Quantikine® ELISA

Human Leptin Immunoassay

Catalog Number DLP00
SLP00
PDLP00

For the quantitative determination of human Leptin 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

Human Leptin (gene name OB) is a 16 kDa, 146 amino acid (aa) residue, non-glycosylated polypeptide that regulates adipose tissue mass and energy balance (1-6). The name Leptin is derived from the Greek (leptos, or "thin") because of its ability to reduce fat stores (7). In mice (ob/ob) and humans, inactivating mutations of the OB gene can cause obesity (1-6). Mature human Leptin shares 87% and 84% aa identity with mouse and rat Leptin, respectively (1, 8). Human Leptin is active in both the mouse and rat systems (9, 10). Leptin is expressed almost exclusively by adipocytes and its production is influenced by hormones, cytokines and nutrients (5, 8, 11). For example, Leptin expression is enhanced by insulin and glucocorticoids, which are associated with positive energy balance, while catecholamines decrease Leptin production during negative energy balance (5). It circulates in the plasma, crosses the blood-brain barrier, and is present in human breast milk (3-6, 12).

The human Leptin receptor (designated ObR or LEPR) is a 150 kDa, 1144 aa residue, type I transmembrane glycoprotein of the IL-6 receptor family of Class I cytokine receptors (13, 14). The gene for ObR undergoes considerable splicing, forming variants a-d with cytoplasmic domains of variable length, plus the potentially soluble form ObRe (14, 15). The long form, ObRb (formerly OB RL), is expressed mainly in the hypothalamic arcuate nucleus and is essential for signal transduction (6, 16, 17). Of the short forms, ObRa is ubiquitous, and ObRa, ObRc, and ObRd are all thought to mediate Leptin binding and endocytosis, but not signal transduction (16). Upon binding of Leptin dimers, ObRb dimers may form signaling tetramers with shorter forms (16). Mutations of ObRb can cause obese phenotypes in both the mouse and rat. The mouse mutation (db/db for diabetes) occurs in the cytoplasmic domain, while the rat mutation (fa/fa for fatty) occurs in the extracellular domain of the receptor (18, 19). In a concentration-dependent manner, Leptin signaling can have diverse effects, causing neurons that express pro-opiomelanocortin (POMC) peptides to reduce food intake, and neurons that express neuropeptide Y and agouti-related protein (NpY and AgRP) to increase food intake (4, 6).

Leptin is fundamentally a "starvation signal" that, when low, prompts increased appetite and decreased energy expenditure (4, 6, 10). Adipocytes increase Leptin expression as cell size increases, which should result in depressed appetite and increased energy expenditure (5). However, obese humans are often resistant to these effects of Leptin (3). Leptin resistance is in part due to saturation of the blood-brain transporter, which is influenced by high circulating triglycerides, and in part due to decreased cellular response to Leptin (6). Rarely, obese humans are genetically Leptin-deficient (3-6). Leptin deficiency also influences the immune system, depressing Th1 responses and causing increased frequency of infections (4). Leptin also regulates puberty, blocking the onset of puberty, or of menses if Leptin deficiency exists due to excessive thinness, such as results from starvation, extreme exercise-induced weight loss, anorexia or cancer-induced cachexia (3, 4).

The Quantikine Human Leptin Immunoassay is a 3.5 hour solid phase ELISA designed to measure soluble human Leptin in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant human Leptin and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate recombinant Leptin accurately. Results obtained measuring natural human Leptin 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 human Leptin.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Leptin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Leptin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for Leptin 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 Leptin 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 fall outside the dynamic range of the assay, dilute samples appropriately with Calibrator Diluent and repeat the assay. If cell culture supernate samples require large dilutions, perform an intermediate dilution with culture media and the final dilution with the appropriate Calibrator Diluent.
- 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, however, 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.

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	CATALOG # DLP00	CATALOG # SLP00	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Leptin Microplate	890573	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against Leptin.	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.*
Leptin Conjugate	890574	1 vial	6 vials	21 mL/vial of mouse monoclonal antibody against Leptin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Leptin Standard	890575	1 vial	6 vials	10 ng/vial of recombinant human Leptin in a buffered protein base with preservative; lyophilized.	
Assay Diluent RD1-19	895467	1 vial	6 vials	11 mL/vial of a buffered protein base with preservatives.	
Calibrator Diluent RD5P Concentrate	895151	1 vial	6 vials	21 mL/vial of a concentrated buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative.	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

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

DLP00 contains sufficient materials to run an ELISA on one 96 well plate.
SLP00 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDLP00). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

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.
- 100 mL and 500 mL graduated cylinders.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human Leptin Controls (optional; available from R&D Systems).

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 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, heparin, or citrate 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.

SAMPLE PREPARATION

Most serum and plasma samples require a 100-fold dilution. A 100-fold dilution may be achieved by adding 10 µL of sample to 990 µL of Calibrator Diluent RD5P (1X).

If samples fall outside the dynamic range of the assay, a lower or higher dilution may be required.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

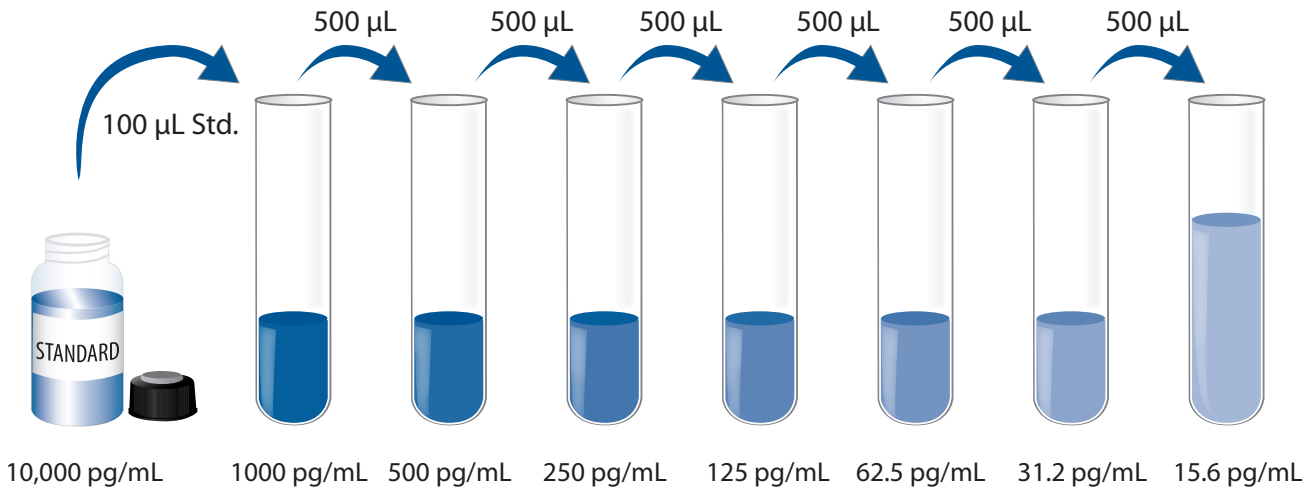
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

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.

Calibrator Diluent RD5P (1X) - Dilute 20 mL of Calibrator Diluent RD5P Concentrate into 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5P (1X).

Leptin Standard - Reconstitute the Leptin Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. 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 RD5P (1X) into the 1000 pg/mL tube. Pipette 500 µL of Calibrator Diluent RD5P (1X) into each of 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 RD5P (1X) 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, 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 RD1-19 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. 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 Leptin Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
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. **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.

*Serum and plasma samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

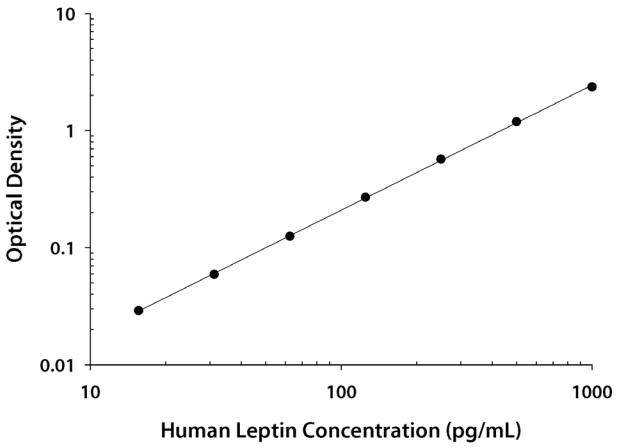
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 Leptin concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If the 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	0.015 0.015	0.015	—
15.6	0.044 0.044	0.044	0.029
31.2	0.075 0.073	0.074	0.059
62.5	0.144 0.136	0.140	0.125
125	0.285 0.282	0.284	0.269
250	0.588 0.581	0.584	0.569
500	1.211 1.195	1.203	1.188
1000	2.415 2.339	2.377	2.362

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 forty separate assays to assess inter-assay precision.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (pg/mL)	64.5	146	621	65.7	146	581
Standard deviation	2.14	4.32	20.0	3.56	6.17	20.6
CV (%)	3.3	3.0	3.2	5.4	4.2	3.5

RECOVERY

The recovery of Leptin spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=5)	98	94 - 102%
Serum* (n=5)	95	89 - 109%
EDTA plasma* (n=5)	99	85 - 112%
Heparin plasma* (n=5)	90	81 - 100%
Citrate plasma* (n=5)	95	87 - 105%

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

SENSITIVITY

The minimum detectable dose (MDD) of Leptin is typically less than 7.8 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.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Leptin were diluted with Calibrator Diluent RD5P (1X) to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=5)	Serum* (n=5)	EDTA plasma* (n=5)	Heparin plasma* (n=5)	Citrate plasma* (n=5)
1:2	Average % of Expected	105	99	99	99	98
	Range (%)	103-107	99-101	97-102	96-104	96-99
1:4	Average % of Expected	109	97	95	97	96
	Range (%)	106-114	94-102	94-99	93-100	93-99
1:8	Average % of Expected	109	92	92	94	93
	Range (%)	107-115	89-95	90-94	90-97	89-97
1:16	Average % of Expected	109	92	91	96	93
	Range (%)	106-113	87-97	86-94	90-100	89-96

*Samples were diluted prior to assay.

CALIBRATION

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

SAMPLE VALUES

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

Sample Type	Range (pg/mL)	Mean (pg/mL)
Male Serum (n = 16)	2205-11,149	4760
Female Serum (n = 36)	3877-77,273	20,676

Five additional male serum samples fell below the lowest standard, 15.6 pg/mL, when diluted 100-fold.

Note: *Values in EDTA and heparin plasma have been found to be comparable to paired serum samples. Values in citrate plasma have been found to be slightly decreased compared to paired serum, EDTA or heparin plasma samples.*

Cell Culture Supernates:
Human peripheral blood mononuclear cells (5 x 10^6 cells/mL) were cultured in RPMI supplemented with 5% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed on days 1 and 5 and assayed for levels of natural Leptin.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	ND	ND
Stimulated	152	75.9

ND=Non-detectable

BeWo human choriocarcinoma cells (1 x 10^6 cells/mL) were cultured in F-12 media supplemented with 15% fetal bovine serum. The cells were cultured unstimulated or stimulated with 2 µM forskolin and 20 µM forskolin. Aliquots of the cell culture supernates were removed on days 1, 2, and 3 and assayed for levels of natural Leptin.

Condition	Day 1 (pg/mL)	Day 2 (pg/mL)	Day 3 (pg/mL)
Unstimulated*	849	1549	1667
2 µM forskolin*	1231	1699	2054
20 µM forskolin*	1137	1725	2747

*Samples were diluted 20-fold prior to assay.

SPECIFICITY

This assay recognizes natural and recombinant human Leptin.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5P (1X) and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range rhLeptin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

ANG
AR
CNTF
β-ECGF
EGF
Epo
FGF acidic
FGF basic
FGF-4
FGF-5
FGF-6
G-CSF
GM-CSF
sgp130
GROα
GROβ
GROγ
HB-EGF
HGF
IFN-γ
IGF-I
IGF-II
IL-1α
IL-1β
IL-1ra
IL-1 sRI
IL-1 sRII
IL-2
IL-2 sRα
IL-3
IL-3 sRα
IL-4
IL-4 sR
IL-5
IL-5 sRβ
IL-6
IL-6 sR
IL-7
IL-8
IL-9
IL-10
IL-11
IL-12
IL-13
KGF
LAP (TGF-β1)
LIF
M-CSF
MCP-1
MIP-1α
MIP-1β
β-NGF
OSM
PD-ECGF
PDGF-AA
PDGF-AB
PDGF-BB
PTN
RANTES
SCF
SLPI
TGF-α
TGF-β1
TGF-β3
TGF-β sRII
TNF-α
TNF-β
sTNF RI
sTNF RII
VEGF

Recombinant mouse:

GM-CSF
IL-1α
IL-1β
IL-3
IL-4
IL-5
IL-5 sRα
IL-6
IL-7
IL-9
IL-10
IL-13
Leptin
LIF
MIP-1α
MIP-1β
SCF
TNF-α

Recombinant amphibian:

TGF-β5

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
human TGF-β1
porcine TGF-β1
porcine TGF-β2

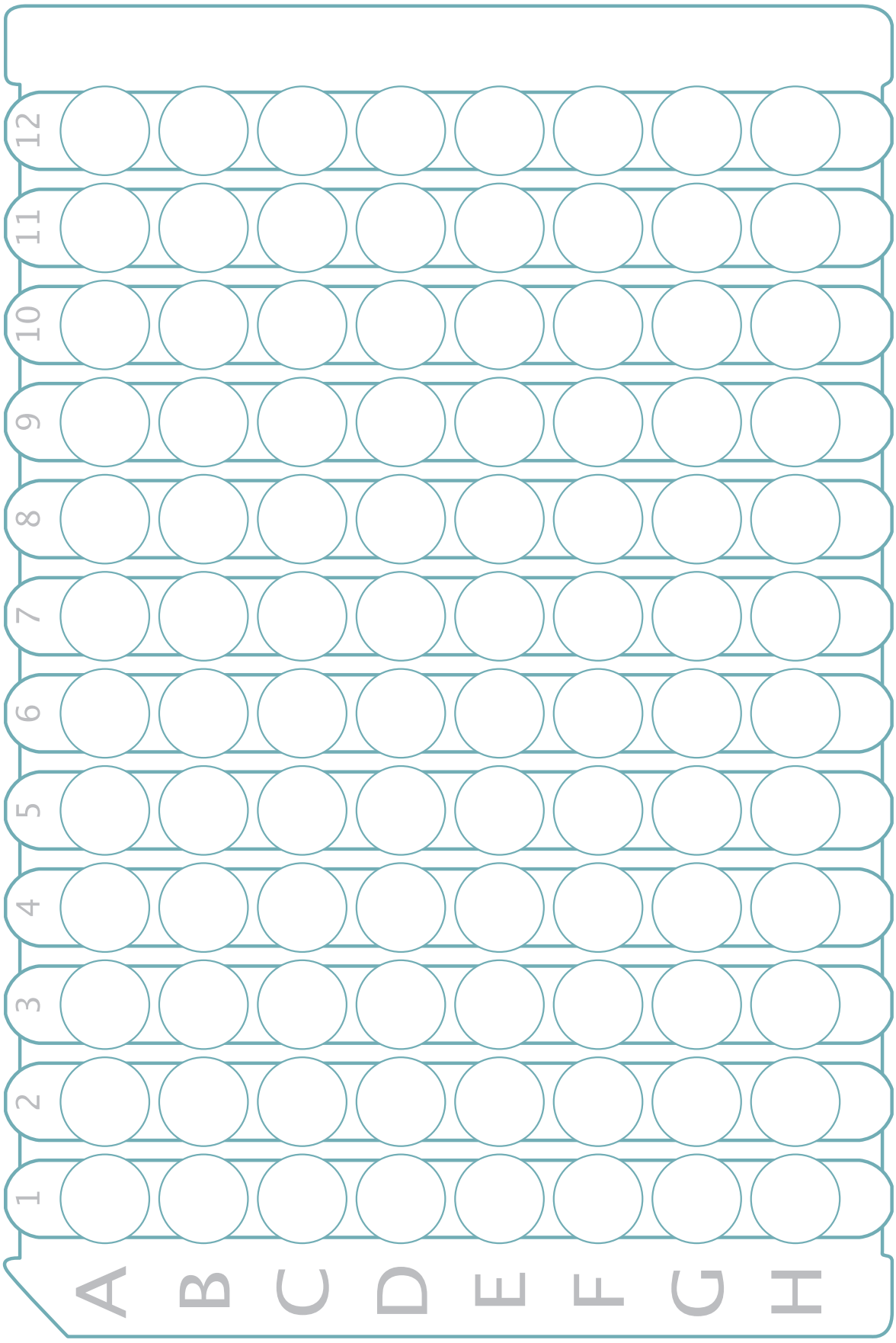
Recombinant human Leptin R/Fc chimera and recombinant mouse Leptin R/Fc chimera do not cross-react in this assay; however, interference was observed at concentrations ≥ 0.78 ng/mL.

REFERENCES

1. Zhang, Y. *et al.* (1994) Nature **372**:425.
2. Cohen, S.L. *et al.* (1996) Nature **382**:589.
3. Friedman, J.M. (2009) Am. J. Clin. Nutr. **89**:973S.
4. Farooqi, I.S. and S. O'Rahilly (2009) Am. J. Clin. Nutr. **89**:980S.
5. Lee, M-J. and S.K. Fried (2009) Am. J. Physiol. Endocrinol. Metab. **296**:E1230.
6. Oswal, A. and G. Yeo (2010) Obesity **18**:221.
7. Halaas, J.L. *et al.* (1995) Science **269**:543.
8. Ogawa, Y. *et al.* (1995) J. Clin. Invest. **96**:1647.
9. Verploegen, S.A.B.W. *et al.* (1997) FEBS Lett. **405**:237.
10. Satoh, N. *et al.* (1997) Neurosci. Lett. **224**:149.
11. Leroy, P. *et al.* (1996) J. Biol. Chem. **271**:2365.
12. Savino, F. *et al.* (2010) Eur. J. Clin. Nutr. **64**:972.
13. Cohen, B. *et al.* (1996) Science **274**:1185.
14. Tartaglia, L.A. *et al.* (1995) Cell **83**:1263.
15. Murakami, T. *et al.* (1997) Biochem. Biophys. Res. Commun. **231**:26.
16. Bacart, J. *et al.* (2010) FEBS Lett. **584**:2213.
17. Tu, H. *et al.* (2007) J. Cell. Physiol. **212**:215.
18. Chen, H. *et al.* (1996) Cell **84**:491.
19. Phillips, M.S. *et al.* (1996) Nat. Genet. **13**:18.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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