DRG® Alpha-Melanocyte Stimulating Hormone (Alpha-MSH) (EIA-2951)

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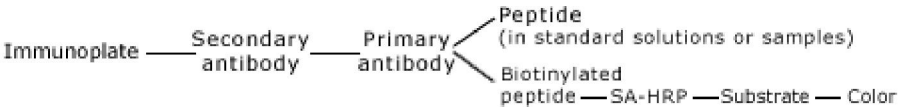
INTRODUCTION

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

RANGE: (0-100ng/ml)

PRINCIPLE OF ENZYME IMMUNOASSAY WITH THIS KIT

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 sample. The biotinylated peptide is able to interact with streptavidin-horseradish peroxidase (SA-HRP) which catalyzes the substrate solution composed of 3,3', 5,5'-tetramethylbenzidine (TMB) and hydrogen peroxidase to produce a blue colored solution. The enzyme-substrate reaction is stopped by hydrogen chloride (HCI) and the solution turns to yellow. 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 and the peptide in standard solutions or samples to the peptide antibody (primary antibody). A standard curve of a peptide with known concentration can be established accordingly. The peptide with unknown concentrations in samples can be determined by extrapolation to this standard curve.



CONTENTS:

1. 20 x assay buffer concentrate (50 ml)
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 µg)
6. Biotinylated peptide (1 vial)
7. Streptavidin-horseradish peroxidase (SA-HRP) (30 µl)
8. Positive Control (2 vials)
9. Substrate solution (12 ml)
10. 2N HCI (15 ml)
11. Assay Diagram (1 sheet)
12. General protocol (1 book)

DRG 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.

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microtiter plate reader capable of absorbance measurement between 405-650 nm.
2. Orbital plate shaker capable of 300-500 rpm (recommended)
3. Microtiter plate washer (recommended)
4. Multi-channel pipette capable of dispensing 50-100 µl (recommended)
5. Solution reservoir (recommended)
6. Absorbent material for blotting
7. EDTA Lavender Vacutaner blood collection tubes (optional)
8. Aprotinin (0.6 TIU/ml of blood) (optional)
9. Buffer A (optional)
10. Buffer B (optional)
11. C18 SEP-COLUMN

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. Recommended blood collection protocol is provided in this booklet. Each kit contains sufficient reagents for 96 wells and is capable of assaying 40 duplicate samples.

STORAGE:

- Store the kit at 2-4°C upon receipt. The kit will be stable for 6 months.
- The kit should be equilibrated to room temperature before assay. It is recommended that the solutions be used on the same day of rehydration.
- Remove any unneeded strips from antibody-coated plate, reseal them in zip lock foil and keep at 4°C.
- Keep rehydrated solution of Standard, Biotinilated peptide, Antibody and HRP at 4°C.

ASSAY PROCEDURE

1. Thoroughly read this protocol before performing assay. Please allow all kit components to return to room temperature before use (25-45 minutes).
2. Dilute the 20X assay buffer concentrate with 950 ml of distilled water. This assay buffer will be used to reconstitute all of the other compounds in this kit and the extract of plasma sample. *Note:* If crystals appear in the 20X assay buffer, the buffer can be placed in a warm water bath for approx. 30 minutes or until no crystals are visible. Mix thoroughly before use.
3. Centrifuge and dilute the standard peptide with 1 ml of 1Xassay buffer. The concentration of this stock solution is 1,000 ng/ml. Allow the standard to sit al least 10 minutes at room temperature (20-23°C) to completely dissolve in solution. Centrifuge and vortex immediately before use.
4. Prepare peptide standard solutions as follows:

Standard No.	Std. Volume	Assay Buffer	Concentrations
Stock	1000 µl	---	1,000 ng/ml
#1	100 µl of stock	900 µl	100 ng/ml
#2	100 µl of #1	900 µl	10 ng/ml
#3	100 µl of #2	900 µl	1 ng/ml
#4	100 µl of #3	900 µl	0.1 ng/ml
#5	100 µl of #4	900 µl	0.01 ng/ml

5. Rehydrate primary antiserum with 5 ml of 1X assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.
6. Rehydrate biotinylated peptide with 5 ml of 1X assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.
7. Centrifuge and rehydrate the positive control with 200µ of 1X assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.
8. Leave wells A-1 and A-2 empty as **Blank**.
9. Add 50 µl of 1X assay buffer into wells B-1 and B-2 as **Total Binding**.
10. Add 50 µl of the prepared peptide standards from #5 to #1 (in reverse order of serial dilution) into the wells from C-1 to C-1 to G-1 to G-2 respectively.
    Note: Peptide standards should be assayed in duplicate.
11. Add 50 µl of rehydrated positive control into wells H-1 and H-1.
    Note: Peptide controls should be assayed in duplicate.
13. Add 50 µl of prepared samples into their designated wells in duplicate.
14. Add 25 µl rehydrated primary antiserum into each well **except the Blank well.**
15. Add 25 µl 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.
16. Seal the immunoplate with acetate plate sealer (APS).
17. 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.
18. Centrifuge the SA-HRP vial provided in this kit (3,000-5,000 r.p.m., 5 seconds, 4°C) and pipet 12 µl of SA-HRP into 12 ml of 1X assay buffer to make SA-HRP solution, vortex thoroughly.
19. Remove APS from the immunoplate.
20. Discard contents of wells.
21. Wash each well with 350 µl of 1X assay buffer, discard the buffer, invert and blot dry the plate Repeat 4 times.
22. Add 100 µl SA-HRP solution into each well.
23. Reseal the immunoplate with APS.
24. Incubate for 1 hour at room temperature (20-23°C). Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.
25. Remove APS from the immunoplate.
26. Wash and blot dry the immunoplate 4 times with 1X assay buffer as described above.

27. 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.
28. Reseal the immunoplate with APS.
29. Incubate for 1 hour at room temperature (20-23°C) .
30. Remove APS from the immunoplate. Add 100 µl 2N HCI into each well to stop the reaction. The color in the well should change from blue to yellow. If the change does not appear to be uniform, gently tap the plate to ensure thorough mixing. Proceed to next step within 20 minutes.
31. Load the immunoplate onto a Microtiter Plate Reader.
32. Read absorbance O.D. at 450 nm.

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 dessicant 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 to obtain the 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 OF RESULTS

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. Because 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.

SUMMARY OF ASSAY PROTOCOL

* Add 50µl/well of standard or sample, 25µl primary antiserum and 25µl biotinylated peptide.
* Incubate at room temperature(20-23°C) for 2 hours.
* Wash immunoplate 4 times with 350 µl/well of 1X assay buffer.
* Add 100 µl/well of SA-HRP solution.
* Incubate at room temperature (20-23°C) for 1 hour
* Wash immunoplate 4 times with 350 µl/well of 1X assay buffer.
* Add 100 µl/well of TMB substrate solution.
* Incubate at room temperature (20-23°C) for 1 hour.
* Terminate reaction with 100µl/well of 2N HCI
* Read absorbance O.D. at 450 nm and calculate results.

SUGGESTEDF METHOD FOR THE EXTRACTION OF PEPTIDES FROM PLASMA

Blood Withdrawal:

Collect blood samples into the Lavender Vacutaner tubes, which contain EDTA and can collect 7ml blood/tube. Gently rock the Lavender Vacutaner tubes several times immediately after collection of blood for anti-coagulation.
Transfer the blood from the Lavender Vacutaner tubes to 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 the Lavender Vacutaner tubes are centrifuge-safe, the aprotinin maybe added into the initial collection step.

Extraction of Peptide from Plasma:

1. Acidify the plasma with an equal amount of Buffer A. For example, if you are using 1 ml of plasma, add 1 ml 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 200 mg of C18 by washing with Buffer B (1 ml, 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-treated C18 SEP-COLUMN.
4. Slowly wash the column with buffer A (3 ml, twice) and discard the wash.
5. Elute the peptide slowly with buffer B (3 ml, once) and collect eluant in 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 assay buffer to reconstitute the dried extract. If the peptide value does not fall within the range of detection, dilute or concentrate the sample as needed.

Tips for Extraction of Plasma:
When using SEP-COLUMN for the first time, use a bulb (not supplied) to apply pressure to the column after addition of 1 ml of Buffer B to facilitate the flow through the column. From step 3-5, no pressure should be applied. Ensure that there is a constant flow for all solutions during the extraction procedure. Do not allow for air bubbles to enter the C-18 matrix for optimal sample processing and recovery.

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

REFERENCES
1. Enzyme Immunoassay Techniques, An Overview, Porstmann, T., and Kiessig, S.T. Journal of Immunological Methods, 150 (1992) 5-21.
2. Amplification Systems in Immunoenzymatic Techniques, Avrameas, S. Journal of Immunological Methods, 150 (1992) 23-32.