DRG

DRG® Human sRANKL (free) ELISA (EIA-4771)

As of 19 Feb. 2008 **RUO**

INTENDED USE

The Human sRANKL (free) ELISA is a sandwich enzyme immunoassay for the quantitative measurement of free sRANKL in serum and plasma samples.

It is intended for in vitro research use only.

Features

– The free assay time is about 4 hours.
– The kit measures free sRANKL

STORAGE, EXPIRATION

Store the kit at 2-8°C.

Under these conditions, the kit is stable until the expiration date (see label on the box).

SUMMARY

sRANKL, receptor activator of nuclear factor (NF)- KB ligand (also: osteoprotegerin ligand, OPGL), is a part of the TNF superfamily with high similarity to other members of that protein species. (SwissProt Nr. O14788).

Three isoforms are produced by alternate splicing, two type II membrane proteins (ISOFORM 1, 317 AA, and ISOFORM 3, 270 AA), and a secreted molecule (ISOFORM 2, 244 AA). ISOFORM 1 is identical to previously reported RANKL and possesses intracellular, transmembrane, and extracellular domains; ISOFORM 2 does not have the intracellular and transmembrane domains, and ISOFORM 3 does not have the intracellular domain. A soluble form arises by proteolytic processing from membrane isoforms.

Although all forms are bioactive, the membrane-bound proteins seem to be the homeostatic forms, while the production of soluble RANKL signals pathological conditions.

RANKL, RANK, and osteoprotegerin (OPG) have been identified as the key molecular regulation system for bone remodelling. RANKL 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. Several studies using animal models have shown that restoring the RANKL/OPG balance (e.g. by administering OPG) reduces the severity of these disorders.

Indication

- Postmenopausal and senile osteoporosis
- Diseases with locally increased bone resorption activity

- Paget´s disease
- Periodontal disease
- Cardiovascular disease, arterial calcification
- Inflammatory diseases
- Immunological disorders
- Arthritis - Oncology

TEST PRINCIPLE

In the Human sRANKL (free) ELISA, standards, quality controls and samples are incubated in microtitration wells coated with OPG. After a 2-hour incubation followed by a wash, biotin-labelled polyclonal antihuman sRANKL antibody is added and incubated with captured sRANKL. After a thorough wash, streptavidin-horseradish peroxidase conjugate is added. After one hour incubation and the last washing step, the remaining conjugate is allowed to react with the substrate H_2O_2-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm.

The absorbance is proportional to the concentration of free sRANKL. A standard curve is constructed by plotting absorbance values versus concentrations of free sRANKL standard, and concentrations of unknown samples are determined using this standard curve.

PRECAUTIONS

- For in vitro research use only.
- This kit contains components of human origin. These materials were found non-reactive for hepatitis B surface antigen and for HIV antibody. However, these materials should be handled as potentially infectious, as no tests can guarantee the complete absence of infectious agents.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide. Wear gloves and eye protection when handling these reagents. In case of contact with the Stop Solution and the Substrate Solution wash skin thoroughly with water and seek medical attention, when necessary.
- Wear gloves and laboratory coats when handling immunodiagnostic materials and serum samples.
- The materials must not be pipetted by mouth.
- Do not drink, eat or smoke in the areas where immunodiagnostic materials are being handled.
- Reagents from kits with different lot numbers should not be mixed.
- Kit should not be used beyond the expiration marked on the label.

REAGENTS SUPPLIED

Kit Components	Quantity
Microtiter Strips, coated with Osteoprotegerin, sealed	96 wells
Biotin labelled Anti-sRANKL **Antibody**, ready to use	13 mL
Streptavidin-Horseradish Peroxidase **Conjugate**, ready to use	13 mL
Human sRANKL **Master Standard**, lyophilized	1 vial
Quality **Control High**, lyophilized	1 vial
Quality **Control Low**, lyophilized	1 vial
Dilution Buffer, ready to use	20 mL
Wash Solution Concentrate (10x)	100 mL
Substrate Solution (TMB), ready to use	13 mL
Stop Solution (0.2 M H_2SO_4)ready to use	13 mL
Instruction Manual + Certificate of Analysis	1 pc

MATERIALS REQUIRED BUT NOT SUPPLIED

- Test tubes for diluting samples
- Precision pipettes to deliver 50-1000 µL and disposable tips
- Multichannel pipette 50-100 µL
- Glassware (graduated cylinder and bottle for Wash Solution)
- Deionized (distilled) water
- Microtitration plate washer (optional) [Manual washing is possible but not preferable.]
- Microplate reader with 450 nm filter
- Software package facilitating data generation and analysis (optional)

PREPARATION OF REAGENTS

All reagents need to be brought to room temperature prior to the assay.

Assay reagents are supplied ready to use, with the exception of Human sRANKL Master Standard, Quality Control and Wash Solution Concentrate (10x).

If you do not use the whole plate, return unused strips in the provided aluminium bag with dessicant and seal the bag carefully. Keep the unused strips at 2-8°C, protected from the moisture.

Preparation of reagents for 1 plate:

Wash Solution:

Dilute 100 mL of Wash Solution concentrate with 900 mL of deionized (distilled) water.

Stability and storage:

The diluted Wash Solution is stable for one month if stored at 2-8°C.

Human sRANKL Standards:

Reconstitute sRANKL Master Standard with 1 mL of Dilution Buffer. The concentration of the human sRANKL in the stock solution is 40 pmol/L. Prepare Standardss as follows:

Standard Volume	Dilution Buffer Volume	Concentration
stock		40 pmol/L
500 µL of stock	500 µL	20 pmol/L
500 µL of std. 20 pmol/L	750 µL	8 pmol/L
500 µL of std. 8 pmol/L	500 µL	4 pmol/L
500 µL of std. 4 pmol/L	500 µL	2 pmol/L
500 µL of std. 2 pmol/L	750 µL	0.8 pmol/L
500 µL of std. 0.8 pmol/L	500 µL	0.4 pmol/L

Prepared standards are ready to use, do not dilute them.

Stability and storage:

Standards are stable until the expiration date (see label on the box) when stored at -20°C.

Human sRANKL Quality Control:

Reconstitute Quality Control with 1 mL of Dilution Buffer. Refer to the Certificate of Analysis for actual Quality Control value.

Reconstituted Quality Controls are ready to use, do not dilute them.

Stability and storage:

Reconstituted Quality Controls are stable until the expiration date (see label on the box) when stored at -20°C.

PREPARATION OF SAMPLES

Dilute serum or plasma samples prior to use **1:25** with Dilution Buffer,

e.g. 5 µL sample + 120 µL Dilution Buffer when assaying samples in singlets or preferably
10 µL sample + 240 µL Dilution Buffer for duplicates.

Stability and storage:

See chapter 15.

Do not store the diluted (1:25) samples

ASSAY PROCEDURE

1. Pipet 100 µL of Standards, diluted Quality Controls, Dilution Buffer (=Blank) and diluted samples, preferably in duplicates, into the appropriate wells.
   See Figure 1 for example of work sheet.
2. **Incubate the plate at room temperature (ca. 25°C) for 2 hours shaking at ca. 300 rpm on an orbital microplate shaker.**
3. Wash the wells 5-times with Wash Solution (0.35 mL per well).
4. Pipet 100 µL of Biotin Labelled Anti-sRANKL Antibody Solution into each well.
5. **Incubate the plate at room temperature (ca. 25°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.**
6. Wash the wells 5-times with Wash Solution (0.35 mL per well).
7. Pipet 100 µL of Streptavidin-HRP Conjugate.
8. **Incubate the plate at room temperature (ca. 25°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.**
9. Wash the wells 5-times with Wash Solution (0.35 mL per well).
10. Pipet 100 µL of Substrate Solution. (Avoid exposing the microtiter plate to direct sunlight.
    Covering the plate with e.g. aluminium foil is recommended.)
11. **Incubate the plate for 10 minutes at room temperature.** (The incubation time may be extended [up to 25 minutes] if the reaction temperature is below than 20°C). No shaking!
12. Stop the colour development by adding 100 µL of Stop Solution.
13. Determine the absorbance by reading the plate at 450 nm. (The absorbance should be read within 5-15 minutes following step 12).

Note: If the plate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine sRANKL concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.)

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	strip 1+ 2	strip 3 + 4	strip 5+ 6	strip 7+ 8	strip 9+10	strip 11+ 12
A	Standard 20	QC High	Sample 7	Sample 15	Sample 23	Sample 31
B	Standard 8	QC Low	Sample 8	Sample 16	Sample 24	Sample 32
C	Standard 4	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
D	Standard 2	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
E	Standard 0.8	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
F	Standard 0.4	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
G	Blank	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
H	Blank	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38

Figure 1: Example of work sheet

CALCULATIONS

Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of Calibrators versus log of the known concentration (X) of Calibrators, using the four-parameter function.

As the Standards and the Quality Controls don't have to be diluted but the samples are diluted 25-times, the values of samples calculated from the calibration curve have to be multiplied by a dilution factor of 25 to obtain the true results!

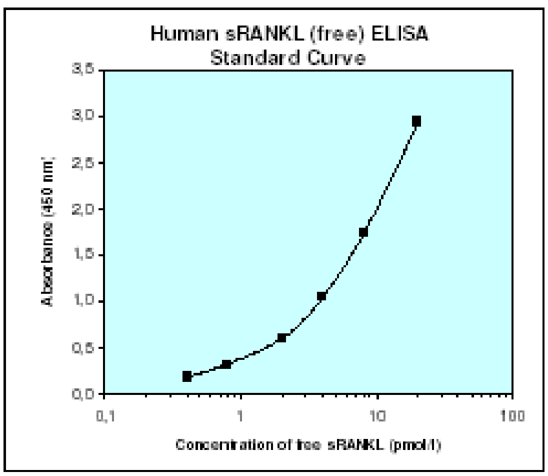

Figure 2: Standard Curve for human free sRANKL is plotted using the four-parameter function as a proportion of human free sRANKL concentration and absorbance at 450 nm and presented in log x lin scale.

LIMITS OF ASSAY

Samples exceeding free sRANKL level of 20 pmol/L should be repeated using higher dilution.

Dilution factors need to be taken into consideration in calculating the free sRANKL concentration.

PERFORMANCE CHARACTERISTICS

Typical analytical data obtained with the Human sRANKL (free) ELISA are presented in this chapter. For actual Calibration curve and Quality Control value see the Certificate of Analysis.

1.1 Sensitivity

The limit of detection (defined as human sRANKL concentration giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: $A_{blank} + 3 \times SD_{blank}$) is defined as follows:

Analytical Limit of Detection (LOD) is calculated from the real sRANKL values in wells and is 0.2 pmol/L

Assay Sensitivity (LOQ) takes the dilution of samples into consideration and is calculated according to the formula:
Assay Sensitivity = Analytical Limit of Detection x sample dilution = 0.2 pmol/L x 25 = 5 pmol/L

*Dilution Buffer is pipetted into blank wells.

1.2 Specificity

The antibody in Human sRANKL (free) ELISA is highly specific to human sRANKL (AA140-AA317 of RANKL protein) with no detectable cross reactivity to human OPG, RANK, COMP, osteocrin, CRP at 50 ng/mL and TNF-alfa, IL-6, IL-11 at 2ng/mL.

1.3 Precision

Serum samples (diluted 1:25 with Dilution Buffer) were assayed. The presented values were corrected with the dilution factor.

Intra-assay (Within-Run) (n=8)

Sample	Mean (pmol/L)	SD	CV (%)
1	62.3	3.9	5.9
2	31.5	2.6	8.2

Inter-assay (Run-to-Run) (n=4)

Sample	Mean (pmol/L)	SD	CV (%)
1	59.5	4.3	7.2
2	32.5	2.8	8.6

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1.4 Dilution Linearity

Serum samples were diluted (see table below) with Dilution Buffer and assayed at a further dilution of 1:25 (see point 8). Results are presented after calculation.

Sample	Dilution	Observed (pmol/L)	Expected (pmol/L)	Recovery O/E (%)
1	-	74.0	-	-
	1:2	37.9	37.0	102
	1:4	19.1	18.5	103
2	-	106.6	-	-
	1:2	56.1	53.3	105
	1:4	28.1	26.6	105

DEFINITION OF sRANKL MASTER STANDARD

In humans serum sRANKL is described as a homotrimeric molecule with MW of 60 kDa (20 kDa for each monomer).

A recombinant sRANKL (AA140-AA317 of RANKL protein) is used as the standard in our assay.

The protein concentration was determined by BCA method (Sigma-Aldrich) and presented in the unit pmol/L (M.W. of homotrimeric protein is used for the calculation).

Unit conversions:

1 pmol/L = 62.5 pg/ml

1 pg/mL = 0.016 pmol/L

PELIMINARY CLINICAL STUDY (UNPUBLISHED DATA)

In our preliminary study, we investigated relations between serum free sRANKL, total sRANKL and OPG level.

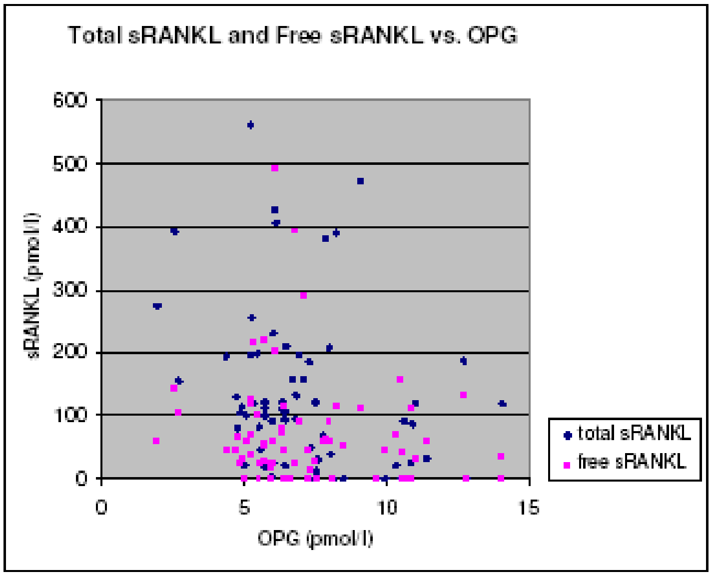

Figure 4: Serum free sRANKLor total sRANKL versus serum OPG level.

TROUBLESHOOTING AND FAQS

1. **Weak signal in all wells**

Possible explanations:

· Omission of a reagent or a step
· Improper preparation or storage of a reagent
· Assay performed before the Substrate Solution was allowed to come to room temperature

2. **High signal and background in all wells**

Possible explanations:

· Improper or inadequate washing
· Overdeveloping; incubation time should be decreased before addition of Stop Solution

3. **High coefficient of variation (CV)**

Possible explanation:

· Improper or inadequate washing

4. **Effect of freezing/thawing on the concentration of free sRANKL in samples**

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No decline was observed in concentration of sRANKL in serum samples after repeated (3x) freezing/thawing cycles. Avoid unnecessary repeated freezing/thawing of the samples.

5. **Stability of samples at 4°C**

Samples should be stored at –80°C. No decline was observed in concentration of free sRANKL in serum and plasma samples in our laboratory when stored at 4°C for 1 week. To avoid microbial contamination, add NaN_3 to a final concentration 0.1% to the samples.

However, poor stability of free sRANKL has been described in the study:

Chan BY, Buckley KA, Durham BH, Gallagher JA, Fraser WD: Effect of anticoagulants and storage temperature on the stability of receptor activator for nuclear factor-kappa B ligand and osteoprotegerin in plasma and serum. Clin Chem. 2003 Dec;49(12):2083-5.

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   Cell (1998), 93:165-176.
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Nature (1999), 402: 304-309.
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J Mol Med (2001), 79: 243-253.
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Arthritis & Rheumatism (2001), 44:253-259.
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J Clin Endocrinol Metab (2000), 85: 2355-2363.
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Science (2000), 289: 1504-1508.

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	A	B	C	D	E	F	G	H

Symbols Used with DRG Assays

Symbol	English	Deutsch	Français	Español	Italiano
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
CE	European Conformity	CE-Konfirmitäts-kennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
IVD	In vitro diagnostic device	In-vitro-Diagnostikum	Ussage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
RUO	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
REF	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
LOT	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
Σ	Contains sufficient for <n> tests/	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos	Contenuto sufficiente per "n" saggi
	Storage Temperature	Lagerungstemperatur	Température de conservation	Temperatura de conservación	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeits-datum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Vertreiber	Distributeur	Distribuidor	Distributore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità

Symbol	Portugues	Dansk	Svenska	Ελληνικά
	Consulte as instruções de utilização	Se brugsanvisning	Se bruksanvisningen	Εγχειρίδιο χρήστη
CE	Conformidade com as normas europeias	Europaeisk overensstemmelse	Europeisk överensstämmelse	Ευρωπαϊκή Συμμόρφωση
IVD	Diagnóstico in vitro	In vitro diagnostik	Diagnostik in vitro	in vitro διαγνωστικό
RUO				
REF	Catálogo n.º	Katalognummer	Katalog nummer	Αριθμός καταλόγου
LOT	No do lote	Lot nummer	Batch-nummer	Αριθμός Παρτίδος
Σ		Indeholder tilstrækkeligt til "n" test	Innehåller tillräckligt till "n" tester	Περιεχόμενο επαρκές για «n» εξετάσεις
	Temperatura de conservação	Opbevarings-temperatur	Förvaringstempratur	Θερμοκρασία αποθήκευσης
	Prazo de validade	Udløbsdato	Bäst före datum	Ημερομηνία λήξης
	Fabricante	Producent	Tillverkare	Κατασκευαστής
Distributed by				
Content	Conteúdo	Indhold	Innehåll	Περιεχόμενο
Volume/No.	Volume/Número	Volumen/antal	Volym/antal	Όγκος/αριθ..

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