



MicroVue™ Complement

C4d Fragment EIA

An enzyme immunoassay for the quantitation of the C4d-containing fragments of activated C4 of the Classical Complement Pathway

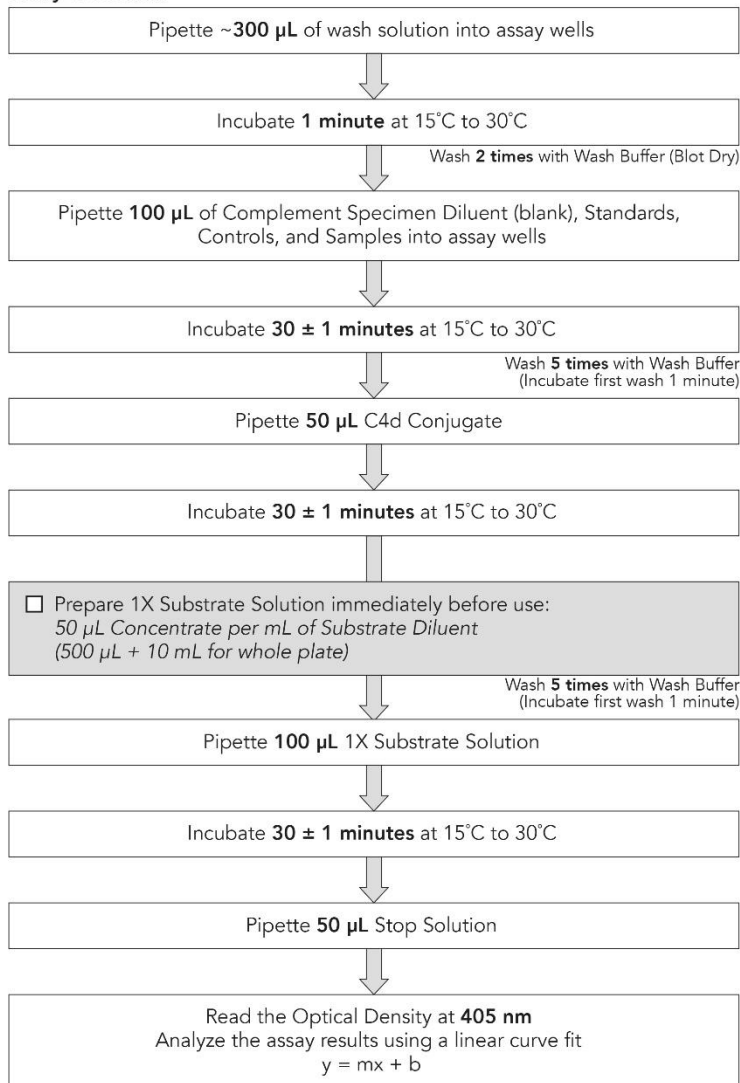
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SUMMARY

Reagent and Sample Preparation

- ☐ Dilute Wash Buffer Concentrate 1:20 with DI Water.
- ☐ Reconstitute each Standard and Control with 2.0 mL of Hydrating Reagent (let sit for 15 minutes, and mix gently before use)
- ☐ Dilute Samples 1:70 with Complement Specimen Diluent (e.g. 10 μ L + 690 μ L) (pipette into assay wells within 30 minutes)

Assay Procedure





The MicroVue CICP Assay is an enzyme immunoassay for the quantitation of the C4d-containing fragments of activated C4 of the Classical Complement Pathway.

SUMMARY AND EXPLANATION

The MicroVue C4d Fragment Enzyme Immunoassay measures the amount of the C4d-containing activation fragments of C4 (C4b, iC4b, and C4d) present in human serum, EDTA plasma, and other biological or experimental samples.

The fourth component of complement is one of the plasma proteins that is unique to the classical pathway of complement activation.¹ Classical pathway activation is triggered upon binding of the C1q subcomponent of C1 to IgG or IgM-containing immune complexes. In addition, C1 is bound and activated by a variety of other substances including retroviruses, certain bacteria, parasites, transformed cells, subcellular membranes, polyanions (DNA), and C-reactive protein in complex with phosphorylcholine.² Binding of C1 to these activators of the classical pathway results in conversion of the zymogen C1s sub-component into an active proteolytic enzyme (C1 \bar{s}). C1 \bar{s} cleaves the C4 α -chain at peptide bond 77 resulting in the production of C4a and C4b fragments with molecular weights of 9,000 and 191,000, respectively. The C4a fragment is one of the complement anaphylatoxins.³ The C4b fragment has numerous important biological activities.^{1,4} These activities include mediation of enhanced phagocytosis of complement-activating targets by white blood cells (opsonization) and participation in classical pathway C3 and C5 convertase assembly, which leads to terminal component activation and subsequent complement-mediated lysis of target microorganisms and other cells.

The expression of the biological activities of C4b is strictly regulated. Thus, the ability of C4b to participate in classical pathway activation and opsonization reactions is inhibited by the single site cleavage of the C4b α -chain by Factor I.⁵ This reaction requires either C4 binding protein (C4bp)⁶ or complement receptor CR1 as a cofactor.^{7,8} C4bp is a complement control protein⁹ and CR1 is the C3b/C4b receptor found on erythrocytes, granulocytes, monocytes, and macrophages.⁸ Factor I cleavage of C4b yields inactivated C4b, termed iC4b.⁷⁻¹⁰ iC4b can be further degraded by Factor I, with the cooperation of C4bp or CR1, to C4c and C4d fragments.⁶⁻¹⁰ The C4c and C4d fragments, which can be produced in fluid phase as well as on target surfaces, appear to be the final physiological degradation products of C4b.^{1,6-10}

C4d has been quantitated in human serum and plasma specimens. The levels of C4d, when normalized for the presence of endogenous C4, can be significantly elevated in plasma specimens obtained from some patients with rheumatoid arthritis, hereditary angioedema, systemic lupus erythematosus, or chronic urticaria with hypocomplementemia.^{11,12} C4d levels may also be elevated in body fluids¹³ and plasma samples obtained from other patients in which classical complement pathway activation is known to occur, e.g., from patients with a variety of humoral autoimmune diseases,¹⁴ septicemia, thermal injury, multiple organ trauma, myocardial infarctions, hereditary angioedema, glomerulonephritis, and acute respiratory distress syndrome. The correlation between C4d activation fragment levels and the clinical status or prognosis for patients with these and other diseases remains to be determined.

The MicroVue C4d Fragment Enzyme Immunoassay provides a rapid, non-radioactive, highly specific and quantitative procedure for measuring C4 activation. Thus, this test is designed for investigations studying the role or status of classical complement pathway activation in numerous research and clinical settings, and for monitoring the generation of C4d-containing fragments *in vitro* or *in vivo*.

PRINCIPLE OF THE PROCEDURE

The MicroVue C4d Fragment Enzyme Immunoassay for the quantitation of C4d in human serum, plasma, or experimental samples is a three-step procedure utilizing (1) a microassay plate coated with a mouse monoclonal antibody that binds specifically to C4d-containing activation fragments of human C4, (2) an HRP-conjugated goat anti-human C4d, and (3) a chromogenic substrate.

In the first step, standards, controls, and test specimens are added to microassay wells pre-coated with an anti-C4d specific monoclonal antibody. C4d present in the standards, controls or specimens will bind to the immobilized anti-C4d. After incubation, a wash cycle removes unbound material.

In the second step, horseradish peroxidase (HRP)-conjugated goat anti-C4d antibody is added to each test well. The enzyme conjugated anti-C4d binds to C4d that was captured by the monoclonal anti-C4d bound on the surface of the microassay wells. After incubation, a wash cycle removes unbound, excess conjugate.

In the third step, a chromogenic enzyme substrate is added to each microassay well. The bound HRP-conjugate reacts with the substrate forming a green color. After incubation the enzyme reaction is stopped chemically, and the color intensity is measured spectrophotometrically at 405 nm. The color intensity of the reaction mixture is proportional to the concentration of C4d present in the test specimens, standards, and controls.

REAGENTS AND MATERIALS PROVIDED

96 Assays for C4d Fragment

MicroVue C4d Fragment EIA Kit contains the following:

A C4d Standards:	Parts A4638 – A4642	2 mL, 1 each
B (Lyophilized) Contains human serum containing known amounts of C4d-containing fragments in PBS,		
C Protein Stabilizers		
D		
E		
L Low/High Controls	Parts A9572, A9573	2 mL, 1 each
H (Lyophilized) Contains human serum with C4d-containing fragments diluted in PBS, Protein Stabilizers		
1 Coated Strips	Part A9567	12 each
12 eight-well strips coated with a mouse monoclonal antibody specific for human C4d in a resealable foil pouch		
2 Stop Solution	Part A3673	6 mL
Contains 250 mM Oxalic Acid		
3 20X Wash Solution Concentrate	Part A9957	50 mL, 2 each
Contains phosphate buffered saline (PBS), 1.0% Tween-20®, and 0.035% ProClin® 300		
4 Specimen Diluent	Part A3670	50 mL
Contains PBS, 0.05% Tween-20, Protein Stabilizers, 0.035% ProClin 300		
5 Substrate Diluent	Part A3672	25 mL
Contains 0.1M Citrate Buffer and 0.05% H ₂ O ₂		
6 Substrate Concentrate	Part A3671	1.5 mL
Contains 0.7% 2-2'-Azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt		
7 C4d Conjugate	Part A9945	3 mL, 2 each
Contains Peroxidase-conjugated (goat) anti-human C4d in HRP stabilizing buffer with preservative		

8 Hydrating Reagent

Part A3675

25 mL

Contains 0.035% ProClin 300

Tween-20® is a registered trademark of ICI Americas Inc.

ProClin® is a registered trademark of Rohm and Haas Company

MATERIALS REQUIRED BUT NOT PROVIDED

- Timer (60 minute range)
- Calculator or other computational method to validate the assay
- Clean, unused microassay plates and/or test tubes and racks
- Container for wash buffer dilution
- Wash bottle or other immunoassay washing system
- Adjustable multichannel pipette (8 or 12 channels) or repeating micropipettes (optional)
- Clean pipettes, 1 mL, 5 mL, and 10 mL
- Micropipettes and pipette tips
- Plate reader capable of optical density readings between 0.0 and 2.0
- Deionized or distilled water

WARNINGS AND PRECAUTIONS

- For Research Use Only in the U.S. Not for use in diagnostic procedures (U.S. only).
- Treat specimen samples as potentially biohazardous material. Follow Universal Precautions when handling contents of this kit and any patient samples.
- Use the supplied reagents as an integral unit prior to the expiration date indicated on the package label.
- Store assay reagents as indicated.
- Do not use Coated Strips if pouch is punctured.
- Test each sample in duplicate.
- ProClin 300 is used as a preservative. Incidental contact with or ingestion of buffers or reagents containing ProClin can cause irritation to the skin, eyes or mouth. Use good laboratory practices to reduce exposure. Seek medical attention if symptoms are experienced.
- Each donor unit used in the preparation of the standards and control sera of this product was tested by an FDA-approved method for the presence of antibody to human immunodeficiency virus (HIV1 and HIV2) and to hepatitis C virus, as well as for hepatitis B surface antigen. Since no test method can offer complete assurance that infectious agents are absent, these reagents should be handled at Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 2007.
- Use of multichannel pipettes or repeat pipettors is recommended to ensure timely delivery of reagents.
- For accurate measurement of samples, add samples and standards precisely. Pipette carefully using only calibrated equipment.
- Proper collection and storage of test specimens are essential for accurate results (see *SPECIMEN COLLECTION AND STORAGE*).
- Avoid microbial or cross-contamination of specimens or reagents.
- Do not use a microassay well for more than one test.
- Decontaminate all specimens, reagents, and materials by soaking for a minimum of 30 minutes in a 1:10 solution of household bleach (sodium hypochlorite) or autoclave at 121°C for 30 minutes at 15 psi.
- Using incubation times and temperatures other than those indicated in the *ASSAY PROCEDURE* section may give erroneous results.
- The Substrate Concentrate must be protected from bright or direct light.
- Do not allow microassay wells to dry once the assay has begun.
- When removing liquid from the microassay wells, do not scrape or touch the bottom of the wells.

- Heat-inactivated specimens may yield erroneous results.
- Hyperlipemic or contaminated specimens may give erroneous results.
- To avoid aerosol formation during washing, use an apparatus to aspirate the wash fluid into a bottle containing household bleach.
- A wash bottle or automated filling device should be used to wash the plate (*ASSAY PROCEDURE*, Step 6). For best results, do not use a multichannel pipette to wash the microassay plate.
- Testing should be performed in an area with adequate ventilation.
- Dispose of containers and unused contents in accordance with Federal, State and Local regulatory requirements.
- Wear suitable protective clothing, gloves, and eye/face protection when handling the contents of this kit.
- Wash hands thoroughly after handling.
- For additional information on hazard symbols, safety, handling and disposal of the components within this kit, please refer to the Safety Data Sheet (SDS) located at quidel.com.

STORAGE

Store the unopened kit at 2°C to 8°C. Equilibrate reagents and materials to 15°C to 30°C before use. Place all unused microassay strips into the storage bag, reseal the bag, and store at 2°C to 8°C.

INDICATIONS OF INSTABILITY OR DETERIORATION OF REAGENTS

The Substrate Concentrate will range in color from colorless to pale or even dark green. However, the freshly prepared Substrate Solution should be colorless to pale green. A dark green color indicates that the reagent has deteriorated, must be discarded, and new Substrate Solution must be prepared in clean glassware.

Cloudiness of the Wash Solution indicates a deterioration of this reagent. If this occurs, the solution should be discarded.

SPECIMEN COLLECTION AND STORAGE

Handle and dispose of all specimens using Universal Precautions.

The proper collection, processing and storage of specimens is essential since C4d is susceptible to proteolysis in improperly collected or stored specimens.

Normal values for serum samples are slightly higher than those obtained with matched EDTA plasma samples. The C4d levels in EDTA plasma may therefore more accurately represent the *in vivo* concentrations.

Serum or EDTA plasma specimens should be collected aseptically using standard techniques. The specimens should be tested immediately or stored at 4°C or on ice until assayed. However, this short-term storage on ice should not exceed four hours.

For longer-term storage, serum or plasma should be frozen at –70°C or below within two hours after collection. Frozen specimens should be tested as soon as possible after thawing or stored on ice (for no longer than four hours) until assayed.

A **Specimen Stabilizing Solution** (Part A9576) can also be used to prepare human serum and plasma specimens for storage. Proper use of this product, available only from Quidel, requires that the specimen be mixed 1:1 with the solution prior to freezing. Additional technical information about the solution is available upon request.

Frozen specimens should be tested as soon as possible after thawing or stored on ice (for no longer than four hours) until assayed. Repeated freezing and thawing is not recommended.

REAGENT PREPARATION

Bring all reagents and materials to 15°C to 30°C before use.

After removing the needed reagents and materials, return the unused items to their appropriate storage temperatures (see *STORAGE*).

Refer to Table 1 for the amounts of reagents and materials required per number of wells.

Wash Solution

Mix the 20X Wash Solution Concentrate by inverting the bottle several times. If the 20X Wash Solution Concentrate has been stored at 2°C to 8°C, crystals may have formed. To dissolve the crystals, warm the bottle in a 37°C to 50°C water bath until all crystals have dissolved and follow by mixing thoroughly. Prepare the Wash Solution by diluting the entire contents of one of the bottles of 20X Wash Solution Concentrate up to one liter with distilled or deionized water. Mix thoroughly. The Wash Solution is stable for 30 days when stored in a clean container at 2°C to 8°C. If discoloration or cloudiness occurs, discard the reagent.

Selecting the Microassay Strips

Determine the number of wells required for the assay. It is recommended that the blank wells, Controls, and Standards be tested in duplicate. Remove the strip retainer from the assembled plate. Remove the unneeded strips and place them in the storage bag, reseal the bag and return it to storage at 2°C to 8°C. Secure the strips to be used in the assay.

C4d Standard and Control Reconstitution

Add 2.0 mL of Hydrating Reagent to each Standard (A–E), and to the Low Control and the High Control. Allow the reconstituted vials to rehydrate for at least 15 minutes at 15°C to 30°C. Mix thoroughly. Avoid formation of foam or bubbles during mixing. Reconstituted standards and controls are stable for 30 days when stored at 2°C to 8°C.

Specimen Dilution

Caution: Treat all specimens using Universal Precautions. Do not use heat-inactivated, contaminated specimens, or improperly stored specimens.

Prepare an appropriate dilution of each specimen using the Complement Specimen Diluent (see *Calculation of Results*). A 1:70 dilution is recommended for normal samples. Mix thoroughly, but avoid formation of foam or bubbles. Do not store or re-use diluted specimens.

Adding Diluted Specimens to the Microtiter Wells

Either of two methods can be used to add diluted specimens, Standards, Controls, and Buffer to the wells (see Step 4 of *ASSAY PROCEDURE*). For small assay runs where only a few specimens are being tested, the diluted specimens and other reagents can be added directly to their assigned wells with a micropipette (100 µL/well). For small or large runs, but especially larger runs, we recommend the use of a multichannel pipettor for adding specimens as follows. (A multi-channel pipettor may be used to conveniently add the Conjugate, Substrate, and Stop Solution, as well.)

In order to load the standards, controls and diluted specimens into the microassay wells as rapidly as possible, a “replica plating” procedure can be employed. Instead of adding 100 µL of each standard, control or diluted

specimen to the antibody-coated wells individually, 120 μ L to 130 μ L of each solution can be added to individual wells in a blank plate (not provided) corresponding to the final EIA pattern desired. After all the solutions to be tested have been added to the microassay wells in the blank plate, rapidly transfer 100 μ L from each blank well to the antibody-coated wells using a multichannel micropipettor. To avoid the possibility of cross-contamination, pipette tips must be changed each time there is a change in the composition of the samples to be transferred.

Preparation of Substrate Solution

Prepare immediately before use. Determine the required volume of Substrate Solution from Table 1. (You will need 1 mL of Substrate Solution per strip or 125 μ L/well.) Prepare the Substrate Solution by adding 50 μ L of Substrate Concentrate to each mL of Substrate Diluent. Mix thoroughly. Do not prepare the Substrate solution until step 8 of the Assay Procedure. If the substrate solution turns dark green prior to use, discard it, and make a fresh solution in a clean container.

Table 1.
Reagent Requirements

Wells¹	8-Well Strips	Substrate Solution Volume (mL)	Substrate Concentrate (μL)
16	2	2.0	100
24	3	3.0	150
32	4	4.0	200
40	5	5.0	250
48	6	5.0	250
56	7	6.0	300
64	8	7.0	350
72	9	8.0	400
80	10	9.0	450
88	11	9.0	450
96	12	10.0	500

¹Determine the number of specimens to be tested and add fifteen (15) wells for the five Standards, the Low and High Controls to be tested (in duplicate) and one blank well. It is recommended that duplicate standards and controls be tested in separate microassay strips when possible.

ASSAY PROCEDURE

Read entire product insert before beginning the assay.

See REAGENT PREPARATION and WARNINGS AND PRECAUTIONS.

- Record the microassay well positions corresponding to the blank well(s), all test samples, standards, and controls as well as the indicated lot numbers from the vial labels. Label one corner of the Microassay Plate for orientation.
- Prepare the microassay strips as follows:
 - Rehydrate microassay wells by adding approximately 250 μ L to 300 μ L of Wash Solution to each well using a wash bottle or automated filling device.

- b. Incubate at 15°C to 30°C for one minute.
- c. Remove the liquid from each well.
- d. Add approximately 250 µL to 300 µL Wash Solution to each well.
- e. Aspirate the contents from each well.
- f. Repeat steps d-e one more time.**
- g. Invert the plate and tap firmly on absorbent paper twice to remove any remaining liquid.
3. Select one or more wells to serve as a blank. Add 100 µL of Specimen Diluent to the well(s) that will be used to blank the plate reader.
4. Add 100 µL of each reconstituted C4d Standard (A, B, C, D, E), Control, or diluted sample to the assigned wells.
5. Incubate at 15°C to 30°C for 30 ± 1 minutes.
6. Wash the microassay wells as follows:
 - a. After the incubation in step 5 (or in step 8 below) remove the liquid from each well.
 - b. Add approximately 300 µL Wash Solution to each well, using a wash bottle or automated filling device.
 - c. Incubate the wells for 1 minute at 15°C to 30°C.
 - d. Remove the liquid from each well.
 - e. Add approximately 300 µL Wash Solution to each well.
 - f. Remove the liquid from each well.
 - g. Repeat steps e-f three additional times.**
 - h. After the fifth wash cycle, invert the plate and tap firmly on absorbent paper twice to remove any remaining liquid.
7. Using a multichannel or repeating pipette, dispense 50 µL of C4d Conjugate into each washed test well, including the blank well(s).
8. Incubate the microassay strips at 15°C to 30°C for 30 ± 1 minutes. Prepare the Substrate Solution during this incubation (see *REAGENT PREPARATION*).
9. Wash the microassay wells after the 30-minute incubation (step 8), as described under *ASSAY PROCEDURES*, step 6.
10. Immediately following the wash procedure, dispense 100 µL of the freshly prepared Substrate Solution into each well, including the blank(s).
11. Incubate the microassay strips at 15°C to 30°C for 30 ± 1 minutes.
12. Add 50 µL of Stop Solution to each well to stop the enzymatic reaction. The Stop Solution should be added to the wells in the same order and at the same rate as was the Substrate Solution. Gently tap the plate to disperse the color development evenly.
13. Determine the absorbance reading at 405 nm (A_{405} value) for each test well within one hour after the addition of the Stop Solution (step 12), making the necessary blank correction.
14. Determine the concentration of samples and controls from the standard curve.
15. Dispose of the remaining diluted specimens and controls and the used microassay strips (see *WARNINGS AND PRECAUTIONS*).

QUALITY CONTROL

Good laboratory practice recommends use of controls to ensure that the assay is performing properly. Each C4d kit contains High and Low Controls that can be used for this purpose. These Controls should be tested at least once for each kit. In addition, the package insert requires that the standard curve generated with the kit Standards meet stringent validation requirements. If the assay does not meet these requirements, repeat the assay or contact Quidel Technical Service.

The Certificate of Analysis included in this kit is lot specific and is to be used to verify that the results obtained by your laboratory are similar to those obtained at Quidel. The optical density values are provided and are to be used as a guideline only. The results obtained by your laboratory may differ.

Quality control ranges are provided. The control values are intended to verify the validity of the curve and sample results. Each laboratory should establish its own parameters for acceptable assay limits. If the control values are NOT within your laboratory's acceptance limits, the assay results should be considered questionable and the samples should be repeated.

INTERPRETATION OF RESULTS

Calculation of Results

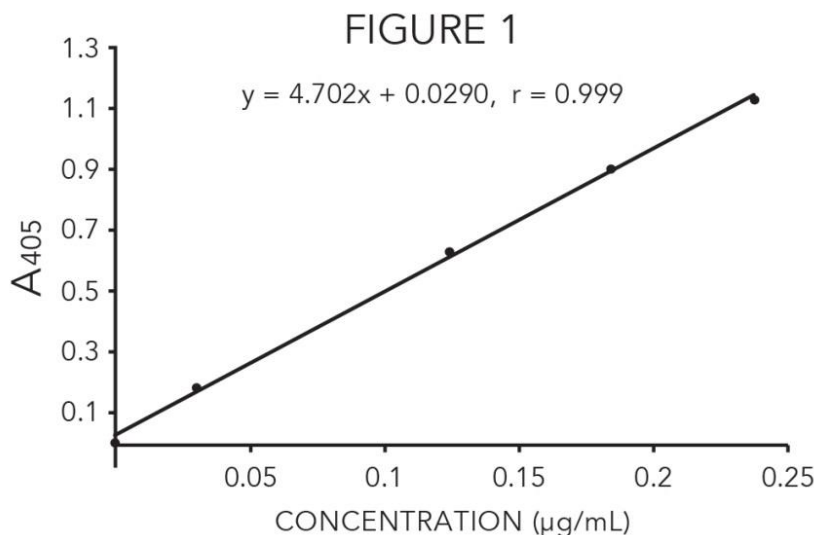
Generally, a 1:70 dilution of normal human plasma or serum has been found to work optimally in the C4d EIA Kit. Whole plasma or serum samples can be diluted from 1:40 to 1:100 with full and accurate quantitation of the C4d analyte. Normal plasma or serum samples diluted more than 1:100 can yield A_{405} values less than 0.1, which may yield inaccurate results.

Depending on the sample concentration and experimental design, samples may need to be diluted further (i.e., higher dilutions) to yield A_{405} values within range of the kit standards in order to calculate the C4d analyte concentration. Alternatively, the C4d level may be reported as greater than or equal to the maximum calculable for the particular dilution tested.

Use of the Standard Curve: The standard curve for the C4d EIA is generated using the blank subtracted A_{405} values for each standard (on the y axis) and the assigned concentration for each standard (on the x axis). After linear regression, the generated standard curve must meet the validation requirements (see below). Most computers and calculators are capable of performing these calculations.

Alternatively, the data may be graphed manually and the values ($\mu\text{g/mL}$) of the test samples read directly from the best-fit line of the standard curve. An example of a typical standard curve is shown in Figure 1.

Representative Standard Curve



Validation

Determine the slope, intercept, and correlation coefficient of the derived best-fit line for the C4d kit Standards. The values must be within the specified ranges to qualify the assay:

correlation coefficient (r): > 0.95
slope (m): between 3.32 and 6.50
y-intercept (b): between (-) 0.056 and 0.063

Refer to the vial labels for the acceptable C4d concentration range for the High and Low Controls.

LIMITATIONS OF THE PROCEDURE

This kit is for research use only and is not intended for use in diagnostic procedures.

The MicroVue C4d Fragment Enzyme Immunoassay has been used to test specimens collected as serum or as plasma in EDTA. Other anticoagulants have not been tested.

EXPECTED VALUES

Eighty (80) EDTA plasma specimens (diluted 1:70 in Specimen Diluent) and fifty-four (54) serum specimens (diluted 1:70 in Specimen Diluent) were tested in the MicroVue C4d Fragment Enzyme Immunoassay. Ranges for plasma and serum specimens are given below.

	n	Mean	Range	
			± 2 SD	± 3 SD
EDTA Plasma	80	3.5	0.7 µg/mL to 6.3 µg/mL	0 µg/mL to 7.7 µg/mL
Serum	54	4.6	1.2 µg/mL to 8.0 µg/mL	0 µg/mL to 9.7 µg/mL

PERFORMANCE CHARACTERISTICS

Limits

LOD: The limit of detection (LOD) for the C4d assay is 0.001 µg/mL, determined by the upper 3SD limit in a zero standard study.

LLOQ: The lower limit of quantification (LLOQ) for the C4d assay is 0.022 µg/mL, the lowest concentration on the standard curve that met NCCLS criteria for accuracy and precision.

Interfering Substances

Sodium Citrate and Tetrasodium EDTA were tested at concentrations of 1000 mg/dL and 800 mg/dL respectively, and were not found to interfere with the assay.

Precision

Within-run and between-run precision was determined by assaying 20 replicates of 3 plasma samples in 9 different runs.

C4d (µg/mL)	Within-run ¹ C.V. (%)	Between-run ² C.V. (%)
4.4	9.7	11.2
4.7	7.4	8.8

7.9	6.1	8.5
¹ n=20 replicates	² n=9 runs	

Linearity

Linearity was performed by mixing a high plasma sample with a low plasma sample in various ratios to create intermediate levels of analyte. The average recovery was 92.7% with an absolute range of 87.0% to 99.0%.

ASSISTANCE

To place an order or for technical support, please contact a Quidel Representative at 800.874.1517 (in the U.S.) or 858.552.1100 (outside the U.S.), Monday through Friday, from 8:00 a.m. to 5:00 p.m., Eastern Time. Orders may also be placed by fax at (740) 592-9820. For e-mail support contact customerservice@quidel.com or technicalsupport@quidel.com.

For services outside the U.S.A., please contact your local distributor. Additional information about Quidel, our products, and our distributors can be found on our website quidel.com.

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REF

A008 – MicroVue C4d Fragment EIA Kit

IVD



EC REP

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Germany



Quidel Corporation
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quidel.com

PIA008000EN00 (10/17)

GLOSSARY

REF

Catalogue number

LOT

Batch code



Use by



Manufacturer



Temperature limitation



Intended use



Consult e-labeling
instructions for use



Biological risks

RUO

For Research use only



Contains sufficient for 96 determinations

CONT

Contents/Contains

CONTROL

Control
