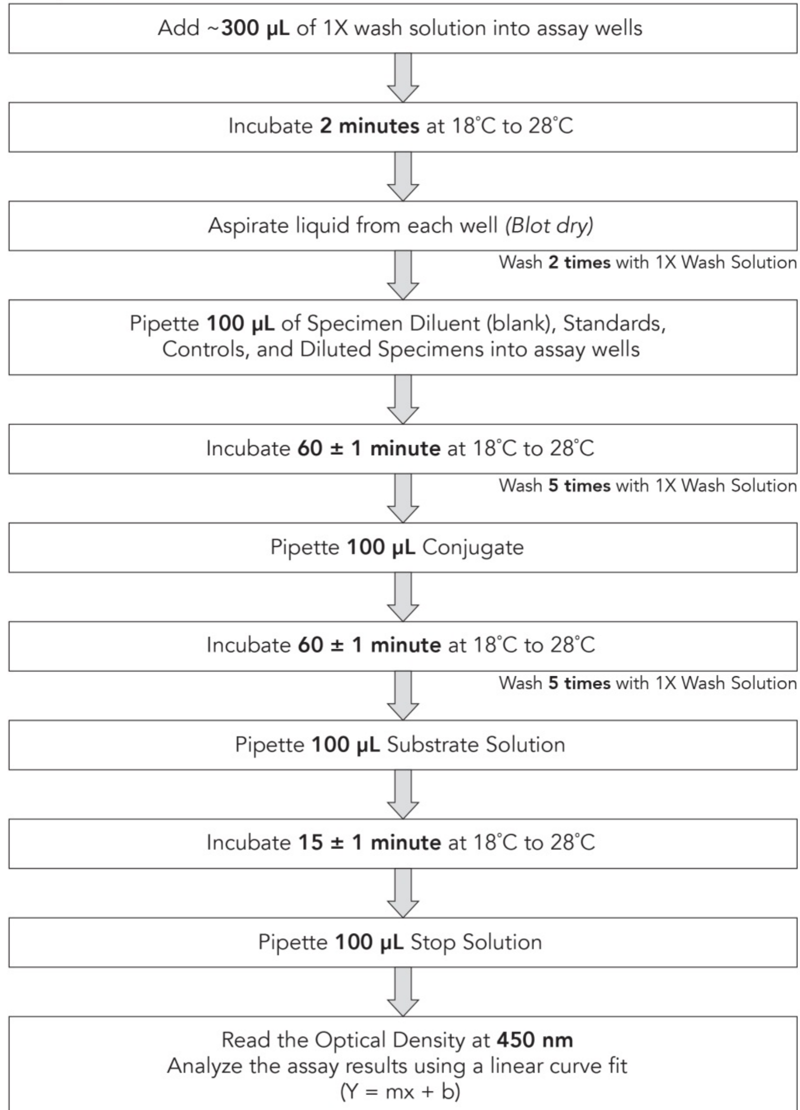
MicroVue™ Complement C5a EIA

The MicroVue C5a Enzyme Immunoassay measures the amount of C5a in human serum andplasma.

For **Research Use Only** in the United States. Not for use in diagnostic procedures.

SUMMARY



SUMMARY AND EXPLANATION

The MicroVue C5a Enzyme Immunoassay is a 96 well, direct-capture immunoassay for the measurement of C5a in human serum, plasma, and other biological or experimental samples.

Under normal conditions, activation of the classical, alternative, or lectin complement pathways results in the formation of a C5 convertase multi-molecular enzyme capable of cleaving C5 to C5a and C5b.[1,2] C5b is a key constituent of the Terminal Complement Complex and has a variety of functions in this role. C5a is a low molecular weight (approximately 9kD) protein fragment of 74 amino acids.[3] C5a is rapidly metabolized by the serum enzyme carboxypeptidase to a more stable, less active, 73 amino acid form, C5a des-Arg.[2,3] For convenience, both forms will be referred to as "C5a" for purposes of this documentation.

The MicroVue C5a assay, which provides a rapid, highly specific and quantitative procedure for measuring C5a levels, is designed for investigations into the role or status of terminal complement pathway activation in numerous research settings, and for monitoring the generation of C5a *in vivo* or *in vitro*. As the most potent of the complement anaphylatoxins, C5a has a host of biologic functions[2] including mast cell degranulation, chemotaxis, leukosequestration, as well as cellular activation via binding to the C5a Receptor[2] (C5aR or CD88). Research has associated elevated levels of fluid phase and adsorbed C5a with hemo-incompatibility of some biomaterials, particularly in extracorporeal circuits.[5-9] Research has also associated levels of C5a with pathogenesis of a variety of disease states including myocardial infarction[10-14], stroke[15,16], as well as vascular leak syndrome and associated kidney injury[17-19]. The role of C5a in the pathogenesis of malaria[20] and other infectious diseases, as well as sepsis,[21-24] is likewise well documented.

PRINCIPLE OF THE PROCEDURE

The MicroVue C5a Enzyme Immunoassay is a three step procedure utilizing (1) a microassay plate coated with murine monoclonal antibody specific for a neo-epitope on human C5a, (2) an HRP conjugated murine monoclonal antibody to the C5a region of C5, and (3) a chromogenic substrate.

In the first step, Standards, Controls and diluted test specimens are added to the assay wells coated with a murine monoclonal antibody to C5a. The monoclonal antibody binds to C5a in the Standards, Controls or Specimens. After an incubation period, a wash cycle removes any unbound material.

In the second step, horseradish peroxidase (HRP) conjugated murine anti-C5(C5a) is added to each assay well. The enzyme conjugated anti-C5(C5a) binds to the immobilized C5a captured in the first step. After an incubation period, a wash cycle removes any unbound conjugate.

In the third step, 3,3',5,5' tetramethylbenzidine (TMB), a ready-to-use, chromogenic substrate solution, is added to the assay wells. The bound HRP reacts with the substrate, forming a blue color. After an incubation period, the reaction is stopped chemically, which results in a color change from blue to yellow, confirming that the reaction has taken place. The color intensity is measured spectrophotometrically at A_{450}. The color intensity of the reaction mixture is proportional to the concentration of C5a present in the diluted test specimens, Standards, and Controls. Results are calculated from the generated standard curve using linear regression analysis.

REAGENTS AND MATERIALS PROVIDED

96 Assays for C5a complex
MicroVue C5a EIA kit contains the following:

A – E C5a Standards: **Parts 5131-5135** **1.5 mL, 1 each**
Ready to use. Contains human serum with assigned C5a concentration (ng/mL), protein stabilizers

L Low Control **Part 5136** **1.5 mL**
Ready to use. Contains human serum with assigned C5a concentration (ng/mL), protein stabilizers

H High Control **Part 5137** **1.5 mL**
Ready to use. Contains human serum with assigned C5a concentration (ng/mL), protein stabilizers

❶ Coated Strips **Part 5129** **12 each**
Eight-well strips coated with a murine monoclonal antibody in a resealable foil pouch

❷ Stop Solution **Part A9947** **12 mL**
Contains 1N (4%) Hydrochloric Acid

❸ 20X Wash Solution Concentrate **Part A9957** **50 mL, 2 each**
Contains phosphate buffered saline (PBS), 1.0% Tween-20® and 0.035% Proclin® 300

❹ Complement Specimen Diluent **Part A3670** **50 mL**
Contains PBS, 0.05% Tween-20, 2.5% protein stabilizers, 0.035% ProClin 300

❺ TMB Substrate **Part 5059** **12 mL**
Ready to use. Contains 3,3',5,5'-tetramethylbenzidene (TMB) and Hydrogen Peroxide (H_2O_2)

❻ Conjugate **Part 5130** **12 mL**
Contains Horseradish Peroxidase-conjugated murine monoclonal antibody to C5a

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
- While not required, a plate reader with auto-mix capability is recommended.

WARNINGS AND PRECAUTIONS

- For Research Use Only. Not for use in diagnostic procedures.
- Treat specimen samples as potentially biohazardous material. Follow Universal Precautions when handling contents of this kit and any patient samples.
- Wear suitable protective clothing, gloves, and eye/face protection when handling contents of this kit.
- 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.
- 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.
- The Stop Solution is considered corrosive and can cause irritation. Do not ingest. Avoid contact with eyes, skin, and clothing. If contact is made, immediately rinse affected area with water. If ingested, call a physician.
- 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 HANDLING AND PREPARATION*).
- Avoid microbial or cross-contamination of specimens or reagents.
- Test each sample in duplicate.
- Do not use a microassay well for more than one test.
- Using incubation times and temperatures other than those indicated in the Procedure section may give erroneous results.
- The TMB Substrate must be protected from light during storage and incubation. Avoid contact with eyes, skin, and clothing. If contact is made, immediately rinse affected area with water.
- 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, 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 8). For best results, do not use a multichannel pipette to wash the microassay plate.
- Dispose of containers and unused contents in accordance with Federal, State and Local regulations.
- 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 unopened kit at 2°C to 8°C. Bring reagents and materials selected for use to 18°C to 28°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

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

SPECIMEN HANDLING AND PREPARATION

Handle and dispose of all specimens using Universal Precautions.

All specimen-handling operations should be carried out at 2°C to 8°C.

Specimen Collection

The proper collection, processing and storage of specimens is essential since C5a may be generated in improperly handled specimens through artifactual complement activation.

Values for normal serum samples will typically be higher than those obtained with EDTA or citrated normal plasma samples. The C5a levels in EDTA or citrated plasma may therefore more accurately represent the *in vivo* concentrations.[25]

Serum, EDTA, or citrated plasma specimens should be collected aseptically using standard techniques.[26] The specimens should be tested immediately or stored at 4°C or on ice for no longer than four hours before being assayed.

If the specimen cannot be tested within four hours under the guidelines detailed above, the specimen should be frozen at –70°C or below.

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.

Thawing Frozen Specimens

Thaw frozen specimens rapidly at 37°C until just thawed. Transfer thawed specimens immediately to ice (for no longer than eight hours) to prevent complement activation prior to dilution. **Do not leave specimens at 37°C**, as complement activation may occur. Do not thaw specimens at room temperature or on ice as this can lead to C5 activation and affect results. Specimens should be tested as soon as possible after thawing. Up to 3 freeze/ thaw cycles may be performed without affecting the samples. If samples need additional freezing for further analysis, Quidel suggests freezing multiple aliquots of the specimen to prevent multiple freeze/thaw cycles.

Specimen Dilution

CAUTION: Treat all specimens as potentially hazardous. Use Universal Precautions. Do not use heat-inactivated, contaminated, or improperly stored specimens.

NOTE: See *SPECIMEN HANDLING AND PREPARATION* for important notes on proper methods to thaw frozen specimens. Proper sample handling is essential for accurate results.

Quidel suggests that normal plasma samples be diluted 1:20 in the provided Specimen Diluent; serum samples should be diluted 1:50. A 1:200 dilution, or greater, may be required for a sample with high levels of C5a. Samples **must** be diluted so that A_{450} values observed are above the LLOQ and do not exceed the A_{450} value of the C5a Kit Standard E. Samples with A_{450} readings outside this range should be re-assayed at a new dilution.

Determine the number (N) of specimens to be tested. Label test tubes #1 through #N, and record which specimen corresponds to each tube. Prepare an appropriate dilution (see preceding paragraph) of each specimen using the Specimen Diluent. Mix thoroughly, but avoid formation of foam and bubbles. Do not store or reuse diluted specimens.

Adding Diluted Specimens to the Microtiter Wells

The addition of diluted specimens to the microtiter wells must be completed within 15 minutes of the application of the first specimen. Either of two methods can be used to add diluted specimens, Standards, Controls, and buffer, to the wells (see Step 6 of *ASSAY PROCEDURE*). For small assay runs where only a few specimens are being tested, the diluted specimens and other reagents may 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.

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, 120–130 µL 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 µL 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.

The “replica plating” procedure may be used to conveniently add the Conjugate, Substrate, and Stop Solution, as well.

REAGENT PREPARATION

Bring all reagents and materials to 18°C to 28°C before use. After removing the needed reagents and materials, return the unused items to their appropriate storage temperatures (see *STORAGE*).

1. **Standards** and Controls
   Standards and Controls are supplied ready to use and do not require dilution or preparation prior to use.

2. **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.

3. **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 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 in the assay plate frame.

ASSAY PROCEDURE

Read entire product Insert before beginning the assay.
See REAGENT PREPARATION and WARNINGS AND PRECAUTIONS before proceeding.

1. 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.

2. Prepare the microassay strips as follows:
    a. Rehydrate microassay wells by adding approximately 300 µL of Wash Solution to each well using a wash bottle or automated filling device.
    b. Incubate at 18°C to 28°C for two minutes.
    c. Remove the liquid from each well.
    d. Add approximately 300 µL Wash Solution to each well.
    e. Remove the liquid from each well.
    f. **Repeat steps d-e one more time for a total of three washes.**
    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 C5a Standard (A, B, C, D, E) to duplicate wells. **NOTE: The Standards are ready to use and do not need dilution.**
5. Add 100 µL of both the C5a Low Control and C5a High Control to duplicate wells. **NOTE: The Controls are ready to use and do not need dilution.**
6. Add 100 µL of each diluted specimen to its assigned microassay well. (See *Specimen Dilution*).
7. Incubate at 18°C to 28°C for 60 ± 1 minutes.
8. Wash the microassay wells as follows:
    a. After the incubation in step 7 (or in step 10 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. Remove the liquid from each well.
    d. **Repeat steps b-c four additional times.**
    e. After the fifth wash cycle, invert the plate, and tap firmly on absorbent paper twice to remove any remaining liquid.
9. Using a multichannel or repeating pipette, dispense 100 µL of C5a Conjugate into each washed test well, including the blank well(s).
10. Incubate the microassay strips at 18°C to 28°C for 60 ± 1 minutes.
11. Wash the microassay wells after the 60-minute incubation (step 10), as described under *ASSAY PROCEDURE*, step 8.
12. Immediately following the wash procedure, dispense 100 µL of the Substrate Solution into each well, including the blank(s).
13. Incubate the microassay strips at 18°C to 28°C for 15 ± 1 minutes.
14. Add 100 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 the Substrate Solution. Gently tap the plate to disperse the color development evenly. **NOTE: Optimal results may be obtained by using the plate reader's auto-mix function (if available) just prior to reading the plate.**
15. Determine the absorbance reading at 450 nm (A_{450} value) for each test well within 60 minutes after the addition of the Stop Solution (step 14), making the necessary blank correction.
16. Determine the concentration of samples and Controls from the standard curve.
17. 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 C5a kit contains High and Low Controls that can be used for this purpose. 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. 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.

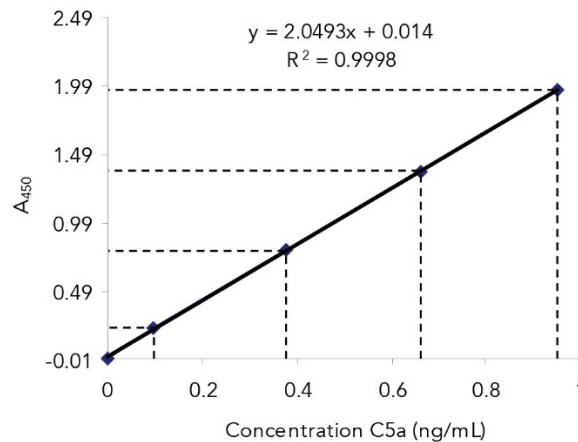
The Certificate of Analysis included in this kit is lot specific and is to be used to verify that the results obtained by your laboratory are similar to those obtained at Quidel Corporation. The optical density values provided are to be used as a guideline only. The results obtained by your laboratory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

INTERPRETATION OF RESULTS

Calculation of Results

Use of the Standard Curve: The standard curve for the C5a EIA is generated using the blank subtracted A_{450} values for each Standard (on the y axis) and the assigned concentration for each Standard (on the x axis). The standard curve must meet the Validation Requirements. Most computers and calculators are capable of performing these calculations. An example of a typical standard curve is shown in Figure 1.

Figure 1
Representative Standard Curve



Sample	A_{450}	ng/mL
Standard A	-0.001	0
Standard B	0.225	0.095
Standard C	0.789	0.378
Standard D	1.369	0.661
Standard E	1.965	0.953
r = 0.9998	m = 2.0493	b = 0.014

Calculation of Actual C5a Concentration in Specimens

The assigned concentration on the certificate of analysis are absolute units of C5a. The concentration of C5a in a specimen is determined by multiplying the determined concentration by the appropriate specimen dilution factor. For example, if an EDTA-plasma specimen is diluted 1:20 for the assay, and the linear regression curve yields a concentration of 0.5 ng C5a/mL, then the concentration of C5a in the specimen would be 10 ng C5a/mL (or 20 x 0.5).

In order to obtain accurate C5a concentration determinations for test specimens that yield A_{450} values greater than that of the C5a Standard E (or that yield values less than the LLOQ), specimens should be re-assayed at a

different dilution so that their new A_{450} values will be within these limits. In all repeat assays, the C5a Standards and Controls must also be retested.

Validation

Determine the slope, intercept, and correlation coefficient of the derived best-fit line for the C5a A, B, C, D, and E Standards. The values must be within the specified ranges to qualify the assay:

correlation coefficient (r):	> 0.98
slope (m):	1.14 to 2.50
y-intercept (b):	(-)0.0145 to (+)0.0532

Refer to the certificate of analysis for the acceptable C3a concentration range for the Low and High Controls.

LIMITATIONS OF THE PROCEDURE

The MicroVue C5a Enzyme Immunoassay has been used to test specimens collected as serum or as plasma in EDTA and citrate. Other anticoagulants have not been tested.

SAMPLE VALUES

The MicroVue C5a Enzyme Immunoassay was used to test twenty (20) EDTA plasma and serum samples. The results are presented below.

	n	**Mean (ng/mL)**	**Range (ng/mL)**
EDTA Plasma	20	20.65	0.37 to 74.33
Serum	20	50.09	13.37 to 179.23

NOTE: The C5a concentrations determined for plasma or serum samples may vary between laboratories; therefore, it is recommended that each laboratory determine its own range. The concentrations provided above should be regarded as a guideline only.

PERFORMANCE CHARACTERISTICS

Limits

LOD: The limit of detection (LOD) for the C5a assay is 0.01 ng/mL, determined by the upper 3SD limit in a zero standard study.

LLOQ: The lower limit of quantitation (LLOQ) for the C5a assay is 0.050 ng/mL, which is the lowest concentration on the standard curve that meets NCCLS criteria for accuracy and precision.

Interfering Substances

The following substances were tested in the C5a assay and found to not interfere with the assay:

Substance	**Concentration**
Bilirubin	40 mg/dL
Hemoglobin	500 mg/dL
Triglycerides	3000 mg/dL
Na + Heparin	14 U/dL
C5 Protein	80 mg/L
Glucose	1200 mg/dL
Cholesterol	500 mg/dL

Neat samples with Albumin concentrations > 118.6 mg/mL or Gamma Globulin > 8.9 mg/dL have been shown to interfere with quantitation in the assay and must be diluted accordingly.

Precision

Within-run and between-run precision was determined by assaying 20 replicates of 2 plasma samples and 2 serum samples in 10 different runs.

Sample	C5a (ng/mL)	Within-run[1] C.V. (%)	Between-run[2] C.V. (%)
EDTA Plasma	12.34	3.8	9.9
	1.41	3.9	13.0
Serum	30.13	3.6	7.1
	21.92	3.5	7.8

[1]n = 20 replicates [2]n = 10 runs

Linearity

Linearity was performed by serially diluting samples with sample diluent and comparing observed values with expected values.

Sample	Dilution Factor	Observed C5a (ng/mL)[3]	Expected C5a (ng/mL)[3]	Recovery (%)
EDTA Plasma	10	1.214	1.266	96
	20	0.667	0.633	105
	40	0.343	0.317	108
	80	0.164	0.158	103
	160	0.077	0.079	98
Serum	25	0.873	0.864	99
	50	0.465	0.432	93
	100	0.236	0.216	92
	200	0.115	0.108	94
	400	0.054	0.054	100

[3]Dilution factor not included

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 A021 – MicroVue C5a EIA Kit

RUO

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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