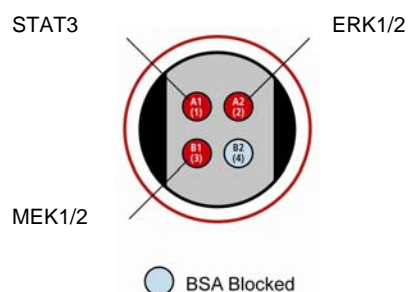


MSD[®] Multi-SPOT[®] ERK-STAT3 Cascade Assay

The following assay protocol has been optimized for quantifying phospho-MEK1/2(Ser217/221), phospho-ERK1/2 (Thr202/Tyr204, Thr185/Tyr187), and phospho-STAT3 (Tyr705) in whole cell lysate.

	Storage
MSD Materials	
<input type="checkbox"/> Read Buffer T (with surfactant), 4X	RT
<input type="checkbox"/> Blocker A	RT
<input type="checkbox"/> MULTI-SPOT ERK-STAT3 Cascade Plate(s)	2-8°C
<input type="checkbox"/> SULFO-TAG [™] Anti-Phospho-STAT3 Antibody (50X)	2-8°C
<input type="checkbox"/> SULFO-TAG Anti-Total ERK Antibody (50X)	2-8°C
<input type="checkbox"/> SULFO-TAG Anti-Phospho-MEK1/2 Antibody (50X)	2-8°C
<input type="checkbox"/> Tris Wash Buffer (10X)	2-8°C
<input type="checkbox"/> Tris Lysis Buffer (1X)	2-8°C
<input type="checkbox"/> Phosphatase Inhibitor I (100X)	2-8°C
<input type="checkbox"/> Phosphatase Inhibitor II (100X)	2-8°C
<input type="checkbox"/> Blocker D-M (2%)	≤-10°C ¹
<input type="checkbox"/> Blocker D-R (10%)	≤-10°C ¹
<input type="checkbox"/> Protease Inhibitor Solution (50X)	≤-10°C
<input type="checkbox"/> PMSF in DMSO (500mM, 250X)	≤-10°C



The SECTOR[®] Imager data file will identify spots according to their well location, not by the coated capture antibody name.

¹ Blockers D-M and D-R can tolerate up to 5 freeze-thaw cycles. Alternatively, an aliquot of blockers D-M and D-R can be stored at 2-8°C for up to 1 month.

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



Notes:

Read the entire detailed instructions before beginning work.

Other Materials & Equipment (not supplied)

- Deionized water for diluting Wash Buffer and Read Buffer
- One 250 mL bottle
- Two 50 mL tube
- One 15 mL tube
- Various microcentrifuge tubes for making serial dilutions of lysates (if desired)
- Adhesive plate seals
- Microtiter plate shaker
- Automated plate washer or other efficient multi-channel pipetting equipment for washing 96-well plates
- Appropriate liquid handling equipment for desired throughput that must accurately dispense 25 μ L and 150 μ L into a 96-well micro plate

Protocol at a Glance

The following protocol describes the most conservative approach toward achieving highly sensitive results using MSD technology to quantify phosphoproteins. The protocol can be completed in approximately 5 1/2 hours if each reagent is prepared during the preceding incubation. All reagents with the exception of diluted lysates can also be prepared ahead of time. This lengthens the overall time required for the assay but frees up time during incubation steps.

Once desired results are achieved, the protocol can be streamlined to eliminate multiple incubation and wash steps to increase throughput.

1. Add Blocking Solution, incubate 1 hour, wash.
2. Add samples or lysate, incubate 3 hours at room temperature, wash.
3. Add Detection Antibody, incubate 1 hour, wash.
4. Add Read Buffer and analyze plate.

Detailed Instructions

Prepare a stock of 1X Tris Wash Buffer:

- a) 1X Tris Wash Buffer will be used throughout the assay to make other reagents as well as wash plates. Approximately 250 mL per plate is required – more if using an automatic plate washer.
- b) In a 250 mL bottle combine:
 - 25 mL 10X Tris Wash Buffer
 - 225 mL deionized water

A larger amount of Tris Wash Buffer may be prepared at once and stored at room temperature for later use.



Notes:**Prepare Blocking Solution-A:**

- a) Prepare 20 mL per plate.
- b) In a 50 ml tube combine:
 - 20 mL 1X Tris Wash Buffer
 - 600 mg Blocker A (30 mg/mL or 3%)

Solutions containing Blocker A should be kept at 4°C and discarded after 14 days.

Prepare Antibody Dilution Buffer:

- a) Prepare 3 mL per plate.
- b) In a 15 mL tube combine:
 - 1 mL Blocking Solution-A
 - 1.82 mL 1X Tris Wash Buffer
 - 150 µL 2% Blocker D-M
 - 30 µL 10% Blocker D-R

Begin with a MULTI-SPOT ERK-STAT3 Cascade Plate.
No pre-treatment is necessary.

STEP 1

Add 150 µL/well of Blocking Solution-A.

Incubate with shaking at room temperature for 1 hour. Prepare Complete Tris Lysis buffer, and prepare samples or dilute cell lysates during this time.

Plates may also be blocked overnight at 4°C.

Prepare Complete Tris Lysis Buffer:

- a) It is important that all of the reagents (including the Tris Lysis Buffer) are room temperature before mixing. To 10 mL of Tris Lysis Buffer, add the following:
 - 100 µL Phosphatase Inhibitor I (100X stock)
 - 100 µL Phosphatase Inhibitor II (100X stock)
 - 200 µL Protease Inhibitor Solution(50X stock)
 - 40 µL PMSF in DMSO (250X stock)
- b) Mix thoroughly for 5 min at room temperature (preferably on a rotator). Keep Complete Tris Lysis Buffer on ice and use buffer as soon as possible following the addition of PMSF.

Complete Tris Lysis Buffer should be made immediately prior to sample dilution and kept on ice at all times.

Prepare samples or positive and negative cell lysates:

(Note: Recommendations for cell lysate handling are provided; however, the suggested concentrations listed below may need to be adjusted depending upon specific samples tested.)

- a) Thaw cell lysate samples on ice and dilute immediately before use. Keep on ice during all manipulations and discard all remaining thawed unused material.



Notes:

- b) Dilute positive and negative cell lysates in Complete Tris Lysis Buffer to a final concentration of 0.8 µg/µL. This will deliver 20 µg/well in 25 µL. A dilution series may also be prepared if desired.

Wash plates four times with Wash Buffer.

STEP 2

Dispense 25 µL/well of samples or diluted lysates.

Incubate with shaking for 3 hours at room temperature. Prepare Detection Antibody Cocktail during this time.

Prepare Detection Antibody Cocktail:

- a) Prepare 3.0 mL per plate.
b) In a 15 mL tube combine:
a. 2.82 mL cold Antibody Dilution Buffer
b. 60 µL 50X Anti-Phospho-STAT3 Antibody
c. 60 µL 50X Anti-Total ERK Antibody
d. 60 µL 50X Anti-Phospho-MEK1/2 Antibody
(Final concentration: 1X for all antibodies)

Wash plates four times with Wash Buffer.

STEP 3

Add 25 µL/well of Detection Antibody Cocktail.

Incubate with shaking at room temperature for 1 hour. Prepare Read Buffer during this time.

Dilute Read Buffer:

- a) Approximately 20 mL per plate is required.
b) Dilute 4X Read Buffer T (with surfactant) to 1X with deionized water.

Wash plates four times with Wash Buffer.

STEP 4

Add 150 µL/well of diluted Read Buffer T (with surfactant).

Analyze with SECTOR Imager instrument.

Shaking a 96-well MULTI-ARRAY[®] or MULTI-SPOT plate accelerates capture at the working electrode.

If certain signals are judged to be too great relative to others in the array, the final concentration of the corresponding detection antibody can be reduced in an effort to equilibrate the signals.

Diluted Read Buffer may be kept in a tightly sealed container at room temperature for later use.

Bubbles in the Read Buffer will interfere with reliable imaging of the plate if carried into the wells.

Plates can be imaged immediately following the addition of read buffer. Most biological interactions tolerate incubation in Read Buffer however each unique assay should be tested for stability in read buffer before being left to sit for extended periods.

