Calculation of Results
Calculate the mean absorbance for each set of duplicate standards, controls and samples. Subtract the mean zero standard absorbance from each.

Plot the standard curve on log-log graph paper, with C5b-9 concentration on the x-axis and absorbance on the y-axis. Draw the best fit curve through the standard points.

To determine the C5b-9 concentration of the unknowns, find the unknown’s mean absorbance value on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the C5b-9 concentration.

Computer data reduction may also be employed, utilizing log-log regression analysis.

Typical Standard Curve
This standard curve is for demonstration only. A standard curve must be run with each assay.

Standardization
This immunoassay is calibrated against cobra venom-factor activated human serum.

Assay Optimization
1. BD OptEIA™ Sets allow flexible assay design to fit individual laboratory needs. To design an immunoassay with different sensitivity and dynamic range, the following parameters can be varied: Capture, Detection Antibody titers, Incubation time, Incubation temperature, Assay Diluent formulation, Buffer pH, ionic strength, protein concentration, Type of substrate, Washing technique (i.e., number of wash repetitions and soak times).

2. “Typical Standard Curve” and 5-plate yield were obtained in the BD Biosciences Pharmingen laboratory, using the recommended procedure and manual plate-washing.

Troubleshooting

Possible Source | Corrective Action
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Poor Precision | Check function of washing system
• Inadequate washing/aspiration of wells | • Check/adapt wash protocol
• Inadequate mixing of reagents | • Ensure adequate mixing
• Improper/inaccurate pipetting | • Check/calibrate pipettes
• Incomplete sealing of plate | • Ensure complete seal on plate

Poor Standard Curve | Corrective Action
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Possible Source | • Improper standard handling/dilution of standards | • Check function of washing system
• Incomplete washing/aspiration of wells | • Check/calibrate pipettes
• Improper buffer/diluent used | • Check buffer/diluent preparation, pH

Low Absorbances | Corrective Action
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Possible Source | • Inadequate reagent volumes added to wells | • Check/calibrate pipettes
• Incorrect incubation times/temperature | • Ensure sufficient incubation times/reagents warmed to RT
• Improper antibody titration | • Check Capture Ab and Working Detector preparation
• Improper buffer/diluent used | • Check buffer/diluent preparation, pH
• Overly high wash/aspiration pressure from automated plate-washer | • Utilize manual washing

Limitations of the Procedure
• Samples that generate absorbance values higher than the standard curve should be diluted with Standard Diluent and re-assayed.
• Interference by drug metabolites, soluble receptors, or other binding proteins in specimens has not been thoroughly investigated. The possibility of interference cannot be excluded.
• BD OptEIA™ Sets are intended for use as an integral unit. Do not mix reagents from different Set batches. Reagents from other manufacturers are not recommended for use in this Set.
Recommended buffers, solutions

Note: Do not use sodium azide in these preparations. Sodium azide inactivates the horseradish peroxidase enzyme.

The BD OptEIA™ Reagent Set B (Cat. No. 550534) containing Coating Buffer, Assay Diluent, Substrate Reagents A and B, Stop Solution and 20X Wash Buffer Concentrate is recommended.

1. Coating Buffer- 0.1 M Sodium Carbonate, pH 9.5

2. Assay Diluent- PBS* + 0.1% Tween-20. The BD Pharmingen™ Assay Diluent (Cat. No. 555213) is recommended.

3. Standards Preparation for Assay:
   - Prepare 30 ng/mL stock standard as described in the Reconstitution section above (step 11). Vortex gently to mix.
   - Add 300 µL Assay Diluent to 6 tubes. Label as 15 ng/mL, 7.5 ng/mL, 3.75 ng/mL, 1.875 ng/mL, 0.938 ng/mL, and 0.469 ng/mL.
   - Perform serial dilutions by adding 300 µL of each standard to the next tube and vortexing between each transfer. Assay Diluent serves as the zero standard (0 ng/mL).

4. Working Detector Preparation
   - Working Detector must be prepared within 15 minutes prior to use.
   - Determine volume needed for experiment: 100 µL per well.
   - Prepare appropriate volumes of 250X detector antibody and 250X Streptavidin-HRP to add to working detector mix.

5. Working Detector Preparation
   - Dilute the Capture Antibody 1:250 in Coating Buffer and coat microwells with 100 µL of diluted Capture Antibody per well. Seal plate and incubate overnight at 4°C. Do not dilute more Capture Antibody than is needed for your experiment.
   - Aspirate wells and wash 3 times with ≥ 300 µL/well Wash Buffer. After last wash, invert plate and blot absorbent paper to remove any residual buffer.

6. Block plates with ≥ 200 µL/well Assay Diluent. Incubate at room temperature (RT) for 1 hour.

7. Add 100 µL standard or sample to each well. Incubate 2 hr RT.

8. Add 100 µL of Substrate Solution to each well. Incubate plate (without plate seals) for 30 minutes RT in the dark.

9. Add 50 µL of Stop Solution to each well. Incubate 2 hours more at RT.

10. Read absorbance at 450 nm within 30 minutes of stopping reaction. If wavelength correction is available, subtract absorbance 570 nm from absorbance 450 nm.

Assay Procedure Summary

1. Dilute the Capture Antibody 1:250 in Coating Buffer and coat microwells with 100 µL of diluted Capture Antibody per well. Seal plate and incubate overnight at 4°C. Do not dilute more Capture Antibody than is needed for your experiment.

2. Aspirate wells and wash 3 times with ≥ 300 µL/well Wash Buffer. After last wash, invert plate and blot absorbent paper to remove any residual buffer.

3. Block plates with ≥ 200 µL/well Assay Diluent. Incubate at room temperature (RT) for 1 hour.


5. Prepare standard and sample dilutions in Assay Diluent. See “Standards Preparation and Handling”. Be sure to record the reconstituted standard concentration for future use.

6. Pipette 100 µL of each standard, sample, and control into appropriate wells. Seal plate and incubate for 2 hours at RT.

7. Aspirate/wash as in step 2, but with 3 total washes.

8. Add 100 µL of Working Detector to each well. Seal plate and incubate for 1 hour at RT.

9. Aspirate/wash as in step 2, but with 7 total washes.

10. Add 100 µL of Substrate Solution to each well. Incubate plate (without plate seals) for 30 minutes RT in the dark.

11. Add 50 µL of Stop Solution to each well.

12. Read absorbance at 450 nm within 30 minutes of stopping reaction. If wavelength correction is available, subtract absorbance 570 nm from absorbance 450 nm.

Recommended Assay Procedure

1. Dilute the Capture Antibody 1:250 in Coating Buffer and coat microwells with 100 µL of diluted Capture Antibody per well. Seal plate and incubate overnight at 4°C. Do not dilute more Capture Antibody than is needed for your experiment.

2. Aspirate wells and wash 3 times with ≥ 300 µL/well Wash Buffer. After last wash, invert plate and blot absorbent paper to remove any residual buffer.

3. Block plates with ≥ 200 µL/well Assay Diluent. Incubate at room temperature (RT) for 1 hour.


5. Prepare standard and sample dilutions in Assay Diluent. See “Standards Preparation and Handling”. Be sure to record the reconstituted standard concentration for future use.

6. Pipette 100 µL of each standard, sample, and control into appropriate wells. Seal plate and incubate for 2 hours at RT.

7. Aspirate/wash as in step 2, but with 3 total washes.

8. Add 100 µL of Working Detector to each well. Seal plate and incubate for 1 hour at RT.

9. Aspirate/wash as in step 2, but with 7 total washes.

10. Add 100 µL of Substrate Solution to each well. Incubate plate (without plate seals) for 30 minutes RT in the dark.

11. Add 50 µL of Stop Solution to each well.

12. Read absorbance at 450 nm within 30 minutes of stopping reaction. If wavelength correction is available, subtract absorbance 570 nm from absorbance 450 nm.

Assay Procedure Summary

1. Dilute the Capture Antibody 1:250 in Coating Buffer and coat microwells with 100 µL of diluted Capture Antibody per well. Seal plate and incubate overnight at 4°C.

2. Aspirate and wash 3 times.

3. Block plates with 200 µL Assay Diluent to each well. Incubate 1 hr RT.

4. Aspirate and wash 3 times.

5. Add 100 µL standard or sample to each well. Incubate 2 hr RT.

6. Aspirate and wash 3 times.

7. Add 100 µL diluted Working Detector to each well. Incubate 1 hr RT.

8. Aspirate and wash 7 times.

9. Add 100 µL Substrate Solution to each well. Incubate 30 min RT in dark.

10. Add 50 µL Stop Solution to each well. Read at 450 nm within 30 min with λ correction at 570 nm.