soluble Semaphorin 4D ELISA

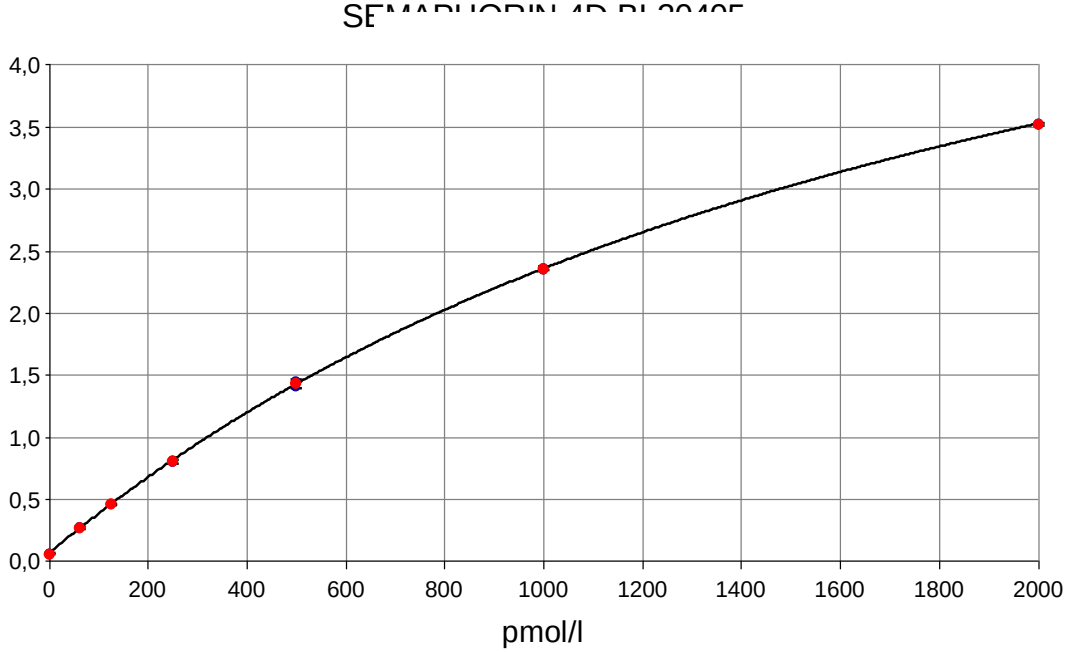
for the quantitative determination of human
soluble Semaphorin 4D in EDTA plasma, heparin plasma, and citrate plasma
Cat. No. BI-20405 . 12 x 8 tests

CONTENTS

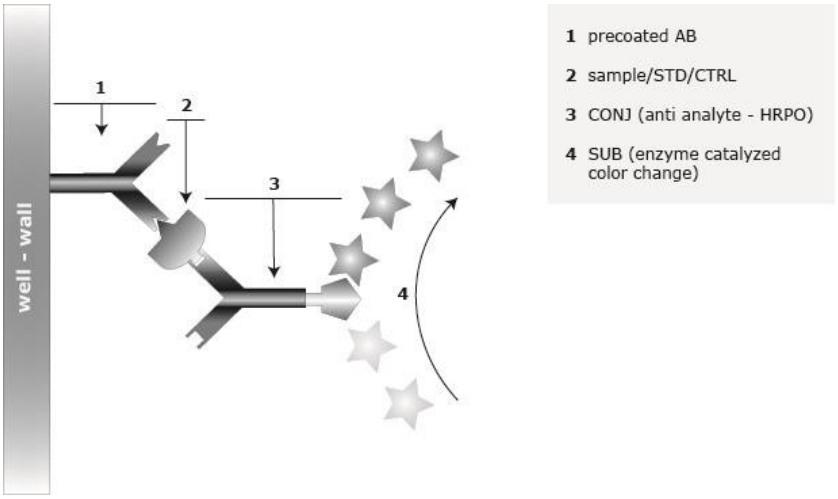
ASSAY CHARACTERISTICS Summary

Method	Sandwich ELISA, HRP/TMB, 12x8-well strips		
Sample type	EDTA plasma, heparin plasma, and citrate plasma		
Standard range	0 to 2,000 pmol/l (0 / 62.5 / 125 / 250 / 500 / 1,000 / 2,000) 7 standards and 2 controls in a human plasma matrix.		
Conversion factor	soluble Semaphorin 4D: 1 pg/ml = 0.0127 pmol/l; 1 pmol/l=78.9 pg/ml (MW: 78.9 kDa)		
Sample volume	10 µl / well		
Incubation time, temp.	3 h / 1 h / 30 min, room temperature		
Sensitivity	LOD: (0 pmol/l + 3 SD): 12 pmol/l; LLOQ: 31 pmol/l		
Specificity	This assay recognizes endogenous and recombinant human soluble Semaphorin 4D.		
Precision	Intra-assay (n=5) ≤ 8% Inter-assay (n=11) ≤ 11%		
Spike/Recovery (recombinant 200 + 1,000 pmol/l Semaphorin 4D)	**Average % recovery**	**200 pmol/l**	**1,000 pmol/l**
	EDTA plasma (n=6)	116	92
	Heparin plasma (n=2)	94	109
	Citrate plasma (n=2)	79	83
Dilution linearity of endogenous soluble Semaphorin 4D	**Average % of expected of dilution:**	**1+1**	**1+3**
	EDTA plasma (n=4):	106	92
	Heparin plasma (n=2):	103	93
	Citrate plasma (n=2):	111	106
Values of apparently healthy individuals	Median EDTA plasma (n=44) = 245 pmol/l Median heparin plasma (n=43) = 192 pmol/l Median citrate plasma (n=28) = 201 pmol/l Each laboratory should establish its own reference range for the samples under investigation. Do not change sample type during the study.		

TYPICAL STANDARD CURVE



PRINCIPLE OF THE ASSAY



CAB Coating Antibody: Monoclonal mouse anti-human Semaphorin 4D IgG antibody
DAB Detection Antibody: Bivalent Fab bacterial alkaline phosphatase fusion antibody-HRP
AG Antigen: human soluble Semaphorin 4D protein (AA22-734 of Q92854 (Uniprot ID))

INFORMATION on the ANALYTE

Human Semaphorin 4D (SEMA4D), also known as CD100, is a type I integral membrane glycoprotein with a molecular weight of 150 kDa. It consists of an extracellular region with a sema domain, a cysteine-rich PSI domain, and an Ig-like C2 type- domain, followed by a transmembrane region, and a cytoplasmatic tail. SEMA 4D forms homodimers due to disulfide linkage, which is essential for successful interaction with its low affinity receptor CD72 primarily found in lymphoid tissues (1) and its high affinity receptors plexin-B1 and plexin-B2 (2, 3). Shedding near the cell membrane releases the extracellular region as 120 kDa bioactive soluble SEMA 4D (sSEMA4D). Shedding can thereby happen spontaneously (4), or it can be the result of proteolytic cleavage by matrix metalloproteases (MMPs) like the Zn-dependent protease ADAM17 (5, 6).

SAMPLE VALUES

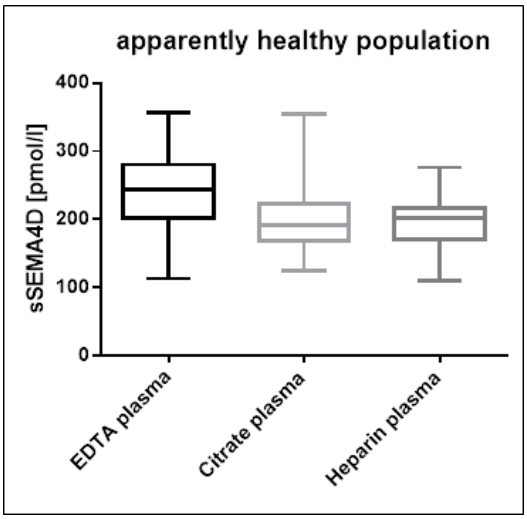
soluble Semaphorin 4D values in an apparently healthy cohort

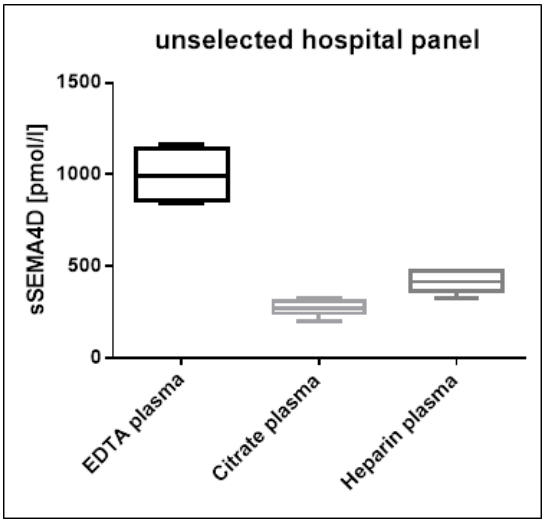
	sSEMA4D [pmol/l]		
	EDTA plasma (n=44)	**Citrate plasma (n=43)**	**Heparin plasma (n=28)**
Mean	239	199	194
Median	**245**	**192**	**201**
5% Percentile	119	130	112
95% Percentile	344	303	270
Minimum	113	125	109
Maximum	357	355	276

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

soluble Semaphorin 4D values in unselected hospital panels

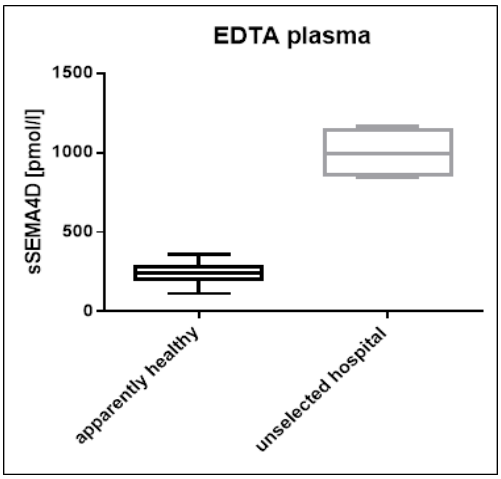
	sSEMA4D [pmol/l]		
	EDTA plasma (n=4)	**Citrate plasma (n=13)**	**Heparin plasma (n=7)**
Mean	997	274	412
Median	**991**	**268**	**417**
5% Percentile	841	199	326
95% Percentile	1165	330	477
Minimum	841	199	326
Maximum	1,165	330	477





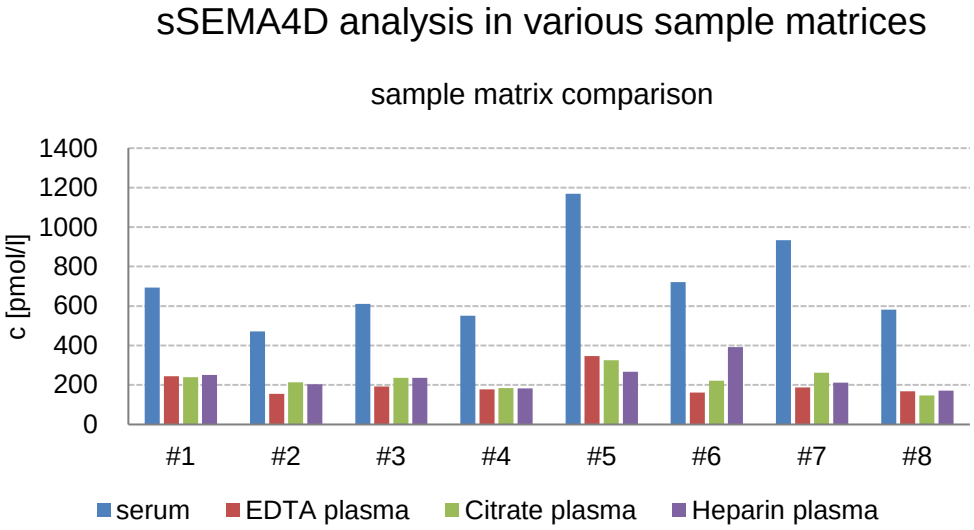
sSEMA4D values in EDTA plasma samples of an apparently healthy cohort compared to a cohort of unselected hospital samples

	EDTA plasma [pmol/l]	
sSEMA4D	**Hopsital panel (n=4)**	**App. healthy (n=44)**
Mean	997	239
Median	**991**	**245**
Minimum	841	113
Maximum	1,165	357



Why we don't recommend serum as matrix to measure soluble Semaphorin 4D?

We analyzed soluble SEMA4D in both serum and plasma samples. Based on our results we do not recommend the use of serum as matrix for sSEMA4D analysis. A comparison between sSEMA4D levels in serum and plasma resulted in significantly elevated sSEMA4D levels in serum. This can be explained that plasma anticoagulants prohibit coagulation-induced platelet activation that might lead to sSEMA4D shedding. *Zhu and colleagues* demonstrated that blood coagulation-related platelet activation, e.g. due to vascular injury in the course of sample collection, leads to increased sSEMA4D surface expression, followed by shedding into the circulation (6). We could demonstrate that plasma is free of shed sSEMA4D and is a suitable matrix for reproducible sSEMA4D quantification (7).



Serum samples were prepared by standard procedure (according to Greiner, Sarstedt blood sample preparation procedure) and showed an approximate tree-fold higher concentration than plasma samples.

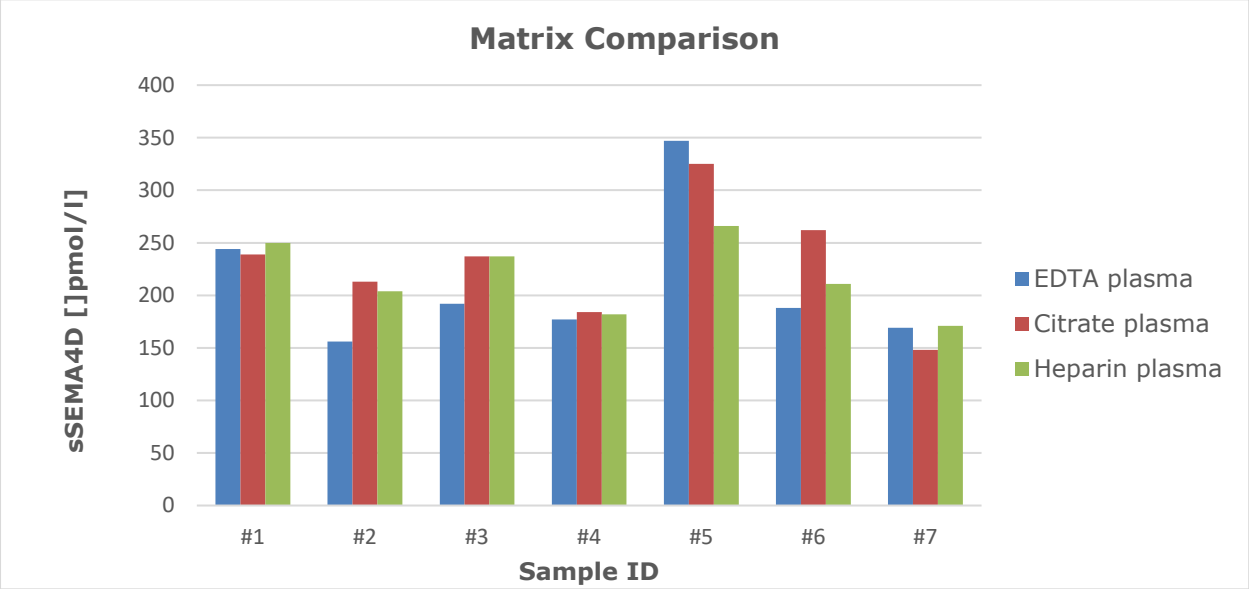
MATRIX COMPARISON

Comparison of Semaphorin 4D plasma sample values from apparently healthy individuals

7 samples of apparently healthy individuals were prepared, each sample derived from one donor. Samples were assayed and the concentrations of the samples were compared.

	soluble Semaphorin 4D [pmol/l]			
Sample ID	**EDTA plasma**	**Citrate plasma**	**Heparin plasma**	**CV [%]**
#1	244	239	250	2
#2	156	213	204	13
#3	192	237	237	9
#4	177	184	182	2
#5	347	325	266	11
#6	188	262	211	14
#7	169	148	171	6
			Mean CV [%]	**8**

Graph showing soluble Semaphorin 4D levels in various sample matrices



ASSAY PERFORMANCE CHARACTERISTICS

RECOVERY

Summary of data showing mean recovery of soluble Semaphorin 4D

Matrix	Mean S/R [%]	
	+200 pmol/l	+1,000 pmol/l
EDTA plasma (n=6)	116	92
Heparin plasma (n=2)	94	109
Citrate plasma (n=2)	79	83

Experiments:
Recovery of spiked samples was tested by adding 2 concentrations of human recombinant Semaphorin 4D (200 + 1,000 pmol/l) to different human plasma sample matrices.

Data showing spike/recovery of human EDTA plasma samples

Sample ID	Spike sSEMA4D [pmol/l]			S/R [%]	
	0	200	1,000	200	1,000
#E1	323	527	1,367	102	104
#E2	244	480	1,032	118	79
#E3	337	608	1,208	136	87
#E4	378	634	1,360	128	98
#E5	413	626	1,322	106	91
#E6	261	469	1,175	104	91
			Mean R [%]	**116**	**92**

Data showing spike/recovery of human heparin plasma samples

Sample ID	Spike sSEMA4D [pmol/l]			S/R [%]	
	0	200	1,000	200	1,000
#H1	297	458	1,117	80	82
#H2	314	469	1,144	78	83
			Mean R [%]	**79**	**83**

Data showing spike/recovery of human citrate plasma samples

Sample ID	Spike sSEMA4D [pmol/l]			S/R [%]	
	0	200	1,000	200	1,000
#C1	258	470	1,399	106	114
#C2	356	520	1,387	82	103
			Mean R [%]	**94**	**109**

LINEARITY

Dilution linearity of samples containing endogenous soluble Semaphorin 4D

Matrix	Mean R of dilution steps [%]		
	1+1	1+3	1+7
EDTA plasma (n=4)	106	92	99
Citrate plasma (n=2)	110	109	121
Heparin plasma (n=2)	103	93	133

- *We recommend diluting high measuring samples (outside of the calibration range) with ASYBUF (assay buffer, supplied in the kit).*

Experiment:
Dilution linearity was assessed by serially diluting samples containing endogenous soluble Semaphorin 4D with assay buffer.

Data showing the dilution of endogenous Semaphorin 4D in **EDTA plasma** samples

Sample ID	sSEMA4D [pmol/l]				R [%]		
	ref	1+1	1+3	1+7	1+1	1+3	1+7
#E1	789	425	210	104	108	106	105
#E2	967	608	200	126	126	83	104
#E3	1,106	515	252	133	93	91	97
#E4	976	471	217	109	96	89	90
			Mean R [%]		**106**	**92**	**99**

Data showing the dilution of endogenous Semaphorin 4D in **citrate plasma** samples

Sample ID	sSEMA4D [pmol/l]				R [%]		
	ref	1+1	1+3	1+7	1+1	1+3	1+7
#C1	274	153	78	41	112	114	119
#C2	272	148	70	42	109	104	124
			Mean R [%]		**110**	**109**	**121**

Data showing the dilution of endogenous Semaphorin 4D in **heparin plasma** samples

Sample ID	sSEMA4D [pmol/l]				R [%]		
	ref	1+1	1+3	1+7	1+1	1+3	1+7
#H1	403	197	77	45	98	77	89
#H2	326	175	90	72	107	110	177
			Mean R [%]		103	93	133

Dilution linearity of samples containing recombinant sSEMA4D

Sample ID	Rec sSEMA4D 1,000 pmol/l					R [%]			
	ref	1+1	1+3	1+7	1+15	1+1	1+3	1+7	1+15
#E1	1,311	678	272	91	71	103	83	56	86
#E2	971	525	279	98	69	108	115	81	114
#E3	1,124	546	297	103	56	97	106	73	80
#E4	1,150	603	310	137	63	105	108	95	88
#E5	1,159	502	293	148	64	87	101	102	88
#E6	1,122	663	349	119	75	118	124	85	107
				Mean R [%]		103	106	82	94

Recommendations for sample dilution:

- High measuring samples outside of the calibration range of the curve should be diluted with ASYBUF (assay buffer, supplied in the kit).

PRECISION

Intra-assay precision & Inter-assay precision

Intra-assay (n=5) ≤ 8%, Inter-assay (n=11) ≤ 11%

Intra-assay: 2 samples of known concentrations were tested 5 times with 1 kit lot by 1 operator.
Inter-assay: 2 samples of known concentrations were tested 3 times with 2 different kit lots on 3 days by 3 different operators.

Intra-assay (n=5)	Sample 1	Sample 2
Mean (pmol/l)	126	1,003
SD (pmol/l)	10.4	63.8
CV (%)	8	6

Inter-assay (n=11)	Sample 1	Sample 2
Mean (pmol/l)	134	1,012
SD (pmol/l)	14.5	55.1
CV (%)	11	5

SENSITIVITY

Limit of detection (LOD)

The LOD is defined as the mean value of the back calculated concentration plus three times the standard deviation. The LOD of the soluble Semaphorin 4D ELISA is **12 pmol/l**.

Lower limit of quantification (LLOQ)
The lower limit of quantification is defined as the accuracy of the back calculated concentrations and shall not exceed ±25% (acc. to ICH [Ref. 1]).
The LLOQ of the soluble Semaphorin 4D ELISA is **31 pmol/l**.

SAMPLE STABILITY

Sample preparation

Collect venous blood samples by using standardized blood collection tubes for plasma. Perform plasma separation by centrifugation according to supplier's instructions of the blood collection devices as soon as possible.
The acquired plasma samples should be measured as soon as possible. For longer storage aliquot samples and store at -25°C or lower for long time storage at -80°C. All samples should undergo only 4 freeze-thaw cycles.

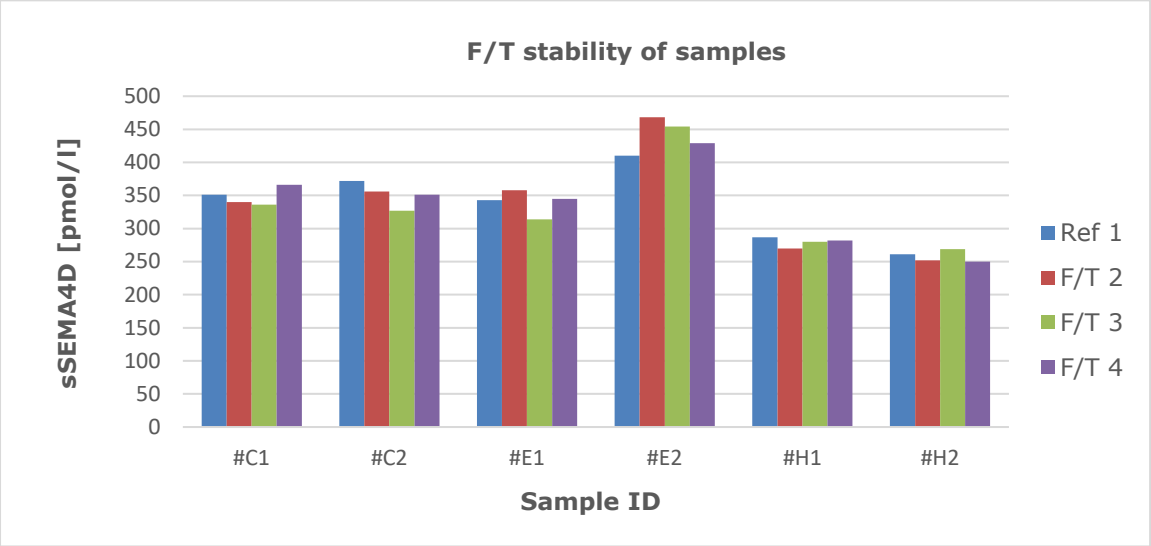
Freeze/thaw of plasma samples containing endogenous soluble Semaphorin 4D

A set of samples (2 EDTA plasma, 2 citrate plasma, 2 heparin plasma) was aliquoted and freeze-thaw stressed. The reference samples are freeze thawed once. Samples can undergo 4 freeze-thaw cycles. The mean recovery of sample concentrations stressed by 4 F/T cycles is 100%.

Plasma samples can undergo 4 freeze-thaw cycles.

Data showing sSEMA4D concentrations of samples after freeze-thaw cycles:

Sample ID	sSEMA4D [pmol/l]				R [%] 4 F/T vs ref
	reference	2x	3x	4x	
#C1	351	340	336	366	96
#C2	372	356	327	351	106
#E1	343	358	314	345	99
#E2	410	468	454	429	96
#H1	287	270	280	282	102
#H2	261	252	269	250	105
				Mean R [%]	**100**



CHARACTERIZATION OF THE ANTIBODIES

CAB Coating Antibody: Monoclonal mouse anti-human Semaphorin 4D IgG antibody binding to region AA30-AA34

DAB Detection Antibody: Bivalent Fab bacterial alkaline phosphatase fusion antibody-HRP binding to region AA 238-241.

SPECIFICITY

The specific interaction of the analyte with the coating and the detection antibody was analysed. This assay recognizes endogenous (natural) and recombinant human soluble Semaphorin 4D.

ISOFORMS
Isoform 1 and 2 are identical between AA 1-554. The epitopes of the antibodies utilized in this ELISA are situated in this area.

CALIBRATION

This immunoassay is calibrated against recombinant human soluble Semaphorin 4D protein (AA22-734 of Q92854 (Uniprot ID)).

VALIDATION GUIDELINES

The assay is fully validated for human plasma samples according to ICH Q2 (R1) (8).

LITERATURE

1. Increased surface expression of a newly identified 150-kDa dimer early after human T-lymphocyte activation. *Bougeret C et al., J Immunol Baltim Md, 1992; 15;148(2):318–323.*
2. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Kumanogoh A et al., Immunity, 2000; 13(5):621–631.*
3. Structural basis of semaphorin-plexin signalling. *Janssen BJC et al., Nature, 2010; 28; 467(7319):1118–1122.*

4. Biological activity of soluble CD100. I. The extracellular region of CD100 is released from the surface of T lymphocytes by regulated proteolysis. *Elhabazi A et al., J Immunol Baltim Md, 2001; 1;166(7):4341–4347.*
5. Identification of a calmodulin-binding domain in Sema4D that regulates its exodomain shedding in platelets. *Mou P et al., Blood, 2013; 16;121(20):4221–4230.*
6. Regulated surface expression and shedding support a dual role for semaphorin 4D in platelet responses to vascular injury. *Zhu L et al., Proc Natl Acad Sci, 2007; 30;104(5):1621–1616.*
7. Analytical performance evaluation of a high-sensitivity enzyme immunoassay for soluble human semaphorin 4D in plasma. *Laber et al., 2018; submitted*.
8. CPMP/ICH/381/95 ICH Topic Q2 (R1) „Validation of Analytical Procedures: Text and Methodology" including: ICH Q2A "Text on Validation of Analytical Procedures" ICH Q2B "Validation of Analytical Procedures: Methodology"

Available on our Website **www.bmgrp.com**

Instructions for use (IFU)
Material Safety Data Sheet
soluble Semaphorin 4D ELISA – Info Leaflet

Date: April 2018