Human TIE-2 ELISA Kit

Enzyme-linked Immunosorbent Assay for quantitative detection of human TIE-2

Catalog Numbers BMS2042 and BMS2042TEN

Pub. No. MAN0016503 Rev. B.0 (31)

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.

Product description

The Human TIE-2 ELISA Kit is an enzyme-linked immunosorbent assay for the quantitative detection of human TIE-2.

Summary

The tyrosine kinase receptor TIE-2 was initially identified as a specific endothelial growth factor receptor that mediated several properties of endothelial cells under both physiologcal and pathological conditions. Angiopoietins are the natural ligands of TIE-2 and they induce TIE-2-depending signalling, including survival and apoptosis of endothelial cells, vascular permeability and regulation of capillary sprouting. In general, the outcome of tie receptor signalling depends on which vascular bed is involved, and crosstalk between different VEGFs has an important modulating effect on the properties of the ligands. TIE-2 plays an important role in several vascular diseases, known as vascular malformation (e.g., venous malformation). Independent of angiogenesis and its involvement in lymphangiogenesis, TIE-2 maintains a long-term, quiescent population of hematopoietc stems cells in the bone marrow. In a tumor model, a subset of monocytes was found to be positve for TIE-2, indicating an important function of TIE-2 in paracrine support of nascent blood vessels.

During cancer formation and spreading, TIE-2 was found to be overexpressed in tumor vessels. However, outside of the vascular compartment, TIE-2 is expressed in several types of cancer, including leukemia, and in gastric tumors, breast tumors, and gliomas. In gliomas, TIE-2 expression in neoplastic glial cells was significantly associated with progression from a lower to a higher grade where it seems to regulate glioma cell adhesion to the extracellular matrix. This, together with the reported fact that malignant gliomas express high levels of Ang1, suggest the existence of an autocrine loop for cell matrix interaction. The possible role of TIE-2 in tumors is currently under investigation. Because it is expressed in several cellular lineages inside the tumor, the receptor may be an attractive target for cancer therapy.

A natually occuring soluble TIE-2 receptor fragment of 75 kDa (sTIE-2) is generated by shedding. Soluble TIE-2 inhibits angiopoietin-mediated TIE-2 phosphorylation and anti-apoptosis. TIE-2 shedding is mediated by PI2K/Akt and p38 MAPK. Soluble TIE-2 was also measured in preterm infants, including the ROP syndrome (retinopathy of prematurity). Elevated plasma levels in active ROP patients were observed for TIE-2. In experimental tumor mouse models, TIE-2 results in tumor regression and prolonged tumor-free survival in 80% of the animals.

For literature update refer to our website.

Principles of the test

An anti-human TIE-2 coating antibody is adsorbed onto microwells.

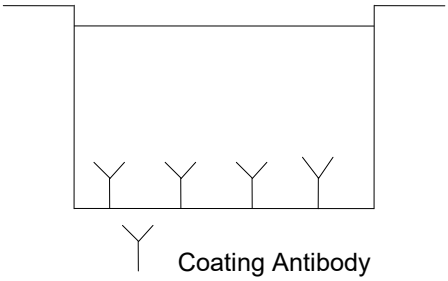

Fig. 1 Coated microwell

Human TIE-2 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human TIE-2 antibody is added and binds to human TIE-2 captured by the first antibody.

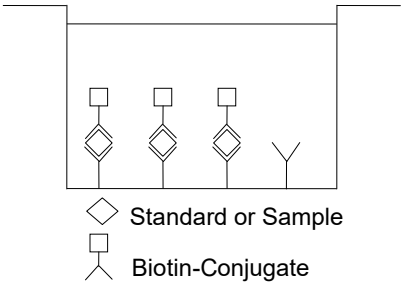

Fig. 2 First incubation

Following incubation, unbound biotinconjugated anti-human TIE-2 antibody is removed during a wash step. Streptavidin- HRP is added and binds to the biotin- conjugated anti-human TIE-2 antibody.

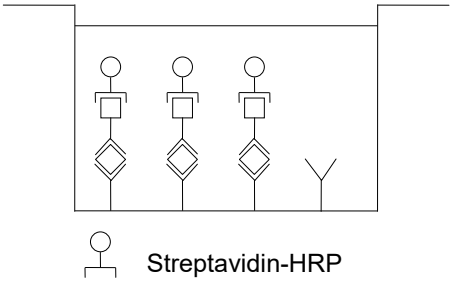

Fig. 3 Second incubation

Following incubation, unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

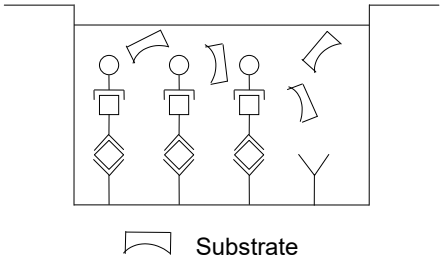

Fig. 4 Third incubation

A colored product is formed in proportion to the amount of human TIE-2 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 TIE-2 standard dilutions and human TIE-2 sample concentration determined.

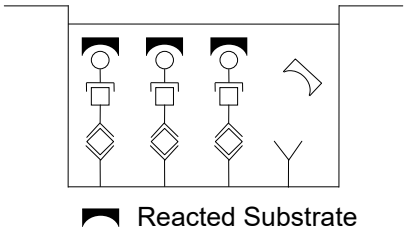

Fig. 5 Stop reaction

Reagents provided

Reagents for human TIE-2 ELISA BMS2042 (96 tests)

1 aluminum pouch with a Microwell Plate (12 strips with 8 wells each) coated with polyclonal antibody to human TIE-2

1 vial (70 µL) Biotin-Conjugate anti-human TIE-2 polyclonal antibody

1 vial (150 µL) Streptavidin-HRP

2 vials human TIE-2 Standard lyophilized, 20 ng/mL upon reconstitution

1 vial (12 mL) Sample Diluent

1 vial (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)

1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)

1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (15 mL) Stop Solution (1M Phosphoric acid)

4 Adhesive Films

Reagents for human TIE-2 ELISA BMS2042TEN (10x96 tests)

10 aluminum pouches with a Microwell Plate (12 strips with 8 wells each) coated with polyclonal antibody to human TIE-2

10 vials (70 µL) Biotin-Conjugate anti-human TIE-2 polyclonal antibody

10 vials (150 µL) Streptavidin-HRP

10 vials human TIE-2 Standard lyophilized, 20 ng/mL upon reconstitution

10 vials (12 mL) Sample Diluent

2 vials (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)

6 bottles (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)

10 vials (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (100 mL) Stop Solution (1M Phosphoric acid)

20 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, serum, and plasma (EDTA, citrate, heparin), were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

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 TIE-2. 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 µL to 1000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL 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. The 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. 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

Biotin-Conjugate

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

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

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

Streptavidin-HRP

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

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

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

Human TIE-2 standard

1. Reconstitute human TIE-2 standard by addition of distilled water. 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 = 20 ng/mL).
2. Allow the standard to reconstitute 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: Pipet 225 µL of Sample Diluent into each tube.
3. Pipet 225 µL of reconstituted standard (concentration of standard = 20 ng/mL) into the first tube, labeled S1, and mix (concentration of standard 1 = 10 ng/mL).
4. Pipet 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 6).

   Sample Diluent serves as blank.

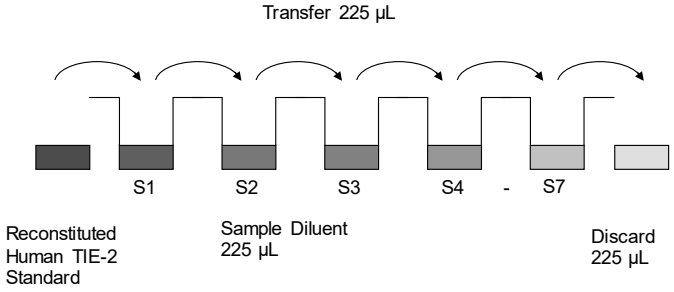

Fig. 6 Dilute standards - tubes

Test protocol

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

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. Pipet 100 µL of prepared standard (see “Human TIE-2 standard” on page 3, concentration = 20.00 ng/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 = 10.00 ng/mL), and transfer 100 µL to wells B1 and B2, respectively (see Figure 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human TIE-2 standard dilutions ranging from 10.00–0.16 ng/mL. Discard 100 µL of the contents from the last microwells (G1, G2) used.

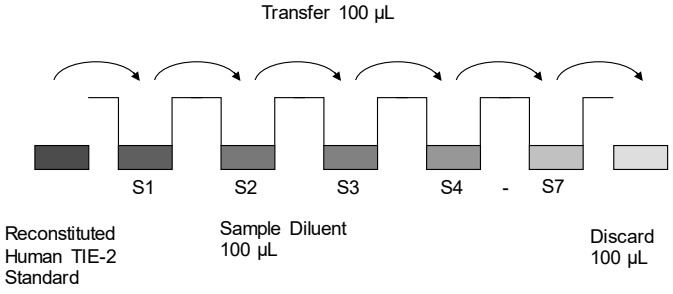

Fig. 7 Dilute standards - microwell plate.

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

	1	2	3	4
A	Standard 1 10.00 ng/mL	Standard 1 10.00 ng/mL	Sample 1	Sample 1
B	Standard 2 5.00 ng/mL	Standard 2 5.00 ng/mL	Sample 2	Sample 2
C	Standard 3 2.50 ng/mL	Standard 3 2.50 ng/mL	Sample 3	Sample 3
D	Standard 4 1.25 ng/mL	Standard 4 1.25 ng/mL	Sample 4	Sample 4
E	Standard 5 0.63 ng/mL	Standard 5 0.63 ng/mL	Sample 5	Sample 5
F	Standard 6 0.31 ng/mL	Standard 6 0.31 ng/mL	Sample 6	Sample 6
G	Standard 7 0.16ng/mL	Standard 7 0.16 ng/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), pipet 100 µL of these standard dilutions (S1–S7) in the standard wells according to Table 1.
4. Add 100 µL of Sample Diluent in duplicate to the blank wells.
5. Add 90 µL of Sample Diluent to the sample wells.
6. Add 10 µL of each sample in duplicate to the sample wells.
7. Prepare Biotin-Conjugate (see “Biotin-Conjugate” on page 3).
8. Add 50 µL of Biotin-Conjugate to all wells.
9. Cover with an adhesive film and incubate over night at 4°C.
10. Prepare Streptavidin-HRP (see “Streptavidin-HRP” on page 3).
11. Remove adhesive film and empty wells. Wash microwell strips 6 times according to step 2. Proceed immediately to the next step.
12. Add 100 µL of diluted Streptavidin-HRP to all wells, including the blank wells.
13. Cover with an adhesive film and incubate at room temperature (18°C to 25°C) for 1 hour.
14. Remove adhesive film and empty wells. Wash microwell strips 6 times according to step 2. Proceed immediately to the next step.
15. Pipet 100 µL of TMB Substrate Solution to all wells.
16. 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 step) 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.
17. 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.
18. Read absorbance of each microwell on a spectrophotometer 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.

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 TIE-2 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 TIE-2 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 TIE-2 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 TIE-2 levels. Such samples require further external predilution according to expected human TIE-2 values with Sample Diluent in order to precisely quantitate the actual human TIE-2 level.
- It is suggested that each testing facility establishes a control sample of known human TIE-2 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 8.

  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.

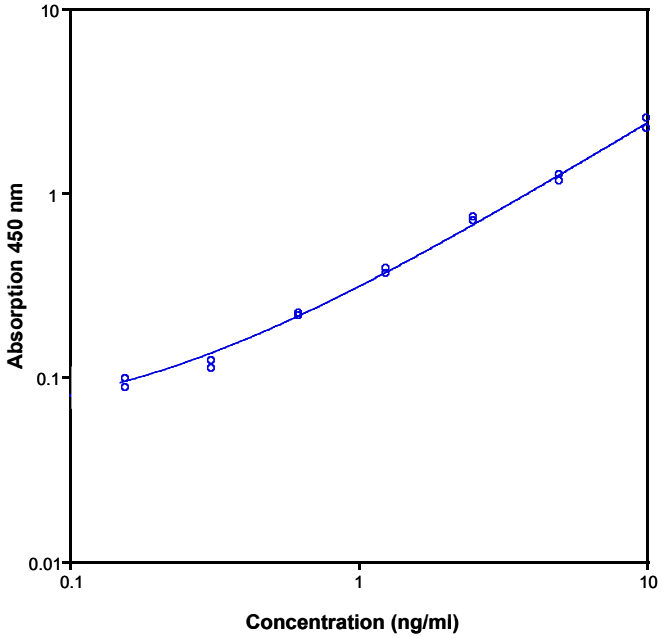

Fig. 8 Representative standard curve for Human TIE-2 ELISA Kit. Human TIE-2 was diluted in serial 2-fold steps in Sample Diluent.

Table 2 Typical data using the Human TIE-2 ELISA Kit (measuring wavelength of 450 nm, reference wavelength of 620 nm).

Standard	Human TIE-2 concentration (ng/mL)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	10.00	2.234 2.528	2.381	6.2
2	5.00	1.143 1.248	1.195	4.4
3	2.50	0.729 0.699	0.714	2.1
4	1.25	0.388 0.364	0.376	3.2
5	0.63	0.221 0.212	0.216	1.9
6	0.31	0.110 0.121	0.115	5.1
7	0.16	0.097 0.087	0.092	5.5
Blank	0.00	0.040 0.043	0.042	2.9

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 TIE-2 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 0.03 ng/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 6 serum samples containing different concentrations of human TIE-2. Two standard curves were run on each plate. Data below show the mean human TIE-2 concentration and the coefficient of variation for each sample. The calculated overall intra-assay coefficient of variation was 7.1%.

Table 3 The mean human TIE-2 concentration and the coefficient of variation for each sample.

Sample	Experiment	Mean human TIE-2 concentration (ng/mL)	Coefficient of variation (%)
1	1 2 3	1.8 2.1 2.0	10.8 10.9 9.7
2	1 2 3	21.6 21.5 23.8	1.6 6.0 1.9
3	1 2 3	11.1 11.7 12.8	7.3 6.3 3.9
4	1 2 3	2.1 2.3 2.5	9.9 9.3 5.7
5	1 2 3	6.1 5.7 6.0	8.4 5.7 5.6
6	1 2 3	29.9 27.3 27.9	10.9 5.8 8.3

Inter-assay

Assay-to-assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of human TIE-2. Two standard curves were run on each plate. Data below show the mean human TIE-2 concentration and the coefficient

of variation calculated on 18 determinations of each sample. The calculated overall inter-assay coefficient of variation was 6.3%.

Table 4 The mean human TIE-2 concentration and the coefficient of variation of each sample.

Sample	Mean human TIE-2 concentration (ng/mL)	Coefficient of variation (%)
1	2.0	8.3
2	22.3	5.9
3	11.9	6.9
4	2.3	8.7
5	5.9	3.4
6	28.4	4.9

Spike recovery

The spike recovery was evaluated by spiking 4 levels of human TIE-2 into serum. Recoveries were determined in 3 independent experiments with 4 replicates each. The amount of endogenous human TIE-2 in unspiked serum was subtracted from the spike values. The recovery ranged from 73–102% with an overall mean recovery of 87%.

Dilution parallelism

Serum samples with different levels of human TIE-2 were analyzed at serial 2-fold dilutions with 4 replicates each. The recovery ranged from 96.8% to 133.7% with an overall recovery of 108.3%.

Sample	Dilution	Expected human TIE-2 concentration (ng/mL)	Observed human TIE-2 concentration (ng/mL)	Recovery of expected human TIE-2 concentration (%)
1	1:2	–	45.5	–
	1:4	22.7	22.0	96.8
	1:8	11.0	11.5	105.0
	1:16	5.8	6.3	108.6
2	1:2	–	43.3	–
	1:4	21.6	21.4	99.0
	1:8	10.7	10.6	98.7
	1:16	5.3	5.3	100.5
3	1:2	–	24.2	–
	1:4	12.1	15.0	124.4
	1:8	7.5	8.4	111.9
	1:16	4.2	4.6	108.8
4	1:2	–	27.5	–
	1:4	13.8	18.4	133.7
	1:8	9.2	10.4	112.9
	1:16	5.2	5.2	99.7

Sample stability

Freeze-Thaw stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 3 times, and the human TIE-2 levels determined. There was no significant loss of human TIE-2 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 TIE-2 level determined after 24 hours. There was no significant loss of human TIE-2 immunoreactivity detected during storage under above conditions.

Specificity

The assay detects both natural and recombinant human TIE-2. The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into serum. No cross-reactivity was detected, notably not with TIE-1.

Expected values

Panels of 40 serum as well as EDTA, citrate, and heparin plasma samples from randomly selected apparently healthy donors (males and females) were tested for human TIE-2. The levels measured may vary with the sample collection used.

Sample matrix	Number of samples evaluated	Range (ng/mL)	Mean (ng/mL)	Standard deviation (ng/mL)
Serum	40	2.9–78.4	33.6	16.9
Plasma (EDTA)	40	5.0–54.4	24.1	10.0
Plasma (citrate)	40	2.1–45.1	26.4	10.8
Plasma (heparin)	40	9.1–47.5	27.2	9.3

Reagent preparation summary

Wash buffer (1x)

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

Number of Strips	Wash Buffer Concentrate Red (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

Biotin-Conjugate

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

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

Streptavidin-HRP

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

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

Human TIE-2 standard

Reconstitute lyophilized human TIE-2 standard with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

Test protocol summary

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).

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 Biotin-Conjugate.
8. Add 50 µL Biotin-Conjugate to all wells.
9. Cover microwell strips and incubate over night at 4°C.
10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 6 times with Wash Buffer.
12. Add 100 µL diluted Streptavidin-HRP to all wells.
13. Cover microwell strips and incubate 1 hour at room temperature (18°C to 25°C).
14. Empty and wash microwell strips 6 times with Wash Buffer.
15. Add 100 µL of TMB Substrate Solution to all wells.
16. Incubate the microwell strips for about 10 minutes at room temperature (18°C to 25°C).
17. Add 100 µL Stop Solution to all wells.
18. Blank microwell reader and measure color intensity at 450 nm.

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