**ProGen Biologics**

*Fixed C5b-9-CIC Proceptor™ ELISA Kit Data Sheet (Product Code CC 005b-9)*

**INTRODUCTION**

The terminal complement pathway involves assembly of C5b with C6, C7 and C8 and polymerization of C9, resulting in the formation of membrane attack complex (MAC) on the cell membrane or soluble non-lytic C5b-9 also referred to as soluble MAC (sMAC). The classical view on the role of MAC is that its insertion into the cell membranes leads to a direct loss of membrane integrity resulting in cell death by necrosis. However, recent data present a much broader role of C5b-9 in disease pathogenesis. C5b-9 is involved in the induction of apoptosis via a caspase dependent pathway. Several studies have suggested that sublytic amounts of C5b-9 can cause apoptosis.

The C5b-9 activates transcription factors NF-κB and AP-1 resulting in the production of IL-6 and TNF α in cardiac myocytes and muscle cells. The role of C5a and sC5b-9 in tissue injury and inflammation has been the focus for many diseases such as lung injury, Cardiomyopathies, neurological disorders, and rheumatologic diseases i.e. SLE and RA.

Proceptor™ (CC-005b-9) ELISA kit measure the total C5b-9 bound to the circulating immune complexes (CICs).

**ASSAY PRINCIPLE**

The assay is based on a unique capture of ICs by proprietary receptors. These purified receptors (Proceptor™) are pre-coated on the microtiter plate. To perform the assay, appropriately diluted samples are incubated in the wells to allow binding of C5b-9 containing CICs to the receptors. The C5b-9 within the CIC is then measured using C5b-9 specific antibody and HRP conjugated secondary antibody. After washing the plate, a substrate solution (TMB) is added to all the wells for color development. Intensity of the color developed is proportionate to the amount of C5b-9 protein bound to CICs.

**REAGENTS**

**Receptor Microplate (1000305b-9):**
Microplate (12X8 wells) coated with receptors specific for CICs. Rows A1-H1 and A2-H2 are coated with varying concentration of purified C5b-9 protein standards. The standards are coated from (1.56 to 200 ng/ml) in doubling dilutions.

**Anti-C5b-9 Antibodies (3000105b-9) (Rabbit):**
One bottle containing 1.2 ml (10X) of anti-C5b-9 antibody (purified globulin fraction) in stabilization and preservative buffer. Dilute with 1X wash buffer before use.

**Antibody-HRP Conjugate (Goat): (10007C5b-9) (Goat):**
One bottle containing 12 ml (1X) of secondary antibody against C5b-9 antibody. Purified immunoglobulin conjugated to Horseradish peroxidase (HRP).

**Color Development Reagent (1000901):** one bottle containing 12 ml of stabilized chromogen TMB (tetramethylbenzidine).

**Stop Solution (1000801):** One bottle containing 7ml of 0.25 N Sulfuric Acid.

**Wash Buffer (1000601):** Two bottles each containing 50 ml of 10X PBS/Tween-20. Prepare a working solution of wash buffer by adding 50 ml of 10X wash buffer to 450 ml of deionized water to make 500 ml of 1X wash buffer. The 1X wash buffer can be used to wash the plate and to dilute samples.

**SAMPLE PREPARATION**

Suitable samples for C5b-9 analysis by Proceptor™ ELISA include human plasma treated with heparin and human serum. EDTA treated plasma is not recommended for the assay. Serum or plasma is diluted 1:10 with wash buffer.

**SAMPLE STORAGE**

Use serum or heparinized plasma samples immediately after collection or store sample preferably below -70º C for future use. Avoid repeated freeze-thaw cycles. Sample stored at -20º C can also be used.
CAUTION

Note: Hemolyzed samples and samples exposed to higher temperatures are not suitable for measurement of C5b-9.

Note: The reagents should be brought to room temperature before using the test kit. Bring all solutions to room temperature (20-25°C).

ASSAY PROCEDURE
Any debris observed should be removed by centrifugation at 1500g for 10 minute at room temperature from the samples prior to sample dilution.

1. Dilute samples with 1X wash buffer. We recommend 1:10 dilution of the samples.
2. In the first step leave the first two rows A1-H1 and A2-H2 containing standards without removing the plate sealer. These wells do not receive any solution in the first step.
3. Wash the wells in the rest of the plate (A3-H3 to A12-H12) with 200 µl of 1X wash buffer.
4. Pipette 100 µl of sample per well. It is recommended to run the assay in duplicate wells.
5. Incubate the plate for ninety minutes at room temperature (25°C).
6. Wash 4-times with 300 µl of wash buffer using a squirt bottle or automated plate washer. Allow buffer to stay in the wells for 2 minutes between each wash.
7. Add 100 µl of anti-C5b-9 primary antibody (diluted to 1X with wash buffer) to each well and incubate the plate for sixty minutes at room temperature (20 to 25°C).
8. Repeat wash as in step 6.
9. Add 100 µl of HRP-Conjugate (1X) and incubate for another sixty minutes at room temperature (20 to 25°C).
11. Add 100 µl of color development reagent and watch for appropriate development of color. It will take about 3 to 10 minute for color development, depending on the amount of C5b-9 present on the CIC. During the development gently tap the plate occasionally to avoid trapping air bubbles in the substrate solution.
12. Stop the reaction by the adding 50 µl of stop reagent when desired optical density is reached.
13. Read the plate using a microplate reader at 450 nm.
14. Plot the standard values against the known concentration and using the linear equation calculate the sample values (y = a + bX).
15. Use the portion of the curve that is linear for calculating the values.

It is preferred to use plate shaker during incubations to obtain uniform binding.

CALCULATION OF RESULTS
Make a spreadsheet and enter the data. Average the duplicate reading for each standard, control and sample. Subtract the average zero standard optical density; if the function is available in the machine the plate reader can do this.

Create a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw the best fit curve through the points on the curve. Discard the values, which distort the curve or add undesired error. Calculate the slope and intercept use these values in a linear equation y = a+bX to calculate the sample.

LIMITATION OF ASSAY
THE ASSAY IS FOR RESEARCH USE ONLY AND NOT FOR USE IN DIAGNOSTIC PROCEDURES.

- The samples should be clear and prepared as described.
- It is important that the sample values fall within the linear part of the standard curve.
- The kit should be used within the expiration date.
- Variations in operator, pipetting technique, washing technique, incubation time, incubation temperature, kit age and presence of interfering agents in the serum and plasma samples may cause variation in binding.
Standard Curve for Fixed C5b-9

<table>
<thead>
<tr>
<th>Conc. ng/ml</th>
<th>OD 450</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5625</td>
<td>0.068</td>
</tr>
<tr>
<td>3.125</td>
<td>0.074</td>
</tr>
<tr>
<td>6.25</td>
<td>0.11</td>
</tr>
<tr>
<td>12.5</td>
<td>0.15</td>
</tr>
<tr>
<td>25</td>
<td>0.27</td>
</tr>
<tr>
<td>50</td>
<td>0.53</td>
</tr>
<tr>
<td>100</td>
<td>1.02</td>
</tr>
<tr>
<td>200</td>
<td>2.17</td>
</tr>
</tbody>
</table>

Slope: 0.010553  
Intercept: 0.023405

Use the linear portion of the curve for calculation.

The insert represents a typical standard curve; however some variation may occur due to the laboratory and user techniques.

Store Refrigerated  
Expiry Date:  
Batch No.:
*ProGen’s proprietary receptors and their use are covered under US patent 7682793 B2. For research use only. Proceptor™ is a trade mark of ProGen Biologics, LLC*