bio-techne®

R&D SYSTEMS

Quantikine™ ELISA

Human Neuropilin-1 Immunoassay

Catalog Number DNRP10

For the quantitative determination of human Neuropilin-1 concentrations in cell culture supernates, cell lysates, serum, plasma, urine, and human milk.

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

TABLE OF CONTENTS

SECTION PAGE

Manufactured and Distributed by:

USA R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413
TEL: 800 343 7475 612 379 2956
FAX: 612 656 4400
E-MAIL: info@bio-techne.com

Distributed by:

Europe | Middle East | Africa Bio-Techne Ltd.
19 Barton Lane, Abingdon Science Park
Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info.emea@bio-techne.com

China Bio-Techne China Co., Ltd.
Unit 1901, Tower 3, Raffles City Changning Office,
1193 Changning Road, Shanghai PRC 200051
TEL: +86 (21) 52380373 (400) 821-3475
FAX: +86 (21) 52371001
E-MAIL: info.cn@bio-techne.com

INTRODUCTION

Neuropilin-1 (Npn-1 or Nrp1), also known as CD304, is an approximately 140 kDa transmembrane glycoprotein that functions as a co-receptor for a number of extracellular ligands. The 923 amino acid (aa) human protein shares 93% aa sequence identity with the mouse and rat orthologs (1). Npn-1 contains a large extracellular domain (ECD) with two N-terminal CUB domains (termed a1a2), two domains with homology to coagulation factors V and VIII (termed b1b2) and a MAM domain (termed c) (1, 2). Its short cytoplasmic tail is believed to mediate signaling by binding to the PDZ domain-containing protein GIPC1 (2-4). Npn-1 is usually expressed as a homodimer, although it can also form heterodimers with Npn-2 (5).Dimerization of Npn-1 is mediated by its MAM domain (4).

Npn-1 is expressed on neurons, melanocytes, and keratinocytes, and on epithelial cells found in breast, uterus, kidney, lung, pancreas, and the gastrointestinal tract (2, 5-8). It has also been shown to be expressed on the surface of several immune cell types including plasmacytoid dendritic cells, resting T cells, natural FoxP3$^+$ regulatory T cells, and a subset of follicular helper T cells (5, 9-12). Npn-1 is involved in a variety of physiological processes including angiogenesis, axon guidance, cell survival and migration, and tumor cell invasion (5, 7). It binds to several class III semaphorins including Sema3A, Sema3B, and Sema3F via its a1a2 domains (14, 15). It also interacts with type-A plexins to induce axonal repulsion and growth cone collapse (13, 16). Npn-1 also interacts with heparin-binding members of the VEGF family ($VEGF_{165}$, VEGF-B, VEGF-E, and PlGF-2) via its b1b2 domains (2, 7, 14, 17, 18). It functions as a co-receptor for VEGF R2/KDR to enhance the affinity of $VEGF_{165}$ for VEGF R2/KDR, thereby enhancing VEGF-stimulated endothelial cell proliferation, migration, survival, and vascular permeability (7, 16, 17, 19). Additionally, Npn-1 has been shown to interact with members of the FGF family (FGF acidic, FGF basic, and FGF-4), HGF and HGF R/c-Met, Galectin-1, PDGF, integrins, and TGF-β1 (5, 7, 8, 18, 20). The interaction between Npn-1 and TGF-β1 results in activation of TGF-β1 and promotion of regulatory T cell activity (7, 20).

Npn-1 is also expressed in multiple cancer types where it has been implicated in mediating the proliferation, survival, migration, and invasion of tumor cells (6, 17, 21-27). Additionally, its expression levels have been shown to correlate with tumor aggressiveness, disease stage, and poor clinical prognosis (5, 23-26). In addition to the transmembrane form of Npn-1, four naturally occurring human soluble isoforms have been identified (28-30). Alternative splicing and premature truncation within introns results in the formation of 704, 644, 609, and 551 aa isoforms (28-30). These soluble Npn-1 proteins contain only the a1a2 and b1b2 domains from the ECD and lack the MAM, transmembrane, and intracellular domains (28-30). While full-length Npn-1 facilitates VEGF signaling and promotes tumor progression, soluble Npn-1 has been shown to function as a $VEGF_{165}$ antagonist and displays antitumor activity (22, 28). In contrast, a distinct circulating Npn-1 protein that contains the full ECD has also been identified (4). Circulating Npn-1 is believed to be produced following ectodomain shedding of the full-length Npn-1, and has been shown to be elevated in the plasma of individuals with breast cancer, colorectal cancer, or non-small cell lung cancer (4, 31).

The Quantikine™ Human Neuropilin-1 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Neuropilin-1 in cell culture supernates, cell lysates, serum, plasma, urine, and human milk. It contains NS0-expressed recombinant human Neuropilin-1 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Neuropilin-1 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 Neuropilin-1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Neuropilin-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Neuropilin-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Neuropilin-1 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 Neuropilin-1 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 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 Neuropilin-1 Microplate	894791	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Neuropilin-1.	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 Neuropilin-1 Standard	894793	2 vials of recombinant human Neuropilin-1 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a new standard for each assay.
Human Neuropilin-1 Conjugate	894792	21 mL of a polyclonal antibody specific for human Neuropilin-1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-34	895265	11 mL of a buffered protein base with blue dye and preservatives.	
Calibrator Diluent RD5-27	895395	2 vials (21 mL/vial) 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	895032	6 mL of 2N 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
- 500 mL graduated cylinder
- 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 Neuropilin-1 Controls (optional; R&D Systems, Catalog # QC187B)

SUPPLIES REQUIRED FOR CELL LYSATE SAMPLES

- Cell Lysis Buffer 2 (R&D Systems, Catalog # 895347)
- PBS

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 and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Cells must be lysed prior to assay as directed in the Cell Lysis Procedure.

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 EDTA or 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: *Citrate plasma has not been validated for use in this assay.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and repeat this process a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Cell lysate samples require a 4-fold dilution. A suggested 4-fold dilution is 50 µL of sample + 150 µL of Calibrator Diluent RD5-27.

Serum and plasma samples require a 200-fold dilution. A suggested 200-fold dilution can be achieved by adding 10 µL of sample to 90 µL of Calibrator Diluent RD5-27. Complete the 200-fold dilution by adding 10 µL of the diluted sample to 190 µL Calibrator Diluent RD5-27.

Human milk samples require a 50-fold dilution. A suggested 50-fold dilution is 10 µL of sample + 490 µL of Calibrator Diluent RD5-27.

CELL LYSIS PROCEDURE

Use the following procedure for the preparation of cell lysate samples.

1. Wash cells three times in cold PBS.
2. Resuspend cells at 1 x 10^7 cells/mL in Cell Lysis Buffer 2.
3. Incubate with gentle agitation for up to 60 minutes at room temperature.
4. Centrifuge at 8000 x g for 10 minutes to remove cell debris.
5. Assay immediately or aliquot the lysis supernates and store at ≤ -70 °C until ready for use.

REAGENT PREPARATION

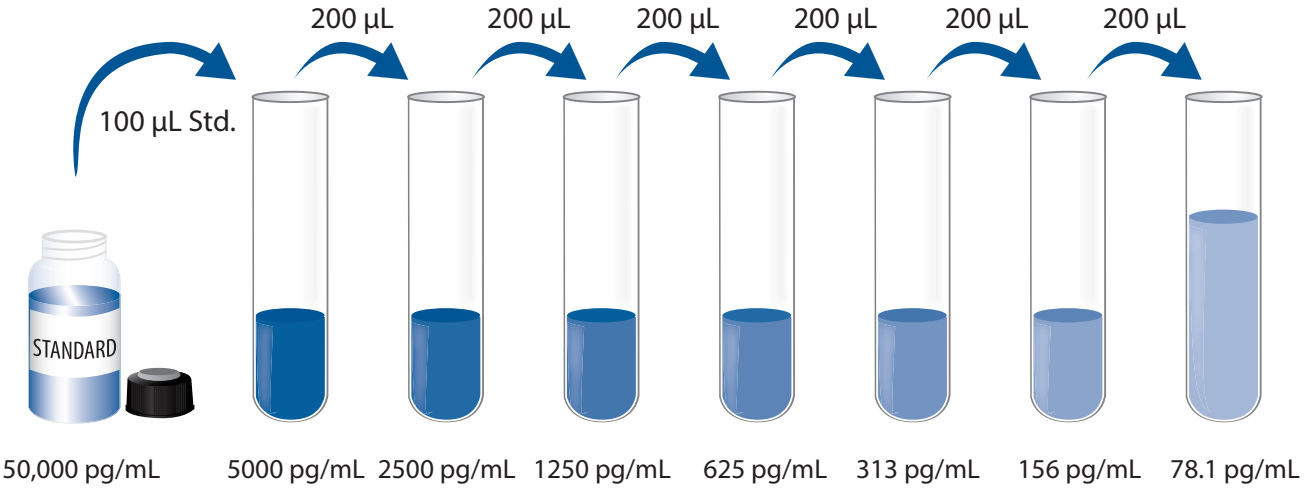
Bring all reagents to room temperature before use.

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 480 mL of 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.

Human Neuropilin-1 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Neuropilin-1 Standard with deionized or distilled water. This reconstitution produces a stock solution of 50,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5-27 into the 5000 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 5000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-27 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, controls, and samples 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 50 µL of Assay Diluent RD1-34 to each well.
4. Add 50 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 Neuropilin-1 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 the Sample Preparation section.

CALCULATION OF RESULTS

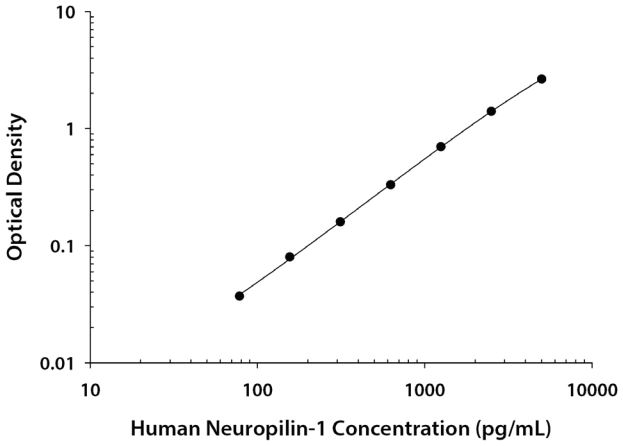
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a 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 human Neuropilin-1 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	0.008 0.008	0.008	—
78.1	0.045 0.045	0.045	0.037
156	0.087 0.088	0.088	0.080
313	0.167 0.168	0.168	0.160
625	0.337 0.341	0.339	0.331
1250	0.694 0.722	0.708	0.700
2500	1.399 1.413	1.406	1.398
5000	2.594 2.710	2.652	2.644

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

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	336	967	1890	329	934	1833
Standard deviation	17.9	70.4	54.7	19.8	48.4	90.4
CV (%)	5.3	7.3	2.9	6.0	5.2	4.9

RECOVERY

The recovery of human Neuropilin-1 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	91	86-99%
Cell lysate* (n=3)	101	94-108%
Urine (n=4)	99	85-117%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Neuropilin-1 were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernate* (n=4)	Cell lysates (n=3)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Urine (n=4)	Human milk* (n=4)
1:2	Average % of Expected	99	102	104	104	102	100	100
	Range (%)	97-101	97-108	100-105	103-106	100-105	92-110	98-100
1:4	Average % of Expected	99	107	108	108	105	101	102
	Range (%)	95-101	104-110	102-111	105-112	99-110	93-116	100-104
1:8	Average % of Expected	101	110	109	108	106	100	103
	Range (%)	90-109	108-114	104-111	104-113	101-111	94-110	101-106
1:16	Average % of Expected	107	111	109	107	107	103	103
	Range (%)	100-112	111-112	104-112	103-111	102-113	95-112	101-108

*Samples were diluted prior to assay.

SENSITIVITY

Twenty-three assays were evaluated and the minimum detectable dose (MDD) of human Neuropilin-1 ranged from 1.88-9.33 pg/mL. The mean MDD was 3.80 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 NS0-expressed recombinant human Neuropilin-1 manufactured at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human Neuropilin-1 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=36)	168,539	118,658-214,221	22,600
EDTA plasma (n=36)	179,599	120,706-235,364	25,663
Heparin plasma (n=36)	181,197	125,953-293,502	31,237
Human milk (n=10)	143,651	5,954-1,166,912	359,779

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Urine (n=11)	420	55	ND-712

ND=Non-detectable

Cell Culture Supernates/Cell Lysates:

HT-29 Human colon adenocarcinoma cells were cultured in McCoy's supplemented with 10% fetal bovine serum and grown until confluent. Cells were unstimulated or stimulated with 50 ng/mL of recombinant human EGF for 1 day. Aliquots of the cell culture supernates were removed and assayed for human Neuropilin-1. Cells were lysed and assayed for human Neuropilin-1. Cell lysate results were normalized to total protein concentration.

Condition	Cell Culture Media Value (pg/mL)	Cell Lysate Value (pg/mg)
Unstimulated	546	1903
Stimulated	665	3508

SPECIFICITY

This assay recognizes natural and recombinant human Neuropilin-1.

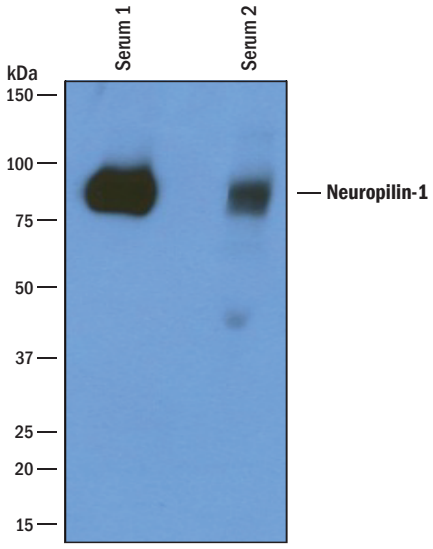
The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human Neuropilin-1 control were assayed for interference. No significant cross-reactivity or interference was observed.

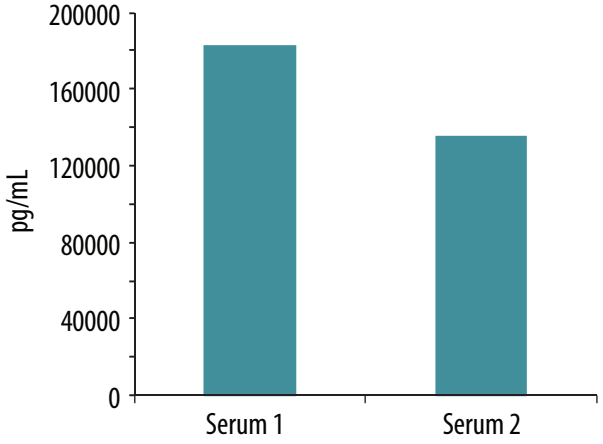
Recombinant human:

Neuropilin-2
Plexin A4
Semaphorin 3A
Semaphorin 3E

Other recombinants:

mouse Neuropilin-1
rat Neuropilin-1





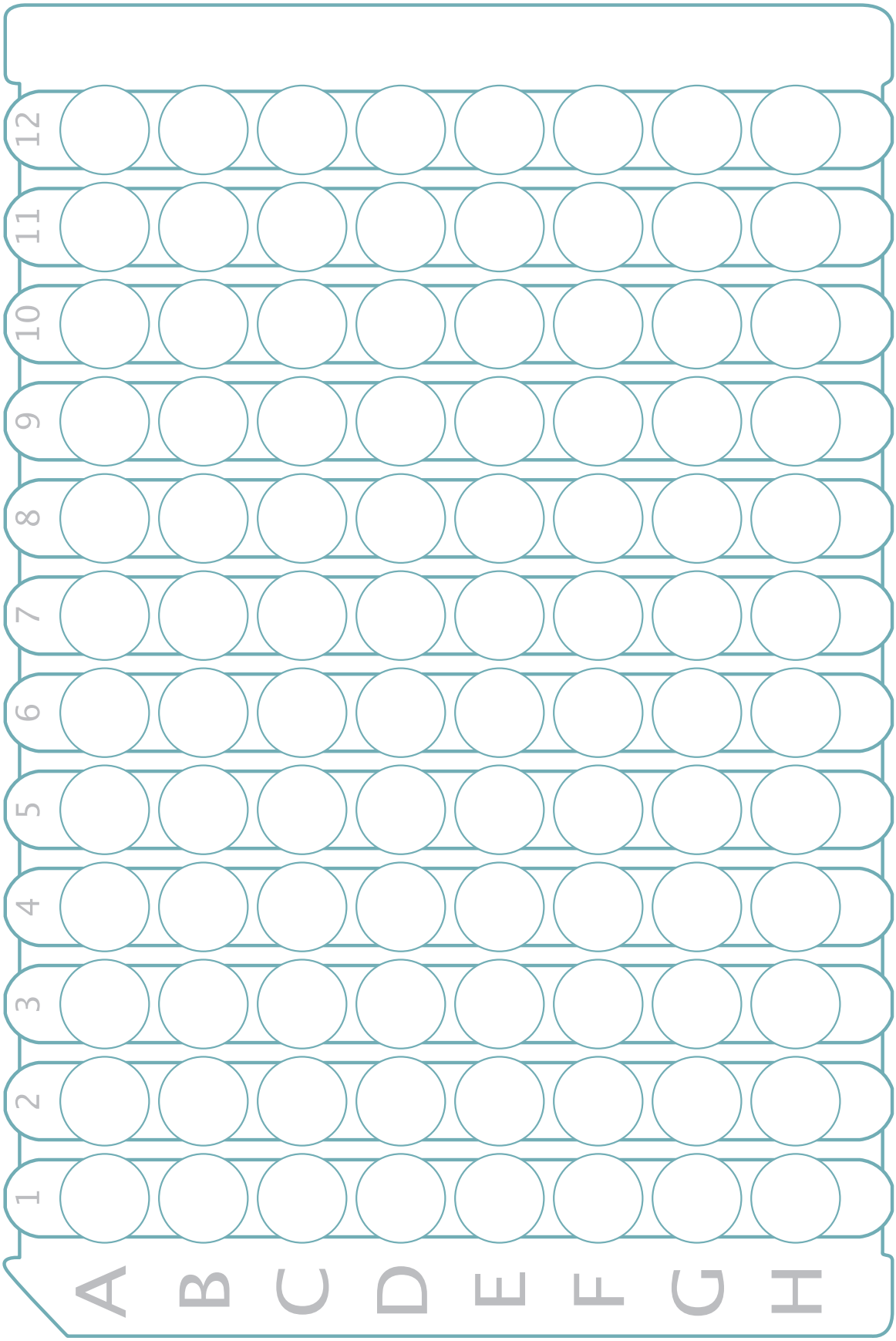
Normal human serum samples were analyzed by Western Blot and Quantikine™ ELISA. Serum samples were resolved under reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with the detection antibody in this kit. The Western Blot correlates well with the ELISA value for these samples.

REFERENCES

1. Takagi, S. *et al.* (1991) Neuron **7**:295.
2. Bielenberg, D.R. *et al.* (2006) Exp. Cell Res. **312**:584.
3. Wang, L. *et al.* (2006) FASEB J. **20**:1513.
4. Lu, Y. *et al.* (2009) mAbs **1**:364.
5. Prud'homme, G.J. and Y. Glinka (2012) Oncotarget **3**:921.
6. Klagsbrun, M. *et al.* (2002) Adv. Exp. Med. Biol. **515**:33.
7. Wild, J.R. *et al.* (2012) Int. J. Exp. Pathol. **93**:81.
8. Campos-Mora, M. *et al.* (2013) Front. Immunol. **4**:405.
9. Tordjman, R. *et al.* (2002) Nat. Immunol. **3**:477.
10. Bruder, D. *et al.* (2004) Eur. J. Immunol. **34**:623.
11. Kumanogoh, A. and H. Kikutani (2013) Nat. Rev. Immunol. **13**:802.
12. Renand, A. *et al.* (2013) PLoS One **8**:e85589.
13. He, Z. and M. Tessier-Lavigne (1997) Cell **90**:739.
14. Gu, C. *et al.* (2002) J. Biol. Chem. **277**:18069.
15. Schwarz, Q. and C. Ruhrberg (2010) Cell Adh. Migr. **4**:61.
16. Gu, C. *et al.* (2003) Dev. Cell **5**:45.
17. Soker, S. *et al.* (1998) Cell **92**:735.
18. Mamluk, R. *et al.* (2002) J. Biol. Chem. **277**:24818.
19. Miao, H.Q. and M. Klagsbrun (2000) Cancer Metastasis Rev. **19**:29.
20. Glinka, Y and G.J. Prud'homme (2008) J. Leukoc. Biol. **84**:302.
21. Hong, T.M. *et al.* (2007) Clin. Cancer Res. **13**:4759.
22. Bagri, A. *et al.* (2009) Clin. Cancer Res. **15**:1860.
23. Zhu, H. *et al.* (2013) Clin. Transl. Oncol. [Epub ahead of print. PMID 24338507].
24. Ben, Q. *et al.* (2014) Pancreas **43**:744.
25. Cheng, W. *et al.* (2014) Tumour Biol. **35**:6089.
26. Zhao, J. *et al.* (2014) Tumour Biol. [Epub ahead of print. PMID 24737589].
27. Shahrabi-Farahani, S. *et al.* (2014) Lab. Invest. [Epub ahead of print. PMID 24791743].
28. Gagnon, M.L. *et al.* (2000) Proc. Natl. Acad. Sci. USA **97**:2573.
29. Rossignol. M. *et al.* (2000) Genomics **70**:211.
30. Cackowski, F.C. *et al.* (2004) Genomics **84**:82.
31. Xin, Y. *et al.* (2012) Clin. Cancer Res. **18**:6040.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES