BioVendor
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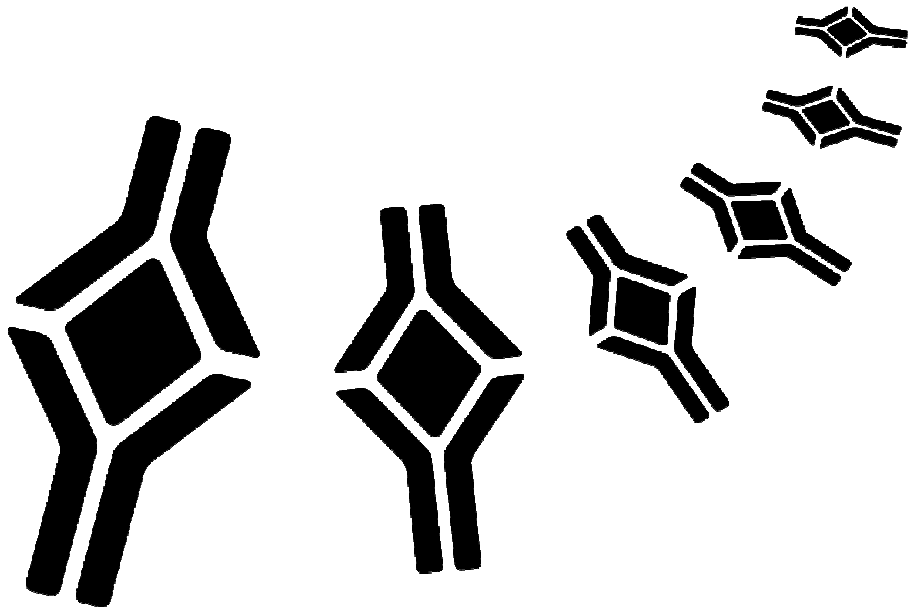

HUMAN ENPP1 ELISA

Product Data Sheet

Cat. No.: RD191124200R

For Research Use Only

CONTENTS

» **This kit is manufactured by:**
Bio Vendor Research and Diagnostic Products, s.r.o.

» **Use only the current version of Product Data Sheet enclosed with the kit!**

1. INTENDED USE

The RD191124200R Human ENPP1 ELISA is a sandwich enzyme immunoassay for the quantitative measurement of human ENPP1.

Features

- **It is intended for research use only**
- The total assay time is less than 3.5 hours
- The kit measures ENPP1 in serum and plasma (citrate)
- Assay format is 96 wells
- 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

Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1, PC-1) is a class II transmembrane glycoprotein that is located both on the plasma membrane and in the endoplasmatic reticulum [6]. Human ENPP1 belongs to the ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) family of proteins which hydrolyze pyrophosphate or phosphodiester bonds in various extracellular compounds, such as nucleotides and lysophospholipids [5,6]. These proteins consist of a short terminal NH_2 intracellular domain, a single transmembrane domain, two somatomedin-B-like domains and COOH-terminal nuclease-like domain [8].

ENPP1 is a 230-260 kDa homodimer, and its reduced form has a molecular size of 115-135 kDa depending on the cell type [11]. Human ENPP1 has 873 amino acids and its gene is located on the long arm of chromosome 6 (6q23.2).

ENPP1 is expressed in various tissues including liver, skeletal muscle and adipose tissue. It is also expressed in heart, brain, kidney, placenta, pancreatic islets, kidney, lung, chondrocytes, lymphocytes, and dermal fibroblasts [11].

The physiological function of ENPP1 is not completely understood. There is evidence that ENPP1 plays a major role in bone and cartilage metabolism by producing pyrophosphate [9]. The latter substance inhibits bone formation. In ENPP1 knockout mice and in humans lacking ENPP1 there is ectopic calcification in the spine, aorta, and other tissues with markedly decreased survival [7]. ENPP1 seems to play a role in glucose metabolism impairment *in vivo.* Insulin resistant rodents and humans show high levels of expression of this protein [3]. Several studies reported that mutations in the ENPP1 gene are associated with idiopathic infantile arterial calcification, ossification of the posterior longitudinal ligament of the spine (OPLL) and insulin resistance.

<u>Areas of investigation:</u>
Bone and cartilage metabolism
Diabetology
Energy metabolism and body weight regulation

4. TEST PRINCIPLE

In the BioVendor Human ENPP1 ELISA, standards and samples are incubated in microplate wells pre-coated with polyclonal anti-human ENPP1 antibody. After 60 minutes incubation and washing, biotin labelled polyclonal anti-human ENPP1 antibody is added and incubated for 60 minutes with captured ENPP1. 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 ENPP1. 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
- 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 Conc. (100x)	concentrated	0.15 ml
Streptavidin-HRP Conjugate	ready to use	13 ml
Master Standard	lyophilized	2 vials
Dilution Buffer	ready to use	20 ml
Biotin-Ab Diluent	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
- Glassware (graduated cylinder and bottle) for Wash Solution (Dilution Buffer)
- Precision pipettes to deliver 5-1000 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, 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 desicant and seal carefully. Remaining Microtiter Strips are stable 3 months stored at 2-8°C and protected from the moisture.
Streptavidin-HRP Conjugate
Dilution Buffer
Biotin-Ab Diluent
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 ENPP1 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 ENPP1 in the stock solution is **16 ng/ml**.

Prepare set of standards using Dilution Buffer as follows:

Volume of Standard	*Dilution Buffer*	*Concentration*
Stock	-	16 ng/ml
250 µl of stock	250 µl	8 ng/ml
250 µl of 8 ng/ml	250 µl	4 ng/ml
250 µl of 4 ng/ml	250 µl	2 ng/ml
250 µl of 2 ng/ml	250 µl	1 ng/ml
250 µl of 1 ng/ml	250 µl	0.5 ng/ml
250 µl of 0.5 ng/ml	250 µl	0.25 ng/ml

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

Stability and storage:
Do not store the diluted Standard solutions.

Biotin Labelled Antibody Conc. (100x)
Prepare the working Biotin Labelled Antibody solution by adding 1 part Biotin Labelled Antibody Concentrate (100x) with 99 parts Biotin-Ab Diluent. Example: 10 µl of Biotin Labelled Antibody Concentrate (100x) + 990 µl of Biotin-Ab Diluent for 1 strip (8 wells).
Stability and storage:
Do not store the diluted Biotin Labelled Antibody solution.

Wash Solution Conc. (10x)
Dilute Wash Solution Concentrate (10x) ten-fold in 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 ENPP1 in serum and plasma (citrate).

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.

Serum samples:
Dilute samples 10x with Dilution Buffer just prior to the assay, e.g. 15 µl of sample + 135 µl of Dilution Buffer for singlets, or preferably 25 µl of sample + 225 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.

*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, 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 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 the microplate 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 ENPP1 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 16**	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	**Standard 8**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	**Standard 4**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	**Standard 2**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	**Standard 1**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	**Standard 0.5**	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	**Standard 0.25**	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
H	**Blank**	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40

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 log of the known concentration (X) of Standards, using the four-parameter algorithm. Results are reported as concentration of ENPP1 ng/ml 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. 1.25 ng/ml (from standard curve) x 10 (dilution factor) = 12.5 ng/ml

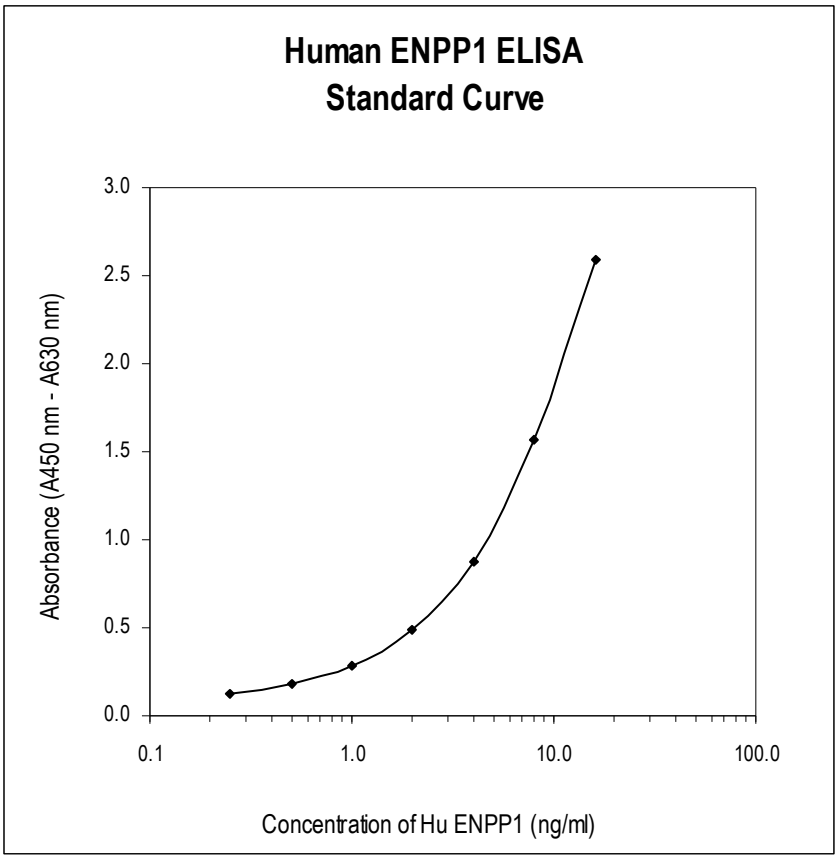

Figure 2: Typical Standard Curve for Human ENPP1 ELISA.

13. PERFORMANCE CHARACTERISTICS

» Typical analytical data of BioVendor Human ENPP1 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 ENPP1 values in wells and is 0.13 ng/ml.

*Dilution Buffer is pipetted into blank wells.

- **Limit of assay**

Samples with absorbances exceeding the absorbance of the highest standard should be measured again with higher dilution. The final concentration of samples calculated from the standard curve must be multiplied by the respective dilution factor.

» Presented results are multiplied by respective dilution factor

- **Precision**

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

Sample	*Mean (ng/ml)*	*SD (ng/ml)*	*CV (%)*
1	23.99	0.31	1.3
2	23.67	0.60	2.5

Inter-assay (Run-to-Run) (n=6)

Sample	*Mean (ng/ml)*	*SD (ng/ml)*	*CV (%)*
1	21.45	0.96	4.5
2	13.40	0.83	6.2

- **Spiking Recovery**

Serum samples were spiked with different amounts of human ENPP1 and assayed.

Sample	***O**bserved (ng/ml)*	***E**xpected (ng/ml)*	*Recovery **O/E** (%)*
1	16.32	-	-
	51.46	56.32	91.4
	33.24	36.32	91.5
	24.24	26.32	92.0
2	16.62	-	-
	54.44	56.62	96.1
	36.04	36.62	98.4
	28.50	26.62	107.1

- **Linearity**

Serum samples were serially diluted with Dilution Buffer and assayed.

Sample	*Dilution*	***O**bserved (ng/ml)*	***E**xpected (ng/ml)*	*Recovery **O/E** (%)*
1	-	28.80	-	-
	2x	14.81	14.40	107.5
	4x	7.49	7.20	108.3
	8x	3.63	3.60	108.7
2	-	26.10	-	-
	2x	14.03	13.05	104.2
	4x	7.07	6.53	108.4
	8x	3.55	3.26	90.3

- **Effect of sample matrix**

Citrate, heparin and EDTA plasmas were compared to respective serum samples from the same 10 individuals. However, we observed low correlation among serum and EDTA plasma and among serum and heparin plasma ENPP1 protein values.
Results are shown below:

Volunteer No.	*Serum (ng/ml)*	*Plasma (ng/ml)*		
		EDTA	*Citrate*	*Heparin*
1	16.27	11.87	13.21	12.99
2	19.20	17.25	18.51	19.96
3	17.97	15.48	14.99	17.04
4	19.30	17.12	17.88	18.82
5	14.16	13.30	13.17	15.40
6	18.03	16.87	16.93	18.45
7	16.17	15.57	14.55	16.91
8	15.18	14.09	13.68	15.27
9	15.83	14.07	13.72	14.68
10	16.13	13.94	14.35	16.53
Mean (*ng/ml*)	**16.82**	**14.96**	**15.10**	**16.61**
Mean Plasma/Serum (%)	**-**	**89**	**90**	**99**
Coefficient of determination R^2	**-**	**0.65**	**0.84**	**0.61**

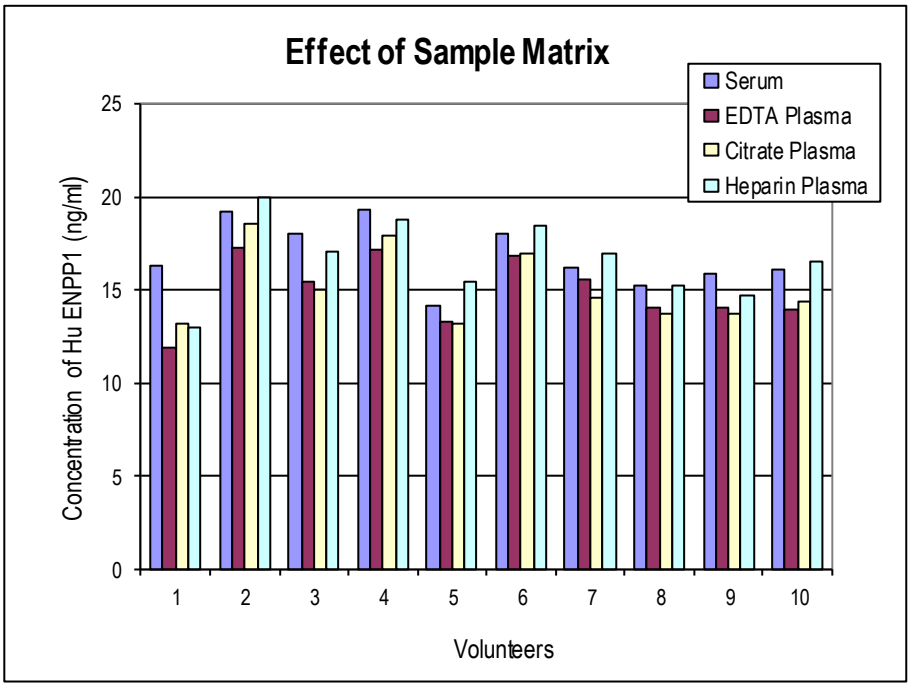

Figure 3: ENPP1 levels measured using Human ENPP1 ELISA from 10 individuals using serum, EDTA, citrate and heparin plasma, respectively.

14. DEFINITION OF THE STANDARD

The recombinant protein is used as the standard in this assay. The recombinant ENPP1, produced in HEK 293 cells, is a 96.5 kDa protein containing 840 amino acids.

15. PRELIMINARY POPULATION AND CLINICAL DATA

The following results were obtained when serum samples from 160 unselected donors (88 men + 72 women) 21 – 65 years old were assayed with the Biovendor Human ENPP1 ELISA in our laboratory.

Each laboratory should establish its own normal and pathological references ranges for ENPP1 levels with the assay.

Sex	*Age (years)*	*n*	*Mean*	*Median*	*SD*	*Min*	*Max*
			ENPP1 (ng/ml)				
Men	21-29	18	16.78	17.10	2.65	12.00	21.50
	30-39	28	17.23	16.75	3.34	11.70	26.00
	40-49	32	18.04	17.80	2.80	13.60	27.70
	50-65	17	15.72	15.30	1.97	12.70	18.50
Women	22-29	13	15.60	15.30	3.01	11.80	20.40
	30-39	28	17.15	16.75	5.03	10.10	25.30
	40-49	23	16.67	16.30	2.45	11.40	23.90
	50-61	9	16.53	16.90	1.60	13.80	18.60

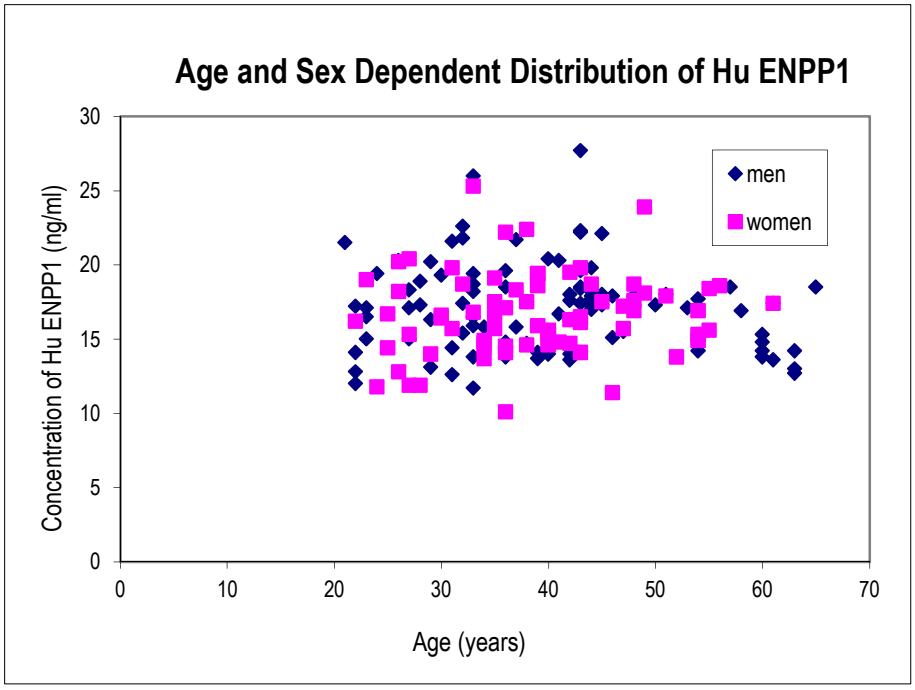

Figure 4: Human ENPP1 concentration plotted against donor age and sex.

16. METHOD COMPARISON

The BioVendor Human ENPP1 ELISA was not compared to the other commercial immunoassays.

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 and samples

18. REFERENCES

References to ENPP1:

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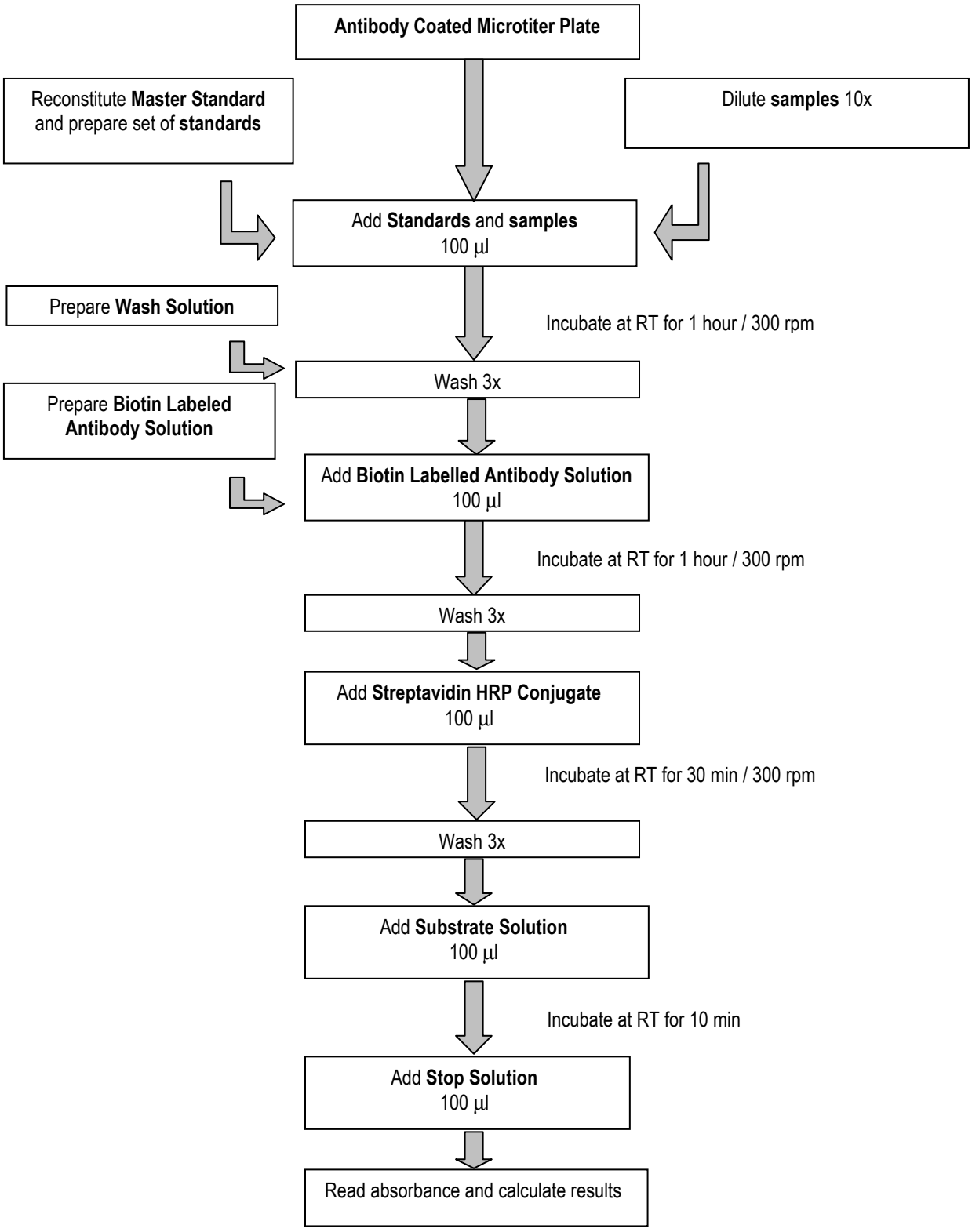
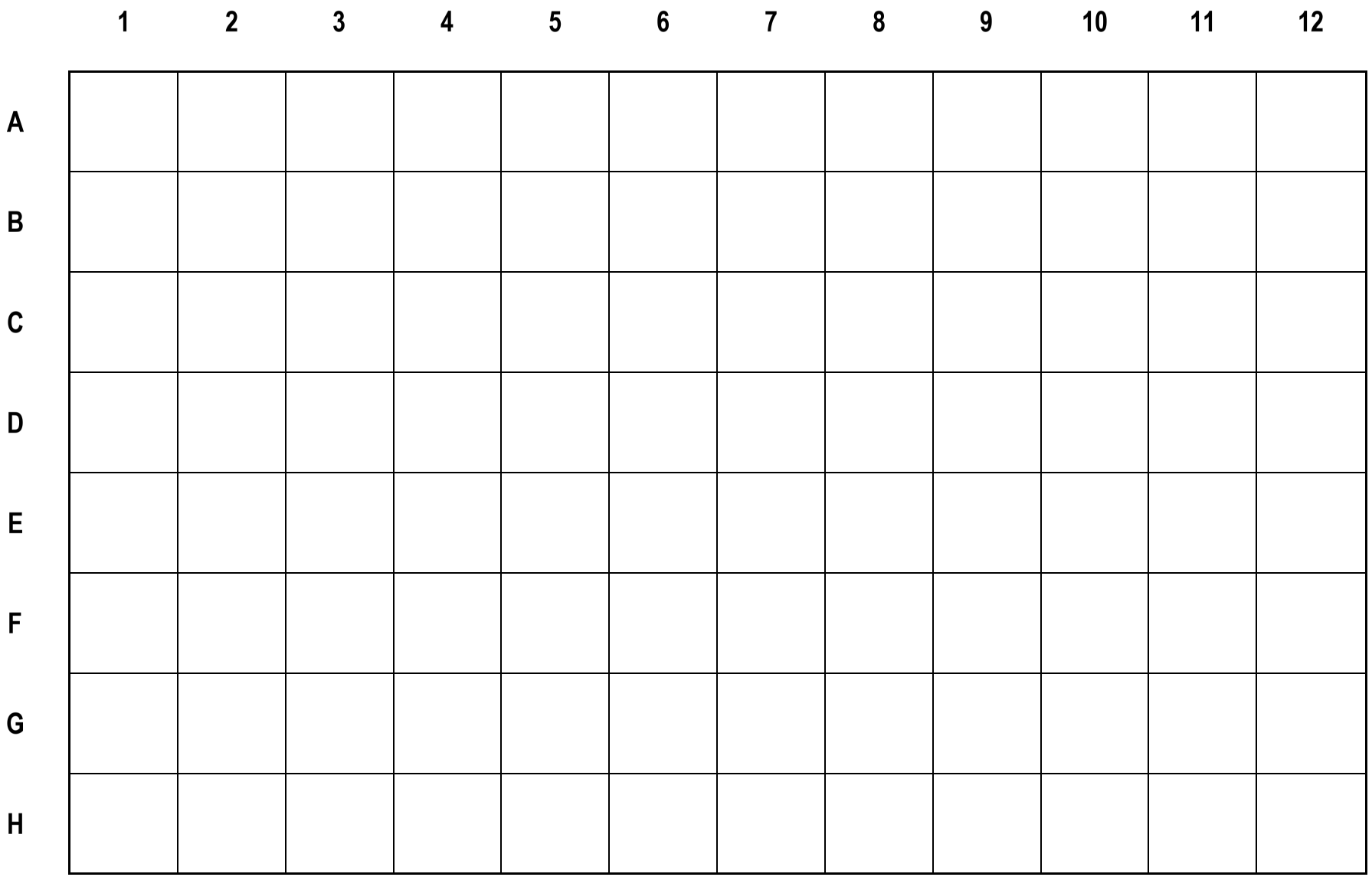
For more references on this product see our WebPages at www.biovendor.com

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

Assay Procedure Summary

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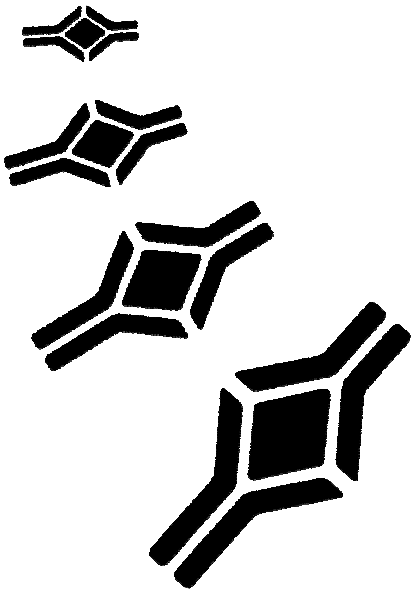

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