AssayLite™
Human Complement C3 EFSIA Kit
(Red Fluorescent Probe)

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This product is manufactured under patent pending technology by
Assaypro LLC

For any questions regarding troubleshooting or performing the assay, please contact our
support team at support@assaypro.com.
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Assay Summary

Step 1. Add 100 µl of Standard or Sample per well. Incubate 2 hours at 37°C.

Step 2. Wash, then add 100 µl of Fluorescent Probe per well. Incubate 1 hour at 37°C.

Step 3. Wash, then add 50 µl of Stabilizing Solution per well.

Step 4. Read at 485/20 EX and 575/15 EM immediately.

Symbol Key

📖 Consult instructions for use.
Assay Mechanism

Figure 1. Standards and samples are added to wells and incubated.

Figure 2. The Antigen A in standards and samples binds to the immobilized Antibody A. All unbound materials are washed away.

Figure 3. The Antibody A Fluorescent Probe is added to wells and binds to the Antigen A.

Figure 4. The microplate is washed and the endpoint fluorescence is measured. The fluorescence intensity is proportional to the concentration of Antigen A in the standard or samples.
Introduction

Complement component 3 (C3) plays a central role in all three complement activation pathways. The C3 precursor contains 1663 amino acids and has a molecular weight of about 180 kDa (1). Human C3 has 77% identity to mouse C3 at the amino acid level (2). C3 is cleaved by C3 convertase into two activated fragments C3a and C3b. The anaphylatoxin C3a is a vasoactive peptide and a mediator of local inflammatory processes (3). The C3b fragment in complex with a receptor can bind covalently to pathogen surfaces to promote phagocytosis (4, 5).

Principle of the Assay

The AssayLite Human Complement C3 EFSIA (Endpoint Fluorescent Sandwich Immunoassay) Kit employs a quantitative sandwich fluorescent probe technique that measures C3 in human plasma, serum, and cell culture samples in less than 4 hours. A polyclonal antibody specific for human C3 has been pre-coated onto a 96-well opaque microplate with removable strips. C3 in standards and samples is sandwiched by the immobilized antibody and a polyclonal antibody specific for C3 conjugated to a fluorescent probe. All unbound material is washed away before the endpoint fluorescence is measured.

Caution and Warning

- This product is for Research Use Only and is not intended for use in diagnostic procedures.
- Prepare all reagents (working diluent buffer, wash buffer, standard, and fluorescent probe) as instructed, prior to running the assay.
- Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this insert. However, the user should determine the optimal dilution factor.
- The kit should not be used beyond the expiration date.
- Avoid direct light exposure to the assay.
- Store the fluorescent probe in a dark place. Do not freeze.
Reagents

- **Human Complement C3 Microplate**: A 96-well black polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against C3.
- **Sealing Films**: Each kit contains 3 precut, pressure sensitive aluminum sealing films that can be cut to fit the format of the individual assay.
- **Human Complement C3 Standard**: Human C3 in a buffered protein base (8000 ng, lyophilized).
- **Red Fluorescent Human Complement C3 Probe**: 6 vials, lyophilized.
- **EIA Diluent Concentrate (10x)**: A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml).
- **Stabilizing Solution**: A ready-to-use solution to stabilize the fluorescent component (8 ml).

Storage Conditions

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store Microplate, Diluent Concentrate (10x), Stabilizing Solution, and Wash Buffer at 2-8°C.
- Unused microplate wells may be returned to the foil pouch with the desiccant packs and resealed. May be stored for up to 30 days in a vacuum desiccator.
- Diluent (1x) may be stored for up to 30 days at 2-8°C.
- Store the Standard at 2-8°C before reconstituting with Diluent and at -20°C after reconstituting with Diluent.
- Store the Fluorescent Probe in a dark place at 2-8°C before and after reconstituting with Diluent. Do not freeze.

Other Supplies Required

- Pipettes (1-20 μl, 20-200 μl, 200-1000 μl, and multiple channel)
- Deionized or distilled reagent grade water
- Incubator (37°C)
- Fluorescent Microplate Reader

**Fluorescent Microplate Reader (BioTek® Synergy H1F, filter-based reader; available for order from Assaypro)**
Sample Collection, Preparation, and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3000 x g for 10 minutes. Dilute samples 1:20000 into EIA Diluent, or within the range of 10000x – 40000x, and assay (*suggested dilution factor only; user should determine optimal dilution factor*). The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA or Heparin can also be used as an anticoagulant).

- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x g for 10 minutes, and remove serum. Dilute samples 1:20000 into EIA Diluent, or within the range of 10000x – 40000x, and assay (*suggested dilution factor only; user should determine optimal dilution factor*). The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

- **Cell Culture Supernatants:** Centrifuge cell culture media at 3000 x g for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Refer to Sample Dilution Guidelines below for further instruction.

### Guidelines for Dilutions of 1:100 or Greater
(for reference only; please follow the insert for specific dilution suggested)

<table>
<thead>
<tr>
<th>1:100</th>
<th>1:10000</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) 4 µl sample: 396 µl buffer = 100 fold dilution</td>
<td>A) 4 µl sample : 396 µl buffer (100x)</td>
</tr>
<tr>
<td><em>Assuming the needed volume is less than or equal to 400 µl.</em></td>
<td><em>Assuming the needed volume is less than or equal to 400 µl.</em></td>
</tr>
<tr>
<td>B) 4 µl of A : 396 µl buffer (100x) = 10000 fold dilution</td>
<td></td>
</tr>
<tr>
<td><em>Assuming the needed volume is less than or equal to 400 µl.</em></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1:1000</th>
<th>1:10000</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) 4 µl sample : 396 µl buffer (100x)</td>
<td>A) 4 µl sample : 396 µl buffer (100x)</td>
</tr>
<tr>
<td>B) 24 µl of A : 216 µl buffer (10x) = 1000 fold dilution</td>
<td>B) 4 µl of A : 396 µl buffer (100x)</td>
</tr>
<tr>
<td><em>Assuming the needed volume is less than or equal to 240 µl.</em></td>
<td>C) 24 µl of B : 216 µl buffer (10x) = 100000 fold dilution</td>
</tr>
<tr>
<td></td>
<td><em>Assuming the needed volume is less than or equal to 240 µl.</em></td>
</tr>
</tbody>
</table>

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **EIA Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.
Dilute the EIA Diluent Concentrate 1:10 with reagent grade water. Store for up to 30 days at 2-8°C.

- **Standard Curve:** Reconstitute the 8000 ng of Human Complement C3 Standard with 2.5 ml of EIA Diluent to generate a 3200 ng/ml standard stock solution. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. The standard stock solution (3200 ng/ml) should be further diluted 1:10 with EIA Diluent to produce a 320 ng/ml standard working solution. Prepare duplicate or triplicate standard points by serially diluting the standard working solution (320 ng/ml) 1:2 with EIA Diluent to generate 125, 62.5, 31.25, 15.63, 7.813, and 3.906 ng/ml solutions. EIA Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within 30 days.

<table>
<thead>
<tr>
<th>Standard Point</th>
<th>Dilution</th>
<th>[C3] (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1 part Standard (3200 ng/ml) + 9 parts EIA Diluent</td>
<td>320.0</td>
</tr>
<tr>
<td>P2</td>
<td>1 part P1 + 1 part EIA Diluent</td>
<td>160.0</td>
</tr>
<tr>
<td>P3</td>
<td>1 part P2 + 1 part EIA Diluent</td>
<td>80.0</td>
</tr>
<tr>
<td>P4</td>
<td>1 part P3 + 1 part EIA Diluent</td>
<td>40.0</td>
</tr>
<tr>
<td>P5</td>
<td>1 part P4 + 1 part EIA Diluent</td>
<td>20.0</td>
</tr>
<tr>
<td>P6</td>
<td>1 part P5 + 1 part EIA Diluent</td>
<td>10.0</td>
</tr>
<tr>
<td>P7</td>
<td>1 part P6 + 1 part EIA Diluent</td>
<td>5.00</td>
</tr>
<tr>
<td>P8</td>
<td>EIA Diluent</td>
<td>0.00</td>
</tr>
</tbody>
</table>

- **Red Fluorescent Human Complement C3 Probe (1x):** Reconstitute the Fluorescent Probe with 1.8 ml EIA Diluent to produce a working solution. Allow the probe to sit for 10 minutes with gentle agitation prior to use. Any remaining solution should be refrigerated at 2-8°C and used within 30 days. Do not freeze.

- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.

**Assay Procedure**

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is incubated at 37°C.
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 100 µl of Human Complement C3 Standard or sample per well. Cover wells with a sealing film and incubate for 2 hours at 37°C. Start the timer after the last addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a machine, wash six times with 300 µl of Wash Buffer and then invert the plate, decanting the contents; hit 4-5 times on absorbent material to completely remove the liquid.
- Add 100 µl of Human Complement C3 Fluorescent Probe per well. Cover wells with a sealing film and incubate for 1 hour at 37°C. Start the timer after the last addition.
- Wash the microplate as described above.
- Immediately add 50 µl of Stabilizing Solution to each well.
- Read the endpoint fluorescence on a microplate reader at an excitation wavelength of 485/20 nm and emission wavelength of 575/15 nm immediately for best results.
- For the Synergy H1F, a gain of 75 is suggested. However, the user should determine the optional gain/amplification.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 575 emitted fluorescence on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Typical Data

- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.
<table>
<thead>
<tr>
<th>Reader</th>
<th>Standard Point</th>
<th>ng/ml</th>
<th>Average Red RFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>BioTek – Synergy H1F</td>
<td>P1</td>
<td>320.0</td>
<td>23601</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>160.0</td>
<td>19700</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>80.0</td>
<td>13798</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>40.0</td>
<td>9247</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>20.0</td>
<td>6048</td>
</tr>
<tr>
<td></td>
<td>P6</td>
<td>10.0</td>
<td>4329</td>
</tr>
<tr>
<td></td>
<td>P7</td>
<td>5.0</td>
<td>2862</td>
</tr>
<tr>
<td></td>
<td>P8</td>
<td>0.0</td>
<td>1373</td>
</tr>
</tbody>
</table>

Sample: Sodium Citrate Plasma (20000x) 10433

**Standard Curve**

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.

**Human C3 Standard Curve**

Note: Standard curve reading with Biotek® Synergy H1F

**Reference Value**

- The normal human plasma level of C3 is 0.5 – 2 mg/ml.
- Human plasma and serum samples from healthy adults were tested (n=40). On average, C3 level was 1081 µg/ml.
Performance Characteristics

- The minimum detectable dose of C3 as calculated by 2SD from the mean of a zero standard was established to be 4 ng/ml.
- Intra-assay precision was determined by testing replicates of three plasma samples in one assay.
- Inter-assay precision was determined by testing three plasma samples in twenty assays.

<table>
<thead>
<tr>
<th>Sample n</th>
<th>Intra-Assay Precision</th>
<th>Inter-Assay Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample 1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.7%</td>
<td>5.0%</td>
</tr>
<tr>
<td>Average CV (%)</td>
<td>4.8%</td>
<td>9.3%</td>
</tr>
</tbody>
</table>

Recovery

<table>
<thead>
<tr>
<th>Standard Added Value</th>
<th>10 – 160 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery %</td>
<td>92 – 110%</td>
</tr>
<tr>
<td>Average Recovery %</td>
<td>95%</td>
</tr>
</tbody>
</table>

Linearity

- Plasma and serum samples were serially-diluted to test for linearity.

<table>
<thead>
<tr>
<th>Average Percentage of Expected Value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Dilution</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>1:100000</td>
</tr>
<tr>
<td>1:200000</td>
</tr>
<tr>
<td>1:400000</td>
</tr>
</tbody>
</table>

Cross-Reactivity

<table>
<thead>
<tr>
<th>Species</th>
<th>Cross Reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>None</td>
</tr>
<tr>
<td>Canine</td>
<td>None</td>
</tr>
<tr>
<td>Mouse</td>
<td>None</td>
</tr>
<tr>
<td>Monkey</td>
<td>None</td>
</tr>
<tr>
<td>Rat</td>
<td>None</td>
</tr>
<tr>
<td>Rabbit</td>
<td>None</td>
</tr>
<tr>
<td>Swine</td>
<td>None</td>
</tr>
<tr>
<td>Human</td>
<td>100%</td>
</tr>
</tbody>
</table>
## Troubleshooting

<table>
<thead>
<tr>
<th>Issue</th>
<th>Causes</th>
<th>Course of Action</th>
</tr>
</thead>
</table>
| **Low Precision** | Use of expired components | • Check the expiration date listed before use.  
• Do not interchange components from different lots. |
| | Improper wash step | • Check that the correct wash buffer is being used.  
• Check that all wells are dry after aspiration.  
• Check that the microplate washer is dispensing properly.  
• If washing by pipette, check for proper pipetting technique. |
| | Splashing of reagents while loading wells | • Pipette properly in a controlled and careful manner. |
| | Inconsistent volumes loaded into wells | • Pipette properly in a controlled and careful manner.  
• Check pipette calibration.  
• Check pipette for proper performance. |
| | Insufficient mixing of reagent dilutions | • Thoroughly agitate the Standard and Fluorescent Probe after reconstitution.  
• Thoroughly mix dilutions. |
| | Improperly sealed microplate | • Check the microplate pouch for proper sealing.  
• Check that the microplate pouch has no punctures.  
• Check that three desiccants are inside the microplate pouch prior to sealing. |
| **Unexpectedly Low or High Signal Intensity** | Microplate was left unattended between steps | • Each step of the procedure should be performed uninterrupted. |
| | Omission of step | • Consult the provided procedure for complete list of steps. |
| | Steps performed in incorrect order | • Consult the provided procedure for the correct order. |
| | Insufficient amount of reagents added to wells | • Check pipette calibration.  
• Check pipette for proper performance. |
| | Wash step was skipped | • Consult the provided procedure for all wash steps. |
| | Improper wash step | • Check that the correct wash buffer is being used. |
| | Improper reagent preparation | • Consult reagent preparation section for the correct dilutions of all reagents. |
| | Insufficient or prolonged incubation periods | • Consult the provided procedure for correct incubation time. |
| | Prolonged exposure of assay or Fluorescent Probe to light | • Overexposure can affect the stability of the Fluorescent Probe, store in a dark location.  
• Cover and cap all reagents when not in use.  
• Cover assay with aluminum sealing film immediately after loading. |
| | Contamination of reagents | • A new tip must be used for each addition of different samples or reagents during the assay procedure. |
| | Contents of wells evaporated | • Verify that the aluminum sealing film is firmly in place before placing the assay in the incubator. |
| | Used filters with an overlapping bandpass | • As an example, do not use a filter combination of 620/20 EX and 660/40 EM, use a 660/20 filter instead. |
| **Standard** | Improper pipetting | • Pipette properly in a controlled and careful manner.  
• Check pipette calibration.  
• Check pipette for proper performance. |
Insufficient mixing of reagent dilutions

- Thoroughly agitate the Standard and Fluorescent Probe after reconstitution.
- Thoroughly mix dilutions.

References


Fluorescent Microplate Reader

- BioTek® Synergy H1F, Filter-Based Reader
- Gen5 Software included
- Includes three filters designed specifically for AssayLite® Multiplex Assays. Additional filters are available for purchase
- Available for order from Assaypro for $19,950