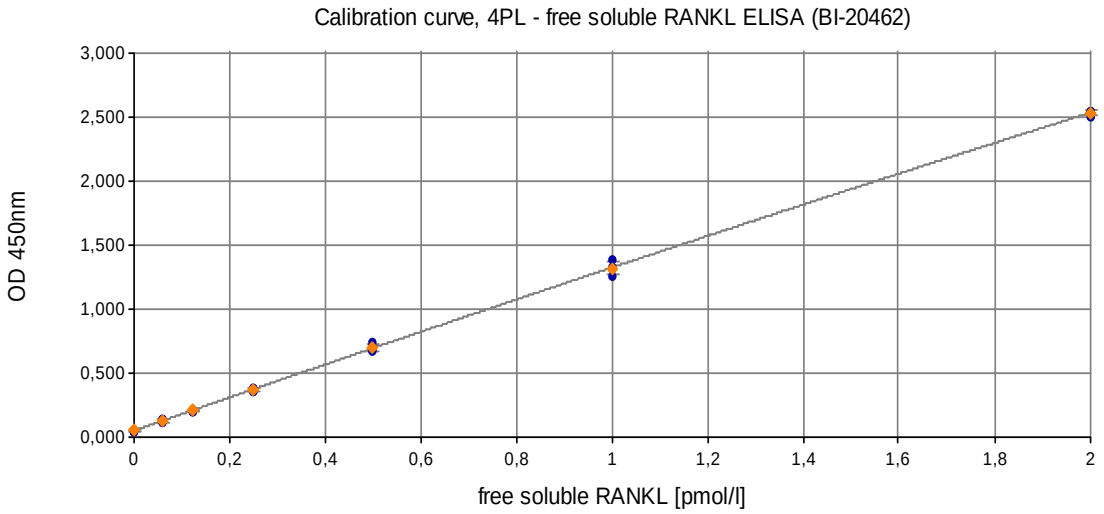
FREE soluble RANKL High Sensitivity ELISA – 3rd Generation (Cat.No. BI-20462)

For the Determination of Free Soluble Uncomplexed RANKL in Human Samples

ASSAY CHARACTERISTICS

Method	Sandwich ELISA, HRP/TMB
Sample type	Serum, Heparin plasma
Standard range	7 standards diluted in a serum matrix ranging from 0-2 pmol/l (0 / 0.0625 / 0.125 / 0.25 / 0.5 / 1 / 2 pmol/l) and 2 serum based controls
Conversion factor	1 pg/ml = 0.05 pmol/l (MW: 20 kDa)
Sample volume	150 µl / well
Detection limit	0.01 pmol/l (0 pmol/l + 3 SD)
LLOQ	0.008 pmol/l
Serum values of apparently healthy individuals	Median 0.14 pmol/l (n=32) ***This value lies between calibration point 3 and 4 of the standard curve.***
Incubation time, temp.	2 h / overnight / 1 h / 30 min room temperature (18-24°C) and 4°C (2-8°C)
Cross reactivity	The sequence homology to various primates is >95%. It is likely that the assay can be used for these species. Internal validations have not been carried out.

Typical standard curve:



Screening of 32 human donor sera from apparently healthy individuals:

Serum	**Free soluble RANKL [pmol/l]**
Mean value	0.17
Median	**0.14**
Percentile 95%	0.43
Percentile 5%	0.04

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

PERFORMANCE CHARACTERISTICS:

Spike recovery of endogenous free soluble RANKL (sRANKL):

The recovery of the back calculated concentration of free sRANKL samples in the mixing proportions is:

Matrix	**Mixing 1+1**	**Mixing 1+4**
Serum	89% (0.15 pmol/l)	85% (0.15 pmol/l)
Heparin plasma	100% (0.15 pmol/l)	98% (0.15 pmol/l)

Experiment: 8 different serum samples were mixed together with two serum samples of known free sRANKL concentrations of 0.15 pmol/l and 0.34 pmol/l, respectively.

Mixing factor		1+1 (0.15 pmol/l)			1+4 (0.15 pmol/l)		
Sample ID	**Reference [pmol/l]**	**Conc [pmol/l]**	**Expected [pmol/l]**	**R [%]**	**Conc [pmol/l]**	**Expected [pmol/l]**	**R [%]**
S#1	0.14	0.15	0.15	103	0.15	0.15	97
S#2	0.03	0.04	0.09	40	0.08	0.13	61
S#3	0.08	0.11	0.12	99	0.12	0.14	88
S#4	0.07	0.11	0.11	99	0.13	0.14	91
S#5	0.04	0.06	0.10	63	0.09	0.13	71
S#6	0.11	0.14	0.13	106	0.13	0.15	89
S#7	0.12	0.13	0.14	96	0.13	0.15	87
S#8	0.14	0.16	0.15	104	0.14	0.15	94
Mean R [%]				**89**			**85**

Mixing factor		1+1 (0.34 pmol/l)		
Sample ID	**Reference [pmol/l]**	**Conc [pmol/l]**	**Expected [pmol/l]**	**R [%]**
S#1	0.18	0.26	0.26	101
S#2	0.34	0.36	0.34	105
S#3	0.17	0.25	0.25	98
S#4	0.14	0.24	0.24	99
S#5	0.07	0.30	0.20	148
S#6	0.17	0.27	0.25	108
S#7	0.15	0.32	0.24	130
S#8	0.19	0.29	0.26	109
			Mean R [%]	**112**

Experiment: 8 different Heparin plasma samples were mixed together with a serum sample of known free sRANKL concentration (0.15 pmol/l).

Mixing factor		1+1 (0.15 pmol/l)			1+4 (0.15 pmol/l)		
Sample ID	**Reference [pmol/l]**	**Conc [pmol/l]**	**Expected [pmol/l]**	**R [%]**	**Conc [pmol/l]**	**Expected [pmol/l]**	**R [%]**
H#1	0.17	0.19	0.16	118	0.16	0.16	101
H#2	0.01	0.04	0.08	55	0.09	0.12	72
H#3	0.17	0.20	0.16	119	0.18	0.16	111
H#4	0.10	0.13	0.13	103	0.13	0.14	93
H#5	0.06	0.11	0.10	107	0.13	0.13	97
H#6	0.12	0.14	0.14	106	0.16	0.15	106
H#7	0.09	0.12	0.12	97	0.14	0.14	99
H#8	0.17	0.16	0.16	98	0.16	0.16	101
Mean R [%]				**100**			**98**

Spike recovery of recombinant free soluble RANKL(sRANKL):

Please note: Recovery of *recombinant* RANKL is a complicated issue in serum/plasma samples as the samples contain a binding factor that influences the recovery. This observation is different from a sample spiked with endogenous soluble RANKL, as the sample has already reached its equilibrium with OPG (and other potential binding factors).

→ We therefore recommend carrying out spike recovery experiments by using samples containing higher endogenous levels of free soluble RANKL as shown in the experiment above: "Spike recovery of endogenous free sRANKL".

However, if samples containing higher endogenous levels of free soluble RANKL are not available please see the following experimental design and data:

Sample matrix	n	Spike [pmol/l]	Mean [%]
Serum	8	0.25	91
	8	1	84
Heparin plasma	8	0.25	91
	8	1	83

Experiment: Recovery of spiked human serum samples was tested by adding 2 defined concentrations of recombinant free sRANKL in 8 different samples.
Data showing recovery of human recombinant free sRANKL in human serum samples:

Matrix	Serum				
	Reference	Spike 0.25 pmol/l		Spike 1 pmol/l	
Sample ID	c [pmol/l]	c [pmol/l]	S/R [%]	c [pmol/l]	S/R [%]
#S1	1.01	1.19	70	1.70	69
#S2	0.47	0.73	104	1.47	100
#S3	0.27	0.53	103	1.20	94
#S4	0.23	0.43	77	1.04	81
#S5	0.05	0.29	98	0.83	78
#S6	0.07	0.25	70	0.74	67
#S7	0.52	0.76	98	1.39	88
#S8	0.27	0.48	85	1.23	96
Mean S/R [%]			**91**		**84**

Experiment: Recovery of spiked human Heparin plasma samples was tested by adding 2 defined concentrations of recombinant free sRANKL in 8 different samples. Data showing recovery of human recombinant free sRANKL in human Heparin plasma samples:

Matrix	**Heparin plasma**				
	Reference	**Spike 0.25 pmol/l**		**Spike 1 pmol/l**	
Sample ID	**c [pmol/l]**	**c [pmol/l]**	**S/R [%]**	**c [pmol/l]**	**S/R [%]**
#H1	0.31	0.55	98	1.29	98
#H2	0.18	0.44	102	0.99	81
#H3	0.39	0.64	98	1.42	103
#H4	0.49	0.79	119	1.55	106
#H5	0.06	0.23	67	0.73	67
#H6	0.04	0.24	79	0.89	85
#H7	0.19	0.37	74	0.96	77
#H8	0.10	0.31	84	0.85	76
Mean S/R [%]			**91**		**83**

Dilution linearity:

The dilution linearity of endogenous free sRANKL with a low measuring serum as dilution medium is:

Matrix	**Dil 1+1**	**Dil 1+3**
Serum	112%	135%
Heparin plasma	121%	no data available

→ We recommend diluting samples with serum samples containing low sRANKL concentrations.

Intra-assay precision & Inter-assay precision:

The intra-assay precision of the free sRANKL ELISA is ≤5%.
The inter-assay precision of the free sRANKL ELISA is ≤3%.

Experiment:
Intra-assay: 2 samples of known concentrations were tested 5 times by 1 operator within 1 kit lot.
Inter-assay: 2 samples of known concentrations were tested 12 times within 3 different assay lots by 2 different operators.

Data showing intra-assay and inter-assay precision:

Intra-assay (n=5)	**Sample 1**	**Sample 2**	**Inter-assay (n=12)**	**Sample 1**	**Sample 2**
Mean (pmol/l)	0.12	0.98	Mean (pmol/l)	0.12	1.00
SD (pmol/l)	0.006	0.009	SD (pmol/l)	0.004	0.02
CV (%)	5	1	CV (%)	3	2

Detection limit:

The detection limit is defined as the mean value of the back calculated concentration plus three times the standard deviation. The **detection limit** of the free sRANKL ELISA is **0.01 pmol/l**.

The 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]).
For the free sRANKL ELISA the **LLOQ is 0.008 pmol/l**.

SAMPLE CHARACTERISTICS:

Effect of sample matrix:

Measurement of free sRANKL in 2 different sample matrices from 18 samples of apparently healthy individuals showed a mean CV of 10% between serum and Heparin plasma.

	c [pmol/l]		
Sample ID	**Serum**	**Heparin plasma**	**CV [%]**
S#1	0.16	0.15	0
S#2	0.20	0.23	12
S#3	0.15	0.17	12
S#4	0.09	0.13	25
S#5	0.07	0.09	16
S#6	0.35	0.40	9
S#7	0.17	0.16	6
S#8	0.17	0.16	7
S#9	0.02	0.02	14
S#10	0.15	0.15	0
S#11	0.08	0.10	14
S#12	0.26	0.28	5
S#13	0.06	0.05	9
S#14	0.20	0.18	5
S#15	0.27	0.29	5
S#16	0.07	0.10	26
S#17	0.15	0.19	16
S#18	0.06	0.05	5
		Mean CV [%]	**10**

Stability of samples:

We recommend performing serum or plasma separation by centrifugation as soon as possible, e.g. 20 min at 2000 x g, preferably at 4°C (2-8°C). If this is not possible store the samples at 4°C (2-8°C) prior to centrifugation (up to one day).

The acquired serum or plasma samples should be measured as soon as possible. For longer storage aliquot samples and store at -25°C, for long time storage at -80°C. All samples should undergo only 3 freeze-thaw cycles.

Whole blood stability:

Human serum sample concentrations which were stored for 20h in whole blood at room temperature show a CV of 3%.
Human Heparin plasma sample concentrations which were stored for 20h in whole blood at room temperature show a CV of 6%.
Free sRANKL is stable in whole blood for 20h at room temperature (18-24°C).

Experiment:
Stability of free sRANKL in whole blood was tested in serum and Heparin plasma samples, directly after collection and after 2h, 4h and 20h.

Matrix serum	Duration between blood draw and sample prep [h]					
	0	2	4	20		
Sample ID	c [pmol/l]				Mean [pmol/l]	CV [%]
#S1	0.33	0.32	0.36	0.31	0.33	6
#S2	0.33	0.33	0.33	0.33	0.33	0
#S3	0.40	0.39	0.37	0.36	0.38	5
#S4	0.22	0.22	0.21	0.22	0.22	2
					Mean CV [%]	**3**

Matrix Heparin plasma	Duration between blood draw and sample prep [h]					
	0	2	4	20		
Sample ID	c [pmol/l]				Mean [pmol/l]	CV [%]
#H1	0.35	0.34	0.34	0.30	0.33	7
#H2	0.36	0.34	0.37	0.33	0.35	5
#H3	0.39	0.39	0.40	0.38	0.39	3
#H4	0.27	0.24	0.23	0.21	0.24	10
					Mean CV [%]	**6**

Freeze/thaw of serum samples containing endogenous free sRANKL:

Serum samples can undergo 3 freeze/thaw cycles.
The mean recovery of sample concentrations stressed by 3 F/T cycles is 92%.
The mean CV of sample concentrations (not stressed, once, and three times stressed of freeze/thaw cycles) is 7%.

No. of F/T cycles	Reference	1x	3x		R [%]
Sample ID	c [pmol/l]			CV [%]	3F/T vs Ref
S#1	0.13	0.10	0.10	17	76
S#2	0.13	0.12	0.11	7	87
S#3	0.27	0.28	0.23	9	87
S#4	0.67	0.62	0.58	7	86
S#5	0.18	0.16	0.18	6	95
S#6	0.78	0.75	0.74	3	94
S#7	0.24	0.22	0.25	6	104
S#8	0.33	0.31	0.34	4	103
S#9	0.23	0.22	0.21	4	92
Mean R [%]				**7**	**92**

Cross reactivity:

Species cross reactivity:

Primates: The sequence homology of human sRANKL (soluble form, aa 140-317) to various primate species is >95%. It is likely that the assay can be used for these species. Internal validations have not been carried out.

http://www.uniprot.org/uniprot/O14788
http://www.uniprot.org/blast/uniprot/2014032690KAKOT1GR

Validation:

The assay is fully validated according to ICH Q2 (R1), Ref. [1].

[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"

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