High Molecular Weight (HMW) & Total Adiponectin ELISA

For the quantitative determination of high molecular weight (HMW) & total adiponectin in human serum and plasma (EDTA, heparin, citrate)

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number:	**80-ADPHU-E01**
Size:	**96 Wells**
Version:	**1.2**

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INTENDED USE

The HMW & Total Adiponectin ELISA is designed for the quantitative determination of HMW and total adiponectin in human serum and plasma (EDTA, heparin, and citrate) samples.

Adiponectin is a research-based biomarker which does not have any established clinical utility to date. This assay has been developed as a life science research tool and is offered For Research Use Only. Not for Use in Diagnostic Procedures.

PRINCIPLE OF THE ASSAY

The HMW & Total Adiponectin ELISA is a simultaneous sandwich assay, which uses two mouse monoclonal antibodies: one as capture that is adsorbed to a 96-well microplate and the other as a primary detector conjugated to biotin. Standards, diluted sample (treated with Sample Treatment Enzyme or untreated), controls and biotinylated detection antibody are added to the plate and the capture-antigen-detection sandwich is incubated at room temperature on a plate shaker set between 700-900 rpm. Post SA-HRP incubation, the plate is washed six times and the TMB substrate is added to each well and the microplate is incubated on a microplate shaker set at 700-900 rpm. Stop solution is added and the optical density (OD) is measured by a spectrophotometer at 450nm. The intensity of color generated is directly proportional to the amount of HMW and/or total adiponectin present in the sample.

MATERIALS SUPPLIED

Component	Quantity	Preparation
Adiponectin Microplate (96 wells)	12 x 8 strips	Ready to use
Sample Treatment Enzyme^	2 vials	Lyophilized
Adiponectin Standard	1 vial	Lyophilized*
Controls (Low and High) and Serum Control	1 vial each	Lyophilized*
Sample Treatment Buffer^	5 mL	Ready to use
Assay Buffer	55 mL	Ready to use
Adiponectin Detector Antibody	70 µL	101X
Detector Antibody Buffer	6 mL	Ready to use
SA-HRP	150 µL	101X
SA-HRP Buffer	12 mL	Ready to use
TMB Substrate	12 mL	Ready to use
Stop Solution	12 mL	Ready to use
Wash Buffer Concentrate	100 mL	21X
Plate Sealers	3	Ready to use

*Please refer to the Certificate of Analysis enclosed with each kit for more information.
^Use when quantifying levels of HMW adiponectin; do not use when quantifying levels of total adiponectin.

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MATERIALS REQUIRED

- Precision pipettes for dispensing up to 1000 µL (with disposable tips)
- Repeating or multi-channel pipette for dispensing up to 100 µL
- Volumetric containers and pipettes for reagent preparation
- Distilled/Deionized water for reagent preparation
- Microplate washer or wash bottle
- Microplate shaker capable of 700-900 rpm
- Microplate reader with 450nm filter
- Centrifuge (10,000 xg to 16,000 xg), vortex, and ice for sample preparation
- Microcentrifuge tubes (1.5 mL or larger) for sample preparation (2 tubes per sample)
- Heat block or water bath capable of reaching 37°C

PRECAUTIONS

1. The human blood products incorporated into this kit have been tested for the presence of HIV (Human Immunodeficiency Virus), HBV (Hepatitis B Virus), and HCV (Hepatitis C Virus). Test methods for these viruses do not guarantee the absence of a virus; therefore, all reagents should be treated as potentially infectious. Handling and disposal should be in accordance with all appropriate national and local regulations for the handling of potentially biohazardous materials.
2. All materials derived from animal sources are bovine spongiform encephalopathy (BSE) negative. However, all materials should be treated as potentially infectious.
3. Avoid direct contact with skin.
4. This product is not for internal use.
5. Avoid eating, drinking, or smoking when using this product.
6. Do not pipette any reagents by mouth.
7. Reagents from this kit are lot-specific and must not be substituted.
8. Do not use reagents beyond the expiration date.
9. Variations to the test procedure are not recommended and may influence the test results.

STORAGE CONDITIONS

The kit should be stored at 2-8°C. The kit is stable until the expiration date on the box label.

If desired, the reconstituted controls and standard can be stored at ≤ -20°C in aliquots for up to 6 months. The reconstituted Sample Treatment Enzyme can be stored at ≤ -20°C for up to 1 month. The frozen reconstituted materials should not be repeatedly frozen and thawed.

SAMPLE HANDLING

Serum and plasma (EDTA, heparin, and citrate) samples are appropriate for use in this assay.

For serum samples, allow collected blood to clot on ice for 20 minutes and then subject the samples to 1,000-2,000 xg centrifugation for 20 minutes at 2-8°C. If testing will occur within 2 hours after collection, keep the serum samples on ice. If the assay is to be performed at a later time, store the serum samples at ≤ -20°C.

If EDTA, heparin, or citrate plasma samples are to be assayed within 2 hours after collection, keep the samples on ice. If the assay is to be performed at a later time, store the samples at

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≤ -20°C.

Upon thawing samples, keep the samples on ice until performing the assay. Avoid repeated freeze/thaw cycles.

It is recommended that all samples be centrifuged at 13,000 RPM at 2-8°C for 10 minutes before starting the sample treatment process.

REAGENT PREPARATION

All reagents must be equilibrated to room temperature prior to preparation and subsequent use in the assay. Prepare enough reagents for only the number of strips used.

Adiponectin Standard is provided in a concentrated lyophilized form. Refer to the Certificate of Analysis included with each kit for information on reconstitution volume. Reconstitute the standard stock with deionized water. Close the vial with the rubber stopper and cap, gently swirl the vial, and allow it to stand for a minimum of 10 minutes prior to use. The contents of the vial should be in solution with no visible particulates. If desired, the reconstituted standard stock can be stored at ≤ -20°C in aliquots for up to 6 months. The reconstituted standard stock should not be repeatedly frozen and thawed. Standard A is prepared by diluting reconstituted standard stock with Assay Buffer. Refer to the Certificate of Analysis for information on dilution volumes. Standards B – G are prepared by serially diluting Standard A with Assay Buffer, as specified in the table below.

Standard	Volume of Source	Volume of Assay Buffer	Standard Concentration (ng/mL)
A	See C of A	See C of A	5.000
B	300 µL Std A	300 µL	2.500
C	300 µL Std B	300 µL	1.250
D	300 µL Std C	300 µL	0.625
E	300 µL Std D	300 µL	0.313
F	300 µL Std E	300 µL	0.156
G	300 µL Std F	300 µL	0.078

Adiponectin Controls (Levels Low and High) are provided in a lyophilized form. Refer to the Certificate of Analysis included with each kit for information on reconstitution volume. Reconstitute each control with deionized water. Close the vial with the rubber stopper and cap, gently swirl the vial, and allow it to stand for a minimum of 30 minutes prior to use. The contents of the vial should be in solution with no visible particulates. If desired, the controls can be stored at ≤ -20°C in aliquots for up to 6 months or at 2-8°C for 1 week. The controls should not be repeatedly frozen and thawed.

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Adiponectin Serum Control is provided in a lyophilized form. Prepare only when quantifying levels of HMW Adiponectin. Not required for quantification of Total Adiponectin. Refer to the Certificate of Analysis included with each kit for information on reconstitution volume. Reconstitute the serum control with deionized water. Close the vial with the rubber stopper and cap, gently swirl the vial, and allow it to stand for a minimum of 30 minutes prior to use. The contents of the vial should be in solution with no visible particulates. If desired, the control can be stored at ≤ -20°C in aliquots for up to 6 months. The control should not be repeatedly frozen and thawed.

Sample Treatment Enzyme is provided in a lyophilized form. Prepare only when quantifying levels of HMW Adiponectin. Not required for quantification of Total Adiponectin. Carefully open stopper on one vial and reconstitute the vial with 2mL of Sample Treatment Buffer, then close the vial and allow it to stand for 20-30 minutes. If desired, the working strength Sample Treatment Enzyme can be stored at ≤ -20°C in aliquots for up to 1 month.

Adiponectin Detector Antibody is to be diluted with Adiponectin Detector Antibody Buffer to make the Working Strength Detector Antibody solution.

Number of plates	Amount of Adiponectin Detector Antibody	Amount of Detector Antibody Buffer
0.25 (3 strips)	15 µL	1.5 mL
0.5 (6 strips)	30 µL	3.0 mL
0.75 (9 strips)	45 µL	4.5 mL
1.0 (12 strips)	60 µL	6.0 mL

Streptavidin-HRP (SA-HRP) is to be diluted with SA-HRP Buffer to make the working strength SA-HRP solution.

Number of plates	Amount of Concentrate	Amount of SA-HRP Buffer
0.25 (3 strips)	30 µL	3.0 mL
0.50 (6 strips)	60 µL	6.0 mL
0.75 (9 strips)	90 µL	9.0 mL
1.0 (12 strips)	120 µL	12.0 mL

Wash Buffer Concentrate is to be diluted with distilled or deionized water to make the working strength Wash Buffer solution.
NOTE: All labs should account for plate washer void volume to prime the machine. The values below do not account for priming automated plate washers.

Number of plates	Amount of Concentrate (manual wash)	Amount of dH_2O (manual wash)
0.25 (3 strips)	12.5 mL	250 mL
0.50 (6 strips)	25.0 mL	500 mL
0.75 (9 strips)	37.5 mL	750 mL
1.0 (12 strips)	50.0 mL	1,000 mL

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QUALITY CONTROL

It is recommended that the controls provided with the High Molecular Weight (HMW) & Total ELISA be included in every assay. The concentration ranges of the controls are provided on the Certificate of Analysis included with each kit; however, it is recommended that each laboratory establishes its own acceptable ranges.

SAMPLE PREPARATION

The HMW & Total Adiponectin ELISA allows for the independent quantification of HMW and Total Adiponectin in samples. Sample preparation is to be performed as follows:

For HMW Adiponectin Samples

1. Combine 5µL of sample with 50µL of working strength Sample Treatment Enzyme (see *Reagent Preparation*) and vortex to mix thoroughly. Incubate for 30 minutes at 37ºC. **In order to ensure an even treatment, samples should be incubated in either a heat block or a water bath.**
2. Add 245µL of Adiponectin Assay Buffer to the treated sample to inhibit the enzymatic activity.
3. In a separate tube, combine 12µL of the diluted treated sample (step 2) with 988µL of Adiponectin Assay Buffer and vortex to mix thoroughly. The sample is now effectively diluted 1:5000. **Samples must be used within 2 hours of dilution when stored at room temperature.**

For Serum Control Treated

1. Combine 5µL of reconstituted serum control with 50µL of working strength Sample Treatment Enzyme and vortex to mix thoroughly. Incubate for 30 minutes at 37ºC. **In order to ensure an even treatment, diluted serum control should be incubated in either a heat block or a water bath.**
2. Add 245µL of Adiponectin Assay Buffer to the treated serum control to inhibit the enzymatic activity.
3. In a separate tube, combine 12µL of the diluted treated serum control (step 2) with 988µL of Adiponectin Assay Buffer and vortex to mix thoroughly. The serum control is now effectively diluted 1:5000. **Treated Serum Control must be used within 2 hours of dilution when stored at room temperature.**

For Serum Control Untreated

1. Combine 5µL of reconstituted serum control with 295µL of Adiponectin Assay Buffer in a tube and vortex to mix thoroughly.
2. In a separate tube, combine 12µL of the previously diluted serum control (step 1) and 988µL of Adiponectin Assay Buffer and vortex to mix thoroughly. The serum control is now effectively diluted 1:5000. **Diluted serum control must be used within 2 hours of dilution when stored at room temperature.**

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For Total Adiponectin Samples

1. Combine 5µL of sample with 295µL of Adiponectin Assay Buffer in a tube and vortex to mix thoroughly.
2. In a separate tube, combine 12µL of the previously diluted sample (step 1) and 988µL of Adiponectin Assay Buffer and vortex to mix thoroughly. The sample is now effectively diluted 1:5000. **Samples must be used within 2 hours of dilution when stored at room temperature.**

ASSAY PROCEDURE

All reagents and microplate strips (while sealed in foil pouch) should be equilibrated to room temperature prior to use. Gently mix all reagents before use. A standard curve and controls must be evaluated in each assay and on each microplate if more than one plate is assayed at one time. All standards, controls, and samples should be run in duplicate.

1. The microplate should be equilibrated to room temperature prior to opening the foil pouch. It is recommended to designate enough microplate strips for duplicate determinations of the standards, controls, and samples. Any remaining microplate strips should be stored at 2-8°C in the tightly sealed foil pouch containing the desiccant.

2. **Pipette 50 µL** of each standard, control, and diluted sample into their respective wells. The Assay Buffer is used for the Zero Standard (blank) in the assay. See *Reagent Preparation* for standard curve generation and control reconstitution instructions. See *Sample Preparation for HMW and Total sample preparation*. A suggested plate layout is included.

3. **Pipette 50 µL** of working strength Detector Antibody solution (see *Reagent Preparation*) into each well.

4. Cover microplate with a plate sealer and **incubate for 2 hours** at room temperature, shaking at 700-900 rpm on a microplate shaker.

5. Decant the contents of the wells and **wash the microplate six times** with 350 µL of Working Strength Wash Buffer per well (see *Reagent Preparation*) using an automated microplate washer.

    a. Manual Wash: Alternatively, fill the wells with Working Strength Wash Buffer using a wash bottle equipped with a wash nozzle or manual washer. (It is not recommended to use a multichannel pipette. Wash buffer must be dispensed with adequate and equal force in order to properly wash the wells.) Soak the wells for 1 minute. Invert the microplate to discard the liquid and firmly tap the inverted microplate on absorbent paper towels. Repeat the wash and soak procedure 2 more times, for a total of 3 washes.

    After the final wash, (automated or manual), remove any residual wash buffer and bubbles from the wells by inverting and firmly tapping the microplate on absorbent paper towels.

6. **Pipette 100 µL** of working strength SA-HRP solution (see *Reagent Preparation*) into each well.

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7. Cover microplate with a plate sealer and **incubate for 30 minutes** at room temperature, shaking at 700-900 rpm on a microplate shaker.

8. Decant the contents of the wells and **wash the microplate six times** as in Step 5 above.

9. **Pipette 100 µL** of TMB Substrate into each well.

10. Cover microplate with a plate sealer and incubate 15 minutes at room temperature, shaking 700-900rpm on a microplate shaker.

11. **Pipette 100 µL** of Stop Solution into each well and gently shake the microplate to mix the contents. Remove any bubbles before proceeding to the next step.

12. Place the microplate in a microplate reader capable of reading the absorbance at 450nm. The microplate should be analyzed immediately after the addition of the Stop Solution, and no longer than 30 minutes after.

CALCULATION OF RESULTS

Construct a standard curve from the standards. It is recommended to use a software program to calculate the standard curve and to determine the concentration of the samples. A 4-Parameter Logistic (4-PL) curve fit with $1/y^2$ weighting is recommended for data analysis.

The HMW & Total Adiponectin ELISA is a ligand binding assay, with responses exhibiting a sigmoidal relationship to the analyte concentration. Currently accepted reference models for such curves use a 4- or 5-parameter logistic (PL) fit, as these models optimize the accuracy and precision across a greater range.

In the example below, a 4-PL curve fit with $1/y^2$ weighting was used to maximize the accuracy and precision of samples with low concentrations. However, the accuracy and precision of all models are limited at the lowest and highest ends of the detectable range due to the influence of individual laboratory conditions. As a result, caution should always be used when interpreting results where the analyte response becomes non-linear.[1]

Multiply the determined sample concentration by 5000 to get the concentration of HMW and Total adiponectin present in the sample.

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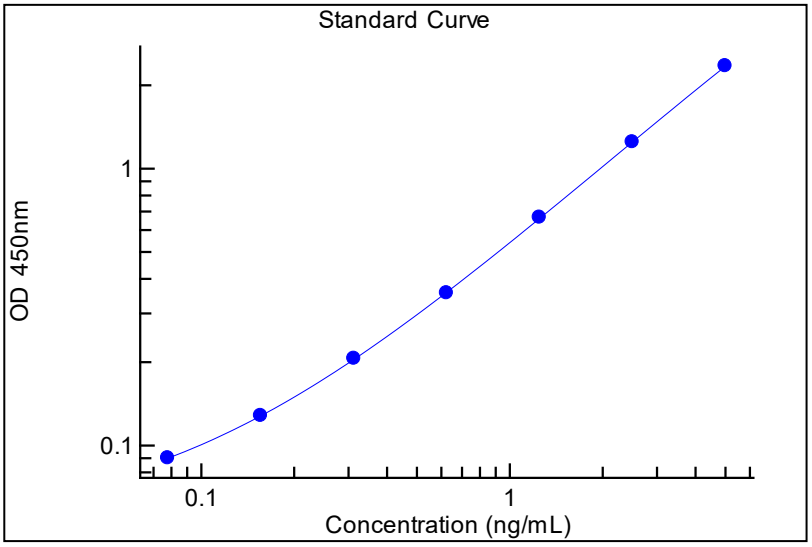
TYPICAL STANDARD CURVE

The following results are provided for demonstration purposes only and cannot be used in place of data obtained with each assay. A standard curve must be performed with each assay run and plate tested. In this example, a 4-parameter curve fit with $1/y^2$ weighting is used for data analysis.

Standard	Standard (ng/mL)	Mean OD	OD CV (%)	S:N	Mean Conc. (ng/mL)	Conc. CV (%)	% Recovery
A	5.000	2.345	1.7	43.0	5.0	1.84	100
B	2.500	1.246	1.6	22.9	2.50	1.74	99.8
C	1.250	0.666	0.6	12.2	1.26	0.64	100.8
D	0.625	0.355	1.0	6.5	0.62	1.19	98.8
E	0.313	0.206	0.6	3.8	0.31	0.75	100.3
F	0.156	0.128	1.3	2.3	0.15	2.14	98.9
G	0.078	0.09	1.8	1.7	0.08	4.32	99.1
Zero	0	0.055	6.2				



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ANALYTICAL PERFORMANCE CHARACTERISTICS:

Sensitivity

Forty replicates of Zero Standard were run to calculate LoB and LoD. The LoB was calculated at 0.015 ng/mL and LoD was calculated at 0.034 ng/mL.

Inter-and intra-assay precision

Three samples were tested at a concentration over the high, mid, and low range of the assay over two days. Acceptance criteria include %CV of OD values and interpolated concentrations of $\leq$ 15% for the high and mid sample while $\leq$ 20% for those of the low sample. Inter- and intra-precision data is as follows:

	Intra-Assay Precision %CV OD		Inter-Assay Precision %CV Concentration
Sample	**Day 1**	**Day 2**	**Day 1/Day 2**
High	1.1	2.6	3.6
Mid	2.0	2.7	4.0
Low	3.9	5.7	6.4

Linearity

The linearity of the assay was determined by diluting various samples and determining the assay response. The samples were prepared using the sample dilution method above. The final dilution was then serially diluted for linearity. The dilutional ranges were reported as a function of the final dilution (1:5000) in the assay. The expected values were compared to the observed values to determine a percent recovery. Total samples exhibited dilutional linearity ranging from 99.4 to 117.9% recovery, while high molecular weight samples exhibited dilutional linearity ranging from 90.4 to 104.2% to a minimum dilution of 1:16 and a maximum dilution of 1:64.

Spike and Recovery

The spike and recovery of the assay was determined by adding various known amounts of adiponectin to samples. The expected values of these spiked samples were compared to the observed values to determine a percent recovery. Data as shown below:

	Total Adiponectin %Recovery				
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
High Spike	104.2%	102.3%	110.3%	101.7%	104.7%
Mid Spike	104.0%	102.3%	106.5%	107.1%	105.1%
Low Spike	98.8%	103.6%	106.4%	101.4%	105.3%
Average	102.3%	102.7%	107.7%	103.4%	105.0%

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	HMW Adiponectin %Recovery				
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
High Spike	107.1%	101.9%	11.2%	104.4%	109.5%
Mid Spike	89.9%	80.9%	86.7%	100.9%	92.5%
Low Spike	101.3%	93.5%	99.5%	82.5%	106.1%
Average	99.4%	92.1%	99.5%	95.9%	102.7%

REFERENCES

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2. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF 1995 A novel serum protein similar to C1q, produced exclusively in adipocytes. J Biol Chem 270:26746-26749

3. Hu E, Liang P, Spiegelman BM 1996 AdipoQ is a novel adipose-specific gene dysregulated in obesity. J Biol Chem 18:10697-10703

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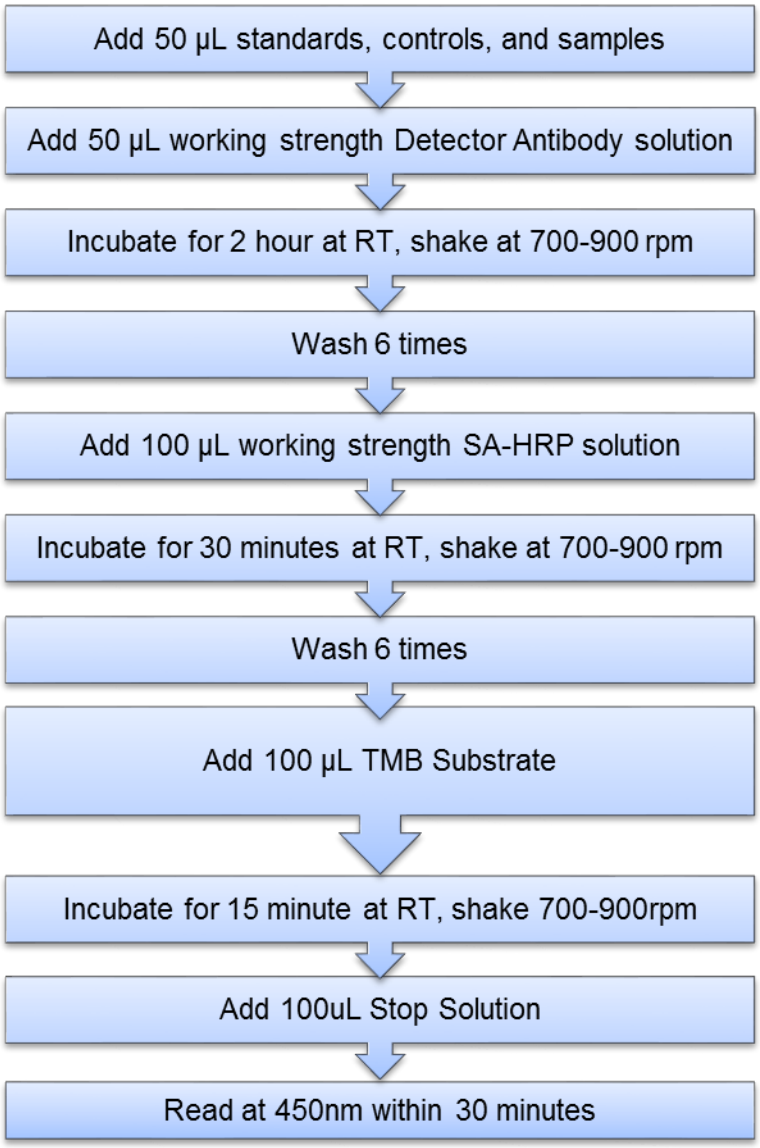
SHORT ASSAY PROTOCOL



Assay Incubation Time = 3 hours 15 minutes*

*** Assay completion time includes the 30 minute sample treatment incubation time.**

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SUGGESTED PLATE LAYOUT

Below are suggested plate layouts for running standards, controls, and samples for HMW & Total Adiponectin in duplicate. If measuring both Total and HMW Adiponectin samples, a total of 18 samples can be run in duplicate. On the other hand, a total of 38 samples can be run in duplicate if measuring only Total Adiponectin or 36 samples can be run duplicate if measuring only HMW Adiponectin.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std A	Std A	Ctrl Low	Ctrl Low	3-U	3-U	7-U	7-U	11-U	11-U	15-U	15-U
B	Std B	Std B	Ctrl High	Ctrl High	3-T	3-T	7-T	7-T	11-T	11-T	15-T	15-T
C	Std C	Std C	Treatment Ctrl (U)	Treatment Ctrl (U)	4-U	4-U	8-U	8-U	12-U	12-U	16-U	16-U
D	Std D	Std D	Treatment Ctrl (T)	Treatment Ctrl (T)	4-T	4-T	8-T	8-T	12-T	12-T	16-T	16-T
E	Std E	Std E	1-U	1-U	5-U	5-U	9-U	9-U	13-U	13-U	17-U	17-U
F	Std F	Std F	1-T	1-T	5-T	5-T	9-T	9-T	13-T	13-T	17-T	17-T
G	Std G	Std G	2-U	2-U	6-U	6-U	10-U	10-U	14-U	14-U	18-U	18-U
H	Zero	Zero	2-T	2-T	6-T	6-T	10-T	10-T	14-T	14-T	18-T	18-T

Std= Standard U = Untreated
Ctrl = Control T = Treated
Numbered wells = Samples

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	1	2	3	4	5	6	7	8	9	10	11	12
A	Std A	Std A	Ctrl Low	Ctrl Low	5-T	5-T	13-T	13-T	21-T	21-T	29-T	29-T
B	Std B	Std B	Ctrl High	Ctrl High	6-T	6-T	14-T	14-T	22-T	22-T	30-T	30-T
C	Std C	Std C	Treatment Ctrl (U)	Treatment Ctrl (U)	7-T	7-T	15-T	15-T	23-T	23-T	31-T	31-T
D	Std D	Std D	Treatment Ctrl (T)	Treatment Ctrl (T)	8-T	8-T	16-T	16-T	24-T	24-T	32-T	32-T
E	Std E	Std E	1-T	1-T	9-T	9-T	17-T	17-T	25-T	25-T	33-T	33-T
F	Std F	Std F	2-T	2-T	10-T	10-T	18-T	18-T	26-T	26-T	34-T	34-T
G	Std G	Std G	3-T	3-T	11-T	11-T	19-T	19-T	27-T	27-T	35-T	35-T
H	Zero	Zero	4-T	4-T	12-T	12-T	20-T	20-T	28-T	28-T	36-T	36-T

Std= Standard U = Untreated
Ctrl = Control T = Treated
Numbered wells = Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std A	Std A	Ctrl Low	Ctrl Low	7-U	7-U	15-U	15-U	23-U	23-U	31-U	31-U
B	Std B	Std B	Ctrl High	Ctrl High	8-U	8-U	16-U	16-U	24-U	24-U	32-U	32-U
C	Std C	Std C	1-U	1-U	9-U	9-U	17-U	17-U	25-U	25-U	33-U	33-U
D	Std D	Std D	2-U	2-U	10-U	10-U	18-U	18-U	26-U	26-U	34-U	34-U
E	Std E	Std E	3-U	3-U	11-U	11-U	19-U	19-U	27-U	27-U	35-U	35-U
F	Std F	Std F	4-U	4-U	12-U	12-U	20-U	20-U	28-U	28-U	36-U	36-U
G	Std G	Std G	5-U	5-U	13-U	13-U	21-U	21-U	29-U	29-U	37-U	37-U
H	Zero	Zero	6-U	6-U	14-U	14-U	22-U	22-U	30-U	30-U	38-U	38-U

Std= Standard U = Untreated
Ctrl = Control
Numbered wells = Samples

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