MANUAL

AlphaLISA® Human Plasminogen Activator Inhibitor-1 (PAI-1) Kit

Product number: AL286 C/F

Research Use Only. Not for use in diagnostic procedures.

Material provided:

Format: AL286C: 500 assay points AL286F: 5 000 assay points

The number of assay points is based on an assay volume of 50 µL in 96- or 384-well assay plates using the kit components at the recommended concentrations.

Document version: 1

Product Information

Kit content: The kit contains 5 components: AlphaLISA Acceptor beads coated with an Anti-Analyte Antibody, Streptavidin-coated Donor beads, Biotinylated Anti-Analyte Antibody, lyophilized analyte and 10X AlphaLISA Immunoassay Buffer.
Assay microplates (96-, 384- or 1536-well plates) must be purchased separately (see page 3 for more details).

Storage: Store kit in the dark at +4˚C. Store reconstituted analyte at -20°C.

Stability: This product is stable for at least 12 months from the manufacturing date when stored in its original packaging and the recommended storage conditions. Note: Once reconstituted, the human PAI-1 analyte is stable for at least 75 days at -20°C (see page 2: Reagents and Materials).

Application: This kit is designed for the quantitative determination of human PAI-1 in serum, plasma, buffered solution or cell culture medium using a homogeneous AlphaLISA assay (no wash steps). The analyte in this kit consists of the active glycosylated human PAI-1.

Sensitivity: Lower Detection Limit (LDL): 39.5 pg/mL (see page 9: Assay Performance Characteristics).
(Higher sensitivity can be achieved using the High sensitivity manual, see page 8: Manual 2.)
A unique assay manual has been developed for this kit (see page 5: Manual)

Dynamic range: 39.5 – 300 000 pg/mL (see page 9: Assay Performance Characteristics).

RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Precautions

- Only the AlphaScreen® Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- All blood components and biological materials should be handled as potentially hazardous. Some analytes are from human source.
- Some analytes are present in saliva. Take precautionary measures to avoid contamination of the reagent solutions.
- The Biotinylated Anti-Analyte Antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Reagents and Materials

The reagents provided in the AlphaLISA kit are listed in the table below:

Kit components	AL286C (500 assay points)	AL286F (5 000 assay points)
AlphaLISA Anti-PAI-1 Acceptor beads stored in PBS, 0.05% Kathon, pH 7.2	50 µL @ 5 mg/mL (1 brown tube, white cap)	500 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon, pH 7.4	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 X 1 mL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated Antibody Anti-PAI-1 stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
AlphaLISA human PAI-1 (1 µg), lyophilized analyte *	1 tube, clear cap	1 tube, clear cap
AlphaLISA Immunoassay Buffer (10X) **	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute human PAI-1 in 100 µL Milli-Q® grade H_2O. The reconstituted analyte should be used within 60 minutes, if possible, or aliquoted into screw-capped polypropylene vials and stored at -20°C for further experiments. Avoid multiple freeze-thaw cycles. It has been demonstrated that reconstituted human PAI-1 is stable for at least 75 days at -20°C. One vial contains an amount of human PAI-1 sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL286S).

** Contains 250 mM HEPES, pH 7.4, 1% Casein, 10 mg/mL Dextran-500, 5% Triton X-100 and 0.5% Kathon. Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).
Note: 10X buffer might be slightly yellow. However, this does not affect the assay results.

Once diluted, 1X AlphaLISA Immunoassay Buffer contains 25 mM HEPES, pH 7.4, 0.1% Casein, 1 mg/mL Dextran-500, 0.5% Triton X-100 and 0.05% Kathon.

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the AlphaLISA signal. Note that sodium azide from the Biotinylated Antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Adhesive Sealing Film	Revvity Inc.	6050195
EnSpire® or EnVision® Multilabel Alpha Reader	Revvity Inc.	-

Manuals have been optimized for 50 µL assays in white OptiPlate™-384 microplates. Other assay volumes can be used with similar manuals and identical final AlphaLISA reagent concentrations:

Format	# of data points	Total assay volume	Sample volume	AlphaLISA beads / Biotin Antibody MIX volume *	SA-Donor beads volume *	Plate recommendation
AL286C	250	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290)
	500	50 µL	5 µL	5 µL	40 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL286F	5 000	50 µL	5 µL	5 µL	40 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)

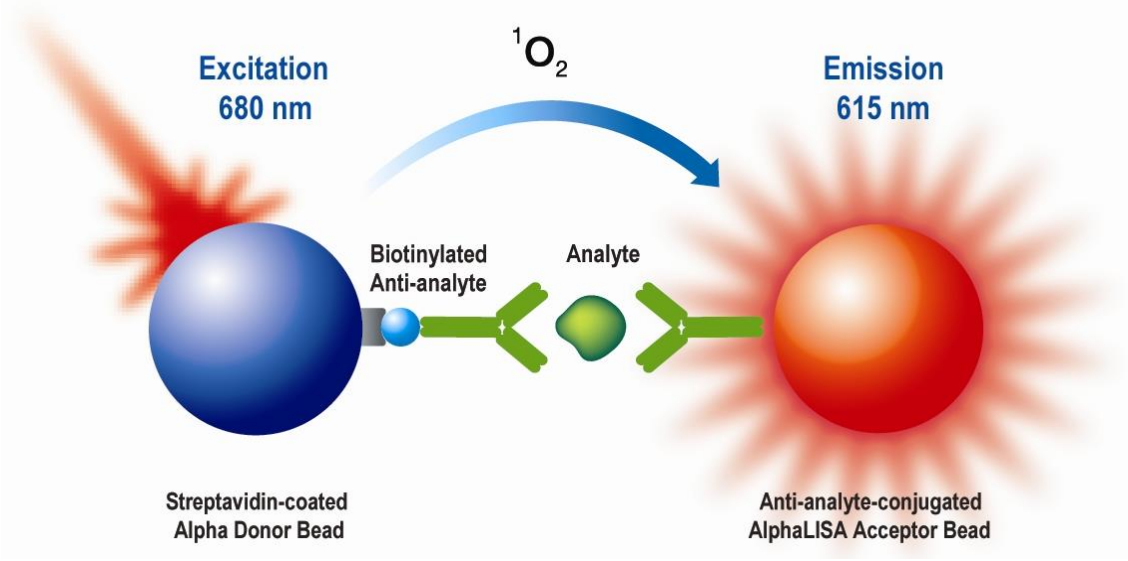
* Volumes based on the Quick manual.

Analyte of Interest

Plasminogen activator inhibitor-1 (PAI-1) is a single chain glycoprotein of 50 kDa. PAI-1 is secreted and is mainly produced by the endothelium, liver, and adipose tissue. It is also produced by certain tumours. PAI-1 activity is tightly regulated on the transcriptional level by transforming growth factor β. PAI-1 is the principal inhibitor of tissue-type and urokinase-type plasminogen activators (tPA and uPA), which convert plasminogen to plasmin. Congenital PAI-1 deficiency can cause hemorrhagic diathesis. PAI-1 is present in larger amounts in various pathologies, such as in a number of cancers or obesity. In breast cancer, high levels of PAI-1 are associated with a high risk of recurrence.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in buffer, cell culture media, serum and plasma in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaLISA assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Donor beads while another Anti-Analyte Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (see figure below).



Recommendations

General recommendations:

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to prewet the tip.

- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2 000 g, 10-15 sec). Resuspend all reagents by vortexing before use.

- Use Milli-Q® grade H_2O (18 MΩ•cm) to dilute 10X AlphaLISA Immunoassay Buffer and to reconstitute the lyophilized analyte.

- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.

- When reagents are added in the microplate, make sure the liquids are at the bottom of the well.

- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film.

- The AlphaLISA signal is detected with an EnVision Multilabel Reader equipped with the ALPHA option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).

- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.

- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment. The standard curve should be performed in a similar matrix as the samples (e.g. FBS for serum samples).

Specific recommendations:

- AlphaLISA assays can be performed in cell culture medium with or without phenol red, with the following recommendations: if possible, avoid biotin-containing medium (e.g. RPMI medium) as lower counts and lower sensitivity are expected. Add at least 1% FBS or 0.1% BSA to cell culture medium.

- When analyzing serum and plasma samples, perform the standard curve in FBS and dilute the samples at least 2-fold with FBS before testing. Serum or plasma should not exceed 10% of final assay volume (i.e. 5 µL serum or plasma sample in 50 µL final assay volume).

Manuals

The two manuals described below are recommended when generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The manuals also include testing samples in 452 wells. If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.

The standard dilution manual is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.

Use of four background points in triplicate (12 wells) is recommended when LDL is calculated. One background point in triplicate (3 wells) can be used when LDL is not calculated.

Manual 1: Quick manual (2 incubation steps) – Dilution of standards in 1X AlphaLISA Immunoassay Buffer, cell culture medium or FBS

Manual 2: High sensitivity manual (3 incubation steps) – Dilution of standards in 1X AlphaLISA Immunoassay Buffer, cell culture medium or FBS

IMPORTANT: PLEASE READ THE RECOMMENDATIONS ABOVE BEFORE USE

Common Steps for Preparing Reagents (Manuals 1 & 2)

If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

1) Preparation of 1X AlphaLISA Immunoassay Buffer:
Add 2.5 mL of 10X AlphaLISA Immunoassay Buffer to 22.5 mL H_2O.

2) Preparation of human PAI-1 analyte standard dilutions:
Reconstitute lyophilized human PAI-1 (1 µg) in 100 µL H_2O.
Prepare standard dilutions as follows (change tip between each standard dilution):

Tube	Vol. of human PAI-1 (µL)	Vol. of diluent (µL) *	[human PAI-1] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted human PAI-1	90	1E-06	1 000 000
B	60 µL of tube A	140	3E-07	300 000
C	60 µL of tube B	120	1E-07	100 000
D	60 µL of tube C	140	3E-08	30 000
E	60 µL of tube D	120	1E-08	10 000
F	60 µL of tube E	140	3E-09	3 000
G	60 µL of tube F	120	1E-09	1 000
H	60 µL of tube G	140	3E-10	300
I	60 µL of tube H	120	1E-10	100
J	60 µL of tube I	140	3E-11	30
K	60 µL of tube J	120	1E-11	10
L	60 µL of tube K	140	3E-12	3
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

* Dilute standards in diluent (e.g. 1X AlphaLISA Immunoassay Buffer, cell culture medium or FBS).
At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

Manual 1: Quick Manual (2 Incubation Steps)

The manual described below is for one standard curve (48 wells) and samples (452 wells). Dilution of standards can be done in 1X AlphaLISA Immunoassay Buffer, cell culture medium or FBS.

If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

3) Preparation of 10X AlphaLISA Anti-PAI-1 Acceptor beads + Biotinylated Antibody Anti-PAI-1 MIX (100 µg/mL / 10 nM):
Add 50 µL of 5 mg/mL AlphaLISA Anti-PAI-1 Acceptor beads and 50 µL of 500 nM Biotinylated Antibody Anti-PAI-1 to 2 400 µL of 1X AlphaLISA Immunoassay Buffer. Prepare just before use.

4) Preparation of 1.25X Streptavidin (SA) Donor beads (50 µg/mL): Keep the beads under subdued laboratory lighting.
Add 200 µL of 5 mg/mL SA-Donor beads to 19 800 µL of 1X AlphaLISA Immunoassay Buffer.

5) Samples: If applicable, dilute samples to be tested in diluent (e.g. 1X AlphaLISA Immunoassay Buffer, cell culture medium or FBS).

6) In a 96- or 384-well microplate:

Add 5 µL of each analyte standard dilution or 5 µL of sample

⇩

Add 5 µL of a 10X MIX (freshly prepared)
AlphaLISA Anti-Analyte Acceptor beads (10 µg/mL final) and Biotinylated Antibody Anti-Analyte (1 nM final)

⇩

Incubate 60 minutes at 23˚C

⇩

Add 40 µL of 1.25X SA-Donor beads (40 µg/mL final)

⇩

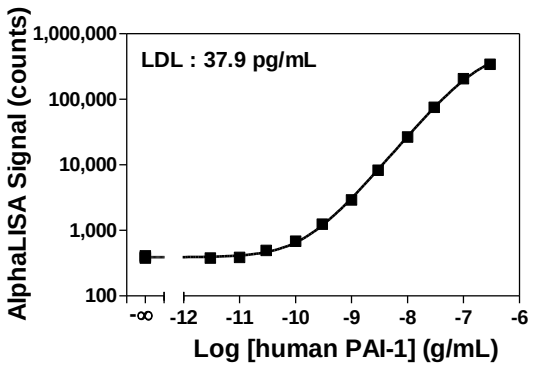
Incubate 30 minutes at 23˚C in the dark

⇩

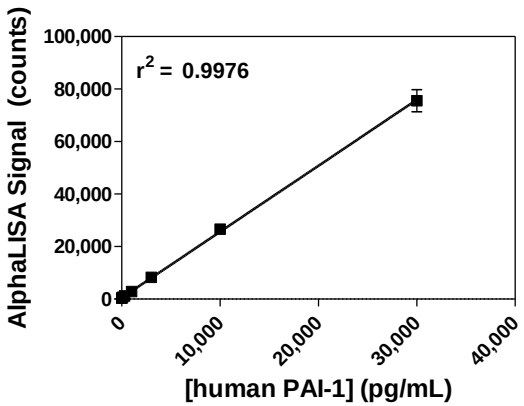
Read using EnSpire or EnVision-Alpha Reader

Manual 1 - Typical results in 1X AlphaLISA Immunoassay Buffer

Log-Log scale:



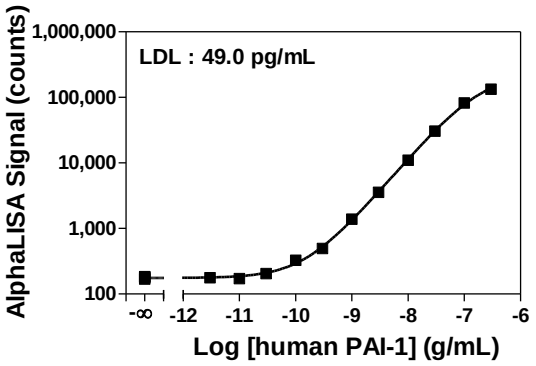
Linear-Linear scale (linear range):



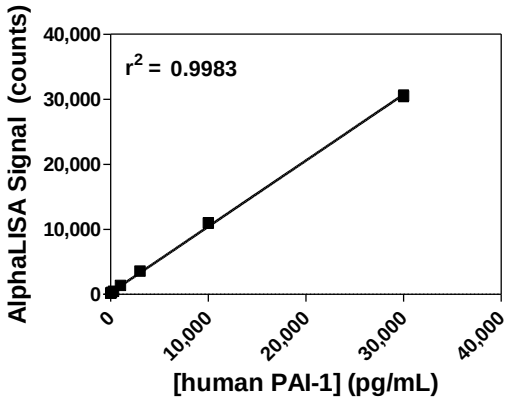
The data was generated using a white Optiplate-384 microplate and an EnVision-Alpha Reader 2102.

Manual 1 - Typical results in FBS

Log-Log scale:



Linear-Linear scale (linear range):



The data was generated using a white Optiplate-384 microplate and an EnVision-Alpha Reader 2102.

Manual 2: High Sensitivity Manual (3 Incubation Steps)

The manual described below is for one standard curve (48 wells) and samples (452 wells). Dilution of standards can be done in 1X AlphaLISA Immunoassay Buffer, cell culture medium or FBS.

If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

3) Preparation of 5X AlphaLISA Anti-PAI-1 Acceptor beads (50 µg/mL):
Add 50 µL of 5 mg/mL AlphaLISA Anti-PAI-1 Acceptor beads to 4 950 µL of 1X AlphaLISA Immunoassay Buffer.

4) Preparation of 5X Biotinylated Antibody Anti-PAI-1 (5 nM):
Add 50 µL of 500 nM Biotinylated Antibody Anti-PAI-1 to 4 950 µL of 1X AlphaLISA Immunoassay Buffer.

5) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL): Keep the beads under subdued laboratory lighting.
Add 200 µL of 5 mg/mL SA-Donor beads to 12 300 µL of 1X AlphaLISA Immunoassay Buffer.

6) Samples: If applicable, dilute samples to be tested in diluent (e.g. 1X AlphaLISA Immunoassay Buffer, cell culture medium or FBS).

7) In a 96- or 384-well microplate:

Add 5 µL of each analyte standard dilution or 5 µL of sample

⇩

Add 10 µL of 5X Anti-Analyte Acceptor beads (10 µg/mL final)

⇩

Incubate 30 minutes at 23˚C

⇩

Add 10 µL of 5X Biotinylated Antibody Anti-analyte (1 nM final)

⇩

Incubate 60 minutes at 23˚C

⇩

Add 25 µL of 2X SA-Donor beads (40 µg/mL final)

⇩

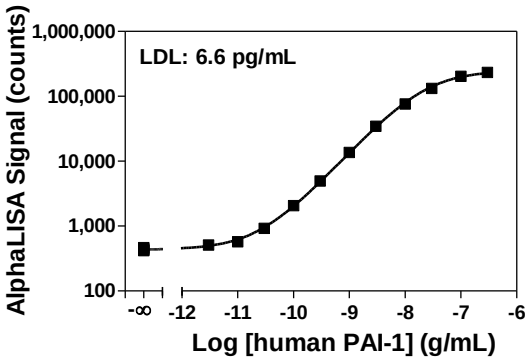
Incubate 30 minutes at 23˚C in the dark

⇩

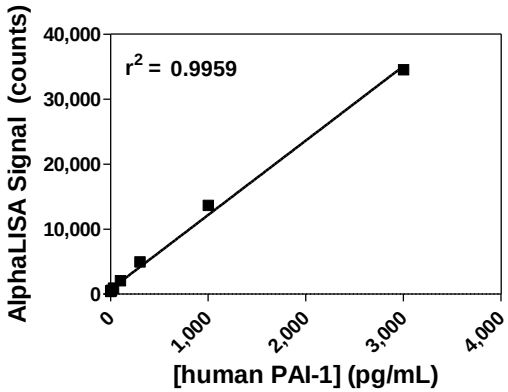
Read using EnSpire or EnVision-Alpha Reader

Manual 2 - Typical results in 1X AlphaLISA Immunoassay Buffer

Log-Log scale:



Linear-Linear scale (linear range):



The data was generated using a white Optiplate-384 microplate and an EnVision-Alpha Reader 2102.

Manuals 1 & 2 - Interpreting the Data

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Assay Performance Characteristics

AlphaLISA assay performance described below was determined using the Quick manual.

Sensitivity:

The LDL was calculated as described above. This value corresponds to the lowest concentration of analyte that can be detected in a volume of 5 µL using the recommended assay conditions.

- Average LDL is 39.5 pg/mL * (using 5 µL of analyte in AlphaLISA Immunoassay Buffer) (mean of 12 independent experiments).
- Average LDL is 53.0 pg/mL (using 5 µL of analyte in FBS) (mean of 4 independent experiments).

* Note that LDL can be decreased (i.e. sensitivity increased) by increasing the volume of analyte in the assay (e.g. use 10 µL of analyte in a final assay volume of 50 µL).

Dynamic range: 39.5 – 300 000 pg/mL (in AlphaLISA Immunoassay Buffer)

Assay precision:

The following assay precision data were calculated from a total of 12 assays. Two operators performed three independent assays using two different kit lots. Each assay consisted of one standard curve and three control samples of high (A), medium (B) and low (C) concentration, assayed in triplicate. The assays were performed in 384-well format using AlphaLISA Immunoassay Buffer.

- Intra-assay precision:

The intra-assay precision was determined using a total of 12 independent determinations in triplicate for each control sample.

Sample	Mean (pg/mL)	SD (pg/mL)	% CV (n = 12)
A	32 463	1 773	5.5
B	2 928	202	6.9
C	319	19.0	6.0

- Inter-assay precision:

The inter-assay precision was determined using a total of 4 independent determinations with 9 measurements for each control sample.

Sample	Mean (pg/mL)	SD (pg/mL)	% CV (n = 6)
A	32 464	1 686	5.2
B	2 928	198	6.8
C	319	23.3	7.3

Human serum and plasma experiments:

In the following experiments, FBS was used as diluent in both the standard curve and dilution of samples. Additionally, all human serum and plasma samples tested were pre-diluted 2-fold with the diluent before being processed. Both EDTA and citrate plasma have been tested for the measurements of PAI-1, with similar results.

- Dilutional linearity:

The dilutional linearity was determined by serial dilutions of a pool of human sera or plasma. The recovery was calculated using the 2-fold diluted sample as the 100% value. The average recovery from two independent measurements is reported.

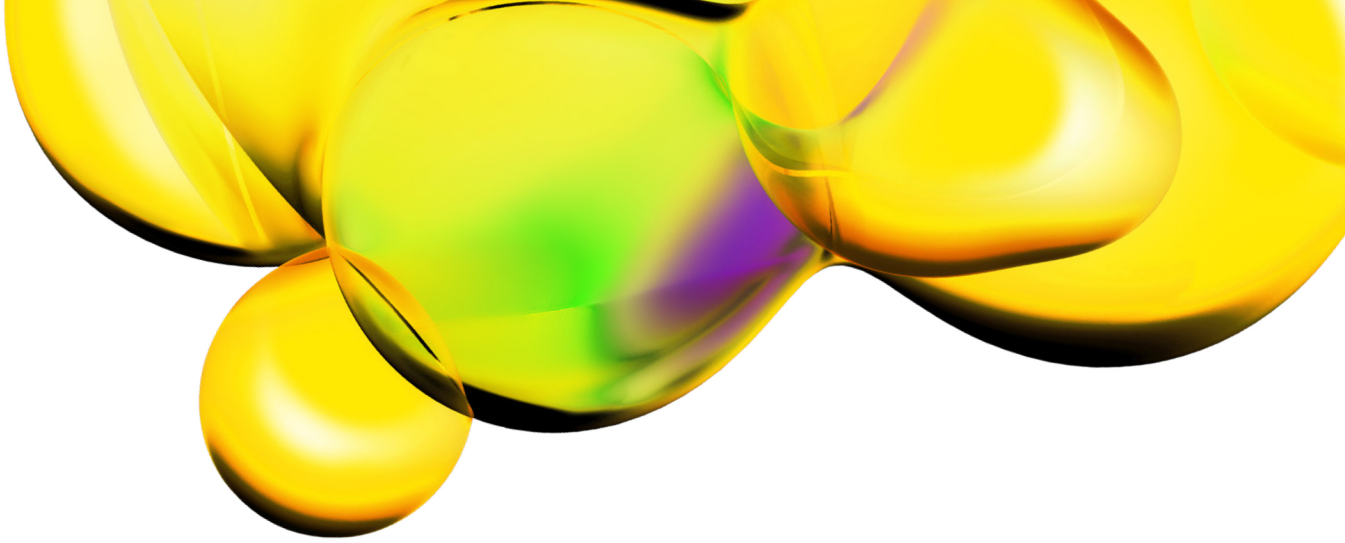
	% Recovery	
Dilution Factor	Serum	Plasma
2	100	100
4	110	76
8	100	88
16	85	84
32	91	81

Specificity:

Cross-reactivity of the AlphaLISA PAI-1 Kit was tested using the following proteins at 0.3 µg/mL in AlphaLISA Immunoassay Buffer.

Protein	% Cross-reactivity	LDL (pg/mL)
Mouse PAI-1	4	135
Rat PAI-1	4	317
Human PAI-1 tPA complex	80	34
Human PAI-1 uPA complex	61	53

The possible interference from human vitronectin was investigated. The human PAI-1 was kept at a constant concentration (EC_{50} value of the standard curve). The binding protein was titrated into the assay. No interference was observed up to 10 µg/mL, which is the maximum concentration tested.



www.revvity.com

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com