

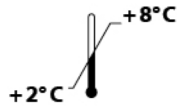
Manual

total sRANKL (human) **ELISA Kit**

*For the in vitro determination of total sRANKL (human)
in serum and plasma*

Valid from 04.04.2011

REF K 1016



IVD **CE**

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1. INTENDED USE

The described Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) is intended for the quantitative determination of *total* sRANKL (human) in serum and plasma. It is for *in vitro* diagnostic use only.

The assay detects free as well as OPG-bound sRANKL in serum and cell culture supernatants. Free sRANKL can be mathematically estimated when the assay is performed once with an excess of OPG (free and OPG-bound sRANKL are determined), and then without any addition of OPG (only OPG-sRANKL-complexes already present in the sample are determined).

2. INTRODUCTION

RANKL (receptor activator of nuclear factor (NF)- κ B ligand; also: osteoprotegerin ligand, OPGL), its cellular receptor, receptor activator of NF- κ B (RANK), and the decoy receptor, osteoprotegerin (OPG) have been identified as the key molecular regulation system for bone remodelling. RANKL, a member of the tumor necrosis factor (TNF) family, is the main stimulatory factor for the formation of mature osteoclasts and is essential for their survival. Therefore, an increase in RANKL expression leads to bone resorption and bone loss. RANKL is produced by osteoblastic lineage cells and activated T lymphocytes. It activates its specific receptor RANK which is located on osteoclasts and dendritic cells.

The effects of RANKL are counteracted by OPG which is secreted by various tissues and acts as an endogenous soluble receptor antagonist.

Imbalances of the RANKL/OPG system have been related to the pathogenesis of Paget's disease, benign and malignant bone tumors, postmenopausal osteoporosis, rheumatoid arthritis, bone metastases and hypercalcemia. It was shown in several studies that in animal models restoring of the RANKL/OPG balance (e.g. by administering OPG) reduces the severity of these disorders.

It has been shown, that RANKL is produced as a membrane-bound protein on murine osteoblasts/stromal cells, and cleaved into a soluble form by a metalloprotease. Stimulators of the osteoclastogenesis such as IL-1 β , IL-6, IL-11, IL-17, and TNF- α , increase the expression of RANKL and decrease OPG expression in osteoblasts/stromal cells. Cytokines inhibiting the osteoclastogenesis such as IL-13, INF- γ , and TGF- β 1, suppress the expression of RANKL and stimulated OPG expression.

Molecular structure:

sRANKL is a part of the TNF superfamily with high similarity to other members of that protein species. (SwissProt Nr. O14788). Two isoforms are produced by alternate splicing, a type II membrane protein (ISOFORM 1, 317 AA, MW 35.5 kD), and a secreted molecule (ISOFORM 2, 244 AA, MW 27.7kD), lacking the cytoplasmic and transmembrane domain. Although both forms are

bioactive, the membrane bound protein seems to be the homeostatic form, while the production of soluble RANKL signals pathological conditions.

Indications

- Postmenopausal and senile osteoporosis
- Diseases with locally increased bone resorption activity
- Paget´s disease
- Periodontal disease
- Inflammatory diseases
- Immunological disorders
- Arthritis
- Oncology

3. MATERIAL SUPPLIED

Catalogue No	Content	Kit Components	Quantity
K 1016MTP	PLATE	One holder with precoated strips	12 x 8 wells
K 1016WP	WASHBUF	ELISA wash buffer concentrate 10x	2 x 100 ml
K 1016LG	SOL	OPG-Solution, ready-to-use	5.5 ml
K 1016ST	STD	Standard, concentrate (for range see specification or label)	1 vial
K 1016KO	CTRL1 CTRL2	Controls, ready-to-use (for range see specification)	2 x 1 vial
K 1016AK2	AB	Detection antibody, biotinylated	1 vial
K 1016K	CONJ	Conjugate, streptavidin peroxidase-labeled	1 vial
K 1016TMB	SUB	TMB substrate (Tetramethylbenzi- dine), ready-to-use	15 ml
K 1016AC	STOP	ELISA stop solution, ready-to-use	15 ml

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Bidistilled water (aqua bidest.)
- Precision pipettors and disposable tips to deliver 10-1000 µl
- Foil to cover the microtiter plate
- A multi-channel dispenser or repeating dispenser
- Centrifuge capable of 3000 x g
- Vortex-Mixer
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader at 450 nm
(reference wave length 620 or 690 nm)

5. PREPARATION AND STORAGE OF REAGENTS

- To run the assay more than one time, make sure that the reagents are stored at the conditions stated on the label. **Prepare just the appropriate amount necessary for the assay.** The kit can be used up to 4 times within the expiry date stated on the label.
- Reagents with a volume less than **100 µl** should be centrifuged before use to avoid loss of volume.
- The **ELISA wash buffer concentrate** (WASHBUF) should be diluted with aqua bidest. **1:10** before use (100 ml concentrate + 900 ml a. bidest.), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals have to be redissolved at room temperature or at 37°C using a water bath before dilution of the buffer solutions. The **buffer concentrate** is stable at **2-8°C** until the expiry date stated on the label. Diluted **buffer solution** could be stored in a closed flask at **2-8°C for one month.**
- Dilute **POD-streptavidin 1:1000** in wash buffer (10 µl POD streptavidin in 10 ml ELISA wash buffer). The streptavidin is stable at 2-8°C until the expiry date stated on the label. **Diluted streptavidin solution is not stable and can not be stored.**
- Dilute **biotinylated detection antibody 1:1000** in wash buffer (10 µl antibody in 10 ml ELISA wash buffer). The antibody is stable at 2-8°C until expiry date given on the label. **Diluted antibody solution is not stable and can not be stored.**
- The **standard concentrate** (STD) and the **controls** (CTRL1, CTRL2) are stable at **2-8°C** until the expiry date stated on the label.

Prepare the solutions for the standard curve from the **total sRANKL standard concentrate** (S6) in **1:3** dilution steps by adding diluted **wash buffer** as follows:

S6 (standard concentrate)

100 µl S6 + 200 µl diluted wash buffer = S5

100 µl S5 + 200 µl diluted wash buffer = S4

100 µl S4 + 200 µl diluted wash buffer = S3

100 µl S3 + 200 µl diluted wash buffer = S2

Diluted wash buffer is used as standard S1, 0 pg/ml.

- All other test reagents are ready to use. The test reagents are stable until the expiry date (see label of test package) when stored at **2-8°C**.

6. SAMPLE PREPARATION

Dilution of samples

Serum/plasma samples

Fresh collected serum/plasma should be centrifuged within one hour. Store samples at -20 °C if not assayed on the same day. Lipemic or hemolytic samples may give erroneous results. Samples should be mixed well before assaying. We recommend duplicate analyses for each sample.

Dilute **serum/plasma** samples **1:10** with **wash buffer** prior to analyses. For example:

50 µl sample + 450 µl diluted wash buffer.

Cell culture supernatants

Cell culture medium is used as a blank. Record the absorbance readings for each sample and subtract the absorbance of the blank background from the absorbance obtained for each sample.

7. ASSAY PROCEDURE

Principle of the test

The assay utilizes the two-site "sandwich" technique with two selected antibodies that bind to human sRANKL and OPG.

Assay standards, controls and prediluted patient samples containing human sRANKL and the OPG-solution are added to wells of microplate coated with a high affine polyclonal anti-human OPG antibody. After the first incubation period, sRANKL is bound to the OPG and the antibody immobilized on the wall of microtiter wells. Then a biotinylated mono-

clonal anti-human sRANKL antibody is added to each microtiter well and a “sandwich” of “capture antibody - human OPG - sRANKL – streptavidin (peroxidase-labeled) is formed. For quantification, a streptavidin horseradish-peroxidase conjugate is added, which specifically binds to biotin. Tetramethylbenzidine (TMB) is used as a substrate for peroxidase. Finally, an acidic stop solution is added to terminate the reaction. The color changes from blue to yellow. The intensity of the yellow color is directly proportional to the concentration of total sRANKL. A dose response curve of the absorbance unit (optical density, OD at 450 nm) vs. concentration is generated, using the values obtained from the standard. *total* sRANKL present in the patient samples, is determined directly from this curve.

Test procedure

1.	Prior to use, allow all reagents and samples to come to room temperature (18-26 °C) and mix well
2.	Mark the positions of STD /SAMPLE/CTRL (Standard/Sample/Control) in duplicate on a protocol sheet
3.	Take microtiter strips out of the kit. Store unused strips covered at 2-8° C. Strips are stable until the expiry date stated on the label
4.	Wash 5 times by dispensing 250 µl of diluted WASHBUF (Wash buffer) into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper
5.	Add 50 µl of STD/SAMPLE/CTRL (Standard/Sample/Control) in duplicate into respective well. Use the wash buffer as STD 0 pg/ml.
6.	Add 50 µl SOL (OPG-solution) into each well
7.	Cover the plate tightly and incubate for 16 - 24 hours at 2 - 8 °C
8.	Discard the contents of each well. Wash 5 times by dispensing 250 µl of diluted WASHBUF (Wash buffer) into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper
9.	Add 100 µl detection antibody into each well
10.	Cover the plate tightly and incubate for 2 hours at room temperature (18-26 °C)
11.	Discard the contents of each well. Wash 5 times by dispensing 250 µl of diluted WASHBUF (Wash buffer) into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper
12.	Add 100 µl CONJ (conjugate) into each well

13.	Cover the plate tightly and incubate for 1 hour at 2 - 8 °C
14.	Discard the contents of each well. Wash 5 times by dispensing 250 µl of diluted WASHBUF (Wash buffer) into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper
15.	Add 100 µl of SUB (substrate) into each well
16.	Incubate for 20-30 minutes at room temperature (18-26°C) in the dark*
17.	Add 50 µl of STOP (stop solution) into each well, mix thoroughly
18.	Determine absorption immediately with an ELISA reader at 450 nm against 620 nm (or 690 nm) as a reference. If no reference wavelength is available, read only at 450 nm. If the extinction of the highest standard exceeds the range of the photometer, absorption must be measured immediately at 405 nm against 620 nm as a reference

*The intensity of the color change is temperature sensitive. We recommend to observe the color change and to stop the reaction upon good differentiation.

8. RESULTS

The following algorithms can be used alternatively to calculate the results. We recommend to use the „4-Parameter-algorithm“.

1. 4-Parameter-algorithm

It is recommended to use a linear ordinate for the optical density and a logarithmic abscissa for the concentration. When using a logarithmic abscissa, the zero calibrator has to be specified with a value less than 1 (e.g. 0.01).

2. Point-to-point-calculation

We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

3. Spline-algorithm

We recommend a linear ordinate for the optical density and a logarithmic abscissa for the concentration. When using a logarithmic abscissa, the zero calibrator has to be specified with a value less than 1 (e.g. 0.01).

The plausibility of the pairs of values should be examined before the automatic evaluation of the results. If this option is not available with the used program, a control of the paired values should be done manually.

Serum/plasma samples

For the calculation of the total sRANKL concentration in **plasma/serum** the result must be multiplied by **10**.

9. LIMITATIONS

Serum/plasma samples with *total sRANKL* levels greater than the highest standard value, should be diluted with wash buffer and re-assayed.

10. QUALITY CONTROL

Immundiagnostik AG recommends the use of commercial control samples for internal quality control if available.

Control samples should be analyzed with each run. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the patient samples may not be valid, if within the same assay one or more values of the quality control sample are outside the acceptable limits.

*Expected values***Conversion factor for pg/ml to pmol/l**

1 pg/ml = 0.016 pmol/l (60 kDa)

Relative molecular mass: sRANKL molecular mass is described in the literature as a trimer molecule of 60 kD; monomer - 20 kDa¹.

We recommend each laboratory to establish its own norm concentration range.

11. PERFORMANCE CHARACTERISTICS*Precision and reproducibility*

Intra-Assay (n=20)		
Sample	total sRANKL [pg/ml]	CV [%]
1	618	0.9
2	2346	3.5

Inter-Assay (n=20)		
Sample	total sRANKL [pg/ml]	CV[%]
1	618	9.3
2	2306	7.1

Recovery

2 samples were spiked with 3 different total sRANKL standards and measured using this assay.

Sample [pg/ml]	Spike [pg/ml]	total sRANKL expected [pg/ml]	total sRANKL measured [pg/ml]
104	1800	1904	1961
	1200	1304	1334
	800	904	835
151	1800	1951	1943
	1200	1351	1259
	800	951	922

Sensitivity

The sensitivity was set as $B_0 + 2SD$. The zero-standard was measured 20 times.

Sample	total sRANKL mean value [OD]	Standard variation (SD)	Detection limit [pg/ml]
1	0.013	0,24	1.56

Linearity

Two patient samples were diluted with sample dilution buffer and analyzed. The results are shown below:

n= 2

Sample	Dilution	Expected [pg/ml]	Measured [pg/ml]
A	1:100	1537	1537
	1:150	1025	1021
	1:200	768	701
B	1:100	1477	1477
	1:150	984	1083
	1:200	731	754

12. PRECAUTIONS

- For *in vitro* diagnostic use only.
- The quality control guidelines should be followed.
- Human material used in the kit components was tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.
- Reagents of the kit package contain sodium azide or thimerosal as bactericides. Sodium azide and thimerosal are toxic. The substrates for the enzymatic color reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- Stop solution is composed of sulfuric acid, which is a strong acid. Even diluted, it still must be handled with care. It can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped out immediately with copious quantities of water.

13. TECHNICAL HINTS

- Do not mix different lot numbers of any kit component.
- Reagents should not be used beyond the expiration date shown on the kit label.
- Substrate solution should remain colourless until use.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Avoid foaming when mixing reagents.
- The assay should always be performed according to the enclosed manual.

14. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and distributed according to the IVD guidelines of 98/79/EC.
- All reagents in the kit package are for *in vitro* diagnostic use only.
- The guidelines for medical laboratories should be followed.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from wrong use.
- Warranty claims and complaints in respect of deficiencies must be lodged within 14 days after receipt of the product. The product shall be sent to Immundiagnostik AG together with a written complaint.

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2. Hein G et al. (2005) Vergleich der Serum- und Synovia-Spiegel von sRANKL und OPG bei rheumatoider Arthritis und nicht erosiven Arthritiden. *Poster, vorgestellt beim 33. Kongress der D. Ges. für Rheumatologie*, September 2005, Dresden
3. Schedler J et al. (2005) Osteoprotegerin, RANK- Ligand und 5b-b TRAP bei Patienten mit Morbus Crohn. *Poster P148 Z Gastroenterol* 43:812

Used Symbols:



Store at



Catalog Number



In Vitro Diagnostic Device



No. of tests



Lot number



Use by



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