A series of dilutions may be needed to determine the correct titer for the antibody (i.e. "determining the functional dilution of an antibody sample necessary to achieve the desired detection range in a given assay" Reference 4).
12. Use goat anti-mouse IgG whole molecule alkaline phosphatase-conjugated antibody at 1:2500 in PBS. If a mouse monoclonal antibody is not used for the primary antibody, be sure to use the appropriate

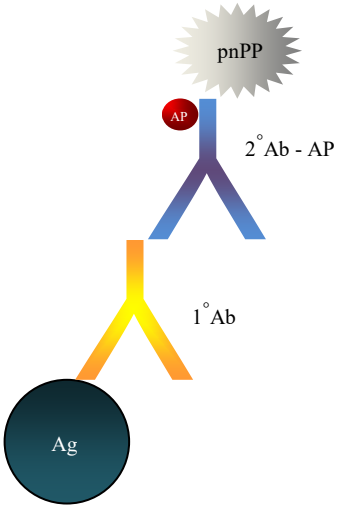
secondary. The secondary must be alkaline phosphatase-conjugated for a colorimetric ELISA (see Table 2).

Table 2: Examples of Primary and Secondary Antibody Pairings (AP-conjugated only)

Primary Antibody	Secondary Antibody
Mouse monoclonal antibody	Goat anti-mouse IgG whole molecule - AP
Rabbit polyclonal antibody	Goat anti-rabbit IgG whole molecule - AP
Guinea pig polyclonal antibody	Goat anti-guinea pig IgG whole molecule - AP

13. Generally, the secondary antibody is incubated for 35 min. A range is given but try to be consistent with incubation times.
14. TBS buffer is used for secondary antibody and subsequent wash.
15. **Make sure to wear lab coat, goggles, and gloves** when handling PNPP and diethanolamine (developer) solution. This solution is highly toxic. **This developer needs to be disposed of properly as hazardous waste.**
16. AP-conjugated Substrate/Developer Directions (per 10 mL of PNPP developer) as according to manufacturer's protocol, with some modifications (see Reference 5):
    - Equilibrate PNPP to room temperature before opening.
    - Dissolve 2 PNPP tablets in 8 mL of DI water (*use water bath sonication to dissolve tablets*).
    - Add 2 mL of 5X Diethanolamine Substrate Buffer to the 8 mL solution. Mix well.
        - "Note: Diethanolamine is effective at concentrations from 10 mM to 1 M."
    - Add solution to reagent reservoir.
        - The Diethanolamine solution is
    - Add 100 µL of the PNPP solution to each microplate well.
    - **Optional step (if not reading kinetics)**:
        - Incubate plate at room temperature for 30 minutes, or until sufficient color develops.
        - Add 50 µL/well of 2 N NaOH to stop the reaction.
        - Place in microplate reader and read at 405 nm (absorbance).
17. Gen 5 Protocol for kinetics read (under Gen5 protocols as "ELISA_protocolKE")
    - Select a generic plate for reader (Dynatech 96-well plate works fine)
    - Absorbance → select endpoint/kinetics
    - Set read wavelength (***was set originally to 450 – change to 405 nm***)
    - Click on kinetics step → Run time = 45 min, Interval = 15 min, should be 4 reads (have to abort read AFTER 45 min to stop run)
    - Add shaking continuously step (orbital at 365 cpm, slow) under kinetics
    - Add read step at 405 nm wavelength under kinetics

Diagram of a colorimetric Indirect ELISA



References:

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6. *Antibodies: A Laboratory Manual*. Ed Harlow and David Lane. Cold Spring Harbor Laboratory, New York. 1988. pp. 553-612.