Quantikine® ELISA

Human LAP (TGF-β1) Immunoassay

Catalog Number DLAP00

For the quantitative determination of human Latency Associated Peptide (LAP) of Transforming Growth Factor beta 1 (TGF-β1) concentrations in cell culture supernates, cell lysates, serum, platelet-poor 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

TGF- β1 (Transforming Growth Factor beta 1) and the closely related TGF-β2 and -β3 are members of the large TGF-β superfamily. TGF β proteins are highly pleiotropic cytokines with distinct, but overlapping, activities regulating processes such as immune function, proliferation, and epithelial mesenchymal transition (1-3). Almost all cells can produce TGF-β1, and a particularly high concentration is found in platelets (1-4). Human TGF-β1 cDNA encodes a 390 amino acid (aa) precursor that contains a 29 aa signal peptide and a 361 aa proprotein (2). A furin-like convertase processes the proprotein within the trans-Golgi to generate an N-terminal 249 aa latency-associated peptide (LAP) and a C-terminal 112 aa mature TGF- β1 (2, 3, 5). Disulfide-linked homodimers of LAP and TGF-β1 remain non-covalently associated after secretion, forming the inactive small latent TGF-β1 complex (5-12). Covalent linkage of LAP to one of three latent TGF-β binding proteins (LTBPs) creates a large latent complex that may interact with the extracellular matrix (3, 5-7, 13). TGF-β activation from latency is controlled both spatially and temporally by multiple pathways that may include the actions of proteases such as plasmin, MMP-2 or MMP-9, adhesion through integrins such as αvβ6 and αvβ8, and/or interaction with Thrombospondin-1 (3, 5, 7, 12-14).

Recombinant TGF-β1 LAP is capable of complexing with and inactivating all other human TGF-β isoforms and those of most other species (15). However, the TGF-β1 LAP is associated *in vivo* with TGF-β1 and binds >98% of the TGF-β1 in circulation (16). Its circulating concentration correlates with that of total TGF-β1, making LAP a convenient surrogate in the measurement of TGF-β1 (16). LAP is accessible for antibody recognition whether or not TGF-β1 is bound. In contrast, latent TGF-β1 is surrounded by a LAP "straightjacket", altering its conformation and sequestering it from recognition by receptors or antibodies (4, 8). Acid dissociation of TGF-β1 from its LAP is thus required to assay total TGF-β1 (4, 16). The LAP portion of human TGF-β1 shares 91%, 92%, 85%, 86% and 88% aa identity with porcine, canine, mouse, rat, and equine TGF-β1 LAP, respectively, while the mature human TGF-β1 portion shares 100% aa identity with porcine, canine, and bovine TGF-β1, and 99% aa identity with mouse, rat, and equine TGF-β1. Assays for TGF-β1 LAP may therefore be more species-specific than assays for mature TGF-β1. Assay of human LAP is an especially convenient way to eliminate interference from bovine serum TGF-β1 and its LAP in tissue culture media.

Deletion of the mouse TGF-β1 gene is lethal, either due to defective prenatal hematopoiesis and endothelial differentiation, or to postnatal overwhelming inflammation (17). Mutations within the human TGF-β1 LAP are associated with Camurati-Engelmann disease, a rare sclerosing bone dysplasia characterized by inappropriate presence of active TGF-β1 (7-9, 18). LAP on the surface of immature dendritic cells promotes differentiation and survival of regulatory T cells (T_{reg}) while inhibiting Th1 cell differentiation (19). Latent TGF-β1 is also expressed on the surface of T_{reg}, where it is immobilized by binding of LAP to the alternate latent TGF binding protein, GARP, and contributes to the immunosuppressive role of T_{reg} (14, 20-23).

The Quantikine Human LAP (TGF-β1) Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human LAP (TGF-β1) in cell culture supernates, cell lysates, serum, platelet-poor plasma, and urine. It contains CHO cell-expressed recombinant human Latent TGF-β1 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human LAP (TGF-β1) 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 naturally occurring LAP (TGF-β1).

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human LAP (TGF-β1) has been pre-coated onto a microplate. Standards and activated samples are pipetted into the wells and any LAP (TGF-β1) present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human LAP (TGF-β1) 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 LAP (TGF-β1) 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 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 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
LAP (TGF-β1) Microplate	894558	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against human LAP (TGF-β1).	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.*
LAP (TGF-β1) Conjugate	894559	21 mL of a monoclonal antibody against human LAP (TGF-β1) conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
LAP (TGF-β1) Standard	894560	15 ng of recombinant human Latent TGF-β1 in a buffered protein base with preservatives; lyophilized.	
Assay Diluent RD1-34	895265	12 mL of a buffered protein base with blue dye and preservatives.	
Calibrator Diluent RD6-31	895323	21 mL of buffered animal serum with preservatives. *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.
- 100 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Polypropylene test tubes for dilution of standards and activation and dilution of samples.
- Human LAP (TGF-β1) Controls (optional; available from R&D Systems).

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

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

ADDITIONAL REAGENTS REQUIRED

Note: *Refer to the vendor supplied MSDS for the following items.*

For sample activation:

- 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)

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® 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.

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 Cell Lysis Procedure.

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.

Platelet-poor Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge at 2-8 °C for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the separated plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. 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.*

LAP is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of LAP, platelet-free plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical Laboratory and Standards Institute (CLSI), result in incomplete removal of platelets from blood. This will cause variable and irreproducible results for assays of factors released by platelet activation.

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.

CELL LYSIS PROCEDURE

Use the following procedure for the preparation of cell lysate samples.

1. Wash cells three times in cold PBS.
2. Resuspend cells at 1 x 10^7 cells/mL of Cell Lysis Buffer 1.
3. Incubate with gentle agitation for up to 60 minutes at room temperature.
4. Centrifuge at 8,000 x g for 5 minutes to remove cell debris.
5. Assay immediately or aliquot the lysis supernates and store at ≤ -20 °C until ready for use.

ACTIVATION REAGENT PREPARATION

To activate LAP (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.*

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.

SAMPLE ACTIVATION PROCEDURE

To activate LAP (TGF-β1) to immunoreactive LAP (TGF-β1) detectable by the Quantikine LAP (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 or controls.***

Cell Culture Supernates/Cell Lysates/Urine	Serum/Platelet-poor Plasma
To 100 µL of sample, add 20 µL of 1 N HCl.	To 20 µL serum/plasma, add 10 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 10 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.

Note: *Discard unused activated samples after use. Do not freeze activated samples.*

Serum samples require a 100-fold dilution. A suggested 100-fold dilution is10 µL of activated sample + 490 µL of Calibrator Diluent RD6-31 (diluted 1:2).* Complete the 100-fold dilution by adding 150 µL of the diluted sample to 150 µL Calibrator Diluent RD6-31 (diluted 1:2) (final dilution factor of sample is 200).

Platelet-poor plasma samples require a 10-fold dilution. A suggested 10-fold dilution is 30 µL of activated sample + 270 µL of Calibrator Diluent RD6-31 (diluted 1:2) (final dilution factor of sample is 20).

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

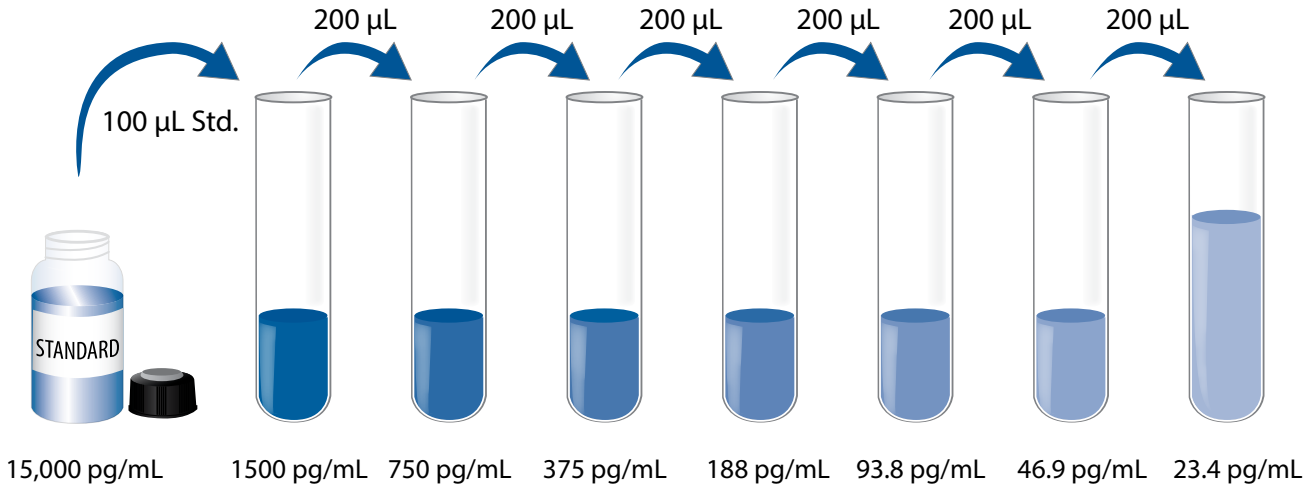
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 RD6-31 (diluted 1:2) - Add 20 mL of Calibrator Diluent RD6-31 to 20 mL of deionized or distilled water to yield 40 mL of Calibrator Diluent RD6-31 (diluted 1:2).

LAP (TGF-β1) Standard - Reconstitute the LAP (TGF-β1) Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 15,000 pg/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 µL of Calibrator Diluent RD6-31 (diluted 1:2) into the 1500 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1500 pg/mL standard serves as the high standard. Calibrator Diluent RD6-31 (diluted 1:2) 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.

1. Prepare all reagents, working standards, 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 RD1-34 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 for a record of 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 LAP (TGF-β1) 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 activation. Some samples may require dilution. See the Sample Activation Procedure section.

CALCULATION OF RESULTS

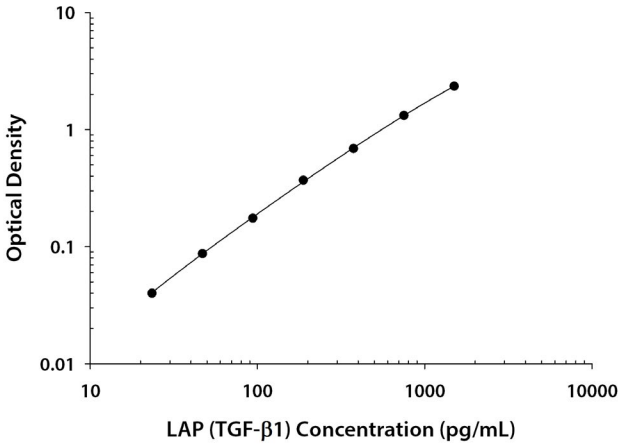
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 LAP (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.

Since samples have been diluted in the Sample Activation Procedure, 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	0.013 0.014	0.014	—
23.4	0.053 0.055	0.054	0.040
46.9	0.098 0.103	0.101	0.087
93.8	0.189 0.189	0.189	0.175
188	0.371 0.395	0.383	0.369
375	0.689 0.714	0.702	0.688
750	1.304 1.362	1.333	1.319
1500	2.331 2.386	2.359	2.345

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 twenty 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	20	20	20
Mean (pg/mL)	218	427	992	216	460	1126
Standard deviation	6.92	17.7	42.1	7.68	21.7	86.9
CV (%)	3.2	4.1	4.2	3.6	4.7	7.7

RECOVERY

The recovery of human LAP (TGF-β1) spiked to levels throughout the range of the assay in various matrices, followed by activation, was evaluated.

Sample Type	Average % Recovery	Range
Cell culture samples (n=4)	98	90-105%
Cell lysates (n=4)	97	90-103%
Urine (n=4)	95	87-101%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human LAP (TGF-β1) were activated and diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates (n=4)	Cell lysates (n=4)	Serum (n=4)	Platelet-poor		Urine (n=4)
					EDTA plasma (n=4)	Heparin plasma (n=4)	
1:2	Average % of Expected	101	108	101	105	105	102
	Range (%)	97-105	104-114	97-105	102-107	101-110	97-105
1:4	Average % of Expected	96	106	104	106	109	103
	Range (%)	90-100	98-114	95-109	99-111	102-113	98-107
1:8	Average % of Expected	93	106	104	108	111	103
	Range (%)	86-101	98-116	97-108	103-115	98-118	96-109
1:16	Average % of Expected	95	105	106	103	111	103
	Range (%)	89-104	94-115	101-112	102-105	101-119	99-110

SENSITIVITY

Fifty-one assays were evaluated and the minimum detectable dose (MDD) of human LAP ranged from 0.521-3.40 pg/mL. The mean MDD was 1.31 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 CHO cell-expressed recombinant human Latent TGF-β1 manufactured at R&D Systems.

SAMPLE VALUES

Serum/Platelet-poor Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human LAP (TGF-β1) 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=19)	155	101-216	32.1
Platelet-poor EDTA plasma (n=19)	7.45	3.58-24.0	4.76
Platelet-poor heparin plasma (n=19)	11.4	5.62-47.0	8.94

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Urine (n=10)	62.9	80	ND-131

ND=Non-detectable

Cell Culture Supernates/Cell Lysates:

JE-3 human choriocarcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 1 mM sodium pyruvate and 100 µg/mL streptomycin sulfate for 4 days until confluent.

CCD-1070Sk human foreskin fibroblast cells were cultured in MEM supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate for 8 days until confluent.

Sample Type	JE-3 (pg/mL)	CCD-1070Sk (pg/mL)
Cell culture supernate	1638	4136
Cell lysate	4065	1263

SPECIFICITY

This assay recognizes natural and recombinant human LAP (TGF-β1) and Latent TGF-β1.

The factors listed below were prepared at 500 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 500 ng/mL in a mid-range recombinant human LAP (TGF-β1) control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Activin A
Activin RIA
Activin RIB
Activin RIIA
Activin RIIB
BMP RIA
BMP RIB
BMP RII
Endoglin
IGF-II R
Inhibin A
Inhibin B
LTBP-1
LTBP-2
LTBP-4
MMP-2
MMP-9
TGF-α
TGF-β1
TGF-β2
TGF-β3
TGF-β RI
TGF-β RII
TGF-β RIII

Other recombinants:

mouse Latent TGF-β2
porcine TGF-β2
amphibian TGF-β5

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.