Version 06-02/25

User's Manual

Human TGF-β1 ELISA Kit

REF **DEIA2649**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The Human TGF-beta 1 Immunoassay is a 4.5 hour solid phase ELISA designed to measure TGF-beta 1 in acid activated cell culture supernates, serum, plasma, and urine. It contains recombinant human TGF-beta 1 expressed by CHO cells and has been shown to quantitate the recombinant factor accurately. Results obtained using natural TGF-beta 1 showed linear curves that were parallel to the standard curves obtained using the recombinant kit standards. These results indicate that this kit will provide accurate quantitation for both recombinant and natural human TGF-beta 1.

General Description

Human TGF-β1 is a 25 kDa, disulfide-linked homodimeric protein involved in a number of key developmental, immunologic, and homeostatic processes (1-4). The molecule is synthesized as a 390 amino acid (aa) precursor that contains a 23 aa signal sequence, a 255 aa pro-region, and a 112 aa mature segment. Processing of the molecule is complex, and it is generally secreted as a latent form (5). Prior to release, the prepro-form is cleaved of its signal sequence, followed by glycosylation of its pro-region. The glycosylation process includes the unusual attachment of mannose-6 phosphate residues. This is followed by furin convertase-mediated cleavage of the prohormone, creating an 80 kDa disulfide-linked proregion (termed LAP for latency-associated protein), plus a 25 kDa disulfidelinked mature segment (termed TGF-β1) (6-8). These two independent disulfide-linked polypeptides associate in a non-covalent interaction that renders TGF-β1 inactive. Although direct secretion of this 80K:25K complex can occur, it does so inefficiently. To facilitate secretion plus extracellular storage, a third 200 kDa component termed LTBP is covalently-linked to the N-terminus of one of the two LAP polypeptide chains. This promotes secretion and subsequent storage within the extracellular matrix (9, 10). After secretion, TGF-β1, via LTBP, covalently links to ECM. This complex is later cleaved by proteases and released, exposing mannose residues on LAP. It is postulated that exposed LAP mannose residues now are able to bind to cell surface IGF-II R, where dissociative events disrupt the LAP-TGF-β1 complex. This results in the release of active, homodimeric TGF-β1 (7, 10). Mature mouse TGF-β1 shares 100% aa sequence identity with rat and cotton rat TGF-β1 (11, 12), 99% aa identity with human, canine, and porcine TGF-β1 (13, 14, 15), and 97% aa identity with guinea pig TGF-β1 (16). Relative to mouse TGF-β2 and β3, mature mouse TGF-β1 shares 72% and 78% aa sequence identity, respectively (17, 18). The traditional high-affinity receptor for TGF-β1 is a heteromeric complex consisting of transmembrane serine/threonine kinases. Two types are involved; a constitutively phosphorylated, ligand-binding 80 kDa glycoprotein termed TβRII and a signal transducing, non-ligand-binding 55 kDa glycoprotein termed TβRI/ALK-5 (19-22). It is suggested that TGF-β1first binds TβRII, which then initiates a crossphosphorylation of TβRI, culminating in signal transduction. There is also a third TGF-β receptor termed TβRIII, which can be either the 250 kDa proteoglycan named betaglycan, or the 180 kDa glycoprotein termed endoglin/CD105 (23, 24). It has been proposed that TβRIII captures TGF-β and "passes" it to TβRII (20). This is perhaps true for betaglycan but not endoglin. Endoglin does not bind TGF-β by itself; only within the context of TβRII ligand binding. Evidence suggests that rather than "passing" on ligand, endoglin may actually enter the receptor complex and modulate TGF-β downstream signaling (25, 26).

Finally, and although ALK-5 has traditionally been assumed to be the only type I signaling receptor for TGF-β1, it is also possible that ALK-1 may serve as a condition-dependent, type I TGF-β receptor (27). TGF-β1 has a wide range of activities. During an immune response, TGF-β1 impacts antibody production by

preferentially induceing IgA production in both mouse and human (28). It also regulates dendritic cell chemotaxis by altering the expression of chemokine receptors (29). Finally, it can downmodulate an inflammatory response by dampening macrophage activity and proinflammatory secretion (30). During wound healing, TGF-β1 is released from activated platelets. This local source of TGF-β1 has marked stimulatory effects on fibroblasts, where it induces matrix synthesis; on monocytes, where it induces proinflammatory mediator and growth factor secretion; and on keratinocytes, where it may promote keratinocyte proliferation by downmodulating its own signaling pathway (31). Finally, during development, TGF-β1 may play a role in endochondral ossification, and its absence results in severely defective yolk sac vasculogenesis and hematopoiesis (32, 33).

Principles of Testing

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TGF-β1 has been pre-coated onto a microplate. Standards, controls and samples are pipetted into the wells and any TGF-β1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β1 is added to the wells to sandwich the TGF-β1 immobilized during the first incubation. 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 TGF-β1 bound in the initial step. The color development is stopped and the intensity of the color is measured.

Materials Required But Not Supplied

ADDITIONAL REAGENTS REQUIRED

For sample activation:

Note: The reagents listed below are available as Sample Activation Kit 1 (CD, Catalog # EZ121).

• Hydrochloric acid (A.C.S. Grade, 12 N)

• Sodium hydroxide (A.C.S. Grade, 10 N)

• HEPES, free acid (Reagent Grade, M.W. 238.3)

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.

• Polypropylene test tubes for dilution of standards and activation of samples.

• Human TGF-β1 Controls (optional).

Storage

2-8°C

Specimen Collection And Preparation

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 (see activation procedure) immediately or aliquot and store samples at ≤ -70°C. Avoid repeated freeze-thaw cycles.

Note: Animal serum used in the preparation of cell culture media may contain high levels of latent TGF-β1. For best results, do not use animal serum for growth of cell cultures when assaying for TGF-β1 production. If animal serum is used as a supplement in the media, precautions should be taken to prepare the appropriate control and run the control in the immunoassay to determine the baseline concentration of TGF-β1.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature. Centrifuge for 15 minutes at 1000 x g. Remove serum and assay (see activation procedure) immediately or aliquot and store at ≤ -70°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. Assay immediately (see activation procedure) or aliquot and store at ≤ -20°C. Avoid repeated freeze/thaw cycles.

Note: Neat unactivated urine samples exhibit a decrease in TGF-β1 concentration in the first 24 hours of storage (frozen or refrigerated). Care should be taken that samples are assayed under identical storage conditions and durations.

Platelet-poor Plasma* - Collect plasma on ice using EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8°C is recommended for complete platelet removal. Assay (see activation procedure) immediately or aliquot and store samples at ≤ -70°C. Avoid repeated freeze-thaw cycles.

*TGF-β1 is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of TGF-β1, platelet-poor plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical Laboratory Standards Institute (CLSI), result in incomplete removal of platelets from blood. This will cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation. The recommended plasma collection protocol is designed to minimize platelet degranulation. However, since even the best methods for plasma collection may result in some platelet degranulation on occasion, it is recommended that markers for platelet degranulation be determined in samples containing elevated TGF-β1 levels.

TGF-β1 SAMPLE ACTIVATION PROCEDURE

To activate latent TGF-β1 to immunoreactive TGF-β1 detectable by the TGF-β1 immunoassay, follow the activation procedure outlined below. Assay samples after neutralization (pH 7.2-7.6). Use polypropylene test tubes.

Note: Do not activate the kit standards. The kit standards contain active recombinant TGF-β1.

Cell Culture Supernates/Urine	Serum/Plasma
To 100 µL of cell culture supernate, add 20 µL of 1 N HCl.	To 40 µL serum/plasma, add 20 µL of 1 N HCl
Mix well.	Mix well.
Incubate 10 minutes at room temperature.	Incubate 10 minutes at room temperature.
Neutralize the acidified sample by adding 20 µL of 1.2 N NaOH/0.5 M HEPES.	Neutralize the acidified sample by adding 20 µL of 1.2 N NaOH/0.5 M HEPES.
Mix well.	Mix well.
Assay immediately.	Prior to the assay, dilute the activated sample with calibrator diluent. *See the following for suggested dilutions*.
The concentration read off the standard curve must be multiplied by the dilution factor, 1.4.	The concentration read off the standard curve must be multiplied by the appropriate dilution factors.

Activated serum and plasma samples may be stored for up to 24 hours at 2-8 °C before use. Activated cell culture supernates/urine samples must be assayed immediately after activation. Do not freeze activated samples.

Serum samples require a 20-fold dilution in Calibrator Diluent CD6-64 (diluted 1:4). A suggested 20-fold dilution is 10 µL of activated sample + 190 µL of Calibrator Diluent CD6-64 (diluted 1:4) (final dilution factor is 40).

Platelet-poor plasma* samples require a 2-fold dilution in Calibrator Diluent CD6-64 (diluted 1:4). A suggested 2-fold dilution is 80 µL of activated sample + 80 µL of Calibrator Diluent CD6-64 (diluted 1:4) (final dilution factor is 4).

Note: Activated serum and EDTA plasma samples may be stored for up to 24 hours at 2-8 °C before use. Activated cell culture supernates/urine samples must be assayed immediately after activation.

Cell Culture Supernates Note

Significant levels of latent TGF-β1 are found in bovine, porcine, equine, and caprine sera. The reported levels of TGF-β1 in bovine and fetal bovine sera can be as high as 16 ng/mL after activation (34, 35). Therefore, conditioned medium containing 10% fetal bovine serum can be expected to have a TGF-β1 concentration of about 1600 pg/mL. The background level of TGF-β1 in control medium can be determined and subtracted from samples of conditioned medium. As an alternative, the background level of TGF-β1 in medium can be lowered using the following procedure prior to assaying (ref. 10 with modifications). After growth to confluence in medium containing 10% serum, the medium is changed to serum-free medium supplemented with 200 µg/mL crystalline BSA with four changes of medium over 12-24 hours. Cells are then switched to medium alone or medium containing 200 µg/mL crystalline BSA. Particular cell lines may require specific additions to the serum-free medium for maintenance. After 24 hours, the serum-free conditioned medium is clarified by centrifugation and samples are stored at ≤ -70 °C. Optionally, 2 µg/mL aprotinin, leupeptin, pepstatin A, and 120 µg/mL PMSF can be added before freezing. Thawed or fresh samples of serum-free or serumcontaining conditioned medium are processed further as described above. If bovine serum added as a supplement to conditioned media exceeds 5%, further dilute the activated sample at least 2-fold using calibrator diluent. The dilution as a result of the sample activation procedure (1.4-fold) should be taken into consideration in the final calculation of the concentration of TGF-β1 in culture media samples.

Reagent Preparation

To activate latent TGF-β1 to the immunoreactive form, prepare the following solutions for acid activation 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 during preparation or use of these reagents. Refer to appropriate MSDS before use.

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

1.2 N NaOH/0.5 M HEPES (100 mL) - To 75 mL of deionized water, slowly add 12 mL of 10 N NaOH. Mix well. Add 11.9 g of HEPES. Mix well. Bring final volume to 100 mL with deionized water.

For each new lot of acidification and neutralization reagents, measure the pH of several representative samples after neutralization to ensure that it is within pH 7.2-7.6. Adjust the volume and corresponding dilution factor of the neutralization reagent as needed.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

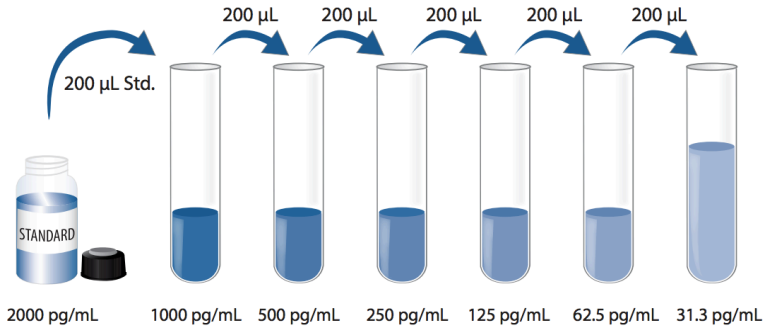
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent CD6-64 (diluted 1:4) - Add 20 mL of Calibrator Diluent CD6-64 Concentrate to 60 mL of deionized or distilled water to prepare 80 mL of Calibrator Diluent CD6-64 (diluted 1:4). May contain a precipitate. Mix well before and during use.

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.

TGF-β1 Standard - Refer to the vial label for reconstitution volume. Reconstitute the TGF-β1 Standard with Calibrator Diluent CD6-64 (diluted 1:4). Do not substitute other diluents. This reconstitution produces a stock solution of 2000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent CD6-64 (diluted 1:4) into each tube. Use the standard stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted TGF-β1 Standard (2000 pg/mL) serves as the high standard. Calibrator Diluent CD6-64 (diluted 1:4) serves as the zero standard (0 pg/mL). Discard any unused reconstituted TGF-β1 Standard after use.



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, standard dilutions, and activated 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 50 µL of Assay Diluent CD2-32 (for cell culture supernate and urine samples) or Assay Diluent CD2-84 (for serum/plasma samples) to each well.
4. Add 50 µL of standard, control, or activated sample* per well. Tap the plate gently to mix. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
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 100 µL of TGF-β1 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.

*Samples may require dilution. See Sample Activation Procedure.

Calculation

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

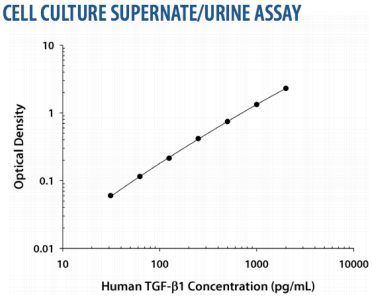
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human TGF-β1 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.

Because samples have been diluted in the activation step prior to the assay, the measured concentrations must be multiplied by the final dilution factor.

Typical Standard Curve

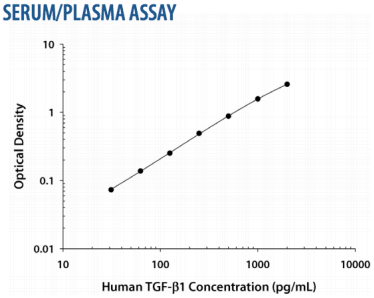
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/URINE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.050 0.052	0.051	—
31.3	0.107 0.114	0.111	0.060
62.5	0.165 0.167	0.166	0.115
125	0.262 0.267	0.265	0.214
250	0.474 0.459	0.467	0.416
500	0.781 0.809	0.795	0.744
1000	1.372 1.390	1.381	1.330
2000	2.322 2.368	2.345	2.294

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.053 0.053	0.053	—
31.3	0.124 0.128	0.126	0.073
62.5	0.189 0.193	0.191	0.138
125	0.303 0.307	0.305	0.252
250	0.542 0.545	0.544	0.491
500	0.924 0.934	0.929	0.876
1000	1.611 1.638	1.625	1.572
2000	2.624 2.630	2.627	2.574

Evaluation

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human TGF-β1 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=23)	39,592	18,289-63,416	9801
Platelet-poor EDTA plasma (n=10)	1165	903-1654	214
Urine* (n=10)	103	ND-257	——

*Only 40% of the urine samples measured detectable levels (> 31.3 pg/mL).
ND=Non-detectable

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 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	22	21	22
Mean (pg/mL)	88	198	816	97	209	826
Standard deviation	3.0	4.9	19.6	8.1	17.3	47.0
CV (%)	3.4	2.5	2.4	8.4	8.3	5.7

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	28	37	30
Mean (pg/mL)	78	180	695	79	185	730
Standard deviation	2.3	3.4	17.3	7.2	17.2	46.8
CV (%)	2.9	1.9	2.5	9.1	9.3	6.4

Sensitivity

Ten assays were evaluated and the minimum detectable dose (MDD) of human TGF-β1 ranged from 1.7-15.4 pg/mL. The mean was 4.61 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.

Specificity

This assay recognizes natural and recombinant human TGF-β1. No significant cross-reactivity or interference was observed.

Cross-reactivity - Latent TGF-β1 complex has approximately 15% cross-reactivity in this assay. This assay also recognizes human TGF-β1.2 and porcine, mouse, rat, and canine TGF-β1.

Linearity

To assess linearity of the assay, activated samples containing and/or spiked with high concentrations of human TGF-β1 were diluted with calibrator diluent and then assayed.

		Cell culture supernates (n=4)	Serum* (n=4)	Platelet-poor EDTA plasma* (n=4)	Urine (n=4)
1:2	Average % of Expected	103	95	104	100
	Range (%)	102-107	94-98	103-106	97-103
1:4	Average % of Expected	107	94	101	97
	Range (%)	105-108	91-97	98-103	94-101
1:8	Average % of Expected	106	91	100	91
	Range (%)	101-109	89-92	98-105	90-100
1:16	Average % of Expected	97	92	100	91
	Range (%)	95-99	89-95	97-107	88-95

*Samples were diluted after activation. See Sample Activation Procedure section.

Recovery

The recovery of human TGF-β1 spiked to levels throughout the range of the assay followed by activation was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n=4)	99	86-115%
Urine (n=3)	96	88-100%

Interferences

Significant interference was observed with recombinant mouse TGF-β RII. However, no naturally occurring soluble mouse TGF-β RII has been reported to date (12), so this observed interference may be of no concern when measuring natural TGF-β1.

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.

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.

Limitations

• 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.

• It is important that the assay diluent selected for the standard curve be consistent with the samples being assayed.

• If samples generate values higher than the highest standard, further dilute the activated 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 other factors present in biological samples. Until all factors have been tested in the Immunoassay, the possibility of interference cannot be excluded.

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