Quantikine® ELISA

Human Thrombospondin-1 Immunoassay

Catalog Number DTSP10

For the quantitative determination of human Thrombospondin-1 concentrations in cell culture supernates, serum, platelet-poor plasma, saliva, and human milk.

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

Thrombospondin-1 (TSP-1 or THBS-1), also known as thrombin-responding protein 1 or thrombin-sensitive protein 1, is a 150 kDa secreted member of the thrombospondin gene family of proteins. It is further a member of the TSR (Thrombospondin type I Repeat) superfamily, and as such, functions as a matricellular protein. Matricellular proteins do not contribute to ECM structural integrity but instead modulate cell-matrix interactions (1-5). Thrombospondin-1 contains a series of diverse modules and exhibits many functions. In conjunction with von Willebrand factor (VWF) and fibrinogen, Thrombospondin-1 contributes to clot formation. In short, and following vascular insult, platelets bind to exposed vessel wall collagen via membrane GP-VI. This results in platelet activation with VWF and Thrombospondin-1 release by platelets (and endothelial cells), and subsequent VWF binding to platelet membrane GPIbα. VWF can cross-link multiple platelet membranes, creating an aggregate. Local Thrombospondin-1 binds to VWF at a site targeted by local proteases, protecting it from proteolysis. This maintains the integrity of the principal inter-platelet molecular linkers in the developing clot (6-8). Thrombospondin-1 is also a well-known inhibitor of angiogenesis. Given the large number of receptors for Thrombospondin-1, multiple pathways may be involved. One possibility involves the co-expression of Thrombospondin-1 with SDF-1 and MMP-9 in platelet α-granules. Both MMP-9 and SDF-1 are pro-angiogenic, and both are regulated by Thrombospondin-1. Whether by direct binding or control of release, Thrombospondin-1 has been shown to minimize new vessel growth (9, 10). A second possibility involves the regulation of NO availability. Low doses of NO promote endothelial cell proliferation and migration. Thrombospondin-1, through binding to CD47 and CD36, interrupts NO-induced generation of pro-angiogenic cGMP (11, 12).

Human Thrombospondin-1 is synthesized as a 1170 amino acid (aa) precursor. It contains an 18 aa signal sequence and a highly modular, 1152 aa mature region (1, 3, 13). The human Thrombospondin-1 precursor shares 95% and 97% aa sequence identity to mouse and canine Thrombospondin-1 precursor, respectively. In order, the human mature molecule contains a 215 aa globular N-terminus, a short 85 aa cysteine-rich segment, a 60 aa VWF type C/procollagen domain, three consecutive type I and type II/EGF-like TSP repeats (aa 379-547, and 549-690, respectively), eight type III, E-F hand-type Ca^{2+}-binding, aspartate-rich repeats (aa 690-955), and a globular, C-terminal lectin-like domain that fluctuates in structure depending on the level of available calcium (1, 2, 4, 14). The signature "domain" that defines Thrombospondin family members consists of the type II and type III repeats, plus the C-terminal globular region. Virtually all modules contain motifs for select ligand binding. For example, the N-terminus binds heparan sulfate plus the α4β1 and α6β1 integrins, the type I TSP repeat binds CD36 and latent TGF-β, and the C-terminus binds CD47 (4). The Cys-rich segment generates two interchain disulfide bonds that form the circulating 450 kDa Thrombospondin-1 trimer. Thrombospondin-1 is produced by megakaryocytes/platelets (3, 7), endothelial cells (1, 15), vascular smooth muscle cells (1, 16), immature astrocytes (17), fibroblasts (1, 18), and dendritic cells (19).

The Quantikine® Human Thrombospondin-1 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Thrombospondin-1 in cell culture supernates, serum, platelet-poor plasma, saliva, and human milk. It contains NS0-expressed recombinant human Thrombospondin-1 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Thrombospondin-1 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 Thrombospondin-1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Thrombospondin-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Thrombospondin-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Thrombospondin-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 Thrombospondin-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, further 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 Thrombospondin-1 Microplate	893298	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Thrombospondin-1.	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 Thrombospondin-1 Conjugate	893299	21 mL of a polyclonal antibody specific for human Thrombospondin-1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Thrombospondin-1 Standard	893300	Recombinant human Thrombospondin-1 in a buffered protein solution with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-56	895102	17 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-33	895813	2 vials (21 mL/vial) 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 preservative. *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.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Collection device for saliva samples that has no protein binding or filtering capabilities such as a Salivette® or equivalent.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human Thrombospondin-1 Controls (optional; R&D Systems®, Catalog # QC170).

PRECAUTIONS

Thrombospondin-1 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

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.

Platelet-poor Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. 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.*

Thrombospondin-1 is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of Thrombospondin-1, platelet-poor plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical Laboratory and Standards Institute (CLSI), result in incomplete removal of platelets from blood. This will cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation.

Saliva - Collect saliva using a collection device such as a Salivette or equivalent, or centrifuge for 5 minutes at 10,000 x g, and collect the aqueous layer. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Saliva collector must not have any protein binding or filtering capabilities.*

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and repeat this process a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Use polypropylene tubes.

Cell culture supernate samples require at least a 2-fold dilution. A suggested 2-fold dilution is 100 μL of sample + 100 μL of Calibrator Diluent RD5-33 (diluted 1:2).

Serum samples require a 100-fold dilution. A suggested 100-fold dilution is 10 μL of sample + 990 μL of Calibrator Diluent RD5-33 (diluted 1:2).

Platelet-poor plasma samples require a 2-fold dilution. A suggested 2-fold dilution is 100 μL of sample + 100 μL of Calibrator Diluent RD5-33 (diluted 1:2).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of Thrombospondin-1 are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

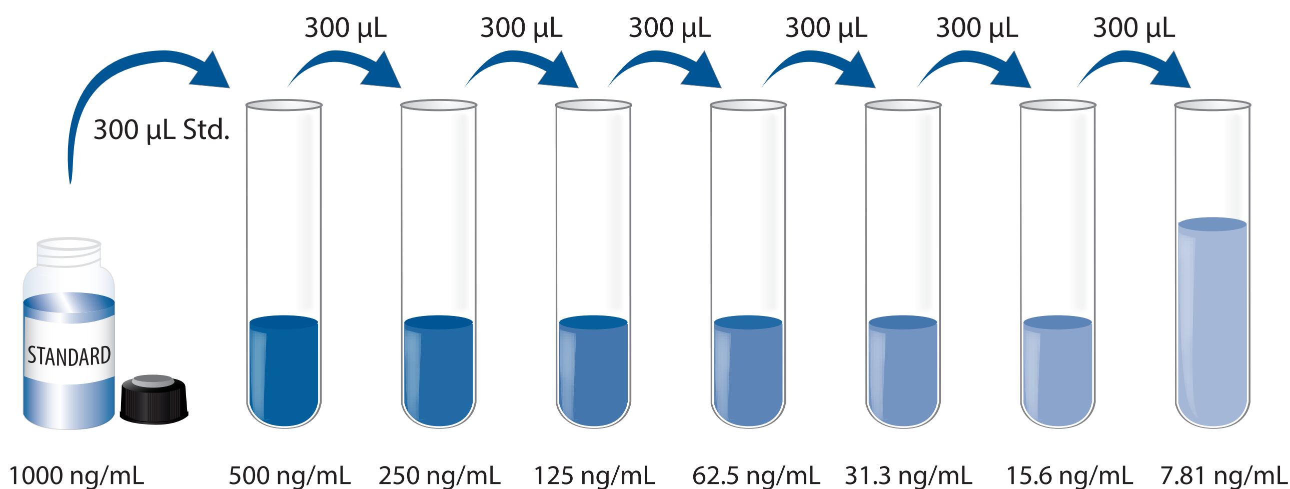
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 RD5-33 (diluted 1:2) - Add 20 mL of Calibrator Diluent RD5-33 to 20 mL of deionized or distilled water to prepare 40 mL of Calibrator Diluent RD5-33 (diluted 1:2).

Human Thrombospondin-1 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Thrombospondin-1 Standard with deionized or distilled water. This reconstitution produces a stock solution of 1000 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.

Use polypropylene tubes. Pipette 300 μL of Calibrator Diluent RD5-33 (diluted 1:2) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 500 ng/mL standard serves as the high standard. Calibrator Diluent RD5-33 (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 samples, controls, and standards be assayed in duplicate.

Note: *Thrombospondin-1 is found in saliva. It is recommended that a face mask and gloves are used to protect kit reagents from contamination.*

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 RD1-56 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 on a horizontal orbital microplate shaker (0.12" orbit) set at 500 rpm ± 50 rpm. 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 Human Thrombospondin-1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
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 **on the benchtop. 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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

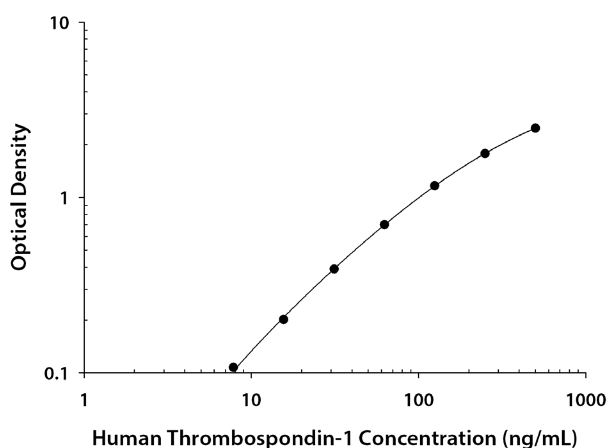
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) 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 the graph. The data may be linearized by plotting the log of the human Thrombospondin-1 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

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.



(ng/mL)	O.D.	Average	Corrected
0	0.046 0.048	0.047	—
7.81	0.153 0.154	0.154	0.107
15.6	0.245 0.250	0.248	0.201
31.3	0.435 0.438	0.437	0.390
62.5	0.731 0.761	0.746	0.699
125	1.194 1.230	1.212	1.165
250	1.805 1.844	1.825	1.778
500	2.521 2.536	2.529	2.482

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)	71.9	142	282	75.8	151	294
Standard deviation	4.34	9.56	18.0	4.71	8.51	17.6
CV (%)	6.0	6.7	6.4	6.2	5.6	6.0

RECOVERY

The recovery of human Thrombospondin-1 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	95	86-101%
Platelet-poor EDTA plasma* (n=4)	94	89-99%
Platelet-poor heparin plasma* (n=4)	93	87-104%

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

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Thrombospondin-1 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

<table>
<tr><th colspan="2" rowspan="2"></th><th rowspan="2">Cell culture supernates* (n=4)</th><th rowspan="2">Serum* (n=4)</th><th colspan="2">Platelet-poor</th><th rowspan="2">Saliva (n=4)</th><th rowspan="2">Human milk* (n=4)</th></tr>
<tr><th>EDTA plasma* (n=4)</th><th>Heparin plasma* (n=4)</th></tr>
<tr><td rowspan="2">1:2</td><td>Average % of Expected</td><td>106</td><td>98</td><td>102</td><td>103</td><td>108</td><td>103</td></tr>
<tr><td>Range (%)</td><td>104-108</td><td>91-103</td><td>100-105</td><td>99-104</td><td>103-112</td><td>90-112</td></tr>
<tr><td rowspan="2">1:4</td><td>Average % of Expected</td><td>107</td><td>101</td><td>103</td><td>104</td><td>107</td><td>107</td></tr>
<tr><td>Range (%)</td><td>102-111</td><td>91-109</td><td>97-107</td><td>98-108</td><td>103-112</td><td>100-113</td></tr>
<tr><td rowspan="2">1:8</td><td>Average % of Expected</td><td>105</td><td>102</td><td>103</td><td>106</td><td>109</td><td>107</td></tr>
<tr><td>Range (%)</td><td>97-112</td><td>90-112</td><td>97-107</td><td>99-114</td><td>102-113</td><td>101-112</td></tr>
<tr><td rowspan="2">1:16</td><td>Average % of Expected</td><td>99</td><td>98</td><td>101</td><td>110</td><td>——</td><td>101</td></tr>
<tr><td>Range (%)</td><td>90-109</td><td>87-112</td><td>97-104</td><td>106-115</td><td>——</td><td>95-108</td></tr>
</table>

*Samples were diluted prior to assay.

SENSITIVITY

Fifty-two assays were evaluated and the minimum detectable dose (MDD) of human Thrombospondin-1 ranged from 0.095-0.944 ng/mL. The mean MDD was 0.355 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.

CALIBRATION

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

SAMPLE VALUES

Serum/Plasma/Saliva/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of human Thrombospondin-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)	16,918	8794-28,335	4388
Platelet-poor EDTA plasma (n=35)	68.4	16.5-259	61.6
Platelet-poor heparin plasma (n=35)	244	80.8-502	109
Saliva (n=12)	66.7	29.5-147	36.1
Human milk* (n=10)	510	ND-1592	——

*One sample measured below the lowest standard, 7.81 ng/mL.
ND=Non-detectable

Cell Culture Supernates:
Human peripheral blood lymphocytes were cultured in DMEM supplemented with 5% fetal bovine 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 human Thrombospondin-1.

Condition	Day 1 (ng/mL)	Day 6 (ng/mL)
Unstimulated	372	358
Stimulated	131	152

MCF 10A human breast epithelial cells were cultured in Ham's F-12 media and DMEM supplemented with 5% equine serum, 100 ng/mL cholera enterotoxin, 10 mg/mL insulin, 0.5 mg/mL hydrocortisol, and 20 ng/mL recombinant human EGF. An aliquot of the cell culture supernate was removed, assayed for human Thrombospondin-1, and measured 1651 ng/mL.

HUVEC human umbilical vein endothelial cells were cultured in EGM®-2 and grown to 90% confluence. An aliquot of the cell culture supernate was removed, assayed for human Thrombospondin-1, and measured 984 ng/mL.

SAMPLE VALUES *CONTINUED*

IMR-90 human lung fibroblasts were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate until confluent. An aliquot of the cell culture supernate was removed, assayed for human Thrombospondin-1, and measured 386 ng/mL.

MRC-5 human embryonic lung fibroblast cells were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 1.0 mM sodium pyruvate. An aliquot of the cell culture supernate was removed, assayed for human Thrombospondin-1, and measured 548 ng/mL.

ME-180 human cervical epithelial carcinoma cells were cultured in McCoy's 5a media supplemented with 10% fetal bovine serum and 2 mM L-glutamine and grown to confluence. An aliquot of the cell culture supernate was removed, assayed for human Thrombospondin-1, and measured 383 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Thrombospondin-1.

The factors listed below were prepared at ≥ 200 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at ≥ 200 ng/mL in a mid-range recombinant human Thrombospondin-1 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
Thrombospondin-2
Thrombospondin-4

Recombinant mouse:
Thrombospondin-1

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

For research use only. Not for use in diagnostic procedures.

NOTES

NOTES

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