Enzyme Linked Immunosorbent Assay (ELISA)

Research Use Only

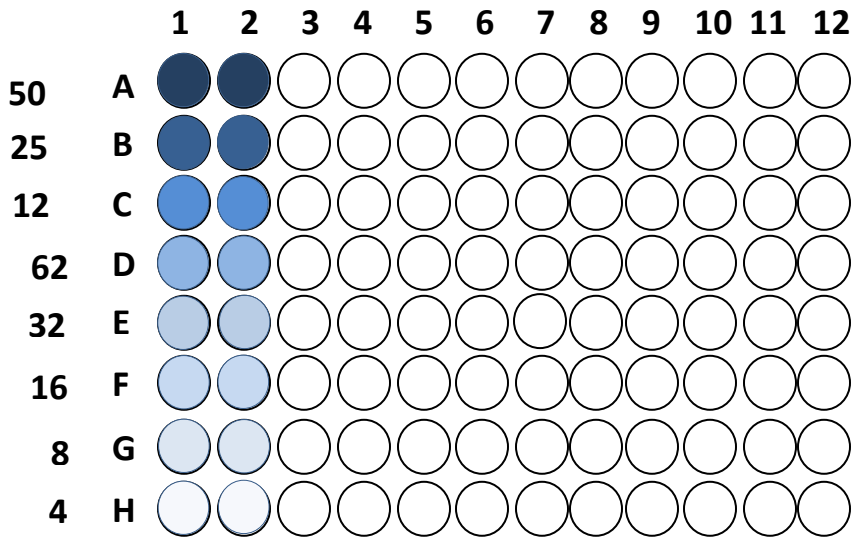
Introduction

The enzyme linked immunosorbent assay (ELISA) is used for the detection and quantification of proteins typically secreted or released from cells. Immobilizing a target-specific capture antibody onto a high protein binding capacity ELISA plate enables capture of target protein. The captured protein is then detected by a protein-specific biotinylated antibody. The target protein is quantified using a colorimetric reaction based on activity of avidin-horseradish peroxidase (bound to the biotinylated detection antibody) on a specific substrate (e.g., ABTS, SuperAqua Blue or TMB). The optical density of the end-product is measured using a spectrophotometer. eBioscience offers a variety of ELISA to cytokines, chemokines, growth factors and protein released upon cell death.

General Notes

A. **The source of the recombinant protein used for the standard:** Antibody pairs used in ELISA are developed and optimized with specific recombinant proteins (form a particular source). Recognition by the antibodies can vary if the protein is made in a different source or from a different supplier. Therefore eBioscience recommends using the prediluted and optimized Single Use ELISA standards (catalog prefix number is 39-, ex: 39-8319 Single-Use ELISA RSG Standard Recombinant Human IFNg (Interferon-gamma, IFN-g). This will assure consistent and accurate ELISA results.

B. **Preparing the standard curve in duplicate or triplicate***: Quantitative analysis requires the generation of a standard curve for each assay using serially diluted protein standard for a total of 8 data points. Please refer to the Technical Data Sheet (TDS) or Certificate of Analysis (CofA) for the standard curve range that is detectable by the antibody pair or READY SET GO ELISA kit. We are using the example of 4-500pg/mL below.*



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1. Prepare a 500 pg/mL standard solution in 1X ELISA/ELISPOT Diluent as instructed in the CofA.
2. Add 100 µL of 1X ELISA/ELISPOT Diluent to wells B1 down to H1 and B2 down to H2 in the high affinity binding ELISA plate.
3. Add 200 µL of the **500 pg/mL** standard to wells A1 and A2.
4. Remove 100 µL from wells A1 and A2 and transfer to wells B1 and B2, respectively, and mix well by pipeting up and down. Wells B1 and B2 contain the **250 pg/mL** standard.
5. Remove 100 µL from wells B1 and B2 and transfer to wells C1 and C2 and mix well by pipeting up and down. These wells contain the **125 pg/mL** standard.
6. Remove 100 µL from wells C1 and C2 and transfer to wells D1 and D2 and mix well by pipeting up and down. The wells contain the **62.5 pg/mL** standard.
7. Continue to transfer and mix well for the remaining dilutions ending at **4 pg/mL** in wells H1 and H2.
8. When you transfer to wells H1 and H2, mix well and then dispose of the last 100 µL so that all wells contain 100 µL total volume.
   Note: The above procedure from Step3-8 should be done quickly. A multichannel pipet can be used to expedite the process.
9. Include two blank wells (A3 and A4) that contain 100 µL of 1X ELISA/ELISPOT Diluent only (No standard).
   Note: Due to the Hook Effect, we recommend using Columns 1 and 12 for blanks rather than for the standard curve or samples when possible.

C. **Calculation of results:** *You will need to generate an equation based on the optical density values obtained for your known standards.*

1. Calculate the average of the optical density measurement for the duplicate wells of standards, blanks, and experimental samples.
2. Subtract the average of the blank wells from the average O.D. reading for your standards and experimental samples.
3. Create a standard curve using computer software capable of generating a four parameter logistic (4-PL) curve fit. An adequate, but less precise fit of the data can be generated by plotting the log of the standard concentrations on the x-axis and the log of each standard's mean OD on the y-axis. The best fit line can be generated by regression analysis.
4. Determine the concentration of your experimental samples using the average O.D. value for each sample (y value) and the equation generated from the standard curve to solve for x which will represent the concentration of that sample.
5. If samples have been diluted prior to assay, the concentration determined must be multiplied by the dilution factor.

D. **Stability and storage instructions.**

1. eBioscience ELISA sets are guaranteed to perform as specified for a minimum of 6 months from the date of receipt if stored and handled as instructed according to the accompanying Technical Datasheet and Certificate of Analysis.
2. The frozen standards are supplied at 20 µL per vial. Upon receipt, the frozen standard should be immediately stored at -80^{0}C and will be stable for at least 6 months. After

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thawing, a quick-spin of the vial prior to opening will reduce the loss of material. Do not aliquot into smaller fractions and refreeze; these are single use vials. Use once and discard any remaining material. For dilution of the standard, please see instruction on the Certificate of Analysis.

3. Plates should be stored at room temperature.
4. Store all other reagents in the kit/set at 4^0C.

Protocol A: ELISA Protocol using Ready-Set-Go! Set

eBioscience Ready-SET-Go! ELISA reagent sets (with or without high-affinity binding microwell plates) contain the necessary reagents, buffers and diluents for performing quantitative enzyme linked immunosorbent assays (ELISA). These ELISA reagent sets are specifically engineered for accurate and precise measurement of protein levels from samples including serum, plasma, and cell culture supernatants (please see product specific datasheets for more information regarding compatible sample type as it can vary). The following protocol is a general guideline for the Ready-SET-Go! Sets.

Materials Provided

- Please refer to the Certificate of Analysis (C of A) for components

Other Materials Needed

- Buffers*
  - Wash Buffer: 1x PBS, 0.05% Tween-20 (or eBioscience ELISA Wash Buffer Powder, Cat. No. 00-0400)
  - Stop Solution: 1M H_3PO_4 (recommended) or 2N H_2SO_4
- Pipettes
- Refrigerator & frost-free -20°C freezer
- 96-well plate (Corning Costar 9018 or NUNC Maxisorp®)
  NOTE: The use of ELISA plates which are not high affinity protein binding plates will result in suboptimal performance, e.g., low signal or inconsistent data. Do not use tissue culture plates or low protein absorption plates. Use only the Corning Costar 9018 or NUNC Maxisorp (Cat. No. 44-2404) 96-well plates
- 96-well ELISA plate reader (microplate spectrophotometer)
- ELISA plate washer (highly recommended)

NOTE: To ensure optimal results from this ELISA Ready-SET-Go! Set, please only use the components included in the set. Exchanging of components is not recommended as a change in signal may occur.

Time Requirements

- 1 overnight incubation
- 4½-hour incubations
- 1 hour washing and analyzing samples

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Experimental Procedure

1. Coat Corning Costar 9018 (or Nunc Maxisorp®) ELISA plate with 100 µL/well of capture antibody in Coating Buffer (dilute as noted on C of A, which is included with the reagent set). Seal the plate and incubate overnight at 4°C.
2. Aspirate wells and wash 3 times with >250 µL/well Wash Buffer*. Allowing time for soaking (~ 1 minute) during each wash step increases the effectiveness of the washes. Blot plate on absorbent paper to remove any residual buffer.
3. Dilute 1 part 5X ELISA/ELISPOT Diluent with 4 parts DI water.* Block wells with 200 µL/well of 1X ELISA/ELISPOT Diluent. Incubate at room temperature for 1 hour.
4. [Optional] Aspirate and wash at least once with Wash Buffer.
5. Using 1X ELISA/ELISPOT Diluent *, dilute standards as noted on the C of A to prepare the top concentration of the standard. Add 100 µL/well of top standard concentration to the appropriate wells. Perform 2-fold serial dilutions of the top standards to make the standard curve for a total of 8 points. Add 100 µL/well of your samples to the appropriate wells. Seal the plate and incubate at room temperature for 2 hours (or overnight at 4°C for maximal sensitivity).
6. Aspirate/wash as in Step 2. Repeat for a total of 3-5 washes**.
7. Add 100 µL/well of detection antibody diluted in 1X ELISA/ELISPOT Diluent * (dilute as noted on C of A). Seal the plate and incubate at room temperature for 1 hour.
8. Aspirate/wash as in Step 2. Repeat for a total of 3-5 washes**.
9. Add 100 µL/well of Avidin-HRP* diluted in 1X ELISA/ELISPOT Diluent (dilute as noted on C of A). Seal the plate and incubate at room temperature for 30 minutes.
10. Aspirate and wash as in Step 2. In this wash step, soak wells in Wash Buffer* for 1 to 2 minutes prior to aspiration. Repeat for a total of 5-7 washes**.
11. Add 100 µL/well of 1X TMB Solution to each well. Incubate plate at room temperature for 15 minutes.
12. Add 50 µL of Stop Solution to each well.
13. Read plate at 450 nm. If wavelength subtraction is available, subtract the values of 570 nm from those of 450 nm and analyze data.

NOTES:

**** Be certain that no sodium azide is present in the solutions used in this assay, as this inhibits HRP enzyme activity.***

*****The number of washes in the protocol was adapted to an automatic plate washer. This can be decreased when using other methods but should be tested empirically. Allowing time for soaking (~ 1 minute) during each wash step increases the effectiveness of the washes.***

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Protocol B: ELISA protocol using Antibody Pairs

The following protocol is a general guideline for using the eBioscience capture and detection antibody pairs.

Materials

- 96-well plate (Nunc Maxisorp flat-bottom, Cat. No. 44-2404)
- Capture and detection antibodies for the cytokine of interest (see the Quick Guide Chart)
- Avidin Horseradish Peroxidase (Avidin HRP, Cat. No. 18-4100)

Buffers

- ELISA/ELISPOT Coating Buffer Powder (Cat. No. 00-0044) or Carbonate buffer
- 5X ELISA/ELISPOT Diluent (Cat. No. 00-4202)
- Wash Buffer: 1X PBS, 0.05% Tween-20 (or eBioscience ELISA Wash Buffer Powder Cat. No. 00-0400)
- Substrate Solution: Super Aqua Blue ELISA Substrate (Cat. No. 00-4203) or 1X TMB Solution (Cat. No. 00-4201)
- Stop Solution: 2N H_2SO_4 or 1M $H_3PO_4$for TMB; 0.625M oxalic acid for Super Aqua Blue

Instruments

- Pipettes and pipettors
- Refrigerator
- 96-well ELISA plate reader (microplate spectrophotometer) capable of reading at 405 nm (Super Aqua Blue) or 450 nm (TMB)
- Plate Washer: Wash bottle or automated wash machine

Experimental Procedure

1. Dilute capture antibody using Coating Buffer. In general, a concentration of 1-4 µg/mL will be sufficient but please refer to the technical data sheet for specific concentration(s) for the clone you are using.
2. Coat plate with 100 µL/well of capture antibody.
3. Seal plate and incubate at 4°C overnight.
4. Aspirate wells and wash 3 times with at least 300 µL/well Wash Buffer. Invert the plate and blot on absorbent paper to remove any residual buffer.
5. Dilute 5X concentrated ELISA/ELISPOT Diluent to final working stock of 1X using DI water (1 part 5X ELISA/ELISPOT Diluent to 4 parts DI water)* Block wells with 200 µL/well of 1X ELISA/ELISPOT Diluent. Incubate at room temperature for 1 hour. ***Important Note: Do not include sodium azide in any buffers, as this will inactivate the HRP.***
6. Aspirate wells and wash 3 times with at least 300 µL/well Wash Buffer. Invert the plate and blot on absorbent paper to remove any residual buffer. Use 1X ELISA/ELISPOT diluent to dilute recombinant standards to recommended high concentration, and perform 2-fold serial dilutions for a total of 8 standard curve points. Please see technical data sheet for specific concentration(s) for the recombinant standard you are using.

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7. Add 100 µL/well of your standard curve and samples to the appropriate wells. Cover or seal the plate and incubate at room temperature for 2 hours (or overnight at 4°C for maximal sensitivity).
8. Aspirate wells and wash 5 times with at least 300 µL/well Wash Buffer. Invert the plate and blot on absorbent paper to remove any residual buffer.
9. Dilute biotinylated detection antibody as indicated on the technical data sheet using 1X ELISA/ELISPOT Diluent. In general, a concentration of 0.5-2 µg/mL will be sufficient but please refer to the technical data sheet for specific concentration(s) for the clone you are using.
10. Add 100 µL/well of detection antibody.
11. Seal plate and incubate at room temperature for 1 hour.
12. Aspirate and wash as in step 4. Repeat for a total of 5 washes.
13. Dilute Avidin-HRP 1:500 using 1X ELISA/ELISPOT Diluent.
14. Add 100 µL/well of Avidin-HRP*.
    Important Note: Do not include sodium azide in any buffers, as this will inactivate the HRP.
15. Seal plate and incubate at room temperature for 30 minutes.
16. Aspirate wells and wash 7 times with at least 300 µL/well Wash Buffer. Invert the plate and blot on absorbent paper to remove any residual buffer. In this wash step, soak wells in wash buffer for 1 to 2 minutes prior to aspiration.
17. Add 100 µL of SuperAqua Blue or TMB* to each well and incubate at room temperature for 10-15 minutes. Absorption can be read and re-read over time to identify optimal color development. If desired, the color reaction can be stopped by the addition of 100 uL of stop solution and read at 405 nm (Super Aqua Blue) or 450 nm (TMB).
    Important Note: Bring TMB Substrate Solution to room temperature prior to use.

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