



LEGEND MAX™

ELISA Kit with Pre-coated Plate



Mouse Isthmin

Cat. No. 438907

ELISA Kit for Accurate Quantitation of Mouse Isthmin from
Cell Culture Supernatant, Serum, Plasma and Other Biological
Fluids

BioLegend, Inc.
biolegend.com

It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

For Research Purposes Only. Not for use in diagnostic or therapeutic procedures. Purchase does not include or carry the right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of BioLegend is strictly prohibited.

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Introduction:

Isthmin, also known as Isthmin-1, is a secreted protein, and it was initially identified in the *Xenopus* midbrain-hindbrain organizer (MHB) or isthmus organizer, where it is highly expressed. Mouse Isthmin is a 454 amino acid protein containing a Thrombospondin Type 1 Repeat (TSR) domain in the central region and an Adhesion-associated domain in MUC4 and Other Proteins (AMOP) domain at the C-terminus. The TSR domains are highly conserved with 98% identity between mouse and human, and 87-88% identity between mouse and *Xenopus*. The C-terminus AMOP domains are also highly conserved with 99% identity between mouse and human, and 91% identity between mouse and *Xenopus*. Recently it has been suggested that Isthmin has angiostatic activity *in vitro* and *in vivo*. Isthmin inhibits EC capillary network formation mainly by interfering with the early stages of *in vitro* angiogenesis on Matrigel. Isthmin inhibits VEGF-induced EC proliferation and induces apoptosis in the presence of VEGF. Overexpression of Isthmin in B16 melanoma inhibits tumor growth and tumor angiogenesis in mice. Knockdown of Isthmin in zebrafish embryos leads to abnormal intersegmental vessel (ISV) formation in the trunk.

BioLegend's LEGEND MAX™ Mouse Isthmin ELISA Kit is a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with a 96-well strip plate that is pre-coated with a monoclonal rat anti-mouse Isthmin capture antibody. Detection antibody is a biotinylated polyclonal goat anti-mouse Isthmin antibody. This kit is specifically designed for the accurate quantitation of mouse Isthmin from cell culture supernatant, serum, plasma and other biological fluids.

Materials Provided:

Description	Quantity	Volume (per bottle)	Part #
Anti-Mouse Isthmin Pre-coated 96-well Strip Microplate	1 plate		77001
Mouse Isthmin Detection Antibody	1 bottle	12 mL	79675
Mouse Isthmin Standard	1 vial	lyophilized	79677
Matrix A (for serum and plasma samples only)	1 vial	lyophilized	78303
Avidin-HRP D	1 bottle	12 mL	78237
Assay Buffer A	1 bottle	25 mL	78232
Wash Buffer (20X)	1 bottle	50 mL	78233
Substrate Solution F	1 bottle	12 mL	79132
Stop Solution	1 bottle	12 mL	79133
Plate Sealers	4 sheets		78101

Materials to be Provided by the End-User:

- Microplate reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 1 µL to 1,000 µL
- Deionized water
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Plate Shaker
- Polypropylene vials

Storage Information:

Store unopened kit components between 2°C and 8°C. Do not use this kit beyond its expiration date.

Opened or Reconstituted Components	
Microplate wells	If not all microplate strips are used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal. Store between 2°C and 8°C for up to one month.
Standard	The remaining reconstituted standard stock solution or Matrix A can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles.
Matrix A	
Detection Antibody	Store opened reagents between 2°C and 8°C and use within one month.
Avidin-HRP D	
Assay Buffer A	
Wash Buffer (20X)	
Substrate Solution F	
Stop Solution	

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online at BioLegend's website for details (www.biolegend.com/msds).
2. Substrate Solution F is harmful if inhaled or ingested. Avoid skin, eye and clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma and other biological fluids in accordance with NCCLS regulations.

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4. Stop Solution contains strong acid. *Wear eye, hand, and face protection.*
5. Before disposing the plate, rinse with an excess amount of tap water.

Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. If possible, unknown samples should be run at a number of dilutions to determine the optimal dilution factor that will ensure accurate quantitation.

Cell Culture Supernatant: If necessary, centrifuge all samples to remove debris prior to analysis. It is recommended that samples be stored at $< -70^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at $1,000 \times g$. Remove serum layer and assay immediately or store serum samples at $< -70^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Plasma: Collect blood samples in citrate, heparin or EDTA containing tubes. Centrifuge for 10 minutes at $1,000 \times g$ within 30 minutes of collection. Assay immediately or store plasma samples at $< -70^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Reagent and Sample Preparation:

Note: All reagents should be diluted immediately prior to use.

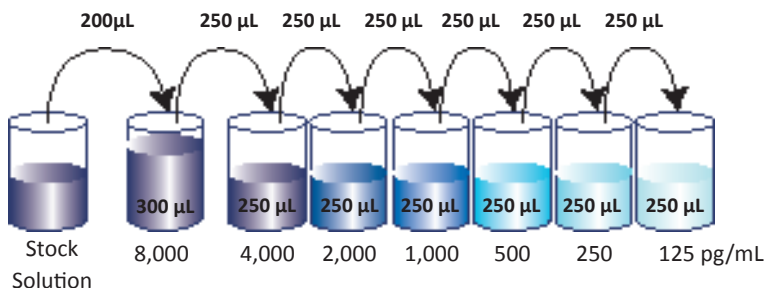
1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and vortex until dissolved.
2. Reconstitute the lyophilized Mouse Isthmin Standard by adding the volume of Assay Buffer A to make the 20 ng/mL standard stock solution (Refer to LEGEND MAX Kit Lot-Specific Certificate of Analysis/LEGEND MAX Kit Protocol). Allow the reconstituted standard to sit at room temperature for 15 minutes, then briefly vortex to mix completely.
3. If serum or plasma samples will be assayed, reconstitute the lyophilized Matrix A by dispensing 2 mL of deionized water into the vial and allow the reconstituted Matrix A to sit at room temperature for 15 minutes, then vortex to mix completely.
4. In general, samples are analyzed without dilutions. However, if dilutions are required, use Assay Buffer A as the diluent for cell culture supernatant and Matrix A as the diluent for serum or plasma samples.

Assay Procedure:

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this kit.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.
2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.
3. **For measuring samples of cell culture supernatant:**

- a) Prepare 500 μL of the 8,000 pg/mL top standard by diluting 200 μL of the standard stock solution in 300 μL of Assay Buffer A. Perform six two-fold serial dilutions of the 8,000 pg/mL top standard in separate tubes using Assay Buffer A as the diluent. Thus, the mouse Isthmin standard concentrations in the tubes are 8,000 pg/mL, 4,000 pg/mL, 2,000 pg/mL, 1,000 pg/mL, 500 pg/mL, 250 pg/mL, and 125 pg/mL, respectively. Assay Buffer A serves as the zero standard (0 pg/mL).

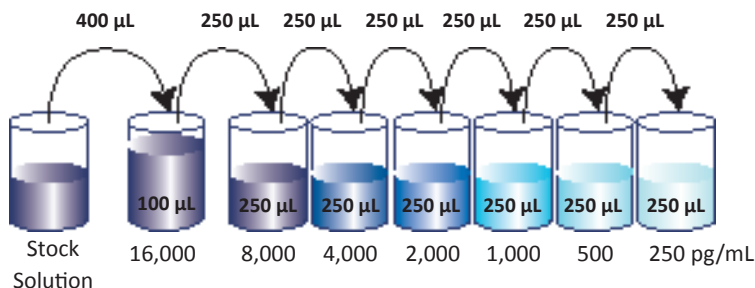


- b) Wash the plate 4 times with at least 300 μL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes should be performed similarly.
- c) Add 50 μL of Assay Buffer A to each well that will contain either standard dilutions or samples.
- d) Add 50 μL of standard dilutions or samples to the appropriate wells.

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For measuring serum or plasma samples:

- a) Prepare 500 μL of the 16,000 pg/mL top standard by diluting 400 μL of the standard stock solution in 100 μL of Assay Buffer A. Perform six two-fold serial dilutions of the 16,000 pg/mL top standard in separate tubes using Assay Buffer A as the diluent. Thus, the mouse Isthmin standard concentrations in the tubes are 16,000 pg/mL, 8,000 pg/mL, 4,000 pg/mL, 2,000 pg/mL, 1,000 pg/mL, 500 pg/mL, and 250 pg/mL, respectively. Assay Buffer A serves as the zero standard (0 pg/mL).



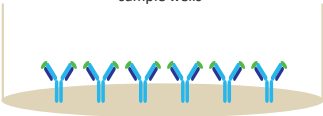
- b) Wash the plate 4 times with at least 300 μL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes should be performed similarly.
- c) Add 50 μL of Matrix A to each well that will contain standard dilutions and 50 μL of Assay Buffer A to each well that will contain samples.
- d) Add 50 μL of standard dilutions to the standard wells and 50 μL of diluted serum or plasma samples to the sample wells.
4. Seal the plate with a Plate Sealer included in the kit and incubate the plate at room temperature for 2 hours while shaking at 200 rpm.
 5. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 3b.
 6. Add 100 μL of Mouse Isthmin Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour while shaking.
 7. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 3b.
 8. Add 100 μL of Avidin-HRP D solution to each well, seal the plate and incubate at room temperature for 30 minutes while shaking.
 9. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 3b. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

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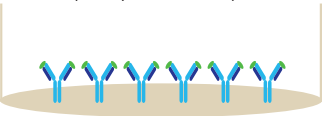
10. Add 100 μ L of Substrate Solution F to each well and incubate for 15 minutes in the dark. Wells containing mouse Isthmin should turn blue in color with an intensity proportional to its concentration. It is not necessary to seal the plate during this step.
11. Stop the reaction by adding 100 μ L of Stop Solution to each well. The solution color should change from blue to yellow.
12. Read absorbance at 450 nm within 30 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

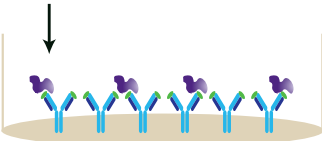
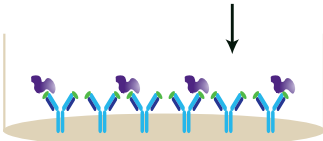
Assay Procedure Summary

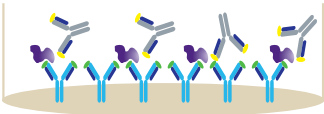
- For measuring cell culture supernatant:**
Wash 4 times
Add 50 μ L Assay Buffer A to standard and sample wells

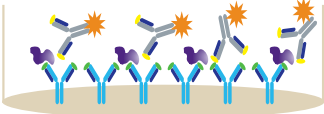


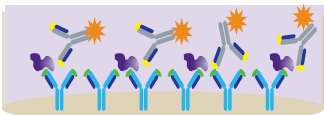
For measuring serum or plasma:
Wash 4 times
Add 50 μ L Matrix A to standard wells
Add 50 μ L Assay Buffer A to sample wells

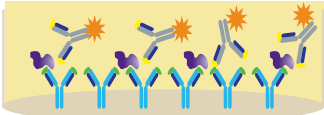

- Add 50 μ L diluted standards to standard wells
Add 50 μ L samples to sample wells
Incubate 2 hr, RT, shaking


- Wash 4 times
Add 100 μ L Detection Antibody solution
Incubate 1 hr, RT, shaking


- Wash 4 times
Add 100 μ L Avidin-HRP D solution
Incubate 30 min, RT, shaking


- Wash 5 times
Add 100 μ L Substrate Solution F
Incubate 15 min, RT, in the dark


- Add 100 μ L Stop Solution


- Read absorbance at 450 nm and 570 nm

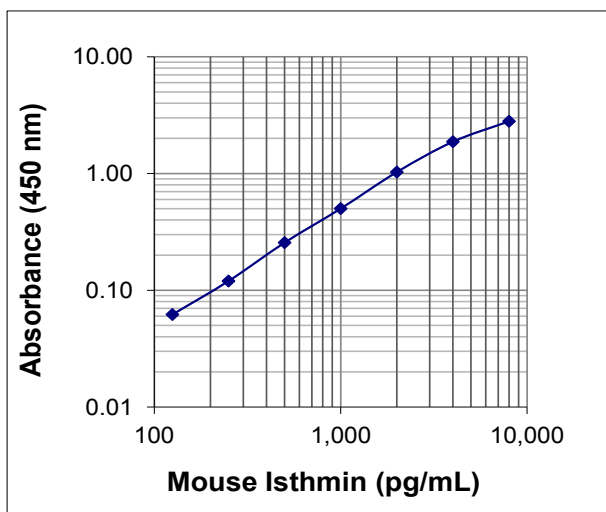
Calculation of Results:

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If an appropriate software is not available, use log-log graph paper to determine sample concentrations. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with cytokine concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown cytokine concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the cytokine concentration.

If cell culture supernatant samples were diluted, multiply the concentration by the appropriate dilution factor. If a test sample's absorbance value falls outside the linear portion of the standard curve, the test sample needs to be re-analyzed at a higher (or lower) dilution as appropriate.

Typical Data:

This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



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Performance Characteristics:

Specificity: This kit 100% cross-reacts with human Isthmin. No cross reactivity was observed when this kit was used to analyze the following recombinant cytokines/chemokines, each at up to 50 ng/mL.

Mouse	IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IL-17A/F, IL-22, IL-23, IL-25, IL-27, IL-33, FGF-basic, GM-CSF, IFN- γ , SCF, TRANCE, TNF- α
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Sensitivity: The minimum detectable concentration is 24 pg/mL in cell culture supernatant and 40 pg/mL in serum and plasma samples.

Recovery: Recombinant mouse Isthmin was spiked into mouse serum samples from 4 different strains at concentrations of 4,000, 2,000 and 1,000 pg/mL. The spiked serum samples were then analyzed with the LEGEND MAX™ Mouse Isthmin ELISA kit. On average, 108.6 % of the protein was recovered from the serum samples.

Linearity: Four murine serum samples, each from a different strain, were spiked with high concentrations of Isthmin then diluted with the appropriate matrix to produce samples with concentrations within the dynamic range and then assayed. On average, 108.9% of the expected protein was detected from the serum samples.

Intra-Assay Precision: Sixteen replicates of each of two samples containing different Isthmin concentrations were tested in one assay.

Concentration	Sample 1	Sample 2
Number of Replicates	16	16
Mean Concentration (pg/mL)	3687.5	890.1
Standard Deviation	195.3	57.3
% CV	4.9	6.0

Inter-Assay Precision: Two samples containing different concentrations of Isthmin were tested in four independent assays.

Concentration	Sample 1	Sample 2
Number of Assays	4	4
Mean Concentration (pg/mL)	3725	420.5
Standard Deviation	108.4	36.4
% CV	2.9	8.7

Biological Samples:

Serum/Plasma - Four pooled mouse serum samples, each from a different strain, were assayed for basal levels of mouse Isthmin. All sample values were below the lowest standard point, 250 pg/mL. Due to novel nature of the molecule, natural level of Isthmin in blood is unclear.

Cell Culture Supernates - 293E cells were transfected with mouse isthmin

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plasmid for 5 days. The cell culture supernates were collected and assayed for the concentration of mouse Isthmin. The concentration of mouse Isthmin was 9 µg/mL in transfected samples, and undetectable in non-transfected samples.

Troubleshooting Guide:

Problem	Probable Cause	Solution
High Background	Background wells were contaminated	Avoid cross-well contamination by using the provided plate sealers. Use multichannel pipettes and change tips between pipetting samples and reagents.
	Insufficient washes	Increase number of washes. Increase soaking time between washes prior to addition of substrate solution.
	TMB Substrate Solution was contaminated	TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.
No or poor signal	Detection Antibody, Avidin-HRP or Substrate solution were NOT added	Rerun the assay and follow the protocol.
	Wrong reagent or reagents were added in wrong sequential order	
	Insufficient plate agitation	The plate should be agitated during all incubation steps using a plate shaker at a speed where solutions in wells are within constant motion without splashing.
	The wash buffer contains Sodium Azide (NaN ₃)	Avoid Sodium Azide contamination in the wash buffer as it inhibits HRP activity.
	Incubations were done at an inappropriate temperature, timing or without agitation	Rerun the assay and follow the protocol.
Low or poor standard curve signal	The standard was incorrectly reconstituted or diluted	Adjust the calculations and follow the protocol.
	Standard was inappropriately stored	Store the reconstituted standard stock solution in polypropylene vials at -70°C. Avoid repeated freeze-thaw cycles.
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and the correct reagent volume.

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Problem	Probable Cause	Solution
Signal is high, standard curves have saturated signal	Standard reconstituted with less volume than required	Reconstitute new lyophilized standard with the correct volume of solution recommended in the protocol.
	Standards/samples, detection antibody, Avidin-HRP or substrate solution were incubated for too long	Rerun the assay and follow the protocol.
Sample readings are out of range	Samples contain no or below detectable levels of the analyte	If samples are below detectable levels, it may be possible to use a larger sample volume. Contact technical support for appropriate protocol modifications.
	Samples contain analyte concentrations greater than highest standard point	Samples may require dilution and analysis.
High variation in samples and/or standards	Multichannel pipette errors	Confirm that pipette calibrations are accurate.
	Plate washing was not adequate or uniform	Ensure pipette tips are tightly secured. Ensure uniformity in all wash steps.
	Non-homogenous samples	Thoroughly mix samples before assaying.
	Samples may have high particulate matter	Remove particulate matter by centrifugation.
	Cross-well contamination	Do not reuse plate sealers. Always change tips for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.

ELISA Plate Template												
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												



The path to legendary discovery™

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