Mouse/Rat ACTH ELISA Kit

Enzyme Immunoassay for the quantification of ACTH in mouse and rat serum, plasma, cell culture supernatants, tissue homogenate, CSF and urine samples

Catalog number: ARG82266

Package: 96 wells

TABLE OF CONTENTS

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INTRODUCTION

Gene POMC encodes a polypeptide hormone precursor that undergoes extensive, tissue-specific, post-translational processing via cleavage by subtilisin-like enzymes known as prohormone convertases. There are eight potential cleavage sites within the polypeptide precursor and, depending on tissue type and the available convertases, processing may yield as many as ten biologically active peptides involved in diverse cellular functions. The encoded protein is synthesized mainly in corticotroph cells of the anterior pituitary where four cleavage sites are used; adrenocorticotrophin, essential for normal steroidogenesis and the maintenance of normal adrenal weight, and lipotropin beta are the major end products. In other tissues, including the hypothalamus, placenta, and epithelium, all cleavage sites may be used, giving rise to peptides with roles in pain and energy homeostasis, melanocyte stimulation, and immune modulation. These include several distinct melanotropins, lipotropins, and endorphins that are contained within the adrenocorticotrophin and beta-lipotropin peptides. The antimicrobial melanotropin alpha peptide exhibits antibacterial and antifungal activity. Mutations in this gene have been associated with early onset obesity, adrenal insufficiency, and red hair pigmentation. Alternatively spliced transcript variants encoding the same protein have been described. [provided by RefSeq, Nov 2014]

ACTH stimulates the adrenal glands to release cortisol.

MSH (melanocyte-stimulating hormone) increases the pigmentation of skin by increasing melanin production in melanocytes.

Beta-endorphin and Met-enkephalin are endogenous opiates. [UniProt]

PRINCIPLE OF THE ASSAY

This is an Enzyme Immunoassay for the quantification ACTH in mouse and rat, serum, plasma, cell culture supernatants, tissue homogenate, CSF and urine 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 ACTH. The primary antibodies in the kit will be competitively bound by biotinylated-ACTH peptides and ACTH peptides in standards or targeted ACTH 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 Streptavidin-HRP reagent, a substrate solution (TMB) is added to the wells and color develops in inverse-proportion to the amount of ACTH peptide 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 ACTH peptide 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
1000X Streptavidin-HRP conjugate	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 (Accept. Range: 0.2-0.5 ng/ml)	2 vials	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 lyophilized 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. This kit is designed for serum and plasma samples but it may be used for other samples when the expression level is fall within the linear range.

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.

Peptide extraction for Serum/plasma–

1. It is recommended samples can through peptide extraction protocol as below.
2. Mix an equal amount of Binding Buffer (1% trifluoroacetic acid (TFA), HPLC grade is recommended) with the plasma or serum samples and vortex the mixture. (E.g. 1ml of plasma/serum + 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- or SPE-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.
5. Load the sample/Binding Buffer mixture solution from step 2 into the washed C18 column from step 4.
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: From steps 5-7, no pressure should be applied to the column.
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. Use 1x wash buffer in the kit to reconstitute the dried extract. If the peptide value does not fall within the range of detection, dilute or concentrate the sample accordingly.

    Note: 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 concentration 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.

Tissue Extraction- Because tissue pretreatment can significantly affect immunoassay results, each laboratory should determine their own appropriate procedure based on (a) the tissue type and (b) the target peptide to be analyzed. In most cases, peptide extraction via C-18 columns (Protocol A) is acceptable. However, for some specific peptides (e.g. somatostatin3, calcitonin4, IGF-15, kisspeptin6), it may be preferable to follow Protocol B to optimize peptide recovery from samples.

Protocol A: Peptide extraction via C-18 SEP-COLUMN

1. Prepare a lysis buffer of 5% acetic acid (5% HoAC): add 5 ml of glacial acetic acid into 95 ml of distilled water and mix thoroughly.
2. Weigh out an appropriate amount of tissue. Dissect tissue with clean tools into small pieces, on ice. Combine this tissue with 3 parts lysis buffer (e.g. 5% HoAc) and boil for 10 minutes at 100°C.
3. Homogenize tissue in lysis buffer by homogenizer.
4. Centrifuge the tissue homogenate at 10,000rpm (~3,400 x g) for 15 minutes at 4°C.
5. Take 1mL of supernatant and combine with 1mL of Binding Buffer (1% trifluoroacetic acid (TFA), HPLC grade is recommended) to acidify sample. *Note: If a separate protein assay is required, designate and remove an aliquot before addition of Binding Buffer. This buffer contains materials which may interfere with protein analysis.*

6. Centrifuge at 6,000 -17,000 × g for 20 minutes at 4°C. Collect the supernatant. This will be loaded onto the C-18 SEP-COLUMN.
7. Slowly equilibrate a C18 column (SEP- or SPE-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).
8. Wash the C18 column with 3 ml of Binding Buffer three times.
9. Load the sample/Binding Buffer mixture solution from step 5 into the washed C18 column from step 8.
10. Wash the column slowly with Binding Buffer (3 ml, twice) and discard the wash.
11. Elute the peptide slowly with Elution Buffer (3 ml, once) and collect eluant into a polystyrene tube.

    Note: From steps 5-7, no pressure should be applied to the column. Ensure there is a constant flow (by gravity) for all solutions during the extraction procedure. For optimal sample processing and recovery, do not allow air bubbles to enter the C-18 matrix.
12. 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)

    Note: A combination of a centrifugal concentrator (i.e. Speedvac) and a lyophilizer (freeze-dryer) produces the best results when drying samples after extraction. If a centrifugal concentrator is not available, the eluent should be frozen at least three hours before using being placed in the lyophilizer.
13. Keep the dried extract at -20°C and perform the assay as soon as possible.

14. Use 1x wash buffer in the kit to reconstitute the dried extract. If the peptide value does not fall within the range of detection, dilute or concentrate the sample accordingly.

    Note: For normal subject extracted from 1 ml original tissue lysate samples, use 250 µl of 1x Wash buffer to reconstitute the dried extract. Aliquot 50 µl into two designated assay wells (150 µl is left over). The concentration factor in this case is 4. (1 ml / 250 ul = 4). The original plasma peptide concentration level is 1/4 of the level of final extracted plasma.

    E.g. If the level of the final extracted sample is 100 pg/ml, then the total level of peptide in the original sample = (100 pg/ml) / 4 = 25 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.

Protocol B: Pretreatment using acid-ethanol

1. Prepare an acid-ethanol solution by combining 1 part concentrated HCl with 7 parts pure ethanol. The final solution should contain 87.5% ethanol and 12.5% HCl (2mol/L).
2. Weigh out an appropriate amount of tissue. Dissect tissue with clean tools into small pieces, on ice. Resuspend this tissue with an equal volume of the acid-ethanol solution and homogenize.
3. Combine this tissue with 5 parts of acidic ethanol with 1 part tissue homogenate and incubate for 30 minutes at 20°C
4. Centrifuge the tissue homogenate at 10,000rpm (~3,400 x g) for 30 minutes at 4°C.
5. Transfer the supernatant into new tubes and evaporate to dryness, either

using a centrifugal concentrator or by a suitable substitute method.

Note: A combination of a centrifugal concentrator (i.e. Speedvac) and a lyophilizer (freeze-dryer) produces the best results when drying samples after extraction. A lyophilizer alone may not be sufficient as the ethanol solution could stay in liquid form and spill. If no centrifugal concentrator is available, please only fill tubes to ¼ of its maximum volume.

6. Keep the dried extract at -20°C and perform the assay as soon as possible.
7. Use 1x wash buffer in the kit to reconstitute the dried extract. If the peptide value does not fall within the range of detection, dilute or concentrate the sample accordingly.

Note: For normal subject extracted from 1 ml original tissue lysate samples,, use 250 µl of 1x Wash buffer to reconstitute the dried extract. Aliquot 50 µl into two designated assay wells (150 µl is left over). The concentration factor in this case is 4. (1 ml / 250 ul = 4). The original plasma peptide concentration level is 1/4 of the level of final extracted plasma.

E.g. If the level of the final extracted sample is 100 pg/ml, then the total level of peptide in the original sample = (100 pg/ml) / 4 = 25 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.

CSF (Cerebrospinal Fluid)-

1. During spinal anesthesia, drops of CSF were collected. 1 ml of collected CSF was dispensed into a 5-mL polypropylene tube, and add 1 ml of Binding Buffer (1% trifluoroacetic acid (TFA), HPLC grade is recommended) to acidify sample immediately.
2. All sample/binding buffer mixture were then frozen and stored at -20°C

until extraction and assayed as a single batch.

3. The 2 ml of sample/binding buffer mixture were thawed and centrifuged at 12,000g for 15 min at 4°C. Collect the supernatant. This will be loaded onto the C-18 SEP-COLUMN.
4. Slowly equilibrate a C18 column (SEP- or SPE-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).
5. Wash the C18 column with 3 ml of Binding Buffer three times.
6. Load the sample/Binding Buffer mixture solution from step 5 into the washed C18 column from step 8.
7. Wash the column slowly with Binding Buffer (3 ml, twice) and discard the wash.
8. Elute the peptide slowly with Elution Buffer (3 ml, once) and collect eluant into a polystyrene tube.
9. Note: From steps 5-7, no pressure should be applied to the column. Ensure there is a constant flow (by gravity) for all solutions during the extraction procedure. For optimal sample processing and recovery, do not allow air bubbles to enter the C-18 matrix.
10. 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)
11. Keep the dried extract at-20°C and perform the assay as soon as possible.
12. Use 1x wash buffer in the kit to reconstitute the dried extract. If the peptide value does not fall within the range of detection, dilute or concentrate the sample accordingly.

Note: For normal subject extracted from 1 ml original CSF samples, use 250 µl of 1x Wash buffer to reconstitute the dried extract. Aliquot 50 µl into two designated assay wells (150 µl is left over). The concentration factor in this case is 4. (1 ml / 250 ul = 4). The original plasma peptide concentration level is 1/4 of the level of final extracted plasma.

E.g. If the level of the final extracted sample is 100 pg/ml, then the total level of peptide in the original sample = (100 pg/ml) / 4 = 25 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.

REAGENT PREPARATION

- **1X Wash Buffer**: Dilute 20X Wash Buffer into **distilled water** to yield 1X Wash buffer. Store the diluted 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**. Allow it to sit for 5 minutes to completely dissolve, mix well and keep rehydrated solution at 4°C before use. Store the reconstituted antibody at 4°C up to a week. For long-term storage, aliquot & store at -20°C for up to 3 months. Avoid repeated freeze-thaw cycles. It is recommended that antibody should only be frozen-thawed once.
- **Biotinylated peptide**: Reconstitute the Biotinylated peptide vial with **5 ml** of **1X Wash Buffer**. Allow it to sit for 5 minutes to completely dissolve, mix well and keep rehydrated solution at 4°C before use. Store the reconstituted peptide at 4°C up to a week. For long-term storage, aliquot

& store at -20°C for up to 3 months. Avoid repeated freeze-thaw cycles. It is recommended that peptide should only be frozen-thawed once.

- **Positive control**: Centrifuge and reconstitute the Positive control vial with **200 µl** of **1X Wash Buffer**. Allow it to sit for 5 minutes to completely dissolve, mix well and keep rehydrated solution at 4°C before use. Store the reconstituted control at 4°C up to a week. For long-term storage, aliquot & store at -20°C for up to 3 months. Avoid repeated freeze-thaw cycles. It is recommended that control should only be frozen-thawed once. (acceptable range dependent on lot#)
- **1X Streptavidin-HRP conjugate:** 1X Streptavidin-HRP conjugate working solution should be prepared freshly before use. Centrifuge 1000X Streptavidin-HRP conjugate briefly and add **12µl** of **Streptavidin-HRP** to **12ml** of **1X wash buffer** to make a 1X Streptavidin-HRP working solution. Vortex thoroughly.
- **Standard peptide**: Centrifuge and reconstitute 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. Store the reconstituted standard at 4°C up to a week. For long-term storage, aliquot & store at -20°C for up to 3 months. Avoid repeated freeze-thaw cycles. It is recommended that standard should only be frozen-thawed once. Dilute peptide standard solutions with 1X Wash buffer to **25 ng/ml, 5 ng/ml, 1 ng/ml, 0.2 ng/ml, 0.04 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	25	975	25 µl of Stock
S2	5	800	200 µl of S1
S3	1	800	200 µl of S2
S4	0.2	800	200 µl of S3
S5	0.04	800	200 µl of S4
S0 (Total binding)	0	800	0

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 20-23°C) 30 minutes before opening and starting the assay. 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 (S0, 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 (20-**

23°C). Orbital shaking on a microplate shaker at 300-400 rpm is recommended.

7. Prepare 1X Streptavidin-HRP conjugate working solution: Mix and centrifuge Streptavidin-HRP concentrate vial (3,000-5,000 rpm for 5 seconds) before use. Pipette 12 µl of Streptavidin-HRP concentrate into 12 ml of 1X Wash Buffer to make a Streptavidin-HRP working solution. 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 (1X) Streptavidin-HRP working 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 of 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 of STOP solution (2N HCl) into each 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 the wells be read 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.

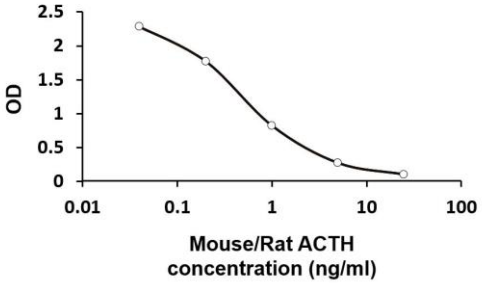
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

5. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for details. (https://www.arigobio.com/elisa-analysis)

6. If samples have been diluted prior to the assay, the measured concentration must be multiplied by their respective dilution factors.

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 ACTH peptide ranged from 0-25 ng/ml.

The mean MDD was 0.09 ng/ml.

Linear Range

0.09 – 2.30 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 (%)
ACTH (Mouse, Rat)	100
ACTH (Human)	100
ACTH (18-39) (Human)	100
ACTH (1-24) (Human)	0.01
ACTH (7-38) (Human)	0
beta-Endorphin (Rat)	0
alpha MSH	0
LH-RH	0
PACAP-38 (Ovine)	0