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ProductInformation

MEK1 ELISA

Product Number **CS0700**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description
MEK1 ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for the quantitative determination of MEK1 protein in cell lysates. A monoclonal antibody specific for MEK1 (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate strips provided. MEK1 standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the MEK1 antigen binds to the immobilized (capture) antibody. After incubation, the excess reagents are washed away and Anti-MEK1 specific for total MEK1 is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized MEK1. 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 MEK1 present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of MEK1.

MEK1 ELISA is designed to detect and quantify the level of MEK1 protein, independent of its phosphorylation state. This assay is intended for the detection of MEK1 from lysates of human, mouse and rat cells, and can be used to normalize the MEK1 content of the samples when examining quantities of phosphorylated sites on MEK1 using Sigma Phospho-MEK1 [pSer^{218}/pSer^{222}] ELISA (Product No. CS0650).

MEK1 (also known as ERK kinase 1, MAPK kinase 1, and MKK1) is a member of the MEK family of dual specificity protein kinases, with MW = 45 kDa. MEK1 shares approximately 80% homology with the closely related dual specificity protein kinase MEK2. MEK family members are homologous with the byr1 gene product of *Schizosaccharomyces pombe* and share the common feature of phosphorylating the threonine and tyrosine residues of a conserved (T-X-Y) motif within the activation loop of substrate proteins. For MEK1, the phosphorylated threonine and tyrosine residues are located within the sequence Thr-Glu-Tyr (TEY). MEK1 contains an N-terminal ERK docking domain (D domain), an N-terminal regulatory region, a consensus catalytic core, and a nuclear export sequence (NES). Under resting conditions, MEK1 localizes to the cytoplasm, but translocates to the nucleus in response to mitogenic stimuli, or to membrane-proximal adhesion structures in response to adhesion.

MEK1 is a key component of the ERK1/2 signaling cascade. The series of events leading to ERK1/2 phosphorylation in this cascade has been an active area of research for over 10 years. In response to growth factor, cytokine, or hormone binding with its specific receptor, membrane-associated Ras is loaded with GTP and thus activated. Activated Ras then recruits members of the Raf protein family (Raf-1, A-Raf, and B-Raf) to the plasma membrane. The interaction of Raf-1 with Ras at the plasma membrane results in phosphorylation of multiple sites on Raf-1 (tyrosines 340 and 341) and interaction with several other proteins, including 14-3-3, Hsp90 and Hsp50. These phosphorylation events and protein:protein interactions relieve the autoinhibitory effect of the Raf N-terminal domain and increase the catalytic activity and membrane localization of Raf. Active Raf family members phosphorylate MEK1 at serines 218 and 222, thereby activating it.

Activated MEK1 (along with MEK2) in turn phosphorylates ERK1 and ERK2 at the conserved threonine and tyrosine residues of the activation loop. ERK1 and ERK2 are the only known substrates for MEK1. The mechanism by which the dual phosphorylation occurs is nonprocessive, i.e., ERK is observed to associate with MEK1 and become phosphorylated at the first residue, then must dissociate from MEK1, and re-associate with MEK1 in order for phosphorylation of the second site to occur, as revealed by the kinetic studies. Upon phosphorylation, ERK then translocates from the cytoplasm to the nucleus,

possibly following dimerization. In the nucleus, ERK phosphorylates and activates Elk-1, Sap-1a, Ets1, c-Myc, Tal, and signal transducer and activator of transcription (STAT) proteins. Phosphorylation of Elk-1 by ERK enhances Elk-1's affinity for the serum response element and enhances transcription of growth related proteins, such as c-Fos. In addition to transcription factors, ERK also phosphorylates p90rsk, which in turn activates another series of transcription factors, as well as other proteins including cytosolic phospholipase A2, the juxtamembrane region of the EGF receptor, and several microtubule-associated proteins (MAP), including MAP-1, MAP-2, MAP-4, and Tau. MEK1 is also phosphorylated by the kinases c-mos, MEK kinase-1, cdk5/p35, and PAK1 (p21-activated protein kinase). PAK1 phosphorylates MEK1 at serine 298 and possibly threonine 292 by a mechanism that does not require membrane recruitment, providing a site of convergence between integrin and growth factor signaling. This may explain the need for cell adhesion in growth factor signaling.

MEK's involvement in regulating the ERK1/2 MAPK signaling cascade is currently under investigation in many areas of research including control of cell proliferation, cell cycle and mitosis, organelle distribution during mitosis, immune responses, protection from apoptosis in response to Fas, TRAIL, ischemia and other stressors, cell differentiation, and in the control of transcription. The signaling cascade is also under intensive investigation in cancer studies, tumorigenesis, and oncogenic transformation. Activation of ERK1 and 2 is correlated with invasiveness and metastatic potential. PD98059 (a MEK1-specific antagonist), used alone and in conjunction with chemotherapeutic agents is currently under investigation in many cancer studies.

Reagents

- **MEK1 Standard, Lyophilized, 2 vials, Product No. M 8568 –** purified full length recombinant MEK1. Refer to vial label for quantity and reconstitution volume.
- **Standard Diluent Buffer, 25 mL, Product No. S 3068,** contains sodium azide as preservative.
- **Sample Treatment Buffer, 10 mL, Product No. S 9694**
- **Monoclonal-Anti-MEK1 Coated 96 well plate, 1EA, Product No. M 9068 -** A plate using break-apart strips coated with monoclonal antibody specific for full-length MEK1 (regardless of phosphorylation state).
- **Anti-MEK1, 5.5 mL, Product No. M 8943** A detection antibody, produced in rabbit. Contains sodium azide. Ready to use.
- **Anti-Rabbit IgG-HRP, Concentrate (100X), 1 vial, Product No. I 1034 -** 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 MEK1. For example, 10^8 Jurkat 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 MEK1.
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

Sample Pre-Treatment:

Incubate each sample and control with an equal volume of *Sample Treatment Buffer* on ice for 20 minutes. Dilute this mixture at least 5-fold in *Standard Diluent Buffer*. For example, for duplicate analyses, add 10 µL sample and 10 µL *Sample Treatment Buffer*, then after incubation, add 80 µL *Standard Diluent Buffer*. The dilution chosen should be optimized for each experimental system.

Reagent Preparation

MEK1 Standard

Note: This MEK1 standard was prepared using purified, full-length, recombinant MEK1 protein

1. Reconstitute MEK1 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 10 ng/mL MEK1. Use standard within 1 hour of reconstitution.
3. Prepare serial standard dilutions as follows:

Tube #	Standard Buffer	Standard from tube #:	Final MEK1 ng/mL
1	Reconstitute according to label instructions		10 ng/mL
2	0.15 mL	0.15 mL (1)	5 ng/mL
3	0.15 mL	0.15 mL (2)	2.5 ng/mL
4	0.15 mL	0.15 mL (3)	1.25 ng/mL
5	0.15 mL	0.15 mL (4)	0.62 ng/mL
6	0.15 mL	0.15 mL (5)	0.31 ng/mL
7	0.15 mL	0.15 mL (6)	0.16 ng/mL
8	0.15 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

MEK1 ELISA Assay Summary

1) 100 µL of MEK1 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-MEK1

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 MEK 1standards, 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-MEK1 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).
c 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 MEK1 may be calculated manually.
4. Calculate the Average 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 MEK1. Draw the best curve through these points to construct the standard curve.
7. The MEK1 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 10 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 MEK1 ng/mL	OD 450 nm
10	3.1
5	1.9
2.5	1.2
1.25	0.70
0.62	0.41
0.31	0.28
0.16	0.21
0	0.15

Limitations

- Do not extrapolate the standard curve beyond the 10 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 MEK1 in various matrices has not been investigated.
- Although MEK1 degradation in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

Performance characteristics

Sensitivity

The analytical sensitivity of this assay is <0.1 ng/mL of MEK1. 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 MEK1 in 6000 cells.

The sensitivity of this ELISA was compared to immunoblotting using known quantities of MEK1. The data presented in Figure 1 show that MEK1 ELISA is approximately twice as sensitive as immunoblotting. The bands shown in the immunoblotting were developed using mouse monoclonal anti-MEK1, an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate.

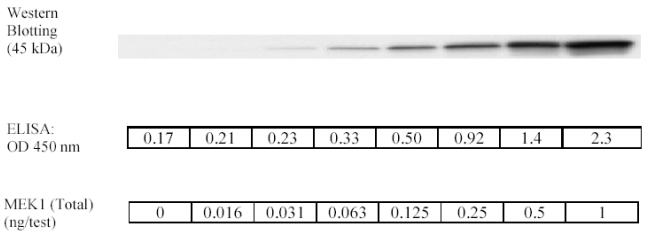

Figure 1 Detection of MEK1 by ELISA vs immunoblot

Precision

1. Intra-Assay Precision

Samples of known MEK1 concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	6.63	4.99	3.42
Standard Deviation (SD)	0.58	0.41	0.19
% Coefficient of Variation	8.7	8.2	5.6

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)	6.95	4.84	3.24
Standard Deviation (SD)	0.57	0.47	0.27
Coefficient of Variation %	8.2	9.8	8.4

Recovery
To evaluate recovery, MEK1 Standard was spiked at 3 different concentrations into 10% cell extraction buffer. The average recovery was 120%.

Parallelism
Natural MEK1 from Jurkat cell lysate was serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against the MEK1 protein standard curve. Parallelism demonstrated by the figure below indicated that the standard accurately reflects MEK1 content.

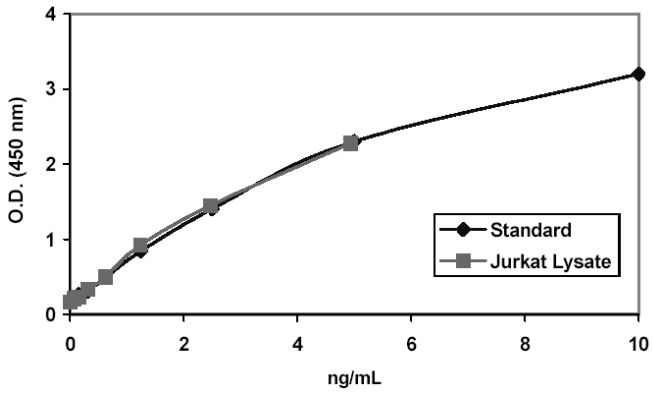

Figure 2 Parallelism: MEK1

Linearity of Dilution
Jurkat cells were grown in cell culture medium containing 10% FCS and lysed with Cell Extraction Buffer. This lysate was pretreated and then diluted with *Standard Diluent Buffer* over the range of the assay and measured for MEK1 content. The neat dilution corresponds to a 1:10 final, in well dilution of cell lysate. 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	4.94	4.94	100
1:2	2.55	2.47	103.2
1:4	1.43	1.24	115.8
1:8	0.65	0.62	105.3
1:16	0.32	0.31	103.6

Specificity

- The MEK1 ELISA recognizes human, mouse and rat MEK1.
- MEK1 ELISA is specific for measurements of MEK1 protein regardless of phosphorylation status.
- To determine the specificity of this kit, cell extracts from different cell lines, each at a final concentration of 200 µg/mL lysate, were analyzed.
- The data presented in Figure 3 show that the kit detects MEK1 in human cell lysates HT1080, MCF7, and Jurkat, mouse cell lysate NIH3T3, and rat cell lysate Y3. The levels of MEK1 protein detected with this ELISA kit are consistent with results obtained by immunoblot analysis (see inset).

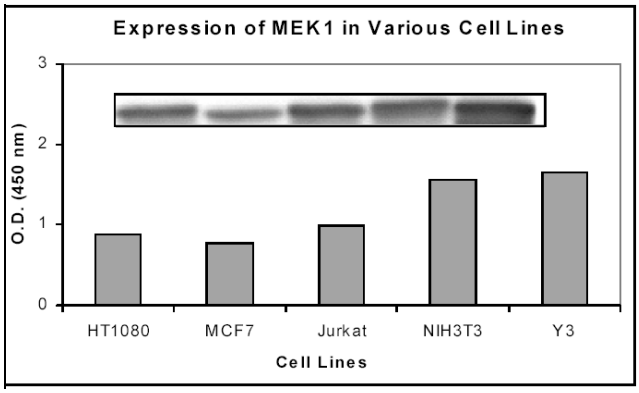

Figure 3

References

1. Burack, W.R. and T.W. Sturgill, The activating dual phosphorylation of MAPK by MEK is nonprocessive., Biochemistry, **36**, 5929-5933 (1997).
2. Mansour, S.J., Interdependent domains controlling the enzymatic activity of mitogen-activated protein kinase kinase 1., Biochemistry, **35**, 15529-15536 (1996).
3. Xu, B.E., Hydrophobic as well as charged residues in both MEK1 and ERK2 are important for their proper docking., J. Biol. Chem., **276**, 26509-26515 (2001).
4. Kolch, W., et al., Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions., Biochem. J. **351**, 289-305 (2000).
5. Bondzi, C., et al., A novel assay of the measurement of the Raf-1 kinases activity. Oncogene, **19**, 5030-5033 (2000).
6. Colanzi, A., et al., RAF-1-activated MEK1 is found on the Golgi apparatus in late prophase and is required for Golgi complex fragmentation in mitosis., J. Cell Biol., **161**, 27-32 (2003).
7. Alpin, A.E., et al., Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk1., J. Cell Biol., **153**, 273-281 (2001).
8. Lee, S.E., et al., Tumor necrosis factor-alpha supports the survival of osteoclasts through the activation of Akt and ERK., J. Biol. Chem. **276**, 49343-49349 (2001).
9. Slack-Davis, J.K., et al., PAK1 phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation., Cell Biol. **162**, 281-291(2003).
10. Soderstrom, T.S., et al., Mitogen-activated protein kinase/ extracellular signal-regulated kinase signaling in activated T cell abrogates TRAIL-induced apoptosis upstream of the mitochondrial amplification loop and caspase-8. J. Immunol. **169**, 2851-2856 (2002).

AH/PHC 09/04