Quantikine®

Mouse Endocan Immunoassay

Catalog Number MEND00

For the quantitative determination of mouse Endocan concentrations in cell culture supernates, mouse 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.

TABLE OF CONTENTS

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INTRODUCTION

Endocan, also known as ESM-1 (endothelial cell specific molecule-1), is a 50 kDa cysteine-rich proteoglycan, of which approximately 30 kDa corresponds to a single dermatan sulfate chain (1 - 3). Endocan contains an IGFBP/EGF-like repeat and intramolecular disulfide bonds but does not form disulfide-linked oligomers (1 - 4). No alternate splice forms have been described in mouse, although a variant of human endocan with a 50 amino acid (aa) internal deletion has been reported (5). Endocan is secreted by vascular endothelial cells (3, 4), epithelial cells lining renal distal tubules, bronchi and lung submucosal glands (4, 6), adipocytes (7, 8), and a subpopulation of splenic monocytes (6). Endocan expression in endothelial and epithelial cells is upregulated by TNF-α, IL-1β, LPS, and VEGF and downregulated by IL-4 and IFN-γ (3, 4, 9, 10). In adipocytes, endocan is positively regulated by phorbol esters and retinoic acid and inhibited by insulin and cortisol (7, 8). Endocan gene transcription is directly repressed by Hhex, a homeodomain transcription factor that also represses VEGF R1, VEGF R2, Tie-1, Tie-2, and Neuropilin-1 expression (11, 12).

Both the protein core and dermatan sulfate moiety of endocan are involved in binding to activated LFA-1 (13). LFA-1 is a non-covalent heterodimer of α_L/CD11a and β_2/CD18 integrin subunits, and it is expressed on virtually all leukocytes (14 - 16). The α_L subunit contains domains termed thigh, calf-1, and calf-2, while the β_2 subunit contains a PSI (plexin-semaphorin-integrin) region and four cysteine-rich I-EGF folds (17). Upon activation by inside-out signaling, clustering, or Mg^{2+} or Mn^{2+} binding, LFA-1 unfolds to expose ligand binding sites for ICAM-1, -2, -3, -4 and JAM-A (16 - 18). The adhesion stabilizes interactions between T cells and antigen-presenting cells, decreases the T cell activation threshold, and facilitates leukocyte extravasation (18 - 20). Endocan functions as a competitive inhibitor of the LFA-1/ICAM-1 interaction (13). Endocan also potentiates the proliferation of HGF-stimulated human embryonic kidney cells *in vitro*, an effect that depends only on its dermatan sulfate chain (2). Pleiotropic HGF is a disulfide-linked dimeric molecule that includes an N-terminal PAN/APPLE-like domain, four Kringle domains, and a serine proteinase-like domain that has no detectable protease activity (21). HGF binds dermatan sulfate and heparan sulfate proteoglycans and the widely expressed receptor tyrosine kinase, HGF R/c-MET (22, 23). HGF regulates epithelial morphogenesis by inducing cell scattering and branching tubulogenesis (24, 25). HGF-dependent c-MET activation is implicated in the development of many human cancers (26).

Endocan is over-expressed in the vascular endothelium of renal carcinoma, breast carcinoma, glioma, and non-small cell lung cancer (5, 6, 10). Local expression levels correlate with vascularity and tumor aggressiveness (5). Endocan is induced by tumor-derived factors, including VEGF, which may involve a VEGF R2-dependent mechanism (5, 6). In return, the dermatan sulfate chain and protein core both contribute to promoting tumor growth (27). In humans, circulating levels of endocan are elevated during septic shock (4, 9) and lung cancer (10, 27). In overweight and obese patients, adipose expression of endocan is increased, while serum levels are reduced (8). A loss of endocan production by other tissue sources is likely responsible for the decrease, as adipose-derived endocan is a minor contributor to total serum levels (8).

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

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse Endocan has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any mouse Endocan present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse Endocan is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of mouse Endocan bound in the initial step. The sample values are then read off the standard curve.

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 the appropriate Calibrator Diluent and repeat the assay.
- Any variation in 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 soluble receptors, binding proteins and other factors present in biological samples. Until all factors have been tested, however, the possibility of interference cannot be excluded.

PRECAUTION

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

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.
- For best results, pipette reagents and samples into the center of each well.
- 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.

MATERIALS PROVIDED

Mouse Endocan Microplate (Part 893656) - One 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse Endocan.

Mouse Endocan Conjugate (Part 893657) - 12 mL of a polyclonal antibody specific for mouse Endocan conjugated to horseradish peroxidase with preservatives.

Mouse Endocan Standard (Part 893658) - 2 vials (30 ng/vial) of recombinant mouse Endocan in a buffered protein base with preservatives; lyophilized.

Mouse Endocan Control (Part 893659) - 2 vials of recombinant mouse Endocan in a buffered protein base with preservatives; lyophilized. The concentration range of mouse Endocan after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.

Assay Diluent RD1-14 (Part 895180) - 12 mL of a buffered protein solution with preservatives. *May contain a precipitate. Mix well before and during use.*

Calibrator Diluent RD5Y (Part 895201) - 21 mL of buffered protein solution with preservatives. *For cell culture supernate samples.*

Calibrator Diluent RD5T (Part 895175) - 21 mL of buffered protein solution with preservatives. *For serum/plasma samples.*

Wash Buffer Concentrate (Part 895024) - 50 mL of a 25-fold concentrated solution of buffered surfactant with preservative.

Color Reagent A (Part 895000) - 12 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895174) - 23 mL of diluted hydrochloric acid.

Plate Covers (Part 640197) - 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="10">Opened/ Reconstituted Reagents</td><td>Mouse Endocan Conjugate</td><td rowspan="8">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Diluted Wash Buffer</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Assay Diluent RD1-14</td></tr>
<tr><td>Calibrator Diluent RD5Y</td></tr>
<tr><td>Calibrator Diluent RD5T</td></tr>
<tr><td>Unmixed Color Reagent A</td></tr>
<tr><td>Unmixed Color Reagent B</td></tr>
<tr><td>Mouse Endocan Standard (3000 pg/mL)</td><td rowspan="2">Discard any unused reconstituted Standard and Control after use. Use a new Standard and Control for each assay.</td></tr>
<tr><td>Mouse Endocan Control</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

- Hydrochloric acid (A.C.S. Grade, 12 N)
- Sodium hydroxide (A.C.S. Grade, 10 N)
- 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.
- 100 mL and 1000 mL graduated cylinders.
- **Polypropylene tubes.**

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.

Serum - Allow blood samples to clot for 2 hours at room temperature or overnight at 2 - 8° C before centrifuging. Centrifuge for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 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. Grossly hemolyzed samples are not suitable for use in this assay.*

SAMPLE PRETREATMENT

Note: *Do not pretreat the kit standard or control. Cell culture supernate samples do not require pretreatment.*

Prepare the following solutions for acid treatment and neutralization. The solutions may be stored in polypropylene bottles at room temperature for up to one month.

Caution: *Wear protective clothing and safety glasses while preparing or using these reagents. Always add acid to water.*

1N HCl (100 mL) - To 91.67 mL of deionized water, slowly add 8.33 mL of 12N HCl. Mix well.

1N NaOH (100 mL) - To 90 mL of deionized water, slowly add 10 mL of 10N NaOH. Mix well.

Serum and plasma samples require pretreatment with 1N HCl followed by neutralization with 1N NaOH.

1. Add 20 µL of 1N HCl to 100 µL of serum/plasma. Mix well.
2. Incubate for 10 minutes at room temperature.
3. Neutralize with 16 µL of 1N NaOH. Mix well.
4. Assay samples after neutralization.

The concentration read off the standard curve must be multiplied by the dilution factor, 1.36.

For each new lot of acidification and neutralization reagents, measure the pH of several representative samples after neutralization to ensure that it is within a pH of 5.0 - 7.0. Adjust the volume and corresponding dilution factor of the 1N NaOH as needed.

Note: *Pretreated serum and plasma samples are stable for up to 3 months at ≤ -20° C.*

REAGENT PREPARATION

Bring all reagents to room temperature before use.

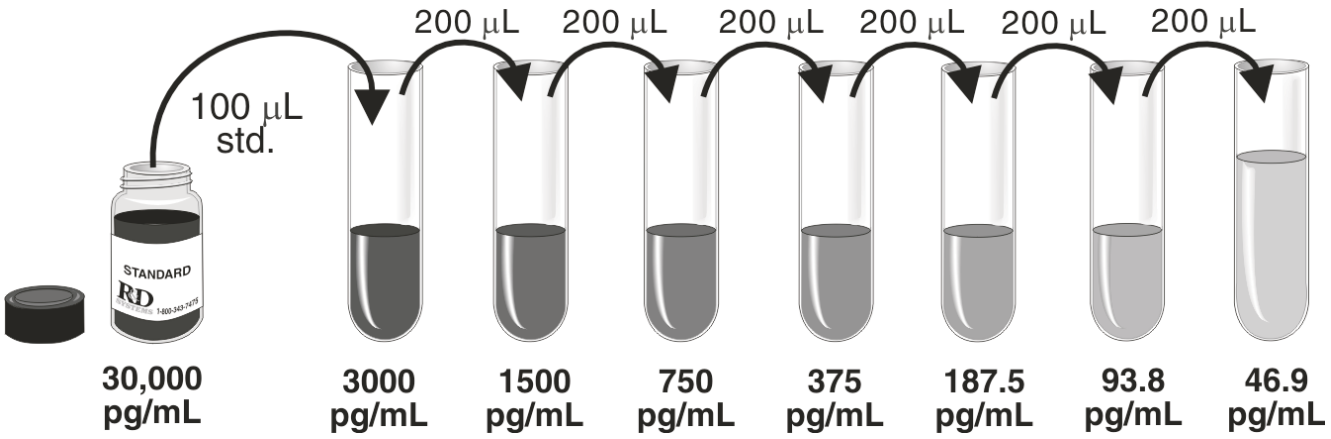
Mouse Endocan Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 625 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. 100 µL of the resultant mixture is required per well.

Mouse Endocan Standard - Reconstitute the mouse Endocan Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 30,000 pg/mL. Allow the stock solution to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 900 µL of Calibrator Diluent RD5T *(for serum/plasma samples)* or Calibrator Diluent RD5Y *(for cell culture supernate samples)* into the 3000 pg/mL tube and 200 µL of the appropriate Calibrator Diluent into the remaining tubes. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube gently but thoroughly before the next transfer. The 3000 pg/mL mouse Endocan Standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

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

1. Prepare all reagents, standard dilutions, control 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, reseal.

3. Add 50 µL of Assay Diluent RD1-14 to each well. Assay Diluent RD1-14 may contain a precipitate. Mix well before and during use.

4. Add 50 µL of Standard, Control, or sample* per well. Gently tap the plate to ensure thorough mixing. 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 four times for a total of five 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 100 µL of mouse Endocan 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 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**

9. Add 100 µL of Stop Solution to each well. 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 and plasma samples require pretreatment. See the Sample Pretreatment section.

PROCEDURE SUMMARY AND CHECKLIST

1. ☐ Bring all reagents to room temperature.
☐ Prepare reagents and samples as instructed.
☐ Return unused components to storage temperature as indicated in the instructions.

2. ☐ Add 50 µL Assay Diluent RD1-14 to each well.

3. ☐ Add 50 µL Standard, Control, or sample* to each well. Gently tap the plate to ensure thorough mixing.
☐ Cover the plate and incubate for 2 hours at room temperature.

4. ☐ Aspirate and wash each well five times.

5. ☐ Add 100 µL Conjugate to each well.
☐ Cover the plate and incubate for 2 hours at room temperature.

6. ☐ Aspirate and wash each well five times.

7. ☐ Add 100 µL Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**

8. ☐ Add 100 µL Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

9. ☐ Read Optical Density at 450 nm (correction wavelength set at 540 nm or 570 nm).

*Serum and plasma samples require pretreatment. See the Sample Pretreatment section.

CALCULATION OF RESULTS

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

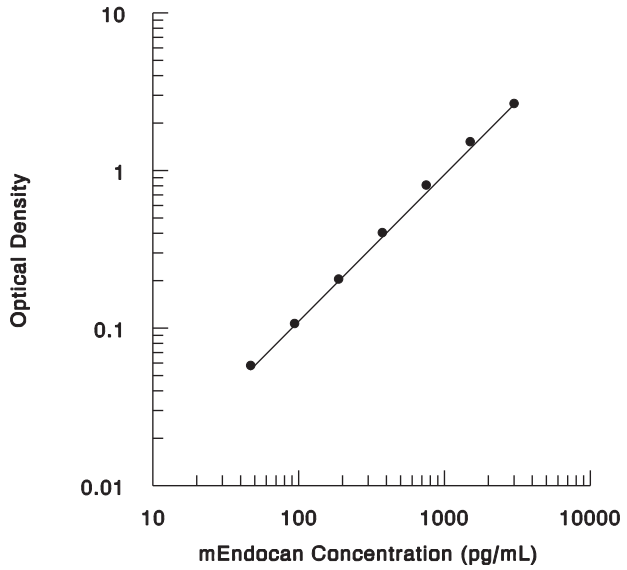
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the mouse Endocan 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 prior to assay, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

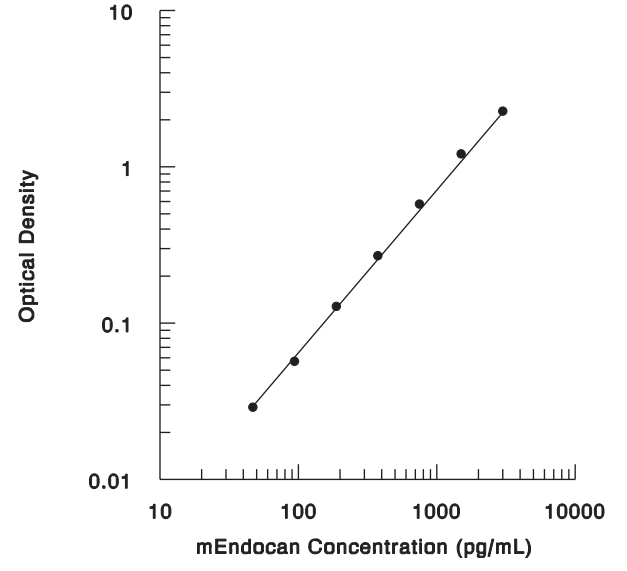
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Calibrator Diluent RD5Y



pg/mL	O.D.	Average	Corrected
0	0.009 0.013	0.011	—
46.9	0.068 0.070	0.069	0.058
93.8	0.117 0.119	0.118	0.107
187.5	0.215 0.216	0.216	0.205
375	0.413 0.416	0.415	0.404
750	0.803 0.837	0.820	0.809
1500	1.508 1.561	1.535	1.524
3000	2.616 2.736	2.676	2.665

Calibrator Diluent RD5T



pg/mL	O.D.	Average	Corrected
0	0.009 0.012	0.011	—
46.9	0.040 0.040	0.040	0.029
93.8	0.066 0.069	0.068	0.057
187.5	0.134 0.143	0.139	0.128
375	0.280 0.281	0.281	0.270
750	0.584 0.593	0.589	0.578
1500	1.205 1.238	1.222	1.211
3000	2.212 2.352	2.282	2.271

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 assays to assess inter-assay precision.

Cell Culture Supernate Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	143	398	1107	137	373	1097
Standard deviation	14.1	25.3	70.7	13.1	23.2	78.4
CV (%)	9.8	6.4	6.4	9.6	6.2	7.1

Serum/Plasma Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	154	515	1439	206	517	1406
Standard deviation	14.8	25.4	86.0	20.8	39.2	99.8
CV (%)	9.6	4.9	6.0	10.1	7.6	7.1

RECOVERY

The recovery of mouse Endocan spiked to three levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture samples (n=4)	99	91 - 120%
Mouse serum* (n=4)	92	81 - 117%
Mouse EDTA plasma* (n=4)	92	81 - 119%
Mouse heparin plasma* (n=4)	108	89 - 119%

*Serum and plasma samples were pretreated prior to assay.

LINEARITY

To assess the linearity of the assay, samples containing or spiked with high concentrations of mouse Endocan in each matrix were diluted with the appropriate Calibrator Diluent and assayed.

		Cell culture samples (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	103	109	105	106
	Range (%)	101 - 108	106 - 113	102 - 109	103 - 109
1:4	Average % of Expected	99	112	107	110
	Range (%)	96 - 102	107 - 119	97 - 115	104 - 115
1:8	Average % of Expected	101	109	115	113
	Range (%)	100 - 104	100 - 116	102 - 121	107 - 120
1:16	Average % of Expected	96	105	114	114
	Range (%)	90 - 104	105 - 105	99 - 119	109 - 118

*Serum and plasma samples were pretreated prior to assay.

SENSITIVITY

Sixty-nine assays were evaluated and the minimum detectable dose (MDD) of mouse Endocan ranged from 1.30 - 21.3 pg/mL. The mean MDD was 6.23 pg/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 mouse Endocan produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse Endocan in this assay.

	Mean (pg/mL)	Range (pg/mL)
Mouse serum* (n=20)	1232	919 - 1614
Mouse heparin plasma* (n=20)	1143	872 - 1606
Mouse EDTA plasma* (n=20)	1340	1053 - 1676

*Samples were pretreated as described in the Sample Pretreatment section.

Cell Culture Supernates -
Tissues from 2 - 3 mice were cut into 1 - 2 mm pieces and cultured in 100 mL of RPMI supplemented with 10% fetal bovine serum, 50 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate for 1 or 3 days. The cell culture supernates were assayed for levels of mouse Endocan.

Tissue Type	Observed Levels (pg/mL)
Kidney (1 day)	337
Lung (1 day)	364
Liver (3 days)	179
Spleen (1 day)	168

SPECIFICITY

This assay recognizes both recombinant and natural mouse Endocan. 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 mouse Endocan control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:
HGF
IFN-γ
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-5
IGFBP-6
IGFBP-Rβ1/IGFBP-7
IGFBP-L1
IGFBP-RP10
IL-1β
IL-4
Integrin αL
Integrin αL/β2
Integrin β2
TNF-α
VEGF
VEGF R1
VEGF R2
Wnt-1

Recombinant human:
Endocan
Integrin αL
Integrin αL/β2
Integrin β2
TNF-β
sTNF RI

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PLATE LAYOUT

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