

Human sRANKL (total) ELISA

Cat. No. RD193004200R

Manufacturer

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Use only the actual version of Product Data Sheet enclosed with the kit!

1. Intended Use

The RD193004200R Human sRANKL (total) ELISA is a sandwich enzyme immunoassay for the quantitative measurement of total sRANKL (free and bound sRANKL) in serum and plasma samples. It is intended for *in vitro* research use only.

Features

- The total assay time is about 18 hours.
- The kit measures total sRANKL

2. 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).

3. Summary

sRANKL, receptor activator of nuclear factor (NF)- κ 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.

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

4. Test Principle

In BioVendor's Human sRANKL (total) ELISA, calibrators, quality controls and samples are incubated in microtitration wells coated with a monoclonal anti-human sRANKL antibody. After a 16-hour incubation followed by a wash, biotin-labelled polyclonal anti-human 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 sRANKL. A standard curve is constructed by plotting absorbance values versus concentrations of sRANKL calibrator, and concentrations of unknown samples are determined using this standard curve.

5. 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.

6. Reagents Supplied

<i>Cat. No.</i>	<i>Kit Components</i>	<i>Quantity</i>
C181221	Microtiter Strips, coated with Anti-sRANKL Antibody, sealed	96 wells
C182511	Biotin labelled Anti-sRANKL Antibody, ready to use	13 ml
C182351	Streptavidin-Horseradish Peroxidase Conjugate, ready to use	13 ml
C183141	Human sRANKL Master Calibrator, lyophilized	1 vial
C184171	Quality Control High, lyophilized	1 vial
C184271	Quality Control Low, lyophilized	1 vial
C005911	Dilution Buffer, ready to use	2x15 ml
C006121	Wash Solution Concentrate (10x)	100 ml
C007111	Substrate Solution (TMB), ready to use	13 ml
C008111	Stop Solution (0.2 M H ₂ SO ₄) ready to use	13 ml
-	Instruction Manual + Certificate of Analysis	1 pc

7. 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)

8. 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 Calibrator, 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 Calibrators:

Reconstitute sRANKL Master Calibrator with 1 ml of Dilution Buffer. The concentration of the human sRANKL in the stock solution is 64 pmol/l.

Prepare Calibrators as follows:

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

Stability and storage:

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

Human sRANKL Quality Control:

Reconstitute Quality Control with 1.5 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.

<i>Calibrator Volume</i>	<i>Dilution Buffer Volume</i>	<i>Concentration</i>
stock	-----	64 pmol/l
500 ul of stock	500 ul	32 pmol/l
500 ul of std.32 pmol/l	500 ul	16 pmol/l
500 ul of std.16 pmol/l	500 ul	8 pmol/l
500 ul of std.8 pmol/l	500 ul	4 pmol/l
500 ul of std.4 pmol/l	750ul	1.6 pmol/l
500 ul of std.1.6 pmol/l	500ul	0.8 pmol/l
500 ul of std.0.8 pmol/l	500ul	0.4 pmol/l

9. Preparation of Samples

Dilute serum or plasma samples prior to use 1:100 with Dilution Buffer, e.g. 5 µl sample + 495 µl 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/ + 150 µl Dilution Buffer

Stability and storage:

See chapter 15.

Do not store the diluted (1:100) samples

10. Assay Procedure

- 1) Pipet 100 μ l of Calibrators, 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 4-6°C for 16 hours, without shaking.**
- 3) Wash the wells 5-times with Wash Solution (0.35 ml per well).
- 4) Pipet 100 μ l of Biotin Labelled Anti-sRANKL Antibody Solution into each well.
Attention: Biotin Labelled Anti-sRANKL Antibody Solution must be brought up to room temperature (incubate at least 2 hours at room temperature before the use).
- 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 μ l 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 μ l 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.)

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

11. 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 Calibrators and the Quality Controls don't have to be diluted but the samples are diluted 100-times, the values of samples calculated from the calibration curve have to be multiplied by a dilution factor of 100 to obtain the true results!

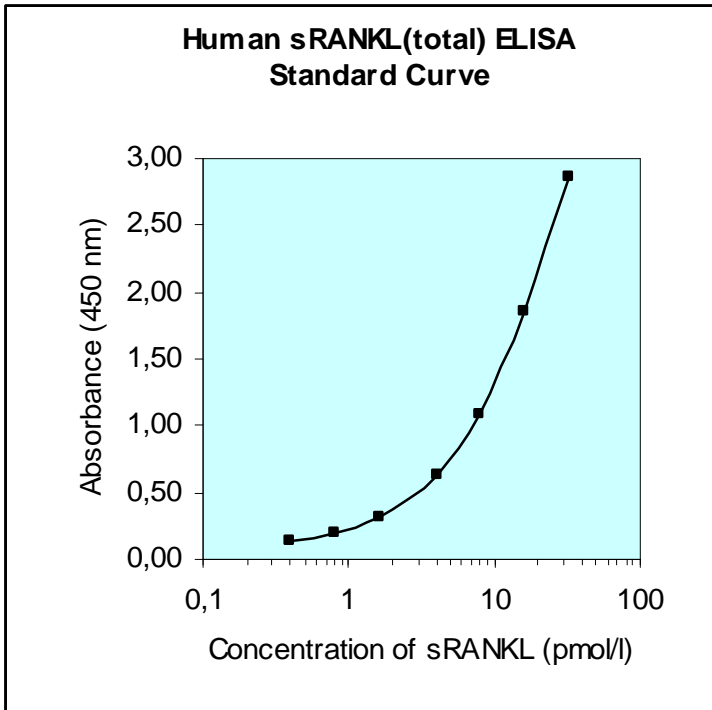


Figure 2: Standard Curve for human sRANKL is plotted using the four-parameter function as a proportion of human sRANKL concentration and absorbance at 450 nm and presented in log x lin scale.

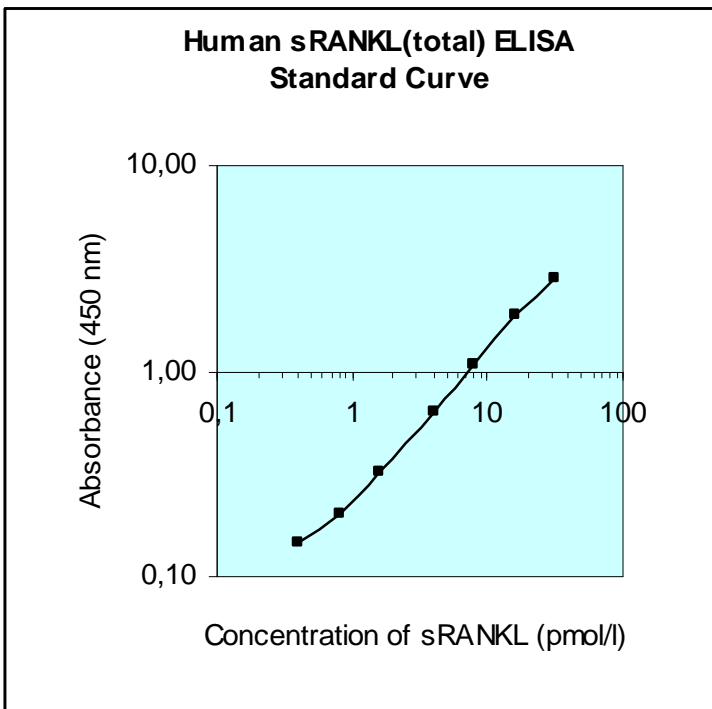


Figure 3: Standard Curve for human sRANKL is plotted as a proportion of human sRANKL concentration and absorbance at 450 nm and presented in log x log scale.

12. Limits of Assay

Samples exceeding total sRANKL level of 32 pmol/l should be repeated using higher dilution. Dilution factors need to be taken into consideration in calculating the total sRANKL concentration.

13. Performance Characteristics

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

- **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: $A_{\text{blank}} + 3 \times \text{SD}_{\text{blank}}$) is defined as follows:

Analytical Limit of Detection (LOD) is calculated from the real sRANKL values in wells and is 0.1pmol/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 = 0.1pmol/l x 100 = 10 pmol/l

*Dilution Buffer is pipetted into blank wells.

- **Specificity**

The antibodies in Human sRANKL (total) ELISA are 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.

- **Precision**

Serum samples (diluted 1:100 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	285	12.8	4.5
2	96	7.6	7.9

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

Sample	Mean (pmol/l)	SD	CV (%)
1	293	17.8	6.0
2	88	7.3	8.3

- **Spiking Recovery**

Serum samples were diluted 1:100 and spiked with different amounts of recombinant sRANKL and assayed. Results are presented after calculation.

Sample	Observed (pmol/l)	Expected (pmol/l)	Recovery O/E (%)
1	58	-	-
	111	108	103
	155	158	98
	189	208	91
2	84	-	-
	129	134	96
	169	184	92
	211	234	90

- **Dilution Linearity**

Serum samples were diluted (see table below) with Dilution Buffer and assayed at a further dilution of 1:100 (see point 8). Results are presented after calculation.

<i>Sample</i>	<i>Dilution</i>	<i>Observed (pmol/l)</i>	<i>Expected (pmol/l)</i>	<i>Recovery O/E (%)</i>
1	-	331	-	-
	1:2	158	166	111
	1:4	74	83	115
2	-	182	-	-
	1:2	94	91	103
	1:4	49	45	108

14. Definition of sRANKL Master Calibrator

In human 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

15. Preliminary clinical study (unpublished data)

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

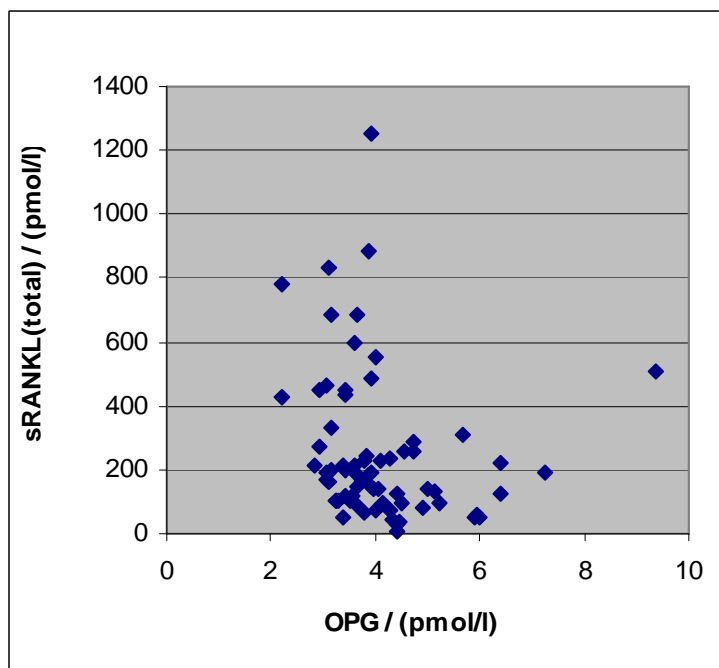


Figure 4: Serum sRANKL (total) is closely related to serum OPG level.

16. 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 total sRANKL in samples

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

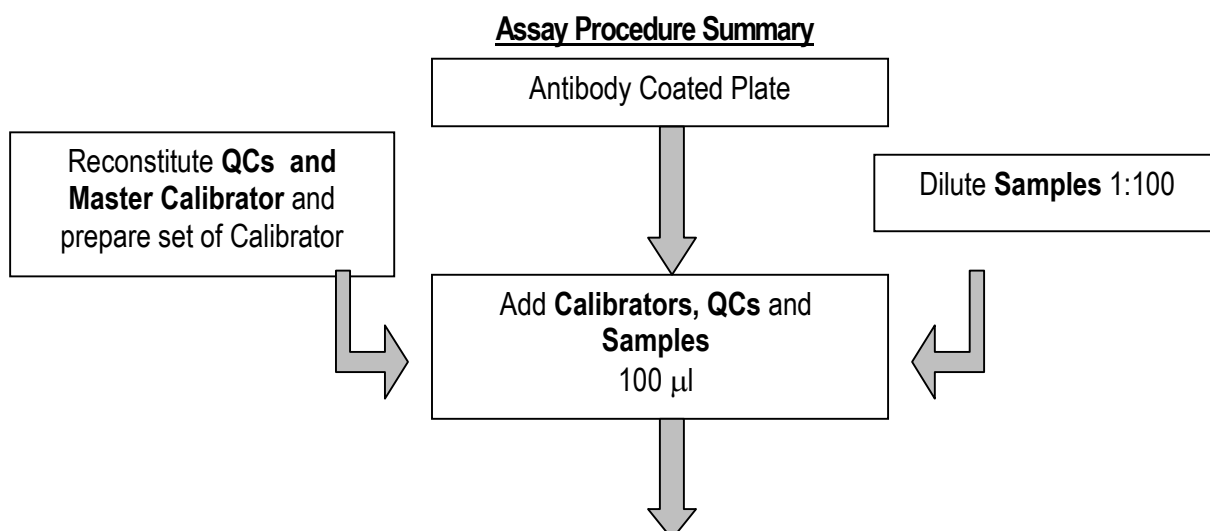
Samples should be stored at -20°C . However, no decline was observed in concentration of sRANKL in serum and plasma samples when stored at 4°C for 1 week. To avoid microbial contamination, add NaN_3 to a final concentration 0.1% to the samples.

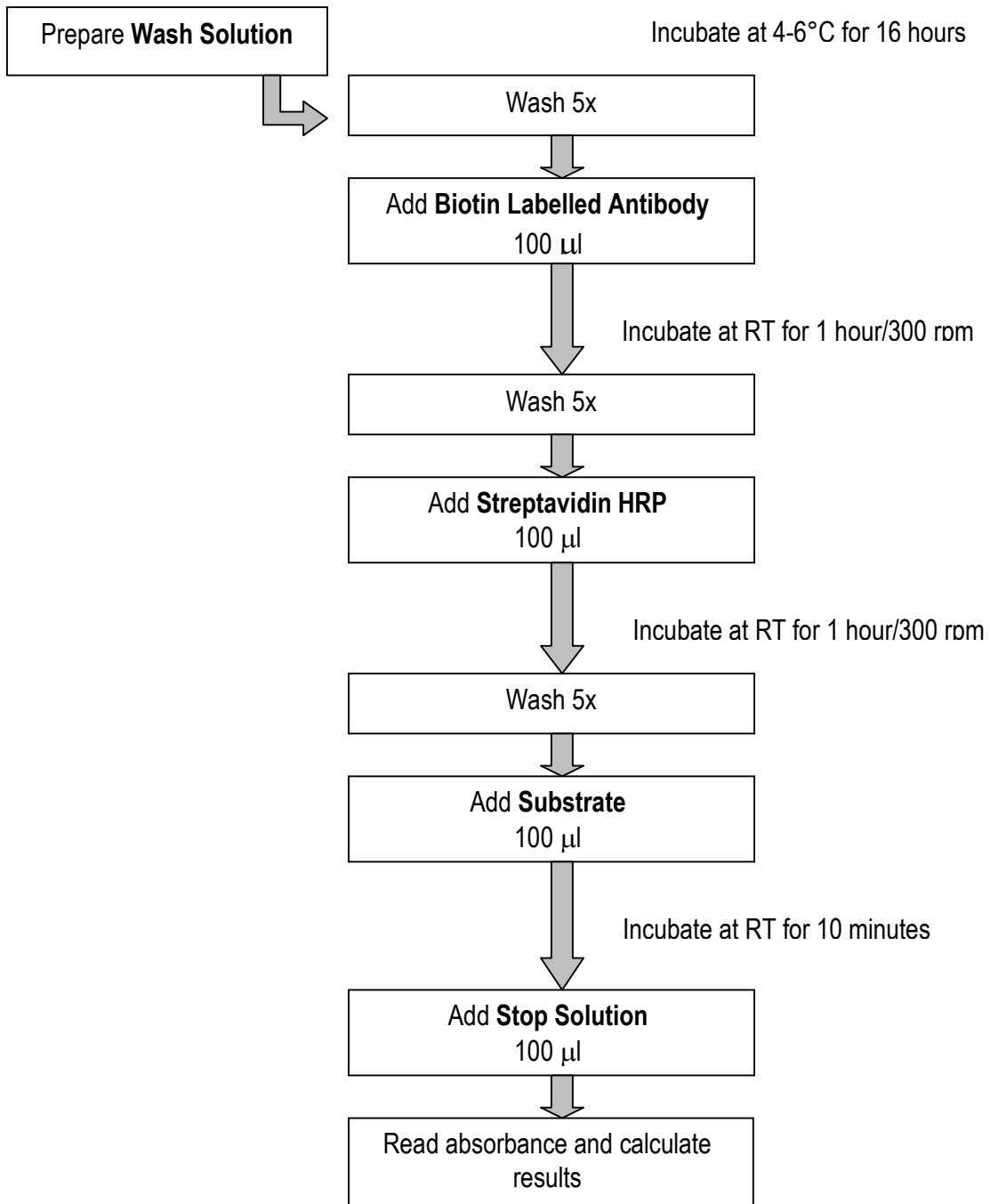
17. References

- Hofbauer L.C et al. Effect of oral contraceptives on circulating osteoprotegerin and soluble RANK ligand serum levels in healthy young women. *Clin Endocrinol* (2004) 60: 214-219
- Ito S. et al. Crystal structure of the extracellular domain of mouse ligand at2.2-A resolution. *J Biol Chem* (2002) 277: 6631-6636.
- Lam J. et al. Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity. *J Clin Invest* (2001) 108: 971-979.
- Lacey D.L, et al., Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* (1998), 93:165-176.
- Kong Y.Y. et al., OPGL is a key regulator of osteoclastogenesis, lymphocyte development and

- lymph-node organogenesis. *Nature* (1999), 397: 315-323.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- Hofbauer L.C. & A.E. Heufelder, The Role of Osteoprotegerin and Receptor Activator of Nuclear Factor κ B Ligand in the Pathogenesis and Treatment of Rheumatoid Arthritis. *Arthritis & Rheumatism* (2001), 44:253-259.
- 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.
- Teitelbaum S.L., Bone resorption by osteoclasts. *Science* (2000), 289: 1504-1508.

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