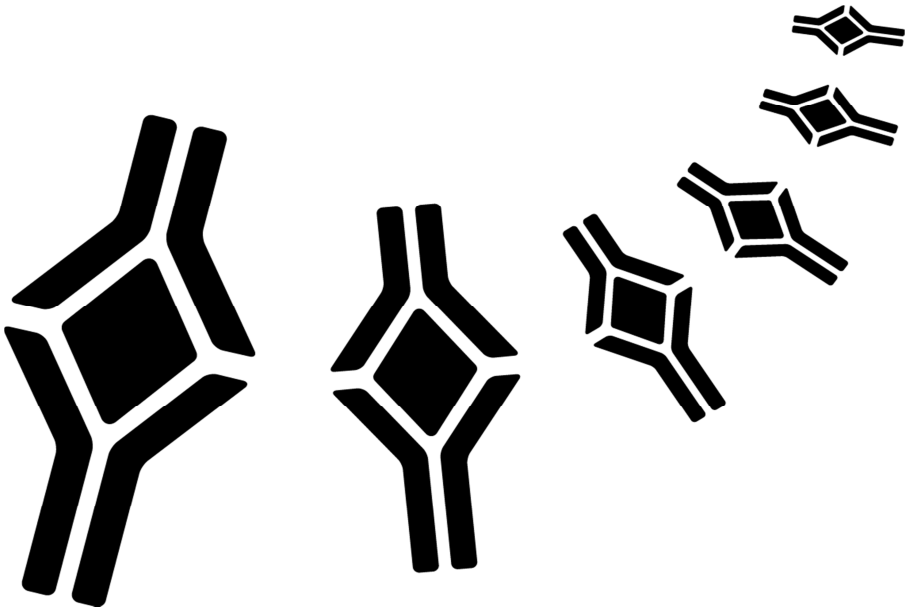
BioVendor
Research
and Diagnostic Products



HUMAN GRANULYSIN ELISA

Product Data Sheet

Cat. No.: RD191327200R

For Research Use Only

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 RD191327200R Human Granulysin ELISA is a sandwich enzyme immunoassay for the quantitative measurement of human granulysin (GNLY).

Features

- **It is intended for research use only**
- The total assay time is less than 3.5 hours
- The kit measures human granulysin in serum, plasma (EDTA, citrate, heparin)
- 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

Granulysin was first identified by subtractive hybridization in a search for genes expressed by human T lymphocytes 'late' (3– 5 days) after activation (1). Granulysin is a product of protein-coding gene with the same name – *Granulysin*, composed of 5 exons (2). This protein is a member of the saposin-like protein family (SAPLIP) which contain a saposin B-type domain. A single mRNA is translated into 15 kDa granulysin, some of which is processed at both the amino and carboxy termini to a 9 kDa protein (3). The two molecules differ in their roles in immune responses and cell localization. The 9 kDa form is sequestered in cytolytic granules and rapidly released after degranulation, while the 15 kDa form is constitutively secreted (4). Both isoform induce expression of proinflammatory cytokines, act as chemoattractants or alarmins and activate immature dendritic cells (iDC).

Studies with recombinant 9 kDa granulysin have demonstrated its cytolytic and proinflammatory properties (3). 9 kDa granulysin is contained in the cytotoxic granules of cytolytic T-cells (CTLs) and natural killers (NKs) and it is directionally released following target cell recognition. This isoform is proinflammatory and has a broad cytotoxic spectrum against gram-negative and gram-positive bacteria, fungi, yeast, parasites and tumors (3, 5, 6, 10, 11, 12, 13). Granulysin contributes to apoptosis of cancer cells via Cytochrome C. Granulysin directly kills *Mycobacterium tuberculosis* and is involved in host defence against leprosy. Recombinant 9 kDa granulysin is dependent on perforin for killing intracellular pathogens. Perforin and granzyme B are colocalized with 9 kDa granulysin. Granulysin is an important mediator of damage in a variety of skin diseases, including folliculitis, psoriasis, acne, lichen planus and viral vesicles.

Recent data also suggest that granulysin may be useful as a diagnostic and therapeutic agent in clinical cancer (3, 5, 6). Granulysin expression has been widely correlated with positive prognosis in variety of cancers. Progression of cancer was associated with decreased granulysin expression in peripheral NK cells in comparison to controls and tumor-free patients. Granulysin is suggested to be a potential biomarker in transplantation; its level increases in severity of graft *vs* host disease (GVHD) (7).

In contrast, 15 kDa granulysin is located in distinct granules negative for perforin and granzyme B (3) and these are released by activated cytolytic cells. The larger isoform is not cytotoxic, but plays an important role in differentiation of monocytes to dendritic cells. Further, 15 kDa granulysin activates both murine and human iDC.

Granulysin binds to and increases permeability of a target cell's plasma membrane resulting in a flux of calcium and potassium (8). Blocking this ion flux protects cells from lysis. The granulysin-mediated increase of intracellular calcium could contribute to mitochondrial damage and induction of apoptosis. Indeed, granulysin has been shown to damage the mitochondrial membrane in the presence of calcium, and cause the release of cytochrome C and production of reactive oxygen species, and in addition to inducing the permeability of lysosomal membranes. It also may induce damage of the endoplasmic reticulum in a caspase 7-dependent manner and contribute to the activation of caspase 3.

No specific receptor for granulysin has been identified to date, however, granulysin has been postulated to activate a G-coupled protein receptor and TLR4 (Toll-like receptor 4), at least in a mouse model (8). Because mice do not express granulysin or an apparent homologue, transgenic mice for human granulysin must be created to establish *in vivo* activity (9). *In* vivo, mice expressing granulysin show markedly improved anti-tumor responses, with an increased numbers of activated dendritic cells and cytokine-producing T cells (3).
Current knowledge suggests that the distinct functions of granulysin isoforms have major implications for diagnosis and potential new therapies for human disease.

Areas of investigation:
Immune Response, Infection and Inflammation
Transplantation
Oncology

4. TEST PRINCIPLE

In the BioVendor Human Granulysin ELISA, standards and samples are incubated in microtitration wells pre-coated with polyclonal anti-human Granulysin antibody. After 60 minutes incubation followed by washing, biotin-labelled polyclonal anti-human Granulysin antibody is added and incubated with the captured Granulysin for 60 minutes. 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 Granulysin. A standard curve is constructed by plotting absorbance values against Granulysin 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 may contain components of human or 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	lyophilized	2 vials
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 desiccant 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 Granulysin 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 granulysin in the stock solution is **4 ng/ml**.

Prepare set of standards using Dilution Buffer as follows:

Volume of Standard	*Dilution Buffer*	*Concentration*
Stock	–	4 ng/ml
250 µl of stock 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
250 µl of 0.25 ng/ml	250 µl	0.125 ng/ml
250 µl of 0.125 ng/ml	250 µl	0.063 ng/ml

Prepared Standards are ready to use, do not dilute them.
Stability and storage:
Do not store the reconstituted Master Standard and diluted standard solutions.

Biotin Labelled Antibody
Refer to the Certificate of Analysis for current volume of Biotin-Ab Diluent needed for reconstitution of Biotin Labelled Antibody!!!
Reconstitute the lyophilized Biotin Labelled Antibody with Biotin-Ab Diluent just prior to the assay. Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam).
Dilute Biotin Labelled Antibody Concentrate 100x with Biotin-Ab Diluent (e.g. 10 µl of Biotin Labelled Antibody Concentrate + 990 µl of Biotin-Ab Diluent for 8 wells).
Stability and storage:
Do not store reconstituted nor diluted Biotin Labelled Antibody solution.

Wash Solution Conc. (10x)
Dilute Wash Solution Concentrate (10x) ten-fold in distilled water to prepare the 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 human granulysin in serum and plasma (EDTA, citrate, heparin).

Samples can be assayed immediately after collection, or after a long-term storage. Thoroughly mix thawed samples just prior to the assay and avoid repeated freeze/thaw cycles, which may cause erroneous results. Avoid using hemolyzed or lipemic samples.

An appropriate dilution should be assessed by the researcher in advance to batch measurement.

Recommended starting dilution for serum and plasma is 5x.

Dilute samples (serum, plasma) 5x with the Dilution Buffer just prior to the assay as follows:
Add 50 µl of sample into 200 µl of Dilution Buffer. **Mix well** (not to foam). Vortex is recommended.

Stability and storage:
Samples should be stored at -20°C, or preferably at -70°C for long-term storage. Avoid repeated freeze/thaw cycles.
Do not store the diluted samples.

See Chapter 13 for effect of sample matrix (serum/plasma) on the concentration of human GRANULYSIN.

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 diluted 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 solution 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 25 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 1: If some samples and standard/s have absorbance 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 GNLY 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 4**	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	**Standard 2**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	**Standard 1**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	**Standard 0.5**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	**Standard 0.25**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	**Standard 0.125**	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	**Standard 0.063**	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 the known concentration (X) of Standards in logarithmic scale, using the four-parameter algorithm. Results are reported as concentration of granulysin (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 they have been diluted prior to the assay, e.g. 0.5 ng/ml (from standard curve) x 5 (dilution factor) = 2.5 ng/ml.

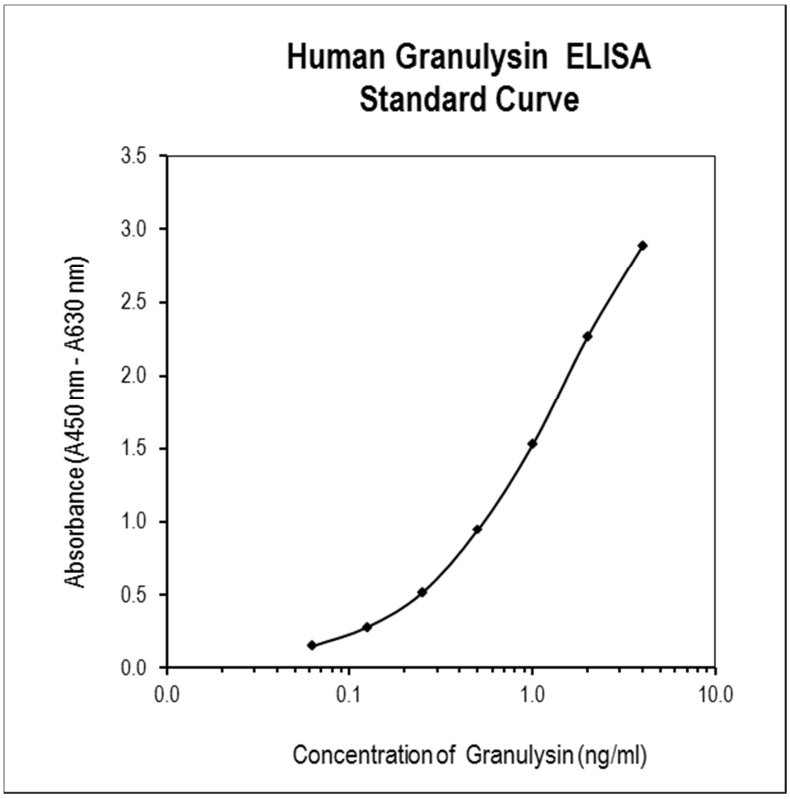


Figure 2: Typical standard curve for Human Granulysin ELISA.

13. PERFORMANCE CHARACTERISTICS

» Typical analytical data of BioVendor Human Granulysin 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 granulysin values in wells and is 0.03 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.

- **Specificity**

The antibodies used in this ELISA are specific for human granulysin with no detectable crossreactivity to human prosaposin at 1000 ng/ml.

» Presented results are multiplied by respective dilution factor

- **Precision**

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

Sample	*Mean (ng/ml)*	*SD (ng/ml)*	*CV (%)*
Serum 1	0.80	0.03	3.6
Serum 2	1.93	0.09	4.5

Inter-assay (Run-to-Run) (n=5)

Sample	*Mean (ng/ml)*	*SD (ng/ml)*	*CV (%)*
Serum 1	0.82	0.03	4.0
Serum 2	1.88	0.17	9.0

- **Spiking Recovery**

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

Sample	***O**bserved (ng/ml)*	***E**xpected (ng/ml)*	*Recovery **O/E** (%)*
Serum 1	0.52	-	-
	0.81	0.83	96.7
	1.10	1.15	96.1
	1.76	1.77	99.2
Serum 2	0.93	-	-
	1.20	1.24	96.6
	1.56	1.55	100.3
	2.19	2.18	100.5

- **Linearity**

Serum samples were serially diluted with Dilution Buffer and assayed.

Sample	*Dilution*	***O**bserved (ng/ml)*	***E**xpected (ng/ml)*	*Recovery **O/E** (%)*
Serum 1	-	2.63	-	-
	2x	1.28	1.31	97.3
	4x	0.65	0.66	98.4
	8x	0.33	0.33	100.5
Serum 2	-	2.99	-	-
	2x	1.47	1.49	98.2
	4x	0.76	0.75	101.2
	8x	0.39	0.37	103.2

- **Effect of sample matrix**

EDTA, citrate and heparin plasma samples were compared to respective serum samples from the same 10 individuals. Results are shown below:

Volunteer No.	*Serum (ng/ml)*	*Plasma (ng/ml)*		
		EDTA	*Citrate*	*Heparin*
1	1.01	0.98	1.15	0.64
2	0.70	0.73	0.66	0.60
3	0.41	0.39	0.51	0.38
4	0.19	0.15	0.16	0.12
5	0.70	0.83	0.77	0.49
6	1.04	0.95	1.02	0.68
7	0.59	0.58	0.56	0.43
8	0.48	0.49	0.44	0.30
9	0.89	0.83	0.88	0.60
10	1.37	1.27	1.07	0.57
Mean (pg/ml)	**0.74**	**0.72**	**0.72**	**0.48**
Mean Plasma/Serum (%)		**97.8%**	**97.8%**	**65.4%**
Coefficient of determination R^2		**0.97**	**0.88**	**0.70**

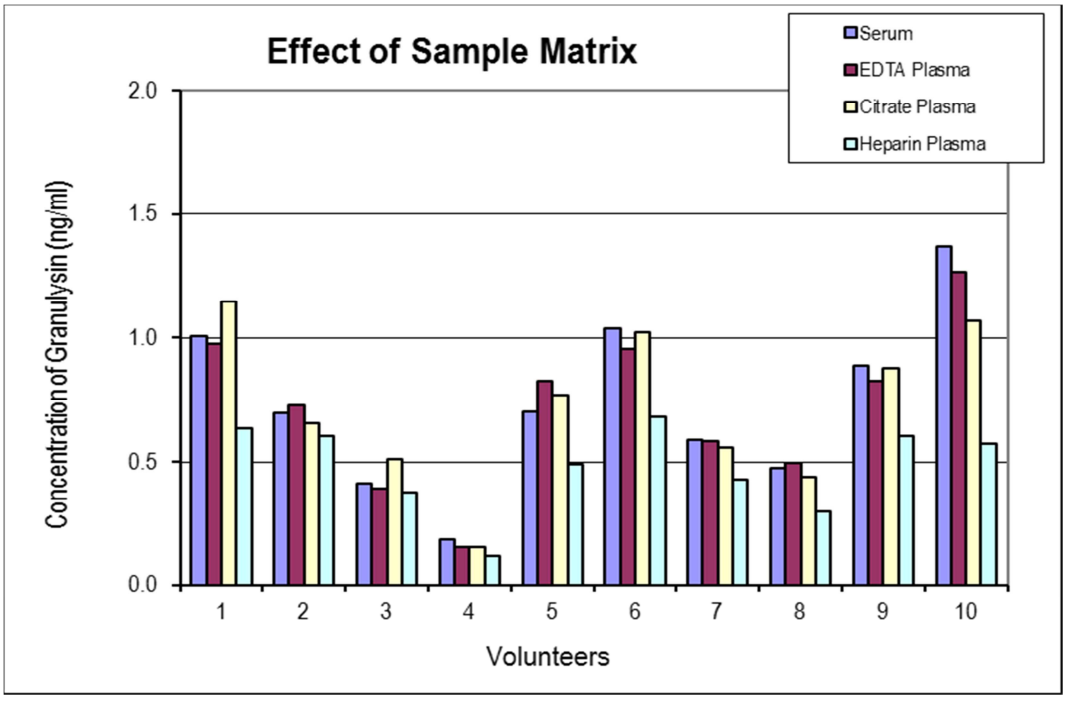


Figure 3: Granulysin levels measured using Human Granulysin ELISA from 10 individuals using serum, EDTA, citrate and heparin plasma, respectively.

14. DEFINITION OF THE STANDARD

In this assay the recombinant protein is used as the standard. The recombinant Granulysin protein is a 15.33 kDa protein consisting of 133 amino acid residues (Arg23-Leu145) of the human Granulysin with a N-terminal 10-His tag.

15. PRELIMINARY POPULATION DATA

The following results were obtained when serum samples from 155 unselected donors (89 men + 66 women) 21–65 years old were assayed with the BioVendor Human Granuylsin ELISA in our laboratory.

Sex	*Age (years)*	*n*	*Granulysin (ng/ml)*				
			Mean	*Median*	*SD*	*Min*	*Max*
Men	20-29	18	0.73	0.69	0.30	0.28	1.60
	30-39	26	0.83	0.77	0.29	0.42	1.64
	40-49	31	0.85	0.86	0.36	0.35	1.82
	50-65	14	0.83	0.78	0.30	0.39	1.69
Women	20-29	12	0.79	0.91	0.21	0.44	1.06
	30-39	26	0.77	0.72	0.26	0.40	1.44
	40-49	20	0.78	0.79	0.32	0.32	1.47
	50-61	8	0.90	0.77	0.41	0.48	1.76

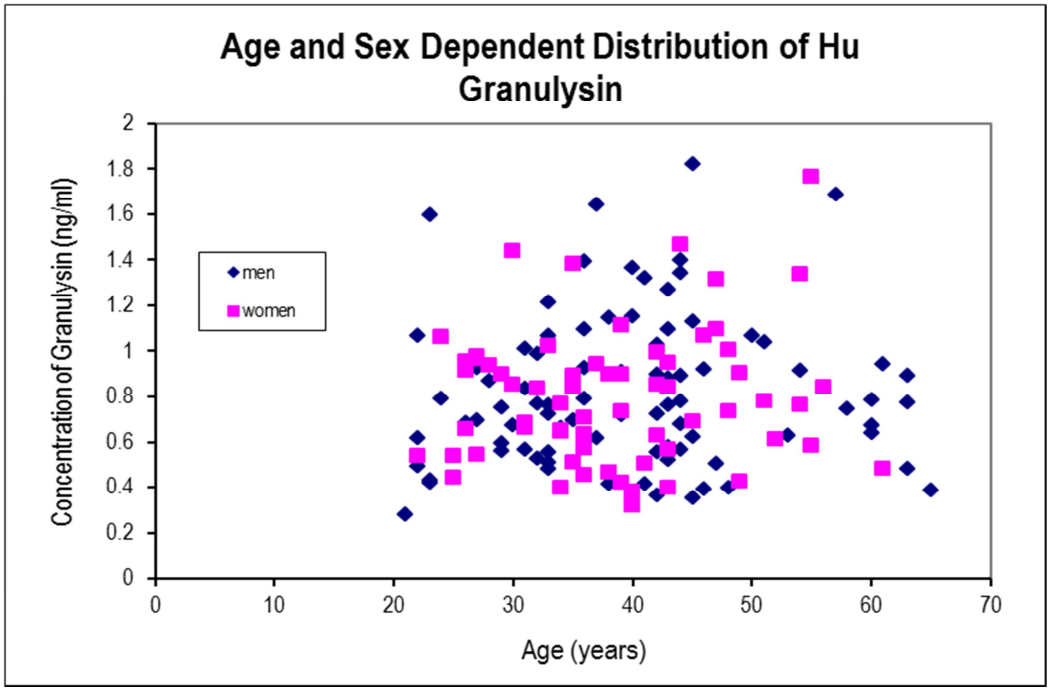


Figure 4: Human Granuylsin concentration plotted against donor age and sex.

- **Reference range**

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 reference ranges granulysin levels with the assay.

16. METHOD COMPARISON

The BioVendor Human Granulysin ELISA has not been compared to another commercial 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
- Manual washing
- 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 or samples

18. REFERENCES

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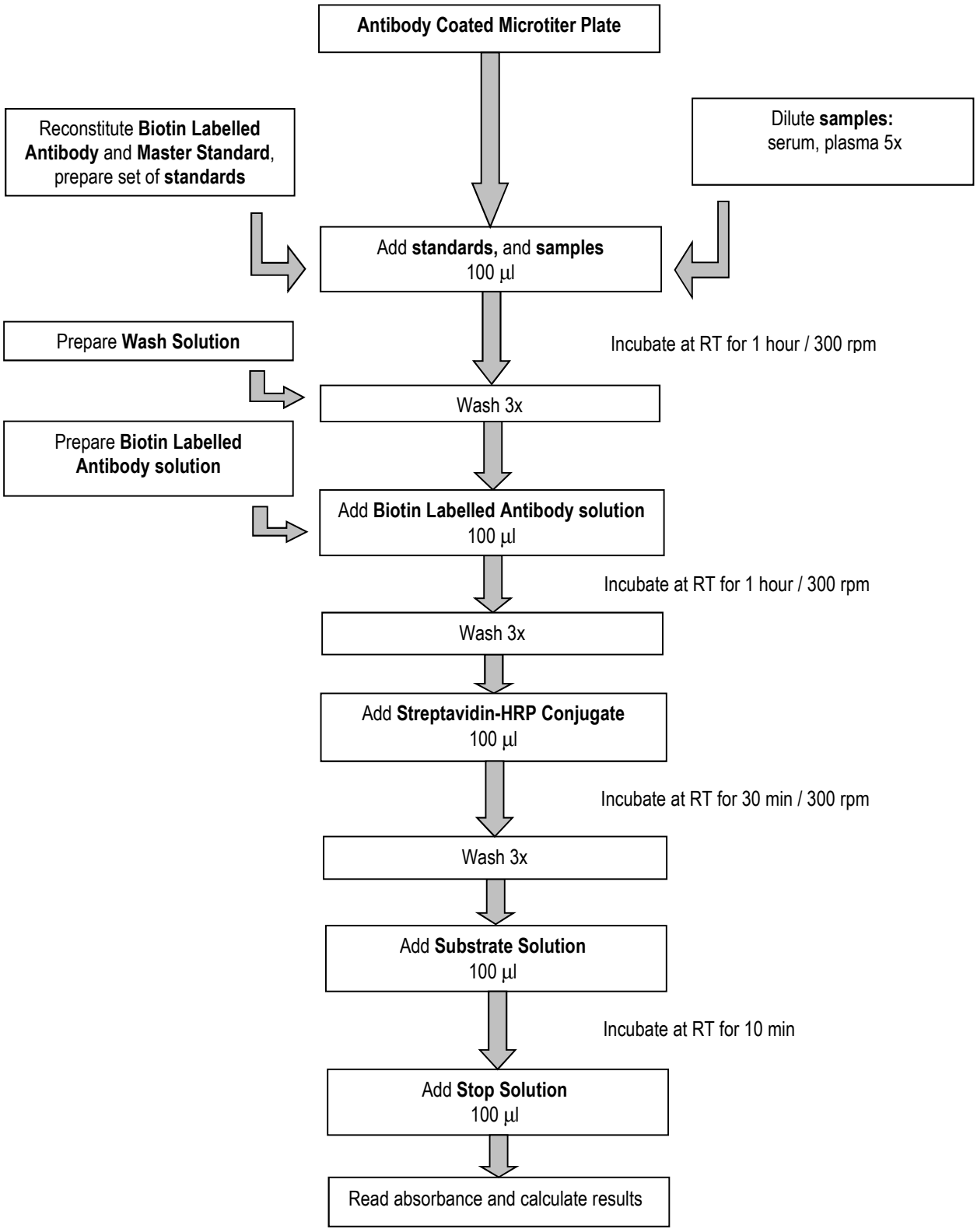
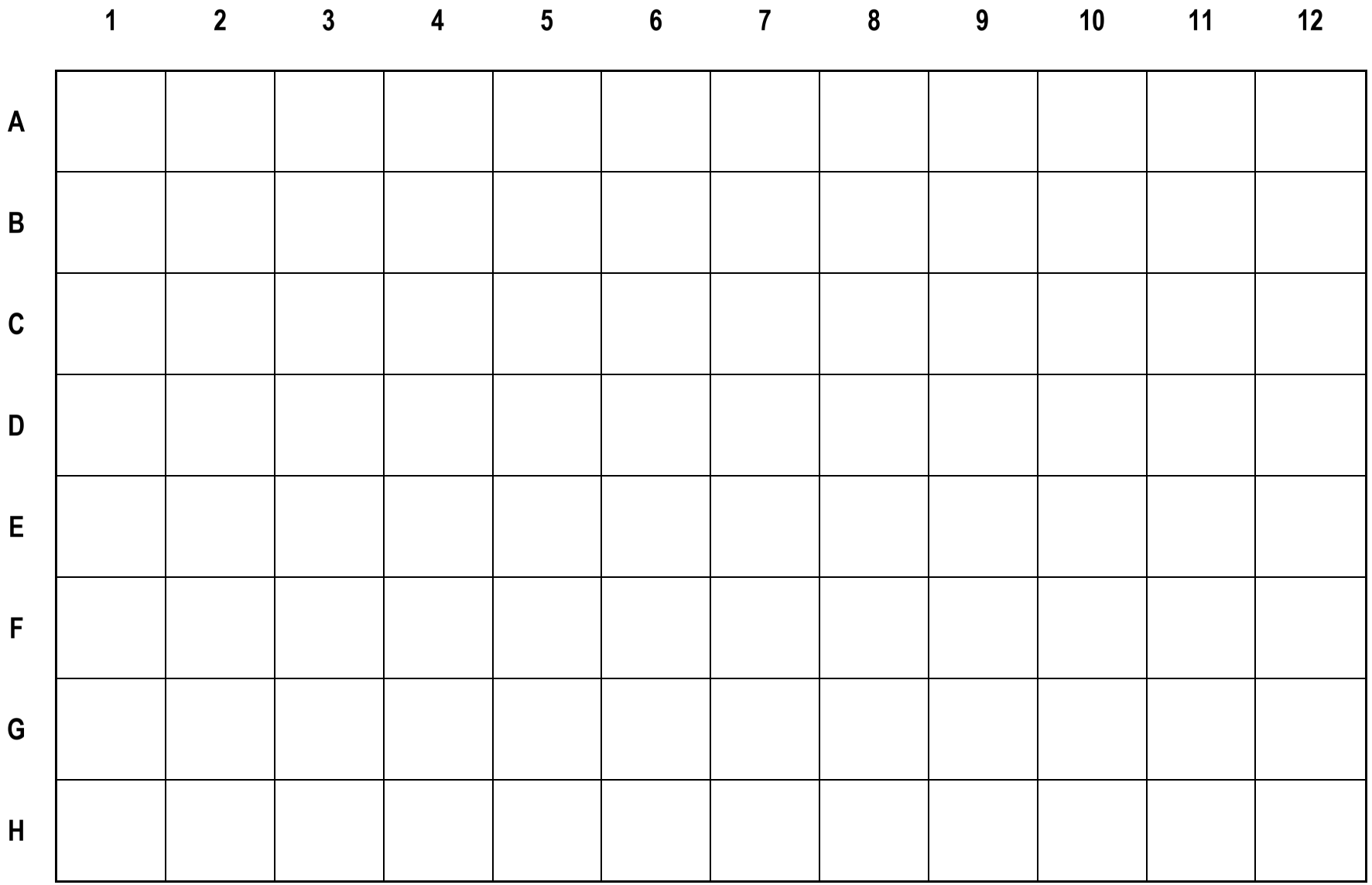
» For more references on this product see our WebPages at www.biovendor.com

19. EXPLANATION OF SYMBOLS

REF	Catalogue number
Cont.	Content
LOT	Lot number
	Attention, see instructions for use
	Potential biological hazard
	Expiry date
	Storage conditions
	Name and registered office of the manufacturer

Assay Procedure Summary

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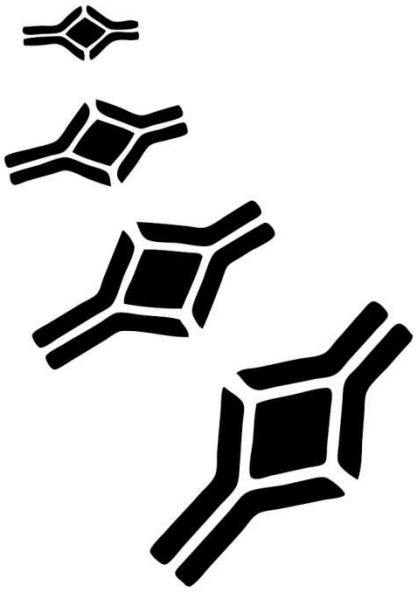

BioVendor – Laboratorní medicína a.s.
Karasek 1767/1, 621 00 Brno, Czech Republic
Phone: +420-549-124-185, Fax: +420-549-211-460
E-mail: info@biovendor.com, sales@biovendor.com
Web: www.biovendor.com

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