Sandwich ELISA Protocol V.3

DOI
dx.doi.org/10.17504/protocols.io.98xh9xn

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

Protocol Citation: Sam Li . Sandwich ELISA Protocol. **protocols.io https://dx.doi.org/10.17504/protocols.io.98xh9xn**

Created: December 06, 2019

Last Modified: December 06, 2019

Protocol Integer ID: 30711

Keywords: sandwich elisa, cytokine elisa

Guidelines

Cytokine ELISA Troubleshooting Tips:

Solutions and Buffers:

- Do not use sodium azide in any buffers or solutions, as sodium azide inactivates the horseradish-peroxidase enzyme.

Poor signal-to-noise ratio:

- Try Capture Antibody at 1-10µg/ml (generally 2µg/ml).
- Try Detection Antibody at 0.25-2µg/ml (generally 1µg/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 (1µg/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 (100ng/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

MATERIALS

ELISA Coating Buffer (5X) **BioLegend Catalog #**421701

PBS 10x Concentrate (Previously Covance catalog# SIG-31020) **BioLegend Catalog #**926201

Stop Solution for TMB Substrate **BioLegend Catalog #**423001

TMB Substrate Set **BioLegend Catalog #**421101

Reagents:

- **PBS/Tween:** 0.5ml of Tween-20 in 1L PBS
- **Blocking Solution:** 10% fetal bovine serum or 1% BSA in PBS. Filter before use to remove particulates.
- **ABTS Substrate Solution:**
  150mg 2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma, Cat. No. A-1888)
  Add to 500ml of 0.1M citric acid in ddH_2O
  Adjust pH to 4.35 with NaOH
  Aliquot 11ml per vial and store at -20° C.
  Avoid light exposure during preparation and storage.
- **ABTS Stop Solution:** Combine 50ml dimethylformamide (DMF; Pierce, Cat. No. 20672) with 50ml ddH_2O Add 20g sodium dodecyl sulfate

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.

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.

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.

11 Wash 3 times with PBS/Tween.

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.

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% H2O2 per 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.

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

OR 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.

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