Protocol for FNDC5, Soluble (Human, Rat, Mouse) ELISA Kit

(Catalog# EK-067-53, Lot 603999) (range: 0.625-320 ng/ml)
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**CAUTION:**

Investigational device. Limited by law to investigational use.
For research use only. Not for use in diagnostic procedures.
KIT CONTENTS

1. 96 well immunoplate (1 plate)........................................... EK-PLATE-067-53
2. 1x assay buffer (60ml).......................................................... EK-BUF-A
3. Soluble FNDC5 standard protein (1 vial).............................. EK-S-067-53
4. 10x soluble FNDC5 detection antibody (1 vial)..................... EK-B-067-53
5. Soluble FNDC5 positive control (1 vial).............................. EK-PC-067-53
6. 200x streptavidin-horseradish peroxidase
   (SA-HRP) (60μl)................................................................ EK-SA-HRP-A
7. HRP diluent buffer (12ml) ..................................................... EK-HRP-DB
8. 10x wash buffer concentrate (50ml).................................... EK-WB
9. Substrate solution (TMB) (11ml)........................................ EK-TMB-A
10. Stop solution (0.5M HCl) (11ml)....................................... EK-HCL-A
11. Acetate plate sealer (APS) (3 pieces)................................. EK-APS

ADDITIONAL MATERIALS, NOT INCLUDED

1. Microtiter plate reader (450nm) (required)
2. Micropipette with disposable pipette tips (required)
3. Absorbent material for blotting (required)
4. Vortex (required)
5. Curve-fitting software capable of
   4 parameter logistics (recommended)
6. Orbital plate shaker (300-400rpm) (recommended)
7. Microtiter plate washer (recommended)
8. Multi-channel pipette (50-100μl) (recommended)
9. Solution reservoir (recommended)
10. Centrifuge (optional)
11. EDTA Lavender Vacutainer blood
    collection tubes (optional).................................................. VT-6450
12. C18 SEP-COLUMN (optional)............................................ RK-SEPCOL-1
13. Aprotinin (30 TIU) (optional)............................................. RK-APRO
14. Buffer A (optional)............................................................. RK-BA-1
15. Buffer B (optional)............................................................. RK-BB-1
STORAGE

1. Store the kit at 4°C upon receipt. Do not freeze.

2. It is highly recommended that solutions be used as soon as possible after reconstitution.

3. If desired, remove any unneeded strips from the antibody-coated plate, reseal them in the zip-lock foil with a dessicant, and keep at 4°C. Do not allow moisture to accumulate on the wells.

4. If necessary, store any reconstituted solutions of standard peptide, positive control, or detection antibody (biotinylated) at -70°C. All other reagents should be stored at 4°C.

REFERENCES

INTRODUCTION

This kit is designed to measure the concentration of soluble FNDC5 (human, rat, mouse) and its related peptides based on the principle of a “sandwich” enzyme immunoassay.

GENERAL DESIGN OF THIS KIT

The immunoplate in this kit is pre-coated with an anti-soluble FNDC5 capture antibody whose non-specific binding sites are blocked. The soluble FNDC5 protein in either the samples or in the standard solutions binds to the immobilized capture antibody in the wells. After washing away any unbound protein, biotinylated anti-soluble FNDC5 detection antibody is then added, which in turn binds to the FNDC5 trapped in the wells. After a second wash, streptavidin-horesradish peroxidase (SA-HRP) is added, which interacts with the biotin conjugate to catalize the substrate solution (TMB) to yield a blue-colored solution. The enzyme-substrate reaction is stopped with the addition of a stop solution (hydrochloric acid, HCl). The intensity of the color measured is directly proportional to the amount of soluble FNDC5 bound in the initial step. A standard curve of known concentration can be established by plotting the measured O.D. as a function of the various known standard peptide concentrations. The unknown peptide concentration in a sample can be determined via extrapolation based on this standard curve.

Phoenix Pharmaceutical’s soluble FNDC5 (Human, Rat, Mouse) ELISA kit is designed to measure the concentration of soluble FNDC5 from serum/plasma.
SUMMARY OF ASSAY PROTOCOL

Add 100μl/well of standard, sample, or positive control

Incubate at room temperature (20-23°C) for 2 hours

Wash immunoplate 4 times with 350μl/well of 1x wash buffer

Add 100μl/well of biotinylated detection antibody

Incubate at room temperature (20-23°C) for 2 hours

Wash immunoplate 4 times with 350μl/well of 1x wash buffer

Add 100μl/well of SA-HRP solution

Incubate at room temperature (20-23°C) for 60 minutes

Wash immunoplate 4 times with 350μl/well of 1x wash buffer

Add 100μl/well of TMB substrate solution

Incubate at room temperature (20-23°C) for 8-12 minutes

Terminate reaction with 100μl/well of 0.5M HCl

Read absorbance O.D. at 450nm and calculate results
Assay Principle

Soluble FNDC5 (Human, Rat, Mouse) Standards or Samples

1. Anti-soluble FNDC5 capture antibody
2. Biotinylated anti-soluble FNDC5 detection antibody
3. Streptadivin-HRP
4. Color Development
5. Substrate (TMB)
Note: Read this protocol in its entirety before starting the assay. Each kit contains sufficient reagents for 96 wells and is capable of assaying 40 duplicate samples.

ASSAY PROTOCOL

Note: The kit and all its components should be equilibrated to room temperature (20-23°C) before opening any vials and starting the assay. Before opening any Eppendorf tubes for reconstitution, briefly centrifuge at ~3000 rpm for 5 seconds to ensure that all the lyophilized material is at the bottom of the tube. It is highly advised that all solutions be used as soon as possible after rehydration.

1. Dilute the 10x wash buffer concentrate with 450ml of distilled water. Store at 4°C. This will be the 1x wash buffer solution used to wash the plate after incubations.

   Note: If crystals appear in the 10x wash buffer, the bottle can be placed in a warm water bath for approximately 30 minutes or until no crystals are visible.

2. Reconstitute the soluble FNDC5 standard protein in 1000μl of 1x assay buffer and vortex thoroughly. Allow the solution to sit at least 15 minutes with gentle agitation at room temperature (20-23°C) to completely dissolve in solution. This will be the standard stock solution.

   Note: Vortex immediately before use.

3. Reconstitute the soluble FNDC5 detection antibody in 1200μl of 1x assay buffer to produce a 10x antibody concentrate and vortex thoroughly. Dilute the antibody further in 10.8ml of 1x assay buffer for a working solution of detection antibody and again, vortex thoroughly.

4. Reconstitute the soluble FNDC5 positive control in 1000μl of 1x assay buffer and vortex thoroughly. Allow the solution to sit for at least 15 minutes at room temperature to completely dissolve.

   Note: Vortex immediately before use. Once reconstituted, the positive control should be used within a few days if stored at -20°C to -70°C.
5. Prepare the standard dilutions from the rehydrated soluble FNDC5 standard protein as shown in Figure 2 and Figure 4 above. Vortex the tube thoroughly after each serial dilution.
Note: For recommended loading positions on the immunoplate, please refer to Figure 3 on the previous page.

6. Add 100μl of 1x assay buffer to wells A1 and A2 as blanks.

7. Add 100μl of the least concentrated peptide standard solution (#5) to wells B1 and B2. Next, add peptide standard #4 into wells C1 and C2, and so forth, continuing in the opposite order of the standard dilution. Note: Standard peptides should always be assayed in duplicate.

8. Add 100μl of rehydrated soluble FNDC5 positive control into wells H1 and H2. Note: Positive controls should always be assayed in duplicate.

9. Add 100μl of any unknown samples into their designated wells, again in duplicate. Note: Each laboratory must determine the appropriate dilution factors for their samples to ensure that peptide levels are within the linear range of the standard curve.

10. Seal the immunoplate with an acetate plate sealer (APS). Incubate the immunoplate for 2 hours at room temperature (20-23°C). Note: Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.

11. Remove the APS from the immunoplate and discard the contents of the wells. Wash each well with 300μl of 1x wash buffer, discard the buffer, invert the immunoplate, and blot the plate dry. Repeat 3 times.

12. Add 100μl of rehydrated soluble FNDC5 detection antibody into each well.

13. Re-seal the immunoplate with an acetate plate sealer (APS). Incubate the immunoplate for 2 hours at room temperature (20-23°C). Note: Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.
14. Centrifuge the 200x SA-HRP concentrate vial (3000-5000 rpm for 5 seconds) to mix. Dilute 60μl of this 200x SA-HRP concentrate in 11.94mL of the HRP Diluent buffer. Vortex the SA-HRP solution thoroughly.

   **Note:** 1x working solution of SA-HRP should be used within a few days and protected from light.

15. Remove the APS from the immunoplate and discard the contents of the wells. Wash each well with 300μl of 1x wash buffer, discard the buffer, invert the immunoplate, and blot the plate dry. Repeat 3 times.

16. Add 100μl of the 1x SA-HRP working solution into each well.

17. Reseal the immunoplate with an APS. Incubate for 60 minutes at room temperature (20-23°C). During incubation, protect the plate from light.

   **Note:** Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.

18. Remove the APS from the immunoplate and discard the contents of the wells. Wash each well with 300μl of 1x wash buffer, discard the buffer, invert the immunoplate, and blot the plate dry. Repeat 3 times.

19. Add 100μl of the substrate solution (TMB) into each well.

   **Note:** TMB is light-sensitive. After the addition of the TMB substrate solution, it is strongly recommended to cover the immunoplate to protect it from light.

20. Reseal the immunoplate with an APS. Incubate for another 6-10 minutes at room temperature (20-23°C).

   **Note:** Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.

21. Remove the APS from the immunoplate. Do **NOT** discard the contents of the wells.

22. Add 100μl of stop solution (0.5M HCl) into each well to stop the reaction. The color in the wells should change from blue to yellow. Gently tap the plate to ensure thorough mixing.

   **Note:** Proceed to the next step within 15 minutes.

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

- Reagents of different lot numbers should never be mixed.
- Plasma, serum, culture media, tissue homogenate, CSF, urine, or any biological fluid can be assayed as long as the level of peptide in the sample is high enough for the sensitivity of the specific kit.
- 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. Please consult the literature for specific methodology.
- When handling the plate, avoid touching the bottom. Any fingerprints or blots may affect the O.D. readings.
- 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.
- 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 its container a few times to wet the pipette walls prior to loading the pipette.
- Avoid submerging the whole pipette tip into reagents and samples. Droplets can accumulate at the end of the tip, causing an excess of solution to be loaded into the well and affecting assay results.
- Performing this procedure outside of the recommended room temperature (20-23°C) may affect assay results.
- Any modifications to the existing protocol (i.e. standard dilutions, pipetting technique, washing technique, incubation time or temperature, storage conditions, and kit expiration) may affect the sensitivity, specificity and results of the assay.
CALCULATION OF RESULTS

1. Label the X-axis (log scale) with the concentration of standards #5 through #1 (0.625 to 320 ng/ml).

2. Label the Y-axis (log scale) as absorbance (O.D.) at 450nm.

3. Average all duplicate readings (standards, positive control, samples) and subtract the average blank O.D. reading.

4. Plot the O.D. for each standard peptide concentration directly above its X-axis coordinate.

5. Draw the best fit curve through these data points. It should show a direct relationship between peptide concentration and absorbance. As the standard peptide concentration increases, the yellow color decreases, thereby reducing absorbance (O.D.).

   Note: We strongly recommend using curve-fitting software capable of 4 parameter logistics or log-logit functionality.

6. To determine the peptide concentration in any unknown samples, first locate its absorbance (O.D.) on the Y-axis. Draw a horizontal line across the graph from that absorbance to the intersection with the standard curve. The X-axis coordinate at this intersection point will correspond to the peptide concentration (ng/ml) in the assayed sample.

   Note: Multiply the measured peptide concentration by any dilution factor(s) used while preparing the original sample.

7. Refer to the QC Data Sheet for acceptable values of the positive controls.
Note: For cell culture supernates, please use animal serum free media. Fetal Bovine serum samples cross-reacts with this ELISA kit.

**Blood Withdrawal and Plasma Collection:**
1. Collect blood samples into Lavendar Vacutainer tubes (Catalog no. VT-6450), which contain EDTA and can hold up to 7ml of blood.
2. Gently rock the Lavendar Vacutainer tubes several times immediately after collection of blood to avoid coagulation.
3. Transfer the blood to centrifuge tubes containing aprotinin (Catalog no. RK-APRO), and gently rock several more times to inhibit the activity of proteases.
   
   Note: We recommend 0.6 TIU, or 100μl, of Aprotinin per 1ml of blood collected. If the Lavendar Vacutainer tubes are centrifuge-safe, the aprotinin may be added directly to them.
4. Centrifuge the blood at 1600g for 15 minutes at 4°C and collect the plasma.
   
   Note: Plasma kept at -70°C will remain stable for up to one month.

**General Tissue Preparation:**
1. Boil tissue in 75% HoAc (Acetic Acid) for 20 minutes at 100°C.
2. Homogenize tissue in lysis buffer, usually with a low pH.
3. Centrifuge the tissue homogenate at 12,000rpm for 20 to 30 minutes at 4°C.