Quantikine® ELISA

Human Endoglin/CD105 Immunoassay

Catalog Number DNDG00

For the quantitative determination of human Endoglin concentrations in cell culture supernates, 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

Endoglin, also known as CD105, is a 180 kDa homodimeric co-receptor for members of the TGF-β superfamily. This large type I integral membrane glycoprotein may have roles in hematopoiesis, cardiovascular development, and angiogenesis. Endoglin has a disulfide-linked extracellular region, and a short, constitutively phosphorylated cytoplasmic tail (1). It shares 71% sequence similarity with the transmembrane and cytoplasmic domains of betaglycan, another TGF-β superfamily co-receptor (1). Two Endoglin splice variants (S and L) have been identified that vary in the length of their cytoplasmic tails (2). The Endoglin-L form consists of a total of 633 amino acids (aa) with a 47 aa cytoplasmic region, while Endoglin-S consists of 600 aa and has a 14 aa cytoplasmic region. Each isoform associates with TGF-β, although differential roles remain to be elucidated. Endoglin is highly expressed on vascular endothelial cells (3), chondrocytes (4), and syncytiotrophoblasts of term placenta (5). It is also found on monocytes (6), erythroid precursors (7), and a subpopulation of hematopoietic stem cells (8). Although its function remains elusive, levels of a circulating soluble form of Endoglin are elevated in patients with atherosclerosis (9) and certain cancers including breast (10), colorectal (11), and myeloid malignancies (12).

Endoglin binds several members of the TGF-β superfamily including TGF-β1, TGF-β3, BMP-2, BMP-7, and Activin A (13). Endoglin does not bind TGF-β ligands by itself, but does so by associating with both ligand and a corresponding receptor (13). For instance, *in vitro*, Endoglin binds TGF-β1 and TGF-β3 by associating with TGF-β type II receptor (TGF-β RII), or interacts with Activin A and BMP-7 via either the Activin type II or type IIB receptors. In addition, Endoglin binds BMP-2 *in vitro* via either BMPR-IA (ALK-3) or BMPR-IB (ALK-6). Although Endoglin does not bind TGF-β by itself, it can bind the receptor in the absence of ligand. For example, TGF-β RI and TGF-β RII each associate with both the intracellular and extracellular domains of Endoglin (14). Binding leads to differential regulation of the phosphorylation state of both TGF-β RI and TGF-β RII. Endoglin appears to have both positive and negative effects as a modulator of TGF-β signaling. *In vitro*, Endoglin enhances phosphorylation of the downstream TGF-β effector, Smad2 (14). When Endoglin expression is suppressed in HUVEC cells, TGF-β1 effects on proliferation and migration are markedly enhanced (15). In addition, over-expression of Endoglin in myelomonocytic cells suppresses TGF-β1 activities (16).

Evidence suggests a role for Endoglin in hematopoiesis, angiogenesis, and cardiovascular development. Endoglin has been used as a marker to define long term repopulating hematopoietic stem cells (8). In addition, *in vitro* differentiation of Endoglin$^{-/-}$ mouse embryonic stem cells into myelopoietic and erythropoietic lineages is impaired (17). Knockout mice die *in utero* at day 10-11, are characterized by the failure of extra-embryonic yolk sack vessels to mature, and exhibit weakened embryonic vasculature leading to the loss of endothelial integrity and hemorrhage (18, 19). Endoglin$^{-/-}$ mice also display abnormal cardiac development including enlarged hearts, necrosis, and defects in valve and septum formation (19). In humans, mutations in the Endoglin gene are responsible for hereditary hemorrhagic telangiectasia type 1 (HHT1), an autosomal dominant disorder characterized by bleeding telangiectases of mucous membranes and the gastrointestinal tract, and arteriovenous malformation in several organ systems including the brain, lungs, and liver (20, 21).

Furthermore, several studies have demonstrated enhanced endothelial expression of Endoglin associated with angiogenesis in several types of cancer (22). The localization of Endoglin to the vasculature of cancer tissues has also made it a potential target for tumor-associated anti-angiogenic therapy (23, 24).

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

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Endoglin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Endoglin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for Endoglin 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 Endoglin 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 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.

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
Endoglin Microplate	892530	96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against human Endoglin.	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.*
Endoglin Conjugate	892531	21 mL of a mouse monoclonal antibody against human Endoglin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Endoglin Standard	892532	100 ng of recombinant human Endoglin in a buffered protein base with preservatives; lyophilized.	
Assay Diluent RD1S	895137	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5K	895119	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.	
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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards.
- Human Endoglin Controls (optional; available from R&D Systems).

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

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 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.*
Do not use grossly hemolyzed or lipemic samples in this assay.

REAGENT PREPARATION

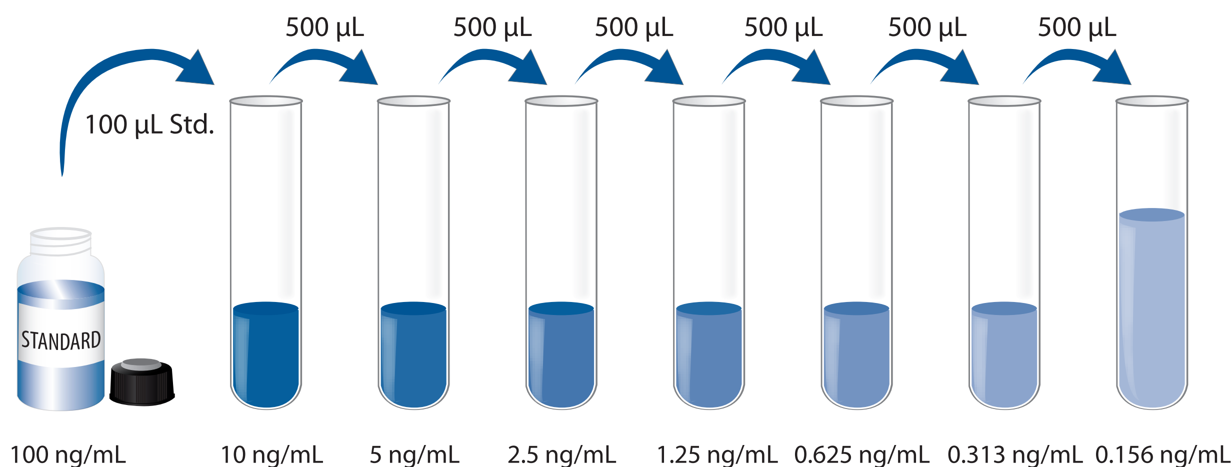
Bring all reagents to room temperature before use.

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.

Endoglin Standard - Reconstitute the Endoglin 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 900 µL of Calibrator Diluent RD5K into the 10 ng/mL tube. Pipette 500 µL 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 Calibrator Diluent serves as the zero standard (0 ng/mL).



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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 RD1S 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 ± 50 rpm.
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 Endoglin 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.

CALCULATION OF RESULTS

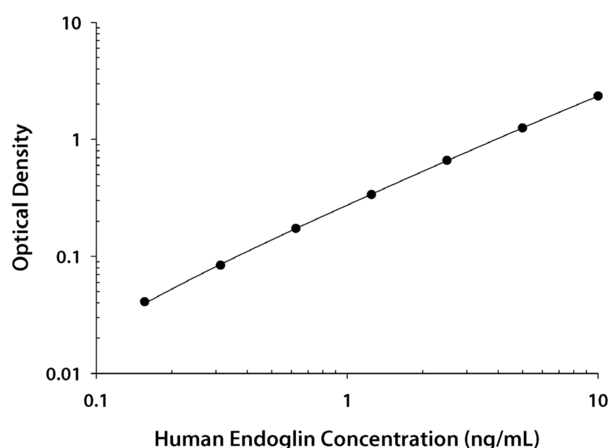
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 Endoglin 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	0.007 0.009	0.008	—
0.156	0.047 0.051	0.049	0.041
0.313	0.091 0.092	0.092	0.084
0.625	0.179 0.182	0.181	0.173
1.25	0.334 0.357	0.346	0.338
2.5	0.649 0.687	0.668	0.660
5	1.252 1.265	1.259	1.251
10	2.335 2.370	2.353	2.345

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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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	0.617	1.67	3.14	0.659	1.80	3.47
Standard deviation	0.017	0.053	0.094	0.044	0.117	0.220
CV (%)	2.8	3.2	3.0	6.7	6.5	6.3

RECOVERY

The recovery of Endoglin spiked to levels throughout the range of the assay was evaluated in cell culture media.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	101	92-111%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Endoglin were serially diluted with 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	99	101	100	101
	Range (%)	97-101	96-104	98-104	98-103
1:4	Average % of Expected	95	104	107	104
	Range (%)	94-97	96-109	104-110	102-107
1:8	Average % of Expected	94	102	109	104
	Range (%)	92-98	95-112	108-111	102-107
1:16	Average % of Expected	94	96	107	103
	Range (%)	92-96	88-100	105-108	100-106

SENSITIVITY

Thirty-three assays were evaluated and the minimum detectable dose (MDD) of Endoglin ranged from 0.001-0.030 ng/mL. The mean MDD was 0.007 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 Endoglin produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of Endoglin 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)	3.96	2.54-7.06	0.92
EDTA plasma (n=37)	4.17	2.99-7.14	0.90
Heparin plasma (n=37)	4.20	2.86-7.32	0.89

Cell Culture Supernates -
HUVEC human umbilical vein endothelial cells were cultured in EGM supplemented with 2% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and bovine brain extract until confluent. An aliquot was removed, assayed for levels of natural human Endoglin, and measured 0.517 ng/mL.

IMR-90 human lung fibroblasts were cultured in MEM supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, and 100 U/mL penicillin. An aliquot was removed, assayed for levels of natural human Endoglin, and measured 0.420 ng/mL.

Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 50 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. Aliquots of the cell culture supernates were removed and assayed for levels of natural human Endoglin.

Condition	Day 1 (ng/mL)	Day 6 (ng/mL)
Unstimulated	ND	ND
Stimulated	ND	0.236

ND=Non-detectable

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SPECIFICITY

This assay recognizes natural and recombinant human Endoglin.

The factors listed below were prepared at 100 ng/mL in Calibrator Diluent RD5K and assayed for cross-reactivity. Preparations of the following factors at 100 ng/mL in a mid-range recombinant human Endoglin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Activin A
Activin RIA
Activin RIIA
Activin RIIB
BMPR-IA
BMPR-IB
BMP-2
BMP-4
BMP-5
BMP-6
Follistatin 288
Follistatin 300
Follistatin 315
Inhibin A
Inhibin B
LAP
TGF-α
TGF-β1
TGF-β1.2
TGF-β2
TGF-β3
TGF-β RII
TGF-β RIII

Recombinant mouse:

BMPR-IA
BMPR-IB

Other recombinants:

rat Agrin
porcine TGF-β2
amphibian TBF-β5

Natural proteins:

human TGF-β1
porcine TGF-β1

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