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

Citation: BioLegend, Inc. (05/27/2016). Sandwich ELISA Protocol. https://dx.doi.org/10.17504/protocols.io.ex9bfr6

This is an open access protocol distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
General References:

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

<table>
<thead>
<tr>
<th>NAME</th>
<th>CATALOG #</th>
<th>VENDOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonate Coating Buffer</td>
<td>421701</td>
<td>BioLegend</td>
</tr>
<tr>
<td>A1888 SIGMA 2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt</td>
<td>A-1888</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Pierce™ Dimethylformamide (DMF), Sequencing grade</td>
<td>20672</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>TMB (tetramethylbenzidine) Substrate Reagent Set</td>
<td>421101</td>
<td>BioLegend</td>
</tr>
<tr>
<td>TMB Stop Solution</td>
<td>423001</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Av-HRP conjugate</td>
<td>405103</td>
<td>BioLegend</td>
</tr>
</tbody>
</table>

STEPS MATERIALS

<table>
<thead>
<tr>
<th>NAME</th>
<th>CATALOG #</th>
<th>VENDOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Av-HRP conjugate</td>
<td>405103</td>
<td>BioLegend</td>
</tr>
</tbody>
</table>

Citation: BioLegend, Inc. (05/27/2016). Sandwich ELISA Protocol. https://dx.doi.org/10.17504/protocols.io.ex9bfr6

This is an open access protocol distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Coat the Plate

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-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.

4. Wash with PBS/Tween (1/3).

5. Wash with PBS/Tween (2/3).

6. Wash with PBS/Tween (3/3).

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

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

9. Wash with PBS/Tween (1/3).

10. Wash with PBS/Tween (2/3).

11. Wash with PBS/Tween (3/3).

12. Firmly blot plate against clean paper towels.

Add Standards and Samples

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.

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

15. Wash with PBS/Tween (1/3).

Washes can be effectively accomplished by filling wells with either a squirt bottle, carboy, manifold dispenser, multichannel 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.

Perform at least 3 washes.

16. Wash with PBS/Tween (2/3).
17 Wash with PBS/Tween (3/3).

Perform at least 3 washes.

Add Detection Antibody

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.

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

01:00:00

20 Wash with PBS/Tween (1/3).

Perform at least 3 washes.

21 Wash with PBS/Tween (2/3).

22 Wash with PBS/Tween (3/3).

Perform at least 3 washes.

Add Avidin-Horseradish Peroxidase (Av-HRP)

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.

Av-HRP conjugate
by BioLegend
Catalog #: 405103

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

00:30:00

25 Wash with PBS/Tween (1/5).

Perform at least 5 washes.

26 Wash with PBS/Tween (2/5).

27 Wash with PBS/Tween (3/5).

28 Wash with PBS/Tween (4/5).
29 Wash with PBS/Tween (5/5).

Perform at least 5 washes.

30 Thaw ABTS Substrate Solution within 20 min of use.

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.

31 Add 11 µl of 30% H2O2 per 11 ml of substrate and vortex.

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

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

This is an open access protocol distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.