INTRODUCTION:

Fibronectin is a major cell surface glycoprotein important in cell-to-cell interactions, adhesion to the extracellular matrix, promotion of cell spreading and morphology[1]. Fibronectin is absent or substantially reduced in some transformed cells, hence the term LETS (large external transformation sensitive) protein[2]. Fibronectin occurs in plasma, also known as cold insoluble globulin (CIG)[3]. Plasma and cellular forms have similar physical and immunological properties.

Recent studies have shown that Fibronectin is important in several aspects of hemostasis and wound healing. Fibronectin is incorporated into the fibrin clot during blood coagulation, and may be covalently crosslinked to fibrin by Factor XIII. Platelet bound fibronectin may be the receptor for collagen in the platelet-collagen interaction following vascular injury[4].

The DRG Human Fibronectin ELISA Kit is a 96 well system, which allows rapid (3 hour) measurement of human fibronectin from most biological samples.

Samples are incubated with specific primary antiserum (rabbit polyclonal) raised against highly purified human fibronectin. A second incubation is then performed with an alkaline phosphatase human fibronectin conjugate. Separation of the antibody bound and a second antibody, which is precoated to the wells, achieves free fractions. The amount of bound enzyme, which is inversely proportional to fibronectin concentration, is estimated by absorption at 400-410 nm, after incubation with substrate. Store at 4°C up to the expiration date on the kit box.

SAMPLE PREPARATION:

Detergent solubilized samples can be accurately measured providing the diluted end concentration of detergent does not exceed the following levels:

0.05% Non-ionic Detergents (Tween-Triton X-100)
0.01% Ionic Detergents (SDS)

All biological samples should be fresh. Fibronectin is very sensitive to proteolysis, include protease inhibitors such as Aprotinin, Phenyl-methylsulfonyl fluoride, and soybean trypsin inhibitor in extraction or dilution media. Plasma specimens must be fresh or fresh frozen. Dilute plasma samples 1000 fold with Diluent buffer prior to assay.

Measurement of Cellular Fibronectin:

Wash cells 3 times with 0.05M Tris Cl, 0.15M NaCl, pH 7.5. Cells in monolayer can be scraped off the plates with a rubber policeman and collected by centrifugation. Resuspend cells in the above buffer with Aprotinin (5ug/ml) and Phenylmethylsulfonyl fluoride (PMSF) (0.5mM) and add urea at 0.6g/ml suspension. Mix to dissolve the urea and incubate at 37°C, 2 hours. Dialyze against 0.01M 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS), pH 11 at 20°C for 24 hours. The solution can be concentrated and/or clarified, then neutralized by the addition of 1/10 volume of 0.5M Tris Cl, pH 7.4. Note: Store samples at 4°C in the CAPS buffer (30 days) until the assay date.

REAGENTS:

1. **Fibronectin stock solution**. 100ug/ml. 0.5ml
2. **Diluent Buffer** 15x Concentrate. 2 vials with 10ml each.
3. **Rabbit anti-Human Fibronectin**. 5ml.

4. **Fibronectin Tracer**. Human fibronectin-alkaline phosphatase adduct. 5ml.
5. **Diethanolamine Buffer**. 60ml.
6. **Enzyme substrate**. p-nitrophenylphosphate (PNPP).
7. **Stopping Reagent**. 4ml.
8. **One 96-well microtiter plate** - 8 well strips. Coated with Goat anti-Rabbit IgG.

MATERIALS REQUIRED BUT NOT SUPPLIED:

- A 96-well plate reader to measure absorbance at 400-410 nm.
- Precision micropipets (Accu-Tips available from DRG).
- Polypropylene culture tubes (DRG catalog number).
- A Multichannel microwell washer is recommended.
- A 37°C incubator.

ASSAY PROCEDURE:

1. Transfer the contents of a vial of Diluent Buffer Concentrate to a graduated cylinder, add distilled water to a volume of 150ml. Store at 4°C when not in use.
2. Preparation of Standards. The Fibronectin stock solution is stable for at least 6 months at 4°C. ***Do not freeze***. Prepare a set of working standards in the range of 25ng/ml to 2000ng/ml with working diluent buffer. These working standards are stable approximately 1 week at 4°C.

Dilution		Concentration
A.	100ul stock + 4.9ml diluent	2000ng/ml
B.	1.0ml Solution **A** + 3.0ml diluent	500ng/ml
C.	1.0ml Solution **B** + 1.0ml diluent	250ng/ml
D.	0.5ml Solution **B** + 2.0ml diluent	100ng/ml
E.	0.1ml Solution **B** + 0.9ml diluent	50ng/ml
F.	0.1ml Solution **C** + 0.9ml diluent	25ng/ml

3. If not using the entire plate, remove strips not in use immediately and store them dry at 4°C. Do all determinations at least in duplicate, using 2 wells for a blank, 2 wells for Bmax and 2 wells for each of six standards. This will use two 8-well strips.
4. Pipet 150ul diluent buffer into blank wells and 100ul into the Bmax wells.
5. Pipet 100ul standards, controls and unknowns into appropriate wells.
6. Pipet 50ul of the Fibronectin Antiserum into all wells except Blanks. Gently mix the plate about 30 seconds, avoid splashing. Cover wells tightly with Parafilm, incubate at 37°C, 1 hour.
7. Add 50ul Fibronectin Tracer to all wells, mix as above, cover, incubate at 37°C, 1 hour.
8. Dissolve PNPP, 4mg/ml, in Diethanolamine Buffer. This solution is best prepared just before use, but may be stored approximately 1 hour at 4°C. Allow a minimum of 200ul per well.
9. Aspirate all wells, fill each with diluent buffer and aspirate. Repeat wash cycle 3 times. Use a multichannel washer if available.

10. Gently tap the empty plate on a paper towel to remove excess liquid. Add 200ul prepared substrate solution to all wells, mix, and incubate at 37°C. After approximately 60 minutes measure absorbance at 400-410 nm in 5-minute intervals until desired range is reached. If desired, 20ul of Stop Solution can be added to all wells and the absorbance measured up to 1 hour later.

CALCULATIONS:

1. Average all duplicates and the blank to obtain net mean absorbances.
2. Construct a standard curve by plotting mean absorbance of each standard versus the concentration or log concentration.
3. Determine concentration of unknowns from this standard curve. Always generate a standard curve for each new assay.

TYPICAL DATA FOR A STANDARD CURVE.

Well No.	Sample	Net Concentration	A_{410nm}	Average
A1	Blank	-	0.166	
A2	Blank	-	0.170	0.168
B1	Bmax	0ng/ml	1.516	
B2	Bmax	0ng/ml	1.553	1.366
C1	Standard F	25ng/ml	1.408	
C2	Standard F	25ng/ml	1.399	1.236
D1	Standard E	50ng/ml	1.244	
D2	Standard E	50ng/ml	1.283	1.096
E1	Standard D	100ng/ml	1.090	
E2	Standard D	100ng/ml	1.104	0.929
F1	Standard C	250ng/ml	0.750	
F2	Standard C	250ng/ml	0.850	0.632
G1	Standard B	500ng/ml	0.528	
G2	Standard B	500ng/ml	0.588	0.390
H1	Standard A	2000ng/ml	0.296	
H2	Standard A	2000ng/ml	0.290	0.125

REACTIVITY OF OTHER FIBRONECTINS

	Useful Range
Mouse	0.2 - 50ug/ml
Bovine	0.5 - 100ug/ml
Rat	0.2 - 20ug/ml

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