Technical Manual

DIY ELISA Kits

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Introduction

Each DIY enzyme linked immunosorbent assay (ELISA) kit contains a capture antibody (unconjugated antibody), a standard (recombinant protein), and a detection antibody (biotinylated antibody) for development of an ELISA. The antibodies have been determined to function in an ELISA with the standard provided. Optimal buffers, concentrations, incubation times, incubation temperatures, and methods for the ELISA have not been determined. This technical guide offers additional insight in ELISA development to help optimize ELISA function.

ELISA Procedure Overview

1. Prepare Antibody Coated Plate
2. Add Standard and Samples and Incubate
3. Wash Plate
4. Add Detection Antibody and Incubate
5. Wash Plate
6. Add Streptavidin-HRP and Incubate
7. Wash Plate
8. Add TMB Substrate and Incubate
9. Stop Reaction
10. Measure Absorbance
11. Calculate Results

Reagents Required

- Specific DIY ELISA kit including:
    I. Capture Antibody (unconjugated antibody)
    II. Standard (recombinant protein)
    III. Detection Antibody (biotinylated antibody)
- DPBS
- Blocking Buffer and Reagent Diluent
- Standard and Sample Diluent
- Wash Buffer
- Streptavidin-Horseradish Peroxidase (HRP)
- 3,3’5,5’-tetramethylbenzidine (TMB) Substrate)
- Stop Solution

Additional Materials Required

- 96-well high binding ELISA plate
- Ultrapure water
- Precision pipettors, with disposable plastic tips
- Polypropylene or polyethylene tubes to prepare standard, samples, and working solutions.
  Note: Do not use polystyrene, polycarbonate or glass tubes. They bind protein and antibodies.
- Containers to prepare buffers
- A wash bottle or an automated 96-well plate washer
- Disposable reagent reservoirs
- Plate sealers/adhesive plate covers
- A standard microtiter plate reader for measuring absorbance at 450 nm

General ELISA Suggestions

- Store all reagents at recommended temperature.
- Do not use reagents if they become visibly contaminated during storage.
- Reagents should be at room temperature before use.
- Vigorous plate washing is essential.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.
- Take care not to contaminate the TMB Solution. Do not expose TMB Substrate solution to glass, foil, or metal. If the solution is blue before use, DO NOT USE IT.
- Individual components may contain preservatives. Wear gloves while performing the assay. Please follow proper disposal procedures.
- Do not use sodium azide as a preservative for buffers. Sodium azide interferes with HRP and TMB substrate reactions.

Reagent and Sampling Handling and Preparation

DPBS Preparation

Recommended buffer: 0.008M sodium phosphate, 0.002M potassium phosphate, 0.14M sodium chloride, 0.01M potassium chloride, pH 7.4

- Alternative buffer may be used as desired. For example, PBS or TBS solutions may be used to replace DPBS.
- Do not use sodium azide as a preservative. Sodium azide interferes with assay.

To prepare 1L of DPBS:

1. Fill a suitable container with ~750 mL ultra-pure water.
2. Dissolve solid reagents in the ultra-pure water. Mix well.
    I. 1.14 g Sodium Phosphate, Dibasic (FW: 142.0)
    II. 0.27 g Potassium Phosphate, Monobasic (FW: 136.0)
    III. 8.18 g Sodium Chloride (FW: 58.44)
    IV. 0.75 g Potassium Chloride (FW: 74.55)
3. Fill to 1L volume.
4. Verify pH.
5. If desired, filter the solution into a sterile container using a 0.2 µm membrane.

Blocking Buffer and Reagent Diluent Preparation

- Recommended buffer: 4% Bovine Serum Albumin (BSA) in DPBS, 0.2 µm filtered
- Alternative buffer may be used as desired. For example:
    I. PBS or TBS solutions may be used to replace DPBS.
    II. BSA may be replaced with alternative blocking reagents.
    III. BSA concentration may be altered as desired.
- Do not use sodium azide as a preservative. Sodium azide interferes with assay.

To prepare 1L of Blocking Buffer and Reagent Diluent:

1. Fill a suitable container with ~750 mL ultra-pure water.
2. Dissolve solid reagents in the ultra-pure water. Mix well.
    i. 1.14 g Sodium Phosphate, Dibasic (FW: 142.0)
    ii. 0.27 g Potassium Phosphate, Monobasic (FW: 136.0)
    iii. 8.18 g Sodium Chloride (FW: 58.44)
    iv. 0.75 g Potassium Chloride (FW: 74.55)
    v. 40 g BSA (4% solution)

3. Fill to 1L volume.
4. Verify pH.
5. If desired, filter the solution into a sterile container using a 0.2 µm membrane.

10X Wash Buffer Preparation

- Recommended buffer: 10X DPBS plus 0.5% Tween®-20
- Alternative buffer may be used as desired. For example, PBS or TBS solutions may be used to replace DPBS.
- Do not use sodium azide as a preservative. Sodium azide interferes with assay.

To Prepare 1L of 10X Wash Buffer:

1. Fill a suitable container with ~750 mL ultra-pure water.
2. Dissolve reagents in the ultra-pure water. Mix well.
    I. 11.4 g Sodium Phosphate, Dibasic (FW: 142.0)
    II. 2.7 g Potassium Phosphate, Monobasic (FW: 136.0)
    III. 81.8 g Sodium Chloride (FW: 58.44)
    IV. 7.5 g Potassium Chloride (FW: 74.55)
    V. 5 mL Tween 20® (0.5% solution)
3. Fill to 1L volume.
4. Verify pH.
5. If desired, filter the solution into a sterile container using a 0.2 µm membrane.

1X Wash Buffer Preparation

- Recommended buffer: 1X DPBS plus 0.05% Tween®-20
- Alternative buffer may be used as desired. For example, PBS or TBS solutions may be used to replace DPBS.
- Do not use sodium azide as a preservative. Sodium azide interferes with assay.

To prepare 500 mL of 1X Wash Buffer:

1. Dilute 50 mL of 10X Wash Buffer into 450 mL of ultrapure water.
2. Mix well.
    Note: A typical wash bottle will hold 500 mL of solution.

Standard and Sample Diluent Preparation

The optimal Standard and Sample Diluent will allow the recombinant protein Standard to mimic natural protein as it would be in the natural sample type. A variety of sample types are available including cell culture supernatants, serum, plasma, synovial fluid, urine, milk, and many more. Optimal results are usually obtained by closely matching the sample type or diluting the sample.

- Complete Medium used to generate cell culture supernatants can be utilized for cell culture supernatant samples.
- Fetal Bovine Serum or Equine Serum can be utilized for serum samples.
- Custom diluents can be created by mixing carrier proteins and buffers to mimic the natural protein response in desired sample type.
- Samples can be diluted to minimize matrix effects. Usually a 1:4 dilution is sufficient to reduce matrix effects.

To verify if a Standard and Sample Diluent works with a desired sample type, a linearity and recovery assay should be performed. Please see **Linearity and Recovery Assay** for more details.

Standard Preparation

• Alternative top standard concentrations can be prepared as desired. Please see **Optimal Standard and Antibody Concentrations** for more details.

To prepare a 25 ng/mL top standard:

1. Reconstitute 5 µg recombinant protein vial with 100 µL of Reagent Diluent to achieve a concentration of 50 µg/mL. Aliquot protein in 10 µL aliquots and store at -20º to minimize freeze-thaw cycles.
2. To prepare a 25 ng/mL (0.025 µg/mL) top standard, dilute 2 µL of 50 µg/mL stock in 4 mL Standard and Sample Diluent. Mix well.
3. Serial dilute the top standard 1:1 with Standard and Sample Diluent. Perform dilution by mixing 250 µL of the previous standard with 250 µL Standard and Sample Diluent. Continue until reach lowest desired standard value.
4. Use Standard and Sample Diluent only as the zero standard.

Sample Handling and Preparation

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions when handling and disposing of infectious agents.
- 100 µL of sample or standard is recommended per well.
- Samples should be assayed in duplicate each time the assay is performed.
- Store samples appropriately prior to analysis. For long-term storage, aliquot and freeze samples at -70°C. Avoid repeated freeze-thaw cycles when storing samples.
- If particulate is present in samples, centrifuge prior to analysis.

- If the integrity of the sample is of concern, make a note on the Plate Template and interpret results with caution.
- If it is suspected that the concentration of the sample exceeds the highest point of the standard curve, prepare one or more dilutions of the sample in Standard and Sample Diluent until the desired concentration is obtained.

To prepare a 1:10 sample dilution:

1. Dilute 25 µL of sample in 225 µL of Standard and Sample Diluent in a clean, fresh tube.
2. Mix well.

Capture Antibody Working Solution Preparation

• Alternative capture antibody concentrations can be prepared as desired.

Please see **Optimal Standard and Antibody Concentrations** for more details.

To prepare a 2.5 µg/mL capture antibody working solution:

1. Dilute 30 µL of 1 mg/mL capture antibody stock in 12 mL DPBS.
2. Mix well.

Detection Antibody Working Solution Preparation

• Alternative detection antibody concentrations can be prepared as desired.

Please see **Optimal Standard and Antibody Concentrations** for more details.

To prepare a 0.2 µg/mL detection antibody working solution:

1. Dilute 2.4 µL of 1 mg/mL detection antibody stock in 12 mL Reagent Diluent.
2. Mix well.

Detailed Assay Procedure

Step 1: Prepare Antibody Coated Plate

1. Prepare Capture Antibody in DPBS at desired working concentration.

Note: Alternative buffers can be used for capture antibody dilution. For example:

i. PBS or TBS solutions may be used to replace DPBS.

ii. Basic or acidic buffers may be used to replace DPBS to enhance antibody binding.

2. Add 100 µL of Capture Antibody Working Solution to appropriate wells. Cover plate with Plate Sealer and incubate at room temperature (20-25°C) for 12-24 hours.

Note: Incubation time and temperature may be altered as desired for optimal antibody binding.

3. Empty Capture Antibody Working Solution from plate. Blot plate onto paper towels or other absorbent material.

4. Add 250 µL of Blocking Buffer to appropriate wells.

   Note: Alternative buffers can be used for plate blocking. For example:

   I. PBS or TBS solutions may be used to replace DPBS.
   II. BSA may be replaced with alternative blocking reagents.
   III. Additional blocking reagents may be used.
   IV. BSA concentration may be altered as desired.
   V. Stabilizing agents, such as sucrose, may be used.

5. Cover plate with Plate Sealer and incubate at room temperature for 1-3 hours.

   Note: Incubation time and temperature may be altered as desired for optimal blocking.

6. Empty Blocking Buffer from plate. Blot plate onto paper towels or other absorbent material.
7. Antibody coated plate is now ready for use. Plate can be dried at room temperature overnight and stored at 2-8ºC with desiccant until ready for use.

Step 2: Add Standard and Samples and Incubate

- Determine the number of strips required. Leave these strips in the plate frame. Place unused strips in pouch with desiccant and seal tightly. Store unused strips at 2-8°C. After completing assay, keep the plate frame for additional assays.
- Use a Plate Template to record the locations of the standards and unknown samples within the wells.

1. Add 100 µL of appropriately diluted standards or samples to each well. Run each standard, sample, or blank in duplicate.
2. Carefully cover wells with a new adhesive plate cover. Incubate for one (1) hour at room temperature.

   Note: Incubation time and temperature can be altered as desired. For example:

   - Standards and samples can be incubated overnight at 2-8ºC.
   - Incubation time can be increased to 2 hours.
   - Incubation temperature can be increased to 37ºC.
   - Plate can be incubated with shaking using an orbital shaker.

Step 3: Wash Plate

1. Carefully remove plate cover.
2. Gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame.
3. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with 1X Wash Buffer, then empty plate contents. Repeat procedure three additional times for a total of FOUR washes. Blot plate onto paper towels or other absorbent material.

   Note: For automated washing, aspirate plate contents from all wells and flood wells with 1X Wash Buffer. Repeat procedure three additional times for a total of FOUR washes. Additional washes may be necessary. Blot plate onto paper towels or other absorbent material.

   Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

Step 4: Add Detection Antibody and Incubate

• Only prepare the required amount of Detection Antibody Working Solution for the number of strips being used.

1. Add 100 µL of Detection Antibody Working Solution to each well containing standard, sample or blank. Mix well by gently tapping the plate several times.

   Carefully attach a new adhesive plate cover. Incubate plate for one (1) hour at room temperature.

   Note: Incubation time and temperature can be altered as desired. For example:

   - Detection Antibody Working Solution can be incubated overnight at 2-8ºC.
   - Incubation time can be increased to 2 hours.
   - Incubation temperature can be increased to 37ºC.
   - Plate can be incubated with shaking using an orbital shaker.

Step 5: Wash Plate

1. Carefully remove plate cover.
2. Gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame.
3. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with 1X Wash Buffer, then empty plate contents. Repeat procedure three additional times for a total of FOUR washes. Blot plate onto paper towels or other absorbent material.

   Note: For automated washing, aspirate plate contents from all wells and flood wells with 1X Wash Buffer. Repeat procedure three additional times for a total of FOUR washes. Additional washes may be necessary. Blot plate onto paper towels or other absorbent material.

   Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

Step 6: Add Streptavidin-HRP and Incubate

• Only prepare the required amount of Streptavidin-HRP Solution for the number of strips being used.

1. Add 100 µL of Streptavidin-HRP Solution to each well containing sample or blank.
2. Carefully attach a new adhesive plate cover. Incubate plate for 30 minutes at room temperature.

Step 7: Wash Plate

1. Carefully remove plate cover.
2. Gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame.
3. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with 1X Wash Buffer, then empty plate contents. Repeat procedure three additional times for a total of FOUR washes. Blot plate onto paper towels or other absorbent material.

Note: For automated washing, aspirate plate contents from all wells and flood wells with 1X Wash Buffer. Repeat procedure three additional times for a total of FOUR washes. Additional washes may be necessary. Blot plate onto paper towels or other absorbent material.

Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

Step 8: Add TMB Substrate and Incubate

- Only remove the required amount of TMB Substrate Solution for the number of strips being used.
- Do NOT use a glass pipette to measure the TMB Substrate Solution. Do NOT cover the plate with aluminum foil or metalized Mylar. Do NOT return leftover TMB Substrate to bottle. Do NOT contaminate the unused TMB Substrate Solution. If the solution is blue before use, DO NOT USE IT!

1. Add 100 µL of TMB Substrate Solution into each well.
2. Allow the enzymatic color reaction to develop at room temperature, in the dark for up to 30 minutes. Do NOT cover plate with a plate sealer. The substrate reaction yields a blue solution.

   Note: Incubation time can be adjusted based on color development.

Step 9: Stop Reaction

• Only remove the required amount of Stop Solution for the number of strips being used.

1. After desired color change, stop the reaction by adding 100 µL of Stop Solution to each well. Tap plate gently to mix. The solution in the wells should change from blue to yellow.

Step 10: Measure Absorbance

Note: Evaluate the plate within 30 minutes of stopping the reaction.

1. Wipe underside of wells with a lint-free tissue.
2. Measure the absorbance on an ELISA plate reader set at 450 nm.

Step 11: Calculate Results

• Duplicate absorbance values should be within 10% of each other. Care should be taken when interpreting data with differences in absorbance values greater than 10%.

1. Prepare a standard curve to determine the amount of protein in an unknown sample. Plot the average absorbance obtained for each standard concentration on the vertical (Y) axis versus the corresponding protein concentration on the horizontal (X) axis using graph paper or curve-fitting software.
2. Calculate the protein concentration in unknown samples using the prepared standard curve. Determine the amount of protein in each unknown sample by noting the protein concentration (X axis) that correlates with the absorbance value (Y axis) obtained for the unknown sample.
3. If the sample was diluted, multiply the concentration obtained by the dilution factor to determine the amount of protein in the undiluted sample.

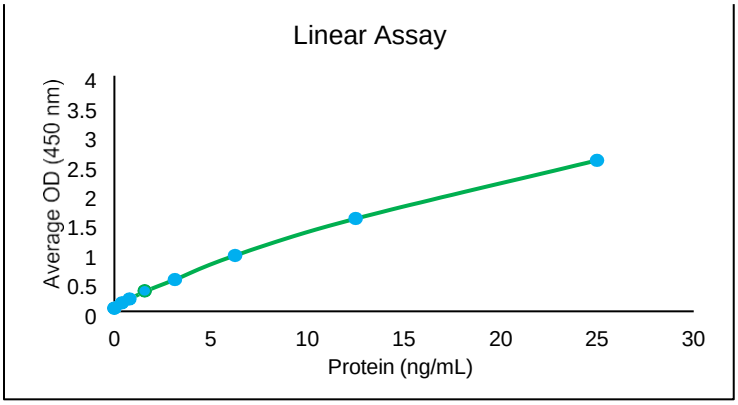
Performance Improvements

The following information is a general guide only. A variety of options may be utilized to obtain optimal results. Assay verification is required.

Optimal Standard and Antibody Concentrations

Antibody Data Sheets included with the DIY ELISA Kit give representative ELISA data. This data can be utilized to help evaluate optimal Standard, Capture Antibody, and Detection Antibody concentrations.

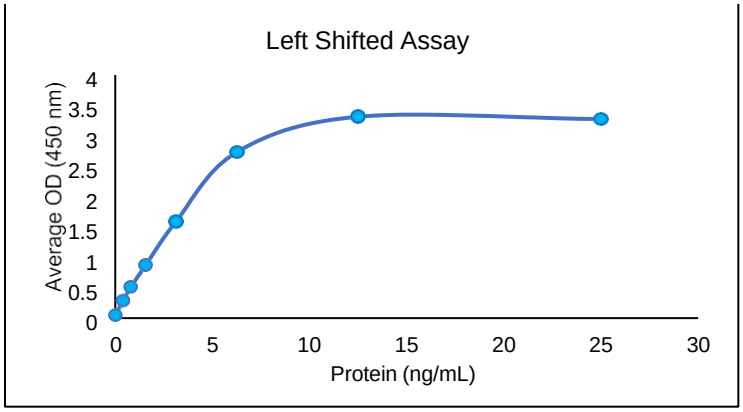
Standard Curve: Optimal Linear Assay



- Optimal Standard curve results should produce a linear line when evaluating the plotted protein concentration (X axis) correlation with the absorbance value (Y axis).
- The top standard should have an absorbance value of 1.8-2.4 optical density (OD) if using TMB Substrate.

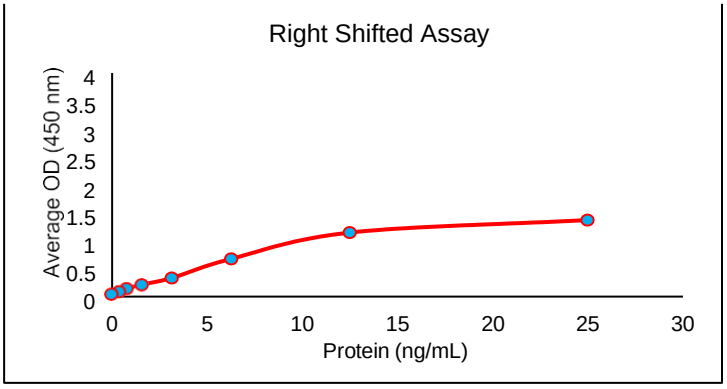
  Note: TMB Substrate typically loses linearity with OD values greater than 2.4. Data above 2.4 OD should be interpreted with caution.
- The zero standard OD values should be less than 0.2 OD (background).
- Discrimination between the lowest standard and the zero standard should be greater than 0.02OD.

Standard Curve: Left Shifted Assay



- The assay is too sensitive and will not produce optimal dynamic range. Suggested options for improving the assay include:
    I. Decreasing Capture Antibody concentration
    II. Decreasing Detection Antibody concentration
    III. Decreasing Standard concentration
    IV. Decreasing TMB Substrate incubation time

Standard Curve: Right Shifted Assay

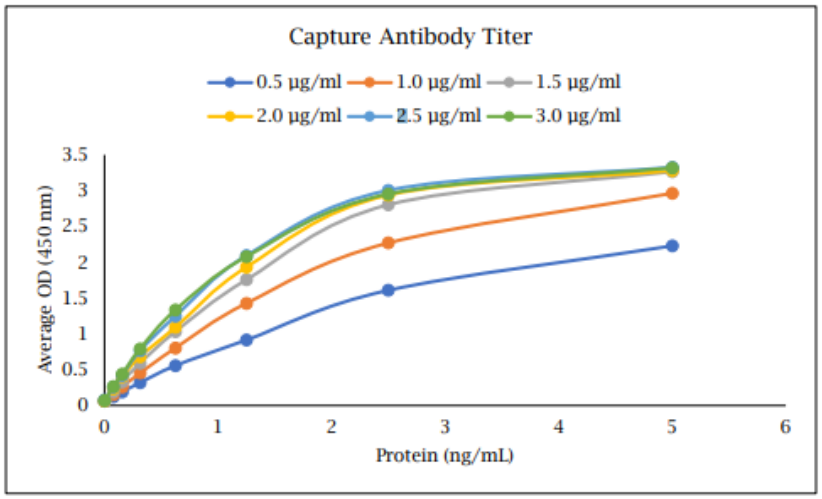


- The assay is not sensitive enough and will not produce optimal dynamic range. Suggested options for improving the assay include:
    I. Increasing Capture Antibody concentration
    II. Increasing Detection Antibody concentration
    III. Increasing Standard concentration
    IV. Increasing incubation times
    V. Increasing incubation temperatures
    VI. Shaking the plate during incubations

Maximizing Assay Sensitivity: Titer Assays

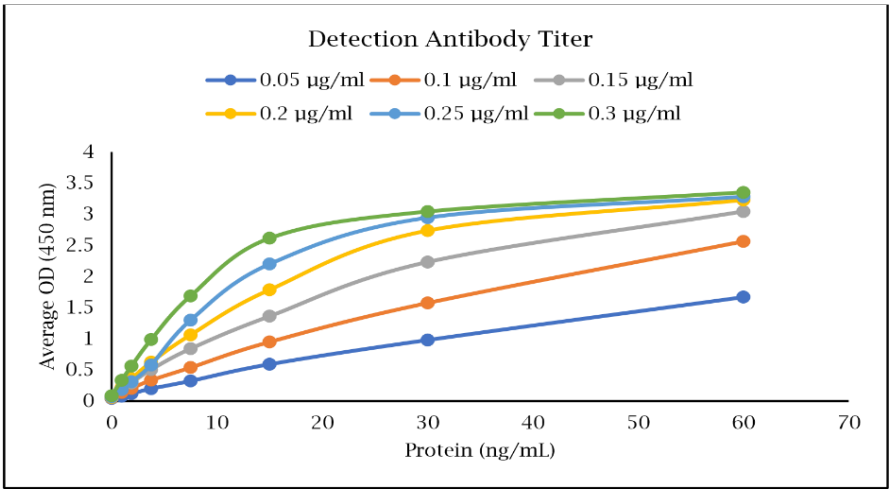
To maximize sensitivity, the optimal Capture Antibody, Detection Antibody, and Standard concentration is required. To verify the optimal concentrations, a titer assay should be performed. This assay requires all components to remain constant except for the one variable being optimized. Each component will need to be verified individually.

Capture Antibody Titer



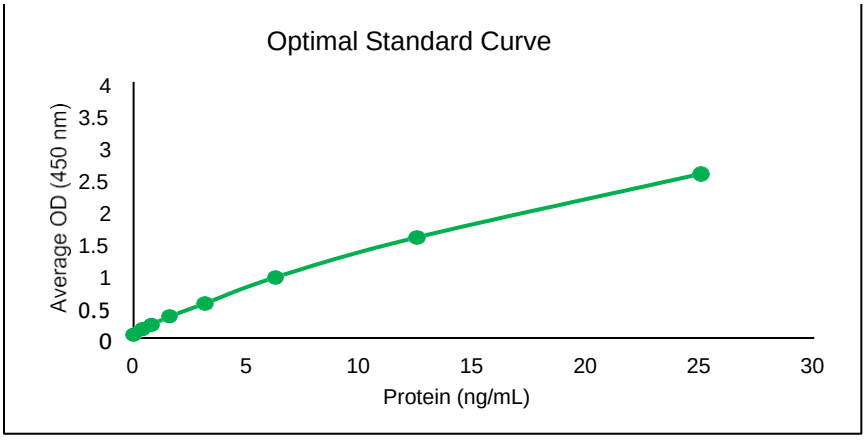
- Optimal capture antibody concentration is obtained when the maximum signal is generated with background values below 0.2 OD.
- In this example, the optimal capture antibody titer is 2.0 µg/mL.

Detection Antibody Titer



- Optimal detection antibody concentration is obtained when the maximum signal is generated with background values below 0.2 OD.
- In this example, the optimal detection antibody titer is 0.3 µg/mL.

Top Standard Concentration Determination



- The optimal top standard concentration should provide linear results.
- The top signal will not plateau so that discrimination between the top two standards can be determined.
- The discrimination between the lowest standard and zero standard will be greater than 0.02 OD.
- In this example, the optimal top standard is 25 ng/mL.

Optimal Standard and Sample Diluent: Linearity and Recovery Assay

Validation of the Standard and Sample Diluent in the assay is suggested. The optimal diluent should provide:

- Linear Results

  Similar values are obtained when correcting for the dilution factor.

- 80-120% Protein Recovery

  When a known quantity of protein is added to a desired sample type, 80-120% of what was added should be measured in the assay.

To verify if a Standard and Sample Diluent works with a desired sample type, a linearity and recovery assay should be performed.

1. Prepare a Standard curve, including a zero Standard, using your test Standard and Sample Diluent.
2. Run a test sample without any added protein in the assay.
   - If possible, choose a negative test sample.
   - Use this unaltered test sample result to subtract out any background/matrix values from the protein added test sample.
3. Add a known quantity of protein into the test sample.
   - Final concentration of the protein in the test sample should be near, but below, the top Standard in your Standard curve.
4. Add the same quantity of protein to the test Standard and Sample Diluent.

   Note: This is the 100% recovery value.
5. Prepare multiple serial dilutions (i.e. 1:2, 1:4, 1:8, 1:16, 1:32, etc.) of the non-protein treated test sample, the protein treated test sample, and the protein treated Standard and Sample Diluent sample and run in the assay.
6. Determine the recovery and linearity of the samples in the assay.
   - Optimal recovery is when the protein added test sample value (subtract out non-protein added test sample values) is 80-120% of the protein added Standard and Sample Diluent value.
   - Optimal linearity should give similar protein values in the protein added test sample when the concentration is corrected for dilution.

Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Notes:

Notes:

Assay Genie 100% money-back guarantee!

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