3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

ATF2 ELISA

Product Number **CS0540**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description
ATF2 ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for quantitative determination of ATF2 protein in cell lysates. A monoclonal antibody specific for ATF2 (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate strips provided. ATF2 standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the ATF2 antigen binds to the immobilized (capture) antibody. After incubation, the excess reagents are washed away and Anti- ATF2 specific for total ATF2, is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized ATF2. After removal of excess detection antibody, horseradish peroxidase-labeled (HRP) anti-rabbit IgG is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove 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 ATF2 present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of ATF2.

ATF2 ELISA is designed to detect and quantify the level of ATF2 protein, independent of its phosphorylation state. Although performance characterization of this ELISA kit was done primarily on human cell lines, cross-reactivity of this kit with mouse cells was observed. This assay is intended for the detection of ATF2 from lysates of cells, and can be used to normalize the ATF2 content of the samples when examining quantities of phosphorylated sites on ATF2 using Sigma Phospho-ATF2 [$pThr^{69}$/$pThr^{71}$] ELISA (Product No. CS0550).

ATF2 (activating transcription factor 2) is a member of the ATF/CREB (cAMP response element-binding protein) family of basic region-leucine zipper proteins. Alternative names for ATF2 include: CREB2, camp responsive element binding protein 2, and CRE-BP1.

The gene for human ATF2 maps to chromosome 2 (band 2q32). Human ATF2 is 94% homologous with mouse ATF2 and 38% homologous with the Y54E10A.6 protein in *C. elegans*. Three isoforms of human ATF2 are observed, designated CRE-BP1, 2, and 3, that result from differential splicing of a single transcript. The full length human ATF2 (CRE-BP1) is a protein with MW 60 kDa, comprised of 487 amino acid residues. ATF2 localizes to the nucleus. Tissues that express the highest levels of ATF2 include brain, lung, liver, and kidney. ATF2 is a transcription factor that regulates expression of genes containing a cAMP-response element contained in the promoter region of regulated genes, including TNF-α, TGF-β, cyclin A, E-selectin, DNA polymerase β, and c-Jun. ATF2 also possesses histone acyltransferase activity, with histones H2B and H4 serving as its substrates. As with Fos, Jun, and CREB, ATF2 contains a C-terminal leucine zipper dimerization motif and a C-terminal basic stretch of amino acids that mediate binding to specific DNA sequences. The N-terminal region of ATF2 contains a transactivation domain. Within this transactivation domain is a putative MAPK docking site, similar to the docking site found in other MAPK substrates.

Under resting conditions, ATF2 possesses low levels of transcriptional activity because of an intramolecular inhibitory interaction in which the C-terminal DNA binding domain is bound to the N-terminal transactivation domain. This intramolecular inhibition can be relieved through protein:protein interactions, including ATF2's interaction with normal cell proteins (pRb, the high mobility group HMG, NF-κB, and c-Jun) and ATF2's interaction with viral proteins (adenovirus E1A, hepatitis B virus protein X, or human T-cell leukemia virus type I protein Tax). Intramolecular inhibition is also relieved through phosphorylation of ATF2 at threonine residues 69 and 71. Phosphorylation of these sites is also correlated with enhanced stability of ATF2. These phosphorylation events afford the protein resistance from ubiquitination and subsequent targeting to the 26S proteasome. Phosphorylation of ATF2 at threonines 69 and 71 is catalyzed by several protein kinases. In response to treatment with

proinflammatory cytokines, UV irradiation, and B-cell and T-cell receptor engagement, both threonine 69 and threonine 71 are phosphorylated by JNK and p38 MAPK by a two-step mechanism in which the MAPK dissociates from the ATF2 after each phosphorylation event. ATF2 is also observed to be phosphorylated in response to stimuli which do not activate JNK or p38 MAPK, including treatment with insulin, epidermal growth factor, and serum. The sequential phosphorylation of ATF2 at threonine 71 by ERK in the Raf/MEK/ERK cascade, followed by phosphorylation of threonine 69 by p38 MAPK in a signaling pathway that includes Ral-RalGDS-Src-p38 MAPK in response to these other stimuli is currently under investigation.

Activated ATF2 forms homodimers with other members of the ATF family or heterodimers with members of the c-Jun family of transcription factors. These ATF2-containing dimers bind to the cAMP response element and with several other proteins including p300/CBP, p50/p65 NF-κB, SMAD3, SMAD4, NFAT family members, and the interferon regulatory factor 1 (IRF1). These ATF-containing protein complexes recruit RNA Pol II, and enhance the transcription of the responsive genes.

Activation of ATF2 is observed in cellular responses to various types of stress and apoptotic signals, and is currently under investigation in studies of growth factor independent cell growth, cell cycle progression, differentiation, cytokine production in response to B-cell and T-cell receptor engagement, and inflammation. ATF2 appears to be especially important in cancer. ATF2 has recently been shown to render melanoma cells resistant to irradiation. A peptide that contains that ATF2 sequence corresponding to amino acid residues 50 through 100 (containing both the phosphorylated threonines and possibly a site for interaction with p300) has been shown to enhance sensitivity of melanoma to chemotherapeutic drugs. The interaction of ATF2 with v-Jun (the avian sarcoma virus 17 transforming protein, a viral homolog of the c-Jun protein) is also implicated in the development of fibrosarcoma.

Reagents

- **ATF2 Standard, Lyophilized, 2 vials, Product No. A 3604-** Full length recombinant ATF2 expressed in *E. coli,* Refer to vial label for quantity and reconstitution volume.
- **Standard Diluent Buffer, 25 mL, Product No. S 7319,** contains sodium azide as preservative.
- **Monoclonal-Anti-ATF2-Coated 96 well plate, 1EA, Product No. A 3354 -** A plate using break-apart strips coated with monoclonal antibody specific for full-length ATF2 (regardless of phosphorylation state).
- **Anti-ATF2, 11 mL, Product No. A 3479** A detection antibody, produced in rabbit. Contains sodium azide. Ready to use.
- **Anti-Rabbit IgG-HRP, Concentrate (100X), 1 vial, Product No. R 7278 -** contains 3.3 mM thymol and 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No. H 5788 -** contains 3.3 mM thymol. Ready to use.
- **Wash Buffer concentrate, 25X, 100 mL, Product No. W 2639 -** *See Reagent Preparation for handling, dilution and storage instructions*
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** –Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm.
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL.
- Cell extraction buffer (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.
- Graph paper: linear, log-log, or semi-log, as desired.

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples of choice – extracts of cell lysates
- 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.

Cell 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
1 mM PMSF (stock is 0.3 M in DMSO) *PMSF is very unstable and must be added prior to use, even if added previously*.
Protease inhibitor cocktail (Sigma Product No. P 2714)
Add 250 µl of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 4 °C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at –20 °C.

Thaw on ice. *Add the protease inhibitors just before use.*

Procedure for Extraction of Proteins from Cells

This protocol has been successfully applied to several cell lines of human origin. Researchers may use the procedures that work best in their hands. They will have to assay their lysates for the satisfactory extraction and/or phosphorylation.

Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash 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 –70 °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.
5. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of ATF2. For example, 108 Colo 201 cells grown in RPMI plus 10% FBS can be extracted in 1 mL of Cell Extraction Buffer. Under these conditions, use of 1-5 µL of the clarified cell extract diluted to a volume of 100 µL/well in Standard Diluent Buffer is sufficient for the detection of ATF2.
6. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 °C.
7. Aliquot the clear lysate to clean microcentrifuge tubes

Before assay: extracted cell lysate samples containing protein should be diluted with Standard Diluent Buffer at least 1:10. This dilution is necessary to reduce the matrix effect of the cell extraction buffer.

Reagent Preparation

Standard

Note: This ATF2 standard was prepared using full length, recombinant ATF2 protein purified from *E. coli*.

1. Reconstitute ATF2 Standard with *Standard Diluent Buffer*. Refer to standard vial label for instructions.
2. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Label as 70 ng/mL Use standard within 1 hour of reconstitution.
3. Prepare serial standard dilutions as follows

Tube #	Standard Buffer	Standard from tube #:	Final ng/mL
1	Reconstitute according to label instructions		70 ng/mL
2	0.25 mL	0.25 mL (1)	35 ng/mL
3	0.25 mL	0.25 mL (2)	17.5 ng/mL
4	0.25 mL	0.25 mL (3)	8.8 ng/mL
5	0.25 mL	0.25 mL (4)	4.4 ng/mL
6	0.25 mL	0.25 mL (5)	2.2 ng/mL
7	0.25 mL	0.25 mL (6)	1.1 ng/mL
8	0.25 mL	-	0 ng/mL

4. Remaining reconstituted standard should be discarded or frozen at –70 °C. for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Anti-rabbit IgG Horseradish Peroxidase (HRP)
Note: The *Anti-rabbit IgG-HRP* 100X concentrate is in 50% glycerol. This solution is viscous. To ensure accurate dilution:

1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Within 1 hour of use, 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.
4. Return the unused concentrate to the refrigerator
5. For more strips use the following amounts:

# of 8 well strips	IgG-HRP Concentrate µL	Diluent mL
2	20	2
4	40	4
6	60	6
8	80	8
10	100	10
12	120	12

Wash Buffer

1. Equilibrate to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25X + 24 volumes of deionized water
3. Label as *Working Wash Buffer.*
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

Storage/Stability
All components of this kit are stable at 2-8 °C. Any unused reconstituted standard should be discarded or frozen at –70 °C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit shelf life. To obtain C of A go to www.sigma-aldrich.com

Procedure
Precautions

- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 °C).
- Use only the coated 96 well capture plate provided with the kit
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2-8°C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of component and reagent addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.

Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle four times, blotting as dry as possible after the 4^{th} wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

ATF2 ELISA Assay Summary

1) **100 µL of ATF2 Standards or Samples (samples diluted 1:10 or higher in Standard Diluent Buffer)**
   Incubate 2 hours at RT
   aspirate and wash 4x

2) **Add 100 µL Anti-ATF2**
   Incubate 1 hour at RT.
   aspirate and wash 4x

3) **Add 100 µL Anti-Rabbit IgG-HRP**
   Incubate 30 min at RT.
   aspirate and wash 4x

4) **Add 100 µL Stabilized Chromogen**
   Incubate 30 minutes at RT
   ***(in the dark)*.**

5) **Add 100 µL of Stop Solution**
   Read at 450nm.

Total Assay Time - 4 hours

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilution wells and 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch

1st incubation

a Add 100 µL Standard Diluent to zero wells.
b Add 100 µL standards, samples or controls to the appropriate wells.
c Samples prepared in cell extraction buffer or brain homogenate buffer must be diluted 1:10 or greater in *Standard Diluent Buffer* (for example, 10 µL sample plus 90 µL buffer). *The dilutions should be optimized for each assay.*
d Cell culture supernatants or buffered solutions; dilute 1:2 in *Standard Diluent Buffer* (50 µL buffer + 50 µL sample).
e Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature.
f Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells for a total of 4 times following washing instructions

2nd incubation

a Add 100 µL Anti-ATF2 detection antibody to all wells (except chromogen blanks).
b Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

3rd incubation

a Add 100 µL Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
b Cover with Plate Cover and incubate 30 minutes at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation

a Add 100 µL of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
b Do not cover the plate
c Incubate approximately 30 minutes at room temperature in the dark (*place plate in a drawer or cabinet).*

Note: *If your multiwell plate reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.*

Stop reaction

a Add 100 µL of Stop Solution to each well. This stops the reaction
b Tap gently to mix. *The solution will turn yellow.*

Absorbance reading

a Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.
b Blank the plate reader against the Chromogen Blank wells (containing Chromogen and Stop Solution).

Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution

Results

1. The results may be calculated using any immunoassay software package.
2. The four-parameter algorithm provides the best curve fit.
3. If the software program is not readily available, the concentrations of ATF2 may be calculated manually.

4. Calculate the Average Net OD (average reading of 2 wells) for each standard dilution and samples as follows:
5. Average Net OD = Average Bound OD – Average Chromogen Blank OD
6. On graph paper plot the Average Net OD of standard dilutions against the standard concentration (ng/mL) of ATF2. Draw the best curve through these points to construct the standard curve.
7. The ATF2 concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
8. Multiply the values obtained for the samples by dilution factor of each sample.
9. Samples producing signals higher than the 70 ng/mL standard should be further diluted and assayed again.

Product Profile

Typical Results

The standard curve below is for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

Standard Curve

Standard ATF2 (ng/mL)	Optical density (450 nm)
0.0	0.139
1.1	0.240
2.2	0.331
4.4	0.411
8.8	0.561
17.5	0.932
35	1.755
70	3.024

Limitations

- Do not extrapolate the standard curve beyond the 70 ng/mL standard point.
- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.
- The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of cell extract and brain homogenate samples have not been thoroughly investigated.
- The rate of degradation of native ATF2 in various matrices has not been investigated.

Performance characteristics

Sensitivity

The analytical sensitivity of this assay is <1 ng/mL of ATF2. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. Using Jurkat cells, this level of sensitivity was equivalent to the detection of ATF2 in 4000 cells.

The sensitivity of this ELISA was compared to immunoblotting using known quantities of ATF2. The data presented in Figure 1 show that the sensitivity of the ELISA is approximately 4x greater than that of immunoblotting. The bands shown in the immuno blotting data were developed using rabbit anti-ATF2, an alkaline phosphatase conjugated anti-rabbit IgG, followed by chemiluminescent substrate and autoradiography.

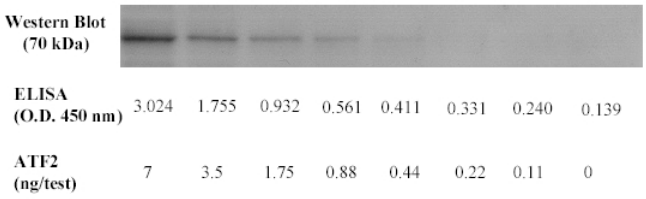

Figure 1 Detection of ATF2 by ELISA vs immunoblot

Precision

1. Intra-Assay Precision

Samples of known ATF2 concentration were assayed in replicates of 12 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	35.9	10.6	3.6
Standard Deviation (SD)	2.0	0.3	0.3
% Coefficient of Variation	5.7	3.0	7.2

2. Inter-Assay Precision

Samples were assayed 36 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	36.7	10.2	3.5
Standard Deviation (SD)	4.1	0.7	0.2
Coefficient of Variation %	11.0	7.3	7.3

Recovery
To evaluate recovery, extraction buffer was diluted 1:50 with *Standard Diluent Buffer*. ATF2 standard was spiked into the Cell Extraction Buffer. The average recovery was 90%.

Parallelism
Natural ATF2 from HeLa and 3T3L1 cells cultured in RPMI + 10% FBS were serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against the ATF2 standard curve. Parallelism demonstrated in Figure 2 indicates that the standard accurately reflects ATF2 content in samples.

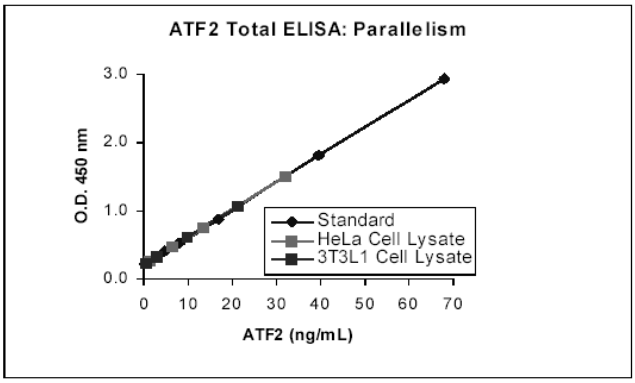

Figure 2

Linearity of Dilution
HeLa cells grown in tissue culture medium containing 10% fetal bovine serum and lysed with Cell Extraction Buffer. This lysate was diluted with *Standard Diluent Buffer* over the range of the assay and measured for ATF2 content. Linear regression analysis of sample values versus the expected concentration yielded a correlation coefficient of 0.99.

Dilution	Cell Lysate		
	Measured ng/mL	Expected ng/mL	% Expected
Neat	32	32	100
1:2	16	16	100
1:4	10	8	125
1:8	5	4	125

Specificity
- The ATF2 ELISA is specific for measurement of total ATF2 protein.
- To determine the specificity of this ELISA kit, cell extracts from different cell lines, each at a concentration of 300 µg/mL total protein, were analyzed.
- The data presented in Figure 3 show that the kit detects ATF2 protein in all cell lysates examined.
- The levels of ATF2 protein detected with this ELISA kit are consistent with results obtained by immunoblot analysis (insert).
- The assay was found to have no cross-reactivity with CREB.
- Cross-reactivity with other ATF/CREB family members was not evaluated.

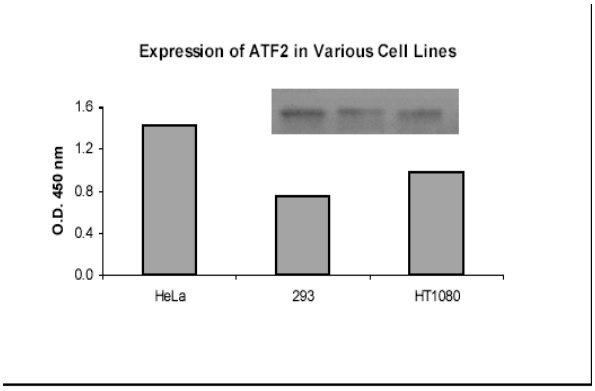

Figure 3

In order to demonstrate that the ATF2 ELISA detects both phosphorylated and non-phosphorylated forms of the protein, studies were conducted using natural and recombinant sources of ATF2.

- HeLa cells were treated with anisomycin at 5 µg/mL for 1 hour, then lysed according to the instructions described previously.
- ATF2 full-length recombinant protein was phosphorylated *in vitro* by active JNK2.
- The assays were performed in parallel for both ATF2 and ATF2 [pThr^{69}/pThr^{71}].
- Figure 4 shows that the amount of ATF2 remained comparable in activated and nonactivated cells or in phosphorylated and non-phosphorylated recombinant proteins.
- In contrast the levels of phosphorylation at threonines 69 and 71 increased in HeLa cells upon anisomycin treatment.
- Recombinant protein phosphorylated by JNK2 showed dramatic increase in ATF2 [pThr^{69}/pThr^{71}].

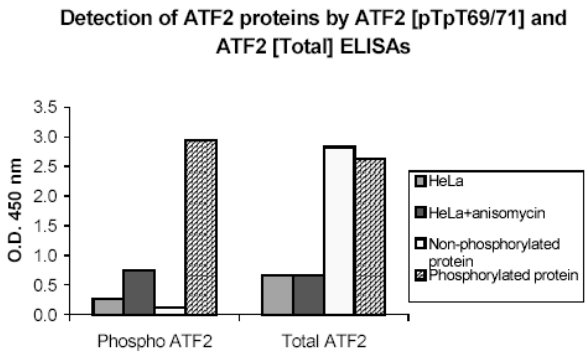

Figure 4

- The data presented in Figure 5 are the levels of ATF2 [pThr^{69}/pThr^{71}], determined with ATF2 [pThr^{69}/pThr^{71}] ELISA (Product No. CS0550) normalized to total ATF2.

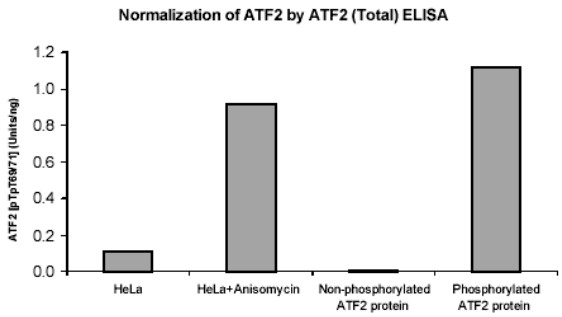

Figure 5

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