bioactive Sclerostin ELISA

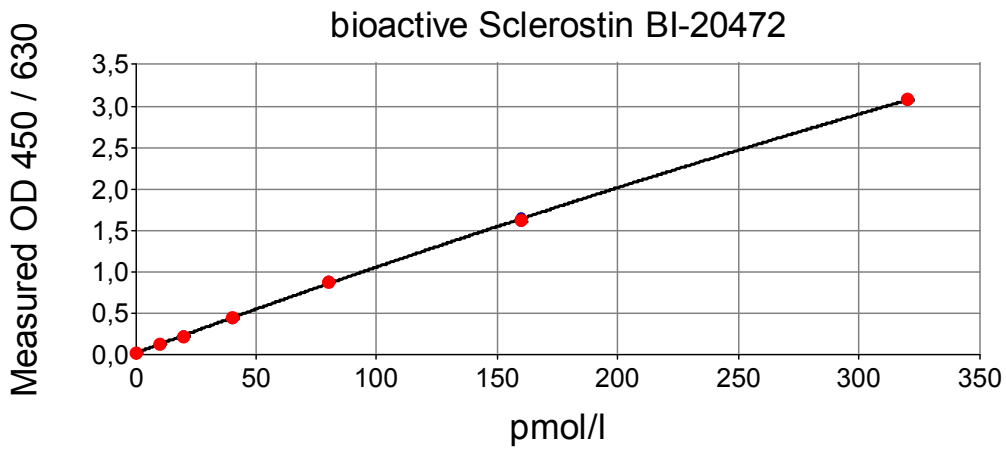
for the quantitative determination of human bioactive Sclerostin
in serum, EDTA plasma, and citrate plasma
Cat. No. BI-20472 . 12 x 8 tests

CONTENTS

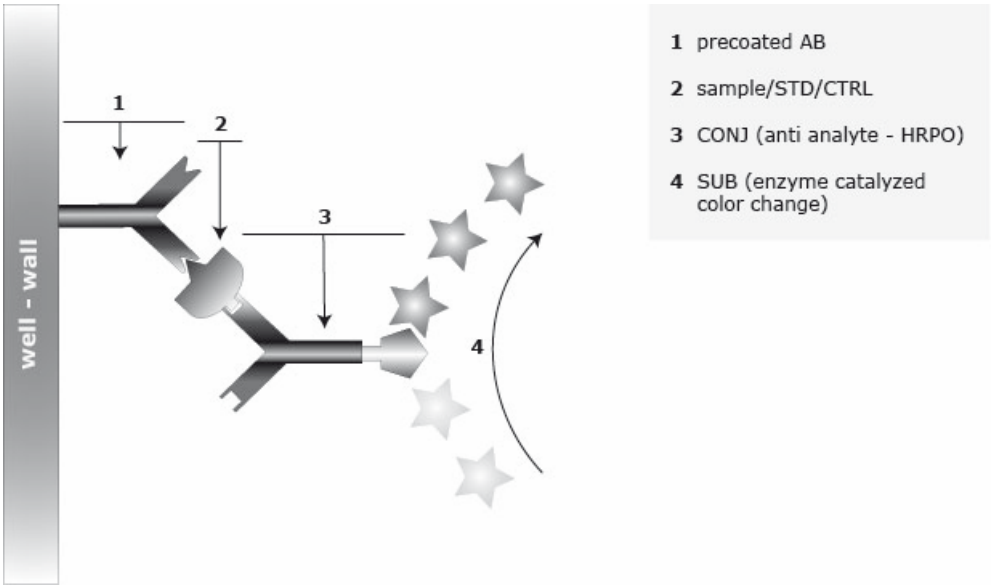
ASSAY CHARACTERISTICS Summary

Method	Sandwich ELISA, HRP/TMB, 12x8-well strips			
Sample type	Serum, EDTA plasma, and citrate plasma			
Standard range	0 to 320 pmol/l (7 standards and 2 controls in a human serum matrix. (Standards: 0/10/20/40/80/160/320 pmol/l)			
Conversion factor	1 pg/ml = 0.044 pmol/l (MW: 22.5 kDa) 1 pmol/l = 22.5 pg/ml			
Sample volume	20 µl / well			
Incubation time, temp.	2 h / 1 h / 30 min, room temperature			
Sensitivity	LOD: (0 pmol/l + 3 SD): 1.9 pmol/l; LLOQ: 1.3 pmol/l			
Specificity	This assay recognizes endogenous and recombinant human bioactive Sclerostin.			
Precision	Intra-assay (n=3) ≤ 1%, Inter-assay (n=7) ≤ 5%			
Spike/Recovery	Average % recovery spiked with 26 and 110 pmol/l, respectively	Serum (n=5): 93%, 86% EDTA plasma (n=5): 94%, 93% Citrate plasma (n=1): 104%, 99%		
Dilution linearity of recombinant bioactive Sclerostin	Average % of expected of dilution:	1+1	1+3	1+7
	Serum (n=6):	98	86	89
	EDTA plasma (n=6):	102	99	91
	Citrate plasma (n=1):	119	132	103
Dilution linearity of endogenous bioactive Sclerostin	Average % of expected of dilution:	1+1	1+3	1+7
	Serum (n=7):	100	103	106
	EDTA plasma (n=6):	105	108	123
	Citrate plasma (n=2):	91	91	103
Bioactive Sclerostin values of apparently healthy individuals	Median serum (n=32): 61.5 pmol/l Median EDTA plasma (n=24): 87 pmol/l Median citrate plasma (n=24): 61.5 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 Capture Antibody: recombinant human monoclonal antibody
DAB Detection Antibody: polyclonal goat antibody
STD Standard: recombinant human bioactive Sclerostin protein (AA24-AA213) in human serum

Detailed information on the antibodies utilized in this ELISA can be found on pages 12-13.

SAMPLE VALUES

bioactive Sclerostin levels in an apparently healthy cohort

	Serum (n=32)	EDTA plasma (n=24)	Citrate plasma (n=24)
Mean	70.8	103.9	72.8
Median	61.5	87	61.5
Percentile 95%	143.4	225.8	165.3
Percentile 5%	12.5	29.2	19.2
Minimum	8	27	18
Maximum	183	235	166

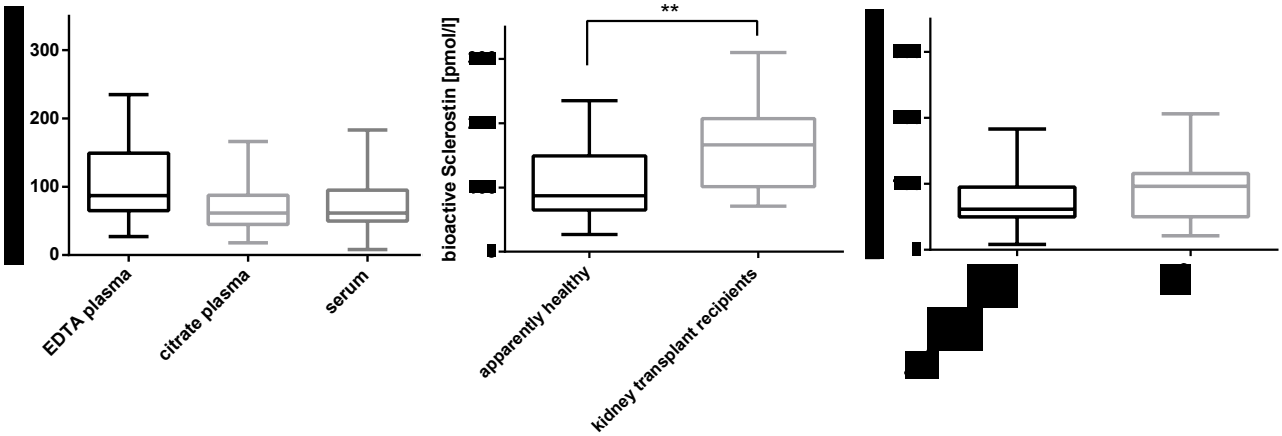
It is recommended to establish the normal range for each laboratory.

Plasma bioactive Sclerostin values in kidney transplant recipients

	apparently healthy subjects (n=24)	kidney transplant recipients (n=16)
Mean	103.9	170.3
Median	**87**	**166.5**
Percentile 95%	225.8	310
Percentile 5%	29.25	71
Minimum	27	71
Maximum	235	310

Serum bioactive Sclerostin values in a CKD patient cohort

	apparently healthy subjects (n=32)	CKD (n=24)
Mean	70.8	94.1
Median	61.5	96
Percentile 95%	143.4	200.3
Percentile 5%	12.5	22.7
Minimum	8	21
Maximum	183	206



Why is heparin plasma not suggested as a sample matrix in this ELISA?

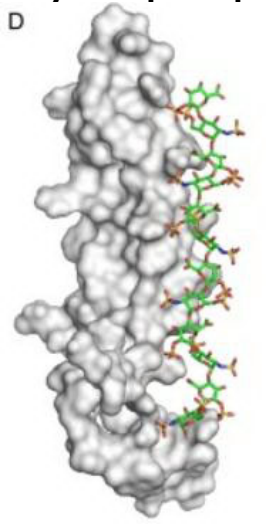


Fig.1: Heparin (green) mainly binds on loop2 and loop3 of the Sclerostin molecule (3).

Heparin disturbs the binding of the detection antibody utilized in this ELISA assay. For this reason, heparin-plasma cannot be measured with this assay.

MATRIX COMPARISON

Comparison of bioactive Sclerostin serum and plasma sample values from apparently healthy individuals

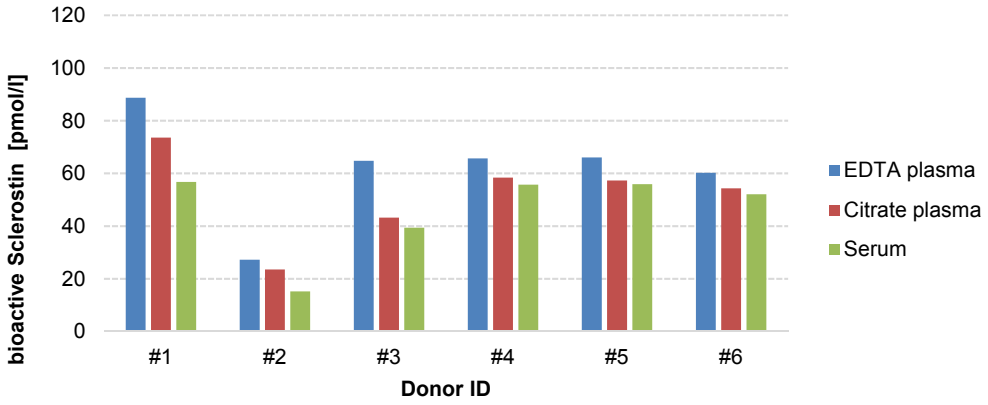
Bioactive human Sclerostin was measured in three matrices from six different individual donors.

	bioactive Sclerostin [pmol/l]		
Donor ID	**EDTA plasma**	**Citrate plasma**	**Serum**
#1	89	74	57
#2	27	23	15
#3	65	43	39
#4	66	58	56
#5	66	57	56
#6	60	54	52

Measured values of human bioactive Sclerostin in serum are lower compared to plasma in an apparently healthy cohort (n=6).

It has been shown that Sclerostin values differ between serum and plasma even when these assays are validated in both matrices (5). Measurements of Sclerostin in plasma are generally higher than in serum. The reasons for this difference is yet unclear, however it is assumed that coagulation process under conditions of serum collection might reduce the accessibility of recognizable determinants (6).

Graph showing matrix comparison of bioactive Sclerostin sample concentrations between serum, EDTA plasma, and citrate plasma in an apparently healthy cohort (n=6).



ASSAY PERFORMANCE CHARACTERISTICS

SPIKE RECOVERY

Summary of data showing mean recovery of bioactive Sclerostin:

Matrix	+26 pmol/l		+110 pmol/l	
	Mean	Range	Mean	Range
Serum (n=5)	93%	76-111%	86%	82-95%
EDTA plasma (n=5)	94%	85-104%	93%	86-98%
Citrate plasma (n=1)	104%	-	99%	-

Experiments:

Recovery of spiked samples was tested by adding 2 concentrations of human recombinant bioactive Sclerostin (26 pmol/l + 110 pmol/l) to different human sample matrices.

Data showing spike/recovery of human **serum** samples:

	bioactive Sclerostin [pmol/l]			S/R [%]	
Sample ID	**Reference**	**+26 pmol/l**	**+110 pmol/l**	**+26 pmol/l**	**+110 pmol/l**
#S1	57	77	147	76	82
#S2	75	104	180	111	95
#S3	55	77	148	84	84
#S4	44	67	140	90	87
#S5	71	97	164	101	84
			Mean [%]	**93**	**86**

Data showing spike/recovery of human **EDTA plasma** samples:

	bioactive Sclerostin [pmol/l]			S/R [%]	
Sample ID	**Reference**	**+26 pmol/l**	**+110 pmol/l**	**+26 pmol/l**	**+110 pmol/l**
#E1	190	212	295	85	96
#E2	152	177	253	95	92
#E3	165	190	259	97	86
#E4	81	108	189	104	98
#E5	67	91	171	91	94
			Mean [%]	**94**	**93**

Data showing spike/recovery of human **citrate plasma** samples:

	bioactive Sclerostin [pmol/l]			S/R [%]	
Sample ID	**Reference**	**+26 pmol/l**	**+110 pmol/l**	**+26 pmol/l**	**+110 pmol/l**
#C1	90	117	199	104	99

LINEARITY

Summary:

Dilution linearity of samples containing endogenous Sclerostin

Matrix	**Recovery of dilution steps [%]**					
	1+1		**1+3**		**1+7**	
	Mean	**Range**	**Mean**	**Range**	**Mean**	**Range**
Serum (n=7)	100	89-108	103	96-108	106	90-120
EDTA plasma (n=6)	105	99-111	108	99-125	123	107-154
Citrate plasma (n=2)	91	89-94	91	86-96	103	102-104

Dilution linearity of samples containing recombinant bioactive Sclerostin

Matrix	**Recovery of dilution steps [%]**					
	1+1		**1+3**		**1+7**	
	Mean	**Range**	**Mean**	**Range**	**Mean**	**Range**
Serum (n=6)	98	93-103	86	73-100	89	75-103
EDTA plasma (n=6)	102	97-106	99	97-103	91	91-109
Citrate plasma (n=1)	119	-	132	-	103	-

- ***All samples were diluted in assay buffer provided in the kit.***

Experiment:
Dilution linearity was assessed by serially diluting samples containing endogenous bioactive Sclerostin with assay buffer.

Data showing the dilution of endogenous bioactive Sclerostin in **serum** samples:

Sample ID	bioactive Sclerostin [pmol/l]				R [%]		
	ref	1+1	1+3	1+7	1+1	1+3	1+7
#S1	139	67	33	17	97	96	95
#S2	122	60	33	18	98	108	120
#S3	114	61	29	13	108	101	90
#S4	139	75	39	20	108	112	116
#S5	103	50	28	15	98	108	116
#S6	199	88	48	25	89	96	101
#S7	89	46	23	12	104	105	106
				Mean R [%]	**100**	**103**	**106**

Data showing the dilution of endogenous bioactive Sclerostin in **EDTA plasma** samples:

Sample ID	bioactive Sclerostin [pmol/l]				R [%]		
	ref	1+1	1+3	1+7	1+1	1+3	1+7
#E1	268	147	66	36	110	99	107
#E2	210	109	52	30	104	99	115
#E3	173	87	50	28	100	116	131
#E4	184	98	47	28	106	102	122
#E5	148	82	46	28	111	125	154
#E6	242	120	66	32	99	110	107
				Mean R [%]	**105**	**108**	**123**

Data showing the dilution of endogenous bioactive Sclerostin in **citrate plasma** samples:

Sample ID	bioactive Sclerostin [pmol/l]				R [%]		
	ref	1+1	1+3	1+7	1+1	1+3	1+7
#C1	175	82	42	23	94	96	104
#C2	171	76	37	22	89	86	102
				Mean R [%]	**91**	**91**	**103**

Experiment:
Dilution linearity was assessed by serially diluting samples containing 110 pmol/l recombinant bioactive Sclerostin with assay buffer.

Data showing the dilution of recombinant bioactive Sclerostin in **serum** samples:

Sample ID	bioactive Sclerostin [pmol/l]				R [%]		
	ref	1+1	1+3	1+7	1+1	1+3	1+7
#S1	147	71	37	17	96	100	93
#S2	180	84	37	19	93	82	83
#S3	259	134	62	27	103	96	82
#S4	148	76	31	19	103	85	100
#S5	140	67	27	18	96	78	103
#S6	164	78	30	15	95	73	75
				Mean R [%]	**98**	**86**	**89**

Data showing the dilution of recombinant bioactive Sclerostin in **EDTA plasma** samples:

Sample ID	bioactive Sclerostin [pmol/l]				R [%]		
	ref	1+1	1+3	1+7	1+1	1+3	1+7
#E1	295	151	72	35	102	97	96
#E2	184	91	45	21	99	98	93
#E3	253	128	62	29	101	97	91
#E4	259	136	67	32	105	103	97
#E5	189	101	46	22	106	97	95
#E6	171	83	44	23	97	103	109
				Mean R [%]	**102**	**99**	**97**

Data showing the dilution of recombinant bioactive Sclerostin in **citrate plasma** samples:

Sample ID	bioactive Sclerostin [pmol/l]				R [%]		
	ref	1+1	1+3	1+7	1+1	1+3	1+7
#C1	174	103	58	22	119	132	103

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=3) ≤ 1%, Inter-assay (n=7) ≤ 5%

Intra-assay: 2 samples of known concentrations were tested 3 times with 1 assay lot by 1 operator.
Inter-assay: 2 samples of known concentrations were tested 7 times with 2 assay lots by 2 different operators.

Intra-assay (n=3)	Sample 1	Sample 2
Mean (pmol/l)	19	153
SD (pmol/l)	0.3	1.0
CV (%)	1	1

Inter-assay (n=7)	Sample 1	Sample 2
Mean (pmol/l)	19	157
SD (pmol/l)	1.0	8.3
CV (%)	5	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 for the bioactive Sclerostin ELISA is **1.9 pmol/l**.

Lower limit of quantification (LLOQ)
The lower limit of quantification is defined as the lowest concentration where the following two criteria are met: 1) back fit of the calibration standards shall be within 75 – 125% and 2) precision shall be ≤ 25% (acc. to ICH [Ref. 1]).
The LLOQ for the bioactive Sclerostin ELISA is **1.3 pmol/l**.

SAMPLE STABILITY

Sample preparation

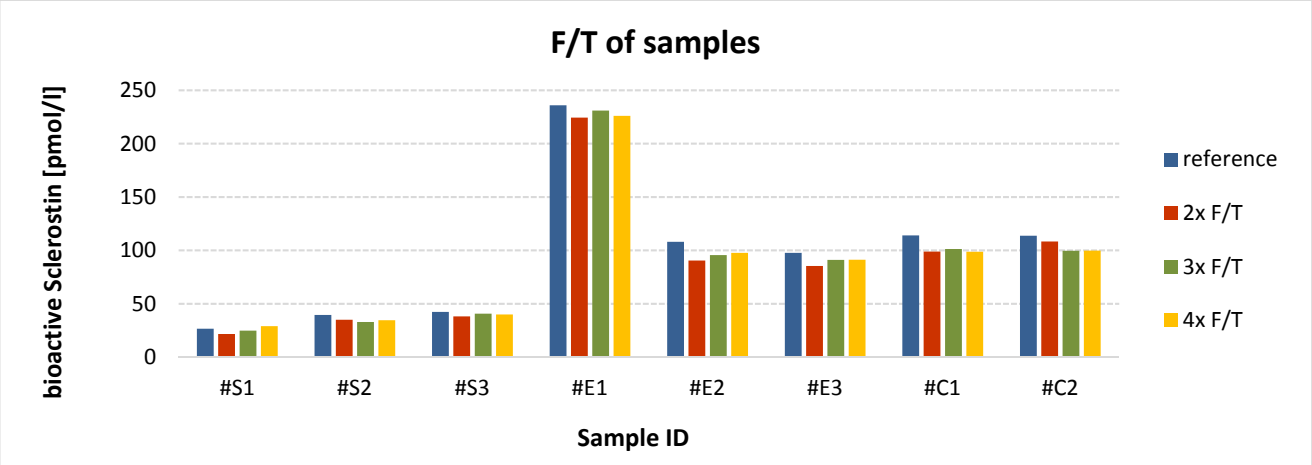
Collect venous blood samples by using standardized blood collection tubes. Perform serum/plasma separation by centrifugation according to supplier's instructions of the blood collection devices as soon as possible. The acquired plasma or serum samples should be measured as soon as possible. For longer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

Freeze/thaw stability of serum samples containing endogenous bioactive Sclerostin

A set of samples (3 sera, 3 EDTA plasma, 2 citrate 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 93%.

Sclerostin concentrations of samples after freeze-thaw cycles:

	bioactive Sclerostin [pmol/l]				R [%]
Sample ID	**reference**	**2x**	**3x**	**4x**	**4 F/T vs ref**
#S1	27	22	25	29	109
#S2	40	35	33	35	87
#S3	42	38	41	40	94
#E1	236	224	231	226	96
#E2	108	90	95	98	90
#E3	98	85	91	91	93
#C1	114	99	101	99	86
#C2	114	108	100	100	88
				Mean R [%]	**93**



SPECIFICITY

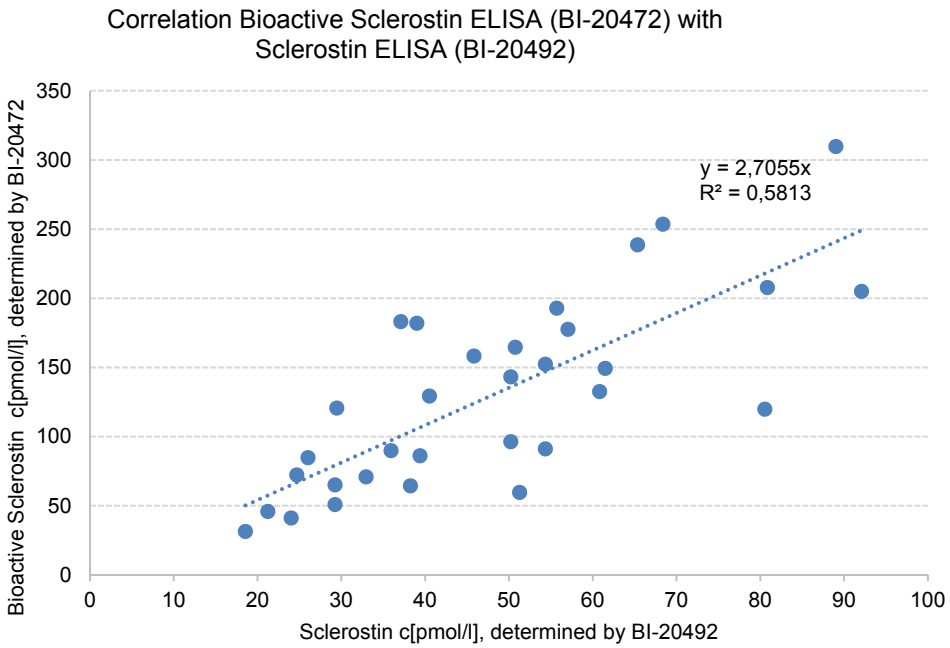
This assay recognizes endogenous (natural) and recombinant human bioactive Sclerostin.

CALIBRATION

This immunoassay is calibrated against recombinant human bioactive Sclerostin (AA24-213).

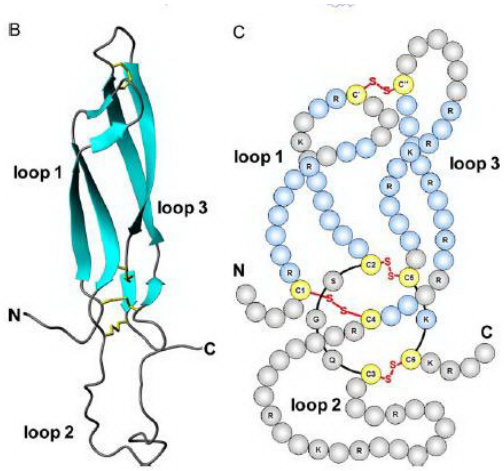
COMPARISON of Biomedica' bioactive Sclerostin ELISA (cat.no. BI-20472) with Biomedica's Sclerostin ELISA (cat.no. BI-20492)

32 samples were compared: EDTA plasma samples (n=16), serum samples (n=16)



Results: The correlation between the two assays resulted in R^2=0.58. Sclerostin sample values measured with the Biomedica "bioactive Sclerostin ELISA" (cat no BI-20472) are higher than in the Biomedica "Sclerostin ELISA" (cat no BI-20492). The results demonstrate that the antibodies utilized in both assays bind to different regions of the Sclerostin molecule. The monoclonal capture antibody of the bioactive Sclerostin ELISA binds to the receptor binding site of Sclerostin; a region that is most probably more robust to cleavage. More information on the characterization of the antibodies see below.

CHARACTERIZATION OF THE ANTIBODIES utilized in the bioactive Sclerostin ELISA



Loop 2 – binding region to LRP5/6 = „active" site (3,4)

Fig.2: Sclerostin (http://www.uniprot.org/uniprot/Q9BQB4.1)

The Sclerostin protein consists of two flexible N- and C-terminal arms and a cystine-knot with three loops, whereas the second loop binds to the LRP5/6 complex of the Wnt-signaling pathway and leads to the inhibition of bone formation (3, 4).

Sclerostin is classically considered to be a monomeric protein, but data from *Hernandez and colleagues* (7) postulate that circulating sclerostin has a dimeric configuration. Furthermore, it is not yet well understood if circulating Sclerostin fragments exist, but the comparison of different ELISAs suggest that those fragments exist as well (8, 9).

As the epitope of the monoclonal capture antibody utilized in the *bioactive Sclerostin ELISA* is located in loop 2 (see Fig.2), the binding region to the LRP 5/6 complex, all Sclerostin molecules (including potential fragments) containing this receptor binding region can be detected.

The characterization of both antibodies utilized in the *bioactive Sclerostin ELISA* comprises epitope mapping with overlapping peptides spotted to a microarray, characterization of binding kinetics with biolayer interferometry measurements and determination of antibody purity with size exclusion chromatography.

Sclerostin Protein Structure - EPITOPES OF COATING AND DETECTION ANTIBODY

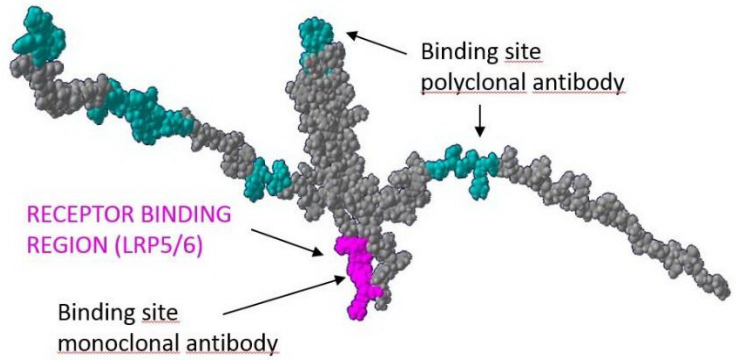


Fig.3: Sclerostin protein structure showing the binding regions of the monoclonal capture antibody (pink) and the polyclonal detection antibody (turquoise).)

AFFINITY OF COATING AND DETECTION ANTIBODY

Both ELISA antibodies utilized in the "bioactive Sclerostin ELISA" bind to Sclerostin with high affinity.

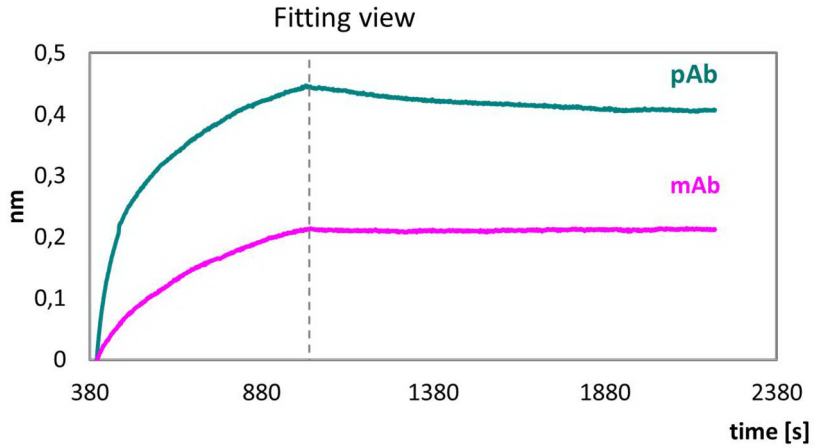


Fig.4: Biolayer interferometry measurements (Octet) of monoclonal coating antibody (mAb, pink) and polyclonal detection antibody (pAb, turquoise) binding to a sensor coated with sclerostin protein.

HPLC ANALYSIS OF COATING AND DETECTION ANTIBODY

HPLC analysis reveals >95% purity of antibody monomers

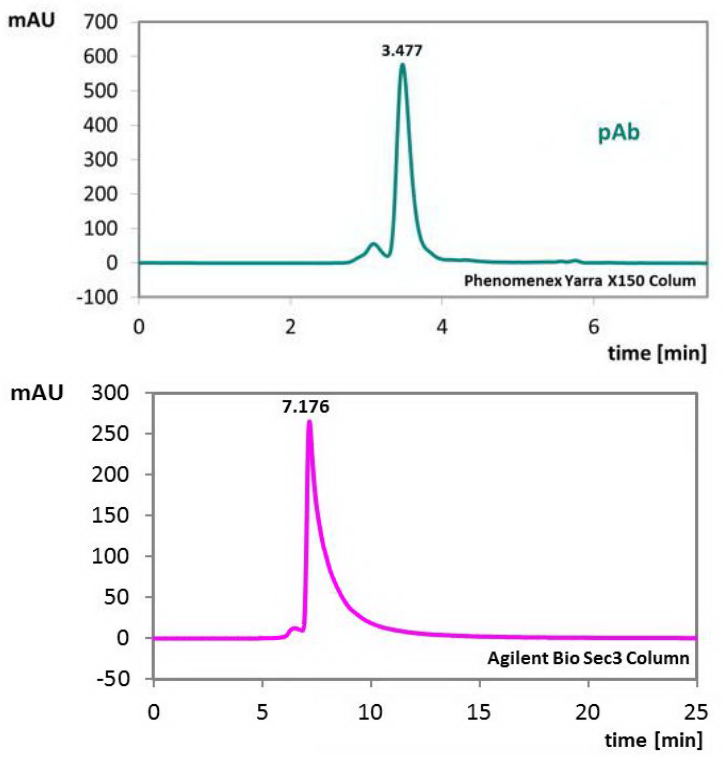


Fig.5: HPLC analysis of both antibodies. Size exclusion chromatography (SEC) of monoclonal antibody (mAb, pink) and polyclonal antibody (pAb, turquoise). The monoclonal antibody was analyzed using an Agilent Bio Sec column, whereas for the polyclonal antibody a Phenomenex Yarra X150 column was used.

VALIDATION GUIDELINES AND LITERATURE

This document is based on the principles of bioanalytical validation defined by ICH Ref. (1), (2) and according to SOP A801.

1. 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"

2. Food and Drug Administration
   Guidance for Industry, Bioanalytical Method Validation, Draft Guidance, September 2013
   Guidance for industry (Draft status), Bioanalytical Validation, FDA Sept2013, Revision 1, chapter IV.

3. Characterization of the structural features and interactions of sclerostin: molecular insight into a key regulator of Wnt-mediated bone formation. *Veverka V et al., J Biol Chem, 2009; 284:10890-10900.*

4. Characterization of the Interaction of Sclerostin with the Low Density Lipoprotein Receptor-related Protein (LRP) Family of Wnt Co-receptors. *Holdsworth G et al., J Biol Chem, 2012; 284(16), 287(32): 26464-26477.*

5. Determination of serum and plasma sclerostin concentrations by enzyme-linked immunoassays. *McNulty M et al., J Clin Endocrinol Metab, 2012; 96 (7), E1159-E1162*

6. Sclerostin measurement in human disease: Validity and current limitations. *Costa A et al., Bone, 2017; 96:24-28.*

7. New insights into the location and form of sclerostin. *Hernandez P et al., Biochem Biophys Res Commun, 2014; 446 (4):1108-1113.*

8. Association of circulating sclerostin with bone mineral mass, microstructure, and turnover biochemical markers in healthy elderly men and women. *Durosier C et al., J Clin Endocrinol Metab, 2013; 98 (9):3873-3883.*

9. Circulating sclerostin levels are decreased in patients with endogenous hypercortisolism and increase after treatment. *van Lierop AH et al., J Clin Endocrinol Metab, 2012; 97:E1953-E1957.*

Available on our Website **www.bmgrp.com**
Instructions for Use (package insert)
Material Safety Data Sheet

MEASUREMENT of BIOACTIVE SCLEROSTIN in CELL CULTURE SUPERNATANTS and URINE SAMPLES

The following experiments have been performed to test the use of the bioactive Slcerostin assay (cat. no. BI-20472) in human urine and cell culture supernatants.

Note: the experiments performed for these samples did not undergo a full validation and are therefore merely a performance check.

1. MEASUREMENT of bioactive Sclerostin in CELL CULTURE SUPERNATANTS

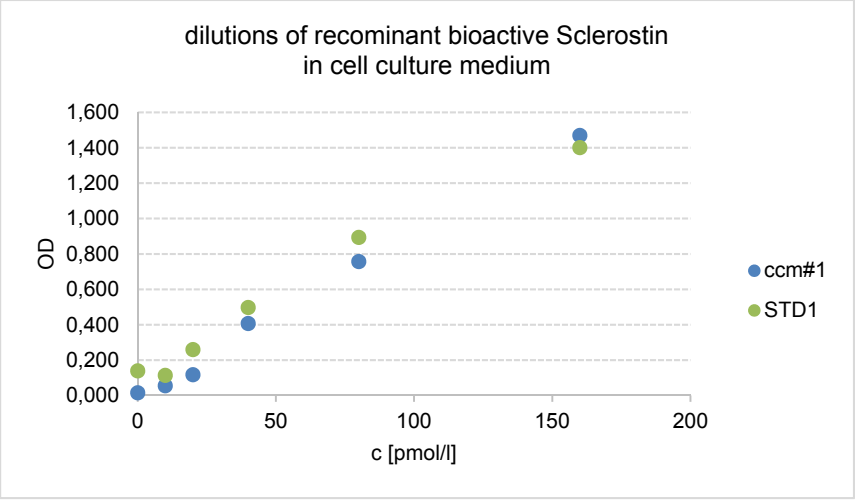
Cell culture medium (ccm: RPMI1640 containing 10% fetal calf serum) was tested undiluted and spiked with a final concentration of 160 pmol/l human Sclerostin protein. The spiked solution was diluted 1+1, 1+3, 1+7, and 1+15 with the cell culture medium.

As a comparison, the spike recovery and dilution linearity of the standard matrix (=STD1) and the dilutions with assay buffer is shown.

OD values of spiked and diluted cell culture medium sample and standard matrix (STD1)

		OD					
Dil medium	**Sample ID**	**Reference**	**+ 160 pmol/l**	**1+1**	**1+3**	**1+7**	**1+15**
ccm#1	ccm#1	0.014	1.468	0.756	0.406	0.116	0.054
ASYBUF	STD1	0.138	1.400	0.892	0.496	0.258	0.113

Graph showing dilution of cell culture medium (ccm) and a comparison to the standard Matrix (STD1), both spiked with spiked with the same amount of recombinant Sclerostin (160 pmol/l).



Suggested protocol for the measurement of human bioactive Sclerostin in cell culture supernatants

Preparation of a cell culture medium (ccm) based standard curve:

Reconstitute STD7 in 250 µl deionized water. Leave at room temperature (18-26°C) for 15 min and mix well prior to making dilutions.

Use polypropylene tubes.
For the preparation of the cell culture based standards *always* use the identical cell culture medium in which the samples are based on.

- Mark tubes e.g. CC STD6, CC STD 5 … CC STD1.
- Prepare a two-fold serial dilution to obtain STD6 to STD2.

e.g.:
Dispense 100 µl cell culture medium into vials labelled with CC STD6 to CC STD1.
Pipette 100 µl of STD 7 into tube marked as CC STD6. Mix thoroughly.
Transfer 100 µl of CC STD6 into vial marked as CC STD5. Mix thoroughly. Continue in the same fashion to obtain CC STD4 to CC STD2.

- ccm serves as the zero standard (=CC STD1, 0 pmol/l).

Attention: Concentrations defined for CTRL A and B are only valid for measuring bioactive Sclerostin in human serum or plasma. The controls cannot be used for cell culture measurements.

2. MEASUREMENT of bioactive Sclerostin in human URINE

The Biomedica ELISA is fully validated for the measurement of human bioactive Sclerostin in serum, EDTA-, and citrate plasma.

The ELISA has not been fully validated for the measurement of bioactive Sclerostin in urine samples.

A small number of experiments with urine samples have been performed

Summary:

Urine samples (n=4) were assayed with the bioactive Sclerostin ELISA (BI-20472) following the standard protocol **using undiluted urine.**

Endogenous bioactive Sclerostin was not detectable in these samples.

Urine samples can be spiked:
The average recovery of 4 human urine samples from hospital donors was 103%.

If required, dilute urine samples 1+1 with ASYBUF (Assay buffer, supplied in the kit).

Suggested protocol for the measurement of human bioactive Sclerostin in urine samples

Follow standard protocol as indicated in the package insert:

Pipette **20 µl** of **undiluted urine sample** directly into the well of the microtiter plate.

If required, dilute samples 1+1 with assay buffer (provided in the kit).