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ab222510
Human VEGF SimpleStep ELISA® Kit

For the quantitative measurement of VEGF in human serum, plasma, milk, saliva, urine, cell culture supernatants, cell and tissue extracts.

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

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1. Overview

VEGF *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of VEGF protein in human serum, plasma, milk, saliva, urine, cell culture supernatants, cell and tissue extracts.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB Development Solution is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

VEGF is a secreted growth factor of PDGF family active in angiogenesis, vasculogenesis and endothelial cell growth both in fetus and adult. Alternative splicing produces many isoforms including major isoforms VEGF121, VEGF165 and VEGF189 in human. VEGF expression is induced by hypoxia. It is regulated by growth factors, cytokines, gonadotropins, nitric oxide, hypoglycemia and oncogenic mutations. VEGF induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels. VEGF dimers bind to the FLT1/VEGFR1 and KDR/VEGFR2 receptors, induce their homodimerization and autophosphorylation. VEGF165 and VEGF145 interact with NRP1/Neuropilin.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed

Add 50 µL standard or sample to appropriate wells

Add 50 µL Antibody Cocktail to all wells

Incubate at room temperature for 1 hour

Aspirate and wash each well three times with 350 µL 1X Wash Buffer PT

Add 100 µL TMB Development Solution to each well and incubate for 10 minutes.

Add 100 µL Stop Solution and read OD at 450 nm

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.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- 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.

6. Materials Supplied

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
Wash Buffer PT 10X	20 mL	+4°C
Cell Extraction Buffer PTR 5X	10 mL	+4°C
Cell Extraction Enhancer Solution 50X	1 mL	+4°C
TMB Development Solution	12 mL	+4°C
Stop Solution	12 mL	+4°C
Sample Diluent NS	50 mL	+4°C
Anti-tag coated microplate (12 x 8 well strips)	96 Wells	+4°C
Plate Seal	1	+4°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform 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).

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 is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.

- The provided Antibody Diluents and Sample Diluents contain protease inhibitor aprotinin. Additional protease inhibitors can be added if required.
- The provided Cell Extraction Buffer 5X contains phosphatase inhibitors and protease inhibitor aprotinin. Additional protease inhibitors can be added if required.
- 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.
- To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.
- 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 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.

9.1 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. Refer to note in the Troubleshooting section.

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

Prepare Sample Diluent NS + 1X Enhancer by diluting 50X Cell Extraction Enhancer Solution to 1X with Sample Diluent NS. To make 10 mL Sample Diluent NS + 1X Enhancer combine 9.8 mL Sample Diluent NS and 200 µL Cell Extraction Enhancer Solution 50X. Mix thoroughly and gently.

9.3 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.

9.4 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.

9.5 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.

10. Standard Preparation

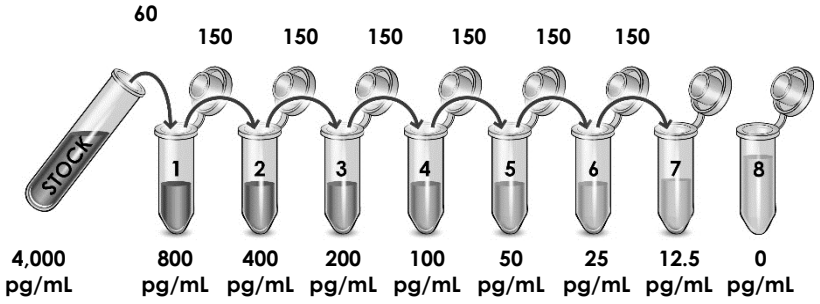
- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 For **serum, plasma, milk, urine, saliva, and cell culture supernatant samples measurements**, reconstitute the VEGF lyophilized protein standard by adding 500 µL of Sample Diluent NS + 1X Enhancer.
For **cell and tissue extract samples measurements**, reconstitute the VEGF lyophilized protein standard by adding 500 µL of 1X Cell Extraction Buffer PTR.
Hold at room temperature for 10 minutes and mix thoroughly and gently. This is the 4,000 pg/mL **Stock Standard** Solution.

10.2 Label eight tubes, Standards 1– 8.

10.3 Add 240 µL of appropriate diluent (see step 10.1) into tube number 1 and 150 µL of appropriate diluent into numbers 2-8.

10.4 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:



11. Sample Preparation

Typical Sample Dynamic Range	
Sample Type	**Range**
Serum	1:16 – 1:2
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
PC-3 Cell Extract (1 Day)	37.5 – 300 µg/mL
PC-3 Cell Extract (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

11.1 Plasma:

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples 2-fold 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.

11.2 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 2-fold 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.

11.3 Cell Culture Supernatants:

Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants. Dilute samples 2-fold 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.

11.4 Urine:

Centrifuge urine at 2,000 x g for 10 minutes to remove debris. Collect supernatants. Dilute samples 2-fold 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.

11.5 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.

11.6 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.

11.7 Preparation of extracts from cell pellets:

11.7.1 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.

11.7.2 Rinse cells twice with PBS.

11.7.3 Solubilize pellet at $2x10^7$ cell/mL in chilled 1X Cell Extraction Buffer PTR.

11.7.4 Incubate on ice for 20 minutes.

11.7.5 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.7.6 Transfer the supernatants into clean tubes and discard the pellets.

11.7.7 Assay samples immediately or aliquot and store at - 80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.7.8 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

11.8 Preparation of extracts from adherent cells by direct lysis (alternative protocol):

11.8.1 Remove growth media and rinse adherent cells 2 times in PBS.

11.8.2 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).

11.8.3 Scrape the cells into a microfuge tube and incubate the lysate on ice for 15 minutes.

11.8.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.8.5 Transfer the supernatants into clean tubes and discard the pellets.

11.8.6 Assay samples immediately or aliquot and store at - 80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.8.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

11.9 Preparation of extracts from tissue homogenates:

11.9.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).

11.9.2 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.

11.9.3 Incubate on ice for 20 minutes.

11.9.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.9.5 Transfer the supernatants into clean tubes and discard the pellets.

11.9.6 Assay samples immediately or aliquot and store at - 80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.9.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

12. 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.

13. 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.

13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

13.2 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.

13.3 Add 50 µL of all sample or standard to appropriate wells.

13.4 Add 50 µL of the Antibody Cocktail to each well.

13.5 Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

13.6 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.

13.7 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.

13.8 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.

13.9 Alternative to 13.7 – 13.8: 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 15 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

Δ **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.

13.10 Analyze the data as described below.

14. Calculations

14.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.

14.2 **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.

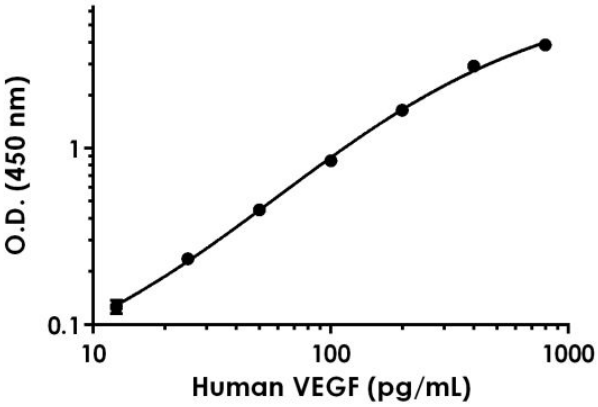
Δ **Note:** Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.

14.3 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.

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

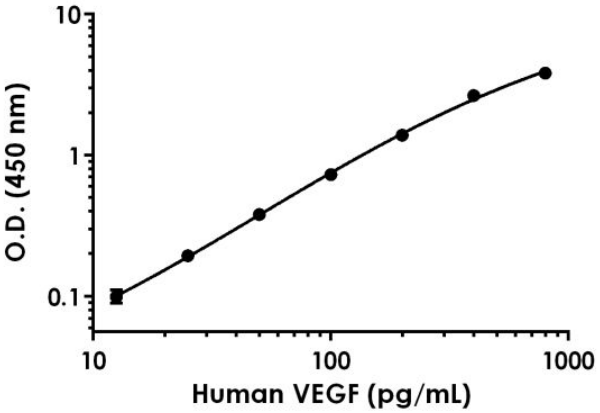
15. Typical Data

Typical standard curve – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
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

Figure 1. Example of human VEGF standard curve in Sample Diluent NS + 1X Enhancer. The VEGF standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.



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

Figure 2. Example of human VEGF standard curve in 1X Cell Extraction Buffer PTR. The VEGF standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. 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).

17. Typical Sample Values

SENSITIVITY –

The 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
1X Cell Extraction Buffer PTR	10	2.9 pg/mL
Sample Diluent NS + 1X Enhancer	14	2.7 pg/mL

RECOVERY –

Three concentrations of recombinant human VEGF protein 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 (%)
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
0.25% Breast Milk (de-fatted)	95	91 - 98
2% Saliva	109	105 - 118
50% HGDMEM Media + 10% FBS	86	81 - 89
150 µg/mL PC-3 Cell Extract (2 Day)	89	80 - 94
75 µg/mL MDA-MB-435S Cell Extract	104	82 - 126

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 VEGF was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS + 1X Enhancer.

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)
Undiluted	pg/mL	692.8	698.8	675.6	665.3
	% Expected value	100	100	100	100
2	pg/mL	365.0	366.1	353.1	332.2
	% Expected value	105	105	105	100
4	pg/mL	166.5	179.0	168.8	166.3
	% Expected value	96	102	100	100
8	pg/mL	75.20	72.75	67.96	80.28
	% Expected value	87	83	80	97
16	pg/mL	34.83	34.80	38.87	41.28
	% Expected value	80	80	92	99

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. Cell culture supernatants are represented by SN.

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 – Non-Detectable. O.D. values below the 7th point of the standard curve.

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. Cell culture supernatants are represented by SN.

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 – Non-Detectable. O.D. values below the 7th point of the standard curve.

PRECISION –

Mean coefficient of variations of interpolated values 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

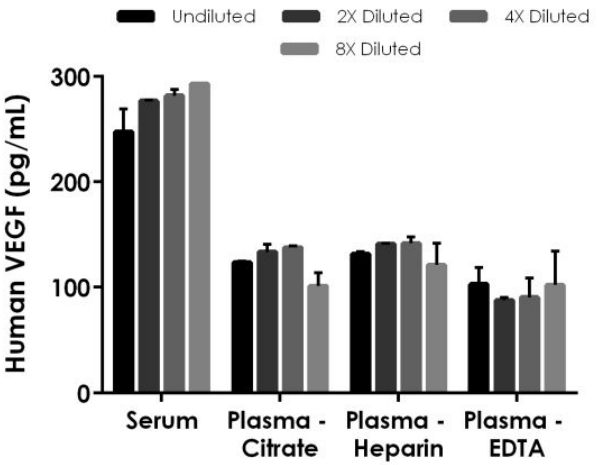


Figure 3. Interpolated concentrations of native VEGF in human serum and plasma samples. The concentrations of VEGF were measured in duplicates, interpolated from the VEGF standard curves and corrected for sample dilution. Undiluted samples are as follows: serum 50%, plasma (citrate) 50%, plasma (heparin) 50% and plasma (EDTA) 50%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean VEGF concentration was determined to be 268.9 pg/mL in neat serum, 123.9 pg/mL in neat plasma (citrate), 133.8 pg/mL in neat plasma (heparin), and 95.76 pg/mL in neat plasma (EDTA).

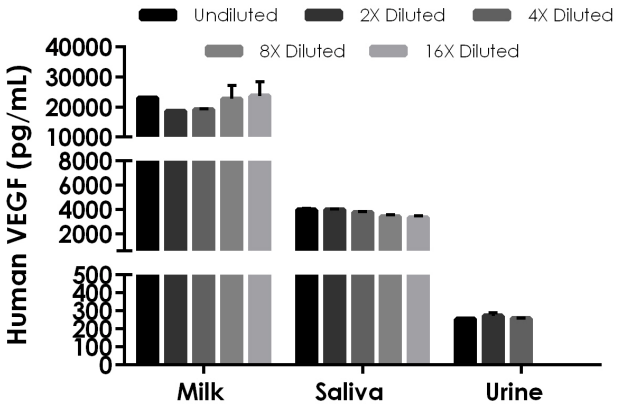


Figure 4. Interpolated concentrations of native VEGF in human breast milk (de-fatted), saliva, and urine samples. The concentrations of VEGF were measured in duplicates, interpolated from the VEGF standard curves and corrected for sample dilution. Undiluted samples are as follows: breast milk 2%, saliva 12.5%, and urine 50%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean VEGF concentration was determined to be 21487 pg/mL in neat human breast milk (de-fatted), 3712 pg/mL in neat human saliva, and 262.0 pg/mL in neat human urine.

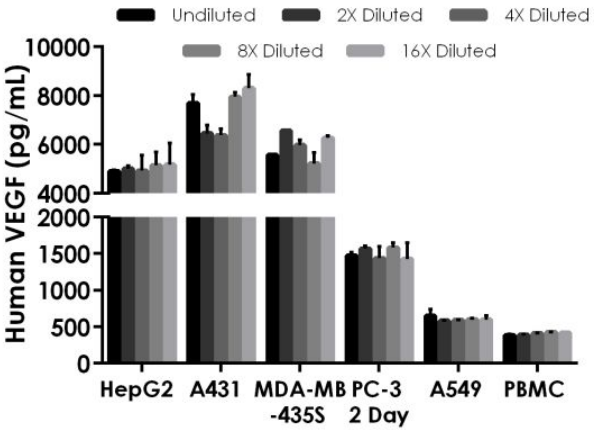


Figure 5. Interpolated concentrations of native VEGF in cell culture supernatant samples. The concentrations of VEGF were measured in duplicates, interpolated from the VEGF standard curves and corrected for sample dilution. Undiluted samples are as follows: HepG2 5%, A431 5%, MDA-MB-435S 12.5%, PC-3 20%, A549 50%, and PBMC 50%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean VEGF concentration was determined to be 5027 pg/mL in neat HepG2 supernatant, 7356 pg/mL in neat A431 supernatant (4 Day), 5918 pg/mL in neat MDA-MB-435S supernatant, 1495 pg/mL in neat PC-3 (2 Day), 603.4 pg/mL in neat A549 supernatant, and 405.3 pg/mL in neat PBMC supernatant (PHA-M, 5 Day).

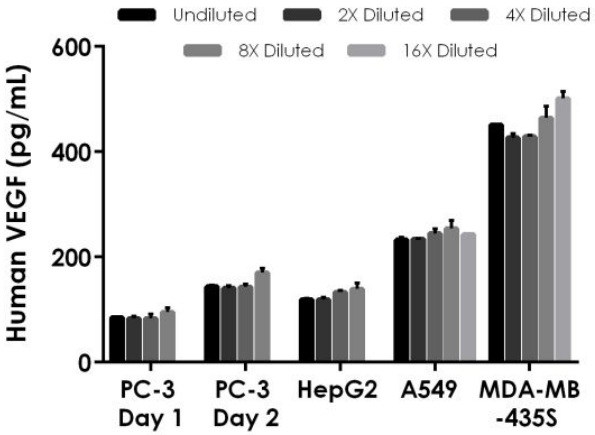


Figure 6. Interpolated concentrations of native VEGF in PC-3 cell extract (1 Day), PC-3 cell extract (2 Day), HepG2 cell extract, A549 cell extract, and MDA-MB-435S cell extract samples based on a 300 µg/mL extract load. The concentrations of VEGF were measured in duplicate and interpolated from the VEGF standard curve and corrected for sample dilution. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean VEGF concentration was determined to be 86.65 pg/mL in PC-3 cell extract (1 Day), 149.6 pg/mL in PC-3 cell extract (2 Day), 127.7 pg/mL in HepG2 cell extract, 241.7 pg/mL in A549 cell extract, and 454.5 pg/mL in MDA-MB-435S cell extract.

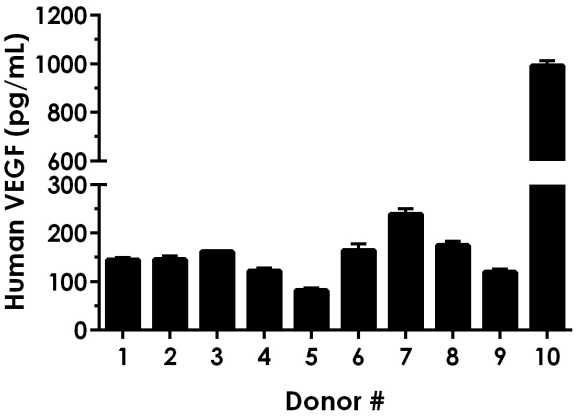


Figure 7. Serum from ten individual healthy human female donors was measured in duplicate. Interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean VEGF concentration was determined to be 235.0 pg/mL with a range of 81.10 – 993.3 pg/mL.

18. Assay Specificity

This kit recognizes both native and recombinant human VEGF protein in serum, plasma, milk, saliva, urine, cell culture supernatant, cell and tissue extract samples only.

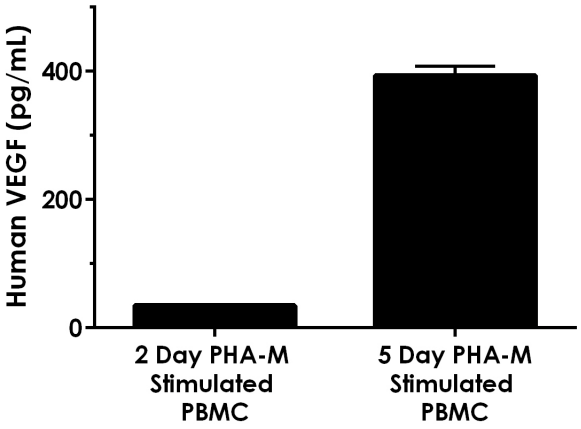


Figure 8. Comparison of VEGF in PHA-M stimulated and unstimulated human PBMC cell culture supernatants stimulated for different durations. Human PBMC (seeded at $10x10^6$/mL) were cultured for 2 or 5 days in the presence or absence of 1.5% PHA-M. The concentrations of VEGF were measured in three different dilutions of the supernatant samples in duplicates and interpolated from the VEGF standard curve. The interpolated values are plotted (mean +/- SD, n=3). The mean VEGF concentration was determined to be 34.76 pg/mL in 2 Day PHA-M stimulated PBMC cell culture supernatant, 393.7 pg/mL in 5 Day PHA-M stimulated PBMC cell culture supernatant, and undetectable in unstimulated PBMC cell culture supernatant at both 2 and 5 days (not shown).

CROSS REACTIVITY

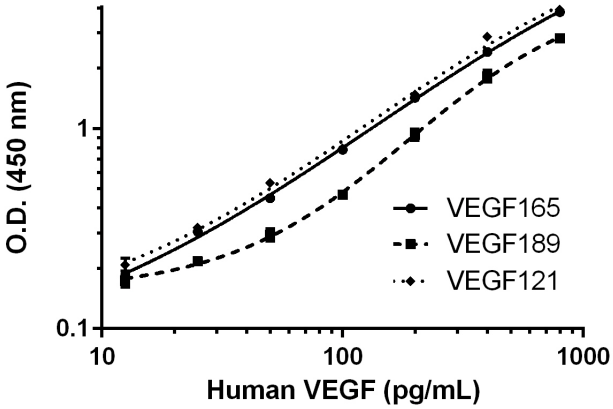


Figure 9. Serial dilutions of recombinant human VEGF189 and VEGF121 were prepared and assayed in parallel with recombinant VEGF165.

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.

19. Species Reactivity

This kit recognizes human VEGF protein.

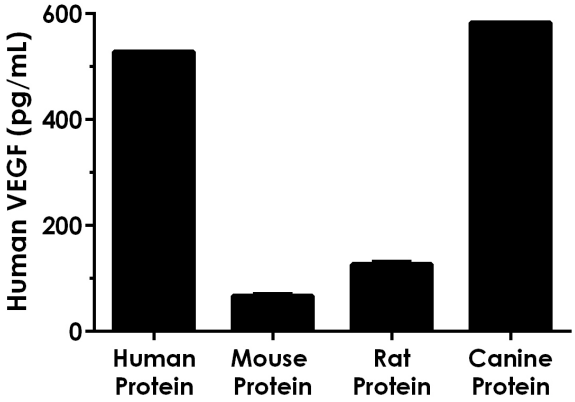


Figure 10. Other species reactivity was determined by measuring a 500 pg/mL protein load of various species recombinant VEGF proteins, interpolating the protein concentrations from the human standard curve, and graphing the interpolated concentrations (mean +/- SD, n=2).

Reactivity < 3% was determined for the following species:

Cow

Please contact our Technical Support team for more information.

20. Troubleshooting

Problem	Reason	Solution
Difficulty pipetting lysate; viscous lysate.	Genomic DNA solubilized	Prepare 1X Cell Extraction Buffer PTR (without enhancer). Add enhancer to lysate after extraction.
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard 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; increase to 2 or 3 hours standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop Solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all parts for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at - 80°C, all other assay components 4°C. Keep TMB Development Solution solution protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

21. Notes

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)