Sandwich ELISA Protocol

BioLegend, Inc.

Abstract

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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 $NaHCO_3$
3.56 g Na_2CO_3
Add ddH_2O up to 1.0 L, pH to 9.5

Phosphate Buffered Saline (PBS):
80.0 g NaCl
14.4 g Na_2HPO_4
2.4 g KH_2PO_4
2.0 g KCl
Add ddH_2O 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 ddH_2O
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 ddH_2O
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

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

Protocol

Coat the Plate

Step 1.

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

ANNOTATIONS

Emily Hsiue 18 Apr 2017

5ug/ml, 50ul per well

Coat the Plate

Step 2.

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

DURATION

16:00:00

Block the Plate

Step 3.

Bring the plate to room temperature, flick off the capture antibody solution.

Block the Plate

Step 4.

Wash with PBS/Tween (1/3).

Block the Plate

Step 5.

Wash with PBS/Tween (2/3).

Block the Plate

Step 6.

Wash with PBS/Tween (3/3).

Block the Plate

Step 7.

Block non-specific binding sites by adding 200 µl of Blocking Solution to each well.

ANNOTATIONS

Emily Hsiue 18 Apr 2017

50ul

Block the Plate

Step 8.

Seal plate and incubate at room temperature for ≥ 1 hour.

DURATION

01:00:00

ANNOTATIONS

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1-2 hours

Block the Plate

Step 9.

Wash with PBS/Tween (1/3).

Block the Plate

Step 10.

Wash with PBS/Tween (2/3).

Block the Plate

Step 11.

Wash with PBS/Tween (3/3).

Block the Plate

Step 12.

Firmly blot plate against clean paper towels.

Add Standards and Samples

Step 13.

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

ANNOTATIONS

Emily Hsiue 18 Apr 2017

in PBS, 50ul

Add Standards and Samples

Step 14.

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

Add Standards and Samples

Step 15.

Wash with PBS/Tween (1/3).

NOTES

Kelsey Knight 10 May 2016

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.

Kelsey Knight 26 May 2016

Perform **at least 3** washes.

Add Standards and Samples

Step 16.

Wash with PBS/Tween (2/3).

Add Standards and Samples

Step 17.

Wash with PBS/Tween (3/3).

NOTES

Kelsey Knight 26 May 2016

Perform **at least 3** washes.

Add Detection Antibody

Step 18.

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

ANNOTATIONS

Emily Hsiue 18 Apr 2017

Or in TBST

Add Detection Antibody

Step 19.

Seal the plate and incubate at room temperature for 1 hour.

DURATION

01:00:00

Add Detection Antibody

Step 20.

Wash with PBS/Tween (1/3).

NOTES

Kelsey Knight 26 May 2016

Perform **at least 3** washes.

Add Detection Antibody

Step 21.

Wash with PBS/Tween (2/3).

Add Detection Antibody

Step 22.

Wash with PBS/Tween (3/3).

NOTES

Kelsey Knight 26 May 2016

Perform **at least 3** washes.

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 23.

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 100 µl per well.

REAGENTS

Av-HRP conjugate 405103 by BioLegend

ANNOTATIONS

Emily Hsiue 18 Apr 2017

NeutrAvidin 1:5000

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 24.

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

DURATION

00:30:00

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 25.

Wash with PBS/Tween (1/5).

NOTES

Kelsey Knight 26 May 2016

Perform **at least 5** washes.

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 26.

Wash with PBS/Tween (2/5).

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 27.

Wash with PBS/Tween (3/5).

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 28.

Wash with PBS/Tween (4/5).

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 29.

Wash with PBS/Tween (5/5).

NOTES

Kelsey Knight 26 May 2016

Perform **at least 5** washes.

Add Substrate (ABTS for slower color development)

Step 30.

Thaw ABTS Substrate Solution within 20 min of use.

DURATION

00:20:00

NOTES

Kelsey Knight 26 May 2016

Alternatively, you can use TMB for faster color development. To do so, substitute steps 30-33 in this section with:

a. 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 100 µl into each well. Incubate at room temperature (4 – 30 min) for color development. To stop the color reaction, add 100 µl of TMB Stop Solution (BioLegend, Cat. No.423001).

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

Add Substrate (ABTS for slower color development)

Step 31.

Add 11 µl of 30% H2O2 per 11 ml of substrateand vortex.

Add Substrate (ABTS for slower color development)

Step 32.

Immediately dispense 100 µl into each well and incubate at room temperature (4-60minutes) for color development. To stop the color reaction, add 50 µl of ABTS Stop Solution.

ANNOTATIONS

Emily Hsiue 18 Apr 2017

50ul Fur?? acid to quench

Add Substrate (ABTS for slower color development)

Step 33.

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