FREE soluble RANKL
High Sensitivity

3rd Generation ENZYME IMMUNOASSAY FOR THE QUANTITATIVE DETERMINATION OF FREE, SOLUBLE, UNCOMPLEXED HUMAN RANKL* IN SERUM OR HEPARIN PLASMA
CAT. NO. BI-20462 12 X 8 TESTS

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

*Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL)

rev.no. 141126

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BIOMEDICA

CONTENT

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1) INTRODUCTION

RANKL, the receptor activator of nuclear factor kappa B ligand, a member of the tumor necrosis factor (TNF) family (http://www.uniprot.org/uniprot/O14788), is the main stimulatory factor for the formation of mature osteoclasts and is essential for their survival. RANKL activates its specific receptor RANK, located on osteoclasts and dendritic cells. The effects are counteracted by OPG which acts as an endogenous soluble receptor antagonist (see: BI-20403 – OPG ELISA).

The major source of RANKL are osteocytes, former osteoblasts that become embedded within the mineralized bone matrix. RANKL is a ~35 kD type II transmembrane-type protein and is cleaved to release a soluble biologically active product that forms a homotrimer.

RANKL and its specific receptor RANK are not only key regulators of bone remodeling but also play an essential role in immunobiology, e.g. lymph node formation, establishment of the thymic microenviroment, mammary gland development during pregnancy, bone metastasis in cancer and sex-hormone, progestin-driven breast cancer, thermoregulation, and finally in the development of type 2 diabetes mellitus.

Possible Indications:

- Postmenopausal and senile osteoporosis
- Glucocorticoid induced osteoporosis
- Disease with locally increased resorption activity
- Arthritis
- Oncology
- Type 2 diabetes mellitus

2) CONTENTS OF THE KIT

CONT	KIT COMPONENTS	QUANTITY
PLATE	Human recombinant OPG pre-coated microtiter strips in strip holder	12 x 8 tests
WASHBUF	Wash buffer concentrate 20x, natural cap	1 x 50 ml
ASYBUF	Assay buffer, red cap, ready to use	1 x 7 ml
AB	Goat polyclonal biotinylated anti sRANKL antibody, green cap, ready to use	1 x 22 ml
STD	Standards (0; 0.0625; 0.125; 0.25; 0.5; 1; 2 pmol/l), white caps	7 vials lyophilised
CTRL	Controls A+B, recombinant human RANKL in human serum, yellow caps, exact concentration after reconstitution see label	2 vials lyophilised
CONJ	Conjugate (streptavidin-polyHRPO), amber cap, ready to use	1 x 22 ml
SUB	Substrate (TMB solution), amber bottle, blue cap, ready to use	1 x 22 ml
STOP	Stop solution, white cap, ready to use	1 x 7 ml

3) ADDITIONAL MATERIAL SUPPLIED IN THE KIT

- 3 self-adhesive plastic films
- QC protocol
- Protocol sheet
- Instruction manual for use

4) MATERIAL AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

- Precision pipettes calibrated to deliver 50 µl, 150 µl, 200 µl, 300 µl and disposable tips
- Distilled or deionised water
- Plate washer is recommended for washing, alternative multichannel pipette or manifold dispenser
- Refrigerator with 4°C (2-8°C)
- ELISA reader capable of measuring absorbance at 450 nm (with correction wavelength at 630 nm)
- Graph paper or software for calculation of results

5) REAGENTS AND SAMPLE PREPARATION

All reagents of the kit are stable at 4°C (2-8°C) until the expiry date stated on the label of each reagent.

Sample preparation:

Collect venous blood samples by using standardized blood collection tubes for serum or Heparin plasma. We recommend performing plasma or serum separation by centrifugation as soon as possible, e.g. 10 min at 2000 x g, preferably at 4°C (2-8°C). If this is not possible, store the samples at 4°C (2-8°C) prior to centrifugation (maximal one day).The acquired plasma or serum samples should be measured as soon as possible. For longer storage aliquot samples and store at -25°C or lower. Samples should undergo 3 freeze-thaw cycles only. Lipemic or haemolysed samples may give erroneous results. Samples should be mixed well before assaying. We recommend duplicates for all values. If samples read higher than the top standard, we recommend diluting with a low-measuring serum sample and re-measuring the samples.

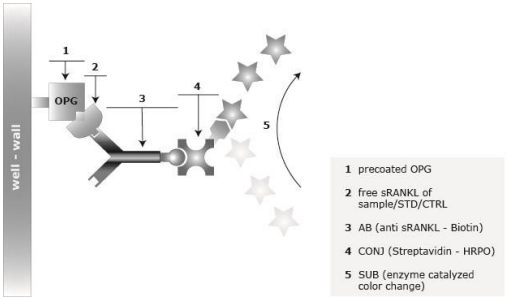
For further information on sample stability please visit our website www.bmgrp.com (see Technical Files) or contact our customer service by e-mail export@bmgrp.com or by phone +43/ 1/ 29107-45.

Reconstitution / Handling:

STD (Standards) and CTRL (Controls): Pipette 700 µl of distilled or deionised water into each vial. Leave at room temperature (18-24°C) for 15 min. Reconstituted STD and CTRL are stable at -25°C or lower until expiry date stated on label. STDs and CTRLs are stable for 3 freeze-thaw cycles.

WASHBUF (Wash buffer): Dilute the concentrate 1:20 (e.g. 50 ml WASHBUF + 950 ml distilled water). Crystals in the buffer concentrate will dissolve at room temperature. The undiluted buffer is stable at 4°C (2-8°C) until expiry date stated on label. The diluted WASHBUF is stable up to one month at 4°C (2-8°C). Only use diluted WASHBUF (Wash buffer) when performing the assay.

6) PRINCIPLE OF THE ASSAY:



7) ASSAY PROTOCOL

All reagents and samples must be at room temperature (18-24°C) before use in the assay.
Mark position for BLANK/STD/SAMPLE/CTRL (Blank/Standard/Sample/Control) on the protocol sheet.
Take microtiter strips out of the aluminium bag, take a minimum of one well as blank. Store unused strips with desiccant at 4°C (2-8°C) in the aluminium bag. Strips are stable until expiry date stated on the label.

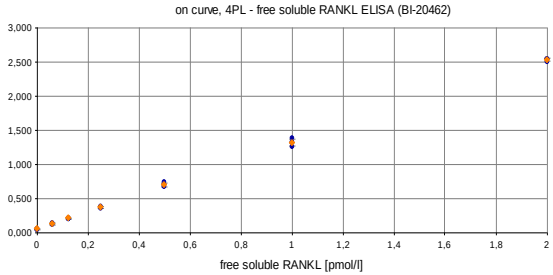
1. **Prewash wells** with 300 µl diluted WASHBUF (wash buffer, natural cap) five times. Remove remaining WASHBUF by tapping plate against paper towel after the last wash.
2. Add 50 µl ASYBUF (assay buffer, red cap) into each well. Pipette additional 150 µl ASYBUF into well marked as blank.
3. Pipette 150 µl STD/SAMPLE/CTRL (Standard/Sample/Control) in duplicate into respective wells, except blank.
4. Cover tightly and incubate at room temperature (18-24°C) for 2 hours.

5. Aspirate and wash wells with 300 µl diluted WASHBUF (wash buffer, natural cap) five times. Remove remaining WASHBUF by tapping plate against paper towel after the last wash.
6. Add 200 µl AB (biotinylated anti sRANKL antibody, green cap) into each well, except blank. Swirl gently. Pipette additional 200 µl ASYBUF (assay buffer, red cap) into well marked as blank.
7. Cover tightly and incubate at 4°C (2-8°C) over night (18-24 hours).
8. Aspirate and wash wells with 300 µl diluted WASHBUF (wash buffer, natural cap) fives times. Remove remaining WASHBUF by tapping plate against paper towel after the last wash.
9. Add 200 µl CONJ (conjugate, amber cap) into each well.
10. Cover tightly and incubate at room temperature (18-24°C) for 1 hour in the dark.
11. Aspirate and wash wells with 300 µl diluted WASHBUF (wash buffer, natural cap) fives times. Remove remaining WASHBUF by tapping plate against paper towel after the last wash.
12. Add 200 µl SUB (substrate, blue cap) into each well.
13. Incubate at room temperature (18-24°C) for 30 min in the dark.
14. Add 50 µl STOP (stop solution, white cap) into each well.
15. Measure absorbance immediately at 450 nm with reference 630 nm if available.

8) CALCULATION OF RESULTS

Read the optical density (OD) of all wells on a plate reader using 450 nm wavelength (correction wavelength 630 nm). Subtract the blank OD from the values of STD, CTRL and sample. Construct the standard curve from the OD values of the STD. Use commercially available software or graph paper. Calculate sample concentration from the standard curve. The assay was evaluated with 4PL algorithm. Different curve-fitting methods need to be evaluated by the user.

Example typical STD-curve:



The quality control protocol supplied with the kit shows the results of the final release QC for each kit lot. Data for optical density obtained by customers may differ due to various influences and/or due to the normal decrease of signal intensity during shelf life. However, this does not affect validity of results as long as an OD of 1.50 or more is obtained for the standard with the highest concentration and the values of the CTRLs are in range (target ranges see labels).

9) ASSAY CHARACTERISTICS

Values of apparently healthy individuals:	Median (serum, n = 32): 0.14 pmol/l Each laboratory should establish its own reference range for the samples under investigation. Do not change sample type during study.
Standard range:	0; 0.0625; 0.125; 0.25; 0.5; 1; 2 pmol/l
Conversion factor pg/ml to pmol/l:	1 pg/ml = 0.05 pmol/l (MW: 20 kD, monomer)
Sample volume:	150 µl human serum or Heparin plasma
Detection limit / LLOQ:	(0 pmol/l + 3 SD): 0.01 pmol/l / 0.008pmol/l
Incubation time:	2 h / overnight / 1 h / 30 min

10) PRECISION

Intra-assay: 2 samples of known concentrations were tested 5 times within one kit lot by one operator.
Inter-assay: 2 samples of known concentrations were tested 12 times within 3 different kit lots and by 3 different operators.

Intra-assay (n=5)	Sample 1	Sample 2	Inter-assay (n=12)	Sample 1	Sample 2
Mean (pmol/l)	0.12	1.00	Mean (pmol/l)	0.12	1.00
SD (pmol/l)	0.005	0.04	SD (pmol/l)	0.004	0.02
CV (%)	4	4	CV (%)	3	2

Further details on validation data and assay characteristics please visit our website www.bmgrp.com (see Technical Files) or contact our customer service by e-mail export@bmgrp.com or by phone +43/ 1/ 29107-45.

11) TECHNICAL HINTS

- Do not mix or substitute reagents with those from other lots or sources.
- Do not mix stoppers and caps from different reagents or use reagents between lots.
- Do not use reagents beyond expiration date.
- Protect reagents from direct sunlight.
- Substrate solution should remain colourless until added to the plate.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Avoid foaming when mixing reagents.

12) PRECAUTIONS

All test components of human source were tested against HIV-Ab and HBsAg and were found negative. Nevertheless, they should be handled and disposed as if they were infectious.
All liquid reagents contain ≤ 0.1% Proclin 300 as preservative. Avoid contact with skin and mucous membrane. Proclin 300 is not toxic in concentrations used in this kit. It may cause allergic skin reactions – avoid contact with skin or eyes.

- Do not pipette by mouth.
- Do not eat, drink, smoke or apply cosmetics where reagents are used.
- Wear gloves, glasses and lab coat while performing this assay.
- Sulfuric acid is irritating to the eyes and skin. Avoid contact with skin and mucous. Irritations are possible. Flush with water if contact occurs!!

13) LITERATURE

- Immunology and bone. Danks L and Takayanagi H, J Biochem 2013; 154: 29-39.
- Physiology and pathophysiology of the RANKL/RANK system. Hanada R et al., Biol Chem 2010; 391(12): 1365-1370.
- Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nakashima T et al., Nat Med 2011; 17(10): 1231-1234.
- RANKL Employs Distinct Binding Modes to Engage RANK and the Osteoprotegerin Decoy Receptor. Nelson CA et al., Structure, Nov 2012; 20(11): 1971-1982.
- Osteoclast differentiation factor RANKL controls development of progestin-driven mammary cancer. Schramek D et al., Nature 2010; 468(7320): 98-102.
- RANK ligand mediates progestin-induced mammary epithelial proliferation and carcinogenesis. Gonzalez-Suarez E et al., Nature 2010; 468: 103-107.
- Central regulation of body temperature by RANKL/RANK pathway. Hanada R and Penninger JM, Clin Calcium 2011; 21(8): 1201-1208.
- Blockade of receptor activator of nuclear factor-κB (RANKL) signaling improves hepatic insulin resistance and prevents development of diabetes mellitus. Kiechl S et al., Nature Medicine 2013; 19(3):358-363.

SYMBOLS

Expiry date / Verfallsdatum / Date de péremption / Data di scadenza /Fecha de caducidad / Data de validade / Uiterste gebruiksdatum / Udløbsdato / Utgångsdatum / Termin Ważności / Lejárati idő / Doba exspirácie / Doba exspirace

Consider instructions for use / Bitte Gebrauchsanweisung beachten / Consultez la notice d'utilisation / Consultare le istruzioni per l'uso / Consulte las instrucciones de utilización / Consulte as instruções de utilização / Raadpleeg de gebruiksaanwijzing / Se brugsanvisningen / Läs anvisningarna före användning / Proszę przeczytać instrukcję wykonania / Vegyük figyelembe a használati utasításban foglaltakat / Postupujte podľa pokynov na použitie / Postupujte dle návodu k použití

In vitro Diagnostic Medical Device (for in Vitro Diagnostic Use)/ In vitro Diagnostikum (zur In-vitro-Diagnostik) / Dispositif médical de diagnostic in vitro (Pour usage diagnostique in vitro) / Dispositivo medico per diagnostica in vitro (per uso diagnostico in vitro) / Dispositivo médico de diagnóstico in vitro (para uso diagnóstico in vitro) / Dispositivo médico para diagnóstico in vitro (Para utilização de diagnóstico "in vitro") / Medisch hulpmiddel voor diagnostiek in vitro (Voor diagnostisch gebruik in vitro) / Medicinsk udstyr til in vitro-diagnostik (Udelukkende til in vitro diagnostisk anvendelse) / Medicinteknisk produkt avsedd för in vitro-diagnostik (För in vitro-diagnostiskt bruk) / Wyrób medyczny do Diagnostyki In Vitro / In vitro orvosdiagnosztikai termék / In vitro diagnostický zdravotnícky materiál (určené pre diagnostiku „in vitro“) / In vitro diagnostický zdravotnicky materiál (určeno pro diagnostiku „in vitro“)

Lot-Batch Number / Charge-Chargennummer / Lot-Code du lot / Lotto-Numero di lotto / Lote-Código de lote / Lote-Código do lote / Lot-Partijnummer / Lot-Batchkode / Lot-Satskod / Numer serii / Lot-Batch szám / Číslo šarže / Číslo šarže

Manufactured by / Hergestellt von / Fabriqué par / Prodotto da / Fabricado por / Fabricado por / Vervaardigd door / Fabrikation af / Tillverkad av / Wyprodukowane pr / Gyártotta / Vyrobené / Vyrobeno

Catalogue Number / Bestellnummer / Numéro de référence / Numero di riferimento / Número de referencia / Número de referência / Referentienummer / Referencenummer / Katalognummer / Numer katalogowy / Katalógusszám / Katalógové číslo / Katalogové číslo

Store at between / Lagerung bei zwischen / Conserver à entre / Conservare a tra / Conservar a temp. entre / Armazene a entre / Bewaar bij tussen / Opbevares mellem / Förvaras vid / Przechowywać w / Tároljuk …… között / Skladujte v rozsahu / Skladujte v rozmezí

Contains sufficient for x tests / Inhalt ausreichend für x Tests / Contient suffisant pour x tests / Contenuto sufficiente per x test / Contiene suficiente para x pruebas / Contém suficiente para x testes / Bevat voldoende voor x bepalingen / Indeholder tilstrækkeligt til x prøver / Innehållet räcker till x analyser / Zawartość na x testów / Tartalma X teszt elvégzésére elegendő / Obsahuje materiál pre x testov / Obsahuje materiál pro x testů

BI-20462 FREE Soluble RANKL HS ELISA

ASSAY PROTOCOL AND CHECKLIST

PREPARATION OF REAGENTS:

- ❑ Bring all reagents to room temperature (18-24°C).
- ❑ Prepare reagents and samples as instructed.
- ❑ Bring unused and prepared components to the storage temperature mentioned in the package insert.
- ❑ Take microtiter strips out of the alu bag and mark positions on the protocol sheet.

- ❑ 1. **Prewash wells** with 300 µl diluted WASHBUF (wash buffer, natural cap) five times. Remove remaining WASHBUF by tapping plate against paper towel after the last wash.
- ❑ 2. Add 50 µl ASYBUF (assay buffer, red cap) into each well. Pipette additional 150 µl ASYBUF into well marked as blank.
- ❑ 3. Add 150 µl STD/SAMPLE/CTRL (standard/sample/control) into all wells except blank.
- ❑ 4. **Cover tightly and incubate at room temperature (18-24°C) for 2 hours.**
- ❑ 5. Aspirate and wash wells with 300 µl diluted WASHBUF (wash buffer, natural cap) five times. Remove remaining buffer by tapping plate against paper towel.
- ❑ 6. Add 200 µl AB (biotinylated anti sRANKL antibody, green cap) into each well, except blank. Swirl gently. Pipette additional 200 µl ASYBUF (assay buffer, red cap) into well marked as blank.
- ❑ **7. Cover tightly and incubate at 4°C (2-8°C) over night (18-24 hours).**
- ❑ 8. Aspirate and wash wells with 300 µl WASHBUF (wash buffer, natural cap) five times. Remove remaining buffer by tapping plate against paper towel.
- ❑ 9. Add 200 µl CONJ (conjugate, amber cap) into each well.
- ❑ **10. Cover tightly and incubate at room temperature (18-24°C) for 1 hour in the dark.**
- ❑ 11. Aspirate and wash wells with 300 µl WASHBUF (wash buffer, natural cap) five times. Remove remaining buffer by hitting plate against paper towel.
- ❑ 12. Add 200 µl SUB (substrate, blue cap) into each well.
- ❑ **13. Incubate at room temperature (18-24°C) for 30 minutes in the dark.**
- ❑ 14. Add 50 µl STOP (stop solution, white cap) into each well.
- ❑ 15. Measure absorbance immediately at 450 nm with reference 630 nm, if available.

Warranty Information

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

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For further information about this kit, its application or the procedures in this kit insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.

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