

# Human t-PA ELISA Kit

Enzyme-linked Immunosorbent Assay for quantitative detection of human t-PA

**Catalog Numbers** BMS258-2 and BMS258-2TEN

**Pub. No.** MAN0016632 **Rev.** A.0 (30)

**WARNING!** Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from [thermofisher.com/support](http://thermofisher.com/support).

## Product description

The Human t-PA ELISA Kit is an enzyme-linked immunosorbent assay for the quantitative detection of human t-PA.

## Summary

Tissue-type plasminogen activator (t-PA) is a serine protease which occurs in blood plasma, serum, other body fluids, tissues and conditioned media of certain cultured cells. It can convert the inactive proenzyme plasminogen to the active protease plasmin. Plasmin can degrade fibrin, the matrix of a blood clot in a process known as fibrinolysis, leading to dissolution of the clot. Furthermore plasminogen activation is implicated in metastatic spread of malignant cells and in tissue remodeling.

Fibrin has been shown to accelerate the conversion of plasminogen to plasmin which is mediated by t-PA. Through this pathway fibrin promotes its own degradation. Inhibitors to t-PA have been found in blood preparations, cell culture media and tissues. These plasminogen activator inhibitors -1 and -2 (PAI-1, PAI-2) react extremely rapidly with t-PA, forming inactive complexes. The availability of free active t-PA is regulated through this interaction.

A correlation between low serum levels of t-PA activity and thrombotic tendency has been described.

An impaired release of t-PA from the endothelium in Graves' disease with significantly lowered basal plasma t-PA levels was described. The clinical evaluation of t-PA levels in patients with liver diseases revealed a change of the t-PA levels in the clinical causes of these pathologies with increased t-PA levels with progression of the liver disease.

Elevated levels of t-PA in serum were shown to occur in relation to retinopathy in type 1 diabetes mellitus.

The major field of clinical interest is the field of diseases of the heart. The t-PA plasma levels have been shown to be altered with the presence of transplant coronary artery disease in cardiac transplant recipients.

t-PA is described as a factor correlating to the risk of development of cardiovascular disease as was shown for controls and long-term dialysis patients.

Changes of t-PA levels were shown in myocardial infarction. In stroke patients, high t-PA antigen concentrations indicate an activation of the fibrinolytic system or a complex formation with the inhibitors.

For literature update refer to our website.

## Principles of the test

An anti-human t-PA coating antibody is adsorbed onto microwells.

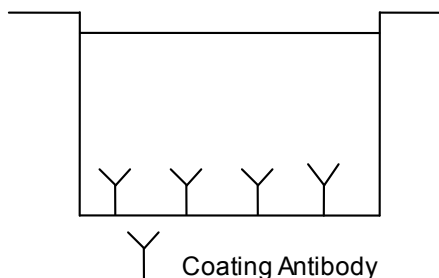


Fig. 1 Coated microwell

Human t-PA present in the sample or standard binds to antibodies adsorbed to the microwells. A HRP-conjugated anti-human t-PA antibody is added and binds to human t-PA captured by the first antibody.

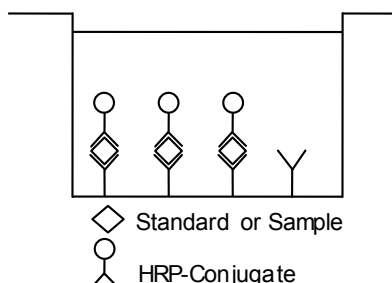


Fig. 2 First incubation

Following incubation unbound HRP-conjugated anti-human t-PA is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

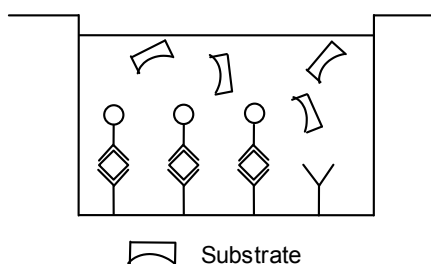


Fig. 3 Second incubation

A colored product is formed in proportion to the amount of human t-PA present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human t-PA standard dilutions and human t-PA concentration determined.

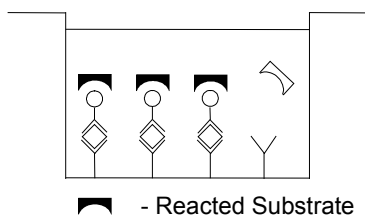


Fig. 4 Stop reaction

## Reagents provided

### Reagents for human t-PA ELISA BMS258/2 (96 tests)

- 1 aluminum pouch with a Microwell Plate (12 strips of 8 wells each) coated with polyclonal antibody to human t-PA
- 1 vial (70  $\mu$ L) HRP-Conjugate anti-human t-PA polyclonal antibody
- 2 vials human t-PA Standard lyophilized, 2 ng/mL upon reconstitution
- 1 vial (12 mL) Sample Diluent
- 1 vial (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween<sup>™</sup> 20, 10% BSA)
- 1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween<sup>™</sup> 20)
- 1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)
- 1 vial (15 mL) Stop Solution (1M Phosphoric acid)
- 2 Adhesive Films

### Reagents for human t-PA ELISA BMS258/2TEN (10x96 tests)

- 10 aluminum pouches with a Microwell Plate (12 strips of 8 wells each) coated with polyclonal antibody to human t-PA
- 10 vials (70  $\mu$ L) HRP-Conjugate anti-human t-PA polyclonal antibody
- 10 vials human t-PA Standard lyophilized, 2 ng/mL upon reconstitution
- 10 vials (12 mL) Sample Diluent
- 1 vial (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween<sup>™</sup> 20, 10% BSA)
- 4 bottles (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween<sup>™</sup> 20)
- 10 vials (15 mL) Substrate Solution (tetramethyl-benzidine)
- 1 vial (100 mL) Stop Solution (1M Phosphoric acid)
- 10 Adhesive Films

## Storage instructions – ELISA kit

Store kit reagents between 2°C and 8°C. Immediately after use remaining reagents should be returned to cold storage (2°C to 8°C). Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

## Sample collection and storage instructions

Cell culture supernatant and serum were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic samples.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human t-PA. If samples are to be run within 24 hours, they may be stored at 2°C to 8°C (for sample stability refer to "Sample stability" on page 6).

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

## Materials required but not provided

- 5 mL and 10 mL graduated pipettes
- 5  $\mu$ L to 1000  $\mu$ L adjustable single channel micropipettes with disposable tips
- 50  $\mu$ L to 300  $\mu$ L adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

## Precautions for use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses, and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipet by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or samples.
- Rubber or disposable latex gloves should be worn while handling kit reagents or samples.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- To avoid microbial contamination or cross-contamination of reagents or samples that may invalidate the test, use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose samples and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

## Preparation of reagents

1. Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.
2. If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

## Wash buffer (1x)

1. Pour entire contents (50 mL) of the Wash Buffer Concentrate (20x) into a clean 1000 mL graduated cylinder. Bring to final volume of 1000 mL with glass-distilled or deionized water.
2. Mix gently to avoid foaming.
3. Transfer to a clean wash bottle and store at 2°C to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.
4. Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

## Assay buffer (1x)

1. Pour the entire contents (5 mL) of the Assay Buffer Concentrate (20x) into a clean 100 mL graduated cylinder. Bring to final volume of 100 mL with distilled water. Mix gently to avoid foaming.
2. Store at 2°C to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.
3. Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

## HRP-Conjugate

**Note:** The HRP-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated HRP-Conjugate solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	HRP-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

## Human t-PA standard

1. Reconstitute human t-PA standard by addition of distilled water.
2. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 2000 pg/mL).
3. Allow the reconstituted standard to sit for 10-30 minutes. Mix well prior to making dilutions.

The standard has to be used immediately after reconstitution and cannot be stored.

## External standard dilution

1. Label 7 tubes, one for each standard point: S1, S2, S3, S4, S5, S6, S7.
2. Prepare 1:2 serial dilutions for the standard curve as follows: Pipette 225 µL of Sample Diluent into each tube.
3. Pipette 225 µL of reconstituted standard (concentration = 2000 pg/mL) into the first tube, labeled S1, and mix (concentration of standard 1 = 1000 pg/mL).
4. Pipette 225 µL of this dilution into the second tube, labeled S2, and mix thoroughly before the next transfer.

5. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 5).

Sample Diluent serves as blank.

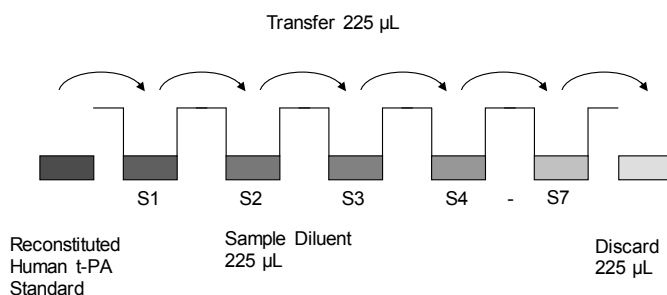


Fig. 5 Dilute standards - tubes

## Test protocol

1. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°C to 8°C sealed tightly.
2. Wash the microwell strips twice with approximately 400 µL Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration. Take care not to scratch the surface of the microwells.  
  
After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.
3. Standard dilution on the microwell plate (Alternatively the standard dilution can be prepared in tubes - see “External standard dilution” on page 3): Add 100 µL of Sample Diluent in duplicate to all standard wells. Pipette 100 µL of prepared standard (see Preparation of Standard “Human t-PA standard” on page 3, concentration = 2000 pg/mL) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 1000 pg/mL), and transfer 100 µL to wells B1 and B2, respectively (see Figure 6). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human t-PA standard dilutions ranging from 1000.0 to 15.6 pg/mL. Discard 100 µL of the contents from the last microwells (G1, G2) used.

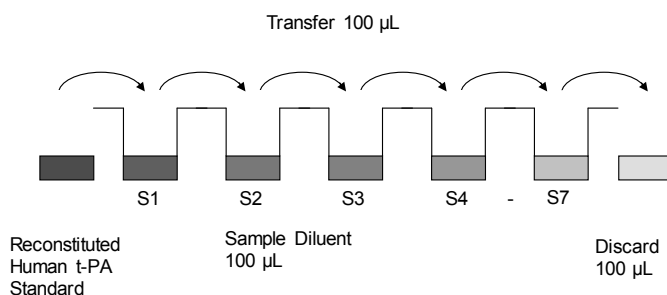


Fig. 6 Dilute standards - microwell plate

**Table 1** Example of the arrangement of blanks, standards and samples in the microwell strips.

	1	2	3	4
A	Standard 1 1000.0 pg/mL	Standard 1 1000.0 pg/mL	Sample 1	Sample 1
B	Standard 2 500.0 pg/mL	Standard 2 500.0 pg/mL	Sample 2	Sample 2
C	Standard 3 250.0 pg/mL	Standard 3 250.0 pg/mL	Sample 3	Sample 3
D	Standard 4 125.0 pg/mL	Standard 4 125.0 pg/mL	Sample 4	Sample 4
E	Standard 5 62.5 pg/mL	Standard 5 62.5 pg/mL	Sample 5	Sample 5
F	Standard 6 31.3 pg/mL	Standard 6 31.3 pg/mL	Sample 6	Sample 6
G	Standard 7 15.6 pg/mL	Standard 7 15.6 pg/mL	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

In case of an external standard dilution (see “External standard dilution” on page 3), pipette 100 µL of these standard dilutions (S1 - S7) in the standard wells according to Table 1.

- Add 100 µL of Sample Diluent in duplicate to the blank wells.
- Add 90 µL of Sample Diluent to the sample wells.
- Add 10 µL of each sample in duplicate to the sample wells.
- Prepare HRP-Conjugate (see Preparation of HRP-Conjugate “HRP-Conjugate” on page 3).
- Add 50 µL of HRP-Conjugate to all wells.
- Cover with an adhesive film and incubate at room temperature (18°C to 25°C) for 2 hours on a microplate shaker.
- Remove adhesive film and empty wells. Wash microwell strips 6 times according to point 2. of the test protocol. Proceed immediately to the next step.
- Pipette 100 µL of TMB Substrate Solution to all wells.
- Incubate the microwell strips at room temperature (18°C to 25°C) for about 10 minutes. Avoid direct exposure to intense light.

The color development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for color development has to be done individually for each assay.

It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

- Stop the enzyme reaction by quickly pipetting 100 µL of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2°C to 8°C in the dark.
- Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

**Note:** In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

## Calculation of results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20% of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human t-PA concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human t-PA for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human t-PA concentration.
- If instructions in this protocol have been followed, samples have been diluted 1:10 (10 µL sample + 90 µL Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 10).
- Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human t-PA levels. Such samples require further external predilution according to expected human t-PA values with Sample Diluent in order to precisely quantitate the actual human t-PA level.
- It is suggested that each testing facility establishes a control sample of known human t-PA concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 7.

**Note:** Do not use this standard curve to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

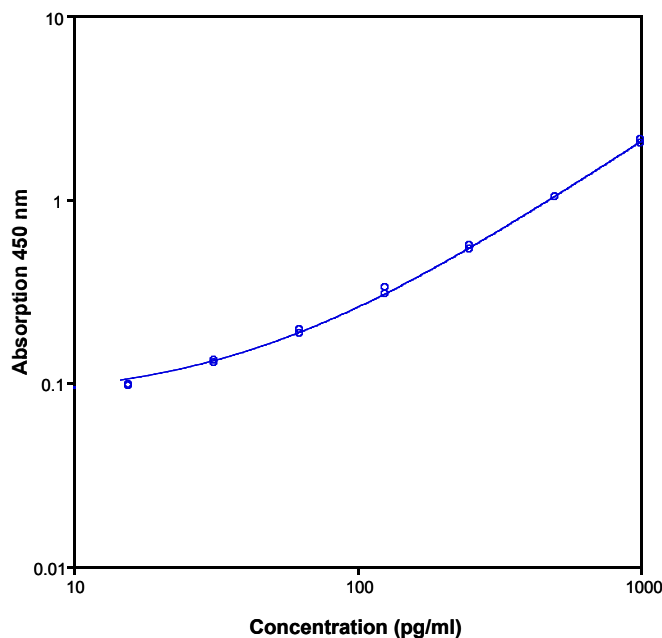


Fig. 7 Representative standard curve for human t-PA ELISA. Human t-PA was diluted in serial 2-fold steps in Sample Diluent.

**Table 2** Typical data using the human t-PA ELISA.

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human t-PA Concentration (pg/mL)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	1000.0	2.008 2.110	2.059	2.5
2	500.0	1.034 1.028	1.031	0.3
3	250.0	0.532 0.558	0.545	2.4
4	125.0	0.327 0.306	0.317	3.3
5	62.5	0.195 0.184	0.190	2.9
6	31.3	0.131 0.128	0.130	1.2
7	15.6	0.096 0.097	0.097	0.5
Blank	0.0	0.053 0.051	0.052	2.7

The OD values of the standard curve may vary according to the conditions of assay performance (e.g., operator, pipetting technique, washing technique, or temperature effects). Furthermore, shelf life of the kit may affect enzymatic activity and thus color intensity. Values measured are still valid.

### Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

### Performance characteristics

#### Sensitivity

The limit of detection of human t-PA defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 6 pg/mL (mean of 6 independent assays).

#### Reproducibility

##### Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human t-PA. Two standard curves were run on each plate. Data below show the mean human t-PA concentration and the coefficient of variation for each

sample (see Table 3). The calculated overall intra-assay coefficient of variation was 3.6%.

**Table 3** The mean human t-PA concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human t-PA Concentration (pg/mL)	Coefficient of Variation (%)
1	1	2231	5.0
	2	2134	3.0
	3	2345	3.0
2	1	1305	4.0
	2	1010	6.0
	3	1065	5.0
3	1	1830	6.0
	2	1999	2.0
	3	2051	6.0
4	1	1067	2.0
	2	1029	8.0
	3	1219	6.0
5	1	1158	4.0
	2	1054	3.0
	3	1206	2.0
6	1	4749	3.0
	2	4300	2.0
	3	4914	6.0
7	1	997	1.0
	2	939	2.0
	3	850	1.0
8	1	2113	1.0
	2	2463	5.0
	3	2211	2.0

#### Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human t-PA. Two standard curves were run on each plate. Data below show the mean human t-PA concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 6.5%.

**Table 4** The mean human t-PA concentration and the coefficient of variation of each sample

Sample	Mean Human t-PA Concentration (pg/mL)	Coefficient of Variation (%)
1	2236	3.9
2	1127	11.3
3	1960	4.8
4	1105	7.5
5	1139	5.6
6	4654	5.6
7	928	6.5
8	2262	6.5

#### Spike recovery

The spike recovery was evaluated by spiking 2 levels of human t-PA into different pooled normal human serum samples. Recoveries were determined in 4 independent experiments with 4 replicates each. The unspiked serum was used as blank in these experiments. The recovery ranged from 77–109% with an overall mean recovery of 98%.

## Dilution parallelism

Four serum samples with different levels of human t-PA were analyzed at serial 2-fold dilutions with 4 replicates each. The recovery ranged from 83–112% with an overall recovery of 100%.

Sample	Dilution	Human t-PA (pg/mL)		Recovery of expected concentration (%)
		Expected concentration	Observed concentration	
1	1:10	–	12,404	–
	1:20	6202	6404	103
	1:40	3101	3002	97
	1:80	1551	1429	92
2	1:10	–	9259	–
	1:20	4269	5053	109
	1:40	2314	2513	109
	1:80	1257	1,529	106
3	1:10	–	6370	–
	1:20	3185	3584	112
	1:40	1593	1634	103
	1:80	796	859	108
4	1:10	–	6717	–
	1:20	3358	2785	83
	1:40	1679	1532	91
	1:80	839	729	87

## Sample stability

### Freeze-Thaw stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the human t-PA levels determined. There was no significant loss of human t-PA immunoreactivity detected by freezing and thawing.

### Storage stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 2°C to 8°C, room temperature, and at 37°C, and the human t-PA level determined after 24 hours. There was no significant loss of human t-PA immunoreactivity detected during storage under above conditions.

## Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human t-PA positive serum. No cross-reactivity was detected, namely not with human PAI-1 (plasminogen activator inhibitor 1, maximum concentration tested 2000 pg/mL).

## Expected values

A panel of 7 serum samples from randomly selected apparently healthy donors (males and females) was tested for human t-PA. The detected human t-PA levels ranged between 500 and 5,500 pg/mL with a mean level of 2060 pg/mL.

## Reagent preparation summary

### Wash buffer (1x)

Add Wash Buffer Concentrate 20x (50 mL) to 950 mL distilled water.

Number of Strips	Wash Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

### Assay buffer (1x)

Add Assay Buffer Concentrate 20x (5 mL) to 95 mL distilled water.

Number of Strips	Assay Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

## HRP-Conjugate

Make a 1:100 dilution of HRP-Conjugate in Assay Buffer (1x):

Number of Strips	HRP-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

## Human t-PA standard

Reconstitute lyophilized human t-PA standard with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

## Test protocol summary

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Standard dilution on the microwell plate: Add 100 µL Sample Diluent, in duplicate, to all standard wells. Pipette 100 µL prepared standard into the first wells and create standard dilutions by transferring 100 µL from well to well. Discard 100 µL from the last wells.  
Alternatively external standard dilution in tubes (see “External standard dilution” on page 3): Pipette 100 µL of these standard dilutions in the microwell strips.
4. Add 100 µL Sample Diluent, in duplicate, to the blank wells.
5. Add 90 µL Sample Diluent to sample wells.
6. Add 10 µL sample in duplicate, to designated sample wells.
7. Prepare HRP-Conjugate.
8. Add 50 µL HRP-Conjugate to all wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18°C to 25°C).
10. Empty and wash microwell strips 6 times with Wash Buffer.
11. Add 100 µL of TMB Substrate Solution to all wells.
12. Incubate the microwell strips for about 10 minutes at room temperature (18°C to 25°C).
13. Add 100 µL Stop Solution to all wells.
14. Blank microwell reader and measure color intensity at 450 nm.

**Note:** If instructions in this protocol have been followed, samples have been diluted 1:10 (10 µL sample + 90 µL Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 10).

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**Note:** For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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