Quantikine® ELISA

Human Sonic Hedgehog/Shh N-Terminus Immunoassay

Catalog Number DSHH00

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

Human Shh encodes a 462 amino acid (aa) precursor protein that is autocatalytically processed to yield a non-glycosylated 19 kDa (173 aa) N-terminal fragment (Shh-N) and a glycosylated 25 kDa (264 aa) 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) 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 Human Sonic Hedgehog/Shh N-Terminus Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Shh-N in cell culture supernates, serum, and heparin plasma. It contains *E. coli*-expressed recombinant human Shh-N and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Shh-N showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human Shh-N.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Shh-N has been pre-coated onto a microplate. Standards 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 human 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 and color develops in proportion to the amount of Shh-N bound in the initial step. The color development is stopped and the intensity of the color is measured.

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, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard 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.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- 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. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate 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
Human Shh-N Microplate	894011	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human 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.*
Human Shh-N Standard	894013	2 vials of recombinant human Shh-N in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a fresh standard for each assay.
Human Shh-N Conjugate	894012	21 mL of a monoclonal antibody specific for human 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 RD5-24	895325	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:2 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives. *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	895032	6 mL of 2 N sulfuric 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.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- Human Shh-N Controls (optional; R&D Systems, Catalog # QC219).

PRECAUTIONS

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

Some components in this kit contain ProClin® 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. Please refer to the MSDS 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 and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 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 15 minutes at 1000 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 suitable for use in this assay.*
Citrate plasma has not been validated for use in this assay.
Grossly hemolyzed samples are not suitable for use in this assay.

SAMPLE PREPARATION

All samples require a 3-fold dilution due to the matrix effect. A suggested 3-fold dilution is 100 µL of sample + 200 µL of Calibrator Diluent RD5-24 (diluted 1:2)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

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 deionized or distilled water to prepare 500 mL of Wash Buffer.

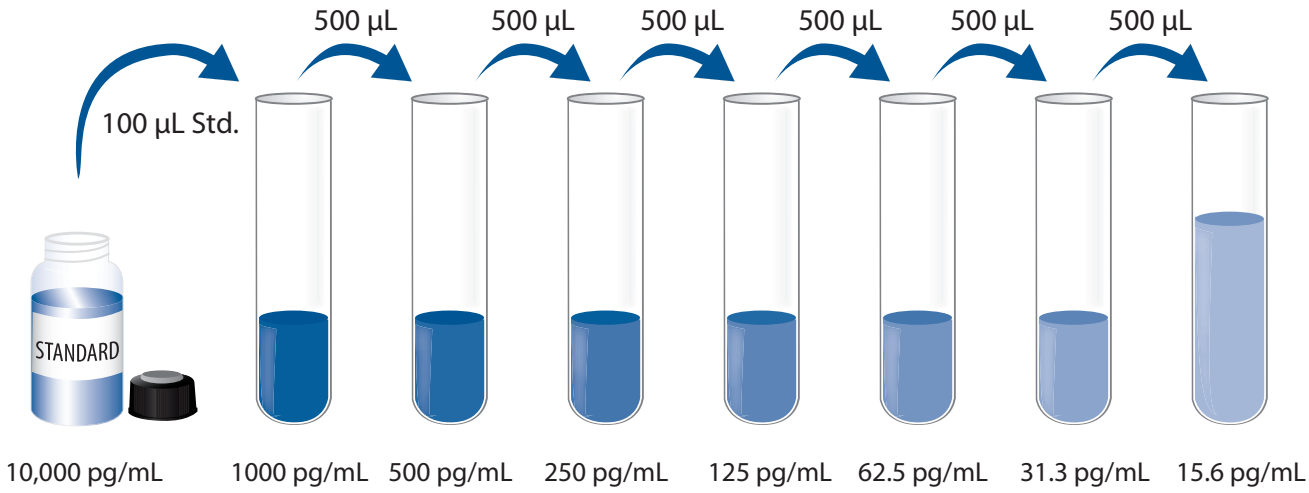
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-24 (diluted 1:2) - Add 20 mL of Calibrator Diluent RD5-24 to 20 mL of deionized or distilled water to prepare 40 mL of Calibrator Diluent RD5-24 (diluted 1:2).

Human Shh-N Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Shh-N Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/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.

Pipette 900 µL of Calibrator Diluent RD5-24 (diluted 1:2) into the 1000 pg/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-24 (diluted 1:2) serves as the zero standard (0 pg/mL).

Note: *Pipette the standard curve within 45 minutes of dilution.*



ASSAY PROCEDURE

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

1. Prepare all reagents, working standards, 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 100 µL of Assay Diluent RD1-63 to each well.
4. Add 100 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four 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 200 µL of Human Shh-N Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, 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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

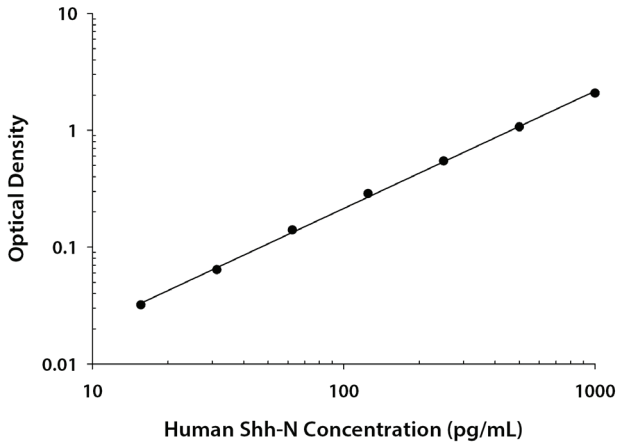
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log 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 a log/log graph. The data may be linearized by plotting the log of the human Shh-N concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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	0.010 0.012	0.011	—
15.6	0.042 0.043	0.043	0.032
31.3	0.074 0.076	0.075	0.064
62.5	0.146 0.155	0.151	0.140
125	0.288 0.306	0.297	0.286
250	0.537 0.577	0.557	0.546
500	1.063 1.093	1.078	1.067
1000	1.969 2.205	2.087	2.076

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 thirty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using three lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	30	30	30
Mean (pg/mL)	99.5	291	532	109	318	612
Standard deviation	5.97	8.15	14.4	10.3	24.5	50.3
CV (%)	6.0	2.8	2.7	9.4	7.7	8.2

RECOVERY

The recovery of human Shh-N spiked to levels throughout the range of the assay in various matrices was evaluated. Samples were diluted prior to assay as directed in the Sample Preparation section.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	108	100-117%
Serum (n=4)	99	81-117%
Heparin plasma (n=4)	97	85-117%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of human Shh-N were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

		Cell culture supernates (n=4)	Serum (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	106	102	108
	Range (%)	104-107	97-109	105-110
1:4	Average % of Expected	108	106	107
	Range (%)	103-117	99-111	104-110
1:8	Average % of Expected	105	109	109
	Range (%)	96-117	99-117	105-112
1:16	Average % of Expected	101	103	103
	Range (%)	92-108	94-113	91-111

SENSITIVITY

Thirty-four assays were evaluated and the minimum detectable dose (MDD) of human Shh-N ranged from 0.406-3.92 pg/mL. The mean MDD was 1.94 pg/mL.

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

CALIBRATION

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

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human Shh-N in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=40)	589	412-907	119
Heparin plasma (n=40)	556	377-870	109

Cell Culture Supernates:

HT-29 human colon adinocarcinoma cells were cultured in McCoy's media supplemented with 10% bovine calf serum, and 4 mM L-glutamine. An aliquot of the cell culture supernate was removed, assayed for human Shh-N, and measured 614 pg/mL.

KATO-III human gastric carcinoma cells were cultured in IMDM supplemented with 20% bovine calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. An aliquot of the cell culture supernate was removed, assayed for human Shh-N, and measured 688 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human 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 at 50 ng/mL in a mid-range recombinant human Shh-N control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

BOC
CDO
Gas1
HIP-14
Megalin-C3
Megalin-C4
Patched 1
Patched 2
SMO

Recombinant mouse:

BOC
CDO
Desert Hedgehog (aa 199-396)
Gas1
HIP
Patched
Patched 2
Sonic Hedgehog/Shh C-Terminus (aa 199-437)

Recombinant mouse Sonic Hedgehog (aa 26-198) cross-reacts approximately 9.5% in this assay.

Recombinant mouse Sonic Hedgehog (aa 25-198) cross-reacts approximately 21% in this assay.

Recombinant human/mouse Indian Hedgehog (aa 67-240) cross-reacts approximately 22.6% in this assay.

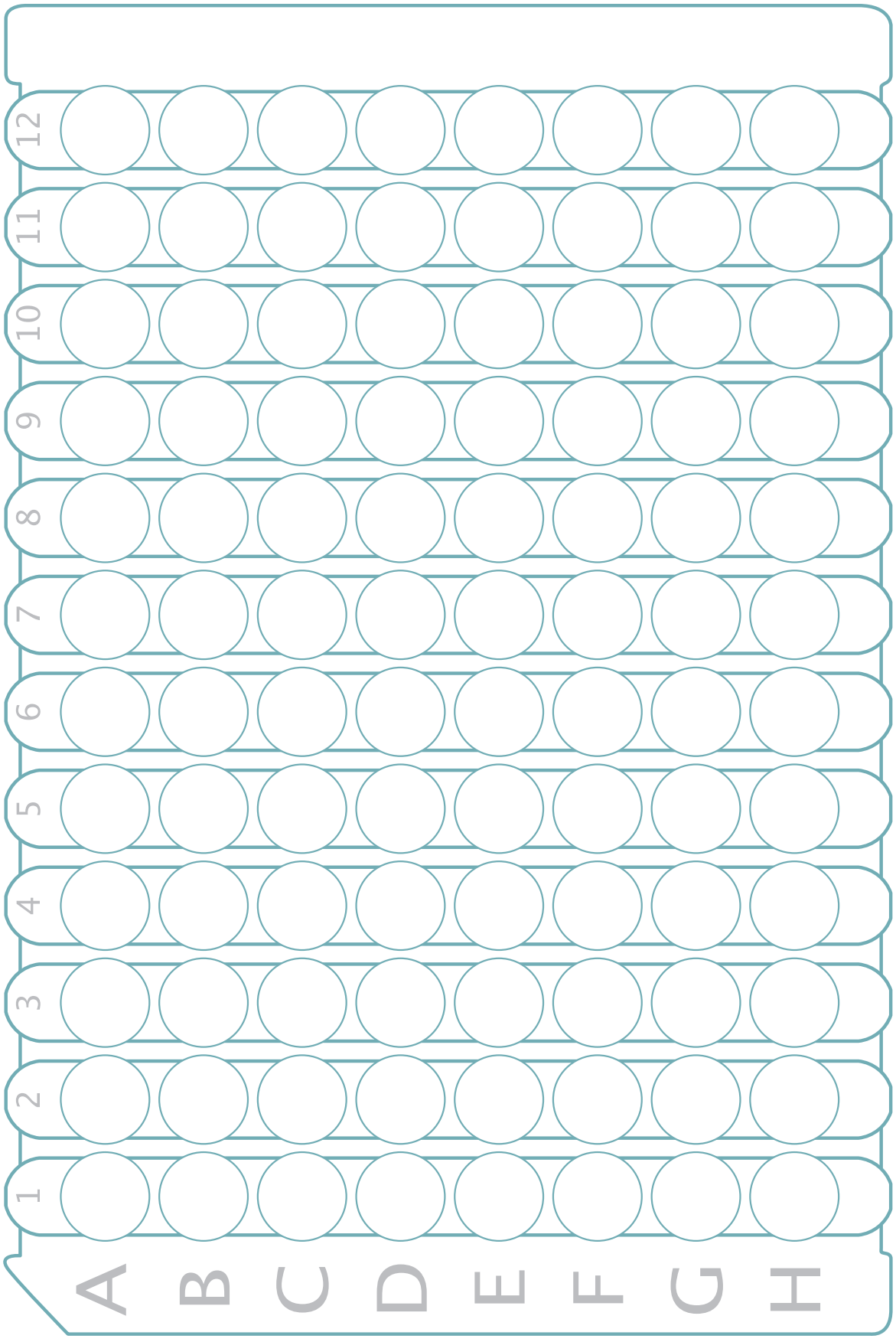
Recombinant mouse (rm) Indian Hedgehog (aa 66-240), rmDesert Hedgehog (aa 23-198), and recombinant human (rh) Desert Hedgehog (aa 24-198) cross-react approximately 1.5% in this assay.

REFERENCES

1. Cohen, M.M. Jr. (2010) Am. J. Med. Genet. Part A **152A**:1875.
2. Jiang, J. and C. Hui (2008) Dev. Cell **15**:801.
3. Traiffort, E. *et al.* (2010) J. Neurochem. **113**:576.
4. Dessaud, E. *et al.* (2008) Development **135**:2489.
5. Farzan, S.F. *et al.* (2008) Am. J. Gastrointest. Liver Physiol. **294**:G844.
6. Chinchilla, P. *et al.* (2010) Cell Cycle **9**:570.
7. Echelard, Y. *et al.* (1993) Cell **75**:1417.
8. Chang, D.T. *et al.* (1994) Development **120**:3339.
9. Roelink, H. *et al.* (1995) Cell **81**:445.
10. Cai, C. *et al.* (2008) Stem Cells **26**:1097.
11. Crompton, T. *et al.* (2007) Nat. Rev. Immunol. **7**:726.
12. Rowbotham, N.J. *et al.* (2009) Blood **113**:5144.
13. Briscoe, J. and P.P. Therond (2013) Mol. Cell. Biol. **14**:416.
14. Goetz, J.A. *et al.* (2002) BioEssays **24**:157.
15. Zeng, X. *et al.* (2001) Nature **411**:716.
16. Choudhry, Z. *et al.* (2014) Ann. Neurosci. **21**:28.
17. Pepinsky, R.B. *et al.* (1998) J. Biol. Chem. **273**:14037.
18. Feng, J. *et al.* (2004) Development **131**:4357.
19. Peters, C. *et al.* (2004) Proc. Natl. Acad. Sci. USA **101**:8531.
20. Chen, M-H. *et al.* (2004) Genes Dev. **18**:641.
21. Goetz, J.A. *et al.* (2006) J. Biol. Chem. **281**:4087.
22. Dierker, T. *et al.* (2009) J. Biol. Chem. **284**:32562.
23. Kawakami, T. *et al.* (2002) Development **129**:5753.
24. Etheridge, L.A. *et al.* (2010) Development **137**:133.
25. Dierker, T. *et al.* (2009) J. Biol. Chem. **284**:8013.
26. Zavros, Y. (2008) Am. J. Gastrointest. Liver Physiol. **294**:G1105.
27. Benameur, T. *et al.* (2010) PLoS ONE **5**:e12688.
28. Carpenter, D. *et al.* (1998) Proc. Natl. Acad. Sci. USA **95**:13630.
29. Renault, M.A. *et al.* (2010) J. Mol. Cell. Cardiol. **49**:490.
30. Ding, X. *et al.* (2013) J. Cereb. Blood Flow Metab. **33**:1015.
31. Renault, M.A. *et al.* (2013) Arterioscler. Thromb. Vasc. Biol. **33**:2858.
32. Sirko, S. *et al.* (2013) Cell Stem Cell **12**:426.
33. Thayer, S.P. *et al.* (2003) Nature **425**:851.
34. Fukaya, M. *et al.* (2006) Gastroenterology **131**:14.
35. Monzo, M. *et al.* (2006) Cancer Lett. **233**:117.
36. El-Zaatari, M. *et al.* (2012) Pancreas **41**:1019.
37. Lei, J. *et al.* (2013) Mol. Cancer **12**:66.
38. Ke, Z. *et al.* (2015) Med. Oncol. **32**:368.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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