Development of a Multiplex Array to Simultaneously Monitor p53 Phosphorylation and the Induction of Apoptosis

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❶ Abstract

The p53 tumor suppressor can induce growth arrest, apoptosis or cell senescence in response to diverse cellular stresses. It is the most commonly mutated gene in human cancers and these traits make this an attractive target for therapeutic intervention. Regulation of p53 function is tightly controlled and is modulated primarily at the level of protein stability. Phosphorylation of p53, in response to DNA damage, blocks interaction with negative regulators that target this protein for degradation via the ubiquitin pathway. Using Meso Scale Discovery's patterned array technology we have developed a multiplex assay for the measurement of p53 phosphorylation as well as the activation of two important apoptotic markers, the cleavage of Caspase-3 and PARP. This assay allows for simultaneous, rapid and sensitive measurement of phospho-p53 levels as well as provides a snapshot into the apoptotic status of the cell, which would be valuable when screening for regulators of p53 function.

❷ MSD MULTI-ARRAY™ Technology and MULTI-SPOT® Plates

Instrument Features

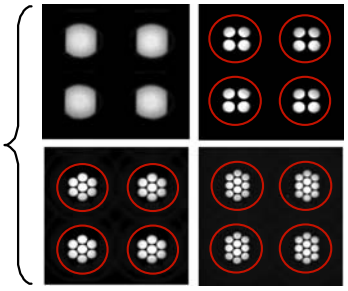
- Highly sensitive imaging detection systems
- Single and multiplex plate formats
- SECTOR Imager 6000 designed for high-throughput screening (HTS)
- Rapid read times
- SECTOR Imager 6000 or SECTOR PR 100 instruments ideal for assay development
- Electrochemiluminescence (ECL) detection

SECTOR PR™ 100 Reader

SECTOR™ Imager 6000

Plate Features

- Disposable plates
- Carbon electrodes with high binding capacity
- Screen printing affords easy patterning
- Suitable electrochemistry for ECL
- A variety of surface treatments, array preparations and coatings are available



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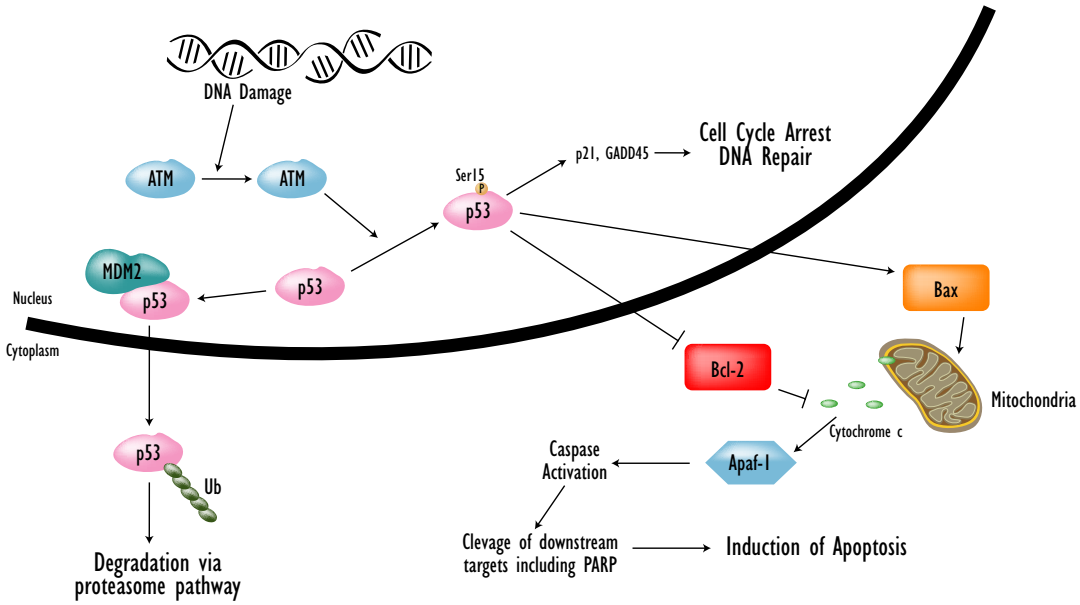
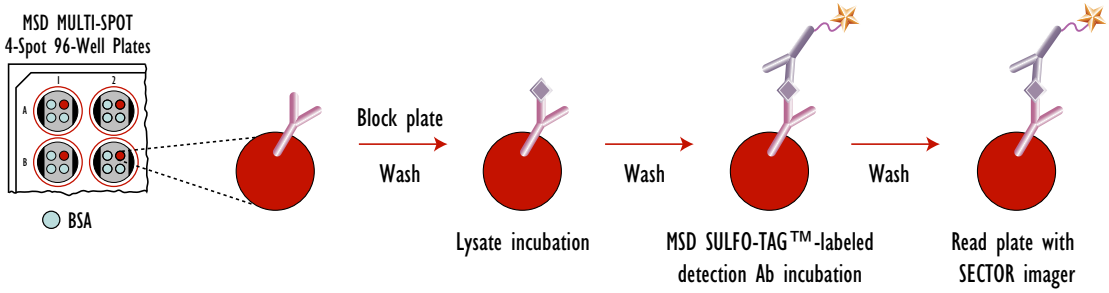
❸ p53 Signaling Pathways



❹ Sandwich Immunoassay Protocol



- Add 150 µL blocking solution/well of MSD 4-spot high bind plate coated with capture antibody, incubate at RT, 2hr
- Wash plates
- Dispense 25 µL/well of prepared lysates, incubate at RT, 1hr
- Wash plates
- Add 25 µL/well of diluted MSD SULFO-TAG-labeled detection antibody, incubate at RT, 1hr
- Wash plates
- Add 150 µL MSD read buffer
- Analyze with SECTOR Imager

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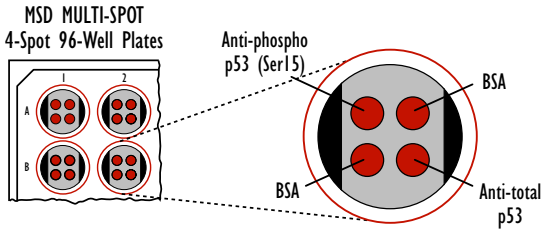
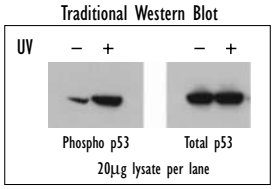
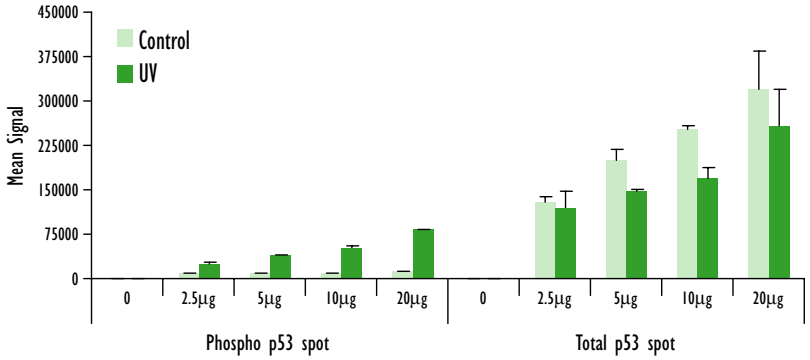
❺ Multiplex p53 Assay: Detection of Phosphorylated and Total p53 in the Same Well



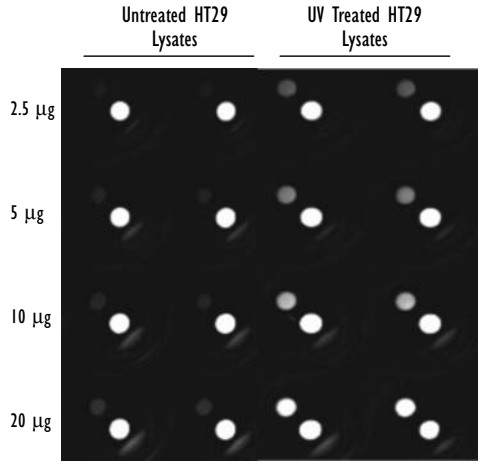


Whole cell lysates were prepared from untreated confluent HT29 cells or cells harvested one hour after UV irradiation (40mJ/cm²). Lysates were added to MSD MULTI-SPOT 4 Spot 96-well high bind plates coated with anti-phospho-p53 antibody (Ser15) and anti-total-p53 antibody (125 fmoles/spot). BSA was coated onto the remaining two electrodes in each well. Phosphorylated and total p53 were detected with 5nM anti-total-p53 antibody labeled with MSD SULFO-TAG reagent.



Lysate [µg]	Control		UV-treated		S/B
	Ave	%CV	Ave	%CV	
0	165	3	141	3	0.9
2.5	8035	16	24992	11	3.1
5	8585	1	40285	2	4.7
10	9011	1	53398	5	5.9
20	11525	5	81312	1	7.1

Lysate [µg]	Control		UV-treated		S/B
	Ave	%CV	Ave	%CV	
0	588	27	695	17	1.2
2.5	129232	7	120236	20	0.9
5	197627	12	147757	1	0.7
10	252273	2	167174	8	0.7
20	316840	26	257884	19	0.8



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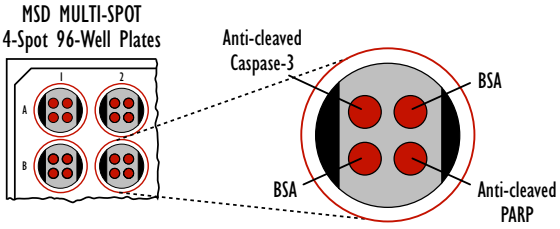
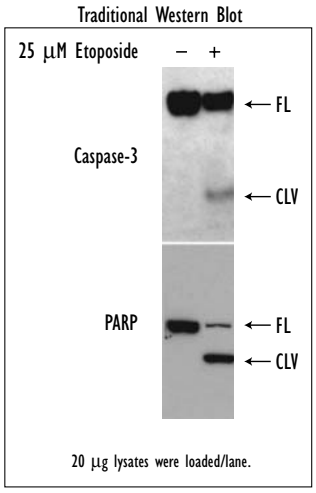
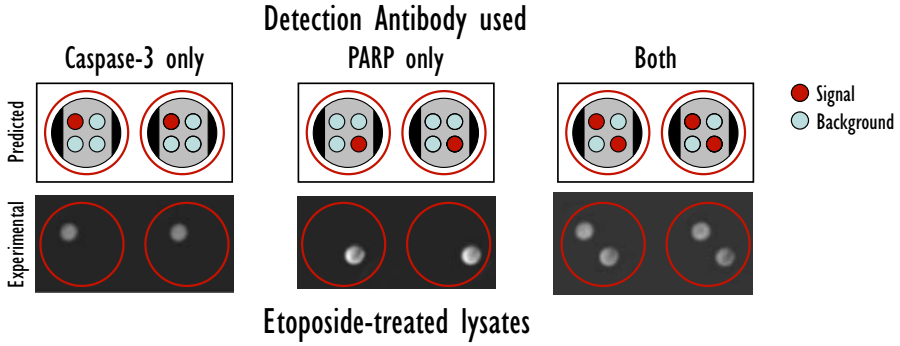
6 Caspase-3 and PARP Duplex Assay





Lysates were prepared from growing Jurkat cells or Jurkat cells treated with 25 µM etoposide for 18 hr. The increasing amounts of Jurkat whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well high bind plates coated with 125 fmoles of the following antibodies: anti-cleaved Caspase-3 or anti-cleaved PARP. BSA was coated onto the remaining electrodes in each well. MSD SULFO-TAG-labeled reporter antibodies were used at the following concentrations: anti-Caspase-3 (0.5nM) and anti-PARP (1nM). Cleaved Caspase-3 and PARP protein levels go up following treatment with etoposide.



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6 Caspase-3 and PARP Duplex Assay (cont.)

Caspase-3 detection antibody only

Caspase-3 Spot

Lysate [µg]	Control		Etoposide		S/B
	Ave	%CV	Ave	%CV	
0	36	36	44	34	1.2
1	90	12	1654	14	8.4
5	153	17	5373	21	35.0
10	196	9	7487	6	38.2

PARP detection antibody only

PARP Spot

Lysate [µg]	Control		Etoposide		S/B
	Ave	%CV	Ave	%CV	
0	19	26	24	9	1.3
1	32	50	36	13	1.1
5	25	41	2996	17	118.3
10	29	19	7667	5	267.5

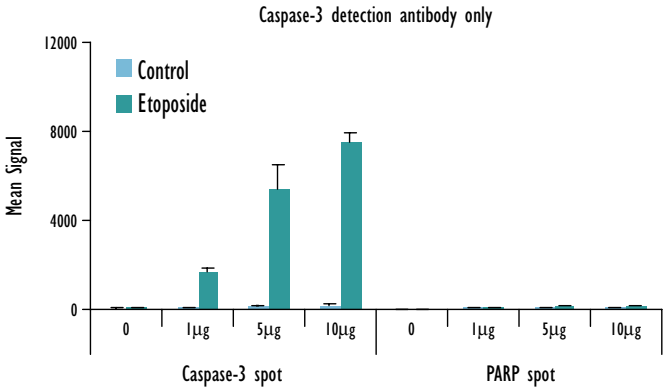
Caspase-3 detection antibody only

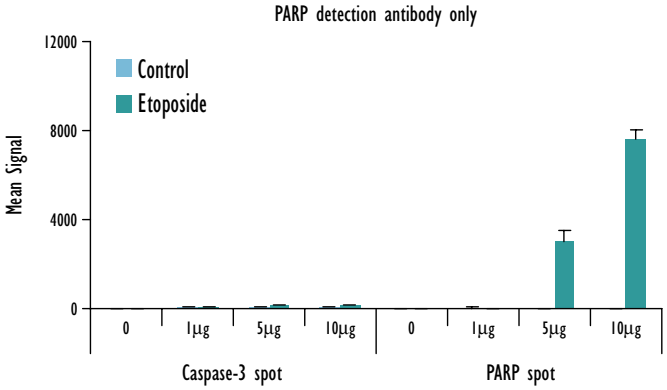
Caspase-3 Spot

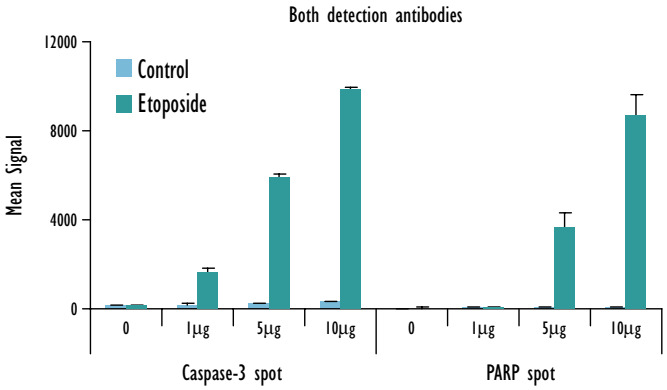
Lysate [µg]	Control		Etoposide		S/B
	Ave	%CV	Ave	%CV	
0	165	9	174	5	1.1
1	200	4	1685	10	8.4
5	235	9	5915	2	25.2
10	326	4	9832	1	30.1

PARP Spot

Lysate [µg]	Control		Etoposide		S/B
	Ave	%CV	Ave	%CV	
0	35	15	39	50	1.1
1	75	2	112	3	1.5
5	102	4	3604	19	35.4
10	87	11	8694	11	99.9







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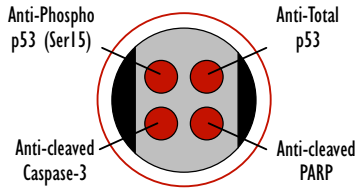
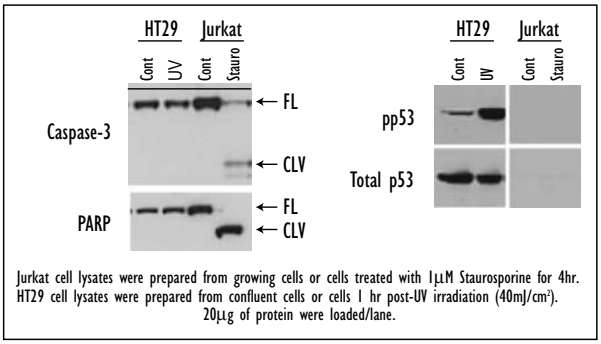
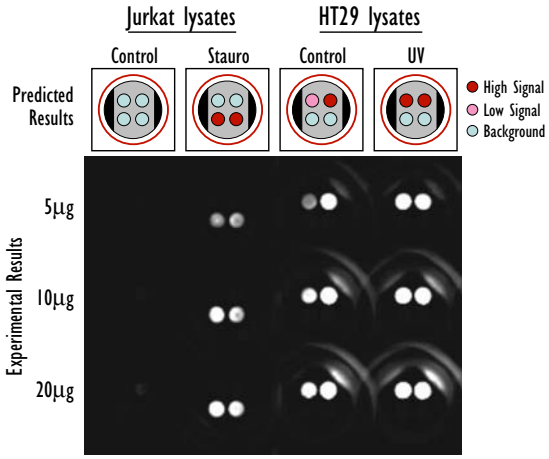
❼ Phospho and total p53, cleaved Caspase-3 and PARP Multiplex Assay

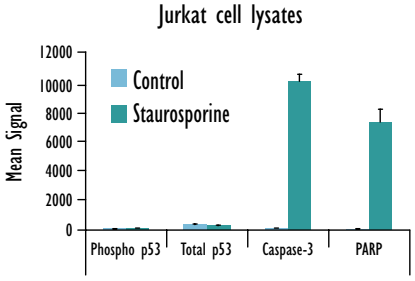




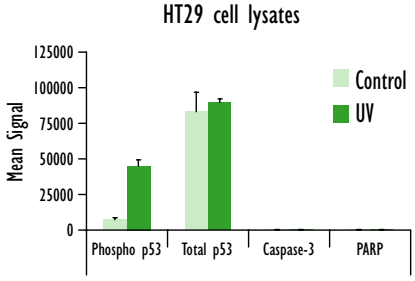
Jurkat cell lysates were prepared from growing cells or cells treated with 1µM Staurosporine for 4hr. HT29 cell lysates were prepared from confluent cells or cells 1 hr post-UV irradiation (40mJ/cm²). 20µg of protein were loaded/lane.



The indicated cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well high bind plates coated with the following antibodies: anti-phospho p53, anti-total p53, anti-cleaved Caspase-3, and anti-cleaved PARP. All capture antibodies were used at 125 fmoles/spot except for total p53 which was 75 fmoles/spot. MSD SULFO-TAG-labeled reporter antibodies were used at the following concentrations: anti-p53 (5nM), anti-Caspase-3 (0.5nM), anti-PARP (1nM).



Spot	Control		Staurosporine		S/B
	Ave	%CV	Ave	%CV	
Phospho p53	76	7	121	5	1.6
Total p53	343	13	262	18	0.8
Caspase-3	129	7	10043	5	78.2
PARP	57	19	7292	12	129.1



Spot	Control		UV-treated		S/B
	Ave	%CV	Ave	%CV	
Phospho p53	7310	16	44899	10	6.1
Total p53	82955	17	89581	3	1.1
Caspase-3	399	0	368	1	0.9
PARP	416	6	356	1	0.9

Data presented in the graphs/tables above are from 10µg of the indicated whole cell lysates.

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8 Conclusions

We have described novel techniques for convenient, rapid and sensitive multiplex measurement of phosphorylated and total p53 protein levels and key players in the apoptotic pathway, active Caspase-3 and cleaved PARP.

The combination of MSD's array technology and electrochemiluminescence detection provides a more sensitive alternative to ELISA while allowing for higher throughput than traditional Western blots. The use of Meso Scale Discovery's MULTI-SPOT plates allows for direct analysis of multiple targets in a single well using a single cell lysate.

The use of this multiplex cell-based sandwich immunoassay format could dramatically improve current methods for high throughput screening, assay development, and drug discovery for regulators of the p53 signaling pathway while simultaneously monitoring the apoptotic status of the cell.

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