Human Fibronectin ELISA Kit (Plasma and Serum Samples)

Introduction

Fibronectin (FN) is a major component of the extracellular matrix and blood plasma, and is a specific ligand for several integrin adhesion receptors (1). FN plays an important role not only in cell adhesion (2) and wound healing (3), but also in embryogenesis (4) and hematopoiesis (5). FN is over-expressed in cardiovascular disease states such as atherosclerosis (6) and myocardial infarction (7). Reduced levels of FN have been reported in patients with Disseminated Intravascular Coagulation (DIC) and low concentrations appear to correlate with a poor prognosis (8).

Principal of the Assay

The Human Fibronectin ELISA (Enzyme-Linked Immunosorbent Assay) kit employs a quantitative competitive enzyme immunoassay technique that measures human plasma, serum and cell culture supernatant FN in less than 3 hours. A murine antibody specific for FN has been pre-coated onto a 96-well microplate with removable strips. FN in standards and samples is competed by a biotinylated FN sandwiched by the immobilized antibody and streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- This kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution.
- All human source materials have been tested and found to be negative to HbsAg, HIV-1 and HCV by FDA approved methods.

Reagents

- **FN Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a murine antibody against FN
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **FN Standard:** Human FN in a buffered protein base (100 μg, lyophilized).
- **Biotinylated FN (8x):** 1 vial, lyophilized.

- **MIX Diluent Concentrate (10x)**: A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (90 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store kit at 2-8^{0}C or -20^{0}C upon arrival up to the expiration date.
- Opened MIX Diluent may be stored for up to 1 month at 2-8^{0}C. Store reconstituted reagents at -20^{0}C or below.
- Opened unused strip wells may return to the foil pouch with the desiccant pack, reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl and multiple channel pipettes)
- Deionized or distilled reagent grade water

Sample Collection, Preparation and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes. Dilute plasma samples 1:100 into MIX Diluent and assay. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (EDTA can also be used as a anticoagulant)
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Dilute samples 1:100 into MIX Diluent and assay. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (EDTA can also be used as a anticoagulant)
- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay. Samples can be stored at -20^{0}C or below. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.
- **MIX Diluent Concentrate (10x):** Dilute the MIX Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8^{0}C.
- **Standard Curve:** Reconstitute the 100 µg of FN Standard with 1 ml of MIX Diluent to generate a 100 µg/ml of solution. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare triplicate standard points by serially diluting the standard solution (100 µg/ml) 1:2 with MIX Diluent to produce 50, 25, 12.5, 6.25, 3.125, 1.565 and 0.78 µg/ml solutions. MIX Diluent serves as the zero standard (0 µg/ml). Any remaining solution should be frozen at < -20^{0}C.

Standard Point	Dilution	[FN] (μg/ml)
P1	Standard (100 μg/ml) + 1 parts MIX Diluent	50.000
P2	1 part P1 + 1 parts MIX Diluent	25.000
P3	1 part P2 + 1 parts MIX Diluent	12.500
P4	1 part P3 + 1 parts MIX Diluent	6.250
P5	1 part P4 + 1 parts MIX Diluent	3.125
P6	1 part P5 + 1 parts MIX Diluent	1.565
P7	1 part P6 + 1 parts MIX Diluent	0.780
P8	MIX Diluent	0.000

- **Biotinylated FN (8x):** Dilute Biotinylated FN with 4 ml MIX Diluent to produce a 8-fold stock solution. Allow the biotin to sit for 10 minutes with gentle agitation prior to making dilutions. The stock solution should be further diluted 1:8 with MIX Diluent. Any remaining solution should be frozen at < -20^{0}C.
- **Wash Buffer Concentrate (20x):** Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen at < -20^{0}C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^{0}C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 25 μl of standard or sample per well and immediately add 25 μl of Biotinylated FN to each well (on top of the Standard or sample) and mix gently. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with 200 μl of Wash Buffer. Invert the plate and decant the contents, and hit it 4-5 times on absorbent paper towel to complete remove liquid at each step.
- Add 50 μl of Streptavidin-Peroxidase Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash five times with 200 μl of Wash Buffer as above.
- Add 50 μl of Chromogen Substrate per well and incubate for about 8 minutes or till the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 μl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. Please note that after the reaction is stopped for about 10 minutes, some black particles may be generated at high absorbance to reduce the readings.

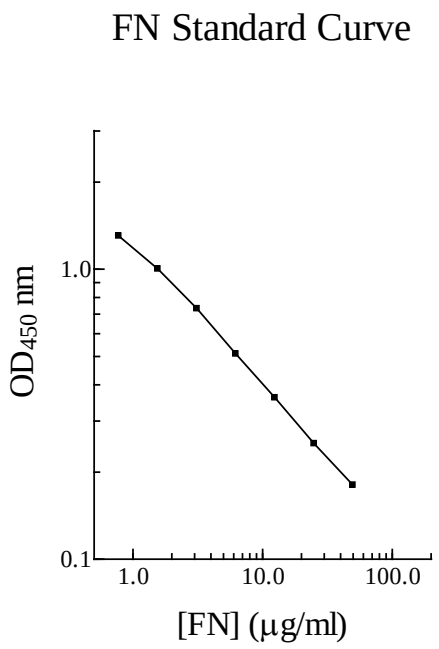
Data Analysis

- Calculate the mean value of the triplicate readings for each standard and sample.
- To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.

- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Performance Characteristics

- The minimum detectable dose of FN is typically 40 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 5.1% and 7.3% respectively.

Linearity

	Average Percentage of Expected Value	
Sample Dilution	**Plasma**	**Serum**
1:50	101 %	97%
1:100	98%	102%
1:200	102%	98%
1:400	101%	101%

Recovery

Standard Added Value	1 - 25 µg
Recovery %	90-112 %
Average Recovery %	101 %

References

(1) Hynes, R.O. (1992) *Cell* 69:11
(2) Wu, C. *et al.* (1995) *Cell* 83:715
(3) Brown, L.F. *et al.* (1993) *Am. J. Pathol.* 142:793
(4) Pagani, F. *et al.* (1991) *J. Cell Biol.* 113:1223
(5) Verfaillie, C.M. *et al.* (1991) *J. Exp. Med.* 174:693
(6) Glukhova M.A. *et al.* (1989) *J. Cell. Biol*. 109:357
(7) Knowlton, A.A. *et al.* (1992) *J. Clin. Invest*. 89:1060
(8) Cembrowski, G.S. and Mosherb, D.F. (1984) *Thrombosis Research* 36:437

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