Miltenyi Biotec

In Vitro MicroFlow® Kits and MACSQuant® Analyzer 10 – Methods

High-throughput genotoxicity assays

In cooperation with

LITRON LABORATORIES

Introduction

Preclinical safety assessments often include the *in vitro* micronucleus assay in order to evaluate chemicals' potential to cause DNA damage. This particular assay provides information on chromosomal damage in cultured cells by detecting the formation of small membrane-bound DNA fragments (micronuclei) in the cytoplasm of interphase cells. Traditionally, the *in vitro* micronucleus assay requires a microscopic analysis of the treated cells which precludes its use as a high-throughput screening tool.

The *In Vitro* MicroFlow® Kit (Litron Laboratories) in combination with the MACSQuant® Analyzer 10 provide a fast, standardized, and automated flow cytometry–based workflow that overcomes the limitations of microscopic analysis by providing high-content information which is both reproducible and reliable.

The *In Vitro* MicroFlow Kits are standardized kits that enable flow cytometric detection of micronuclei derived from both adherent and suspension-cultured cells. It allows for the sequential staining and liberation of nuclei and micronuclei from each sample, prior to the analysis on a flow cytometer. When conducted following proper procedures, results from the *In Vitro* MicroFlow Kits are suitable for regulatory safety submissions.

The MACSQuant Analyzer 10 flow cytometer is a platform that can deliver high-throughput, high content information on genotoxicity when combined with the *In Vitro* MicroFlow Kits. The MACSQuant Analyzer 10 provides automated sampling for 96-well plate analysis and is equipped with a syringe needle capable of volumetric pipetting, thereby enabling absolute counting. In addition, the easy-to-use MACSQuantify™ Software (21 CFR part 11 compliant) provides direct access to instrument parameters for data collection and immediate analysis of results. Paired with the *In Vitro* MicroFlow Kit assay, pre-made templates and settings are available for download, significantly reducing, or even removing, set-up, and optimization time.

To demonstrate the compatibility, human lymphoblastoid TK6 cells were exposed to a range of concentrations of methyl methanesulfonate (MMS), a well-known chromosomal damaging agent. After 27 continuous hours, the samples were processed and analyzed as described.

Materials and methods

Materials

- Cell line of choice*
- Cell culture medium
- *In Vitro* MicroFlow Kit
- MACSQuant Analyzer 10
- (Optional) Benchtop centrifuge with swinging rotors for microtiter plates

* Both attachment and suspension cells have been investigated. Go to www.litronlabs.com for advice on tested cell lines.

Methods

Cell handling, exposure, and processing using the *In Vitro* MicroFlow® Kit

The general scheme is outlined in figure 1.

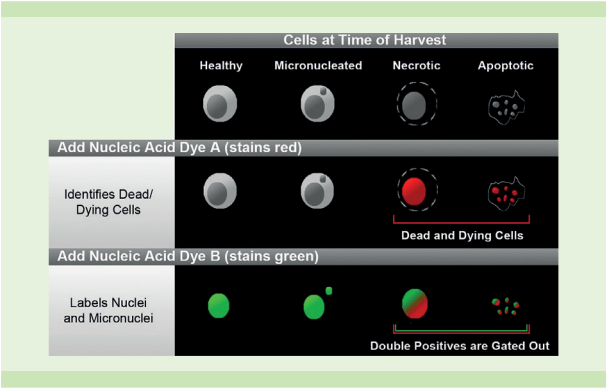

Figure 1: *In Vitro* MicroFlow Kit sequential staining procedure.

1. Plate cells at appropriate density to achieve approximately 1.5–2 population doublings during exposure period (generally 24–30 hours).
2. Expose cells for desired period of time and then remove cell culture medium.

   Note: Centrifugation may be necessary if using a suspension cell line.
3. Add Nucleic Acid Dye A and incubate cells under appropriate light source.
4. Wash cells and add Complete Lysis Solution 1 to wells. Incubate for 1 hour.
5. Add Complete Lysis Solution 2 to wells and incubate for 30 minutes.
6. Proceed with analysis of cells by flow cytometry using the MACSQuant Analyzer 10.

   Note: Processed plates can be stored at 4 °C until analysis. For more details visit www.litronlabs.com.

Micronucleus analysis on the MACSQuant® Analyzer 10

Allow plate to come to room temperature prior to analysis.

The MACSQuant® Analyzer 10 templates are available at www.litronlabs.com.

1. Prepare and prime the MACSQuant Analyzer 10.

   For further information refer to the MACSQuant Instrument user manual and the MACSQuantify™ Software user guide.
2. Open *MACSQuant® In Vitro MicroFlow Analysis Template* and *MACSQuant® In Vitro MicroFlow Expt Template* with the MACSQuantify Software.
3. In the **Channels** tab, make the following adjustments to the instrument settings:

Instrument settings	
FSC	hlog
SSC	log3
B1	hlog
B3	log4
Trigger	B1; value near 1.00
(Optional) Turn off	B2, B4, V1, V2, R2
Advanced	Height and Width
Secondary trigger	SSC; value 1.50

4. Place plate on the MACS® MiniSampler Plus and acquire data from a negative control well. Adjust instrument settings as follows:
   - Adjust **FSC** and **SSC** voltages to bring nuclei into view on the Light Scatter plot (figure 2A).
   - Adjust **B3** voltage to resolve nuclei from dead and healthy cells on the EMA positive plot (figure 2B).
   - Adjust **B1** voltage so that the G1 peak is positioned high enough so that 1/100th the MFI will still fall on scale in the Nucleic Acid Dye B Range plot (figure 2C).

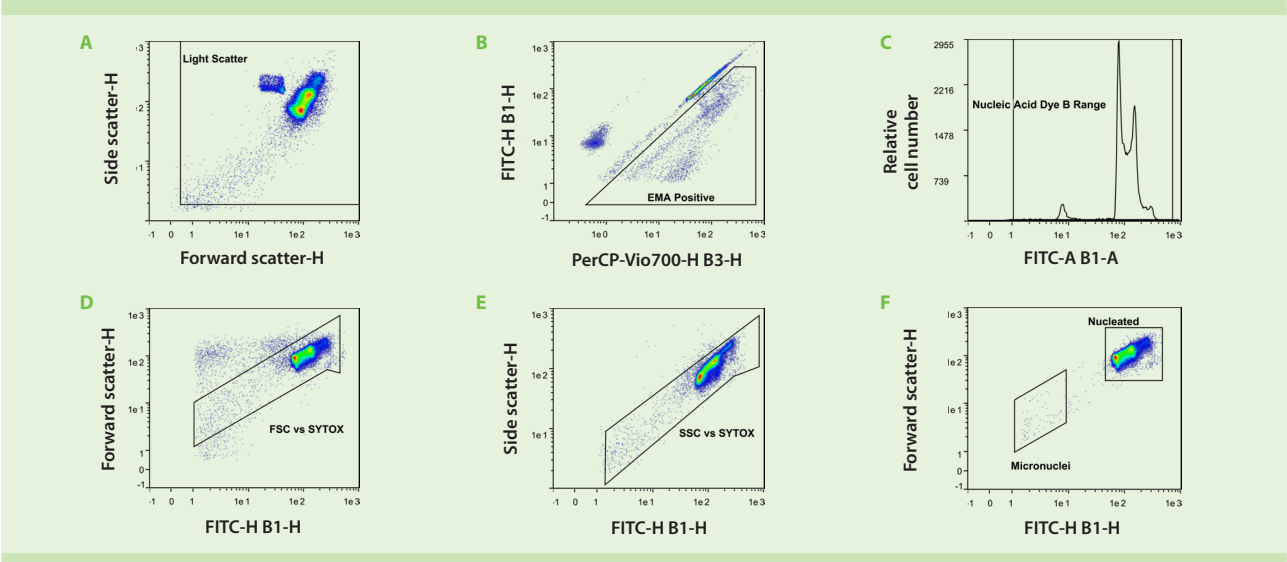

Figure 2: Representative plots for the setup of the MACSQuant Analyzer 10 using a negative control sample prepared with the *In Vitro* MicroFlow Kit.

- Adjust the position of the **FSC vs SYTOX®** and **SSC vs SYTOX** regions until nuclei are positioned (figures 2D and 2E).
- Adjust the position of the **nucleated** and **micronuclei** regions as needed (figure 2F).

5. Analyze the remainder of the plate.

Results

Micronuclei are small, nucleic acid dense particles that form following division of cells that have experienced double-strand DNA breaks or lagging whole chromosomes. These particles are readily discriminated from whole nuclei via the liberation and staining procedure of the *In Vitro* MicroFlow® Kit and visualized and enumerated by the MACSQuant® Analyzer 10.

The resolution of such small events – typically 1/10th to 1/100th the size of a nucleus – is easily achieved on the MACSQuant Analyzer 10 which utilizes a syringe pump for a constant flow rate, PMTs for forward and side scatter, and hydrodynamic focusing and direct light interrogation of particles. Figure 3 shows dot plots derived from TK6 cells exposed to a vehicle control sample (figure 3a) or to MMS (figure 3b) . The increased number of events in the micronucleus gate is clearly evident indicating the genotoxic activity of the compound. The micronucleus response was tested across the full concentration range of MMS revealing a dose-dependent increase in combination with increased cytotoxicity (figure 4).

The cytotoxicity information is critical to understanding if a sufficient exposure has occurred that would result in genotoxic activity. The *In Vitro* MicroFlow Kit utilizes fluorescent particles to generate these cytotoxicity data, and the MACSQuant Analyzer 10 enables comparative indices of cytotoxicity based on the absolute counts acquired natively by the instrument. This permits the calculation of cytotoxicity metrics such as relative increased cell counts (RICC) or relative population doubling (RPD) that can aid in generating information for regulatory safety submission (figure 4).

Conclusions

The results demonstrate that the combination of the *In Vitro* MicroFlow Kits with the MACSQuant Analyzer 10 enable a rapid, simple collection of chromosomal damage information that is required for preclinical safety studies. This methodology provides a much faster, more objective approach than traditional methods accomplished by manual scoring. This alignment provides a turn-key solution for automation and activation for easy conversion from current methods and marks the advantage of an improved methodology for genetic toxicology screening.

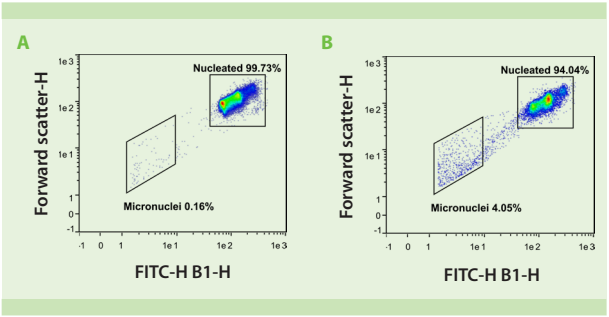

Figure 3: Dot plots from TK6 cells exposed to vehicle control (3a) or MMS (3b).

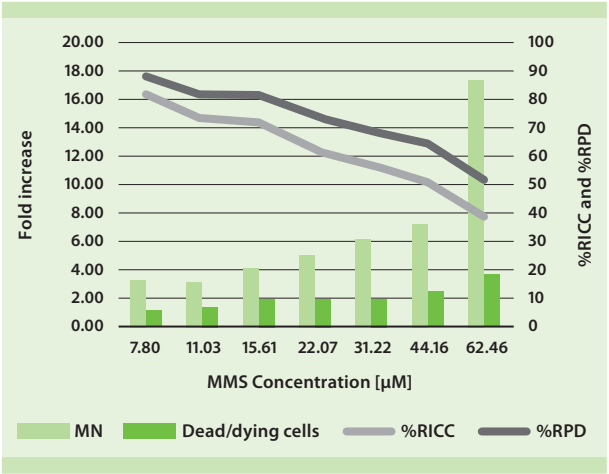

Figure 4: The micronucleus response was tested across the full dose range of MMS, revealing a dose-dependent increase in micronucleus (MN) frequency. Along with the primary endpoint, several measures of cytotoxicity are simultaneously collected, including frequency of dead/dying cells, relative increased cell counts (RICC), and relative population doublings (RPD).

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