Sandwich ELISA Protocol

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[1]BioLegend

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BioLegend
Version 2
Working

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EXTERNAL LINK

https://www.biolegend.com/protocols/sandwich-elisa-protocol/4268/

PROTOCOL STATUS

Working

GUIDELINES

Solutions and Buffers:
Note: Do not use sodium azide in any buffers or solutions, as sodium azide inactivates the horseradish-peroxidase enzyme.

Carbonate Coating Buffer
BioLegend Cat. No. 421701 or…
8.4 g NaHCO3
3.56 g Na2CO3
Add ddH2O up to 1.0 L, pH to 9.5

Phosphate Buffered Saline (PBS):
80.0 g NaCl
14.4 g Na2HPO4
2.4 g KH2PO4
2.0 g KCl
Add ddH2O up to 10 L, pH to 7.2 with HCl

PBS/Tween:
0.5 ml of Tween-20 in 1 L PBS

Blocking Solution:
10% fetal bovine serum or 1% BSA in PBS. Filter before use to remove particulates.

ABTS Substrate Solution:
150 mg 2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma, Cat. No. A-1888)
Add to 500 ml of 0.1M citric acid in ddH2O
Adjust pH to 4.35 with NaOH
Aliquot 11 ml per vial and store at -20˚ C.
Avoid light exposure during preparation and storage.

ABTS Stop Solution:
Combine 50 ml dimethylformamide (DMF; Pierce, Cat. No. 20672) with 50 ml ddH2O
Add 20 g sodium dodecyl sulfate

TMB (tetramethylbenzidine) Substrate Reagent Set:
BioLegend Cat. No. 421101

TMB Stop Solution:
BioLegend Cat. No. 423001 or 1M H3PO4 or 2N H2SO4

General References:

1. Davies, C. 1994. The Immunoassay Handbook. D. Wild, Ed. Stockton Press, New York.
2. Abrams, J.S. 1995. Immunoenzymetric assay of mouse and human cytokines using NIP-labeled anti-cytokine antibodies. Current Protocols in Immunology (J. Coligan, A. Kruisbeek, D. Margulies, E. Shevach, W. Strober, Eds). John Wiley and Sons, New York. Unit 6.20.
3. Sander, B., et al. 1993. J. Immunol. Meth. 166:201.
4. Abrams, J.S., et al. 1992. Immunol. Rev. 127:5.

Cytokine ELISA Troubleshooting Tips

Poor signal-to-noise ratio

• Try Capture Antibody at 1 – 10 µg/ml (generally 2 µg/ml).
• Try Detection Antibody at 0.25 – 2 µg/ml (generally 1 µg/ml).
• Titrate against each other to obtain optimal dilutions.

Low Sensitivity

• Try overnight incubations of standards and samples at 4°C.

Poor Signal

• If using HRP, avoid sodium azide in wash buffers and diluents, as sodium azide inhibits HRP.
• Verify that appropriate antibody pairs were used and the activity of the samples and/or standards.
• Check the activity of enzyme and substrate by coating with Detection Antibody (1 µg/ml), adding biotinylated avidin and revealing with the appropriate substrate. If the enzyme/substrate is active, a strong signal should be observed.

Poor Standard Curve

• Handling Instructions for standards are lot-specific. Refer to product information for proper handling.
• Recombinant protein vials should be quick-spun for maximum recovery.
• BioLegend suggests that cytokines be stored in a concentrated format (>100 ng/ml) and in the presence of a protein carrier.

High Background

• Increase stringency of washing steps by soaking plates for ~1 minute during washes.
• Determine optimum Capture and Detection Antibody dilutions.
• Increase the dilution of Detection Antibody and/or increase the number of washes after Av-HRP incubation.

MATERIALS

NAME	CATALOG #	
Carbonate Coating Buffer	421701	by BioLegend
A1888 SIGMA 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt	A-1888	by Sigma Aldrich
Pierce™ Dimethylformamide (DMF), Sequencing grade	20672	by Thermo Fisher Scientific
TMB (tetramethylbenzidine) Substrate Reagent Set	421101	by BioLegend
TMB Stop Solution	423001	by BioLegend
Av-HRP conjugate	405103	by BioLegend

Coat the Plate

1 Dilute unlabeled capture antibody to a final concentration of 0.5-8µg/ml in Coating Buffer (BioLegend, Cat. No.421701) and transfer 100µl to each well of a high affinity, protein-binding ELISA plate (*e.g.*, BioLegend Cat. No.423501).

2 Seal plate to prevent evaporation. Incubate at 4°C overnight.

Block the Plate

3 Bring the plate to room temperature, flick off the capture antibody solution, wash 3 times with PBS/Tween, and block non-specific binding sites by adding 200µl of Blocking Solution to each well.

Note: You may need to experiment with different blocking solutions, such as gelatin or milk, to find one that will give you the lowest background noise.

4 Seal plate and incubate at room temperature for 1 hour. 01:00:00

5 Wash 3 times with PBS/Tween. Firmly blot plate against clean paper towels.

Add Standards and Samples

6 Dilute standards and samples to desired concentrations in Blocking Solution (perform dilutions in polypropylene tubes or plate) and add 100µl per well to the ELISA plate.

Note: Try to match the diluent of the standards as closely as you can to the matrix in your samples. For example, if your samples are cell culture supernatants, use the same media to dilute the standards.

7 Seal the plate and incubate at room temperature for 2-4 hours or at 4°C overnight.
04:00:00

8 Wash 3 times with PBS/Tween. Washes can be effectively accomplished by filling wells with either a squirt bottle, carboy, manifold dispenser, multi-channel pipettor or automatic plate washer. For increased stringency, after each wash, let the plate stand briefly, flick off the buffer, and blot plates on paper towels before refilling.

Note: It is very important to use clean paper towels between each wash. This will help avoid any possible cross-well contamination.

Add Detection Antibody

9 Dilute the biotin-labeled detection antibody to 0.25-2µg/ml in Blocking Solution. Add 100µl of diluted antibody to each well.

10 Seal the plate and incubate at room temperature for 1 hour.
01:00:00

11 Wash 3 times with PBS/Tween.

Add Avidin-Horseradish Peroxidase (Av-HRP)

12 Dilute the Av-HRP conjugate (Cat. No.405103) or other enzyme conjugate to its pre-determined optimal concentration in Blocking Buffer (usually between 1/500-1/2000). Add 100µl per well.

13 Seal the plate and incubate at room temperature for 30 minutes.

00:30:00

14 Wash 5 times with PBS/Tween.

Add Substrate (ABTS for slower color development)

15 Thaw ABTS Substrate Solution within 20 min of use. Add 11µl of 30% H_2O_2per 11 ml of substrate and vortex. Immediately dispense 100µl into each well and incubate at room temperature (4-60 min) for color development. To stop the color reaction, add 50µl of ABTS Stop Solution. 00:20:00

16 Read the optical density (OD) for each well with a microplate reader set to 405 nm.

Add Substrate (TMB for faster color development)

17 For each plate, mix 6 ml of TMB Reagent A with 6 ml TMB Reagent B (BioLegend TMB Substrate Reagent Set, Cat. No.421101) immediately prior to use. Transfer 100µl into each well. Incubate at room temperature (4 – 30 min) for color development. To stop the color reaction, add 100µl of TMB Stop Solution. 00:30:00

18 Read the optical density (OD) for each well with a microplate reader set to 450 nm.