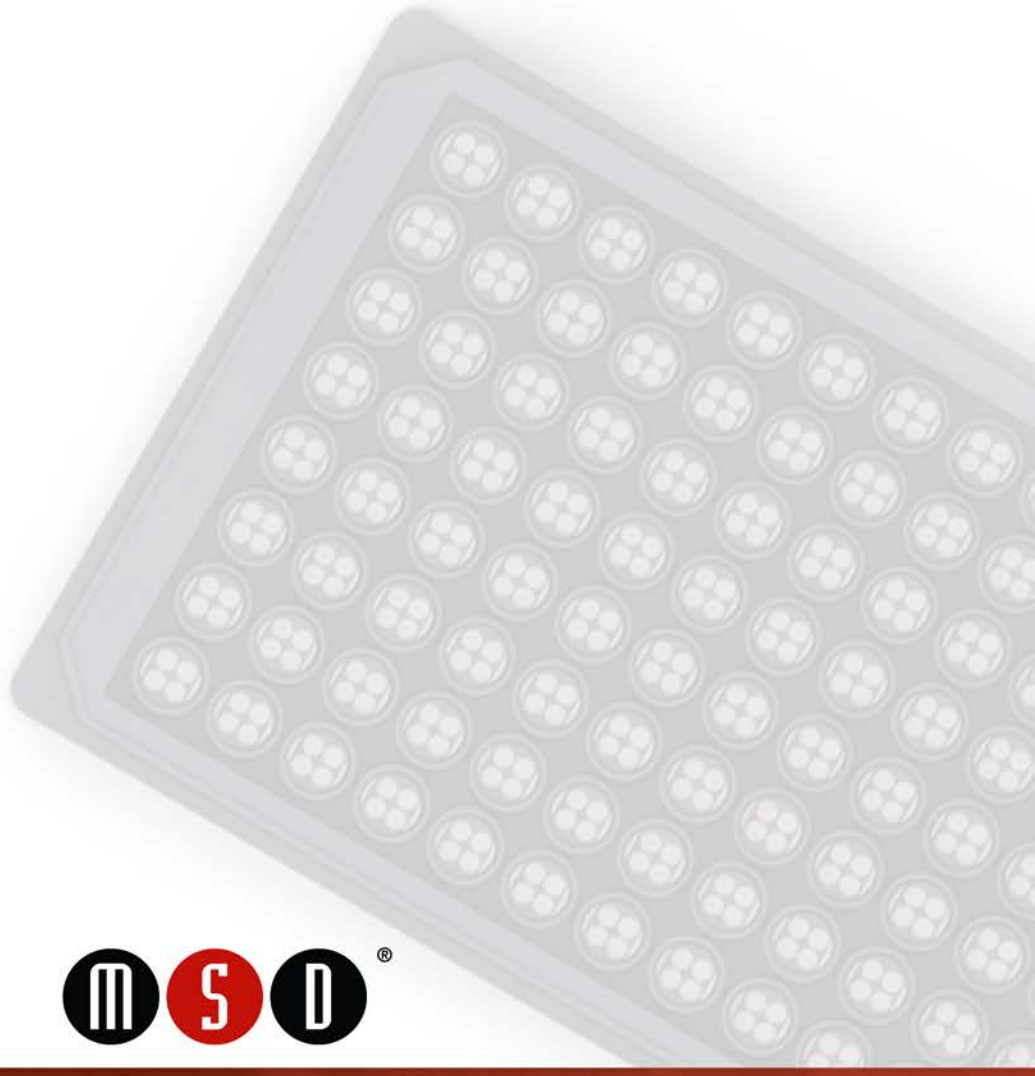
MSD® MULTI-SPOT Assay System

Apoptosis Panel Base Kit

20-Plate Kit K15102A-3



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MSD Phosphoprotein Assays

Apoptosis Panel

Base Kit

Cleaved PARP (Asp214), Cleaved Caspase-3 (Asp175), Phospho-p53 (Ser15), Total p53

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

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

MESO SCALE DISCOVERY®
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MSD Advantage

MESO SCALE DISCOVERY'S unique spot patterns are a hallmark of our MULTI-ARRAY® technology, which enables the measurement of biomarkers utilizing the next generation of electrochemiluminescent detection. In an MSD assay, specific capture antibodies for the analytes are coated in arrays in each well of a 96-well carbon electrode plate surface. The detection system uses patented SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of the MULTI-ARRAY and MULTI-SPOT® plates. The electrical stimulation is decoupled from the output signal, which is light, to generate assays with minimal background. MSD labels can be conveniently conjugated to biological molecules, are stable, and are non-radioactive. Additionally, only labels near the electrode surface are detected, enabling non-washed assays.

One of the advantages of MSD assays is the minimal sample volume required as compared to a traditional ELISA, which is also limited by its inability to measure more than a single analyte. With an MSD assay, up to ten different biomarkers can be analyzed simultaneously using as little as 10-25 µL of sample. These assays have high sensitivity, up to five logs of linear dynamic range, and excellent performance in complex biological matrices. Combined, these advantages enable the measurement of native levels of biomarkers in normal and diseased samples without multiple dilutions. Further, the simple and rapid protocols of MSD assays provide a powerful tool to generate reproducible and reliable results. The MSD product line offers a diverse menu of assay kits for profiling biomarkers, cell signaling pathways, and other applications, as well as a variety of plates and reagents for assay development.

Introduction

Apoptosis is the controlled or regulated destruction of a cell. Multiple systems are involved to ensure that the appropriate cells are destroyed, and healthy (normal) cells are allowed to live. The central players in apoptosis are the caspase family of cysteinyl aspartate-specific proteases, which can be sub-divided into two groups, initiator and effector caspases. Caspases 2, 8, 9, and 10 are initiator caspases and contain N-terminal adaptor domains allowing for auto-cleavage and activation of downstream effector capases, caspases 3, 6, and 7.[1] There are over 100 substrates of the caspase family of proteases, most of which are inactivated due to caspase cleavage. A few of the substrates are activated due to cleavage of inhibitory domains.[2] One of the substrates of caspase-3 is poly (ADP-ribose) polymerase (PARP). PARP is a nuclear enzyme involved in DNA repair.[3] When PARP is cleaved by caspase-3, the N-terminal 24 kDa DNA binding domain is separated from the C-terminal 89 kDa catalytic domain, rendering PARP inactive, but still able to bind DNA, thereby further inhibiting DNA repair.[4] PARP normally promotes cell survival by repairing DNA damage, but when cellular damage is beyond repair, the apoptotic pathway mediated by the caspases inactivates PARP and ensures that severely damaged cells are destroyed. p53 is the most commonly mutated gene in cancer, and a functional copy of p53 is required to maintain a non-tumorigenic phenotype.[5] p53 restricts tumor development by sensing cellular stress and responding to signals such as DNA damage, hypoxia, oncogene expression, and nutrient deprivation by inhibiting cell cycle progress.[6] p53 is negatively regulated by MDM2, and phosphorylation of p53 at Ser15 removes this regulation. During times of cellular stress and DNA damage, p53 is phosphorylated by kinases such as ATR, ATM, and DNAPK, resulting in dissociation from MDM2. p53 acts as a transcription factor of many genes.[7] When cell repair is possible, p53 activates genes which pause the cell cycle allowing time for DNA repair, but in cases of extensive damage, p53 activates the BCL-2 family of proteins leading to apoptosis.[8]

Much research has focused on cleaved caspase-3, cleaved PARP, and phosphorylated p53, to better understand their roles in apoptosis and for the development of pharmacologic treatments capable of inducing or preventing apoptosis.

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. The assays are available in both singleplex and multiplex formats. In a singleplex assay, an antibody for a specific protein target is coated on one electrode (or "spot") per well. In a multiplex assay, an array of capture antibodies against different targets is patterned on distinct spots in the same well. The Apoptosis Panel is a sandwich immunoassay (Figure 1). MSD provides a plate that has been pre-coated with capture antibodies for cleaved PARP (Asp214), cleaved caspase-3 (Asp175), phospho-p53 (Ser15), and total p53 on spatially distinct spots. The user adds the sample and a solution containing the detection antibodies—anti-total PARP, anti-total caspase-3, and anti-total p53 conjugated with an electrochemiluminescent compound, MSD SULFO-TAG label—over the course of one or more incubation periods. Analytes in the sample bind to the capture antibodies immobilized on the working electrode surface; recruitment of the conjugated detection antibody by bound analytes complete the sandwich. The user adds an MSD read buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into an MSD SECTOR® Imager for analysis. Inside the SECTOR Imager, a voltage applied to the plate electrodes causes the labels bound to the electrode surface to emit light. The instrument measures intensity of emitted light to provide a quantitative measure of cleaved PARP (Asp214), cleaved caspase-3 (Asp175), phospho-p53 (Ser15), and total p53 present in the sample.

1. Cleaved PARP (Asp214)
2. Total p53
3. Phospho-p53 (Ser15)
4. Cleaved Caspase-3 (Asp175)

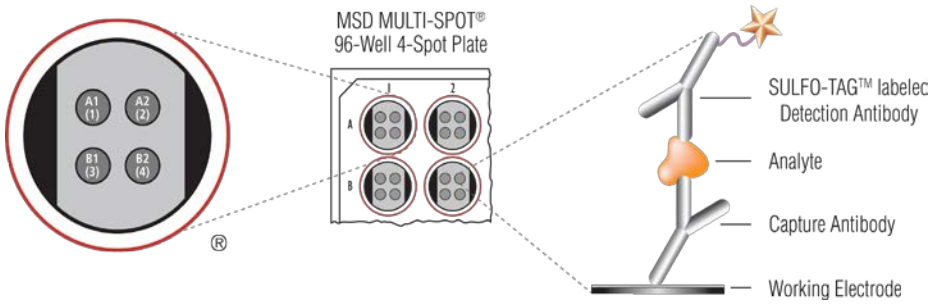

Figure 1. Spot diagram showing placement of analyte capture antibody. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing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

Reagents Supplied

Product Description	Storage	Quantity per Kit K15102A-3
MULTI-SPOT 96-Well 4-Spot Apoptosis Panel Plate N45102B-1	2–8°C	20 plates
SULFO-TAG Anti-PARP Antibody[1] (50X)	2–8°C	4 vials (375 µL ea)
SULFO-TAG Anti-Caspase-3 Antibody[1] (50X)	2–8°C	4 vials (375 µL ea)
SULFO-TAG Anti-Total p53 Antibody[1] (50X)	2–8°C	4 vials (375 µL ea)
Read Buffer T (4X) R92TC-3 (50 mL), R92TC-2 (200 mL)	RT	1 bottle (200 mL)

Required Materials and Equipment - not supplied

- Deionized water for diluting Tris Wash Buffer (10X) and Read Buffer T (4X)
- 500 mL bottle for reagent preparation
- 50 mL tubes for reagent preparation
- 15 mL tubes for reagent preparation
- Microcentrifuge tubes for preparing serial dilutions
- Appropriate liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL into a 96-well microtiter plate
- Plate washing equipment: automated plate washer or multichannel pipette
- Adhesive plate seals
- Microtiter plate shaker

Optional Material — not supplied

- Phosphoprotein Reagent Support Pack (K0000D-3)

[1] Some SULFO-TAG conjugated detection antibodies may be light-sensitive, so they should be stored in the dark.

Safety

Safe laboratory practices and personal protective equipment such as gloves, safety glasses, and lab coats should be used at all times during the handling of all kit components. All hazardous samples should be handled and disposed of properly, in accordance with local, state, and federal guidelines.

Additional product-specific safety information is available in the safety data sheet (SDS), which can be obtained from MSD Customer Service or at www.mesoscale.com.

Reagent Preparation

Note

The instructions below will prepare the reagents needed as described in the assay protocol. All supplemental reagents (inhibitors, buffers, and blocking reagents) are available for purchase in the MSD Phosphoprotein Reagent Support Pack, or alternatively can be purchased and prepared separately by the end user. Please see the enclosed assay development insert for purchasing and preparation instructions.

Prepare Tris Wash Buffer

Dilute 10X stock of 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 one plate, combine:

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

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

Prepare Blocking Solution-A

For one plate, combine:

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

Prepare Antibody Dilution Buffer

For one plate, combine:

- ❑ 30 µL 10% Blocker D-R
- ❑ 1 mL Blocking Solution-A
- ❑ 1.97 mL 1X Tris Wash Buffer

Set aside on ice.

Prepare Complete Lysis Buffer

To 10 mL of Tris Lysis Buffer, add the following supplemental materials to prepare the Complete Lysis Buffer (sufficient for 2-3 plates):

- ❑ 100 µL Protease Inhibitor Solution (100X stock)
- ❑ 100 µL Phosphatase Inhibitor Solution I (100X stock)
- ❑ 100 µL Phosphatase Inhibitor Solution II (100X stock)

The Complete Lysis Buffer should be ice cold before use.

Prepare 5% Blocking Solution-B

For one plate, combine:

- ❑ 50 mg Blocker B (dry powder)
- ❑ 1 mL Complete Lysis Buffer

Mix well. The 5% Blocking Solution-B should be ice cold before use.

Prepare Detection Antibody Solution

For one plate, combine:

- ❑ 2.82 mL Antibody Dilution Buffer
- ❑ 60 µL 50X SULFO-TAG Anti-PARP Antibody (1X final concentration)
- ❑ 60 µL 50X SULFO-TAG Anti-Caspase-3 Antibody (1X final concentration)
- ❑ 60 µL 50X SULFO-TAG Anti-Total p53 Antibody (1X final concentration)

Prepare Read Buffer T

For one plate, combine:

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

Diluted Read Buffer T (1X) may be stored at room temperature in a tightly sealed container for later use.

Prepare MSD Plate

This plate has been pre-coated with antibodies for the analytes shown in Figure 1. The plate can be used as delivered; no additional preparation (e.g., pre-wetting) is required. The plate has also been exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies.

Sample Preparation and Storage

This cell lysis protocol is provided as a reference. Specific cell types or targets may benefit from alternative buffer components or techniques, depending upon the particular research application. Most lysis buffers are compatible with MSD MULTI-SPOT plates, although high concentrations of denaturing detergents (>0.1%) and reducing agents (DTT >1mM) should be avoided. Please contact MSD Scientific Support with any questions regarding lysate preparation options.

All manipulations should be performed on ice. The amount of complete lysis buffer required will vary depending on scale of preparation and type of cells. Larger cells (e.g. NIH3T3, HeLa) should be lysed at concentrations of 1-5 x 10^6 cells per mL of lysis buffer. Smaller cells (e.g. Jurkat) should be lysed at concentrations of 1-5 x 10^7 cells per mL of lysis buffer.

Analysis of proteins in their activated state (i.e. phosphorylated) usually requires stimulation prior to cell lysis. Verification of cell stimulation and sample preparation should be performed prior to using this kit. Phosphate Buffered Saline (PBS) should be ice-cold prior to use.

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 the 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 centrifugation greater than or equal to 10000 x g, at 2-8°C for 10 minutes. Discard the pellet and determine protein concentration in the lysate using a detergent compatible protein assay such as BCA. Unused lysates should be aliquoted and quickly frozen in a dry ice-ethanol bath and stored at ≤-70°C.

Adherent Cells

All volumes are determined for cells plated in 15 cm dishes. Remove media from the plates and wash cells one time with 5 mL cold PBS. Add 2 mL PBS to the plates and 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. 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 centrifugation greater than or equal to 10000 x g, at 2-8°C for 10 minutes. Discard the pellet and determine protein concentration in the lysate using a detergent compatible protein assay such as BCA. Unused lysates should be aliquoted and quickly frozen in a dry ice-ethanol bath and stored at ≤-70°C.

Refer to Appendix I for cell lysate preparation protocol modifications that accommodate the use of 96-well culture plates.

Assay Protocol

The following protocol describes the most conservative approach to achieving optimal results with the MULTI-SPOT Apoptosis Panel. The entire assay, including plate analysis on the MSD reader, can be completed in 3.5 hours. Once desired results are achieved, the protocol can be streamlined to eliminate multiple incubations and wash steps. Samples may be prepared for testing in the manner outlined in the Sample Preparation and Storage section.

1. Block Plate and Prepare Samples:
   a. Add 150 µL of Blocking Solution-A into each well. Seal the plate with an adhesive plate seal and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.
   b. Prepare Complete Lysis Buffer just prior to sample dilution.

      Note: Samples, including cell lysates, etc., may be used neat or after dilution.

      - MSD plates are compatible with most sample matrices. Avoid reagents that will denature the capture antibodies (e.g. high concentrations of reducing agents such as DTT should be avoided, and also SDS and other ionic detergents should be 0.1% or less in the sample applied to the well).
      - Depending on the stability of the target in the matrix, additional protease and phosphatase inhibitors may be required in the matrix or diluent.
      - 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.
      - Keep diluted samples on ice until use

   c. Prepare positive and negative cell lysates:

      (if purchased separately).

      - Thaw cell lysate samples on ice, and dilute them immediately before use. Keep on ice during all manipulations, and discard all remaining thawed, unused material.
      - Dilute cell lysate and 5% Blocking Solution-B together in Complete Lysis Buffer to a final concentration of 0.8 µg/µL. This will deliver 20 µg/well lysate in 25 µL and 0.5% Blocking Solution-B.

Notes

Read entire protocol prior to beginning the assay.

Solutions containing MSD Blocker A should be stored at 2-8°C and discarded after 14 days.

Complete lysis buffer should be kept ice-cold during all experimental manipulations.

The sensitivity of MSD immunoassays rivals that of ELISAs and Western blots. 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.

Samples and standards cannot be serially diluted in the MSD plate. Use microcentrifuge tubes or a separate 96-well polypropylene plate to prepare dilutions.

- For example, to prepare 200 µL of 0.8 µg/µL lysate-0.5% Blocking Solution-B, combine the following:
  - ❑ 20 µL 5% Blocking Solution-B
  - ❑ 80 µL 2 mg/mL lysate
  - ❑ 100 µL Complete Lysis Buffer
- A dilution series may also be prepared if desired. Use a stock of Complete Lysis Buffer-0.5% Blocking Solution-B (dilute 5% Blocking Solution-B 1:10 in Complete Lysis Buffer) for any subsequent lysate dilutions.

2. **Wash and Add Samples:** Wash the plate 3 times with 300 µL/well of Tris Wash Buffer. Add 25 µL of samples per well. Seal the plate with an adhesive plate seal and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.

   Prepare Detection Antibody Solution during this time.

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

   Prepare 1X Read Buffer T during this time.

4. **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 of the MSD plate.

   Analyze the plate on the MSD instrument:

   a. Double click on DISCOVERY WORKBENCH® icon on computer desktop (if not already open).
   b. Click the imager icon in upper left corner of screen (if not already open to plate reading screen).
   c. From the pull down menu select "Read From Barcode."
   d. If only reading one plate check "Return Plate to Input Stack." Then check "Read Plates(s)" checkbox and enter 1.
   e. If reading multiple plates, check the "Read Plate(s)" checkbox and enter number of plates to be read in the text field. For example, if five plates need to be read, type in "5."
   f. Click the "Run" button. The "Run Options" window will be displayed.

Notes

Shaking a 96-well MSD MULTI-ARRAY or MULTI-SPOT plate during an incubation step will typically accelerate capture at the working electrode.
The lysate sample incubation time provided is optimized for the use of MSD cell lysates. Samples from other sources may require a longer incubation.

Excess diluted read buffer may be kept in a tightly sealed container at room temperature for later use.

Bubbles introduced during the read buffer addition will interfere with imaging of the plate and produce unreliable data.

Plate should be imaged within 5 minutes following the addition of read buffer. Due to the varying nature of each research application, assay stability should be investigated prior to allowing plates to sit with read buffer for extended periods.

An all-inclusive indelible copy of the data and associated instrument information will be saved on the internal database, regardless of data file export selection. Additional copies of the data can be exported in any layout at a later time using this database. Consult the instrument user manual for more information.

g. If the data from each microplate is to be exported as individual files, select “Separate Files” in the “Export” area of the “Run Options” window. Select “Appended File” if all data from the entire stack run is to be exported to one file. Select “Default” in the “Export Format” area. Check the box to export default data file.

h. If desired, make selections to export a custom data file.

i. Browse and select the location to export data files.

j. Click OK to initiate the run.

k. Data will be automatically saved in the software database. Text versions of the requested data files will be exported to the designated folder.

Analysis of Results

The percent phosphoprotein in a sample can be calculated using independent MSD phosphoprotein and total protein singleplex assays or MSD phospho-/total multiplex phosphoprotein assays.

INDEPENDENT ASSAY FORMAT: Anti-Total Singleplex and Anti-Phospho-Singleplex Assays
% Phosphoprotein = (Phospho-signal / Total signal) x 100

MULTIPLEX ASSAY FORMAT: Anti-Total and Anti-Phospho-Assay in the same well
% Phosphoprotein = ((2 x Phospho-signal) / (Phospho-signal + Total signal)) x 100

Note:

1. The above calculation assumes that the capture antibodies on the anti-phospho and anti-total spots have very similar binding affinities.
2. The numerator in the equation contains a distribution factor of 2 based on the assumption that the phosphorylated isoform of the protein binds with a similar affinity to the phospho-specific and total capture antibodies. Given equivalent binding of the phosphorylated isoform to both capture antibodies, half of the phosphorylated species will be captured by the phospho-specific and the other half will be captured by the phosphorylation-independent (total) antibody. Therefore, the phospho-specific signal can be referred to as 2X of the phospho spot.
3. The denominator is "phospho + total" because this represents the total of all the analyte captured on both of the spots.
4. If the % phosphorylation is > 100%, then the distribution factor in the numerator may be adjusted to less than 2X such that the % phosphorylation with the control lysates is 100%.

Example:

Phosphoprotein Assay							
Lysates (µg)	Positive Control Lysate			Negative Control Lysate			P/N
	Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
0	245	4	1.4	242	6	26.0	
5.0	19,235	2,342	12.2	461	3	0.6	42

Total Protein Assay							
Lysates (µg)	Positive Control Lysate			Negative Control Lysate			P/N
	Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
0	561	18	3.2	569	19	3.4	
5.0	7,304	1,227	16.8	14,530	585	4.0	0.5

% Phosphoprotein = [(2 x Phospho signal) / (Phospho signal + Total signal)] x 100

Therefore, % phosphoprotein with 5 µg of positive lysate will be:

[(2 x 19,235) / (19,235 + 7,304)] x 100 = 144% phosphorylation

In this case, the constant in the numerator may be adjusted using the control lysates as follows:

[(1.38 x 19,235) / (19,235 + 7,304)] x 100 = 100% phosphorylation

1.38 should be used as the numerator for further calculations in the same experiment.

Typical Data

Representative results for the MULTI-SPOT Apoptosis Panel are illustrated below. The signal and ratio values provided below are example data; individual results may vary depending upon the samples tested.

Results with Jurkat cells treated with etoposide: Control Lysates for Cleaved Caspase-3 and Cleaved PARP

Logarithmically growing Jurkat cells (negative) were treated with etoposide (25 µM; 18 hours) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-cleaved PARP (Asp214), anti-cleaved Caspase-3 (Asp175), anti-total p53, and anti-phospho-p53 (Ser15) antibodies on the four spatially distinct electrodes per well. Cleaved PARP, cleaved Caspase-3, total p53, and phospho-p53 were detected with anti-PARP, anti-Caspase-3 and anti-total p53 antibodies conjugated with MSD SULFO-TAG reagent. Western blot analyses of each lysate type were performed with phospho-p53, total p53, cleaved and full-length Caspase-3, and cleaved and full-length PARP antibodies and are shown below for comparison.

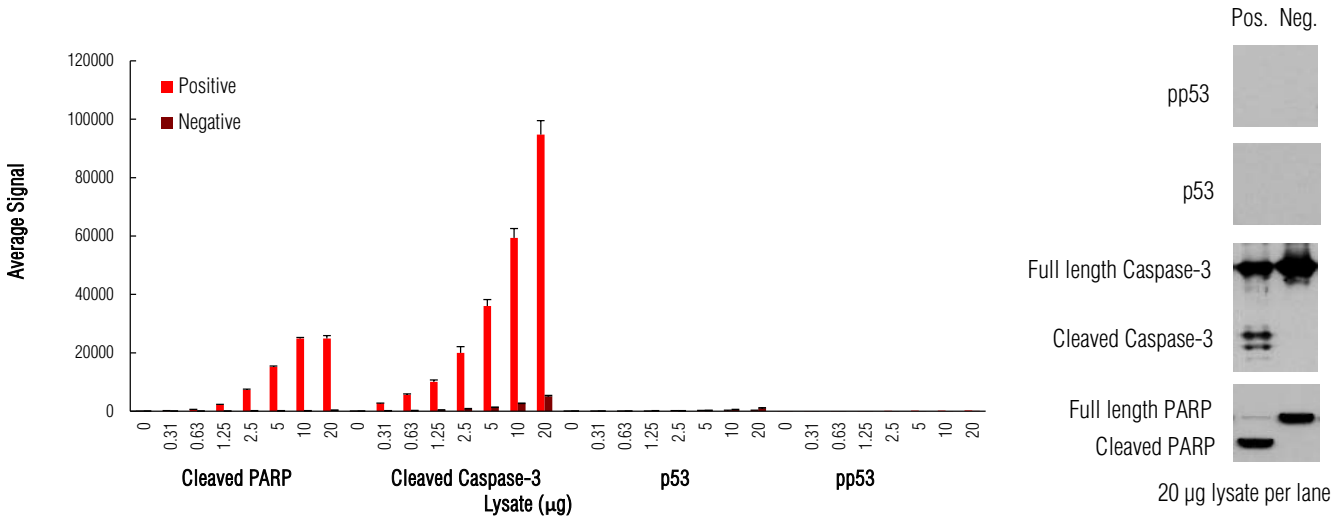

Figure 2: *Sample data generated with MULTI-SPOT Apoptosis Panel. Increased signals for cleaved PARP and cleaved Caspase-3 were observed with only Apoptosis Panel positive cell lysate. Signals for phospho- and total p53 remained low throughout the titration of positive lysate. Signals for negative lysate were also low throughout the titration for all assays. The Apoptosis Panel provides a quantitative measure of the data obtained with the traditional Western blot.*

Lysate Titration

Data for positive and negative Jurkat cell lysates using the MULTI-SPOT Apoptosis Panel are presented below.

	Lysate (µg)	Positive			Negative			P/N
		Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
Cleaved PARP	0	70	15	21.1	72	7	9.1	
	0.31	216	20	9.3	76	16	21.2	2.8
	0.63	582	39	6.6	67	17	24.7	8.7
	1.3	2,248	82	3.7	75	15	19.9	30
	2.5	7,259	304	4.2	102	16	15.7	71
	5.0	15,184	286	1.9	118	10	8.6	129
	10	24,894	382	1.5	157	17	11.1	159
	20	24,883	1,008	4.1	325	41	12.7	76
Cleaved Caspase-3	0	45	11	23.4	47	9	19.2	
	0.31	2,724	93	3.4	143	17	12.2	19
	0.63	5,655	248	4.4	221	21	9.4	26
	1.3	10,085	597	5.9	400	46	11.5	25
	2.5	19,978	2,132	10.7	764	79	10.4	26
	5.0	36,055	2,163	6.0	1,373	24	1.7	26
	10	59,357	3,203	5.4	2,660	86	3.2	22
	20	94,707	4,845	5.1	5,038	308	6.1	19
p53	0	43	17	38.2	53	18	33.8	
	0.31	65	19	28.7	64	7	10.2	1.0
	0.63	77	12	15.0	83	4	4.2	0.9
	1.3	92	7	7.7	101	18	18.1	0.9
	2.5	171	29	16.9	167	9	5.6	1.0
	5.0	234	29	12.5	293	8	2.6	0.8
	10	281	49	17.6	500	52	10.4	0.6
	20	350	44	12.7	986	153	15.5	0.4
pp53	0	42	2	4.1	42	13	31.0	
	0.31	64	33	51.3	47	7	14.9	1.4
	0.63	76	21	28.2	45	10	22.2	1.7
	1.3	86	13	15.2	53	13	23.7	1.6
	2.5	155	17	11.1	71	9	12.2	2.2
	5.0	212	49	23.3	88	10	11.3	2.4
	10	213	45	21.3	93	4	4.3	2.3
	20	258	37	14.4	121	9	7.1	2.1

Results with HT-29 cells treated with UV irradiation: Control Lysates for Phosphorylated and Total p53

Growing HT29 cells (negative) were harvested 1 hour after UV irradiation (40 mJ/cm^2) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-cleaved PARP (Asp214), anti-cleaved Caspase-3 (Asp175), anti-total p53, and anti-phospho-p53 (Ser15) antibodies on the four spatially distinct electrodes per well. Cleaved PARP, cleaved Caspase 3, total p53, and phospho-p53 were detected with anti-PARP, anti-Caspase-3 and anti-total p53 antibodies conjugated with MSD SULFO-TAG reagent. Western blot analyses of each lysate type were performed with phospho-p53, total p53, cleaved and full-length Caspase-3, and cleaved and full-length PARP antibodies and are shown below for comparison.

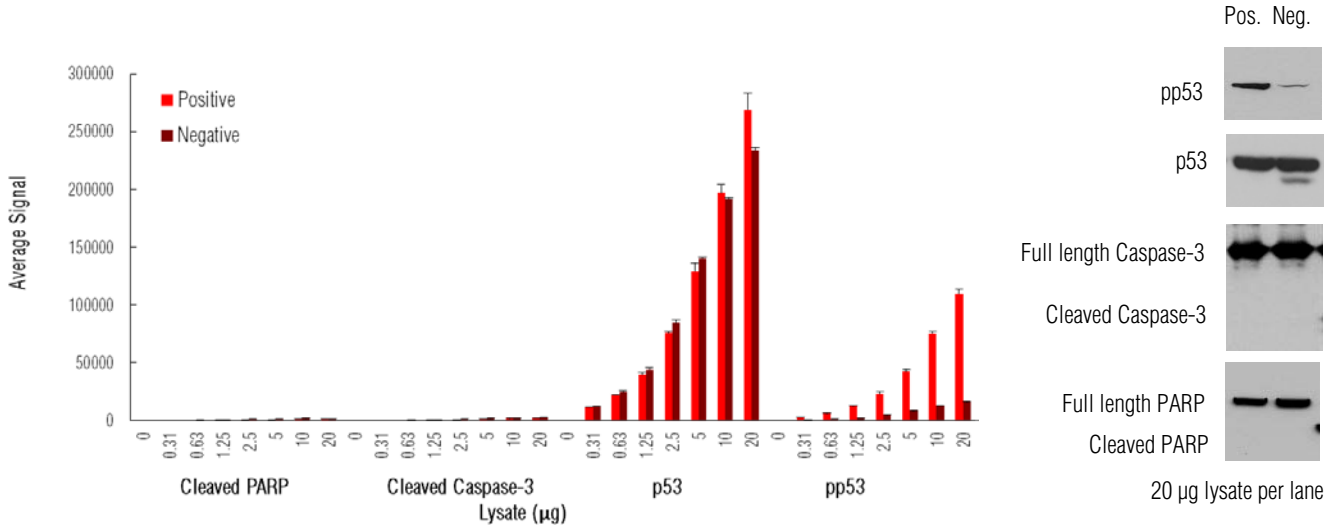

Figure 3 *Sample data generated with MULTI-SPOT Apoptosis Panel. Increased signals for phospho-p53 was observed with only Apoptosis Panel positive cell lysate, whereas total p53 signals increased throughout the titration of both positive and negative cell lysates. Cleaved PARP and cleaved Caspase-3 signals were low for both positive and negative lysates. The Apoptosis Panel provides a quantitative measure of the data obtained with the traditional Western blot.*

Lysate Titration

Data for positive and negative HT29 cell lysates using the MULTI-SPOT Apoptosis Panel are presented below.

	Lysate (µg)	Positive			Negative			P/N
		Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
Cleaved PARP	0	74	11	14.3	79	15	18.7	
	0.31	338	3	0.9	473	24	5.1	0.7
	0.63	510	10	2.0	754	88	11.7	0.7
	1.3	627	13	2.0	919	62	6.7	0.7
	2.5	930	77	8.2	1,384	121	8.8	0.7
	5.0	1,131	65	5.8	1,710	132	7.7	0.7
	10	1,392	139	10.0	1,964	208	10.6	0.7
	20	1,346	137	10.1	1,747	161	9.2	0.8
Cleaved Caspase-3	0	46	4	7.7	50	7	14.0	
	0.31	273	9	3.2	390	26	6.5	0.7
	0.63	457	27	5.8	646	31	4.8	0.7
	1.3	607	25	4.0	880	98	11.1	0.7
	2.5	960	81	8.4	1,319	173	13.1	0.7
	5.0	1,336	141	10.6	1,896	189	10.0	0.7
	10	1,975	52	2.6	2,473	172	6.9	0.8
	20	2,504	140	5.6	2,990	321	10.7	0.8
p53	0	47	9	19.2	54	17	31.4	
	0.31	11,357	117	1.0	12,347	236	1.9	0.9
	0.63	21,965	602	2.7	24,519	1,247	5.1	0.9
	1.3	39,540	1,953	4.9	44,097	1,470	3.3	0.9
	2.5	75,930	1,404	1.8	84,456	2,425	2.9	0.9
	5.0	128,996	7,564	5.9	140,694	713	0.5	0.9
	10	197,309	7,096	3.6	192,061	507	0.3	1.0
	20	269,034	14,612	5.4	234,171	1,861	0.8	1.1
pp53	0	36	7	19.4	47	3	6.9	
	0.31	3,037	324	10.7	836	23	2.7	3.6
	0.63	6,221	328	5.3	1,568	132	8.4	4.0
	1.3	12,464	594	4.8	2,402	60	2.5	5.2
	2.5	23,293	1,849	7.9	4,616	509	11.0	5.0
	5.0	42,591	1,774	4.2	8,316	168	2.0	5.1
	10	75,668	1,267	1.7	12,872	186	1.4	5.9
	20	110,010	3,671	3.3	16,155	543	3.4	6.8

Assay Components

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

	Source Species	
Analyte	MSD Capture Antibody	MSD Detection Antibody
Cleaved PARP	Rabbit Monoclonal	Rabbit Polyclonal
Cleaved Caspase-3	Rabbit Polyclonal	Goat Polyclonal
Phospho-p53	Mouse Monoclonal	Goat Polyclonal
p53	Mouse Monoclonal	Goat Polyclonal

Limitations of the Procedure

The following points should be noted with the MULTI-SPOT Apoptosis Panel to maximize assay sensitivity and performance.

- A no-wash assay format may be employed, however lower sensitivity may be observed.
- All buffers containing phosphate should be avoided when detecting phosphoproteins.
- Due to the unstable nature of phosphoproteins, cell lysates should be thawed immediately prior to use, and any remaining thawed material should be subsequently discarded.

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Appendix

96-well Culture Plate Modifications

Successful adaptation to a 96-well culture format is cell type and target-dependent. The number of cells to be plated per well should be determined for each cell type. General recommended plating concentrations for adherent cells range from 1 x 10^4 – 5 x 10^4 cells per well and approximately 2 x 10^6 cells per mL (50 - 75 µL per well) for suspension cells. These numbers are provided as a guide, and the optimal concentrations will vary depending upon cell line used.

Suspension Cells

For flat bottom plates, experiments should be designed such that the final volume per well is 50 – 75 µL. Perform cell lysis using a 4X complete lysis buffer concentrate, supplemented with protease and phosphatase inhibitors at 4X concentrations. Add 4X complete lysis buffer directly to cells in the growth medium for a final 1X concentration in the well.

Note: With some effort, a 10X complete lysis buffer can also be prepared.

(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 biologically treated tissue culture ware [such as BD BioCoat Cellware (Becton, Dickinson and Company, Franklin Lakes, NJ)] to reduce variability due to cells lost as growth medium is removed. Treat cells as desired. Gently aspirate growth medium from microwell plate. A PBS wash step is not required and can introduce variability. Add 50-100 µL 1X complete lysis buffer per well.

Cell lysis time should be determined by the end user. Some targets are immediately available for detection. Other targets may require an incubation step at room temperature, 45°C, or on ice with gentle agitation.

Carefully pipet cell lysate onto prepared capture plate, and proceed with assay protocol.

It is important to transfer a constant volume and avoid pipetting too vigorously, as the introduction of air bubbles may result. (Targets can be captured from a volume greater than 25 µL).

Summary Protocol

MSD 96-well MULTI-SPOT Apoptosis Panel Assay Kit

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol prior to performing the MULTI-SPOT Apoptosis Panel Assay.

Step 1 : Block Plate and Prepare Samples

Add 150 µL/well of blocking solution.

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

Prepare Complete Lysis Buffer just prior to sample dilution.

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

Step 2 : Wash and Add Sample

Wash the plate 3 times with 300 µL/well of 1X Tris Wash Buffer.

Dispense 25 µL/well samples.

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

Step 3 : Wash and Add Detection Antibody Solution

Wash the plate 3 times with 300 µL/well of 1X Tris Wash Buffer.

Dispense 25 µL/well of 1X Detection Antibody Solution.

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

Step 4 : Wash and Read Plate

Wash the plate 3 times with 300 µL/well of 1X Tris Wash Buffer.

Dispense 150 µL/well of 1X Read Buffer T.

Analyze plate on MSD instrument within 5 minutes of read buffer addition.

Plate Diagrams