

Quantikine[®]

Human Endostatin Immunoassay

Catalog Number DNST0

For the quantitative determination of human endostatin concentrations in cell culture supernates, serum, plasma, and saliva.

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

Endostatin is well known for its anti-growth and anti-migratory effects on endothelial cells (ECs) (1). In this capacity it has received much attention for its potential use as an angiogenesis inhibitor capable of reducing the blood supply necessary for the maintenance and growth of tumors (2). Endostatin was originally isolated from murine hemangioendothelioma (EOMA) cells as a 20 kDa proteolytic fragment of type XVIII Collagen (2). Human type XVIII Collagen consists of an N-terminal region containing at least two splice variants, followed by a central triple helical domain, and a C-terminal, non-collagenous (NC1) domain from which Endostatin is derived (3, 4). The process leading to the release of human Endostatin from the NC1 domain is not fully understood (5). However, Cathepsin L and Elastase have been implicated as proteinases responsible for its generation in EOMA cells (6, 7). Mean concentrations in serum samples obtained from healthy human donors are somewhat variable but are estimated at approximately 20 ng/mL (8 - 11).

Endostatin is perhaps best known for its anti-tumor activity in animal models. Effects were first described for mouse Endostatin where an almost complete regression of several tumor types was reported including Lewis lung carcinoma, fibrosarcoma, melanoma, and EOMA (2, 12). Since then, experiments carried out using human Endostatin suggest that, like the murine form, it can also suppress tumor growth in animal models (13 - 18). Some studies using gene transfer techniques paradoxically report no effect on the vasculature or on implanted tumors in mice despite relatively high levels of circulating Endostatin (19, 20). The underlying reason for the conflicting results is unclear and suggests that some questions may still remain (21, 22).

In vitro studies demonstrate that Endostatin can regulate EC physiology in ways that could affect angiogenesis. For instance, soluble Endostatin inhibits EC migration and leads to rearrangements of the cytoskeleton that include the loss of Actin stress fibers and focal adhesions (13, 23, 24). This is shown to involve several binding partners including $\alpha_5\beta_1$ integrins, Tropomyosin, and putative heparan sulfate proteoglycans (25 - 27). Effects on the human EC cytoskeleton are accompanied by downregulation of Mitogen-activated Protein Kinase (MAPK), Focal Adhesion Kinase (FAK), the Urokinase Plasminogen Activator (uPA) System, and the RhoA GTPase (24, 27, 28). In addition, Endostatin inhibition of the Wnt signaling pathway has been shown to suppress VEGF and FGF-2-induced EC migration *in vitro* (29). It should be noted that differential effects on EC migration have been reported depending upon whether Endostatin was in a soluble form, or immobilized to a substrate (23). Human Endostatin has also been shown in some studies to inhibit EC proliferation. Endostatin-induced cell cycle arrest in G1 phase is accompanied by Cyclin D1 downregulation (29). Human Endostatin can initiate EC apoptosis, and in C-PAE cells, this is accompanied by a reduction in the anti-apoptotic proteins Bcl-2 and Bcl-x_L (30, 31).

Elevated circulating levels of Endostatin are associated with many forms of cancer including breast, renal, ovarian, and endometrial cancers, soft tissue sarcoma, acute myelogenous leukemia, non-Hodgkins lymphoma, non-small cell lung cancer, hepatocellular carcinoma, and head and neck squamous cell carcinoma (9 - 11, 32 - 38). Additionally, human Endostatin treatment in murine xenograft models of rheumatoid arthritis (RA) has been effective, and increased circulating levels of Endostatin have been found in patients following the commencement of treatment for RA (39 - 41). Endostatin is also found upregulated in amyloid plaques associated with Alzheimer's disease (42).

Endostatin is expressed in tissue from mouse brain, skeletal muscle, heart, kidney, testes, and liver, and is found in serum from healthy human donors (8 - 11, 43). However, the physiological role for Endostatin remains unclear. Collagen XVIII knockout mice display abnormal outgrowth of retinal capillaries and delays in the normal regression of hyaloid vessels that could suggest a role in development of the vasculature (44). Knockout mice do not exhibit excessive tumor growth indicating that normal circulating levels of Endostatin may not be sufficient to inhibit tumor progression (44).

In addition to ECs, Endostatin may affect other cell types as well. For instance, overexpression of mouse Endostatin can cause developmental abnormalities in early *Xenopus* embryos, potentially due to deficient Wnt signaling and subsequent promotion of β -Catenin degradation (29). In addition, the Glypicans, cell surface proteoglycans, are low affinity binding partners for mouse Endostatin (25). This interaction is important for Endostatin-mediated inhibition of renal tubular epithelial cell branching and morphogenesis, and ureteric bud branching (25, 45). Lastly, ectopic expression of the Endostatin homolog in *C. elegans* leads to neuronal migratory and axon pathfinding defects (46).

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

PRINCIPLE OF THE ASSAY

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

Endostatin Microplate (Part 892533) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against Endostatin.

Endostatin Conjugate (Part 892534) - 21 mL of a mouse monoclonal antibody against Endostatin conjugated to horseradish peroxidase with preservatives.

Endostatin Standard (Part 892535) - 100 ng of recombinant human Endostatin in a buffer with preservatives; lyophilized.

Assay Diluent RD1W (Part 895117) - 11 mL of a buffered protein base with preservatives.

Calibrator Diluent RD5P Concentrate (Part 895151) - 21 mL of a buffered protein base 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

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Assay Diluent RD1W	
	Calibrator Diluent RD5P	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	
Microplate Wells	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.*	

*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.
- 100 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 Salivette™ or equivalent.
- Human Endostatin Controls (optional; available from R&D Systems).

PRECAUTIONS

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

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

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ 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 $\leq -20^{\circ}$ 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 $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

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

Saliva - Collect saliva using a collection device such as an Salivette or equivalent. Assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

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

SAMPLE PREPARATION

Serum and plasma samples require a 50-fold dilution. A suggested 50-fold dilution is 20 μ L sample + 980 μ L Calibrator Diluent RD5P (1X).

Cell culture supernate samples may require dilution with Calibrator Diluent RD5P (1X).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: High concentrations of endostatin are found in saliva. We recommend using a face mask and gloves to protect kit reagents from contamination.

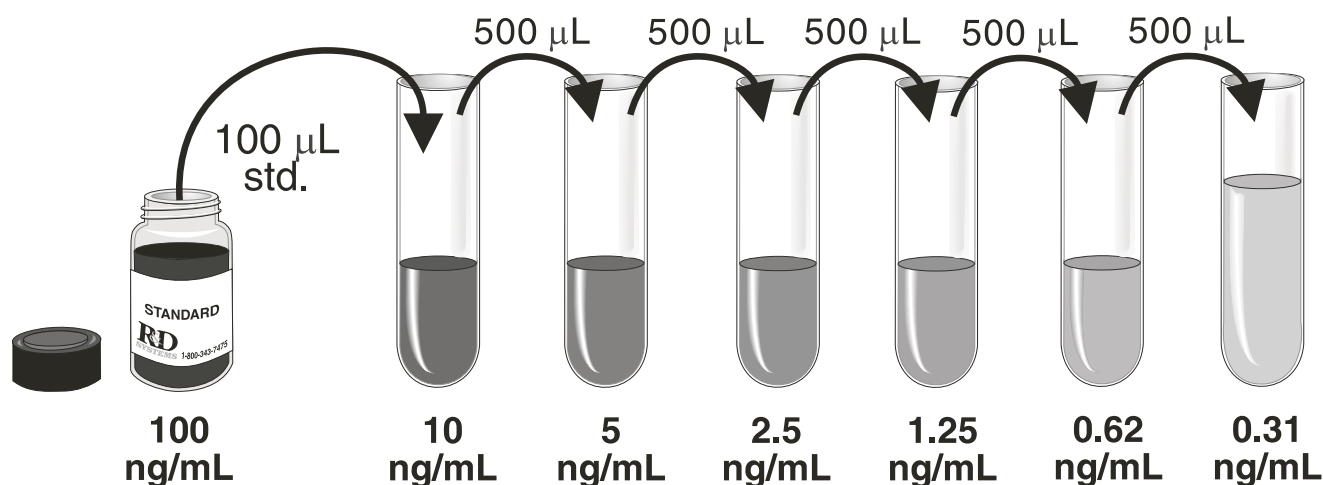
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

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

Calibrator Diluent RD5P (1X) - Dilute 20 mL of Calibrator Diluent RD5P Concentrate into 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5P (1X).

Endostatin Standard - Reconstitute the Endostatin 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 μ L of Calibrator Diluent RD5P (1X) into the 10 ng/mL tube. Pipette 500 μ L of Calibrator Diluent RD5P (1X) 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 (1X) 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 RD1W 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. 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 Endostatin 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.

*Serum/plasma samples require dilution. See Sample Preparation section.

ASSAY PROCEDURE SUMMARY

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



2. Add 100 μL Assay Diluent RD1W to each well.



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



4. Aspirate and wash 4 times.



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



6. Aspirate and wash 4 times.



7. Add 200 μL Substrate Solution to each well. Incubate 30 minutes **on the benchtop. 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 Sample Preparation.

CALCULATION OF RESULTS

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

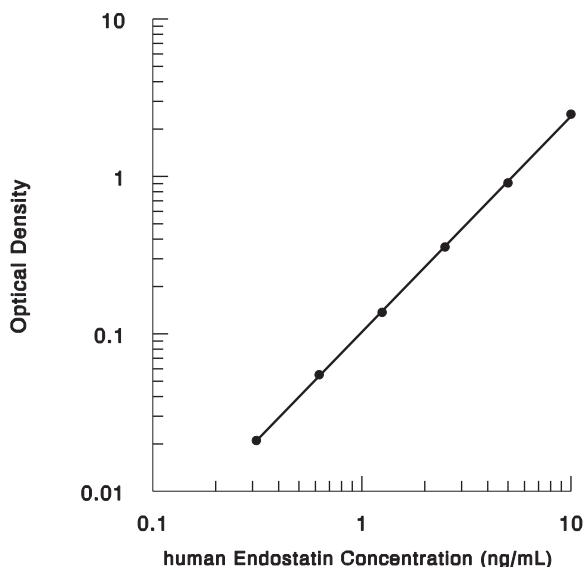
Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation.

To determine the Endostatin 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 Endostatin concentration.

Since 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	0.019 0.019	0.019	—
0.31	0.041 0.074	0.040	0.021
0.62	0.074 0.152	0.074	0.055
1.25	0.159 0.366	0.156	0.137
2.5	0.383 0.922	0.375	0.356
5	0.934 2.433	0.928	0.909
10	2.573	2.503	2.484

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.

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	0.70	2.02	4.14	0.76	2.13	4.21
Standard deviation	0.05	0.12	0.15	0.06	0.13	0.24
CV (%)	6.9	6.0	3.6	7.9	6.1	5.7

RECOVERY

The recovery of Endostatin spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample	Average % Recovery	Range
Cell culture media (n=4)	101	91 - 114%
Serum (n=4)	100	93 - 110%
EDTA plasma (n=4)	100	92 - 110%
Heparin plasma (n=4)	98	86 - 108%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Endostatin were serially diluted with Calibrator Diluent RD5P (1X) to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	Heparin plasma (n=4)	EDTA plasma (n=4)
1:2	Average % of Expected	95	98	103	97
	Range (%)	94 - 96	91 - 101	97 - 115	93 - 101
1:4	Average % of Expected	91	102	104	99
	Range (%)	89 - 92	92 - 109	97 - 115	92 - 105
1:8	Average % of Expected	92	101	103	98
	Range (%)	91 - 94	93 - 109	93 - 113	94 - 103

SENSITIVITY

Fifty-one assays were evaluated and the minimum detectable dose (MDD) of Endostatin ranged from 0.001 - 0.063 ng/mL. The mean MDD was 0.023 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 *E. coli*-expressed recombinant human Endostatin produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples drawn from apparently healthy volunteers were evaluated for the presence of Endostatin 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)	122	58 - 232	30
EDTA Plasma (n=35)	120	69 - 172	26
Heparin Plasma (n=35)	128	65 - 196	28

Saliva - Seven saliva samples were evaluated for the presence of Endostatin in this assay. One of the samples measured below the lowest standard (0.31 ng/mL). The other six samples had a mean value of 0.91 ng/mL and a range of 0.39 - 1.87 ng/mL.

Cell Culture Supernates - Human umbilical vein endothelial cells (HUVEC) 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. Two samples were tested for the presence of Endostatin and measured 34.0 ng/mL and 70.9 ng/mL.

SPECIFICITY

This assay recognizes both natural and recombinant human Endostatin. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5P (1X) and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human Endostatin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Angiopoietin-1	HB-EGF
Angiopoietin-2	HGF
Angiostatin	HRG-2
β-ECGF	IGF-I
β-NGF	IGF-II
EGF	KGF (FGF-7)
FGF acidic	M-CSF
FGF basic	MSP
FGF-4	MSP β-chain
FGF-5	PDGF-AA
FGF-6	PDGF-AB
FGF-9	PDGF-BB
FGF-10	PD-ECGF
FGF-18	P/IGF
Flt-3/Flk-2 ligand	VEGF ₁₂₁
Flt-4	VEGF ₁₆₅
G-CSF	VEGF/P/IGF
GM-CSF	VEGF-D

Recombinant mouse:

FGF-8b
FGF-8c
Flt-3/Flk-2 ligand
G-CSF
GM-CSF
M-CSF
P/IGF-2
VEGF₁₂₀
VEGF₁₆₄

Recombinant porcine:

GM-CSF

Recombinant rat:

GM-CSF
β-NGF
PDGF-BB

Other:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF

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PLATE LAYOUT

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

	A	B	C	D	E	F	G	H
1								
2								
3								
4								
5								
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8								
9								
10								
11								
12								