Quantikine® ELISA

Human HB-EGF Immunoassay

Catalog Number DHBEG0

For the quantitative determination of human Heparin-Binding EGF-like Growth Factor (HB-EGF) concentrations in cell culture supernates, serum, plasma, saliva, 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.

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INTRODUCTION

Human Heparin-Binding EGF-like growth factor (HB-EGF), also known as DTR, is a 12-16 kDa member of the EGF family of peptide growth factors (1-3). It is further classified as a group 2 ErbB ligand based on its ability to activate both the EGF R/ErbB1 and ErbB4 receptors (4, 5). HB-EGF is synthesized as a 208 amino acid (aa) type I transmembrane preproprecursor (1). It contains a 19 aa signal sequence, a 43 aa prosegment, an 86 aa mature region, an 11 aa juxtamembrane cleavage peptide, a 24 aa transmembrane segment, and a 25 aa cytoplasmic tail. HB-EGF is expressed as a 19-27 kDa protein in mammalian cells (6-8). The variability in molecular weight is attributed to heterogeneity in glycosylation and/or the utilization of multiple proteolytic cleavage sites during maturation. Mature HB-EGF is a soluble peptide that arises from proteolytic processing of the transmembrane form. It possesses an EGF-like domain between aa 104-144, and a heparin-binding motif between aa 93-113. Although the aa range for "mature" HB-EGF is typically stated to be Asp63-Leu148, potential N-terminal start (cleavage) sites also exist at Gly32, Arg73, Val74, Ser77, and Ala82 (7, 9-11). Thus, differential processing likely accounts, at least in part, for the 16-23 kDa range noted for mammalian-derived mature HB-EGF. Proteases suggested to contribute to HB-EGF processing include TACE/ADAM17, MMP-3 and -7, and ADAM12 (10, 12-15). Over aa 63-148, human HB-EGF shares 76% and 73% aa sequence identity with rat and mouse HB-EGF, respectively (1, 16).

Cells known to express HB-EGF include bronchial epithelium, visceral and vascular smooth muscle, $CD4^+$ T cells, cardiac muscle, glomerular podocytes, keratinocytes, and IL-10-secreting regulatory macrophages (12, 17-23). HB-EGF exerts mitogenic and/or chemotactic effects on a variety of cell types, including monocytes/macrophages, fibroblasts, smooth muscle cells, endothelial cells, and astrocytes (1, 20, 24-26). Accordingly, HB-EGF has been linked to many cellular processes, including proliferation, apoptosis, cell migration/invasion, differentiation, morphogenesis, and development (1, 27-38). HB-EGF also appears to be involved in several aspects of cancer development and progression (39-41).

The Quantikine Human HB-EGF Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human HB-EGF in cell culture supernates, serum, plasma, saliva, urine, and human milk. It contains *Sf* 21-expressed recombinant human HB-EGF and antibodies raised against the recombinant factor. Results obtained using natural human HB-EGF 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 HB-EGF.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human HB-EGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any HB-EGF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human HB-EGF 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 HB-EGF 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.
- Samples, controls, and standards must be pipetted within 15 minutes.
- 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 HB-EGF Microplate	894905	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human HB-EGF.	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 HB-EGF Standard	894907	2 vials of recombinant human HB-EGF 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 HB-EGF Conjugate	894906	21 mL of a polyclonal antibody specific for human HB-EGF conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895117	11 mL of a buffered protein base with and preservatives.	
Calibrator Diluent RD6-47	895570	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	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.
- Refrigerator (2-8 °C).
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human HB-EGF Controls (optional; R&D Systems, Catalog # QC215).

PRECAUTIONS

HB-EGF is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

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 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.

Platelet-poor Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -70 °C. Avoid repeated freeze-thaw cycles.

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

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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

Use polypropylene tubes.

Cell culture supernate and saliva samples require a 2-fold dilution due to matrix effect. A suggested 2-fold dilution is 150 µL of sample + 150 µL of Calibrator Diluent RD6-47.

REAGENT PREPARATION

The Conjugate must remain at 2-8 °C. Bring all remaining reagents to room temperature before use.

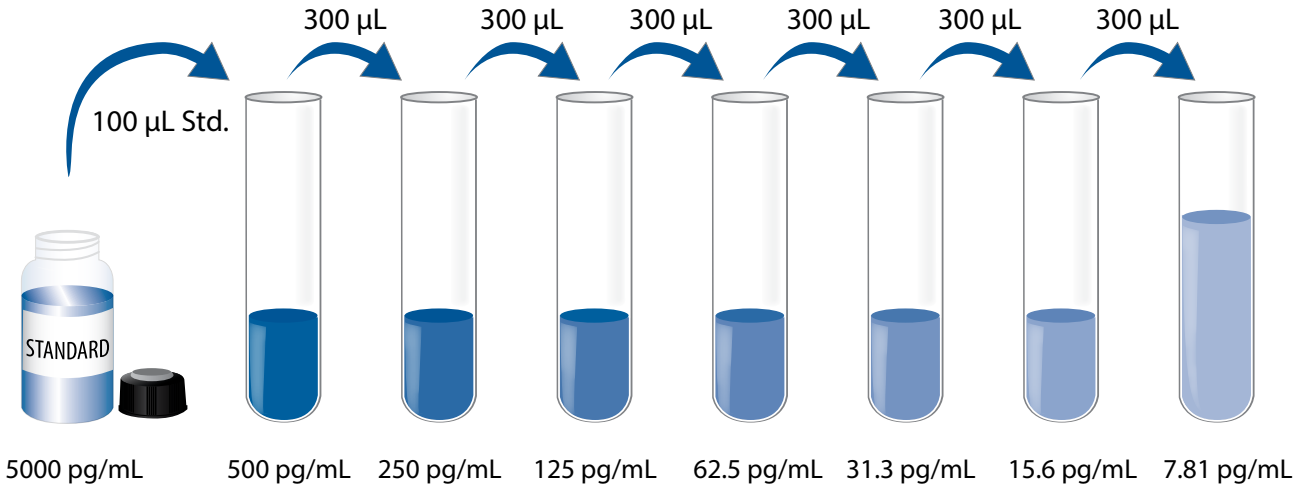
Note: *HB-EGF is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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.

Human HB-EGF Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human HB-EGF Standard with deionized or distilled water. This reconstitution produces a stock solution of 5000 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.

Use polypropylene tubes. Pipette 900 µL of Calibrator Diluent RD6-47 into the 500 pg/mL tube. Pipette 300 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 500 pg/mL standard serves as the high standard. Calibrator Diluent RD6-47 serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

The Conjugate must remain at 2-8 °C. Bring all remaining reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

Note: *HB-EGF is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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 RD1W 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 2-8 °C.** A plate layout is provided to record standards and samples assayed.

   Note: *Samples, controls, and standards must be pipetted within 15 minutes.*
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 HB-EGF Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours **at 2-8 °C.**
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

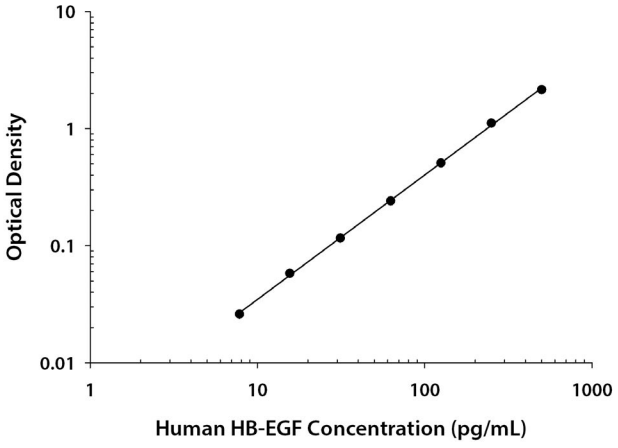
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a 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 HB-EGF 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	0.006 0.006	0.006	—
7.81	0.030 0.033	0.032	0.026
15.6	0.062 0.065	0.064	0.058
31.3	0.121 0.122	0.122	0.116
62.5	0.244 0.250	0.247	0.241
125	0.513 0.515	0.514	0.508
250	1.111 1.124	1.118	1.112
500	2.146 2.169	2.158	2.152

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)	82.0	186	328	76.0	171	313
Standard deviation	2.82	2.30	2.50	7.31	10.1	22.2
CV (%)	3.4	1.2	0.8	9.6	5.9	7.1

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media* (n=4)	120	113-125%
Serum (n=4)	95	79-107%
EDTA plasma (n=4)	99	83-114%
Heparin plasma (n=4)	84	75-96%
Platelet-poor EDTA plasma (n=4)	101	84-119%
Platelet-poor heparin plasma (n=4)	98	75-111%
Urine (n=4)	98	81-121%

*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 HB-EGF were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Platelet-poor		Urine (n=4)
						EDTA plasma (n=4)	Heparin plasma (n=4)	
1:2	Average % of Expected	102	106	99	106	104	108	114
	Range (%)	99-107	102-109	94-103	102-110	95-115	104-114	112-119
1:4	Average % of Expected	111	112	96	109	98	108	106
	Range (%)	101-122	106-117	87-106	100-118	89-113	97-118	105-111
1:8	Average % of Expected	99	109	97	112	100	109	102
	Range (%)	90-108	98-116	87-113	102-124	89-119	98-119	98-109
1:16	Average % of Expected	89	97	96	105	101	108	98
	Range (%)	79-98	85-115	75-114	87-120	83-122	102-121	95-101

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

SENSITIVITY

Nineteen assays were evaluated and the minimum detectable dose (MDD) of human HB-EGF ranged from 0.243-1.74 pg/mL. The mean MDD was 0.787 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 highly purified *Sf* 21-expressed recombinant human HB-EGF produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of human HB-EGF 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=72)	139	45.9-376	49.0
Saliva (n=10)	53.5	35.0-86.2	17.4
Human milk (n=10)	19.1	13.3-28.6	5.73

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
EDTA plasma (n=36)	28.1	94	ND-95.2
Heparin plasma (n=36)	17.6	89	ND-34.7
Platelet-poor EDTA plasma (n=36)	———	———	ND
Platelet-poor heparin plasma (n=36)	11.0	25	ND-17.2

Sample Type	Mean of Detectable (µg/g Creatinine)	% Detectable	Range (pg/mL)
Urine (n=10)	0.007	10	ND-9.47

ND=Non-detectable

Cell Culture Supernates:

Human peripheral blood leukocytes (PBL) were cultured in DMEM and supplemented with 5% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured stimulated with 10 µg/mL PHA for 1 or 5 days. Aliquots of the cell culture supernates were removed, assayed for levels of human HB-EGF, and measured 133 pg/mL and 530 pg/mL, respectively.

Human PBMC derived monocytes were stimulated with 10 ng/mL of recombinant human GM-CSF and recombinant human IL-4 for 7-9 days. 1 µg/mL LPS was added to mature cells. An aliquot of the cell culture supernate was removed, assayed for human HB-EGF, and measured 206 pg/mL.

U937 human histiocytic lymphoma cells were cultured in RPMI supplemented with 10% fetal bovine serum and PSG. Cells were cultured stimulated with 60 nM PMA for 1 day. An aliquot of the cell culture supernate was removed, assayed for human HB-EGF, and measured 798 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human HB-EGF.

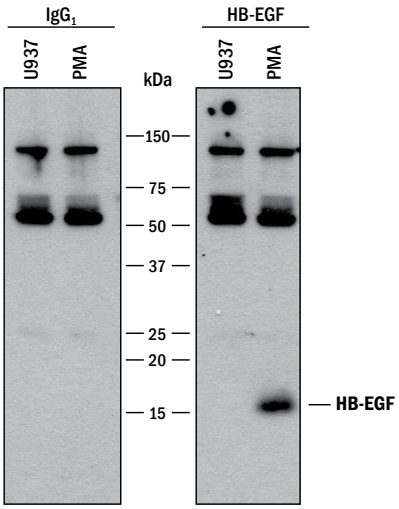
The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range control were assayed for interference. No significant cross-reactivity or interference was observed.

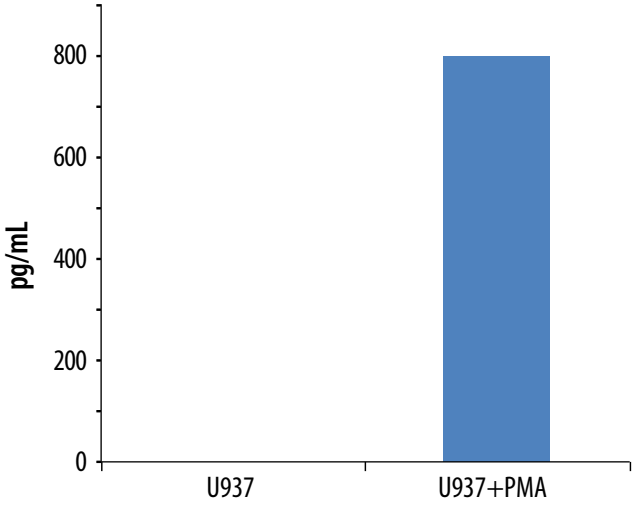
Recombinant human:
Amphiregulin
Betacellulin
EGF
EGF R
Epigen
Epiregulin
ErbB4
NRG-1
NRG-1α
NRG-1β1
NRG-2
TGF-α

Recombinant mouse:
EGF
EGF R
Epigen
Epiregulin
ErbB4
NRG-3

Recombinant rat:
EGF

Recombinant mouse HB-EGF cross-reacts approximately 0.29% in this assay.





U937 conditioned media samples were analyzed by Immunoprecipitation/Western blot and Quantikine ELISA. Immunoprecipitated samples were resolved under reducing SDS-PAGE conditions, transferred to PVDF membrane, and immunoblotted with the detection antibody used in this kit. The immunoprecipitation/Western blot shows direct correlation with the ELISA value for these samples.

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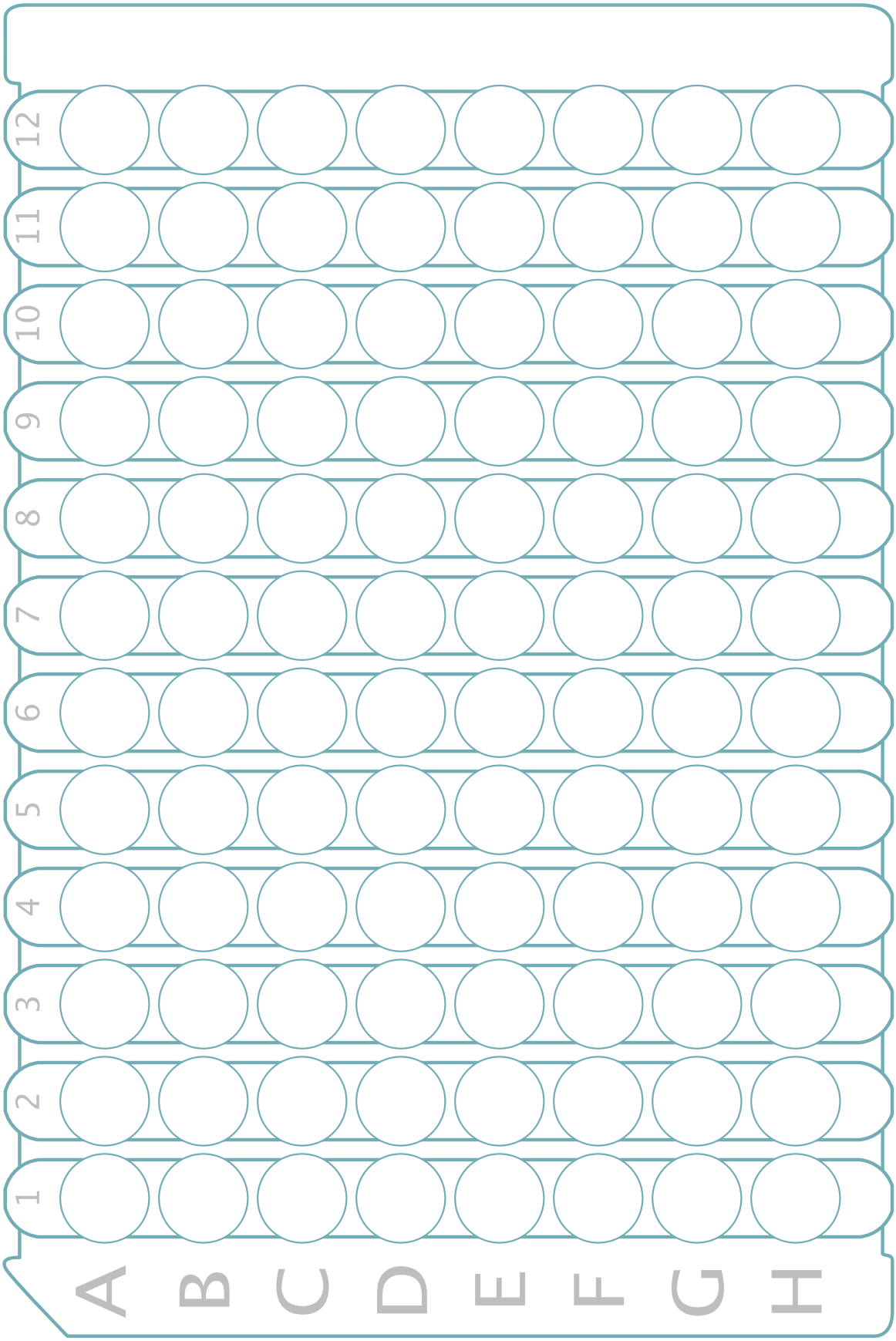
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES