DRG® Adiponectin Human (EIA-4646)

As of 2 Jan. 2007 (Vers. 1.0) **RUO**

1 INTENDED USE

The DRG Adiponectin (Human) ELISA provides a method for the quantitative determination of human adiponecin in serum or plasma.

2 SUMMARY AND EXPLANATION OF THE TEST

Adiponectin is also called: Acrp30 (30 kDa adipocyte complement-related protein), GBP28 (Gelatin-binding protein), adipoQ, apM 1 (Adipose most abundant gene transcript 1) [1, 2].

Adiponectin is an adipocyte-secreted hormone, consisting of 244 amino acids with a molecular weight of approximately 30kDa (28-30kDa). It is one of the most abundant proteins in human blood, with a concentration of 5-30 µg/ml which accounts for approximately 0,01% of total plasma protein [2].

The protein consists of four domains: one globular C-terminal, one collagen-like N-terminal, one signalling peptide and one hyper variable domain. The globular domain has significant sequence and structural similarities to the complement factor C1q [2, 3]. The globular domain also has structural similarities to TN F-alfa, [3-5].

Two receptors, AdipoR1 and AdipoR2 have been cloned. The receptors are expressed in muscle, liver and human fat cells [2,6]. Recently T-cadherin was identified as a receptor for the hexameric and high molecular weight forms of adiponectin [2, 7].

Adiponectin concentration is reversely associated with type 2 diabetes, coronary disease and obesity, all together called the metabolic syndrome. Adiponectin decreases blood glucose and free fatty acid serum concentrations and increases insulin sensitivity [8]. Adiponectin has also been shown to have anti-inflammatory [2] and anti-apoptotic effects [9].

Adiponectin has been suggested to exist in different forms in circulation: monomers, isolated globular form (the globular domain), trimers, hexamers and larger oligomers [10-13]. Monomers are believed to associate in circulation to a trimer through the globular domain. Trimers are associated to larger oligomers through the collagen like domain [8]

However, recent studies indicate that adiponectin may not be present in circulation as monomer or isolated globular form, but rather as multimeric structures. The dominant forms of adiponectin that circulates in human serum and plasma are hexamers (LMW) and larger oligomers (HMW) [6,14-16]. The LMW adiponectin levels does not seem to differ between insulin sensitive- and insulin resistant subjects, nor does LMW adiponectin differ between men and women. The increased levels of total adiponectin in insulin sensitive subjects and women was caused by increased amounts of HMW adiponectin. Both total and HMW adiponectin showed significant differences between the insulin sensitive- and insulin resistant subjects [6]. Another finding showed that it is the HMW form and not the LMW form of adiponectin that is increased by addition of pioglitazone [14].

Several isoforms of adiponectin do circulate in blood. It is yet to be determined whether all isoforms are secreted by the adipocytes, whether there is a posttranscriptional assembly of HMW adiponectin in blood or whether the HMW form is secreted and degraded in blood. The individual metabolic significance of each adiponectin isoforms also remains unclear [6].

3 PRINCIPLE OF THE PROCEDURE

The DRG Adiponectin ELISA is a solid phase two-site enzyme immunoassay. It is based on the sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the adiponectin molecule. During incubation, adiponectin in the sample react with anti-adiponectin antibodies bound to microtitration well. After washing, peroxidase conjugated anti-adiponectin antibodies are added and after the second incubation and a simple washing step that removes unbounded enzyme labelled antibody, the bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

4 WARNINGS AND PRECAUTIONS

- **For research use only.** Not for use in diagnostic procedures. Not for internal or external use in humans or animals.
- The content of this kit and their residues must not be allowed to come into contact with ruminating animal or swine.
- The Stop Solution in this kit contains 0.5 M H_2SO_4. Follow routine precautions for handling hazardous chemicals.
- All patient samples should be handled as capable of transmitting infections.

5 MATERIAL REQUIRED BUT NOT PROVIDED

- Pipettes for 25, 50, 100, 200 and 1000 µl (repeat pipettes preferred for addition of enzyme conjugate solution, Substrate TMB and Stop Solution)
- Beakers and cylinders for reagent preparation
- Redistilled water
- Microplate reader (450 nm filter)
- Plate shaker (The recommended velocity is 700-900 cycles per minute, orbital movement)
- Microplate washing device

6 REAGENTS

Each DRG Adiponectin ELISA kit contains reagents for 96 wells, sufficient for 42 samples and one calibration curve in duplicate. For larger series of assays, use pooled reagents from packages bearing identical lot numbers. The expiry date for the complete kit is stated on the outer label. The recommended storage temperature is +2-8°C.

1.	**Coated Plate** (Mouse monoclonal anti-human adiponectin) For unused microtitration strips, reseal the bag using adhesive tape and store at +2-8°C for two months.	1 plate, 8-well strips	96 wells	Ready for use
2.	**Standards 1, 2, 3, 4, 5** (Recombinant human adiponectin) Concentration stated on vial label.	5 vials	1000 µl	Ready for Use
3.	**Assay Buffer** Color coded red	1 vial	12 ml	Ready for use

4.	**Sample Buffer 2X** Dilute with 50 ml redistilled water to make sample buffer. Color coded yellow Storage after dilution: +2-8°C for two months	1 bottle	50 ml	
5.	**Enzyme Conjugate 11X** (Peroxidase conjugated mouse monoclonal anti-human adiponectin) Note! Light sensitive!	1 vial	1.3 ml	Preparation, see below
6.	Enzyme Conjugate Buffer Color coded blue	1 vial	13 ml	Ready for use
7.	**Wash Buffer 21X** Dilute with 800 ml redistilled water to make wash buffer. Storage after dilution: +2-8°C for two months	1 bottle	40 ml	
8.	**TMB Substrate** (TMB) Colorless solution Note! Light sensitive!	1 bottle	22 ml	Ready for use
9.	**Stop Solution** 0.5 M H_2SO_4	1 vial	7 ml	Ready for use

6.1 Preparation of enzyme conjugate solution

Prepare the needed volume of enzyme conjugate solution by dilution of Enzyme Conjugate 1 1X, (1+10) in Enzyme Conjugate Buffer according to the table below. Mix gently.

When preparing enzyme conjugate solution for the whole plate or if the reagents are to be used within two months, pour all of the Enzyme Conjugate Buffer into the Enzyme Conjugate 11X vial.

Number of strips	Enzyme Conjugate 11X	Enzyme Conjugate Buffer
12 strips	1 vial	1 vial
8 strips	700 µl	7 ml
6 strips	500 µl	5 ml
4 strips	400 µl	4 ml

Storage after dilution: +2-8°C for two months.

7 SPECIMEN COLLECTION AND HANDLING

Serum

Collect blood by venipuncture, allow to clot. Separate the serum by centrifugation at X 4 300g for 15 minutes at 2-8°C.

Specimen can be stored at 2-8°C up to 14 days.

For longer periods, store samples at –20°C. Avoid repeated freezing and thawing.

Plasma

Collect blood by venipuncture into tubes containing heparin, citrate or EDTA as anticoagulant, and separate the plasma fraction.

Samples can be stored at 2-8°C up to 14 days.

For longer periods store samples at –20°C. Avoid repeated freezing and thawing.

7.1 PREPARATION OF SAMPLES

Samples should be diluted **1/101** v/v with sample buffer (20 µl sample + 2.0 ml sample buffer).

Diluted samples can be stored at 2-8°C up to 14 days.

Note! Buffers containing sodium azide (NaN_3) cannot be used for sample dilution.

8 TEST PROCEDURE

All reagents and samples must be brought to room temperature before use. Prepare a Standard curve for each assay run.

1. Prepare enzyme conjugate solution (according to table on previous page), sample buffer, wash buffer and samples.
2. Prepare sufficient microplate wells to accommodate Standards and samples in duplicate.
3. Pipette 25 µl each of sample buffer (used as blank)/Standards and samples into appropriate wells.
4. Add 100 µl of Assay Buffer into each well.
5. Incubate on a plate shaker for 1 hour at room temperature (18-25°C).
6. Wash plate 6 times with automatic plate washer
   or
   Aspirate the reaction volume completely and fill each well with 350 µl wash buffer. Aspirate liquid completely. Repeat 5 times. After final wash, invert and tap the plate firmly against absorbent paper.
7. Add 100 µl of enzyme conjugate solution into each well.
8. Incubate on a plate shaker for 1 hour at room temperature (18-25°C).
9. Wash plate 6 times with automatic plate washer
   or
   Aspirate the reaction volume completely and fill each well with 350 µl wash buffer. Aspirate liquid completely. Repeat 5 times. After final wash, invert and tap the plate firmly against absorbent paper.
10. Add 200 µl Substrate TMB into each well.
11. Incubate for 15 minutes at room temperature (18-25°C).
12. Add 50 µl Stop Solution to each well.
    Place the plate on the shaker for approximately 5 seconds to ensure mixing.

13. Read optical density at 450 nm and calculate results.
    Read within 30 minutes.

Note! To prevent contamination between the conjugate and substrate, separate pipettes are recommended.

9 INTERNAL QUALITY CONTROL

Internal serum pools with low, intermediate and high adiponectin concentrations should routinely be assayed as unknowns, and results charted from day to day.

It is good laboratory practice to record the following data for each assay: kit lot number; reconstitution dates of kit components; OD values for the blank (sample buffer), Standards and controls.

10 CALCULATION OF RESULTS

10.1 Computerized calculation

The concentration of adiponectin is obtained by computerized data reduction of the absorbance for the Standards 1-5 versus the concentration using cubic spline regression.

10.2 Manual calculation

1. Plot the absorbance values obtained for the Standards 1-5 against the adiponectin concentration on a log log paper and construct a Standard curve.
2. Read the concentration of the unknown samples from the Standard curve.
3. Multiply the concentration with the dilution factor.

Example of results

Wells	Identity	A_{450}	Mean conc. ng/ml	x 101 µg/ml
1A-B	Sample buffer	0.059/0.056		
1C-D	Standard 1 (5 ng/ml)*	0.106/0.102		
1 E-F	Standard 2 (15 ng/ml)*	0.207/0.216		
1G-H	Standard 3 (50 ng/ml)*	0.563/0.559		
2A-B	Standard 4 (150 ng/ml)*	1.477/1.567		
2C-D	Standard 5 (300 ng/ml)*	2.602/2.681		
2E-F	Unknown 1	0.374/0.367	29.825	3.012
2G-H	Unknown 2	0.754/0.758	67.178	6.785
3A-B	Unknown 3	1.385/1.373	133.340	13.467

*Exact concentration indicated on vial label.

11 LIMITATIONS OF THE PROCEDURE

As with all diagnostic tests, a definitive diagnosis should not be based on the results of a single test, but should be made by the physician after all clinical findings have been evaluated.

Grossly lipemic, icteric or haemolyzed samples do not interfere in the assay.

12 EXPECTED VALUES

Good practice dictates that each laboratory establishes its own expected range of values.

DRG Adiponectin ELISA detects LMW(Hexamer 230kDa) and HMW(Oligomer >420 kDa) adiponectin, as determined by size exclusion gel chromatography, see figure 2.

The different multimeric forms of endogenous adiponectin was studied and separated in serum from a healthy individual by a three step method, ammonium sulphate precipitation followed by ion exchange and gel filtration chromatography.

With ion exchange chromatography, proteins binds to the matrix with electrostatic forces and separation is obtained since different proteins/isoforms have different total net charge, isoelectric points. The ion exchange column that was used was Mono Q HR 5/5 (GE Health Care). Triethylamine buffer was used for eluting the proteins.

The isoforms of adiponectin has been shown to have different isoelectric points and post-translational patherns [17] Proline hydroxylation and lysine hydroxylation/ glycosylation are believed to have great importance on the assembly of the oligomers [14, 17].

Elution profile of serum adiponectin from ion exchange chromatography and as identified by the DRG Adiponectin ELISA:

Five distinct peaks were visible when the serum adiponectin was separated by ion exchange chromatography, indicating the presence of different post-translational patterns and/or different multimeric forms, with different isoelectric points, or total net charge. Pools A-E, were further analysed by size exclusion gel chromatography to determine the apparent size of the multimeric adiponectin forms.

Elution profile of serum adiponectin from size exclusion gel filtration chromatography and as identified by the DRG Adiponectin ELISA:

(Size exclusion gel filtration chromatography separates proteins according to apparent globular size. The gel filtration column that was used was HiLoad 16/60 Superdex 200 prep grade (GE Health Care) and PBS was used for eluting the proteins.)

Three dominant multimeric forms were visible when the serum adiponectin was seperated by size exclusion gel chromatography, with apparent sizes of 230 kDa, 420 kDa and > 600 kDa respectively, and interpreted as LMW (hexamer 230 kDa) and HMW (420 kDa and >600 kDA).

The serum adiponectin analyzed displayed three dominant multimeric forms based on size, and five different forms based on isoelectric points, or total net charge.

13 PERFORMANCE CHARACTERISTICS

13.1 Detection limit

Detection limit is defined as the Capability of Detection according to ISO11 843-Part 1. Capability of Detection should be seen as part of a method validation, rather than the lowest concentration that can be measured.

The detection limit is 1.25 (ng/ml) as determined by the methodology described in ISO11843-Part 4.

Concentration of samples with absorbance below Standard 1 should not be calculated, instead expressed as less or equal to (≤) the concentration indicated on the vial for Standard 1.

13.2 Recovery

Recovery upon addition is 92-109% (mean 101%)

Recovery upon dilution is 89-111% (mean 98%)

13.3 Hook effect

There is no existing hook effect.

13.4 Precision

Each sample was analyzed in 4-replicates on 39 different occasions.

Sample	Mean value (ng/ml)	Coefficient of variation		
		within assay %	between assay %	total assay %
1	29.7	3.0	5.3	5.5
2	65.9	2.7	5.0	5.2
3	13.0	3.0	5.8	6.0

13.5 Specificity

The following crossreactions have been found:

C1q ≤ 0.007%

TNF-alfa n.s

14 CALIBRATION

Adiponectin ELISA is calibrated against a highly purified, fully validated, commercial adiponectin preparation. The concentration of adiponectin is expressed in ng/ml.

15 WARRANTY

The performance data presented here was obtained using the procedure indicated. Any change or modification in the procedure not recommended by DRG may affect the results, in which event DRG disclaims all warranties expressed, implied or statutory, including the implied warranty of merchantability and fitness for use. DRG and its authorized distributors, in such event, shall not be liable for damages indirect of consequential.

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SYMBOLS USED WITH DRG ELISA´S

Symbol	English	Deutsch	Francais	Español	Italiano
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
CE	European Conformity	CE-Konfirmitäts-kennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
IVD	In vitro diagnostic device	In-vitro-Diagnostikum	Ussage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
RUO	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
REF	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
LOT	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
	Contains sufficient for <n> tests/	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos	Contenuto sufficiente per "n" saggi
	Storage Temperature	Lagerungstemperatur	Temperature de conservation	Temperatura de conservación	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeits-datum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Distributeur	Distributeur	Distribuidor	Distributtore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità

Symbol	Portugues	Dansk	Svenska	Ελληνικά
	Consulte as instruções de utilização	Se brugsanvisning	Se bruksanvisningen	Εγχειρίδιο χρήστη
CE	Conformidade com as normas europeias	Europaeisk overensstemmelse	Europeisk överensstämmelse	Ευρωπαϊκή Συμμόρφωση
IVD	Diagnóstico in vitro	In vitro diagnostik	Diagnostik in vitro	in vitro διαγνωστικό
REF	Catálogo n.º	Katalognummer	Katalog nummer	Αριθμός καταλόγου
LOT	No do lote	Lot nummer	Batch-nummer	Αριθμός Παρτίδος
		Indeholder tilsttrækkeligt til "n" test	Innehåller tillräckligt till "n" tester	Περιεχόμενο επαρκές για «n» εξετάσεις
	Temperatura de conservação	Opbevarings-temperatur	Förvaringstempratur	Θερμοκρασία αποθήκευσης
	Prazo de validade	Udløbsdato	Bäst före datum	Ημερομηνία λήξης
	Fabricante	Producent	Tillverkare	Κατασκευαστής
Content	Conteúdo	Indhold	Innehåll	Περιεχόμενο
Volume/No.	Volume/Número	Volumen/antal	Volym/antal	Όγκος/αριθ..