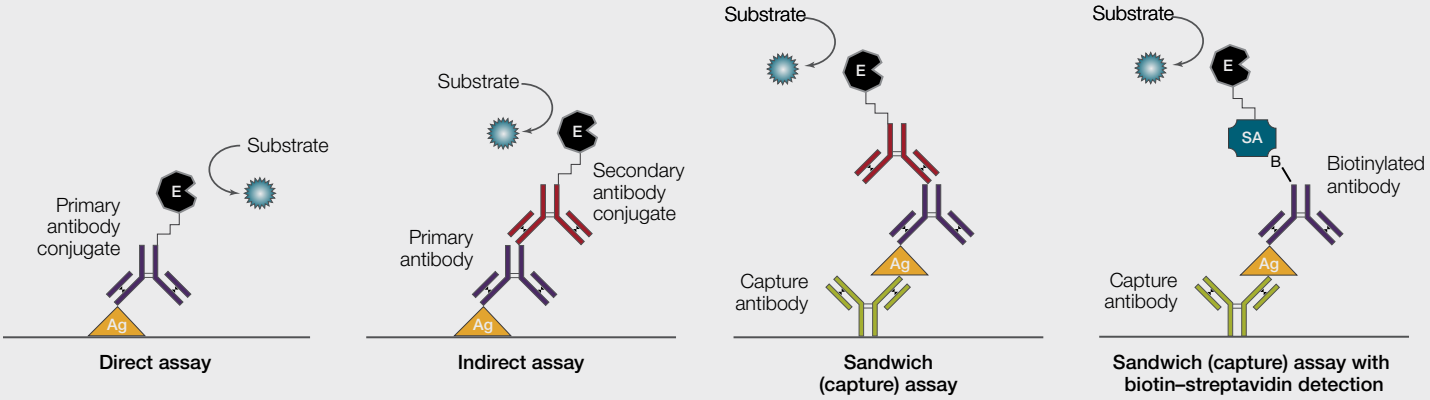

Protein biology

General ELISA protocols

This document provides general protocols for performing a sandwich ELISA using colorimetric, chemiluminescent, and fluorescent detection. Guidance is provided for direct and indirect assays.

Colorimetric sandwich ELISA protocol

This protocol represents an example of a colorimetric sandwich ELISA using indirect detection with a biotinylated antibody and streptavidin–HRP. TMB (tetramethyl benzene) is the HRP substrate used for detection.

Materials required	Suggested products	Cat. No.
Clear 96-well plate	Thermo Scientific™ Pierce™ 8-well strip polystyrene microplates	15031
Coating buffer (50 mM carbonate buffer, pH 9.4, or 10 mM phosphate buffer, pH 7.4)	Thermo Scientific™ BupH™ Carbonate-Bicarbonate Buffer Packs	28382
Blocking buffer	Thermo Scientific™ SuperBlock™ (TBS) Blocking Buffer	37535
Wash buffer (TBS or PBS with 0.05% Tween 20 detergent)	Thermo Scientific™ Pierce™ 20X TBS Tween™ 20 Buffer or Pierce™ 20X PBS Tween™ 20 Buffer	28360 or 28352
Plate sealers	Thermo Scientific™ Sealing Tape for 96-Well Plates	15036
Reagent reservoirs	Thermo Scientific™ ELISA Reagent Reservoirs	15075
Streptavidin–HRP	Thermo Scientific™ Pierce™ High Sensitivity Streptavidin–HRP	21130
TMB substrate solution	Thermo Scientific™ 1-Step™ Turbo TMB-ELISA Substrate Solution	34022
Stop solution (0.16 M sulfuric acid)	Thermo Scientific™ Stop Solution for TMB Substrates	N600
Additional materials required		
Capture (coating) antibody, biotinylated detection antibody, standards and samples, distilled or deionized water, absorbance-based microplate reader (e.g., Thermo Scientific™ Multiskan™ FC Microplate Photometer)		

Procedure

1. Prepare coating solution by diluting the capture antibody in coating buffer. Refer to “Antibody dilution recommendations” (page 5) or the manufacturer’s instructions.
2. Coat plate with 100 µL per well of coating solution. Cover plate and incubate 1 hr at room temperature or overnight (12–18 hr) at 2–8°C.
3. Aspirate contents and wash wells 1 time with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
4. Block plate with 300 µL per well of blocking buffer for 1 hr at room temperature.
5. Aspirate blocking buffer, then invert and tap plate on absorbent paper to remove excess liquid.
6. Prepare standards and sample dilutions in blocking buffer.
7. Pipette 100 µL of standards (in duplicate) and samples into designated wells. Incubate for 1–2 hr at room temperature with gentle continual shaking (~500 rpm).
8. Aspirate contents and wash wells 5 times with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
9. Prepare detection antibody solution by diluting the detection antibody in blocking buffer. For the recommended antibody dilution, refer to the manufacturer’s instructions.
10. Add 100 µL of the detection antibody solution to each well. Incubate for 2 hr at room temperature with gentle continual shaking (~500 rpm).
11. Aspirate contents and wash wells 5 times with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
12. Make a working solution of streptavidin–HRP with blocking buffer by diluting 1:5,000. For example, to make enough for 1 plate, add 2 µL of streptavidin–HRP to 9.998 mL of blocking buffer.
13. Add 100 µL of working streptavidin–HRP solution to each well. Incubate for 1 hr at room temperature with gentle continual shaking (~500 rpm).
14. Aspirate contents and wash wells 5 times with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
15. Add 100 µL of TMB substrate solution to each well. Incubate plate for 30 min at room temperature or until the desired color intensity is reached.
16. Add 100 µL of stop solution to each well.
17. Measure absorbance at 450 nm within 30 min of adding stop solution.
18. Calculate results using a log–log or 4-parameter curve fit.

Using an alkaline phosphatase system

If alkaline phosphatase (AP) is to be used instead of HRP for the enzyme conjugate, an AP-specific substrate must be used. Substitute the TMB substrate solution in step 15 with p-nitrophenyl phosphate (PNPP) (Thermo Scientific™ 1-Step™ PNPP Substrate Solution, Cat. No. 37621). Incubate at room temperature for 15–30 min. Substitute the stop solution with 50 µL 2 N NaOH to stop the reaction. Measure absorbance of each well at 405 nm.

Chemiluminescent sandwich ELISA protocol

This protocol represents an example of a chemiluminescent sandwich ELISA using direct detection with an HRP-conjugated antibody, or indirect detection with a biotinylated antibody and streptavidin–HRP. A luminol-based substrate is used for detection.

Materials required	Suggested products	Cat. No.
Opaque 96-well plate*	Thermo Scientific™ Pierce™ 96-well polystyrene plates, white opaque	15042
Coating buffer (50 mM carbonate buffer, pH 9.4, or 10 mM phosphate buffer, pH 7.4)	Thermo Scientific™ BupH™ Carbonate-Bicarbonate Buffer Packs	28382
Blocking buffer	Thermo Scientific™ StartingBlock™ T20 TBS Blocking Buffer or StartingBlock™ T20 PBS Blocking Buffer	37543 or 37539
Wash buffer (TBS or PBS with 0.05% Tween 20 detergent)	Thermo Scientific™ Pierce™ 20X TBS Tween™ 20 Buffer or Pierce™ 20X PBS Tween™ 20 Buffer	28360 or 28352
Chemiluminescent substrate	Thermo Scientific™ SuperSignal™ ELISA Pico Chemiluminescent Substrate	37070
Streptavidin–HRP (if detection antibody is biotinylated)	Thermo Scientific™ HRP-Conjugated Streptavidin	N100
Reagent reservoirs	Thermo Scientific™ ELISA Reagent Reservoirs	15075
Plate sealers	Thermo Scientific™ Sealing Tape for 96-Well Plates	15036
Additional materials required		
Capture (coating) antibody, detection antibody, distilled or deionized water, luminometer-based microplate reader (e.g., Thermo Scientific™ Luminoskan™ Microplate Luminometer)		

* **Tip:** White or black plates can be used for chemiluminescent detection. White plates typically display higher signal than black plates, and black plates should be used when background signal is an issue.

Procedure

1. Prepare coating solution by diluting the capture antibody in coating buffer. Refer to “Antibody dilution recommendations” (page 5) or the manufacturer’s instructions.
2. Coat plate with 100 µL per well of coating solution. Cover plate and incubate 1 hr at room temperature or overnight (12–18 hr) at 2–8°C.
3. Aspirate contents and wash wells 1 time with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
4. Block plate with 300 µL per well of blocking buffer for 1 hr at room temperature.
5. Aspirate blocking buffer, then invert and tap plate on absorbent paper to remove excess liquid.
6. Prepare standards and sample dilutions in blocking buffer.
7. Pipette 100 µL of standards (in duplicate) and samples into designated wells. Incubate for 1–2 hr at room temperature with gentle continual shaking (~500 rpm).
8. Aspirate contents and wash wells 5 times with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
9. Prepare detection antibody solution by diluting detection antibody in blocking buffer. For the recommended antibody dilution, refer to the manufacturer’s instructions.
10. Add 100 µL of the detection antibody solution to each well. Incubate for 2 hr at room temperature with gentle continual shaking (~500 rpm).
11. Aspirate contents and wash wells 5 times with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid. If the detection antibody is HRP-conjugated, proceed to step 15.
12. If the detection antibody is biotinylated: Make a working solution of streptavidin–HRP with blocking buffer by diluting 1:5,000–1:20,000. For example, to make enough for 1 plate, add 2 µL of streptavidin–HRP to 9.998 mL of blocking buffer. The optimal dilution should be determined empirically.
13. If the detection antibody is biotinylated: Add 100 µL of working streptavidin–HRP solution to each well. Incubate for 1 hr at room temperature with gentle continual shaking (~500 rpm).
14. If the detection antibody is biotinylated: Aspirate contents and wash wells 5 times with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
15. Make a working solution of chemiluminescent substrate solution by mixing equal parts of luminol and stable peroxide solution.
16. Add 100 µL of working chemiluminescent substrate solution to each well. Incubate for 1 min at room temperature.
17. Use a luminometer to measure relative light units (~425 nm) from 1 to 5 min after adding the substrate. Longer periods between adding the substrate and measuring the plate may result in decreased signal intensity.

Using an alkaline phosphatase system

If alkaline phosphatase (AP) is to be used instead of HRP for the enzyme conjugate, an AP-specific substrate must be used. Substitute the working luminol substrate solution in step 16 with 100 µL Invitrogen™ CDP-Star™ Substrate (0.4 mM Ready-To-Use, with Sapphire-II™ Enhancer, Cat. No. T2214). Incubate at room temperature for 5–10 min and then measure at 5-min intervals until light emission has peaked (usually 20–30 min after substrate addition).

Fluorescent sandwich ELISA protocol

This protocol represents an example of a fluorescent sandwich ELISA using direct detection with an HRP-conjugated antibody, or indirect detection with a biotinylated antibody and streptavidin–HRP. A fluorogenic peroxidase substrate is used for detection.

Materials required	Suggested products	Cat. No.
Black 96-well plate	Thermo Scientific™ Black 96-Well Immuno Plates	437111
Coating buffer (50 mM carbonate buffer, pH 9.4, or 10 mM phosphate buffer, pH 7.4)	Thermo Scientific™ BupH™ Carbonate-Bicarbonate Buffer Pack	28382
Blocking buffer	Thermo Scientific™ StartingBlock™ T20 TBS Blocking Buffer or StartingBlock™ T20 PBS Blocking Buffer	37543 or 37539
Wash buffer (TBS or PBS with 0.05% Tween 20 detergent)	Thermo Scientific™ Pierce™ 20X TBS Tween™ 20 Buffer or Pierce™ 20X PBS Tween™ 20 Buffer	28360 or 28352
Fluorogenic peroxidase substrate	Thermo Scientific™ QuantaBlu™ Fluorogenic Peroxidase Substrate Kit	15169
Streptavidin–HRP (if detection antibody is biotinylated)	Thermo Scientific™ HRP-Conjugated Streptavidin	N100
Reagent reservoirs	Thermo Scientific™ ELISA Reagent Reservoirs	15075
Plate sealers	Thermo Scientific™ Sealing Tape for 96-Well Plates	15036
Additional materials required		
Capture (coating) antibody, detection antibody, distilled or deionized water, microplate fluorometer (e.g., Thermo Scientific™ Fluoroskan™ FL Microplate Fluorometer and Luminometer)		

Procedure

1. Prepare coating solution by diluting the capture antibody in coating buffer to 5–10 µg/mL.
2. Coat plate with 50–100 µL per well of coating solution. Cover plate and incubate 1 hr at room temperature or overnight (12–18 hr) at 2–8°C.
3. Aspirate contents and wash wells 1 time with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
4. Block plate with 300 µL per well of blocking buffer for 1 hr at room temperature.
5. Aspirate blocking buffer, then invert and tap plate on absorbent paper to remove excess liquid.
6. Prepare standards and sample dilutions in blocking buffer.
7. Pipette 100 µL of standards (in duplicate) and samples into designated wells. Incubate for 1–2 hr at room temperature with gentle continual shaking (~500 rpm).
8. Aspirate contents and wash wells 5 times with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
9. Prepare detection antibody solution by diluting detection antibody to 0.05–0.1 µg/mL in wash buffer.
10. Add 100 µL of the detection antibody solution to each well. Incubate for 1–2 hr at room temperature with gentle continual shaking (~500 rpm).
11. Aspirate contents and wash wells 5 times with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid. If the detection antibody is HRP-conjugated, proceed to step 15.
12. If the detection antibody is biotinylated: Make a working solution of streptavidin–HRP in wash buffer by diluting to 0.05–0.1 µg/mL. The optimal dilution should be determined empirically.
13. If the detection antibody is biotinylated: Add 50–100 µL of working streptavidin–HRP solution to each well. Incubate for 1 hr at room temperature with gentle continual shaking (~500 rpm).
14. If the detection antibody is biotinylated: Aspirate contents and wash wells 5 times with 300 µL of wash buffer per well. Following the wash, invert and tap plate on absorbent paper to remove excess liquid.
15. Make a working solution of QuantaBlu substrate by mixing 9 parts of QuantaBlu Substrate Solution and 1 part of QuantaBlu Stable Peroxide Solution.
16. Add 100 µL of working QuantaBlu solution to each well. Incubate for 1.5–90 min at room temperature or 37°C.
17. Add 100 µL of QuantaBlu Stop Solution. The enzymatic activity is immediately stopped (incubation is not required).
18. Avoid bubbles, which cause light scattering and erroneous signals. Briefly centrifuge the microplate, or pop large bubbles with a pipette tip.
19. Measure relative fluorescence. The excitation and emission maxima for the QuantaBlu substrate are 325 nm and 420 nm. Wavelengths between 315 and 340 nm for excitation and 370 and 470 nm for emission can also be used for detection.

Using a fluorophore-conjugated detection antibody

With a fluorophore-conjugated detection antibody, no enzyme conjugate or substrate is required. The plate fluorescence can be measured directly after step 11. The working concentration of labeled antibody or protein is typically 2–4 µg/mL.

Performing direct antigen immobilization

When immobilizing the antigen-containing sample directly to the plate, there is obviously no need for a capture antibody. Different concentrations of the sample should be prepared in coating buffer and identical volumes added directly to the plate. The rest of the protocol should be performed as previously described using a detection antibody and enzyme conjugate plus substrate.

Using an enzyme-labeled secondary antibody

When using a nonbiotinylated detection antibody followed by an enzyme-labeled secondary antibody, there will be slightly less amplification of enzyme signal compared to using a biotinylated detection antibody with streptavidin-HRP. Therefore, it may be necessary to use a slightly higher concentration of a secondary antibody–enzyme conjugate than one would normally use for a streptavidin–enzyme conjugate.

Antibody dilution recommendations

The following tables provide recommended ranges for different ELISA components. Concentrations are guidelines only; for best results, optimize each component individually.

Recommended starting concentration ranges for coating and detection antibodies for ELISA optimization. Unpurified antibodies will work but may result in higher background. It is generally recommended that affinity-purified antibodies be used for optimal signal-to-noise ratios.

Source	Coating antibody	Detection antibody
Polyclonal serum	5–15 µg/mL	1–10 µg/mL
Crude ascites	5–15 µg/mL	1–10 µg/mL
Affinity-purified polyclonal antibody	1–12 µg/mL	0.5–5 µg/mL
Affinity-purified monoclonal antibody	1–12 µg/mL	0.5-5 µg/mL

Recommended detection antibody concentrations for different ELISA systems. Check the user guide for the substrate, as it may recommend a more defined concentration range for the enzyme conjugate.

Enzyme	System	Concentration
HRP	Colorimetric system	20–200 ng/mL
	Fluorescent system	25–50 ng/mL
	Chemiluminescent system	10–100 ng/mL
AP	Colorimetric system	100–200 ng/mL
	Chemiluminescent system	40–200 ng/mL

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