Protocol

Indirect Competitive Enzyme-Linked Immunosorbent Assay (ELISA)

Thomas O. Kohl and Carl A. Ascoli

The indirect competitive ELISA (indirect cELISA) pits plate-immobilized antigen against antigens in solution for binding to antigen-specific antibody. The antigens in solution are in the test sample and are first incubated with antigen-specific antibody. These antibody–antigen complexes are then added to microtiter plates whose wells have been coated with purified antigen. The wells are washed to remove unbound antigen–antibody complexes and free antigen. A reporter-labeled secondary antibody is then added followed by the addition of substrate. Substrate hydrolysis yields a signal that is inversely proportional to antigen concentration within the sample. This is because when antigen concentration is high in the test sample, most of the antibody is bound before adding the solution to the plate. Most of the antibody remains in solution (as complexes) and is thus washed away before the addition of the reporter-labeled secondary antibody and substrate. Thus, the higher the antigen concentration in the test sample, the weaker the resultant signal in the detection step. The indirect cELISA is often used for competitive detection and quantification of antibodies against viral diseases in biological samples.

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 material 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 HRP-based assays)
Antibody, antigen-specific
Antibody diluent solution (1% [v/v] fish gel [Sigma-Aldrich, #G7765] in PBS or TBS)
Antibody, reporter-labeled antihost, and dilution buffer (e.g., Rockland Immunochemicals, #MB-061-100 or #MB-076-0100)
Antigen standards (used in the second plate)
Antigen test solutions (used in the second plate)
Antigens, purified (used to coat the first plate)
Blocking buffer (3% [w/v] fish gel in PBS or TBS)
Carbonate buffer for immunoassays <R> (optional; see Step 1)
para-nitrophenyl phosphate (pNPP) (Rockland Immunochemicals, #NPP-10) (optional; for alkaline phosphatase [AP]-based assays)

From the Antibodies collection, edited by Edward A. Greenfield.

Cite this protocol as *Cold Spring Harb Protoc;* doi:10.1101/pdb.prot093757

Phosphate-buffered saline (PBS) for immunoassays (pH 7.4) <R> (optional; for horseradish peroxidase [HRP]-based assays)
Stop solution (1.0 M HCl) (for HRP-based assays only; see Step 15)
Tris-buffered saline (TBS) for immunoassays <R> (optional; for AP-based assays)
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/well) with appropriate surface chemistry (e.g., Immulon 4 HBX; Thermo Scientific, #3855 or equivalent)
Multichannel pipettes (8- 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.

Preparing the Antigen-Coated First Plate

1. Dilute purified antigen in PBS or carbonate buffer to an optimal concentration determined previously by checkerboard titration (see Protocol: **Indirect Immunometric Enzyme-Linked Immunosorbent Assay (ELISA)** [Kohl and Ascoli 2017]). Add 100 µL of antigen to each well.
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 antigen solution. Wash the plate three times by filling each well with 300 µL of wash buffer containing detergent. Gently 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 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. Set this plate aside until Step 10.

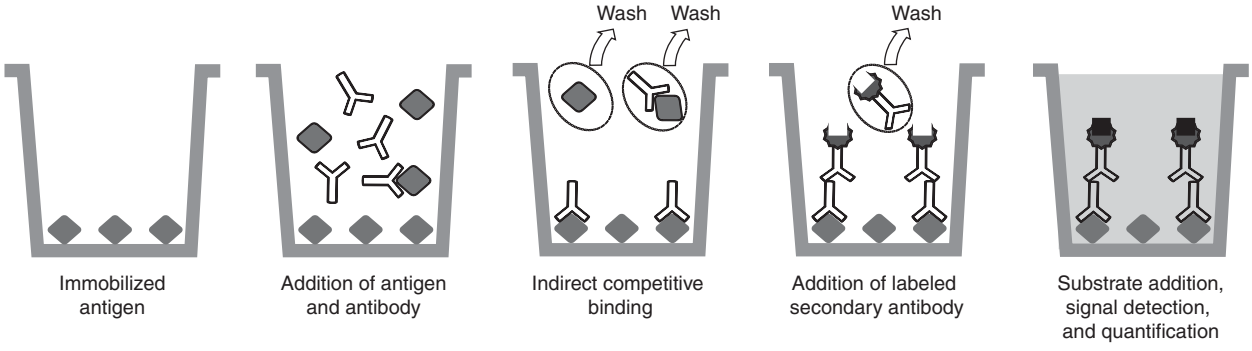

FIGURE 1. Indirect competitive ELISA for determining the degree of antigen competition in a sample.

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot093757

Preparing the Second Plate

6. On a second plate, use a permanent marker to mark wells identical to those used on the first plate. Block the wells using 3% (v/v) fish gel as described in Steps 4 and 5.
7. Add 100 µL of standards or test antigen solution diluted in PBS or carbonate buffer to the wells in Column 1. Titrate by twofold or another serial dilution across the length of the plate.

   Different dilutions of the test antigen solution might be necessary to facilitate inhibition within the dynamic range of the assay set by the standards.
8. Dilute antigen-specific antibody to twice the optimal concentration—as previously determined—in 1% (v/v) fish gel. Add 100 µL of diluted antibody to select experimental and control wells (including the positive [purified antigen] and negative [buffer-only] controls).

   This step will result in dilution of the reporter-labeled antibody (used in Step 12) to the desired optimal dilution applicable to the assay.
9. Cover the plate with a lid or plastic adhesive. Incubate for 1 h at room temperature.

Running the Indirect cELISA

10. Transfer 100 µL of the antigen–antibody complex solution from the second plate to corresponding wells on the antigen-coated first plate. Incubate for 1 h at room temperature.
11. Aspirate the antigen–antibody complex solution using the plate washer. Wash the plate as described in Step 3.
12. Prepare reporter-labeled antihost antibody at a recommended dilution of 1:20,000 using the diluent supplied with the antibody. Add 100 µL to each well, including the assay control wells. Incubate for 30 min at 37°C.

    *Dilution of the reporter-labeled antibody to a final concentration of 1:20,000 serves as an indicator only and is dependent on the concentration of the reporter antibody. It is best to titrate the antibody by indirect ELISA using the checkerboard titration format (see Protocol: **Indirect Immunometric Enzyme-Linked Immunosorbent Assay (ELISA)** [Kohl and Ascoli 2017]) to determine the optimal working concentration.*
13. Using the plate washer, aspirate the reporter-labeled antibody solution. Wash the plate as described in Step 3.
14. Prepare or equilibrate the substrate solution (TMB for HRP or pNPP for ALP) to room temperature. Add 100 µL to each well. Incubate the enzyme-based reactions for 30 min at room temperature in the dark.

    Avoid introducing air bubbles when adding substrate to the wells, because these will affect the absorbance readings.
15. Stop the HRP reaction by adding 100 µL of 1 M HCl per well to each well. Alkaline phosphatase-based substrate development does not require a stopping reagent.

    For further quantification of low-level reactions, skip Step 15 and perform plate measurements at set time intervals.
16. Read plates on a microplate reader set to 405 nm for AP-based and 450 nm for HRP-based substrate development.

    The use of chemiluminescent or fluorescent reporters will result in the determination of relative light units (RLUs) instead of absorbance.
17. Prepare a standard curve from the data by plotting standard antigen concentration on the *x*-axis versus absorbance on the *y*-axis, and determine the antigen concentration of unknown test samples. Alternatively, calculate the percentage inhibition and plot B/B_0 on the *y*-axis versus antigen concentration on the *x*-axis.

    Please see Table 1 for a detailed troubleshooting guide.

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.
	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.
Excessive signal observed	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.
	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.
Plate edge effect	Environmental temperature fluctuations	Perform assay within a temperature-controlled environment. Seal the plates completely.
Positive result in negative control	Contaminated assay-specific reagents	Prepare fresh reagents and buffers.
	Cross-reactive antibodies (sandwich ELISA)	Ensure no cross-reactivity between antibodies used in the ELISA.
Plate absorbance inconsistencies	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.
	Dirty plate bottom	Clean the bottom of the plate to allow for accurate absorbance readings.
Poor interassay reproducibility	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.
	Protocol variations	Ensure adherence to the same protocol for all assays performed.
	Contaminated buffers	Prepare fresh buffers.
Slow color development	Incorrect incubation temperature	Ensure that all assay-associated steps are performed at the recommended temperatures.
	Reporter antibody concentration too low	Decrease antibody dilution; titrate antibody if required.
	Contaminated assay-specific reagents	Prepare fresh reagents and buffers.

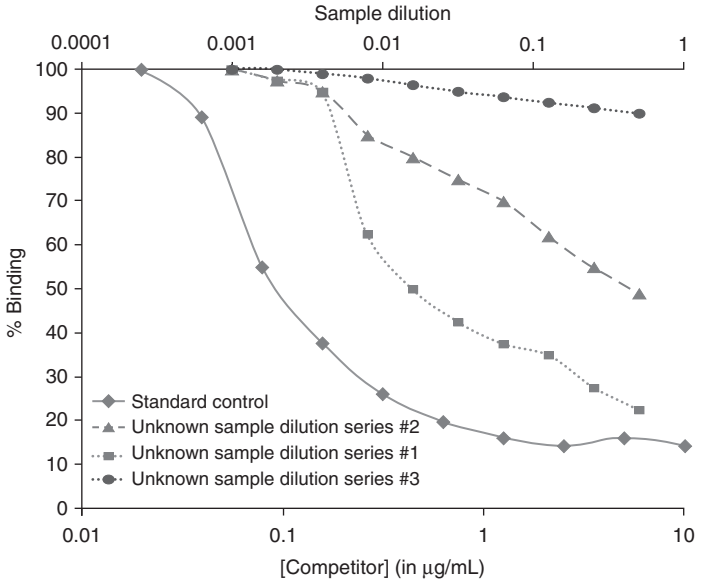

FIGURE 2. Competitive ELISA for antigen detection.

DISCUSSION

Determination of the analyte detection limit is facilitated by this method in which titration series prepared from differently diluted unknown samples are analyzed by competition against known amounts of antigen. Figure 2 shows a decreased percentage in binding of the antibody to the plate-immobilized antigen in the presence of increasing free antigen (bottom *x*-axis). Similarly, a titration series of an unknown sample from different starting dilutions (top *x*-axis) indicates the presence of antigen competitor within the sample as indicated by reduced binding of the antibody to the immobilized antigen.

RECIPES

Carbonate Buffer for Immunoassays

Reagent	Amount per 1 L of solution
Na_2CO_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_2HPO_4	1.44 g
KH_2PO_4	0.24 g

Store at ambient temperature.

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 enzyme-linked immunosorbent assay (ELISA). *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot093708.

Indirect Competitive Enzyme-Linked Immunosorbent Assay (ELISA)

Thomas O. Kohl and Carl A. Ascoli

Cold Spring Harb Protoc; doi: 10.1101/pdb.prot093757