Development of MULTI-ARRAY® and MULTI-SPOT® Immunoassays for Cell Cycle Targets

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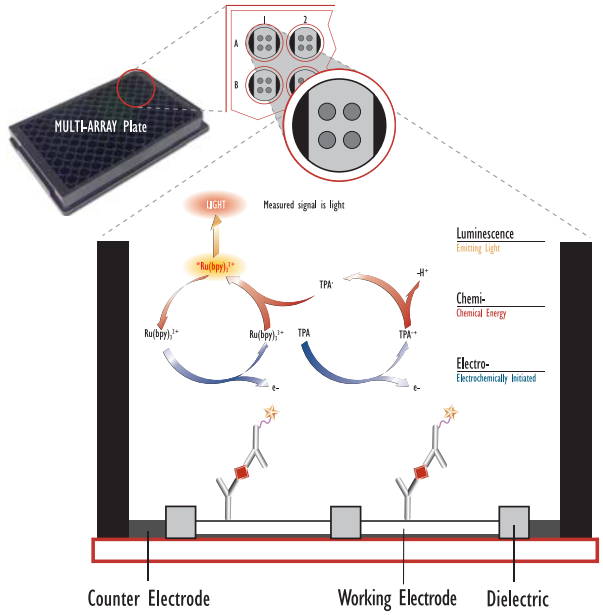
The cell cycle is controlled by an ordered set of interactions among signaling molecules and structural proteins. Deregulation of the cell cycle can result in an uncontrolled increase in cell divisions, a hallmark of carcinogenesis. Many molecules involved in cell cycle regulation can serve as biomarkers for cancer progression and for effects of anti-cancer therapies. We developed highly sensitive electrochemiluminescence immunoassays for the detection of post-translational modifications of several key proteins involved in cell proliferation – phospho-Histone H3 (Ser10), phospho-Rb (Ser608), phospho-Rb (Ser780), phospho-Aurora A (Thr288), phospho-p53 (Ser15), ubiquitinated p53 and ubiquitinated MDM2. We also present an assay to detect DNA-binding activity of p53 in nuclear extracts. The assays employ Meso Scale Discovery's patterned array technology in 96-well MULTI-SPOT® plates. In many cases we demonstrate parallel detection of the post-translationally modified and total protein pools in the same well. The assays are sensitive, quantitative, involve simple protocols, and results obtained from treated and untreated cells agree with those obtained by traditional Western blot analysis.

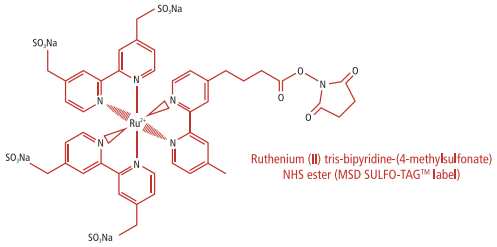
The MSD® Platform

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY and MULTI-SPOT microplates.



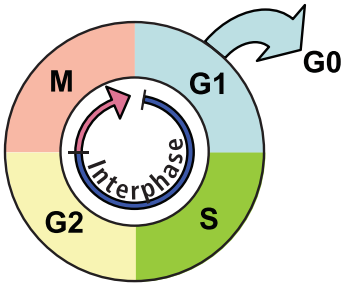
Electrochemiluminescence Features:

- Minimal background signals and high signal to background ratios - the stimulation mechanism (electricity) is decoupled from the signal (light)
- Proximity - only labels bound near the electrode surface are detected, enabling non-washed assays
- Flexibility - labels are stable, non-radioactive, and are conveniently conjugated to biological molecules
- Emission at ~620 nm - eliminating problems with color quenching
- Signal amplification - multiple excitation cycles of each label enhance light levels and improve sensitivity



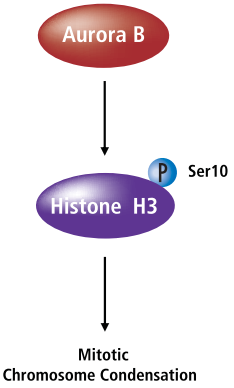
The Cell Cycle

Cell cycle is a fundamental biological process governing cell proliferation and accurate division of the genetic information between daughter cells. Cell cycle in eukaryotes consists of four stages. In the G1 phase cells are growing and preparing for duplication of the genome. In the S phase DNA is replicated to generate a copy for each daughter cell. In the G2 phase the cell prepares for mitosis. In the M (mitosis) phase the nuclear envelope temporarily breaks down, matching sets of chromosomes are distributed between two daughter cells, and nuclear envelopes are newly assembled.



Histone Modifications

Histones are a family of positively charged proteins found in chromatin of all eukaryotic cells. Histones associate with nuclear DNA to form nucleosomes. Post-transcriptional modifications of histones play an important role in regulation of transcription and chromatin rearrangements throughout the cell cycle. Phosphorylation of Ser10 of Histone H3 is a distinctive marker of dividing cells since highly condensed metaphase chromosomes contain this modification. Mitosis-specific Histone H3 (Ser10) phosphorylation is carried out by Aurora B kinase. Phosphorylation of Histone H3 (Ser10) can also occur at lower levels in interphase and is linked to transcriptional activation of mitogen-stimulated immediate-early response genes, and some other inducible genes.



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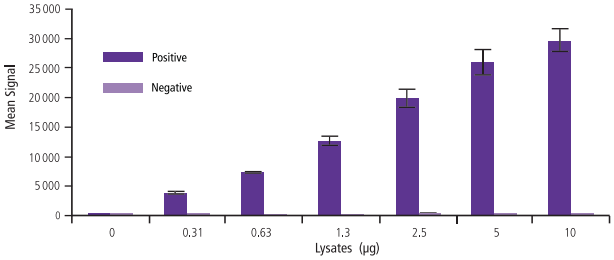
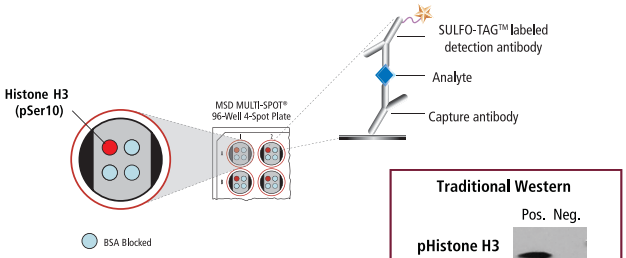
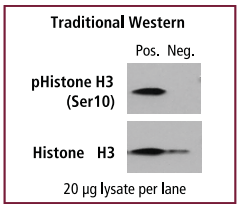
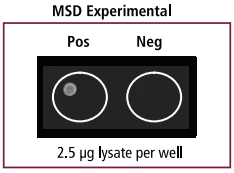
Detection of Phospho-Histone H3 (Ser10)





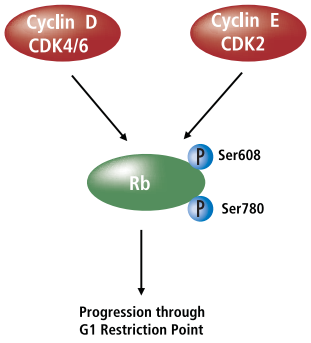




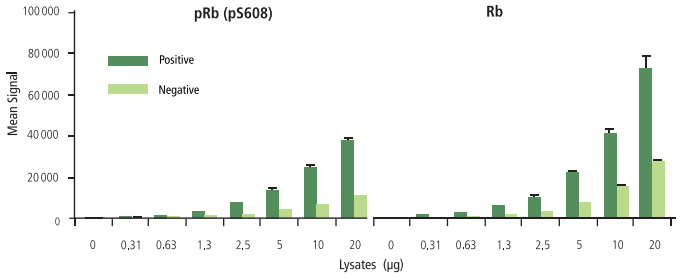
Growing HeLa cells (negative) were treated with nocodazole (1 µg/mL; 19 hours) and calyculin A (50 nM; final 30 minutes) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with an anti-total Histone H3 antibody on one of the four spatially distinct electrodes per well. Phosphorylated Histone H3 was detected with anti-phospho-Histone H3 (Ser10) antibody labeled with MSD SULFO-TAG reagent.

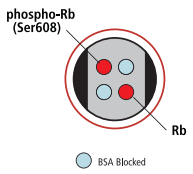
Retinoblastoma (Rb)

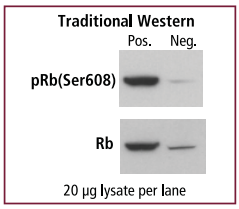
Rb was initially characterized as a tumor suppressor protein, which is lost in childhood retinoblastoma. It is also inactivated or lost in many other human tumors. Rb is a nuclear phosphoprotein regulating cell proliferation by controlling the commitment to enter the S phase of the cell-cycle (G1 restriction point). In early G1, Rb exists in a hypophosphorylated state capable of binding to and inactivating the E2F family of transcription factors. This interaction between Rb and E2F prevents activation of S-phase-specific genes and causes cells to remain in G1. Inactivation of Rb through phosphorylation by the cyclin D/CDK4/6 and cyclin E/CDK2 kinase complexes results in expression of E2F target genes, which is essential for entry into S phase. In addition, Rb is involved in interaction with other nuclear proteins, including many transcription factors. Phosphorylation at multiple sites scattered throughout the protein has been implicated in cell senescence, apoptosis and differentiation.

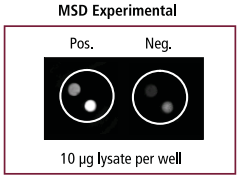


Simultaneous Detection of Phospho-Rb (Ser608) and Total Rb in the Same Well



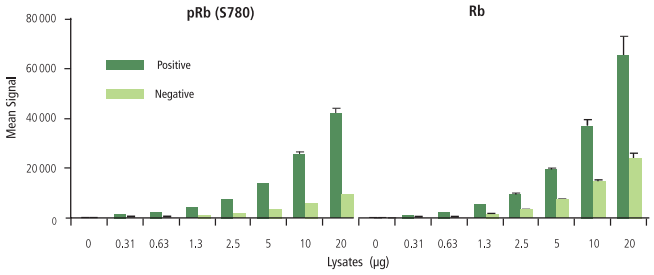


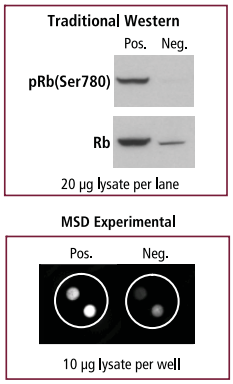




Growing HT29 cells were treated with tetrandrine (30 µM; 18 hours) and harvested 8 hours after a feed with complete medium (negative) or treated with nocodazole (0.2 µg/mL; 18 hours) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-phospho-Rb antibody and anti-total Rb antibody on two of the four spatially distinct electrodes per well. Phosphorylated and total Rb were detected with anti-total Rb antibody labeled with MSD SULFO-TAG reagent.

Simultaneous Detection of Phospho-Rb (Ser780) and Total Rb in the Same Well

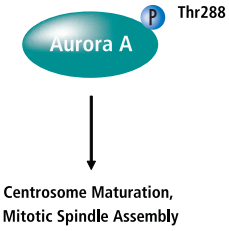




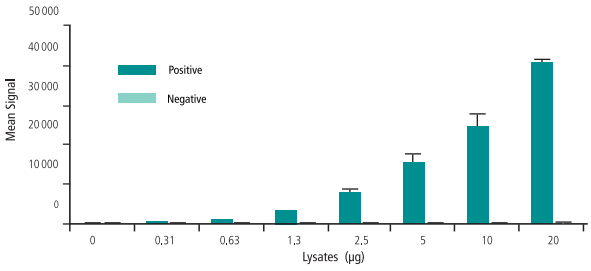
Growing HT29 cells were treated with tetrandrine (30 µM; 18 hours) and harvested 8 hours after a feed with complete medium (negative) or treated with nocodazole (0.2 µg/mL; 18 hours) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-phospho-Rb antibody and anti-total Rb antibody on two of the four spatially distinct electrodes per well. Phosphorylated and total Rb were detected with anti-total Rb antibody labeled with MSD SULFO-TAG reagent.

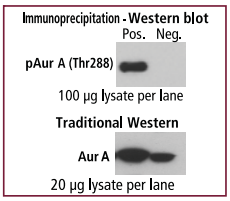
Aurora A

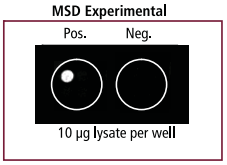
Aurora A is a cell cycle-regulated kinase, which is overexpressed in many tumors and functions as an oncogene when exogenously expressed in various cell lines. In normal cells, Aurora A localizes to the centrosome and is abundant between the G2 and M phases of the cell cycle. Phosphorylation of Aurora A at Thr288 is required for the activation of kinase activity. Upon activation, Aurora A phosphorylates multiple substrates to promote mitotic spindle assembly and cytokinesis. At the final stages of mitosis, Aurora A is degraded through ubiquitin-proteasome pathway.



Detection of Phospho-Aurora A (Thr288)



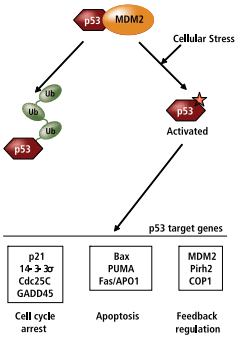




Growing HeLa cells (negative) were treated with nocodazole (1 µg/mL; 19 hours) and calyculin A (50 nM; final 30 minutes) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-total Aurora A antibody on one of the four spatially distinct electrodes per well. Phosphorylated Aurora A was detected with anti-phospho-Aurora A antibody labeled with MSD SULFO-TAG reagent.

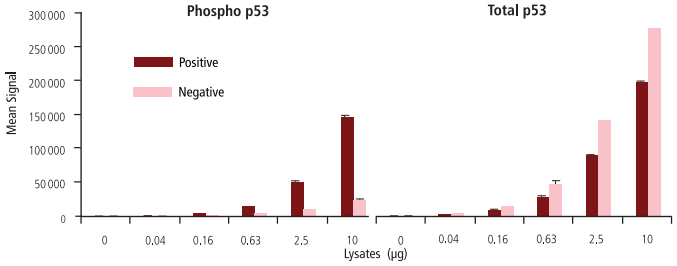
p53 and MDM2

The tumor suppressor p53 responds to DNA damage and other types of cellular stress to induce cell cycle arrest in G1 and G2 stages, or apoptosis. Under normal conditions p53 is functionally inactive due to ubiquitination by the ubiquitin ligase MDM2 and rapid degradation by the proteasome. Metabolic stability of MDM2 is in turn affected by it's own self-ubiquitination. Activity of p53 is regulated by phosphorylation and dephosphorylation of different residues. For example, phosphorylation of Ser15 is induced by DNA damage. P53 mediates G1 arrest caused by ionizing radiation through transcriptional induction of p21, the cyclin-dependent kinase inhibitor. P21 inhibits the activity of cyclin D-CDK4/6 complex resulting in accumulation of hypophosphorylated Rb. Other transcriptional targets of p53 include 14-3-3σ, CDC25C, GADD45, MDM2, BAX, PUMA, FAS/APO1.



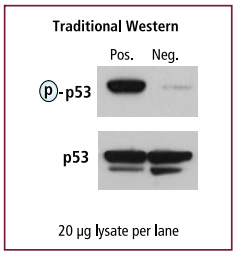
Multiplex p53 Assays

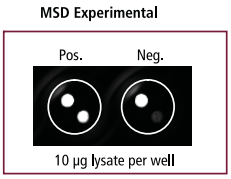
Simultaneous Detection of Phosphorylated and Total p53 in the Same Well



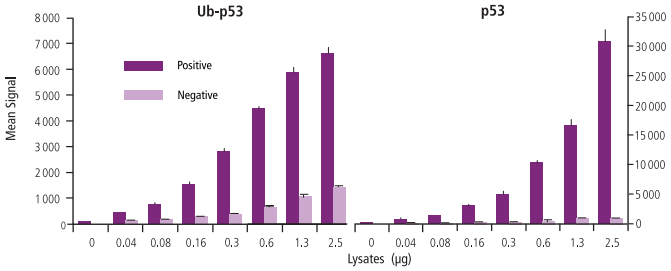
	Lysate (µg)	Positive Ave	Positive StdDev	Positive %CV	Negative Ave	Negative StdDev	Negative %CV	P/N
Phospho p53	0	30	5	17	28	9	33	
	0.04	1334	23	2	382	2	1	3.5
	0.16	4583	436	10	1219	25	2	3.8
	0.63	15193	319	2	4102	290	7	3.7
	2.5	51045	522	1	10424	85	1	4.9
	10	146814	3160	2	23871	822	3	6.2
Total p53	0	28	8	28	29	1	5	
	0.04	2634	206	8	4331	164	4	0.6
	0.16	9054	1136	13	14867	629	4	0.6
	0.63	28498	698	2	46364	5786	12	0.6
	2.5	89099	2449	3	141111	187	0	0.6
	10	199210	722	0	277193	28	0	0.7

Growing HT29 cells (negative) were harvested 1 hour after UV irradiation (40 mJ/cm2) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-phospho-p53 antibody and anti-total p53 antibody on two of the four spatially distinct electrodes per well. Phosphorylated and total p53 were detected with anti-total p53 antibody labeled with MSD SULFO-TAG reagent.

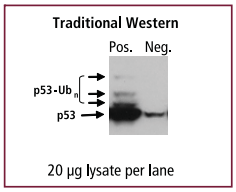


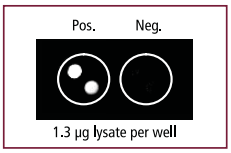


Simultaneous Detection of Ubiquitinated p53 and Total p53 in the Same Well

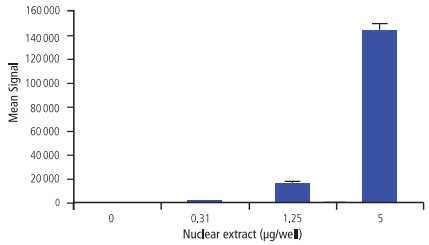


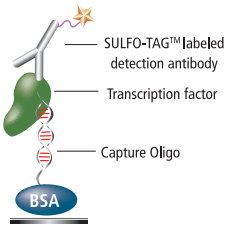
Growing HCT116 cells (negative) were treated with doxorubicin (1 µM, 21 hours) and epoxomicin (1 µM, 6 hours) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-p53 antibody and antibody against ubiquitinated proteins on two of the four spatially distinct electrodes per well. Total and ubiquitinated p53 were detected with anti-total p53 antibodies labeled with MSD SULFO-TAG reagent. Note that Ub-p53 electrode allows detection of directly ubiquitinated p53, and also potentially of p53 complexed with other ubiquitinated proteins.

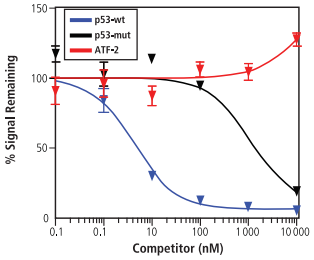




A p53 Promoter DNA Binding Assay





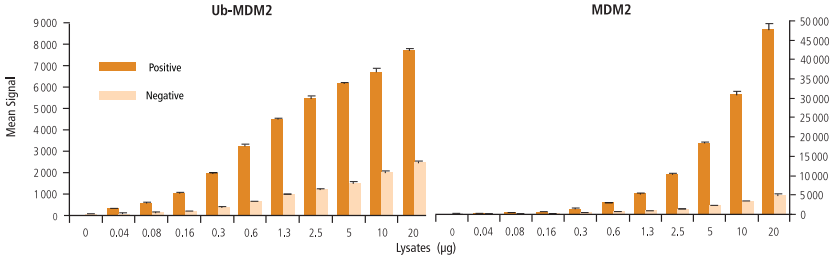


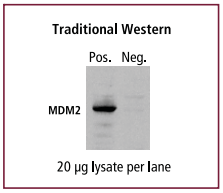
Different amounts of nuclear extracts from MCF-7 cells treated with H2O2 were added to MSD MULTI-SPOT 4 Spot plates coated with an oligonucleotide containing a binding site for p53 conjugated to BSA on one of the four spatially distinct electrodes per well. Bound p53 is detected with anti-p53 antibody labeled with MSD SULFO-TAG reagent.

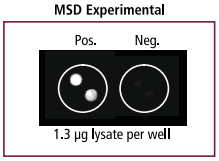
Competitor	IC50 (nM)
ATF-2	-
p53-wt	4.2
p53-mut	1100

Competitor oligos containing the identical p53 binding site, p53 mutant or an unrelated ATF-2 binding sites were co-incubated in solution with the transcription factor to show the specificity of binding to the immobilized oligo.

Simultaneous Detection of Ubiquitinated MDM2 and Total MDM2 in the Same Well

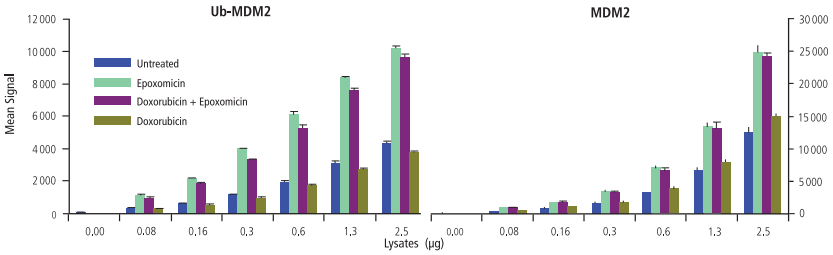


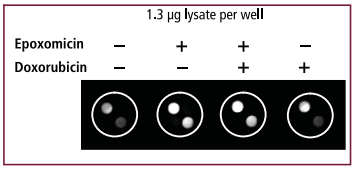




Growing HCT116 cells (negative) were treated with doxorubicin (1 µM, 21 hours) and epoxomicin (1 µM, 6 hours) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-MDM2 antibody and antibody against ubiquitinated proteins on two of the four spatially distinct electrodes per well. Total and ubiquitinated MDM2 were detected with anti-total MDM2 antibodies labeled with MSD SULFO-TAG reagent. Note that Ub-MDM2 electrode allows detection of directly ubiquitinated MDM2, and also potentially of MDM2 complexed with other ubiquitinated proteins.

Ubiquitinated, Total MDM2 Assay: a Cell Line with MDM2 Overexpression





SJSA-1 cells were harvested after (1) no treatment, or treatment with (2) epoxomicin (6 hours), or with (3) doxorubicin (20 hours) and epoxomicin (6 hours), or with (4) doxorubicin (20 hours). Total MDM2 and Ub-MDM2 levels were measured using Ubiquitinated, Total MDM2 assay.

Conclusions

- We present sensitive MSD assays for the detection of various cell cycle markers, including phosphorylated Histone H3, Rb, Aurora A and p53, ubiquitinated and total p53, and MDM2.
- Many assays can be run in a duplex format allowing simultaneous detection of both modified (phosphorylated or ubiquitinated) and total levels of the target protein.
- We also present a p53 promoter DNA-binding assay allowing detection of p53 DNA-binding activity in nuclear extracts. The signal in this assay can be specifically competed by oligonucleotides containing p53 binding sites, but not with unrelated oligonucleotides.
- Our assays achieve results that agree with gold-standard methods like Western blots and electrophoretic mobility shift assays (EMSA) but offer advantages in that they are highly sensitive, quantitative, easily automated, and suitable for handling large numbers of samples.

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