

ab325445 –Human Fibronectin SimpleStep ELISA® Kit, Chemiluminescent

For the quantitative measurement of Fibronectin in human serum, plasma (EDTA), plasma (citrate), cell culture supernatant, milk, cell extract, and tissue extract.

For research use only - not intended for diagnostic use.
Patent pending.

For overview, typical data and additional information please visit: www.abcam.com/ab325445

Storage and Stability: Store kit at 2-8°C immediately upon receipt. Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Standard Preparation and Reagent preparation sections.

Limitations: All data, except Typical Standard Curve, sensitivity, individual healthy human donors, and individual human donors with rheumatoid arthritis were collected using the colorimetric version of this kit (ab219046).

Materials Supplied

Item	Quantity 1 x 96 tests	Storage Condition
Human Fibronectin Capture Antibody 10X	600 µL	+4°C
Human Fibronectin Detector Antibody 10X	600 µL	+4°C
Human Fibronectin Lyophilized Recombinant Protein	2 Vials	+4°C
Antibody Diluent 4BR	6 mL	+4°C
Cell Extraction Buffer PTR 5X	10 mL	+4°C
Cell Extraction Enhancer Solution 50X	1 mL	+4°C
Sample Diluent NS	50 mL	+4°C
Wash Buffer PT 10X	20 mL	+4°C
ChemiHRP Reagent A	3 mL	+4°C
ChemiHRP Reagent B	3 mL	+4°C
SimpleStep Pre-Coated Black 96-Well Microplate	96 Wells	+4°C
Plate Seal	1	+4°C

Materials Required, Not Supplied

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

- Luminometer with the following settings: 0.5-1 second/well read time; summation mode (all wavelengths)
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.

Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

Reagent Preparation

Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.

Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

The provided Cell Extraction Enhancer Solution 50X may precipitate when stored at + 4°C. To dissolve, warm briefly at + 37°C and mix gently. The Cell Extraction Enhancer Solution 50X can be stored at room temperature to avoid precipitation.

1X Cell Extraction Buffer PTR (For cell and tissue extracts only): Prepare 1X Cell Extraction Buffer PTR by diluting Cell Extraction Buffer PTR 5X and 50X Cell Extraction Enhancer Solution to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 7.8 mL deionized water, 2 mL Cell Extraction Buffer PTR 5X and 200 µL Cell Extraction Enhancer Solution 50X. Mix thoroughly and gently. If required protease inhibitors can be added.

1X Wash Buffer PT: Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

Antibody Cocktail: Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BR. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent 4BR. Mix thoroughly and gently.

Lumi HRP Development Solution: Just prior to use, prepare Lumi HRP Development Solution by mixing equal volume of the ChemiHRP Reagent A and the ChemiHRP Reagent B. To make 3 mL of the Lumi HRP Development Solution combine 1.5 mL of ChemiHRP Reagent A and 1.5 mL of ChemiHRP Reagent B. Mix thoroughly and gently by inversion or slow pipetting (Avoid shaking or vortexing). Protect the prepared solution from light until use.

Standard Preparation

Always prepare a fresh set of standards for every use. Discard working standard dilutions after use as they do not store well. The following section describes the preparation of a standard curve for duplicate measurements (recommended).

1. Reconstitute the **Fibronectin** protein standard by adding the volume indicated on the protein vial label. For **serum, plasma, milk, and cell culture supernatant sample measurements**, use Sample Diluent NS. For **cell and tissue extract sample measurements**, use 1X Cell Extraction Buffer PTR. Hold at room temperature for 10 minutes and mix thoroughly and gently. This is the 32,000 pg/mL **Stock Standard** Solution.
2. Label eight tubes, Standards 1– 8.
3. Use the same Sample Diluent as used to resuspend the Stock Standard to prepare the standard curve. Add 57 µL of Sample Diluent into tube number 1 and 150 µL of Sample Diluent into numbers 2-8.
4. Use the **Stock Standard** to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:

Standard #	Dilution Sample	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	Stock Standard	171	57	32,000	24,000
2	Standard#1	75	150	24,000	8,000
3	Standard#2	75	150	8,000	2,667.7
4	Standard#3	75	150	2,667.7	888.9
5	Standard#4	75	150	888.9	296.3
6	Standard#5	75	150	296.3	98.8
7	Standard#6	75	150	98.8	32.9
8	Blank Control	0	150	0	0

Sample Preparation

Typical Sample Dynamic Range	
Sample Type	Range
Serum	1:320,000 - 1:20,000
Plasma – Citrate	1:640,000 - 1:20,000
Plasma – EDTA	1:640,000 - 1:40,000
HepG2 Cell Culture Supernatant	1:32,000 - 1:2,000
Milk	1:800 - 1:100
HepG2 Cell Extract	1.56 - 25 µg/mL
SW 480 Cell Extract	3.125 - 100 µg/mL
U87-MG Cell Extract Other	0.3125 - 5 µg/mL
Liver Tissue Extract	6.25 - 100 µg/mL
Heart or Skeletal Muscle Tissue Extract	3.125 - 100 µg/mL
Colon Tissue Extract	0.63 - 5 µg/mL
Placenta Tissue Extract	0.16 - 1.25 µg/mL

Serum Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples at least 1:20,000 into 1X Wash Buffer PT and Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

Plasma Collect plasma using citrate or EDTA. Centrifuge samples at 2,000 x g for 10 minutes. Dilute plasma (citrate) samples at least 1:20,000 and dilute plasma (EDTA) samples at least 1:40,000 into 1x Wash Buffer PT and Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles. Note: This kit is incompatible with plasma (heparin) samples.

Δ **Note:** Due to the high dilutions required for serum and plasma samples, we recommend to pre-dilute the samples sequentially. As an example, the table below demonstrates the steps suggested to generate a final sample dilution of 1:40,000.

Tube #	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Diluent	Starting Dilution	Final Dilution
1	Neat serum/ plasma	5	245	1X Wash Buffer PT	Neat	1:50
2	Tube # 1	5	195	1X Wash Buffer PT	1:50	1:2,000
3	Tube # 2	7.5	142.5	Sample Diluent NS	1:2,000	1:40,000

Cell Culture Supernatants Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants and assay. Or dilute samples at least 1:2,000 into Sample Diluent NS and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Milk De-fat milk samples as follows. Centrifuge milk samples at 500 x g for 15 minutes at 4°C and collect the aqueous fraction using syringe attached to needle. Centrifuge the aqueous fraction at 3,000 x g for 15 minutes at 4°C and collect the final aqueous fraction (de-fatted milk) using syringe attached to needle. Dilute the de-fatted milk samples at least 1:100 into Sample Diluent NS and assay. Store un-diluted de-fatted milk at -20°C or below. Avoid repeated freeze-thaw cycles.

Preparation of extracts from cell pellets Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4°C. Rinse cells twice with PBS. Solubilize pellet at 2×10^7 cell/mL in chilled 1X Cell Extraction Buffer PTR. Incubate on ice for 20 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay. Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

Preparation of extracts from adherent cells by direct lysis (alternative protocol) Remove growth media and rinse adherent cells 2 times in PBS. Solubilize the cells by addition of chilled 1X Cell Extraction Buffer PTR directly to the plate (use 750 µL - 1.5 mL 1X Cell Extraction Buffer PTR per confluent 15 cm diameter plate). Scrape the cells into a microfuge tube and incubate the lysate on ice for 15 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay. Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

Preparation of extracts from tissue homogenates Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (Dounce homogenizer recommended). Homogenize 100 to 200 mg of wet tissue in 500 µL - 1 mL of chilled 1X Cell Extraction Buffer PTR. For lower amounts of tissue adjust volumes accordingly. Incubate on ice for 20 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay. Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or "edge effects" have not been observed with this assay.

Assay Procedure

Equilibrate all materials and prepared reagents to room temperature prior to use. We recommend that you assay all standards, controls and samples in duplicate.

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, resealed and return to 4°C storage.
3. Add 50 µL of all sample or standard to appropriate wells.
4. Add 50 µL of the Antibody Cocktail to each well.
5. Seal the plate and incubate for 30 minutes at room temperature on a plate shaker set to 750 rpm.
6. Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Wash Buffer PT should remain in wells for at least 30 seconds. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and tap gently against clean paper towels to remove excess liquid.
7. Add 50 µL of prepared Lumi HRP Development Solution to each well and incubate for 1 minute in the dark on a plate shaker set to 750 rpm. Further optimization of incubation time vs signal strength can be performed if needed. Avoid introducing bubbles into the wells.
8. Measure the produced light of each well using a microplate luminometer with the following settings: 0.5-1 second/well read time in summation mode (all wavelengths). Relative light unit (RLU) readings may vary between luminometer models. It is recommended to configure instrument settings according to the manufacturer's specifications. Note: Relative light unit (RLU) values may change over the course of the 15-minute reading window.
9. Analyze the data as described below.

Mode:	Luminescence
Instrument settings:	Endpoint
Detection Mode:	All wavelengths
Read Time:	0.5-1 sec
Read:	Top

Note For microplate readers with Pre-Read Optimization option, the Read Height as well as Microplate Optimization is recommended before the first read.

Download our ELISA guide for technical hints, results, calculation, and troubleshooting tips:
www.abcam.com/protocols/the-complete-elisa-guide

For technical support contact information, visit: www.abcam.com/contactus

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Additional information

ASSAY SPECIFICITY

This kit is designed for the quantification of human Fibronectin.

The standard protein in this kit is a fragment representing approximately 7% of the full-length mass of human Fibronectin calibrated to natively purified protein.

Native signal was detected in serum, plasma, cell culture supernatant, milk, cell extract, and tissue extract sample types.

CSF samples have not been tested with this kit.

This kit is incompatible with plasma (heparin), saliva, and urine samples.

CROSS REACTIVITY

Recombinant human Anastrozole was prepared within the working range of the assay and assayed for cross reactivity. No cross-reactivity was observed.

SPECIES REACTIVITY

Other species reactivity was determined by measuring 1:100,000 serum (mouse, rat, cow) or plasma (Rhesus macaque) samples, interpolating the protein concentrations from the human standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in human serum or plasma assayed at the same dilution.

Reactivity was determined for the following species: Mouse serum (98%), Rhesus macaque plasma (111%)

Reactivity < 3% was determined for the following species: Rat, Cow

Other species reactivity not determined.

CALCULATION

- Preconfigured protocols are available when using SoftMax Pro software from Molecular Devices.
- Calculate the average chemiluminescence value for the blank control (zero) standards. Subtract the average blank control standard chemiluminescence value from all other chemiluminescence values.
- Create a standard curve by plotting the average blank control subtracted chemiluminescence value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.
- Note: Most chemiluminescence reader software or graphing software will plot these values and fit a curve to the data. A four-parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4-parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.
- Determine the concentration of the target protein in the sample by interpolating the blank control subtracted chemiluminescence values against the standard curve. Multiply the

resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

- Samples generating chemiluminescence values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at chemiluminescence values less than that of the lowest standard should be retested in a less dilute form.

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			
Concentration (pg/mL)	RLU		Mean RLU
	1	2	
0	5,571	5,783	5,677
32.9	11,080	10,646	10,863
98.8	19,901	20,317	20,109
296.3	54,125	50,371	52,248
888.9	148,810	147,270	148,040
2,666.7	440,130	438,300	439,215
8,000	1,348,000	1,303,100	1,325,550
24,000	3,750,900	3,762,000	3,756,450

Table 1. Example of human Fibronectin standard curve in Sample Diluent NS. The Fibronectin standard curve was prepared as described in the Standard Preparation section. The table shows raw data values.

Standard Curve Measurements			
Concentration (pg/mL)	RLU		Mean RLU
	1	2	
0	5,880	5,863	5,872
32.9	9,744	10,651	10,198
98.8	21,800	23,001	22,401
296.3	53,253	54,164	53,709
888.9	158,930	163,920	161,425
2,666.7	472,500	488,420	480,460
8,000	1,454,000	1,432,800	1,443,400
24,000	3,587,200	3,757,800	3,672,500

Table 2. Example of human Fibronectin standard curve in 1X Cell Extraction Buffer PTR. The Fibronectin standard curve was prepared as described in the Standard Preparation section. The table shows raw data values.

TYPICAL SAMPLE VALUES

Sensitivity:

The minimal detectable dose (MDD) was determined by calculating the mean of zero standard replicates and adding 2 standard deviations then extrapolating the corresponding concentration.

Sample Diluent Buffer	N=	Minimal Detectable Dose
Sample Diluent NS	8	18.5 pg/mL
1X Cell Extraction Buffer PTR	8	15.3 pg/mL

Recovery

Three concentrations of Fibronectin were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
1:166,700 Serum	93	87 - 102
1:200,000 Plasma – Citrate	99	87 - 120
1:200,000 Plasma – EDTA	86	81 - 94
1:10,000 HepG2 Cell Culture Supernatant (4 Days)	102	90 - 117
1:500 Defatted Milk	102	91 - 111
3 µg/mL HepG2 Cell Extract	89	83 - 100
10 µg/mL Liver Tissue Extract	117	99 - 131
20 µg/mL SW 480 Cell Extract	90	83 - 100
2.5 µg/mL U87-MG Cell Extract	92	80 - 105
30 µg/mL Heart Muscle Tissue Extract	97	81 - 118
30 µg/mL Skeletal Muscle Tissue Extract	106	91 - 122
2 µg/mL Colon Tissue Extract	99	89 - 112
1 µg/mL Placenta Tissue Extract	90	80 - 104

Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native Fibronectin was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	1:20,000 Serum	1:20,000 Plasma (Citrate)	1:40,000 Plasma (EDTA)	1:100 De-fatted Milk	1:2,000 HepG2 SN (4 days)
Undiluted	pg/mL	6,832	6,984	5,890	5,398	5,408
	% Expected value	100	100	100	100	100
2	pg/mL	3,349	3,836	2,901	2,520	2,289
	% Expected value	98	110	99	93	85
4	pg/mL	1,459	1,700	1,335	1,144	1,174
	% Expected value	85	97	91	85	87
8	pg/mL	765.2	838.8	733.9	638.1	616.5
	% Expected value	90	96	100	95	91
16	pg/mL	383.3	450.1	381.8	NL	287.1
	% Expected value	90	103	104	NL	85

NL – Non-Linear

Native Fibronectin was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in 1X Cell Extraction Buffer PTR.

Dilution Factor	Interpolated value	25 µg/mL HepG2 Extract	100 µg/mL SW 480 Extract	5 µg/mL U87-MG Extract
Undiluted	pg/mL	6,602	6,291	1,860
	% Expected value	100	100	100
2	pg/mL	3,423	3,508	909.3
	% Expected value	104	112	98
4	pg/mL	1,735	1,587	428.9
	% Expected value	105	101	92
8	pg/mL	913.2	767.8	213.6
	% Expected value	111	98	92
16	pg/mL	483.2	360.7	102.7
	% Expected value	117	92	88

Native Fibronectin was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in 1X Cell Extraction Buffer PTR.

Dilution Factor	Interpolated value	100 µg/mL Liver Tissue Extract	100 µg/mL Heart Tissue Extract	100 µg/mL Skeletal Muscle Tissue Extract	5 µg/mL Colon Tissue Extract	1.25 µg/mL Placenta Tissue Extract
Undiluted	pg/mL	7,282	4,817	4,758	2,973	1,405
	% Expected value	100	100	100	100	100
2	pg/mL	4,040	2,273	2,109	1,481	607.0
	% Expected value	111	94	89	100	86
4	pg/mL	1,801	1,199	1,020	608.6	281.5
	% Expected value	99	100	86	82	80
8	pg/mL	929.9	662.7	499.6	295.8	143.8
	% Expected value	102	110	84	80	82
16	pg/mL	510.3	318.3	270.9	NL	NL
	% Expected value	112	106	91	NL	NL

NL – Non-Linear

Precision

Mean coefficient of variations of interpolated values of Fibronectin from three concentrations of serum within the working range of the assay.

	Intra-assay	Inter-assay
N=	5	3
CV (%)	5.0	8.3

Download our ELISA guide for technical hints, results, calculation, and troubleshooting tips:

www.abcam.com/protocols/the-complete-elisa-guide

For technical support contact information, visit: www.abcam.com/contactus

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