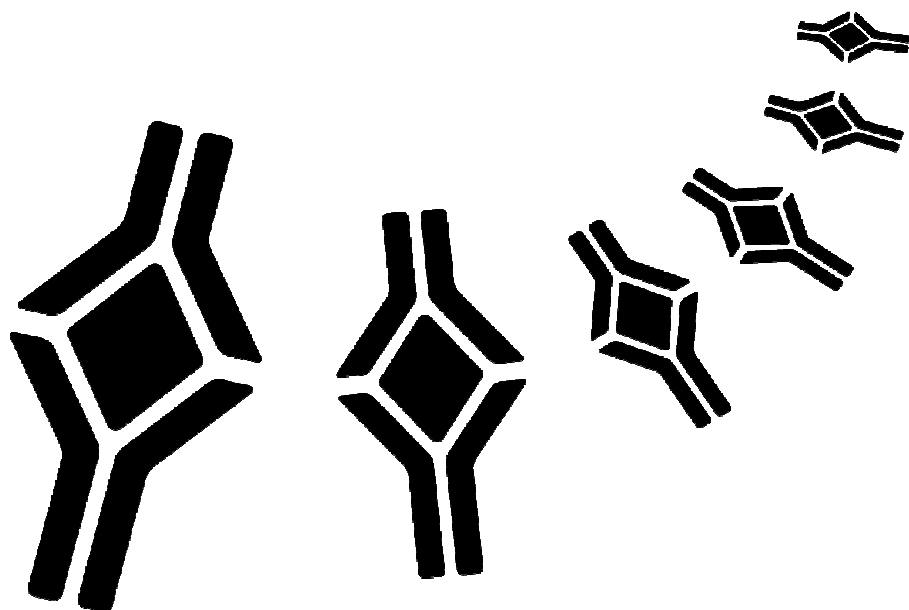


BioVendor

Research
and Diagnostic Products



HUMAN sRANKL (TOTAL) ELISA

Product Data Sheet

Cat. No.: RD193004200R

For Research Use Only

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**»» This kit is manufactured by:
BioVendor – Laboratorní medicína a.s.**

»» Use only the current 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.

»» 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 human serum based
- Standard is human serum based native protein
- Components of the kit are provided ready to use, concentrated or lyophilized

2. 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.

3. INTRODUCTION

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.

Areas of investigation:

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 the BioVendor 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. 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.

5. 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
- This kit contains components of human origin. These materials were found non-reactive for HBsAg, HCV antibody and for HIV 1/2 antigen and antibody. However, these materials should be handled as potentially infectious, as no test can guarantee the complete absence of infectious agents
- This kit contains components of animal origin. These materials should be handled as potentially infectious
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and 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

6. TECHNICAL HINTS

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

7. REAGENT SUPPLIED

<i>Kit Components</i>	<i>State</i>	<i>Quantity</i>
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	2 vials
Quality Control HIGH	lyophilized	2 vials
Quality Control LOW	lyophilized	2 vials
Dilution Buffer	ready to use	2 x 15 ml
Wash Solution Conc. (10x)	concentrated	100 ml
Substrate Solution	ready to use	13 ml
Stop Solution	ready to use	13 ml
Product Data Sheet + Certificate of Analysis	-	1 pc

8. MATERIAL 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 5-1000 μ l with disposable tips
- Multichannel pipette to deliver 100 μ l with disposable tips
- Absorbent material (e.g. paper towels) for blotting the microtiter 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, preferably with reference wavelength 630 nm (alternatively another one from the interval 550-650 nm)
- Software package facilitating data generation and analysis (optional)

9. 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

- 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 desiccant 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.

- **Assay reagents supplied concentrated or lyophilized:**

Human sRANKL Master Standard

Refer to the Certificate of Analysis for current 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 occasional gentle shaking (not to foam). The resulting concentration of the human sRANKL in the stock solution is **32 pmol/l**.

Prepare set of standards using Dilution Buffer as follows:

<i>Volume of Standard</i>	<i>Dilution Buffer</i>	<i>Concentration</i>
Stock	-	32 pmol/l
250 µl of stock	250 µl	16 pmol/l
250 µl of std. 16 pmol/l	250 µl	8 pmol/l
250 µl of std. 8 pmol/l	250 µl	4 pmol/l
250 µl of std. 4 pmol/l	250 µl	2 pmol/l
250 µl of std. 2 pmol/l	250 µl	1 pmol/l
250 µl of std. 1 pmol/l	250 µl	0.5 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.

Do not store the diluted Standard solutions.

Quality Controls HIGH, LOW

Refer to Certificate of Analysis for current volume of Dilution Buffer needed for reconstitution and for current Quality Control concentration!!!

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.

Note:

Concentration of analyte in Quality Controls need not be anyhow associated with normal and/or pathological concentrations in serum or another body fluid. Quality Controls serve just for control that the kit works in accordance with PDS and CoA and that ELISA test was carried out properly.

Wash Solution Conc. (10x)

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.

10. PREPARATION OF SAMPLES

The kit measures sRANKL (total) in serum and plasma samples (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 serum or plasma samples 100x with Dilution Buffer just prior to the assay, 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

Mix well (not to foam). Vortex is recommended.

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 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.

11. ASSAY PROCEDURE

1. Pipet **100 µl** of Standards, Quality Controls, Dilution Buffer (=Blank) and 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**, shaking at ca. 300 rpm on an orbital microplate shaker.
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 into each well. **Attention: Biotin Labelled Antibody 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 **25 minutes** at room temperature. The incubation time may be extended [up to 40 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 of each well using a microplate reader set to 450 nm, preferably with the reference wavelength set to 630 nm (acceptable range: 550-650 nm). Subtract readings at 630 nm (550-650 nm) from the readings at 450 nm. **The absorbance should be read within 5 minutes following step 12.**

Note: If some samples and standard/s have absorbances above the upper limit of your microplate reader, 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 standards and samples. The readings at 405 nm should not replace the readings for samples that were "in range" at 450 nm.

Note 2: Manual washing: Aspirate wells and pipet 0.35 ml Wash Solution into each well. Aspirate wells and repeat four times. 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 2	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
F	Standard 1	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
G	Standard 0.5	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.

12. CALCULATIONS

Most microplate readers perform automatic calculations of analyte concentration. The Standard curve is constructed by plotting the absorbance (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.

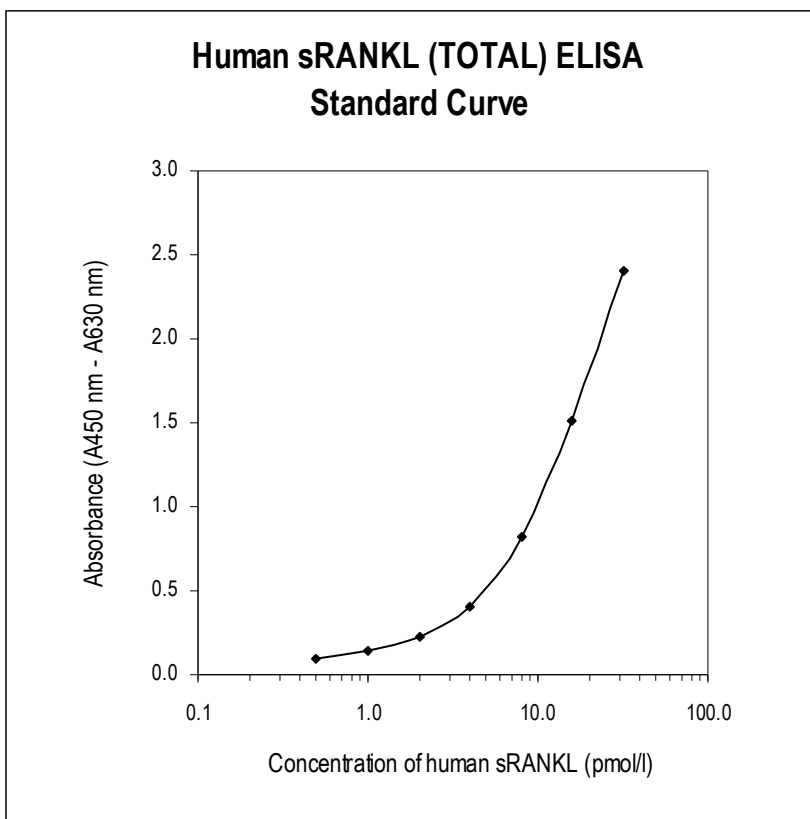


Figure 2: Typical Standard Curve for Human sRANKL (total) ELISA.

13. PERFORMANCE CHARACTERISTICS

➤➤ Typical analytical data of BioVendor Human sRANKL (total) ELISA are presented in this chapter

- **Sensitivity**

Limit of Detection (LOD) (defined as concentration of analyte 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 calculated from the real sRANKL values in wells and is 0.4 pmol/l.

*Dilution Buffer is pipetted into blank wells.

- **Limit of assay**

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

- **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 2 ng/ml.

Sera of several mammalian species were measured in the assay. See results below.

For details please contact us at info@biovendor.com.

<i>Mammalian serum sample</i>	<i>Observed crossreactivity</i>
Bovine	no
Cat	no
Dog	no
Goat	no
Hamster	no
Horse	no
Monkey	no
Mouse	no
Pig	yes
Rabbit	no
Rat	no
Sheep	no

➤➤ **Presented results are multiplied by respective dilution factor**

• **Precision**

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

<i>Sample</i>	<i>Mean (pmo/l)</i>	<i>SD (pmo/l)</i>	<i>CV (%)</i>
1	698.2	50.6	7.25
2	373.3	43.0	11.51

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

<i>Sample</i>	<i>Mean (pmo/l)</i>	<i>SD (pmo/l)</i>	<i>CV (%)</i>
1	141.82	15.90	11.21
2	310.17	39.61	12.77

• **Spiking Recovery**

Serum samples were spiked with different amounts of sRANKL and assayed.

<i>Sample</i>	<i>Observed (pmo/l)</i>	<i>Expected (pmo/l)</i>	<i>Recovery O/E (%)</i>
1	167.2	-	-
	945.1	967.2	97.7
	559.2	567.2	98.6
	332.8	367.2	90.6
2	381.1	-	-
	1 206.4	1 181.1	102.1
	790.7	781.1	101.2
	508.6	581.1	87.5

• **Linearity**

Serum samples were serially diluted with Dilution Buffer and assayed.

<i>Sample</i>	<i>Dilution</i>	<i>Observed (pmo/l)</i>	<i>Expected (pmo/l)</i>	<i>Recovery O/E (%)</i>
1	-	536.81	-	-
	2x	306.81	268.41	114.3
	4x	147.52	134.20	109.9
2	-	440.57	-	-
	2x	248.83	220.29	113.0
	4x	125.39	110.14	113.8

- **Effect of sample matrix**

EDTA, citrate and heparin plasmas were compared to respective serum samples from the same 10 individuals.

Results are shown below:

Volunteer No.	Serum (pmol/l)	Plasma (pmol/l)		
		EDTA	Citrate	Heparin
1	138.34	111.74	160.44	148.91
2	92.23	83.44	101.12	109.68
3	76.72	68.90	87.82	84.64
4	172.49	159.99	186.37	184.63
5	906.13	862.17	1 088.41	1 031.33
6	79.09	65.03	70.85	75.95
7	370.04	292.98	335.43	352.91
8	164.74	141.71	163.42	173.76
9	43.48	35.41	44.20	41.25
10	664.04	626.53	656.37	747.02
Mean (pmol/l)	270.73	244.79	289.44	295.01
Mean Plasma/Serum (%)	-	90.42	118.24	101.92
Coefficient of determination R²	-	0.998	0.991	0.999

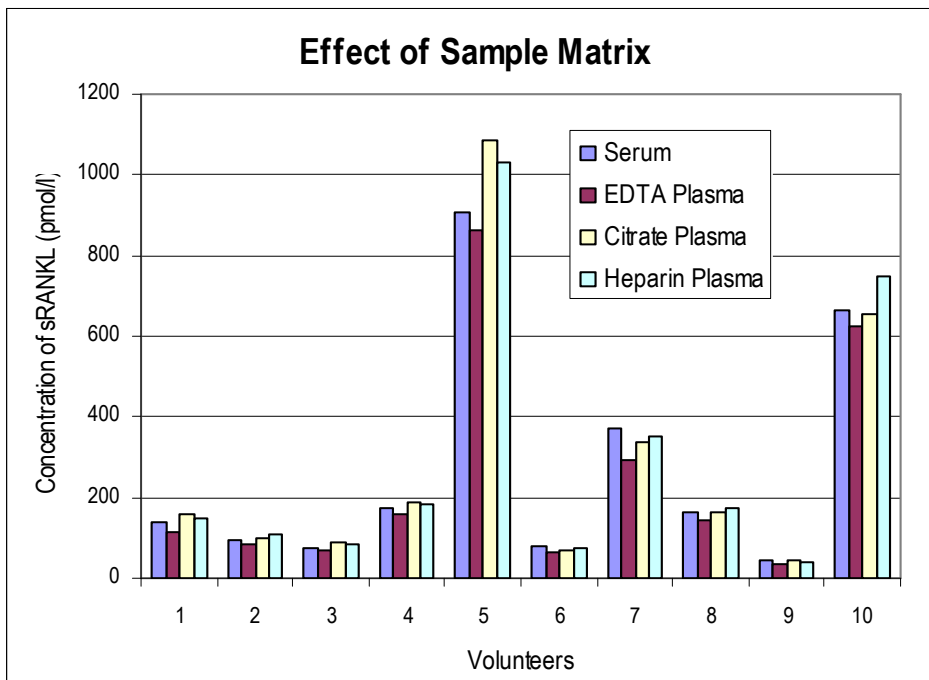


Figure 3: sRANKL levels measured using Human sRANKL (total) ELISA from 10 individuals using serum, EDTA, citrate and heparin plasma, respectively.

- **Stability of samples stored at 2-8C**

Samples should be stored at -20°C. However, no decline in concentration of sRANKL was observed in serum and plasma samples after 14 days when stored at 2-8°C. To avoid microbial contamination, samples were treated with ε-aminocaproic acid and sodium azide, resulting in the final concentration of 0.03% and 0.1%, respectively.

- **Effect of Freezing/Thawing**

No decline was observed in concentration of human sRANKL in serum and plasma samples after repeated (5x) freeze/thaw cycles. However it is recommended to avoid unnecessary repeated freezing/thawing of the samples.

Sample	Number of f/t cycles	Serum (pmol/l)	Plasma (pmol/l)		
			EDTA	Citrate	Heparin
1	1x	437.4	406.2	412.5	442.6
	3x	427.0	424.9	344.5	459.4
	5x	448.9	430.7	435.3	462.0
2	1x	199.8	197.0	184.4	220.1
	3x	211.6	203.1	184.4	230.6
	5x	214.9	205.0	192.8	239.3
3	1x	122.3	128.9	108.2	131.8
	3x	113.0	102.8	110.3	103.6
	5x	116.3	103.2	103.3	116.3

14. DEFINITION OF THE STANDARD

In human serum sRANKL is described as a homotrimeric molecule with MW of 60 kDa (20 kDa for each monomer). The Standard used in the kit is a serum-based protein.

Unit conversions:

1 pmol/l = 62.5 pg/ml

1 pg/ml = 0.016 pmol/l

15. PRELIMINARY POPULATION AND CLINICAL DATA

- **Normal value in human serum**

The mean value study with sera samples from 39 women and men, 25-65 years old, has been established with the Biovendor Human sRANKL (total) ELISA in our laboratory (n=39, mean ±SEM): 339.34 ± 42.30 pmol/l.

- **Reference range**

The data quoted in these instructions should be used for guidance only. It is recommended that each laboratory include its own panel of control sample in the assay. Each laboratory should establish its own normal and pathological references ranges for sRANKL levels with the assay.

16. METHOD COMPARISON

BioVendor Human sRANKL (total) ELISA has not been compared to any other immunoassay.

17. 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







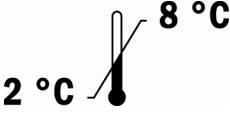

18. REFERENCES

»» References to sRANKL:

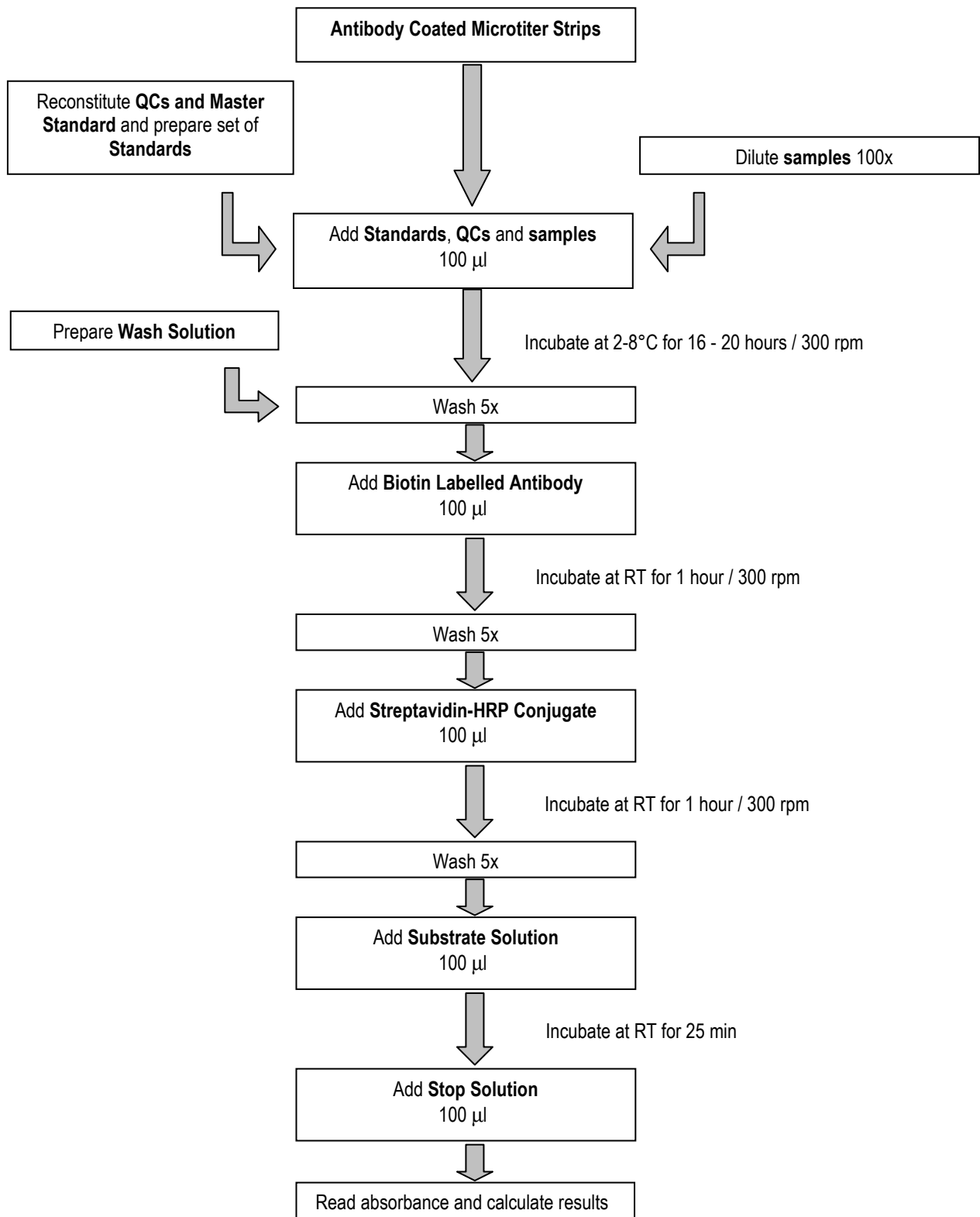
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»» For more references on this product see our WebPages at www.biovendor.com

19. EXPLANATION OF SYMBOLS

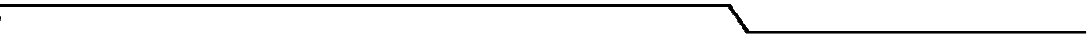
	Catalogue number
	Content
	Lot number
	See instructions for use
	Biological hazard
	Expiry date
	Storage conditions
	Identification of packaging materials

Assay Procedure Summary



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NOTES

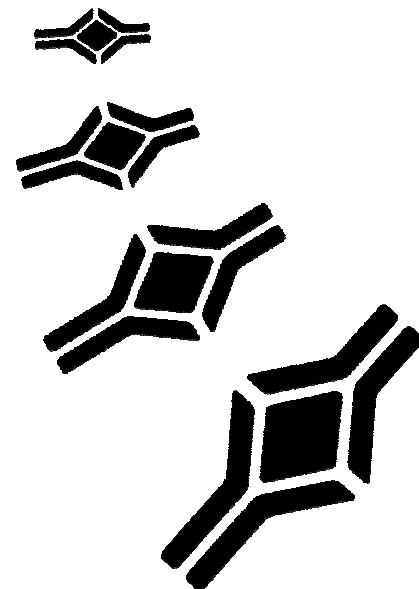


NOTES



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