Quantikine™ ELISA

Mouse Sonic Hedgehog/Shh N-Terminus Immunoassay

Catalog Number MSHH00

For the quantitative determination of mouse Sonic Hedgehog N-Terminus (Shh-N) concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Hedgehog proteins are essential for development in both vertebrates and invertebrates (1-4). The three mammalian hedgehog genes, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh), share ~ 60% amino acid (aa) identity but differ in expression pattern and function (5, 6). Shh is expressed in embryonic tissues that are critical for the patterning of the developing central nervous system, somite, and limb (7-9). Shh is also involved in whisker, hair, foregut, tooth, and bone development (2-4, 10-12). Shh regulates neural and hematopoietic stem cell fate and is important for thymocyte differentiation and proliferation, as well as T cell determination. In adult tissue Shh is associated with cancer development and tissue remodeling following injury (13).

Mouse Shh is autocatalytically processed to yield a non-glycosylated 20 kDa N-terminal fragment (Shh-N) and a glycosylated 27 kDa C-terminal protein (Shh-C) (7-9, 14). Shh-N retains all known signaling capabilities and is thought to cluster in lipid rafts on the surface of the producing cell. Shh-C, which is responsible for the intramolecular processing of Shh, is rapidly degraded following Shh proteolysis (9, 13, 15). Shh-N is highly conserved, sharing >98% aa identity between mouse, human, rat, canine, porcine, and chicken Shh-N. Addition of a palmitoyl group onto the N-terminal cysteine residue (p-Shh-N) and cholesterol onto the C-terminus greatly increases Shh-N receptor binding affinity and signaling potency (16-19). At the cell surface, heparan sulfate and transglutaminase activity can crosslink membrane-tethered p-Shh-N to form multimers of varying size (19-24). Monomeric and multimeric Shh can be released from the plasma membrane by the cooperative action of Dispatched and SCUBE2 (13). Non-lipidated Shh-N can be freely secreted (25, 26). Shh can also be released in membrane microparticles, which have been implicated in neovascularization after ischemic injury (27).

Shh can act as both a short-range, contact dependent factor and as a long-range, diffusible morphogen (16). During development Shh forms gradients that promote different cell fates depending on distance from the Shh source (13). Shh signaling can be enhanced or blunted through interactions with specific cell surface molecules (1, 4, 15). Canonical signaling of Shh is mediated by a multicomponent receptor complex that includes Patched (PTCH1, PTCH2) and Smoothened (SMO) (28). The binding of Shh to PTCH releases the basal repression of SMO by PTCH. SMO then regulates the activity of Gli transcription factors (13). Gli proteins promote angiogenesis by increasing cell production of VEGF and angiopoietins. Shh can also promote angiogenesis via "non-canonical" activation of RhoA, a regulator of the actin cytoskeleton, or by inhibiting PTCH1-mediated activation of caspases to promote endothelial cell survival (6, 29). PTCH and LRP2 mediate endocytotic degradation of Shh (1).

Shh expression is elevated by oxidative insult and following ischemic injury, where it promotes stem cell recruitment, engraphment and tissue plasticity (30-32). Dysregulation of Shh signaling is associated with tumor development, including basal cell carcinoma, medulloblastoma, and breast cancer (1-3, 13). Changes in serum and plasma Shh are associated with the occurrence of pancreatic, gastric, and colorectal cancer (33-36). Shh signaling is also involved in the progression of epithelial-to-mesenchymal transition associated with cancer development (37, 38).

The Quantikine™ Mouse Sonic Hedgehog/Shh N-Terminus Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse Shh-N in cell culture supernates, serum, and plasma. It contains recombinant mouse Shh-N and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant factor accurately. Results obtained using natural mouse Shh-N showed dose-response curves that were parallel to the standard curves obtained using the recombinant Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural mouse Shh-N.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse Shh-N has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any Shh-N present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for mouse Shh-N is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of Shh-N bound in the initial step. The sample values are then read off the standard curve.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine™ Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- For best results, pipette reagents and samples into the center of each well.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse Shh-N Microplate	893937	96 well microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse Shh-N.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Mouse Shh-N Standard	893939	2 vials of recombinant mouse Shh-N in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard and control for each assay. Discard after use.
Mouse Shh-N Control	893940	2 vials of recombinant mouse Shh-N in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Mouse Shh-N Conjugate	893938	12 mL of a monoclonal antibody specific for mouse Shh-N conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-63	895352	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6-12	895214	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	23 mL of diluted hydrochloric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- **Polypropylene** test tubes for dilution of standards

PRECAUTIONS

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

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *EDTA plasma is not recommended for use in this assay*
Citrate plasma has not been validated for use in this assay.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

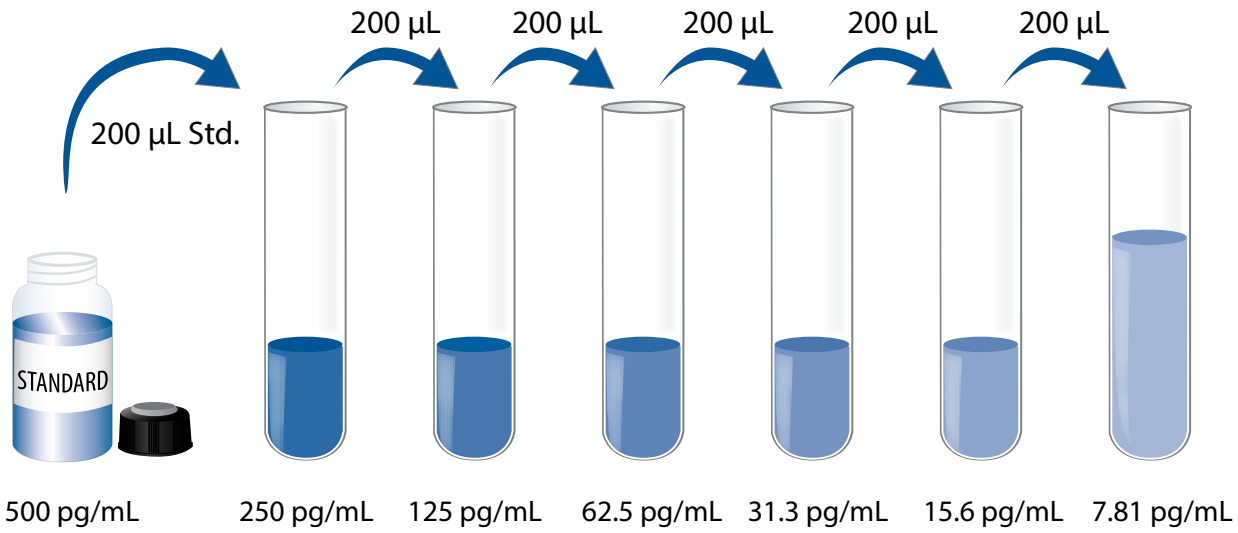
Mouse Shh-N Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Mouse Shh-N Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse Shh-N Standard with Calibrator Diluent RD6-12. Do not substitute other diluents. This reconstitution produces a stock solution of 500 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent RD6-12 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse Shh-N Standard (500 pg/mL) serves as the high standard. Calibrator Diluent RD6-12 serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, control, and samples be assayed in duplicate.

1. Prepare reagents, standards, control, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1-63 to each well.
4. Add 50 µL of standard, control, or sample per well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 µL of Mouse Shh-N Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

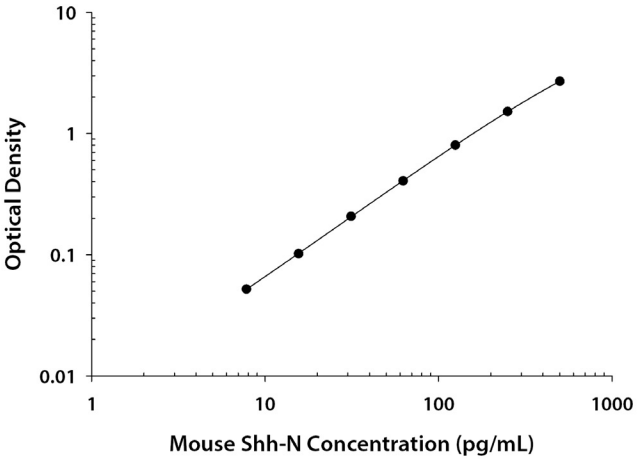
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the mouse Shh-N concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.027 0.029	0.028	—
7.81	0.079 0.080	0.080	0.052
15.6	0.129 0.131	0.130	0.102
31.3	0.233 0.237	0.235	0.207
62.5	0.431 0.439	0.435	0.407
125	0.824 0.835	0.830	0.802
250	1.538 1.550	1.544	1.516
500	2.709 2.737	2.723	2.695

PRECISION

Intra-Assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Three samples of known concentration were tested in separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of kit components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	44	44
Mean (pg/mL)	22.7	54.6	212	21.9	50.4	188.7
Standard deviation	1.08	1.31	10.6	2.28	2.96	9.21
CV (%)	4.8	2.4	5.0	10.4	5.9	4.9

RECOVERY

The recovery of mouse Shh-N spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture samples (n=6)	101	96-109%
Serum (n=4)	98	86-109%
Heparin plasma (n=4)	97	86-108%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of mouse Shh-N were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture samples (n=4)	Serum (n=6)	Heparin plasma (n=6)
1:2	Average % of Expected	99	104	110
	Range (%)	98-100	96-108	105-115
1:4	Average % of Expected	100	107	104
	Range (%)	98-101	100-112	95-114
1:8	Average % of Expected	101	104	96
	Range (%)	98-105	94-116	83-106
1:16	Average % of Expected	103	96	99
	Range (%)	94-111	83-111	95-104

SENSITIVITY

Sixty-eight assays were evaluated and the minimum detectable dose (MDD) of mouse Shh-N ranged from 0.032-2.37 pg/mL. The mean MDD was 1.22 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant mouse Shh-N produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse Shh-N in this assay.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=20)	131	82.3-238	37.2
Heparin plasma (n=20)	102	75.6-148	18.7

Cell Culture Supernates - D3 mouse embryonic stem cells were seeded in DMEM with 2% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 7 and 14 days. Aliquots of the cell culture supernate were removed, assayed for levels of mouse Shh-N, and measured 8.74 pg/mL after 7 days and 22.7 pg/mL after 14 days.

SPECIFICITY

This assay recognizes natural and recombinant mouse Shh-N.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-level mouse Shh-N control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

Dhh (C-Terminal)
HIP
Shh (C-Terminal)

Recombinant mouse Dhh and recombinant mouse Ihh does not interfere but does cross-react approximately 0.2% in this assay.

Recombinant human Shh-N does not interfere but does cross-react approximately 4% in this assay.

This kit also recognizes low levels of Shh-N in rat serum.

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