Quantikine® ELISA

Human Tie-2 Immunoassay

Catalog Number DTE200

For the quantitative determination of human Tie-2 concentrations in cell culture supernates, serum, and plasma.

Note: The standard reconstitution method has changed. Please read this package insert in its entirety before using this product.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Tie-2 (also known as Tek) is a receptor tyrosine kinase that is expressed primarily on endothelial cells (ECs) and plays a critical role in vascular development. The ligands for Tie-2 include angiopoietins (Ang) -1, -2, -3 and -4 (1-3). Ang-1, -2, and -4 are expressed in humans while Ang-3 is likely a divergent mouse counterpart to human Ang-4 (3). The Tie-2 gene encodes a protein of 1122 amino acids (aa) (4). The extracellular region has three distinct structural motifs including two immunoglobulin (Ig)-like loops separated by three EGF-like repeats, and three repeats with fibronectin type III homology located after the second Ig loop (4). The intracellular portion of Tie-2 contains two tyrosine kinase domains that, when phosphorylated, interact with a number of binding partners including Grb2, Grb7, Grb14, Shp2, the p85 subunit of phosphatidylinositol 3-kinase (PI3K), and Dok-R (5, 6). Deletion of the last 16 aa of the intracellular C-terminus results in increased levels of autophosphorylation, suggesting that it may play an autoinhibitory role (7). Tie-2 is ~46% identical overall to the other member of the Tie receptor family, Tie-1, although the cytoplasmic tyrosine kinase domains are the most highly conserved, exhibiting ~80% aa sequence identity (4).

Several *in vivo* studies demonstrate the importance of Tie-2 in vascular development. On average, Tie-2-deficient mice die at embryonic day 10.5 and exhibit profound vascular defects including abnormal dilation of the head vasculature, lack of vessel sprouting into the neuroectoderm, decreased complexity of myocardial vessels, and decreases in endothelial cell number (8, 9). Moreover, a Tie-2 mutation that results in a 6- to 10-fold increase in receptor activity is associated with a form of vascular dysmorphogenesis in humans (10).

In vitro studies have revealed some of the signaling mechanisms that may underlie Tie-2 regulation of vascular development. Activation of Tie-2 by Ang-1 can stimulate EC migration, sprout formation, and survival (5, 11, 12). Ang-1/Tie-2 mediated sprouting requires the activation of PI3K and is accompanied by focal adhesion kinase (FAK) phosphorylation and secretion of the proteases plasmin and matrix metalloproteinase 2 (MMP-2) (11). Ang-1/Tie-2-mediated EC migration has been shown to involve two signaling pathways. The first requires stimulation of PI3K and downstream activation of FAK, (5, 11) and the second, recruitment of the protein Dok-R and downstream activation of the p21-activating kinase (Pak) (6). Ang-1/Tie-2-mediated cell survival also requires the activity of PI3K followed by stimulation of its downstream effector, Akt (12). Ang-2 was initially described as a competitive inhibitor of Ang-1/Tie-2 signaling (2), although in some contexts, Ang-2 can stimulate Tie-2 activity as well (13-15). Additionally, Ang-2/Tie-2 signaling leads to EC death and regression, but in the presence of VEGF can promote new vessel formation (16-18). Although the signaling is not as well understood, Ang-4 appears to act as an agonist of Tie-2 (3).

Although primarily thought of as an EC protein, Tie-2 is also expressed by some hematopoietic stem cells (HSCs) and neurons (19, 20). In mice, Ang-1/Tie-2 can mediate the mobilization of HSCs to the peripheral circulation, and when combined with VEGF, is associated with the induction of hematopoiesis and angiogenesis (21). Tie-2 is expressed in human HSCs as well, and *in vitro*, Ang-1/Tie-2 signaling promotes HSC adhesion to fibronectin (22, 23). In addition, *in vitro* studies show that Ang1/Tie2 signaling can protect neurons from apoptosis in a PI3K-dependent manner (20).

The Quantikine® Human Tie-2 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Tie-2 in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant human Tie-2 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Tie-2 showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural human Tie-2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Tie-2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Tie-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Tie-2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Tie-2 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with calibrator diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Tie-2 Microplate	892400	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Tie-2.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Tie-2 Standard	892402	Recombinant human Tie-2 in a buffered protein base with preservative; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store for 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
Human Tie-2 Conjugate	892401	21 mL of a monoclonal antibody specific for human Tie-2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895117	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5P Concentrate	895151	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:2 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Test tubes for dilution of standards and samples.
- Human Tie-2 Controls (optional; R&D Systems®, Catalog # QC172).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

SAMPLE PREPARATION

Serum and plasma samples require a 10-fold dilution. A suggested 10-fold dilution is 25 µL of sample + 225 µL of Calibrator Diluent RD5P (diluted 1:2)*.

* See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

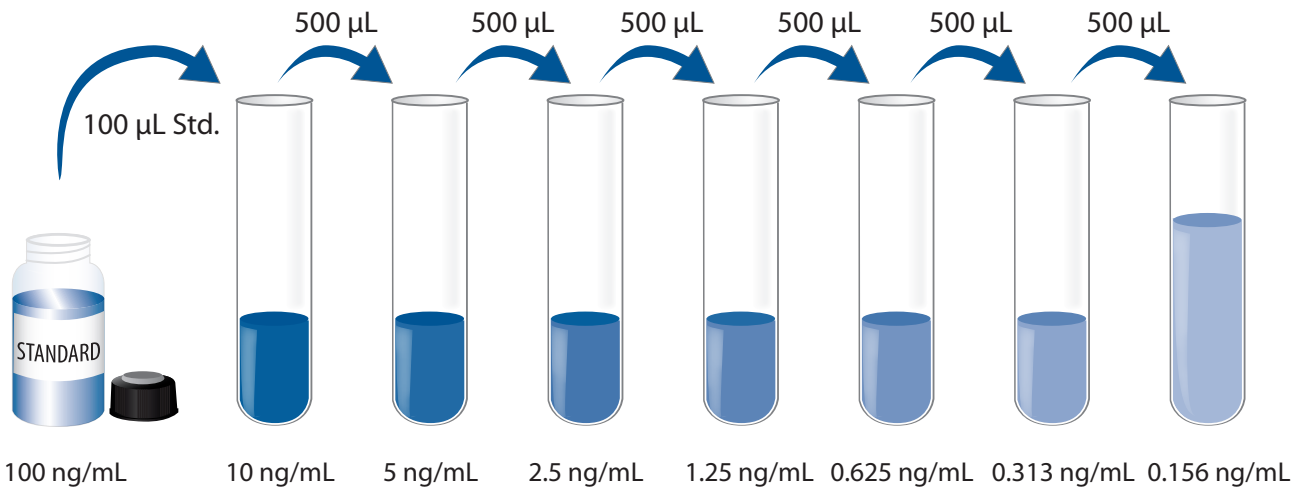
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5P (diluted 1:2) - Add 20 mL of Calibrator Diluent RD5P Concentrate to 20 mL of deionized or distilled water to prepare 40 mL of Calibrator Diluent RD5P (diluted 1:2).

Human Tie-2 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Tie-2 Standard with deionized or distilled water. This reconstitution produces a stock solution of 100 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5P (diluted 1:2) into the 10 ng/mL tube. Pipette 500 µL of Calibrator Diluent RD5P (diluted 1:2) into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 10 ng/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:2) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1W to each well.
4. Add 100 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human Tie-2 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. Protect from light.
9. Add 50 µL of Stop Solution to each well. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

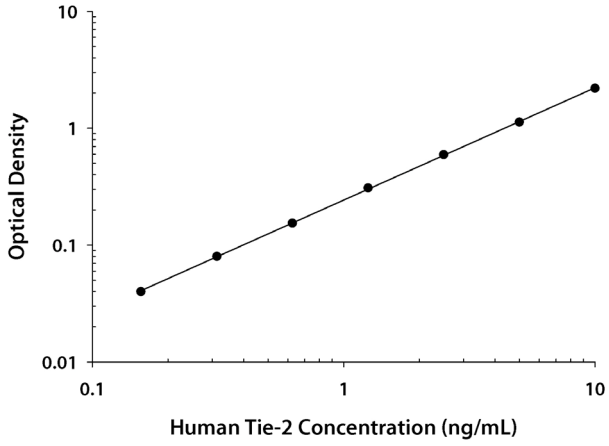
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human Tie-2 concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected
0	0.016 0.017	0.017	—
0.156	0.056 0.058	0.057	0.040
0.313	0.096 0.097	0.097	0.080
0.625	0.169 0.172	0.171	0.154
1.25	0.318 0.332	0.325	0.308
2.5	0.604 0.618	0.611	0.594
5	1.124 1.168	1.146	1.129
10	2.164 2.261	2.213	2.196

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	0.76	2.57	5.14	0.82	2.65	5.15
Standard deviation	0.04	0.11	0.26	0.07	0.17	0.27
CV (%)	5.3	4.3	5.1	8.5	6.4	5.2

RECOVERY

The recovery of human Tie-2 spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	91	85-101%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Tie-2 were serially diluted with the calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	105	99	98	95
	Range (%)	102-107	94-102	95-100	90-99
1:4	Average % of Expected	103	99	99	98
	Range (%)	100-105	95-103	94-104	94-100
1:8	Average % of Expected	99	97	97	91
	Range (%)	92-103	91-104	91-106	90-93
1:16	Average % of Expected	96	96	94	86
	Range (%)	94-98	85-107	87-101	85-88

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human Tie-2 produced at R&D Systems®.

SENSITIVITY

Forty-eight assays were evaluated and the minimum detectable dose (MDD) of human Tie-2 ranged from 0.001-0.066 ng/mL. The mean MDD was 0.014 ng/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human Tie-2 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=60)	27.4	18.6-75.3	8.69
EDTA plasma (n=35)	23.9	16.5-35.7	4.18
Heparin plasma (n=35)	23.4	17.9-34.2	4.04

Cell Culture Supernates - HUVEC human umbilical vein endothelial cells were cultured in EGM supplemented with 2% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate and bovine brain extract until confluent. Two samples were tested for human Tie-2 and measured 0.226 ng/mL and 0.337 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Tie-2.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human Tie-2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Angiogenin
Angiopoietin-1
Angiopoietin-2
Angiopoietin-4
Angiopoietin-like 3
Angiopoietin-like 4
Angiopoietin-like 5
Angiopoietin-like 7
Tie-1

Recombinant mouse:

Angiopoietin-3
Angiopoietin-like 3
Angiopoietin-like 4
Angiopoietin-like 7
Tie-2

Recombinant rat:

Tie-2

Recombinant zebrafish:

Tie-2

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