Quantikine®

Human Tie-1 Immunoassay

Catalog Number DTE100

For the quantitative determination of human Tie-1 concentrations in cell culture supernates, cell lysates, serum, and plasma.

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-1 is a 130 kDa type I transmembrane protein and member of the protein kinase superfamily, the tyrosine protein kinase family, and the Tie subfamily of receptor tyrosine kinases (1). Human Tie-1 is synthesized as an 1138 amino acid (aa) precursor with a 21 aa signal sequence, a 759 aa extracellular domain (ECD), a 25 aa transmembrane sequence, and a 354 aa cytoplasmic domain (SwissProt #: P35590). The ECD contains two immunoglobulin-like (Ig-like C2-type) domains, three EGF-like domains, and three fibronectin type III domains (2). In addition, there are five sites for potential N-linked glycosylation and one Ca^{2+} binding site (2). The cytoplasmic domain contains one tyrosine kinase domain (2). Mature human Tie-1 is 93% aa identical to mature mouse Tie-1. The expression of the Tie-1 gene is restricted to endothelial cells and some hematopoietic cell lineages (1, 3).

Targeted Tie-1 disruption results in embryonic lethality due to edema, hemorrhage, and microvascular rupture (4 - 7). Tie-1 deficient embryos also contain hyperactive endothelial cells that exhibit a large number of extensions and filopodia projecting into vessel lumen and have increased capillary densities, suggesting that Tie-1 may act as a brake during embryonic angiogenesis (4, 7). Upregulation of Tie-1 has been observed during wound healing, ovarian follicle maturation, and tumor angiogenesis (3, 8, 9). Tie-1 is expressed in gastrointestinal stromal tumors, leiomyoma, and schwannoma (10), is an independent prognostic marker for gastric cancer (11), and has also been detected in epithelial tumors in breast and thyroid (12). Recently, an alternative splice variant in Tie-1 has been shown to reduce rheumatoid arthritis severity (13).

Tie-1 shares a high degree of homology with Tie-2, the receptor for three members of the angiopoietin family (Ang-1, Ang-2, and Ang-3/4) (3, 14 - 17). A soluble Ang-1 chimeric protein, COMP-Ang-1, and native Ang-1 and Ang-4 also activate Tie-1 (3). They all induce Tie-1 phosphorylation, although native Ang-1 and Ang-4 are less efficient than COMP-Ang-1 (3). It is reported that Ang-1 induces Tie-1 phosphorylation; however, it is Tie-2 dependent (4). The research shows that Ang-1 fails to induce Tie-1 phosphorylation when Tie-2 is down-regulated in endothelial cells. Tie-1 phosphorylation is induced in the absence of Ang-1 by either a constitutively active form of Tie-2 or a Tie-2 agonistic antibody. In HEK293 cells, Ang-1 phosphorylates a form of Tie-1 without kinase activity when co-expressed with kinase-defective Tie-2 (4). Research also shows that a main role for Tie-1 is to modulate blood vessel morphogenesis by virtue of its ability to down-regulate Tie-2-driven signaling and endothelial survival (4). Both Tie-1 and Tie-2 are also needed for postnatal bone marrow hematopoiesis (3, 18).

The Quantikine Human Tie-1 immunoassay is a 4.5 hour solid phase ELISA designed to measure human Tie-1 in cell culture supernates, cell lysates, serum, and plasma. It contains NS0-expressed recombinant human Tie-1 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Tie-1 showed dose-response curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine Human Tie-1 kit can be used to determine relative mass values for naturally occurring human Tie-1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Tie-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Tie-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for Tie-1 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-1 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 samples with the appropriate 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.
- This assay is designed to eliminate interference by ligands, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Tie-1 Microplate (Part 893245) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against Tie-1.

Tie-1 Conjugate (Part 893246) - 21 mL of polyclonal antibody against Tie-1 conjugated to horseradish peroxidase with preservatives.

Tie-1 Standard (Part 893247) - 100 ng of recombinant human Tie-1 in a buffered protein solution with preservatives; lyophilized.

Assay Diluent RD1-89 (Part 895881) - 11 mL of a buffered protein solution with preservatives.

Calibrator Diluent RD5-17 (Part 895512) - 21 mL of a buffered protein solution with preservatives.

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895032) - 6 mL of 2 N sulfuric acid.

Plate Covers - 4 adhesive strips.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="9">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="8">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Assay Diluent RD1-89</td></tr>
<tr><td>Calibrator Diluent RD5-17</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>Unmixed Color Reagent A</td></tr>
<tr><td>Unmixed Color Reagent B</td></tr>
<tr><td>Standard</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*</td></tr>
</table>

*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.
- Human Tie-1 Controls (optional; available from R&D Systems).

If using cell lysate samples, the following supplies are also required:

- Cell Lysis Buffer 3 (R&D Systems, Catalog # 895366) or equivalent.
- Phosphate-buffered saline (PBS).

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

SAMPLE COLLECTION AND STORAGE

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

Cell Lysates - Cells must be lysed before assaying. Refer to the Cell Lysis Procedure below.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes 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.*

CELL LYSIS PROCEDURE

Use the following procedure for the preparation of cell lysate samples.

1. Perform a 5-fold dilution of Cell Lysis Buffer 3 (available from R&D Systems) with deionized or distilled water.
2. Wash cells one time in cold PBS.
3. Resuspend cells at 1 x 10^6 cells/mL in diluted lysis buffer.
4. Incubate with gentle agitation for 30 minutes at 37° C.
5. Centrifuge to remove cell debris.
6. Assay immediately or aliquot the lysis supernates and store at ≤ -20° C until ready for use.

SAMPLE PREPARATION

Serum and plasma samples require a 10-fold dilution. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD5-17.

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. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

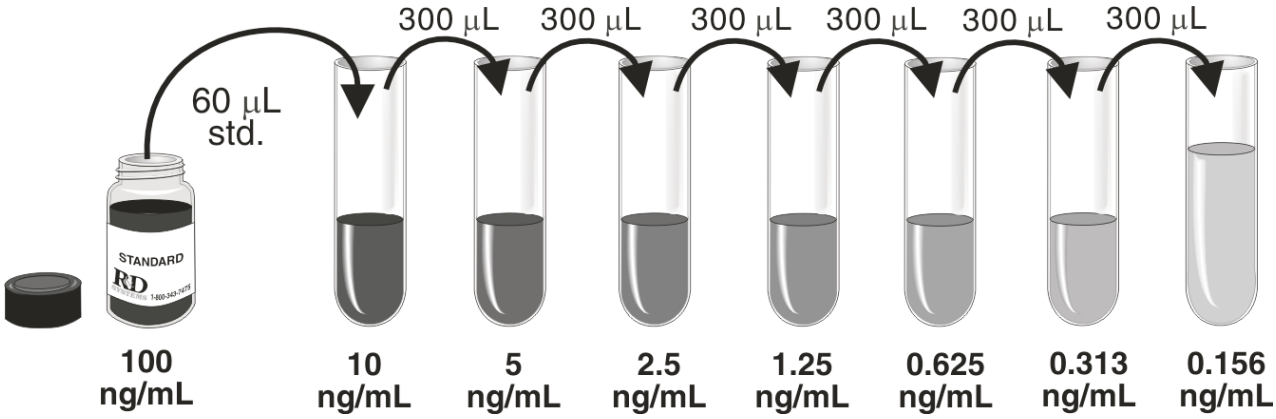
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-17 (1:2) - A 2-fold dilution is required for use with cell culture supernate samples and cell lysates. A suggested 2-fold dilution is 5.0 mL of Calibrator Diluent RD5-17 + 5.0 mL of deionized or distilled water.

Note: *Do not dilute Calibrator Diluent RD5-17 for use with serum or plasma samples.*

Tie-1 Standard - Reconstitute the Tie-1 Standard with 1.0 mL of 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 540 µL of Calibrator Diluent RD5-17 *(for serum and plasma samples)* or Calibrator Diluent RD5-17 (1:2) *(for cell culture supernate/cell lysate samples)* into the 10 ng/mL tube. Pipette 300 µL of the appropriate Calibrator Diluent RD5-17 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. The appropriate Calibrator Diluent 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 samples, controls, and standards 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 RD1-89 to each well.

4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

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 Tie-1 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. The color in the wells should change from blue to yellow. 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.

*Serum/Plasma samples require dilution. See the Sample Preparation section.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and standards as instructed.

2. Add 100 µL Assay Diluent RD1-89 to each well.

3. Add 50 µL Standard, control, or sample* to each well. Incubate 2 hours at RT.

4. Aspirate and wash 4 times.

5. Add 200 µL Conjugate to each well. Incubate 2 hours at RT.

6. Aspirate and wash 4 times.

7. Add 200 µL Substrate Solution to each well. Incubate 30 minutes. **Protect from light.**

8. Add 50 µL Stop Solution to each well. Read at 450 nm within 30 min. λ correction 540 or 570 nm

*Serum/Plasma samples require dilution. See the Sample Preparation section.

CALCULATION OF RESULTS

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

Plot the optical density for the standards versus the concentration of the standards and draw the best fit curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation.

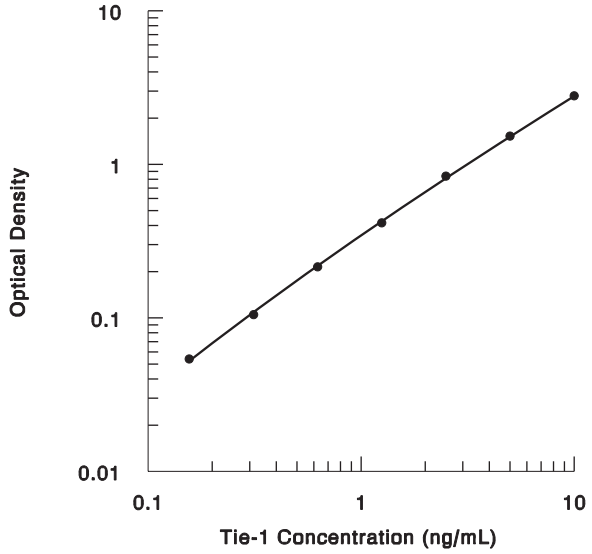
To determine the Tie-1 concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding Tie-1 concentration.

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

TYPICAL DATA

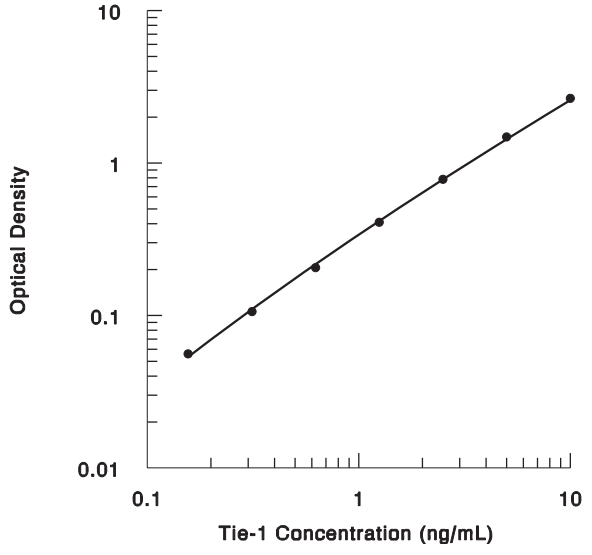
This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Calibrator Diluent RD5-17



ng/mL	O.D.	Average	Corrected
0	0.010 0.013	0.012	—
0.156	0.064 0.067	0.066	0.054
0.313	0.116 0.118	0.117	0.105
0.625	0.225 0.228	0.227	0.215
1.25	0.421 0.434	0.428	0.416
2.5	0.826 0.874	0.850	0.838
5	1.536 1.545	1.541	1.529
10	2.763 2.854	2.809	2.797

Calibrator Diluent RD5-17 (1:2)



ng/mL	O.D.	Average	Corrected
0	0.010 0.011	0.011	—
0.156	0.054 0.057	0.056	0.045
0.313	0.101 0.111	0.106	0.095
0.625	0.199 0.212	0.206	0.195
1.25	0.376 0.442	0.409	0.398
2.5	0.773 0.790	0.782	0.771
5	1.460 1.505	1.483	1.472
10	2.596 2.719	2.658	2.647

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.
- 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.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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.

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.

Serum/Plasma Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	1.17	2.23	4.84	1.21	2.20	4.84
Standard deviation	0.06	0.07	0.16	0.08	0.14	0.17
CV (%)	5.1	3.1	3.3	6.6	6.4	3.5

Cell Culture Supernate/Cell Lysate Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	1.41	2.55	5.73	1.34	2.42	5.27
Standard deviation	0.08	0.09	0.26	0.10	0.15	0.23
CV (%)	5.7	3.5	4.5	7.5	6.2	4.4

RECOVERY

The recovery of Tie-1 spiked to levels throughout the range of the assay was evaluated.

Sample	Average % Recovery	Range
Cell culture media (n=4)	99	90 - 108%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Tie-1 were serially diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Cell lysates (n=3)	Serum* (n=4)	Heparin plasma* (n=4)	EDTA plasma* (n=4)
1:2	Average % of Expected	104	95	101	106	102
	Range (%)	99 - 110	85 - 103	98 - 105	100 - 109	99 - 104
1:4	Average % of Expected	105	92	102	106	102
	Range (%)	94 - 114	85 - 97	99 - 105	98 - 111	97 - 106
1:8	Average % of Expected	102	98	101	103	99
	Range (%)	92 - 109	——	95 - 105	97 - 109	93 - 104
1:16	Average % of Expected	98	97	101	102	96
	Range (%)	87 - 108	——	91 - 110	89 - 111	90 - 100

*Samples were diluted prior to assay, as directed in the Sample Preparation section.

SENSITIVITY

One hundred ten assays were evaluated and the minimum detectable dose (MDD) of Tie-1 ranged from 0.003 - 0.021 ng/mL. The mean MDD was 0.010 ng/mL.

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

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human Tie-1 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples drawn from apparently healthy volunteers were evaluated for the presence of Tie-1 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=35)	61.2	30.2 - 88.0	12.1
EDTA plasma* (n=35)	58.8	32.1 - 92.3	11.0
Heparin plasma* (n=35)	59.4	28.4 - 96.3	11.9

*Samples were diluted prior to assay, as directed in the Sample Preparation section.

Cell Culture Supernates -
Human peripheral blood lymphocytes (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal calf serum, 5 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 μg/mL PHA for 1 and 6 days. Aliquots of the cell culture supernates were removed and assayed for levels of natural Tie-1.

Condition	Day 1 (ng/mL)	Day 6 (ng/mL)
Unstimulated	ND	0.340
Stimulated	ND	0.226

ND = Non-detectable

Human umbilical vein endothelial cells (HUVEC) were grown in EGM-2 media at 37° C, 95% humidity, and 5% CO_2 for 3 days. Cells were cultured unstimulated or stimulated with 40 ng/mL of recombinant human TNF-α and 50 ng/mL of recombinant human IFN-γ. Aliquots of the cell culture supernates were removed and assayed for levels of natural Tie-1.

Condition	Day 3 (ng/mL)
Unstimulated	6.16
Stimulated	7.92

Human lung microvascular endothelial cells (HMVEC) were grown in EGM-2 media at 37° C, 95% humidity, and 5% CO_2. An aliquot of the cell culture supernate was removed, assayed for levels of natural Tie-1, and measured 1.190 ng/mL.

Cell Lysates -
Human umbilical vein endothelial cells (HUVEC) were grown in EGM-2 media at 37° C, 95% humidity, and 5% CO_2 for 3 days. Cells were cultured unstimulated or stimulated with 40 ng/mL of recombinant human TNF-α and 50 ng/mL of recombinant human IFN-γ for 1 day. The cell culture supernate was removed, and cells were lysed as described in the Cell Lysis Procedure. The cell lysates were assayed for levels of natural Tie-1.

Condition	Day 1 (ng/mL)
Unstimulated	1.13
Stimulated	0.186

Human umbilical vein endothelial cells (HUVEC) were grown in EGM-2 media at 37° C, 95% humidity, and 5% CO_2 for 5 days. Cells were then stimulated with 10 ng/mL of recombinant human IL-1β for 1 day. The cell culture supernate was removed, and cells were lysed as described in the Cell Lysis Procedure. The cell lysate was assayed for levels of natural Tie-1 and measured 0.886 ng/mL.

Human lung microvascular endothelial cells (HMVEC) were grown in EGM-2 media at 37° C, 95% humidity, and 5% CO_2. The cell culture supernate was removed, and cells were lysed as described in the Cell Lysis Procedure. The cell lysate was assayed for levels of natural Tie-1 and measured 2.60 ng/mL.

SPECIFICITY

This assay recognizes recombinant and natural human Tie-1. The factors listed below were prepared at 100 ng/mL in the appropriate Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 100 ng/mL in a mid-range recombinant human Tie-1 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
Ang-1
Ang-2
Ang-4
ANGPT-L1
ANGPT-L7
EGF
EG-VEGF
HB-EGF
Tie-2
$VEGF_{121}$
$VEGF_{162}$
$VEGF_{165}$
VEGF-C
VEGF/P*l*GF

Recombinant mouse:
Ang-3
EGF
EG-VEGF
Tie-1
Tie-2
$VEGF_{120}$
$VEGF_{164}$
$VEGF\text{-}B_{167}$
$VEGF\text{-}B_{186}$

Recombinant rat:
EGF
$VEGF_{164}$

Recombinant zebrafish:
Tie-2
$VEGF_{165}$

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PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.