ab219046 – Human Fibronectin SimpleStep ELISA® Kit

For the quantitative measurement of Fibronectin in human serum, plasma, milk, cell culture supernatant, cell extract, and tissue extract.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab219046

This kit is available in a 384-well plate format. This plate utilises smaller volumes of standards and samples per well. Directions for using this format can be found on pages 6-7.

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.

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
TMB Development Solution	12 mL	+4°C
Stop Solution	12 mL	+4°C
SimpleStep Pre-Coated 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:

Microplate reader capable of measuring absorbance at 450 or 600 nm.
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.

Alternative – Enhancer may be added to 1X Cell Extraction Buffer PTR after extraction of cells or tissue.

Sample Diluent NS + 1X Enhancer: Prepare Sample Diluent NS + 1X Enhancer by combining Sample Diluent NS and 50X Cell Extraction Enhancer Solution. To make 5 mL Sample Diluent NS + 1X Enhancer, combine 4.9 mL Sample Diluent NS and 100 µL Cell Extraction Enhancer Solution 50X. Mix thoroughly and gently.

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.

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. **IMPORTANT**: If the protein standard vial has a volume identified on the label, reconstitute the Fibronectin standard by adding that volume of Diluent indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the Fibronectin standard by adding 500 µL Diluent. Hold at room temperature for 10 minutes and mix thoroughly and gently. This is the 32,000 pg/mL **Stock Standard** Solution.

   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.

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 225 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**	75	225	32,000	8,000
2	Standard#1	150	150	8,000	4,000
3	Standard#2	150	150	4,000	2,000
4	Standard#3	150	150	2,000	1,000
5	Standard#4	150	150	1,000	500
6	Standard#5	150	150	500	250
7	Standard#6	150	150	250	125
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
De-fatted Human Milk	1:800 – 1:100
HepG2 Cell Culture Supernatant	1:32,000 – 1:2,000
HepG2 Cell Extract	1.56 – 25 µg/mL
SW 480 Cell Extract	3.125 – 100 µg/mL
U87-MG Cell Extract	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 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 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 $2x10^7$ 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, reseal 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 1 hour at room temperature on a plate shaker set to 400 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 10 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 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.
   Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.
   Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.
8. Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.
9. Alternative to 7 – 8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode	Kinetic
Wavelength:	600 nm
Time:	up to 20 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

Note that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450 nm.

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, milk, cell culture supernatant, 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 Anastelin 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
- Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.
- Create a standard curve by plotting the average blank control subtracted absorbance 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.

  Δ Note: Most microplate 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 absorbance 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 absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at absorbance 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)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.148	0.161	0.154
125	0.234	0.264	0.249
250	0.298	0.340	0.319
500	0.468	0.555	0.511
1,000	0.816	0.874	0.845
2,000	1.453	1.443	1.448
4,000	2.622	2.704	2.663
8,000	3.687	3.760	3.724

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)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.157	0.153	0.155
125	0.275	0.260	0.267
250	0.369	0.342	0.356
500	0.563	0.510	0.537
1,000	0.926	0.833	0.880
2,000	1.668	1.537	1.603
4,000	2.996	2.686	2.841
8,000	3.821	3.733	3.777

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	16	20.6 pg/mL
1X Cell Extraction Buffer PTR	32	22.3 pg/mL

Recovery

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

Sample Type	Average % Recovery	Range (%)
1:166,700 Human Serum	93	87 - 102
1:200,000 Human Plasma – Citrate	99	87 - 120
1:200,000 Human Plasma – EDTA	86	81 - 94
1:500 Human De-fatted Milk	102	91 - 111
1:10,000 HepG2 Cell Culture Supernatant (4 days)	102	90 - 117
3 µg/mL HepG2 Cell Extract	89	83 - 100
20 µg/mL SW 480 Cell Extract	90	83 - 100
2.5 µg/mL U87-MG Cell Extract	92	80 - 105
10 µg/mL Human Liver Tissue Extract	117	99 - 131
30 µg/mL Human Heart Tissue Extract	97	81 - 118
30 µg/mL Human Skeletal Muscle Tissue Extract	106	91 - 122
2 µg/mL Human 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 Human Serum	1:20,000 Human Plasma (Citrate)	1:40,000 Human Plasma (EDTA)	1:100 Human 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.

Dilution Factor	Interpolated value	25 µg/mL HepG2 Cell Extract	100 µg/mL SW 480 Cell Extract	5 µg/mL U87-MG Cell 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.

Dilution Factor	Interpolated value	100 µg/mL Liver Extract	100 µg/mL Heart Extract	100 µg/mL Skeletal Muscle Extract	5 µg/mL Colon Extract	1.25 µg/mL Placenta Extract
Undiluted	pg/mL	7282	4817	4758	2973	1405
	% Expected value	100	100	100	100	100
2	pg/mL	4040	2273	2109	1481	607.0
	% Expected value	111	94	89	100	86
4	pg/mL	1801	1199	1020	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=	3	5
CV (%)	5.0	8.3

DIRECTIONS FOR 384-WELL PLATE FORMAT:

Materials Supplied for 384-well Format

Item	Quantity	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	50 mL	+4°C
Cell Extraction Enhancer Solution 50X	6 x 1 mL	+4°C
Sample Diluent NS	2 x 50 mL	+4°C
Wash Buffer PT 10X	2 x 20 mL	+4°C
TMB Development Solution	2 x 12 mL	+4°C
Stop Solution	2 x 12 mL	+4°C
SimpleStep Pre-Coated 384-Well Microplate	384 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:

Microplate reader capable of measuring absorbance at 450 or 600 nm in a 384-well plate.
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).
Optional: Automated liquid handler.

Reagent Preparation

Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for one full plate. The sample volumes below are sufficient for running all 384 wells; adjust volumes as needed for the number of samples and dilution scheme for 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 250 mL 1X Cell Extraction Buffer PTR combine 195 mL deionized water, 50 mL Cell Extraction Buffer PTR 5X and 5 mL 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 6 mL of the Antibody Cocktail combine 600 µL 10X Capture Antibody and 600 µL 10X Detector Antibody with 4.8 mL Antibody Diluent 4BR. Mix thoroughly and gently.

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. **IMPORTANT**: If the protein standard vial has a volume identified on the label, reconstitute the Fibronectin standard by adding that volume of Diluent indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the Fibronectin standard by adding 500 µL Diluent. Hold at room temperature for 10 minutes and mix thoroughly and gently. This is the 32,000 pg/mL **Stock Standard** Solution.

   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.

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 150 µL of Sample Diluent into tube number 1 and 75 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**	50	150	32,000	8,000
2	Standard#1	75	75	8,000	4,000
3	Standard#2	75	75	4,000	2,000
4	Standard#3	75	75	2,000	1,000
5	Standard#4	75	75	1,000	500
6	Standard#5	75	75	500	250
7	Standard#6	75	75	250	125
8	Blank Control	0	75	0	0

Plate Preparation

The 384-well plate included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
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 for 384-well Plate Format

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. Add 12.5 µL of all sample or standard to appropriate wells.
3. Add 12.5 µL of the Antibody Cocktail to each well.
4. Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 700 rpm.
5. Wash each well with 3 x 100 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 100 µL 1X Wash Buffer PT into each well. Wash Buffer PT should remain in wells for at least 10 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.
6. Add 25 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 700 rpm.
   Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.
   Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.
7. Add 25 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading. Proper mixing of the Stop Solution is required for proper measurement.
8. Alternative to 6 – 7: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode	Kinetic
Wavelength:	600 nm
Time:	up to 20 min
Interval:	20 sec – 1 min
Shaking:	Shake between readings

Note that an endpoint reading can also be recorded at the completion of the kinetic read by adding 25 µL Stop Solution to each well and recording the OD at 450 nm.

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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