



User's Manual

Mouse Agouti-Related Peptide (AgRP) (82-131) amide ELISA Kit



DEIA-PP2



96T



This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

This Enzyme Immunoassay kit is designed to detect a specific peptide and its related peptides based on the principle of "competitive" enzyme immunoassay.

General Description

Agouti (ASIP) and Agouti-related protein (AgRP) are endogenous antagonists of melanocortin receptors that play critical roles in the regulation of pigmentation and energy balance, respectively, and which arose from a common ancestral gene early in vertebrate evolution. The N-terminal domain of ASIP facilitates antagonism by binding to an accessory receptor. Computational analysis reveals similar patterns of evolutionary constraint in the ASIP and AgRP C-terminal domains, but fundamental differences between the N-terminal domains.

Principles of Testing

The immunoplate in this kit is pre-coated with secondary antibody and the nonspecific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the primary antibody (peptide antibody) whose Fab fragment will be competitively bound by both biotinylated peptide and peptide standard or targeted peptide in samples. The biotinylated peptide interacts with streptavidin-horseradish peroxidase (SA-HRP) which catalyzes the substrate solution. The intensity of the yellow is directly proportional to the amount of biotinylated peptide-SA-HRP complex but inversely proportional to the amount of the peptide in standard solutions or samples. This is due to the competitive binding of the biotinylated peptide with the standard peptide or samples to the peptide antibody (primary antibody). A standard curve of known concentration can be established accordingly. The unknown concentration in samples can be determined by extrapolation to this standard curve.

Reagents And Materials Provided

1. 20x assay buffer concentrate (50ml).
2. 96 well immunoplate (1 plate).
3. Acetate plate sealer (APS), (3 pieces).
4. Primary antiserum (rabbit anti-peptide IgG) (1 vial).
5. Standard peptide (1 vial).
6. Biotinylated peptide (1 vial).
7. Streptavidin-horseradish peroxidase (SA-HRP) (30µl).
8. Positive control (2 vials).
9. Substrate solution (TMB) (12ml).
10. 2N HCl (15ml).
11. Assay diagram (1 sheet).



12. General protocol (1 book).

NOTE: CD guarantees that its products conform to the information contained in this publication. The purchaser must determine the suitability of the product for their particular needs and establish optimum sample concentration.

Materials Required But Not Supplied

1. Microtiter plate reader capable of absorbance measurement of 450nm.
2. Orbital plate shaker capable of 300-400rpm (recommended)
3. Microtiter plate washer (recommended)
4. Multi-channel pipette capable of dispensing 50-100µl (recommended)
5. Solution reservoir (recommended)
6. Absorbent material for blotting.
7. EDTA Lavender Vacutainer blood collection tubes (optional).
8. Aprotinin (0.6TIU/ml of blood) (optional).
9. Buffer A (optional).
10. Buffer B (optional).
11. C18 SEP-COLUMN (optional).

Note: The kit should be equilibrated to room temperature (20-23°C) before opening any vials and starting the assay. It is highly recommended that the solutions be used as soon as possible after rehydration. Each kit contains sufficient reagents for 96 wells and is capable of assaying 40 duplicate samples.

Storage

1. Store the kit at 4°C upon receipt.
2. It is highly recommended that solutions be used as soon as possible after rehydration.
3. Store 1x assay buffer at 4°C.
4. Remove any unneeded strips from antibody-coated plate, reseal them in zip-lock foil and keep at 4°C.
5. Keep rehydrated solution of Standard, Biotinylated peptide, Antibody and HRP at 4°C.

Specimen Collection And Preparation

SUGGESTED METHOD FOR THE EXTRACTION OF PEPTIDES FROM PLASMA:

Blood Withdrawal:

Collect blood samples into the Lavender Vacutainer tubes which contain EDTA and can collect up to 7ml of blood. Gently rock the Lavender Vacutainer tubes several times immediately after collection of blood for anti-coagulation. Transfer the blood from the Lavender Vacutainer tubes to the centrifuge tubes containing aprotinin (0.6TIU/ml of blood) and gently rock for several times to inhibit the activity of proteinases. Centrifuge the blood at 1,600 x g for 15 minutes at 4°C and collect the plasma. Plasma kept at -70°C may be stable for one month. If Lavender Vacutainer tubes are centrifuge-safe, the aprotinin may be added into the initial collection step.

Extraction of Peptides from Plasma:

1. Acidify the plasma with an equal amount of buffer A. For example, if you are using 1ml of plasma, add 1ml of buffer A. Mix and centrifuge at 6,000 to 17,000 x g for 20 minutes at 4°C.
2. Equilibrate a SEP-COLUMN containing 200mg of C18 by washing with buffer B (1ml, once) followed by buffer A (3ml, 3 times).

Note: From steps 3-5, no pressure should be applied to the column.

3. Load the acidified plasma solution onto the pre-equilibrated C-18 SEP- Column.
4. Slowly wash the column with buffer A (3ml, twice) and discard the wash.
5. Elute the peptide slowly with buffer B (3ml, once) and collect the eluant into a polystyrene tube.
6. Evaporate eluant to dryness in a centrifugal concentrator or by a suitable substitute method.
7. Keep the dried extract at -20°C and perform the assay as soon as possible. Use 1x assay buffer to reconstitute the dried extract. If the peptide value does not fall within the range of detection, dilute or concentrate the sample accordingly.

Tips for extraction of plasma:

When using a C-18 SEP-COLUMN for the first time, use a bulb (not supplied) to apply pressure to the column after the addition of 1ml of buffer B to facilitate the flow through the column. From steps 3-5, no pressure should be applied.

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

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.

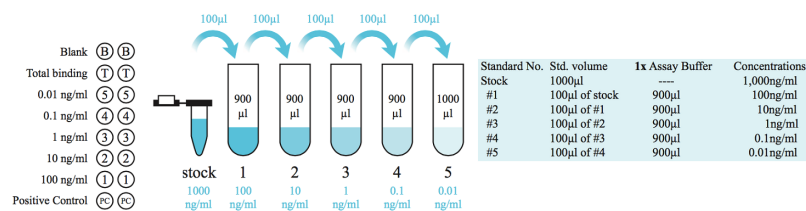
Assay Procedure

1. Thoroughly read this protocol before performing an assay. Please allow all kit components to return to room temperature before use (25-45 minutes).
2. Dilute the 20x assay buffer concentrate with 950ml of distilled water. This will be the 1x assay buffer solution and used to dilute or reconstitute all other reagents in this kit and samples.

Note: If crystals appear in the 20x assay buffer, the buffer can be placed in a warm water bath for approximately 30 minutes or until no crystals are visible. Mix thoroughly before use.

3. Centrifuge and dilute the standard peptide with 1ml of 1x assay buffer, vortex. The concentration of this stock solution is 1,000ng/ ml. Allow the solution to sit at least 10 minutes at room temperature (20-23°C) to completely dissolve in solution. Centrifuge and vortex immediately before use.

Prepare peptide standard solutions as follows:



- Rehydrate primary antibody with 5ml of 1x assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.
- Rehydrate biotinylated peptide with 5ml of 1x assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.
- Centrifuge and rehydrate the positive control with 200 µl of 1x assay buffer. Allow it to sit at least 5 minutes to completely dissolve. Mix thoroughly.
- Leave wells A-1 and A-2 empty as Blank.
- Add 50 µl of 1x assay buffer into wells B-1 and B-2 as Total Binding.
- Add 50 µl of prepared peptide standards from #5 to #1 (in reverse order of serial dilution) into wells from C-1 and C-2 to G-1 and G-2 respectively.

Note: Peptide standards should be assayed in duplicate.

- Add 50 µl of rehydrated positive control into wells H-1 and H-2.

Note: Positive controls should be assayed in duplicate.

- Add 50 µl of prepared samples into their designated wells in duplicate.
- Add 25 µl of rehydrated primary antibody into each well except the Blank well.
- Add 25 µl of rehydrated biotinylated peptide into each well except the Blank well.

Note: A multi-channel pipette is NOT recommended to load the biotinylated peptide or primary antiserum.

- Seal the immunoplate with acetate plate sealer (APS). Incubate the immunoplate for 2 hours at room temperature (20-23°C). Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.
- Centrifuge the SA-HRP vial provided in this kit (3,000-5,000 rpm, 5 seconds) and pipette 12 µl of SA-HRP into 12 ml of 1x assay buffer to make SA-HRP solution, vortex thoroughly.
- Remove APS from the immunoplate. Discard contents of the wells.
- Wash each well with 350 µl of 1x assay buffer, discard the buffer, invert and blot dry plate. Repeat 4 times.
- Add 100 µl of SA-HRP solution into each well.
- Reseal the immunoplate with APS. Incubate for 1 hour at room temperature (20-23°C). Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.
- Remove APS from the immunoplate.
- Wash and blot dry the immunoplate 4 times with 1x assay buffer as described above in step 17.
- Add 100 µl of TMB substrate solution provided in this kit into each well. Orbital shaking at 300-400 rpm is recommended for the duration of the incubation. After the addition of TMB solution, it is strongly recommended to cover the immunoplate to protect from light.
- Reseal the immunoplate with APS. Incubate for 1 hour at room temperature (20-23°C).
- Remove APS from the immunoplate. Add 100 µl 2N HCl into each well to stop the reaction. The color in the well should change from blue to yellow. If the color change does not appear to be uniform, gently tap the plate to ensure thorough mixing. Proceed to the next step within 20 minutes.

25. Load the immunoplate onto a Microtiter Plate Reader. Read absorbance O.D. at 450nm.

Additional Recommended Procedural Notes:

- Reagents of different lot numbers should not be mixed.
- Recheck the reagent labels when loading the plate to ensure that everything is added correctly.
- 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.
- When handling the plate, avoid touching the bottom.
- Manual washing may cause high duplicate coefficient variations. To reduce this factor, liquid from the plate should be removed by inverting and blotting the plate on an absorbent material.
- If the room temperature is not within the suggested range (20-23°C), variations in results may occur.
- The same reservoir for the reagents may be reused if the reservoir is washed well with distilled water before each use.
- Each laboratory must determine the appropriate dilution factors for the samples to be measured to ensure that the samples are within the dynamic range of the standard curve.
- High levels of interfering proteins may cause variations within sample results; therefore, it is imperative to select the appropriate sample preparation procedure to obtain optimal results.
- 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.
- Modification of the existing protocol (i.e. standard dilutions, pipetting technique, washing technique, incubation time or temperature, storage conditions, and kit expiration) may affect the sensitivity and specificity of the test.

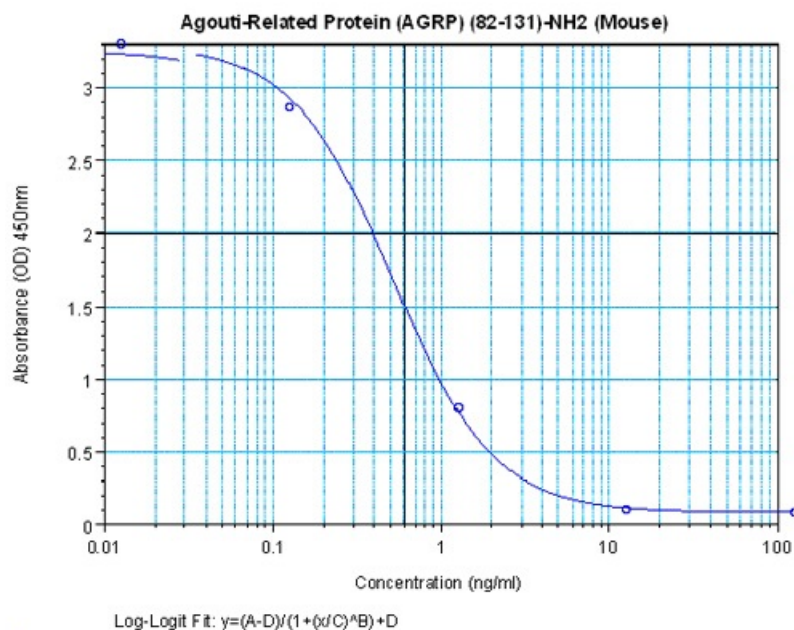
Calculation

Plot the standard curve on semi-log graph paper. A standard curve is constructed by plotting the known concentrations of standard peptide on the log scale (X-axis), and its corresponding O.D. reading on the linear scale (Y-axis). It is strongly recommended to use curve-fitting software capable of 4 parameter logistics or log-logit to quantify the concentration of standard peptide. The standard curve shows an inverse relationship between peptide concentrations and the corresponding absorbance. As the standard concentration increases, the yellow color decreases, thereby reducing the O.D. absorbance.

The concentration of peptide in a sample is determined by locating the sample's O.D. on the Y-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line drawn from this point will intersect the X-axis at a coordinate corresponding to the peptide concentration in the sample. If samples have been diluted prior to the assay, the measured concentration must be multiplied by their respective dilution factors.

The standard curve will be a reverse sigmoidal shape.

Refer to QC Data Sheet for acceptable values of the positive controls.



Precision

Intra-assay Precision (Precision within an assay): CV%<10%

Inter-assay Precision (Precision between assays): CV%<15%

Detection Range

0-100 ng/ml

Sensitivity

0.09 ng/ml

Specificity

Peptide Cross-reactivity

Agouti-Related Protein (82-131)-NH2 (Mouse): 100%

Agouti-Related Protein (83-132)-NH2 (Human): 100%

Agouti-Related Protein Form C: 100%

Agouti-Related Protein (79-129)-NH2 (Rat): 94.8%

Orexin A: 0

Orexin B (Human): 0

Orexin B (Rat, Mouse): 0

Orexin B (3-28): (Rat, Mouse): 0

Leptin (Human): 0

Leptin (Mouse): 0

α-MSH: 0

GLP-1 (7-37): 0

GLP-2: 0

NPY (Human): 0

Linearity

0.09 - 0.49 ng/ml