CELL APPLICATIONS, INC.

Rat Fibronectin ELISA Kit

Cat. No. CL0350
96-wells

Target Protein Species: Rat
Range 156 pg/ml – 10000pg/ml
Specificity No detectable cross-reactivity with other cytokines

COMPONENTS

Kit Component	Amount
96-well plate precoated with anti-rat Fibronectin antibody	1 Plate
Protein Standard: Lyophilized recombinant rat Fibronectin	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-rat Fibronectin)	130 µl (100x)
Antibody Diluent Buffer	12ml
Avidin-Biotin-Peroxidase Complex (ABC) Solution	130 µl (100x)
ABC Diluent Buffer	12 ml
Tetramethyllbenzidine (TMB) Color Developing Agent	10 ml
TMB Stop Solution	10 ml

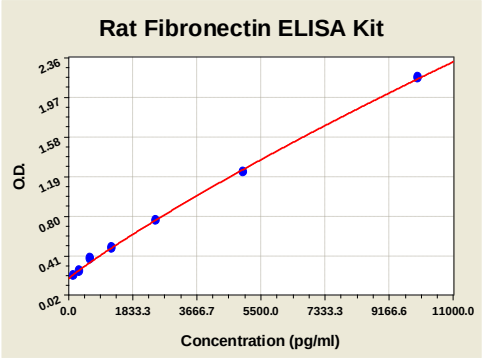
Washing Buffer (not provided): TBS or PBS

0.01M TBS: Add 1.2g Tris, 8.5g NaCl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 900ml H_2O and adjust pH to 7.2-7.6. Adjust the total volume to 1L.

0.01M PBS: Add 8.5g sodium chloride, 1.4g Na_2HPO_4 and 0.2g NaH_2PO_4 to 900ml distilled H_2O and adjust pH to 7.2-7.6. Adjust the total volume to 1L.

Storage

Store at 4°C. Cell Applications, Inc. recommends using the kit within 6 months of order.



X	pg/ml	0.0	156	313	625	1250	2500	5000	10000
Y	O.D.450	0.169	0.216	0.256	0.384	0.487	0.758	1.229	2.162

Figure 1: Fibronectin Standard Curve. Using the rat Fibronectin ELISA Kit, O.D. data was graphed against Fibronectin protein concentration. The TMB reaction was incubated at 37°C for 10 min.

BACKGROUND

Fibronectin (FN) is a high molecular weight glycoprotein present in a soluble dimeric form in plasma, and in a dimeric or multimeric form at the cell surface and in extracellular matrix. FN is widely expressed by multiple cell types and is critically important in vertebrate development, as demonstrated by the early embryonic lethality of mice with targeted inactivation of the FN gene. Fibronectin (FN) mediates a wide variety of cellular interactions with the extracellular matrix (ECM) and plays important roles in cell adhesion, migration, growth and differentiation processes including embryogenesis, wound healing, blood coagulation, host defense, and metastasis.[1]

FN usually exists as a dimer composed of two nearly identical 250 kDa subunits linked covalently near their C-termini by a pair of disulfide bonds (see poster). Each monomer consists of three types of repeating units (termed FN repeats): type I (purple rectangles), type II (green octagons) and type III (red ovals). FN contains 12 type I repeats, two type II repeats and 15-17 type III repeats, which together account for approximately 90% of the FN sequence. Although FN molecules are the product of a single gene, the resulting protein can exist in multiple forms that arise from alternative splicing of a single pre-mRNA that can generate as many as 20 variants in human FN. FN can be a ligand for a dozen members of the integrin receptor family. In addition, FN has a remarkably wide variety of functional activities besides binding to cell surfaces through integrins. It binds to a number of biologically important molecules that include heparin, collagen/gelatin, and fibrin. These interactions are mediated by several distinct structural and functional domains.[2] Anastellin binds fibronectin and induces fibril formation. This fibronectin polymer, named superfibronectin, exhibits enhanced adhesive properties. Both anastellin and superfibronectin inhibit tumor growth, angiogenesis and metastasis. Anastellin activates p38 MAPK and inhibits lysophospholipid signaling.[3]

References

1. Hynes, R.O. & Yamada, K.M.: J Cell Biol. 95:369-77, 1982
2. Pankov, R. & Yamada, K.M.: J. Cell Sci. 115:3861-3, 2002
3. Morla, A. et al: Nature367:193-6, 1997

ELISA OVERVIEW

Cell Applications ELISA Kits are based on standard sandwich enzyme-linked immunosorbent assay technology. Freshly prepared standards, samples, and solutions are recommended for best results.

1. Prepare test samples.
2. Prepare a protein standard of the target protein.
3. Add test samples and standards to the pre-coated 96-well plate. Do not wash.
4. Add biotinylated detection antibodies. Wash.
5. Add Avidin-Biotin-Peroxidase Complex (ABC) Solution. Wash.
6. Add Tetramethyllbenzidine (TMB) Color Developing Agent, containing HRP substrate.
7. Add TMB Stop Solution
8. Subject the plate to analysis.

NOTES:

- Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.
- Duplicate assay wells are recommended for both standard and sample testing.
- Do not let the 96-well plate dry, this will lead to inactivation of plate components.
- When diluting samples and reagents, ensure that they are mixed completely and evenly.
- Pre-warm diluted ABC and TMB solutions at 37°C for 30 min before use to avoid variable temperature effects.
- For washes, use TBS or PBS. Do not touch well walls.
- A protein standard is included in the kit. A protein standard detection curve should be generated with each experiment, no more than 2 hours prior to the experiment.
- The user will determine sample dilution fold by estimation of target protein amount in samples.

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PROTOCOL

I. Plate Washing

Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of three washes.

II. Preparation of Test Samples

Test Sample Processing

- **Cell culture supernate, tissue lysate or body fluids**: Remove particulates by centrifugation.
- **Serum**: Allow the serum to clot in a serum separator tube (about 2 hours) at room temperature. Centrifuge at approximately 1000 X g for 10 min.
- **Plasma**: Collect plasma using EDTA, citrate as an anticoagulant. Centrifuge for 20 min at 1000 x g within 30 min of collection. Analyze immediately or aliquot and store frozen at -20°C. Heparin is not recommended as the anticoagulant.

Sample Dilution Guideline

Estimate the concentration of the target protein in the sample and select a proper dilution factor such that the diluted target protein concentration falls within the standard curve range. Depending on the sample, several trial dilutions may be necessary. Dilute the sample using the provided diluent buffer, mixing well. Suggested working dilutions of samples are as follows:

Target Protein Concentration Range	Sample Working Dilution	Sample Vol.	Diluent Buffer Vol.
100-1000 ng/ml	1:100	1 µl	99 µl
10-100 ng/ml	1:10	10 µl	90 µl
156-10000 pg/ml	1:2	50 µl	50 µl
≤156 pg/ml	n/a	100µl	n/a

If samples will be assayed within 24 hours, store at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

III. Preparation of Reagents

Reconstitution of the Standard

The standard solutions should be prepared no more than 2 hours prior to the experiment. Two tubes of the standard are included in each kit. Use one tube for each experiment.

1. 10,000pg/ml of rat Fibronectin standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly. The 10 ng/ml standard solution may be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.
2. 4000pg/ml of rat Fibronectin standard solution: Add 0.4 ml of the above 10ng/ml Fibronectin standard solution into 0.6 ml sample diluent buffer and mix thoroughly.
3. 2000pg/ml→62.5pg/ml of rat Fibronectin standard solutions: Label 6 Eppendorf tubes with 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 4000pg/ml Fibronectin standard solution into 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3 ml from 2nd tube to 3rd tube and mix, and so on.

Preparation of Biotinylated Antibody Working Solution

The solution should be prepared no more than 2 hours prior to the experiment.

1. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
2. Biotinylated antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly.

Preparation of the Avidin-Biotin-Peroxidase Complex (ABC) Working Solution

The solution should be prepared no more than 1 hour prior to the experiment.

1. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
2. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly.

IV. ELISA

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. A standard detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of target protein amount in samples.

1. Aliquot 0.1ml per well of the 10,000pg/ml, 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 313pg/ml, 156pg/ml rat Fibronectin standard solutions into the precoated 96-well plate. Add 0.1ml of the sample diluent buffer into the control well (Blank well). Add 0.1ml of each properly diluted sample of rat sera, plasma, body fluids, tissue lysates or cell culture supernatants to each empty well. **See "Sample Dilution Guideline"** for details. We recommend that each rat Fibronectin standard solution and each sample is measured in duplicate.
2. Seal the plate with the cover and incubate at 37°C for 90 min.
3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 0.1ml of biotinylated anti-rat Fibronectin antibody working solution into each well and incubate the plate at 37°C for 60 min.
5. Wash the plate three times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
6. Add 0.1ml of prepared ABC working solution into each well and incubate the plate at 37°C for 30 min.
7. Wash plate 5 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
8. Add 90 µl of prepared TMB color developing agent into each well and incubate plate at 37°C for 20-25 min (shades of blue can be seen in the wells with the four most concentrated rat Fibronectin standard solutions; the other wells show no obvious color).
9. Add 0.1ml of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution.

V. Calculating Protein Concentration

- For all wells, determine O.D.450(Relative):
  O.D.450(Relative) = O.D.450(Reading) – O.D.450(Blank)

- Plot the standard curve:
  Plot O.D.450(Relative) of each standard solution (Y) vs. the respective concentration of the standard solution (X). See **Figure 1** for a typical standard curve.

- The target protein concentration in samples can be interpolated from the standard curve. Multiply the interpolated concentration by the dilution factor to obtain the target protein concentration in the sample.