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Manual

total sRANKL (human) ELISA

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

Valid from 2022-08-08

REF **K 1016**

Σ 96

+2°C +8°C

IVD

CE

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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)-kB ligand; also: osteoprotegerin ligand, OPGL), its cellular receptor, receptor activator of NF-kB (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-1beta, IL-6, IL-11, IL-17, and TNF-alpha, increase the expression of RANKL and decrease OPG expression in osteoblasts/stromal cells. Cytokines inhibiting the osteoclastogenesis such as IL-13, INF-gamma, and TGF-beta1, 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 transmemb-

rane 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	Microtiter plate, pre-coated	12 x 8 wells
K 0001.C.100	WASHBUF	Wash buffer concentrate, 10 x	2 x 100 ml
K 1016	SOL	OPG solution, ready-to-use	5.5 ml
K 1016	STD	Standard, concentrate (concentration 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-labelled	1 vial
K 0002.15	SUB	Substrate (tetramethylbenzidine), ready-to-use	15 ml
K 0003.15	STOP	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

- Ultrapure water*
- Calibrated precision pipettors and 10–1 000 µl tips
- Foil to cover the microtiter plate
- Multi-channel pipets or repeater pipets
- Centrifuge, 3 000 *g*
- Vortex
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader (required filters see chapter 7)

* Immundiagnostik AG recommends the use of ultrapure 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.
- **Preparation of the wash buffer:** The **wash buffer concentrate (WASHBUF)** has to be diluted with ultrapure water **1:10** before use (100 ml WASHBUF + 900 ml ultrapure water), mix well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals have to be redissolved at room temperature or in a water bath at 37 °C. The **WASHBUF** can be used until the expiry date stated on the label when stored at **2–8 °C**. **Wash buffer** (1:10 diluted WASHBUF) can be stored in a closed flask at **2–8 °C for 1 month**.
- **Preparation of the conjugate:** Before use, the **conjugate concentrate (CONJ)** has to be diluted **1:1001** in **wash buffer** (10 µl CONJ + 10 ml wash buffer). The **CONJ** can be used until the expiry date stated on the label when stored at **2–8 °C**. **Conjugate** (1:1001 diluted CONJ) **is not stable and cannot be stored.**
- **Preparation of the detection antibody:** detection antibody concentrate (AB) must be diluted **1:1 001 in wash buffer** (10 µl AB + 10 ml wash buffer). an be used until the expiry date stated on the label when stored at **2–8 °C**. **detection antibody** (1:1001 diluted AB) **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 can be used until the expiry date (see label) when stored at **2–8 °C**.

6. STORAGE AND PREPARATION OF SAMPLES

Sample storage

Freshly collected EDTA plasma or serum should be centrifuged within one hour. Samples can be stored for 1 day at room temperature (15–30 °C) or for longer storage at -20 °C.

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.

EDTA plasma or serum samples must be diluted **1:10** before performing the assay,

e. g. 50 µl sample + 450 µl wash buffer., mix well.

50 µl of the dilution are used in the test.

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 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

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

Mark the positions of standards/controls/samples 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.	**Before use**, wash the wells **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
2.	Add **50 µl of standards/controls/diluted samples** 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 content of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate 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 content of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate 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 content of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
12.	Add **100 µl substrate** (SUB) into each well.
13.	Incubate for **20–30 minutes*** at room temperature (15–30 °C) in the dark.
14.	Add **50 µl stop solution** (STOP) into each well 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 by 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 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 kDa[1].

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	2 346	3.5

Inter-Assay (n = 20)

Sample	total sRANKL [pg/ml]	CV [%]
1	618	9.3
2	2 346	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	1 800	1 904	1 961
		1 200	1 304	1 334
		800	904	835
B	151	1 800	1 951	1 943
		1 200	1 351	1 259
		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	1 537	1 537
	1:150	1 025	1 021
	1:200	768	701
B	1:100	1 477	1 477
	1:150	984	1 083
	1:200	731	754

Analytical Sensitivity

The Zero-standard was measured 20 times. The detection limit was set as B_0 + 2 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 harmful to health and the environment. Substrates for enzymatic color reactions can also cause skin and/or respiratory irritation. Any contact with the substances should be avoided. Further safety information can be found in the safety data sheet, which is available from Immundiagnostik AG on request.
- The 10x Wash buffer concentrate (WASHBUF) contains surfactants which may cause severe eye irritation in case of eye contact.

  Warning: Causes serious eye irritation. **IF IN EYES:** Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: get medical Advice/attention.

- 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

1. Lacey D.L, et al., Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* (1998), **93**:165-176.
2. Kong Y.Y. et al., OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* (1999), **397**: 315-323.
3. Hsu H. et al., Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci* (1999), **96**:3540-3545.
4. Josien R, et al, TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells in vivo. *J Exp Med* (2000), **191**: 495-502.
5. Fuller K. et al., TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *J Exp Med* (1998), **188**: 997-1001.
6. Nakashima T , et. al., Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. *Biochem Biophys Res Commun* (2000), **275**(3):768-75.
7. Kong Y.Y. et al., Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* (1999), **402**: 304-309.
8. Hofbauer L.C. & A.E. Heufelder, Role of receptor activator of nuclear factor-KB ligand and osteoprotegerin in bone cell biology. *J Mol Med* (2001), **79**: 243-253.
9. Hofbauer L.C. & A.E. Heufelder, The Role of Osteoprotegerin and Receptor Activator of Nuclear Factor KB Ligand in the Pathogenesis and Treatment of Rheumatoid Arthritis. *Arthritis & Rheumatism* (2001), **44**:253-259.
10. Hofbauer LC, et al.,. The role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in the pathogenesis and treatment of metabolic bone diseases. *J Clin Endocrinol Metab* (2000), **85**: 2355-2363.
11. Teitelbaum S.L., Bone resorption by osteoclasts. *Science* (2000), **289**: 1504-1508.

Literature using K1016

12. Boumans, M.J.H. et al., 2012. Rituximab abrogates joint destruction in rheumatoid arthritis by inhibiting osteoclastogenesis. *Annals of the rheumatic diseases*, **71**(1), pp.108–13.
13. Dovio, A. et al., 2008. Circulating osteoprotegerin and soluble RANK ligand in systemic sclerosis. *The Journal of rheumatology*, **35**(11), pp.2206–13.
14. Dovio, A. et al., 2007. Increased osteoprotegerin levels in Cushing's syndrome are associated with an adverse cardiovascular risk profile. *The Journal of clinical endocrinology and metabolism*, **92**(5), pp.1803–8.
15. Findlay, D. et al., 2008. Circulating RANKL is inversely related to RANKL mRNA levels in bone in osteoarthritic males. *Arthritis research & therapy*, **10**(1), p.R2.
16. González-Alvaro, I. et al., 2007. Baseline serum RANKL levels may serve to predict remission in rheumatoid arthritis patients treated with TNF antagonists. *Annals of the rheumatic diseases*, **66**(12), pp.1675–8.
17. Hein, G. et al., 2000. Vergleich der Serum- und Synovia-Spiegel von sRANKL und OPG bei rheumatoider Arthritis und nicht erosiven Arthritiden. Poster at *Kongress der Deutschen Gesellschaft für Rheumatologie*. Dresden.
18. Hein, G.E. et al., 2008. sRANKL and OPG in serum and synovial fluid of patients with rheumatoid arthritis in comparison to non-destructive chronic arthritis. *Rheumatology international*, **28**(8), pp.765–9.
19. Hofbauer, L.C. et al., 2004. Effects of oral contraceptives on circulating osteoprotegerin and soluble RANK ligand serum levels in healthy young women. *Clinical endocrinology*, **60**(2), pp.214–9.
20. Kamiya, N. et al., 2011. Significance of serum osteoprotegerin and receptor activator of nuclear factor κB ligand in Japanese prostate cancer patients with bone metastasis. *International journal of clinical oncology*, **16**(4), pp.366–72.
21. Kerschan-Schindl, K. et al., 2008. Serum levels of receptor activator of nuclear factor kappaB ligand (RANKL) in healthy women and men. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association*, **116**(8), pp.491–5.
22. Li, E.K. et al., 2009. High prevalence of asymptomatic vertebral fractures in Chinese women with systemic lupus erythematosus. *The Journal of rheumatology*, **36**(8), pp.1646–52.

23. Nielen, M. et al., 2004. Markers of bone formation and resorption in preclinical rheumatoid arthritis are associated with radiographic progression. Poster at *American College of Rheumatology Meeting*. San Diego.

24. Oelzner, P. et al., 2009. Beziehung zwischen löslichen Komponenten des IL-6-Systems und des RANKL-OPG-Systems bei postmenopausalen Frauen mit Rheumatoider Arthritis. Poster at *Osteologie*. Frankfurt.

25. Oelzner, P. et al., 2006. RANKL , Osteoprotegerin und IL-6-System bei Rheumatoider Arthritis – Einfluß von Alter , Erkrankungsdauer , Menopause und entzündlicher Aktivität. Poster at *Osteologie*. Köln.

26. Schederl, J. et al., 2005. P148 - Osteoprotegerin, RANK-Ligand und 5b-b TRAP bei Patienten mit Morbus Crohn. *Zeitschrift für Gastroenterologie*, **43**, p.812.

27. Secchiero, P. et al., 2006. An increased osteoprotegerin serum release characterizes the early onset of diabetes mellitus and may contribute to endothelial cell dysfunction. *The American journal of pathology*, **169**(6), pp.2236–44.

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


Attention


Consult instructions for use

Consult specification data sheet


Irritant