Quantikine® ELISA

Human Coagulation Factor III/Tissue Factor Immunoassay

Catalog Number DCF300

For the quantitative determination of human Coagulation Factor III concentrations in cell culture supernates, cell lysates, plasma, and urine.

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

Blood clotting can be triggered through the intrinsic or extrinsic coagulation pathways. Coagulation Factor III (also known as Tissue Factor, Thromboplastin, and CD142) is the primary initiator of the extrinsic coagulation pathway. Coagulation Factor III is a glycoprotein expressed in the subendothelial structure throughout the vasculature. When the lining of the blood vessel wall is damaged, Coagulation Factor III comes in contact with the bloodstream and binds to Coagulation Factor VII. Once Coagulation Factor VII is complexed with Coagulation Factor III, it is converted to the active form (FVIIa). The Coagulation Factor III-FVIIa complex then proteolytically activates downstream coagulation factors, including Coagulation Factor IX and Coagulation Factor X, ultimately leading to the conversion of prothrombin to thrombin and fibrin clot formation. Coagulation Factor III is a 47 kDa transmembrane protein, which consists of an extracellular domain, a transmembrane domain, and a cytoplasmic tail (1-3). In the vessel wall, Coagulation Factor III is constitutively expressed in subendothelial cells, such as vascular smooth muscle cells. Other cell types, such as endothelial cells and monocytes, are also able to express Coagulation Factor III when stimulated with cytokines and growth factors (4-5). Coagulation Factor III can be released into the circulation in association with microparticles (6). Because of its essential function in the coagulation cascade, it is well established that Coagulation Factor III plays an important role in pathological conditions related to hemostasis, such as thrombotic diseases and disseminated intravascular coagulation (7, 8). In atherosclerosis, Coagulation Factor III is abundantly expressed in the plaque and may contribute to thrombus formation once the plaque is ruptured (9). Besides coagulation, it has now been recognized that Coagulation Factor III is also involved in non-hemostatic processes, such as inflammation, angiogenesis, and tumor growth and metastasis. Carmeliet *et al.* have reported that mice lacking Coagulation Factor III die at the embryonic stage due to abnormal development of vasculature (10). *In vitro* assays show that Coagulation Factor III-FVIIa can facilitate cell migration (11-13). Furthermore, Coagulation Factor III-FVIIa has been found to contribute to apoptosis resistance and increased survival of cancer cell lines (14). How Coagulation Factor III modulates these cellular processes is not yet fully understood. Accumulating experimental evidence suggests that Coagulation Factor III is able to communicate with other intracellular signaling pathways. Coagulation Factor III-FVIIa complex can proteolytically activate protease-activated receptors, through which it is connected to other signaling pathways. Alternatively, Coagulation Factor III can directly interact with other signaling molecules through its cytoplasmic tail (15-17).

The Quantikine® Human Coagulation Factor III/Tissue Factor Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Coagulation Factor III in cell culture supernates, cell lysates, plasma, and urine. It contains NS0-expressed recombinant human Coagulation Factor III and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Coagulation Factor III 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 Coagulation Factor III.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Coagulation Factor III has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Coagulation Factor III present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Coagulation Factor III 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 Coagulation Factor III 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 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 Coagulation Factor III Microplate	893667	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Coagulation Factor III.	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 Coagulation Factor III Conjugate	893668	21 mL of a polyclonal antibody specific for human Coagulation Factor III conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Coagulation Factor III Standard	893669	Recombinant human Coagulation Factor III in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-89	895881	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-20	895346	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 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.
- 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 and samples.
- Human Coagulation Factor III Controls (optional; R&D Systems®, Catalog # QC65).

If using cell lysate samples, the following are also required:

- Cell Lysis Buffer 1 (R&D Systems®, Catalog # 890713).
- PBS

PRECAUTIONS

Cell Lysis Buffer 1 contains sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

High concentrations of Coagulation Factor III are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.

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.

Note: *Serum is not a valid sample type in this assay due to its role in coagulation.*

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Prior to assay, cells must be lysed according to the directions in the Sample Values section.

Plasma - Collect plasma using EDTA , heparin, or citrate 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.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

All samples require at least a 2-fold dilution prior to assay. A suggested 2-fold dilution is 150 µL of sample + 150 µL of Calibrator Diluent RD5-20.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

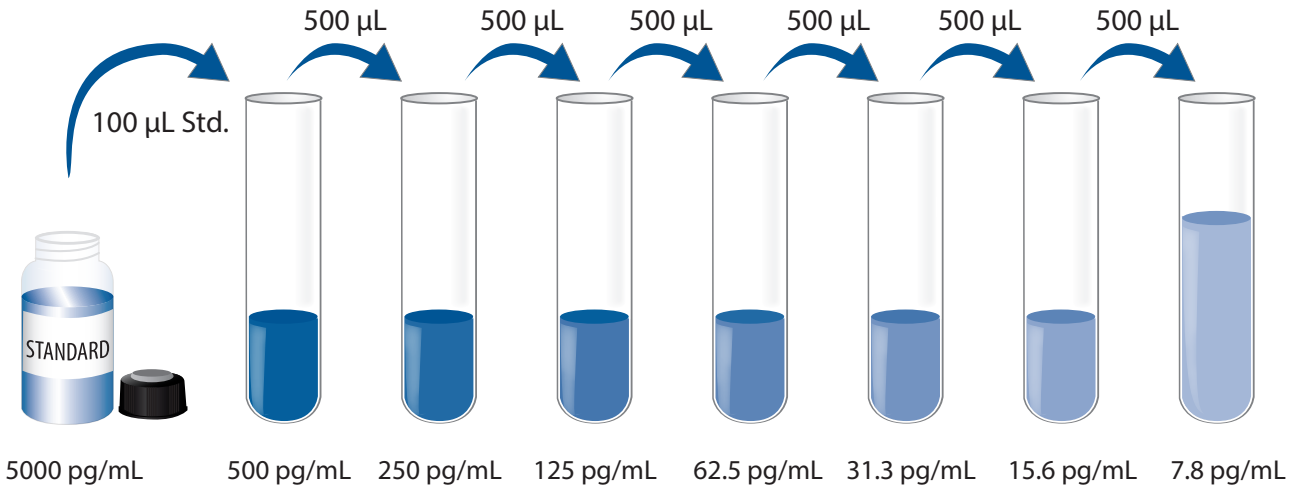
Note: *High concentrations of Coagulation Factor III are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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.

Human Coagulation Factor III Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Coagulation Factor III Standard with deionized or distilled water. This reconstitution produces a stock solution of 5000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5-20 into the 500 pg/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 500 pg/mL standard serves as the high standard. Calibrator Diluent RD-20 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, controls, and standards be assayed in duplicate.

Note: *High concentrations of Coagulation Factor III are found in saliva. It is recommended that a face mask and gloves be 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 RD1-89 to each well.
4. Add 100 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 Human Coagulation Factor III 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 require dilution prior to assay. See the Sample Preparation section.

CALCULATION OF RESULTS

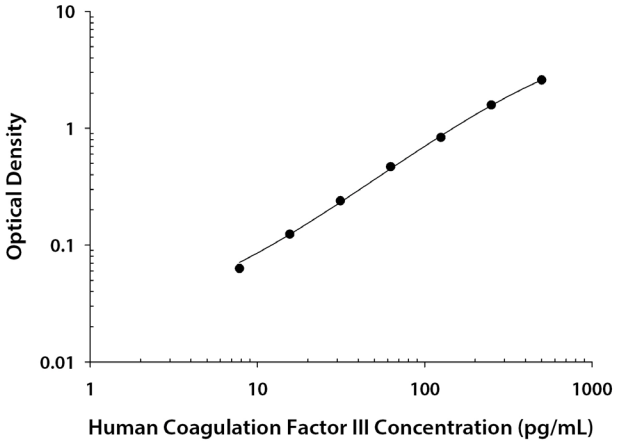
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 Coagulation Factor III 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.

Since samples have been diluted prior to assay, 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.



(pg/mL)	O.D.	Average	Corrected
0	0.016 0.018	0.017	—
7.8	0.080 0.080	0.080	0.063
15.6	0.141 0.141	0.141	0.124
31.3	0.247 0.264	0.256	0.239
62.5	0.480 0.489	0.485	0.468
125	0.797 0.902	0.850	0.833
250	1.555 1.638	1.597	1.580
500	2.591 2.609	2.600	2.583

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 (pg/mL)	33.4	65.4	148	71	144	301
Standard deviation	0.78	1.81	5.0	4.5	7.8	17.1
CV (%)	2.3	2.8	3.4	6.3	5.4	5.7

RECOVERY

The recovery of human Coagulation Factor III spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	104	95-110%
EDTA plasma* (n=4)	91	86-97%
Heparin plasma* (n=4)	95	88-109%
Citrate plasma* (n=4)	92	86-102%
Urine* (n=4)	102	96-112%

*Samples were diluted prior to assay.

LINEARITY

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

		Cell culture media (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Citrate plasma* (n=4)	Urine* (n=4)
1:2	Average % of Expected	99	100	103	101	93
	Range (%)	97-100	95-102	102-103	100-103	92-94
1:4	Average % of Expected	102	105	108	103	92
	Range (%)	101-103	98-110	105-111	102-104	90-94
1:8	Average % of Expected	104	107	111	103	92
	Range (%)	100-106	99-110	108-115	102-104	89-96
1:16	Average % of Expected	100	110	110	97	91
	Range (%)	96-103	106-112	104-114	95-100	88-93

*Samples were diluted prior to assay.

SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of human Coagulation Factor III ranged from 0.16-2.05 pg/mL. The mean MDD was 0.69 pg/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 Coagulation Factor III produced at R&D Systems®.

SAMPLE VALUES

Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human Coagulation Factor III 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)
EDTA plasma (n=36)	39.2	25.0-57.8	7.5
Heparin plasma (n=36)	36.1	22.6-53.6	7.2
Citrate plasma (n=36)	33.3	21.0-49.4	6.6
Urine (n=11)	79.9	20.7-151	47.5

Cell Culture Supernates:
U-87 MG human glioblastoma/astrocytoma cells were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 1 mM sodium pyruvate, and 100 mg/mL of streptomycin sulfate in a T75 flask until confluent. An aliquot of the cell culture supernate was removed, assayed for human Coagulation Factor III, and measured 282 pg/mL.

HASMC human aortic smooth muscle cells were cultured in Cascade™ medium 231 supplemented with smooth muscle cell growth supplement in a T75 flask until confluent. An aliquot of the cell culture supernate was removed, assayed for human Coagulation Factor III, and measured 27.8 pg/mL.

Cell Lysates - U-87 MG human glioblastoma/astrocytoma cells and HASMC human aortic smooth muscle cells were cultured as described above in a T75 flask until confluent. After the supernate was removed, 10 mL of Cell Lysis Buffer 1 was added and incubated for 30-60 minutes at room temperature with gentle agitation. The cell lysate was centrifuged at 12,000 rpm for 10 minutes to remove cell debris. An aliquot of the lysis supernate was removed and assayed for human Coagulation Factor III. The U-87 MG sample measured 6500 pg/mL and HASMC sample measured 286 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Coagulation Factor III.

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 recombinant human Coagulation Factor III control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Coagulation Factor VII
Coagulation Factor Xa
Coagulation Factor XI
Coagulation Factor XIV/Protein C
Protein S
TFPI
TFPI-2
Thrombin

Recombinant mouse:

Coagulation Factor III
Coagulation Factor VII
Coagulation Factor XI
TFPI
TFPI-2

REFERENCES

1. Camerer, E. *et al.* (1996) Thromb. Res. **81**:1.
2. Morrissey, J.H. *et al.* (1987) Cell **50**:129.
3. Spicer, E.K. *et al.* (1987) Proc. Natl. Acad. Sci. USA **84**:5148.
4. Napoleone, E. *et al.* (1997) Blood **89**:541.
5. Bavendiek, U. *et al.* (2002) J. Biol. Chem. **277**:25032.
6. Giesen, P.L. *et al.* (1999) Proc. Natl. Acad. Sci. USA **96**:2311.
7. Mackman, N. *et al.* (2006) Blood Cells Mol. Dis. **36**:104.
8. Levi, M. (2007) Crit. Care Med. **35**:2191.
9. Wilcox, J.N. *et al.* (1989) Proc. Natl. Acad. Sci. USA **86**:2839.
10. Carmeliet, P. *et al.* (1996) Nature **383**:73.
11. Contrino, J. *et al.* (1996) Nature Med. **2**:209.
12. Bromberg, M.E. *et al.* (1995) Proc. Natl. Acad. Sci. USA **92**:8205.
13. Mueller, B.M. *et al.* (1998) J. Clin. Invest. **101**:1372.
14. Sorensen, B.B. *et al.* (2003) Blood **102**:1708.
15. Camerer, E. *et al.* (2000) Proc. Natl. Acad. Sci. USA **97**:5255.
16. Rao, L.V. *et al.* (2005) Arterioscler. Thromb. Vasc. Biol. **25**:47.
17. Belting, M. *et al.* (2004) Nature Med. **10**:502.