Quantikine® ELISA

Human Fibronectin Immunoassay

Catalog Number DFBN10

For the quantitative determination of human Fibronectin concentrations in cell culture supernates, cell lysates, tissue lysates, serum, plasma, saliva, urine, and human milk.

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

Fibronectin (FN) is a large modular glycoprotein that is found as a polymeric fibrillar network in the extracellular matrix (ECM) and as soluble disulfide-linked dimeric protomers in plasma and other body fluids (1-4). Fibronectin is a ligand for many molecules including fibrin, heparin, chondroitin sulfate, collagen/gelatin, various integrins and growth factors, myocilin, and Apolipoprotein A (4-10). It is involved in multiple cellular processes such as cell adhesion/migration, blood clotting, morphogenesis, tissue repair, and cell signaling. Fibronectin functions are mediated by the insoluble polymeric fibrils. Conversion of soluble Fibronectin to Fibronectin fibrils in the ECM is initiated by binding to cell surface integrins, resulting in exposure of cryptic epitopes necessary for polymerization (4). Mature human Fibronectin shares 88% aa sequence identity with mouse and rat Fibronectin.

Fibronectin is made up of three types of homologous structural motifs termed FN type I, type II, and type III repeats (11-13). Alternative splicing generates multiple isoforms of Fibronectin which may have insertions of extra type III domains (EDA and EDB) or alteration of the type III connecting segment (IIICS) (3, 13). Differential splicing within the IIICS domain determines the presence of CS1 and CS2 sequences and the sensitivity to proteases (14, 15). The IIICS domain contains two sites (CS1 and CS2) that interact with Integrin α4β1 (14, 16, 17). The CS1 sequence is not accessible in full length Fibronectin but is exposed by protease digestion, thereby enabling cell adhesion *via* Integrin α4β1 (17). A fragment from the first type III repeat, known as Anastellin, binds to Fibronectin and induces the formation of high molecular weight disulfide-linked superfibronectin (sFN) (18-20). sFN exhibits increased protease sensitivity and cellular adhesiveness compared to Fibronectin (18, 20). Anastellin acts *in vivo* to inhbit angiogenesis, tumor growth, and metastasis (19).

The Quantikine® Human Fibronectin Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Fibronectin in serum-free cell culture supernates, cell lysates, tissue lysates, serum, plasma, saliva, urine, and human milk.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Fibronectin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Fibronectin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Fibronectin 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 Fibronectin 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 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 Fibronectin Microplate	894764	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Fibronectin.	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 Fibronectin Standard	894766	2 vials of human Fibronectin in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.* **Note:** *Human sourced material. See Precautions section.*	Use a new standard for each assay. Discard after use.
Human Fibronectin Conjugate	894765	21 mL of a polyclonal antibody specific for human Fibronectin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-9	895167	11 mL of a buffered protein base with preservatives. *May contain a precipitate. Warm to room temperature, and mix gently to dissolve. If the precipitate does not completely dissolve, mix well during use.*	
Calibrator Diluent RD5P	895151	21 mL of a buffered protein base with preservatives. *Use diluted 1:10 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold 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.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards and samples.
- Human Fibronectin Controls (optional; R&D Systems®, Catalog # QC200).

SUPPLIES REQUIRED FOR TISSUE LYSATE SAMPLES

- RIPA buffer plus protease inhibitors

PRECAUTIONS

The Human Fibronectin Standard is derived from human plasma. The plasma was tested at the donor level using an FDA licensed method and found to be non-reactive for anti-HIV-1/2 and Hepatitis B surface antigen. As no testing can offer complete assurance of freedom from infectious agents, this reagent should be handled as if capable of transmitting infection.

Fibronectin is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

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

Some components in this kit contain a preservative 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. Refer to the SDS 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.

Cell Lysates/Tissue Lysates - Cells must be lysed prior to assay as directed in the Sample Values section.

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.

Plasma - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Sample values differences of 4-64% were observed when samples underwent multiple freeze/thaw cycles. It is recommended to use a sample aliquot that has been subjected to a maximum of one freeze/thaw cycle for each assay.*

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and repeat this process a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Serum and plasma samples require a 10,000-fold dilution. A suggested 10,000-fold dilution can be achieved by adding 10 μL of sample to 990 μL of Calibrator Diluent RD5P (diluted 1:10)*. Complete the 10,000-fold dilution by adding 10 μL of the diluted sample to 990 μL Calibrator Diluent RD5P (diluted 1:10)*.

Saliva and urine samples require a 3-fold dilution. A suggested 3-fold dilution is 100 μL of sample + 200 μL of Calibrator Diluent RD5P (diluted 1:10)*.

Human milk samples require a 30-fold dilution. A suggested 30-fold dilution is 10 μL of sample + 290 μL of Calibrator Diluent RD5P (diluted 1:10)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *Fibronectin is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

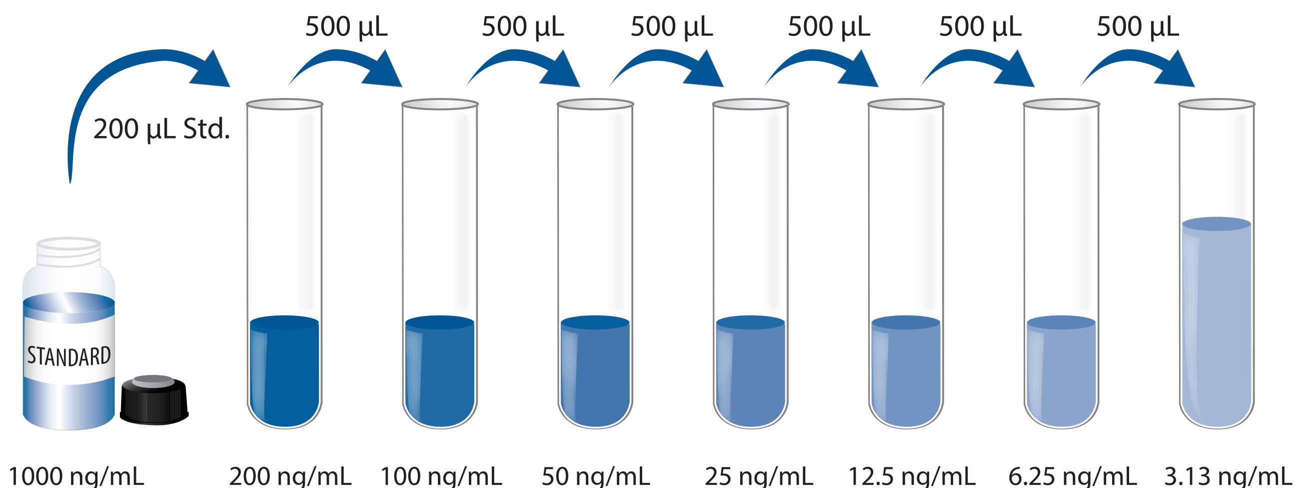
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

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.

Calibrator Diluent RD5P (diluted 1:10) - Add 5.0 mL of Calibrator Diluent RD5P to 45 mL of deionized or distilled water to prepare 50 mL of Calibrator Diluent RD5P (diluted 1:10).

Human Fibronectin Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Fibronectin Standard with deionized or distilled water. This reconstitution produces a stock solution of 1000 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 800 µL of Calibrator Diluent RD5P (diluted 1:10) into the 200 ng/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 200 ng/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:10) serves as the zero standard (0 ng/mL).



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ASSAY PROCEDURE

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

Note: *Fibronectin is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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 RD1-9 to each well. *Warm assay diluent to room temperature and mix well if precipitate is present.*
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. A plate layout is provided to record standards and samples assayed.
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 Fibronectin 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

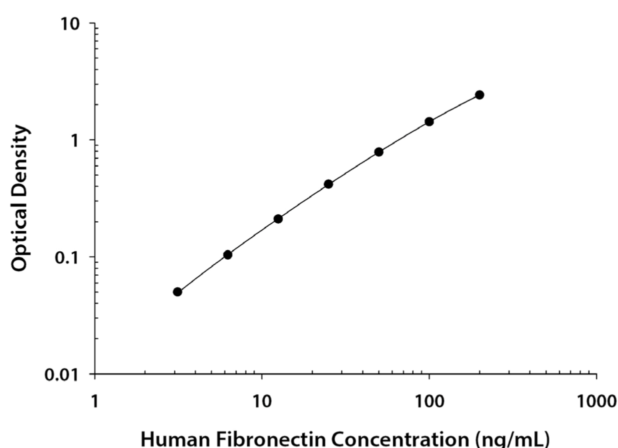
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 Fibronectin 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	0.031 0.034	0.033	—
3.13	0.082 0.083	0.083	0.050
6.25	0.134 0.139	0.137	0.104
12.5	0.243 0.244	0.244	0.211
25	0.447 0.452	0.450	0.417
50	0.811 0.825	0.818	0.785
100	1.451 1.467	1.459	1.426
200	2.427 2.478	2.453	2.420

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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)	24.0	58.1	123	23.3	56.5	115
Standard deviation	1.40	2.78	6.35	1.31	2.98	6.38
CV (%)	5.8	4.8	5.2	5.6	5.3	5.5

RECOVERY

The recovery of human Fibronectin spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	97	89-115%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Fibronectin were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

		Cell culture supernate (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Citrate plasma (n=4)	Saliva (n=4)	Urine (n=4)	Human milk (n=4)
1:2	Average % of Expected	106	105	107	107	103	103	101	99
	Range (%)	100-111	101-108	102-109	103-113	100-106	101-106	98-106	94-103
1:4	Average % of Expected	106	106	111	110	106	105	101	103
	Range (%)	101-114	98-113	106-117	105-115	104-109	100-107	98-103	102-106
1:8	Average % of Expected	107	107	110	111	110	104	101	102
	Range (%)	103-112	96-113	104-115	105-117	105-118	99-107	98-104	100-105
1:16	Average % of Expected	110	108	111	113	113	107	108	102
	Range (%)	102-115	100-113	103-118	110-117	110-119	100-110	103-113	98-105

SENSITIVITY

Twenty-eight assays were evaluated and the minimum detectable dose (MDD) of human Fibronectin ranged from 0.062-0.579 ng/mL. The mean MDD was 0.187 ng/mL.

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

CALIBRATION

This immunoassay is calibrated against highly purified Fibronectin from human plasma.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of human Fibronectin in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (mg/mL)	Range (mg/mL)	Standard Deviation (mg/mL)
Serum (n=35)	0.259	0.083-0.539	0.114
EDTA plasma (n=35)	0.322	0.116-0.601	0.132
Heparin plasma (n=35)	0.192	0.048-0.527	0.100
Citrate plasma (n=35)	0.283	0.138-0.503	0.095

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Saliva (n=10)	104	11.6-356	102
Urine (n=11)	49.1	18.2-107	30.0
Human milk (n=10)	1658	555-3316	933

Cell Culture Supernates:
A549 human lung carcinoma cells were cultured in F12 supplemented with 10% fetal bovine serum. Cells were unstimulated or stimulated with 10 ng/mL of recombinant human TGF-β for 2 days. Aliquots of the cell culture supernates were removed, assayed for human Fibronectin, and measured 33.1 ng/mL and 87.6 ng/mL, respectively.

HepG2 human hepatocellular carcinoma cells were cultured in MEM(NEAA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. An aliquot of the cell culture supernate was removed, assayed for human Fibronectin, and measured 11,338 ng/mL.

Tissue Lysates - Human liver tissue was placed in cold RIPA buffer plus protease inhibitors and homogenized on ice with a polytron for 10-15 seconds. Tissue was then centrifuged to remove debris. An aliquot of the tissue lysate was removed, assayed for human Fibronectin, and measured 19.1 ng/mL.

Cell Lysates - HepG2 human hepatocellular carcinoma cells were harvested by trypsinization, neutralized, pelleted, and washed in PBS. The cell pellet was resuspended in 1% NP-40 alternative, 20 mM Tris, 137 mM NaCl, 10% Glycerol, 1 mM EDTA, and 1 mM activated Sodium Orthovanadate (R&D Systems®, Catalog # DYC002) and incubated on ice for 15 minutes. The lysate was then centrifuged to remove debris. An aliquot of the cell lysate was removed, assayed for human Fibronectin, and measured 5.17 ng/mL.

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SPECIFICITY

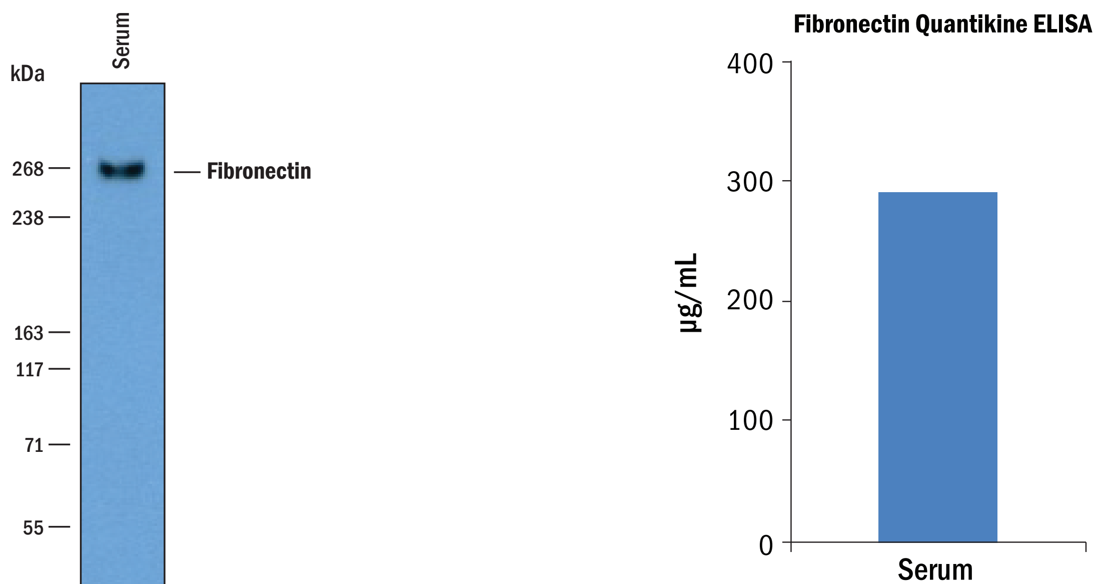
This assay recognizes natural and recombinant human Fibronectin and Fibronectin fragments aa 32-1908 and aa 607-1265. Fibronectin fragment aa 631-705 cross-reacts approximately 0.415%. Fibronectin fragments aa 1266-1908, aa 1722-1811, and aa 1913-2477 are not detected in this kit.

The factors listed below were prepared at 2500 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 2500 ng/mL in a mid-range human Fibronectin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Integrin α1β1
Integrin α2β1
Integrin α2β3
Integrin α3β1
Integrin α4β1
Integrin α4β7
Integrin α5β1
Integrin α6β1
Integrin α6β4 X1 isoform
Integrin α9β1
Integrin α10β1
Integrin α11β1
Integrin αEβ7
Integrin αLβ2
Integrin αMβ2
Integrin αVβ1
Integrin αVβ3
Integrin αVβ5
Integrin αVβ6
Integrin αVβ8
Integrin αXβ2

Bovine Fibronectin does not interfere but does cross-react approximately 1% in this assay.



A human serum sample was resolved by reducing SDS-PAGE, transferred to PVDF membrane, and immunoblotted with the detection antibody in this kit. A human serum sample was diluted 1:10,000 for the Western Blot.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

NOTES

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