Quantikine®

Human Complement Factor D Immunoassay

Catalog Number DFD00

For the quantitative determination of human Complement Factor D concentrations in cell culture supernates, serum, plasma, and urine.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTRODUCTION

The complement system comprises approximately 30 circulating plasma proteins as well as cell-surface receptors that function as part of the innate and adaptive immune system to eliminate pathogens. The system is organized into multiple proteolytic cascades where proteases exist as inactive zymogens and are activated via the action of an upstream active protease. Three pathways of complement activation: classical, lectin, and alternative, exist. These pathways converge in the generation of the C3 convertase, which is responsible for the initiation of a series of events leading to the generation of bacterial opsonin (that facilitates the phagocytosis of opsonized pathogens), anaphylatoxins (that mediate inflammation), and the formation of the terminal membrane attack complex (that induces the lysis of pathogens or cells) (1).

Complement factor D, also known as adipsin, is a serine protease that is indispensable for the initiation of complement activation via the alternative pathway. Upon activation through reversible substrate-induced conformational change into an active enzyme, factor D functions to cleave the C3b-bound factor B, resulting in the formation of C3bBb complex, which is the alternative pathway C3 convertase. Human complement factor D is synthesized as a 253 amino acid (aa) precursor that contains a signal peptide (aa 1 - 20), a five-residue activation-/pro-peptide (aa 21 - 25), and the mature chain (aa 26 - 253). Under physiological conditions, mature factor D lacking the activation peptide circulates as an inactive enzyme and requires interaction with its natural substrate, C3b-bound factor B, for activation of its catalytic activity (2). Mature human factor D shares 98%, 96%, 84%, and 66% aa sequence homology with the chimpanzee, rhesus monkey, porcine, and mouse protein, respectively. Factor D is expressed in multiple tissues, including monocyte/macrophages, muscle, sciatic nerve, endometrium, kidney, intestine, and at especially high levels in adipocytes (3). Even though the level of factor D expression is reduced in various mouse models of obesity, a role for factor D in fat metabolism or systemic energy balance has not been demonstrated so far (4 - 6).

Serum complement factor D concentration is regulated through catabolism in the kidney where factor D is filtered by the glomerulus and reabsorbed by the proximal tubule (7). In patients with renal failure, circulating levels of factor D are elevated. Similarly in patients with Fanconi syndrome, a disorder in which the proximal tubular function of the kidney is impaired, urinary factor D concentrations are also highly elevated (8). Complement factor D deficiency is associated with low activity of the alternative complement pathway and low capacity to opsonize bacteria. In patients with mutations in the factor D gene resulting in complete factor D deficiency, recurrent bacterial infections were observed (9, 10).

The Quantikine Human Complement Factor D Immunoassay is a 3.5 hour sandwich ELISA designed to measure human Complement Factor D in cell culture supernates, serum, plasma, and urine. It contains NS0-expressed recombinant human Complement Factor D and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Complement Factor D showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human Complement Factor D.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Complement Factor D has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Complement Factor D present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for Complement Factor D is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Complement Factor D bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Complement Factor D Microplate (Part 893176) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against Complement Factor D.

Complement Factor D Conjugate (Part 893177) - 12 mL of polyclonal antibody against Complement Factor D conjugated to horseradish peroxidase with preservatives.

Complement Factor D Standard (Part 893178) - 40 ng of recombinant human Complement Factor D in a buffered protein solution with preservatives; lyophilized.

Assay Diluent RD1W (Part 895117) - 11 mL of a buffered protein solution with preservatives.

Calibrator Diluent RD5-26 Concentrate (Part 895525) - 21 mL of a buffered protein solution with preservatives.

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895174) - 23 mL of diluted hydrochloric acid.

Plate Covers - 4 adhesive strips.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Assay Diluent RD1W	
	Calibrator Diluent RD5-26	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	
	Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Test tubes for dilution.
- Human Complement Factor D Controls (optional; available from R&D Systems).

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20° C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Serum and plasma samples require a 400-fold dilution. A suggested 400-fold dilution can be achieved by adding 20 μL of sample to 380 μL of Calibrator Diluent RD5-26 (1X). Complete the 400-fold dilution by adding 20 μL of the diluted sample to 380 μL of Calibrator Diluent RD5-26 (1X).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

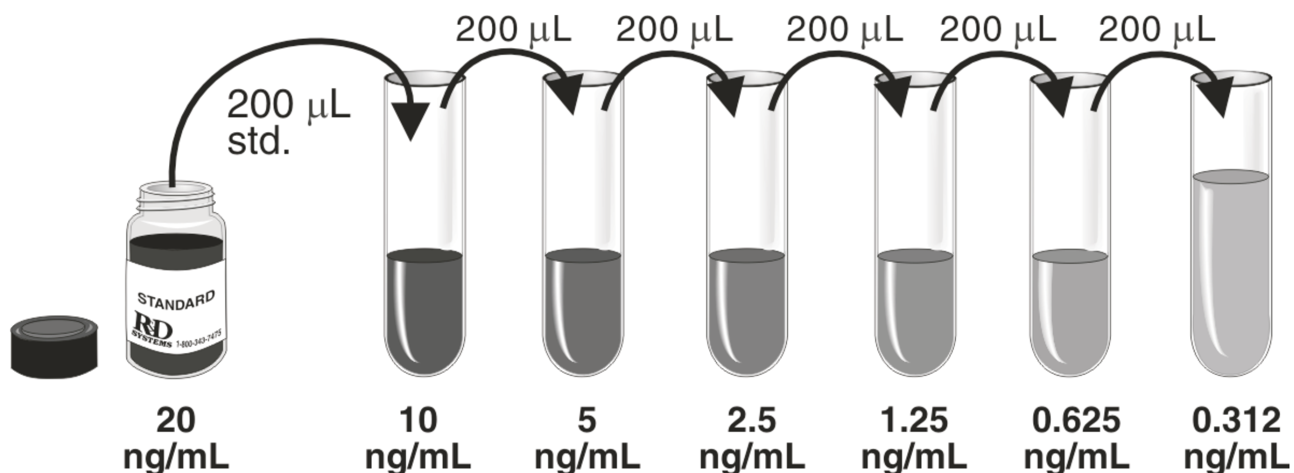
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 μL of the resultant mixture is required per well.

Calibrator Diluent RD5-26 (1X) - Dilute 20 mL Calibrator Diluent RD5-26 Concentrate into deionized or distilled water to yield 80 mL of Calibrator Diluent RD5-26 (1X).

Complement Factor D Standard - Reconstitute the Complement Factor D Standard with 2.0 mL of Calibrator Diluent RD5-26 (1X). This reconstitution produces a stock solution of 20 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Pipette 200 μL of Calibrator Diluent RD5-26 (1X) into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Complement Factor D standard serves as the high standard (20 ng/mL). Calibrator Diluent RD5-26 (1X) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

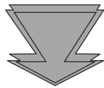
Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 μL of Assay Diluent RD1W to each well.
4. Add 50 μL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 μL of Complement Factor D Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 μL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

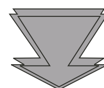
*Serum and plasma samples require dilution as directed in the Sample Preparation section.

ASSAY PROCEDURE SUMMARY

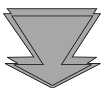
1. Prepare reagents, samples, and standards as instructed.



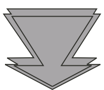
2. Add 50 µL Assay Diluent RD1W to each well.



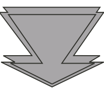
3. Add 50 µL Standard, control, or sample* to each well. Incubate 2 hours at RT.



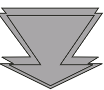
4. Aspirate and wash 4 times.



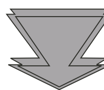
5. Add 100 µL Conjugate to each well. Incubate 1 hour at RT.



6. Aspirate and wash 4 times.



7. Add 100 µL Substrate Solution to each well. Incubate 30 minutes. **Protect from light.**



8. Add 100 µL Stop Solution to each well. Read at 450 nm within 30 minutes. λ correction 540 or 570 nm

*Serum and plasma samples require dilution as directed in the Sample Preparation section.

CALCULATION OF RESULTS

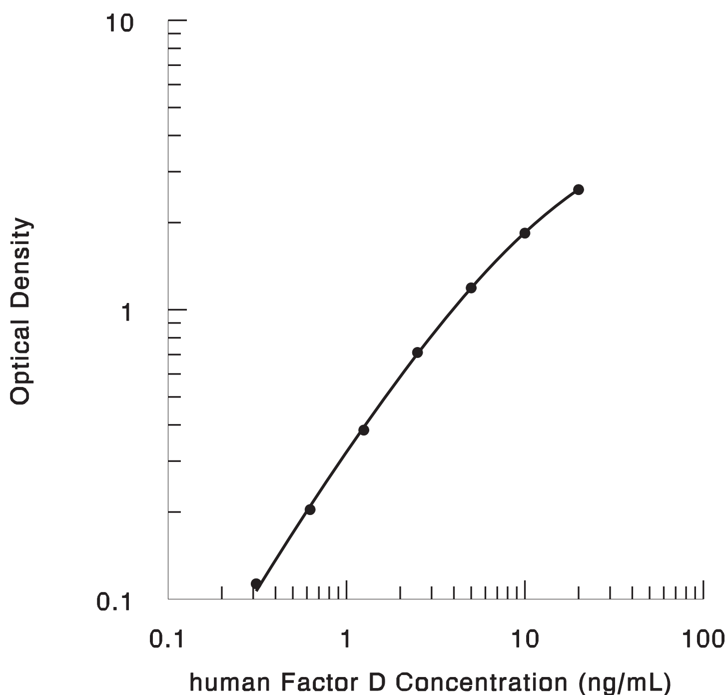
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Complement Factor D concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



ng/mL	O.D.	Average	Corrected
0	0.015 0.017	0.016	—
0.312	0.125 0.132	0.129	0.113
0.625	0.217 0.223	0.220	0.204
1.25	0.394 0.406	0.400	0.384
2.50	0.720 0.736	0.728	0.712
5.00	1.200 1.210	1.200	1.186
10.0	1.840 1.880	1.860	1.844
20.0	2.590 2.650	2.620	2.604

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	0.860	3.12	8.13	0.831	3.04	7.73
Standard deviation	0.047	0.183	0.552	0.074	0.211	0.506
CV (%)	6.4	5.9	5.8	9.0	6.9	6.6

RECOVERY

The recovery of Complement Factor D spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample	Average % Recovery	Range
Cell culture media (n=4)	104	97 - 117%
Urine (n=4)	100	87 - 115%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Complement Factor D were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates (n=4)	Serum* (n=4)	Heparin plasma* (n=4)	EDTA plasma* (n=4)	Urine (n=4)
1:2	Average % of Expected	100	103	96	103	103
	Range (%)	98 - 106	99 - 110	90 - 101	95 - 106	97 - 110
1:4	Average % of Expected	98	105	92	102	102
	Range (%)	89 - 113	94 - 112	91 - 94	89 - 115	96 - 104
1:8	Average % of Expected	92	100	92	101	101
	Range (%)	87 - 100	89 - 111	90 - 96	90 - 111	96 - 105
1:16	Average % of Expected	82	94	—	99	100
	Range (%)	—	85 - 103		93 - 105	99 - 100

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of Complement Factor D ranged from 0.005 - 0.025 ng/mL. The mean MDD was 0.013 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human Complement Factor D produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of Complement Factor D in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (ng/mL)	% Detectable	Range (ng/mL)
Serum (n=35)	2339	100	1729 - 3238
EDTA plasma (n=35)	2297	100	1468 - 3657
Heparin plasma (n=35)	1667	100	906 - 2545
Urine (n=12)	1.83	75	ND - 7.32

ND = Non-detectable

Cell Culture Supernates -
Human peripheral blood leukocytes (1 x 10^6 cells/mL) were cultured for 6 days in RPMI supplemented with 10% fetal bovine serum, 5 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. An aliquot of the cell culture supernate was assayed for levels of natural Complement Factor D, and measured 13.2 ng/mL.

Human peripheral blood mononuclear cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 2 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 ng/mL of rhIL-2 for 1 day. Aliquots of the cell culture supernate were removed and assayed for levels of natural Complement Factor D.

Condition	Day 1 (ng/mL)
Unstimulated	1.46
Stimulated	1.60

HT-29 cells (0.25 x 10^5 cells/mL) were cultured in McCoy's 5a media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate for 7 days. An aliquot of the cell culture supernate was removed, assayed for levels of natural Complement Factor D, and measured 0.798 ng/mL.

U937 cells (1 x 10^5 cells/mL) were cultured for 1 and 3 days in RPMI supplemented with 10% fetal bovine serum, and 2 mM L-glutamine. Aliquots of the cell culture supernate were removed and assayed for levels of natural Complement Factor D.

Condition	Day 1 (ng/mL)	Day 3 (ng/mL)
Unstimulated	3.17	5.61

Dendritic cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 5 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. Cells were stimulated with 1000 U/mL of rhGM-CSF and 500 U/mL of rhIL-4 for 7 days. An aliquot of the cell culture supernate was assayed for levels of natural Complement Factor D, and measured 2.89 ng/mL.

SPECIFICITY

This assay recognizes recombinant and natural human Complement Factor D. The factors listed below were prepared at 200 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 200 ng/mL in a mid-range recombinant human Complement Factor D control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
Coagulation Factor II/Thrombin
Coagulation Factor VII
Coagulation Factor X
Coagulation Factor Xa
Coagulation Factor XI
Complement Component C1r
Complement Component C1s
Complement Component C2
Complement Component C3
Complement Factor B
Complement Factor H-related 1/CFHR1
Complement Factor H-related 5/CFHR5

Other:
bovine Coagulation Factor Xa
human Coagulation Factor II/Thrombin

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PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.

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