Rat Adiponectin ELISA

For the quantitative determination of adiponectin in rat serum and plasma.

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

Catalog Number: 22-ADPRT-E01

Size: 96 wells

Version: 02.12.2019 Version 2.0 - ALPCO 2.0

1. Intended Use
Measurement of Adiponectin in rat serum and plasma samples for research use only.

2. Introduction
Adiponectin was described for the first time in the early 1990s as an endocrine factor produced by adipocytes. Adiponectin is involved in regulation of energy and fat metabolism. So, its concentration in the circulation is said to reflect the risk of atherosclerosis and the degree of insulin resistance. Based on the high incidence of these diseases, adiponectin was and still is an object of intensive research regarding the underlying biological mechanisms and its value as a biomarker. Mice and rats are suitable model organisms for basic research and pre-clinical studies.

Although the comparability of rats and humans is limited we offer some background information on *human* adiponectin physiology in the following section:

Adiponectin is a 30kDa protein comprising 0.01% of serum protein. It is mainly synthesized by adipocytes, but muscle cells and hepatocytes also have the ability to synthesize adiponectin. Until now, IGF-I is the only known natural inductor of the synthesis of adiponectin. It consists of a collagen-like N-terminal and a globular C-terminal domain [1]. In vivo adiponectin appears with different oligomers. Trimer, ditrimer, and high molecular multimers exist [1-3]. Up to now two different receptors are known, both receptors are ubiquitously expressed, though the distribution in the tissues varies. The Adiponectin Receptor 1 (AdipoR1) can be found in muscle tissue and AdipoR2 in liver tissue [4].

Adiponectin is associated with glucose- and lipo-metabolism [5, 6]. Studies show that adiponectin correlates negatively with BMI and thus it could have relevance for energy metabolism; for example, through the regulation of fatty acid oxidation. Adiponectin level is also associated with Insulin-Resistance [7-9] and so it is also linked with Type II Diabetes. HMW adiponectin has been shown to have similar ability to total adiponectin in assessing the presence of insulin resistance. [10]. Blueher et al. clearly demonstrated that regarding the diagnosis of insulin resistance, measured by whole body glucose uptake below 40 µmol/kg*min, total adiponectin as determined with the kit, was of greater diagnostic value [10].

Furthermore, adiponectin is involved in inflammatory processes [11-15]. It is of importance in arteriosclerosis [4, 7, 16] and coronaritis [17, 18], thus the determination of adiponectin level in plasma could serve to estimate the risk of coronary disease [19, 20]. Adiponectin also influences other physiological processes such as angiogenesis [21, 22].

3. Principle of the Assay
This assay is a sandwich assay using two specific and high affinity antibodies. The adiponectin in the samples binds to the first antibody coated on the microtiter plate. In the following step the second specific anti-adiponectin antibody binds in turn to the immobilized adiponectin. The second antibody is biotinylated and will be applied in a mixture with a Streptavidin-Peroxidase-Enzyme Conjugate. In the last substrate reaction, the color of the mixture will quantitatively depend on the adiponectin level in the samples.

4. WARNINGS AND PRECAUTIONS

For research use only. For professional use only.
Do not use obviously damaged, contaminated, or spilled material.

Disposal of containers and unused contents should be done in accordance with federal and local regulatory requirements.

Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.

Caution: This kit contains material of human and/or animal origin. Therefore, all components and specimens should be treated as potentially infectious.

Rat Serum is contained in the following components: **KS1 and KS2, STD A-F**

Reagents

A –F, AK, VP, WP contain as preservatives 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one (<0.015 %)

H317	May cause an allergic skin reaction.
P280	Wear protective gloves/ protective clothing/ eye protection/ face protection.
P272	Contaminated work clothing should not be allowed out of the workplace.
P261	Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
P333+P313	If skin irritation or rash occurs: Get medical advice/ attention.
P302+P352	IF ON SKIN: Wash with plenty of soap and water.
P501	Dispose of contents/ container in accordance with local/ regional/ national/ international regulations.

Substrate Solution (S)

The TMB-Substrate (S) contains 3.3´,5.5´ Tetramethylbencidine (<0.05 %)

H315	Causes skin irritation.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.
P261	Avoid breathing dust/ fume/ gas/ mist/ vapors/ spray.
P305+P351+	IF IN EYES: Rinse cautiously with water for several minutes.
P338	Remove contact lenses, if present and easy to do. Continue rinsing.

Stopping Solution (SL)

The Stopping solution contains 0.2 M acid sulfuric acid (H_2SO_4)

H290	May be corrosive to metals.
H314	Causes severe skin burns and eye damage.
P280	Wear protective gloves/ protective clothing/eye protection/ face protection.
P301+P330+	IF SWALLOWED: rinse mouth.
P331	Do NOT induce vomiting.
P305+P351+	IF IN EYES: Rinse cautiously with water for several minutes.
P338	Remove contact lenses, if present and easy to do. Continue rinsing.
P309+P310	IF exposed or if you feel unwell: Immediately call a POISON CENTER or doctor/physician.

4.1 General first aid procedures:
Skin contact: Wash affected area rinse immediately with plenty of water for at least 15 minutes. Remove contaminated cloths and shoes.
Eye contact: In case of contact with eyes, rinse immediately with plenty of water for at least 15 minutes. In order to assure an effectual rinsing, spread the eyelids.
Ingestion: After swallowing the product, if the affected person is conscious, rinse out the mouth with plenty of water: seek medical advice immediately.

5. Samples

5.1 Sample Type

Serum and plasma samples of rats can be used in this assay.
Influence of Heparin (30 IE/mL), EDTA (6.8 mM) and NaCitrat (0.015 M) on the measurement of adiponectin has been investigated by recovery experiments. PBS was enriched with recombinant rat adiponectin and the above-mentioned substances. No significant influence on the recovery of adiponectin was detected.

5.2 Specimen collections
Hemolyzed samples must be avoided. For serum samples, blood should be clotted completely, and serum separated by centrifugation.

5.3 Required sample volume

10 µL recommended, minimum volume 5 µL

5.4 Sample stability
In firmly sealed sample vials:

- Storage at Room Temperature 20-25°C: max. 2 days
- Storage at -20°C: 2 years
- Freeze/Thaw cycles: max. 5

Freeze-Thaw cycles should be minimized. Up to 5 cycles showed no effect on the measured Adiponectin concentration.
Samples should be stored at 4°C as quickly as possible. For long-term storage, the sample must be frozen and stored at ≤ -20°C.

5.5 Sample dilution
Samples **must be diluted** prior to measurement.

Dilution: **1:1,500** with Dilution Buffer **VP**

We recommend a dilution in 2 steps:
Pipette **990 µL** Dilution Buffer **VP** in PE-/PP-Tubes (application of a multi-stepper is recommended in larger series), add **10 µL** Serum- or Plasma (dilution 1:100) and mix each tube immediately. Pipette **100 µL** of this dilution into another PE/PP vessel with **1,400 µL** of Dilution Buffer **VP** and mix immediately. This results in a final dilution of 1:1,500. After mixing, use 100 µL per well in the assay

If sample size is **limiting, a minimum of 5 µL** sample might be used alternatively, dilution in 495 µL Dilution Buffer **VP** yields a dilution of **1:100** Pipette **100 µL** of this dilution into another PE/PP

vessel with **1,400 µL** of Dilution Buffer **VP** and mix immediately. After mixing use 100 µL per assay in the assay of this solution.

Where required, depending on the expected adiponectin values, the dilution with **Dilution Buffer VP** can be higher or lower.

6. Materials

6. Materials Provided

The reagents listed below are sufficient for 96 wells including the standard curve.

MTP	**Microtiter plate,** ready for use, coated with human anti-Rat Adiponectin antibody. Well strips can be separated.	**12 x 8 wells**
A-F	**Standards A-F**, lyophilized, (native Rat Adiponectin) concentrations are given on vial labels and on the QC-certificate.	**6 x 1 mL**
KS1	**Control Serum 1,** lyophilized, (rat serum), concentration is given on the QC-certificate.	**1 x 250 µL**
KS2	**Control Serum 2,** lyophilized, (rat serum), concentration is given on the QC-certificate.	**1 x 250 µL**
AK	**Antibody-POD-Conjugate,** ready for use, contains a mixture of biotinylated anti-Adiponectin antibody and HRP (Horseradish Peroxidase)-labelled Streptavidin.	**1 x 12 mL**
VP	**Dilution Buffer,** ready for use, **Please shake before use!**	**1 x 125 mL**
WP	**Washing Buffer,** 20-fold concentrated solution	**1 x 50 mL**
S	**Substrate,** ready for use, horseradish-peroxidase-(HRP) substrate, stabilized Tetramethylbenzidine.	**1 x 12 mL**
SL	**Stopping Solution,** ready for use, 0.2 sulfuric acid.	**1 x 12 mL**
-	**Sealing Tape,** for covering the **microtiter plate.**	**2 x**
-	**Instructions for Use**	**1 x**
--	**Quality Control Certificate**	**1 x**

6.2 Materials Required but not provided

Precision pipettes and multichannel pipettes with disposable plastic tips
Graduated cylinder for diluting Wash Buffer (WP)
Distilled or deionized water for dilution of the Wash Buffer (WP), 950 mL
Vortex-mixer
Microtiter plate shaker (350 rpm)
Microtiter plate washer (recommended)
Micro plate reader ("ELISA-Reader") with filter for 450 and ≥ 590 nm.
Polyethylene PE/Polypropylene PP tubes for dilution of samples
Timer

7. TECHNICAL NOTES

Storage Conditions

Store the kit at 2-8°C after receipt until its expiry date.

Storage Life

The shelf life of the components after initial opening is warranted for **4 weeks**, store the unused strips and microtiter wells airtight together with the desiccant at 2-8°C in the clip-lock bag, use in the frame provided. The reconstituted components standards **A-F** and Control Sera **KS1** and **KS2** must be stored at –20°C (max. 4 weeks). **Attention: Standards should be thawed only once** – where required please store aliquoted in adequate volumes. For further use, thaw quickly but gently (avoid temperature increase above room temperature and avoid excessive vortexing). Avoid repeated thawing and freezing. The 1:20 diluted Washing Buffer **WP** is stable up to 4 weeks at 2-8°C

Preparation of reagents

Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming. Reagents with different lot numbers cannot be mixed.

Reconstitution

The Standards **A – F** and Controls **KS1** and **KS2** are reconstituted with the Dilution Buffer **VP**. It is recommended to keep reconstituted reagents at room temperature for 15 minutes and then to mix them thoroughly but gently (no foam should result) with a Vortex mixer.

Dilution

After reconstitution dilute the Control Sera **KS1** and **KS2** with the Dilution Buffer **VP** in the same ratio (1:1500) as the sample.
The required volume of Washing Buffer **WP** is prepared by 1:20 dilution of the provided 20X concentrate with DI water

Assay Procedure

When performing the assay, Standards **A-F**, Control Serum **KS1 and KS2** and the samples should be pipetted as quickly as possible (e.g. <15 minutes). To reduce variability due to differences in incubation times Antibody-POD-Conjugate **AK,** as well as the succeeding Substrate Solution **S** should be added to the plate in the same order and in the same time interval as the samples. Stopping Solution **SL** should be added to the plate in the same order as Substrate Solution **S**. All determinations (Standards **A-F**, Control Sera **KS1 and KS2** and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.

Incubation

Incubation at room temperature means: Incubation at 20 - 25°C. The Substrate Solution **S** stabilized Tetramethylbenzidine is photosensitive; store and incubate in the dark.

Shaking

The recommended shake rate for a plate shaker is 350 rpm. However, adjustments may be necessary depending upon the plate shaker model used. Insufficient shaking may lead to

inadequate mixing of the solutions and thereby to low optical densities, high variations and/ or false values, excessive shaking may result in high optical densities and/ or false values.

Washing
Proper washing is of basic **importance** for a secure, reliable and precise performance of the test. Incomplete washing is common and will adversely affect the assay outcome. Improper washing technique can increase variability e.g. high value of blank, potentially leading to erroneous results. Effects like high background values or high variations may indicate washing problems.
All washing must be performed with the provided Washing Buffer **WP** diluted to usage concentration. Washing volume per washing cycle and well must be at least 300 µL.

Manual washing is recommended. Washing Buffer may be dispensed via a multi-stepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamically swinging out the microtiter plate over a basin. If aspirating devices are used, care has to be taken that the inside well surface is not scratched. Subsequent to every single washing step, the remaining fluid should be removed by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

When using an **automatic microtiter plate washer**, the respective instructions for use must be carefully followed. Device adjustments, e.g. for plate geometry and the provided washing parameters, must be performed. Dispensing and aspirating manifold must not scratch the inside well surface. Provisions must be made that the remaining fluid volume of every aspiration step is minimized. Following the last aspiration step of each washing cycle, this could be controlled, and possible remaining fluid could then be removed, by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

The danger of handling with potentially infectious material must be considered.

8. ASSAY PROCEDURE

Preparation of reagents		**Reconstitution:**	**Dilution**
A-F	**Standards**	in **1 mL** Dilution Buffer **VP**	-
KS1	**Control Serum 1**	in **250 µL** Dilution Buffer **VP**	**1:1,500** with Dilution Buffer **VP**
KS2	**Control Serum 2**	in **250 µL** Dilution Buffer **VP**	**1:1,500** with Dilution Buffer **VP**
WP	**Washing Buffer**	-	**1:20** with **DI Water.**
Sample dilution: with Dilution Buffer **VP 1:1500. Don´t use samples undiluted!**			
Before assay procedure bring all reagents to room temperature **20-25°C**.			

Assay Procedure in Double Determination:		
Pipette	**Reagents**	**Position**
100 µL	Dilution Buffer **VP**	A1/A2
100 µL	Standard **A (0.25 ng/mL**)	B1/B2
100 µL	Standard **B (0.75 ng/mL**)	C1/C2
100 µL	Standard **C (1.5 ng/mL**)	D1/D2
100 µL	Standard **D (3.0 ng/mL**)	E1/E2
100 µL	Standard **E** (**6.5 ng/mL**)	F1/F2
100 µL	Standard **F (10 ng/mL**)	G1/G2
100 µL	Control Serum **KS 1** (1:1,500 diluted)	H1/H2
100 µL	Control Serum **KS 2** (1:1,500 diluted)	A3/A4
100 µL	Sample (1:1,500 diluted)	In the remainin wells according to the requirements
Cover the wells with the sealing tape.		
Sample Incubation: Shake at 350 rpm for 1 hour at 20-25°C		
3 x 300 µL	Aspirate the contents of the wells and wash **3 x** with **300 µL** each Washing Buffer **WP/ well.**	In each well
100 µL	Antibody-POD- Conjugate **AK**	In each well
Cover the wells with the sealing tape.		
Incubation: Shake at 350 rpm for 1 hour at 20-25°C		
3 x 300 µL	Aspirate the contents of the wells and wash **3 x** with **300 µL** each Washing Buffer **WP/ well.**	In each well
100 µL	Substrate Solution **S**	In each well
Incubation: 30 minutes in the Dark at 20-25°C		
100 µL	Stopping Solution **SL**	In each well
	Measure the absorbance within 30 min at **450 nm** with ≥ 590 nm as reference wavelength.	

9. EVALUATION OF RESULTS

For the evaluation of the assay it is required that the absorbance values of the Blank should be below 0.25 and the absorbance of standard F should be above 1.00.

Samples which yield higher absorbance values than Standard F should be re-tested at a higher dilution.

9.1 Establishing the Standard Curve

The standards provided contain the following concentrations of rat adiponectin:

Standard	A	B	C	D	E	F
ng/mL	0.25	0.75	1.5	3	6.5	10

1) Calculate the **mean absorbance** value for the blank from the duplicate determination (wells A1/A2).

2) Subtract the mean absorbance of the blank from the mean absorbances of all other samples and standards.

3) Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis.

4) Recommendation: Calculation of the standard curve should be done by using a computer program because the curve is in general (without respective transformation) not ideally described by linear regression. **A higher-grade polynomial, or four parametric logistic (4-PL) curve fit** or **non-linear regression** are usually suitable for the evaluation (as might be spline or point-to-point alignment in individual cases).

5) The Rat Adiponectin concentration in ng/mL of the samples and controls can be calculated by **multiplication** with the respective **dilution factor.**

9.2 Example of a Typical Standard Curve

The example data and the standard curve in Figure 1 cannot be used for the calculation of the test results. A standard curve should be generated for each test conducted.

Table 1 Data which describe a typical standard curve.

Standard	A	B	C	D	E	F
ng/mL	0.25	0.75	1.5	3	6.5	10
OD450-620 nm	0.047	0.152	0.326	0.585	1.227	1.805

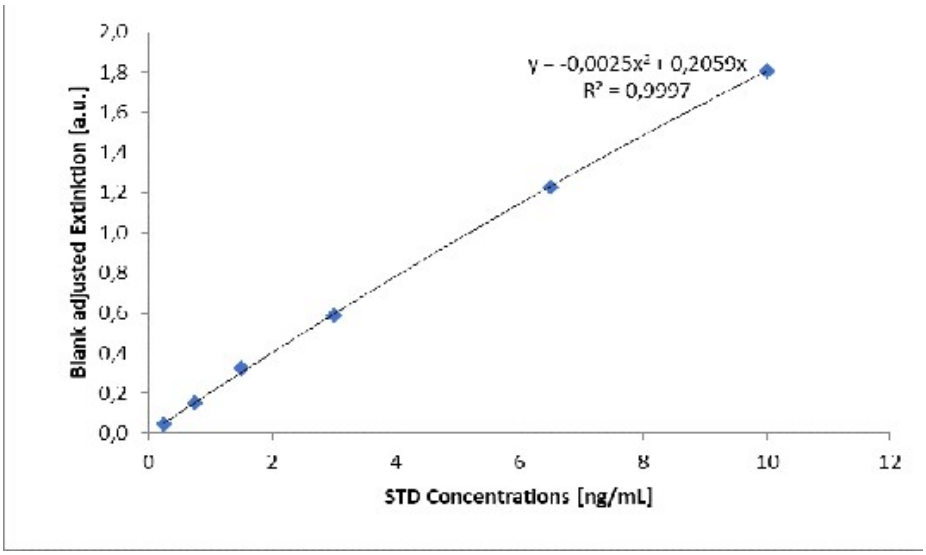

Figure 1. Exemplary Standard Curve

9.3 Example evaluation of sample concentrations

Sample dilution: 1:1500

Measured extinction (OD) of sample: 0.6075
Measured extinction (OD) of the Blank: 0.0227

An assay measurement program will calculate the adiponectin concentration of the diluted sample automatically by using the difference of sample and Blank for the calculation. The user should determine the most suitable curve fit. In the example, the following equation is solved by the program to calculate the adiponectin concentration in the sample:

$y = -0.0025x^2 + 0.2059x$
$R^2 = 0.9997$
$x = 2.65$

If the dilution factor (**1500**) is taken into account the Adiponectin concentration of the undiluted sample is:

2.65 ng/mL x 1500 = 3975 ng/mL
= 3.975 µg/mL

10. PERFORMANCE CHARACTERISTICS

10.1 Standards

The Standards are prepared from native adiponectin in concentrations of 0.25, 0.75, 1.5, 3, 6.5 and 10 ng/ml. The native adiponectin was calibrated against recombinant protein.

10.2 Sensitivity

The analytical sensitivity of the kit was determined as 2SD of 16 replicates of the zero standard. The analytical sensitivity is 0.081 ng/mL.

10.3 Precision

The **Inter**- and **Intra-Assay** variation coefficients were on average ≤ **10%.** Precision data is summarized in Table 2 and 3.

Table 2
Intra-Assay Variation**, n= 20**

	Sample 1	**Sample 2**
Mean [µg/ml]	**12.702**	**5.709**
SD	0.197	0.191
CV %	1.55	3.34

Table 3
Inter-Assay Variation, **n= 6**

	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**	**Sample 7**
Mean [µg/mL]	**1.62**	**7.06**	**7.97**	**7.22**	**11.23**	**8.55**	**7.24**
SD	0.03	0.40	0.27	0.28	1.25	0.42	0.35
CV%	2.16	5.70	3.39	3.82	11.16	4.93	4.85

10.4 Linearity

Table 4
Linearity of the Sample Dilution (typical results for two different sera)

Dilution	**Sample 1**	**Dilution**	**Sample 2**
	µg/mL		µg/mL
1:600	5.2	1:1000	7.70
1:1200	5.09	1:2000	7.46
1:2400	5.29	1:4000	7.66
1:4800	5.12	1:8000	7.60
1:9600	5.01	1:16000	7.07

A sample dilution of 1:1,500 is generally suitable. However, individual Adiponectin levels can vary significantly. it is therefore recommended to check this and adjust the dilution accordingly.

Literature

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