Quantikine® ELISA

Human Follistatin Immunoassay

Catalog Number DFN00

For the quantitative determination of human Follistatin concentrations in follicular fluid, 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

Follistatin (FS) was first identified as a follicle-stimulating hormone inhibiting substance (*i.e.* structurally distinct from inhibin) present in ovarian follicular fluid (1, 2). It has since been shown to be a multifunctional regulatory protein exerting a majority of its effects via neutralization of activin. FS is a monomeric binding protein that regulates activin activity by forming an inactive complex. FS plays a role as a tissue regulator in the gonad, pituitary gland, pregnancy membranes, vasculature, and liver (for reviews, see references 3 and 4). It is also essential for normal development, as FS-knockout mice die shortly after birth with a range of defects including insufficient muscle development and skeletal abnormalities (5).

FS is a single-chain polypeptide with a structure unlike activin or inhibin. Alternative splicing events, proteolytic cleavage and variable glycosylation lead to a vast array of possible FS isoforms found within various biological fluids (for reviews, see references 3 and 4). Differential mRNA processing produces core proteins of FS that vary from 32-40 kDa. Removal of the signal peptide yields mature polypeptides of 315 (FS315) and 288 (FS288) amino acids. The longer FS315 variant is predominant whereas FS288 accounts for less than 5% of the encoded mRNA (6). FS315 may also be proteolytically cleaved *in vivo* at the C-terminal end to yield an ~300 amino acid form (1).

FS is a member of a larger group of proteins that contain a well-conserved secondary structure that has been termed the "follistatin" domain. This domain consists of a cysteine-rich sequence with similarity to EGF and the Kazal family of enzyme inhibitors. FS family members have an insertion in the ovomucoid-like inhibitory loop that prevents protease activity within the FS domain (7). Family members have between one and nine FS domains. FS itself contains three. Other members of this family include SPARC, agrin, testican, hevin, QR1, FRP, X7365, mac25, Ovomucoid, Factor I, Complement C6, and Complement C7 (3).

There is a broad distribution of FS in adult tissues. It is not confined solely to those involved in reproduction. FS has been detected in pituitary, placenta, ovary, testis, prostate, adrenal glands, thyroid, brain, bone marrow, endochondral bone, pancreas, liver, kidney, and blood vessels (*i.e.* endothelial cells and smooth muscle cells). FS is commonly found co-localized within a tissue with activin subunits or activin receptors. Activin/FS complexes may also bind extracellular matrix components thus forming reservoirs of activin/FS. Within the circulation, 70-90% of FS exists in the bound state (8).

FS binds to the common β_A and β_B subunits of activin and inhibin. Therefore, activin has two binding sites for FS (*i.e.* one activin dimer and two FS monomers make up the activin-FS complex), whereas inhibin has only one (9, 10). Activin A, AB, or B each binds FS with similar affinity (11). Since inhibin contains only one β-subunit, it binds FS with a lower affinity (10, 12, 13).

FS does not bind activin exclusively. It can bind other members of the TGF-β superfamily, including BMP-4 and BMP-7 (14, 15). It is also capable of binding the serum protein α_2-macroglobulin, itself another binding protein for activin (16). FS has a strong affinity for heparan sulfate and can associate with heparan sulfate proteoglycans on cell surfaces (17, 18). Following binding to cell surface proteoglycans, activin/FS complexes can be rapidly endocytosed by lysosomal enzymes (19, 20).

The Quantikine Human Follistatin Immunoassay is a 5.5 hour solid-phase ELISA designed to measure human Follistatin (FS288, FS300 and FS315) in follicular fluid, cell culture supernates, serum, and plasma. It contains *Sf* 21-expressed recombinant human Follistatin and antibodies raised against the recombinant factor. It has been shown to accurately quantitate the recombinant human Follistatin. Results obtained using natural Follistatin 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 Follistatin.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Follistatin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Follistatin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Follistatin 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 Follistatin 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 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.
- 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 Follistatin Microplate	890807	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Follistatin.	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 Follistatin Conjugate	890808	21 mL of a monoclonal antibody specific for human Follistatin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Follistatin Standard	890809	Recombinant human Follistatin in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for recontitution volume.*	
Assay Diluent RD1-8	895465	11 mL of a buffered protein base with blue dye and preservatives.	
Calibrator Diluent RD5-21	895348	21 mL of a buffered protein base with preservatives. *For cell culture supernate samples.*	
Calibrator Diluent RD5L	895028	21 mL of a concentrated buffered protein base with preservatives. *For follicular fluid/serum/plasma samples. 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.
- 2-8 °C refrigerator for plate incubation.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human Follistatin Controls (optional; available from R&D Systems).

PRECAUTIONS

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. Please 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.

Follicular Fluid - Aliquot and store samples at ≤ -70 °C. Avoid repeated freeze-thaw cycles.

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.

Plasma - Collect plasma using 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: *Heparin plasma is not suitable for use in this assay.*
Citrate plasma has not been validated for use in this assay.

SAMPLE PREPARATION

Follicular fluid samples require a 40-fold dilution. A suggested 40-fold dilution is 10 µL of sample + 390 µL of Calibrator Diluent RD5L (diluted 1:2)*.

Cell culture supernate samples may require up to a 10-fold dilution. A suggested 10-fold dilution is 25 µL of sample + 225 µL of Calibrator Diluent RD5-21.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents (except Conjugate) to room temperature before use.

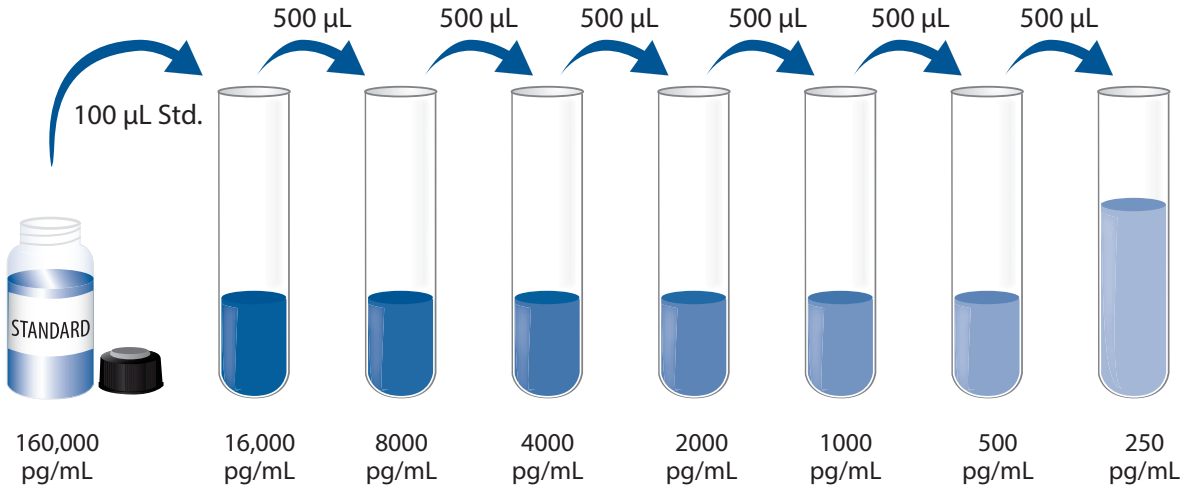
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 RD5L (diluted 1:2) - Dilute 20 mL of Calibrator Diluent RD5L concentrate into deionized or distilled water to prepare 40 mL of Calibrator Diluent RD5L (diluted 1:2).

Human Follistatin Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Follistatin Standard with deionized or distilled water. This reconstitution produces a stock solution of 160,000 pg/mL. Allow the standard to sit for a minimum of 30 minutes with gentle mixing to ensure complete reconstitution prior to making dilutions.

Use polypropylene tubes. Pipette 900 µL of Calibrator Diluent RD5-21 (*for cell culture supernate samples*) or Calibrator Diluent RD5L (diluted 1:2) (*for follicular fluid/serum/plasma samples*) into the 16,000 pg/mL tube. Pipette 500 µL of the appropriate Calibrator Diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 16,000 pg/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Conjugate should remain at 2-8 °C until use. Bring all other reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate.

1. Prepare all reagents and working standards 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 Assay Diluent RD1-8 to each well.
4. Add 100 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 3 hours at **2-8 °C.**
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 toweling.
6. Add 200 µL of **cold** Human Follistatin Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at **2-8 °C.**
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. **Protect from light.**
   For cell culture supernate samples: Incubate 20 minutes at **room temperature.**
   For follicular fluid/serum/plasma samples: Incubate 30 minutes at **room temperature.**
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.).

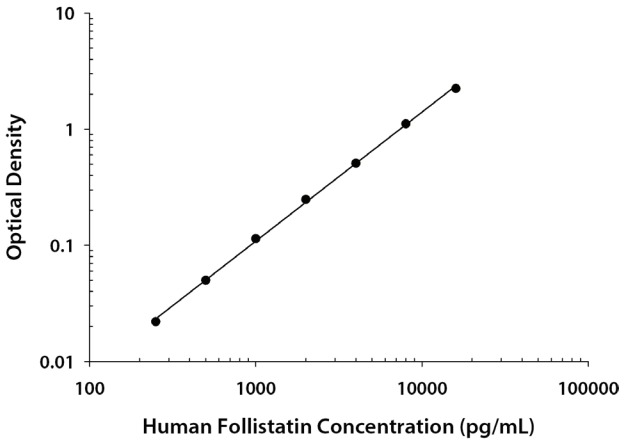
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human Follistatin concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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

TYPICAL DATA

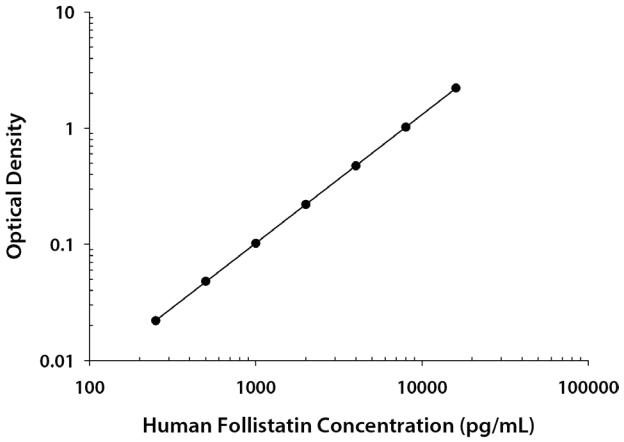
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.005 0.007	0.006	—
250	0.029 0.028	0.028	0.022
500	0.057 0.055	0.056	0.050
1000	0.121 0.119	0.120	0.114
2000	0.255 0.253	0.254	0.248
4000	0.514 0.515	0.514	0.508
8000	1.143 1.088	1.116	1.110
16,000	2.284 2.200	2.242	2.236

FOLLICULAR FLUID/SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.005 0.007	0.006	—
250	0.029 0.028	0.028	0.022
500	0.055 0.054	0.054	0.048
1000	0.109 0.106	0.108	0.102
2000	0.229 0.223	0.226	0.220
4000	0.479 0.479	0.479	0.473
8000	1.044 1.008	1.026	1.020
16,000	2.179 2.258	2.218	2.212

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.

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)	1850	5363	10734	1645	4850	10256
Standard deviation	59	136	181	139	312	584
CV (%)	3.2	2.5	1.7	8.4	6.4	5.7

FOLLICULAR FLUID/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)	1996	6047	11999	1888	5604	11626
Standard deviation	53	144	239	173	399	918
CV (%)	2.7	2.4	2.0	9.2	7.1	7.9

RECOVERY

The recovery of human Follistatin spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	97	91-107%
EDTA plasma (n=5)	96	87-102%
Serum (n=5)	95	87-104%

SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of human Follistatin ranged from 10-83 pg/mL. The mean MDD was 29 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.

LINEARITY

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

		Follicular fluid* (n=6)	Cell culture media (n=6)	Serum (n=5)	EDTA plasma (n=5)
1:2	Average % of Expected	103	97	100	99
	Range (%)	101-108	94-99	97-104	97-101
1:4	Average % of Expected	105	97	102	99
	Range (%)	100-109	94-100	95-110	92-102
1:8	Average % of Expected	105	97	101	98
	Range (%)	99-112	95-98	89-113	90-103
1:16	Average % of Expected	106	99	99	96
	Range (%)	101-114	92-103	87-115	82-109

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

CALIBRATION

This immunoassay is calibrated against a highly purified *Sf* 21-expressed recombinant human Follistatin (FS300) produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human Follistatin in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=54)	2483	889-11,123	1676
EDTA Plasma (n=29)	2636	954-10,902	2066

Cell Culture Supernates - PC-3 human prostate cancer cells were cultured in RPMI supplemented with 5% fetal calf serum and stimulated with 10 ng/mL of recombinant human TNF-α until confluent. Samples were diluted 1:10 and 1:20 prior to assay and the results were averaged.

Cell Line	Unstimulated (ng/mL)	Stimulated (ng/mL)
PC-3	185	170

Follicular Fluid - Twelve follicular fluid samples were evaluated for the presence of human Follistatin in this assay. The sample values ranged from 244-480 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Follistatin (isoforms FS288, FS300, and FS315).

The factors listed below were prepared at 10 ng/mL and 100 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 150 ng/mL in a mid-range recombinant human Follistatin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Activin RI
Activin RIIα
Activin RIIβ
BMP-4
BMP-5
BMP-6
Inhibin A
Inhibin B
α_2-Macroglobulin
TGF-β RI
TGF-β RIII

Recombinant rat:

Agrin

Recombinant mouse Follistatin cross-reacts approximately 26% in this assay.

Recombinant human Activin A, recombinant human Activin AB, and recombinant human Activin B were added to a serum sample at concentrations ranging from 80-100 ng/mL. No cross-reactivity was observed, but the Activins interfered at concentrations ≥ 2 ng/mL.

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