

total sRANKL (human) ELISA Kit

Manufactured by Immundiagnostik AG.

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Global Headquarters

ENZO LIFE SCIENCES, INC.
10 Executive Blvd.
Farmingdale, NY 11735
Ph: 800.942.0430
Fax: 631.694.7501
info-usa@enzolifesciences.com

European Sales Office

ENZO LIFE SCIENCES (ELS) AG
Industriestrasse 17
CH-4415 Lausen, Switzerland
Ph: +41 61 926 8989
Fax: +41 61 926 8979
info-eu@enzolifesciences.com

LOCAL EUROPEAN OFFICES

Belgium, The Netherlands & Luxembourg

Enzo Life Sciences BVBA
Avenue Louise 65 / Box 11
1050 Bruxelles
Belgium
Ph: +32 3 466 0420
Fax: +32 3 808 7033
info-be@enzolifesciences.com

Germany

Enzo Life Sciences GmbH
Basler Strasse 57a
DE-79540 Lörrach
Germany
Ph: +49 7621 5500 526
Fax: +49 7621 5500 527
info-de@enzolifesciences.com

France

Enzo Life Sciences (ELS) AG
Branch Office Lyon
13, avenue Albert Einstein,
F-69100 Villeurbanne, France
Ph: +33 472 440 655
Fax: +33 481 680 254
info-fr@enzolifesciences.com

UK & Ireland

Enzo Life Sciences (UK) Ltd.
1 Colleton Crescent
Exeter EX2 4DG
Ph: 0845 601 1488 (UK customers)
Ph: +44 1392 825900
Fax: +44 1392 825910
info-uk@enzolifesciences.com

www.enzolifesciences.com

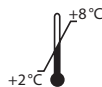
For laboratory use only. Not for human or diagnostic use.

total sRANKL (human) ELISA Kit

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

Valid from 2015-03-27

REF K 1016



IVD **CE**



Immundiagnostik AG, Stubenwald-Allee 8a, D-64625 Bensheim

Tel.: +49 6251 70190-0

Fax: + 49 6251 849430

e.mail: info@immundiagnostik.com

www.immundiagnostik.com

1. INTENDED USE

The described 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 plasma. 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, OPL), 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 No. 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 27.7 kD), 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

Cat. No.	Label	Kit components	Quantity
K 1016	PLATE	Holder with precoated strips	12 x 8 wells
K 1016	WASHBUF	ELISA wash buffer concentrate 10x	2 x 100 ml
K 1016	SOL	OPG solution, ready to use	5.5 ml
K 1016	STD	Standard, concentrate (for range see specification or label)	1 vial
K 1016	CTRL1	Control, ready-to-use (for range see specification)	1 vial
K 1016	CTRL2	Control, ready-to-use (for range see specification)	1 vial
K 1016	AB	Detection antibody, biotinylated	1 vial
K 1016	CONJ	Conjugate, streptavidin peroxidase-labeled	1 vial
K 1016	SUB	TMB substrate (tetramethylbenzidine), ready to use	15 ml
K 1016	STOP	ELISA stop solution, ready to use	15 ml

For reorders of single components, use the catalogue number followed by the label as product number.

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultra pure water*
- Calibrated precision pipettors and 10–1000 µl tips
- Foil to cover the microtiter plate
- Multi-channel pipets or repeater pipets
- Centrifuge, 3000 g
- Vortex
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader (required filters see chapter 7)

* Immundiagnostik AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25 °C (≥ 18.2 MΩ cm).

5. STORAGE AND PREPARATION OF REAGENTS

- To run the assay more than once, ensure that reagents are stored at the conditions stated on the label. **Prepare only the appropriate amount necessary for each run.** 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 **1:10 in ultra pure water** before use (100 ml concentrate + 900 ml ultra pure water), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals must be redissolved at room temperature or 37 °C 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** (wash buffer) can be stored in a closed flask at **2–8 °C for one month**.
- The **conjugate concentrate** (CONJ) must be diluted **1:1001 in wash buffer** (10 µl CONJ + 10 ml wash buffer). The concentrate is stable at **2–8 °C** until the expiry date stated on the label. **Diluted conjugate is not stable and cannot be stored.**
- The **detection antibody concentrate** (AB) must be diluted **1:1001 in wash buffer** (10 µl AB + 10 ml wash buffer). The concentrate is stable at **2–8 °C** until the expiry date stated on the label. **Diluted antibody is not stable and cannot 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 wash buffer as follows:

S6 (standard concentrate)

100 µl S6 + 200 µl wash buffer = S5

100 µl S5 + 200 µl wash buffer = S4

100 µl S4 + 200 µl wash buffer = S3

100 µl S3 + 200 µl wash buffer = S2

Wash buffer is used as standard **S1, 0 pg/ml**.

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

6. STORAGE AND PREPARATION 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 wash buffer.

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, 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 monoclonal 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 termi-

nate 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

Bring all **reagents and samples to room temperature** (15–30 °C) and mix well.

Mark the positions of STD /SAMPLE/CTRL (standards/sample/controls) on a protocol sheet.

Take as many **microtiter strips** as needed from kit. Store unused strips covered at 2–8 °C. Strips are stable until expiry date stated on the label.

For automated ELISA processors, the given protocol may need to be adjusted according to the specific features of the respective automated platform. For further details please contact your supplier or Immundiagnostik AG.

We recommend to carry out the tests in duplicate.

1.	Wash 5 times with 250 µl of ELISA wash buffer . After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
2.	Add 50 µl of STD/CTRL/SAMPLE into the respective wells.
3.	Add 50 µl of SOL (OPG solution) into the respective wells
4.	Cover the strips and incubate for 16–24 hours at 2–8 °C .
5.	Discard the contents of each well and wash 5 times with 250 µl of diluted wash buffer . After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
6.	Add 100 µl detection antibody (diluted AB) in each well.
7.	Cover the strips and incubate for 2 hours at room temperature (15–30 °C).
8.	Discard the contents of each well and wash 5 times with 250 µl of diluted wash buffer . After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
9.	Add 100 µl conjugate (diluted CONJ) in each well.
10.	Cover the strips and incubate for 1 hour at 2–8 °C.

11.	Discard the contents of each well and wash 5 times with 250 µl of diluted wash buffer . After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
12.	Add 100 µl SUB (TMB substrate) in each well.
13.	Incubate for 20–30 minutes* at room temperature (15–30°C) in the dark.
14.	Add 50 µl STOP (ELISA stop solution) and mix well.
15.	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 observing the color change and stopping the reaction upon good differentiation.

8. RESULTS

The following algorithms can be used alternatively to calculate the results. We recommend using 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 standard must be specified with a value less than 1 (e.g. 0.001).

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 linear abscissa for the concentration.

The plausibility of the duplicate values should be examined before the automatic evaluation of the results. If this option is not available with the programme used, the duplicate values should be evaluated manually.

Serum/Plasma samples

For the calculation of the total sRANKL concentration in plasma/serum, the result

must be multiplied by **10**.

In case **another dilution factor** has been used, multiply the obtained result with the dilution factor used.

9. LIMITATIONS

Samples with concentrations above the measurement range must be further diluted and re-assayed. Please consider this greater dilution when calculating the results.

Samples with concentrations lower than the measurement range cannot be clearly quantified.

The upper limit of the measurement range can be calculated as:

highest concentration of the standard curve × sample dilution factor to be used

The lower limit of the measurement range can be calculated as:

analytical sensitivity × sample dilution factor to be used

10. QUALITY CONTROL

Immundiagnostik recommends the use of external controls for internal quality control, if possible.

Control samples should be analysed 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.

Reference range

Conversion factor for pg/ml to pmol/l

$$1 \text{ pg/ml} = 0.016 \text{ pmol/l (60 kDa)}$$

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 reference 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	2346	7.1

Spiking Recovery

Two samples were spiked with different total sRANKL concentrations and measured using this assay (n = 2).

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

Dilution recovery

Two patient samples were diluted and analyzed. The results are shown below (n = 2):

Sample	Dilution	total sRANKL expected [pg/ml]]	total sRANKL 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

Analytical Sensitivity

The Zero-standard was measured 20 times. The detection limit was set as $B_0 + 2 \text{ SD}$ and estimated to be 1,56 pg/ml.

12. PRECAUTIONS

- All reagents in the kit package are for *in vitro* diagnostic use only.
- Human materials used in kit components were 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.
- Kit reagents contain sodium azide or ProClin as bactericides. Sodium azide and ProClin are toxic. Substrates for the enzymatic color reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- The stop solution consists of diluted sulphuric acid, a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped up immediately with copious quantities of water. Do not breath vapour and avoid inhalation.

13. TECHNICAL HINTS

- Do not interchange different lot numbers of any kit component within the same assay. Furthermore we recommend not assembling wells of different microtiter plates for analysis, even if they are of the same batch.
- Control samples should be analyzed with each run.
- Reagents should not be used beyond the expiration date stated on 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.
- Do not mix plugs and caps from different reagents.
- The assay should always be performed according 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.
- 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 incorrect use.
- Warranty claims and complaints regarding deficiencies must be logged within 14 days after receipt of the product. The product should be send to Immundiagnostik AG along with a written complaint.

15. REFERENCES

General literature

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Used symbols:

	Temperature limitation		Catalogue Number
	In Vitro Diagnostic Medical Device		To be used with
	Manufacturer		Contains sufficient for <n> tests
	Lot number		Use by