Adropin ELISA Kit

Enzyme Immunoassay for the quantification of Adropin in Human, Mouse, Rat Serum/Plasma extraction, Tissue extraction and CSF extraction samples

Catalog number: ARG80915

For research use only. Not for use in diagnostic procedures.

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MANUFACTURED BY:

Arigo Biolaboratories Corporation

Address: No. 22, Ln. 227, Gongyuan Rd., Hsinchu City 300, Taiwan

Phone: +886 (3) 562 1738

Fax: +886 (3) 561 3008

Email: info@arigobio.com

INTRODUCTION

Adropin is a metabolic hormone encoded by Energy Homeostasis Association (ENHO) gene that serves in lipid metabolism regulation. It was first isolated from mouse liver and brain tissues by Kumar et al. 2008. The concerned experiment showed that lean C57BL/6J mice fed on a high-fat diet (HFD) had a rapid increase in adropin expression compared to control values; conversely, adropin expression of fasting mice dropped in comparison to control values. Elevated amounts of adropin in circulation reduce insulin resistance and glucose intolerance that occur in response to metabolic stress [PMID: 24721335]

PRINCIPLE OF THE ASSAY

This is an Enzyme Immunoassay for the quantification Adropin in Human, Mouse, Rat, Guinea pig, Pig, Sheep plasma samples. This assay employs the competitive quantitative enzyme immunoassay technique. A secondary antibody has been pre-coated onto a microtiter plate. The secondary antibody can bind to the Fc fragment of the primary antibody which recognizes Adropin. The primary antibodies in the kit will be competitively bound by both biotinylated Adropin peptides and Adropin peptides in standards or targeted Adropin peptides in samples. The wells are washed and then incubated with Streptavidin-HRP reagent. The biotinylated peptide interacts with streptavidin-horseradish peroxidase to form a complex. After washing away any unbound antibody conjugate, a substrate solution (TMB) is added to the wells and color develops in inverse-proportion to the amount of Adropin present in the samples. The color development is stopped by the addition of STOP solution

and the intensity of the color is measured at a wavelength of 450nm±2 nm. The concentration of Adropin in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Secondary antibody coated microplate	12 x 8 wells	4°C
20X Wash Buffer	50 ml	4°C
Primary antibody	1 vial	4°C
Biotinylated peptide	1 vial	4°C
Standard	1 vial	4°C
Streptavidin-HRP complex	30 µl	4°C
TMB substrate	12 ml (Ready-to-use)	4°C (Protect from light)
STOP solution (2N HCl)	15 ml (Ready-to-use)	4°C
Positive Controls (Acc. range: 0.8-1.5 ng/ml)	2 vial	4°C
Plate sealer	3 pieces	Room Temperature

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- Pipettes and pipette tips
- Deionized or distilled water
- Microplate shaker (300-400rpm)
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times.
- Before opening any Eppendorf tubes for reconstitution, briefly centrifuge at ~3,000rpm for 5 seconds to ensure that all the lypholized material is at the bottom of the tube.
- If crystals are observed in the 20X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- Ensure complete reconstitution and dilution of reagents prior to use.
- It is highly recommended that all solutions be used as soon as possible after reconstitution.
- Unused microplate strips should be placed back in the foil pouch with a desiccant and stored at 4°C. Do not allow moisture to enter the wells.
- All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- Each time a new tip is used, make sure the tip is secure and free of air bubbles. For better intra-assay variation, aspirate and expel a reagent or sample back into the container a few times prior to loading.
- Avoid submerging the whole tip into reagents because droplets can accumulate at the end of the tip causing an excess of reagent to be loaded into the well. This can lead to poor results.
- For optimal results, an orbital plate shaker capable of 300-400 rpm is recommended for all incubations.

- It is highly recommended that the standards, samples and controls be assayed in duplicates.

SAMPLE COLLECTION & STORAGE INFORMATION

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 before centrifugation for 15 minutes at 1600 x g at 4°C. Collect serum and assay immediately or aliquot and store samples at ≤ -80 °C up to 1 month. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge for 15 minutes at 4°C at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -80 °C up to 1 month. Avoid repeated freeze-thaw cycles.

Note: We recommended add Aprotinin (enzyme inhibitor) for ALL sample collection to prevent sample degradation. 0.6 TIU or 100 µl of Aprotinin per mL of sample solution.

Tissues lysate - Weigh out an appropriate amount of tissue. Dissect tissue with clean tools into small pieces, preferably on ice. Combine this tissue with 3 parts lysis buffer (e.g. 5% Acetic Acid) and boil for 20 minutes at 100°C. Homogenize tissue in lysis buffer using homogenizer. Centrifuge the tissue homogenate at 10,000rpm (~3,400 x g) for 15 minutes at 4°C. Collect the supernatant. Assay immediately or aliquot and store samples at ≤ -80 °C up to 1 month. Avoid repeated freeze-thaw cycles.

Note: If a separate protein assay is required, designate and remove an aliquot before addition of Binding Buffer for peptide extraction process. This buffer contains materials which may interfere with protein analysis.

Peptide extraction –

1. It is recommended through peptide extraction protocol.
2. Mix an equal amount of Binding Buffer (1% trifluoroacetic acid (TFA), HPLC grade is recommended) with the plasma/serum or tissue lysate samples and vortex the mixture (Ex. 1ml of sample + 1 ml of Binding Buffer). Centrifuge at 6,000 -17,000 × g for 20 minutes at 4°C. Collect the supernatant.
3. Slowly equilibrate a C18 column (SEP-COLUMN containing 200 mg of C18) by washing the C18 column with 1 ml Elution Buffer (60% acetonitrile, 1% TFA, and 39% distilled water, HPLC grade of acetonitrile and TFA are recommended).
4. Wash the C18 column with 3 ml of Binding Buffer three times.
   Note: It is recommended do not use pressure from steps 5-7,
5. Load the sample/Binding Buffer mixture solution from step 2 into the washed C18 column from step 3.
6. Wash the column slowly with Binding Buffer (3 ml, twice) and discard the wash.
7. Elute the peptide slowly with Elution Buffer (3 ml, once) and collect eluant into a polystyrene tube.

   Note: Ensure there is a constant flow for all solutions during the extraction procedure. For optimal sample processing and recovery, do not allow air bubbles to enter the C-18 matrix.

8. Evaporate eluant to dryness in a centrifugal concentrator or by a suitable substitute method to dryness the eluant. (Freeze-dry the resulting water/TFA solution to dryness)
9. Keep the dried extract at-20°C and perform the assay as soon as possible.
10. For normal subject extracted from 1 ml original plasma/serum, use 125 µl 1X Wash buffer to reconstitute the dried extract. Aliquot 50 µl into two designated assay wells (25 µl is left over). The concentration factor in this case is 8. (1 ml / 125 ul = 8). The original plasma peptide level is 1/8 of the level of final extracted plasma.

    e.g. If the level of the final extracted plasma is 100 pg/ml, then the total level of peptide in the original plasma = (100 pg/ml) / 8 = 12.5 pg/ml. After performing assay, if the peptide value exceeds or does not fall in the range of detection, dilute or concentrate the samples accordingly.

Drying Sample After Extraction:

A combination of a centrifugal concentrator (i.e. Speedvac) and a lyophilizer (freeze-dryer) produces the best results for drying the sample after extraction. First, use a Speedvac to dry sample for approximately 15 minutes to remove the organic layer. Then snap-freeze the remaining sample, and freeze-dry overnight using a lyophilizer. This two-step procedure produces a more consistent fluffy powder that is easier to rehydrate than a sample dried only with a centrifugal concentrator. However, if a centrifugal concentrator is not accessible, freeze-drying overnight using a lyophilizer will be sufficient.

REAGENT PREPARATION

- 1X Wash Buffer: Dilute 20X Wash Buffer into distilled water to yield 1X Wash buffer. Keep 1X Wash Buffer at 4°C. If crystals appear in 20X Wash Buffer, warm the buffer in warm water bath (not higher than 50°C) for 30 minutes or until crystals disappear. Mix well before use.
- Primary antibody: Reconstitute the Primary antibody vial with 5 ml of 1X Wash Buffer, mix thoroughly. Allow it to sit for 5 minutes to completely dissolve, mix well before use. Keep rehydrated solution at 4°C.
- Biotinylated peptide: Reconstitute the Biotinylated peptide vial with 5 ml of 1X Wash Buffer, mix thoroughly. Allow it to sit for 5 minutes to completely dissolve, mix well before use. Keep rehydrated solution at 4°C.
- Positive control: Centrifuge the vial before opening. Reconstitute the Positive control vial with 200 µl of 1X Wash Buffer, mix thoroughly. Allow it to sit for 5 minutes to completely dissolve, mix well before use. Keep rehydrated solution at 4°C. (acceptable range dependent on lot#)
- Streptavidin-HRP complex: Centrifuge Streptavidin-HRP complex briefly before use. Add 12µl of Streptavidin-HRP to 12ml 1X wash buffer to make Streptavidin-HRP solution. Vortex thoroughly.
- Standard peptide: Centrifuge and dilute the standard with 1 ml of 1X Wash buffer and vortex. The concentration of this stock solution is 1000 ng/ml. Allow the solution to sit for at least 10 minutes at room temperature to completely dissolve. Dilute peptide standard solutions with 1X Wash buffer to 100 ng/ml, 10 ng/ml, 1 ng/ml, 0.1 ng/ml, 0.01 ng/ml as follows:

The example of the dilution of standards

Standard No.	Standard Conc. (ng/ml)	1X Wash Buffer (µl)	Standard (µl)
Stock	1000	-	-
S1	100	900	100 µl of Stock
S2	10	900	100 µl of S1
S3	1	900	100 µl of S2
S4	0.1	900	100 µl of S3
S5	0.01	900	100 µl of S4
S0 (Total binding)	0	150	0

Vortex the tube thoroughly after each serial dilution.

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 20-23°C) 20-30 min before use. Standards, samples and blank should be assayed in duplicates.

1. Remove excess microtiter strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add **50 µl of 1X Wash Buffer** as Total Binding (zero standard). Two empty wells should be left as blank.
3. Add **50 µl of prediluted peptide standards** (add from S5 to S1), **50 µl positive controls** or **50 µl samples** into corresponding wells. It is advisable to assay each condition in duplicates.
4. Add **25 µl of primary antibody** into each well **except the Blank wells**.
5. Add **25 µl of Biotinylated peptide** into each well **except the Blank wells**. It is not recommended to use a multi-channel pipette to load the primary antibody and biotinylated peptide.
6. Seal the microtiter plate with plate sealer. Incubate for **2 hours at RT.** Orbital shaking on a microplate shaker at 300-400 rpm is recommended.

7. Mix and centrifuge Streptavidin-HRP complex (concentrate) vial (3,000-5,000 rpm for 5 seconds) before use. Pipette 12 µl of Streptavidin-HRP into 12 ml of 1X Wash Buffer. Vortex thoroughly. Prepare freshly.
8. Remove sealer from plate.
9. Aspirate each well and wash, repeating the process 3 times for a **total 4 washes**. Wash by filling each well with **1× Wash Buffer (350 µl)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
10. Add **100 µl of diluted Streptavidin-HRP** solution into **each well**.
11. Reseal the plate with sealer. Incubate for **1 hour at RT.** Orbital shaking on a microplate shaker at 300-400 rpm is recommended.
12. Remove sealer from plate. **Wash** as according to step 9.
13. Add **100 µl TMB substrate** solution into each well.
14. Reseal the plate with sealer. Incubate for **1 hour at RT in dark.** Orbital shaking on a microplate shaker at 300-400 rpm is recommended.
15. Remove sealer from plate. (**DO NOT** wash or discard the contents of the wells)
16. Add **100 µl 2N HCl** into all wells to stop the reaction. Gently tap the plate to ensure thorough mixing. The color of the solution should change from blue to yellow.
17. Read the OD with a microplate reader at **450 nm** immediately. It is recommended that read the absorbance within 20 minutes after adding the Stop Solution.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

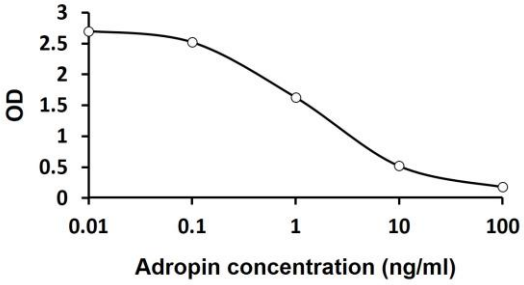
2. Using log-log, semi-log or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

The standard of Adropin ranged from 0-100 ng/ml.

The mean MDD was 0.4 ng/ml.

Linear Range

0.3 – 8.5 ng/ml

Precision:

Intra-assay: < 10%

Inter-assay: < 15%

Cross Reactivity

The cross reactivity ratio of the tested peptide as the table:

Peptide	Cross Reactivity (%)
Adropin (34-76) (Human, Mouse, Rat)	100
Adropin Recombinant Protein (8.2 kD)	100
MCH (Human, Mouse, Rat)	39.4
GLP-1 (7-36) Amide (Human, Bovine, Canine, Mouse, Ovine, Porcine, Rat)	25.2
AGRP (83-132) Amide (Human)	0
Apelin-36 (Human)	0
CCK (26-33) (Non-sulfated) (Human, Mouse, Rat)	0
CRF (Human, Canine, Feline, Mouse, Rat)	0
Ghrelin (Human)	0