ab203367 – VEGF R3 (FLT4) Mouse SimpleStep ELISA® Kit

Instructions for Use

For the quantitative measurement of VEGF R3 (FLT4) in mouse serum, plasmas, and cell culture supernatants.

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

Version 2a Last Updated 5 December 2023

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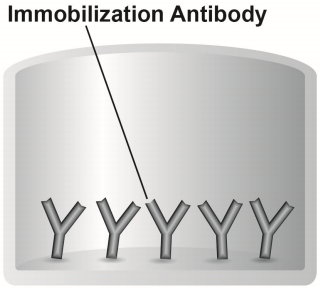
1. BACKGROUND

Abcam's VEGF R3 (FLT4) in *vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of VEGF R3 protein in mouse serum, plasma, and cell culture supernatants.

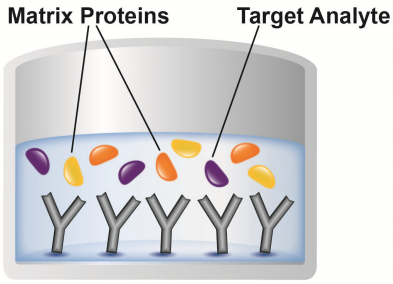
The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating 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.

The VEGF receptor family includes three members VEGF R1, VEGF R2, and VEGF R3 (FLT4). These receptors have a characteristic structure that includes 7 Ig-like domains in the extracellular domain and a cytoplasmic tyrosine kinase domain with a long kinase insert region. VEGF R3 is a Tyrosine-protein kinase that acts as a cell-surface receptor for VEGFC and VEGFD. It plays an essential role in adult lymphangiogenesis, and in the development of the vascular network and cardiovascular system during embryonic development. VEGF R3 promotes proliferation, survival and migration of endothelial cells, and regulates angiogenic sprouting. Signaling by activated VEGF R3 leads to enhanced production of VEGFC and VEGFA, creating a positive feedback loop that enhances VEGF R3 signaling.

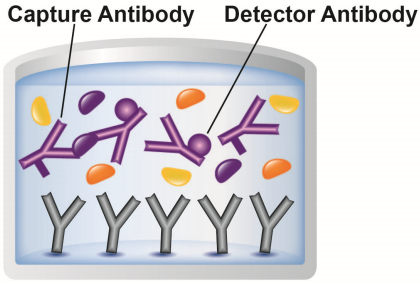
2. ASSAY SUMMARY



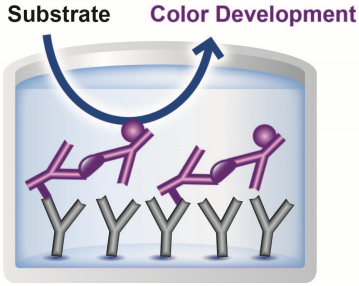
Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all reagents, samples, and standards as instructed.



Add standard or sample to appropriate wells.



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



Aspirate and wash each well. Add TMB Development Solution 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 2-8ºC 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. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
10X Mouse VEGF R3 Capture Antibody	600 µL	+2-8ºC
10X Mouse VEGF R3 Detector Antibody	600 µL	+2-8ºC
Mouse VEGF R3 Lyophilized Protein	2 Vials	+2-8ºC
Antibody Diluent CP2	6 mL	+2-8ºC
10X Wash Buffer PT	20 mL	+2-8ºC
TMB Development Solution	12 mL	+2-8ºC
Stop Solution	12 mL	+2-8ºC
Sample Diluent NS*	50 mL	+2-8ºC
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	+2-8ºC
Plate Seal	1	+2-8ºC

Note: Antibody Diluent CP2- This buffer has been reformulated to enhance stability after freeze-thaw cycles while producing data equivalent to the original formulation of antibody diluent CP previously used in this kit.

While we run stock down, you may receive kits containing antibody diluent CP. This does not affect the way you should use the kit.

If you have any questions please contact Abcam Scientific Support.

6. 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.
- PBS (1.4 mM KH2PO4, 8 mM Na2HPO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4).
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

7. 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.

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.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- **Incubate ELISA plates without shaking during all incubation steps.**
- **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 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 Antibody Cocktail

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent CP2. 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 CP2. 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.

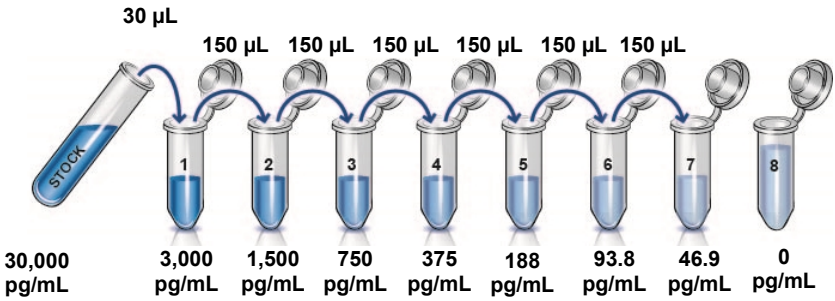
The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 **IMPORTANT:** If the protein standard vial has a volume identified on the label, reconstitute the VEGF R3 standard by adding that volume of Sample Diluent NS indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the VEGF R3 standard by adding 500 µL Sample Diluent NS. Hold at room temperature for 10 minutes and mix gently. This is the 30,000 pg/mL **Stock Standard** Solution

10.2 Label eight tubes, Standards 1– 8.

10.3 Add 270 µL Sample Diluent NS into tube number 1 and 150 µL of Sample Diluent NS 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**
Mouse Serum	0.039 – 5%
Mouse Plasma - Citrate	0.039 - 2.5%
Mouse Plasma - Heparin	0.039 - 2.5%
Mouse Plasma - EDTA	0.078 - 2.5%
Stimulated Mouse Lung Supernatant	3.13 – 50%
Unstimulated Mouse Lung Supernatant	3.13 – 50%
Unstimulated Mouse Liver Supernatant	3.13 – 25%

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 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 and assay. Or dilute samples into Sample Diluent NS 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 been observed with this assay when plate is shaken during sample incubation. Do not shake plate during 1 hour sample incubation step. Only shake plate during 10 minute TMB development step.

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 with no shaking.

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 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.

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.

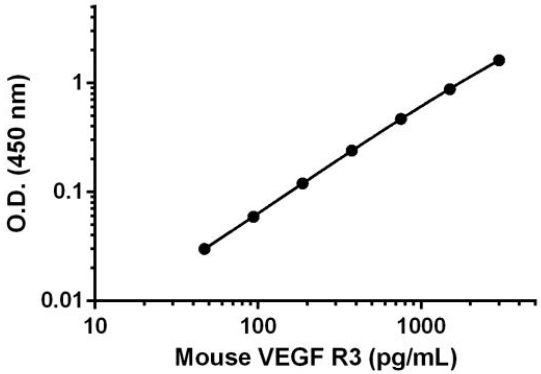
13.9 Analyze the data as described below.

14. CALCULATIONS

Subtract the average zero standard absorbance value from all measurements to determine the background-corrected absorbance value. Determine the average value of the duplicate background-subtracted measurements of the positive control dilutions and plot these values against their concentrations. Draw the best smooth curve through these points to construct a standard curve. Software supplied with microplate readers have or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to determine the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). The concentrations of unknown samples can be interpolated from the curve using the background-corrected absorbance values and the dilution factor, if used. Absorbance values of samples which are greater than that of the highest standard should be diluted until the absorbance value falls within the linear part of the curve. The concentration of the sample can then be determined by applying the dilution factor.

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	0.059	0.058	0.058
46.9	0.091	0.087	0.089
93.8	0.119	0.117	0.118
188	0.181	0.175	0.178
375	0.300	0.296	0.298
750	0.534	0.520	0.527
1,500	0.920	0.947	0.933
3,000	1.698	1.659	1.678

Figure 1. Example of VEGF R3 standard curve. The VEGF R3 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 17.97 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=16) and adding 2 standard deviations then extrapolating the corresponding concentration.

RECOVERY –

Three concentrations of VEGF R3 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 (%)
0.313% Mouse Serum	85	81 - 88
0.313% Mouse Plasma - Citrate	103	101 - 107
0.313% Mouse Plasma - Heparin	99	95 - 101
0.313% Mouse Plasma - EDTA	100	96 - 104
50% Cell Culture Media	87	87 - 88

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 R3 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	5% Mouse Serum	2.5% Mouse Plasma (Citrate)	2.5% Mouse Plasma (EDTA)	2.5% Mouse Plasma (Heparin)
Undiluted	pg/mL	7838	4076	4952	5286
	% Expected value	**100**	**100**	**100**	**100**
2	pg/mL	4455	2024	2566	2602
	% Expected value	**114**	**99**	**104**	**98**
4	pg/mL	2215	1065	1271	1294
	% Expected value	**113**	**104**	**103**	**98**
8	pg/mL	1072	495	609	640
	% Expected value	**109**	**97**	**98**	**97**
16	pg/mL	504	229	285	289
	% Expected value	**103**	**90**	**92**	**88**

Dilution Factor	Interpolated value	50% Stimulated Lung Supernatant	50% Unstimulated Lung Supernatant	25% Stimulated Liver Supernatant
Undiluted	pg/mL	599.3	534.5	192.1
	% Expected value	**100**	**100**	**100**
2	pg/mL	304.6	275.1	113.5
	% Expected value	**102**	**103**	**118**
4	pg/mL	146.5	137.5	59.3
	% Expected value	**98**	**103**	**124**
8	pg/mL	72.7	72.1	28.8
	% Expected value	**97**	**108**	**120**
16	pg/mL	32.5	32.9	NL
	% Expected value	**87**	**99**	**NL**

NL – Non-Linear

PRECISION –

Mean coefficient of variations of interpolated values from 3 concentrations of VEGF R3 within the working range of the assay.

	Intra-Assay	Inter-Assay
n=	8	3
CV (%)	4.59	7.14

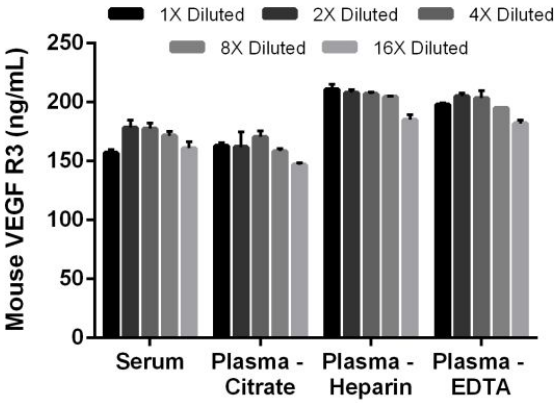


Figure 2. Titration of mouse serum, citrate plasma, heparin plasma, and EDTA plasma within the working range of the assay. The 1X dilution is 5% for mouse serum, and 2.5% for each of the plasmas. Interpolated data values (mean +/- SD, n = 2) are graphed.

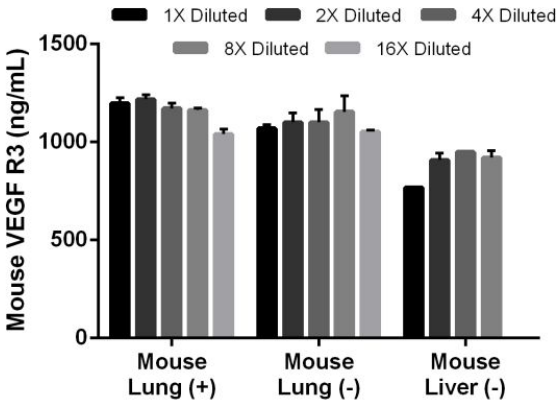


Figure 3. Titration of mouse stimulated (100 ng/mL LPS + 50 ng/mL PMA, 3 days (+)) lung supernatant, and unstimulated (-) lung and liver cell culture supernatants within the working range of the assay. The 1X dilution is 50.0% for the lung supernatants, and 25.0% for the liver supernatant. Interpolated data values (mean +/- SD, n = 2) are graphed.

17. SPECIES REACTIVITY

This kit recognizes mouse VEGF R3 protein.

Other species reactivity was determined by measuring 0.625% (1:160 dilution) serum samples of various species, interpolating the protein concentrations from the mouse standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in mouse serum assayed at the same dilution.

Reactivity < 3% was determined for the following species:

- Human
- Guinea Pig
- Rabbit
- Dog
- Pig
- Cow

Please contact our Scientific Support team for more information.

18. 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.
	High assay variability	Do not shake plate during 1 hour sample incubation step.
	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 protected from light.

19. **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)