

Validation of a Non-Radioactive Flow Cytometry-based Unscheduled DNA Synthesis Assay (FL-UDS)

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ABSTRACT

The genotoxic potential of new chemicals and drugs drives the growing need for an inexpensive, reliable genotoxicity screening assay. The Unscheduled DNA Synthesis (UDS) assay has been proven to identify and characterize genotoxic chemicals by detecting repair of damaged DNA via measurement of the incorporation of ³H-thymidine. Since this DNA repair is distinct from *de novo* DNA synthesis observed in normally dividing cells, it is commonly referred to as "Unscheduled" DNA Synthesis. MB Research has developed and optimized a high-throughput and non-radioactive UDS assay using flow cytometry, termed FL-UDS. This assay measures incorporation of fluorescently-labeled or conjugated thymidine analogs, rather than radioactive nucleotides. The FL-UDS assay is run on an automated microplate-driven platform, which is more cost-efficient, shortens study time (1 wk vs. 16 wks), can interrogate a much larger number of cells (10,000 vs. 50 - 100), and increases both accuracy and throughput. Another key improvement of FL-UDS over the radioactive UDS is that FL-UDS methodology can resolve three types of genotoxic agents: activation-dependent, activation-independent and those that are detoxified by biotransformation.

We have evaluated 15 known genotoxins and non-genotoxins and successfully classified 14 of the 15 test chemicals (Accuracy = 93%). Due to its many improvements over the standard UDS assay in assessment of genotoxicity potential (especially lower cost and faster turn-around), the FL-UDS assay is proving to be of considerable commercial value to the Pharmaceutical, Biotech, Chemical, Cosmetic and Consumer Products industries.

INTRODUCTION

The *in vitro* Unscheduled DNA Synthesis (UDS) assay is extremely useful for identifying and characterizing chemicals that induce damage in genomic DNA. To conduct this assay, monolayer cultures of primary rodent hepatocytes, incubated in the presence of tritiated thymidine, are treated with a suspected genotoxic agent for 24 hours. During this period, the agent induces DNA damage and repair, while the radiolabeled nucleotide becomes incorporated into the newly repaired DNA strands. Following the treatment period, the hepatocytes are fixed, coated with a photographic emulsion, and processed using standard autoradiographic methods. The processed cell monolayers are then visually inspected using microscopy and the extent of radionucleotide incorporation into hepatocytes is determined. Increased numbers of labeled cells in treated samples relative to untreated monolayers indicate that the agent of concern has induced DNA repair and therefore exhibits genotoxic potential. Although this assay has been used extensively to identify genotoxic agents, several technical aspects have limited its common application in the laboratory as a routine screen for genotoxicants. For example, the standard form of the UDS assay is technically intricate. Laboratory personnel must conduct a variety of complex tasks that require moderate technical skill. Furthermore, distinguishing cells that exhibit UDS from cells synthesizing DNA (*de novo*) on autoradiographic slides can be subjective. The assay is also time-consuming; the autoradiographic exposure period alone takes approximately 2 weeks before the results can be determined.

To address these shortcomings, our group has made several technical improvements to the standard UDS assay. In FL-UDS, metabolically active chicken embryo hepatocytes (vs. rodent hepatocytes in the conventional assay) are easily liberated from liver tissue using a brief treatment with a mixture of specific proteases instead of an elaborate (rodent) liver perfusion technique. Primary cultures of these hepatocytes are then used in FL-UDS studies.

In contrast to the standard version of the assay, which utilizes radioactive thymidine to label the repaired DNA, the method selected by our group incorporates a biotinylated analog of thymidine into damaged/repaired DNA. The incorporation of this modified nucleotide is then quantified immediately following the treatment period (vs. a 2-week post-treatment autoradiographic period required by the standard method). Flow cytometry is used to quantify cells that exhibit UDS in the samples, which has been shown to be more objective than the methods employed by the standard assay. In addition, the large sample size that can be analyzed by the cytometer (> 10,000 cells can be interrogated by the cytometer within a short time period) is expected to improve the ability of the assay to identify weak genotoxicants.

METHODS AND MATERIALS

Embryonic Chicken Hepatocyte Cultures

Livers from chick embryos are isolated and placed in unsupplemented media containing proteases and DNase I. The solution is vortexed thoroughly to disrupt the livers and placed on a rotary shaker for 15 minutes. Following centrifugation and washing, the cells are seeded into tissue culture plates in serum-free Dulbecco's Modified Eagles Medium (DMEM) and incubated at 37°C/5% CO₂. The tissue culture media is changed following a short incubation, and the cells are then dosed with genotoxicants dissolved in DMEM with between 0 and 0.5% DMSO.

Keratinocytes Cultures

Keratinocytes are cultured in a similar manner to the chick hepatocytes (post-seeding), except that the keratinocyte tissue culture media is supplemented with 5% charcoal-stripped fetal bovine serum.

Treatment with Genotoxicants

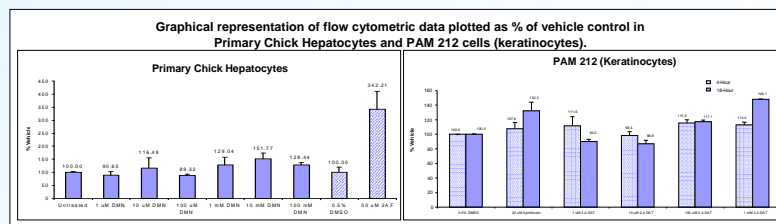
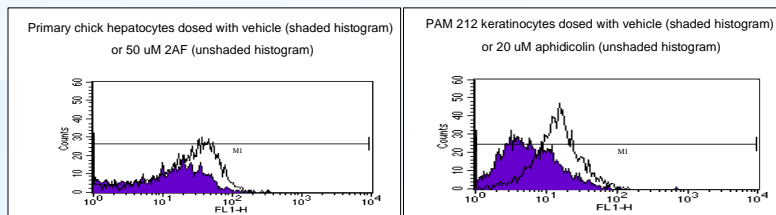
After viable cell cultures have been established, they are treated with test chemicals and processed for FL-UDS analysis. The medium of >80% confluent plates of hepatocytes or keratinocytes is replaced with medium containing increasing concentrations of the genotoxic test agents.

Following an 18-24 hour incubation/treatment period, the cells are washed and trypsinized to remove them from the plates. The cells are centrifuged and washed, and are then permeabilized on ice for 20 minutes. After again pelleting by centrifugation, the cells are resuspended and DNA repair synthesis is initiated by addition of dNTPs and the biotinylated thymidine analog biotin-dUTP. The solutions are incubated in a humidified CO₂ incubator at 37°C for 1 hour, washed, and pelleted.

The cells are next fixed with cold 90% methanol and refrigerated overnight. Another wash is performed before staining the cells with a FITC-conjugated anti-biotin monoclonal antibody, followed by propidium iodide. Acquisition is performed using an enhanced FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with Cytek Development Automatic Microsampling System. The histograms generated in these experiments were analyzed using CellQuest Flow Cytometry Software (Becton Dickinson, San Jose, CA).

RESULTS

15 known genotoxic and non-genotoxic chemicals were evaluated in 2 cell types: primary hepatocytes (embryonic chicken or adult rat) and keratinocyte cell lines (PAM 212 or HaCat). Hepatocytes are necessary to detect chemicals which require bioactivation by metabolic enzymes (most notably Cytochrome P450) to damage DNA and elicit Unscheduled DNA Synthesis (UDS). Keratinocytes (which have low metabolic potential) are employed to identify genotoxicants which are either direct-acting or may be detoxified by the hepatic metabolic processes. A positive UDS response is defined as a 30% or greater increase in DNA repair using biotin-dUTP and a fluorescent antibody to biotin (measured as increased mean fluorescence intensity - MFI) for test samples when compared to vehicle control. A positive result in either (or both) cell types categorizes a test agent as a genotoxin in the FL-UDS assay. All known non-genotoxins tested by this method were confirmed to have no UDS-inducing properties; all known genotoxic chemicals tested, with the exception of DEN, elicited a positive response in the FL-UDS assay. DEN belongs to a class of chemicals (alkylating agents), which induce "short-patch" repair in cells, meaning only 0-6 nucleotides (nt) are replaced during nucleotide excision repair. Conversely, "long-patch" repair processes involve the excision and replacement of 30-100 nt. Short-patch repair is notoriously difficult to detect in UDS assays, and after advisement by our panel of expert consultants, we have decided to postpone testing most of the alkylating agents on our proposed chemical list until a modified method for detecting short-patch repair-inducing genotoxins can be explored.



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SUMMARY

Combined Results of UDS testing in hepatocytes and keratinocytes

Genotoxins	Hepatocytes	Keratinocytes	Combined
2-acetylaminofluorene (2AAF)	+	ND	+
2-aminofluorene (2AF)	+	-	+
Aphidicolin	+	+	+
2,4-Diaminotoluene (2,4-DAT)	-	+	+
Diethylnitrosamine (DEN)	+	-	-
7,12-Dimethylbenzanthracene (DMBA)	+	-	+
Dimethylnitrosamine (DMN)	+	+	+
Ethidium Bromide (EtBr)	-	+	+
Formaldehyde (Formalin)	-	+	+
Isophorone	-	+	+
Mitomycin C	-	+	+
Non-Genotoxins			
Aniline	-	-	-
Dimethylsulfoxide (DMSO)	-	-	-
Ethanol (0.1-2%)	-	-	-
Fluorene	-	-	-

A flow cytometry-based Unscheduled DNA Synthesis assay has been optimized and utilized in feasibility studies designed to examine the usefulness and commercial value of the assay as a screen for genotoxic agents. The current radiometric UDS assay, though widely accepted, has several key aspects which limit its more widespread use. Initial validation studies indicate that the FL-UDS assay can be used to identify and characterize agents that damage genomic DNA.

Due to its use of flow cytometry and the absence of radionuclide use, the FL-UDS assay would be a much cheaper and faster method for determining the genotoxic potential of various test agents than is the standard UDS assay. An entire experiment in the FL-UDS assay can be conducted in approximately 30 hours, versus at least 2 weeks in the standard UDS assay. Unlike primary chick hepatocytes, keratinocytes do not efficiently metabolize xenobiotics, due to very low levels of Phase I and II enzymes; thus, it is possible to classify 3 different types of genotoxicants using the FL-UDS assay: those that are activation-dependent, those that require metabolic activation, and those that are inactivated by biotransformation. This mechanistic information also was not obtainable using the standard UDS assay.

Finally, due to its ability to be conducted in 24-well plates, the FL-UDS assay is much more high-throughput than the standard *in vitro* UDS assay.

CONCLUSIONS

- Initial studies of our *in vitro*, flow cytometry-based UDS assay (FL-UDS) demonstrate the feasibility of replacement of the animal-based radiometric UDS assay.
- FL-UDS is considered to be a non-animal alternative assay, thus reducing the number of animals used in toxicological research.
- Mechanistic characterization of genotoxic effects of substances can be determined using FL-UDS to characterize:
 - Direct-acting chemicals
 - Agents that require metabolic activation
 - Agents that are inactivated by biotransformation.
- FL-UDS has several advantages over the standard radiometric UDS assay, specifically
 - Cost efficiency (less than 1/2 the cost)
 - Shorter time of performance (3 days vs. up to 16 weeks)
 - The ability to analyze a much larger cell set (10,000 cells vs. 50-100 per sample)
 - High throughput 96-well plates allow more compounds can be examined per assay