Quantikine® ELISA

Human CXCL4/PF4 Immunoassay

Catalog Number DPF40

For the quantitative determination of human Platelet Factor 4 (PF4) concentrations in cell culture supernates, serum, and platelet-poor plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Platelet Factor 4 (PF4), also known as CXCL4, is an 8 kDa member of the CXC chemokine family that shares features with CXCL8/IL-8 and CXCL7/thymus chemokine-1/NAP-2/β-thromboglobulin (1-3). Mature human PF4 shares 65-76% amino acid sequence identity with mouse, rat, bovine, ovine and porcine PF4. The active protein is a tetramer of PF4 subunits that forms a ring of heparin-binding positive charges from sites at the C-terminal region of each monomer (4). Megakaryocytes synthesize PF4 and store it in platelet α-granules (5, 6). Secretion from activated platelets can produce micromolar levels in serum and over 100-fold higher within clots (2). In contrast to other CXC chemokines, PF4 does not contain an ELR motif and lacks binding to nearly all chemokine receptors (2, 3). A potential high-affinity G-protein-coupled receptor for PF4, the CXCR3 isoform CXCR3B, is expressed in human but not mouse (2, 3). In most cases, it is likely that cell surface binding and signaling properties of PF4 are due to binding of glycosaminoglycan chains, particularly chondroitin sulfates (2, 7-9).

Release of PF4 from activated platelets affects coagulation. It binds and regulates thrombin/thrombomodulin complexes. PF4 also regulates and enhances production of activated Protein C (APC), which limits the coagulation cascade (10-12). It binds and influences the enzymatic activity of coagulation factor Xa (13). It binds fibrin and affects clot structure (14). PF4 is thought to aid platelet aggregation by adhering to the platelet surface after its release. Deletion of mouse PF4 impairs platelet aggregation in response to low doses of thrombin. However, transgenic overexpression of PF4 can inhibit platelet aggregation and thrombus formation (3, 15). Therapeutic doses of the anticoagulant heparin neutralize PF4 procoagulant effects (15). The complex between heparin and PF4 can be immunogenic, and circulating PF4-heparin antibodies cause the pathological syndrome HITT (heparin-induced thrombocytopenia and thrombosis, also called HIT) (16). In addition, immunogenic complexes of PF4 with apolipoprotein H can contribute to antiphospholipid syndrome (APS) (17).

Many other functions have been observed for PF4. In a feedback mechanism, PF4 interaction with megakaryocyte LRP1 downregulates further platelet production (18, 19). In hematopoietic progenitors, heterodimers of PF4 and CXCL8/IL-8 can slow proliferation and promote adhesion (9, 20). PF4 can be proinflammatory. It is involved in monocyte survival, macrophage differentiation, TNF-α induction and induction of endothelial cell apoptosis (7, 21-23). It promotes atherogenesis by downregulating macrophage CD163, and by binding endothelial cell LRP1, which results in upregulation of E-selectin surface expression (24, 25). It also affects neutrophil transmigration through endothelial cells, but is not chemotactic for neutrophils (2, 3, 26-29). PF4 can be antiproliferative and antiangiogenic, at least in part via interfering with FGF basic and VEGF heparin binding and thus inhibiting their signaling (30-32). In acute myelogenous leukemia (AML), increased circulating PF4 may indicate induction of remission, while in rheumatoid arthritis patients treated with infliximab (anti-TNF-α), high serum PF4 is thought to indicate lack of response (33, 34). Finally, an antimicrobial effect has been shown for PF4, both by direct binding of microbes and by stimulating macrophage respiratory bursts and cytokine production (35, 36).

The Quantikine Human CXCL4/PF4 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human PF4 in cell culture supernates, serum, and platelet-poor plasma. It contains *E. coli*-expressed recombinant human PF4 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human PF4 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human PF4.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human PF4 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any PF4 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human PF4 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of PF4 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human PF4 Microplate	894555	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human PF4.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human PF4 Conjugate	894556	21 mL of polyclonal antibody specific for human PF4 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human PF4 Standard	894557	Recombinant human PF4 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-15	895424	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6-13	895491	2 vials (21 mL/vial) of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human PF4 Controls (optional; available from R&D Systems).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Platelet-poor Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -70 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

PF4 is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of PF4, platelet-poor plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical and Laboratory Standards Institute (CLSI), result in incomplete removal of platelets from blood.

SAMPLE PREPARATION

Serum samples require at least a 400-fold dilution. A suggested 400-fold dilution can be achieved by adding 20 µL of sample to 380 µL of Calibrator Diluent RD6-13. Complete the 400-fold dilution by adding 20 µL of the diluted sample to 380 µL Calibrator Diluent RD6-13.

Platelet-poor plasma samples require a 10-fold dilution. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD6-13.

REAGENT PREPARATION

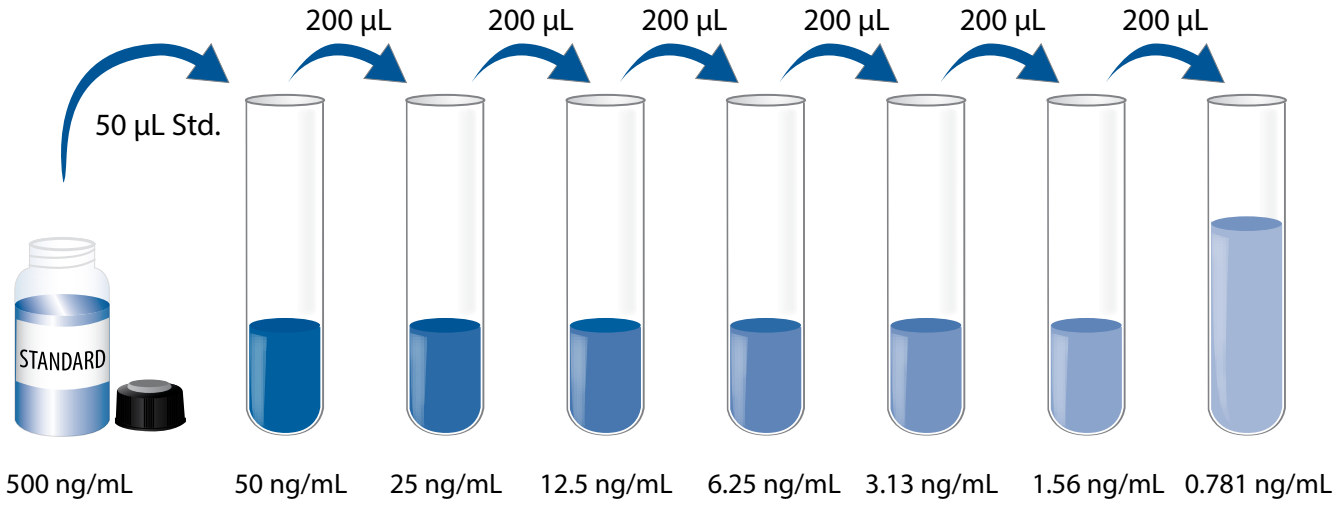
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Human PF4 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human PF4 Standard with deionized or distilled water. This reconstitution produces a stock solution of 500 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 30 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD6-13 into the 50 ng/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 50 ng/mL standard serves as the high standard. Calibrator Diluent RD6-13 serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1-15 to each well.
4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human PF4 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

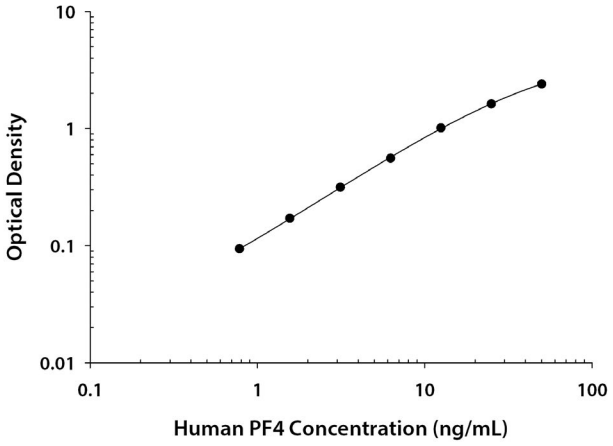
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human PF4 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected
0	0.027 0.029	0.028	—
0.781	0.121 0.122	0.122	0.094
1.56	0.198 0.199	0.199	0.171
3.13	0.341 0.344	0.343	0.315
6.25	0.576 0.596	0.586	0.558
12.5	1.020 1.060	1.040	1.012
25	1.634 1.667	1.651	1.623
50	2.416 2.450	2.433	2.405

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	5.80	14.1	26.8	5.61	14.3	27.2
Standard deviation	0.472	0.840	1.85	0.558	1.70	3.20
CV (%)	8.1	6.0	6.9	9.9	11.9	11.8

RECOVERY

The recovery of human PF4 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	110	99-117%
Platelet-poor EDTA plasma* (n=4)	100	89-117%

*Samples were diluted prior to assay.

LINEARITY

To assess linearity of the assay, samples containing and/or spiked with high concentrations of human PF4 were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum* (n=4)	Platelet-poor EDTA plasma* (n=4)	Platelet-poor heparin plasma* (n=4)
1:2	Average % of Expected	99	102	101	113
	Range (%)	94-104	96-112	95-106	109-116
1:4	Average % of Expected	100	103	96	115
	Range (%)	93-103	91-116	92-108	112-120
1:8	Average % of Expected	101	101	93	112
	Range (%)	96-111	95-110	86-103	103-117
1:16	Average % of Expected	97	96	86	106
	Range (%)	93-100	92-100	81-90	87-120

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Twenty-seven assays were evaluated and the minimum detectable dose (MDD) of human PF4 ranged from 0.010-0.100 ng/mL. The mean MDD was 0.032 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human PF4 (aa 32-101 of accession # P02776.2) produced at R&D Systems.

SAMPLE VALUES

Serum/Platelet-Poor Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human PF4 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=36)	8965	3166-13,275	2144
Platelet-poor EDTA plasma (n=36)	78.8	12.5-344	66.2
Platelet-poor heparin plasma (n=36)	292	54.2-1286	227

Cell Culture Supernates - Human monocytes were cultured in RPMI supplemented with 10% fetal bovine serum for 6 days. An aliquot of the cell culture supernate was removed, assayed for natural human PF4, and measured 6.17 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human PF4.

The factors listed below were prepared at 500 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 500 ng/mL in a mid-range recombinant human PF4 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Apolipoprotein H
Coagulation Factor X/Xa
CXCL1/GROα
CXCL2/GROβ
CXCL7/NAP-2
CXCL8/IL-8
CXCL9/MIG
CXCL10/IP-10/CRG-2
CXCL12/SDF-1
CXCL16
IL-3
LRP-1 Cluster II
LRP-1 Cluster III
LRP-1 Cluster IV
Pro-Factor II/Prothrombin
VEGF
Thrombomodulin/BDCA-3

Recombinant human CXCL4LI/PF4V cross-reacts approximately 43.8% in this assay.

Recombinant mouse CXCL4/PF4 interferes at concentrations > 50 ng/mL.

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