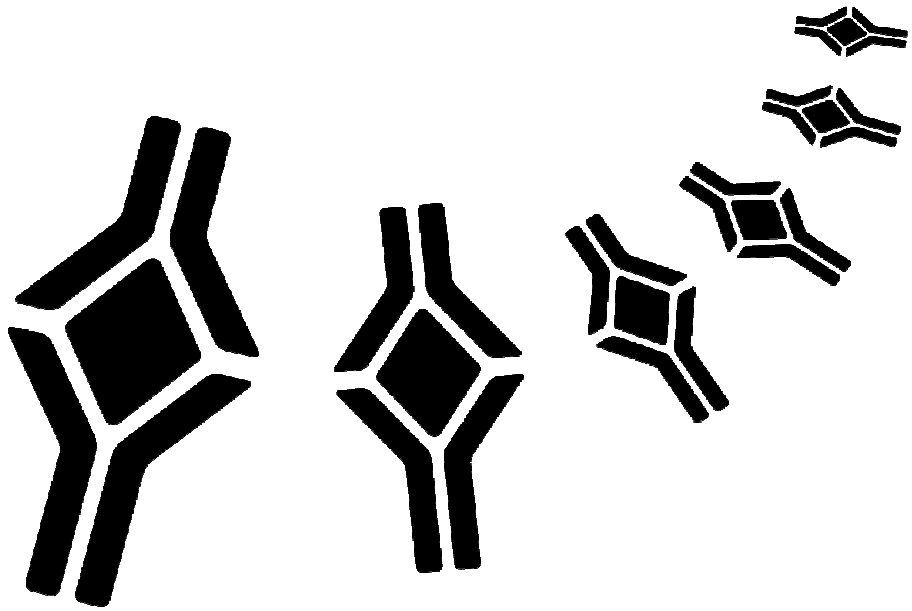
BioVendor
Research
and Diagnostic Products



HUMAN ALLOGRAFT INFLAMMATORY FACTOR 1 ELISA

Product Data Sheet

Cat. No.: RD191204200R

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 RD191204200R Human Allograft Inflammatory Factor 1 ELISA is a sandwich enzyme immunoassay for the quantitative measurement of human Allograft Inflammatory Factor 1 (AIF-1).

Features

- **For research use only!**
- The total assay time is less than 3 hours
- The kit measures total Allograft Inflammatory Factor 1 in serum and plasma (EDTA, citrate)
- Assay format is 96 wells
- Quality Controls are human serum based
- Standard is recombinant 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

Allograft inflammatory factor 1 (AIF-1), is a 17kDa cytoplasm, calcium-binding protein that, in humans, is encoded by the AIF1 gene. This gene is induced by cytokines and interferon. Three transcript variants encoding different isoforms have been found for this gene. The AIF1 gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, and rat.

AIF-1 is thought to be involved in the negative growth regulation of vascular smooth muscle cells, which contributes to the anti-inflammatory response caused by vessel wall trauma. AIF-1 also plays an important role in immune response and vasculopathy in allografts. AIF-1 was found in both cardiac allografts and hearts with other cardiac cellular diseases. In cardiac allografts, expression levels of AIF-1 in both cardiomyocytes and mononuclear cells directly correlated with the severity of cardiac cellular rejection. In cardiac transplantation, AIF-1 was associated with the severity of cardiac allograft rejection and Quilty B lesions, which could predict subsequent increases in rejection grade. Thus, AIF-1 shows promise that it can be a potential biomarker for cardiac allograft rejection. AIF-1 is a protein whose expression in transplanted human hearts correlates with rejection and development of coronary artery vasculopathy (CAV).

AIF-1 is crucial for the survival and pro-inflammatory activity of macrophages. Pro-inflammatory cytokines induced p-regulation of AIF-1 in macrophages. AIF-1 promote the activation and proliferation of T-lymphocytes and enhances lymphocyte migration. In human coronary artery vasculopathy, AIF-1 is expressed in T lymphocytes, and expression increases when T cells are activated.

In the brain, a subset of microglial cells constitutively expresses AIF-1. Increased numbers of AIF-1 immunoreactive macrophages/microglial cells were observed in focal human brain infarctions and human traumatic brain injury, inflammatory lesions due to autoimmune disease in a rat model and other disorders. Functional studies revealed that AIF-1 is secreted into the blood stream during experimental autoimmune neuritis.

The results of several studies suggest that enhanced AIF-1 expression leads to augmented incorporation of degenerated LDL by macrophages and promotes development of atherosclerotic vasculopathy. AIF-1 is also expressed in tissues from patients with systemic sclerosis.

<u>Areas of investigation:</u>
Immune Response, Infection and Inflammation
Autoimmunity
Transplantation
Traumatic Brain Injury
Cardiovascular Disease

4. TEST PRINCIPLE

In the BioVendor Human Allograft Inflammatory Factor 1 ELISA, standards, quality controls and samples are incubated in a microtitrate plate wells pre-coated with polyclonal anti-human Allograft Inflammatory Factor 1 antibody. After 60 min incubation and a washing, biotin-labelled polyclonal anti-human AIF-1 antibody is added and incubated with captured AIF-1 for 60 min. After another washing, the streptavidin-HRP conjugate is added. After 30 min 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 Allograft Inflammatory Factor 1. A standard curve is constructed by plotting absorbance values against concentrations of Allograft Inflammatory Factor 1 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 Conc. (100x)	concentrated	0.13 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
Biotin-Ab Diluent	ready to use	13 ml
Dilution Buffer	ready to use	20 ml
Wash Solution Conc. (10x)	concentrate	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 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 dessicant and seal carefully. Remaining Microtiter Strips are stable 3 months stored at 2-8°C and protected from the moisture.

Streptavidin-HRP Conjugate
Biotin-Ab Diluent
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 Allograft Inflammatory Factor 1 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 (do not foam).
The resulting concentration of AIF-1 in the stock solution is **1000 pg/ml**.

Prepare set of standards using Dilution Buffer as follows:

Volume of Standard	*Dilution Buffer*	*Concentration*
Stock	-	1000 pg/ml
250 µl of std. 1000 pg/ml	250 µl	500 pg/ml
250 µl of std. 500 pg/ml	250 µl	250 pg/ml
250 µl of std. 250 pg/ml	250 µl	125 pg/ml
250 µl of std. 125 pg/ml	250 µl	62.5 pg/ml
250 µl of std. 62.5 pg/ml	250 µl	31.3 pg/ml

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

Quality Controls HIGH, LOW
Refer to the Certificate of Analysis for current volume of Dilution Buffer needed for reconstitution and for current Quality Control concentrations!!!
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 (do not foam).
The reconstituted Quality Controls are ready to use, do not dilute them. Mix well (not to foam).
Stability and storage:
Do not store the reconstituted Quality Controls.
Note:
Concentration of analyte in Quality Controls is not 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.

Biotin Labelled Antibody Conc. (100x)
Prepare the working Biotin Labelled Antibody solution by adding 1 part Biotin Labelled Antibody Concentrate (100x) to 99 parts Biotin-Ab Diluent.
Example: 60 µl of Biotin Labelled Antibody Concentrate (100x) + 5 940 µl of Biotin-Ab Diluent for 6 strips (48 wells).
Stability and storage:
Opened Biotin Labelled Antibody Concentrate (100x) is stable 3 months when stored at 2-8°C.
Do not store the diluted Biotin Labelled Antibody solution.

Wash Solution Conc. (10x)

Dilute Wash Solution Concentrate (10x) ten-fold in 900 ml of distilled water to prepare a 1x working solution, e.g. 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 Allograft Inflammatory Factor 1 in serum and plasma (EDTA, 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 and plasma samples:
Samples should be assayed immediately after collection or should be stored at -20°C or -70°C. 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.

Preparation of samples:
Dilute samples 5x with Dilution Buffer just prior to the assay (e.g. 30 µl of sample + 120 µl of Dilution Buffer for singlets, or preferably 60 µl of sample + 240 µl of Dilution Buffer for duplicates). **Mix well** (do not 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 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 human AIF-1.

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 5-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against a paper towel.
4. Pipet **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. Incubation without shaking is the alternative that requires to extend incubation with substrate – see point 11.
6. Wash the wells 5-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against a paper towel.
7. Pipet **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 5-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against a 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 less than 20°C. Do not shake with 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 Allograft Inflammatory Factor 1 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: Remove liquid from wells completely and pipet 0.35 ml Wash Solution into each well. ***Allow Wash Solution to stay in each well for 30 seconds.*** *Remove solution from wells completely and repeat washing four times. After final wash, invert and tap the plate strongly against paper towel.* ***Make certain that all liquid has been removed from the wells after each washing step.***

	strip 1+2	strip 3+4	strip 5+6	strip 7+8	strip 9+10	strip 11+12
A	**Standard 1000**	**Blank**	Sample 8	Sample 16	Sample 24	Sample 32
B	**Standard 500**	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	**Standard 250**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	**Standard 125**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	**Standard 62.5**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	**Standard 31**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	**QC HIGH**	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
H	**QC LOW**	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 Allograft Inflammatory Factor 1 (pg/ml) in samples.

Alternatively, the *logit log* function can be used to linearize the standard curve (i.e. *logit* of 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. 300 pg/ml (from standard curve) x 5 (dilution factor) = 1500 pg/ml = 1.5 ng/ml.

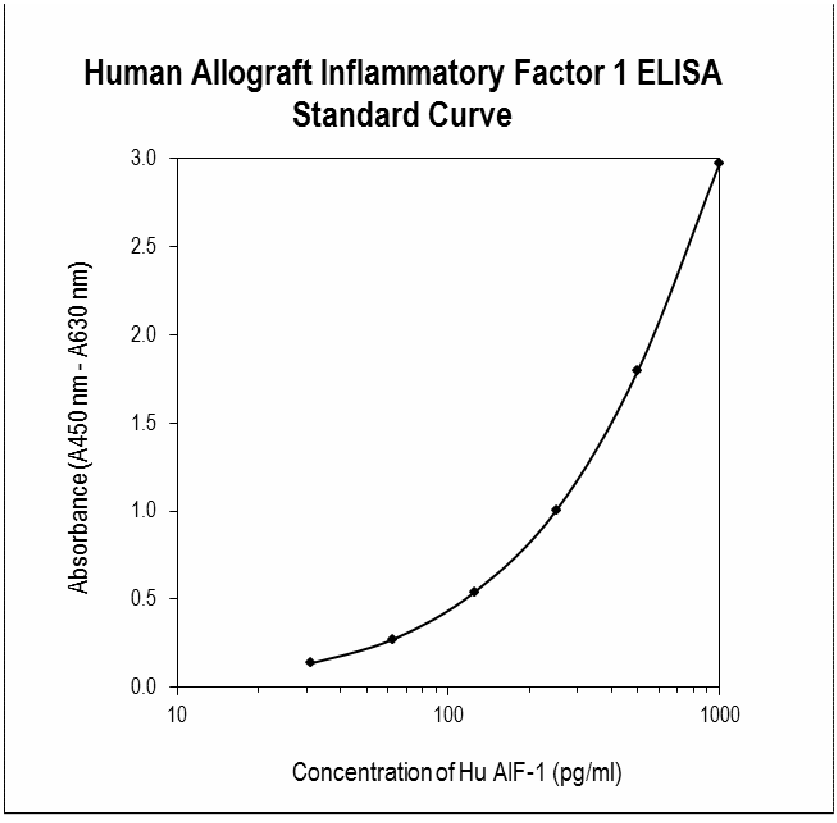


Figure 2: Typical Standard Curve for Human Allograft Inflammatory Factor 1 ELISA.

13. PERFORMANCE CHARACTERISTICS

Typical analytical data of BioVendor Human Allograft Inflammatory Factor 1 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 Allograft Inflammatory Factor 1 values in wells and is 4.0 pg/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 Allograft Inflammatory Factor 1.

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	yes
Pig	yes
Rabbit	no
Rat	yes
Sheep	no

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

- **Precision**

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

Sample	*Mean (pg/ml)*	*SD (pg/ml)*	*CV (%)*
1	570.1	10.9	1.9
2	945.8	18.6	2.0

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

Sample	*Mean (pg/ml)*	*SD (pg/ml)*	*CV (%)*
1	357.3	26.4	7.4
2	572.9	33.1	5.8

- **Spiking Recovery**

Serum samples were spiked with different amounts of human AIF-1, diluted with Dilution Buffer 5x and assayed.

Sample	***O**bserved (pg/ml)*	***E**xpected (pg/ml)*	*Recovery **O/E** (%)*
1	377.28 519.12 1030.71 2949.57	- 533.78 1002.28 2877.28	- 97.3 102.8 102.5
2	614.01 703.07 1262.73 3225.46	- 770.51 1239.01 3114.01	- 91.2 101.9 103.6

- **Linearity**

Serum samples were serially diluted with Dilution Buffer after primary dilution 5x and assayed.

Sample	*Dilution*	***O**bserved (pg/ml)*	***E**xpected (pg/ml)*	*Recovery **O/E** (%)*
1	- 2x 4x 8x	839.88 430.26 205.79 94.30	- 419.94 209.97 104.99	- 102.5 98.0 89.8
2	- 2x 4x 8x	1532.63 727.00 374.18 181.03	- 766.31 383.16 191.58	- 94.9 97.7 94.5

- **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 (pg/ml)*	*Plasma (pg/ml)*		
		EDTA	*Citrate*	*Heparin*
1	205.24	209.49	151.91	483.64
2	318.21	308.70	234.70	699.91
3	387.03	338.40	294.41	578.06
4	219.68	222.11	181.10	559.03
5	214.30	299.18	198.18	675.34
6	333.05	408.96	342.55	815.59
7	484.82	521.01	403.03	817.41
8	281.91	227.51	182.91	494.90
9	573.31	587.59	488.37	1620.10
10	292.04	390.59	228.70	767.27
Mean (pg/ml)	**330.96**	**351.35**	**270.59**	**751.12**
Mean Plasma/Serum (%)	**-**	**106.2**	**81.8**	**227.0**
Coefficient of determination R^2	**-**	**0.83**	**0.92**	**0.65**

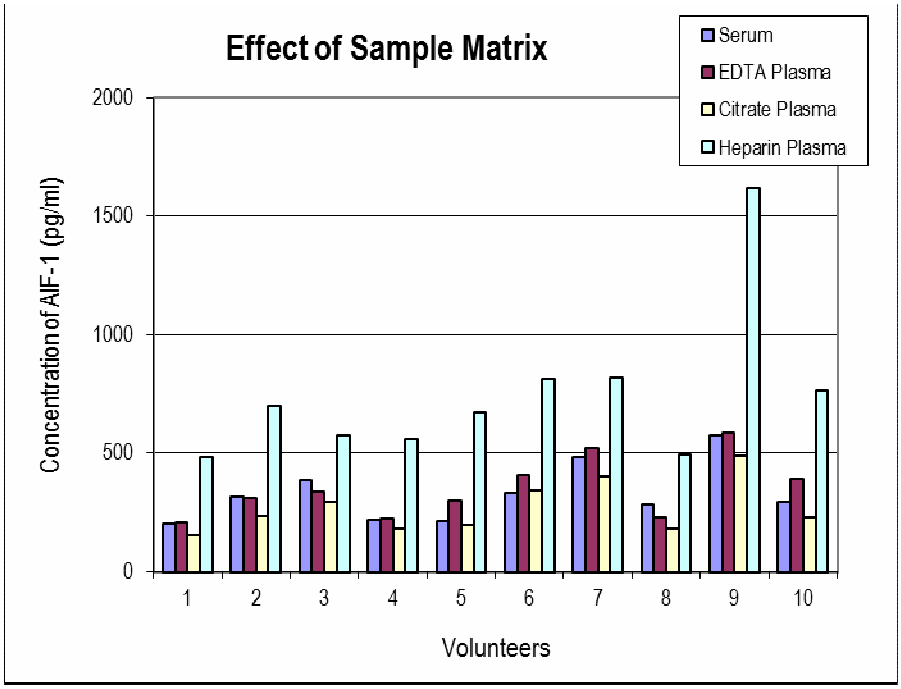


Figure 3: Allograft Inflammatory Factor 1 levels measured using Human Allograft Inflammatory Factor 1 ELISA from 10 individuals using serum, EDTA, citrate and heparin plasma, respectively.

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

Samples should be stored at -20°C. However, no decline in concentration of Allograft Inflammatory Factor 1 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 (pg/ml)*	*Plasma (pg/ml)*		
			EDTA	*Citrate*	*Heparin*
1	-20°C	185.32	200.87	153.31	408.92
	2-8°C, 1 day	182.33	209.82	155.74	412.39
	2-8°C, 7 days	183.53	203.86	159.99	421.64
2	-20°C	277.63	302.75	234.12	672.57
	2-8°C, 1 day	288.74	303.33	235.30	676.08
	2-8°C, 7 days	281.14	299.25	235.89	676.08
3	-20°C	309.75	388.68	353.92	766.37
	2-8°C, 1 day	302.75	389.83	364.83	752.81
	2-8°C, 7 days	299.84	385.78	352.18	751.63

- **Effect of Freezing/Thawing**

No decline was observed in concentration of human Allograft Inflammatory Factor 1 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 (pg/ml)*	*Plasma (pg/ml)*		
			EDTA	*Citrate*	*Heparin*
1	1x	214.61	254.35	214.08	351.15
	3x	213.56	271.09	217.75	362.69
	5x	216.70	305.59	247.04	394.22
2	1x	287.29	274.22	226.64	369.00
	3x	291.99	275.79	223.50	392.64
	5x	300.89	323.39	262.20	384.23
3	1x	254.35	251.22	265.33	488.29
	3x	316.06	311.87	354.30	627.64
	5x	250.17	271.60	278.92	503.71

14. DEFINITION OF THE STANDARD

The Standard used in this kit is recombinant protein. Recombinant human AIF-1, produced on E. Coli, is 17.7 kDa protein consisting of 155 amino-acid residues of human AIF-1 and 9 additional amino-acids.

15. PRELIMINARY POPULATION AND CLINICAL DATA

The following results were obtained when serum samples from 165 unselected donors (90 men + 75 women) 20 - 69 years old were assayed with the Biovendor Human Allograft Inflammatory Factor 1 ELISA in our laboratory:

- **Age and Sex dependent distribution of human Allograft Inflammatory Factor 1**

Sex	Age (years)	n	Mean	SD	Min	Max
			AIF-1 (pg/ml)			
Men	20-39	47	583	208	266	1533
	40-69	43	659	327	282	2478
Women	20-39	42	577	152	257	1475
	40-69	33	608	206	322	1125

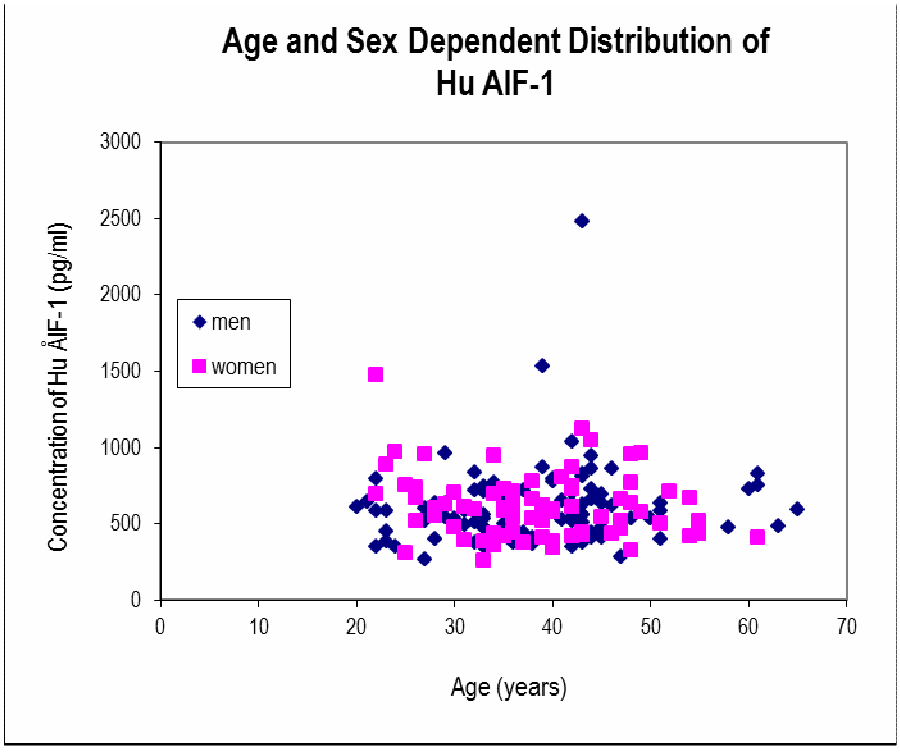


Figure 4: Human Allograft Inflammatory Factor 1 concentration plotted against donor age and sex.

- **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 AIF-1 levels with the assay.

16. METHOD COMPARISON

The BioVendor Human Allograft Inflammatory Factor 1 ELISA has not been compared to any 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, Quality Controls or samples

18. REFERENCES

References to Allograft Inflammatory Factor 1:

References to Allograft Inflammatory Factor 1:

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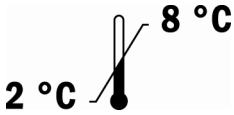
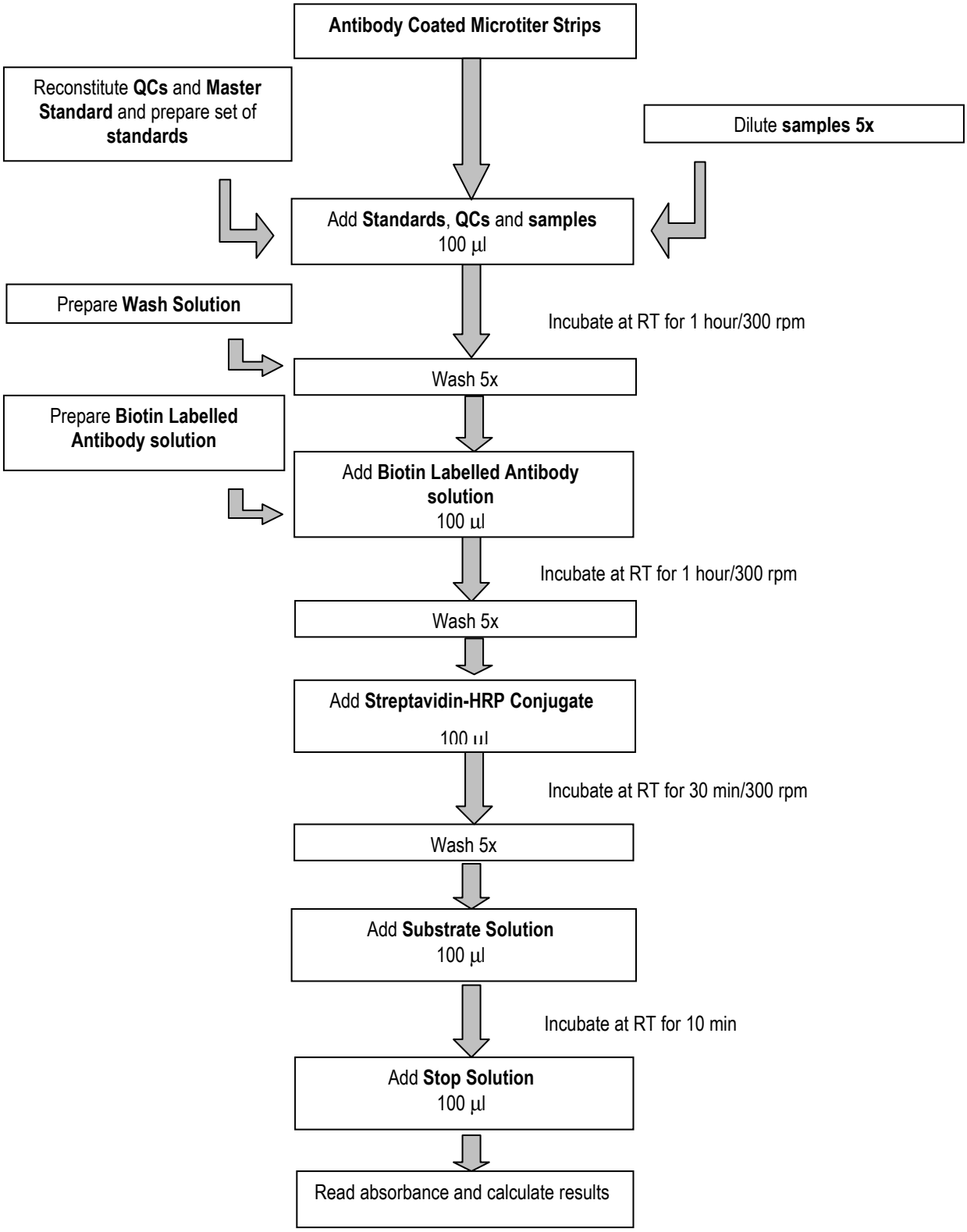
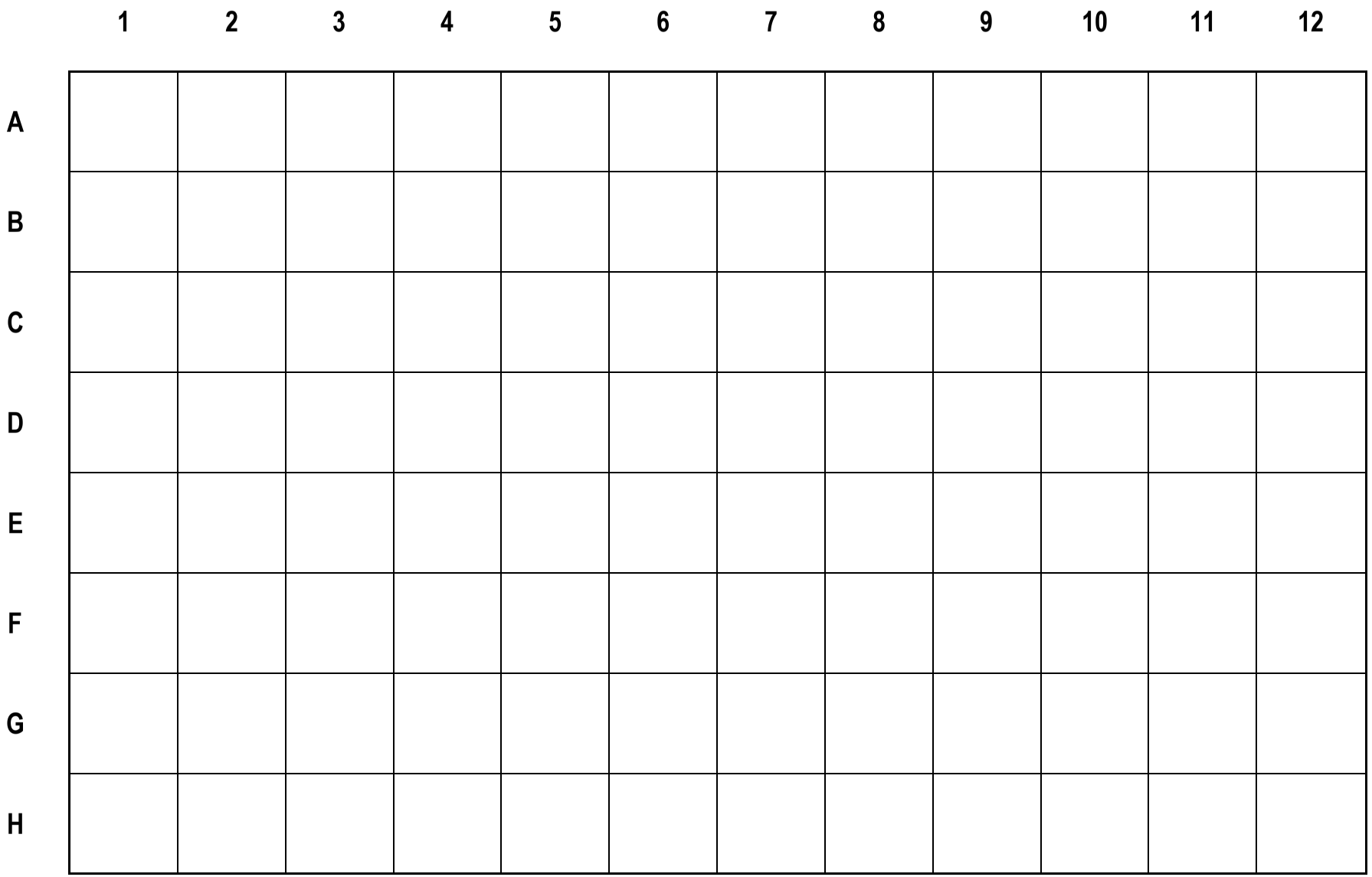
For more references on this product see our WebPages at www.biovendor.com

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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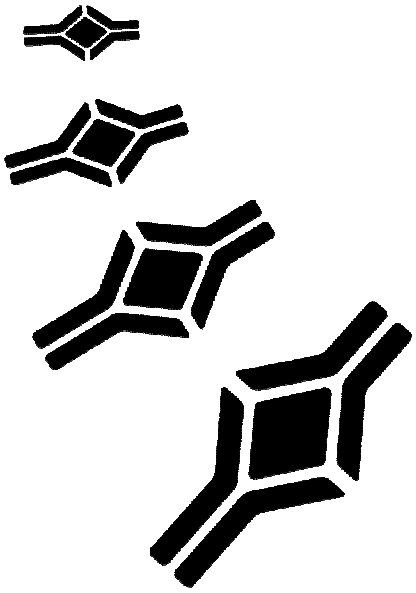

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