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ab282311 Human Tissue Plasminogen Activator CatchPoint[®] SimpleStep ELISA[®] Kit

For the quantitative measurement of Tissue Plasminogen Activator in human serum, plasma, milk, urine, saliva and cell culture supernatant.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Tissue Plasminogen Activator *in vitro* CatchPoint SimpleStep ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Tissue Plasminogen Activator protein in human serum, plasma, milk, urine, saliva and cell culture supernatant.

The CatchPoint SimpleStep ELISA employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. CatchPoint HRP Development Solution containing the Stoplight Red Substrate is added. During incubation, the substrate is catalyzed by HRP generating a fluorescent product. Signal is generated proportionally to the amount of bound analyte and the intensity is measured in a fluorescence plate reader at 530/570/590 nm Excitation/Cutoff/Emission.

Tissue Plasminogen Activator is a circulating serine protease involved in the breakdown of clots. Tissue Plasminogen Activator converts inactive plasminogen to active plasmin; in turn plasmin degrades the fibrin matrix in clots. In addition, plasmin can cleave Tissue Plasminogen Activator at Arg310 resulting in two chains (A and B) of Tissue Plasminogen Activator that are linked by disulfide bond. The two chains structure of Tissue Plasminogen Activator has even greater proteolytic activity. Tissue Plasminogen Activator is synthesized in many tissues and is secreted into most extracellular body fluids. Recombinant Tissue Plasminogen Activator is used medically to resolve or prevent blood clots in ischemic stroke or myocardial infarction.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed



Add 50 μ L standard or sample to appropriate wells



Add 50 μ L Antibody Cocktail to all wells



Incubate at room temperature for 1 hour



Aspirate and wash each well three times with 350 μ L 1X Wash Buffer
PT



Add 100 μ L of prepared CatchPoint HRP Development Solution to
each well and incubate for 10 minutes



Read fluorescence at Ex/Cutoff/Em 530/570/590 nm

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
- All data, except Typical Standard Curve and Sensitivity were collected using the colorimetric version of this kit (ab282306).

6. Materials Supplied

Item	Quantity	Storage Condition
Human Tissue Plasminogen Activator Capture Antibody 10X	600 µL	+4°C
Human Tissue Plasminogen Activator Detector Antibody 10X	600 µL	+4°C
Human Tissue Plasminogen Activator Lyophilized Recombinant Protein	2 Vials	+4°C
Antibody Diluent 5BI	6 mL	+4°C
Wash Buffer PT 10X	20 mL	+4°C
Stoptlight Red Substrate Buffer	12 mL	+4°C
100X Stoptlight Red Substrate	120 µL	+4°C
500X Hydrogen Peroxide (H ₂ O ₂ , 3%)	50 µL	+4°C
Sample Diluent NS	50 mL	+4°C
SimpleStep Pre-Coated Black 96-Well Microplate	96 Wells	+4°C
Plate Seal	1	+4°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Fluorescence microplate reader Ex/Cutoff/Em 530/570/590 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

8. Technical Hints

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.

- The incubation times provided in this protocol were optimized for fastest results with good signal. It is possible to increase the signal with longer incubation times, further optimization might be necessary.
- Keep in mind any RFU values shown are relative, NOT absolute. RFU from one plate reader are not comparable to another, especially if different make or model.
- To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.
- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

9.1 1X Wash Buffer PT:

Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

9.2 Antibody Cocktail:

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 5BI. To make 3 mL of the Antibody Cocktail combine 300 μ L 10X Capture Antibody and 300 μ L 10X Detector Antibody with 2.4 mL Antibody Diluent 5BI. Mix thoroughly and gently.

9.3 CatchPoint HRP Development Solution

Just prior to use prepare CatchPoint HRP Development Solution by diluting the 100X Stoplight Red Substrate and the 500X Hydrogen Peroxide in Stoplight Red Substrate Buffer.

For example, to make 6 mL of the CatchPoint HRP Development Solution combine 60 μ L 100X Stoplight Red Substrate and 12 μ L of 500X Hydrogen Peroxide with 5.928 mL Stoplight Red Substrate Buffer. Mix thoroughly and gently.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 IMPORTANT: If the protein standard vial has a volume identified on the label, reconstitute the Tissue Plasminogen Activator by adding that volume of Sample Diluent NS indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the Tissue Plasminogen Activator standard by adding 500 μL Sample Diluent NS. Hold at room temperature for 10 minutes and mix gently. This is the 35,200 ng/mL **Stock Standard** Solution.

10.2 Label 11 tubes, Standards 1– 11.

10.3 Add 222 μL Sample Diluent NS into tube number 1 and 150 μL of Sample Diluent NS into numbers 2-11.

10.4 Use the Stock Standard to prepare the following dilution series. Standard #11 contains no protein and is the Blank control.

Standards will be added to the plate in step 13.3. If desired all 11 standards can be used for a full standard curve.

Alternatively, to commit fewer wells to the standard curve, select a subset of at least 7 standards plus the blank control. If 7 standards are desired, we recommend standards #3-9.

Standard #	Dilution Sample	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	Stock	185	222	35,200	16,000
2	Standard#1	150	150	16,000	8,000
3	Standard#2	150	150	8,000	4,000
4	Standard#3	150	150	4,000	2,000
5	Standard#4	150	150	2,000	1,000
6	Standard#5	150	150	1,000	500
7	Standard#6	150	150	500	250
8	Standard#7	150	150	250	125
9	Standard#8	150	150	125	62.5
10	Standard#9	150	150	62.5	31.25
11	None	0	150	0.0	0.0

11. Sample Preparation

Typical Sample Dynamic Range	
Sample Type	Range
Serum	3 - 50%
Plasma - Citrate	1.6 - 25%
Plasma - EDTA	3 - 50%
Plasma - Heparin	1.6 - 25%
Milk	3 - 25%
Urine*	≤50%
Saliva*	≤25%
HUVEC Cell Culture Supernatant	1:3,200 - 1:200

*Based on spiked sample

11.1 Plasma:

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute plasma (citrate and heparin) samples at least 1:4 into Sample Diluent NS and assay. Dilute plasma (EDTA) samples at least 1:2 into Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.2 Serum:

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples at least 1:2 into Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

11.3 Cell Culture Supernatants:

Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants and assay. Or dilute samples into Sample Diluent NS and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

11.4 Urine:

Centrifuge urine at 2,000 x g for 10 minutes to remove debris. Dilute samples at least 1:2 into Sample Diluent NS and assay. Store un-diluted urine samples at -20°C or below. Avoid repeated freeze-thaw cycles.

11.5 Saliva:

Centrifuge saliva at 800 x g for 10 minutes to remove debris. Collect supernatants. Dilute samples at least 1:4 into Sample Diluent NS and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

11.6 Milk:

De-fat milk samples as follows. Centrifuge milk samples at 500 x g for 15 minutes at 4°C and collect the aqueous fraction using syringe attached to needle. Centrifuge the aqueous fraction at 3,000 x g for 15 minutes at 4°C and collect the final aqueous fraction (de-fatted milk) using syringe attached to needle. Dilute the de-fatted milk samples at least 1:4 into Sample Diluent NS and assay. Store un-diluted de-fatted milk at -20°C or below. Avoid repeated freeze-thaw cycles.

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well fluorescence or "edge effects" have not been observed with this assay.
- Ensure plate and all materials are equilibrated to room temperature during use.
- Cover the plate with a plate seal during incubation steps.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
 - We recommend that you assay all standards, controls and samples in duplicate.
 - Prepare all reagents, working standards, and samples as directed in the previous sections.
- 13.1** Prepare all reagents, working standards, and samples as directed in the previous sections.
 - 13.2** Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.
 - 13.3** Add 50 µL of all sample or standard to appropriate wells.
 - 13.4** Add 50 µL of the Antibody Cocktail to each well.
 - 13.5** Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.
 - 13.6** Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.
 - 13.7** Add 100 µL of prepared CatchPoint HRP Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm. Further optimization of incubation time vs signal strength can be performed if needed.
 - 13.8** Record the fluorescence at Ex/Cutoff/Em 530/570/590 nm. If using a Molecular Devices' plate reader supported by SoftMax® Pro software, a preconfigured protocol for these CatchPoint SimpleStep ELISA Kits is available with all the protocol and analysis settings at www.softmaxpro.org.

Mode:	Fluorescence
Instrument settings:	Endpoint
Excitation:	530 nm
Cutoff:	570 nm
Emission:	590 nm
Sensitivity:	6 flashes/read or 200ms
PMT:	Auto
Auto calibrate:	On
Read:	Top
Read Height:	1*

*For microplate readers with Pre-Read Optimization option, the Read Height as well as Microplate Optimization is recommended before the first read.

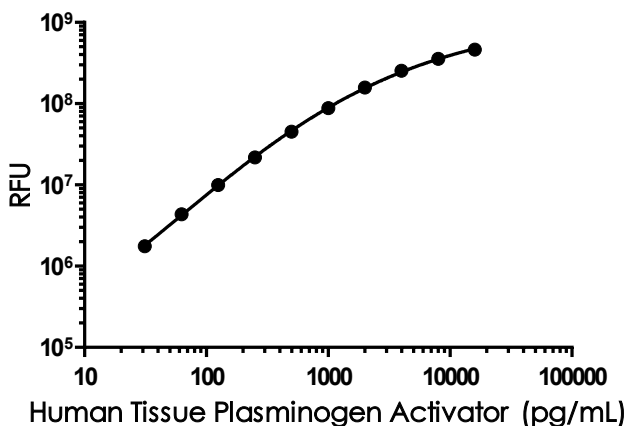
13.9 Analyze the data as described below.

14. Calculations

- 14.1 Preconfigured protocols are available when using SoftMax Pro software from Molecular Devices
 - 14.2 Calculate the average fluorescence value for the blank control (zero) standards. Subtract the average blank control standard fluorescence value from all other fluorescence values.
 - 14.3 **Create a standard curve** by plotting the average blank control subtracted fluorescence value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.
- Δ **Note:** Most **fluorescence** reader software or graphing software will plot these values and fit a curve to the data. A four-parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4-parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.
- 14.4 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted fluorescence **values against the standard curve**. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.
 - 14.5 Samples generating fluorescence values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at fluorescence values less than that of the lowest standard should be retested in a less dilute form.

15. Typical Data

Typical standard curve – data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Concentration (pg/mL)	RFU		Mean RFU
	1	2	
0.0	529,476	529,508	529,492
31.25	2,246,955	2,317,175	2,282,065
62.5	4,861,436	4,912,680	4,887,058
125	10,305,767	10,709,681	10,507,724
250	22,444,478	22,288,956	22,366,717
500	44,346,192	46,783,444	45,564,818
1,000	86,395,800	90,337,656	88,366,728
2,000	159,307,088	157,362,240	158,334,664
4,000	251,142,960	258,009,968	254,576,464
8,000	356,845,472	354,606,688	355,726,080
16,000	465,052,928	462,476,032	463,764,480

Figure 1. Example of human Tissue Plasminogen Activator standard curve in Sample Diluent NS. The Tissue Plasminogen Activator standard curve was prepared as described in Section 10. Raw data generated on SpectraMax M4 Multi-Mode Microplate Reader is shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Typical Sample Values

SENSITIVITY –

The calculated minimal detectable dose (MDD) is 13 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=22) and adding 2 standard deviations then extrapolating the corresponding concentration.

RECOVERY –

Three concentrations of Tissue Plasminogen Activator recombinant protein were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
20% Serum	97	92 - 101
20% Plasma - Citrate	96	91 - 103
20% Plasma - EDTA	88	81 - 93
20% Plasma - Heparin	93	90 - 95
10% Milk	82	81 - 84
50% Urine	84	82 - 86
25% Saliva	84	81 - 87
0.25% HUVEC Cell Culture Supernatant	106	93 - 113
100% Cell Culture Media*	96	94 - 98

*Media is RPMI 1640 containing 10% fetal bovine serum.

Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native Tissue Plasminogen Activator was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	50% Human Serum	25% Human Plasma (Citrate)	50% Human Plasma (EDTA)	25% Human Plasma (Heparin)
Undiluted	pg/mL	1,028	856.8	758.0	651.0
	% Expected value	100	100	100	100
2	pg/mL	556.5	484.2	440.3	370.0
	% Expected value	108	113	116	114
4	pg/mL	290.4	236.9	227.0	191.6
	% Expected value	113	111	120	118
8	pg/mL	149.5	122.3	115.1	96.32
	% Expected value	116	114	121	118
16	pg/mL	71.63	57.45	50.73	42.27
	% Expected value	111	107	107	104

Dilution Factor	Interpolated value	25% Human Milk	0.5% Human HUVEC Sup.
Undiluted	pg/mL	239.4	911.5
	% Expected value	100	100
2	pg/mL	143.4	413.3
	% Expected value	120	91
4	pg/mL	67.41	197.6
	% Expected value	113	87
8	pg/mL	33.43	96.9
	% Expected value	112	85
16	pg/mL	ND	46.6
	% Expected value	ND	82

ND – Not Determined, interpolated value below standard 7th point

Recombinant Tissue Plasminogen Activator was spiked into the following biological samples and diluted in a 2-fold dilution series in Sample Diluent NS.

Dilution Factor	Interpolated value	50% Human Urine	25% Human Saliva
Undiluted	pg/mL	602.3	507.4
	% Expected value	100	100
2	pg/mL	322.0	271.7
	% Expected value	107	107
4	pg/mL	159.5	140.6
	% Expected value	106	111
8	pg/mL	81.79	69.07
	% Expected value	109	109
16	pg/mL	36.01	35.80
	% Expected value	96	113

50% pooled urine and 25% pooled saliva samples from healthy donors was measured in duplicate. All values were below the detectable range of the assay.

PRECISION –

Mean coefficient of variations of interpolated values of Tissue Plasminogen Activator from two concentrations of human serum within the working range of the assay.

	Intra-Assay	Inter-Assay
n =	8	3
CV(%)	3.2	2.4

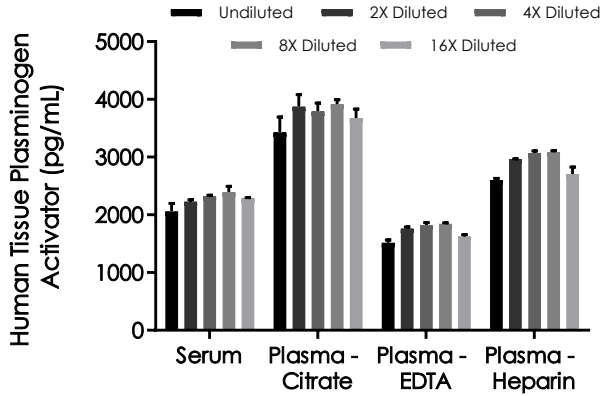


Figure 2. Interpolated concentrations of native Tissue Plasminogen Activator in human serum and plasma samples. The concentrations of Tissue Plasminogen Activator were measured in duplicates, interpolated from the Tissue Plasminogen Activator standard curves and corrected for sample dilution. Undiluted samples are as follows: serum 50%, plasma (citrate) 25%, plasma (EDTA) 50% and plasma (heparin) 25%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean Tissue Plasminogen Activator concentration was determined to be 2,258 pg/mL in neat serum, 3,736 pg/mL in neat plasma (citrate) 1,712 pg/mL in neat plasma (EDTA) and 2,883 pg/mL in neat plasma (heparin).

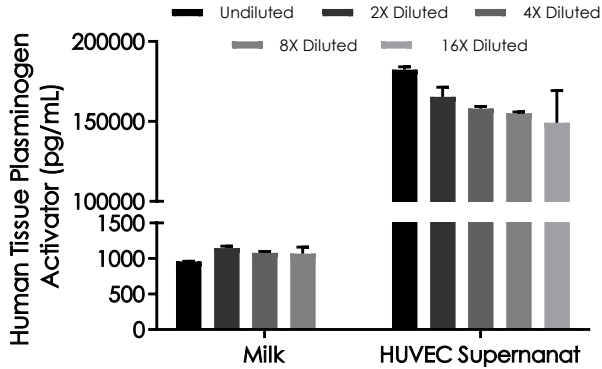


Figure 3. Interpolated concentrations of native Tissue Plasminogen Activator in human milk and HUVEC cell culture supernatant samples. The concentrations of Tissue Plasminogen Activator were measured in duplicates, interpolated from the Tissue Plasminogen Activator standard curves and corrected for sample dilution. Undiluted samples are as follows: milk 25%, and HUVEC supernatant 0.5%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean Tissue Plasminogen Activator concentration was determined to be 1,063 pg/mL in neat milk and 161,985 pg/mL in neat HUVEC supernatant.

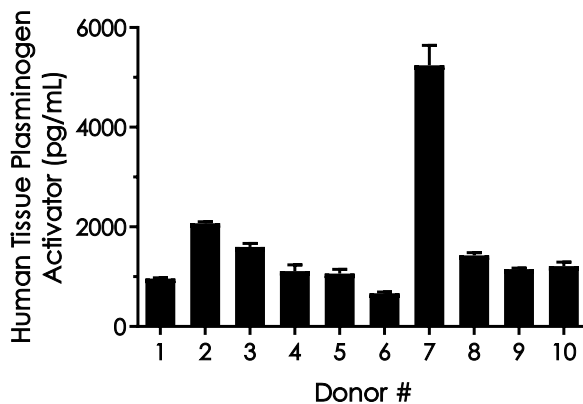


Figure 4. Serum from ten individual healthy human female donors was measured in duplicate. Interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean Tissue Plasminogen Activator concentration was determined to be 1,651 pg/mL with a range of 669 – 5,244 pg/mL.

17. Assay Specificity

This kit recognizes both native and recombinant human Tissue Plasminogen Activator protein in in serum, plasma (citrate, EDTA and heparin), cell culture supernatant, urine, milk and saliva samples only.

Cerebrospinal fluid, cell and tissue extract samples have not been tested with this kit.

CROSS REACTIVITY

2 ng/mL of recombinant human TPA-PAI1 complex protein and 2 ng/mL of recombinant human PAI1 protein were tested for cross reactivity. 66% cross reactivity with human TPA-PAI1 complex protein was observed. No cross reactivity with human PAI protein was observed.

INTERFERENCE

1 ng/mL and 50 ng/mL of recombinant human PAI1 protein was tested for interference with 1 ng/mL of recombinant human Tissue Plasminogen Activator protein. No interference was observed.

1 ng/mL of recombinant human TPA-PAI1 complex protein increased interpolated value of 1 ng/mL of recombinant human Tissue Plasminogen Activator protein 1.55-fold (55%).

18. Species Reactivity

This kit recognizes human Tissue Plasminogen Activator protein.

2.2 ng/mL of recombinant mouse Tissue Plasminogen Activator protein was tested for reactivity. No reactivity was observed.

Other species reactivity was determined by measuring 50% serum samples of various species, interpolating the protein concentrations from the human standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in human serum assayed at the same dilution

Species	% Reactivity
Rhesus Macaque Monkey	32

Reactivity < 3% was determined for the following species:

- Mouse
- Rat
- Cow

Other species reactivity not determined.

Please contact our Technical Support team for more information.

19. Troubleshooting

Problem	Reason	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with CatchPoint HRP Development Solution too brief	Read plate again after longer incubation time
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep Stoplight Red Substrate protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

20. Notes

Technical Support

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