Human LAP ELISA Kit

Enzyme-linked Immunosorbent Assay for quantitative detection of human LAP (TGF ß 1)

Catalog Numbers BMS2065 and BMS2065TEN

Pub. No. MAN0016519 **Rev.** A.0 (30)

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Human LAP ELISA Kit is an enzyme-linked immunosorbent assay for the quantitative detection of human LAP.

Summary

Many different cells produce TGF β and it mediates effects on the proliferation, differentiation, and function of many cell types. TGF β is synthesized as a precursor that contains Latency Associated Peptide (LAP) at the N-terminus and mature TGF β at the C-terminus forming a complex called Small Latent Complex (SLC). This complex remains in the cell until it is bound to LTBP (latent TGF-b binding protein) to form a large latent complex (LLC). LTBP does not confer latency but is for efficient secretion of the complex to extracellular sites. It is LLC that get secreted to the Extra Cellular Matrix (ECM).

The initially sequestered, inactive LTGF β (latent TGF β) requires activation (cleavage and dissociation of its TGF β 1) before it can exert biological activity.

The non-covalent interactions between these molecules can be disrupted by heat, extremes of pH (e.g., acid treatment denatures LAP) and other chaotropic factors in vitro.

Human LAP is a homodimer of 65-75 kDa that is important in regulating the activity of TGF β. Processing and cleavage of the precursor protein between amino acids 278 and 279 results in the formation of LAP dimers and TGF β dimers that then non-covalently associate with each other to form the small latent TGF β complex. LAP is secreted and can be found in the extracellular matrix. LAP can induce epithelial cell migration and promote chemotaxis of monocytes and block inflammation. LAP also can enhance hepatocyte regeneration and reduce fibrosis. LAP is also a surface marker of activated regulatory T-cells. In addition, LAP can also be expressed on platelets and activated regulatory T-cells. It is believed that this surface-expressed LAP is due to the binding of LAP to GARP (LRRC32), which is a transmembrane protein that is also found at high levels on platelets and activated regulatory T-cells.

Mutations within the LAP are associated with Camurati Engelmann disease, a rare sclerosing bone dysplasia characterized by inappropriate presence of active TGF β 1.

Measuring LAP has the advantage that samples do not need to be pretreated with acid. The antibodies in this ELISA recognize the LAP/TGF β complex, hence allow to draw conclusion upon TGF β levels.

For literature update refer to our website.

Principles of the test

An anti-human LAP coating antibody is adsorbed onto microwells.

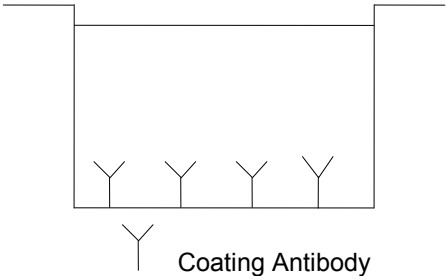

Fig. 1 Coated microwell

Human LAP present in the sample or standard binds to antibodies adsorbed to the microwells and a biotin-conjugated anti-human LAP antibody is added and binds to human LAP captured by the first antibody.

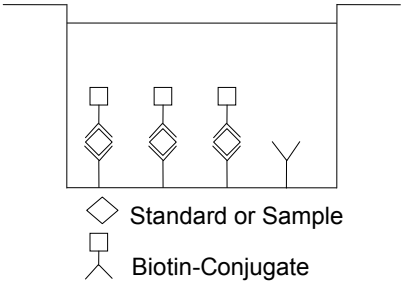

Fig. 2 First incubation

Following incubation unbound biotin-conjugated anti-human LAP antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human LAP antibody.

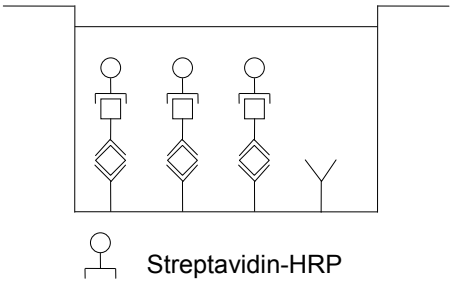

Fig. 3 Second incubation

Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

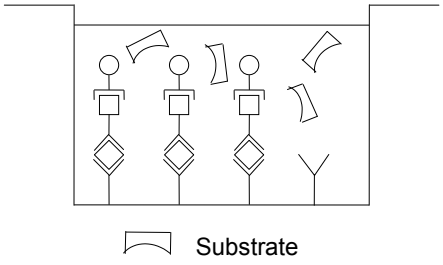

Fig. 4 Third incubation

A colored product is formed in proportion to the amount of human LAP present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from [number std dilutions] human LAP standard dilutions and human LAP sample concentration determined.

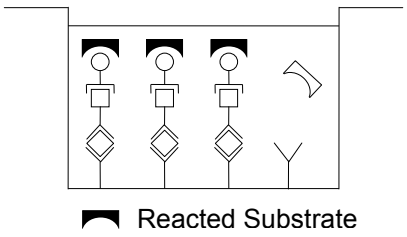

Fig. 5 Stop reaction

Reagents provided

Reagents for human LAP (TGF ß 1) ELISA BMS2065 (96 tests)

1 aluminum pouch with a Microwell Plate (12 strips with 8 wells each) coated with monoclonal antibody to human LAP

1 vial (70 µL) Biotin-Conjugate anti-human LAP antibody

1 vial (150 µL) Streptavidin-HRP

2 vials human LAP Standard lyophilized, 20 ng/mL upon reconstitution

1 vial (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)

1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)

1 vial (12 mL) Sample Diluent

1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (15 mL) Stop Solution (1M Phosphoric acid)

4 Adhesive Films

Reagents for human LAP (TGF ß 1) ELISA BMS2065TEN (10x96 tests)

10 aluminum pouches with a Microwell Plate (12 strips with 8 wells each) coated with monoclonal antibody to human LAP

10 vials (70 µL) Biotin-Conjugate anti-human LAP antibody

10 vials (150 µL) Streptavidin-HRP

10 vials human LAP Standard lyophilized, 20 ng/mL upon reconstitution

2 vials (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)

5 bottles (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)

10 vials (12 mL) Sample Diluent

10 vials (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (100 mL) Stop Solution (1M Phosphoric acid)

20 Adhesive Films

Storage instructions – ELISA kit

Store kit reagents between 2°C and 8°C. Immediately after use remaining reagents should be returned to cold storage (2°C to 8°C). Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

Sample collection and storage instructions

Cell culture supernatant, serum, and plasma (heparin) were tested with this assay. Other biological samples might be suitable for use in the assay.

Pay attention to a possible *Hook Effect* due to high sample concentrations (see "Calculation of results" on page 4)

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic samples.

Samples should be aliquoted and must be stored frozen at –20°C to avoid loss of bioactive human LAP. Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

Materials required but not provided

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate shaker
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

Precautions for use

- All reagents should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipet by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or samples.
- Rubber or disposable latex gloves should be worn while handling kit reagents or samples.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- To avoid microbial contamination or cross-contamination of reagents or samples that may invalidate the test, use disposable pipette tips and/or pipettes.

- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose samples and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

Preparation of reagents

1. Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.
2. If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

Wash buffer (1x)

1. Pour entire contents (50 mL) of the Wash Buffer Concentrate (20x) into a clean 1000 mL graduated cylinder. Bring to final volume of 1000 mL with glass-distilled or deionized water. Mix gently to avoid foaming.
2. Transfer to a clean wash bottle and store at 2°C to 25°C. The Wash Buffer (1x) is stable for 30 days.
3. Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1–6	25	475
1–12	50	950

Assay buffer (1x)

1. Pour the entire contents (5 mL) of the Assay Buffer Concentrate (20x) into a clean 100 mL graduated cylinder. Bring to final volume of 100 mL with distilled water. Mix gently to avoid foaming.
2. Store at 2°C to 8°C. The Assay Buffer (1x) is stable for 30 days.
3. Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1–6	2.5	47.5
1–12	5.0	95.0

Biotin-Conjugate

Note: The Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	3.97
1 - 12	0.06	5.94

Streptavidin-HRP

Note: The Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:200 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

Human LAP standard

1. Reconstitute human LAP standard by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 20 ng/mL).
2. Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

The standard has to be used immediately after reconstitution and cannot be stored.

External standard dilution

1. Label 7 tubes, one for each standard point: S1, S2, S3, S4, S5, S6, S7.
2. Prepare 2-fold serial dilutions for the standard curve as follows: Pipet 225 µL of Sample Diluent into each tube.
3. Pipet 225 µL of reconstituted standard (concentration = 20 ng/mL) into the first tube, labeled S1, and mix (concentration of S1 = 10 ng/mL.
4. Pipet 225 µL of this dilution into the second tube, labeled S2, and mix thoroughly before the next transfer.
5. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 6).

   Sample Diluent serves as blank.

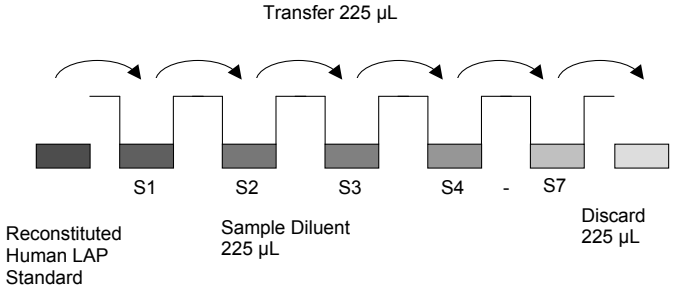

Fig. 6 Dilute standards - tubes

Test protocol

Note: If instructions of this protocol have been followed samples have been diluted 1:10, the concentration read from the standard curve must be multiplied by the dilution factor (x 10).

Shaking is absolutely necessary for an optimal test performance.

1. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°C to 8°C sealed tightly.
2. Prepare Biotin-Conjugate (see “Biotin-Conjugate“ on page 3).

3. Wash the microwell strips twice with approximately 400 µL Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10–15 seconds before aspiration. Take care not to scratch the surface of the microwells.

   After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.
4. Standard dilution on the microwell plate (alternatively, the standard dilution can be prepared in tubes, see "External standard dilution" on page 3):

   Add 100 µL of Sample Diluent in duplicate to all standard wells. Pipet 100 µL of prepared standard (see Preparation of Standard "Human LAP standard" on page 3, concentration = 20 ng/mL), in duplicate, into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1 S1 = 10 ng/mL), and transfer 100 µL to wells B1 and B2, respectively (see Figure 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human LAP standard dilutions, ranging from 10–0.16 ng/mL. Discard 100 µL of the contents from the last microwells (S7) used.

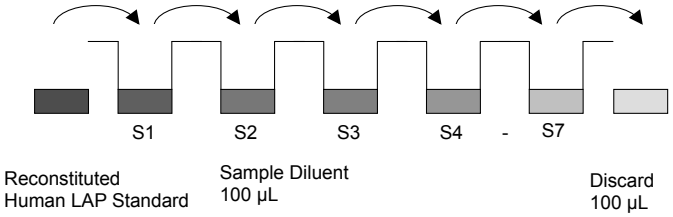

Fig. 7 Dilute standards - microwell plate.

Table 1 Example of the arrangement of blanks, standards, and samples in the microwell strips.

	1	2	3	4
A	Standard 1 10.00 ng/mL	Standard 1 10.00 ng/mL	Sample 1	Sample 1
B	Standard 2 5.00 ng/mL	Standard 2 5.00 ng/mL	Sample 2	Sample 2
C	Standard 3 2.50 ng/mL	Standard 3 2.50 ng/mL	Sample 3	Sample 3
D	Standard 4 1.25 ng/mL)	Standard 4 1.25 ng/mL	Sample 4	Sample 4
E	Standard 5 0.63 ng/mL	Standard 5 0.63 ng/mL	Sample 5	Sample 5
F	Standard 6 0.31 ng/mL	Standard 6 0.31 ng/mL	Sample 6	Sample 6
G	Standard 7 0.16 ng/mL	Standard 7 0.16 ng/mL	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

   In case of an external standard dilution (see "External standard dilution" on page 3), pipet 100 µL of these standard dilutions (S1–S7) in the standard wells according to Table 1.
5. Add 100 µL of Sample Diluent in duplicate to the blank wells.
6. Add 90 µL of Sample Diluent to the sample wells.
7. Add 10 µL of each sample in duplicate to the sample wells.
8. Add 50 µL of diluted Biotin-Conjugate to all wells, including the blank wells.
9. Cover with an adhesive film and incubate at room temperature (18°C to 25°C) for 2 hours on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance.)
10. Prepare Streptavidin-HRP (see "Streptavidin-HRP" on page 3).
11. Remove adhesive film and empty wells. Wash microwell strips 4 times according to step 2. Proceed immediately to the next step.
12. Add 100 µL of diluted Streptavidin-HRP to all wells, including the blank wells. Cover with an adhesive film and incubate at room temperature (18°C to 25°C) for 1 hour on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance.)
13. Remove adhesive film and empty wells. Wash microwell strips 4 times according to step 2. Proceed immediately to the next step.
14. Pipet 100 µL of TMB Substrate Solution to all wells.
15. Incubate the microwell strips at room temperature (18°C to 25°C) for 30 minutes. Avoid direct exposure to intense light.

    The color development on the plate should be monitored and the substrate reaction stopped (see next step) before positive wells are no longer properly recordable. Determination of the ideal time period for color development has to be done individually for each assay.

    It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9–0.95.
16. Stop the enzyme reaction by quickly pipetting 100 µL of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2°C to 8°C in the dark.
17. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Calculation of results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20% of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human LAP concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human LAP for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human LAP concentration.
- If instructions in this protocol have been followed, samples have been diluted 1:10 (10 µL sample + 90 µL Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 10).
- Calculation of samples with a concentration exceeding standard 1 will result in incorrect, low human LAP levels (Hook Effect). Such samples require further external predilution according to expected human LAP values with Sample Diluent in order to precisely quantitate the actual human LAP level.
- It is suggested that each testing facility establishes a control sample of known human LAP concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.

- A representative standard curve is shown in Figure 8.

  Note: Do not use this standard curve to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

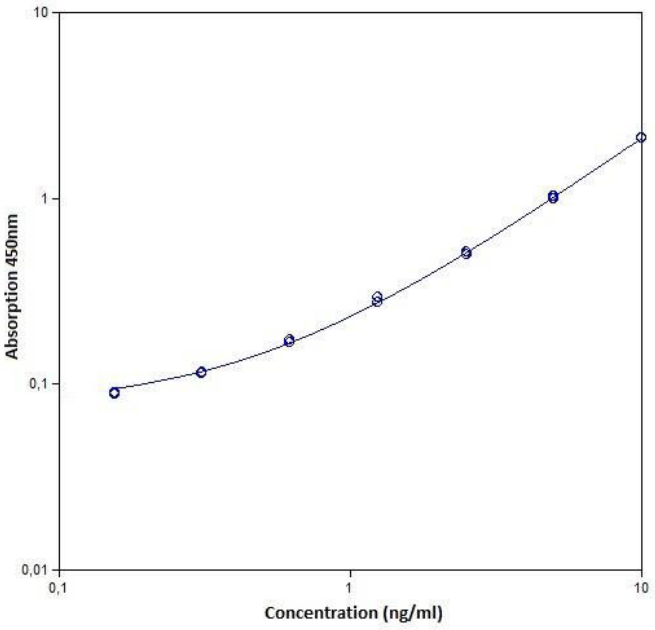

Fig. 8 Representative standard curve for Human LAP ELISA Kit. Human LAP was diluted in serial 2-fold steps in Sample Diluent.

Table 2 Typical data using the Human LAP ELISA Kit.

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human LAP concentration (ng/mL)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	10.00	2.064 2.025	2.044	0.9
2	5.00	0.993 0.963	0.978	1.5
3	2.50	0.546 0.510	0.528	3.4
4	1.25	0.299 0.280	0.289	3.3
5	0.63	0.195 0.190	0.193	1.2
6	0.31	0.129 0.124	0.126	1.7
7	0.16	0.095 0.094	0.095	0.8
Blank	0.0	0.075 0.067	0.071	5.5

The OD values of the standard curve may vary according to the conditions of assay performance (e.g., operator, pipetting technique, washing technique, or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus color intensity. Values measured are still valid.

Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Performance characteristics

Sensitivity

The limit of detection of human LAP defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.098 ng/mL (mean of 4 independent assays).

Reproducibility

Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum, heparin plasma and cell culture supernatant samples containing different concentrations of human LAP. Two standard curves were run on each plate. Data below show the mean human LAP concentration and the coefficient of variation for each sample. The calculated overall intra-assay coefficient of variation was 7.6%.

Table 3 The mean human LAP concentration and the coefficient of variation for each sample.

Sample	Experiment	Mean human LAP concentration (ng/mL)	Coefficient of variation (%)
1	1 2 3	80.35 79.94 84.87	6.3 4.0 4.1
2	1 2 3	31.12 26.71 30.11	2.2 10.2 7.5
3	1 2 3	27.09 29.28 30.50	8.8 5.7 6.5
4	1 2 3	3.38 3.65 3.03	10.0 9.1 11.2
5	1 2 3	11.40 12.43 11.91	7.8 7.5 8.8
6	1 2 3	10.42 10.66 8.64	9.3 8.3 9.2
7	1 2 3	21.19 22.37 22.04	5.4 5.7 10.2
8	1 2 3	15.03 16.06 15.32	8.8 6.4 8.3

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum, heparin plasma and cell culture supernatant samples containing different concentrations of human LAP. Two standard curves were run on each plate. Data below show the mean human LAP concentration and the coefficient of variation calculated

on 18 determinations of each sample. The calculated overall inter-assay coefficient of variation was 6.0%.

Table 4 The mean human LAP concentration and the coefficient of variation of each sample.

Sample	Mean human LAP concentration (ng/mL)	Coefficient of variation (%)
1	81.72	4.8
2	29.31	6.6
3	28.96	7.0
4	3.35	10.1
5	11.91	8.0
6	9.91	9.0
7	21.87	7.1
8	15.47	7.9

Spike recovery

The spike recovery was evaluated by spiking 3 levels of human LAP into serum, heparin plasma, and cell culture supernatant. Recoveries were determined with 2 replicates each. The amount of endogenous human LAP in unspiked samples was subtracted from the spike values.

Sample matrix	Spike high		Spike medium		Spike low	
	Mean (%)	Range (%)	Mean (%)	Range (%)	Mean (%)	Range (%)
Serum	81	76-88	114	108-117	92	81-105
Plasma (heparin)	82	75-88	99	88-107	99	77-112
Cell culture supernatant	79	75-82	109	103-115	95	89-101

Dilution parallelism

Serum, heparin plasma and cell culture supernatant samples with different levels of human LAP were analyzed at serial 2 fold dilutions with 4 replicates each.

Sample matrix	Recovery of Exp. Val.		
	Dilution	Mean (%)	Range (%)
Serum	1:20	100	89-106
	1:40	109	96-123
	1:80	103	90-117
Plasma (heparin)	1:20	114	105-120
	1:40	120	115-125
	1:80	118	105-147
Cell culture supernatant	1:20	83	75-91
	1:40	95	94-96
	1:80	95	88-101

Sample stability

Freeze-Thaw stability

Aliquots of serum, plasma, cell culture supernatant samples (spiked or unspiked) were stored at -20°C and thawed 3 times, and the human LAP levels determined.

A significant increase/decrease of human LAP immunoreactivity was detected with thawing the sample three times. Therefore samples should be stored in aliquots at -20°C and thawed only once.

Storage stability

Aliquots of serum, plasma, and cell culture supernatant samples (spiked or unspiked) were stored at –20°C, 2°C to 8°C, room temperature, and at 37°C, and the human LAP level determined after 24 hours. There was no significant loss of human LAP immunoreactivity detected during storage under above conditions.

Specificity

The assay detects both natural and recombinant human LAP. The cross-reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human LAP positive sample. No cross-reactivity or interference was detected.

Expected values

Panels of 40 serum and heparin plasma samples from randomly selected apparently healthy donors (males and females) were tested for human LAP. The levels measured may vary with the sample collection used.

Sample matrix	Number of samples evaluated	Mean (ng/mL)	Range (ng/mL)	Standard deviation (ng/mL)
Serum	40	23.7	5.0-41.4	7.5
Plasma (heparin)	40	2.6	1.0-6.1	1.1

Reagent preparation summary

Wash buffer (1x)

Add Wash Buffer Concentrate 20x (50 mL) to 950 mL distilled water.

Number of Strips	Wash Buffer Concentrate (mL)	Distilled Water (mL)
1–6	25	475
1–12	50	950

Assay buffer (1x)

Add Assay Buffer Concentrate 20x (5 mL) to 95 mL distilled water.

Number of Strips	Assay Buffer Concentrate (mL)	Distilled Water (mL)
1–6	2.5	47.5
1–12	5.0	95.0

Biotin-Conjugate

Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

Streptavidin-HRP

Make a 1:200 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

Human LAP standard

Reconstitute human LAP standard with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

Test protocol summary

Note: If instructions in this protocol have been followed, samples have been diluted 1:10 (10 µL sample + 90 µL Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 10).

1. Determine the number of microwell strips required.
2. Prepare Biotin-Conjugate.
3. Wash microwell strips twice with Wash Buffer.

4. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to all standard wells. Pipette 100 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.

   Alternatively, external standard dilution in tubes (see "External standard dilution" on page 3): Pipette 100 µl of these standard dilutions in the microwell strips.
5. Add 100 µL of Sample Diluent in duplicate to the blank wells.
6. Add 90 µL of Sample Diluent to the sample wells.
7. Add 10 µL of each sample in duplicate to the sample wells.
8. Add 50 µL diluted Biotin-Conjugate to all wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18°C to 25°C) on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance).
10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 4 times with Wash Buffer.
12. Add 100 µL diluted Streptavidin-HRP to all wells.
13. Cover microwell strips and incubate 1 hours at room temperature (18°C to 25°C) on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance).
14. Empty and wash microwell strips 4 times with Wash Buffer.
15. Add 100 µL of TMB Substrate Solution to all wells.
16. Incubate the microwell strips for about 30 minutes at room temperature (18°C to 25°C)
17. Add 100 µL Stop Solution to all wells.
18. Blank microwell reader and measure color intensity at 450 nm.

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