Protocol

Direct Competitive Enzyme-Linked Immunosorbent Assay (ELISA)

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The competitive enzyme-linked immunosorbent assay (ELISA) (cELISA; also called an inhibition ELISA) is designed so that purified antigen competes with antigen in the test sample for binding to an antibody that has been immobilized in microtiter plate wells. The same concept works if the immobilized molecule is antigen and the competing molecules are purified labeled antibody versus antibody in a test sample. Direct cELISAs incorporate labeled antigen or antibody, whereas indirect assay configurations use reporter-labeled secondary antibodies. The cELISA is very useful for determining the concentration of small-molecule antigens in complex sample mixtures. In the direct cELISA, antigen-specific capture antibody is adsorbed onto the microtiter plate before incubation with either known standards or unknown test samples. Enzyme-linked antigen (i.e., labeled antigen) is also added, which can bind to the capture antibody only when the antibody's binding site is not occupied by either the antigen standard or antigen in the test samples. Unbound labeled and unlabeled antigens are washed away and substrate is added. The amount of antigen in the standard or the test sample determines the amount of reporter-labeled antigen bound to antibody, yielding a signal that is inversely proportional to antigen concentration within the sample. Thus, the higher the antigen concentration in the test sample, the less labeled antigen is bound to the capture antibody, and hence the weaker is the resultant signal.

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 HRP-based assays)
Antigen diluent solution (1% [v/v] fish gel [Sigma-Aldrich, #G7765] in PBS or TBS)
Antigens, reporter-labeled
Blocking buffer (3% [w/v] fish gel in PBS or TBS)
Capture antibody, purified, or host-specific secondary antibody (see note at Step 1)
Carbonate buffer for immunoassays <R> (optional; see Step 1)
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> (optional; for horseradish peroxidase [HRP]-based assays)
Stop solution (1.0 M HCl) (optional; for HRP-based assays only; see Step 11)
Test samples containing the antigen(s) of interest
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 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 purified capture antibody in PBS or carbonate buffer to an optimal concentration that was previously determined by checkerboard titration of the capture antibody against the antigen (see Protocol: **Indirect Immunometric Enzyme-Linked Immunosorbent Assay (ELISA)** [Kohl and Ascoli 2017a]). Add 100 µL of diluted antibody to each well.

   If purified primary capture antibody is unavailable, coat the wells with a purified host-specific secondary antibody directed against the capture antibody before adding the antigen-specific capture antibody.

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 antibody 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 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.

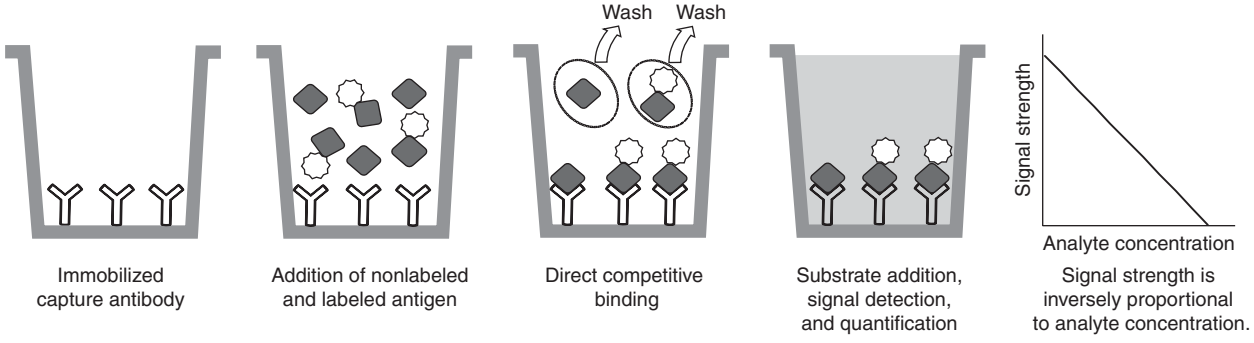

FIGURE 1. Direct competitive ELISA for determining the degree of antigen competition in a sample.

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 at 2°C–8°C for up to 2 wk 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.

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6. Prepare antigen standards by dilution in 1% (v/v) fish gel. Apply 50 µL to select coated wells. (These standards will be used in the preparation of a standard curve.) Antigen blanks and assay blanks do not receive any antigen solution.

   For an accurate determination of antigen concentration in unknown samples, dilutions of the antigen standard need to cover the dynamic range of the assay, defined as the range in which the sensitivity of the immunoassay is determined by the interplay between antigen concentration and signal strength. A derived standard curve allows for the accurate determination of antigen concentrations within unknown samples. Standards and unknown samples should be analyzed in duplicate or triplicate.

7. Prepare dilutions of the test sample(s) in 1% (v/v) fish gel. Add 50 µL to selected wells containing capture antibody.

8. Add 50 µL of reporter-labeled standard antigen in 1% (v/v) fish gel to the wells used in Step 7. Mix thoroughly. Incubate the samples for 2 h at room temperature.

   Determine the optimal working concentration that results in maximum signal strength by checkerboard titration of the reporter-labeled antigen standard (see Protocol: ***Indirect Immunometric ELISA*** *[Kohl and Ascoli 2017a]).*

9. Aspirate the antigen solution using the plate washer. Wash the plate as described in Step 3.

10. Prepare the substrate solution (TMB for HRP or pNPP for AP) or equilibrate it to room temperature. Add 100 µL to each of the wells. 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.

11. Stop the HRP reaction by adding 100 µL of 1 M HCl per well to each of the wells. Alkaline phosphatase-based substrate development does not require a stopping reagent.

    For further quantification of low-level reactions, skip Step 11 and perform plate measurements at set time intervals.

12. 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.

13. Prepare a standard curve from the data by plotting standard antigen concentration on the *x*-axis versus absorbance on the *y*-axis. Use this standard curve to 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.

RELATED INFORMATION

For an indirect assay configuration using reporter-labeled secondary antibodies, see Protocol: **Indirect Competitive Enzyme-Linked Immunosorbent Assay (ELISA)** (Kohl and Ascoli 2017b).

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.

RECIPES

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. 2017a. Indirect immunometric enzyme-linked immunosorbent assay (ELISA). *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot093708.

Kohl TO, Ascoli CA. 2017b. Indirect competitive enzyme-linked immunosorbent assay (ELISA). *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot093757.

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