TECHNICAL MANUAL

Phospho-4E-BP1 (T36) ELISA Kit

- **SKU CODE:** SBRS1736
- **SIZE:** 96 Wells
- **DETECTION PRINCIPLE:** Sandwich ELISA
- **RUO:** Research-Use-Only

Revised: 09/26

Phospho-4E-BP1 (T36) ELISA Kit

Please read entire manual carefully before starting experiment.

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1. Product Description

The Assay Genie Phospho-4E-BP1 (Thr36) ELISA Kit is a rapid, convenient and sensitive assay kit for monitoring the activation or function of important biological pathways in human and mouse cell lysates. By determining phosphorylated EIF4EBP1 protein in your experimental model system, you can verify pathway activation in your cell lysates. Numerous different cell lysates can be measured simultaneously without spending excess time and effort in performing a Western Blot analysis.

This Sandwich ELISA kit is an in vitro enzyme-linked immunosorbent assay for the measurement of human and mouse phospho-EIF4EBP1. An anti-pan EIF4EBP1 antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and EIF4EBP1 present in a sample is bound to the wells by the immobilised antibody. The wells are washed and rabbit anti-phospho-4E-BP1 (Thr36) antibody is used to detect phosphorylated EIF4EBP1. After washing away unbound antibody, HRP-conjugated anti-rabbit IgG is pipetted into the wells. The wells are again washed, a TMB substrate solution is added to the wells and colour develops in proportion to the amount of 4E-BP1 (Thr36) bound. The Stop Solution changes the colour from blue to yellow, and the intensity of the colour is measured at 450 nm.

2. Storage

The entire kit may be stored at -20°C for up to 6 months from the date of shipment. Avoid repeated freeze-thaw cycles. For the storage of prepared reagents, see the Materials Provided table above.

3. Materials Provided

Name	Size / Quantity	Description	Storage / Stability After Preparation
Anti-Pan-EIF4EBP1 Microplate	96 wells	Microplate coated with anti-pan-EIF4EBP1 antibody	1 month at -20°C*
Positive Control	1 vial	Lyophilised powder from treated HeLa cell lysate.	1 week at -80°C
Anti-Phospho 4E-BP1 (Thr36) Detection Antibody	2 vials	Rabbit anti-phospho-4E-BP1 (Thr36); 1 vial is enough to assay half a microplate	5 days at 4°C
HRP-conjugated anti-rabbit IgG	1 vial	25 µL of 1000X concentrated HRP-conjugated anti-rabbit IgG.	Do not store and reuse
Wash Buffer (20X)	25 mL	20X concentrated wash buffer	1 month at 4°C
Assay Diluent B (5X)	15 mL	5X concentrated assay diluent	1 month at 4°C
Lysis Buffer (2X)	5 mL	2X cell lysis buffer	1 month at 4°C
TMB One-Step Substrate Reagent	12 mL	3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution	N/A
Stop Solution	8 mL	0.2 M sulfuric acid	N/A

*Return unused wells to the pouch containing the desiccant pack and reseal along the entire edge.

4. Other Supplies Required

1. Microplate reader capable of measuring absorbance at 450 nm
2. Protease and phosphatase inhibitors
3. Shaker
4. Precision pipettes to deliver 2 µL to 1 mL volumes
5. Adjustable 1-25 mL pipettes for reagent preparation
6. 100 mL and 1 L graduated cylinders
7. Absorbent paper
8. Distilled or deionized water

9. Log-log graph paper or computer and software for ELISA data analysis
10. Tubes to prepare the positive control or sample dilutions

5. Sample Preparation

Cell lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding the lysis buffer. Solubilise cells at 4 x 10^7 cells/mL in Lysis Buffer (1X) (we recommend adding protease and phosphatase inhibitors to the lysis buffer prior to sample preparation). Pipette up and down to resuspend and incubate the lysates with shaking at 2 - 8°C for 30 minutes. Microcentrifuge at 13,000 rpm for 10 minutes at 2 - 8°C, and transfer the supernatants into a clean test tube. Lysates should be used immediately or aliquoted and stored at -70°C. Avoid repeated freeze-thaw cycles. Thawed lysates should be kept on ice prior to use. For detailed sample preparation recommendations, please contact Assay Genie technical support.

For the initial experiment, we recommend a serial dilution, such as a 5-fold to 50-fold dilution, for your cell lysates with prepared Assay Diluent (see Reagent Preparation step 2) before use.

Note: *The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be determined empirically. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.*

6. Reagent Preparation

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Assay Diluent B should be diluted 5-fold with deionized or distilled water before use.
3. Lysis Buffer (2X) should be diluted 2-fold with deionized or distilled water (for cell lysate and tissue lysate). We also recommend the addition of protease and phosphatase inhibitors (not included) to the Lysis Buffer prior to use.
4. Preparation of Positive Control: Briefly spin the Positive Control vial. Add 400 µL of prepared Assay Diluent (1X) into the Positive Control. Gently mix the powder to allow it to dissolve thoroughly. If a precipitate is seen in the solution after mixing, this can be removed by a quick centrifugation of the positive control vial, and then pipetting the supernatant only for the assay.
5. If the Wash Concentrate (20X) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 mL of Wash Buffer Concentrate (20X) into deionized or distilled water to yield 400 mL of Wash Buffer (1X).
6. Preparation of rabbit anti-phospho-4E-BP1 (Thr36) antibody: Briefly spin the vial of rabbit anti-phospho-4E-BP1 (Thr36). Add 100 µL of Assay Diluent (1X) into the vial to prepare a phospho detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days or at -80°C for one month). The concentrate should then be diluted 55-fold with Assay Diluent (1X) and used in step 5 of the Assay Procedure.
7. Preparation of HRP-conjugated anti-rabbit IgG: Briefly spin the vial of HRP-conjugated anti-rabbit IgG concentrate before use. HRP-conjugated anti-rabbit IgG should be diluted 1000X with Assay Diluent (1X) and used in step 7 of the Assay Procedure.

7. Assay Procedure

Please read the entire manual carefully before starting the assay. Bring all reagents and samples to room temperature before use.

1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is strongly recommended to run all positive controls and samples in at least duplicate.
2. Label removable 8-well strips as appropriate for your experiment.
3. Add 100 µL of positive control (see Reagent Preparation step 4) or sample into the appropriate wells. Cover the wells and incubate for 2.5 hours at room temperature or overnight at 4°C with gentle shaking.
4. Discard the solution and wash 4 times with 1X Wash Solution. Wash by filling each well with Wash Buffer (300 µL) using a multi-channel pipette or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100 µL of prepared 1X rabbit anti-phospho-4E-BP1 (Thr36) (see Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.
6. Discard the solution. Repeat the wash as in step 4.
7. Add 100 µL of prepared HRP-conjugated anti-rabbit IgG solution (see Reagent Preparation step 7) to each well. Incubate for 1 hour at room temperature with gentle shaking.
8. Discard the solution. Repeat the wash as in step 4.
9. 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.
10. Add 50 µL of Stop Solution to each well. Read at 450 nm immediately.

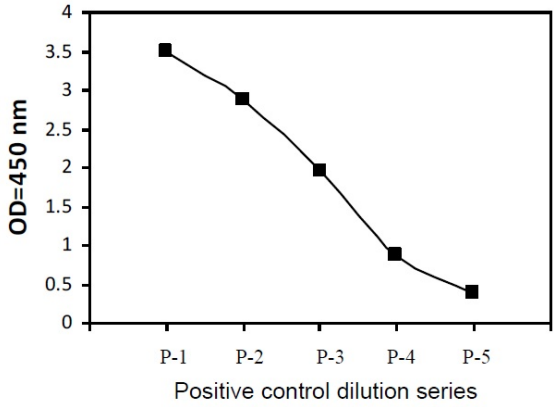
8. Calculation of Results

Calculate the mean absorbance for each set of duplicate positive controls and samples, and then subtract the average zero (blank) optical density.

The typical data shown below are provided for demonstration purposes only and should not be used to calculate results for your own samples.

Positive Control

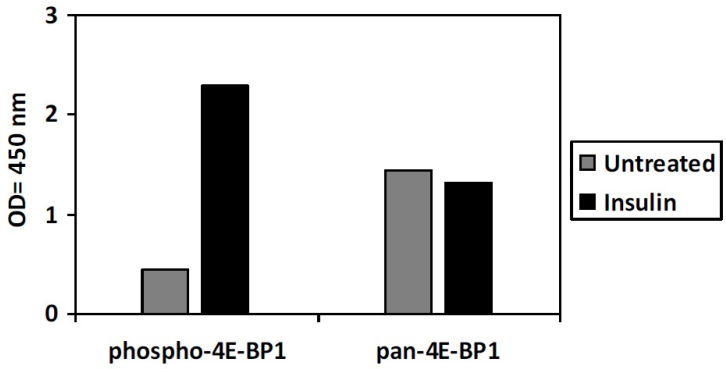
HeLa cells were treated with FBS. Cells were solubilised at 4 x 10^7 cells/mL in Cell Lysate Buffer.



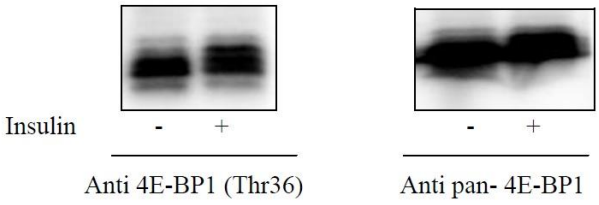
Insulin Stimulation of 293 Cell Line

293 cells were untreated or treated with Insulin. Cell lysates were analysed using this phosphoELISA and Western Blot.

ELISA



Western Blot Analysis



9. Troubleshooting Guide

Problem	Cause	Solution
Low signal in samples	Sample concentration is too low	Increase the sample concentration.
	Improper preparation of detection antibody	Briefly spin down vials before opening. Dissolve the powder thoroughly.
	Too brief incubation times	Ensure sufficient incubation time; Assay Procedure step 3 may be done overnight.
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation.
High signal in samples	Sample concentration is too high	Reduce the sample concentration.
Large CV	Inaccurate pipetting	Check pipettes.
	Air bubbles in wells	Remove bubbles in wells.
High background	Plate is insufficiently washed	Review the manual for proper washing. If using a plate washer, ensure that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer.
Low sensitivity	Improper storage of the ELISA kit	Store your positive control at <-70°C after reconstitution, others at 4°C. Keep the substrate solution protected from light.
	Stop solution	Add stop solution to each well before reading the plate.
	Improper primary or secondary antibody dilution	Ensure correct dilution.

Notes:

Assay Genie 100% money-back guarantee!

If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.