BIOMEDICA IMMUNOASSAYS

VEGF ELISA

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.

CONTENTS

ASSAY CHARACTERISTICS OVERVIEW

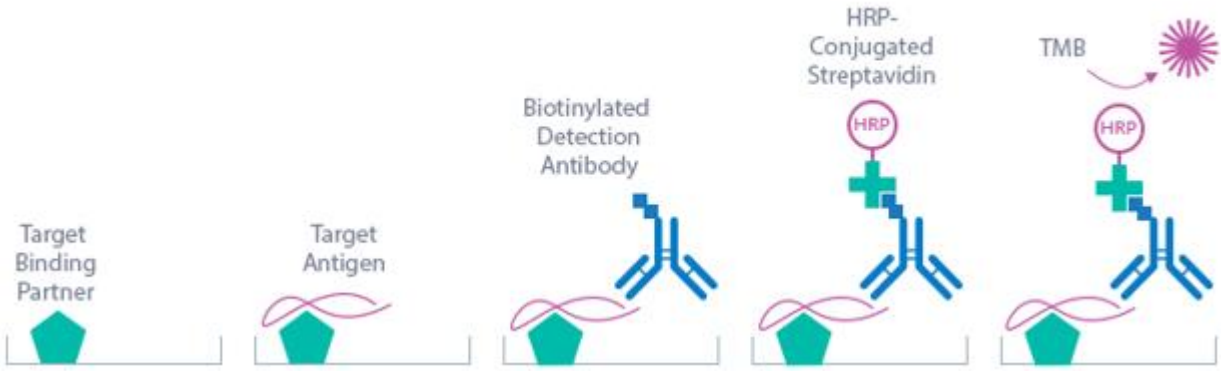
Method	Sandwich ELISA, HRP/TMB, 12x8-well detachable strips			
Sample type(s)	Serum, EDTA plasma, citrate plasma, cell culture supernatants, urine			
Sample volume	10 µl sample / well			
Standard range	0 – 2000 pg/ml (0 / 31.25 / 62.5/ 125 / 250/ 500 / 1000 / 2000)			
Sensitivity	LOD: 2.5 pg/ml; LLOQ: 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 recombinant 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. $VEGF_{165b}$).			
Use	Research use only			
Values of apparantely healthy donors		**n**	**Median VEGF (pg/ml)**	
	Serum	23	540	
	EDTA plasma	23	111	
	Citrate plasma	*23*	79	
	Urine	3	198	

Abbreviation: n.d.: not determined

ASSAY PRINCIPLE

The Biomedica 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. 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.

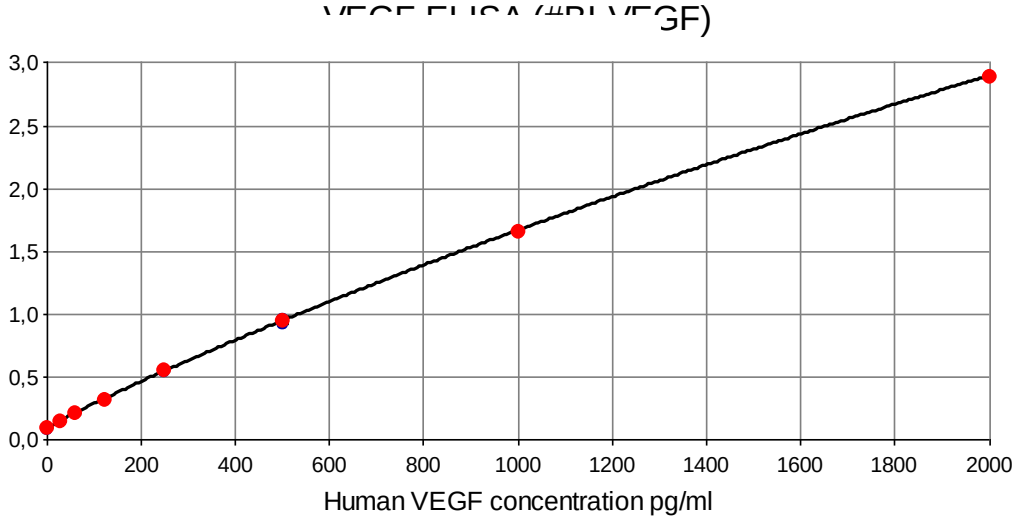
The figure below explains the principle of the human VEGF sandwich ELISA:



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 a 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 next 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 colour 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.

TYPICAL DATA

This standard curve and the displayed Optical Density (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

CALIBRATION

The Biomedica human Vascular Endothelial Growth Factor (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).

DETECTION LIMIT & SENSITIVITY

To determine the sensitivity of the VEGF ELISA, experiments measuring the Lower Limit of Detection (LOD) and the Lower Limit of Quantification (LLOQ) were conducted.

The LOD, also called the detection limit, is the lowest point at which a signal can be distinguished from the background signal, *i.e.,* the signal that is measured in the absence of VEGF, with a confidence level of 99%. It is defined as the mean back-calculated concentration of standard 1 (0 pgl/ml of VEGF, five independent measurements) plus three times the standard deviation of the measurements.

The LLOQ, or sensitivity of an assay, is the lowest concentration at which an analyte can be accurately quantified. The criteria for accurate quantification at the LLOQ are an analyte recovery between 75% and 125% and a coefficient of variation (CV) of less than 25%. To determine the LLOQ, standard 2, *i.e.,* the lowest standards containing VEGF, is diluted, measured five times and its concentration back calculated. The lowest dilution, which meets both criteria, is reported as the LLOQ.

The following values were determined for the human VEGF ELISA:

LOD	2.5 pg/ml
LLOQ	15.6 pg/ml

PRECISION

The precision of an ELISA is defined as its ability to measure the same concentration consistently within the same experiments carried out by one operator (within-run precision or repeatability) and across several experiments using the same samples but conducted by several operators at different locations using different ELISA lots (in-between-run precision or reproducibility).

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.

ID	n	Mean VEGF [pg/ml]	SD VEGF [pg/ml]	CV [%]
Sample 1	3	67.8	1.8	3
Sample 2	3	491.5	9.2	2

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.

ID	n	Mean VEGF [pg/ml]	SD VEGF [pg/ml]	CV [%]
Sample 1	3	67.5	4.07	6
Sample 2	3	500.3	20.31	4

ACCURACY

The accuracy of an ELISA is defined as the precision with which it can recover samples of known concentrations.
The recovery of the human Vascular Endothelial Growth Factor (VEGF) ELISA was measured by adding recombinant human VEGF to samples containing a known concentration of endogenous VEGF. The %recovery of the spiked concentration was calculated as the percentage of measured over the expected value.

This table shows the summary of the recovery experiments in the VEGF ELISA in different sample matrices:

Sample Matrix	n	% Recovery			
		+500 pg/ml rec. VEGF		+250 pg/ml rec. VEGF	
		Mean	Range	Mean	Range
Serum	6	94	89 - 97	94	81 - 129
EDTA plasma	6	102	94 - 114	92	81 - 104
Citrate plasma	2	105	101- 110	92	92 - 92
				+125 pg/ml	
Cell culture supernatant	2	94	89 - 99	77	74 - 81
				+1000 pg/ml	
Urine	6	n.d.	n.d.	119	111 - 134

n.d.: not determined

Experiments:
Recovery of spiked samples was tested by adding 2 concentrations of human recombinant VEGF to different human sample matrices.

Data showing % recovery of recombinant VEGF in human serum samples:

ID	VEGF [pg/ml]			% Recovery	
	Reference	+500 pg/ml	+250 pg/ml	+500 pg/ml	+250 pg/ml
S1	130	n.d.	374	n.d.	104
S2	971	n.d.	1173	n.d.	129
S3	414	685	566	96	82
S4	652	811	779	97	83
S5	164	529	350	89	82
S6	364	646	520	93	81
n.d.: not determined			**Mean R[%]**	**94**	**94**

Data showing % recovery of recombinant VEGF in human EDTA plasma samples:

ID	VEGF [pg/ml]			% Recovery	
	Reference	+500 pg/ml	+250 pg/ml	+500 pg/ml	+250 pg/ml
E1	95	532	303	97	88
E2	166	551	349	94	81
E3	48	514	258	98	87
E4	125	551	343	98	93
E5	40	580	280	112	98
E6	75	605	327	114	104
			Mean R[%]	**102**	**92**

Data showing % recovery of recombinant VEGF in human citrate plasma samples:

ID	VEGF [pg/ml]			% Recovery	
	Reference	+500 pg/ml	+250 pg/ml	+500 pg/ml	+250 pg/ml
C1	20	559	247	110	92
C2	50	529	275	101	92
			Mean R[%]	**105**	**92**

DILUTION LINEARITY & PARALLELISM

Tests of dilution linearity and parallelism ensure that both, endogenous and recombinant samples containing VEGF, behave in a dose-dependent manner and are not affected by matrix effects. Dilution linearity assesses the accuracy of measurements in diluted clinical samples spiked with known concentrations of recombinant analyte. By contrast, parallelism refers to dilution linearity in clinical samples and provides evidence that endogenous analyte behaves in the same way as the recombinant one/likewise to the recombinant analyte. Dilution linearity and parallelism are assessed for each sample type and should be within 20% of the expected concentration.

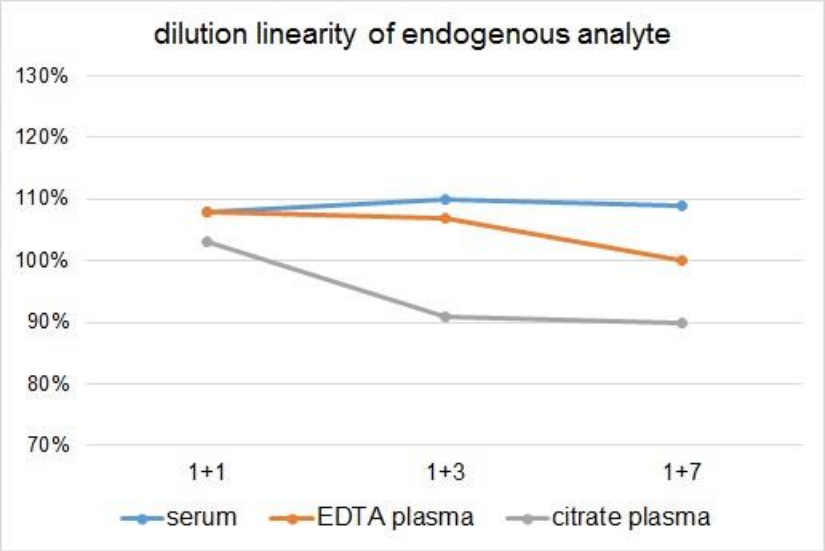
Parallelism

Experiment:
Parallelism was assessed by serially diluting human samples containing **endogenous** VEGF with assay buffer.

Summary table below shows the mean recovery and range of serially diluted endogenous VEGF in several sample matrices:

Sample Matrix	n	% Recovery of endogenous human VEGF in diluted samples					
		1+1		1+3		1+7	
		Mean	Range	Mean	Range	Mean	Range
Serum	6	108	102 - 110	110	103 - 113	109	102 - 115
EDTA plasma	5	108	97 - 114	113	107 - 116	100	93 - 112
Citrate plasma	2	103	99 - 108	91	80 - 102	90	85 - 96
Cell culture supernatant	2	93	89 - 98	109	108 - 110	114	108 - 119
Urine	6	85	70 - 94	n.d.	n.d.	n.d.	n.d.

n.d.: not determined



Data showing dilution linearity of endogenous VEGF in human serum samples:

ID	VEGF [pg/ml]				% Recovery		
	Reference	1+1	1+3	1+7	1+1	1+3	1+7
S1	1153	643	337	159	112	117	110
S2	1002	521	282	144	104	113	115
S3	460	249	123	59	108	107	102
S4	845	465	227	114	110	108	108
S5	591	322	167	85	109	113	115
S6	559	285	144	73	102	103	104
				Mean R[%]	**108**	**110**	**109**

Data showing dilution linearity of endogenous VEGF in human EDTA plasma samples:

ID	VEGF [pg/ml]				% Recovery		
	Reference	1+1	1+3	1+7	1+1	1+3	1+7
E1	324	179	94	38	110	116	93
E2	739	420	213	104	114	115	112
E3	524	291	140	61	111	107	93
E4	55	26.4	n.d.	n.d.	97	n.d.	n.d.
E5	983	532	274	123	108	111	100
E6	324	179	94	38	110	116	93
				Mean R[%]	**108**	**113**	**100**

n.d.: not determined

Data showing recovery of endogenous VEGF in a human citrate plasma samples:

ID	VEGF [pg/ml]				% Recovery		
	Reference	1+1	1+3	1+7	1+1	1+3	1+7
C1	943	467	240	113	99	102	96
C2	198	106	40	21	108	80	85
				Mean R[%]	**103**	**91**	**90**

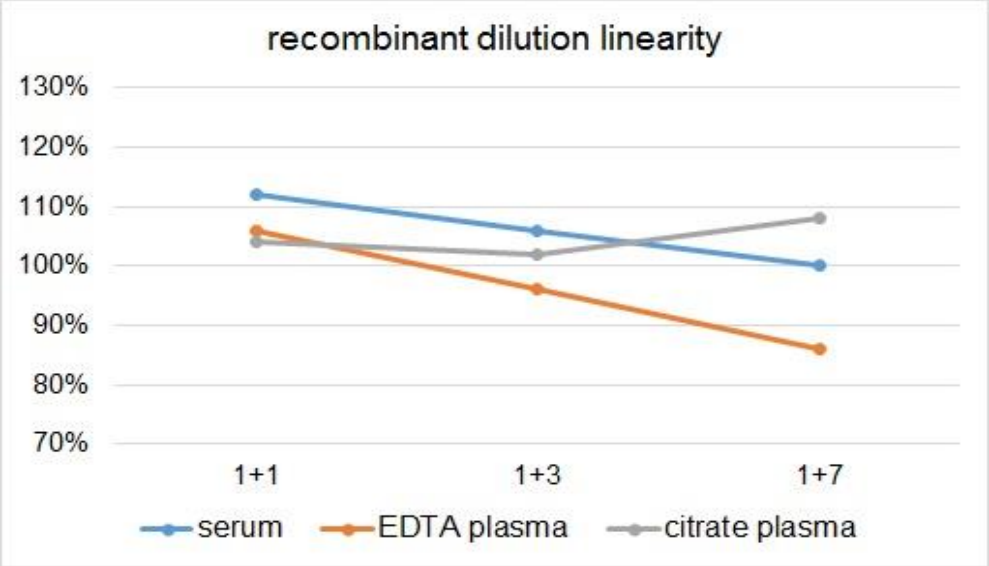
Dilution Linearity

Experiment:
Dilution linearity was assessed by serially diluting samples containing **recombinant** human VEGF with assay buffer.

The figure and table below show the mean recovery and range of serially diluted recombinant VEGF in several sample matrices:

Sample Matrix	n	% Recovery of recombinant VEGF in diluted samples					
		1+1		1+3		1+7	
		Mean	Range	Mean	Range	Mean	Range
Serum	5	112	99 - 118	106	97- 110	100	88- 109
EDTA plasma	5	96	84 - 108	93	83- 102	85	81 - 92
Citrate plasma	2	102	100 - 104	108	105- 111	100	97-103
Cell culture supernatant	2	103	100 - 106	98	96- 99	105	102-107
Urine	2	106	106 - 106	105	98- 113	n.d.	n.d.

n.d.: not determined



Data showing dilution linearity of recombinant VEGF spiked into human serum and plasma samples (ref) containing endogenous VEGF.

Calculation of dilution linearity of spiked serum samples:

ID	VEGF [pg/ml]				% Recovery		
	Reference	1+1	1+3	1+7	1+1	1+3	1+7
S1	1497	885	404	199	118	108	106
S2	1157	660	310	127	114	107	88
S3	1329	770	357	176	116	107	106
S4	1466	724	357	170	99	97	93
S5	1619	915	447	220	113	110	109
n.d.: not determined				**Mean R[%]**	**112**	**106**	**100**

Calculation of dilution linearity of spiked EDTA plasma samples:

ID	VEGF [pg/ml]				% Recovery		
	Reference	1+1	1+3	1+7	1+1	1+3	1+7
E1	580	244	148	67	84	102	92
E2	605	298	142	62	98	94	82
E3	659	282	137	67	86	83	81
E4	1298	702	303	132	108	93	81
E5	1265	653	297	139	103	94	88
				Mean R[%]	**96**	**93**	**85**

Calculation of dilution linearity of spiked citrate plasma samples:

ID	VEGF [pg/ml]				% Recovery		
	Reference	1+1	1+3	1+7	1+1	1+3	1+7
C1	519	17	270	144	67	104	111
C2	560	55	279	148	68	100	105
				Mean R[%]	**102**	**108**	**100**

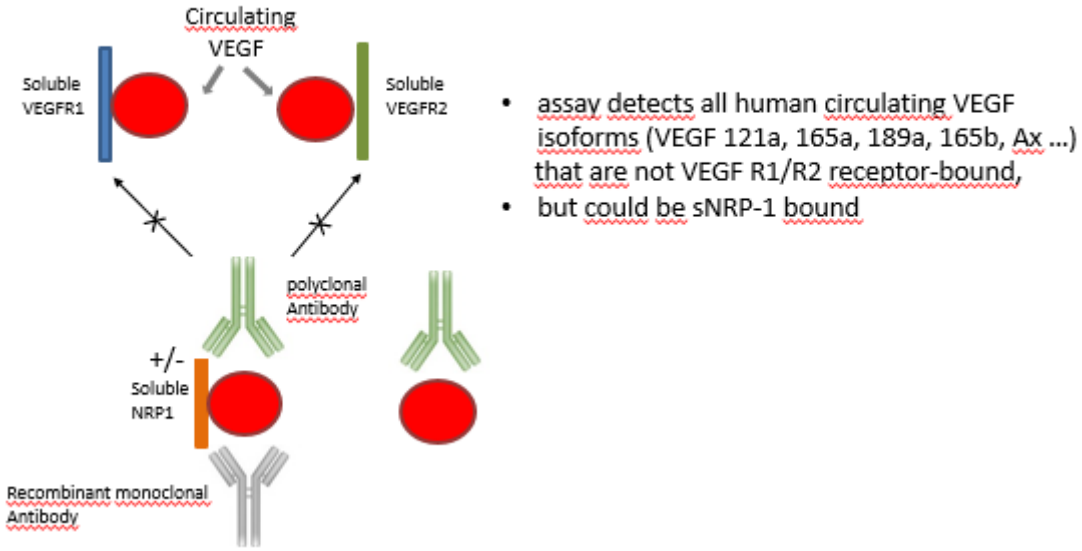
SPECIFICITY

The specificity of an ELISA is defined as its ability to exclusively recognize the analyte of interest.

The specificity of the VEGF ELISA was shown by characterizing both the capture and the detection antibodies through epitope mapping. In addition, the specificity of the ELISA was established through competition experiments, which measure the ability of the antibodies to exclusively bind VEGF.

This assay recognizes recombinant and endogenous (natural) human VEGF including all circulating VEGF isoforms (incl. $VEGF_{165b}$).

The Biomedica human VEGF ELISA detects all human VEGF isoforms that are found in circulation and that are not bound to the soluble decoy receptors VEGFR1 and VEGFR2. However, Neuropilin-1-bound VEGF forms can be quantified, if present. This is due to the binding properties of the employed antibodies. The recombinant anti-human VEGF capture antibody detects a structural epitope near the receptor binding site of the VEGF molecule and hinders the binding of soluble VEGFR1/2 receptors but does not affect the NRP1 epitope. Indeed, no interference at high NRP1 concentrations (10x higher than physiological levels) was observed.
Linear epitopes of the polyclonal anti-human VEGF detection antibody are concentrated within the N-terminal region of the VEGF protein.
As all VEGF isoforms posess conserved N-termini and both antibody epitopes fall within this region, all VEGF isoforms will be detected. This includes all circulating pro- and anti-angiogenic VEGF isoforms (incl. $VEGF_{165b}$ and $VEGF_{Ax}$).



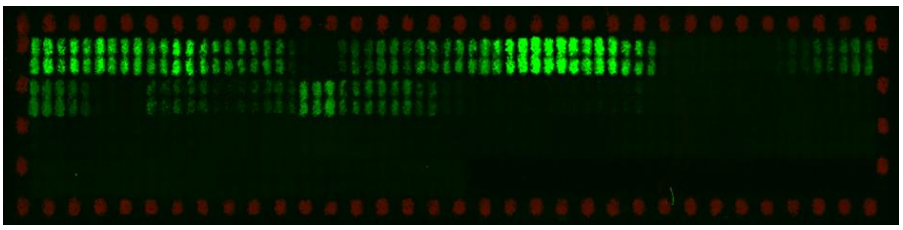
Epitope Mapping

Antibodies were characterized by epitope mapping of linear epitopes with microarray technology and by the determination of binding kinetics with biolayer interferometry.

Capture Antibody: the peptide-specific recombinant capture antibody recognizes a structural epitope in the conserved receptor binding-site of VEGF and thus, specifically binds to all isoforms of VEGF.

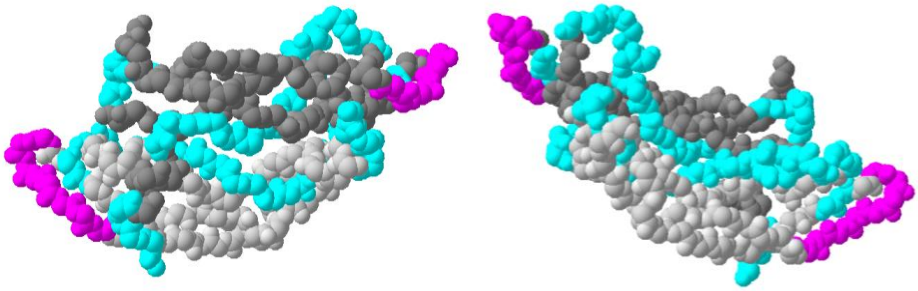
Detection Antibody: Multiple linear epitopes recognized by the polyclonal detection antibody are concentrated in the first 120 amino acids of the VEGF molecule. The polyclonal detection antibody recognizes linear epitopes N-terminal of VEGF.

Microarray of detection antibody



High resolution epitope mapping of the polyclonal detection antibody on human VEGF.
The canonical sequence of VEGF (P15692-1) was printed as 15mers with an 14 amino acid overlap in duplicates on a glass chip. Green fluorescent signals on the microarray illustrate binding of the polyclonal detection antibody to VEGF and corresponds to its epitopes. Red fluorescent signals mark the position of control peptides. The polyclonal detection antibody recognizes linear epitopes of VEGF.

Antibody epitopes on human VEGF molecule



3D structure of human VEGF dimer (V14-K107, pdb 1BJ1) with antibody epitopes. The recombinant capture antibody recognizes a structural epitope (purple) in the conserved receptor binding-site of VEGF (shown as dimer, dark and light grey) and thus, specifically binds to all bioactive isoforms of VEGF. Linear epitopes (blue) of the detection antibody are concentrated in the first 120 amino acids of the VEGF molecule.

Competition of Signal

Competition experiments were carried out by pre-incubating human samples containing endogenous VEGF with an excess of capture antibody (AB). The concentration measured in this mixture was then compared to a reference value, which was obtained from the same sample without the pre-incubation step. Mean competition in serum and plasma samples was 97%.

		VEGF [pg/ml]		% Competition
Sample Matrix	**ID**	**Reference**	**+ capture AB**	
Serum	S1	694	48	93
Serum	S2	242	7	97
Serum	S3	90	0	100
Serum	S4	699	32	95
EDTA plasma	E1	186	0	100
EDTA plasma	E2	740	34	95
Citrate plasma	C1	15	0	100
			Mean R[%]	**97**

Isomer Forms

17 described (+15 computationally mapped) isoforms produced by alternative promoter usage, alternative splicing and alternative initiation with distinct biological activity.

There are 5 dominant isoforms (121, 145, 165, 189, 206). Of these VEGF 165 is the most common VEGF isoform.

The Biomedica human VEGF ELISA detects all human circulating VEGF that are not VEGF R1/R2 receptor-bound.

Ligands

VEGF R1 (FLT1), VEGF R2 (KDR), NRP-1, heparan sulfate, heparin

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.

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.

Porcine VEGF: The sequence homology of the capture antibody utilized in the kit (recombinant human VEGF antibody) to the porcine VEGF sequence is 100%.
The presence of endogenous VEGF signal was analyzed in pig samples; its specificity was determined with a competition experiment by pre-incubating porcine samples containing endogenous VEGF with an excess of capture antibody (AB). The concentration measured in this mixture was then compared to the reference value, obtained from the same sample without the pre-incubation step.

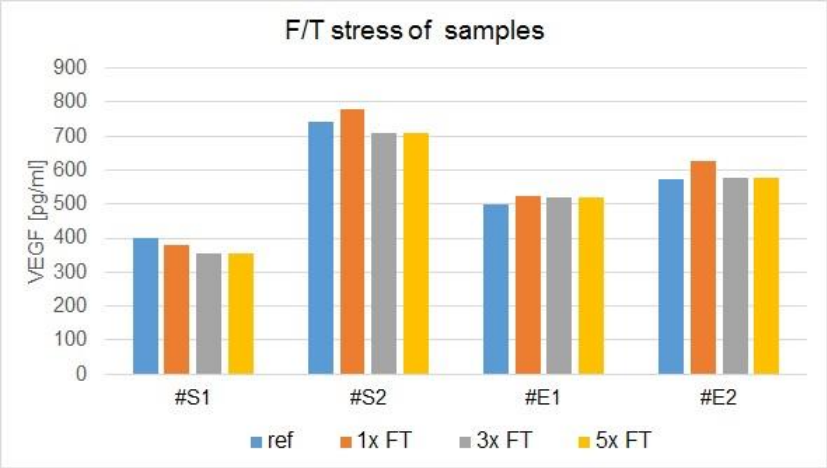
Sample matrix	ID	OD		VEGF [pg/ml]		R [%]
		reference	competition	reference	competition	
pig serum	P1	0.109	0.078	12.96	0.0	100
pig plasma	P2	0.135	0.095	32.43	0.0	100
					Mean R [%]	**100**

Porcine sample values: 12 samples from healthy pigs measured with this assay showed a mean VEGF concentration of 33 pg/ml (range: 13-53 pg/ml).

SAMPLE STABILITY

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. We recommend duplicate measurements for all samples, standards and controls. The sample collection and storage conditions listed are intended as general guidelines.

Freeze-thaw Stability
The stability of endogenous Vascular Endothelial Growth Factor (VEGF) was tested by comparing samples that had undergone five freeze-thaw cycles (F/T).
For freeze-thaw experiments, samples were collected according to the supplier's instruction using blood collection devices and stored at -80°C. Reference samples were freeze-thawed once. The mean recovery of sample concentration after five freeze-thaw cycles is 97%.



VEGF concentrations of samples after freeze-thaw (F/T) cycles:

		VEGF [pg/ml]			% Recovery	
Sample Matrix	**ID**	**Reference**	**1x F/T**	**5x F/T**	**1x F/T**	**5x F/T**
Serum	S1	402	382	355	95	88
Serum	S2	744	778	708	105	95
EDTA plasma	E1	499	523	518	105	104
EDTA plasma	E2	572	626	577	109	101
				Mean R[%]	**103**	**97**

All samples should undergo a maximum of five freeze-thaw cycles.

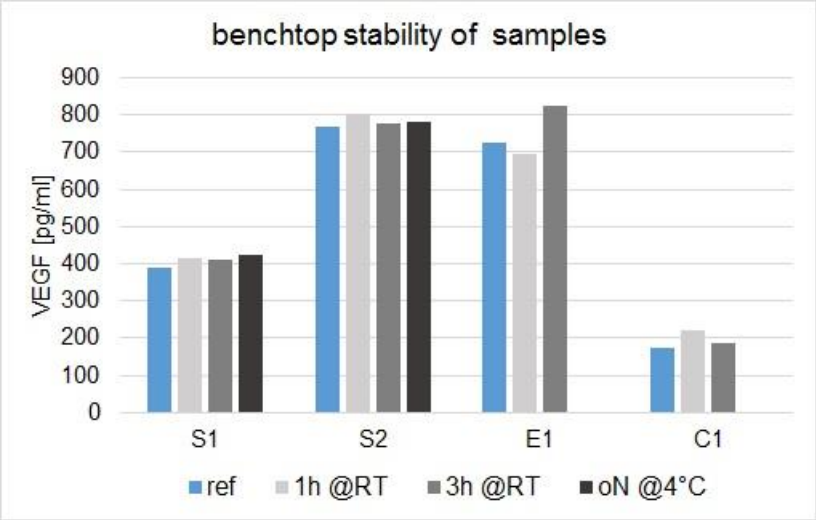
Benchtop Stability

The benchtop stability of endogenous Vascular Endothelial Growth Factor (VEGF) was tested by comparing VEGF measurements in human samples that had been stored at different temperatures.

For the assessment of the benchtop stability, a set of human samples was aliquoted and stored at room temperature or at 4°C. Samples can be stored for at least three hours at room temperature as well as overnight at 4°C. The mean recovery of sample concentrations after overnight storage at 4°C is 102%.

VEGF concentrations of samples stored at -25°C (reference), at room temperature (RT) or overnight (o.n.) at 4°C:

		VEGF [pg/ml]			% Recovery	
Sample Matrix	**ID**	**Reference**	**3h @RT**	**o.n. @4°C**	**3h @RT**	**o.n. @4°C**
Serum	S1	389	411	423	99	103
Serum	S1	771	778	783	97	101
EDTA plasma	E1	727	827	n.d.	119	n.d.
Citrate plasma	C1	175	187	n.d.	85	n.d.
n.d.: not determined				**Mean R [%]**	**100**	**102**



SAMPLE VALUES

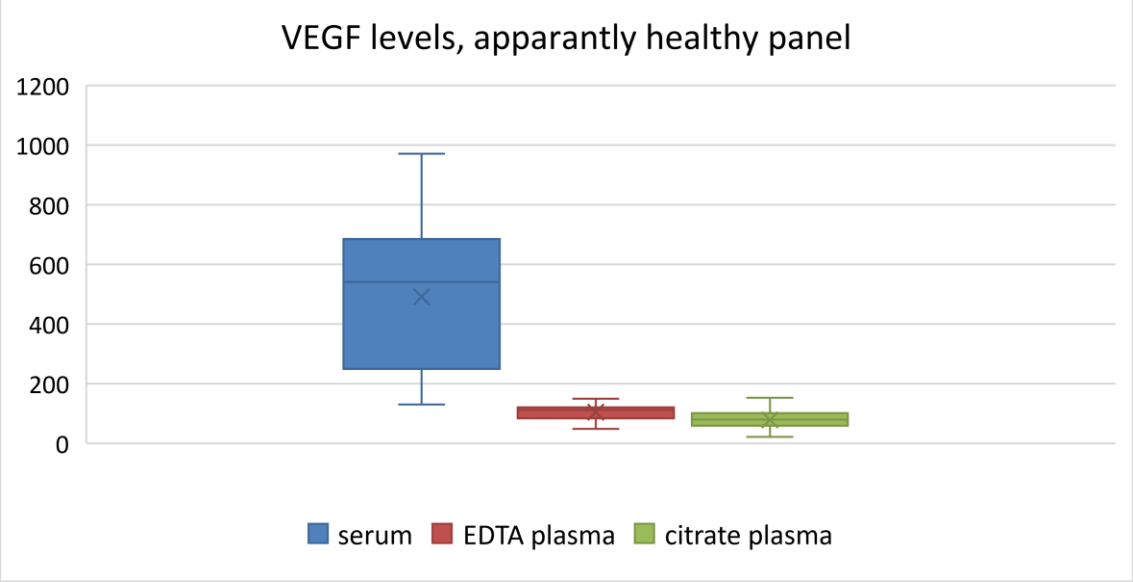
VEGF Values in Apparently Healthy Individuals

To provide values for circulating Vascular Endothelial Growth Factor (VEGF), a panel of samples from apparently healthy donors was tested (no medical histories were available). Each individual donated blood for all tested sample matrices.

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

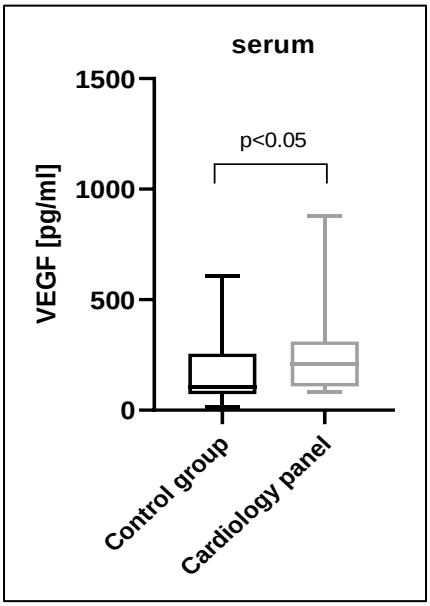
**Platelets and leukocytes can release VEGF during blood clotting which is reflected in higher serum sample concentrations compared to plasma samples levels (Banks RE et al., Gunsilius E et al.).*

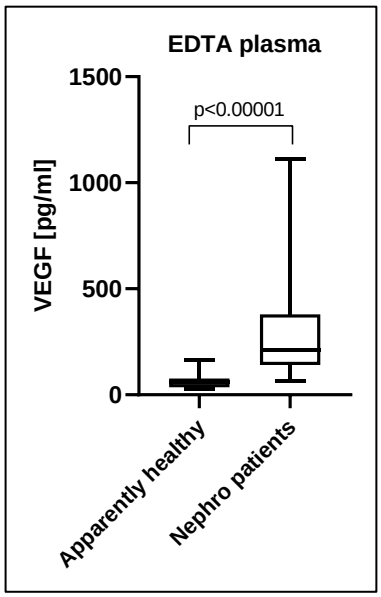
It is recommended to establish the normal range for each laboratory.



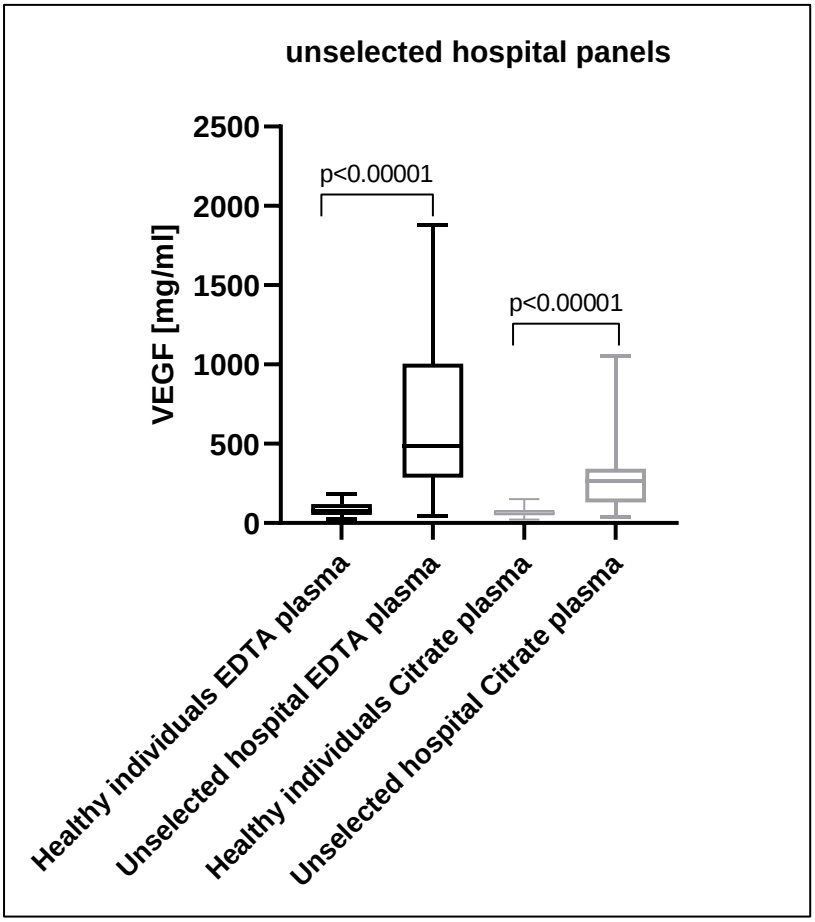
VEGF Values in Disease Panels

In addition to samples of apparently healthy donors, panels of samples from patients with heart disease, kidney disease, as well as a panel of unselected hospital patients were tested.
VEGF values measured in apparently healthy subjects and patients with heart and kidney disease:





VEGF values measured in apparently healthy subjects and in an unselected hospital panel:



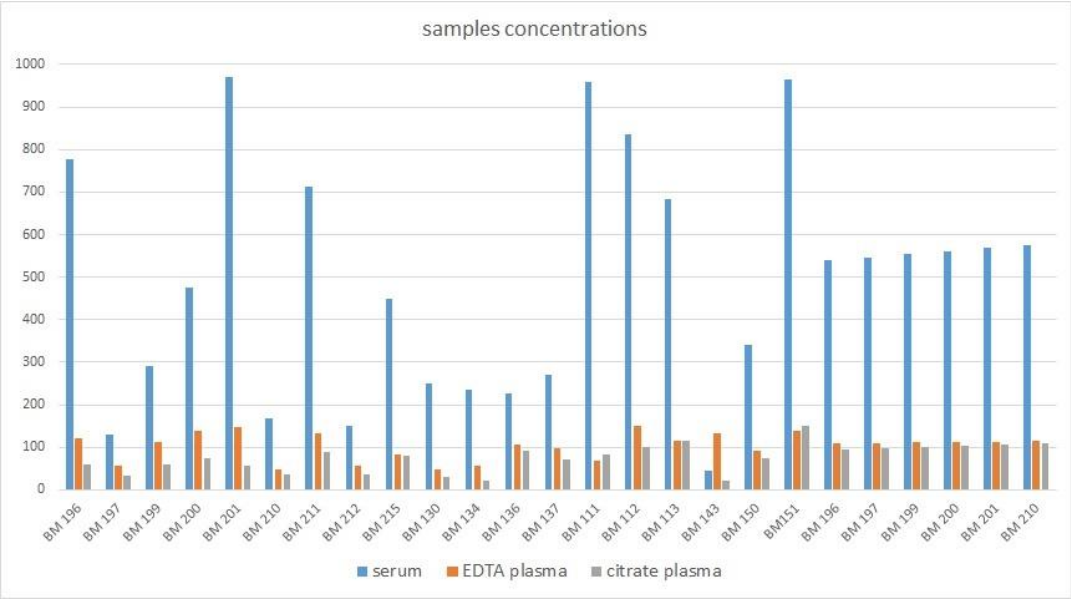
Summary of the results:

		VEGF [pg/ml]		
Samples / Matrix	**n**	**Mean**	**Range**	**Median**
Controls Serum	17	181	17-609	105
HD NHYA 3. 4. Serum	22	268	82-881	210
Apparently healthy EDTA plasma	22	66	29-164	59
Nephro Panel EDTA plasma	32	287	63-1110	212
Apparently healthy EDTA plasma	51	88	29-182	77
Unselected hospital panel EDTA plasma	18	628	45-1878	486
Apparently healthy Citrate plasma	33	66	21-152	62
Unselected hospital panel Citrate plasma	12	302	37-1054	302

MATRIX COMPARISON

To assess whether all tested matrices behave the same way Vascular Endothelial Growth Factor (VEGF) was measured in serum, EDTA plasma, and citrate plasma samples prepared from 23 apparently healthy donors. Each individual donated blood in all tested sample matrices.

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



Measurement of Human VEGF in Urine Samples

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. Urine samples were not normalized to creatinine values.

Sample Matrix Urine	VEGF [pg/ml]
Donor 1. Apparently healthy	171
Donor 2. Apparently healthy	267
Donor 3. Apparently healthy	158
Donor 4. Kidney disease	372
Donor 5. Kidney disease	265
Donor 6. Kidney disease	312
Donor 7. Kidney disease	343

Acccuracy: Data showing % recovery of recombinant VEGF in human urine samples:

ID	VEGF [pg/ml]		% Recovery
	Reference	+1000 pg/ml	
U1	372	1355	117
U2	265	1272	114
U3	312	1348	119
U4	343	1359	119
U5	171	1425	134
U6	267	1246	111
		Mean R [%]	**119**

Dilution linearity, parallelism

Data showing dilution linearity of recombinant VEGF in human urine samples:

Sample ID	VEGF [pg/ml]			R [%]	
	Ref	1+1	1+3	1+1	1+3
U1	1219	646	298	106	98
U2	1129	596	319	106	113
			Mean R [%]	**106**	**105**

Data showing dilution linearity of endogenous VEGF in human urine samples:

Sample ID	VEGF [pg/ml]		R [%]
	Reference	1+1	
U1	372	175	94
U2	265	116	87
U3	312	109	70
U4	343	141	82
U5	171	78	91
U6	267	112	84
		Mean R [%]	**85**

Competition experiments were carried out by pre-incubating human urine samples containing endogenous VEGF with an excess of capture antibody (AB). The concentration measured in this mixture was then compared to a reference value, which was obtained from the same sample without the pre-incubation step. Mean competition in urine samples was 99%.

Competition of endogenous signal:

Sample ID	VEGF [pg/ml]		R [%]
	Reference	+AB	
U1	372	0	100
U2	265	0	100
U3	312	13.6	96
U4	343	3.9	99
U5	171	0	100
U6	267	0	100
		Mean R [%]	**99**

Measurment of Human VEGF in 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
DM-F-12 (with supplements)	0
RPMI (with supplements)	0

Accuracy

Recombinant VEGF was spiked into samples by using STD7 (1000pg/ml). Two concentration levels were generated.

CCS	ID	VEGF [pg/ml]			R [%]	
		Reference	500	125	500	125
MDA-MB-231	CCS#1	150	570	232	99	81
MCF-7	CCS#2	699	793	703	89	74
				Mean R [%]	**94**	**77**

Dilution linearity

Dilution linearity of recombinant analyte of cell culture supernatants.
Samples were spiked with STD 7 (1000pg/ml, +500pg/ml) and diluted with dilution medium (ASYBUF).

CCS	ID	VEGF [pg/ml]				% Recovery		
		Ref	1+1	1+3	1+7	1+1	1+3	1+7
MDA-MB-231	CCS#1	570	286	137	73	100	96	102
MCF-7	CCS#2	793	421	196	108	106	99	109
					Mean R [%]	**103**	**98**	**105**

Dilution linearity of endogenous analyte was tested in conditioned media (48h) of MCF7 and MDA 231 cell lines. Dilution medium is assay buffer .

CCS	ID	VEGF [pg/ml]				% Recovery		
		Ref	1+1	1+3	1+7	1+1	1+3	1+7
MDA-MB-231	CCS#1	150	66	41	22	89	110	119
MCF-7	CCS#2	699	344	189	94	98	108	108
					Mean R [%]	**93**	**109**	**114**

Competition of VEGF in cell culture supernatants

Competition experiments were carried out by pre-incubating human cell culture supernatnant samples containing endogenous VEGF with an excess of capture antibody (AB). The concentration measured in this mixture was then compared to a reference value, which was obtained from the same sample without the pre-incubation step. Mean competition in cell culture supernatants was 93%.

Calculated according to calibration curve prepared from ELISA s standards:

CCS	ID	VEGF (pg/ml)		R [%]
		Ref	+ CAB	
MDA-MB-231	CCS#1	150	15	90
MCF-7	CCS#2	699	23	97
			Mean R [%]	**93**

COMPARISON with another Human VEGF ELISA assay

Assay Characteristics of two different human VEGF ELISA assays

	BIOMEDICA	Another MANUFACTURER
Method	Sandwich ELISA Streptavidin-HRPO/TMB, 12x8-well detachable strips	Sandwich ELISA HRPO/TMB, 12x8-well detachable strips
Sample type	Serum, EDTA plasma, citrate plasma, cell culture supernatants, urine	Serum, plasma, cell culture supernates
Sample volume	**10 µl** sample / well	100 µl (serum/plasma) / well 200 µl (cell culture) / well
Assay time	2 h / 1 h / 1 h / 30 min	2 h / 2 h / 30 min
Assay range	0 – 2000 pg/ml (0 / 31.25 / 62.5 / 125 / 250 / 500 / 1000 / 2000)	0 – 2000 pg/ml
Sensitivity	LOD: 2.5 pg/ml; LLOQ: 15.6 pg/ml *(measurable concentrations in serum AND plasma samples)*	MDD: 5 pg/ml
Specificity	Assay recognizes recombinant and endogenous (natural) human VEGF including all circulating VEGF isoforms (incl. $VEGF_{165b}$).	Assay recognizes natural and recombinant human VEGF. This assay also recognizes recombinant human $VEGF_{165b}$.
Antibodies	Epitope-mapped antibodies Capture antibody: recombinant VEGF antibody specific for human VEGF Detection antibody: polyclonal VEGF antibody specific for human VEGF, streptavidin-HRPO-labeled	Capture antibody: monoclonal antibody specific for human VEGF Detection antibody: polyclonal VEGF antibody specific for human VEGF, HRPO-labeled
Standard matrix	Serum based matrix containing recombinant $VEGF_{165}$ *8 ready to use standards, lyophilized*	Protein based matrix containing recombinant $VEGF_{165}$ *1 stock standard, lyophilized*
Values of apparently healthy samples	Serum median (n=23): 491 pg/ml 100 % detectable EDTA-plasma mean (n=23): 103 pg/ml **100 % detectable**	Serum median (n=37): 220 pg/ml 100 % detectable EDTA-plasma mean (n=37): 61 pg/ml 24 % detectable
Controls	2 controls (high and low) included	Not included
Validation	According to FDA/ICH/EMEA guidelines	Not indicated
Use	RUO	RUO

Comparison of human sample concentrations measured with different human VEGF ELISA Assays

The Biomedica human VEGF ELISA kit (Cat. No. BI-VEGF) was compared with an ELISA kit from another manufacturer. The same panel of samples, consisting of 46 samples (healthy and diseased), were tested.

Pearson coefficient	0.979
P value	0.00001
P value summary	****

Conclusion – Comparison between two VEGF ELISA assays

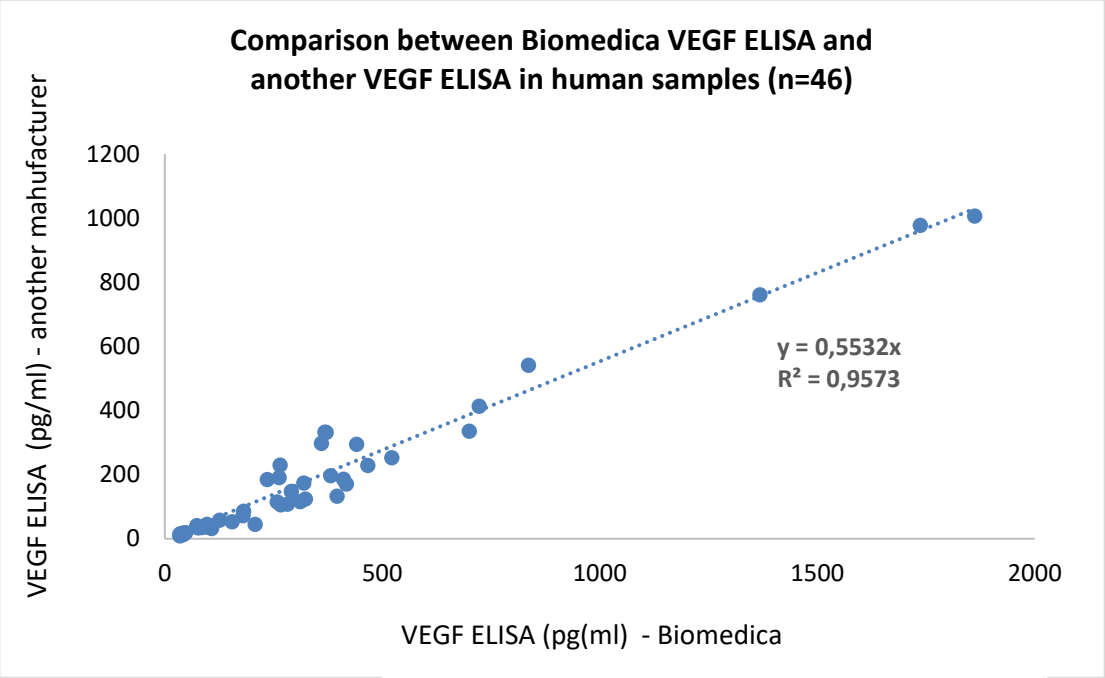
The two commercial ELISA kits tested in the present experiment yielded different absolute values of VEGF in the same serum and plasma samples. Overall the correlation between both kits is very high. The standards in the Biomedica #BI-VEGF kit contain human serum to minimize matrix differences between samples and standards.

a) Apparently healthy EDTA plasma samples (n=12) with the Biomedica ELISA are approximately 2-2.5 x higher than in other assay.
b) Apparently healthy serum samples (n=8) with the Biomedica ELISA are approximately 1.5 x higher than in other assay.
c) Excellent correlation for human serum and plasma samples (healthy and diseased): Pearson correlation coefficient R = 0.979, $p < 0.00001$
d) Cell culture supernatants (n=2): Biomedica and other assay show nearly identical pg/ml concentrations (read-out Biomedica standard curve with standard curve-cell culture from the other manufacturer – following instuctions for use)
e) Concentrations obtained in both kits were converted to approximate NIBSC/WHO 02/286 units. Their comparison shows that Biomedica human VEGF values (U/ml) are approximately 0.5 fold higher for serum samples and 0.7 - 0.8 higher fold for EDTA plasma samples – when compared with other assay.

Table showing human VEGF concentrations measured with the Biomedica human VEGF ELISA and a human VEGF ELISA assay from another manufacturer:

	ALL SAMPLES		
	n = 46		
	Sample ID	Biomedica (BI-VEGF)	Other
Cohorts:		VEGF [pg/ml]	VEGF [pg/ml]
Apparently healhty (AH) samples	AH s1	368	333
	AH s2	723	413
	AH s3	236	185
	AH s4	836	541
	AH s5	360	297
	AH s6	265	230
	AH s7	263	190

	AH s8	371	331
	AH ep9	83	37
	AH ep10	84	35
	AH ep11	76	33
	AH ep12	74	36
	AH ep13	48	19
	AH ep14	97	45
	AH ep15	98	36
	AH ep16	74	41
	AH ep17	42	13
	AH ep18	126	58
	AH ep19	34	14
	AH ep20	180	71
	AH cp21	42	17
	AH cp22	43	14
	AH cp23	34	9
	AH cp24	35	15
Unspecific hospital panel (UHP) samples	UHP ep25	466	229
	UHP ep26	291	147
	UHP ep27	700	336
	UHP ep28	411	185
	UHP ep29	417	171
	UHP ep30	181	86
	UHP ep31	1737	977
	UHP cp32	319	173
	UHP cp33	381	197
	UHP cp34	259	115
	UHP s35	441	294
	UHP s36	1368	760
	UHP s37	1862	1007
	UHP s38	208	44
	N ep39	107	32
Nephrology (N) Samples	N ep40	311	115
	N ep41	323	123
	N ep42	154	53
	N ep43	396	132
	N ep44	282	108
	N ep45	266	105
	N ep46	522	252
		Pearson	**0.979**
		p value	< 0.00001



Comparison of cell culture supernatant sample concentrations in human breast cancer cell lines measured with different human VEGF ELISA Assays

The Biomedica VEGF ELISA (#BI-VEGF) was compared with an ELISA kit from another manufacturer.

Cell Culture Supernatatants (CCS)		
n = 2		
Sample ID	Biomedica (# BI-VEGF)	Other ELISA
	VEGF [pg/ml]	VEGF [pg/ml]
CCS - MDA-231	121.5	101.8
CCS - MCF-7	546.2	624.4

REFERENCES & DOCUMENTS

Validation Literature

The assay is fully validated according to:

1. ICH Topic Q2 (R1) „Validation of Analytical Procedures: Text and Methodology"
2. EMEA/CHMP/EWP/192217/2009 Guideline on bioanalytical method validation
3. Bioanalytical Method Validation, Guidance for Industry, FDA, May 2018

Additional Literature

Banks RE, Forbes MA, Kinsey SE, Stanley A, Ingham E, et al. Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF. Br J Cancer. 1998; 77:956–964. https://pubmed.ncbi.nlm.nih.gov/9528841/

Gunsilius E, Petzer A, Stockhammer G, Nussbaumer W, Petra Schumacher P, Clausen J, Gast G. Thrombocytes Are the Major Source for Soluble Vascular Endothelial Growth Factor in Peripheral Blood. Oncology. 2000; 58, 2: 169–74. https://doi.org/10.1159/000012095.

Additional Documents Available Online (www.bmgrp.com)

Instructions for Use (IFU, package insert)
Material Safety Data Sheet (MSDS)

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