IMUCLONE® Platelet Factor 4 ELISA

REF 634

For Research Use Only

Sekisui Diagnostics, LLC
500 West Avenue, Stamford, CT 06902
Tel. (203) 602-7777  Fax (203) 602-2221

INTENDED USE

The IMUCLONE® Platelet Factor 4 ELISA is an enzyme-linked immunosorbent assay for measuring human Platelet Factor 4 in platelet depleted plasma or any biological fluid where Platelet Factor 4 may be present. The assay is For Research Use Only and is not intended for in vitro diagnostic use.

EXPLANATION OF THE TEST

Platelet Factor 4 (PF4) is a 70 amino acid, 30,000 D molecular ratio protein. PF4 is released from activated platelet α granules in a tetrameric form complexed with platelet proteoglycan. Upon release, the half-life of PF4 is very short, less than 5 minutes, as it quickly binds to endothelial cell glycosaminoglycans where it is stored. PF4 possesses a powerful anti-heparin activity as it binds to heparin, forming a stochiometric complex, where 1 mg of PF4 will inhibit 27 IU of heparin.

ASSAY PRINCIPLE

Diluted plasma samples, biological fluid or PF4 standards are added to microwells precoated with an affinity purified rabbit polyclonal antibody specific for human PF4. The antibody captures the PF4 protein present in the solutions during an incubation period. Following a wash step, an affinity purified rabbit polyclonal antibody specific for human PF4 coupled to horseradish peroxidase (HRP) is added to the microwells and binds to the immobilized PF4. Following another wash step, the peroxidase substrate 3,3',5,5' – tetramethylbenzidine (TMB), in presence of hydrogen peroxide (H2O2), is added to the microwell and the subsequent enzymatic reaction yields a blue colored solution. Last, the addition of sulphuric acid stops the reaction and turns the solution color to yellow. The absorbance of the solution is measured at 450 nm. The absorbance is directly proportional to the amount of PF4 present in the tested sample.

REAGENTS

12 strips of 8 Anti-Human PF4 coated microwells (96 wells total)
2 vials of PF4 Sample Diluent, ready to use (50 mL)
3 vials of Human PF4 Standard (lyophilized)
1 vial of PF4 Control Plasma I, High (lyophilized)
1 vial of PF4 Control Plasma II, Low (lyophilized)
3 vials of Anti-PF4-HRP Immunoconjugate (lyophilized)
1 vial of Conjugate Diluent, ready to use (25 mL)
1 vial of Wash Solution, 20 fold concentrate (50 mL)
1 vial of TMB Substrate, ready to use (25 mL)
1 vial of Stop Solution, 0.45M H2SO4 (6 mL)

WARNING

Source material for some of the reagents in this kit is of human origin. This material has been found to be non-reactive for Hepatitis B Surface Antigen (HBsAg), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus Type 1 and Type 2 (HIV-1, HIV-2). As no known test method provides complete assurance that products derived from human blood will not transmit HBsAg, HCV, HIV-1, HIV-2 or other blood-borne pathogens, reagents should be handled as recommended for any potentially infectious human specimen. Discard all waste associated with test specimens and human source reagents in a biohazard waste container.

For Research Use Only. For in vitro use only. Not for internal use in humans or animals. Do not use the kit components beyond the stated expiration date. Do not mix reagents from different kits. Avoid microbial contamination of the reagents. Do not smoke, eat or drink in areas in which specimens or kit reagents are handled. Do not pipette reagents by mouth. Wear laboratory coat and disposable gloves throughout the test procedure and wash hands thoroughly afterwards. Avoid splashing or aerosol formation.

REAGENT PREPARATION AND STORAGE

Unopened and lyophilized reagents are stable until the expiration date printed on the box when properly stored at 2°-8°C. Allow the reagents to warm to room temperature for at least 30 minutes before using.

1. Anti-Human PF4 Coated Microwells: Once removed from the aluminum pouch, the microwell strips must be used within 30 minutes. Unused strips may be stored at 2°-8°C for 4 weeks when sealed in the original pouch with the desiccant present, protected from any moisture, and stored in the provided storage bag.
2. PF4 Sample Diluent: Supplied ready to use, once opened, the diluent may be used for up to 4 weeks when stored at 2°-8°C. This diluent contains 0.05% Kathon CG and a rheumatoid factor inhibitor.
3. PF4 Standard: Reconstitute each vial with 2.0 mL of PF4 Sample Diluent. The standard concentration is approximately 10 IU/mL. See the included flyer for the exact standard concentration. Reconstituted standard is stable for at least 8 hours at room temperature (18°-25°C) or 24 hours at 2°-8°C.
4. PF4 Control I (High): Reconstitute this vial 0.5 mL of filtered deionized water. This control is a high plasma control. See the enclosed data for the acceptable range. Reconstituted control is stable for 8 hours at room temperature (18°-25°C), 24 hours at 2°-8°C or 2 months at –20°C providing bacterial contamination is avoided.
5. PF4 Control II (Low): Reconstitute this vial 0.5 mL of filtered deionized water. This control is a low plasma control. See the enclosed data for the acceptable range. Reconstituted control is stable for 8 hours at room temperature (18°-25°C), 24 hours at 2°-8°C or 2 months at –20°C providing bacterial contamination is avoided.
6. Anti-Human PF4-HRP Immunoconjugate: Reconstitute each vial with 7.5 mL of Conjugate Diluent. Shake the vial gently to homogenize the content. Reconstituted immunoconjugate is stable for at least 24 hours at room temperature (18°-25°C) or for at least 4 weeks at 2°-8°C.
7. Conjugate Diluent: Supplied ready to use. Once opened, it may be used for up to 4 weeks when stored at 2°-8°C.
8. Wash Solution: If solids are present, incubate the vial for 15-30 minutes in a 37°C water bath. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 mL is sufficient to prepare 1 Liter of Wash Solution). The Wash Solution may be used for up to 4 weeks after opening when stored at 2°-8°C in its original vial. Diluted Wash Solution may be used for up to 7 days when stored at 2°-8°C. This reagent contains 0.05% Kathon CG.
9. TMB Substrate: Supplied ready to use. Once opened, it may be used for up to 4 weeks when stored at 2°-8°C.
10. Stop Solution, 0.45M H2SO4: Supplied ready to use. Warning: Sulphuric acid, although diluted to 0.45M is caustic. As for any similar chemical, handle with great care. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.

SPECIMEN COLLECTION AND PREPARATION

Either CTAD (citrate, theophylline, adenosine, diprydamole) or ETP (EDTA, theophylline, prostaglandin E1) collected platelet depleted plasma may be used for this assay. Blood must be collected, with a venipuncture without tourniquet, as follows:

1. Collect 9 parts of blood into 1 part of 3.2% (0.109M) trisidum citrate anticoagulant solution containing theophylline, adenosine and diprydamole (CTAD) and immediately cooled.
2. Centrifuge the blood sample at 2,500 x g, at 2°-8°C, for 30 minutes.
3. Plasma should be stored at 2°-8°C and assayed within 4 hours. Alternatively, plasma may be stored at –20°C for up to 6 months.
4. Frozen plasma should be thawed rapidly at 37°C. Thawed plasmas should be stored at 2°-8°C and assayed within 4 hours.

Plasma must be prepared properly in order to avoid false elevated PF4 concentrations resulting from the presence of residual platelets or platelet activation.

Test samples must be diluted 1:2 or 1:5 in the PF4 Sample Diluent. The diluent contains a rheumatoid factor inhibitor. For expected PF4 concentrations >50 ng/mL, samples must be assayed at higher dilutions, 1:10 or 1:20. The PF4 Controls must be diluted 1:2 in the PF4 Sample Diluent.
PROCEDURE

Materials Provided – See Reagents

Materials Required But Not Provided

- 0.22 µm filtered deionized H₂O
- 50-300 µL eight channel multi-pipette
- 0-200 µL, 200-1000 µL single pipettes
- Microwell plate reader for reading absorbance at 450 nm
- Microwell plate washer (optional)

Preparation of the Standards

Using the PF4 Standard with a concentration "C" as indicated on the flyer included in the kit, prepare the following standard solutions. The PF4 standard has been calibrated against the 1st International Standard for PF4 (NIBSC 83/505, 400 IU per ampoule). One IU/mL is equivalent to 1 ng/mL.

PREPARATION OF THE STANDARDS

<table>
<thead>
<tr>
<th>PF4 Concentration, IU/mL</th>
<th>C</th>
<th>C/2</th>
<th>C/5</th>
<th>C/10</th>
<th>C/20</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol. of PF4 Standard at &quot;C&quot; IU/mL</td>
<td>1.0 mL</td>
<td>0.50 mL</td>
<td>0.20 mL</td>
<td>0.10 mL</td>
<td>0.05 mL</td>
<td>0 mL</td>
</tr>
<tr>
<td>Vol. of PF4 Sample Diluent</td>
<td>0 mL</td>
<td>0.50 mL</td>
<td>0.80 mL</td>
<td>0.90 mL</td>
<td>0.95 mL</td>
<td>1.0 mL</td>
</tr>
</tbody>
</table>

Mix gently for a complete homogenization. The standard dilutions are stable for at least 8 hours at room temperature (18°-25°C).

Assay Procedure

Remove the required number of strips from the aluminium pouch sufficient for the number of assays to be performed. Place the strips in the frame provided. To the appropriate wells, add the reagents and perform the various assay steps as indicated on the following table:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF4 Standard, diluted control or diluted test sample</td>
<td>200 µL</td>
<td>Add the standard, diluted control or diluted test samples to an appropriate microwell. Incubate for 1 hour at room temperature (18°-25°C).</td>
</tr>
<tr>
<td>Wash Solution</td>
<td>300 µL</td>
<td>Wash the wells 5 times.</td>
</tr>
<tr>
<td>Anti-PF4-HRP Immunocojugate</td>
<td>200 µL</td>
<td>Add the immunocojugate to each microwell. Incubate for 1 hour at room temperature (18°-25°C).</td>
</tr>
<tr>
<td>Wash Solution</td>
<td>300 µL</td>
<td>Wash the wells 5 times.</td>
</tr>
<tr>
<td>TMB Substrate</td>
<td>200 µL</td>
<td>Add the substrate immediately after washing the wells. Incubate for exactly 5 minutes at room temperature (18°-25°C).</td>
</tr>
<tr>
<td>0.45M H₂SO₄</td>
<td>50 µL</td>
<td>Following exactly the same time intervals used for adding the substrate, stop the reaction by adding 0.45M H₂SO₄. Wait for 10 minutes to allow the color to stabilize and measure the solution absorbance at 450 nm (A450).</td>
</tr>
</tbody>
</table>

Notes:
1. Avoid leaving the plate in bright sunlight during incubations and particularly during color development.
2. Incubation temperatures of 18°-25°C must be maintained. Temperatures >25°C or <18°C will cause the measured A450 to be too high or too low, respectively.
3. Use of a microplate shaker should be limited to 1-2 minutes at the beginning of an incubation step.
4. Do not allow the microwells to dry out between the addition of reagents or following a washing step. Add the next reagent within 3 minutes to prevent the microwells from drying, which could damage the immobilized components. If necessary, fill the microwells with Wash Solution and empty them just before the introduction of the next reagent.
5. When adding the TMB substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.
6. For bichromatic absorbance readings, a reference wavelength of 690 nm or 620 nm may be used.

RESULTS

Construct a standard curve by plotting the mean absorbance value for each PF4 standard (ordinate) versus its corresponding concentration in IU/mL (abscissa). A standard curve should be generated each time the assay is performed. The following standard curve is for demonstration purposes only.

CALCULATIONS

From the standard curve generated, directly deduce the PF4 concentration in the diluted sample. To obtain the actual PF4 concentration in the sample, multiply the deduced value by the dilution factor (i.e. multiply the concentration by 2 for a 1:2 sample dilution or by 5 for a 1:5 sample dilution). Alternatively, an ELISA software (i.e. Dynex, etc.) can be used for the calculation of concentrations.

LIMITATIONS OF THE PROCEDURE

Plasma must be prepared properly in order to avoid false elevated PF4 concentrations resulting from the presence of residual platelets or platelet activation.

EXPECTED VALUES

The PF4 concentration in normal human plasma is < 10 IU/mL.

PERFORMANCE CHARACTERISTICS

The IMUCLONE® PF4 ELISA measures PF4 homogeneously and is insensitive to heparin.

REFERENCES