BioVendor Group

ENG

Instructions for Use:

HUMAN ISTHMIN-1 ELISA

Catalogue number:

RAG031R

For research use only.

BioVendor R&D

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CONTENT

HISTORY OF CHANGES

Previous version	Current version
	ENG.001.A
New edition	

1. INTENDED USE

The Isthmin-1 (human) ELISA Kit is to be used for the in vitro quantitative determination of human Isthmin-1 in plasma and cell culture supernatant. This ELISA Kit is for research use only.

2. STORAGE, EXPIRATION

- Reagent must be stored at 2-8°C when not in use.
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

3. INTRODUCTION

Isthmin-1 (ISM1) was first identified as a gene expressed in the Xenopus midbrain hind brain organizer called isthmus, with a proposed role during early brain development. Isthmin-1 encodes a predicted ~50-kDa protein containing a signal peptide, a thrombospondin domain and an adhesion-associated domain [1]. Isthmin-1 is important for embryonic and postnatal development. Growing evidence has shown that aberrant expression of Isthmin-1 can also affect the biological behavior of cancer. The Ism1 gene is conserved in mice and humans [2].
A recent study showed that Isthmin-1 is an adipokine that induces glucose uptake in human and mouse adipocytes. Isthmin-1 is secreted by mature adipocytes and triggers a signaling cascade similar to that of insulin, regulating glucose uptake while suppressing lipid accumulation [3].
Recombinant Isthmin-1 or overexpression of Isthmin-1 causes a robust increase in GLUT4-dependent glucose uptake in cultured primary murine and immortalized human adipocytes as well as in primary human muscle cells and prevents insulin resistance and hepatic steatosis in a diet-induced obesity mouse model. Ablation of Isthmin-1 causes glucose intolerance and impaired insulin-stimulated adipocyte glucose uptake [4]. Isthmin-1 suppresses de novo lipogenesis and increases protein synthesis in hepatocytes whereas Isthmin-1 knockdown in adipocytes reduces glucose uptake and insulin-dependent phosphorylation of protein kinase AKT at serine residue 473 (p-AKTSer473). Isthmin-1 signaling is dependent on PI3K and shares downstream phosphorylation targets with insulin signaling, such as p-AKTSer473, p-AKTThr308, p-ERK1/2Thr202/Tyr204 and p-S6Ser235/236. Isthmin-1 does not seem to act through the insulin receptor or the insulin-like growth factor 1 receptor; it is most likely to signal through another, yet-to-be-identified, receptor tyrosine kinase [3,4].
Over the past few years, multiple studies have focused on the functional analysis of ISM1 in several events, including angiogenesis, metabolism, organ homeostasis, immunity and cancer. In most processes, Isthmin-1 plays a role in homeostasis and in suppressing inflammation via Isthmin-1-GRP78 signaling by controlling macrophages populations and functions [5]. Isthmin-1 is a potential biomarker for diseases such as obesity, diabetes type II, metabolic diseases and some cancers [6].

4. TEST PRINCIPLE

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human Isthmin-1 in biological fluids. A monoclonal antibody specific for human Isthmin-1 has been precoated onto the 96-well microtiter plate. Standards and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, human Isthmin-1 is recognized by the addition of a biotinylated monoclonal antibody specific for human Isthmin-1 (Detection Antibody). After removal of excess biotinylated antibody, streptavidin-peroxidase (STREP-HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of human Isthmin-1 in the samples.

5. TECHNICAL HINTS

- It is recommended that all standards, controls and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Substrate Solution protected from light.
- The Stop Solution consists of sulfuric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

6. REAGENT SUPPLIED

Kit Components	Quantity
1 vial Isthmin-1 Standard (lyophilized)	100 ng
1 vial Detection Antibody	30 µl
1 vial HRP Labeled Streptavidin (lyophilized)	2 µg
2 bottles Wash Buffer 10X	2 x 30 ml
1 bottle Sample Buffer 10X	1 x 30 ml
1 bottle TMB Substrate Solution	12 ml
1 bottle Stop Solution	12 ml
1 plate coated with Isthmin-1 Antibody	6 x 16-well strips
2 plate sealers (plastic film)	
2 silica Gel Minibags	

7. MATERIAL REQUIRED BUT NOT SUPPLIED

- Microtiter plate reader at 450 nm
- Calibrated precision single and multi-channel pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. PREPARATION OF REAGENTS

NOTE: Prepare just the appropriate amount of the buffers necessary for the assay.

8.1 Wash Buffer 10X

has to be diluted with deionized water 1:10 before use (e.g. 50 ml Wash Buffer 10X + 450 ml water) to obtain Wash Buffer 1X.

8.2 Sample Buffer 10X

has to be diluted with deionized water 1:10 before use (e.g. 20 ml Sample Buffer 10X + 180 ml water) to obtain Sample Buffer 1X.

8.3 Detection Antibody

has to be diluted to 1:500 in Sample Buffer 1X (20 µl DET + 10 ml Sample Buffer 1X).
NOTE: The diluted Detection Antibody is not stable and cannot be stored!

8.4 HRP Labeled Streptavidin

has to be reconstituted with 100 µl of Sample Buffer 1X.

- After reconstitution of STREP-HRP, prepare aliquots and store them at -20°C. **Avoid freeze/thaw cycles**.
- Dilute the reconstituted STREP-HRP to the working concentration by adding 50 µl in 10 ml of Sample Buffer 1X (1:200).

NOTE: The diluted STREP-HRP is not stable and cannot be stored!

8.5 Human Isthmin-1 Standard

has to be reconstituted with 100 µl of Sample buffer 1x.

- This reconstitution produces a stock solution of 1µg/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.
  NOTE: The reconstituted standard is aliquoted and stored at -20°C.
- Dilute the standard protein concentrate (**1 µg/ml**) in Sample Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in Sample Buffer 1X is recommended.
- Suggested standard points are:
  40, 20, 10, 5, 2.5, 1.25, 0.625 and **0** ng/ml

Dilute for the standard curve:

To obtain	Add	Into
40 ng/ml	40 µl of Isthmin-1 (1 µg/ml)	960 µl of Sample Buffer 1X
20 ng/ml	300 µl of Isthmin-1 (40 ng/ml)	300 µl of Sample Buffer 1X
10 ng/ml	300 µl of Isthmin-1 (20 ng/ml)	300 µl of Sample Buffer 1X
5 ng/ml	300 µl of Isthmin-1 (10 ng/ml)	300 µl of Sample Buffer 1X
2.5 ng/ml	300 µl of Isthmin-1 (5 ng/ml)	300 µl of Sample Buffer 1X
1.25 ng/ml	300 µl of Isthmin-1 (2.5 ng/ml)	300 µl of Sample Buffer 1X
0.625 ng/ml	300 µl of Isthmin-1 (1.25 ng/ml)	300 µl of Sample Buffer 1X
0 ng/ml	300 µl of Sample Buffer 1X	Empty tube

9. PREPARATION OF SAMPLES

9.1 Plasma

Collect plasma using heparin, EDTA, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at ≤ -20°C for later use. Avoid repeated freeze/ thaw cycles.

9.2 Plasma or Cell Culture Supernatant

have to be diluted in Sample Buffer 1X. Samples containing visible precipitates must be clarified before use.

NOTE: As a starting point, 1/2 dilution of plasma is recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required!

10. ASSAY PROCEDURE

1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. **NOTE:** Remaining 16-well strips coated with human Isthmin-1 antibody when opened can be stored at 4°C for up to 1 month.

2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted plasma or cell culture supernatant samples in duplicate to the wells **(see Preparation of Reagents and Preparation of Samples)**.

3. Cover the plate with plate sealer and incubate for **1 hour at 37°C.**

4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.

5. Add 100 µl to each well of the Detection Antibody.

6. Cover the plate with plate sealer and incubate for **1 hour at room temperature**.

7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.

8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin **(see Preparation of Reagents)**.

9. Cover the plate with plate sealer and incubate for **30 min at room temperature**.

10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.

11. Add 100 µl to each well of TMB Substrate Solution

12. Allow the color reaction to develop **at room temperature (RT) in the dark for 25 - 30 minutes**.

13. Stop the reaction by adding 100 µl of Stop Solution. Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.

! CAUTION: CORROSIVE SOLUTION!

14. Measure the OD at 450 nm in an ELISA reader within 30 minutes.

11. CALCULATIONS

- Average the duplicate readings for each standard, control and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding Isthmin-1 concentration (ng/ml) on the vertical (Y) axis (Chapter TYPICAL DATA).
- Calculate the Isthmin-1 concentrations of samples by interpolation of the regression curve formula in a form of a quadratic equation.
- If the test samples were diluted, multiply the interpolated values by the dilution factor to calculate the concentration of human Isthmin-1 in the samples.

12. TYPICAL DATA

The following data are obtained using the different concentrations of standard as described in this protocol:

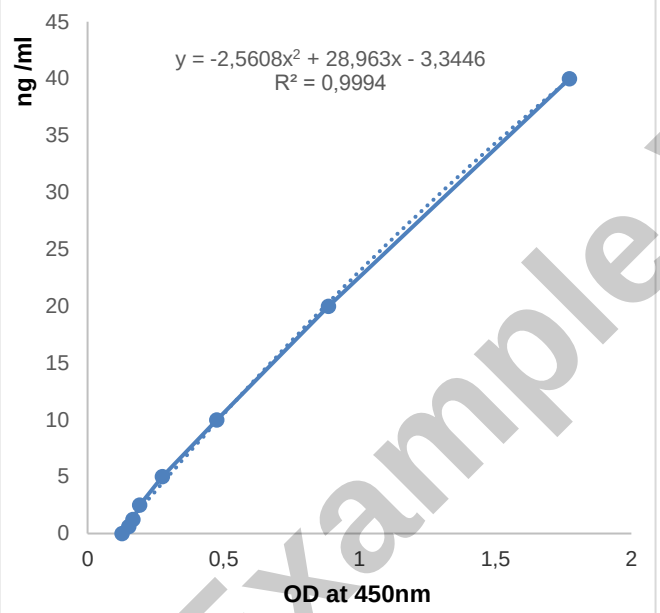


Figure 1: Standard curve

Standard Isthmin-1 (ng/ml)	Optical Density (mean)
40	1.7725
20	0.886
10	0.4755
5	0.2755
2.5	0.1915
1.25	0.167
0.625	0.1525
0.00	0.127

13. PERFORMANCE CHARACTERISTICS

13.1 Sensitivity (Limit of detection):

The lowest level of human Isthmin-1 that can be detected by this assay is 0.4 ng/ml.
NOTE: The Limit of detection was measured by adding three standard deviations to the mean value of 50 zero standard.

13.2 Assay range

0.625 ng/ml - 40 ng/ml

13.3 Specificity:

This ELISA Kit is specific for the measurement of natural and recombinant human and mouse Isthmin-1.

13.4 Intra-assay precision:

Four samples of known concentrations of human Isthmin-1 were assayed in replicates 5 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
1	9.47	0.47	4.36	5
2	1.03	5.65	5.37	5
3	2.91	0.19	6.68	5
4	39.89	2.32	5.81	5

13.5 Inter-assay precision:

Four samples of known concentrations of human Isthmin-1 were assayed in 5 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
1	40.32	0.94	2.33	5
2	19.72	0.87	4.39	5
3	0.86	0.074	8.54	5
4	4.62	0.33	7.23	5

13.6 Recovery:

When samples (serum or plasma) are spiked with known concentrations of human Isthmin-1, the recovery averages range from 70% to 99% (average 83%).

13.7 Linearity:

Different human plasma samples were diluted 1/4 to 1/8 and the measured linearity ranged from 103% to 131% (average 120 %).

13.8 Expected values:

Human Isthmin-1 protein levels range in plasma (healthy patients) from **non detectable to > 150ng/ml.**

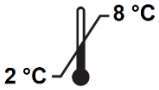
14. TROUBLESHOOTING AND FAQS

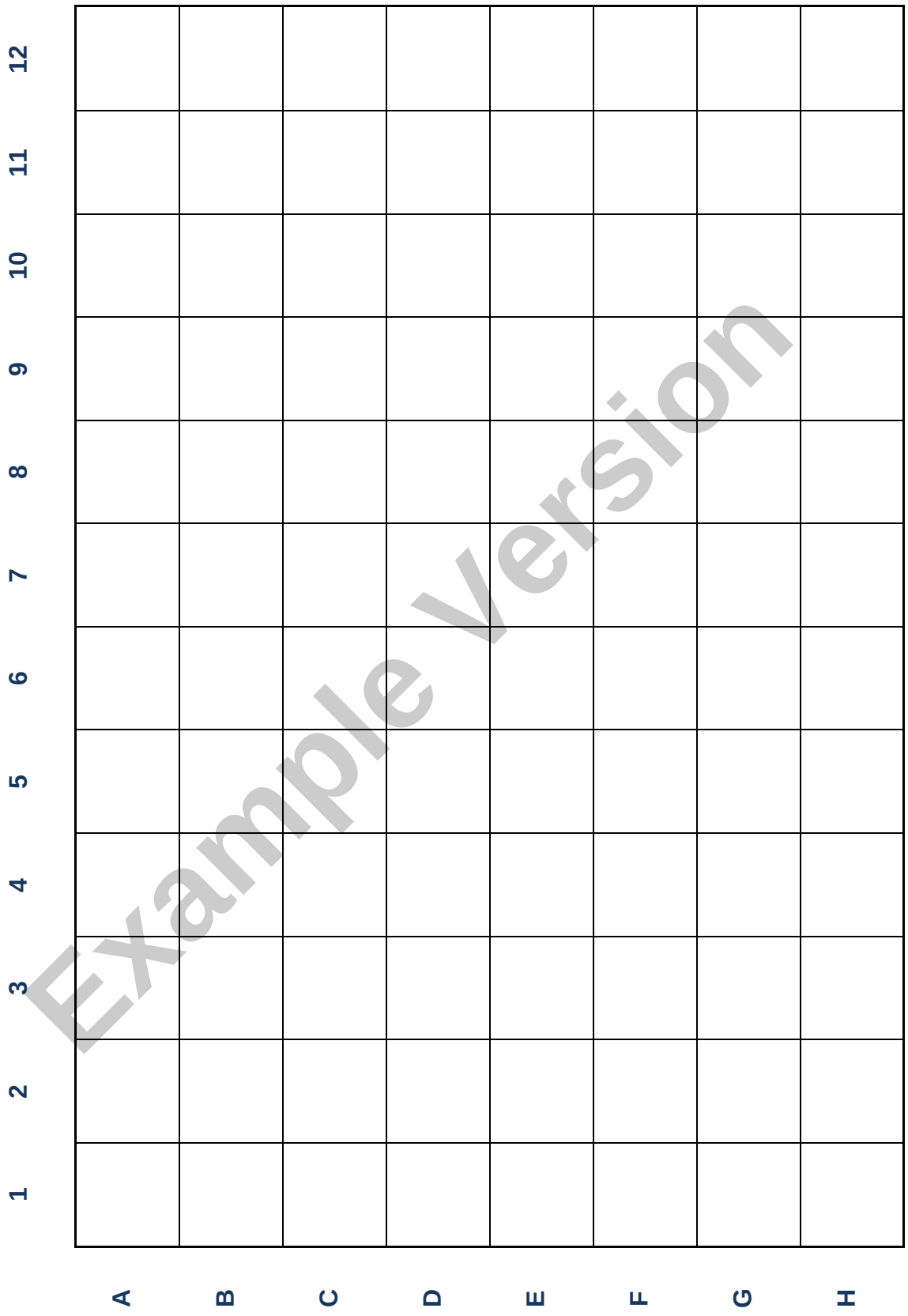
PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of STREP-HRP too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

15. REFERENCES

1 Isthmin - A Multifaceted Protein Family: H.M. Shakhawat, et al.; Cells 12, 17 (2023)
2 Isthmin 1 is a secreted protein expressed in skin, mucosal tissues, and NK, NKT, and th17 cells: R. Valle-Rios, et al.; J. Interf. Cytok. Res. 34, 795 (2014)
3 Isthmin-1 is an adipokine that promotes glucose uptake and improves glucose tolerance and hepatic steatosis: Z. Jang, et al.; Cell Metab. 33, 1836 (2021)
4 Isthmin 1 - a novel insulin-like adipokine: J. Heerin & L. Scheja; Nat. Rev. Endocrinol. 17, 709 (2021)
5 ISM1 protects lung homeostasis via cell-surface GRP78-mediated alveolar macrophage apoptosis: T.Y.W. Lam, et al.; PNAS 119, e2019161119 (2022)
6 Isthmin: A multifunctional secretion protein: J.Y. Liang, et al. ; Cytokines 173, 156423 (2023)

16. EXPLANATION OF SYMBOLS

	Catalogue number
	Batch code
	Caution
	Use by date
	Temperature limit
	Manufacturer
	Read electronic instructions for use - eIFU
	The content is sufficient for 96 tests
	Biological risks



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