Calculation of Results

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

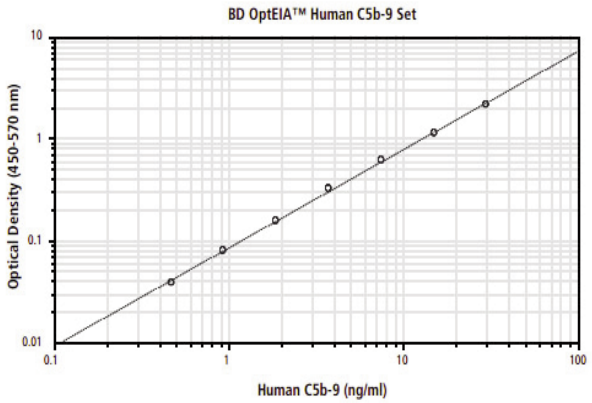
Plot the standard curve on log-log graph paper, with C5b-9 concentration on the x-axis and absorbance on the y-axis. Draw the best fit curve through the standard points.

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. If samples were diluted, multiply the C5b-9 concentration by the dilution factor.

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.



Specificity

Cross Reactivity: The following factors were tested in the BD OptEIA™ assay at ≥ 5 µg/mL and no cross-reactivity (value ≥ 470 pg/mL) was identified.

Purified Native Human:

C3, C4, C5, C6, C7, C8 and C9.

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

Poor Precision

Possible Source	*Corrective Action*
• Inadequate washing/ aspiration of wells	• Check function of washing system
• Inadequate mixing of reagents	• Ensure adequate mixing
• Imprecise/ inaccurate pipetting	• Check/ calibrate pipettes
• Incomplete sealing of plate	• Ensure complete seal on plate

Poor Standard Curve

Possible Source	*Corrective Action*
• Improper standard handling/ dilution	• Ensure correct preparation, storage of standards
• Incomplete washing/ aspiration of wells	• Check function of washing system
• Imprecise/ inaccurate pipetting	• Check/ calibrate pipettes
• Improper buffer/ diluent used	• Check buffer/ diluent preparation, pH

Low Absorbances

Possible Source	*Corrective Action*
• Inadequate reagent volumes added to wells	• Check/ calibrate pipettes
• Incorrect incubation times/ temperature	• Ensure sufficient incubation times/ reagents warmed to RT
• Incorrect 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.

558315 Rev.2

BD OptEIA™

Technical Data Sheet

Human C5b-9 ELISA Set

Cat. No. 558315

Materials Provided

The BD OptEIA™ Set for Human C5b-9 contains the components necessary to develop enzyme-linked immunosorbent assays (ELISA) for natural C5b-9 in serum, plasma, and other biological samples. Sufficient materials are provided to yield approximately 5 plates of 96-wells if the recommened storage, materials, buffer preparation, and assay procedure are followed as specified in this package.

Capture Antibody

Purified Anti-Human C5b-9 (250X)

Detection Antibody

Biotin Anti-Human C5b-9 (250X)

Enzyme Reagent

Streptavidin-horseradish peroxidase conjugate (SAv-HRP) (250X)

Standard

Human C5b-9 Standard, lyophilized (5 vials)

United States
877.232.8995

Canada
866.979.9408

Europe
32.2.400.98.95

Japan
0120.8555.90

Asia/Pacific
65.6861.0633

Latin America/Caribbean
55.11.5185.9995

BD Biosciences

BD Biosciences Pharmingen
10975 Torreyana Road
San Diego, CA 92121
Customer/Technical Service
Tel 877.232.8995 (US)
Fax 858.812.8888
www.bdbiosciences.com

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
   7.13 g $NaHCO_3$, 1.59 g Na_2CO_3; q.s. to 1.0 L; pH to 9.5 with 10 N NaOH. Freshly prepare or use within 7 days of preparation, stored at 2-8°C.
2. **Assay Diluent-** PBS* with 10% FBS#, pH 7.0. The BD Pharmingen™ Assay Diluent (Cat. No. 555213) is recommended.
   *Phosphate-Buffered Saline: 80.0 g NaCl, 11.6 g Na_2HPO_4, 2.0 g KH_2PO_4, 2.0g KCl, q.s. to 10 L; pH to 7.0.
   #Fetal Bovine Serum: Hyclone Cat. No. SH30088 (heat-inactivated) recommended.
   Freshly prepare or use within 3 days of preparation, with 2-8°C storage.
3. **Wash Buffer-** PBS* with 0.05% Tween-20. Freshly prepare or use within 3 days of preparation, with 2-8°C storage.
4. **Substrate Solution-** Tetramethylbenzidine (TMB) and Hydrogen Peroxide. The BD Pharmingen™ TMB Substrate Reagent Set (Cat. No. 555214) is recommended.
5. **Stop Solution -** 1 M H_3PO_4 or 2 N H_2SO_4

Additional Materials Required

1. 96-well Nunc-Immuno™ polystyrene Maxisorp ELISA flat bottom plates (ThermoFisher Scientific Cat. No. 442404) are recommended
2. Microplate reader capable of measuring absorbance at 450 nm
3. Precision pipettes
4. Graduated cylinder, one liter
5. Deionized or distilled water
6. Wash bottle or automated washer
7. Log-log graph paper or automated data reduction
8. Tubes to prepare standard dilutions
9. Laboratory timer
10. Plate sealers or parafilm

Storage Information

1. Store unopened reagents at 2-8°C. Do not use reagents after expiration date, or if precipitation or turbidity is evident.
2. Before use, bring all reagents to room temperature (18-25°C). Immediately after use, return to proper storage conditions.

Specimen Collection and Handling

Specimens should be clear, non-hemolyzed and non-lipemic.

Cell culture supernatants: Remove any particulate material by centrifugation and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

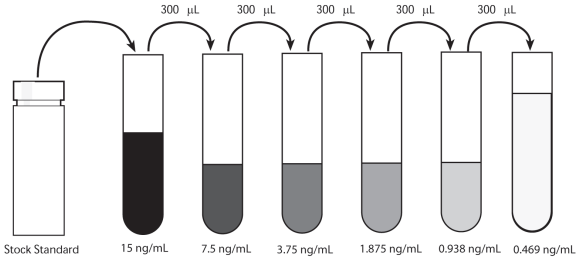
Serum: Use a serum separator tube and allow samples to clot for 30 minutes, then centrifuge for 10 minutes at 1000 x g. Remove serum and assay immediately or store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using citrate, EDTA, or heparin as anticoagulant. Centrifuge for 10 minutes at 1000 x g within 30 minutes of collection. Assay immediately or store samples at ≤-20°C. Avoid repeated freeze-thaw cycles.

Standards Preparation and Handling

1. **Reconstitution:** After warming to room temperature, carefully open vial to avoid loss of material. Reconstitute lyophilized standard with appropriate amount of assay diluent to yield a 30 ng/mL stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Vortex gently to mix.
2. **Storage/ handling of reconstituted standard:** Use reconstituted standard immediately. Use one vial of standard per 96-well plate.
3. **Standards Preparation for Assay:**
   a. Prepare 30 ng/mL stock standard as described in the Reconstitution section above (step 1). Vortex gently to mix.
   b. 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.
   c. 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).

Serial dilutions within the plate may also be performed, by pipetting 100 µL of Assay Diluent into each standard well except the highest (30 ng/mL), then adding 100 µL of the 30 ng/mL standard to both that well and the 15 ng/mL well, mixing the well contents by rinsing the pipette tip, and adding 100 µL of the 15 ng/mL standard to the 7.5 ng/mL well. Continue these dilutions to the 0.469 ng/mL standard well, out of which the extra 100 µL should be discarded.



Working Detector Preparation

Note: Working Detector must be prepared within 15 minutes prior to use.

1. Determine volume needed for experiment: 100 µL per well.

2. Determine amount of 250X detector antibody and 250X Streptavidin-HRP to add to working detector mix.

3. Add appropriate volumes of 250X detector antibody and 250X Streptavidin-HRP to Assay Diluent to prepare required volume of Working Detector. Do not dilute more Detection Antibody than is needed for your experiment.

4. Add Working Detector to each well as described in the assay procedure.

Warnings and Precautions

1. Human C5b-9 Standard contains human serum, a potentially biohazardous material. Use universal precautions when handling. Handle as if product were capable of transmitting disease. Materials used in this product have been tested using FDA approved methods and found negative for Human Immunodeficiency Virus (HIV-1/HIV-2), Hepatitis B Surface Antigen (HBSAG) and antibody to Hepatitis C Virus (HCV). However, no known test method can offer complete assurance that specimens of human origin will not transmit infectious disease. When handling or disposing, follow precautions described in CDC and FDA recommendations and OSHA Bloodborne Pathogen recommendations.
2. Reagents which contain preservatives may be toxic if ingested, inhaled, or in contact with skin.
3. Handle all serum and plasma specimens in accordance with NCCLS guidelines for preventing transmission of blood-borne infections.
4. Capture Antibody contains ≤ 0.09% sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
5. Detection Antibody contains BSA, ≤ 0.09% sodium azide and ProClin®-150 as a preservative.
6. Human C5b-9 Standard contains BSA and ProClin®-150 as a preservative.
7. Source of all serum proteins is from USDA inspected abattoirs located in the United States.
8. ***Warning:*** Human C5b-9 lyophilized standard (component 51-9004086) contains 0.03% (w/w) and Detection Antibody Biotin Anti-Human C5b-9 (component 51-9004167) contains 0.002% (w/w) of a CMIT/MIT mixture (3:1), which is a mixture of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC No 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC No 220-239-6] (3:1).
   Hazard statements
   May cause an allergic skin reaction.
   Precautionary statements
   Wear protective gloves / eye protection.
   Wear protective clothing.
   Avoid breathing mist/vapours/spray.
   If skin irritation or rash occurs: Get medical advice/attention.
   IF ON SKIN: Wash with plenty of water.
   Dispose of contents/container in accordance with local/regional/national/international regulations.

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 on absorbent paper to remove any residual buffer.
3. Block plates with ≥ 200 µL/well Assay Diluent. Incubate at room temperature (RT) for 1 hour.
4. Aspirate/wash as in step 2.
5. Prepare standard and sample dilutions in Assay Diluent. See "Standards Preparation and Handling". Be sure to record the reconstituted standard concentraiton 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 sealer) 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. Add 100 µL diluted Capture Ab to each well. Incubate overnight at 4°C.
2. Aspirate and wash 3 times.
3. Block plates: 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 TMB 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.