MSD® MULTI-SPOT Assay System

Total eIF2α Kit

1-Plate Kit	K150NGD-1
5-Plate Kit	K150NGD-2
25-Plate Kit	K150NGD-4

MSD Phosphoprotein Assays

Total eIF2α Kit

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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Ordering Information

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Introduction

Eukaryotic protein synthesis is a tightly coordinated process consisting of initiation, elongation, and termination phases. Initiation of mRNA translation requires binding of Met-tRNA to the 40S ribosomal subunit.

Eukaryotic translation initiation factor 2 (eIF2), a heterotrimer consisting of alpha, beta, and gamma subunits, is an essential regulator of translational initiation. Active eIF2-GTP, Met-tRNA, and the 40S subunit form the ternary 43S preinitiation complex.[1,2] Once initiation is completed, inactive eIF2-GDP complex is ejected from the ribosome, and the guanine nucleotide exchange reaction catalyzed by eIF-2β recycles eIF2 to an active state, permitting additional rounds of initiation.[1,2] The alpha subunit, eIF2α, is the regulatory domain of the eIF2 heterotrimer; phosphorylation of eIF2α(Ser51) stabilizes the eIF2-GDP-eIF2β complex, globally repressing translation.[1,2]

Four eIF2α kinases have been identified to date, and each kinase responds to a different stressor. General control non-depressible-2 (GCN2) is activated during amino acid starvation;[3] protein kinase R (PKR) is activated in response to dsRNA;[4] PKR-like endoplasmic reticulum kinase is activated in response to accumulation of misfolded proteins in the endoplasmic reticulum;[5] and heme-regulated inhibitor (HRI) limits protein synthesis in heme-deficient cells.[6]

This assay is used to qualify total eIF2α levels in human, rat, and mouse cell lysates.

Principle of the Assay

MSD phosphoprotein assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample Total eIF2α is a sandwich immunoassay (Figure 1). MSD provides a plate pre-coated with capture antibodies. The user adds the sample and a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™) over the course of one or more incubation periods. Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the detection antibodies by the bound analytes completes the sandwich. The user adds an MSD buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into a SECTOR® Imager where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light to provide a quantitative measure of analytes in the sample.

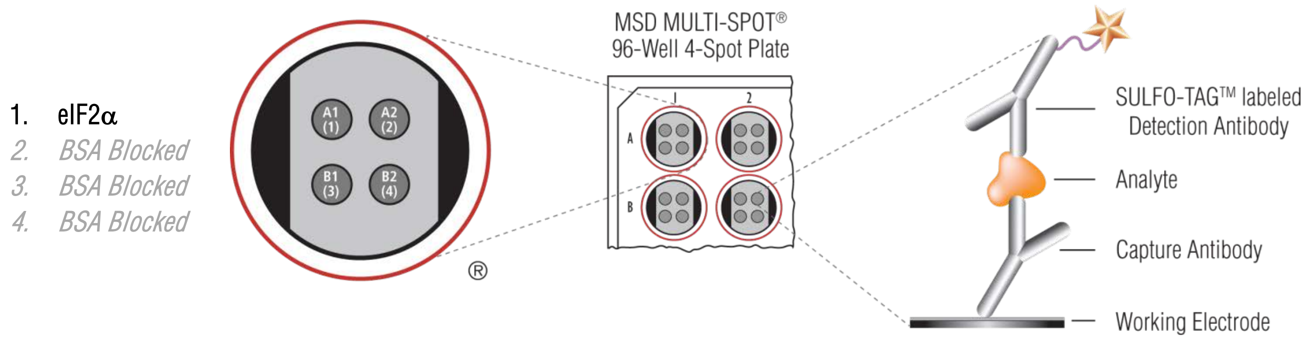


Figure 1. Spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing records.

Reagents Supplied

Product Description	Storage	Quantity per Kit K150NGD-1	K150NGD-2	K150NGD-4
MULTI-SPOT 96-Well 4-Spot Total eIF2α Plate N450NGA-1	2–8°C	1 plate	5 plates	25 plates
SULFO-TAG Anti-Total eIF2α Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
Tris Lysis Buffer (1X) R60TX-3 (50 mL)	2–8°C	1 bottle (50 mL)	1 bottle (50 mL)	5 bottles (50 mL ea)
Tris Wash Buffer (10X) R61TX-2 (200 mL)	2–8°C	1 bottle (200 mL)	1 bottle (200 mL)	5 bottles (200 mL ea)
Phosphatase Inhibitor I (100X)	2–8°C	1 vial (0.1 mL)	1 vial (0.5 mL)	5 vials (0.5 mL ea)
Phosphatase Inhibitor II (100X)	2–8°C	1 vial (0.1 mL)	1 vial (0.5 mL)	5 vials (0.5 mL ea)
Protease Inhibitor Solution (100X)	2–8°C	1 vial (0.1 mL)	1 vial (0.5 mL)	5 vials (0.5mL ea)
Blocker A (dry powder) R93BA-4	RT	1 vial (15 g)	1 vial (15 g)	5 vials (15 g ea)
Read Buffer T (4X) R92TC-3 (50 mL)	RT	1 bottle (50 mL)	1 bottle (50 mL)	5 bottles (50 mL ea)

Required Material and Equipment (not supplied)

- ❑ Appropriately sized tubes and bottles for reagent preparation
- ❑ Microcentrifuge tubes for preparing serial dilutions
- ❑ Liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL/well into a 96-well plate
- ❑ Plate washing equipment: automated plate washer or multichannel pipette
- ❑ Adhesive plate seals
- ❑ Microtiter plate shaker
- ❑ Deionized water

Safety

Use safe laboratory practices and wear gloves, safety glasses, and lab coats when handling kit components. Handle and dispose of all hazardous samples properly in accordance with local, state, and federal guidelines.

Additional safety information is available in the product Material Safety Data Sheet, which can be obtained from MSD Customer Service.

[1] SULFO-TAG–conjugated detection antibodies should be stored in the dark.

Reagent Preparation

Prepare Tris Wash Buffer

Dilute the 10X Tris Wash Buffer to 1X as shown below. Tris Wash Buffer (1X) will be used throughout the assay to make additional reagents and wash plates. Approximately 350 mL per plate is required—more if using an automatic plate washer.

For 1 plate, combine:

- ❑ 35 mL of Tris Wash Buffer (10X)
- ❑ 315 mL of deionized water

Excess Tris Wash Buffer may be stored at room temperature in a tightly sealed container.

Prepare Blocking Solution

For 1 plate, combine:

- ❑ 600 mg of Blocker A (dry powder)
- ❑ 20 mL of 1X Tris Wash Buffer

Prepare Antibody Dilution Buffer

For 1 plate, combine:

- ❑ 1 mL of blocking solution
- ❑ 2 mL of 1X Tris Wash Buffer

Set aside on ice.

Prepare Complete Lysis Buffer

Prepare complete lysis buffer just prior to use. The working solution is 1X.

For 1 plate, combine:

- ❑ 50 µL of Protease Inhibitor Solution (100X stock)
- ❑ 50 µL of Phosphatase Inhibitor Solution I (100X stock)
- ❑ 50 µL of Phosphatase Inhibitor Solution II (100X stock)
- ❑ 4.85 mL of 1X Tris Lysis Buffer

Immediately place the complete lysis buffer on ice; it should be ice cold before use.

Prepare Detection Antibody Solution

MSD provides detection antibody as a 50X stock solution. The working detection antibody solution is 1X.

For 1 plate, combine:

- ❑ 60 µL of 50X SULFO-TAG Anti-Total eIF2α Antibody
- ❑ 2.94 mL of cold antibody dilution buffer

Prepare Read Buffer

MSD provides Read Buffer T as a 4X stock solution. The working solution is 1X.

For 1 plate, combine:

- ❑ 5 mL of Read Buffer T (4X)
- ❑ 15 mL of deionized water

You may prepare diluted 1X read buffer in advance and store it at room temperature in a tightly sealed container.

Prepare MSD Plate

MSD plates are pre-coated with capture antibodies (see Figure 1) and exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies. Plates can be used as delivered; no additional preparation (e.g., pre-wetting) is required.

Sample Preparation and Storage

Most lysis buffers and sample matrices are compatible with MSD plates, although high concentrations of denaturing reagents should be avoided. Keep SDS and other ionic detergents to a concentration of 0.1% or less in the sample applied to the well and avoid reducing agents (DTT >1mM). Please contact MSD Scientific Support if you have any questions about lysate preparation options.

Analysis of proteins in their activated state (i.e. phosphorylated) usually requires stimulation prior to cell lysis. Verify cell stimulation and sample preparation prior to using this kit.

Perform all manipulations on ice; keep PBS wash buffer and complete lysis buffer ice cold. Cell concentrations for lysis can range from 0.5 to 5 x 10^7 cells per mL of lysis buffer. Protein yields will vary by cell line. To get your desired final protein concentration, you will need to optimize the number of cells used and the amount of complete lysis buffer added. Depending on the stability of the target in the matrix, you may need additional protease and phosphatase inhibitors in the matrix or diluent.

MSD provides suggested cell lysis protocols in the appendix; however, specific cell types or targets may benefit from alternative buffer components or techniques, depending upon the particular research application.

Protocol

1. **Block Plate:** Add 150 µL of blocking solution to each well. Seal the plate with an adhesive plate seal and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.

   Prepare complete lysis buffer immediately prior to sample dilution.

2. **Prepare Positive and Negative Cell Lysates:** Thaw cell lysate samples on ice and dilute them immediately before use in ice cold complete lysis buffer. Keep on ice during all manipulations and discard any unused thawed material.

   Lysate samples should be diluted to a working concentration of 50–800 µg/mL using complete lysis buffer as prepared above. This will provide 1.25–20 µg of lysate per well.

   You may prepare a dilution series at this point if desired.

3. **Wash and Add Samples:** Wash the plate 3 times with 300 µL/well of Tris Wash Buffer. Add 25 µL of sample (standards, controls, or unknowns) per well. Seal the plate with an adhesive plate seal and incubate for 3 hours with vigorous shaking (300–1000 rpm) at room temperature.

   You may prepare detection antibody solution during incubation.

4. **Wash and Add Detection Antibody Solution:** Wash the plate 3 times with 300 µL/well of Tris Wash Buffer T. Add 25 µL of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.

   You may prepare diluted read buffer during incubation.

5. **Wash and Read:** Wash the plate 3 times with 300 µL/well of Tris Wash Buffer. Add 150 µL of 1X Read Buffer T to each well. Analyze the plate on the SECTOR Imager. No incubation in read buffer is required.

Notes

Shaking the plate typically accelerates capture at the working electrode.

Store solutions containing MSD Blocker A at 2-8°C; discard after 14 days.

If working with purified protein, only a few nanograms per well will generally provide a strong assay signal. Purified recombinant proteins may exhibit differences in both signal and background as compared to native proteins in cell lysates.

Samples, including cell lysates, may be used neat or diluted.

MSD recommends preparing serial dilutions in microcentrifuge tubes or a separate 96-well polypropylene plate.

The amount of sample required for a given assay will depend on the abundance of the analyte in the matrix and the affinities of the antibodies used.

You may keep excess diluted read buffer in a tightly sealed container at room temperature for later use.

Bubbles introduced when adding read buffer will interfere with plate imaging and produce unreliable data. Use reverse pipetting technique to avoid creating bubbles.

Due to the varying nature of each research application, you should assess assay stability before allowing plates to sit with read buffer for extended periods.

Typical Data

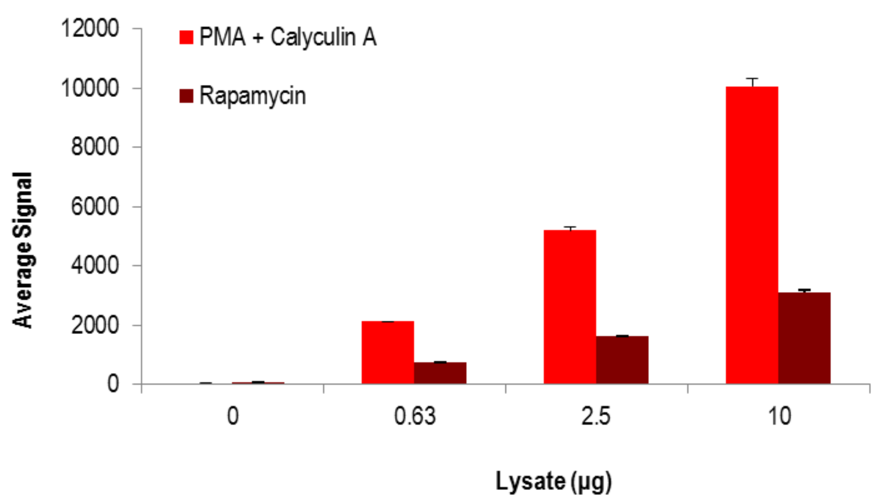
Representative results for the Total eIF2α Kit are illustrated below. The signal and ratio values provided are examples; individual results will vary depending on the samples tested.

Growing Jurkat cells were treated with either PMA (200 nM) and calyculin A (50 nM) for 15 minutes or with rapamycin (1 µM) for 3 hours. Whole cell lysates were added to MSD MULTI-SPOT 4-spot plates coated with anti-total eIF2α antibody on one of the four spatially distinct electrodes in each well. Total eIF2α was detected with anti-total eIF2α antibody conjugated with MSD SULFO-TAG reagent.

Lysate Titration

Data for each treatment using the Total eIF2α Kit is presented below.

Lysate	200 nM PMA+50 nM Calyculin A			1 µM Rapamycin		
(µg/Well)	Average Signal	StdDev	%CV	Average Signal	StdDev	%CV
0	42	5	11.9	57	6	9.9
0.63	2119	19	0.9	736	5	0.7
2.5	5196	122	2.3	1633	7	0.4
10	10060	273	2.7	3118	88	2.8



Assay Components

The capture and detection antibodies used in this assay are listed below. They cross-react with human, mouse, and rat cell lysates.

	Source Species	
Analyte	MSD Capture Antibody	MSD Detection Antibody
Total eIF2α	Mouse Monoclonal	Rabbit Monoclonal

References

1. Hershey JW. Protein phosphorylation controls translation rates. J Biol Chem. 1989 Dec 15; 264(35):20823-6.
2. Kimball SR. Eukaryotic initiation factor eIF2. Int J Biochem Cell Biol. 1999 Jan;31(1):25-29.
3. Grallert B, Boye E. The Gcn2 kinase as a cell cycle regulator. Cell Cycle. 2007 Nov 15;6(22):2768-72.
4. Nalagatla SR, et al. Regulation of innate immunity through RNA structure and the protein kinase PKR. Curr Opin Struct Biol. 2011 Feb; 21(1):119-27.
5. Raven JF, Koromilas AE. PERK and PKR: Old kinases learn new tricks. Cell Cycle. 2008 May 1;7(9):1146-50.
6. Chen JJ. Regulation of protein synthesis by the heme regulated eIF2alpha kinase: relevance to anemias. Blood. 2007 Apr 1;109(7):2693-9.

Appendix: Suggested Cell Lysis Protocols

Preparation in Culture Flask or Petri Dish

Suspension Cells. Pellet cells by centrifugation at 500 x g for 3 minutes at 2–8°C. Discard supernatant and wash the pellet once with cold PBS. Pellet cells again, discard supernatant, and resuspend in complete lysis buffer at 1–5 x 10^7 cells per mL. Incubate on ice for 30 minutes. (A shorter incubation time of 15 minutes may be adequate for many targets.) Clear cellular debris from the lysate by centrifuging (≥10 000 x g) for 10 minutes at 2–8°C. Discard the pellet and determine the protein concentration in the lysate using a detergent-compatible protein assay such as a bicinchoninic acid (BCA) assay. Unused lysates should be aliquoted, quickly frozen in a dry ice-ethanol bath, and stored at ≤-70°C.

Adherent Cells. All volumes given are for cells plated on 15 cm dishes. Remove media from the dish and wash cells once with 5 mL cold PBS. Add 2 mL PBS to each dish, scrape the cells from the surface of the dish, and transfer into 15 mL conical tubes. Pellet the cells by centrifugation at 500 x g for 3 minutes at 2–8°C. Discard supernatant and resuspend cells in 0.5–2 mL of complete lysis buffer per dish. (Alternatively, cells can be lysed by adding 1–2 mL of complete lysis buffer per 15 cm dish after completely removing the PBS wash buffer. Cell lysate can be collected by snapping the dish surface prior to the clarifying spin.) Incubate on ice for 30 minutes. A shorter incubation time of 15 minutes may be adequate for many targets. Clear cellular debris from the lysate by centrifuging (≥10 000 x g) for 10 minutes at 2–8°C. Discard the pellet and determine protein concentration in the lysate using a detergent compatible protein assay such as BCA. Unused lysates should be aliquoted, quickly frozen in a dry ice-ethanol bath, and stored at ≤-70°C.

Preparation in 96-well Culture Plate

Successful adaptation to a 96-well culture format depends on cell type and target. First, determine the number of cells of each cell type to be plated per well. MSD generally recommends plating concentrations ranging from 1 x 10^4 to 10^5 cells per well; however, the optimal concentrations will vary depending on cell line used.

Suspension Cells. You may lyse many cell types without removing growth medium. For flat bottom plates, design the experiment so that the final suspension cell volume per well is such that a concentrated complete lysis buffer (prepared by the user) can be added to the well to achieve a final a 1X lysis buffer concentration in the well. For example, 40 µL of 5X complete lysis buffer added to a well containing 160 µL of cell culture medium would provide a 1X concentration of complete lysis buffer.

For conical microwell plates, perform lysis by pelleting the cells, removing most of the growth medium, and adding a constant amount of 1X complete lysis buffer.

Adherent Cells. Plate cells on coated tissue culture plates to reduce variability due to cells lost as growth medium is removed. Treat cells as desired. Gently aspirate growth medium from the microwell plate to avoid disrupting the cell monolayer. A PBS wash step is not required and can introduce variability as cells may detach during the wash step. Add 100 µL of 1X complete lysis buffer per well. You may modify lysis volume for different cell types or applications.

You will need to determine the optimum cell lysis time. Some targets are immediately available for detection. Other targets may require an incubation step at room temperature or on ice with gentle agitation.

Carefully pipet cell lysate onto prepared plate and proceed with assay protocol. Note: It is important to transfer a constant volume and to avoid introducing air bubbles by pipetting too vigorously..

Summary Protocol

Total eIF2α Kit

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol prior to performing the Total eIF2α assay.

Reagent Preparation

Prepare Tris Wash Buffer.

Prepare blocking solution.

Prepare antibody dilution buffer.

Prepare detection antibody solution by diluting 50X detection antibody 50-fold in antibody dilution buffer.

Prepare 1X Read Buffer T by diluting 4X Read Buffer T 4-fold with deionized water.

Step 1: Block Plate

Add 150 µL/well of blocking solution.

Incubate at room temperature with vigorous shaking (300-1000 rpm) for 1 hour.

Step 2: Prepare Samples

Prepare complete lysis buffer just prior to sample dilution.

Prepare positive and negative cell lysates and keep on ice until use.

Step 3: Wash and Add Sample

Wash plate 3 times with 300 µL/well of Tris Wash Buffer.

Add 25 µL/well of sample (standards, controls, or unknowns).

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 3 hours.

Step 4: Wash and Add Detection Antibody Solution

Wash plate 3 times with 300 µL/well of of Tris Wash Buffer.

Add 25 µL/well of 1X detection antibody solution.

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 1 hour.

Step 5: Wash and Read Plate

Wash plate 3 times with 300 µL/well of Tris Wash Buffer.

Add 150 µL/well of 1X Read Buffer T.

Analyze plate on SECTOR Imager within 5 minutes of adding read buffer.

Plate Diagrams