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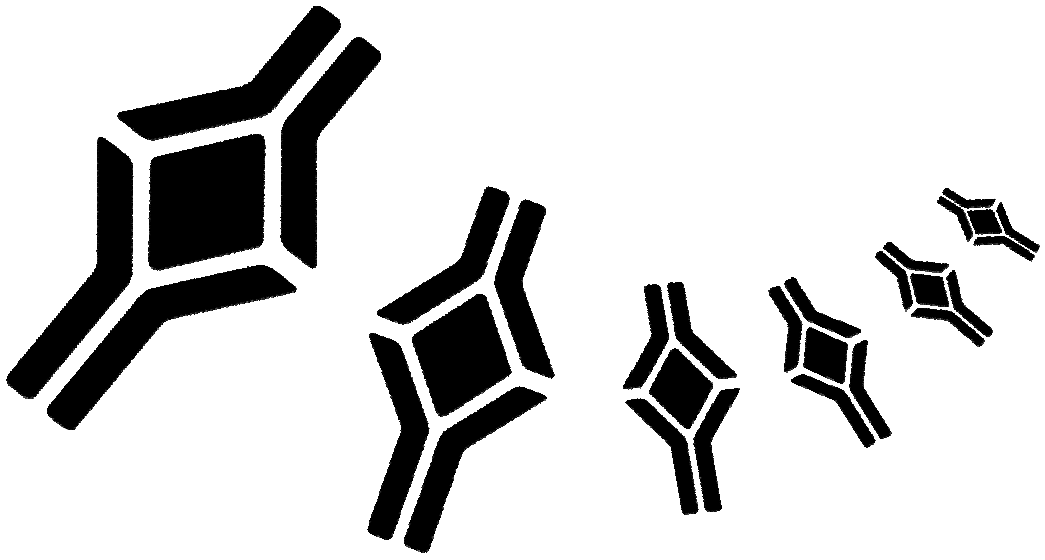
Research
and Diagnostic Products



HUMAN OSTEOPROTEGERIN ELISA

Product Data Sheet

Cat. No.: RD194003200

European Union: IVD CE

Rest of the world:
For research use only!

VERSION 06 090211 07

CONTENTS

» **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 RD194003200 Human Osteoprotegerin ELISA is a sandwich enzyme immunoassay for the quantitative measurement of human osteoprotegerin.

Features

- **European Union: for *in vitro* diagnostic use**
  Rest of the world: for research use only!
- The total assay time is less than 3.5 hours
- The kit measures osteoprotegerin in serum and plasma (EDTA, citrate, heparin)
- Assay format is 96 wells
- Quality Controls are human serum based
- Standard is recombinant protein based
- 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

Osteoprotegerin (OPG, osteoclastogenesis inhibitory factor, OCIF), a product of TNFRSF11B gene located in chromosome 8q24, is a member of the TNF receptor superfamily that plays a key role in bone remodeling. Human OPG is a secreted glycoprotein composed of 401 amino acid residues that exists naturally as either a 120 kDa disulfide-linked homodimer or 60 kDa monomer. Both these OPG forms are active, with the dimer showing more potent bioactivity. Unlike most members of the TNF receptor superfamily, OPG apparently exists only in a soluble form as a decoy receptor for its ligands, RANKL (TNFSF11, OPGL, ODF, TRANCE) and TRAIL. Human OPG shares 85% and 86% amino acid identity to mouse and rat OPG respectively. In adult humans, OPG mRNA is highly expressed in various tissues, e.g. bone, skin, liver, stomach, intestine, heart, brain and lung.
Osteoprotegerin inhibits the binding of RANKL to RANK (osteoclast differentiation and activation receptor, ODAR) on the osteoclast surface and thus, OPG is a natural antagonist to RANKL-induced osteoclastogenesis and bone resorption. Since OPG exhibits an inhibitory effect on osteoclasts, it acts as a soluble factor in the regulation of bone mass.
Osteoclast formation activity may be monitored principally by determination of concentration ratio of RANKL/OPG. Alteration of this ratio may be the cause of bone loss in many imbalances in bone metabolism such as osteoporosis, osteopetrosis, hypercalcemia, metastatic osteolytic lesions and rheumatic bone degradation.

Areas of investigation:
Arthritis
Diseases with changed bone resorption activity
Oncology

4. TEST PRINCIPLE

In the BioVendor Human Osteoprotegerin ELISA, standards, quality controls and samples are incubated in microplate wells pre-coated with monoclonal anti-human OPG antibody. After 60 minutes incubation and washing, biotin labelled polyclonal anti-human OPG antibody is added and incubated for 60 minutes with captured OPG. After another washing, streptavidin-HRP conjugate is added. After 30 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 OPG. 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

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	13 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
- Vortex mixer
- Glassware (graduated cylinder and bottle) for Wash Solution
- Precision pipettes to deliver 10-1000 µl with disposable tips
- Multichannel pipette to deliver 100 µl with disposable tips
- Orbital microplate shaker capable of approximately 300 rpm
- Microplate washer (optional). [Manual washing is possible but not preferable.]
- Absorbent material (e.g. paper towels) for blotting the microtitrate plate after washing
- 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.

Dilution Buffer
Biotin Labelled Antibody
Streptavidin-HRP Conjugate
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 Osteoprotegerin Master Standard
Refer to the Cetrificate 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 OPG in the stock solution is **60 pmol/l**.

Prepare set of standards using Dilution Buffer as follows:

Volume of Standard	*Dilution Buffer*	*Concentration*
Stock	-	60 pmol/l
150 µl of stock	150 µl	30 pmol/l
150 µl of 30 pmol/l	150 µl	15 pmol/l
120 µl of 15 pmol/l	180 µl	6 pmol/l
150 µl of 6 pmol/l	150 µl	3 pmol/l
150 µl of 3 pmol/l	150 µl	1.5 pmol/l

Dilute prepared Standards (60 - 1.5 pmol/l) 3x with Dilution Buffer just prior to the assay, e.g. 100 µl of Standard + 200 µl of Dilution Buffer for duplicates.

Stability and storage:

Standard stock solution (60 pmol/l) should be aliquoted and frozen at –20°C for 3 months. Avoid repeated freeze/thaw cycles.

Do not store the diluted Standard solutions.

Quality Controls HIGH, LOW

Refer to the Certificate of Analysis for current volume of deionized water needed for reconstitution and for current Quality Control concentration!!!

Reconstitute each Quality Control (HIGH and LOW) with deionized water just prior to the assay. Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam).

Dilute reconstituted Quality Controls 3x with Dilution Buffer, e.g. 50 µl of Quality Control + 100 µl of Dilution Buffer when assaying samples in singlets, or preferably 100 µl of Quality Control + 200 µl of Dilution Buffer for duplicates.

Stability and storage:

The reconstituted Quality Controls must be used immediately or stored frozen at -20°C for 1 month. Avoid repeated freeze/thaw cycles.

Do not store the diluted Quality Controls.

Wash Solution Conc. (10x)

Dilute Wash Solution Conc. (10x) ten-fold in distilled water to prepare a 1x working solution. Example: 100 ml of Wash Solution Conc. (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 Conc. (10x) is stable 3 months when stored at 2-8°C.

10. PREPARATION OF SAMPLES

The kit measures OPG in serum or 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 3x with Dilution Buffer just prior to the assay, e.g. 50 µl of sample + 100 µl of Dilution Buffer for singlets, or preferably 100 µl of sample + 200 µl of Dilution Buffer for duplicates. **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 when stored at 2-8°C, effect of freezing/thawing and effect of sample matrix (serum/plasma) on the concentration of OPG.

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 diluted 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 room temperature (ca. 25°C) for **1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.
3. Wash the wells 3-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.
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 3-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 **30 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.
9. Wash the wells 3-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 **10 minutes** at room temperature. The incubation time may be extended [up to 20 minutes] if the reaction temperature is below 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 1: 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 OPG 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 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 60**	**QC LOW**	Sample 8	Sample 16	Sample 24	Sample 32
B	**Standard 30**	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	**Standard 15**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	**Standard 6**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	**Standard 3**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	**Standard 1.5**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	**Blank**	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
H	**QC HIGH**	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39

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 mean absorbance (Y) of Standards against the known concentration (X) of Standards in logarithmic scale, using the four-parameter algorithm. Results are reported as concentration of OPG 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.

Samples, Quality Controls and Standards are all diluted 3x prior to analysis, so there is no need to take this dilution factor into account.

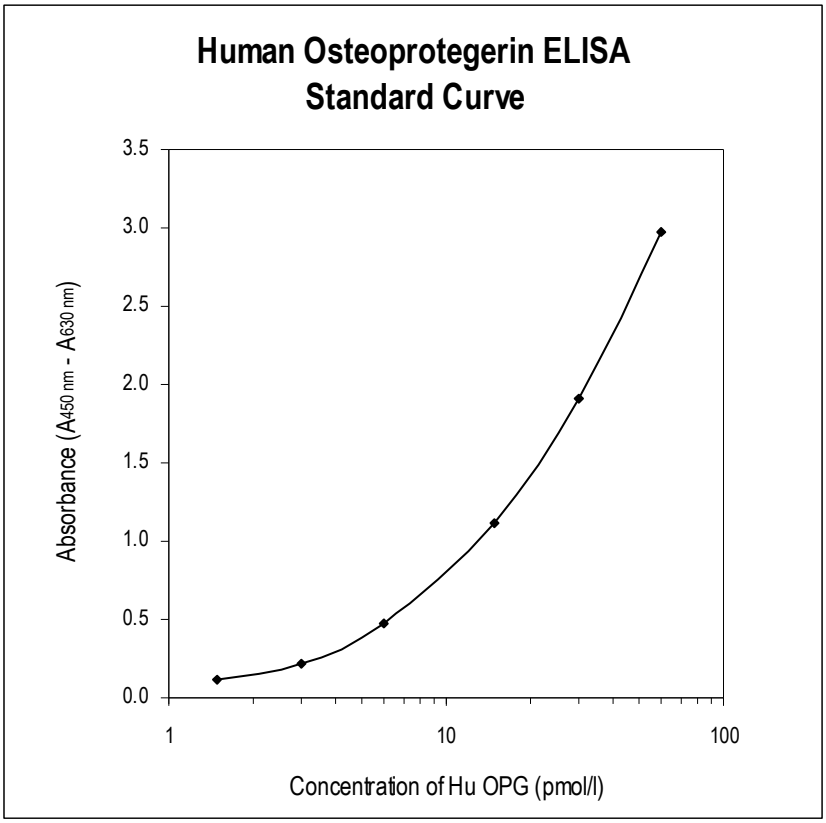


Figure 2: Typical Standard Curve for Human Osteoprotegerin ELISA.

13. PERFORMANCE CHARACTERISTICS

Typical analytical data of BioVendor Human Osteoprotegerin 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_{blank} + $3xSD_{blank}$, is calculated from the real human OPG values in wells and is 0.10 pmol/l.
*Dilution Buffer is pipetted into blank wells.

- **Limit of assay**

Results exceeding OPG level of 60 pmol/l should be repeated with more diluted samples. Dilution factor needs to be taken into consideration in calculating the OPG concentration.

- **Specificity**

The antibodies used in this ELISA are specific for human OPG with no detectable crossreactivities to human sRANKL and TRAIL at 120 pmol/l.
Approximately 1% crossreactivity with recombinant mouse OPG, less than 0.06% with recombinant human CD40, rec. human sTNF RI and sTNF RII has been observed.

Sera of several mammalian species were measured in the assay. See results below.
For details please contact us at info@biovendor.com.

Mammalian serum sample	*Observed crossreactivity*
Bovine	no
Cat	no
Dog	no
Goat	no
Hamster	no
Horse	no
Monkey	yes
Mouse	no
Pig	no
Rabbit	no
Sheep	no

- **Precision**

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

Sample	*Mean (pmol/l)*	*SD (pmol/l)*	*CV (%)*
1	14.26	0.41	2.9
2	4.82	0.18	3.8
3	12.72	0.31	2.5
4	15.28	0.74	4.9

Inter-assay (Run-to-Run) (n=3)

Sample	*Mean (pmol/l)*	*SD (pmol/l)*	*CV (%)*
1	4.38	0.34	7.1
2	6.18	0.55	9.0
3	12.93	0.69	5.3
4	14.33	0.25	1.7

- **Spiking Recovery**

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

Sample	***O**bserved (pmol/l)*	***E**xpected (pmol/l)*	*Recovery **O/E** (%)*
1	5.38	-	-
	8.12	6.89	117.9
	14.73	13.92	105.8
	20.48	20.71	98.9
2	11.38	-	-
	13.57	12.89	105.3
	20.93	19.92	105.1
	28.62	26.71	107.2

- **Linearity**

Serum samples were serially diluted with Dilution Buffer and assayed.

Sample	*Dilution*	***O**bserved (pmol/l)*	***E**xpected (pmol/l)*	*Recovery **O/E** (%)*
1	-	12.88	-	-
	2x	7.11	6.44	110.4
	4x	3.51	3.22	109.0
	8x	1.64	1.61	101.9
2	-	14.68	-	-
	2x	7.90	7.34	107.6
	4x	4.06	3.67	110.6
	8x	1.95	1.84	106.3

- **Effect of sample matrix**

Citrate, heparin and EDTA 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	8.47	9.52	7.97	8.37
2	5.42	4.89	4.47	4.95
3	6.81	6.88	6.61	6.23
4	10.99	12.48	10.25	10.35
5	8.68	9.58	7.95	9.57
6	6.11	5.66	6.46	5.72
7	6.98	7.59	6.95	7.29
8	8.29	8.07	6.70	7.27
9	7.85	8.10	6.73	7.13
10	9.59	8.49	7.00	7.97
Mean (pmol/l)	**7.92**	**8.12**	**7.11**	**7.49**
Mean Plasma/Serum (%)	**-**	**102.6**	**89.8**	**94.5**
Coefficient of determination R^2	**-**	**0.88**	**0.83**	**0.75**

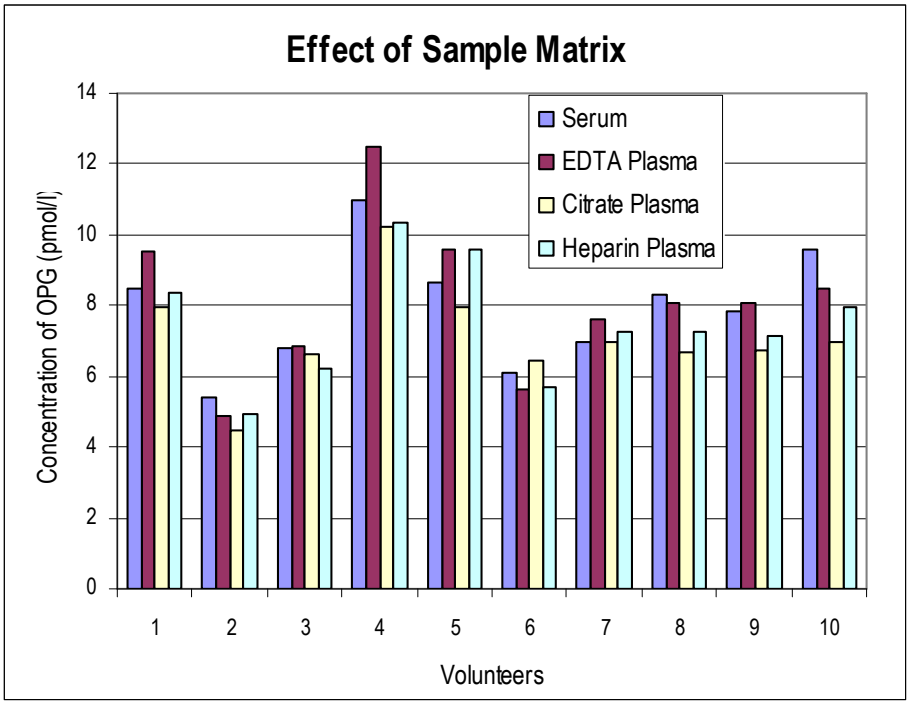


Figure 3: OPG levels measured using Human Osteoprotegerin ELISA from 10 individuals using serum, heparin, citrate and EDTA plasma, respectively.

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

Samples should be stored at –20°C. However, no decline in concentration of OPG was observed in serum and plasma samples after 7 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.

Sample	*Incubation Temp, Period*	*Serum (pmol/l)*	*Plasma (pmol/l)*		
			EDTA	*Citrate*	*Heparin*
1	-20°C	9.37	9.06	7.96	9.47
	2-8°C, 7 days	9.26	8.86	8.05	9.71
	2-8°C, 14 days	9.26	9.53	7.56	9.67
2	-20°C	6.76	6.58	5.64	7.35
	2-8°C, 7 days	6.68	6.69	5.00	7.35
	2-8°C, 14 days	6.70	6.54	5.39	7.19
3	-20°C	8.48	9.33	7.94	10.11
	2-8°C, 7 days	9.66	9.34	8.18	9.52
	2-8°C, 14 days	9.11	8.97	8.31	9.12

- **Effect of Freezing/Thawing**

No decline was observed in concentration of human OPG 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	8.28	7.26	6.69	8.06
	3x	7.74	6.43	6.66	8.00
	5x	7.06	6.11	6.36	7.14
2	1x	7.85	7.13	6.73	8.09
	3x	7.85	6.27	6.66	7.41
	5x	7.68	6.20	6.01	6.69
3	1x	9.28	7.97	6.99	8.48
	3x	8.11	6.54	7.14	7.84
	5x	7.60	6.43	6.50	7.36

14. DEFINITION OF THE STANDARD

A recombinant chimeric protein composed of human osteoprotegerin and Fc-domain of human IgG (OPG/Fc) is used as the Standard. Mature OPG/Fc is a disulfide-linked homodimeric protein. Each monomer contains 380 residues from mature OPG and 243 residues from the Fc protein and linker. As a result of glycosylation, the OPG/Fc migrates as a 77 kDa protein in SDS-PAGE under reducing conditions.
Since the native serum OPG is a protein of 60 kDa (for monomer) differing significantly from our standard, we used to employ the unit U/l. From the lot number RD-738 we started to use the unit pmol/l.
1 pmol OPG / l = 1.5 U OPG / l (previously used). It is possible to recalculate previous results with factor 1.5. For example: concentration of the sample 15 U/l measured in previous assays corresponds to 10 pmol/l of OPG measured in this assay.
Conversion factor for pmol/l to pg/ml:
1 pmol/l = 120 pg/ml
(Relative molecular mass of OPG as a glycosylated dimeric molecule is 120 kDa.)

15. PRELIMINARY POPULATION AND CLINICAL DATA

- **Normal range**

The mean value study with serum samples from young unselected donors has been established with the BioVendor Human Osteoprotegerin ELISA (n=17, mean ± SEM): **4.7 ± 0.33 pmol/l.**
See reference for details:
Naylor KE et al.: *J Clin Endocrinol Metab Nov*; **88**(11): 5361-5 (2003)

The normal range with serum samples from unselected donors (N=70, age: 35-65 years) has been established with the Human Osteoprotegerin ELISA in our laboratory:
Normal range (mean ± 2SD): **4.1 ± 2.3 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 samples in the assay. Each laboratory should establish its own normal and pathological reference ranges for human OPG levels with the assay.

16. METHOD COMPARISON

The BioVendor Human Osteoprotegerin ELISA was compared to another commercial immunoassay, by measuring 49 serum samples. The following correlation graph was obtained. Linear regression analysis of the results yielded the following results.
ELISA (Competitor) = 0.77 x ELISA (BioVendor) + 1.20 $r^2 = 0.8$

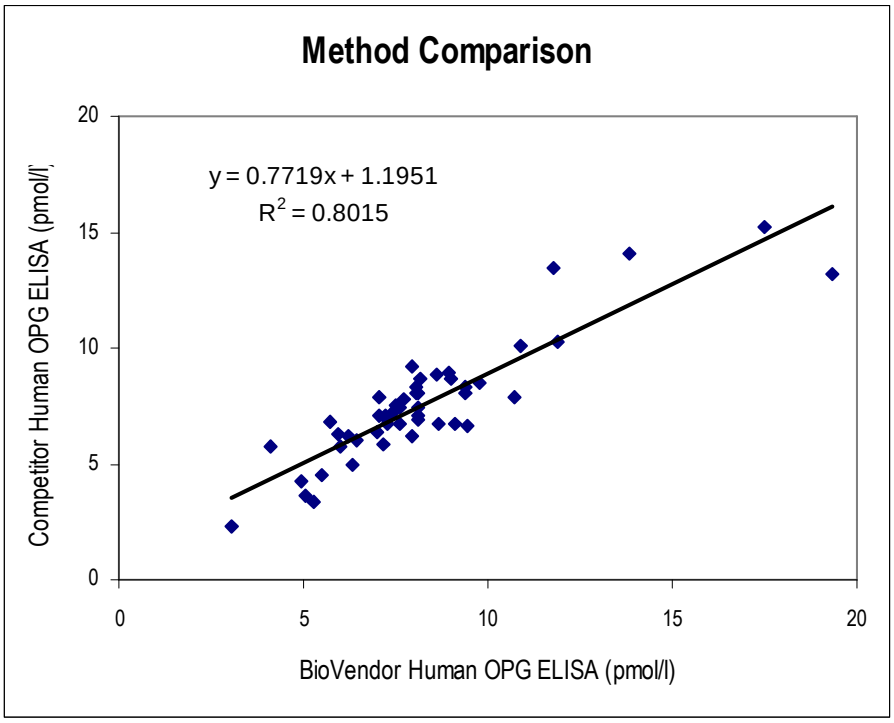


Figure 4: Method comparison.

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

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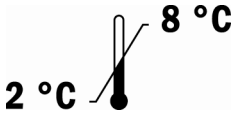
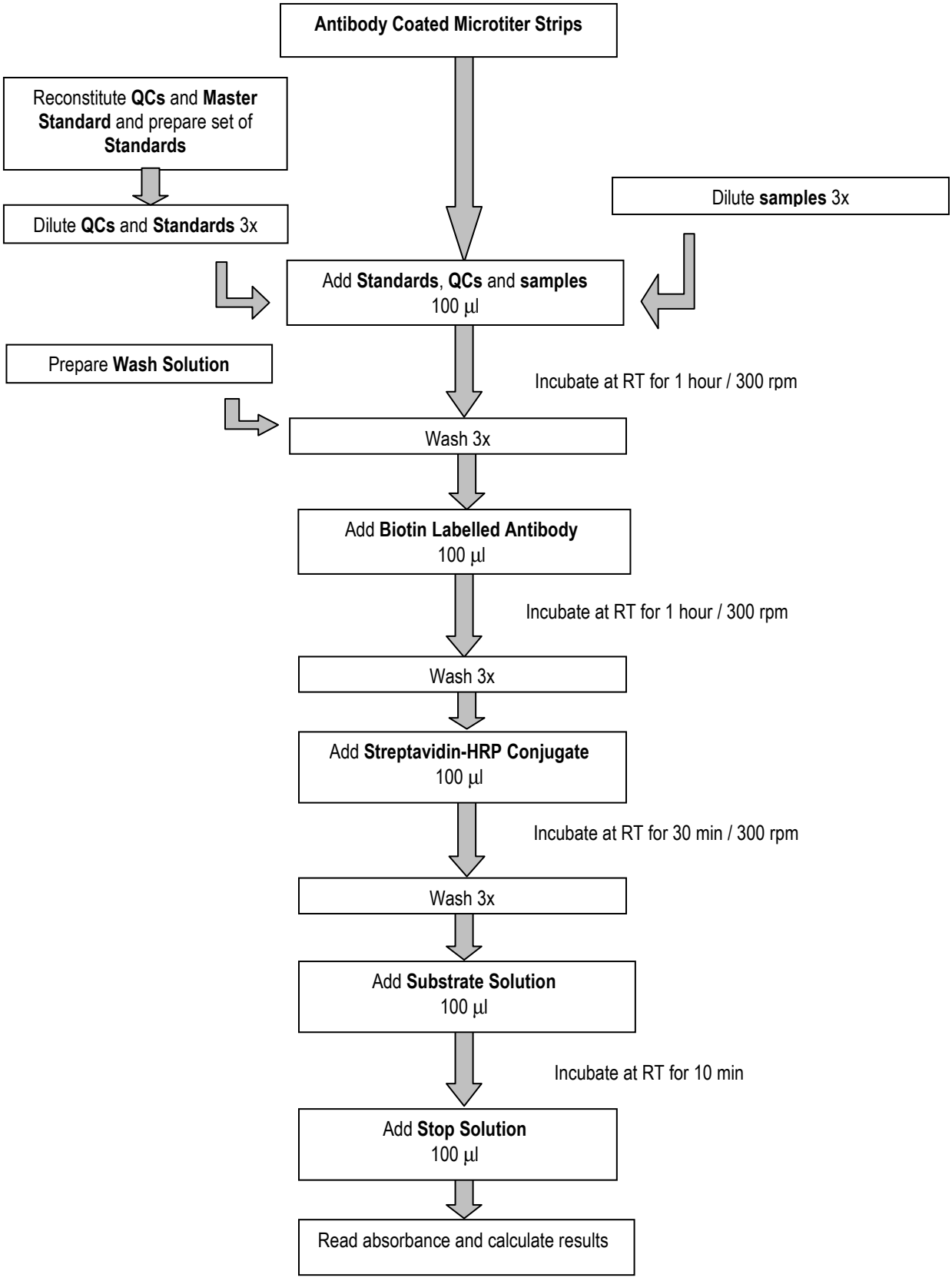
19. EXPLANATION OF SYMBOLS

REF	Catalogue number
Cont.	Content
LOT	Lot number
	See instructions for use
	Biological hazard
	Expiry date
	Storage conditions
	Identification of packaging materials
IVD CE	In vitro diagnostic medical device

Assay Procedure Summary



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A												
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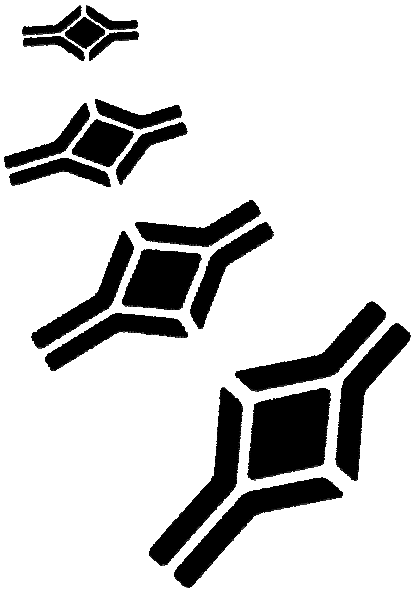

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