Instruction Manual For

MEK1 STAR ELISA Kit
Catalog # 17-473

Sufficient reagents for 96 assays per kit

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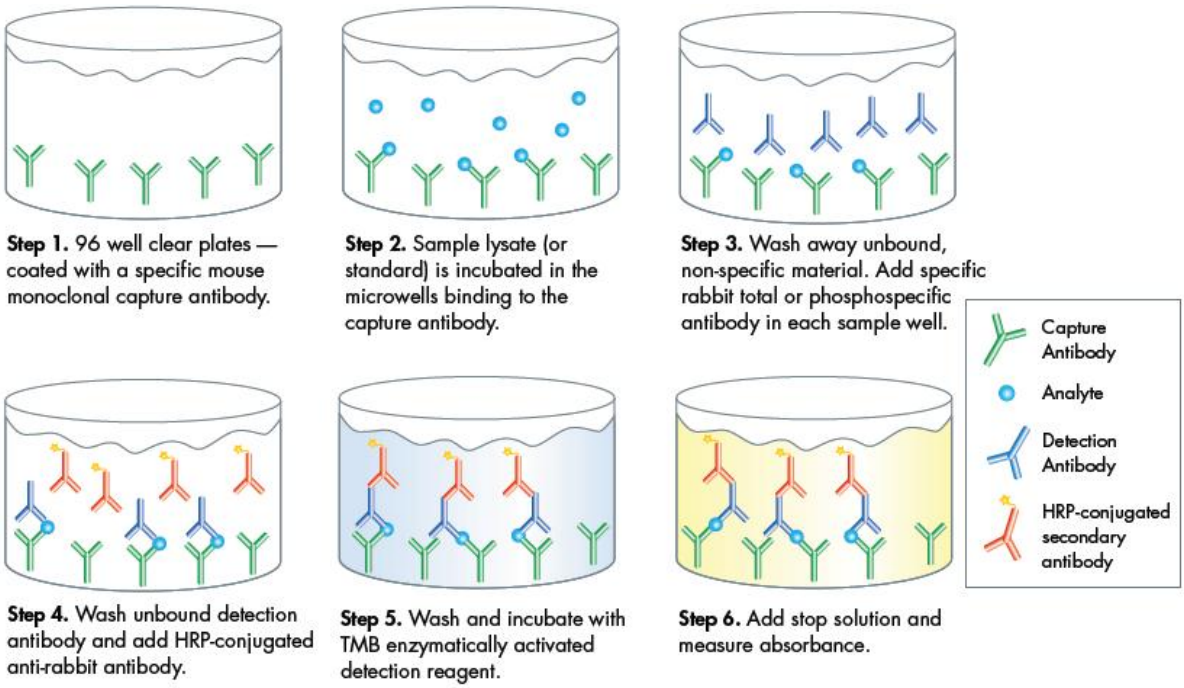
I. TEST PRINCIPLE

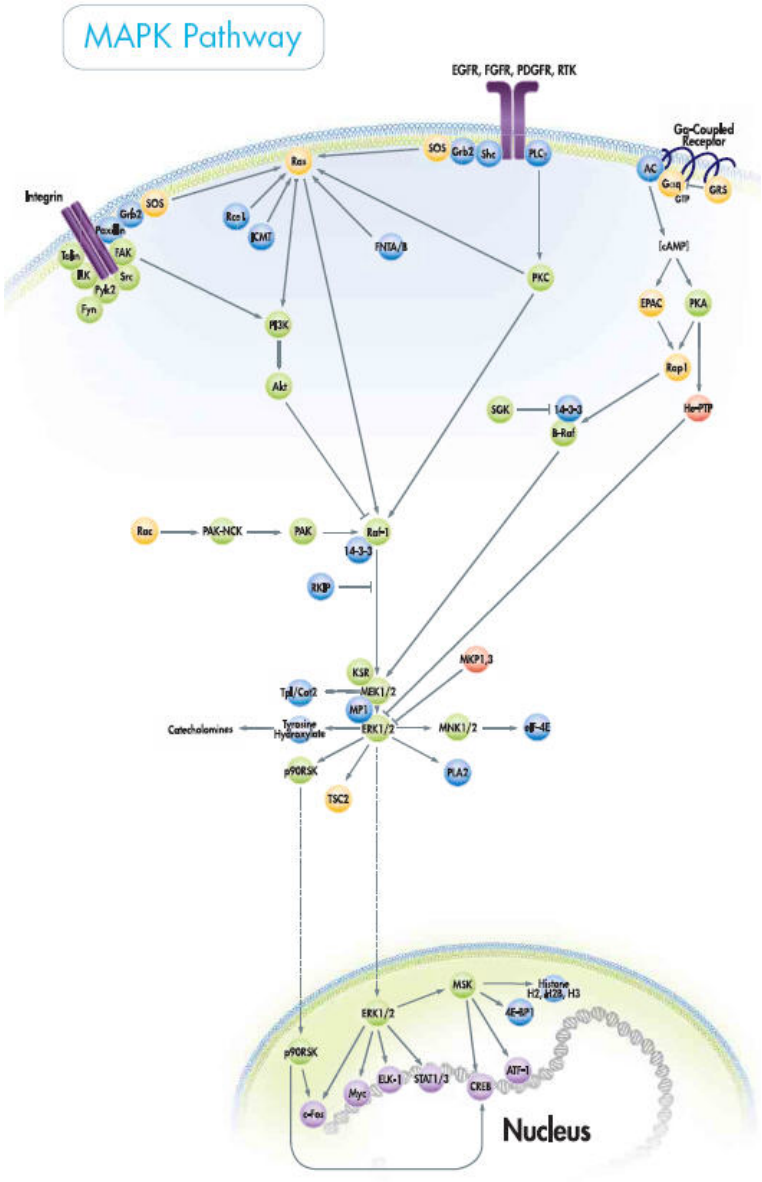
The UPSTATE® colorimetric STAR (Signal Transduction Assay Reaction) ELISA kit is a solid phase sandwich enzyme linked immunosorbent assay that provides a fast, sensitive method to detect specific levels of signaling targets in denatured cell extracts. The MEK1 plate is coated with a specific mouse monoclonal anti-MEK1 capture antibody on the microwells of the 96-well clear plate. Sample lysate or the standard included in the kit are incubated in the microwells allowing MEK1 antigen to be captured in the plate wells. The plate is then washed to remove any non-bound unspecific material. The wells are then incubated with a specific rabbit anti-MEK1 antibody to detect the captured MEK1 on the plate well. The unbound detection antibody is washed away followed by incubation with an HRP-conjugated anti-rabbit antibody. This allows for a sensitive enzymatic detection of the sample. After the addition of TMB substrate and stop solution the absorbance is measured at 450 nm using a plate reader.

The entire assay takes less than 5 hours to complete with minimal hands-on time. Many of the reagents are supplied in ready-to use formulations for ease of use. The kit also includes a standard that is run as both a positive control and to develop a standard curve.



II. MEK1 BACKGROUND

MEK (MAPK Kinase/Erk Kinase) has been implicated in a large number of human cancers. MEK1 and MEK2 are activated by phosphorylation of two serine residues (Ser218/222 in MEK1 and Ser222/226 in MEK2), which are substrates for the Raf family of kinases. Mutation of the phosphorylation sites from Ser to Asp creates a protein with constitutive kinase activity, which when expressed in cells is able to cause transformation. The two highly homologous MEK proteins, MEK1 and MEK2, are true dual-specificity kinase, in that they contain two consensus kinases motifs with one involved in the phosphorylation of serine/threonine residues and the other in the phosphorylation of tyrosine residues. MEK's only known substrate is ERK1/2 which it subsequently phosphorylate and activates in its TEY motif, phosphorylation sites Tyr185 followed by Thr183 on Erk 2 (Tyr204 and Thr202 in Erk1) that lay in the ERK activation loop.



III. ASSAY SENSITIVITY, DETECTION LIMITS and SPECIES REACTIVITY

Sensitivity: 0.16 ng/mL.

Range of Detection: 0.16 to 10 ng/mL

Species Reactivity Human, mouse and rat

***NOTE**: This data is presented for reference use only and should not be used to interpret actual assay results. A standard curve must be generated for each assay.*

IV. STORAGE OF KIT COMPONENTS

Kit components are stable for a minimum of 3 months from date of shipment if stored and handled correctly.

V. KIT COMPONENTS

1. **Capture Plate pre-coated with anti-MEK1 antibody**: (Part No. 17-473A) One pre-coated 96-stripwell immunoplate sealed in a foil pouch.
2. **Anti-MEK1 detection antibody:** (Part No. 17-473B) One bottle (6 mL) of anti-MEK1 detection antibody containing sodium azide, ready to use.
3. **ELISA Diluent:** (Part No. 17-473C) One bottle (25 mL) of ELISA Diluent containing sodium azide, ready to use.
4. **25X ELISA Wash Buffer:** (Part No. 17-473D) One bottle (50 mL) of 25X ELISA Wash Buffer.
5. **Anti-Rabbit IgG HRP conjugate:** (Part No. 17-473E) One vial (125 µL) of 100X anti-rabbit HRP conjugate.
6. **HRP Diluent:** (Part No. 17-473F) One bottle (25 mL) of HRP Diluent.
7. **TMB Solution:** (Part No. 17-473G) One bottle (25 mL) of stabilized tetramethylbenzidine (TMB), ready to use.
8. **Stop Solution:** (Part No. 17-473H) One bottle (25 mL) of stop solution, ready to use.
9. **MEK1 Standard:** (Part No. 17-473I) Two vials of MEK1 standard, lyophilized.
10. **Sample Incubation Buffer:** (Part No. 17-73J) One bottle (10 mL) of a sample incubation (denaturing) buffer, ready to use.
11. **Plate Covers:** Three plate covers.

Materials Not Supplied

1. Multi-channel or repeating pipettes
2. Plate shaker (optional)
3. Pipettors & tips capable of accurately measuring 1-1000 µL
4. Graduated serological pipettes
5. 96-well microtiter Plate Reader with 450 nm filter
6. Graphing software for plotting data or graph paper for manual plotting of data
7. Microfuge tubes for standard and sample dilutions
8. Mechanical vortex
9. 1 liter container
10. Distilled or deionized water

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and the failure to produce accurate data.
- Caustic Material: Stop Solution. **Caution: Eye, hand, face, and clothing protection should be worn when handling this material.**
- **Safety Warnings and Precautions:** This kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.
- The Detection Antibody and ELISA Diluent contain sodium azide. Sodium azide may react with copper and lead plumbing to form highly explosive metal azides. Upon disposal, flush with large amounts of water to prevent azide build-up. Avoid contact with skin.

Technical Notes

- All kit reagents should be at room temperature (20°C to 25°C) prior to use.
- Do not use reagents beyond the expiration date of the kit.
- Do not mix or interchange reagent from various kit lots.
- Manual Plate Washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values. Gentle agitation during the wash steps or a 2-3 minute soak may reduce background values.
- The desiccant enclosed in the 96-well capture plate pouch will keep the plate stable when stored at 2° to 8°C should the plate loose its seal during shipping.

VI. PREPARATION OF SAMPLE

1. Culture cells and stimulate MEK1 activation as desired.
2. Remove culture media and wash cells twice with ice-cold 1X TBS (Tris Buffered Saline) or PBS (Phosphate Buffered Saline). Discard supernatant.
3. Add 5-10 mL of cold 1X RIPA containing 0.1% SDS and protease inhibitors per 150 mm tissue culture plate.
   Note: 10 mL of 1X RIPA containing 0.1% SDS can be prepared by adding 10 µL of 1 µg/µL Leupeptin, 10 µL of 1 µg/µL Aprotinin, 10 µL of 1 µg/µL Pepstatin, 100 µL of 100mM PMSF, 100 µL 10% SDS, 1 mL 10X RIPA (Cat. No. 20-188) to 8.77 mL of distilled or deionized water.
4. Scrape cells from plate with a rubber policeman.
5. Transfer cells in RIPA buffer to a microcentrifuge tube and incubate on ice for 15 minutes.
6. Vortex tube for 10 seconds or sonicate briefly for 10 seconds.
7. Clarify lysate by centrifugation at 12,000 rpm for 10 minutes at 4ºC in a microcentrifuge prior to use.
8. Samples prepared in the RIPA buffer containing 0.1% SDS should be mixed with an equal volume of the Sample Incubation Buffer and incubated on ice for 20 minutes.
9. Cell extract containing SDS must be diluted to 0.01% SDS using ELISA Diluent prior to use.
10. Collect the supernatant and calculate protein concentration using a Bradford Assay or by densitometry.
11. It is suggested that the cell lysate be used immediately following preparation. However, samples can be frozen and stored at -80º C for later use. Frozen samples should be used within 6 months of preparation. Avoid repeated freeze thaws.

Further information regarding denatured lysate preparation protocols can be obtained at *http://www.millipore.com*.

VII. REAGENT PREPARATION

1. 1X Wash Buffer

Warm the 25X ELISA Wash Buffer to room temperature and mix to ensure that any precipitated salts have re-dissolved. For 500 mL of Wash Buffer, combine 20 mL of 25X ELISA Wash Buffer and 480 mL distilled or deionized water. Stir to homogeneity. Wash Buffer can be stored for up to 4 weeks at 2-8°C. Discard the Wash Buffer if it becomes turbid or if a precipitate develops.

2. Anti-Rabbit IgG HRP Conjugate

Dilute the anti-Rabbit IgG HRP Conjugate 100-fold with HRP Diluent immediately before use. Prepare 1 mL for each strip used.

3. Standard

***Note**: When opening lyophilized Standard, remove rubber stopper gently as the lyophilizate may have become dislodged during shipping.*

Reconstitute the standard with the volume of ELISA Diluent specified on the vial label to give a concentration of 10ng/mL. Gently swirl the vial and allow the vial to sit for 10 minutes to ensure the material is completely reconstituted. The standard should be reconstituted immediately before the assay. This stock material (tube #1) is then used to generate a standard curve. Any remaining standard can be stored at -80°C. A suggested 2-fold dilution scheme is as follows:

a) Label 7 test tubes #2-7 and "0 dose". Add 150 µL of the ELISA Diluent to tubes #2-7 and "0 dose".
b) Add 150 µL of the stock Standard solution [10 ng/mL] to tube #2 and vortex. This is Standard tube #2 with a concentration of 5 ng/mL.
c) Standards #3-7 are then prepared by performing a 2-fold serial dilution of the preceding standard. Refer to Fig. 1. For example, to make Standard #3, remove 150 µL of Standard #2 and add it to tube #3 and vortex and so on. Do not add any Standard to the "0 Dose" Standard tube.

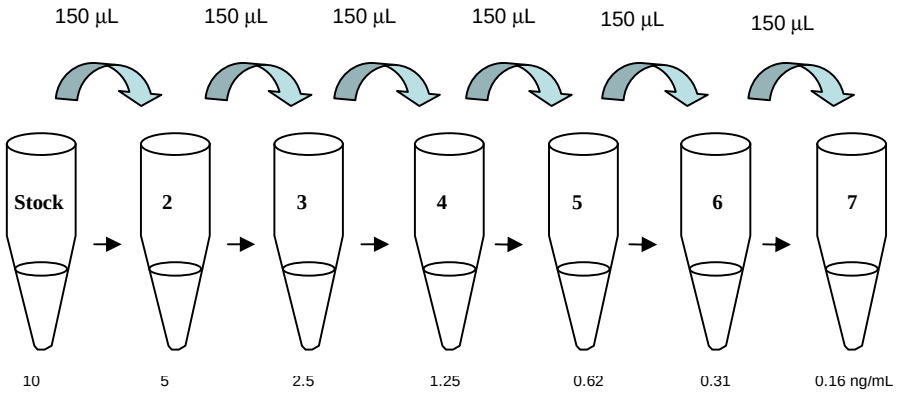


Figure 1: Recommended 2-fold Serial Dilution of Standard

Note: The Standard curve can set up with a different serial dilution scheme by making appropriate adjustments to the dilution pattern.

VII. ASSAY PROTOCOL

1. Prepare the reagents as described in the Reagent Preparation section.
2. Place the desired number of strips in the strip well plate holder. (Re-bag the extra strips and return unused strips to refrigerator for future use.)
3. Add 50 µL of either the Standards 1 through 7 or the samples to wells. Add 50 µL of the zero dose to the control wells. It is recommended that standards and samples be run in duplicate. Wells reserved for TMB blanks should be left empty.

   Note: *It is recommended that standards and samples be run in at least duplicate.*

   Note: *Do not add standard or sample lysate to wells reserved for TMB blanks.*

   Note: *A standard curve must be generated with each assay.*
4. Add 50 µL of the detection antibody to each well except the TMB blank wells.
5. Seal the plate with a plate sealer. Incubate the plate for 2 hours at room temperature (on a shaker if possible).
6. **IMPORTANT WASH STEP:**

   Gently remove the plate sealer and wash the plate at least 4 times. A thorough washing of the plate is extremely important to reduce background. We recommend using a multi-channel pipette to fill each well with 250 µL of diluted Wash Buffer. Fluid removal from the wells is best accomplished by inverting the plate over a sink and flicking the fluid out of the wells and then blotting the plate on clean paper towels. Using the multichannel pipet add 250 µL of Wash Buffer to each well; flick and blot the plate. Repeat this procedure for a total of 4 times.

   For users of automatic plate washers: It is important to ensure that the wash apparatus is properly maintained and operating correctly. Tubing and tips can easily become clogged, leading to incomplete washing and inadequate aspiration of wells. The result may be poor precision and an unsuitable standard curve. For best results, we recommend at least 4 wash cycles.
7. Add 100 µL of a 1:100 dilution of the anti-Rabbit IgG HRP Conjugate to each well. Cover the plate and incubate at room temperature for 30-45 minutes (on shaker with mild agitation if possible). Wash as described in Step 6. Remove all fluid from the wells and blot the wells dry.
8. Add 100 µL of the TMB Solution to each well. Incubate at room temperature in the dark for 10 to 45 minutes, monitor the color development. Stop the reaction by adding 100 µL of Stop Solution to each well. Immediately read the plate at 450 nm. Plate should be read within 1 hour of adding the stop solution.
9. The plate reader may be blanked against a TMB blank prepared by adding 100 µL of stop solution to 100 µL of the TMB solution.

   CAUTION: *Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.*

   NOTE: *For very low starting protein levels, samples can be placed at 37^{o}C during the final incubation to obtain greater sensitivity.*

IX. CALCULATION OF RESULTS

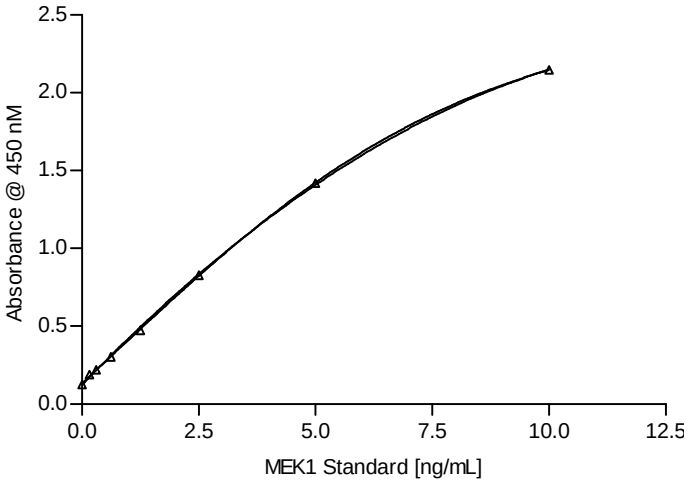


Figure 2. Typical MEK1 Standard Curve

50 µL of progressive 2 fold dilutions of the MEK1 standard included in the kit and run as described in the assay instructions.

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