invitrogen™

phosphoELISA™ Kit

Catalog # KHO0681 (96 tests)

4E-BP1 (Total)

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Contents and Storage

Storage

Store at 2 to 8°C.

Contents

Reagents Provided	96 Test Kit
4E-BP1 (Total) Standard, lyophilized. Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer.* Contains 0.1% sodium azide; red dye**; 25 mL per bottle.	1 bottle
*4E-BP1 Antibody Coated Well*s. 12 x 8 Well Strips.	1 plate
4E-BP1 (Total) Detection Antibody. Contains 0.1% sodium azide; blue dye**; 11 mL per bottle.	1 bottle
Anti-Rabbit IgG HRP (100X). Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial
HRP Diluent. Contains 3.3 mM thymol; yellow dye**; 25 mL per bottle.	1 bottle
Wash Buffer Concentrate (25X). 100 mL per bottle.	1 bottle
Stabilized Chromogen, *Tetramethylbenzidine (TMB).* 25 mL per bottle.	1 bottle
Stop Solution. 25 mL per bottle.	1 bottle
Plate Covers, adhesive strips.	3

* If precipitates are found in standard diluent buffer, they should be completely dissolved by warming to room temperature before use.

** In order to help our customers avoid any mistakes in pipetting the ELISAs, we provide colored *Standard Diluent Buffer*, *Detection Antibody*, and *HRP Diluent* to help monitor the addition of solution to the reaction well. This does not in any way interfere with the test results.

Disposal Note

This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

Safety

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

Introduction

Purpose

The Invitrogen 4E-BP1 (Total) ELISA is designed to detect and quantify the levels of 4E-BP1 protein independent of its phosphorylation state. This assay is intended to detect 4E-BP1 in human, mouse and rat cell lysates. This kit can be used to normalize the phosphorylated 4E-BP1 content of samples when using the Invitrogen 4E-BP1 [pT46] ELISA kit (Cat. # KHO0691).

For Research Use Only. CAUTION: Not for human or animal therapeutic or diagnostic use.

Principle of the Method

The Invitrogen 4E-BP1 (Total) kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A mouse monoclonal antibody specific for 4E-BP1 (regardless of phosphorylation state) has been coated onto the wells of the microtiter strips provided. Samples, including a standard containing 4E-BP1, control specimens, and unknowns, are pipetted into these wells. During the first incubation, the 4E-BP1 antigen binds to the immobilized (capture) antibody. After washing, a rabbit antibody specific for 4E-BP1 is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized 4E-BP1 protein captured during the first incubation. After removal of excess detection antibody, a horseradish peroxidase-labeled Anti-Rabbit IgG (Anti-Rabbit IgG HRP) is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove all the excess Anti-Rabbit IgG HRP, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of 4E-BP1 present in the original specimen.

Background Information

Eukaryotic initiation factor 4E binding protein 1 (4E-BP1, MW: 20 kDa), also known as PHAS-I, is the predominant member of a family of eIF4E-binding proteins whose binding affinity to eIF4E is regulated by its phosphorylation. Besides its eIF4E-binding domain, two other motifs are important for 4E-BP1 function in vivo. One of these is a TOS motif (Phe-Glu-Met-Asp-Ile) located at the end of the carboxy-terminus, and the other is a RAIP motif (Arg-Als-Ile-Pro) at the amino-terminus.

4E-BP1 is an important effector of mTOR signaling. Hypophosphorylated 4E-BP1 acts as a translational repressor by binding and inhibiting the eIF4E. Translational control is exerted predominantly by regulating the quantity, activity and integrity of the cap-dependent translation initiation apparatus. In mammals, a tri-molecular complex designated eukaryotic initiation factor 4F (eIF4F) initiates cap-dependent translation. It consists of three proteins: eIF4E, the mRNA cap binding protein; eIF4A, an mRNA helicase; and eIF4G, which serves as a docking protein for eIF4E and eIF4A. eIF4G also binds the adapter protein eIF3 which targets the intact eIF4F complex to the 40S subunit of the ribosome. The translational function of eIF4E is negatively regulated by 4E-BP1. Binding of 4E-BP1 to eIF4E prevents the latter protein from engaging with other partners, such as the scaffold eIF4G, and therefore blocks cap-dependent mRNA translation initiation. Upon stimulation, 4E-BP1 undergoes phosphorylation at multiple sites, which results in the dissociation of 4E-BP1 from eIF4E, thereby relieving the inhibitory effect of 4E-BP1 on eIF4E-dependent translation initiation. Release of 4E-BP1 from eIF4E is generally blocked by rapamycin, indicating an essential role for mTOR signaling. mTOR-dependent phosphorylation of 4E-BP1

might represent one of the underlying mechanisms by which mTOR regulates cell growth, cell size and cell fate in mammalian cells.

In response to exogenous stimuli, such as growth factors and insulin, 4E-BP1 appears to be phosphorylated on at least six serine and threonine sites (Thr37, Thr46, Ser65, Thr70, Ser83, and Ser112). The phosphorylation of these sites is believed to occur in an orderly fashion, with phosphorylation of the two amino-terminal threonines (Thr37 and Thr46) functioning together to promote subsequent phosphorylation of the carboxy-terminal sites. The phosphorylation of 4E-BP1 increases in response to activated PI-3K or its downstream effector Akt and mTOR. mTOR mediates phosphorylation of 4E-BP1 at residues Thr37 and Thr46 and also impacts phosphorylation of Ser65 and Thr70. Evidence for mTOR-mediated phosphorylation of 4E-BP1 came from studies demonstrating that rapamycin reduces the phosphorylation of 4E-BP1 and partially prevents its dissociation from eIF4E. Phosphorylation of Thr70 and Ser65 is more sensitive to rapamycin treatment than is phosphorylation of Thr37 and Thr46. Subsequently, it was shown that overexpression of rapamycin-insensitive mTOR resulted in 4E-BP1 phosphorylation even in the presence of rapamycin.

Methods

Materials Needed But Not Provided

- Microtiter plate reader (at or near 450 nm) with software
- Calibrated adjustable precision pipettes
- Distilled or deionized water
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.)
- Glass or plastic tubes for diluting solutions
- Absorbent paper towels
- Calibrated beakers and graduated cylinders

Procedural Notes

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.
3. Samples should be collected in pyrogen/endotoxin-free tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
5. When possible, avoid use of badly hemolyzed or lipemic sera. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
6. It is recommended that all standards, controls and samples be run in duplicate.
7. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
8. **Do not mix or interchange different reagent lots from various kit lots**.
9. Do not use reagents after the kit expiration date.
10. Absorbances should be read immediately, but can be read up to 2 hours after assay completion. For best results, keep plate covered in the dark.
11. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
12. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. ***Never*** insert absorbent paper directly into the wells.
13. Because Stabilized *Chromogen* is light sensitive, avoid prolonged exposure to light. Avoid contact between chromogen and metal, or color may develop.

Directions for Washing

- **Incomplete washing will adversely affect the test outcome**. All washing must be performed with the *Wash Buffer Concentrate (25X)* provided.
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip into the bottom of each well. Take care not to scratch the inside of the well. After aspiration, fill the wells with at least 0.4 ml of diluted *Wash Buffer*. Let soak for 15 to 30 seconds, and then aspirate the liquid. Repeat as directed under Assay Procedure. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
- Alternatively, the diluted *Wash Buffer* may be put into a squirt bottle. If a squirt bottle is used, flood the plate with the diluted *Wash Buffer*, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
- If using an automated washer, follow the washing instructions carefully.

Procedure For Extraction Of Proteins From Cells Or Tissues

Recommended Formulation of Cell and Tissue Extraction Buffer:

- 10 mM Tris, pH 7.4
- 100 mM NaCl
- 1 mM EDTA
- 1 mM EGTA
- 1 mM NaF
- 20 mM $Na_4P_2O_7$
- 2 mM Na_3VO_4
- 1% Triton X-100
- 10% glycerol
- 0.1% SDS
- 0.5% deoxycholate

This Cell Extraction Buffer (Invitrogen, Cat. # FNN0011) needs the following reagents to be added:

- 1 mM PMSF (stock is 0.3 M in DMSO)
- Protease inhibitor cocktail (e.g., Sigma Cat. # P-2714) (reconstituted according to manufacturer's guideline). Add 500 µl per 5 ml Cell Extraction Buffer.

The Cell Extraction Buffer is stable for 2 to 3 weeks at 4°C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at -20°C. When stored frozen, the buffer should be thawed on ice. **Important:** add the protease inhibitors just before using. The stability of protease inhibitor supplemented Cell Extraction Buffer is 24 hours at 4°C. PMSF is very unstable and must be added prior to use, even if added previously.

The following protocol has been applied to several cell lines using this Cell Extraction Buffer. Researchers may optimize the cell extraction procedures that work best in their hands.

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash cells twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at -80°C and lysed at a later date).
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10 minute intervals. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of 4E-BP1. For example, 5 x 10^6 HEK 293 cells grown in DMEM plus 10% FBS can be extracted in 1 ml of Extraction Buffer. Under these conditions, use of 1-10 µL of the clarified cell extract diluted to a volume of 100 µL/well in *Standard Diluent Buffer* (See **Assay Procedure**) is sufficient for the detection of 4E-BP1.
5. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4°C.
6. Aliquot the clear lysate to clean microcentrifuge tubes. These samples are ready for assay. Lysates can be stored at -80°C. Avoid multiple freeze-thaw cycles.

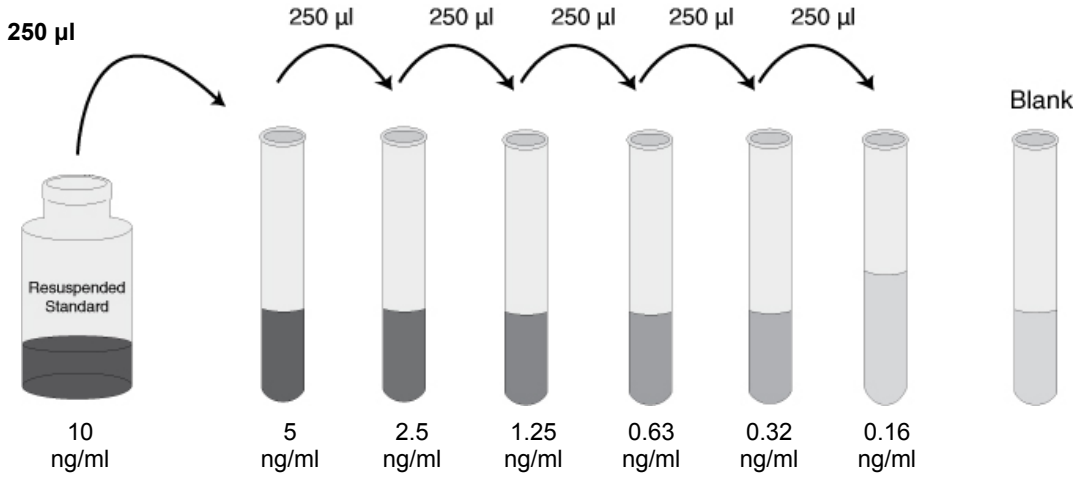
Preparation of Reagents

Dilution of Standard

Note: The *4E-BP1 (Total) Standard* is prepared from recombinant 4E-BP1 protein.

1. Reconstitute *4E-BP1 (Total) Standard* with *Standard Diluent Buffer*. Refer to standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Label as 10 ng/ml 4E-BP1. Use the standard within 1 hour of reconstitution.
2. Add 0.25 ml of *Standard Diluent Buffer* to each of 6 tubes labeled 5, 2.5, 1.25, 0.63, 0.32, and 0.16 ng/ml of 4E-BP1.
3. Make serial dilutions of the standard as described in the following dilution diagram. Mix thoroughly between steps.

Remaining reconstituted standard should be discarded or frozen in aliquots at -80°C for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.



Preparing IgG HRP

Note: Prepare within 15 minutes of usage. The *Anti-Rabbit IgG HRP (100X)* is in 50% glycerol, which is viscous. To ensure accurate dilution, allow the *Anti-Rabbit IgG HRP (100X)* to reach room temperature. Gently mix. Pipette the *Anti-Rabbit IgG HRP (100X)* slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Dilute 10 µl of this 100X concentrated solution with 1 ml of *HRP Diluent* for each 8-well strip used in the assay. Label as Anti-rabbit IgG-HRP Working Solution.
2. Return the unused *Anti-Rabbit IgG HRP (100X)* to the refrigerator.

# of 8-Well Strips	Volume of Anti-Rabbit IgG HRP (100X)	Volume of Diluent
2	20 µl solution	2 ml
4	40 µl solution	4 ml
6	60 µl solution	6 ml
8	80 µl solution	8 ml
10	100 µl solution	10 ml
12	120 µl solution	12 ml

Dilution of Wash Buffer

1. Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* with 24 volumes of deionized water (e.g., 50 ml may be diluted up to 1.25 liters, 100 ml may be diluted up to 2.5 liters). Label as Working Wash Buffer.
2. Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

Assay Procedure

Be sure to read the *Procedural Notes* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Add 100 µl of the *Standard Diluent Buffer* to the zero standard wells. Well(s) reserved for chromogen blank should be left empty.
3. Add 100 µl of standards and diluted samples or controls to the appropriate microtiter wells. Samples prepared in Cell Extraction Buffer must be diluted 1:10 or greater in *Standard Diluent Buffer* (for example, 10 µl sample into 90 µl buffer). While a 1:10 sample dilution has been found to be satisfactory, higher dilutions such as 1:25 or 1:50 may be optimal. The dilution chosen should be optimized for each experimental system. Tap gently on side of plate to mix.
4. Cover wells with *plate cover* and incubate for **2 hours at room temperature.**
5. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **Directions for Washing**.
6. Pipette 100 µL of *4E-BP1 (Total) Detection Antibod*y solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
7. Cover wells with *plate cover* and incubate for **1 hour at room temperature.**
8. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **Directions for Washing**.
9. Add 100 µl Anti-Rabbit IgG HRP Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in **Preparing IgG-HRP)**.
10. Cover wells with the *plate cover* and incubate for **30 minutes at room temperature**.
11. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **Directions for Washing**.
12. Add 100 µl of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
13. Incubate for **30 minutes at room temperature and in the dark. *Please Note*: Do not cover the plate with aluminum foil or metalized mylar**. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceeds the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.

14. Add 100 µl of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
15. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µl each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 2 hours after adding the *Stop Solution*.
16. Use a curve fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit.
17. Read the concentrations for unknown samples and controls from the standard curve. Samples producing signals greater than that of the highest standard should be diluted in *Standard Diluent Buffer* and reanalyzed. Multiply value(s) obtained for sample(s) by the appropriate dilution factor to correct for the dilution in step 3.

Typical Data (Example)

The following data were obtained for the various standards over the range of 0 to 10 ng/ml 4E-BP1.

Standard 4E-BP1 (Total) (ng/ml)	Optical Density (450 nm)
10	3.18
5	2.04
2.5	1.44
1.25	0.82
0.63	0.54
0.32	0.40
0.16	0.28
0	0.20

Performance Characteristics

Sensitivity

The analytical sensitivity of this assay is < 0.10 ng/ml of 4E-BP1. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times.

The sensitivity of this ELISA was compared to Western blotting using known quantities of human 4E-BP1. The data presented below show that the sensitivity of the ELISA is approximately 2x greater than that of Western blotting. The bands shown in the Western blot data were developed using rabbit anti-4E-BP1, and an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

Detection of 4E-BP1 (Total) by ELISA vs Western Blot:

ELISA: O.D. 450 nm	2.22	1.28	0.77	0.54	0.35	0.31	0.26	0.21
HEK 293 lysate (µg / test)	20	10	5	2.5	1.25	0.63	0.31	0

Precision

1. Intra-Assay Precision

Samples of known 4E-BP1 concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (ng/ml)	5.75	1.20	0.57
SD	0.35	0.09	0.04
%CV	6.00	7.52	6.54

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays

	Sample 1	Sample 2	Sample 3
Mean (ng/ml)	5.72	1.21	0.60
SD	0.46	0.10	0.05
%CV	8.02	7.91	8.09

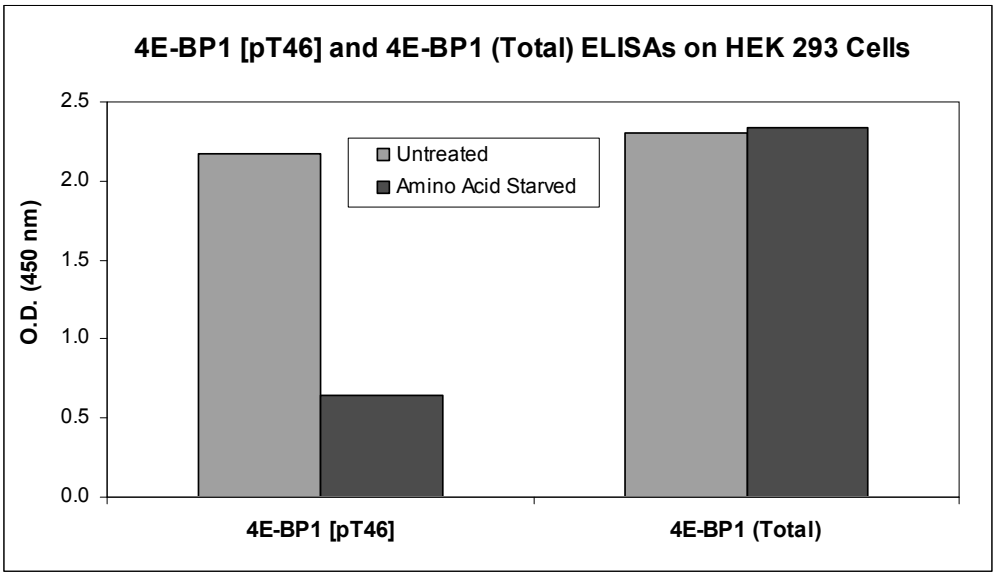
SD = Standard Deviation
CV = Coefficient of Variation

Recovery

To evaluate recovery, 4E-BP1 (Total) Standard was spiked at 3 different concentrations into 10% Cell Extraction Buffer. The percent recovery was calculated as an average of 92%.

Specificity

The 4E-BP1 (Total) ELISA kit is specific for the measurement of 4E-BP1 protein in human, mouse and rat cell lysates. Other species have not been tested. In the figure below HEK 293 cells were serum starved overnight, then deprived of amino acids in PBS for 60 minutes. Untreated HEK 293 cells were used as control. Cell extracts were prepared and analyzed with the 4E-BP1 [pT46] and 4E-BP1 (Total) ELISA. The results show that compared to untreated control, the Thr46 phosphorylation of 4E-BP1 is down-regulated in amino acid starved HEK 293 cells, whereas the total level of 4E-BP1 remains relatively constant.



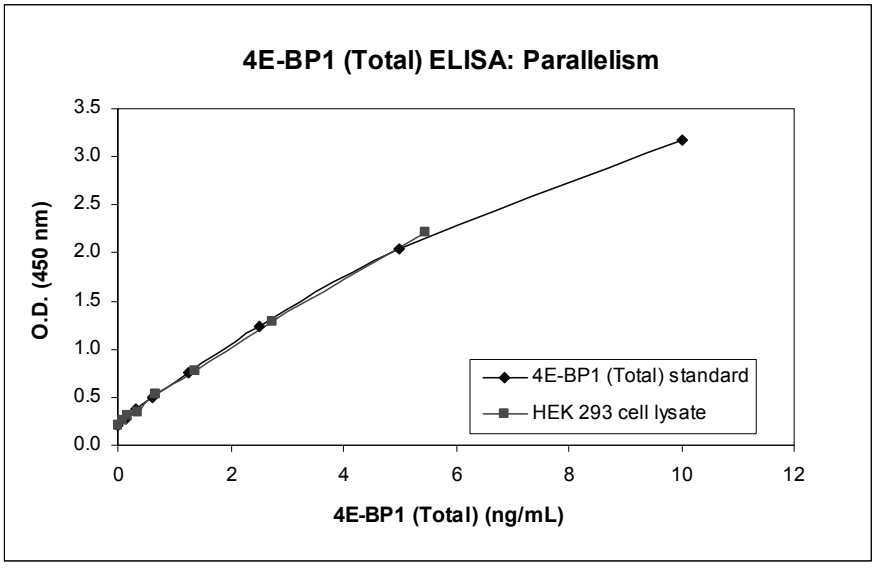
Linearity of Dilution

HEK 293 cells were grown in DMEM (Invitrogen Cat. # 10313-021) containing 10% fetal bovine serum at 37°C, and lysed with Cell Extraction Buffer. This lysate was diluted in *Standard Diluent Buffer* over the range of the assay and measured for 4E-BP1. Linear regression analysis of sample values versus the expected concentrations yielded a correlation coefficient of 0.99.

	Cell Lysate		
Dilution	**Measured (ng/ml)**	**Expected (ng/ml)**	**% Expected**
Neat	6.08	6.08	100
1/2	2.98	3.04	98
1/4	1.57	1.52	103
1/8	0.97	0.86	113

Parallelism

Natural 4E-BP1 from HEK293 cell lysate was serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against the 4E-BP1 (Total) standard curve. Parallelism was demonstrated by the figure below and indicated that the standard accurately reflects 4E-BP1 (Total) content in samples.



Limitations of the Procedure

Do not extrapolate the standard curve beyond the 10 ng/ml standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples >10 ng/ml with *Standard Diluent Buffer;* reanalyze these and multiply results by the appropriate dilution factor.

The influence of various extraction buffers has not been thoroughly investigated. The rate of degradation of native 4E-BP1 in various matrices has not been investigated. Although 4E-BP1 degradation in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

Appendix

Troubleshooting Guide

Standard curve wells develop, but sample wells produce weak or no signal.	*Cause:* Improper sample preparation. *Solution 1:* Make sure to prepare cell extracts in the protease inhibitor-supplemented Cell Extraction Buffer recommended in the protocol booklet. Other buffer formulations have not been evaluated. *Cause:* Samples contain materials that interfere with the assay. *Solution 1:* The Cell Extraction Buffer recommended in the protocol booklet contains SDS. This detergent can potentially interfere with the immunoassay. For this reason, we recommend that all samples be diluted by a factor of at least 1:10 using the Standard Diluent Buffer provided in the kit. This buffer also contains blocking proteins that will reduce background signal. *Cause:* The concentration of the target analyte is too dilute. *Solution 1:* When preparing the cell extracts, increase the number of cells per volume of protease inhibitor supplemented Cell Extraction Buffer. Ideally, the concentration of protein in each cell extract, as determined by the Quant-iT™ protein assay kit, will be between 1 and 10 mg/ml (Method 1) or 1 and 5 mg/ml (Method 2). It is recommended that 5-10 µg of total cellular protein as a starting point be loaded into each well. *Solution 2:* Optimize the stimulation procedure and time. *Cause:* A sample treatment step was not performed. *Solution 1:* Certain analytes (e.g., ERK1/2 [pTpY185/187] and ERK1/2 Total) require a sample treatment step to improve performance with Invitrogen phosphoELISA™ kits. Please see the analyte-specific protocol booklet for information on sample treatment procedures. *Cause:* Samples deteriorated during storage. *Solution 1:* Make sure that the Cell Extraction Buffer is supplemented with phosphatase inhibitors and protease inhibitors just prior to use. *Solution 2:* All samples should be stored frozen at –80°C. *Solution 3:* Samples should be subjected to only one freeze-thaw cycle. *Solution 4:* Some proteins can be lost by adsorption when stored in containers made of polystyrene or certain kinds of glass. Polypropylene tubes are best for storing samples.
Sample wells develop, but standard wells produce weak or no signal.	*Cause:* Improper dilution of standards. *Solution 1:* Check reconstitution volume of standard. *Solution 2:* Standard curves are generated by serially diluting the reconstituted standard. Check the serial dilution method. *Solution 3:* Standards should be used within an hour of reconstitution and serial dilution. *Cause:* Improper storage of standards. *Solution 1:* Standards are provided as lyophilized powders that should be stored at 2–8°C. Once reconstituted, standard should be stored at –80°C.

Neither the standard curve wells nor the sample wells develop.	*Cause:* Insufficient Anti-Rabbit IgG HRP secondary antibody activity. *Solution 1:* Check the dilution of the Anti-Rabbit IgG HRP (100X). *Solution 2:* The Anti-Rabbit IgG HRP (100X) must be freshly diluted for each assay. *Solution 3:* The Anti-Rabbit IgG HRP (100X) must be stored at 2–8°C. *Solution 4:* Sodium azide is an irreversible inhibitor of horseradish peroxidase enzyme activity. Make sure to dilute the Anti-Rabbit IgG HRP (100X) in the correct buffer. A quick test can be performed to determine if the Anti-Rabbit IgG HRP (100X) is active. Into a clean test tube, dispense 200 µl of the TMB substrate solution provided in the kit. This TMB substrate solution should be clear to slightly blue-green tinted. Next, pipette 2 µl of the Anti-Rabbit IgG HRP (100X). The color of the TMB will change to an intense aqua blue instantaneously if the HRP has retained its enzyme activity. *Cause:* Insufficient Detector Antibody. *Solution 1:* The Detector Antibody must be stored at 2–8°C. *Solution 2:* Improper dilution of Detector Antibody. *Cause:* TMB solution lost activity. *Solution 1:* The TMB solution should be clear before it is dispensed into the wells of the microtiter plate. An intense aqua blue color indicates that the product is contaminated. Please contact Technical Support if this problem is noted. To avoid contamination, we recommend that the quantity required for an assay be dispensed into a disposable trough for pipetting. Any TMB solution left in the trough should be discarded. *Solution 2:* Avoid contact of the TMB solution with items containing metal ions.
Standard curves are not developing consistently in between different runs.	*Cause:* Improper dilution of Secondary antibody. *Solution 1:* The Anti-Rabbit IgG HRP (100X) is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow Anti-Rabbit IgG HRP (100X) to reach room temperature. Gently mix. Pipette concentrate slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper. *Solution 2*: Check plate washing technique. Results can be effected if some liquid remains after aspiration.

Technical Support

Contact Us

For more troubleshooting tips, information, or assistance, please call, email, or go online to www.invitrogen.com/ELISA.

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Limited Warranty

Licensing Information

Explanation of symbols

Symbol	Description	Symbol	Description
REF	Catalogue Number	LOT	Batch code
RUO	Research Use Only	IVD	*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer	EC REP	European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

NOTES

4E-BP1 (Total) Assay Summary

Incubate 100 µL Standard or Cell Extract (>1:10) for 2 hours at RT

aspirate and wash 4x

Incubate 100 µL of Detection Antibody for 1 hour at RT

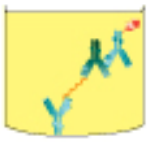
aspirate and wash 4x

Incubate 100 µL of HRP Anti-Rabbit Antibody for 30 minutes at RT

aspirate and wash 4x

Incubate 100 µL of Stabilized Chromogen for 30 minutes at RT

Add 100 µL of Stop Solution and read at 450 nm



Total time: 4 hours