

Quantikine™ ELISA

Human HMW Adiponectin/Acrp30 Immunoassay

Catalog Number DHWAD0

SHWAD0

PDHWAD0

For the quantitative determination of human High Molecular Weight Adiponectin (HMW Adiponectin) concentrations in 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

Adiponectin, also known as Acrp30, apM1, AdipoQ, and GBP28, is a 30 kDa glycoprotein that is secreted primarily by adipocytes and induces wide ranging paracrine and endocrine effects on metabolism and inflammation (1-3). Mature human Adiponectin consists of a 60 amino acid (aa) N-terminal collagenous region and a 137 aa C-terminal C1q/TNF- α -like globular domain and shares approximately 85% aa sequence identity with mouse and rat Adiponectin (4).

Adiponectin forms 90 kDa homotrimers that contain two disulfide-linked monomers and a third subunit which is noncovalently associated. Two trimers can be covalently linked to create a 180 kDa hexamer which associates into >300 kDa high molecular weight (HMW) Adiponectin (5-7). The various forms of Adiponectin do not interconvert in the serum (7). Adiponectin is O-glycosylated on four hydroxylated lysines in its collagen domain, a modification which is required for the intracellular formation of HMW Adiponectin and its insulin-sensitizing activity (8, 9). The ratio between different forms of Adiponectin may be biologically significant; a much greater amount of HMW Adiponectin circulates in females compared to males, although the levels of trimeric and hexameric Adiponectin are comparable between genders (7, 10, 11). A cleaved form of Adiponectin, known as gAdiponectin, consists of the globular domains in trimeric complexes (12, 13). Circulating Adiponectin levels are high, comprising approximately 0.01% of total plasma protein (10). Adiponectin exerts its bioactivity through interactions with the 7-transmembrane receptors AdipoR1 and AdipoR2 (14-16). The widely expressed AdipoR1 binds gAdiponectin with high affinity but binds full length Adiponectin with very low affinity (14). AdipoR2 binds both the full length and globular forms with intermediate affinity and is relatively restricted to the liver (14). The various forms of Adiponectin also differentially interact with Cadherin-13 in muscle and with several growth factors (17, 18).

Adiponectin promotes insulin sensitivity through multiple actions on glucose and fatty acid metabolism, frequently in opposition to the actions of TNF- α (19-23). It induces a decrease in serum glucose and triglyceride levels, an increase in serum glucagon, but no change in insulin levels (20, 22, 24). In the liver, Adiponectin enhances the insulin-dependent inhibition of gluconeogenesis (22, 24). In skeletal muscle, Adiponectin promotes fatty acid uptake and oxidation, glucose uptake, and lactate production (12, 19, 20, 25, 26). HMW Adiponectin is the most potent isoform at inducing insulin sensitization in liver, and gAdiponectin is more potent than the full length molecule at inducing metabolic effects in muscle (8, 9, 12, 25-27). The various isoforms of Adiponectin differentially trigger the activation of AMPK and NF κ B in liver and muscle (6, 8, 25, 26, 28). In the adult (but not in the fetus), elevated levels of circulating total Adiponectin, and particularly HMW Adiponectin, are negatively correlated with conditions related to metabolic syndrome (10, 29). Decreased plasma HMW Adiponectin levels are associated with upper body obesity, insulin resistance, reduced fatty acid oxidation, dyslipidemia, coronary artery disease, and atherogenesis (30-33). Plasma HMW Adiponectin levels increase in response to treatment with insulin-sensitizing thiazolidinediones (27, 34).

INTRODUCTION *CONTINUED*

Adiponectin inhibits inflammation by antagonizing TNF- α induced vascular endothelial cell apoptosis and the upregulation of leukocyte adhesion proteins on the vascular endothelium (32, 35, 36). In macrophages, Adiponectin promotes polarization toward the M2 anti-inflammatory phenotype, inhibits TNF- α production, and interacts with C1qR_p to promote the clearance of Adiponectin-opsonized apoptotic cell debris (37-39). It protects against atherosclerosis by suppressing nitric oxide formation, the progression of macrophages into foam cells, and the migration of adventitial fibroblasts to the intima (40, 41). In nonmetabolic disorders such as rheumatoid arthritis and inflammatory bowel disease, however, Adiponectin levels are elevated and it can promote inflammation (42-45). Adiponectin also negatively regulates myelomonocytic progenitor cell growth (38).

The Quantikine™ Human HMW Adiponectin/Acrp30 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human HMW Adiponectin in serum and plasma. It contains NS0-expressed recombinant human HMW Adiponectin and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human HMW Adiponectin 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 natural human HMW Adiponectin.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human HMW Adiponectin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any HMW Adiponectin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human HMW Adiponectin 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 HMW Adiponectin 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 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 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 # | CATALOG # DHWAD0 | CATALOG # SHWAD0 | DESCRIPTION | STORAGE OF OPENED/ RECONSTITUTED MATERIAL |
|----------------------------------|--------|---------------------|---------------------|--|--|
| Human HMW Adiponectin Microplate | 893766 | 1 plate | 6 plates | 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human HMW Adiponectin. | 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 HMW Adiponectin Conjugate | 893767 | 1 vial | 6 vials | 21 mL /vial of a monoclonal antibody specific for human HMW Adiponectin conjugated to horseradish peroxidase with preservatives. | May be stored for up to 1 month at 2-8 °C.* |
| Human HMW Adiponectin Standard | 893768 | 1 vial | 6 vials | Recombinant human HMW Adiponectin in a buffer with preservatives; lyophilized. <i>Refer to the vial label for reconstitution volume.</i> | |
| Assay Diluent RD1W | 895117 | 1 vial | 6 vials | 11 mL/vial of a buffered protein solution with preservatives. | |
| Calibrator Diluent RD6-61 | 895975 | 3 vials | 18 vials | 21 mL/vial of a 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 preservatives. <i>May turn yellow over time.</i> | |
| 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 2N sulfuric acid. | |
| Plate Sealers | N/A | 4 strips | 24 strips | Adhesive strips. | |

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

DHWAD0 contains sufficient materials to run an ELISA on one 96 well plate.

SHWAD0 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PDHWAD0). Refer to the PharmPak Contents section for specific vial counts.

PHARMPAK CONTENTS

Each PharmPak contains reagents sufficient for the assay of 50 microplates (96 wells/plate). The package inserts supplied are the same as those supplied in the single kit packs and because of this, a few minor differences related to the number of reagents and their container sizes should be noted.

- Sufficient material is supplied to perform at least 50 standard curves; reuse of each vial may be required. The number of vials, and the number of standard curves obtained per vial will vary with the analyte.
- Wash Buffer 25X Concentrate is bulk packed in 125 mL bottles containing 100 mL.

Note: Additional wash buffer is available for purchase (R&D Systems®, Catalog # WA126).

The reagents provided in this PharmPak are detailed below.

| PART | PART # | QUANTITY |
|----------------------------------|--------|------------|
| Human HMW Adiponectin Microplate | 893766 | 50 plates |
| Human HMW Adiponectin Conjugate | 893767 | 50 vials |
| Human HMW Adiponectin Standard* | 893768 | 25 vials |
| Assay Diluent RD1W | 895117 | 50 vials |
| Calibrator Diluent RD6-61 | 895975 | 150 vials |
| Wash Buffer Concentrate | 895126 | 9 bottles |
| Color Reagent A | 895000 | 50 vials |
| Color Reagent B | 895001 | 50 vials |
| Stop Solution | 895032 | 50 vials |
| Plate Sealers | N/A | 100 sheets |

*If additional standard vials are needed, contact Technical Service at techsupport@bio-technie.com

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
- Test tubes for dilution of standards and samples
- Human HMW Adiponectin Controls (Optional; R&D Systems®, Catalog # QC235)

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.

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 $\leq -20^{\circ}\text{C}$. 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 $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

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

SAMPLE PREPARATION

Serum and plasma samples require a 100-fold dilution. A suggested 100-fold dilution is 10 μL of sample + 990 μL of Calibrator Diluent RD6-61.

REAGENT PREPARATION

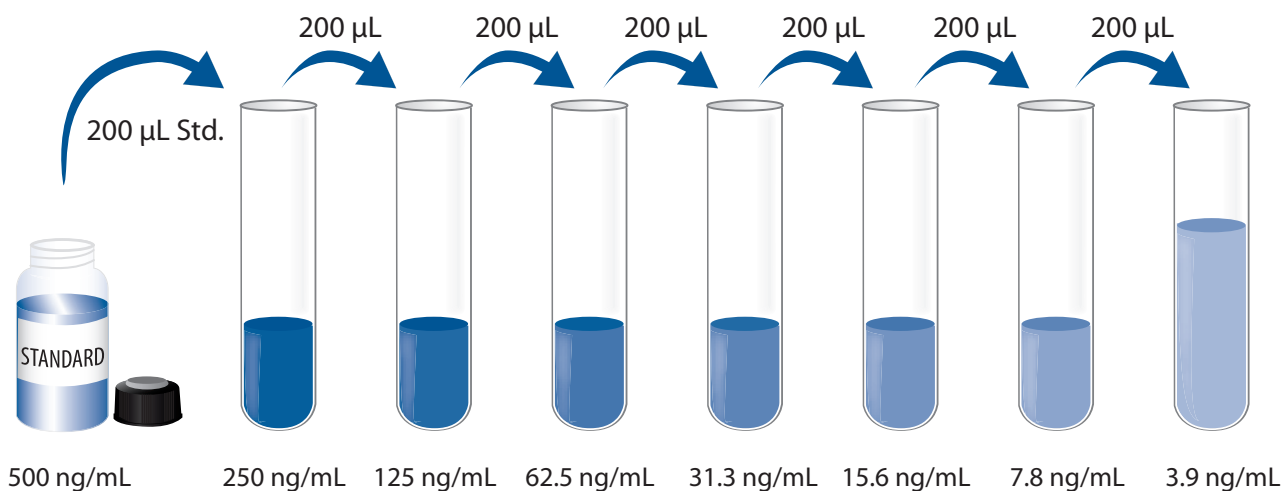
Bring all reagents to room temperature before use.

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 μ L of the resultant mixture is required per well.

Human HMW Adiponectin Standard - Refer to the vial label for reconstitution volume. Reconstitute the Human HMW Adiponectin Standard with deionized or distilled water. This reconstitution produces a stock solution of 500 ng/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 200 μ L of Calibrator Diluent RD6-61 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 250 ng/mL standard serves as the high standard. Calibrator Diluent RD6-61 serves as the zero standard (0 ng/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 100 μL of Assay Diluent RD1W to each well.
4. Add 50 μL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 3 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 Human HMW Adiponectin 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.

*Samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

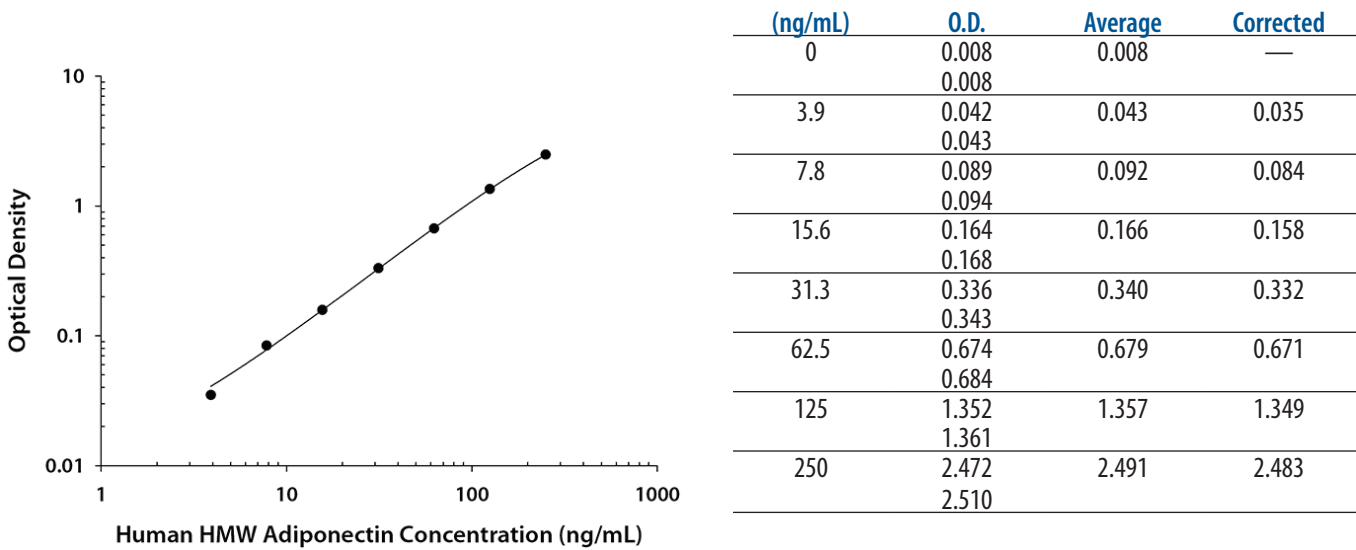
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 HMW Adiponectin 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.

Since 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.



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 (ng/mL) | 25.6 | 70.6 | 141 | 27.7 | 70.4 | 134 |
| Standard deviation | 0.67 | 2.64 | 3.97 | 2.4 | 6.0 | 11.1 |
| CV (%) | 2.6 | 3.7 | 2.8 | 8.6 | 8.5 | 8.3 |

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of human HMW Adiponectin were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. All samples were diluted prior to assay as directed in the Sample Preparation section.

| | | Serum (n=4) | EDTA plasma (n=4) | Heparin plasma (n=4) |
|------|-----------------------|----------------|-------------------------|----------------------------|
| 1:2 | Average % of Expected | 100 | 96 | 97 |
| | Range (%) | 96-104 | 92-101 | 91-103 |
| 1:4 | Average % of Expected | 95 | 95 | 95 |
| | Range (%) | 92-102 | 88-106 | 88-110 |
| 1:8 | Average % of Expected | 92 | 92 | 90 |
| | Range (%) | 87-102 | 87-104 | 85-104 |
| 1:16 | Average % of Expected | 94 | 92 | 90 |
| | Range (%) | 90-103 | 85-106 | 83-104 |

SENSITIVITY

Thirty-four assays were evaluated and the minimum detectable dose (MDD) of human HMW Adiponectin ranged from 0.086-0.989 ng/mL. The mean MDD was 0.195 ng/mL.

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

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human HMW Adiponectin produced at R&D Systems®.

SAMPLE VALUES

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

| Sample Type | Mean (ng/mL) | Range (ng/mL) | Standard Deviation (ng/mL) |
|------------------------------|--------------|---------------|----------------------------|
| Male serum (n=12) | 4103 | 1686-6751 | 1892 |
| Female serum (n=24) | 7215 | 2595-11,882 | 2321 |
| Male EDTA plasma (n=12) | 3962 | 1539-6985 | 1926 |
| Female EDTA plasma (n=24) | 6842 | 2524-10,906 | 2215 |
| Male heparin plasma (n=12) | 3889 | 1590-6507 | 1807 |
| Female heparin plasma (n=24) | 6848 | 2445-11,948 | 2286 |

SPECIFICITY

This assay recognizes natural and recombinant human HMW Adiponectin.

The factors listed below were prepared at 2500 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 2500 ng/mL in a mid-range recombinant human HMW Adiponectin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

LMW Adiponectin
MMW Adiponectin
C1qTNF9
SP-D

Recombinant mouse:

Adiponectin
gAdiponectin
HMW Adiponectin
LMW Adiponectin

Natural proteins:

human C1q

Rat Adiponectin interferes at levels > 1250 ng/mL.

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 HMW Adiponectin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

4-1BB Ligand
APRIL
BAFF/BLyS
CD27 Ligand
CD30 Ligand
CD40 Ligand
Fas Ligand
GITR Ligand
LIGHT
Lymphotoxin- α 1/ β 2
Lymphotoxin- α 2/ β 1
OX40 Ligand
TNF- α
TNF- β
TRAIL
TRANCE
TWEAK
VEGI

Recombinant mouse:

CD27 Ligand
CD30 Ligand
Fas Ligand
Lymphotoxin α 1/2
Lymphotoxin α 2/ β 1
OX40 Ligand
TNF- α
TRANCE

Recombinant rat:

TNF- α

Recombinant porcine:

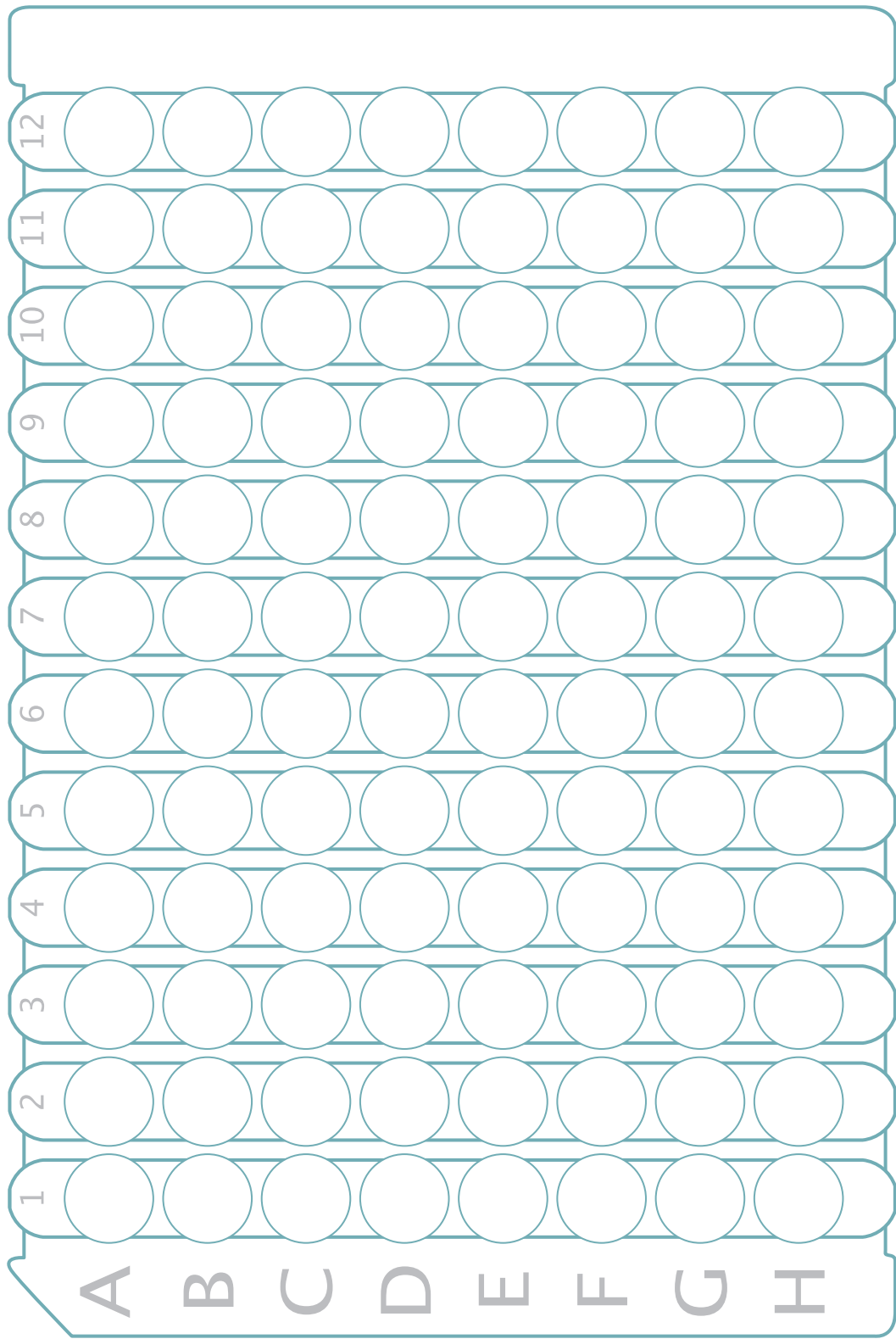
TNF- α

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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