Manual

total sRANKL (human) ELISA Kit

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

Valid from 2015-03-27

REF **KR1016**

Σ 96

+8°C +2°C

RUO

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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 Research Use Only. Not For Use in Diagnostic Procedures.

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.

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 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.

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
KR1016	PLATE	Holder with precoated strips	12 x 8 wells
KR1016	WASHBUF	ELISA wash buffer concentrate 10 x	2 x 100 ml
KR1016	SOL	OPG solution, ready to use	5.5 ml
KR1016	STD	Standard, concentrate (for range see specification or label)	1 vial
KR1016	CTRL1	Control, ready-to-use (for range see specification)	1 vial
KR1016	CTRL2	Control, ready-to-use (for range see specification)	1 vial
KR1016	AB	Detection antibody, biotinylated	1 vial
KR1016	CONJ	Conjugate, streptavidin peroxidase-labeled	1 vial
KR1016	SUB	TMB substrate (tetramethylbenzidine), ready to use	15 ml
KR1016	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 subject samples containing human sRANKL and the OPG solution are added to wells of microplate coated with a high affine poly-clonal 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 bio-tinylated monoclonal anti-human sRANKL antibody is added to each microtiter well and a sandwich of capture antibody – human OPG – sRANKL – streptavidin (per-oxidase-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 ge-nerated, using the values obtained from the standard. total sRANKL present in the subject 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 subject samples may not be valid if within the same assay one or more values of the quality control sample are outside the accep-table limits.

Reference range

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

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 research 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

- The guidelines for 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

For research use only

To be used with


Manufacturer

Contains sufficient for <n> tests

Lot number


Use by


Attention


Consult instructions for use

Consult specification data sheet