Protocol

Immunometric Double-Antibody Sandwich Enzyme-Linked Immunosorbent Assay

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The double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) is preferentially used to determine the concentration of unknown antibody in a sample. Pure antigen is not required in this assay; however, the use of a reporter-labeled detection antibody is essential. The double-antibody sandwich ELISA is suitable for epitope mapping of different monoclonal antibodies that have been generated against a single antigen. First, plates are coated with a capture antibody specific for immunoglobulins generated by immunization of a host species. Next, the test antibody solution (e.g., serum) is incubated with the capture antibody to facilitate binding. The plates are washed to remove unbound antibody, and then antigen is added. The plates are washed again followed by the addition of an antigen-specific reporter-labeled antibody. Following incubation, unbound reporter antibody is washed off, and reporter-specific substrate is added. Reporter-mediated substrate hydrolysis is visualized and measured. The signal is proportional to the number of test antibodies present in the serum.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

3,3',5,5'-tetramethylbenzidine (TMB) (Rockland Immunochemicals, #TMBE-1000) (for horseradish peroxidase [HRP]-based assays)

Antibody diluent solution (1% [v/v] fish gel [Sigma-Aldrich, #G7765] in PBS)

Antigen

Antigen-specific reporter-labeled detection antibody and dilution buffer (e.g., Rockland Immunochemicals, #MB-061-100 or #MB-076-0100)

It is vitally important that the reporter-labeled antibody does not cross-react with the capture antibodies used in this assay.

Blocking buffer (3% [w/v] fish gel in PBS or TBS)

Carbonate buffer for immunoassays <R> (optional; see Step 1)

Immunoglobulin-specific capture antibody at a known concentration

It is imperative that the capture antibody does not display nonspecific cross-reactivity to the antigen or antigen-specific reporter-labeled antibody used in the assay.

para-nitrophenyl phosphate (pNPP) (Rockland Immunochemicals, #NPP-10) (for alkaline phosphatase [AP]-based assays)

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Phosphate-buffered saline (PBS) for immunoassays (pH 7.4) <R>
 Stop solution (1 M HCl) (for HRP-based assays only; see Step 13)
 Test antibody solutions (e.g., tissue culture supernatants, host antisera, or ascites fluids)
 Tris-buffered saline (TBS) for immunoassays <R> (optional)
 Wash buffer with detergent (PBS or TBS containing 0.05% [v/v] Tween 20)

Equipment

Microplate reader (Bio-Rad, Model #680) with Microplate Manager Software (5.2.1) or equivalent
 Microplate sealing tape, polyester, nonsterile (Corning, #4612 or equivalent)
 Microtiter plates (flat bottom, 96-well, 0.3 mL per well) with appropriate surface chemistry (e.g., Immulon 4 HBX, Thermo Scientific, #3855 or equivalent)
 Multichannel pipettes (eight- or 12-channel) (Costar, #4888 or equivalent)
 Plate washer (Dynex Ultrawash PLUS or equivalent)
 Reagent reservoirs (VWR, #12577-027 or equivalent)

METHOD

The procedure is illustrated schematically in Figure 1.

1. Dilute the immunoglobulin-specific capture antibody to a final concentration of 1 $\mu\text{g/mL}$ in PBS or a carbonate buffer. Coat a microtiter plate by pipetting 100 μL of diluted antibody into individual wells.

Dilution of the immunoglobulin-specific capture antibody to a final concentration of 1 $\mu\text{g/mL}$ serves as an indicator only. Dilute the capture antibody to a working concentration of 1 $\mu\text{g/mL}$ for the analysis of hybridoma supernatant, ascites fluid, or host antiserum.

2. Cover the plate with a lid or plastic adhesive. Incubate for 1 h at room temperature or overnight at 2°C–8°C.
3. Using a plate washer, aspirate the coating solution. Wash the plate three times by filling each well with 300 μL of wash buffer containing detergent. Remove retained liquid by patting the plate on a paper towel.
4. Add 200 μL of 3% (v/v) fish gel in PBS or TBS blocking buffer to block the remaining protein-binding sites. Incubate for 1 h at room temperature or 30 min at 37°C.
5. Aspirate the blocking solution using the plate washer. Wash the plate as described in Step 3.
6. Prepare dilutions of the test antibody solutions in 1% (v/v) fish gel in PBS. Pipette 100 μL into each coated well. Incubate for 2 h at room temperature.

Preparation of 1:5 dilutions for the analysis of tissue culture supernatant and 1:200 dilutions for analysis of host antiserum or ascites fluid is recommended.

FIGURE 1. Double-antibody sandwich ELISA for the colorimetric detection of specific antibodies.

TABLE 1. ELISA troubleshooting guide

Problem	Possible cause	Recommendation
High background	Insufficient washing	Ensure proper performance of the plate washer for complete removal of residual liquid following the washing steps. Increase the number of washes or lengthen the soaking time between washes.
	Insufficient blocking	Increase blocking time or apply different blocking buffer. Fish gelatin can be stored for up to 2 wk at 2°C–8°C and should be discarded thereafter.
	Secondary antibody dilution too low; increased presence of reporter	Increase antibody dilution, titrate antibody if required.
No signal observed	Incorrect preparation or addition of reagents	Review protocol and repeat the assay. Ensure proper calculation of all dilutions and prepare fresh assay-associated reagents.
	Insufficient antibody applied	Increase antibody concentrations.
	Insufficient antigen presence and binding	Select an alternative microtiter plate displaying different binding characteristics. Dilute antigen in PBS devoid of additional proteins. Increase the antigen amount or change to a more sensitive assay.
Excessive signal observed	Contaminated buffers	Prepare fresh buffers.
	Contaminated enzyme-labeled secondary antibody	Apply fresh reagents.
	Expired substrate	Prepare fresh substrate.
	Insufficient incubation temperature and time	Ensure that all assay-associated steps are performed at the recommended temperatures.
	Insufficient washing; unbound enzyme remaining	Ensure proper performance of the plate washer for complete removal of residual liquid following the washing steps. Increase the number of washes or lengthen the soaking time between washes.
	Insufficient blocking	Increase the blocking time or apply a different blocking buffer.
	Cross-well contamination	Ensure correct use of pipette tips for reagent addition and appropriate application of unused disposable plate sealers.
	Reporter antibody dilution too low; increased presence of reporter	Increase the antibody dilution; titrate the antibody if required. Reduce the incubation time.
	Reporter contamination as a result of reused consumable materials	Use disposable reagent reservoirs and plate sealers. Do not reuse them.
	Contaminated buffers	Prepare fresh buffers.
Plate edge effect Positive result in negative control Plate absorbance inconsistencies	Reaction was not stopped as recommended.	Stop reaction as directed.
	Substrate development occurred in the light.	Perform substrate development in the dark.
	Incubation temperature too high	Ensure that all assay-associated steps are performed at the recommended temperatures.
	Environmental temperature fluctuations	Perform assay within a temperature-controlled environment. Seal the plates completely.
	Contaminated assay-specific reagents	Prepare fresh reagents and buffers.
	Contaminated assay-specific reagents (sandwich ELISA)	Ensure no cross-reactivity between antibodies used in the ELISA.
	Plates stacked during incubations	Avoid stacking plates.
	Pipetting inconsistencies	Ensure careful pipetting using calibrated micropipettes.
	Excessive drying	Perform assay within a humidity-controlled environment.
	Insufficient washing	Ensure proper performance of the plate washer for complete removal of residual liquid following the washing steps. Increase the number of washes or lengthen the soaking time between washes.
Poor interassay reproducibility	Dirty plate bottom	Clean the bottom of the plate to allow for accurate absorbance readings.
	Insufficient washing	Ensure proper performance of the plate washer for complete removal of residual liquid following the washing steps. Increase the number of washes or lengthen the soaking time between washes.
	Insufficient incubation temperature	Ensure that all assay-associated steps are performed at the recommended temperatures.
Slow color development	Protocol variations	Ensure adherence to the same protocol for all assays performed.
	Contaminated buffers	Prepare fresh buffers.
	Incorrect incubation temperature	Ensure that all assay-associated steps are performed at the recommended temperatures.
	Reporter antibody concentration too low Contaminated assay-specific reagents	Decrease antibody dilution; titrate antibody if required. Prepare fresh reagents and buffers.

7. Aspirate the antibody solution. Wash the plate as described in Step 3.
8. Dilute the antigen to a recommended concentration of 1 µg/mL in PBS. Add 100 µL to each well. Incubate for 2 h at room temperature. Antigen blanks and assay blanks do not receive any antigen solution.
For an accurate determination of the antibody concentration in unknown samples (and although purified antigen preparations are not essential), the limit of detectability for most protein antigens in this type of system is 2–20 ng/mL. A concentration of 20–200 ng of antigen/mL is recommended.
9. Aspirate the antigen solution. Wash the plate as described in Step 3.
10. Dilute the reporter-labeled antigen-specific detection antibody to a final concentration of 1 µg/mL in PBS using the diluent supplied with the antibody. Pipette 100 µL of the detection antibody into each well, including the antigen blank control wells. Incubate the plates for 2 h at room temperature.
*Dilution of the reporter-labeled antibody to a final concentration of 1 µg/mL serves as an indicator only; it is best to titrate the antibody by indirect ELISA (see Protocol: **Indirect Immunometric ELISA** [Kohl and Ascoli 2017]) using the checkerboard titration format to determine the optimal working concentration.*
11. Using the plate washer, aspirate the reporter-labeled detection antibody solution. Wash the plate as described in Step 3.
12. Prepare or equilibrate the substrate solution (TMB for HRP or pNPP for AP) to room temperature. Add 100 µL per well to each of the wells. Incubate the enzyme-based reactions for 1 h at room temperature in the dark.
Avoid introducing air bubbles when adding substrate to the wells, because these will affect the absorbance readings.
13. Stop the HRP reaction by adding 100 µL of 1 M HCl per well. Alkaline phosphatase-based substrate development does not require a stopping reagent.
For further quantification of low-level reactions, skip Step 13 and perform plate measurements at set time intervals.
14. Read the plates on a microplate reader set to 405 nm for AP-based and 450 nm for HRP-based substrate development.
The results from ELISAs using chemiluminescent or fluorescent reporters will be in relative light units (RLUs) instead of absorbance in the standard curve. Standard curves are only required to accurately determine the amount of antigen in test samples; they are redundant for qualitative answers. Please see Table 1 for a detailed troubleshooting guide.

RECIPES

Carbonate Buffer for Immunoassays

Reagent	Amount per 1 L of solution
Na ₂ CO ₃	1.59 g
NaHCO ₃	2.93 g
Adjust to pH 9.5 using 1 N NaOH. Store at ambient temperature.	

Phosphate-Buffered Saline (PBS) for Immunoassays (pH 7.4)

Reagent	Amount per 1 L of solution
NaCl	8 g
KCl	0.2 g
Na ₂ HPO ₄	1.44 g
KH ₂ PO ₄	0.24 g
Store at ambient temperature.	

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Tris-Buffered Saline (TBS) for Immunoassays

Reagent	Amount per 1 L of solution
NaCl	8 g
KCl	0.2 g
Tris-HCl	3 g
Adjust to pH 7.6 using 1 N NaOH. Store at ambient temperature.	

REFERENCES

Kohl TO, Ascoli CA. 2017. Indirect immunometric ELISA. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot093708.

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