ab213476 – Human VEGF Receptor 2 SimpleStep ELISA® Kit

Instructions for use:

For the quantitative measurement of human VEGF Receptor 2 in serum, plasmas, and cell culture supernatant.

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

Version 3a Last Updated 09 December 2022

Table of Contents

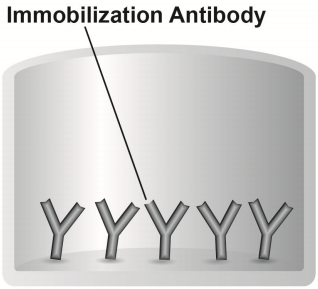
1. BACKGROUND

VEGF Receptor 2 *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of VEGF Receptor 2 protein in serum, plasma, and cell culture supernatant.

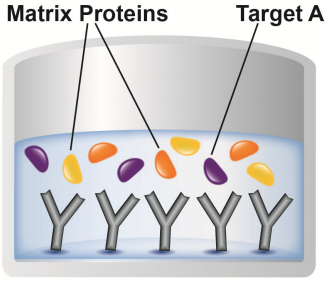
The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating 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 substrate 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 R2 (vascular endothelial growth factor receptor 2) is 1356 amino acids long and is a class III receptor tyrosine kinase. VEGF R2 acts as a cell surface receptor for VEGFA, VEGFC, and VEGFD, where binding of these VEGFs to VEGF R2 activates several signaling cascades, including activation of PLCG1 (leading to the activation of protein kinase C), the MAP kinase signaling pathways, and the AKT1 signaling pathway. Furthermore, VEGF R2 plays an essential role in the regulation of angiogenesis, vascular development and permeability, embryonic hematopoiesis, and reorganization of the actin cytoskeleton. Finally, VEGF R2 is a major growth factor for endothelial cells and is the main facilitator of VEGF-induced endothelial proliferation, differentiation, and migration.

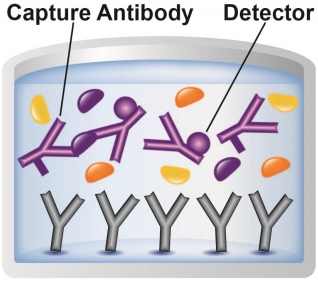
2. ASSAY SUMMARY



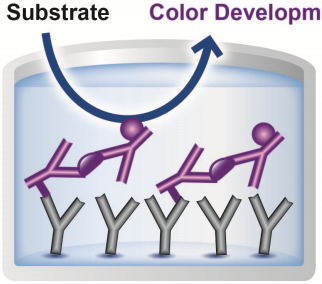
Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all reagents, samples, and standards as instructed.



Add standard or sample to appropriate wells.



Add Antibody Cocktail to all wells. Incubate at room temperature.



Aspirate and wash each well. Add TMB Substrate to each well and incubate. Add Stop Solution at a defined endpoint. Alternatively, record color development kinetically after TMB substrate addition.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

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

4. STORAGE AND STABILITY

Store kit at +4ºC 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 Reagent and Standard Preparation sections.

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	Amount	Storage Condition (Before Preparation)
10X Human VEGF Receptor 2 Capture Antibody	600 µL	+4ºC
20X Human VEGF Receptor 2 Detector Antibody	300 µL	+4ºC
Human VEGF Receptor 2 Lyophilized Recombinant Protein	2 Vials	+4ºC
Antibody Diluent 4BI	6 mL	+4ºC
Sample Diluent NBS	2 mL	+4ºC
10X Wash Buffer PT	20 mL	+4ºC
TMB Development Solution	12 mL	+4ºC
Stop Solution	12 mL	+4ºC
Sample Diluent NS	50 mL	+4ºC
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	+4ºC
Plate Seal	1	+4ºC

7. 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).

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.
- **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 and 20X formulations.

9.1 1X Wash Buffer PT

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

9.2 Sample Diluent 3.75BS

Prepare Sample Diluent 3.75BS if analysing Plasma Heparin or Cell Culture Supernatant samples. To make 15 mL of Sample Diluent 3.75BS mix 563 µL Sample Diluent NBS and 14.437 mL Sample Diluent NS. Mix thoroughly and gently.

9.3 Antibody Cocktail

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 150 µL 20X Detector Antibody with 2.55 mL Antibody Diluent 4BI. Mix thoroughly and gently.

10. STANDARD PREPARATION

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

The following section describes the preparation of a standard curve for duplicate measurements (recommended).

IMPORTANT: If the protein standard vial has a volume identified on the label, reconstitute the VEGF Receptor 2 standard by adding that volume of Diluent indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the VEGF Receptor 2 standard by adding 0.5 mL Diluent. Hold at room temperature for 10 minutes and mix gently. This is the 30,000 pg/mL **Stock Standard** Solution.

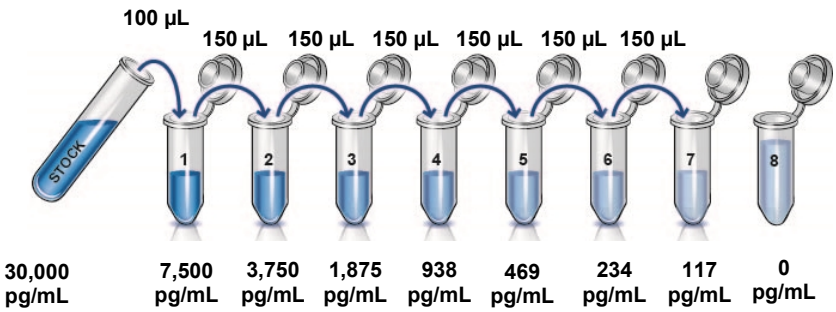
10.1 For **serum, citrate plasma, and EDTA plasma sample** measurements, reconstitute the VEGF Receptor 2 standard by adding Sample Diluent NS.

For **heparin plasma and cell culture supernatant samples** measurements, reconstitute the VEGF Receptor 2 standard by adding 3.75% BS Diluent.

10.2 Label eight tubes, Standards 1– 8.

10.3 Add 300 µL of appropriate diluent (see step 10.1) into tube number 1 and add 150 µL of appropriate diluent (see step 10.1) 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:



ASSAY PREPARATION

11. SAMPLE PREPARATION

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	**Range**
Human Plasma - Heparin	0.39% - 12.5%
Human Plasma - EDTA	0.47% - 7.5%
Human Plasma - Citrate	0.47% - 7.5%
Human Serum	0.47% - 7.5%
HUVEC Cell Culture Supernatant	5% - 80%

11.1 **Plasma**

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples into Sample Diluent NS for citrate or EDTA plasmas and Sample Diluent 3.75BS for heparin plasma and assay. Store un-diluted plasma samples at -20ºC 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 into Sample Diluent NS and assay. Store un-diluted serum at -20ºC 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 into Sample Diluent 3.75BS, and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

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.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

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

13.2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4ºC 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. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.

13.7. Add 100 µL of TMB Substrate 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.

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 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.

13.9. 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.

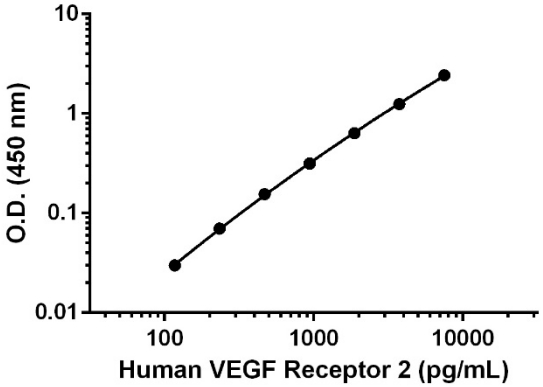
Note: Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better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

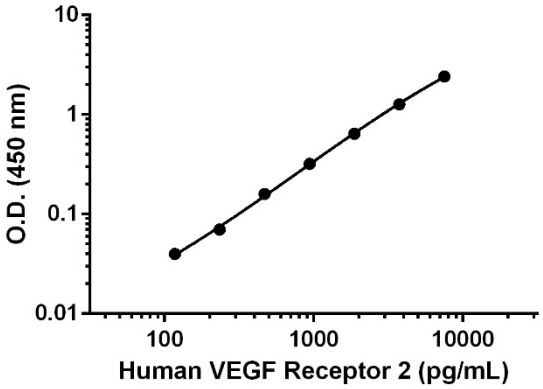
15. TYPICAL DATA

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



Standard Curve Measurements			
Conc. (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.06	0.06	0.06
117	0.09	0.09	0.09
234	0.13	0.13	0.13
469	0.22	0.21	0.22
938	0.38	0.37	0.38
1,875	0.70	0.69	0.70
3,750	1.32	1.30	1.31
7,500	2.51	2.47	2.49

Figure 1. Example of human VEGF Receptor 2 standard curve in Sample Diluent NS. The VEGF Receptor 2 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			
Conc. (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.06	0.08	0.07
117	0.11	0.11	0.11
234	0.14	0.14	0.14
469	0.23	0.23	0.23
938	0.38	0.39	0.39
1,875	0.71	0.72	0.71
3,750	1.33	1.34	1.33
7,500	2.50	2.47	2.49

Figure 2. Example of human VEGF Receptor 2 standard curve in Sample Diluent 3.75BS. The VEGF Receptor 2 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. TYPICAL SAMPLE VALUES

SENSITIVITY –

The calculated minimal detectable dose (MDD) is 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	41.6 pg/mL
Sample Diluent 3.75BS	16	10 pg/mL

RECOVERY –

Three concentrations of human recombinant VEGF Receptor 2 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 (%)
Mouse Plasma - Heparin	97	95 - 99
Mouse Plasma - EDTA	95	89 - 98
Mouse Plasma - Citrate	114	109 - 122
Mouse Serum	96	94 - 97
HUVEC Culture Supernatant	97	95 - 98

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 human VEGF Receptor 2 was measured in all but one of the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS (serum, plasma citrate, and plasma EDTA) or Sample Diluent 3.75BS (plasma heparin and HUVEC culture supernatant).

For HUVEC culture supernatant, native signal was detectable but to expand testing of the linear range, recombinant VEGF Receptor 2 was spiked into the sample and it was diluted in a 2-fold dilution series in Sample Diluent 3.75BS.

Dilution Factor	Interpolated value	7.5% Human Serum	7.5% Human Plasma (Citrate)	7.5% Human Plasma (EDTA)	12.5% Human Plasma (Heparin)	80% HUVEC Culture Supernatant
Undiluted	pg/mL	4425	3103	3785	5691	3931
	% Expected value	**100**	**100**	**100**	**100**	**100**
2	pg/mL	2225	1477	1706	2800	1977
	% Expected value	**101**	**95**	**90**	**98**	**101**
4	pg/mL	1112	740	769	1346	1030
	% Expected value	**100**	**95**	**81**	**95**	**105**
8	pg/mL	562	345	379	677	512
	% Expected value	**102**	**89**	**80**	**95**	**104**
16	pg/mL	294	209	196	342	247
	% Expected value	**106**	**108**	**83**	**96**	**100**

PRECISION –

Mean coefficient of variations of interpolated values of VEGF Receptor 2 measured in 3 concentrations of pooled normal human serum within the working range of the assay.

	Intra-Assay	Inter-Assay
n=	8	3
CV (%)	2.5	5.8

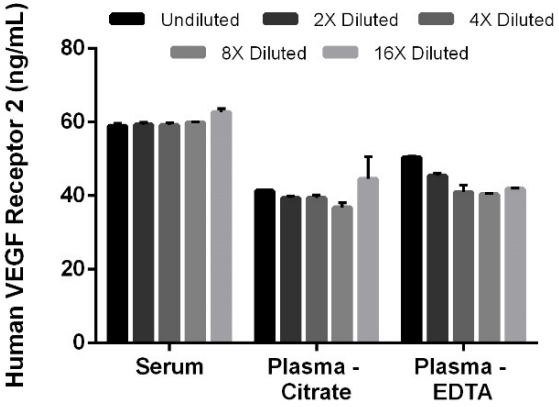


Figure 3. Interpolated concentrations of native VEGF Receptor 2 in human serum, plasma citrate, and plasma EDTA samples. The concentrations of VEGF Receptor 2 were measured in duplicate, interpolated from the VEGF Receptor 2 standard curves and corrected for sample dilution. Undiluted samples are as follows: serum 7.5%, plasma (citrate) 7.5%, and plasma (EDTA) 7.5%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean VEGF Receptor 2 concentration was determined to be 60 ng/mL in serum, 42 ng/mL in plasma (citrate) and 49 ng/mL in plasma (EDTA).

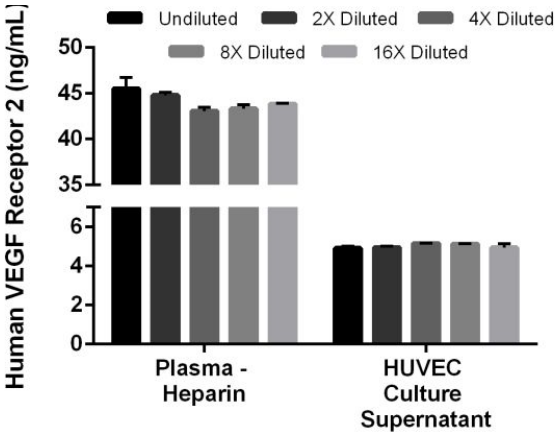


Figure 4. Interpolated concentrations of native VEGF Receptor 2 in human plasma heparin, and spiked recombinant VEGF Receptor 2 in HUVEC culture supernatant samples. The concentrations of VEGF Receptor 2 were measured in duplicate, interpolated from the VEGF Receptor 2 standard curve and corrected for sample dilution. Undiluted samples are as follows: plasma (heparin) 12.5% and HUVEC culture supernatant 80%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean VEGF Receptor 2 concentration was determined to be 44 ng/mL in plasma (heparin). Native signal of HUVEC culture supernatant at 80% was determined to be 0.4 ng/mL.

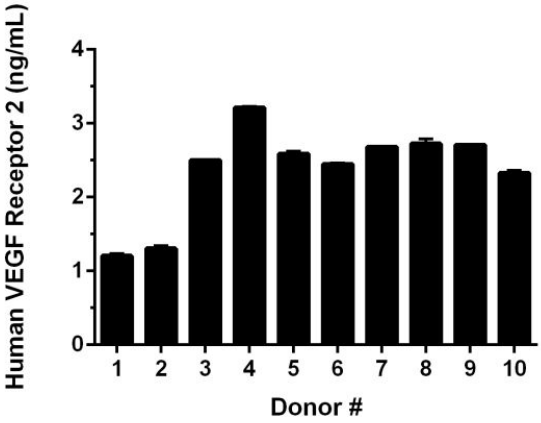


Figure 5. Serum from ten individual healthy human male donors was measured in duplicate. Interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean VEGF Receptor 2 concentration was determined to be 2.37 ng/mL with a range of 1.19 – 3.23 ng/mL.

17. ASSAY SPECIFICITY

This kit recognizes both native and recombinant human VEGF Receptor 2 protein in serum, plasma, and cell culture supernatant samples only.

Urine, milk, saliva, cerebrospinal fluid, and synovial fluid samples have not been tested with this kit.

CROSS REACTIVITY

Recombinant human VEGF Receptor 1 and VEGF Receptor 3 were prepared at 50 ng/mL and 3.75 ng/mL and assayed for cross reactivity. No cross-reactivity was observed.

INTERFERENCE

Recombinant VEGF Receptor 2 was diluted 2-fold for 7 points in Sample Diluent NS prepared with 2.7 ng/mL VEGF and tested for interference. No interference with was observed. Average raw signal compared to VEGF Receptor 2 diluted in unspiked Sample Diluent NS was 97%, ranging from 94-100%.

18. SPECIES REACTIVITY

This kit recognizes human VEGF Receptor 2 protein.

Other species reactivity was determined by measuring 7.5% and 0.75% 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 < 3% was determined for the following species:

- Mouse
- Rat
- Cow

Please contact our Technical Support team for more information.

19. TROUBLESHOOTING

Problem	Cause	Solution
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 hour 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 ports 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 substrate 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 37ºC.

20. NOTES

RESOURCES

RESOURCES

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)