ELISA

BETHYL LABORATORIES, INC

Direct ELISA

Required Reagents:
Antigen (preferably purified)
HRP-Conjugated Primary Antibody
Coating Buffer, 0.05 M Carbonate-Bicarbonate, pH 9.6
Wash Solution, 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0
Blocking (Postcoat) Solution, 50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0
Conjugate Diluent, 50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0
Enzyme Substrate, TMB
Stop Solution, 0.18 M H2SO4 or other appropriate solution
High Protein Binding Microtiter Plate (i.e. Nunc C bottom Immunoplate 96 well, Cat. No. 446612)

Preparation of Reagents:
Bethyl Laboratories ELISA Accessory Kit may be used or prepare the following reagents as specified below.

Coating Buffer
3.7 g Sodium Bicarbonate (NaHCO3)
0.64 g Sodium Carbonate (Na2CO3)
1 L of distilled water

Tris Buffered Saline (TBS)
6.06 g Tris Base
8.2 g NaCl
6.0 ml 6 M HCl
1 L of distilled water
pH should be 7.2 to 7.8, conductivity should be 14,000 to 16,000

Wash Solution
1 L of TBS
5 ml of 10% Tween 20

Blocking (Postcoat) Solution
1 L of TBS
10 g BSA

Conjugate Diluent
1 L of TBS
10 g of BSA
5 ml of 10% Tween 20

0.18 M H2SO4
Stock is 18 M
10 ml of Stock solution
1L distilled water

Procedure:
(Perform all steps at room temperature.)

Coat with Antigen

1. Dilute the antigen in Coating Buffer to the desired concentration. For purified antigens 1 – 2 mcg/ml is sufficient. For non-purified antigens, optimal concentration needs to be determined. Transfer 100 mcl of diluted antigen to each well.
2. Incubate coated plate for 60 minutes.
3. After incubation, aspirate the antigen solution from each well.
4. Wash each well with Wash Solution as follows:
5. Fill each well with Wash Solution
6. Remove Wash Solution by aspiration
7. Repeat for a total of 3 washes.

Blocking (Postcoat)

1. Add 200 mcl of Blocking (Postcoat) Solution to each well.
2. Incubate 30 minutes.
3. After incubation, remove the Blocking (Postcoat) Solution and wash each well three times.

HRP Conjugated Primary Antibody

1. Dilute the HRP Conjugate in Conjugate Diluent.
2. Transfer 100 mcl to each well.
3. Incubate 60 minutes.
4. After incubation, remove HRP Conjugate and wash each well 5 times.

Enzyme Substrate Reaction

1. Prepare the substrate solution according to the manufacturer's recommendation. TMB is highly recommended but OPD or ABTS can be used.
2. Transfer 100 mcl of substrate solution to each well.
3. Incubate plate 5 - 30 minutes.
4. To stop the TMB reaction, apply 100 mcl of 0.18 M H2SO4 to each well. If using another substrate, use the stop solution recommended by manufacturer.
5. Using a microtiter plate reader, read the plate at the appropriate wavelength for the substrate. (450 nm for TMB)

Notes: This procedure is suitable for alkaline phosphatase conjugated antibody. Use an appropriate substrate for alkaline phosphatase.

Indirect ELISA

Required Reagents:
Antigen
Primary Antibody
HRP Conjugated Secondary Conjugate
Coating Buffer, 0.05 M Carbonate-Bicarbonate, pH 9.6
Wash Solution, 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0
Blocking (Postcoat) Solution, 50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0
Sample/Conjugate Diluent, 50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0
Enzyme Substrate, TMB
Stop Solution, 0.18 M H_2SO_4 or other appropriate solution
High Protein Binding Microtiter Plate (i.e. Nunc C bottom Immunoplate 96 well, 446612)

Preparation of Reagents:
Bethyl Laboratories ELISA Accessory Kit may be used or prepare the following reagents as specified below.

Coating Buffer
3.7 g Sodium Bicarbonate ($NaHCO_3$)
0.64 g Sodium Carbonate (Na_2CO_3)
1 L of distilled water

Tris Buffered Saline (TBS)
6.06 g Tris Base
8.2 g NaCl
6.0 ml 6 M HCl
1 L of distilled water
pH should be 7.2 to 7.8, conductivity should be 14,000 to 16,000

Wash Solution
1 L of TBS
5 ml of 10% Tween 20

Blocking (Postcoat) Solution
1 L of TBS
10 g BSA

Sample/Conjugate Diluent
1 L of TBS
10 g of BSA
5 ml of 10% Tween 20

0.18 M H_2SO_4
Stock is 18 M
10 ml of Stock solution
1 L distilled water

Procedure:
(Perform all steps at room temperature.)

Coat with Antigen

1. Determine the number of single wells needed. Standards, samples, blanks and/or controls should be analyzed in duplicate.
2. Dilute the antigen in Coating Buffer to the desired concentration. For purified antigens 1 – 2 mcg/ml is sufficient. For non-purified antigens, optimal concentration needs to be determined. Transfer 100 mcl of diluted antigen to each well.
3. Incubate coated plate for 60 minutes.
4. After incubation, aspirate the Antigen solution from each well.
5. Wash each well with Wash Solution as follows:
    1. Fill each well with Wash Solution
    2. Remove Wash Solution by aspiration
    3. Repeat for a total of 3 washes.

Blocking (Postcoat)

1. Add 200 mcl of Blocking (Postcoat) Solution to each well.
2. Incubate 30 minutes.
3. After incubation, remove the Blocking (Postcoat) Solution and wash each well three times.

Primary Antibody

1. Dilute primary antibody samples in Sample/Conjugate Diluent as per manufacturer's recommendation or determine optimal concentration empirically.
2. Add 100 ul of diluted primary antibody to each well.
3. Incubate 60 minutes.
4. After incubation, remove the Blocking (Postcoat) Solution and wash each well three times.

HRP-Conjugated Secondary Antibody

1. Dilute the HRP Conjugate Secondary Antibody in Conjugate Diluent to the optimal concentration.
2. Transfer 100 mcl to each well.
3. Incubate 60 minutes.
4. After incubation, remove HRP Conjugate and wash each well 5 times.

Enzyme Substrate Reaction

1. Prepare the substrate solution according to the manufacturer's recommendation. TMB is highly recommended but OPD or ABTS can be used.
2. Transfer 100 mcl of substrate solution to each well.
3. Incubate plate 5 - 30 minutes.
4. To stop the TMB reaction, apply 100 mcl of 0.18 M H2SO4 to each well. If using another substrate, use the stop solution recommended by manufacturer.
5. Using a microtiter plate reader, read the plate at the appropriate wavelength for the substrate. (450 nm for TMB)

Notes: This procedure is suitable for alkaline phosphatase conjugated antibody. Use an appropriate substrate for alkaline phosphatase

Sandwich ELISA Using Two Primary Antibodies

Required Reagents:
Capture Antibody (preferably affinity purified)
Standard
HRP-Conjugated Primary Antibody
Coating Buffer, 0.05 M Carbonate-Bicarbonate, pH 9.6
Wash Solution, 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0
Blocking (Postcoat) Solution, 50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0
Sample/Conjugate Diluent, 50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0
Enzyme Substrate, TMB
Stop Solution, 0.18 M H_2SO_4 or other appropriate solution
High Protein Binding Microtiter Plate (i.e. Nunc C bottom Immunoplate 96 well, 446612)

Preparation of Reagents:
Bethyl Laboratories ELISA Accessory Kit is strongly recommended for best results.

Coating Buffer
3.7 g Sodium Bicarbonate ($NaHCO_3$)
0.64 g Sodium Carbonate (Na_2CO_3)
1 L of distilled water

Tris Buffered Saline (TBS)
6.06 g Tris Base
8.2 g NaCl
6.0 ml 6 M HCl
1 L of distilled water
pH should be 7.2 to 7.8, conductivity should be 14,000 to 16,000

Wash Solution
1 L of TBS
5 ml of 10% Tween 20

Blocking (Postcoat) Solution
1 L of TBS
10 g BSA

Sample/Conjugate Diluent
1 L of TBS
10 g of BSA
5 ml of 10% Tween 20

0.18 M H_2SO_4
Stock is 18 M
10 ml of Stock solution
1 L distilled water

Procedure:
(Perform all steps at room temperature.)

Coat with Capture Antibody

1. Determine the number of single wells needed. Standards, samples, blanks and/or controls should be analyzed in duplicate.
2. Dilute capture antibody to a concentration of 2 – 10 mcg/ml in Coating Buffer. Transfer 100 mcl to each well.
3. Incubate coated plate for 60 minutes.
4. After incubation, aspirate the Capture Antibody solution from each well.
5. Wash each well with Wash Solution as follows:
    1. Fill each well with Wash Solution
    2. Remove Wash Solution by aspiration
    3. Repeat for a total of 3 washes.

Blocking (Postcoat)

1. Add 200 mcl of Blocking (Postcoat) Solution to each well.
2. Incubate 30 minutes.
3. After incubation, remove the Blocking (Postcoat) Solution and wash each well three times.

Standards and Samples

1. Dilute the standards in Sample Diluent according to desired concentration.
2. Dilute the samples, based on the expected concentration of the analyte, to fall within the concentration range of the standards.
3. Transfer 100 mcl of standard or sample to assigned wells.
4. Incubate plate 60 minutes.
5. After incubation, remove samples and standards and wash each well 3 times.

HRP-Conjugated Primary Antibody

1. Dilute the HRP Conjugate in Conjugate Diluent.
2. Transfer 100 mcl to each well.
3. Incubate 60 minutes.
4. After incubation, remove HRP Conjugate and wash each well 5 times.

Enzyme Substrate Reaction

1. Prepare the substrate solution according to the manufacturer's recommendation. TMB is highly recommended but OPD or ABTS can be used.
2. Transfer 100 mcl of substrate solution to each well.
3. Incubate plate 5 - 30 minutes.
4. To stop the TMB reaction, apply 100 mcl of 0.18 M H2SO4 to each well. If using another substrate, use the stop solution recommended by manufacturer.
5. Using a microtiter plate reader, read the plate at the appropriate wavelength for the substrate. (450 nm for TMB)

Calculation of Results

1. Average the duplicate readings from each standard, control, and sample.
2. Subtract the zero reading from each averaged value above.
3. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. Other curve fits may also be used.
4. A standard curve should be generated for each set of sample.

Troubleshooting

Problem: Low absorbance
Incorrect dilutions or pipetting errors
Improper incubation times
Improper preparation of the TMB substrate.
Wrong filter on microtiter reader. Wavelength should be 450 nm for TMB, 490 nm for OPD, or 405 nm for ABTS.
Reagents are contaminated or expired.
Incorrect reagents used.

Problem: High Absorbance
Cross contamination from other samples or positive control
Incorrect dilutions or pipetting errors
Improper washing
Wrong filter on microtiter reader. Wavelength should be 450 nm for TMB, 490 nm for OPD, or 405 nm for ABTS.
Contaminated buffers or enzyme substrate
Improper incubation times
Reagents are contaminated or expired.

Problem: Poor Duplicates
Poor mixing of specimens
Incorrect dilutions or pipetting errors
Technical error
Inconsistency in following ELISA protocol
Inefficient washing

Problem: All wells are positive
Contaminated buffers or enzyme substrate
Incorrect dilutions or pipetting errors
Kit materials or reagents are contaminated or expired.
Inefficient washing

Problem: All wells are negative
Procedure not followed correctly
Contaminated buffers or enzyme substrate
Contaminated Conjugate
Kit materials or reagents are contaminated or expired.

Technical Hints
When preparing coating buffer from the gel capsule, break the capsule apart and pour ingredients into water. Do not place gel capsule into water. The gelatin from the capsule interferes with the binding of the coating antibody to the plate.
Capture antibody diluted with coating buffer should be added to wells immediately.
Coated (covered) plates are stable overnight at 4oC.
Check all buffers for contamination and expiration. When trouble shooting, it may be helpful to start with all new buffers. Make buffers in new or properly cleaned vessels.
Sodium Azide should not be added to any of the buffers.
Dilutions should be made shortly before application and immediately applied to appropriate wells.
Wash buffer should be aspirated from wells. Pouring/Dumping wash buffer from wells may lead to cross contamination.
Excess antibody/analyte should be wiped from pipettes tips when making dilutions.
Incubation time of the Enzyme Substrate will depend on the substrate used and the intensity of the color change. The high standard should have an O.D. reading of about 2.0 and the O.D. reading of the low standard should be above background.
Stop solution should be added to the plate in the same order as the Enzyme Substrate.

ELISA FAQs

Can I use other ELISA buffers (ex. PBS) and substrates (ex. ABTS or OPD) with Bethyl's ELISA kits?
Yes, our ELISA kits contain antibodies and a reference standard to establish a curve. We test and qualify our kits using the reagents that are listed in the product specification sheet. However, other standard ELISA reagents will work with our antibodies.

Do Bethyl's ELISA kits contain polyclonal or monoclonal antibodies?
Polyclonal

What type of samples can I use with Bethyl's ELISA kits?
Samples can be of biological (i.e. serum, plasma, urine, feces) or cell culture origin.

Are there any stopping points during the ELISA?
Yes, after adding the coating antibody or blocking solution to the wells, the covered plates can be incubated overnight at 4 C. The coating antibody or blocking solution must be left on the plate during the incubation period. We do not recommend leaving these solutions on the plate for more than 24 hours.

How should I store my samples before use?
We recommend that you store your samples at 4 C. However, biological or cell culture samples can be initially frozen until use but should not be repeatedly freeze-thawed. Once the sample is thawed, it should be stored at 4 C.

How do I dilute my samples?
Since all experimental designs are different, it is difficult to determine an overall dilution for all samples. If you have a general idea of the concentration of your samples, you will want to make dilutions so that your samples will fall in the middle range (or linear range) of the standard curve. Otherwise, you may want to take a few of your samples and make several dilutions (ex. 1:100, 1:1000, and 1:10,000) to determine a dilution range for your samples. Each investigator must determine the best dilution for their samples.

What if my raw OD values are too low or too high?
If the raw OD values are too low, then the dilution of the HRP conjugate should be decreased (ex. Used a 1:50,000 dilution, then try a 1:25,000 dilution). If the raw OD values are too high, then the dilution of the HRP conjugate should be increased (ex. Used a 1:50,000 dilution, then try a 1:100,000 dilution).

What can I do to decrease my background?
Usually background is caused by cross contamination of samples or reagents. Make sure to always use a plate washer or wash by hand with a multi-channel pipette. Do not use the "squirt and dump" method for washing. You may also add another wash to each step. Make new reagents if background problems continue and make sure that you are not using a BSA product with an ELISA kit that may cross react (Bovine Albumin ELISA, Bovine Transferrin ELISA, Bovine IgG ELISA, Goat IgG ELISA, and others)

The quantitation of my sample is not consistent or seems to be incorrect. What can I do to correct this problem?
Make sure that you are quantitating your samples in the linear region of the curve. Samples that quantitate too close to the extreme ends of the curve may be inaccurate and difficult to reproduce.

Where can I buy the stop solution (2 M Sulfuric Acid)?
Sulfuric Acid is sold by chemical companies such as Sigma or VWR. The stock solution is 18 M and should be diluted to 2 M with distilled water.

What type of software is needed to graph a 4-parameter curve?
Some of the software choices are SoftMax Pro by Molecular Devices, KC Jr., SigmaPlot, or others. You cannot directly plot a 4-parameter curve with Microsoft EXCEL.

I do not have software that will perform a 4 parameter standard curve. What should I use to analyze my data?
You can use a linear regression curve in Microsoft EXCEL. If you use this type of curve, only use a maximum of 5 points on the curve. We recommend that you discard the upper and lower points and plot your standard curve with the 5 middle points.