DRG® Human sRANKL (total) ELISA (EIA-4772)

Revised 3 Aug. 2010 rm (Vers. 3.1) **RUO**

Please use only the valid version of the package insert provided with the kit.

INTENDED USE

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

Features

- **It is intended for research use only.**
- The total assay time is about 20 hours.
- The kit measures total sRANKL in serum and plasma (EDTA, citrate, heparin).
- Assay format is 96 wells.
- Quality Controls are recombinant protein based. No animal sera are used.
- Standard is recombinant protein based.
- Components of the kit are provided ready to use, concentrated or lyophilized.

STORAGE, EXPIRATION

Store the complete kit at 2-8°C.

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

For stability of opened reagents see Chapter 9.

INTRODUCTION

sRANKL, receptor activator of nuclear factor (NF)- $_{K}$B 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.

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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 (total) ELISA, standards, quality controls and samples are incubated in microplate wells pre-coated with monoclonal anti-human sRANKL antibody. After 16 - 20 hours incubation and washing, biotin-labelled polyclonal anti-human sRANKL antibody is added and incubated with captured sRANKL for 60 minutes. After another washing, streptavidin-HRP conjugate is added. After 60 minutes incubation and the last washing step, the remaining conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured spectrophotometrically at 450 nm. The absorbance is proportional to the concentration of sRANKL. A standard curve is constructed by plotting absorbance values against concentrations of standards, and concentrations of unknown samples are determined using this standard curve.

PRECAUTIONS

- **For professional use only.**
- Wear gloves and laboratory coats when handling immunodiagnostic materials
- Do not drink, eat or smoke in the areas where immunodiagnostic materials are being handled.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves, eye and clothing protection when handling these reagents. Stop and/or Substrate Solutions may cause skin/eyes irritation. In case of contact with the Stop Solution and the Substrate Solution wash skin/eyes thoroughly with water and seek medical attention, when necessary.
- The materials must not be pipetted by mouth.

TECHNICAL HINTS

- Reagents with different lot numbers should not be mixed.
- Use thoroughly clean glassware.

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- Use deionized (distilled) water, stored in clean containers.
- Avoid any contamination among samples and reagents. For this purpose, disposable tips should be used for each sample and reagent.
- Substrate Solution should remain colourless until added to the plate. Keep Substrate Solution protected from light.
- Stop Solution should remain colourless until added to the plate. The colour developed in the wells will turn from blue to yellow immediately after the addition of the Stop Solution.
- Wells that are green in colour indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.
- Dispose of consumable materials and unused contents in accordance with applicable national regulatory requirements.

REAGENTS SUPPLIED

Kit Components	State	Quantity
Antibody Coated Microtiter Strips	ready to use	96 wells
Biotin Labelled Antibody	ready to use	13 ml
Streptavidin-HRP Conjugate	ready to use	13 ml
Master Standard	lyophilized	1 vial
Quality Control High	lyophilized	1 vial
Quality Control Low	lyophilized	1 vial
Dilution Buffer	ready to use	2 x 15 ml
Wash Solution Concentrate (10x)	concentrated	100 ml
Substrate Solution	ready to use	13 ml
Stop Solution	ready to use	13 ml
Instruction Manual + Certificate of Analysis		1 pc

MATERIALS REQUIRED BUT NOT SUPPLIED

- Deionized (distilled) water
- Test tubes for diluting samples
- Glassware (graduated cylinder and bottle) for Wash Solution (Dilution Buffer)
- Precision pipettes to deliver 10-1000 µl with disposable tips
- Multichannel pipette to deliver 100 µl with disposable tips
- Absorbent material (e.g. paper towels) for blotting the microtitrate plate after washing
- Vortex mixer
- Orbital microplate shaker capable of approximately 300 rpm
- Microplate washer (optional). [Manual washing is possible but not preferable.]
- Microplate reader with 450 ± 10 nm filter
- Software package facilitating data generation and analysis (optional)

PREPARATION OF REAGENTS

- **All reagents need to be brought to room temperature prior to use.**
- **Always prepare only the appropriate quantity of reagents for your test.**
- **Do not use components after the expiration date marked on their label.**

1.1 Assay reagents supplied ready to use

Antibody Coated Microtiter Strips

Stability and storage:

Return the unused strips to the provided aluminium zip-sealed bag with desicant and seal carefully. Remaining Microtiter Strips are stable 3 months stored at 2-8°C and protected from the moisture.

Biotin Labelled Antibody

Streptavidin-HRP Conjugate

Dilution Buffer

Substrate Solution

Stop Solution

Stability and storage:

Opened reagents are stable 3 months when stored at 2-8°C.

1.2 Assay reagents supplied concentrated or lyophilized

Human sRANKL Master Standard

Refer to Certificate of Analysis for actual volume of Dilution Buffer needed for reconstitution of standard!!!

Reconstitute the lyophilized Master Standard with Dilution Buffer just prior to the assay. Let it dissolve at least 15 minutes with occasionally gently shaking (not to foam). The resulting concentration of the human sRANKL in the stock solution is **64 pmol/l.**

Prepare set of standards using Dilution Buffer as follows:

Volume of Standard	Standard Diluent	Concentration
stock		64 pmol/L
400 µL of stock	400 µL	32 pmol/L
400 µL of std. 32 pmol/L	400 µL	16 pmol/L
400 µL of std. 16 pmol/L	400 µL	8 pmol/L
400 µL of std. 8 pmol/L	400 µL	4 pmol/L
400 µL of std. 4 pmol/L	600 µL	1.6 pmol/L
400 µL of std. 1.6 pmol/L	400 µL	0.8 pmol/L
400 µL of std. 0.8 pmol/L	400 µL	0.4 pmol/L

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

Stability and storage:

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

Quality Controls High, Low

Refer to Certificate of Analysis for actual volume of Dilution Buffer for reconstitution and for actual Quality Controls concentrations!!!

Reconstitute each Quality Control (High and Low) with Dilution Buffer just prior to the assay.

Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam).

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.

Wash Solution

Dilute Wash Solution Concentrate (10x) ten-fold in 900 ml of distilled water to prepare a 1x working solution.

Example: 100 ml of Wash Solution Concentrate (10x) + 900 ml of distilled water for use of all 96-wells.

Stability and storage:

The diluted Wash Solution is stable 1 month when stored at 2-8°C. Opened Wash Solution Concentrate (10x) is stable 3 months when stored at 2-8°C.

PREPARATION OF SAMPLES

The kit measures sRANKL (total) in serum and plasma (EDTA, citrate, heparin).

Samples should be assayed immediately after collection or should be stored at -20°C. Mix thoroughly thawed samples just prior to the assay and avoid repeated freeze/thaw cycles, which may cause erroneous results. Avoid using hemolyzed or lipemic samples.

Dilute samples just prior to the assay 100x with Dilution Buffer, e.g. 5 µl of sample + 495 µl of Dilution Buffer when assaying samples in duplicates, or in two steps when assaying samples in singlets, e.g.

A/ 5 µl sample + 95 µl Dilution Buffer

B/ 50 µl prediluted sample from the step A/ + 200 µl Dilution Buffer

Stability and storage:

Samples should be stored at -20°, or preferably at -70°C for long-term storage. Avoid repeated freeze/ thaw cycles.

Do not store the diluted samples.

See Chapter 13 for stability of serum and plasma samples if stored at 2-8°C, effect of freezing/thawing and effect of sample matrix (serum/plasma) on the concentration of sRANKL.

Note: It is recommended to use a precision pipette and a careful technique to perform the dilution in order to get precise results.

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 2-8°C for 16-20 hours, without shaking.**
3. Wash the wells 5-times with Wash Solution (0.35 mL per well). After final wash, invert and tap the plate strongly against paper towel.
4. Add **100 µL** of Biotin Labelled Antibody Solution into each well.
   Attention: Biotin Labelled Antibody Solution must be brought up to room temperature (incubate at least 2 hours or over night at 25-29°C before the use). Inappropriate temperature may influence resulting sRANKL concentration in samples.
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). After final wash, invert and tap the plate strongly against paper towel.
7. Add **100 µL** of Streptavidin-HRP Conjugate into each well.
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). After final wash, invert and tap the plate strongly against paper towel.
10. Add **100 µL** of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminium foil is recommended.
11. Incubate the plate for **15 minutes** at room temperature**.** The incubation time may be extended [up to 20 minutes] if the reaction temperature is below than 20°C. Do not shake the plate during the incubation.
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 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.)

Note 2:
Manual washing: Aspirate wells and pipet 0.35 ml Wash Solution into each well. Aspirate wells and repeat twice. After final wash, invert and tap the plate strongly against paper towel. Make certain that Wash Solution has been removed entirely.

	strip 1+ 2	strip 3 + 4	strip 5+ 6	strip 7+ 8	strip 9+10	strip 11+ 12
A	**Standard 32**	**QC High**	Sample 7	Sample 15	Sample 23	Sample 31
B	**Standard 16**	**QC Low**	Sample 8	Sample 16	Sample 24	Sample 32
C	**Standard 8**	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
D	**Standard 4**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
E	**Standard 1,6**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
F	**Standard 0,8**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
G	**Standard 0,4**	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 a work sheet

CALCULATIONS

Most microplate readers perform automatic calculations of analyte concentration. The Standard curve is constructed by plotting the mean absorbance at 450 nm (Y) of Standards against log of the known concentration (X) of Standards, using the four-parameter algorithm. Results are reported as concentration of sRANKL pmol/l in samples.

Alternatively, the logit log function can be used to linearize the standard curve (i.e. logit of the mean absorbance (Y) is plotted against log of the known concentration (X) of Standards.

The measured concentration of samples calculated from the standard curve must be multiplied by their respective dilution factor, because samples have been diluted prior to the assay. e.g. 1.68 pmol/l (from standard curve) x 100 (dilution factor) = 168 pmol/l.

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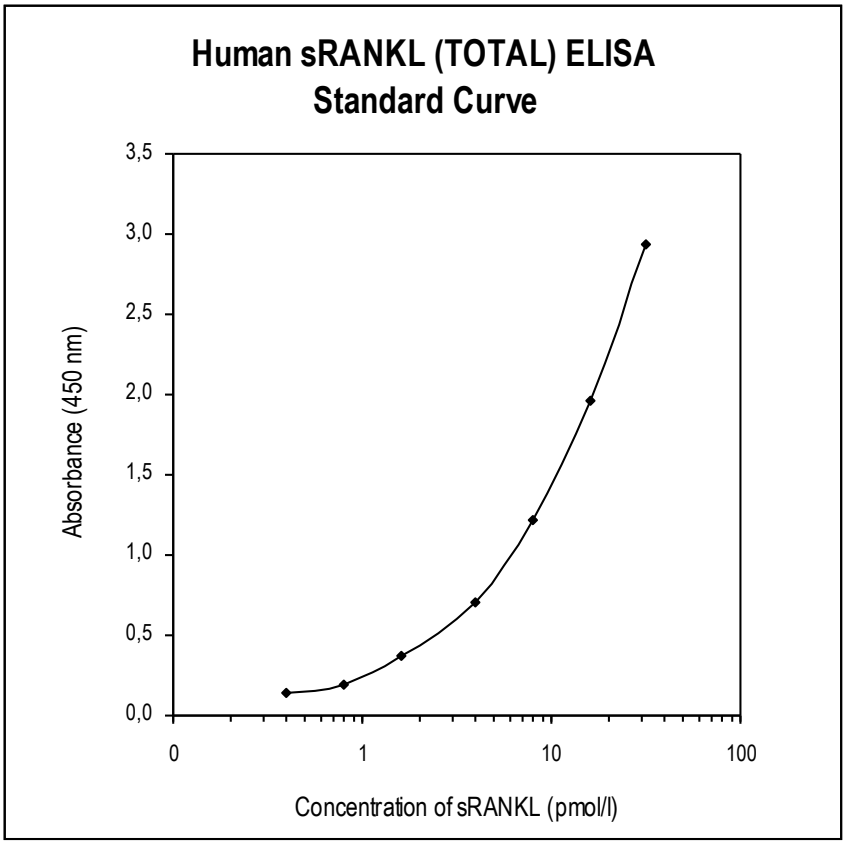

Figure 2: Typical Standard Curve for Human sRANKL (total) ELISA

TROUBLESHOOTING AND FAQS

Weak signal in all wells

Possible explanations:

- Omission of a reagent or a step
- Improper preparation or storage of a reagent
- Assay performed before reagents were allowed to come to room temperature
- Improper wavelength when reading absorbance

High signal and background in all wells

Possible explanations:

- Improper or inadequate washing
- Overdeveloping; incubation time with Substrate Solution should be decreased before addition of Stop Solution
- Incubation temperature over 30°C

High coefficient of variation (CV)

Possible explanation:

- Improper or inadequate washing
- Improper mixing Standards, Quality Controls or samples

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REFERENCES

1. Hofbauer L.C et al. Effect of oral contraceptives on circulatingosteoprotegerin and soluble RANK ligand serum levels in healthy young women.
   Clin Endocrinol (2004) 60: 214-219
2. Ito S. et al. Crystal structure of the extracellular domain of mouse ligand at2.2-A resolution.
   J Biol Chem (2002) 22: 6631-6636.
3. Lam J. et al. Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specifity.
   J Clin Invest (2001) 108: 971-979.
4. Lacey D.L, et al., Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and · activation.
   Cell (1998), 93:165-176.
5. Kong Y.Y. et al., OPGL is a key regulator of osteoclastogenesis, lymphocyte development and · lymph-node organogenesis. Nature (1999), 397: 315-323.
6. 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.
7. 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.
8. 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.
9. 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):768-775.
10. 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.
11. 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.
12. Hofbauer L.C. & A.E. Heufelder, The Role of Osteoprotegerin and Receptor Activator of Nuclear Factor B Ligand in the Pathogenesis and Treatment of Rheumatoid Arthritis.
    Arthritis & Rheumatism (2001), 44:253-259.
13. 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.
14. Teitelbaum S.L., Bone resorption by osteoclasts.
    Science (2000), 289: 1504-1508.

ASSAY PROCEDURE SUMMARY

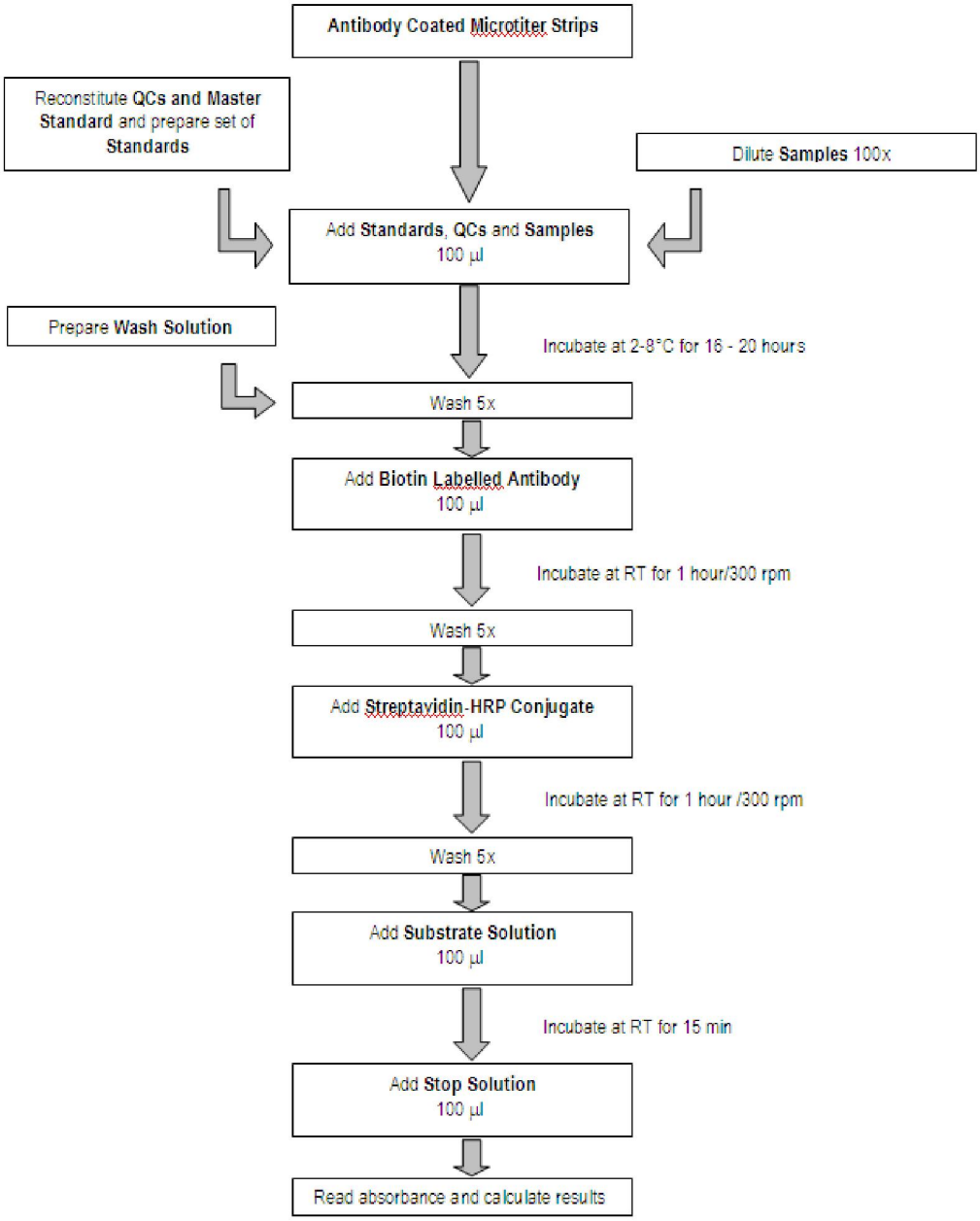

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Plate Layout

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	A	B	C	D	E	F	G	H

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