White Paper

Exploring DNA Damage/Genotoxicity Using the MILLIPLEX® MAP 7-plex DNA Damage/Genotoxicity Kit

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Introduction

DNA damage in cells is inevitable. It has been estimated that up to one million DNA changes occur per cell per day, in response to environmental insults and byproducts of normal metabolism[1]. If not repaired, the lesions in critical genes (such as tumor suppressor genes) can impede the normal functions of a cell and increase the likelihood of tumor formation, as in the case of skin cancer. Similarly, genotoxicity describes the capacity of chemical agents to cause DNA damage within a cell, leading to mutations and, potentially, cancer. Genotoxicity tests are routinely used in the pharmaceutical industry to determine whether a pharmaceutical compound induces genetic damage, which can cause a wide range of problems including cancer and inherited birth defects.

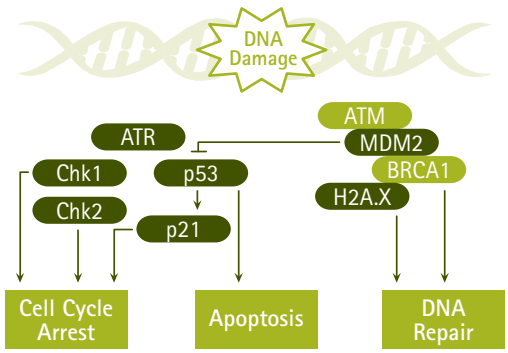
A cell's response to DNA damage involves many complex pathways and mechanisms, collectively called the DNA damage response. Once initiated, these pathways ultimately lead to the repair of the DNA damage or the initiation of apoptosis. The DNA damage response plays a crucial role in maintaining the function, genomic stability and viability of the cell and organism at large. Dysfunctions in the DNA damage response are implicated in many disease states, including cancer, premature aging, tissue toxicity and neurodegenerative disease.

FDA regulations require testing drug candidates for safety, efficacy, pharmacokinetics, toxicology, carcinogenicity and genotoxicity. The ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) guidelines on genotoxicity testing recommend two *in vitro* assays (such as the Ames test and the comet assay) and one *in vivo* assay (such as a micronucleus test (MNT)). These are the gold standards for genotoxicity testing and are offered as assay services from various companies. In addition, cell-based assays, such as the ATAD5 assay[2] and high content assay/screening (HCA/HCS) services are also readily available. Although these assays are broadly used to determine if a drug is genotoxic, they provide limited mechanistic understanding of the cellular response to genotoxic compounds. To meet this need for mechanistic understanding of drug-induced DNA damage response, a high-throughput assay to elucidate pathway changes within cells can be of high utility.

Signaling molecules in the DNA damage/genotoxicity pathway are coordinately regulated by phosphorylation, and understanding the role of this pathway requires the ability to simultaneously measure the phosphorylation status of multiple protein targets. Several assays to examine phosphorylation status are currently available, including Western blotting, ELISA, reverse phase arrays,

quantitative cell imaging and mass spectrometry. Although some of these platforms yield absolute, quasi-quantitative data, the assays are either limited to measuring only one analyte at a time, or are excessively difficult or expensive. In recent years, bead-based multiplex assays, such as those using Luminex xMAP® technology, have enabled the high-throughput measurement of phosphorylation levels of multiple proteins simultaneously, which give the advantages of reduced sample volume, time and cost compared to traditional methods.

Merck Millipore's MILLIPLEX® MAP DNA Damage/Genotoxicity Kit (Figure 1) is a magnetic bead-based immunoassay that simultaneously detects seven proteins in the DNA damage/genotoxicity pathway in a single sample, enabling the measurement of phosphorylation changes in this pathway. Here, we demonstrate the utility of this assay in the analysis of DNA damage/genotoxicity in two cancer cell lines, HepG2 and HEK293. All analytes were detected with good specificity, sensitivity and precision. In addition, genotoxic compound screening shows the utility of this kit in drug discovery and development research.



MILLIPLEX® MAP 7-plex DNA Damage/Genotoxicity Kit Analytes
ATR (Total)
Chk1 (Ser345)
Chk2 (Thr68)
H2A.X (Ser139)
MDM2 (Total)
p21 (Total)
p53 (Ser15)

Figure 1.
Simplified schematic showing the DNA damage/genotoxicity pathway. Analytes detected using the MILLIPLEX® MAP 7-plex DNA Damage/Genotoxicity Kit are listed in the table above and highlighted in the schematic.

Methods

Tissue Culture

HepG2 and HEK293 cells were cultured according to ATCC® guidelines in recommended media. Cells were plated at 50,000 cells per well in a 96-well plate. Twenty-four hours after plating, cells were treated with fresh complete media. After another 24 hours, cells were treated with designated genotoxic and nongenotoxic compounds (listed in Table 1) for a predetermined time period.

Compound	Abbreviation	Genotoxicity
ethylnitrosourea	ENU	G
methyl methanesulfonate	MMS	G
cisplatin	CIS	G
p-chloroaniline	PCL	G
etoposide	ETO	G
hydroquinone	HQU	G
sodium arsenite	SOD	G
taxol	TAX	G
chloramphenicol	CLA	G
D-limonene	LIM	NG
diethanolamine	DIA	NG

Table 1.
Genotoxic (G) and nongenotoxic (NG) compounds.

Sample Preparation

Immediately prior to harvest, media were collected and centrifuged (10,000 g for 10 minutes at 4 °C). Cells were lysed and samples collected according to the MILLIPLEX® MAP 7-plex DNA Damage/Genotoxicity Kit (Cat. No. 48-621MAG) protocol. Samples were then incubated with gentle rocking at 4 °C for 15 minutes and centrifuged (10,000 g for 10 minutes at 4 °C). Lysate supernatants were transferred into new tubes. Protein concentration in untreated samples was determined by bicinchoninic acid (BCA) assay. Using unstimulated sample protein concentration as an estimator, samples were diluted in assay buffer to provide a concentration of approximately 20 µg/well of a 96-well plate. Signals from the compound screening studies were all normalized to β-Tubulin signal using the β-Tubulin MAPmate™ assay (Cat. No. 46-713MAG).

Microspheres

We developed the MILLIPLEX® MAP 7-plex DNA Damage/Genotoxicity Kit by conjugating specific capture antibodies to magnetic microsphere beads purchased from Luminex Corporation. Each set of beads is

distinguished by different ratios of two internal dyes, yielding a unique fluorescent signature to each bead set. Capture antibodies were covalently coupled to the carboxylate-modified magnetic microsphere beads.

Immunoassay Protocol

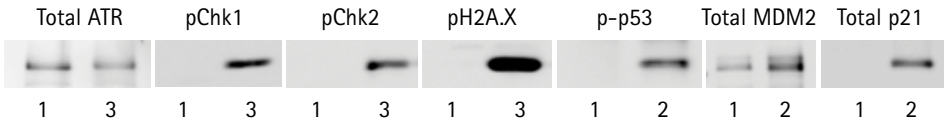
The multiplex assay was performed in a 96-well plate according to product instructions supplied for the MILLIPLEX® MAP DNA Damage/Genotoxicity 7-plex Kit. The plate was first rinsed with 100 µL assay buffer. 25 µL of controls and samples and 25 µL beads were added to each well. Plates were incubated overnight at 4 °C (alternatively, the plates may be incubated for 2 hours at room temperature (RT)). Beads were washed twice with assay buffer, and then incubated for 1 hour at RT with the biotinylated detection antibody cocktail. The detection antibody cocktail was replaced with 25 µL streptavidin-phycoerythrin (SAPE) and incubated for 15 minutes at RT. 25 µL of amplification buffer was added and incubated for another 15 minutes at RT. Then, the SAPE/amplification buffer was removed and beads were resuspended in 150 µL assay buffer. The assay plate was read and analyzed in a Luminex 200™ system. This is a compact unit consisting of an analyzer, a computer and software (Luminex Corporation, Austin, TX).

Results and Discussion

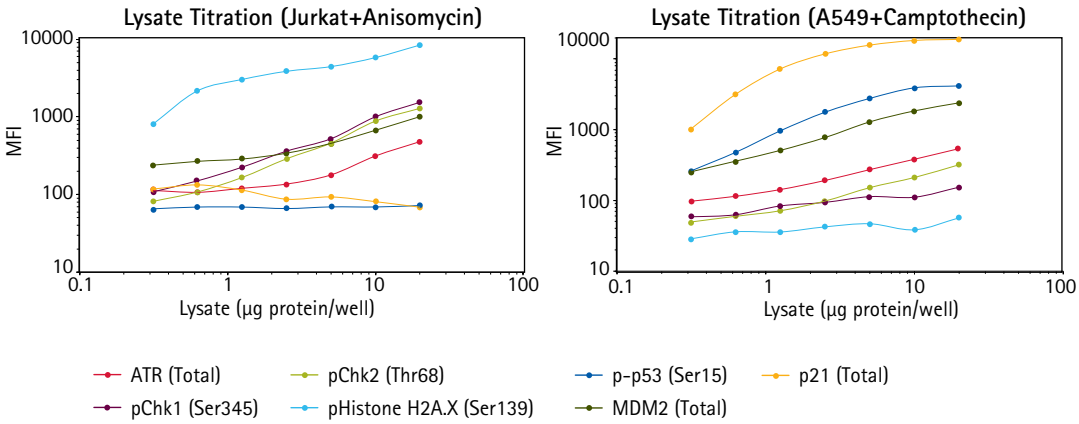
The MILLIPLEX® MAP 7-plex DNA Damage/Genotoxicity Kit enabled the detection of phosphorylated Chk1, Chk2, H2A.X and p53, and total ATR, MDM2 and p21 with good specificity, sensitivity and precision (Figure 2). The assay provided high specificity, indicated by the detection of proteins at the expected molecular weights as shown by immunoprecipitation/Western blot (Figure 2A). In addition, demonstrations of high signal-to-noise ratios (data not shown), sample linearity (Figure 2B) and precision (Figure 2C) lent support to the robustness of this kit. All analytes in the MILLIPLEX® MAP 7-plex DNA Damage/Genotoxicity Kit could be detected in human cell lines and tissues (data not shown).

Using the DNA Damage/Genotoxicity Kit, changes in levels of phosphorylated Chk1, Chk2, H2A.X and p53, and total ATR, MDM2 and p21 were tested in HepG2 and HEK293 cells treated with genotoxic and nongenotoxic

2A. IP/Westerns



2B. Lysate Titrations



2C. Precision	Intra-CV (%) n=16	Inter-CV (%) n=16
ATR (Total)	4.5	11.0
pChk1 (Ser345)	3.4	8.5
pChk2 (Thr68)	4.7	7.6
pHistone H2A.X (Ser139)	4.8	13.9
MDM2 (Total)	9.4	13.8
p21 (Total)	4.8	12.8
p-p53 (Ser15)	7.2	5.7

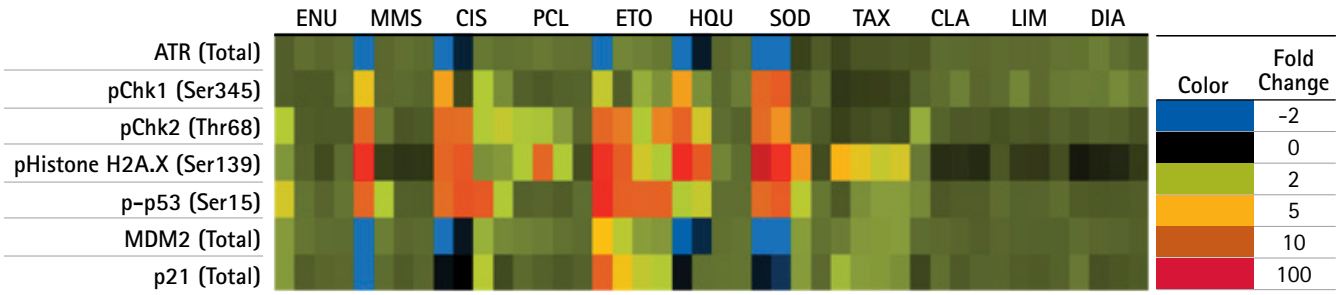
Figure 2.
Specificity, sensitivity and precision of the MILLIPLEX® MAP 7-plex DNA Damage/Genotoxicity Kit. Phosphorylated proteins were simultaneously detected in HeLa, Jurkat and A549 cells. (2A) Immunoprecipitation (IP) of proteins was performed with capture beads and detected by Western blotting with the biotinylated detection antibodies. Lanes correspond to: (1) untreated HeLa cell lysate, (2) camptothecin-treated A549 cell lysate and (3) anisomycin-treated Jurkat cell lysate. (2B) Lysate titrations were performed on Jurkat cells treated with 25 µM anisomycin (4 hours) and A549 cells treated with 5 µM camptothecin (overnight). The signal is represented as Median Fluorescent Intensity (MFI). (2C) Intra- and inter-assay coefficients of variation (CVs) were calculated and reported as percentages (n=16).

carcinogens (Table 1). Changes in the DNA damage response were detected in a dose- (Figure 3) and time-dependent (Figure 4) manner.

Because the panel enabled the simultaneous measurement of multiple proteins, we could distinguish the varying effects of compounds that exerted their genotoxicity through varying mechanisms, as has been reported using gene expression profiling[3]. For example, treatment with compounds that caused DNA double-strand breaks, such as ETO, HQU and CIS, resulted in greatly increased phosphorylation of the cell cycle regulating kinases Chk1 and Chk2, indicating activation of checkpoint-mediated pathways (Figure 3)[4,5].

On the other hand, compounds that exerted genotoxic effects through other means, such as the microtubule-binding agent TAX or the DNA alkylators, ENU and MMS, showed different patterns of pathway activation. Dose response data for ENU, MMS and TAX, for example, showed less dramatic phosphorylation of the Chk kinases accompanied by phosphorylation of p53 or histone H2A.X (Figure 3). The p53 and histone H2A.X proteins are important players in the DNA repair pathway and can be phosphorylated in response to multiple types of DNA damage.

3A.



3B.

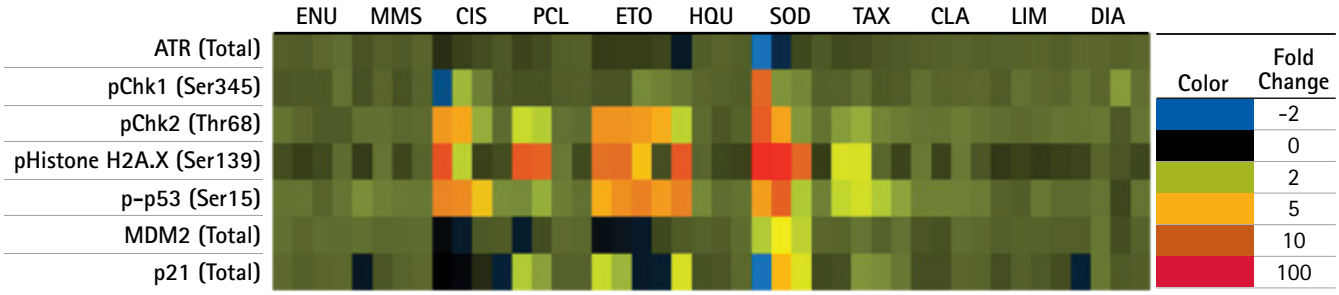
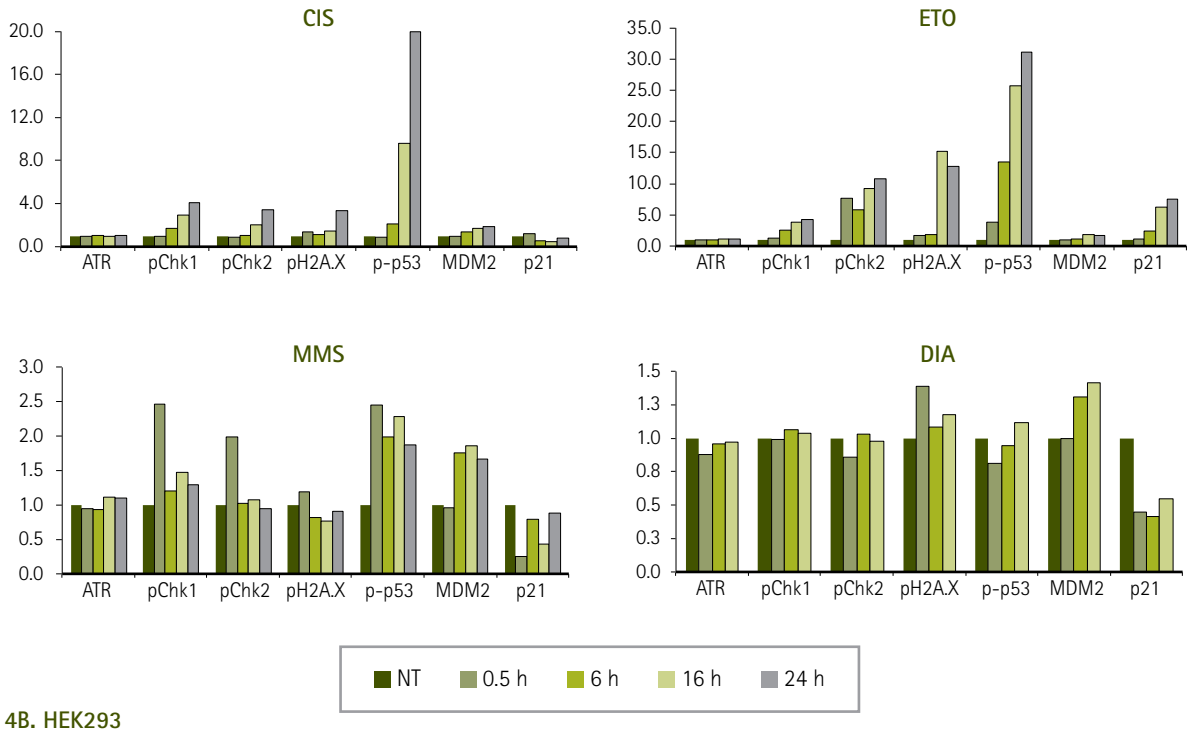


Figure 3.
Dose Response in HepG2 and HEK293 Cells. DNA Damage/Genotoxicity Panel analytes were detected in HepG2 (3A) and HEK293 (3B) cells treated with genotoxic and nongenotoxic compounds ranging in concentration from 1 µM to 1 mM (on diagram, decreasing from left to right; 0.01 µM to 10 µM for TAX) for 48 hours. Median Fluorescent Intensities (MFI) were normalized to β-Tubulin and reported as fold change over the untreated control.

Measurement of time-dependent DNA damage response (Figure 4) also revealed differences in mechanism between different genotoxic compounds. While the double-strand break-inducers, ETO and CIS, caused increasing phosphorylation of Chk1, Chk2, p53 and histone H2A.X with respect to time, the DNA alkylator, MMS, caused an initial spike in Chk kinase and histone H2A.X phosphorylation that then diminished over time, but was accompanied by increased activation of p53. Again, this pattern may indicate initial checkpoint activation, which cells might have overcome, but was followed by checkpoint-independent DNA damage response.

4A. HepG2



4B. HEK293

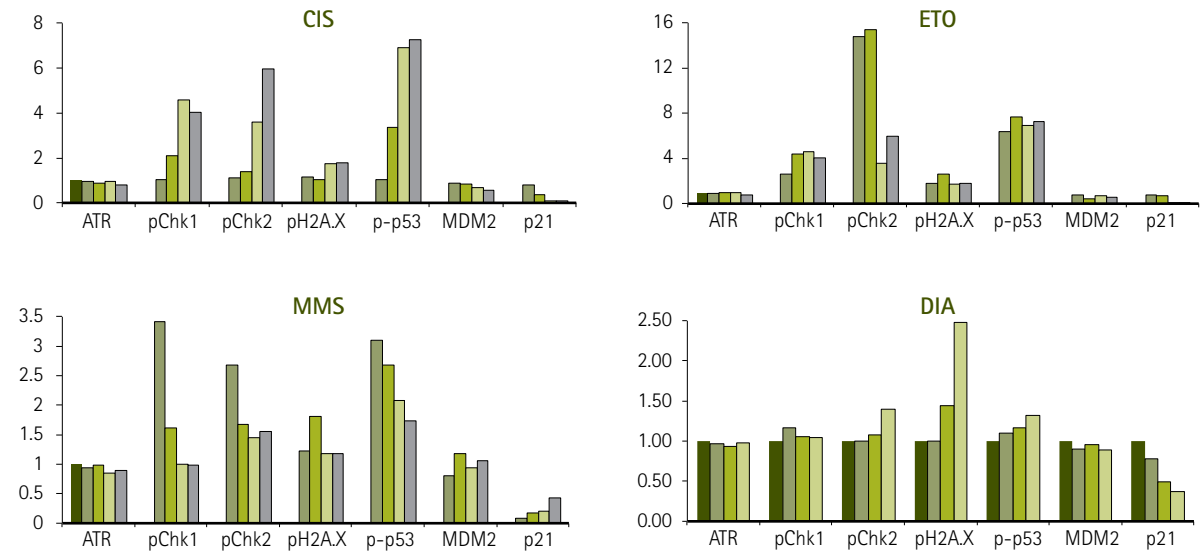


Figure 4.

Time Course in HepG2 and HEK293 Cells. DNA Damage/Genotoxicity Panel analytes were detected in HepG2 (4A) and HEK293 (4B) cells treated with genotoxic and nongenotoxic compounds for 0, 0.5, 6, 16 and 24 hours. Median Fluorescent Intensities (MFI) were normalized to β-Tubulin and reported as fold change over the untreated control.

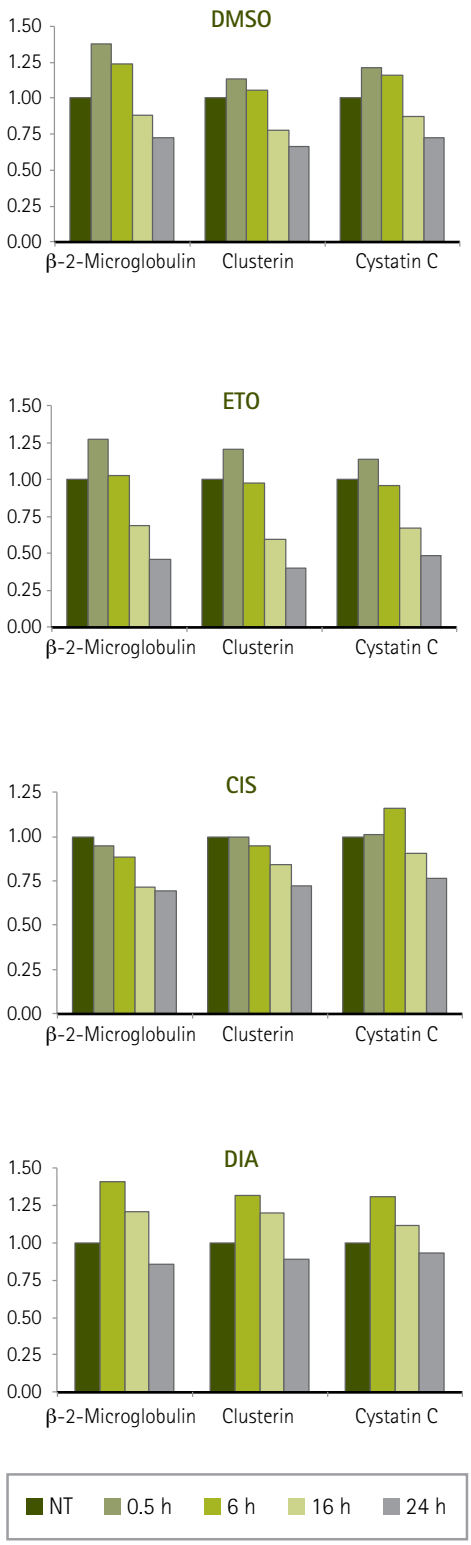


Figure 5.

Time course of toxicity biomarker expression in HEK293 cells using MILLIPLEX® MAP Human Kidney Toxicity Panel 2. Analytes were detected in HEK293 cells treated with 1% DMSO, nongenotoxic compound (DIA) or genotoxic compounds (CIS and ETO) for 0, 0.5, 6, 16 and 24 hours. Analyte protein concentrations are reported as fold change over the untreated control.

As expected, LIM and DIA (nongenotoxic carcinogens) did not result in any significant changes in the analytes. Also as expected, little effect was seen with any of the compounds on total ATR, except at high doses of compounds that may have caused a general decline in cell health.

No significant changes in apoptosis were detected (data not shown) using the MILLIPLEX® MAP 7-plex Early Apoptosis Kit (Cat. No. 48-669MAG). Cell toxicity was also not observed in HEK293 cells (Figure 5) using the MILLIPLEX® MAP Human Kidney Toxicity Panel 2 (Cat. No. HKTX2MAG-38K), as shown by the absence of any increase in the measured analytes with respect to time.

These studies demonstrate that the DNA damage response is complex, and involves more than the dysregulation of a single pathway[6]. This conclusion further underscores the importance of simultaneous measurement of multiple phosphoprotein targets and demonstrates the utility of the MILLIPLEX® MAP 7-plex DNA Damage/Genotoxicity Kit in elucidating the mechanism of action of DNA damaging compounds.

References

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Features of Merck Millipore's MILLIPLEX® MAP Cell Signaling Assays

As shown by the DNA damage/genotoxicity data in this publication, the complexity and number of protein targets involved in signaling events, as well as cellular responses, are best addressed using multiplexed analysis of samples to achieve a complete, accurate picture of a signaling network. Merck Millipore's MILLIPLEX® MAP Cell Signaling Assays enable the analysis of a greater number of intracellular analytes per well, saving valuable time and resources. Flexible assay formats include preconfigured multiplex kits as well as single plex MAPmate™ assays, which can be mixed and matched to meet individual needs.

MILLIPLEX® MAP Cell Signaling Assays offer:

- Simultaneous measurement of multiple analytes in a single well
- Flexible configurations of multiplexing analytes to meet specific needs
- Options for both preconfigured multiplex kits and single plex MAPmate™ kits
- Largest selection of intracellular analytes for detection with the Luminex® system
- Kits for detecting both phosphorylated and total protein
- β-Tubulin or GAPDH MAPmate™ kits can be purchased separately and plexed with other analytes for protein normalization
- All kits include lyophilized positive and negative control lysates

Featured Products

Description	Catalogue No.
MILLIPLEX® MAP 7-plex Human DNA Damage/Genotoxicity Kit	48-621MAG
MILLIPLEX® MAP Human Kidney Toxicity Panel 2	HKTX2MAG-38K
MILLIPLEX® MAP β-Tubulin (Total) Magnetic Bead MAPmate™	46-713MAG
MILLIPLEX® MAP Early Apoptosis Kit	48-669MAG
Methyl methanesulfonate	820775
Cisplatin	232120
p-chloroaniline	802613
Etoposide	341205
Hydroquinone	822333
Sodium arsenite	106277
Paclitaxel (Taxol)	580555
Chloramphenicol	220551

Related Products

Description	Catalogue No.
MILLIPLEX® MAP Cell Signaling Buffer and Detection Kit	48-602MAG
MILLIPLEX® MAP H2A.X (Ser139) Magnetic Bead MAPmate™ Assay	46-692MAG
MILLIPLEX® MAP p53 (Ser15) Magnetic Bead MAPmate™ Assay	46-663MAG
MILLIPLEX® MAP p21 (Total) Magnetic Bead MAPmate™ Assay	46-621MAG
MILLIPLEX® MAP GAPDH (Total) Magnetic Bead MAPmate™ Assay	46-667MAG

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