VEGF ELISA

VASCULAR ENDOTHELIAL GROWTH FACTOR

Cat.No. BI-VEGF
12x8 Tests

IMMUNOASSAY FOR THE QUANTITATIVE DETERMINATION OF HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN SERUM, PLASMA, CELL CULTURE SUPERNATANTS, AND URINE

For research use only. Not for use in diagnostic procedures.

This kit was developed and manufactured by:

BIOMEDICA

This package insert must be read entirely before using this product.

Detailed information on the human VEGF ELISA, e.g. assay validation data, sample matrix comparisons, and stability data is available on our website.

Related Products

- Human Angiopoietin-2 ELISA (#BI-ANG2)
- Total soluble Neuropilin-1 ELISA (#BI-20409)
- Soluble Semaphorin 4D ELISA (#BI-20405)

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INTRODUCTION

VEGF PROTEIN

Vascular endothelial growth factor (VEGF or VEGF-A), is a growth hormone secreted by endothelial cells, fibroblasts, smooth muscle cells, platelets, macrophages, and many other cell types (https://ebi16.uniprot.org/uniprot/P15692). It belongs to the cysteine-knot growth factor superfamily (1) and has a molecular weight of about 40 kDa. Currently, 17 different VEGF isoforms have been described to be expressed from one single gene. They are produced by alternative promoter usage/initiation or alternative splicing/proteolysis after protein translation. The N-terminal region is responsible for receptor binding and conserved among all VEGF isoforms. In contrast, residues of the C-terminus differ between isoforms and determine protein length and properties: binding to co-receptor Neuropilin-1 (NRP1) or to extracellular matrix (ECM), agonist/antagonist of angiogenesis. Most isoforms result from the common transcripts: VEGF111, VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206. Additionally, a third VEGF variant (VEGFAx), that demonstrates pro- and anti-apoptotic properties, was described. Thus, vascularization is tightly controlled by the balance of various splice variants, their availability and concentration, whereas isoforms linked to the ECM constitute a reservoir of VEGF that can quickly be shed to circulating forms (1). One of the most potent pro-angiogenic isoforms is VEGF165a. After secretion, 50-70% of VEGF165a is attached to the extracellular matrix (via heparin binding site), the rest is freely diffusible (1) . It is the most abundant isoform and enhances signaling over the VEGFR2 receptor by additionally binding to its co-receptor Neuropilin-1. VEGF A isoforms are glycosylated, homodimeric proteins. Two anti-parallel monomers are linked by intermolecular disulfide bonds (2) whereas eight cysteine residues form a knot-like structure at one end of each monomer (3). However, heterodimerization with PLGF has been described as well (4).

VEGF FUNCTION

Vascular Endothelial Growth Factor (VEGF) has an important role in vasculogenesis/angiogenesis stimulating cell survival, migration and proliferation of endothelial cells (1, 5). Apart from its vascular role, VEGF is also involved in skeletal bone formation and bone repair (6) and in the development/homeostasis of many other organs (respiratory, nervous, renal, heart, reproductive system etc.) (3, 7). Although VEGF is essential for physiologic vascular homeostasis in diverse cells and tissues, it has been demonstrated to be important in the molecular pathogenesis of tumor growth and metastasis and in retinopathy associated with several blinding eye diseases, including age-related macular degeneration (AMD) and diabetic and hypertensive retinopathy (8). Changes of the cellular environment (e.g. oxygen level, presence of other growth factors, cytokines, shear stress etc.) induce VEGF A and VEGF A receptor expression.

VEGF receptors VEGFR1 and VEGFR2 are tyrosine kinases on vascular/non-vascular cells that are essential for transducing VEGF-A signals. Upon receptor homo-/heterodimerization, VEGF-A binds and leads to the phosphorylation of the intracellular receptor domains, thus activating the signal transduction cascade (9) . VEGF induces pro-angiogenic signals mainly via VEGFR2: sprouting angiogenesis and endothelial cell survival. In contrast, VEGFR1 works as a decoy receptor for circulating VEGF and exists as soluble form as well. Furthermore, it can dimerize and inactivate other receptor monomers as it lacks the cytoplasmatic, transducing domain. VEGF has a higher affinity for its decoy receptor than for the main signaling receptor.

INTRODUCTION CONTINUED

Additionally, there are several matrix proteins in the extracellular space that contain VEGF binding sites. The glycoproteins NRP1 and NRP2 are considered as co-receptors that are non-signaling (9).

VEGF-mediated pathogenic effects are primarily due to its effects on vascular permeability and neoangiogenesis-neovascularization (5). A number of therapeutic approaches have thus targeted one or more isoforms of VEGF, the VEGF receptors, or signaling pathways, and some have since led to approval of drugs by regulatory authorities around the world (10).

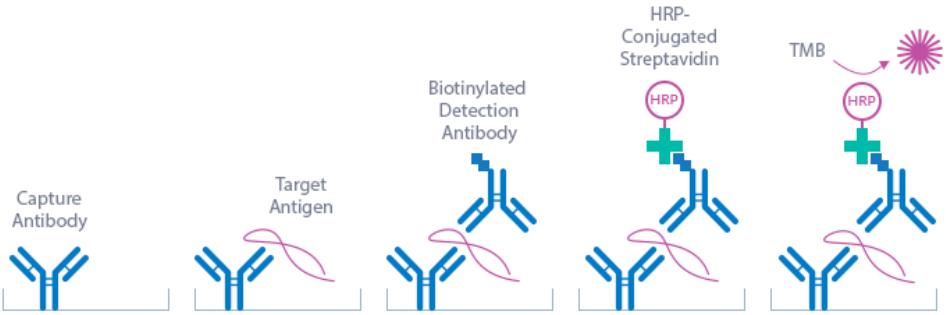
AREAS OF INTEREST

- Cancer
- Metabolic diseases (diabetes and diabetic kidney disease, diabetic retinopathy, obesity)
- Retinal diseases
- Autoimmune & inflammatory diseases (rheumatoid arthritis, psoriasis, psoriatic arthritis)
- Heart and cardiovascular diseases
- Skeletal bone formation and bone repair

ASSAY PRINCIPLE

The Biomedica human Vascular Endothelial Growth Factor (VEGF) ELISA kit is a sandwich enzyme immunoassay that has been optimized and fully validated for the quantitative determination of human VEGF in serum, EDTA plasma, and citrate plasma. Validation experiments have been performed according to international quality guidelines (ICH/ FDA/ EMEA). Cell culture supernatant and urine samples are compatible with this ELISA (data download: www.bmgrp.com). The VEGF ELISA assay recognizes both natural and recombinant human VEGF. The assay employs highly purified epitope mapped antibodies as well as human serum-based standards and controls.

The figure below explains the principle of the human VEGF sandwich ELISA:



In a first step, assay buffer is pipetted into the wells of the microtiter strips. Thereafter, STD/ sample/CTRL are pipetted into the wells, which are pre-coated with the recombinant anti-human VEGF antibody. Any soluble VEGF present in the STD/sample/CTRL binds to the pre-coated anti-VEGF antibody in the well. After incubation, a washing step is applied where all non-specific unbound material is removed. In the next step, the biotinylated anti-VEGF antibody (AB) is pipetted into the wells and reacts with the VEGF present in the sample, forming a sandwich.

Next, all unbound antibody is removed during another washing step. In the following step, the conjugate (streptavidin-HRPO) is added and reacts with the biotinylated anti-VEGF antibody. After another washing step, the substrate (tetramethylbenzidine; TMB) is pipetted into the wells. The enzyme catalysed color change of the substrate is directly proportional to the amount of VEGF present in the sample. This color change is detectable with a standard microtiter plate ELISA reader.

A dose response curve of the absorbance (optical density, OD at 450 nm) versus standard concentration is generated, using the values obtained from the standards. The concentration of soluble VEGF in the sample is determined directly from the dose response curve.

ELISA KIT COMPONENTS

All reagents supplied in the human VEGF ELISA kit are stable at 2-8°C until the expiry date stated on the label of each reagent.

CONTENT	DESCRIPTION	QUANTITY
PLATE	Microtiter strips coated with recombinant VEGF antibody specific for human VEGF in strip holder packed in an aluminum bag with desiccant	12 x 8 tests
WASHBUF	20x wash buffer concentrate, transparent cap	1 x 50 ml
ASYBUF	Assay buffer, **red** cap, ready to use	1 x 18 ml
STD	Recombinant VEGF165 standards (0 / 31.25 / 62.5 / 125 / 250 / 500 / 1000 / 2000 pg/ml), human serum based, white caps, lyophilized	8 vials
CTRL	Control A and B, human serum based, **yellow** cap, lyophilized, exact concentration is stated on labels	2 vials
AB	Polyclonal VEGF antibody specific for human VEGF, biotin-labeled, **green** cap, ready to use	1 x 13 ml
CONJ	Conjugate (streptavidin-HRPO), **brown** cap, ready to use	1 x 13 ml
SUB	Substrate (TMB solution), **blue** cap, ready to use	1 x 13 ml
STOP	STOP solution, white cap, ready to use	1 x 7 ml

ADDITIONAL KIT COMPONENTS

Three self-adhesive plastic films
Quality control protocol
Instruction for use
Plate layout sheet

OTHER SUPPLIES REQUIRED

Precision and multichannel pipettes calibrated to deliver 10 µl, 100 µl, 200 µl, and disposable tips.
Distilled or deionized water.
A plate washer is recommended for washing. Alternatively use a multichannel pipette or manifold dispenser.
A microplate reader capable of measuring absorbance at 450nm (optionally with a correction wavelength at 630nm).
Software for the calculation of results or, alternatively, graph paper.

SAMPLE COLLECTION AND STORAGE

Serum, EDTA plasma, citrate plasma, cell culture supernatants, and urine samples are suitable for use in this assay. Do not change sample type during studies. The sample collection and storage conditions listed are intended as general guidelines.

SERUM & PLASMA

Collect venous blood samples by using standardized blood collection tubes. Perform plasma or serum separation by centrifugation according to supplier's instructions of the blood collection devices. Assay the acquired samples immediately or aliquot and store at -25°C or lower. Lipemic or haemolyzed samples may give erroneous results. Samples are stable for up to five freeze-thaw cycles.

CELL CULTURE SUPERNATANT

Cell culture supernatants should contain at least 1% fetal bovine serum for stability of the VEGF. Remove particles by centrifugation and assay immediately or aliquot and store samples at -25°C or lower. Avoid repeated freeze-thaw cycles.

URINE

Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particles, assay immediately or aliquot and store at -25°C or lower. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

WASH BUFFER

1.	Bring the WASHBUF concentrate to room temperature. Crystals in the buffer concentrate will dissolve at room temperature (18-26°C).
2.	Dilute the WASHBUF concentrate 1:20, e.g., 50 ml WASHBUF + 950 ml distilled or deionized water. Only use diluted WASHBUF when performing the assay.

The diluted WASHBUF is stable up to one month at 4°C (2-8°C).

STANDARDS & CONTROLS FOR SERUM, PLASMA, CELL CULTURE SUPERNATANTS, AND URINE MEASUREMENTS

1.	Pipette **200 µl** of distilled or deionized water into each standard (STD) and control (CTRL) vial. The exact concentration is printed on the label of each vial.
2.	Leave at room temperature (18-26°C) for 15 min. Vortex gently.

Reconstituted STDs and CTRL are stable at -25°C or lower until the expiry date stated on the label. STDs and CTRLs are stable for up to five freeze-thaw cycles.

The standards and controls provided in the kit are suitable for all sample types.

ASSAY PROTOCOL

Read the entire instructions for use before beginning the assay.

All reagents and samples must be at room temperature (18-26°C) before use in the assay.

Mix samples gently to ensure the samples are homogenous. We recommend performing duplicate measurements for all samples, standards and controls.

Mark position for STD/CTRL/SAMPLE (standard/control/sample) on the plate layout sheet.

Take microtiter strips out of the aluminum bag. Store unused strips with desiccant at 4°C (2-8°C) in the aluminum bag. Strips are stable until the expiry date stated on the label.

1.	Pipette **100 µl** ASYBUF (Assay buffer, **red** cap) into each well.
2.	Add **10 µl** of STD/SAMPLE/CTRL (Standard/Sample/Control) in duplicate into respective well. Swirl gently.
3.	Cover tightly and incubate for **2 hours** at room temperature (18-26°C).
4.	Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer, transparent cap). After final wash, remove remaining WASHBUF by strongly tapping plate against paper towel.
5.	Add **100 µl** AB (biotinylated anti-VEGF antibody, **green** cap) into each well. Swirl gently.
6.	Cover tightly and incubate for **1 hour** at room temperature (18-26°C).
7.	Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer, transparent cap). After final wash, remove remaining WASHBUF by strongly tapping plate against paper towel.
8.	Add **100 µl** CONJ (Conjugate, **brown** cap) into each well. Swirl gently.
9.	Cover tightly and incubate for **1 hour** at room temperature (18-26°C).
10.	Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer, transparent cap). After final wash, remove remaining WASHBUF by strongly tapping plate against paper towel.
11.	Add **100 µl** SUB (Substrate, **blue** cap) into each well. Swirl gently.
12.	Incubate for **30 min** at room temperature (18-26°C), in the dark.
13.	Add **50 µl** STOP (Stop solution, white cap) into each well. Swirl gently.
14.	Measure absorbance immediately at 450 nm with reference 630 nm, if available.

PRECAUTIONS

Do not pipette by mouth.
Do not eat, drink, smoke or apply cosmetics where reagents are used.
Refer to the Material Safety Data Sheet (MSDS) available for download at www.bmgrp.com.
All test components of human origin were tested against HIV-Ab, HCV-Ab, and HBsAg and were found negative. Nevertheless, they should be handled and disposed of as if they were infectious.
Avoid all contact with reagents by using protective gloves, clothing and eye protection.
Sulfuric acid contained in the STOP solution may cause irritations to eyes and skin. Avoid contact with skin and mucous membrane. – Flush with water if contact occurs!
Liquid reagents in this assay contain ≤0.1% Proclin 950 as a preservative. Proclin 950 is not toxic in concentrations used in this kit but may cause allergic skin reactions – avoid contact with skin or eyes.

TECHNICAL HINTS

Do not mix or substitute reagents with those from other lots or sources.
Do not mix stoppers and caps from different reagents or use reagents between lots.
Do not use reagents beyond the expiration date.
Protect reagents from direct sunlight.
Substrate solution should remain colorless until added to the plate.
Properly seal plates with the self-adhesive films during incubation steps to ensure accurate results.
Avoid foaming when mixing reagents.

CALCULATION OF RESULTS

Construct a standard curve from the absorbance read-outs of the standards using commercially available software capable of generating a four-parameter logistic (4-PL) fit. Alternatively, plot the standards' concentration on the x-axis against the mean absorbance for each standard on the y-axis and draw a best fit curve through the points on the graph. Curve fitting algorithms other than 4-PL have not been validated and will need to be evaluated by the user.

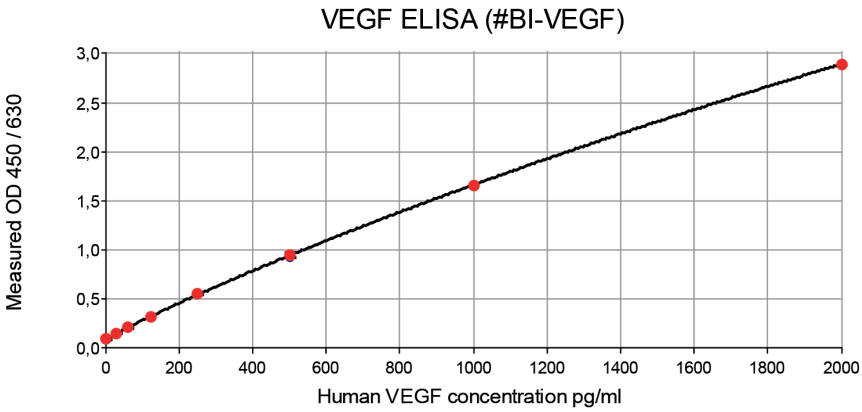
Obtain sample concentrations from the standard curve. If required, pg/ml can be converted into pmol/l by applying a conversion factor (1 pg/ml = 0.052 pmol/l, MW: 19.20 kDa).

Samples with analyte concentrations outside of the calibration range of the assay (2000 pg/ml) should be diluted with assay buffer.

Concentrations of high-measuring samples that have been diluted during sample preparation must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve and the displayed OD values are for demonstration only. A standard curve should be generated for each assay run.



STANDARD	VEGF pg/ml	OD			CV%
		#1	#2	AVERAGE	
STD1	0	0.098	0.090	0.094	6
STD2	31.25	0.151	0.157	0.154	3
STD3	62.5	0.210	0.218	0.214	3
STD4	125	0.322	0.322	0.322	0
STD5	250	0.557	0.545	0.551	2
STD6	500	0.969	0.928	0.949	3
STD7	1000	1.577	1.660	1.619	4
STD8	2000	2.894	2.894	2.894	0

The quality control protocol supplied with the kit shows the results of the final release QC for each kit at the production date. ODs obtained by customers may differ due to various influences including a normal decrease of signal intensity throughout shelf life. However, this does not affect the validity of the results provided an OD of 1.50 or higher is obtained for the standard with the highest concentration, and the measured control values fall into their target range (see labels).

ASSAY CHARACTERISTICS OVERVIEW

Method	Sandwich ELISA, HRP/TMB, 12x8-well detachable strips				
Sample type(s)	Serum, EDTA plasma, citrate plasma, cell culture supernatants, urine				
Sample volume	10 µl sample / well				
Standard range	0 – 2000 pg/ml (0 / 31.25 / 62.5 / 125 / 250 / 500 / 1000 / 2000)				
Sensitivity	LOD: 2.5 pg/ml; LLOQ: 15.6 pg/ml (measurable concentrations in serum AND plasma samples)				
Assay time	2 h / 1 h / 1 h / 30 min				
Precision			**n**	**Average % CV**	
	Within-run		3	≤3	
	In-between-run		3	≤6	
Accuracy (Spike/Recovery of rec human VEGF)			**n**	**Average % recovery**	
				+500 pg/ml	**+250 pg/ml**
	Serum		6	94	94
	EDTA plasma		6	102	92
	Citrate plasma		2	105	92
	Cell culture		2	105	92
	Urine		6	119	n.d.
Parallelism of endogenous human VEGF		**n**	**Average % of expected dilution**		
			1+1	**1+3**	**1+7**
	Serum	6	108	110	109
	EDTA plasma	5	108	107	100
	Citrate plasma	2	103	91	90
	Cell culture	2	93	109	114
	Urine	6	85	n.d	n.d
Specificity	This assay recognizes recombinant and endogenous (natural) human VEGF including all circulating VEGF isoforms (incl. VEGF165b).				
Use	Research use only.				
Values of apparently healthy blood donors			**n**	**Median VEGF (pg/ml)**	
	Serum		23	540	
	EDTA plasma		23	111	
	Citrate plasma		23	79	
	Urine		3	198	

n.d.: not determined

PRECISION

WITHIN-RUN PRECISION

Within-run precision was tested by measuring two samples of known concentrations three times within one VEGF ELISA lot by one operator.

IN-BETWEEN-RUN PRECISION

Within-run precision was tested by measuring two samples of known concentrations three times within different VEGF ELISA lots by different operators.

Within-run (n=3)	Sample 1	Sample 2	In-between-run (n=3)	Sample 1	Sample 2
Mean (pg/ml)	67.8	491.5	Mean (pg/ml)	67.5	500.3
SD (pg/ml)	1.8	9.2	SD (pg/ml)	4.07	20.31
CV (%)	3	2	CV (%)	6	4

SENSITIVITY

LOWER LIMIT OF DETECTION (LOD) & LOWER LIMIT OF QUANTIFICATION (LLOQ)

The LOD is defined as the mean back-calculated concentration of standard 1 (0 pg/ml of VEGF, five independent measurements) plus three times the standard deviation of the measurements.

The LLOQ is defined as the lowest concentration at which an analyte can be accurately quantified. To determine the LLOQ, standard 2, i.e., the lowest standard containing human VEGF is diluted, measured five times and its concentration back calculated.

The following values were determined for the human VEGF ELISA:

LOD	2.5 pg/ml
LLOQ	15.6 pg/ml

CALIBRATION

The human VEGF ELISA kit is calibrated against a highly purified recombinant human VEGF165 protein (Ala27-Arg191; expressed in spodoptera frugiperda 21).
The human serum based calibrator is provided in eight lyophilized glass vials in the following concentrations: 0 / 31.25 / 62.5 / 125 / 250 / 500 / 1000 / 2000 pg/ml.

CALIBRATION using WHO standard

The WHO reference reagent VEGF165/NIBSC code 02/286 (recombinant DNA, human sequence) was analysed in this human VEGF ELISA kit.
The equation below can be used to convert the sample values obtained with this kit to approximate WHO/VEGF165/NIBSC 02/286 units:

WHO/NIBSC (02/286) reference (U/ml) = 0.0006 x BI-VEGF value (pg/ml).

SAMPLE VALUES

SERUM/PLASMA

VEGF was measured in samples from apparently healthy donors (no medical histories were available).

		VEGF [pg/ml]			
Sample Matrix	**n**	**Mean**	**Range**	**Median**	**% Detectable**
Serum*	23	491	130-971	540	100
EDTA plasma	23	103	47-149	111	100
Citrate plasma	23	78	21-152	79	100

**Platelets and leukocytes can release VEGF during blood clotting which is reflected in higher serum sample concentrations compared to plasma samples levels (11, 12).*

It is recommended to establish the normal range for each laboratory.

CELL CULTURE SUPERNATANTS (CCS)

Two human breast cancer cell lines MDA-MB-231, MCF-7 and a human macrophage cell line 4TL9.R were cultured in DMEM/Ham's F12 and RPMI, respectively and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 for 48 hours. Aliquots of the cell culture supernatants were removed, centrifuged to remove particles, and assayed for levels of human VEGF.

Sample Matrix CCS	**VEGF [pg/ml]**
CCS - MDA-MB-231	150
CCS - MCF-7	699
CCS - 4TL9.R	788
DMEM-F-12 (with supplements)	0
RPMI (with supplements)	0

URINE

Seven human urine samples from several donors (apparently healthy and diseased) were measured with this assay and showed a VEGF concentrations between 158 – 372 pg/ml.

For more information please visit our website www.bmgrp.com.

SPECIFICITY

This human VEGF ELISA recognizes recombinant and endogenous (natural) human VEGF including all circulating VEGF isoforms (including VEGF165).

CROSS REACTIVITY with other VEGF Family Members

The low level of sequence homology between the different VEGF family members indicates that other VEGF family members e.g. VEGF-B / VEGF-C / VEGF-D, are not recognized using this ELISA.

For more information please visit our website www.bmgrp.com.

CROSS REACTIVITY with non-human samples

This ELISA was tested in rat, mouse and porcine samples. According to our data the kit cannot be used for the detection of rat and mouse VEGF.

The sequence homology of the capture antibody utilized in the kit (recombinant human VEGF antibody) to the porcine VEGF sequence is 100%. We therefore analysed the presence of endogenous VEGF signal in porcine samples; the specificity was determined by competition.

12 samples from healthy pigs measured with this assay showed a mean VEGF concentration of 33 pg/ml (range: 13 – 53 pg/ml). The mean competition in porcine samples was 100%.

For more information please visit our website www.bmgrp.com.

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NOTES

NOTES

SYMBOLS

	Expiry date / Verfallsdatum / Date de péremption / Data di scadenza /Fecha de caducidad / Data de validade / Uiterste gebruiksdatum / Udløbsdato / Utgångsdatum / Termin Ważności / Lejárati idö / Doba exspirácie / Doba exspirace
	Consider instructions for use / Bitte Gebrauchsanweisung beachten / Consultez la notice d'utilisation / Consultare le istruzioni per l'uso / Consulte las instrucciones de utilización / Consulte as instruções de utilização / Raadpleeg de gebruiksaanwijzing / Se brugsanvisningen / Läs anvisningarna före användning / Proszę przeczytać instrukcję wykonania / Vegyük figyelembe a használati utasításban foglaltakat / Postupujte podl'a pokynov na použitie / Postupujte dle návodu k použití
	Lot-Batch Number / Charge-Chargennummer / Lot-Code du lot / Lotto-Numero di lotto / Lote-Código de lote / Lote-Código do lote / Lot-Partijnummer / Lot-Batchkode / Lot-Satskod / Numer serii / Lot-Batch szám / Číslo šarže / Číslo šarže
	Manufactured by / Hergestellt von / Fabriqué par / Prodotto da / Fabricado por / Fabricado por / Vervaardigd door / Fabrikation af / Tillverkad av / Wyprodukowane pr / Gyártotta / Vyrobené / Vyrobeno
	Catalogue Number / Bestellnummer / Numéro de référence / Numero di riferimento / Número de referencia / Número de referência / Referentienummer / Referencenummer / Katalognummer / Numer katalogowy / Katalógusszám / Katalógové číslo / Katalogové číslo
	Store at between / Lagerung bei zwischen / Conserver à entre / Conservare a tra / Conservar a temp. entre / Armazene a entre / Bewaar bij tussen / Opbevares mellem / Förvaras vid / Przechowywać w / Tároljuk ..... között / Skladujte v rozsahu / Skladujte v rozmezí
	Contains sufficient for x tests / Inhalt ausreichend für x Tests / Contient suffisant pour x tests / Contenuto sufficiente per x test / Contiene suficiente para x pruebas / Contém suficiente para x testes / Bevat voldoende voor x bepalingen / Indeholder tilstrækkeligt til x prøver / Innehållet räcker till x analyser / Zawartość na x testów / Tartalma X teszt elvégzésére elegendö / Obsahuje materiál pre x testov / Obsahuje materiál pro x testu

ASSAY PROTOCOL & CHECKLIST - FOR ALL SAMPLE TYPES

Human VEGF ELISA - # BI-VEGF

REAGENT PREPARATION

	Read the entire instruction for use before beginning the assay.
☐	Bring all reagents to room temperature (18-26°C).
☐	Prepare reagents and samples as instructed.
☐	Bring unused and prepared components to the storage temperature mentioned in the package insert.
☐	Take microtiter strips out of the aluminum bag and mark STD, CTRL, and SAMPLE positions on the plate layout sheet.

ASSAY PROCEDURE

☐	1. Pipette **100 µl** ASYBUF (Assay buffer, red cap) into each well.
☐	2. Add **10 µl of STD/SAMPLE/CTRL** (Standard/Sample/Control) in duplicate into respective well. Swirl gently.
☐	3. Cover tightly and **incubate for 2 hours** at room temperature (18-26°C).
☐	4. Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer, transparent cap). After final wash, remove remaining WASHBUF by strongly tapping plate against paper towel.
☐	5. Add **100 µl** AB (biotinylated anti-VEGF antibody, green cap) into each well. Swirl gently.
☐	6. Cover tightly and **incubate for 1 hour** at room temperature (18-26°C).
☐	7. Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer, transparent cap). After final wash, remove remaining WASHBUF by strongly tapping plate against paper towel.
☐	8. Add **100 µl** CONJ (Conjugate, amber cap) into each well. Swirl gently.
☐	9. Cover tightly and **incubate for 1 hour** at room temperature (18-26°C).
☐	10. Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer, transparent cap). After final wash, remove remaining WASHBUF by strongly tapping plate against paper towel.
☐	11. Add **100 µl** SUB (Substrate, blue cap) into each well. Swirl gently.
☐	12. **Incubate for 30 min** at room temperature (18-26°C), **in the dark**
☐	13. Add **50 µl** STOP (Stop solution, white cap) into each well. Swirl gently.
☐	14. Measure absorbance immediately at 450 nm with reference 630 nm, if available.