Soluble TWEAK (Human)

ELISA KIT PROTOCOL

(Catalog No.: EK-054-85)
(range: 62.5 - 4000 pg/ml)

PHOENIX PHARMACEUTICALS, INC.

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INTRODUCTION AND PROTOCOL OVERVIEW

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK, also named TNFSF12) is a multiple functional cytokines playing a key role in tissue regenernation and remodeling. It is expressed as a 249 amino acid type II transmembrane protein. Upon specific proteolysis by the serine protease furin, an active soluble 18 kD fragment (sTWEAK) is released into the interstitium. In contrast to other TNF superfamily members, TWEAK is a widely expressed cytokine in many different tissue and tumor specimens. To date, the widely expressed tansmembrane protein, Fn14 and a scavenger receptor, CD163 were discovered as receptors of sTWEAK. The involvement of the TWEAK-Fn14 axis in beneficial as well as hazardous processes, make both ligand and receptor potential targets for novel therapeutics. The TNF Receptor-Associated Factor 1 also has been proposed as the major target of sTWEAK. Therefore, the level of sTWEAK has been investigated as a potential biomarker for diseases such as diabetes[1], obesity[2,3], cardiomyopathy[4], and rheumatoid arthritis[5].

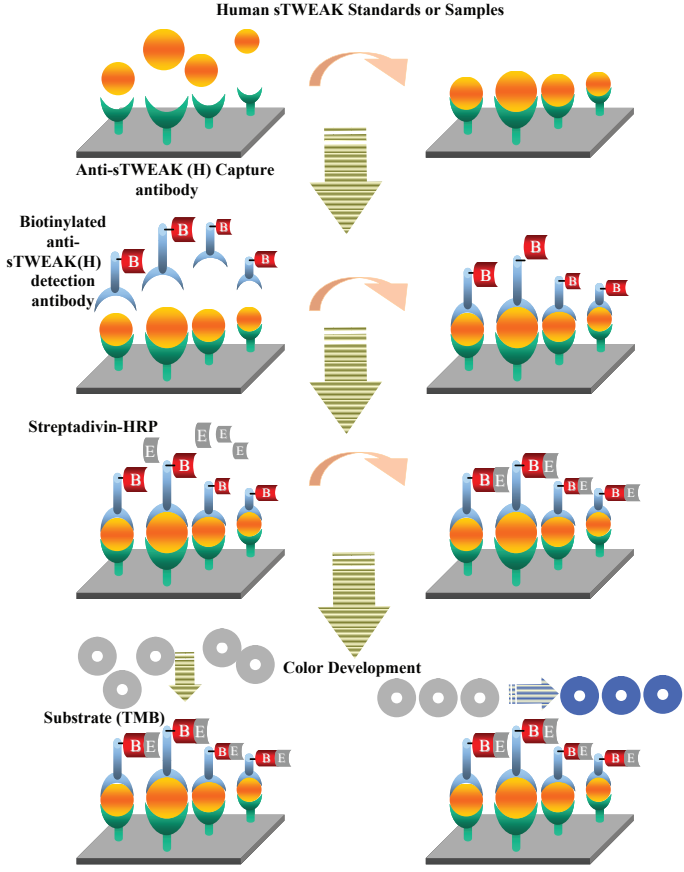
However, many studies also have indicated that sTWEAK levels are reduced in diseases with an inflammatory component. Additionally, sTWEAK hampers TNFα activity in human cells. The sTWEAK level was found to be lower but the interleukin-17A (IL-17A) levels in hypertensive patients with/without asymptomatic organ damage (AOD) was higher[6]. Therefore, it may imply a role of sTWEAK to prevent the target organ damage produced by IL-17A. There is a potential value of sTWEAK levels for the prediction of mortality in patients with chronic dialysis or chronic stable heart failure or dilated cardiomyopathy. However, it is still unclear whether sTWEAK has the potential as a biomarker for disease monitoring in patients with heart failure similar to NT-proBNP.

ASSAY CONDITIONS

The immunoplate in this kit is pre-coated with capture antibody. Plasma, culture media, tissue homogenate, CSF, urine or any biological fluid can be assayed as long as the level of the sample is high enough the for the sensitivity of the kit to detect it.

CAUTION: Phoenix Pharmaceuticals guarantees that its products conform to the information contained in this publication. The purchaser must determine the suitability of the product for its particular use and establish optimum sample concentrations.

Assay Principle



LIST OF COMPONENTS

Store all components at 4°C. DO NOT FREEZE.

1. Wash Buffer Concentrate *(20x, 50ml)*.....**Catalog No. EK-BUF**
2. Assay Diluent (PBS and BSA) .....**Catalog No. EK-BUF-054-85** *(1x, 75ml)*
3. 96 Well anti-sTWEAK Capture.....**Catalog No. EK-Plate-054-85** Antibody-Coated Plate *(1 plate)*
4. sTWEAK (Human) Standard.....**Catalog No. EK-S-054-85** *(lyophilized powder, 20ng/vial)*
5. Biotinylated anti-sTWEAK.....**Catalog No. EK-D-054-85** Detection Antibody *(1 vial)*
6. sTWEAK (Human) Positive Control.....**Catalog No. EK-PC-054-85** *(2 vials)*
7. Streptavidin-Horseradish Peroxidase.....**Catalog No. EK-HRP** (SA-HRP) *(2000x, 15µl)*
8. Substrate Solution (TMB) *(12ml)*.....**Catalog No. EK-SS**
9. Stop Solution 2N HCl *(15ml)* .....**Catalog No. EK-HCL**
10. Acetate Plate Sealer (APS) *(3 pieces)*.....**Catalog No. EK-APS**
11. Assay Diagram *(1 sheet)*

MATERIALS REQUIRED BUT NOT SUPPLIED

- Micropipettor(s) and disposable pipette tips
- Multi-channel pipette capable of dispensing 50-100µl
- Solution Reservoir *(recommended)*
- Microtiter plate washer *(recommended)*
- Orbital plate shaker capable of 300-500 rpm *(recommended)*
- Microtiter plate reader capable of absorbance measurement 450nm
- Well-closed containers (15ml tubes or more in capacity)
- Absorbent material for blotting

REAGENT PREPARATION

Note: *The kit should be equilibrated to room temperature (20-23°C) before opening any vials and starting the assay. It is highly recome nended that the solutions be used as soon as possible after rehydra tion.*

1. **1x Wash Buffer:** Dilute the **20x** Wash Buffer Concentrate with 950ml of distilled water. If crystals are observed in the **20x** Wash Buffer, warm the bottle in a 37°C water bath for approximately 30 minutes or until the crystals disappear. After preparation, store **1x** Wash Buffer at 4°C. This buffer is used to wash the plate wells.
2. **Biotinylated anti-sTWEAK (Human) Detection Antibody:** Rehydrate Biotinylated anti-sTWEAK (Human) Detection Antibody with 200µl of **1x** Assay Diluent, vortex (centrifuge the tube to dislodge powder from the cap or walls).
   Further dilute Biotinylated anti-sTWEAK (Human) Detection Antibody to 1:100 as needed with Assay Diluent and mix thoroughly before use.
3. **Streptavidin-Horseradish Peroxidase (SA-HRP):** Centrifuge the HRP vial (15µl) provided in this kit (3,000-5,000 rpm, 5 seconds) and dilute HRP with **1x** Assay Diluent to 1:6000 before use. Vortex thoroughly.
4. **Human sTWEAK Positive Control:** Rehydrate Human sTWEAK Positive Control with 1000µl of **1x** Assay diluent. Vortex thoroughly.

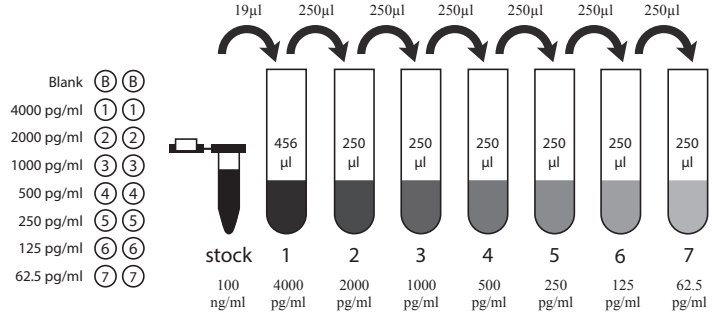
SAMPLE PREPARATION

The samples of plasma, serum, culture media, and tissue homogenate can be assayed. However, the serum samples may be overestimated and has more variations due to the proteolysis occur during the assay.

HUMAN sTWEAK STANDARD PREPARATION

1. Rehydrate recombinant Human sTWEAK Standard with 200 µl **1x** Assay Diluent, vortex. Allow the solution to sit at least 10 minutes at room temperature (20-23°C) to completely dissolve in solution. Vortex and centrifuge before use. The concentration of this stock solution is 100ng/ml.
2. Prepare Human sTWEAK standard solutions as follows:

Standard No.	Std. volume	Assay Buffer	Concentrations
Stock	Powder	200µl	100 ng/ml
#1	19µl of Stock	456µl	4000 pg/ml
#2	250µl of #1	250µl	2000 pg/ml
#3	250µl of #2	250µl	1000 pg/ml
#4	250µl of #3	250µl	500 pg/ml
#5	250µl of #4	250µl	250 pg/ml
#6	250µl of #5	250µl	125 pg/ml
#7	250µl of #6	250µl	62.5 pg/ml



sTWEAK (Human) ELISA PROTOCOL

1. Thoroughly read this protocol before performing an assay. Allow all reagents to come to room temperature (20-23°C) prior to the start of the assay.

2. Remove Capture Antibody-Coated Plate from its zip-lock foil pouch. Remove unneeded strips from the plate frame, reseal them in the foil pouch, and return the foil pouch to 4°*C*.

3. Prepare the test samples by dilute the sample with equal of dilution buffer.

4. Leave wells A-1 and A-2 empty as **Blank**.

5. Add 100µl of the prepared Human sTWEAK standard solutions from **#7** to **#1** (reverse order of serial dilution) in duplicate to each well.

6. Add 100µl of Human sTWEAK Positive Control solution in duplicate.

7. Add 100µl diluted samples in duplicate into their designated wells.

8. Seal the immunoplate with Acetate Plate Sealer (APS). Incubate for 2 hours at room temperture on a plate shaker (300-400 rpm).

9. Before washing the plate, remove the plate sealer carefuly. Completely discard the liquid from wells. Wash each well with 300-350µl Wash Buffer four times. At the end of each the wash, discard the buffer, invert the plate, and tap on a clean absorbent towel. We recommend using muti-channel pipette and automatic plate washers.

10. Add 100µl diluted biotinylated anti-Human sTWEAK Detection Antibody into each well **EXCEPT THE BLANK**. Reseal the immunoplate with plate sealer and incubate for 2 hours at room temperture on a plate shaker (300-400rpm).

11. Wash 4 times with the **1x** Wash Buffer as described in step 9.

12. Add 100µl diluted SA-HRP solution into each well. Reseal the immunoplate with plate sealer and incubate the plate for 40 minutes at room temperture on plate shaker (300-400rpm).

13. Wash 4 times with the **1x** Wash Buffer as described in step 9.

14. Add 100µl Substrate Solution (TMB) provided in this kit into each well. Reseal the plate with plate sealer to protect from light and incubate the plate for 1 hour at room temperature (20-23°C) on a plate shaker (300-400 rpm). After incubation, blue coloring should be observed in the 4000 pg/ml standard wells.

15. Add 100µl Stop Solution (2N Hydrochloric Acid) 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 10 minutes.

16. Read Absorbance O.D. at 450nm using a Microtiter Plate Reader.

ADDITIONAL RECOMMENDED PROCEDURAL NOTES:

- Reagents of different lot numbers shoud not be mixed.
- Recheck the reagent labels when loading the plate to ensure that everything is added correctly.
- Unused microplate strips should be placed 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 the sample results, therefore, it is imperative to select the appropriate sample preparation procedure 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 300-500 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.

SUMMARY OF ASSAY PROTOCOL

Add 100µl/well of Human sTWEAK Standard, Positive Control or Samples

▼

Incubate at room temperture for 2 hours

▼

Wash immunoplate 4 times with 350µl/well of **1x** Wash Buffer

▼

Add 100µl/well of Biotinylated Anti-Human sTWEAK Detection Antibody

▼

Incubate at room room temperture for 2 hours

▼

Wash immunoplate 4 times with 350µl/well of **1x** Wash Buffer

▼

Add 100µl/well of SA-HRP solution

▼

Incubate at room temperture for 40 minutes

▼

Wash immunoplate 4 times with 350µl/well of **1x** Wash Buffer

▼

Add 100µl/well of Substrate Solution (TMB)

▼

Incubate at room temperature (20-23°C) for 10 mins

▼

Terminate reaction with 100µl/well of 2N HCl

▼

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

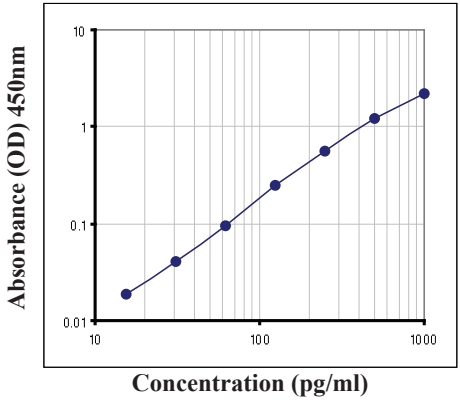
CALCULATION OF RESULTS

Plot the standard curve on log-log graph paper. Known concentration of Human sTWEAK Standard and its corresponding O.D. reading is plotted on the log scale (X-axis) and the log scale (Y-axis), respectively. The standard curve shows a correlated relationship between Human sTWEAK concentrations and the corresponding O.D. absorbance. As the standard concentration increases, the intensity of the yellow color, and in turn the O.D. absorbance, increases.

The concentration of Human sTWEAK within a sample is determined by plotting the sample's O.D. on the Y-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line dropped from this point will intersect the X-axis at a coordinate corresponding to the Human sTWEAK concentration in the unknown sample.

Refer to QC Data sheet for acceptable values of the positive conrtol.

Human sTWEAK Standard Curve



STORAGE

1. Store the kit at 4°C upon receipt. The kit should be equilibrated to room temperature (20-23°C) before assay.

2. Store **1x** Assay Buffer at 4°C.

3. Remove any unneeded strips from Human sTWEAK antibody-Coated plate, reseal them in zip-lock foil and keep at 4°C.

4. Keep rehydrated solution of Human sTWEAK Standard, Biotinylated anti-Human sTWEAK Detection Antibody and SA-HRP at -20°C. Prepare only the required amount.
   After reconstitution, maintain the sTWEAK Standard at -20°C for up to 30 days. The standard may be frozen and thawed three times without loss of immunoreactivity. Do not store reconstituted standard at 4°C.

NOTE:

1. It is recommended that the solutions be used on the same day of rehydration.
2. After adding Stop Solution, read the plate within 10 minutes.

REFERENCES

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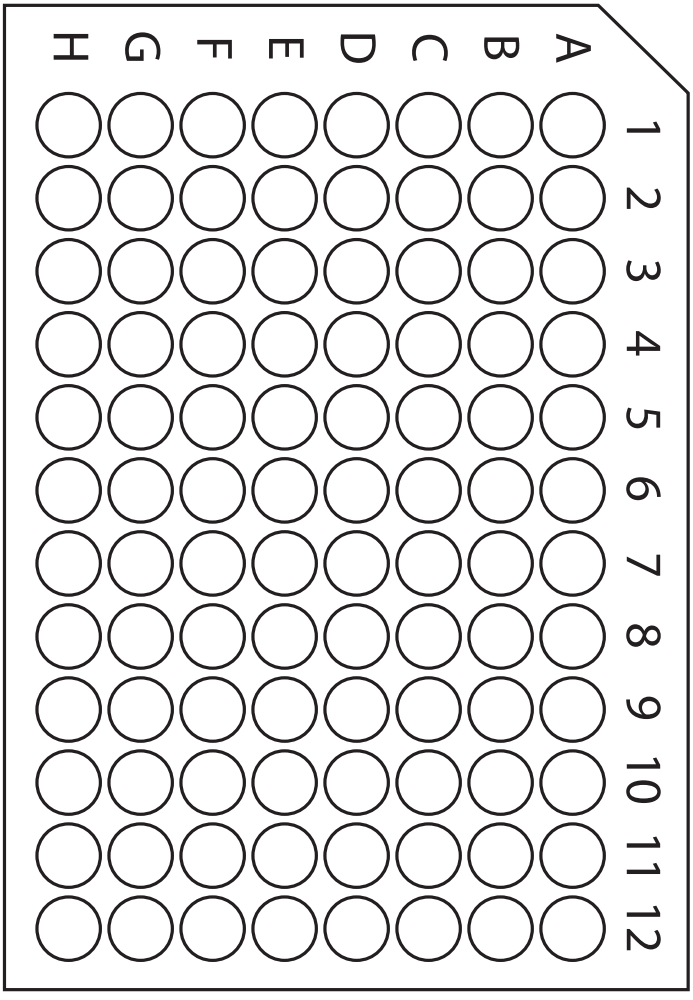
NOTES

ASSAY DIAGRAM



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