ab222510 – Human VEGF SimpleStep ELISA® Kit

For the quantitative measurement of VEGF in human serum, plasma (citrate), plasma (EDTA), plasma (heparin), cell culture supernatant, urine, saliva, milk, CSF, and cell extract.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab222510

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 7-8.

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 VEGF Capture Antibody 10X	600 µL	+4°C
Human VEGF Detector Antibody 10X	600 µL	+4°C
Human VEGF Lyophilized Recombinant Protein	2 Vials	+4°C
Antibody Diluent 4BI	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.

Sample Diluent NS + 1X Enhancer (see sample preparation instructions before preparing): 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.

Sample Diluent NS + 2X Enhancer (see sample preparation instructions before preparing): Prepare Sample Diluent NS + 2X Enhancer by diluting 50X Cell Extraction Enhancer Solution to 2X with Sample Diluent NS. To make 5 mL Sample Diluent NS + 2X Enhancer, combine 4.8 mL Sample Diluent NS and 200 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 4BI. 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 4BI. 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. Reconstitute the VEGF standard sample by adding the volume indicated on the protein vial label. For **serum, plasma, supernatant, saliva, urine, milk, and CSF samples measurements**, use Sample Diluent NS + 1X Enhancer. For **cell extract samples measurements**, use 1X Cell Extraction Buffer PTR. Hold at room temperature for 10 minutes. Mix thoroughly and gently. This is the 4,000 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 240 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**	60	240	4,000	800
2	Standard#1	150	150	800	400
3	Standard#2	150	150	400	200
4	Standard#3	150	150	200	100
5	Standard#4	150	150	100	50
6	Standard#5	150	150	50	25
7	Standard#6	150	150	25	12.5
8	Blank Control	0	150	N/A	0

Sample Preparation

Typical Sample Dynamic Range	
Sample Type	**Range**
Serum, Plasma	1:16 – 1:2
Milk	1:400 – 1: 50
Saliva	1:256 – 1: 8
Urine	1:8 – 1: 2
PC-3 Cell Culture Supernatant (1 Day)	1:16 – 1:2
PC-3 Cell Culture Supernatant (2 Day)	1:32 – 1:2
HepG2 Cell Culture Supernatant	1:320 – 1:20
A549 Cell Culture Supernatant	1:32 – 1:2
MDA-MB-435S Cell Culture Supernatant	1:128 – 1:8
A431 Cell Culture Supernatant (4 Day)	1:320 – 1:20
PHA-M Stimulated PBMC Cell Culture Supernatant (5 Day)	1:32 – 1:2
Cerebrospinal Fluid	≤ 25%
PC-3 Cell Extract (1 Day or 2 Day)	37.5 – 300 µg/mL
HepG2 Cell Extract	37.5 – 300 µg/mL
A549 Cell Extract	18.75 – 300 µg/mL
MDA-MB-435S Cell Extract	18.75 – 300 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 1:2 into Sample Diluent NS + 2X Enhancer and assay. Further dilutions can be made in Sample Diluent NS + 1X Enhancer. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

Plasma Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples 1:2 into Sample Diluent NS + 2X Enhancer and assay. Further dilutions can be made in Sample Diluent NS + 1X Enhancer. Store un-diluted plasma samples at -20°C or below for up to 3 months. 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 1:50 in Sample Diluent NS + 1X Enhancer. Store un-diluted de-fatted milk at -20°C or below. Avoid repeated freeze-thaw cycles.

Saliva Centrifuge saliva at 800 x g for 10 minutes to remove debris. Dilute samples 1:8 in Sample Diluent NS + 1X Enhancer. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Urine Centrifuge urine at 2,000 x g for 10 minutes to remove debris. Collect supernatants. Dilute samples 1:2 into Sample Diluent NS + 2X Enhancer and assay. Further dilutions can be made in Sample Diluent NS + 1X Enhancer. Store un-diluted samples at - 20°C or below. Avoid repeated freeze-thaw cycles.

Cell Culture Supernatants Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants. Dilute samples 1:2 into Sample Diluent NS + 2X Enhancer and assay. Further dilutions can be made in Sample Diluent NS + 1X Enhancer. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Cerebrospinal Fluid (CSF) Dilute cerebrospinal fluid at least 1:4 into Sample Diluent NS + 1X Enhancer and assay. Store un-diluted samples 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

Version 4a | 2024-10-24

ab222510 – Human VEGF SimpleStep ELISA® Kit

Additional information

ASSAY SPECIFICITY
This kit is designed for the quantification of human VEGF.

The standard protein in this kit is mature full length human VEGF165 (isoform 4).

Native signal was detected in serum, plasma (citrate), plasma (EDTA), plasma (heparin), cell culture supernatant, urine, saliva, milk, and cell extract sample types.

Spiked protein experiments were used to validate in serum, plasma (citrate), plasma (EDTA), plasma (heparin), and CSF sample types.

Tissue extract samples have not been tested with this kit.

CROSS REACTIVITY
Serial dilutions of recombinant human VEGF189 and VEGF121 were prepared and assayed in parallel with recombinant VEGF165 and cross reactivity was observed.

INTERFERENCE
Serial dilutions of recombinant human VEGFR1, VEGFR2, and VEGFR3 were prepared starting at 4 ng/mL and tested for interference. No interference was observed.

SPECIES REACTIVITY
Species reactivity was tested by measuring 500 pg/mL of recombinant mouse, rat, and canine VEGF. Reactivity was as follow: 13% (mouse), 24% (rat), and 110% (canine).

Other species reactivity was determined by measuring 1:2 serum samples of various species, interpolating the protein concentrations from the human standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in human serum assayed at the same dilution. Reactivity was as follow: pig 20%, guinea pig 3.4% and canine 28%. No signal was observed with 1:2 serum samples from the following species: Rabbit, hamster, rat, and mouse.

Non-human primate cross-reactivity was determined by measuring 1:2 plasma samples of various species, interpolating the protein concentrations from the human standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in human plasma assayed at the same dilution. Reactivity was as follow: rhesus macaque 29% and cynomolgus macaque 60%.

Other species reactivity not determined.

CALIBRATION

This immunoassay is calibrated against a highly purified human VEGF. The NIBSC/WHO unclassified purified human VEGF preparation 02/286 was evaluated in this kit.

The dose response curve of the unclassified standard 02/286 parallels the SimpleStep standard curve. To convert sample values obtained with the SimpleStep human VEGF kit to approximate NIBSC 02/286 units, use the equation below.

NIBSC 02/286 approximate value (IU/mL) = 0.0011 x SimpleStep human VEGF value (pg/mL).

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	0.104	0.100	0.102
12.5	0.236	0.221	0.229
25	0.345	0.332	0.338
50	0.558	0.540	0.549
100	0.962	0.940	0.951
200	1.740	1.750	1.745
400	3.068	2.987	3.027
800	3.959	3.954	3.957

Table 1. Example of human VEGF standard curve in Sample Diluent NS + 1X Enhancer. The VEGF 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	0.101	0.099	0.100
12.5	0.207	0.192	0.199
25	0.304	0.284	0.294
50	0.495	0.467	0.481
100	0.853	0.805	0.829
200	1.547	1.425	1.486
400	2.879	2.634	2.757
800	3.962	3.895	3.928

Table 2. Example of human VEGF standard curve in 1X Cell Extraction Buffer PTR. The VEGF 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 + 1X Enhancer	14	2.7 pg/mL
1X Cell Extraction Buffer PTR	10	2.9 pg/mL

Recovery

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

Sample Type	Average % Recovery	Range (%)
50% Serum	109	98 - 116
50% Plasma (Citrate)	101	95 - 107
50% Plasma (Heparin)	89	84 - 92
50% Plasma (EDTA)	98	95 - 99
25% Urine	95	91 - 102
1: 400 Breast Milk (de-fatted)	95	91 - 98
2% Saliva	109	105 - 118
50% Cell Culture Media*	86	81 - 89
25% Cerebrospinal Fluid	92	89 - 95
150 µg/mL PC-3 Cell Extract (2 Day)	89	80 - 94
75 µg/mL MDA-MB-435S Cell Extract	104	82 - 126

*Media is HGDMEM containing 10% fetal bovine serum.

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.

Recombinant human VEGF was spiked into the following biological samples and diluted in a 2-fold dilution series in Sample Diluent NS + 2X Enhancer and in Sample Diluent NS + 1X Enhancer, see Sample Preparation section for details.

Dilution Factor	Interpolated value	50% Human Serum	50% Human Plasma (Citrate)	50% Human Plasma (EDTA)	50% Human Plasma (Heparin)	25% CSF
Undiluted	pg/mL	692.8	698.8	675.6	665.3	384.5
	% Expected value	100	100	100	100	100
2	pg/mL	365.0	366.1	353.1	332.2	409.0
	% Expected value	105	105	105	100	106
4	pg/mL	166.5	179.0	168.8	166.3	360.6
	% Expected value	96	102	100	100	94
8	pg/mL	75.20	72.75	67.96	80.28	427.1
	% Expected value	87	83	80	97	111
16	pg/mL	34.83	34.80	38.87	41.28	407.3
	% Expected value	80	80	92	99	106

Native VEGF was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS + 2X Enhancer and in Sample Diluent NS + 1X Enhancer, see Sample Preparation section for details.

Dilution Factor	Interpolated value	2% Human Breast Milk	12.5% Human Saliva	50% Human Urine	20% PC-3 (1 Day) SN	20% PC-3 (2 Day) SN
Undiluted	pg/mL	461.4	497.3	127.4	123.8	294.4
	% Expected value	100	100	100	100	100
2	pg/mL	187.1	250.2	68.12	69.22	156.5
	% Expected value	81	101	107	112	106
4	pg/mL	96.03	118.3	32.34	35.23	71.87
	% Expected value	83	95	102	114	98
8	pg/mL	56.86	53.86	ND	18.31	39.46
	% Expected value	99	87	ND	118	107
16	pg/mL	29.63	26.14	ND	ND	17.81
	% Expected value	103	84	ND	ND	97

ND – Not Detected – below product dynamic range

Native VEGF was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS + 2X Enhancer and in Sample Diluent NS + 1X Enhancer, see Sample Preparation section for details.

Dilution Factor	Interpolated value	50% A549 SN	12.5% MDA-MB-435S SN	5% HepG2 SN	5% A431 SN	50% PHA-M PBMC SN (5 Day)
Undiluted	pg/mL	325.8	696.7	245.2	384.6	191.2
	% Expected value	100	100	100	100	100
2	pg/mL	145.3	409.8	125.1	161.1	97.32
	% Expected value	89	118	102	84	102
4	pg/mL	73.49	186.7	61.51	79.70	51.17
	% Expected value	90	107	100	83	107
8	pg/mL	37.53	81.58	32.11	49.70	26.66
	% Expected value	92	94	105	103	112
16	pg/mL	18.61	48.93	16.16	25.98	13.10
	% Expected value	91	112	105	108	110

Native VEGF 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	300 µg/mL PC-3 Cell Extract (1 Day)	300 µg/mL PC-3 Cell Extract (2 Day)	300 µg/mL HepG2 Cell Extract	300 µg/mL A549 Cell Extract	300 µg/mL MDA-MB-435S Cell Extract
Undiluted	pg/mL	85.16	143.9	119.0	232.5	451.4
	% Expected value	100	100	100	100	100
2	pg/mL	41.55	70.46	59.64	117.1	213.3
	% Expected value	98	98	100	101	95
4	pg/mL	20.77	35.78	33.36	61.19	107.2
	% Expected value	98	99	112	105	95
8	pg/mL	11.90	21.31	17.39	31.75	58.03
	% Expected value	112	118	117	109	103
16	pg/mL	ND	ND	ND	15.19	31.34
	% Expected value	ND	ND	ND	105	111

ND – Not Detected – below product dynamic range

Precision

Mean coefficient of variations of interpolated values of VEGF from three concentrations of A431 cell culture supernatant within the working range of the assay.

	Intra-assay	Inter-assay
N=	5	3
CV (%)	5.4	5.5

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

Version 4a | 2024-10-24

DIRECTIONS FOR 384-WELL PLATE FORMAT:

Materials Supplied for 384-well Format

Item	Quantity	Storage Condition
Human VEGF Capture Antibody 10X	600 µL	+4°C
Human VEGF Detector Antibody 10X	600 µL	+4°C
Human VEGF Lyophilized Recombinant Protein	2 Vials	+4°C
Antibody Diluent 4BI	6 mL	+4°C
Cell Extraction Buffer PTR 5X	50 mL	+4°C
Cell Extraction Enhancer Solution 50X	11 x 1 mL	+4°C
Sample Diluent NS	2 x 50 mL	+4°C
Wash Buffer PT 10X	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.

Sample Diluent NS + 1X Enhancer: Prepare Sample Diluent NS + 1X Enhancer by combining Sample Diluent NS and 50X Cell Extraction Enhancer Solution. To make 50 mL Sample Diluent NS + 1X Enhancer, combine 49 mL Sample Diluent NS and 1 mL Cell Extraction Enhancer Solution 50X. Mix thoroughly and gently.

Sample Diluent NS + 2X Enhancer: Prepare Sample Diluent NS + 2X Enhancer by diluting 50X Cell Extraction Enhancer Solution to 2X with Sample Diluent NS. To make 5 mL Sample Diluent NS + 2X Enhancer, combine 4.8 mL Sample Diluent NS and 200 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 4BI. 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 4BI. 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. Reconstitute the VEGF standard sample by adding the volume indicated on the protein vial label. For **serum, plasma, supernatant, saliva, urine, milk, and CSF samples measurements**, use Sample Diluent NS + 1X Enhancer. For **cell extract samples measurements**, use 1X Cell Extraction Buffer PTR. Hold at room temperature for 10 minutes. Mix thoroughly and gently. This is the 4,000 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 120 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**	30	120	4,000	800
2	Standard#1	75	75	800	400
3	Standard#2	75	75	400	200
4	Standard#3	75	75	200	100
5	Standard#4	75	75	100	50
6	Standard#5	75	75	50	25
7	Standard#6	75	75	25	12.50
8	Blank Control	0	75	N/A	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

Version 4a | 2024-10-24