ZYMUTEST PF4

 # RK006A

Platelet Factor 4 antigen

(Complete ELISA kit for the assay of human platelet factor 4)

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

HYPHEN BioMed

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INTENDED USE:

The ZYMUTEST PF4 kit is a two-site immuno-assay for measuring human PF4 in platelet depleted plasma, or in any fluid where PF4 must be measured.
This kit is for research use only and should not be used for patient diagnosis or treatment.

ASSAY PRINCIPLE:

ZYMUTEST PF4 is a sandwich ELISA designed with affinity purified rabbit polyclonal antibodies specific for human platelet factor 4.
The diluted tested plasma or biological fluid is introduced in a microwell, coated with affinity purified rabbit polyclonal antibodies specific for human PF4. When present, this protein is captured onto the solid phase. Following a washing step, the immunoconjugate, which is an affinity purified rabbit polyclonal antibody coupled to horse radish peroxidase (HRP), is introduced, and binds to the free epitopes of immobilized PF4. Following a new washing step, the peroxidase substrate, Tetramethylbenzidine (TMB) in presence of Hydrogen Peroxide (H_2O_2), is introduced and a blue colour develops. The colour turns yellow when the reaction is stopped with Sulfuric Acid. The amount of colour developed is directly proportional to the concentration of human PF4 in the tested sample.

TEST SAMPLE:

- Human plasma especially collected on an anticoagulant containing platelet inhibitors, such as CTAD tubes or ETP.
- Any biological fluid where PF4 must be measured.

REAGENTS:

1. **COAT: Micro ELISA plate**, containing 12 strips of 8 wells, coated with affinity purified rabbit polyclonal antibodies specific for human PF4, then stabilized; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
2. **SD:** 2 vials containing 50 ml of **PF4-Sample Diluent***, ready to use, containing a Rhumatoïd factor inhibitor.
3. **STD:** 3 vials of **PF4 Standard**, lyophilised. When restored with **2 ml of PF4-Sample Diluent**, a solution containing about 10 IU**/ml (ng/ml) of human PF4 is obtained. .The exact PF4-Ag concentration is indicated on the flyer provided in the kit.
4. **CI:** 1 vial containing 0.5 ml of lyophilised **PF4 Control I (High).**
5. **CII:** 1 vial containing 0.5 ml of lyophilised **PF4 Control II (Low).**

   The PF4 concentrations and acceptance ranges for controls are indicated on the flyer provided in the kit.
6. **IC:** 3 vials of **Anti-(h)-PF4-HRP immunoconjugate**, an affinity purified rabbit polyclonal antibody coupled to HRP, lyophilised.
7. **CD:** 1 vial of 25 ml of **Conjugate Diluent**, ready to use.
8. **WS:** 1 vial of 50 ml of 20 fold concentrated **Wash Solution**.
9. **TMB**: 1 vial of 25 ml peroxidase substrate: **3,3',5,5' – Tetramethylbenzidine** containing hydrogen peroxide. Ready to use.
10. **SA**: 1 vial of 6 ml of **0.45M Sulfuric acid** (Stop solution), ready to use.

> ***Note:***
> Use only components from a same kit lot number. Do not mix components from different lots when running the assay.

NOTE: *Use a Rheumatoid factor Inhibitor in a sample diluent allows to avoid the interference of Rheumatoid Factor.
**** IU: International Unit**

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:

- **8-channel** or repeating **pipette** allowing dispensing 50-300 µl.
- **1-channel pipettes** at variable volumes from 0 to 20 µl, 20 to 200 µl and 200 to 1000 µl.
- **Micro ELISA plate** washing equipment and shaker.
- Micro ELISA plate **reader** with a wavelength set up at 450 nm.
- Distilled water.

REAGENTS PREPARATION, STORAGE AND STABILITY:

In their original packaging box, before use, when stored at 2-8°C, the unopened reagents are stable until the expiration date printed on the box.

1. **Micro ELISA plate:** open the plastic pouch and take off the required amounts of 8 well strips for the test series. When out of the pouch, the strips must be used within 30 minutes. Unused strips can be stored at **2-8°C** for **4 weeks** in their original aluminium pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided microplate storage bag (minigrip).
2. **PF4-Sample Diluent:** It is ready to use. When open, it can be used for **4 weeks**, stored at **2-8 °C**, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG and a Rheumatoid factor inhibitor.
3. **PF4 Standard:** restore each vial with **2 ml** PF4-Sample Diluent This solution is stable for at least **8 hours** at room temperature, or for at least **24 hours at 2-8°C**.
4. **PF4 Control I (High)**: restore with **0.5 ml** distilled water.
5. **PF4 Control II (Low)**: restore with **0.5 ml** distilled water.

   > ***Note:*** when restored, PF4 controls are stable for **8 hours** at room temperature**, 24 hours at 2-8°C** or **2 months** frozen at **–20°C** or below.

> ***Warning:***
> PF4 standard (3) and controls (4&5) are prepared with normal human plasma. This latter was tested with registered methods and found negative for HIV antibodies, HBs Ag and HVC antibodies. However, no assay may warrant the total absence of infectious agents. Any product of human origin must then be handled with all the required cautions, as being potentially infectious.

6. **Anti-(h)-PF4-HRP immunoconjugate**: each vial must be restored with **7.5 ml of Conjugate Diluent**. Let the pellet completely dissolve before use, and shake the vial gently in order to homogenize the contents. The restored conjugate is stable for at least **24 hours at room temperature** or for at least **4 weeks at 2-8°C**.
7. **Conjugate Diluent**: It is ready to use. When open, it can be used for **4 weeks**, stored at **2-8°C**, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG.
8. **Wash Solution**: Incubate the vial for 15-30 minutes in a water bath **at 37°C** until complete dissolution of solids, when present. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 ml contained in the vial allow preparing 1 liter of Wash Solution). The Wash Solution must be stored at **2-8°C** in its original vial and used within **4 weeks** following opening. The diluted Wash Solution must be used within **7 days**, when protected from any contamination and stored at **2-8°C**. This reagent contains 0.05% Kathon CG.
9. **TMB substrate:** It is ready to use. When open, it can be used for **4 weeks**, stored at **2-8°C**, and provided that any bacterial contamination is avoided during use.
10. **Stop solution:** It is ready to use.

    > ***Cautions:*** Sulfuric acid, although diluted to 0.45M is caustic. As for any similar chemical, handle Sulfuric acid with great care. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.

> ***Note:***
> - Bring the kit at room temperature, at least 30 min. before use. Store the unused reagents at 2-8°C.
> - The stability studies at 30°C show that the reagents can be shipped at room temperature without damage.

PROCEDURE:

Specimen collection:

Blood plasma (9 vol.) must be collected, with a net venipuncture without tourniquet, on 0.109M citrate anticoagulant containing theophylline, adenosine and dipyridamole (CTAD tubes) (1 vol.) and immediately cooled in a melting ice bath; one third of plasma supernatant is collected following a 30 min. centrifugation at 2,500 g at 2-8°C; plasma should be tested within 4 hours or stored frozen at –20°C or below for up to 6 months, and thawed for 15 min. at 37°C just before use. Thawed specimen must be tested within 4 hours. ETP (EDTA, theophylline, prostaglandin E1) collected human plasma may also be used.

Warning:

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

Tested plasma or sample or controls:

The sample must be tested diluted **two- (1:2) or five- (1:5) fold** in the PF4-Sample Diluent. For expected PF4 concentrations > 50 ng/ml, plasma or samples can be diluted **1:10, or 1:20**.
Controls I and II must be tested diluted **two fold (1:2)** as for plasmas.

Calibration:

Using the PF4 Standard (reconstituted by 2 ml) provided in the kit, with a PF4 Ag concentration "**C**" indicated, for each lot of reagents, on the flyer provided in the kit), prepare the following standard solutions:

PF4 concentration IU/ml	C	C/2	C/5	C/10	C/20	0
Vol. of PF4 Std at "C" IU/ml	1 ml	0.5 ml	0.20 ml	0.1 ml	0.05 ml	0 ml
Vol. of PF4-Sample Diluent	0 ml	0.5 ml	0.80 ml	0.9 ml	0.95 ml	1 ml

Mix gently for a complete homogenization.

The standard dilutions are stable for at least **8 hours** at room temperature.

Assay procedure:

Remove the required number of strips from the aluminium pouch, for the series of measures to be performed. Then put the strips in the frame provided. In the different wells of the micro ELISA plate introduce the reagents and perform the various assay steps as indicated on the following table:

Reagent	Volume	Procedure
PF4 Standard solution or tested sample	**200 µl**	Introduce the standard solutions or the tested samples in the corresponding micro ELISA plate well.
Incubate for 1 hour at room temperature (18-25 °C) (a)		
Wash Solution (20 fold diluted in distilled water)	**300 µl**	Proceed to 5 successive washings using the washing instrument. **(b)**
Conjugate (anti PF4 polyclonal antibody coupled with peroxidase. Restored with 7.5 ml of Conjugate Diluent)	**200 µl**	Introduce the Anti-(h)-PF4-HRP immunoconjugate in the micro ELISA plate wells.
Incubate for 1 hour at room temperature (18-25 °C) (a)		
Wash Solution (20 fold diluted in distilled water)	**300 µl**	Proceed to 5 successive washings using the washing instrument. **(b)**
TMB/H_2O_2 Substrate	**200 µl**	Immediately after the washing, introduce the substrate into the wells. **Note**: The substrate distribution, row by row, must be accurate and at exact time intervals **(b, c)**.
Incubate for exactly 5 min. at room temperature (18-25 °C)		
0.45M Sulfuric Acid	**50 µl**	Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the 0.45M sulfuric acid**.**
Wait for **10 min.** in order to allow the colour to stabilise and measure absorbance at **450 nm**. **(d)**		

Note:

a) Avoid letting the plate in the bright sunlight during incubations, and more particularly during colour development. A micro-ELISA plate shaker can be used. An incubation temperature of 18-25°C must be respected. Results are affected by a too high (>25°C) or too low (<18°C) temperature, and measured A450 are then too high or too low. It has to be considered when analyzing the results. In the same way, if a microplate shaker is used, it should be used only at the beginning of each step (sample introduction, immunoconjugate introduction, stop solution introduction), for 1 to 2 minutes, in order to obtain a good homogeneity. A450 values generated in the assay are significantly increased if shaking is used throughout the incubation steps.

b) Never let the plates empty between the addition of the reagents or following the washing step. The next reagent must be added within 3 minutes, in order to prevent the plate from drying, which could damage the immobilized components. If necessary, keep the plate filled with Wash Solution and empty it just before the introduction of the next reagent. The washing instrument must be adjusted in order to wash the plates gently, and to avoid a too drastic emptying, which could lower plate reactivity.

c) For addition of the TMB substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.

d) For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

RESULTS:

Users must construct their own calibration curve, obtained using their standard dilutions.

- On a linear graph paper plot the **PF4 concentration** on abscissae (**IU/ml**) and the corresponding absorbance (**A450**) on ordinates.
- From the curve obtained, deduce the PF4 concentration for the tested dilution. For obtaining the PF4 concentration in the tested sample, this value must be multiplied by the dilution factor (**i.e., 2, 5, 10, or 20**).
- Alternatively, an ELISA software (i.e., Dynex, Biolise, etc.) can be used for the calculation of concentrations.

The results obtained should be for research purposes only and not used for patient diagnosis or treatment.

BIOCHEMISTRY OF PLATELET FACTOR 4:

PF4 is a 70 amino acid platelet specific protein with a molecular weight of about 30 Kd for the tetramer. It is released from platelet α granules upon activation in the tetrameric form as a complex with a platelet proteoglycan (Ref. 1, 3, 4). The half-life in plasma is short (< 5 min.), as PF4 binds to endothelial cell glycosaminoglycans where it is stored. PF4 has a strong anti-heparin activity. It forms stoichiometric macromolecular complexes with heparin with an optimal ratio of 27 IU of heparin per mg of PF4 (Ref. 2). PF4 concentration in normal human plasma is < 10 IU/ml (Ref. 6).

STANDARDISATION:

ZYMUTEST PF4 standard is calibrated against the 1st International Standard for PF4 (NIBSC, 83/505, 400 IU per ampoule) (Ref. 5). One IU/ml PF4 corresponds to about 1 ng/ml.

ASSAY REACTIVITY:

ZYMUTEST PF4 measures PF4 homogeneously, whatever its presentation.
It is insensitive to the presence of heparin.

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