GE Healthcare

Amersham Biotrak Vascular Endothelial Growth Factor [(h)VEGF], Human ELISA System

for the quantitative measurement of (h)VEGF in serum; EDTA, heparin and sodium citrate plasma; and culture supernatants.

Product Booklet

Code: RPN2779

Page finder

1. Legal

http//www.gelifesciences.com

GE Healthcare UK Limited.
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2. Handling

2.1. Safety warnings and precautions

Warning: *For research use only.* Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

2.2. Storage

Store all reagents at 2–8°C upon receipt. Do not freeze reagents.

2.3. Expiry

Refer to the expiry date stated on the kit box. Do NOT use kit beyond the stated expiry date.

3. Components

Standard: human recombinant VEGF, lyophilized, × 2.

Plate: anti-human VEGF precoated stripwell plate (96 well).

Antibody: biotinylated antibody reagent, 12 ml, with 0.1% (w/v) Sodium Azide.

Streptavidin-HRP reagent: reagent, 14 ml.

Substrate: Premixed TMB substrate solution, 12–13 ml.

Sample diluent: 14 ml, with 0.1% (w/v) Sodium Azide.

Wash buffer: 30 fold concentration, 50 ml.

Stop solution: <1% Sulfuric Acid, 13–15 ml.

Plate covers: adhesive, × 8.

Instructions for use

4. Other materials required

- Precision pipettes with disposable plastic tips to deliver 5–1000 µl
- Plastic pipettes to deliver 5–15 ml
- Distilled or deionized water
- A glass or plastic 2 litre container for preparation of wash buffer
- A squirt wash bottle, or an automated immunoplate washer
- 1.5 ml polypropylene or polyethylene tubes. Do not use polystyrene, polycarbonate or glass tubes
- Disposable reagent reservoirs
- A standard ELISA reader for measuring absorbance at 450 nm and 550 nm. If a 550 nm filter is not available, the absorbance can be read only at 450 nm. Refer to the instruction manual supplied with the instrument used.
- Graph paper or a computerized curve-fitting statistical software package.
- Assays may be performed with commercially available microplate washers to aid convenience and assay throughput.

5. Description

The Biotrak™ human VEGF ELISA from GE Healthcare is an enzyme-linked immunosorbent assay for the quantitative measurement of (h)VEGF in serum; EDTA, heparin and Sodium Citrate plasma and culture supernatants.

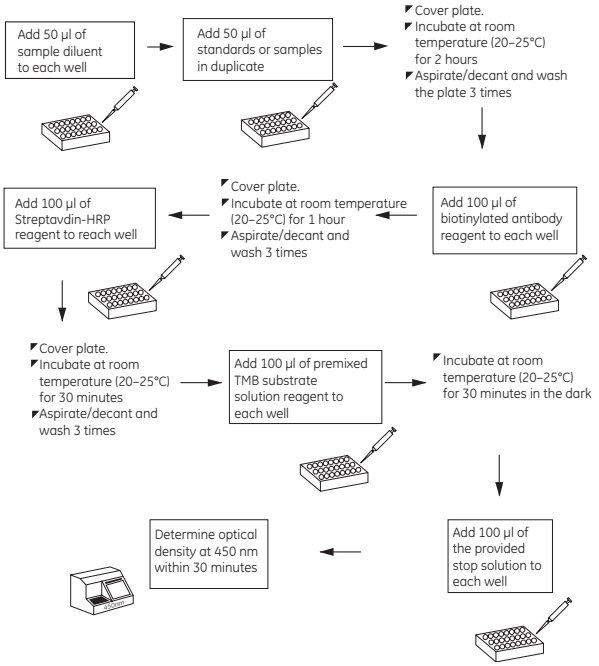

Figure 1. Summary of procedure

6. Critical parameters

The following points are critical:

- **All specimens and reagents must be at room temperature (20–25°C) before use in the ELISA.**
- Vigorous washing of plate is essential.
- Do not use a 37°C water bath to thaw samples. Thaw samples at room temperature and mix thoroughly before use. If using samples that are clotted, grossly hemolyzed, lipemic or microbially contaminated, or if there is any question about the integrity of the sample, make a note and interpret results with caution.
- If using a multichannel pipette, always use a new disposable reagent reservoir for the addition of each reagent
- Use fresh disposable pipette tips for each contamination.
- Use a new adhesive plate cover for each incubation step in the ELISA.
- Do not mix reagents from different kit lots. Discard unused ELISA components after use.
- Avoid handling the tops of the wells both before and after filling.
- Avoid exposure of reagents to excessive heat or light during storage and incubation. Avoid microbial contamination.
- Individual components of this assay may contain antibiotics and preservatives. Gloves must be worn while performing the assay to avoid contact with samples and reagents. Please follow proper disposal procedures.
- Do not use glass pipettes to measure the TMB substrate solution. Care must be taken not to contaminate the TMB substrate solution. If the solution is blue prior to use, **DO NOT USE.**

- The total dispensing time for each plate should not exceed 20 minutes.
- Some components contain sodium azide, which may react with copper and lead plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

7. Sample preparation

Handling and storage

Serum; EDTA, heparin and sodium citrate plasma; and culture supernatant may be tested in this ELISA.

- 50 µl of sample or standard per well is required in this assay.
- Samples that are to be assayed within 2–3 hours should be stored at 2–8°C. When storing samples for longer periods of time, freeze them in aliquots at -70°C.
- Test samples and standards must be assayed in duplicate each time the ELISA is performed.
- Avoid repeat freeze thaw cycles when storing samples.
- Bring samples gradually to room temperature before running the assay. **Do not use heated water baths to thaw or warm samples.**
- Mix samples by gently inverting the tubes.
- If samples are clotted, grossly hemolyzed, lipemic or microbially contaminated, make a note and interpret results with caution.
- If testing culture supernatants and using a medium other than RPMI with up to 20% FCS, we recommend that you validate the medium. To perform this validation, prepare two standard curves as described in the assay procedure section, one using the culture medium and the other using sample diluent. If the mean absorbance values for each point on the two curves are within 10% of each other, the medium will not interfere with the assay. If the absorbance values of the two curves differ by more than 10% request further advice from your GE Healthcare representative.

Sample dilution

If it is suspected that the (h)VEGF concentration of a sample will exceed the highest point of the standard curve, 2000 pg/ml, prepare one or more five-fold dilutions of the test sample. For example, a five-fold dilution is prepared by adding 50 µl of test sample to 200 µl of appropriate diluent. When testing culture supernatants, prepare the serial dilutions using your culture medium. When testing serum or plasma, prepare the serial dilutions using the sample diluent provided. Mix thoroughly between dilutions before assaying.

8. Reagent preparation

1. Wash buffer

Label a clean glass or plastic 2 litre container 'Wash buffer.' 30x wash buffer may have a cloudy appearance. Add the entire contents of the 30x wash buffer bottle to the 2 litre container and dilute to a final volume of 1.5 litres with distilled or deionized water. Mix thoroughly. Wash buffer should be stored at room temperature. **Do not use wash buffer if it becomes visibly contaminated during storage.**

2. Standards

a. Two vials of lyophilized standard are provided with this kit. Reconstitute and use one vial per assay. Use standards within one hour of reconstitution. **Do not store reconstituted standards.**

b. Reconstitute standard in distilled or deionized water. The reconstitution volume is stated on the VEGF standard vial label. The standard will take approximately 1 minute to dissolve. Mix by gently inverting the vial until the contents have completely dissolved.

c. Label 8 tubes, one for each standard curve point: 2000, 1000, 500, 250, 125, 62.5, 31.3 and 0 pg/ml. Prepare serial dilutions to generate the standard curve points as follows:

d. Pipette 200 µl of appropriate diluent into each tube. If testing serum, plasma or culture supernatants generated with RPMI containing 0–20% FCS, use the sample diluent provided to prepare standard dilutions.

Note: If testing culture supernatants generated with other types of media or media containing additives, we recommend that the media be validated in order to confirm that it will not interfere with the assay. (See page 11 for validation procedure.)

e. Pipette 200 µl of the reconstituted standard into the first tube, 2000 pg/ml and mix.

f. Pipette 200 µl of this dilution into the next tube, 1000 pg/ml and mix.

g. Repeat serial dilutions (using 200 µl) five more times to complete the standard curve. Do not add standard to the tube marked 0 pg/ml.

9. Protocol

9.1. Sample incubation

a. Determine the number of strips required. Leave these strips in the plate frame. Place the remaining unused strips back in the foil pouch with the desiccant provided. Store the reserved strips at 2–8°C, making sure the foil pouch is tightly sealed. After running the assay, retain the plate frame for the second partial plate. When running the second partial plate, place the reserved strips securely in the plate frame.

b. Record the locations of the human VEGF standards and test samples. Seven standards and one zero must be run in duplicate with each series of unknown samples.

c. Add 50 ml of sample diluent to each well.

d. Add 50 ml standard or sample to each well in duplicate. **If the VEGF concentration in any test sample exceeds the highest point on the standard curve,** 2000 pg/ml, see sample preparation section. Mix well by gently tapping the plate several times.

e. Carefully cover the plate with an adhesive plate cover. Ensure that all edges and strips are sealed tightly by running a thumb over the edges and down each strip. Incubate for 2 hours at room temperature (20–25°C).

f. At the end of the incubation period, carefully remove the adhesive plate cover. Wash the plate **THREE** times with wash buffer, using the procedure outlined in 9.2. below.

9.2. Plate washing

Gently squeeze the long sides of the plate frame when washing the plate to ensure that all strips remain securely in the frame.

Manual wash: Decant the contents of the plate into a sink or other receptacle. Using a squirt bottle, **vigorously fill each well completely with wash buffer,** then decant the contents into a sink or other receptacle. Repeat the procedure two more times for a total of **THREE** washes. Blot on to paper towels or other absorbent material.

Automated wash: Aspirate all wells and wash **THREE** times with wash buffer making sure wells are filled uniformly with wash buffer. Blot on to paper towels or other absorbent material.

9.3. Biotinylated antibody incubation

a. If using a multichannel pipette, use a new reagent reservoir to add the biotinylated antibody reagent. Remove from the vial only the amount required for the number of strips being used.

b. Add 100 µl of biotinylated antibody reagent to all wells being used.

c. Carefully cover the plate with an adhesive plate cover. Ensure all edges and strips are tightly sealed by running a thumb over the edges and down each strip. Incubate for one (1) hour at room temperature, 20–25°C.

d. At the end of the incubation period, carefully remove the adhesive plate cover. Wash the plate **THREE** times with wash buffer, using the procedure outlined in section 9.2.

9.4. Streptavidin-HRP reagent incubation

a. Add 100 µl of streptavidin-HRP reagent to each well. If using a multichannel pipette, use a new reagent reservoir and pipette tips when adding the prepared streptavidin-HRP reagent. Remove from the vial only the amount required for the number of strips used.

b. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate the plate at room temperature (20–25°C) for 30 minutes.

c. At the end of the incubation, carefully remove the adhesive plate cover, decant the plate then wash **THREE** times using the procedure outlined in section 9.2.

9.5. Substrate incubation and stop step

a. Use new disposable reagent reservoirs and pipette tips when adding the TMB substrate solution and stop solution.

b. Dispense from the bottle ONLY the amount required for the number of strips being run, 100 µl per well. Do not use a glass pipette to measure the TMB substrate solution. Do not combine leftover substrate with that reserved for the second half plate. Care must be taken to ensure that the remaining TMB substrate solution is not contaminated.

c. Pipette 100 µl of TMB substrate solution into each well.

d. Allow enzymatic color reaction to develop at room temperature for 30 minutes. **THE PLATE SHOULD BE DEVELOPED IN THE DARK. Do not cover the plate with aluminum foil or a plate sealer.** The substrate reaction yields a blue solution that turns yellow when stop solution is added.

e. After 30 minutes, stop the reaction by adding 100 µl of stop solution to each well.

9.6. Reading the absorbance

Measure the absorbance on an ELISA reader set at 450 and 550 nm. Subtract readings at 550 nm from the readings at 450 nm. Reading at dual wavelengths will correct for optical imperfections in the microplate. If a wavelength correction is not available read the plate at 450 nm.

Note: When the 550 nm adjustment is omitted, OD values will be higher. **THE PLATE MUST BE READ WITHIN 30 MINUTES OF STOPPING THE REACTION.**

10. Calculation of results

a. The standard curve is used to determine the amount of (h)VEGF in an unknown sample. The standard curve is generated by plotting the average absorbance (450–550 nm) obtained for each of the standard concentrations on the vertical (Y) axis versus the corresponding (h)VEGF concentration on the horizontal (X) axis.

b. Calculate results manually using graph paper or with a curve-fitting statistical software package. If using curve-fitting software, plot a four parameter logistic curve fit. If four parameter is not available, a point-to-point curve fit may be used. The amount of VEGF in each sample is determined by interpolating the VEGF concentration (X axis) from the absorbance value (Y axis). If a dilution was performed on a test sample, multiply the value interpolated from the standard curve by the dilution factor to calculate the amount of (h)VEGF in the sample.

c. Optical density values obtained for duplicates should be within 10% of the mean. **Duplicate values that differ from the mean by greater than 10% should be considered suspect and should be repeated.**

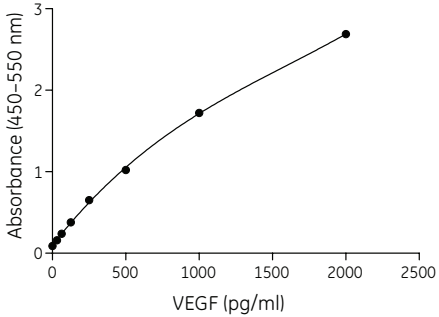

Figure 2. Typical standard curve

11. Additional information

11.1. Sensitivity

<8.0 pg/ml

The sensitivity of this assay, or lower limit of detection (LLD)[1], was determined by assaying replicates of zero and the standard curve. The mean signal of zero + 2 standard deviations read in dose from the standard curve is the LLD. This value is the smallest dose that is not zero with 95% confidence.

11.2. Assay range

31.3–2000 pg/ml

Standard curve points are 0, 31.3, 62.5, 125, 250, 500, 1000 and 2000 pg/ml.

11.3. Calibration

The standards in this ELISA are calibrated to an in-house human VEGF reference preparation.

11.4. Reproducibility

Reproducibility of the Biotrak human VEGF was evaluated in each sample matrix. To determine within-assay precision, 20 replicates of samples containing two levels of recombinant human VEGF were run on a single plate. To evaluate between-assay precision, samples were tested by three operators who performed at least three separate assays on more than one day. Twelve duplicate sample values were used to calculate within-assay precision data for each level of human VEGF. Data is shown in table 1.

[1]*Immunoassay: A Practical Guide*, ed. Chan and Perlstein, Academic Press. p.71, 1987

Table 1. Assay precision

		Within-assay precision			Between-assay precision		
Sample	**Level**	**Mean (pg/ml)**	**SD (pg/ml)**	**CV (%)**	**Mean (pg/ml)**	**SD (pg/ml)**	**CV (%)**
Serum	1	95.8	6.7	7.0	70.7	6.6	9.3
	2	1174	56	4.8	1224	83	6.8
EDTA	1	86.6	8.1	9.3	86.0	7.9	9.2
plasma	2	1161	82	7.1	1337	80	6.0
Citrate	1	80.2	6.2	7.8	71.8	6.8	9.5
plasma	2	1021	61	6.0	1262	107	8.5
Heparin	1	73.8	4.8	6.4	70.2	6.3	9.0
plasma	2	1075	51	4.7	1219	119	9.7
Cell culture	1	72.0	6.3	8.9	74.5	7.3	9.8
supernatant	2	1147	67	5.8	1244	113	9.1

11.5. Specificity

This ELISA is designed for the measurement of natural and recombinant human VEGF165. Isoform VEGF121 cross-reacts 100% in the assay. Other isoforms were not available to assess cross-reactivity at the time of assay development. Recombinant human VEGF/PIGF heterodimer cross-reacts at ~ 20% throughout the standard curve range. This is reflective of the VEGF portion of the heterodimer.

VEGF soluble receptors R1 (Flt-1) and R2 (KDR/Fc chimera) do not exhibit interference at concentrations up to 1500 pg/ml and 15 000 pg/ml respectively.

The following cytokines, tested at 1 µg/ml, did not interfere with or cross-react in the Biotrak (h)VEGF ELISA: human IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12, IL-12p40, IL-13, IL-15,

IL-16, IL-17, IL-18, Eotaxin, G-CSF, GM-CSF, GROα, GROβ, IFNγ, IFNα, MCP-1, MCP-2, MCP-3, MCP-4, MIP-1α, MIP-1β, RANTES, TGFβ, TNF-β or VEGF-D; mouse $VEGF_{120}$ or $VEGF_{164}$.

The following substances, tested at a concentration of 20 µg/ml, did not interfere with this human VEGF ELISA:

Concanavalin A (Con A), phytohaemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), aprotinin, hemoglobin (HGB), beta-mercaptoethanol (β-ME).

11.6. Expected values

A total of 30 serum and plasma samples collected from apparently healthy individuals were run in this assay. The levels of human VEGF found in each sample type are reported below.

Table 2. Expected values

Sample type	n	Samples with detectable VEGF	Mean of detectable samples (pg/ml)	Median (pg/ml)	Range (pg/ml)
Serum	30	30	182.1	196.5	22–420
EDTA plasma	30	12	59.5	ND	ND–86.5
Heparin plasma	30	15	43.4	ND	ND–89.0
Citrate plasma	30	2	10.4	ND	ND–11.9

ND = Not detectable

11.7. Spike and recovery

Human serum and plasma samples and sample diluent controls were spiked with recombinant human VEGF or with natural (h)VEGF from previously assayed synovial fluid. Expected values were calculated by adding endogenous VEGF levels, from unspiked samples, to those of spiked diluent controls. Percent(%) recovery was found by dividing

observed by expected values. Results for representative individual samples and populations are shown in table 3 below.

Table 3. Recombinant VEGF spike

Sample	Level	Expected value range (pg/ml)	Mean recovery (%)	Median recovery (%)	Recovery range (%)	n
Serum	Low	120–163	94.0	93.6	83.4–102.6	8
	High	454–605	103.5	104.0	90.1–114.3	13
EDTA	Low	135–151	89.0	90.0	80.9–99.3	8
plasma	High	432–559	107.0	105.7	91.9–119.6	13
Citrate	Low	136–178	91.6	91.4	82.4–106.0	8
plasma	High	454–547	106.1	102.8	94.2–119.6	13
Heparin	Low	143–214	92.0	86.3	80.4–114.4	8
plasma	High	465–1117	93.5	88.0	84.3–114.7	8

Table 4. Natural VEGF spike

Sample	Expected value range (pg/ml)	Mean recovery (%)	Median recovery (%)	Recovery range (%)	n
Serum	280–699	95.8	93.6	80.5–120.2	11
EDTA plasma	165–495	94.2	94.1	85.8–109.4	10
Citrate plasma	255–661	94.2	94.9	83.0–105.4	12
Heparin plasma	215–636	100.5	98.9	89.7–111.3	10
Cell culture supernatant	270–548	87.9	88.7	80.0–97.7	5

11.8. Linearity of dilution

Twelve human serum and plasma samples spiked with recombinant or natural human VEGF and six cell culture supernatants spiked with natural human VEGF were serially diluted in sample diluent, and evaluated in the human VEGF ELISA kit. Results for a typical evaluation are shown below. Results for heparin and citrate plasma were similar to those shown for EDTA plasma. Observed values were divided by expected values to calculate % recovery and demonstrate the linearity of dilution of the assay.

Table 5. Recombinant VEGF spike

Sample	Dilution	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
Serum 1	Neat	496	–	–
	1:2	248	257	104
	1:4	124	127	102
	1:8	62	55	87
	1:16	31	32	102
EDTA	Neat	446	–	–
plasma 1	1:2	233	222	95
	1:4	117	120	103
	1:8	58	55	94
	1:16	29	32	109

Table 6. Natural VEGF spike

Sample	Dilution	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
Serum	1:2*	538	–	–
	1:4	269	259	96
	1:8	135	148	110
	1:16	67	72	108
EDTA plasma	1:2*	184	–	–
	1:4	92	95	103
	1:8	46	47	102
	1:16	23	25	109
Cell culture supernatant	1:2*	222	–	–
	1:4	111	115	104
	1:8	56	54	98
	1:16	28	31	112

*Due to the viscous properties of synovial fluid used for the natural VEGF spikes, baseline VEGF determinations were made for samples diluted 1:2 with sample diluent.

12. Related products

Biotrak range of Easy ELISA systems

Interferon-alpha [(h)IFNα]	RPN5960
Interferon-gamma [(h)IFNγ]	RPN5961
Interleukin 1β [(h)IL-1β]	RPN5971
Interleukin-2 [(h)IL-2]	RPN5965
Interleukin-2 [(m)IL-2]	RPN5966
Interleukin-6 [(h)IL-6]	RPN5968
Interleukin-8/Neutrophil-activating peptide-1-1 [(h)IL-8/NAP-1]	RPN5969
Interleukin-10 [(m)IL-10]	RPN5963
Interleukin-13 [(h)IL-13]	RPN5972
Monocyte chemoattractant protein-1 [(h)MCP-1]	RPN5964
Transforming growth factor beta-1 [(h)TGFβ_1]	RPN5970
Tumor necrosis factor-alpha [(h)TNFα]	RPN5967

Biotrak range of human cytokine and growth factor ELISA systems

Interleukin-1α [(h)IL-1α]	RPN2750
Interleukin-1β [(h)IL-1β]	RPN2751
Interleukin-6 [(h)IL-6]	RPN2754
Interleukin-8 [(h)]IL-8]	RPN2764
Interleukin-10 [(h)IL-10]	RPN2755
Interleukin-12 p40/70 [(h)IL-12]	RPN2765
Interferon-gamma [(h)IFNγ]	RPN2757
Tumor necrosis factor-alpha [(h)TNFα]	RPN2758

Biotrak range of high sensitivity human cytokine and growth factor ELISA systems

Interferon-gamma [(h)IFNγ]	RPN2787
Interleukin-6 [(h)IL-6]	RPN2784
Interleukin-10 [(h)IL-10]	RPN2785

Tumor necrosis factor-alpha [(h)TNFα]	RPN2788

Biotrak range of mouse cytokine and growth factor ELISA systems

Interferon-gamma [(m)IFNγ]	RPN2707
Interleukin 1β [(m)IL-1β]	RPN2720
Interleukin-4 [(m)IL-4]	RPN2712
Interleukin-6 [(m)IL-6]	RPN2708
Interleukin-12 [(m)IL-12]	RPN2702
Tumor necrosis factor-alpha [(m)TNFα]	RPN2718

Biotrak range of rat cytokine and growth factor ELISA systems

Cytokine-induced neutrophil chemoattractant [(r)GRO/C1NC-1]	RPN2730
Interleukin-1β [(r)IL-1β]	RPN2743
Interleukin-6 [(r)IL-6]	RPN2742
Monocyte chemoattractant protein-1 [(r)MCP-1]	RPN2740
Tumor necrosis factor-alpha [(r)TNFα]	RPN2744

Cell Proliferation assay system and reagents

Cell Proliferation Biotrak ELISA	RPN250
Cell proliferation kit for immunocytochemistry	RPN20
Monoclonal anti-bromodeoxyuridine	RPN202
Cell Proliferation Labeling Reagent	RPN201

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RPN2779PL Rev AE 01-2009