

# **ab119524 – Fibronectin Human ELISA Kit**

## Instructions for Use

For the quantitative measurement of Human Fibronectin concentrations in cell culture supernatant, serum, plasma (EDTA, heparin) and urine

This product is for research use only and is not intended for diagnostic use.

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## 1. BACKGROUND

Abcam's Fibronectin Human *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for accurate quantitative measurement of Human fibronectin concentrations in cell culture supernatant, serum, plasma (EDTA, heparin) and urine.

Fibronectin specific antibodies have been precoated onto 96-well plates. Standards and test samples are added to the wells along with a biotinylated fibronectin detection antibody and the microplate is incubated at room temperature. Following washing with wash buffer a Streptavidin-HRP conjugate is added to each well, incubated at room temperature and then unbound conjugates are washed away using wash buffer. TMB is then added and catalyzed by HRP to produce a blue color product that changes into yellow after addition of an acidic stop solution. The density of yellow coloration is directly proportional to the amount of fibronectin captured on the plate.

Fibronectin is a ubiquitous extracellular glycoprotein that exists in a soluble form in body fluids and in an insoluble form in the extracellular matrix. It plays a major role in many important physiological processes, such as embryogenesis, wound healing, hemostasis and thrombosis. Fibronectin is secreted as a dimer with a monomer molecular weight of 220-250 kDa. Fibronectin is composed of three types of modules organized into functional domains that are resistant to proteolysis and contain binding sites for extracellular matrix proteins, cell surface receptors and circulating blood proteins.

In general, fibronectin is synthesized by and present around fibroblasts, endothelial cells, chondrocytes, glial cells, and myocytes. Fibronectin is abundant in the connective tissue matrix and in basement membranes. Extremely high levels of the glycoprotein are found in plasma.

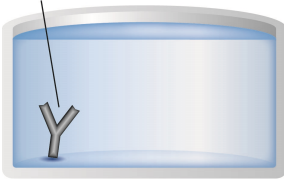
## INTRODUCTION

Fibronectin has been reported to play a major role in the cardiovascular system, being functionally involved in blood clotting, atherosclerosis, hypertension and myocardial infarction.

Fibronectin's importance during cancer progression has further been shown. It represents an active element in the process of T cell activation in the immune cascade triggered by organ transplantation. Fetal fibronectin has been described as a predictor of term labor.

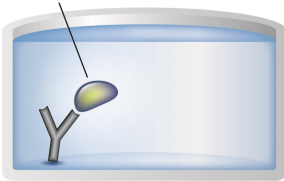
## 2. ASSAY SUMMARY

**Primary Capture Antibody**



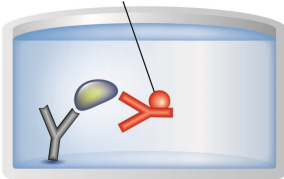
Prepare all reagents, samples and standards as instructed.

**Sample**



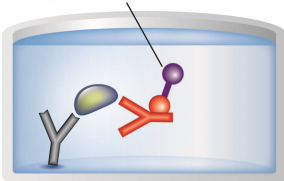
Add standard or sample to each well used.

**Detection Antibody**



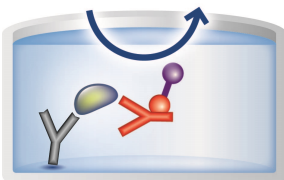
Add prepared detection antibody to each well. Incubate at room temperature.

**Streptavidin-HRP**



Wash and add prepared Streptavidin-HRP conjugate. Incubate at room temperature.

**Substrate**      **Colored Product**



Add TMB Substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

## 3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

## 4. STORAGE AND STABILITY

Upon receipt, store kit immediately at 2-8°C.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9 Reagent Preparation.

## 5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Microplate coated with polyclonal antibody to fibronectin (12 x 8 wells)	96 wells	2-8 °C
Biotin-Conjugate anti-fibronectin polyclonal antibody	70 µL	2-8 °C
Streptavidin-HRP	200 µL	2-8 °C
Fibronectin Standard lyophilized	2 Vials	2-8 °C
20X Assay Buffer Concentrate	3 vials x 5 mL	2-8 °C
20X Wash Buffer Concentrate	50 mL	2-8 °C
TMB Substrate Solution	15 mL	2-8 °C
Stop Solution (1M Phosphoric acid)	15 mL	2-8 °C
Adhesive Films	4 units	2-8 °C

### **6. MATERIALS REQUIRED, NOT SUPPLIED**

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 5 mL and 10 mL graduated pipettes
- 5  $\mu$ L to 1000  $\mu$ L adjustable single channel micropipettes with disposable tips
- 50  $\mu$ L to 300  $\mu$ L adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

### **7. LIMITATIONS**

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

### 8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers
- Avoid foaming or bubbles when mixing or reconstituting components
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps
- Complete removal of all solutions and buffers during wash steps.
- As exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radio immunotherapy has significantly increased the number of patients with Human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine polyclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analyzed in such assays when murine immunoglobulins (serum, ascitic fluid, or polyclonal antibodies of irrelevant specificity) are added to the sample.
- **This kit is sold based on number of tests.** A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions

## 9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18-25°C) prior to use.

### 9.1 **1X Wash Buffer**

Prepare 1X Wash Buffer by diluting the 20X Wash Buffer Concentrate with distilled or deionized water. To make 500 mL 1X Wash Buffer, combine 25 mL 20X Wash Buffer Concentrate with 475 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

*Note: The 1X Wash Buffer should be stored at 2-8 °C and is stable for 30 days.*

### 9.2 **1X Assay Buffer**

Prepare 1X Assay Buffer by diluting the 20X Assay Buffer Concentrate with distilled or deionized water. To make 50 mL 1X Assay Buffer, combine 2.5 mL 20X Assay Buffer Concentrate with 47.5 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

*Note: The 1X Assay Buffer should be stored at 2-8 °C and is stable for 30 days.*

## 9.3 1X Biotin Conjugated Antibody

To prepare the Biotin Conjugated Antibody, dilute the anti-Human Fibronectin polyclonal antibody 100-fold with 1X Assay Buffer. Use the following table as a guide to prepare as much 1X Biotin Conjugated Antibody as needed by adding the required volume ( $\mu\text{L}$ ) of the Biotin Conjugated Antibody to the required volume (mL) of 1X Assay Buffer. Mix gently and thoroughly.

Number of strips	Volume of Biotin-Conjugated Fibronectin antibody ( $\mu\text{L}$ )	1X Assay Buffer (mL)
1 - 6	30	2.97
7 - 12	60	5.94

*Note: The 1X Biotin-Conjugated Antibody should be used within 30 minutes after dilution.*

## 9.4 1X Streptavidin-HRP Conjugate

To prepare the Streptavidin-HRP Conjugate, dilute the anti-Streptavidin-HRP Conjugate 200-fold with 1X Assay Buffer. Use the following table as a guide to prepare as much 1X Streptavidin-HRP Conjugate as needed by adding the required volume ( $\mu\text{L}$ ) of the Streptavidin-HRP Conjugate to the required volume (mL) of 1X Assay Buffer. Mix gently and thoroughly.

Number of strips	Volume of Streptavidin-HRP ( $\mu\text{L}$ )	1X Assay Buffer (mL)
1 - 6	30	5.97
7 - 12	60	11.94

*Note: The 1X Streptavidin-HRP should be used within 30 minutes after dilution.*

## 10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use.

- 10.1 Prepare a 40 ng/mL **Stock Standard** by reconstituting one vial of the fibronectin standard with the volume of distilled water stated on the label. Hold at room temperature for 10-30 minutes. The 40 ng/mL **Stock Standard** cannot be stored for later use.
- 10.2 Label eight tubes with numbers 1 - 8.
- 10.3 Add 225  $\mu$ L 1X Assay Buffer into all tubes.
- 10.4 Prepare a 20 ng/mL **Standard 1** by transferring 225  $\mu$ L of the 40 ng/mL Stock Standard to tube 1. Mix thoroughly and gently.
- 10.5 Prepare **Standard 2** by transferring 225  $\mu$ L from Standard 1 to tube 2. Mix thoroughly and gently.
- 10.6 Prepare **Standard 3** by transferring 225  $\mu$ L from Standard 2 to tube 3. Mix thoroughly and gently.
- 10.7 Using the table below as a guide, repeat for tubes number 4 through to 7.
- 10.8 **Standard 8** contains no protein and is the Blank control

## ASSAY PREPARATION

Standard	Sample to Dilute	Volume to Dilute ( $\mu\text{L}$ )	Volume of Diluent ( $\mu\text{L}$ )	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Stock	225	225	40	20
2	Standard 1	225	225	20	10
3	Standard 2	225	225	10	5
4	Standard 3	225	225	5	2.5
5	Standard 4	225	225	2.5	1.25
6	Standard 5	225	225	1.25	0.63
7	Standard 6	225	225	0.63	0.31
8	None	-	225	-	0



## 11. SAMPLE COLLECTION AND STORAGE

- Cell culture supernatant, serum, plasma (EDTA, heparin) and urine were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.
- Possible “Hook Effects” may be observed due to high sample concentrations. It is recommended to run several dilutions of your sample to ensure an accurate reading.
- Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.
- Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive Human Fibronectin. If samples are to be run within 24 hours, they may be stored at 2° to 8°C.
- Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.
- Aliquots of serum samples (undiluted and 1:10.000 prediluted) were stored at -20°C and thawed 5 times, and the Human Fibronectin levels determined. There was no significant loss of Human Fibronectin immunoreactivity detected by freezing and thawing in undiluted samples. A decrease of ~30% of Human Fibronectin immunoreactivity was detected in prediluted serum samples after 1 freeze/thaw cycle. Therefore samples should be stored in aliquots at -20°C and thawed only once.

- Aliquots of serum (undiluted and 1:10.000 prediluted) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the Human Fibronectin level determined after 72 h. There was no significant loss of Human Fibronectin immunoreactivity detected during storage under above conditions.

### **12. PLATE PREPARATION**

- The 96 well plate strips included with this kit are supplied ready to use.
- Unused well strips should be returned to the plate packet and stored at 2-8°C
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay.

## **13. ASSAY PROCEDURE**

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
  - **It is recommended to assay all standards, controls and samples in duplicate.**
- 13.1. Prepare all reagents, working standards, and samples as directed in the previous sections. Determine the number of microplate strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards.
  - 13.2. Wash the microplate twice with approximately 400  $\mu$ L 1X Wash Buffer per well with thorough aspiration of microplate contents between washes. Allow the 1X Wash Buffer to remain in the wells for about 10 - 15 seconds before aspiration. Take care not to scratch the surface of the microplate.
  - 13.3. After the last wash step, empty wells and tap microplate on absorbent pad or paper towel to remove excess 1X Wash Buffer. Use the microplate strips immediately after washing. Alternatively the microplate strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.
  - 13.4. Add 100  $\mu$ L of prepared standards (including the standard blank control) to the appropriate wells.
  - 13.5. Add 50  $\mu$ L of 1X Assay Buffer to the sample wells.
  - 13.6. Add 50  $\mu$ L of each sample (prediluted serum or plasma samples, undiluted serum-free cell culture supernatants or urine) to the sample wells.
  - 13.7. Add 50  $\mu$ L of 1X Biotin Conjugated Antibody to all wells.
  - 13.8. Cover with adhesive film and incubate at room temperature (18° to 25°C) for 2 hours (microplate can be incubated on a shaker set at 400 rpm).

- 13.9. Remove adhesive film and empty wells. Wash microplate strips 6 times according to step 13.2. Proceed immediately to step 13.10.
- 13.10. Add 100  $\mu$ L of 1X Streptavidin-HRP to all wells, including the blank wells.
- 13.11. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour (microplate can be incubated on a shaker set at 400 rpm).
- 13.12. Remove adhesive film and empty wells. Wash microplate strips 6 times according to step 13.2. Proceed immediately to the next step.
- 13.13. Pipette 100  $\mu$ L of TMB Substrate Solution to all wells.
- 13.14. Incubate the microplate strips at room temperature (18 to 25°C) for 10 minutes. Avoid direct exposure to intense light.

*Note:* The color development on the plate should be monitored and the substrate reaction stopped (see step 13.15) before the signal in the positive wells becomes saturated. Determination of the ideal time period for color development should be done individually for each assay. It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 - 0.95.

- 13.15. Stop the enzyme reaction by adding 100  $\mu$ L of Stop Solution into each well.

*Note:* It is important that the Stop Solution is mixed quickly and uniformly throughout the microplate to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microplate strips are stored at 2 - 8°C in the dark.

- 13.16. Read absorbance of each microplate on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

*Note:* In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

## 14. CALCULATIONS

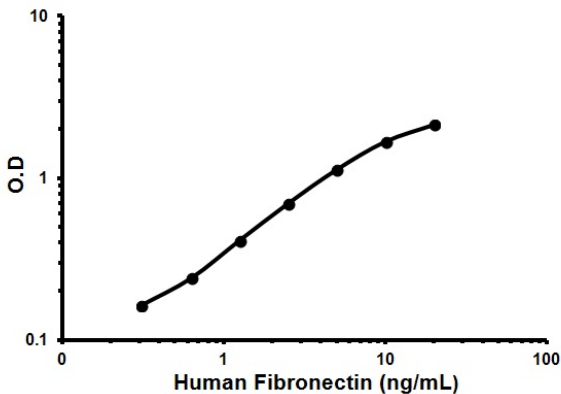
Average the duplicate readings for each standard, sample and control blank. Subtract the no protein control blank from all mean readings. Plot the mean standard readings against their concentrations and draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4-parameter logistic). Extrapolate protein concentrations for unknown and control samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

If serum or plasma samples have been diluted 1:20,000, and serum-free cell culture supernatants or urine samples 1:2 as stated, the concentration obtained from the standard curve must be multiplied by the dilution factor ( $\times 20,000$  or  $\times 2$ , respectively) to obtain an accurate value, in addition to any initial sample dilution factor.

Calculation of samples with a concentration exceeding standard 1 will result in incorrect, low human Fibronectin levels (Hook Effect). Such samples require further external predilution according to expected human Fibronectin values with 1X Assay Buffer in order to precisely quantitate the actual human Fibronectin level.

## 15. TYPICAL DATA

**TYPICAL STANDARD CURVE** – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Conc. (ng/mL)	O.D. 450 nm		Mean
	1	2	O.D.
0.00	0.035	0.031	0.033
0.31	0.157	0.169	0.163
0.63	0.235	0.245	0.240
1.25	0.395	0.422	0.409
2.50	0.675	0.710	0.693
5.00	1.125	1.118	1.122
10.00	1.661	1.680	1.671
20.00	2.103	2.149	2.126

**Figure 1.** Example of Human fibronectin protein standard curve.

## 16. TYPICAL SAMPLE VALUES

### **SERUM/PLASMA –**

A panel of 40 sera and 8 plasma and urine samples from randomly selected apparently healthy donors (males and females) was tested for Human Fibronectin. The detected Human Fibronectin level in urine is 4-11 ng/mL.

<b>Sample Type</b>	<b>n=</b>	<b>Range (µg/mL)</b>
Serum	40	12-124
Plasma (EDTA)	8	117-338
Plasma (Heparin)	8	19-106

### **SENSITIVITY -**

The limit of detection of Human Fibronectin defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.1 ng/mL (mean of 6 independent assays).

### **RECOVERY –**

The spike recovery was evaluated by spiking 4 levels of Human Fibronectin into serum. Recoveries were determined in 4 independent experiments with 4 replicates each. The amount of endogenous Human Fibronectin in unspiked serum was subtracted from the spike values. The overall mean recovery was 110.6%.

### **LINEARITY OF DILUTION –**

Serum samples with different levels of Human Fibronectin were analyzed at serial 2 fold dilutions with 4 replicates each. The recovery ranged from 93.2% to 122.6% with an overall recovery of 109.4%.

## PRECISION –

Intra- and Inter-assay reproducibility was determined by measuring samples containing different concentrations of Human fibronectin.

	<b>Intra-Assay</b>	<b>Inter-Assay</b>
n=	8	8
%CV	5.3	6.7%

## 17. ASSAY SPECIFICITY

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into positive serum. No cross reactivity was detected.

## 18. TROUBLESHOOTING

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed.

19. NOTES

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