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ab193761
Human Midkine SimpleStep ELISA® Kit

For the quantitative measurement of Midkine in human serum, plasma, cell culture supernatant, cell and tissue extract.

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

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1. Overview

Midkine *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Midkine protein in human serum, plasma, cell culture supernatant, cell and tissue extract.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB Development Solution is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

Midkine (MK), also known as neurite growth-promoting factor 2 (NEGF2), is a 13kDa heparin-binding growth factor or cytokine composed of two domains: The N-terminally located N-domain and the C-terminally located C-domain, which are connected by a hinge. The C-domain plays a role in neuronal development, whereas the N-domain is important for protein stability and dimerization. MK is involved in development, reproduction, repair, inflammation, innate immunity, control of blood pressure and angiogenesis.

MK is strongly expressed during embryogenesis and due to its distribution in the embryo it has been proposed to play a role in neurogenesis, epithelial-mesenchymal interactions and mesoderm remodeling. Expression in adult tissues is restricted to the kidney, epidermis, bronchial epithelium, lymphocytes and macrophages, but it is strongly expressed in the brain, kidney, blood vessels and heart after tissue injury as well as during inflammation, infection and oncogenesis. During inflammation, substratum-bound MK enhances

neutrophil and macrophage migration directly and through induction of chemokine expression. On the other hand, soluble MK is associated with differentiation of regulatory T-cells, induction of epithelial-mesenchymal transition, angiogenesis, fibrinolytic and anti-microbial activity.

MK signaling is mediated by cell surface receptors as well as membrane proteins such as Protein Tyrosine Phosphatase ζ (PTPζ), low density lipoprotein receptor-related proteins (LRP), Notch2, integrins, anaplastic lymphoma kinase (ALK) and neuroglycan C. Binding of MK to PTPζ induces tyrosine phosphorylation in β-catenin and Wnt signaling inhibition. Furthermore, it induces phosphorylation of PI3K, MAPK, PKC and Src family kinase. Binding of MK to LRP leads to embryonic neuronal survival and prevention of hypoxic injury via Akt and HIF1a. Binding of MK to integrins activates focal adhesion kinase, paxillin and STAT1a pathway leading to increase invasiveness of cancer cells. Activation of ALK by MK leads to phosphorylation of IRS-1, MAPK, PI3K and activation of NF-κB.

Due to its multifunctionality, MK has become an emerging target of drug development for the treatment of multiple diseases. On the one hand, administration of MK ameliorates ischemic injury, enhances oocyte maturation and promotes neurogenesis therefore limiting the progression of neurodegenerative diseases. However, on the other hand, due to its over-expression in malignant tumors and in inflammation, MK inhibitors may be useful in the treatment of cancer, multiple sclerosis, hypertension and osteoporosis.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed

Add 50 µL standard or sample to appropriate wells

Add 50 µL Antibody Cocktail to all wells

Incubate at room temperature for 1 hour

Aspirate and wash each well three times with 350 µL 1X Wash Buffer PT

Add 100 µL TMB Development Solution to each well and incubate for 10 minutes.

Add 100 µL Stop Solution and read OD at 450 nm

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition
Human Midkine Capture Antibody 10X	600 µL	+4°C
Human Midkine Detector Antibody 10X	600 µL	+4°C
Human Midkine Lyophilized Recombinant Protein	2 Vials	+4°C
Antibody Diluent CPI	6 mL	+4°C
Cell Extraction Buffer PTR 5X	10 mL	+4°C
Sample Diluent NS	50 mL	+4°C
Wash Buffer PT 10X	20 mL	+4°C
TMB Development Solution	12 mL	+4°C
Stop Solution	12 mL	+4°C
SimpleStep Pre-Coated 96-Well Microplate	96 Wells	+4°C
Plate Seal	1	+4°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

8. Technical Hints

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.

- The provided Antibody Diluents and Sample Diluents contain protease inhibitor aprotinin. Additional protease inhibitors can be added if required.
- The provided Cell Extraction Buffer 5X contains phosphatase inhibitors and protease inhibitor aprotinin. Additional protease inhibitors can be added if required.
- To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.
- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

9.1 1X Cell Extraction Buffer PTR (For cell and tissue extracts only):

Prepare 1X Cell Extraction Buffer PTR by diluting Cell Extraction Buffer PTR 5X to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 8 mL deionized water and 2 mL Cell Extraction Buffer PTR 5X. Mix thoroughly and gently. If required protease inhibitors can be added.

9.2 1X Wash Buffer PT:

Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

9.3 Antibody Cocktail:

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent CPI. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent CPI. Mix thoroughly and gently.

10. Standard Preparation

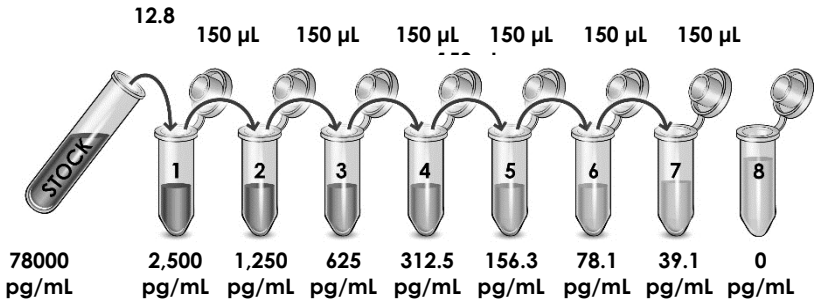
- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 For **serum, plasma, and cell culture supernatant sample measurements**, reconstitute the Midkine protein standard by adding 500 µL of Sample Diluent NS.
For **cell and tissue extract sample measurements**, reconstitute the Midkine protein standard by adding 500 µL of 1X Cell Extraction Buffer PTR.
Hold at room temperature for 10 minutes and mix thoroughly and gently. This is the 78,000 pg/mL **Stock Standard** Solution.

10.1.1 Label eight tubes, Standards 1– 8.

10.1.2 Add 387.2 µL of appropriate diluent (see step 10.1) into tube number 1 and 150 µL of appropriate diluent into numbers 2-8.

10.1.3 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:



11. Sample Preparation

Typical Sample Dynamic Range	
Sample Type	**Range**
Serum	≤50%
Plasma - EDTA	≤50%
Plasma - Citrate	≤25%
Plasma - Heparin	≤25%
HepG2 Cell culture supernatant	0.3 - 2.5%
HepG2 Cell extract	3.13 - 50 µg/mL
Cell culture media	≤50%

11.1 Plasma:

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples at least 1:2 into Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.2 Serum:

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples at least 1:2 into Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

11.3 Cell Culture Supernatants:

Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants and assay. Or dilute samples at least 1:2 into Sample Diluent NS and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

11.4 Preparation of extracts from cell pellets:

11.4.1 Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4°C.

11.4.2 Rinse cells twice with PBS.

11.4.3 Solubilize pellet at $2x10^7$ cell/mL in chilled 1X Cell Extraction Buffer PTR.

11.4.4 Incubate on ice for 20 minutes.

11.4.5 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.4.6 Transfer the supernatants into clean tubes and discard the pellets.

11.4.7 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.4.8 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

11.5 Preparation of extracts from tissue homogenates:

11.5.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).

11.5.2 Homogenize 100 to 200 mg of wet tissue in 500 µL – 1 mL of chilled 1X Cell Extraction Buffer PTR. For lower amounts of tissue adjust volumes accordingly.

11.5.3 Incubate on ice for 20 minutes.

11.5.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.5.5 Transfer the supernatants into clean tubes and discard the pellets.

11.5.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.5.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or "edge effects" have not been observed with this assay.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.

13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

13.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

13.3 Add 50 µL of all sample or standard to appropriate wells.

13.4 Add 50 µL of the Antibody Cocktail to each well.

13.5 Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

13.6 Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.

13.7 Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.

13.8 Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

13.9 Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode	Kinetic
Wavelength:	600 nm
Time:	up to 15 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

Δ **Note**: that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450 nm.

13.10 Analyze the data as described below.

14. Calculations

14.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.

14.2 **Create a standard curve** by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

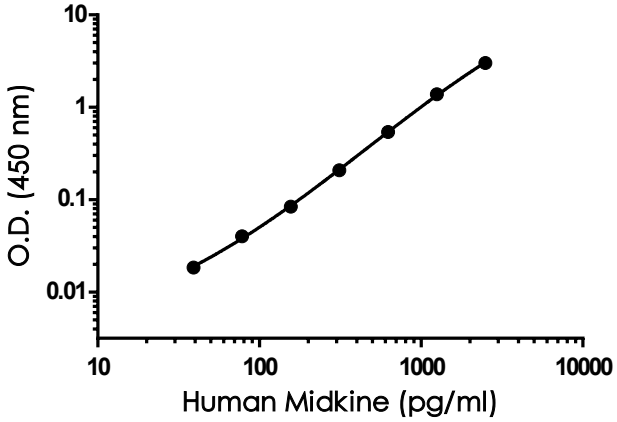
Δ **Note:** Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four- parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4- parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.

14.3 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted **absorbance values against the standard curve**. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

14.4 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

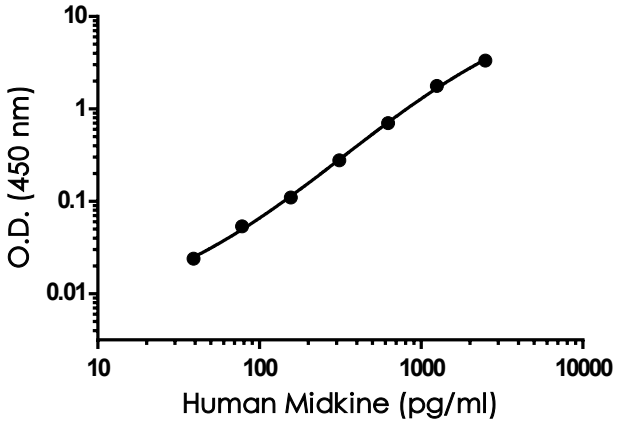
15. Typical Data

Typical standard curve – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Concentration (pg/mL)	O.D 450 nm		Mean O.D
	1	2	
0	0.058	0.057	0.058
39.1	0.077	0.076	0.076
78.1	0.103	0.093	0.098
156.3	0.142	0.142	0.142
312.5	0.268	0.264	0.266
625.0	0.598	0.596	0.597
1250	1.446	1.437	1.442
2500	3.092	3.066	3.079

Figure 1. Example of human Midkine standard curve in Sample Diluent NS. The Midkine standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.



Standard Curve Measurements			
Concentration (pg/mL)	O.D 450 nm		Mean O.D
	1	2	
0	0.058	0.057	0.058
39.1	0.081	0.081	0.081
78.1	0.111	0.112	0.111
156.3	0.170	0.164	0.167
312.5	0.335	0.335	0.335
625.0	0.759	0.761	0.760
1250	1.808	1.851	1.830
2500	3.388	3.388	3.388

Figure 2. Example of human Midkine standard curve in 1X Cell Extraction Buffer PTR. The Midkine standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Typical Sample Values

SENSITIVITY –

The MDD was determined by calculating the mean of zero standard replicates and adding 2 standard deviations then extrapolating the corresponding concentration.

Sample Diluent Buffer	n=	Minimal Detectable Dose
Sample Diluent NS	36	14.4 pg/mL
1X Cell Extraction Buffer PTR	20	13.6 pg/mL

RECOVERY –

Three concentrations of Midkine recombinant protein were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
50% Serum	114	113 - 115
50% Plasma - EDTA	112	109 - 114
25% Plasma - Citrate	128	115 - 137
50% Plasma - Heparin	83	73 - 90
1% HepG2 Cell culture supernatant	100	82 - 115
6.25 µg/mL HepG2 Cell extract	156	152 - 160
50% Cell culture media	89	81 - 98

*Media is DMEM containing 10% fetal calf serum containing 10% fetal calf serum.

Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native Midkine was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	2.5% HepG2 Cell Culture Supernatant
Undiluted	pg/mL	1486
	% Expected value	100
2	pg/mL	780
	% Expected value	105
4	pg/mL	396
	% Expected value	107
8	pg/mL	198
	% Expected value	107
16	pg/mL	99.0
	% Expected value	107

Native Midkine was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in 1X Cell Extraction Buffer PTR.

Dilution Factor	Interpolated value	50 µg/mL HepG2 Cell Extract	50 µg/mL SH-SY5Y Cell Extract
Undiluted	pg/mL	2365	1021
	% Expected value	100	100
2	pg/mL	1268	558
	% Expected value	107	109
4	pg/mL	658	307
	% Expected value	111	120
8	pg/mL	339	154
	% Expected value	115	121
16	pg/mL	185	81.4
	% Expected value	125	127

Recombinant Midkine was spiked into the following biological samples and diluted in a 2-fold dilution series in Sample Diluent NS.

Dilution Factor	Interpolated value	50% Human Serum	50% Human Plasma (EDTA)	25% Human Plasma (Citrate)	50% Human Plasma (Heparin)
Undiluted	pg/mL	812	750	497	588
	% Expected value	100	100	100	100
2	pg/mL	449	407	250	359
	% Expected value	111	109	101	122
4	pg/mL	241	216	125	164
	% Expected value	119	115	100	112
8	pg/mL	130	113	60.3	NL
	% Expected value	128	122	97	
16	pg/mL	62	59.6	NL	
	% Expected value	122	123		

NL – Non-Linear

PRECISION –

Mean coefficient of variations of interpolated values of Midkine from one concentrations of HepG2 Supernatant within the working range of the assay.

	Intra-Assay	Inter-Assay
n =	8	3
CV(%)	2.0	3.9

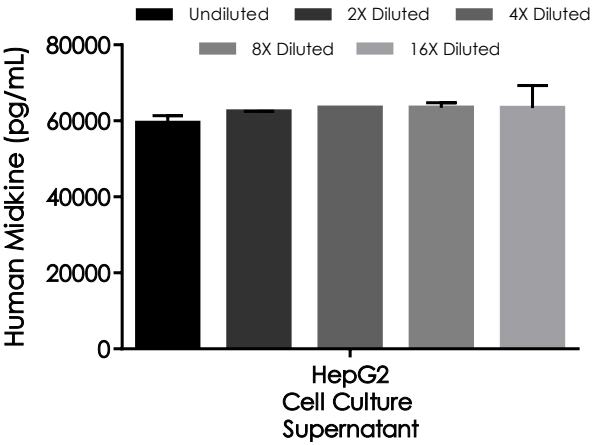


Figure 3. Interpolated concentrations of native Midkine in human cell culture supernatant samples. The concentrations of Midkine were measured in duplicates, interpolated from the Midkine standard curves and corrected for sample dilution. Undiluted samples are as follows: HepG2 cell culture supernatant 2.5%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean Midkine concentration was determined to be 6,242 pg/mL in serum.

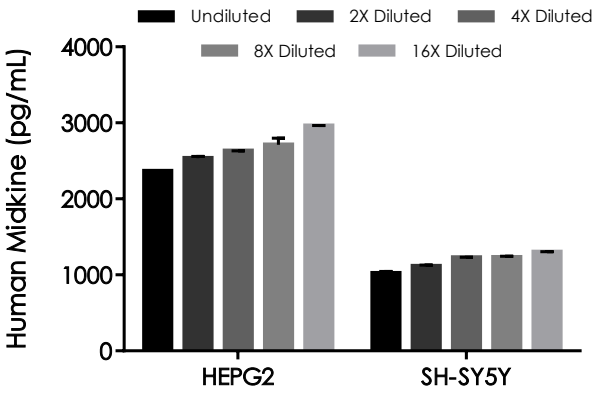


Figure 4. Interpolated concentrations of native Midkine in human HepG2 and SH-SY5Y cell extract samples and samples based on a 50 µg/mL extract load. The concentrations of Midkine were measured in duplicate and interpolated from the Midkine standard curve and corrected for sample dilution. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean Midkine concentration was determined to be 2641 pg/mL in HepG2 cell extract and 1179 pg/mL in SH-SY5Y cell extract.

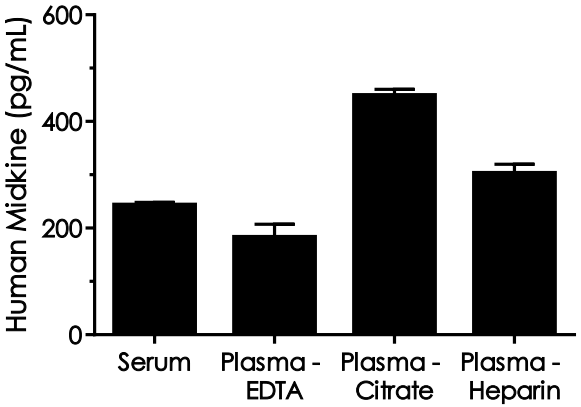


Figure 5. Interpolated concentrations of native Midkine in human serum, plasma samples. The concentrations of Midkine were measured in duplicates, interpolated from the Midkine standard curves and corrected for sample dilution. Undiluted samples are as follows: serum 50%, plasma (EDTA) 50%, plasma (citrate) 25%, and plasma (heparin) 50%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean Midkine concentration was determined to be 243 pg/mL in serum, 183 pg/mL in plasma (EDTA), 449 pg/mL plasma (citrate) and 303 pg/mL in plasma (heparin).

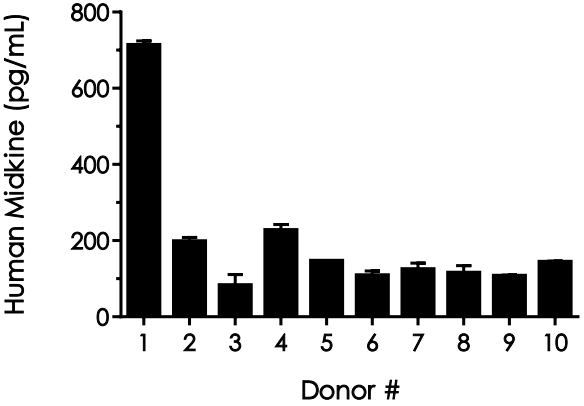


Figure 6. Serum from ten individual healthy human male donors was measured in duplicate. Interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean Midkine concentration was determined to be 164 pg/mL with a range of 69 – 594 pg/mL.

17. Assay Specificity

This kit recognizes both native and recombinant human Midkine protein in serum, plasma, and cell culture supernatant, cell and tissue extract samples only.

Urine, saliva and milk samples have not been tested with this kit.

18. Species Reactivity

This kit recognizes human Midkine protein.

Other species reactivity was determined by measuring 50% serum samples of various species, interpolating the protein concentrations from the human standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in human serum assayed at the same dilution.

Reactivity < 3% was determined for the following species:

- Mouse
- Rat
- Cow
- Pig

100% cross reactivity with Dog serum was observed.

Other species reactivity not determined.

Please contact our Technical Support team for more information.

19. Troubleshooting

Problem	Reason	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB Development Solution protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

20. Notes

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