User Guide

Inhibin-B EIA Kit

RAB0325

Storage Temperature: –20 °C

Introduction

The Inhibin B Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Inhibin B peptide based on the competitive ELISA principle.

In this assay, a biotinylated Inhibin B peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated Inhibin B peptide competes with endogenous (unlabeled) Inhibin B for binding to the anti-Inhibin B antibody. After a wash step, any bound biotinylated Inhibin B then interacts with horseradish peroxidase (HRP)-streptavidin, which catalyzes a color development reaction. The intensity of the colorimetric signal is directly proportional to the amount of captured biotinylated Inhibin B peptide and inversely proportional to the amount of endogenous Inhibin B in the standard or samples. A standard curve of known concentration of Inhibin B peptide can be established and the concentration of Inhibin B peptide in the samples can be calculated accordingly.

Storage

The entire kit may be stored at -20°C to -80°C for up to 6 months from the date of shipment. For extended storage, it is recommended to store at -80°C. Avoid repeated freeze-thaw cycles. For prepared reagent storage, see the 'Reagents' table on the next page.

Components

- Microplate: 96 wells (12 strips x 8 wells) coated with secondary antibody. Store at 4 °C for up to a month after opening. (Return unused wells to the pouch containing desiccant pack, reseal along entire edge).
- Wash Buffer: 25 mL of 20X concentrated solution. Store at 4 °C for up to a month after opening.
- Standard Inhibin B Peptide: 2 vials of Inhibin B Peptide. 1 vial is enough to run each standard in duplicate. The first standard can be stored at 4 °C for 2-3 days. Do not store and reuse additional dilutions.
- Anti-Inhibin B Polyclonal Antibody: 2 vials of anti-Inhibin B. Store at 4 °C for up to a month after opening.
- Assay Diluent A: 30 mL. contains 0.09% sodium azide as preservative. Diluent for standards and serum or plasma.
- Assay Diluent B: 15 ml of 5X concentrated buffer. Diluent for standards, cell culture media or other sample types, and HRP-Streptavidin. Store at 4 °C for up to a month after opening.
- Biotinylated Inhibin B Peptide: 2 vials of Biotinylated Inhibin B Peptide, 1 vial is enough to assay the whole plate. Store for 2-3 days at 4 °C.
- HRP-Streptavidin Concentrate: 600 µL of 200X concentrated HRP-conjugated streptavidin. Do not store and reuse.
- Positive Control: 1 vial of Positive Control. Store for 2-3 days at 4 °C.
- TMB One-Step Substrate Reagent: 12 mL of 3,3’,5,5’-tetramethylbenzidine (TMB) in buffer solution.
- Stop Solution: 8 mL of 0.2 M sulfuric acid.

Additional Materials Required (Not Provided)

- Microplate reader capable of measuring absorbance at 450 nm
- Precision pipettes to deliver 2 µL to 1 mL volumes
- Adjustable 1-25 mL pipettes for reagent preparation
- 100 mL and 1-liter graduated cylinders
- Absorbent paper
- Distilled or deionized water
- SigmaPlot® software (or other software which can perform four-parameter logistic regression models)
- Tubes to prepare standard or sample dilutions
- Orbital shaker
- Aluminum foil
- Plastic wrap

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the safety data sheet for information regarding hazards and safe handling practices.

Reagent Preparation

Keep kit reagents on ice during reagent preparation steps.

Note: Assay Diluent A should be used for dilution of samples, biotinylated peptide, and standard peptide when testing plasma or serum samples. 1X Assay Diluent B should be used for dilution of samples, biotinylated peptide, and standard peptide when testing cell culture media or other sample types.

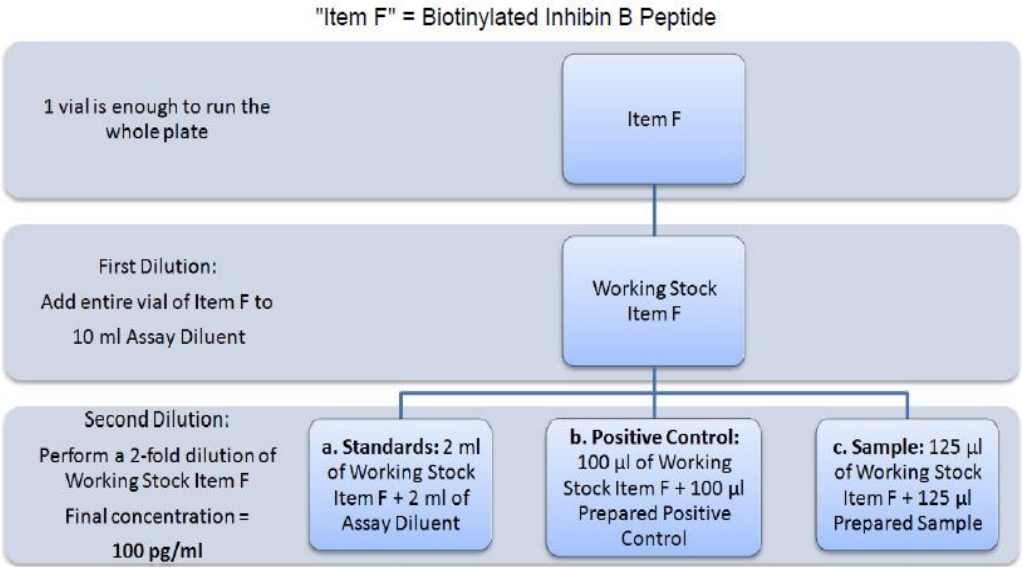
A. Preparation of Plate and Anti-Inhibin B Antibody

1. Equilibrate plate to room temperature before opening the sealed pouch.
2. Label removable 8-well strips as appropriate for your experiment.
3. 5X Assay Diluent B should be diluted 5-fold with deionized or distilled water.
4. Briefly centrifuge the anti-Inhibin B antibody vial. Then add 50 µL of 1X Assay Diluent B to the vial to prepare the antibody concentrate. Pipette up and down to mix gently
5. The antibody concentrate should then be diluted 100-fold with 1X Assay Diluent B. This is your anti-Inhibin B antibody working solution, which will be used in step 2 of Assay Procedure.

Note: The following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure).

B. Preparation of Biotinylated Inhibin B

6. Briefly centrifuge the vial of Biotinylated Inhibin B before use.
7. See the image below for proper preparation of Biotinylated Inhibin B Peptide. Transfer the entire contents of the biotinylated peptide vial into a tube containing 10 mL of the appropriate Assay Diluent. This is your Working Stock. Pipette up and down to mix gently. The final concentration of biotinylated Inhibin B will be 200 pg/mL.
    a. Second Dilution of Biotinylated Inhibin B Peptide for Standards: Add 2 mL of Working Stock Biotinylated Inhibin B Peptide to 2 mL of the appropriate Assay Diluent. The final concentration of biotinylated Inhibin B will be 100 pg/mL.
    b. Second Dilution of Biotinylated Inhibin B Peptide for Positive Control: Add 100 µL of Working Stock Biotinylated Inhibin B Peptide to 100 µL of the prepared Positive Control (See section D for Positive Control preparation) The final concentration of biotinylated Inhibin B will be 100 pg/mL.
    c. Second Dilution of Biotinylated Inhibin B Peptide for samples: Add 125 µL of Working Stock Biotinylated Inhibin B Peptide to 125 µL of prepared sample (see section E for sample preparation). This is a 2-fold dilution of your sample. The final concentration of biotinylated Inhibin B will be 100 pg/mL.

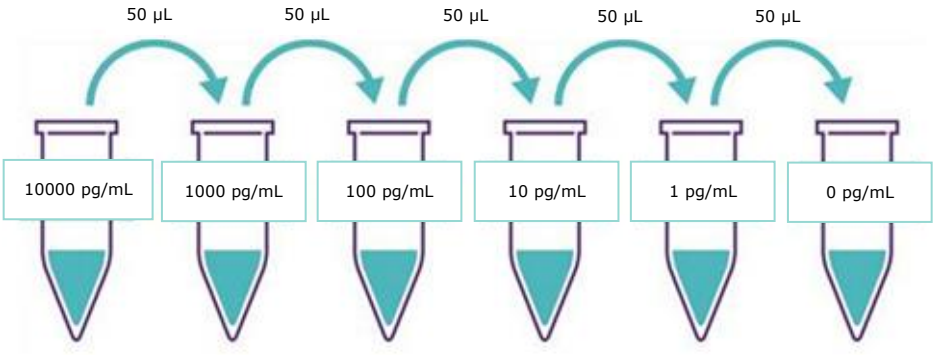


C. Preparation of Standards

8. Label 6 microtubes with the following concentrations: 10,000 pg/mL, 1,000 pg/mL, 100 pg/mL, 10 pg/mL, 1 pg/mL and 0 pg/mL. Pipette 450 µL of biotinylated Inhibin B peptide working solution (prepared in step 7a) into each tube, except the 10,000 pg/mL (leave this one empty).

It is very important to make sure the concentration of the biotinylated Inhibin B is 10 pg/mL in all standards.

9. Briefly centrifuge the vial of Inhibin B Standard. Pipette 8 µL of Standard Peptide and 792 µL of 100 pg/mL biotinylated Inhibin B working solution (prepared in step 7a) into the tube labeled 10,000 pg/mL. Mix thoroughly. This solution serves as the first standard (10,000 pg/mL Inhibin B standard, 10 pg/mL biotinylated Inhibin B).
10. To make the 1000 pg/mL standard, pipette 50 µL of the 10,000 pg/mL Inhibin B standard into the tube labeled 1,000 pg/mL. Mix thoroughly.
11. Repeat this step with each successive concentration, preparing a dilution series as shown in the illustration below. Each time, use 450 µL of biotinylated Inhibin B and 50 µL of the prior concentration until the 1 pg/mL is reached. Mix each tube thoroughly before the next transfer.



D. Positive Control Preparation

12. Briefly centrifuge the Positive Control vial.
13. Refer to step 7b. This is a 2-fold dilution of the Positive Control. The final concentration of biotinylated Inhibin B should still be 100 pg/mL.

The Positive Control is a cell culture media sample that serves as a system control to verify that the kit components are working. The resulting OD will not be used in any calculations; if no positive competition is observed please contact our Technical Support. The Positive Control may be diluted further if desired, but be sure the final concentration of biotinylated Inhibin B is 100 pg/mL.

E. Sample Preparation

14. If you wish to perform a 2-fold dilution of your sample, proceed to step 7c. If you wish to perform a higher dilution of your sample, dilute your sample with the appropriate Assay Diluent before performing step 7c.

    EXAMPLE (to make a 4-fold dilution of sample):

    a. Dilute sample 2-fold (62.5 µL of sample + 62.5 µL of the appropriate Assay Diluent).
    b. Perform step 7c (125 µL of working solution Biotinylated Inhibin B Peptide + 125 µL of sample prepared above).

The total volume is 250 µL, enough for duplicate wells on the microplate. It is very important to make sure the final concentration of the biotinylated Inhibin B is 100 pg/mL.

Note: Optimal sample dilution factors should be determined empirically, however you may reference below for recommended dilution factors for serum: Human = 2x Mouse = 2x Rat = 2x. If you have any questions regarding the recommend ended dilutions, please contact technical support.

F. Preparation of Wash Buffer and HRP

15. If Wash Buffer contains visible crystals, warm to room temperature and mix gently until dissolved.
16. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to yield 400 mL of 1X Wash Buffer.
17. Briefly centrifuge the HRP-Streptavidin vial before use.
18. Dilute the HRP-Streptavidin concentrate 200-fold with 1X Assay Diluent B.

    Note: do not use Assay Diluent A for HRP-Streptavidin preparation in step 18.

Assay Procedure

1. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 µL of anti-Inhibin B antibody (see Preparation, step 5) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1–2 cycles/sec) or incubate overnight at 4 °C.
3. Discard the solution and wash wells 4 times with 1X Wash Buffer (200–300 µL each). Washing may be done with a multichannel pipette or an automated plate washer. Complete removal of liquid at each step is essential to good assay performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 100 µL of each standard (see Reagent Preparation Section C), positive control (see Reagent Preparation Section D), and sample (see Reagent Preparation Section E) into appropriate wells. Be sure to include a blank well (Assay Diluent only). Cover wells and incubate for 2.5 hours at room temperature with gentle shaking (1–2 cycles/sec) or overnight at 4 °C.
5. Discard the solution and wash 4 times as directed in Step 3.
6. Add 100 µL of prepared HRP-Streptavidin solution (see Reagent Preparation step 18) to each well. Incubate with gentle shaking for 45 minutes at room temperature or overnight at 4 °C. It is recommended that incubation time should not be shorter or longer than 45 minutes.
7. Discard the solution and wash 4 times as directed in step 3.
8. Add 100 µL of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking (1–2 cycles/sec).
9. Add 50 µL of Stop Solution to each well. Read absorbances at 450 nm immediately.

Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed.
2. Add 100 µL anti-Inhibin B to each well. Incubate 1.5 hours at room temperature or overnight at 4°C.
3. Add 100 µL standard or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.
4. Add 100 µL prepared Streptavidin solution. Incubate 45 minutes at room temperature.
5. Add 100 µL TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.
6. Add 50 µL Stop Solution to each well. Read at 450 nm immediately.

Calculation of Results

Calculate the mean absorbance for each set of duplicate standards, controls, and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot® software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit curve through the standard points.

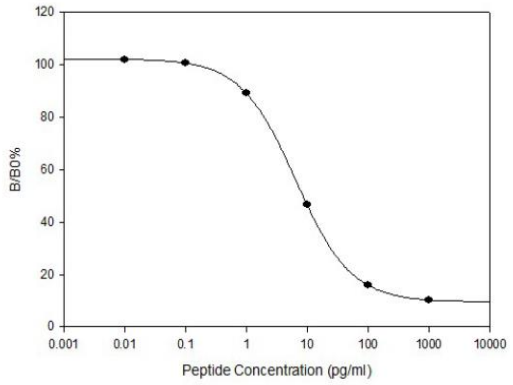
Percentage absorbance = (B – blank OD)/(B_o – blank OD)

Where, B = OD of sample or standard

B_o = OD of zero standard (total binding)

Typical Data

Standard curves are for demonstration only. Standard curves must be run with each assay.



Sensitivity

The minimum detectable concentration of Inhibin B is 2 pg/mL.

Standard Curve Range

1-10,000 pg/mL

Reproducibility

- Intra-Assay: CV < 10%
- Inter-Assay: CV < 15%

Specificity

- This kit targets the beta B subunit, and therefore theoretically detects Activin B and Activin AB in addition to Inhibin B.
- Cross Reactivity = This EIA kit shows no cross-reactivity with any of the cytokines tested: Ghrelin, Nesafatin, Angiotensin II, NPY and APC.

Assay Diagram

Blank	Blank	SA1	SA1	SA9	SA9	SA17	SA17	SA25	SA25	SA33	SA33
Total Binding	Total Binding	SA2	SA2	SA10	SA10	SA18	SA18	SA25	SA25	SA34	SA34
Standard 1	Standard 1	SA3	SA3	SA11	SA11	SA19	SA19	SA26	SA26	SA35	SA35
Standard 2	Standard 2	SA4	SA4	SA12	SA12	SA20	SA20	SA27	SA27	SA36	SA36
Standard 3	Standard 3	SA5	SA5	SA13	SA13	SA21	SA21	SA28	SA28	SA37	SA37
Standard 4	Standard 4	SA6	SA6	SA14	SA14	SA22	SA22	SA29	SA29	SA38	SA38
Standard 5	Standard 5	SA7	SA7	SA15	SA15	SA23	SA23	SA30	SA30	SA38	SA38
Pos Control	Pos Control	SA8	SA8	SA16	SA16	SA24	SA24	SA31	SA31	SA40	SA40

Key:

Blank = Buffer Only

Total Binding = Biotin-Inhibin B Only

Standard 1 = 10000 pg/mL

Standard 2 = 1000 pg/mL

Standard 3 = 100 pg/mL

Standard 4 = 10 pg/mL

Standard 5 = 1 pg/mL

Pos Control = Biotin with Positive Control

Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes.
	Improper standard dilution	Ensure a brief spin of Item C and dissolve the powder thoroughly with gentle mixing.
Low signal	Too brief incubation times	Ensure sufficient incubation time; Procedure, step 2 may change to overnight.
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation.
Large CV	Inaccurate pipetting	Check pipettes.
High background	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer.
Low sensitivity	Improper storage of the ELISA kit	Store the standard at ≤ –20 °C after reconstitution, others at 4 °C. Keep substrate solution protected from light.
	Stop solution	Stop solution should be added to each well before measurement.

Notice

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