ELISA Protocol

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Abstract

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying peptides, proteins, antibodies, and hormones. In an ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measurable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction.

This protocol describes the steps for performing sandwich ELISA.

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Protocol

Sample Preparation

Step 1.

The procedure below provides a general guide for preparing commonly tested samples used in ELISA assays. Please check the literature for experiments similar to yours for your new assay development. In this protocol, the following samples are covered:

1. Cell Culture Supernatant
2. Cell Culture (Conditioned) Media
3. Cell Lysate
4. Tissue Homogenate
5. Tissue (Bone)
6. Serum
7. Plasma
8. Urine
9. Saliva
10. Milk

NOTES
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General tips for ELISA sample preparation:

- Serum, plasma, cell and tissue extracts are typically diluted by 50% with binding buffer.
- Total protein concentration of homogenate should be at least 1 mg/mL. However, 2 mg/mL or more would be better.
- The collected samples can be kept for different periods: 48 hours (2-8°C), 1 month (-20°C), or 6 months (-70°C).
- The protein concentration of your lysates can be determined by a total protein assay not inhibited by detergents such as the Bicinchoninic acid (BCA) assay. The volume of each sample can also be normalized to deliver the same amount of total protein for each assay.

Sample Preparation - Cell Culture Supernatant

Step 2.

Centrifuge cell culture media at 1,500 rpm and 4°C for 10 minutes.

TEMPERATURE

4 °C Additional info:

Sample Preparation - Cell Culture Supernatant

Step 3.

Assay immediately or aliquot supernatant and hold at -80°C. Avoid freeze/thaw cycles.

TEMPERATURE

-80 °C Additional info:

Sample Preparation - Cell Culture (Conditioned) Media

Step 4.

Since serum tends to contain cytokines which may produce significant background signals, we suggest the preparation of serum-free or low-serum medium samples. If it is necessary to test serum containing medium, we also suggest running an uncultured medium blank to obtain the baseline signals which can then be subtracted from the cultured media sample data.

Sample Preparation - Cell Culture (Conditioned) Media - Day 1

Step 5.

Plate cells in complete growth media (with serum) until the desired level of confluence is achieved.

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The optimal number of seeded cells varies from one cell type to another and may need to be empirically determined. We suggest seeding around 1 million cells in 100 mm tissue culture plate with complete growth medium.

Sample Preparation - Cell Culture (Conditioned) Media - Day 4

Step 6.

Remove growth media and gently wash cells using 2-3 mL of warm PBS.

Sample Preparation - Cell Culture (Conditioned) Media - Day 4

Step 7.

Repeat the wash step.

Sample Preparation - Cell Culture (Conditioned) Media - Day 4

Step 8.

Remove PBS and replace the medium with serum-free or low serum containing medium (e.g. medium containing 0.2% calf serum).

Sample Preparation - Cell Culture (Conditioned) Media - Day 4

Step 9.

Incubate for 1-2 days.

Sample Preparation - Cell Culture (Conditioned) Media - Day 6

Step 10.

Collect medium.

Sample Preparation - Cell Culture (Conditioned) Media - Day 6

Step 11.

Centrifuge at 1,500 rpm and 4°C for 10 minutes.

TEMPERATURE

4 °C Additional info:

Sample Preparation - Cell Culture (Conditioned) Media - Day 6

Step 12.

Aliquot the supernatant and keep it at -80ºC until experiment. Avoid freeze/thaw cycles. Most samples prepared this way can be stored for at least 1 year.

TEMPERATURE

-80 °C Additional info:

Sample Preparation - Cell Lysate

Step 13.

Collect and rinse cells in PBS.

Sample Preparation - Cell Lysate

Step 14.

Homogenize and lyse cells thoroughly in lysis buffer (e.g. Mammal Cell Protein Extraction Reagent: AR0103, Boster Bio).

REAGENTS

Mammal Cell Protein Extraction Reagent AR0103 by Boster Bio

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Guidelines on lysis buffer

- Avoid more than 0.1% SDS or other strongly denaturing detergents. In general, non-ionic detergents such as Triton X-100 or NP-40 are best. Zwitterionic detergents such as CHAPS or mild ionic detergents such as sodium deoxycholate will work.
- Do not use more than 2% v/v total detergent.
- Avoid the use of sodium azide.
- Avoid more than 10 mM reducing agents (e.g. dithiothreitol, mercaptoethanols).

Sample Preparation - Cell Lysate

Step 15.

Centrifuge cell lysate at approximately 10,000 x g and 4°C for 5 minutes.

TEMPERATURE

4 °C Additional info:

Sample Preparation - Cell Lysate

Step 16.

Assay immediately or aliquot supernatant and hold at -80°C. Avoid freeze/thaw cycles.

TEMPERATURE

-80 °C Additional info:

Sample Preparation - Tissue Homogenate

Step 17.

Rinse tissue with PBS to remove excess blood.

Sample Preparation - Tissue Homogenate

Step 18.

Chop tissue into 1-2 mm pieces on ice in ice-cold buffer. Keep on ice for immediate homogenization or at -80°C for later use.

Sample Preparation - Tissue Homogenate

Step 19.

Prepare the extraction buffer. It can be prepared ahead of time and stored at 4°C.

- 100 mM Tris, pH 7.4
- 150 mM NaCl
- 1 mM EGTA
- 1 mM EDTA
- 1% Triton X-100 0.5%
- 0.5% sodium deoxycholate

Sample Preparation - Tissue Homogenate

Step 20.

Immediately before use, the extraction buffer must be supplemented with the following to generate a complete extraction buffer:

- Phosphatase inhibitor cocktail (as directed by manufacturer)
- Protease inhibitor cocktail (as directed by manufacturer)
- PMSF (Phenyl Methyl Sulfonyl Floride) to 1 mM

Sample Preparation - Tissue Homogenate

Step 21.

For every 0.1 mg of tissue, add 500 µL of complete extraction buffer to the tube and homogenize.

Sample Preparation - Tissue Homogenate

Step 22.

Rinse the blade of the homogenizer 2X with 500 µL extraction buffer.

Sample Preparation - Tissue Homogenate

Step 23.

Place the sample on a shaker at 4°C for 1 hour.

TEMPERATURE

4 °C Additional info:

Sample Preparation - Tissue Homogenate

Step 24.

Centrifuge the sample at approximately 10000 X g for 5 minutes.

Sample Preparation - Tissue Homogenate

Step 25.

Assay immediately or aliquot supernatant (soluble protein extract) and hold at -80°C. Avoid freeze/thaw cycles.

TEMPERATURE

-80 °C Additional info:

Sample Preparation - Tissue (Bone)

Step 26.

Extract de-mineralized bone samples in 4M Guanidine-HCl and protease inhibitors.

Sample Preparation - Tissue (Bone)

Step 27.

Dissolve the final sample in 2M Guanidine-HCl.

Sample Preparation - Serum

Step 28.

Collect whole blood into a tube without additives.

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Hemolysis should be avoided while collecting serum samples. Samples that have undergone hemolysis may increase non-specific staining in HRP-conjugated ELISA assays.

Sample Preparation - Serum

Step 29.

Keep the blood at room temperature for 30 minutes.

Sample Preparation - Serum

Step 30.

Centrifuge at 3,000 rpm and 4°C for 10 minutes.

TEMPERATURE

4 °C Additional info:

Sample Preparation - Serum

Step 31.

Assay immediately or aliquot supernatant (serum) and hold at -80°C. Avoid freeze/thaw cycles.

TEMPERATURE

-80 °C Additional info:

Sample Preparation - Plasma

Step 32.

Collect whole blood into a tube containing anticoagulant. Different proteins may require different anticoagulants. See datasheet for details on which anticoagulant to use.

Sample Preparation - Plasma

Step 33.

Centrifuge at 3,000 rpm and 4°C for 10 minutes.

TEMPERATURE

4 °C Additional info:

Sample Preparation - Plasma

Step 34.

Assay immediately or aliquot supernatant (plasma) and hold at -80°C. Avoid freeze/thaw cycles.

TEMPERATURE

-80 °C Additional info:

Sample Preparation - Urine

Step 35.

Collect urine into a sterile or disposable container. Fresh urine samples must be used immediately or saved to avoid reproduction of bacteria which produce endogenous HRP that may potentially give false positive results.

Sample Preparation - Urine

Step 36.

Centrifuge sample at 10,000 x g for 1 minute.

Sample Preparation - Urine

Step 37.

Assay immediately or aliquot supernatant and hold at -80°C. Avoid freeze/thaw cycles.

TEMPERATURE

-80 °C Additional info:

Sample Preparation - Saliva

Step 38.

Collect saliva using a collection device without any protein binding or filtering capabilities (e.g.

Salivette).

Sample Preparation - Saliva

Step 39.

Centrifuge at 10,000 x g and 4°C for 2 minutes.

TEMPERATURE
4 °C Additional info:

Sample Preparation - Saliva

Step 40.

Assay immediately or aliquot supernatant and hold at -80°C. Avoid freeze/thaw cycles.

TEMPERATURE
-80 °C Additional info:

Sample Preparation - Milk

Step 41.

Centrifuge at 1500 x g and 4°C for 15 minutes.

TEMPERATURE
4 °C Additional info:

Sample Preparation - Milk

Step 42.

Collect the aqueous fraction and repeat this process 3 times.

Sample Preparation - Milk

Step 43.

Filter through a 0.2 µm filter.

Sample Preparation - Milk

Step 44.

Assay immediately or aliquot supernatant and hold at -80°C. Avoid freeze/thaw cycles.

TEMPERATURE
-80 °C Additional info:

Reagent Preparation - Standard Solutions

Step 45.

- 10,000 pg/mL: Add 1 mL of sample diluent buffer into one tube of standard (10 ng per tube) and mix thoroughly. Store this solution at 4°C for up to 12 hours (or -20°C for 48 hours) and avoid freeze-thaw cycles.
- 5,000 pg/mL: Mix 0.3 mL of 10,000 pg/mL with 0.3 mL of sample diluent buffer and mix thoroughly.
- 2,500 pg/mL: Mix 0.3 mL of 5,000 pg/mL with 0.3 mL of sample diluent buffer and mix thoroughly.
- Perform similar dilutions until the standard solutions with these concentrations (pg/mL) are

made: 1250, 625, 312, 156, and 78.
- Add 100 µL of each of the diluted standard solutions to the appropriate empty wells. Repeat in duplicate or triplicate for accuracy.

NOTES
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The standard solutions are best used within 2 hours.

Reagent Preparation - Biotinylated Antibody

Step 46.

- Calculate the total volume needed for the assay by multiplying 0.1 mL/well and the number of wells required. Add 2-3 extra wells to the calculated number of wells to account for possible pipetting errors.
- Generate the required volume of diluted antibody by performing a 1:100 dilution (For each 1 µL concentrated antibody, add 99 µL antibody dilution buffer) and mixing thoroughly.

Reagent Preparation - Avidin-Biotin-Peroxidase Complex (ABC)

Step 47.

- Calculate the total volume needed for the assay by multiplying 0.1 mL/well and the number of wells required. Add 2-3 extra wells to the calculated number of wells to account for possible pipetting errors.
- Generate the required volume of diluted ABC solution by performing a 1:100 dilution (For each 1 µL concentrated ABC solution, add 99 µL ABC dilution buffer) and mixing thoroughly.

NOTES
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The diluted ABC solution should not be prepared more than 1 hour prior to the experiment.

Sandwich ELISA Protocol

Step 48.

All of the ELISA kits from Boster use the sandwich format and biotin-streptavidin chemistry. Our ELISA assays require the dilutions of standard solutions, biotinylated antibody (detection antibody), and avidin-biotin-peroxidase complex.

Sandwich ELISA Protocol - Capture Antibody Coating (Not required if using Boster's pre-adsorbed PicoKine ELISA kits)

Step 49.

Dilute the capture antibody to a final concentration of 1-10 µg/mL in bicarbonate/carbonate antigen-coating buffer (100 mM $NaHCO_3$ in deionized water; pH adjusted to 9.6).

Sandwich ELISA Protocol - Capture Antibody Coating (Not required if using Boster's pre-adsorbed PicoKine ELISA kits)

Step 50.

Pipette 100 µL of diluted antibody to each well of a microtiter plate.

Sandwich ELISA Protocol - Capture Antibody Coating (Not required if using Boster's pre-adsorbed PicoKine ELISA kits)

Step 51.

Cover the plate with adhesive plastic and incubate at 4°C overnight (or 37°C for 30 minutes).

Sandwich ELISA Protocol - Capture Antibody Coating (Not required if using Boster's pre-adsorbed PicoKine ELISA kits)

Step 52.

Remove the coating solution and wash the plate 3X with 200 µL PBS (Phosphate Buffered Saline) buffer (10 mM Na2HPO4 and 1.8 mM NaH2PO4 in deionized water with 0.2% Tween 20; pH Adjusted to 7.4) for 5 minutes each time. The coating/washing solutions can be removed by flicking the plate over a sink. The remaining drops can be removed by patting the plate on a paper towel or by aspiration. Do not allow the wells to dry out at any time.

Sandwich ELISA Protocol - Blocking (Not required if using Boster's pre-adsorbed PicoKine ELISA kits)

Step 53.

Pipette 200 µL blocking buffer (5% w/v non-fat dry milk in PBS buffer) per well to block residual protein-binding sites. Alternatively, BSA or BlockACE can be used to replace non-fat dry milk.

Sandwich ELISA Protocol - Blocking (Not required if using Boster's pre-adsorbed PicoKine ELISA kits)

Step 54.

Cover the plate with adhesive plastic and incubate for 1-2 hours at 37°C (or at 4°C overnight).

Sandwich ELISA Protocol - Blocking (Not required if using Boster's pre-adsorbed PicoKine ELISA kits)

Step 55.

Remove the blocking solution and wash the plate 2X with 200 µL PBS for 5 minutes each time. Flick the plate and pat the plate as described in the coating step.

Sandwich ELISA Protocol - Reagent Preparation

Step 56.

Prepare the diluted standard solutions, biotinylated antibody and ABC solutions as described in the above Reagent Preparation section.

GOTO

ABC Solution Preparation -> go to step #47

Sandwich ELISA Protocol - Sample (Antigen) Incubation

Step 57.

Serially dilute the sample with blocking buffer immediately before use. The optimal dilution should be determined by a titration assay according to the antibody manufacturer.

Sandwich ELISA Protocol - Sample (Antigen) Incubation

Step 58.

Pipette 100 µL of each of the diluted sample solutions and control to each empty well. Repeat in duplicate or triplicate for accuracy. The negative control should be species- and isotype-matched as well as non-specific immunoglobulin diluted in PBS buffer.

Sandwich ELISA Protocol - Sample (Antigen) Incubation

Step 59.

Cover the plate with adhesive plastic and incubate for 2 hours at room temperature.

Sandwich ELISA Protocol - Sample (Antigen) Incubation

Step 60.

Remove the content in the wells and wash them 3X with 200 µL PBS buffer for 5 minutes each time. Flick the plate and pat the plate as described in the coating step.

Sandwich ELISA Protocol - Biotinylated Antibody Incubation

Step 61.

Pipette 100 µL of diluted antibody to the wells with the control, the standard solutions, and the diluted samples.

Sandwich ELISA Protocol - Biotinylated Antibody Incubation

Step 62.

Cover the plate with adhesive plastic and incubate for 1 hour at 37°C (or 2 hours at room temperature). These incubation times should be sufficient to receive a strong signal. However, if a weak signal is observed, perform incubation overnight at 4°C for a stronger signal.

Sandwich ELISA Protocol - Biotinylated Antibody Incubation

Step 63.

Remove the content in the wells and wash them 3X with 200 µL PBS for 5 minutes each time. Flick the plate and pat the plate as described in the coating step.

Sandwich ELISA Protocol - ABC Incubation

Step 64.

Pipette 100 µL of diluted ABC solution to the wells with the control, the standard solutions, and the diluted samples.

Sandwich ELISA Protocol - ABC Incubation

Step 65.

Cover the plate with adhesive plastic and incubate for 0.5 hour at 37°C.

TEMPERATURE

37 °C Additional info:

Sandwich ELISA Protocol - ABC Incubation

Step 66.

Remove the content in the wells and wash them 3X with 200 µL PBS buffer for 5 minutes each time. Flick the plate and pat the plate as described in the coating step.

Sandwich ELISA Protocol - Substrate Preparation

Step 67.

Prepare the substrate solution immediately before use or bring the pre-made substrate to room temperature. The two widely used enzymes for signal detection are horseradish peroxidase (HRP) and alkaline phosphatase (AP), and their corresponding substrates, stopping solutions, detection absorbance wavelengths and color developed are as follows:

Enzyme	Substrate*	Stop Solution	Absorbance (nm)	Color Developed
HRP	TMB	2M H_2SO_4	450	Yellow
AP	pNPP	0.75M NaOH	405	Yellow

* TMB: 3,3’,5,5’-tetramethylbenzidine; pNPP: p-nitrophenyl-phosphate

NOTES

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- The TMB substrate must be kept at 37°C for 30 minutes before use.
- Hydrogen peroxide can also act as a substrate for HRP.
- Sodium azide is an inhibitor of HRP. Do not include the azide in buffers or wash solutions if HRP-labeled conjugate is used for detection.

Sandwich ELISA Protocol - Signal Detection

Step 68.

Pipette 90 µL of substrate solution to the wells with the control, the standard solutions, and the diluted samples.

Sandwich ELISA Protocol - Signal Detection

Step 69.

Incubate the plate at 37°C in the dark. If TMB is used, shades of blue will be observed in the wells with the most concentrated solutions. Other wells may show no obvious color.

TEMPERATURE

37 °C Additional info:

Sandwich ELISA Protocol - Signal Detection

Step 70.

Color should be developed in positive wells after 15 minutes. After sufficient color development, pipette 100 µL of stop solution to the appropriate wells (if necessary).

Sandwich ELISA Protocol - Signal Detection

Step 71.

Read the absorbance (OD: Optical Density) of each well with a plate reader.

Sandwich ELISA Protocol - Data Analysis

Step 72.

Prepare a standard curve using the data produced from the diluted standard solutions. Use absorbance on the Y-axis (linear) and concentration on the X-axis (log scale).

Sandwich ELISA Protocol - Data Analysis

Step 73.

Interpret the sample concentration from the standard curve.