MSD™ Technology: A New Way to Measure Classic Markers of Apoptosis

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Abstract

Apoptosis is a genetically determined, biochemically-ordered process in which cells are induced to initiate a cellular suicide program in response to physiologic signals, cellular damage or viral infection. In normal cells, the pathway is very tightly controlled, but deregulation of the apoptotic pathway contributes to the pathogenesis of many human diseases including cancer, AIDS, neurodegenerative diseases and autoimmune disorders. Using Meso Scale Discovery's MULTI-SPOT™ plates, we have developed a multiplex assay in which we can simultaneously measure the protein levels of three important members of the apoptotic pathway. Here we show data in which active caspase-3, cleaved PARP and total Bcl-2 proteins are measured in a single well from a single whole cell lysate. This sandwich immunoassay uses capture antibodies that are coated onto the surface of the plates, whole cell lysates are then incubated with the plates, followed by detection of the captured protein with an antibody labeled with an electrochemiluminescent tag. The results of this assay closely mimic those seen in western blot analysis. The use of MULTI-SPOT plates and MSD electrochemiluminescence technology offers many advantages over other traditional technologies such as ELISA by allowing for convenient, rapid and sensitive multiplex measurement of intracellular targets.

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

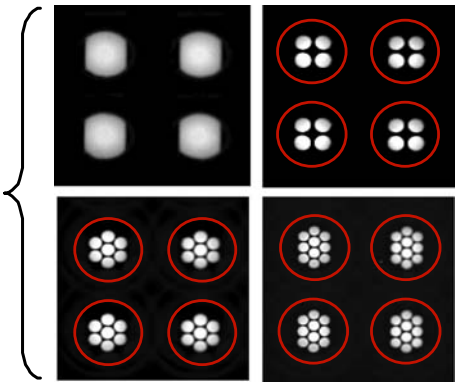
Instrument Features

- 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™ Imager 6000

SECTOR™ PR 100 Reader

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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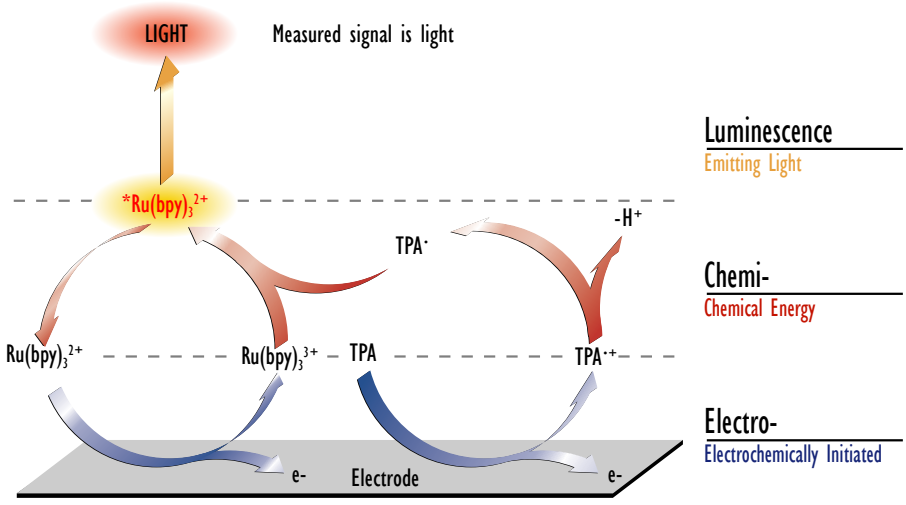
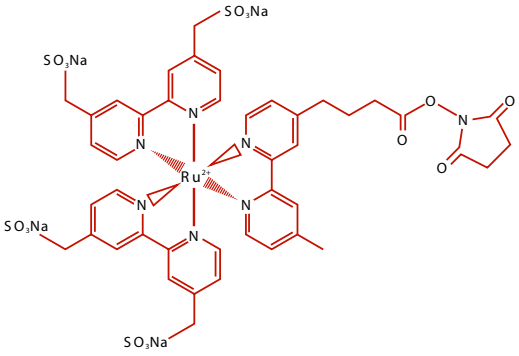
❸ Electrochemiluminescence (ECL)



Ruthenium (II) tris-bipyridine-(4-methylsulfone) NHS ester (MSD SULFO-TAG™ label)



- Selective
- Convenient chemistry
- Robust, stable
- Few interferences

- Size, MW: ~1200 daltons
- Stability: Years
- Solubility: Aqueous, DMSO
- Functionality: Hydrophilic
- Specificity: High

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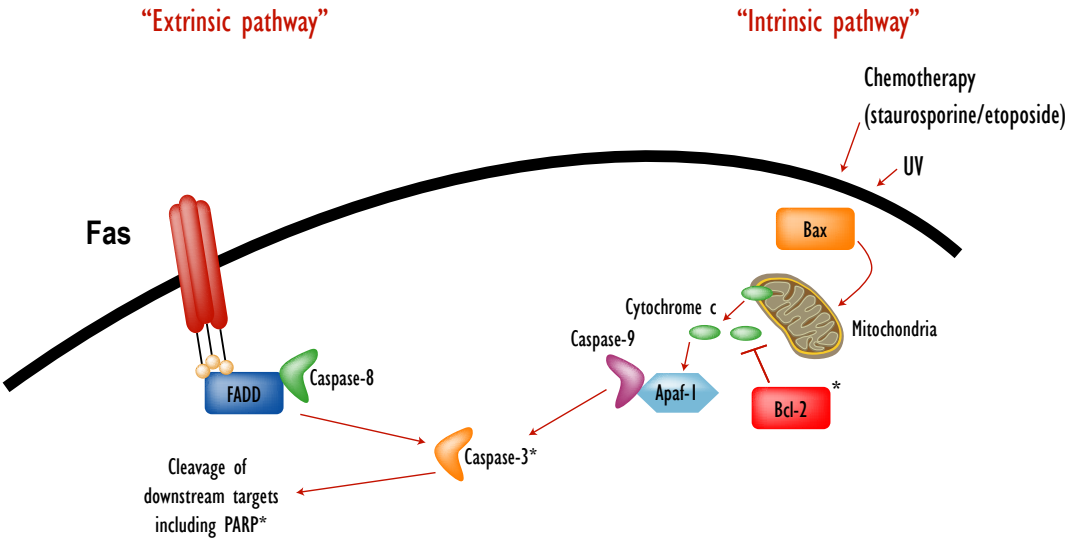
❹ Apoptotic Pathways



There are two principal convergent pathways that lead to the activation of apoptosis- the so-called extrinsic and intrinsic pathways. The extrinsic pathway is exemplified by a receptor-initiated pro-apoptotic signal, such as that initiated by Fas. The intrinsic pathway is initiated by cellular stress such as chemotherapy or UV irradiation which results in the translocation of Bax to the mitochondria and the subsequent release of cytochrome C. Bcl-2 opposes the activity of Bax and inhibits apoptosis by preventing cytochrome C release from the mitochondria. Both of these pathways result in the activation of a family of cysteine proteases called the caspases. Caspases are synthesized as inactive precursors and are activated by proteolytic cleavage. Upstream caspases such as caspases-8 and -9 are cleaved and activated and then go on to activate the common downstream caspase, caspase-3. Cleaved, activated caspase-3 has a number of cellular targets including poly-ADP ribose polymerase or PARP.

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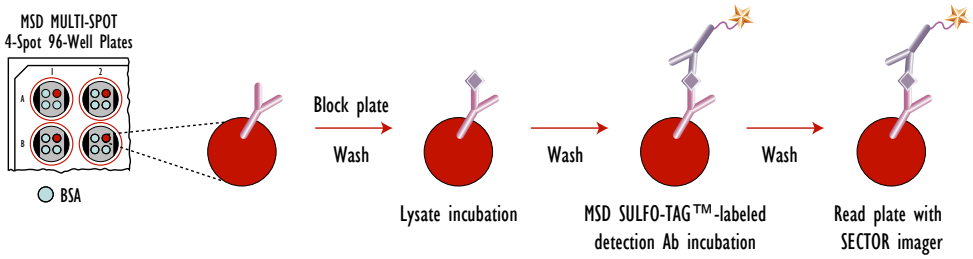
5 Sandwich Immunoassay Protocol



- 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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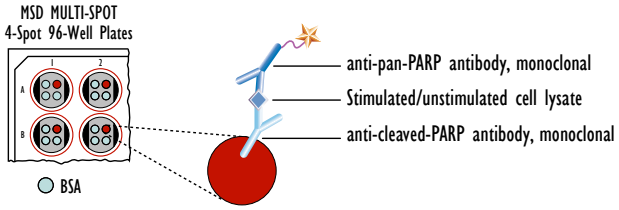
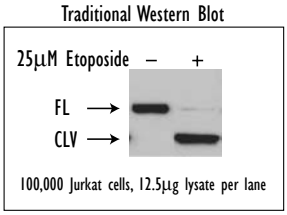
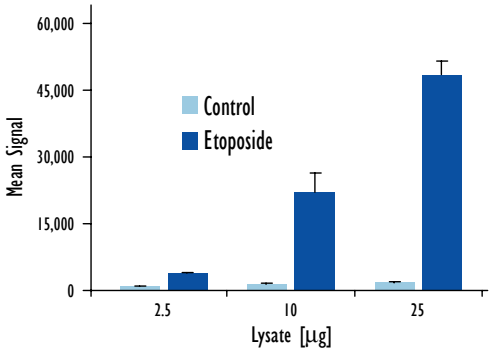
6 Detection of Cleaved PARP in Whole Cell Lysates







Lysate [µg]	Control			18h Etoposide			S-B	S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
2.5	803	22	3	3853	171	4	3051	4.8
10	1,241	7	1	21,960	4,337	20	20,719	17.7
25	1,918	240	13	48,644	2,688	6	46,726	25.4

Logarithmically growing Jurkat cells were treated with etoposide for 18h. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with anti-cleaved-PARP antibody on one of the four spatially distinct electrodes per well. BSA was coated onto the remaining three electrodes in each well. Cleaved PARP was detected with 10nM anti-pan-PARP antibody labeled with MSD SULFO-TAG label.

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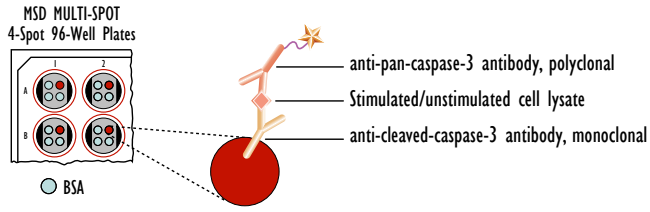
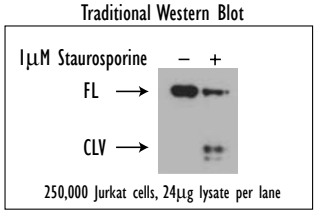
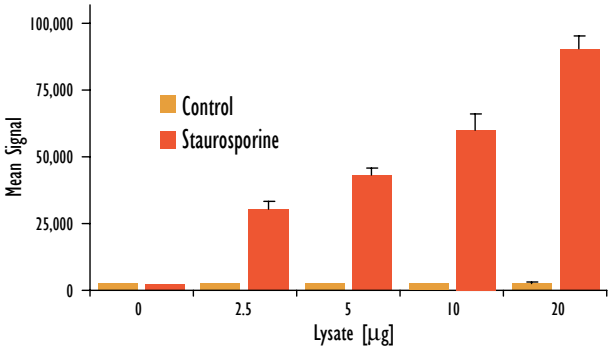
7 Detection of Active Caspase-3 in Whole Cell Lysates







Lysate [µg]	Control			Staurosporine			S-B	S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
0	2,495	22	1	2,435	192	8	-60	1.0
2.5	2,181	133	6	30,398	3,156	10	28,217	13.9
5	2,385	254	11	43,607	2,109	5	41,222	18.3
10	2,359	219	9	60,355	6,422	11	57,996	25.6
20	2,535	701	28	90,822	4,930	5	88,287	35.8

Logarithmically growing Jurkat cells were treated with staurosporine for 4h. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with anti-cleaved-caspase-3 antibody on one of the four spatially distinct electrodes per well. BSA was coated onto the remaining three electrodes in each well. Cleaved active caspase-3 was detected with 10nM anti-pan-caspase-3 antibody labeled with MSD SULFO-TAG label.

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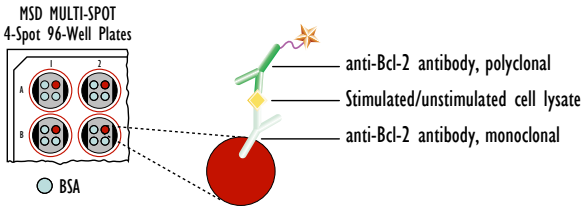
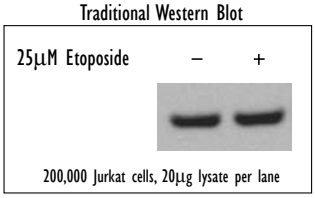
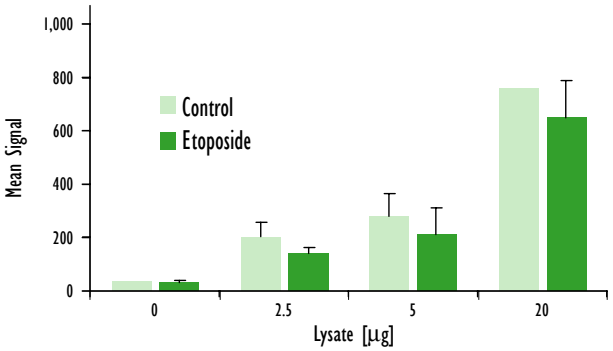
8 Detection of Bcl-2 in Whole Cell Lysates







Logarithmically growing Jurkat cells were treated with etoposide for 18h. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with anti-Bcl-2 antibody on one of the four spatially distinct electrodes per well. BSA was coated onto the remaining three electrodes in each well. Total Bcl-2 protein was detected with 5nM anti-Bcl-2 antibody labeled with MSD SULFO-TAG label.

Lysate [mg]	Unreated			Etoposide			S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV	
0	37	5	12	35	3	9	0.9
2.5	204	55	27	141	25	17	0.7
5	284	86	30	215	99	46	0.8
20	756	5	1	648	138	21	0.9

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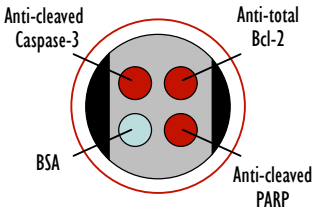
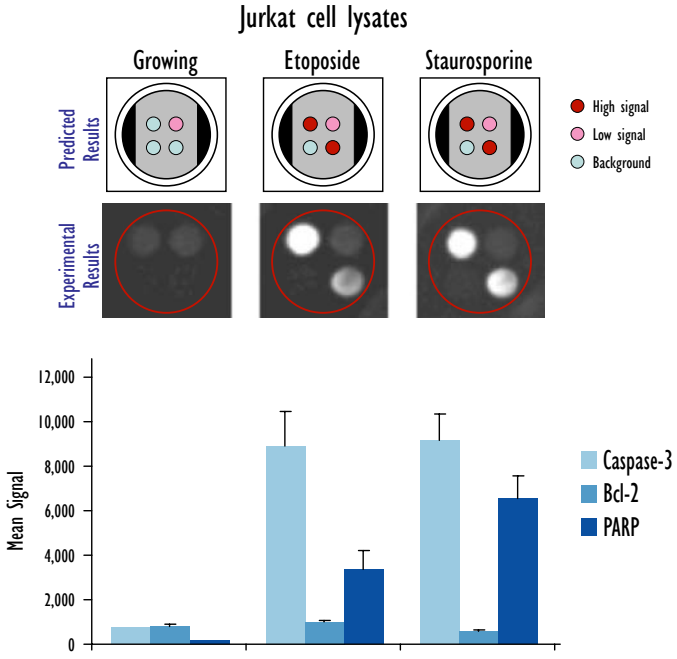
❾ Caspase-3, Bcl-2 and PARP Multiplex



The indicated Jurkat whole cell lysates (10µg/well) were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with the following antibodies: anti-cleaved caspase-3, anti-Bcl-2 and anti-cleaved PARP. BSA was coated onto the remaining electrode in each well. MSD SULFO-TAG-labeled reporter antibodies were used at the following concentrations: anti-caspase-3 (0.5nM), anti-Bcl-2 (5nM) and anti-PARP (1nM). Cleaved caspase-3 and PARP protein levels go up following drug treatment while Bcl-2 protein levels remain the same.



	S/B Ratios (treated/growing)	
	Etoposide	Staurosporine
Caspase-3	11.9	12.3
Bcl-2	1.2	0.7
PARP	24.5	47.5

❿ Conclusions

We have described novel techniques for convenient, rapid and sensitive multiplex measurement of key players in the apoptotic pathway, cleaved, active caspase-3, total Bcl-2 and cleaved PARP. 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 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 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 apoptotic pathway.

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