PRODUCT INFORMATION

Total MEK 1

Enzyme Immunometric Assay Kit

Catalog No. IB09644

96 Well Kit

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FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Description

The IBL-America Total MEK 1 Enzyme Immunometric Assay (EIA) kit is a complete kit for the determination of pan or total MEK 1 in cell lysates. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to MEK1 immobilized on a microtiter plate to bind the MEK 1 in the standards or samples. A recombinant phosphorylated MEK1 Standard is provided in the kit. After a short incubation the excess sample or standard is washed out and a rabbit polyclonal antibody to MEK 1/2 is added. This antibody binds to the MEK 1 captured on the plate. After a short incubation the excess antibody is washed out and anti-rabbit IgG conjugated to Horseradish peroxidase is added, which binds to the polyclonal MEK 1/2 antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of MEK 1 in either standards or samples. For further explanation of the principles and practices of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

Introduction

MEK1 is also known by a variety of other names. They include dual specificity mitogen-activated protein kinase kinase (MAP kinase kinase 1 or MAPKK 1), ERK activator kinase 1, MAPK/ERK kinase 1, ERK kinase 1 and MAP kinase kinase. MEK1 is a 393 amino acid, 43.5kD protein that is highly conserved in evolution[3]. MEK phosphorylates threonine and tyrosine residues on MAP kinases ERK 1 and 2 (p44 and p42 MAP kinase)[4]. MEK participates in a wide range of cellular processes including cell proliferation[5], differentiation[6] and apoptosis[7]. MEK1 is activated by phosphorylation of Ser^{218} and Ser^{222} by the serine-threonine kinase RAF1, which is part of the p21ras signal transduction pathway. Constitutive activation of MEK1 results in cellular transformation. This protein kinase has been reported to be a likely target for pharmacological intervention in proliferative diseases[8]. Recent literature reviews cover MEK activity in great detail[9-10].

Precautions

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1. Stop Solution 2 is a 1N hydrochloric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of buffers, however it is possible that high levels of interfering substances may cause variation in assay results.
4. The phospho-MEK 1 Standard provided should be handled with care because of the known and unknown effects of MEK.
5. The phospho-MEK 1 Standard should be stored at or below -20 °C. Do not repeatedly freeze-thaw.

Materials Supplied

1. **MEK Microtiter Plate, One Plate of 96 Wells**
   A plate using break-apart strips coated with a mouse monoclonal antibody specific to MEK1.
2. **Total MEK Antibody, 10 mL**
   A yellow solution of rabbit polyclonal antibody to Total MEK 1 and 2.
3. **Assay Buffer 21, 100 mL**
   Tris Buffer Saline containing proteins, detergents and phosphatase inhibitors.
4. **Total MEK Conjugate, 10 mL**
   A blue solution of anti-rabbit IgG conjugated to Horseradish peroxidase.
5. **Cell Lysis Buffer 3, 100 mL**
   100 mM Tris, pH 7.5, 2 mM EDTA, 2 mM EGTA, 5 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 0.1% Triton X-100, 0.1% Tween 20 and 0.1% Hydorol M.
6. **Wash Buffer Concentrate, 100 mL**
   Tris buffered saline containing detergents.
7. **phospho-MEK 1 Standards, 2 each**
   Two vials containing 5,000 pg each lyophilized recombinant human phospho-MEK1.
8. **TMB Substrate, 10 mL**
   A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. **Protect from prolonged exposure to light.**
9. **Stop Solution 2, 10 mL**
   A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: **Caustic.**
10. **Total MEK 1 Assay Layout Sheet, 1 each**
11. **Plate Sealer, 3 each**

Storage

All components of this kit, **except the Standard**, are stable at 4 °C until the kit's expiration date. The Standard **must** be stored at or below -20 °C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Phenylmethanesulfonyl Fluoride (PMSF), Sigma # P7626 or equivalent.
3. Activated Sodium Orthovanadate, Sigma # S6508 or equivalent.
4. Protease Inhibitor Cocktail (PIC), Sigma # P8340 or equivalent.
5. Precision pipets for volumes between 100 µL and 1,000 µL.
6. Repeater pipet for dispensing 100 µL.
7. Disposable beakers for diluting buffer concentrates.
8. Graduated cylinders.
9. A microplate shaker.
10. Adsorbent paper for blotting.
12. Microplate reader capable of reading at 450 nm, preferably with correction between 570 nm and 590 nm.
12. Graph paper for plotting the standard curve.

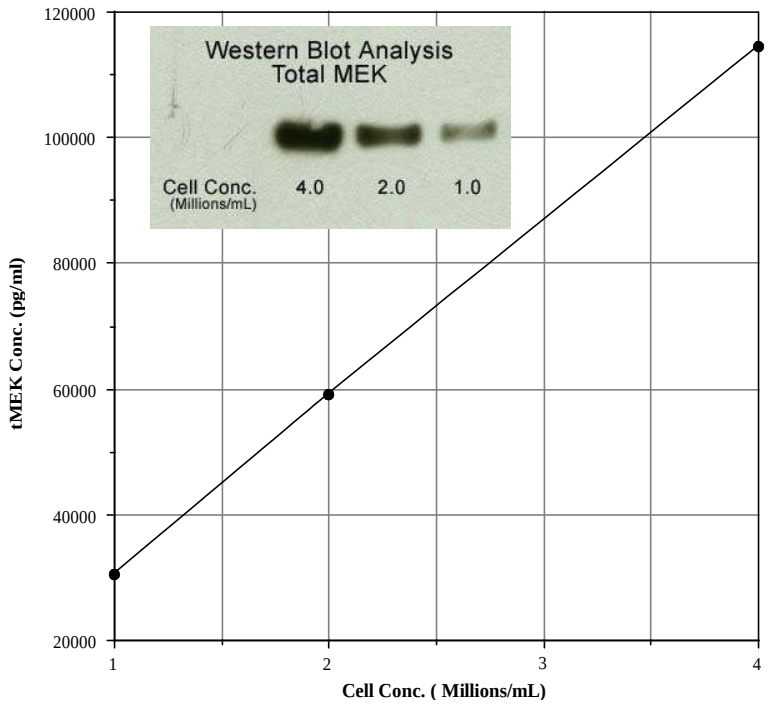
Sample Handling

This assay is compatible with MEK 1 samples in a wide range of cell lysates. Samples diluted sufficiently into Assay Buffer 21 plus Inhibitors (see Reagent Preparation, page 5, # 4) can be read directly from a standard curve. It is recommended that all samples be lysed with the provided Cell Lysis Buffer 3 (equivalent to Assay Buffer 22 in phospho-MEK1 EIA Kit) modified by the addition of PMSF and activated Sodium Orthovanadate (see Reagent Preparation, page 5, # 2 and # 3) immediately prior to use. Samples lysed with Cell Lysis Buffer 3 plus Inhibitors require further dilution with Assay Buffer 21 plus Inhibitors prior to running the assay. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions.

If the end user chooses to use another lysis buffer, or a greater number of cells, it is up to the end user to determine the appropriate dilution of samples and assay validation. Only standard curves generated in Assay Buffer 21 plus Inhibitors should be used to calculate the concentration of MEK 1. Samples must be stored frozen at or below -70 °C to avoid loss of bioactive MEK 1. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen samples should be brought to 4 °C slowly and gently mixed.

Jurkat Cell Experiment

This experiment was adapted from a protocol outlined in reference # 11. The number of non-stimulated Jurkat cells used in this experiment were: 4, 2 and 1 million per mL. Cells were centrifuged at 1,700 rpm for 7 minutes and the supernatant discarded. The cell pellets were resuspended and washed with PBS. Cells were pelleted at 1,700 rpm for 7 minutes and the supernatant dicarded. The cell pellets were resuspended with modified Cell Lysis Buffer 3, vortexed and placed at room temperature for 5 minutes. The lysates were vortexed and centrifuged at 600 rpm for 5 minutes. The supernantants were split for the Western blot and EIA to generate the data illustrated. Note that 15 µL of sample was used per lane in the Western blot. The exposure time for development was 6 minutes.



Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards must be made up in polypropylene tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag. The wells should be used in the frame provided.
8. **Prior to addition of antibody, conjugate and substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. Wash Buffer

Prepare the Wash Buffer by diluting 50 mL of the supplied concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. Activated Sodium Orthovanadate

Prepare a 200 mM solution of Sodium Orthovanadate using Sigma # S6508 or equivalent. Adjust the pH to 10.0 using either 1N NaOH or 1N HCl (at pH 10.0 the solution will be yellow). Boil the solution until it turns colorless (approximately 10 minutes). Cool the solution to room temperature. Readjust the pH to 10.0. Repeat the boiling and pH readjustment steps until the solution remains colorless and the pH stabilizes at 10.0. Aliquot and store the solution at -20 °C.

3. Cell Lysis Buffer 3 plus Inhibitors

Allow to come to room temperature. Ensure Cell Lysis Buffer 3 is completely in solution prior to use. Immediately prior to cell lysis, PMSF and Activated Sodium Orthovanadate must be added to the buffer. Add PMSF, such as Sigma # P7626, to a final concentration of 1 mM. Add Activated Sodium Orthovanadate to a final concentration of 2 mM.

Do not add Protease Inhibitor Cocktail (PIC) to this buffer if the same lysates will be used in the phospho MEK 1 EIA kit (Catalog No. 900-119).

Fresh Cell Lysis Buffer 3 plus Inhibitors must be made each time the cells are lysed.

4. Assay Buffer 21 plus Inhibitors

Immediately prior to use in the assay, PMSF and PIC must be added to the buffer. If using Sigma Protease Inhibitor Cocktail # P8340, add 0.5 µL/mL or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma # P7626, to a final concentration of 1 mM.

This modified Assay Buffer 21 must be used for standard reconstitution and all sample and standard dilutions to ensure optimal integrity of MEK 1. Fresh Assay Buffer 21 plus Inhibitors must be made for each assay.

5. phospho-MEK 1 Standards

Allow the lyophilized phospho-MEK 1 standard to warm to room temperature. Add 500 µL of Assay Buffer 21 plus Inhibitors to the lyophilized phospho-MEK 1 vial and vortex. Wait 5 minutes and vortex again prior to use. Label the vial standard #1. Label five 12x75 mm polypropylene tubes #2 through #6. Pipet 250 µL of Assay Buffer 21 plus Inhibitors into tubes #2 through #6. Add 250 µL of reconstituted standard to tube #2 and vortex. Add 250 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #6.

The concentration of phospho-MEK1 in tubes #1 through #6 will be 10,000, 5,000, 2,500, 1,250, 625, and 312.5 pg/mL respectively. See Total MEK 1 Assay Layout Sheet for dilution details.

Reconstituted and diluted standards should be used within 20 minutes of preparation.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.
All standards, controls and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.
2. Pipet 100 µL of Assay Buffer 21 plus Inhibitors into the S0 (0 pg/mL standard) wells.
3. Pipet 100 µL of Standards #1 through #6 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~ 500 rpm.
7. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100 µL of yellow Antibody into each well, except the Blank.
9. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~ 500 rpm.
10. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 100 µL of blue Conjugate to each well, except the Blank.
12. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~ 500 rpm.
13. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
14. Pipet 100 µL of Substrate Solution into each well.
15. Incubate for 30 minutes at room temperature on a plate shaker at ~ 500 rpm.
16. Pipet 100 µL Stop Solution 2 to each well. This stops the reaction and the plate should be read immediately.
17. Blank the plate reader against the Blank wells, read the optical density at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all the readings.

Calculation of Results

Several options are available for the calculation of the concentration of MEK 1 in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of MEK 1 can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

2. Using linear graph paper, plot the Average Net OD for each standard versus MEK 1 concentration in each standard. Approximate a straight line through the points. The concentration of MEK 1 in the unknowns can be determined by interpolation.

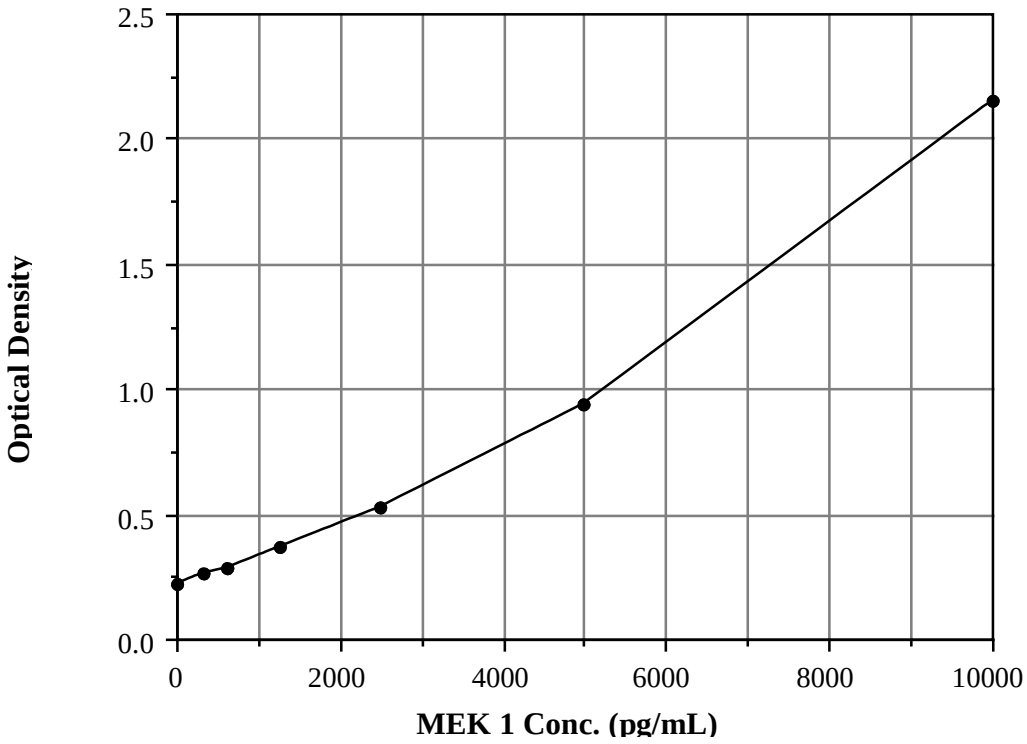
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

Sample	Average OD	Net OD	**MEK 1 (pg/mL)**
Blank	0.075		
S0	0.299	0.224	**0**
S1	2.229	2.154	**10,000**
S2	1.015	0.940	**5,000**
S3	0.604	0.529	**2,500**
S4	0.440	0.365	**1,250**
S5	0.362	0.287	**625**
S6	0.336	0.261	**312.5**
Unknown # 1	1.167	1.092	**5,636**
Unknown # 2	0.411	0.336	**1,163**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate MEK 1 concentrations; each user must run a standard curve for each assay.



Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[12].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run with 0 pg/mL Standard, and comparing to the average optical density for sixteen (16) wells run with Standard # 6. The detection limit was determined as the concentration of MEK 1 measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Mean OD for S0 =	0.254 ± 0.009 (3.5%)
Mean OD for Standard # 6 =	0.285 ± 0.015 (5.2%)
Delta Optical Density (312.5 - 0 pg/mL) = 0.285-0.254=	0.031
2 SD's of 0 pg/mL Standard = 2 x 0.009 =	0.018
Sensitivity = $\frac{0.018}{0.031}$ x 312.5 pg/mL =	**181.4 pg/mL**

Linearity

A sample containing 5,927 pg/mL MEK 1 was serially diluted 4 times 1:2 in the Assay Buffer 21 plus Inhibitors supplied in the kit and measured in the assay. The data was plotted graphically as actual MEK 1 concentration versus measured MEK 1 concentration.

The line obtained had a slope of1.161 with a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of MEK 1 and running these samples multiple times (n= 16) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium, and high concentrations of MEK 1 in multiple assays (n= 11).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of MEK 1 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	MEK 1 (pg/mL)	Intra-assay % CV	Inter-assay % CV
Low	2,022	4.9	
Medium	3,835	1.7	
High	5,658	2.1	
Low	1,921		7.5
Medium	3,660		6.7
High	5,544		5.4

Cross Reactivities.

The cross reactivities for a number of related compounds were determined by dissolving the cross reactant in the kit assay buffer. These samples were then measured in the Total MEK 1 assay and measured MEK 1 concentration calculcated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	Cross Reactivity
pMEK 1	100%
pMEK 2	25.5%
pJNK	< 0.1%
ERK2	< 0.1%
GSK-3β	< 0.1%
p38	< 0.1%

Sample Recoveries

Please refer to pages 4 through 6 for Sample Handling recommendations and Standard preparation.

Total MEK 1 concentrations were measured in cell lysates diluted with Assay Buffer 21 plus Inhibitors and assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
1 million Jurkat cells per mL	98.7	1:4 -1:32
2 million Jurkat cells per mL	96.4	1:8 -1:32
4 million Jurkat cells per mL	96.4	1:16 -1:32

WARNING: If the end user chooses to not use the provided Assay Buffer 21 plus Inhibitors or Cell Lysis Buffer 3 plus Inhibitors, it is up the end user to determine the appropriate dilution of samples and assay validation for their chosen buffers.

* See Sample Handling instructions on page 4 for details.

References

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LIMITED WARRANTY

IBL-America warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

IBL-America must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if IBL-America is not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

For more details concerning the information within this kit insert, or to order any IBL-America products, please call (763) 780-2955 between 8:00 a.m. and 5:00 p.m. CST.

Material Safety Data Sheet (MSDS) available on our web site or by fax.

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