General Protocol

Peptide Enzyme Immunoassay

Note: In order to choose the correct range for your standard curve, please check the accompanying data sheet.

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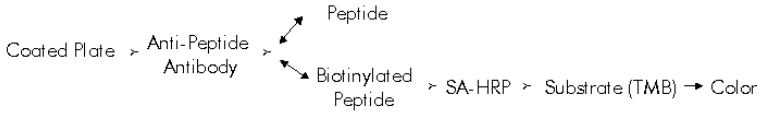
Introduction

This competitive enzyme immunoassay kit is designed to detect the specific peptide indicated on the enclosed accompanying data sheet. It provides sufficient reagents for a total of 96 determinations, enabling the generation of duplicate six or ten point standard curves and the analysis of up to 41 samples in duplicate.

Principle of the Assay

Peptide antibody, biotinylated peptide, and non-biotinylated peptide (either standard or unknown) are placed in a well and mixed. The peptide antibodies bind to the specially treated walls of the well. The biotinylated peptide competes for the antibody binding sites with the standard peptide and the unknown sample peptide (analyte). After incubation, unbound biotinylated peptide is removed by washing and streptavidin-conjugated horseradish peroxidase (SA-HRP) is added and allowed to bind to the immobilized primary antibody/biotinylated peptide complex. After washing away excess SA-HRP, TMB (3,3',5,5'-tetramethyl benzidine dihydrochloride) is allowed to react with the bound HRP. The color intensity that develops depends on the quantity of biotinylated peptide bound to the immobilized antibody. When more non-biotinylated peptide competes for the limited amount of antibody, less biotinylated peptide/SA-HRP can be immobilized and less color is produced by the substrate.



Materials Provided

- Assay buffer concentrate.
- One 96 well immunoplate with acetate plate sealer (APS).
- One vial of peptide antibody (lyophilized powder).
- One vial of peptide standard (lyophilized powder).
- One vial of biotinylated peptide (lyophilized powder).
- Streptavidin-HRP concentrate (15 µl).
- Substrate solution (11ml of TMB and H_2O_2).
- 2 N HCl (15 ml).
- Assay diagram.

Materials Required but not Provided

- Multi-channel or repeating pipettes.
- Pipettors and tips capable of accurately measuring 10 to 1000 µl.
- Graduated serological pipettes, 10 ml and 25 ml.
- 96 well microtiter plate reader set up to measure 450 nm.
- 96 well plate washer and shaker (optional).
- Semi-log graph paper for manual plotting of data or appropriate computer software.
- 12 x 75 mm polypropylene test tubes, 15 and 50 ml polypropylene or polystyrene tubes.
- Mechanical vortex mixer.
- Sterile deionized or USP water.

Assay Procedure

General Considerations

- Carefully read all of the instructions before starting the assay.
- Carefully check the components listed on the inside of the box or page 3 to ensure that all items are included.
- Each kit is especially formulated. Therefore the cross-usage of components between different kits (from different lots) is strongly discouraged.
- All procedures and incubations are performed at room temperature. The reagents, samples, and plate should be brought to room temperature before use.
- Shakers are optional. Shakers may help lower the c.v. between duplicates (recommended at 60 rpm).

Note: We strongly recommend doing an extraction on all samples. The extraction procedure for plasma is provided for your reference (materials needed for extraction are sold separately). Other extraction protocols may be used. Peninsula Laboratories, Inc. guarantees this product to perform as expected provided the purchaser follows the enclosed protocol and accompanying datasheet. The purchaser must determine the suitability of the product for his/her particular research use.

Step 1: Preparation of Reagents

A) Preparation of the assay buffer:
Dilute assay buffer concentrate to 1,000 ml with sterile deionized or USP pure water (18 MOhm) and mix well.

B) Preparation of the standard peptide:
Add 1 ml of the assay buffer to the vial of lyophilized standard peptide and mix vigorously (vortex). The concentration of analyte in this stock solution is 1,000 ng/ml.

C) Preparation of the primary antibody:
Add 5 ml of assay buffer and mix vigorously (vortex).

D) Preparation of the tracer:
Add 5 ml of assay buffer to the vial of lyophilized biotinylated peptide and mix vigorously (vortex).

Step 2: Dilution of Standard

Note: See accompanying datasheet to choose the right range for your standard curve and follow procedures for either A) Dilution of Standard (Standard Curve Range 0-10 ng/ml) or B) Dilution of Standard (Standard Curve Range 0-25 ng/ml). Select the number of points on the curve as needed.

A) Dilution of Standard (Standard Curve Range 0-10 ng/ml):

Set up 6 (Table 1) or 10 (Table 2) 12 x 75 mm test tubes depending on the desired standard curve resolution. Label tubes as shown in the appropriate table. To each tube add the indicated respective volume of assay buffer. To tube #S1 add 10 µl of stock (1,000 ng/ml) peptide solution as indicated in column 2 of the tables. Mix vigorously (vortex). Transfer 200 µl from #S1 to #S2 tube (use a fresh pipette tip to reduce a carryover). Mix vigorously (vortex). Prepare the subsequent serial dilutions as indicated in the appropriate table. (*Change pipette tips after each addition to reduce cross contamination.*)

Table 1: Six Point Standard Curve

Std. No.	Std. Vol.	Assay Buffer	Concentration
Stock	1,000 µl	----	1,000 ng/ml
S1	10 µl of stock	990 µl	10 ng/ml
S2	200 µl of S1	800 µl	2.0 ng/ml
S3	200 µl of S2	800 µl	0.4 ng/ml
S4	200 µl of S3	800 µl	0.08 ng/ml
S5	200 µl of S4	800 µl	0.016 ng/ml
S0	----	1000 µl	0 ng/ml

Table 2: Ten Point Standard Curve

Std. No.	Std. Vol.	Assay Buffer	Concentration
Stock	1,000 µl	----	1,000 ng/ml
S1	10 µl of stock	990 µl	10 ng/ml
S2	200 µl of S1	800 µl	2.0 ng/ml
S3	500 µl of S2	500 µl	1.0 ng/ml
S4	500 µl of S3	500 µl	0.5 ng/ml
S5	500 µl of S4	500 µl	0.25 ng/ml
S6	500 µl of S5	500 µl	0.13 ng/ml
S7	500 µl of S6	500 µl	0.063 ng/ml
S8	500 µl of S7	500 µl	0.031 ng/ml
S9	500 µl of S8	500 µl	0.016 ng/ml
S0	----	1000 µl	0 ng/ml

B) Dilution of Standard (Standard Curve Range 0-25 ng/ml):

Set up 6 (Table 3) or 10 (Table 4) 12 x 75 mm test tubes depending on the desired standard curve resolution. Label tubes as shown in the appropriate table. To each tube add the indicated respective volume of assay buffer. To tube #S1 add 25 µl of stock (1,000 ng/ml) peptide solution as indicated in column 2 of the tables. Mix vigorously (vortex). Transfer 200 µl from #S1 to #S2 tube (use a fresh pipette tip to reduce a carryover). Mix vigorously (vortex thoroughly). Prepare the subsequent serial dilutions as indicated in the appropriate table. (*Change pipette tips after each addition to reduce cross contamination.*)

Table 3: Six Point Standard Curve

Std. No.	Std. Vol.	Assay Buffer	Concentration
Stock	1,000 µl	----	1,000 ng/ml
S1	25 µl of stock	975 µl	25 ng/ml
S2	200 µl of S1	800 µl	5.0 ng/ml
S3	200 µl of S2	800 µl	1.0 ng/ml
S4	200 µl of S3	800 µl	0.2 ng/ml
S5	200 µl of S4	800 µl	0.04 ng/ml
S0	----	1000 µl	0 ng/ml

Table 4: Ten Point Standard Curve

Std. No.	Std. Vol.	Assay Buffer	Concentration
Stock	1,000 µl	----	1,000 ng/ml
S1	25 µl of stock	975 µl	25 ng/ml
S2	200 µl of S1	800 µl	5.0 ng/ml
S3	500 µl of S2	500 µl	2.5 ng/ml
S4	500 µl of S3	500 µl	1.25 ng/ml
S5	500 µl of S4	500 µl	0.63 ng/ml
S6	500 µl of S5	500 µl	0.31 ng/ml
S7	500 µl of S6	500 µl	0.16 ng/ml
S8	500 µl of S7	500 µl	0.08 ng/ml
S9	500 µl of S8	500 µl	0.04 ng/ml
S0	----	1000 µl	0 ng/ml

Step 3: Initiation of Assay

A) Remove acetate plate sealer (APS) from the immunoplate and save it for reuse.

B) Leave 2 wells open for the blank OD reading.

Note: Only 100 µl of TMB solution and 100 µl of HCl should be added to the blank wells (at steps 4 I and 4 K, pages 7 and 8, respectively).

C) Dispense 50 µl of each of the prepared standards (from lowest to highest concentration) into designated wells. Please refer to the example plate in Appendix 2, page 12 (*Change tips after each addition to reduce cross contamination.*)

D) Dispense 50 µl of the unknown samples into the designated wells.

E) Dispense 25 µl of primary antiserum (from step 1 C on page 4) into each well.

F) Dispense 25 µl of biotinylated peptide solution (from step 1 D on page 4) into each well.

G) Gently agitate.

H) Reseal the immunoplate with APS.

I) Incubate for 2 hours at room temperature.

Step 4: Enzymatic Detection

A) Due to the possibility of the contents of the SA-HRP vial spreading throughout the tube during transport, tap or centrifuge the SA-HRP vial for two minutes and mix gently (vortex). Transfer 12 ml of assay buffer to a 15 ml tube. Add 6 µl of SA-HRP to the 12 ml of assay buffer and mix vigorously (vortex). SA-HRP can be stored at 4 °C and should be protected from a prolonged exposure to light.

B) Remove APS from plate and save for re-use.

C) Discard the contents of the wells prior to first wash.

D) Wash the plate 5 times with assay buffer and blot dry (see Endnotes, page 15).

E) Dispense 100 µl of the diluted streptavidin-HRP solution into each well.

F) Reseal the immunoplate with APS.

G) Incubate for 60 minutes at room temperature.

H) Wash immunoplate 5 times with the assay buffer as described above and blot dry.

I) Dispense 100 µl of the TMB solution into each well, including the “blank” well. TMB can be stored at 4 °C and should be protected from a prolonged exposure to light.

J) Reseal the immunoplate with APS. Incubate for 0.5-1 hour at room temperature (solution color should turn blue). Do not exceed the OD range of your individual OD reader.

Note: Due to different experimental conditions such as room temperature, washing intensity, etc., results may vary slightly from lab to lab. We suggest that users check the color every 5-10 minutes. If the color reaches the desired extent, or turns dark blue too quickly, one should go to the next step (4K) immediately to terminate the reaction. This will prevent the reading from going beyond the limitations of the customer's OD reader. **Air bubbles should also be avoided, as they will give false readings**.

K) Dispense 100 µl of 2 N HCl into each well including the "blank" well to stop the reaction (solution color should turn yellow) and **go to next step within 10 minutes**.

Step 5: Reading the Immunoplate

A) Wipe the bottom of the immunoplate clean with 70% ethanol.
B) Remove the APS and load the immunoplate onto the microtiter plate reader
C) Read the absorbance at 450 nm. Use 100 µl of TMB solution plus 100 µl of 2 N HCl as a blank control.

Note: When using software packages that automatically subtract the blanks from all other readings, use the wells marked in Appendix 2 to measure background (A1, A2).

Calculations of Results

A) Manual Plotting: Plot the standard curve on semi-log graph paper. The known concentrations of peptide are plotted on a log scale of the X-axis and the corresponding OD (optical density) on a linear scale of the Y-axis. The standard curve should have a sigmoidal shape that shows an inverse relationship between the peptide concentration and the corresponding absorbance. The concentration of peptide in the unknown sample is determined by locating the sample's OD on the Y-axis, drawing a horizontal line to intersect with the standard curve, and dropping a vertical line from the point of intersection between the standard curve and the horizontal line. This vertical line will intersect the X-axis at the coordinate corresponding to the concentration of peptide in the unknown sample.

B) Computer plotting: An appropriate computer software program can be used to plot and calculate the results. The plotted data should form the sigmoidal curve. A standard curve must be generated at the time of assay for calculations of sample value.

Notes and Warnings

A) Handling of pipettes: Since incubation volumes are small, careful pipetting is crucial to the success of the assay. Poor technique may cause droplets to stick to the upper wall of the wells and spray (aerosols) may contaminate adjacent wells causing variation in results.
B) Handling the immunoplate: Do not touch the bottom of the plate. This leaves grease, dirt, etc. that will adversely affect the optical absorbance reading.
C) Water purity: The performance of the kit is sensitive to contaminants in water. If possible, use sterile deionized water or USP pure water (18 MOhm). When drawing water from a water purification system, we recommend purging the system before using the water to eliminate any contaminants, which may have built up in the water lines.
D) Storage: Store the kit in a cool (2-4 °C) dry area upon receipt. If the kit will be stored for more than one month prior to use, transfer primary antiserum, standard peptide and biotinylated peptide to –20 °C. The lyophilized components will be stable for at least four months when stored properly. Since all incubations and procedures are performed at room temperature, the reagents, immunoplate, and samples should be equilibrated to room temperature before use. Do not open reagents and immunoplate while they are cold. If you wish to save a partially used kit and reuse it later, it is best to store all the components at –20 °C, with the exception of the plate (**4 °C and dry location**), SA-HRP (4 °C), TMB (4 °C), buffer (4 °C), and HCl (4 °C). Although we have had success with this method of storage, we cannot guarantee the performance of our kits on a second or subsequent use. Avoid numerous freezing and thawing by aliquoting reagents.
E) Shelf life: Four months after shipment date.

Summary of Assay Protocol

1. Add 50 µl/well of standard/sample, 25 µl of primary antibody and 25 µl of biotinylated peptide (incubate at room temperature for 2 hours).
2. Wash immunoplate 5 times with 300 µl/well of assay buffer.
3. Add 100 µl/well of streptavidin-HRP (incubate at room temperature for one hour).
4. Wash immunoplate 5 times with 300 µl/well of assay buffer.
5. Add 100 µl/well of TMB solution (incubate at room temperature for 0.5-1 hour).
6. Terminate with 100 µl/well of 2 N HCl.
7. Read absorbance at 450 nm within ten minutes and calculate results.

Total assay time is 4.5 hours.

Note: This procedure is not intended to replace the preparation steps, precautions and procedures shown in this booklet. Please consult all those sections for complete directions.

Appendix 1:
Sample Preparation and Extraction

Measurement of Peptides in Biological Fluids

This kit is designed to quantify peptides in the enzyme immunoassay buffer. It may not give accurate results when the peptides are suspended in a different assay matrix such as plasma, serum, or urine. This problem is traditionally addressed by extracting the peptide from the biological fluid.

A good extraction procedure serves two purposes:

1. It separates the peptide from potentially interfering substances.
2. It allows the investigator to concentrate the sample.

Unfortunately, as with any purification technique, recovery of the analyte is never 100%. Therefore, it is recommended that the extraction procedure is optimized on an individual basis. Analyte recovery can best be determined by evaluating the extraction of control samples paired with the same samples spiked with known quantities of the peptide of interest. The difference in the measured quantity of peptide between spiked and non-spiked samples will allow the user to determine the recovery percentage of an extraction procedure.
In the following pages, we describe a generic plasma extraction procedure to help researchers get started. If a better extraction method is found in the literature, we encourage the researcher to use it.

C18 Sep-Column Extraction Method

Blood Withdrawal and Preparation of Plasma:

Collect blood samples (2.0 ml to 6.0 ml) using a chilled syringe and transfer into a polypropylene tube containing EDTA (1mg/ml of blood) and aprotinin (500 KIU/ml of blood) at 0 °C. Centrifuge blood at 1,600 g for 15 minutes at 0 °C. Transfer the plasma to a fresh polypropylene tube. For maximum stability, store unused portion at –70 °C.

Required Chromatography Solvents:

1. Buffer A (Y-1040): 1% trifluoroacetic acid (TFA, HPLC Grade) in 99% distilled water.
2. Buffer B (Y-1045): 60% acetonitrile (HPLC Grade), 1% TFA in 39% distilled water.

Extraction Procedure:

1. Add an equal volume of buffer A to the plasma sample. Clarify by centrifugation at 6,000 to 17,000 g for 20 minutes at 4 °C. Save the supernatant and discard any pellets.
2. Equilibrate a Sep-column containing 200 mg of C18 (Cat. No. Y-1000) by washing with 100% acetonitrile (1 ml, once) followed by buffer A (3 ml, 3 times).
3. Load the clarified and acidified biological fluid onto the pre-treated C18 Sep-column. (Do not apply pressure to the column).

Note: For steps 4 and 5, a light vacuum (10 sec/drop) may be applied to the column.

4. Slowly wash the column with buffer A (3 ml, 3 times) and discard the wash.
5. Elute the peptide slowly with buffer B (3 ml, once) and collect eluate in a polypropylene tube.
6. Evaporate eluate to dryness in a centrifugal concentrator or by a suitable substitute method.
7. Store the dried extract at –20 °C and assay as soon as possible. The reconstituting volume is determined by the expected value of peptide in the sample. If the subsequently established peptide concentration does not fall within the usable linear range of the kit, dilute or concentrate the samples accordingly.
8. For low concentration levels of analyte, reconstitute in 200 µl of assay buffer. If subsequently measured duplicate 50 µl samples reveal the analyte concentration to be above the useable range, the remaining 100 µl of sample can be further diluted in assay buffer and retested accordingly.

Appendix 2:
Schematic Maps for the 96 Well Plate

Suggested Plate Layout for Six Point Standard Curve

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	UK2	UK2	UK10	UK10	UK18	UK18	UK26	UK26	UK34	UK34
B	S1	S1	UK3	UK3	UK11	UK11	UK19	UK19	UK27	UK27	UK35	UK35
C	S2	S2	UK4	UK4	UK12	UK12	UK20	UK20	UK28	UK28	UK36	UK36
D	S3	S3	UK5	UK5	UK13	UK13	UK21	UK21	UK29	UK29	UK37	UK37
E	S4	S4	UK6	UK6	UK14	UK14	UK22	UK22	UK30	UK30	UK38	UK38
F	S5	S5	UK7	UK7	UK15	UK15	UK23	UK23	UK31	UK31	UK39	UK39
G	S0	S0	UK8	UK8	UK16	UK16	UK24	UK24	UK32	UK32	UK40	UK40
H	UK1	UK1	UK9	UK9	UK17	UK17	UK25	UK25	UK33	UK33	UK41	UK41

Suggested Plate Layout for Ten Point Standard Curve

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	S8	S8	UK6	UK6	UK14	UK14	UK22	UK22	UK30	UK30
B	S1	S1	S9	S9	UK7	UK7	UK15	UK15	UK23	UK23	UK31	UK31
C	S2	S2	S10	S10	UK8	UK8	UK16	UK16	UK24	UK24	UK32	UK32
D	S3	S3	UK1	UK1	UK9	UK9	UK17	UK17	UK25	UK25	UK33	UK33
E	S4	S4	UK2	UK2	UK10	UK10	UK18	UK18	UK26	UK26	UK34	UK34
F	S5	S5	UK3	UK3	UK11	UK11	UK19	UK19	UK27	UK27	UK35	UK35
G	S6	S6	UK4	UK4	UK12	UK12	UK20	UK20	UK28	UK28	UK36	UK36
H	S7	S7	UK5	UK5	UK13	UK13	UK21	UK21	UK29	UK29	UK37	UK37

Where:
S = serial dilution
UK = your unknown samples

Safety Information

The physical and chemical properties of the reagents contained in this kit have been tested individually.

It is strongly recommended that laboratory employees wear lab coats, gloves, and safety glasses when handling the reagents provided with this kit. Reagents do not contain ingredients which have been determined to be health hazards and which comprise greater than 1% of the mixture or which could be released from the mixture in concentrations that would exceed OSHA permissible exposure limits.

Hazardous Ingredients

Standards are supplied in lyophilized form. The lyophilized antiserum contains Tween 20 and thimerosal. The assay buffer concentrate contains Tris and thimerosal. The buffer is in liquid form.

Physical and Chemical Data

Components are stable in closed containers under normal temperatures and pressures. No hazardous polymerization is known.

Fire and Explosion Data

Components are non-combustible with negligible fire hazard when exposed to heat or flame. Fire fighting media should be appropriate to burning material.

Health Hazards

Components may be harmful by inhalation, ingestion, or skin absorption and may cause skin irritation or eye irritation. In case of eye contact, flush eye with water and contact a physician. In case of skin contact, wash skin with soap and water.

Reactivity Data

Components are stable in closed containers under normal temperatures and pressures.

Spill and Disposal Procedures

For spills, ventilate area and wash spill site. For disposal, please dispose in accordance with local regulation.

Handling and Storage Information

Safety glasses, gloves, and a full-length lab coat should be worn to prevent unnecessary contact.

The above information is believed to be correct but does not purport to be all-inclusive and shall be used only as a guide. It is the user's responsibility to determine the suitability of this information for the adoption of safety precautions as may be necessary. Peninsula Laboratories, Inc. shall not be held liable for any damage resulting from the handling or use of the above product.

References

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3. E. Bucht et al.
   A rapid extraction method for serum calcitonin.
   Clin. Chim. Acta, **195**, 115-124 (1991)

Ordering Information

For additional kits or our most current catalog products, please visit our web site at www.bachem.com or call us at:

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Technical Support

If you need technical information or assistance with assay procedures, please call our Technical Service Department at (800) 922 1516 or (650) 592-5392. Our staff will be happy to answer your questions about this or any of our other products.

Guarantee and Limitation of Remedy

The Peninsula Laboratories, Inc. makes no guarantee of any kind, expressed or implied, which extends beyond the description of the material in this kit, except that these materials and this kit will meet our specifications at the time of delivery. The customer's remedy and Peninsula Laboratories, Inc.'s sole liability hereunder are limited to either refund of the purchase price or the replacement of material(s) that does not meet our specifications. By the acceptance of our products, the customer indemnifies and holds Peninsula Laboratories, Inc. harmless against, and assumes all liability for, the consequences of its use or misuse by the customer, its employees or others.

Endnotes

Manual vs. Automatic Washing

Manual Washing

1. Dispense 300 µl of assay buffer into each well with a multichannel or repeating pipette. Avoid splashing which could cross-contaminate the neighboring wells. Gently shake by hand for five seconds.
2. With a rapid flicking motion of the wrist, discard the contents of the plate into the sink. "Flicking" the plate means inverting the plate using a rapid wrist motion to "flick" the contents of the plate into the sink. Do not let go of the plate!
3. Gently blot dry the top of plate on paper towels or an absorbent pad.
4. Repeat washing, "flicking" and blotting.

Automatic Plate Washer

Prepare sufficient assay buffer for washing. At the end of the wash cycle, "flick" the plate twice and blot dry the top of the plate as in manual washing.

In both cases, "flicking" and blotting should be done quickly, and the next solution should be dispensed into the wells as soon as possible.