MicroFlow®

Quality Counts!

Micronucleus Analysis Kit

MicroFlow (In Vitro, 96 well)

Appendices

For Research Use Only. Not for use in diagnostic procedures.

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Appendix D – Cell Culture and Treatment Conditions

- **Treatment Conditions**

  - Please refer to the most recent guidance documents (e.g., OECD 487).

  - Short-term treatment (with or without metabolic activation) is achieved by exposing logarithmically dividing cells to test article for 3 to 6 hours. After exposure, cells are washed and transferred to fresh growth medium for a duration of time that approximates **1.5 to 2 normal cell cycles**. At this time, cells are harvested and processed for MN scoring. See Thougaard et al., (2014) for an example of how to incorporate S9 treatment as part of *In Vitro* MicroFlow.

  - Long-term treatment (without metabolic activation) is accomplished by exposing logarithmically dividing cells to test article for a duration of time that approximates **1.5 to 2 normal cell cycles**. At this time, cells are harvested and processed for MN scoring.

  - The use of cytochalasin B is advised when performing MN assays with mitogen-stimulated primary lymphocytes. For cell lines that divide continuously in culture, most guidance documents suggest that this reagent is not required. **For standard cell line work, we recommend avoiding the use of cytochalasin B.**

- **Limit Concentration**

  - Please refer to the most recent guidance documents (e.g., OECD 487), but a generally accepted principle is that test articles should be evaluated at concentrations that cause minimal toxicity, modest toxicity, and distinct toxicity.

  - Current recommendations suggest that distinct toxicity is indicated by approximately 55 % ± 5 % toxicity at the time of harvest (relative to concurrent solvent or negative control). The manner in which cell counts are made can affect relative survival values. We recommend using a sensitive method based on Counting Beads (see Appendix B).

  - For non-cytotoxic agents, the top concentration is usually specified by expert working groups and/or regulatory bodies. As of the most recent (2014) update to the OECD 487 guideline for *in vitro* micronucleus, the recommended top concentration criteria for those compounds that do not achieve limiting cytotoxicity is 10 mM, 2 mg/mL or 2 μL/mL, which ever is the lowest. OECD also recommends the lowest precipitating concentration as a valid concentration limit, however precipitation can clog flow cells and caution must be used when analyzing precipitating concentrations on a flow cytomter.

- **Other Methodological Considerations**

  For information on assay validation, generation and maintenance of historical controls, substitution of a new cell line, etc., please refer to the appropriate regulatory guideline or other established practice as described in the literature.

- **Specific Advice for 96 Well Plate Format**

  - **Suspension Cells**

    - U-bottom 96 well plates are recommended when using suspension cells.

    - In order to achieve proper and consistent washing of cells in 96 well plate format, we strongly recommend the use of a multi-channel aspirator with a fixed bridge in order to control depth. See Section 2 and Appendix D.

    - In order to obtain optimal cytotoxicity measurements, addition of microspheres to Complete Lysis Solution 1 is recommended. See Appendix B for discussions of the various cytotoxicity measurements that can be obtained with *In Vitro* MicroFlow kits.

    - Efficient treatment of cells over a range of concentrations can take several forms. We recommend closely spacing test article concentrations, and laying out plates as described in Appendix E.

- **Attachment Cells**

  - The use of flat-bottom 96 well plates are recommended when using attachment cells.

  - In order to achieve proper and consistent washing of cells in 96 well plate format, we strongly recommend the use of a multi-channel aspirator with a fixed bridge in order to control depth. See Section 2 and Appendix D.

  - The use of attachment cell lines offers certain advantages, including the elimination of centrifugation steps. By culturing cells in multi-well plates, treatment and washing steps can occur in the same vessel by simply aspirating the various supernatants and replacing them with the appropriate reagents. *In Vitro* MicroFlow reagents are added sequentially to cells while they are still attached to the plate bottom and not trypsinization is required.

  - In order to obtain optimal cytotoxicity measurements, addition of microspheres to Complete Lysis Solution 1 is recommended. See Appendix B for discussions of the various cytotoxicity measurements that can be obtained with *In Vitro* MicroFlow kits.

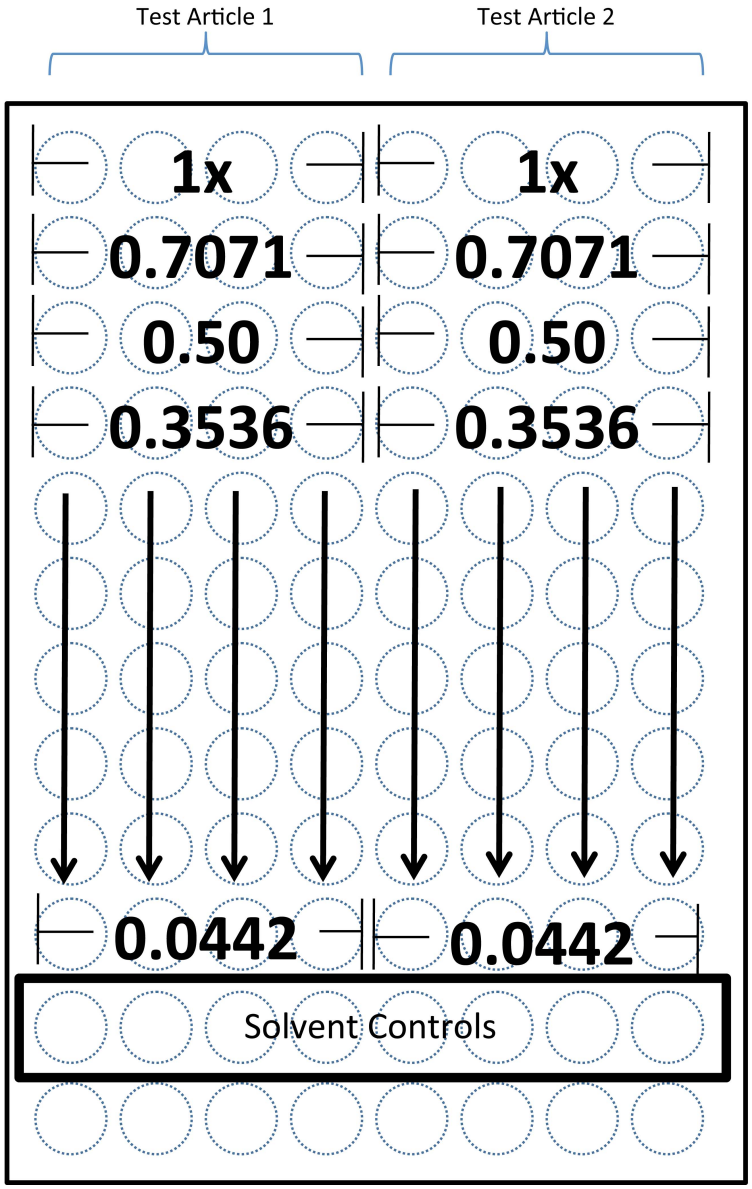
  - Efficient treatment of cells over a range of concentrations can take several forms. We recommend closely spacing test article concentrations. One option is to use an approach similar to the layout described in Appendix E.

Appendix E – Suggested Plate Layout

Some classes of chemicals exert minimal cytotoxicity but then become extremely cytotoxic over a relatively narrow range of concentrations. MN-induction can also be restricted to narrow concentration ranges, as is the case with aneugenic compounds. This is a rationale for closely spacing test article concentrations in the *in vitro* MN assay. While a commonly employed approach is for each concentration to be separated by a factor of one-half, plate-based analysis provides greater flexibility in the study design and in the range of concentrations that can be practically examined.

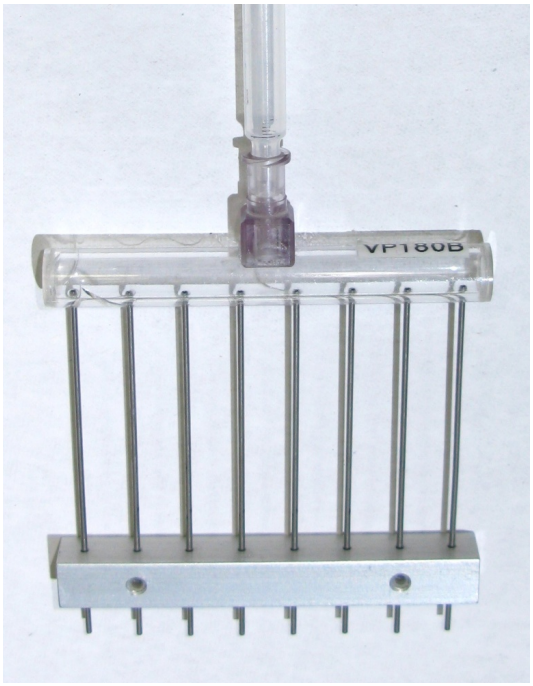
An example of a plate layout that incorporates fine dose spacing is shown below. The top concentration of test article is depicted as 1x and each successively lower concentration is 70.71% of the previous one, i.e., square root 2 dilution scheme. In this way, every other concentration differs by a factor of 2. This dilution scheme can be efficiently performed and covers a broad range of concentrations. In this case the exposures are studied in quadruplicate cultures (except for solvent control).

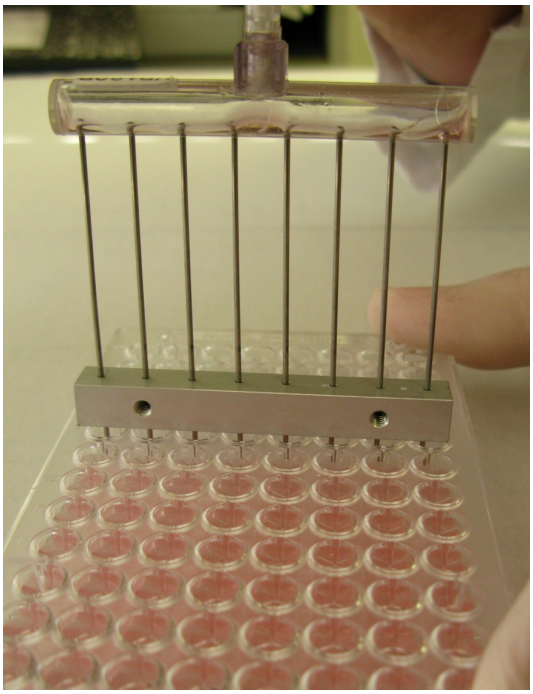
Plate layout may also be dictated by other variables, such as availability of robotic liquid handlers that perform chemical dilutions and/or cell plating.



Appendix F – Multichannel Aspirator with Bridge

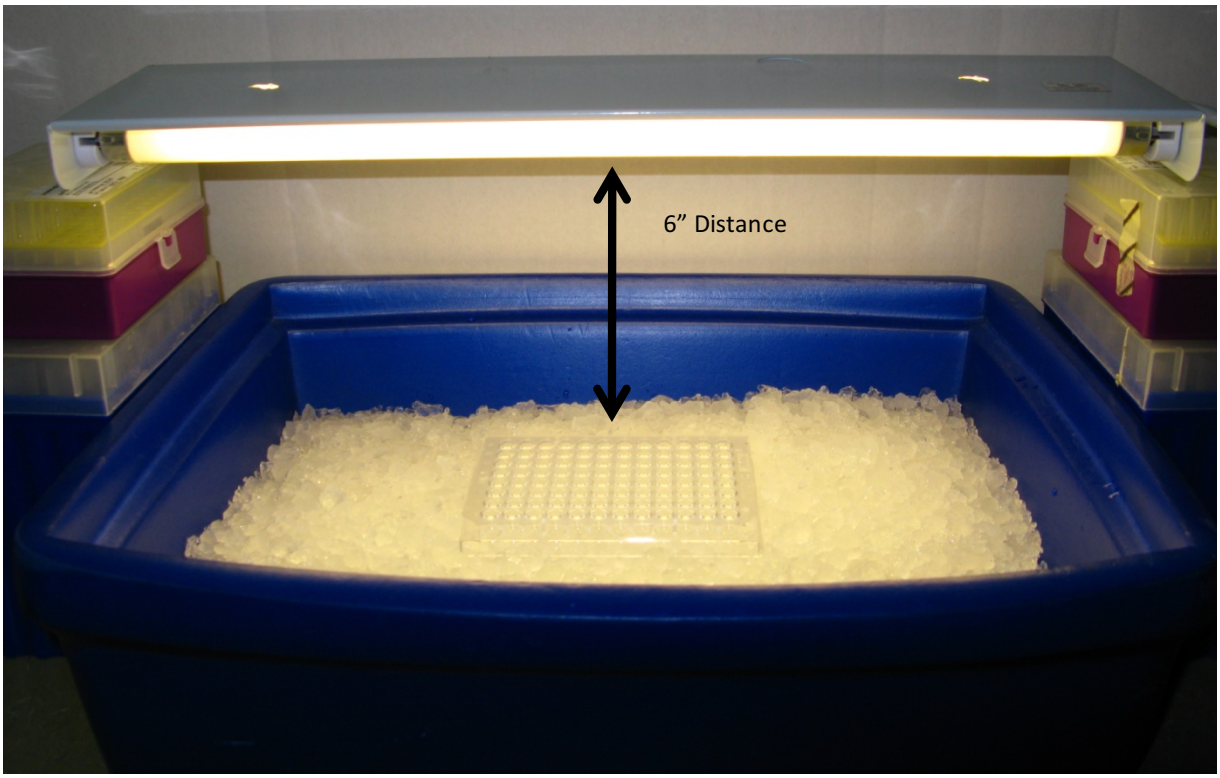
In order to achieve efficient and consistent aspiration across 96 well plates, we recommend the use of a multichannel device that is fitted with a bridge in order to control depth. One example is a product manufactured by V&P Scientific (cat. nos. VP 180B and VP 180S).





Appendix G – Plate Placement During Nucleic Acid Dye A Photoactivation

Ideal plate placement during Nucleic Acid Dye A photoactivation is shown below.



Appendix H – Mode of Action Signatures (CHO-K1 and V79 Cells)

There are some advantages to the use of certain cell lines. As with other mammalian cell lines, MN induction can be observed in CHO-K1 or V79 cells when exposed to aneugenic or clastogenic agents. When using these specific cell lines, it is possible to discriminate between aneugenic and clastogenic modes of action. Two characteristics of the MicroFlow data can help investigators differentiate between these activities. These responses are referred to herein as clastogenic and aneugenic signatures.

❖ **Advantages of Using CHO-K1 or V79 Cells**

- The shift in median fluorescence intensity of MN events is likely related to the whole chromosome nature of aneugen-induced MN. It is interesting that this shift has not been evident in Litron's work with L5178Y or TK6. We attribute this to the larger average size of hamster chromosomes compared to mouse and human chromosomes. This characteristic would be expected to enhance the difference in MN size generated by aneugens versus clastogens.

- Robust increases in the frequency of hypodiploid nuclei upon aneugen treatment was first noted with TK6 cells, although more recent work with that cell line suggest that it does not discriminate between modes of action as reliably as when hamster cell lines such as CHO-K1 or V79 are studied. We attribute induction of hypodiploidy to aneuploidization, possibly via failed cytokinesis and/or extra centrosome content. These are situations that lead to multipole division, and this is a mechanism that promotes rapid acquisition of aneuploidy.

- We hypothesize that the lysis procedure used for flow cytometric MN scoring is capable of revealing aneuploidization, as hypodiploid nuclei are apparent as sub-2n DNA events with the light scatter and DNA staining characteristics of healthy cells' nuclei: EMA-negative nuclei with SYTOX-intensity slightly lower than 2n.

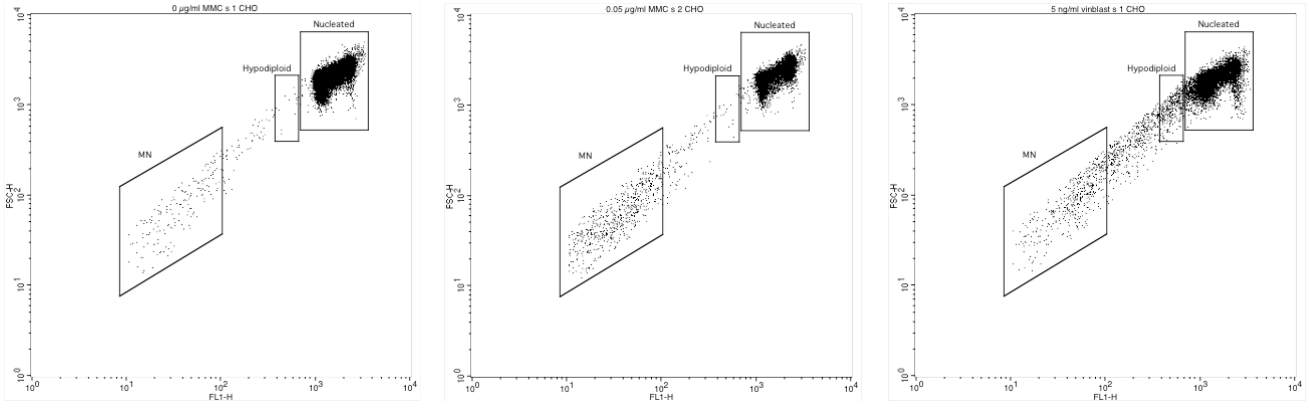
❖ **Comparison of Bivariates**

- Examples of vehicle control and clastogenic and aneugenic signatures are shown below. In these cases, CHO-K1 cells were treated with the clastogen mitomycin C or the aneugen vinblastine. In order to enumerate hypodiploid nuclei, a region should be created on PLOT G that is adjacent to the Nucleated region.

Vehicle Control

Clastogenic Signature:
- Significant MN induction
- Minimal shift in median fluorescence intensity of MN events
- No robust increase in the frequency of hypodiploid nuclei

Aneugenic Signature:
- Significant MN induction
- Increased median fluorescence intensity of MN events
- Robust increase in the frequency of hypodiploid nuclei



❖ **Calculate Percent Hypodiploid Nuclei**

$$\% \text{ Hypodiploid} = \frac{\#\text{Events Hypodiploid}}{\#\text{Events Nucleated}} \times 100$$