Rat Blood Study Phase Plan (M56MF-2010) for MicroFlow^{BASIC} Analysis Kit

An original signed document is required.

A. Contact Information

Test Facility Name and Address:

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Study Director:

Name ____

Phone ____

Fax ____

Email ____

Test Site Name and Address:

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Lead QA Auditor:

Name ____

Phone ____

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Email ____

B. Study Information

Study ID: ____________________________________

GLP Number: ______________________________________
(if not provided, Litron will assign one)

☐ For analysis in compliance with GLP regulations, initial here. Provide the agency name that the data will be submitted to: ___________________. Also, indicate which GLP regulations should be followed (FDA and/or OECD): _____________________ A copy of your protocol is required prior to sample analysis. For FDA GLP analyses, label samples with Sample ID, Study ID, Date Collected, Source (i.e., rat) and Type (i.e., blood). For OECD GLP, label samples with Unique ID and Sample ID.

☐ For Non-GLP analysis, initial here if a final report is requested in addition to the electronic data file.

☐ Initial here for statistical analysis of data. Additional charges apply. Contact Litron for details.

☐ Initial here to have study phase specific records sent to your test facility after study phase completion, otherwise records will be retained at Litron (see Section 8).

C. If applicable, please indicate any requested modifications to this Study Phase Plan:

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D. Study Phase Plan Approval

Study Director Signature: ____ **Date:** _____________

For Litron use only

Principal Investigator ____

Principal
Investigator's Signature ____ Date_____________________________

1. Objective

This MicroFlow® Study Phase Plan describes procedures for analyzing test facility-prepared rat blood samples for the presence of micronuclei (MN) using the MicroFlow procedure. Micronuclei will be analyzed in the high CD71-positive reticulocyte (RET) population to provide an indication of genotoxicity. The frequency of high CD71-positive RET (% RET) among total red blood cells (RBCs) is also measured to provide an indication of bone marrow toxicity. The analysis can be performed under US Food and Drug Administration (FDA) and/or Organisation for Economic Co-Operation and Development (OECD) Good Laboratory Practice (GLP) guidelines (see Section B).

2. Introduction

The *in vivo* Micronucleus Assay is capable of detecting clastogenic (chromosome-breaking) and aneugenic (whole chromosome loss) activity. When cell division occurs, the chromosome fragments or whole chromosomes that are not included in the main nucleus become a micronucleus. Erythrocytes expel their main nucleus before entering the bloodstream, making them ideal for measuring fragmented DNA. Flow cytometry is used for this analysis, as it provides a high-speed method for objective scoring of these rare events. Stained blood cells fluoresce as they pass through a focused laser beam, and the collected data is then sent to a computer for analysis. Micronuclei occur spontaneously, but clastogens and aneugens cause an increase in the number of MN relative to the background (spontaneous) level.

3. Proposed Study Dates

The experimental start and end dates will be documented in the MicroFlow report.

4. Experimental Procedures (performed at Test Facility)

The test facility is responsible for following the procedures detailed in the Litron-provided manual. Substitutions of kit components or deviations from the procedures described in the manual are not recommended. Modifications not previously approved by Litron may result in samples that are incompatible with flow cytometric analysis.

5. Flow Cytometric Analysis (performed at Test Site)

- **Sample Receipt**

  Upon receipt at Litron, the fixed samples will be stored in a freezer (-85 °C ± 5 °C) until preparation for analysis.

- **Sample Preparation**

  The fixed samples will be washed with a cold, balanced salt solution (will include Fetal Bovine Serum if samples are stored in Long Term Storage Solution) and isolated by centrifugation. The resulting cell pellets will be stored at 5 °C ± 3 °C or on ice until staining.

- **Staining for Identification of Cell Populations**

  Samples will be incubated with RNase (to degrade RNA), a fluorescently labeled antibody to the transferrin receptor (anti-CD71-FITC) to stain RETs, and a fluorescently labeled antibody to label platelets (anti-CD61-PE). After incubation, cells will be stored at 5 °C ± 3 °C or on ice until analysis. A propidium iodide solution will be added to each sample before flow cytometric analysis to stain the DNA of micronuclei.

- **Flow Cytometer Calibration**

  Methanol-fixed blood from rats infected with *Plasmodium berghei* will be used to configure the flow cytometer before analysis. Whereas MN are relatively rare and exhibit a heterogeneous DNA content, parasitized cells are prevalent and have a homogenous DNA content. These characteristics make them ideal for calibrating the flow cytometer for the micronucleus scoring application.

- **Analysis of Samples**

  Samples will be analyzed by flow cytometry. The stained cells are moved past a laser set to provide 488 nm excitation. The fluorescence emitted by each cell is collected by photomultiplier tubes. Using the previously described staining procedure, the propidium iodide-stained DNA of the micronuclei emit a red fluorescence, the anti-CD71-FITC antibody emits a high green fluorescent signal, and platelets are excluded based on anti-CD61-PE fluorescence.

6. Data Provided

When possible, twenty thousand RET are analyzed per blood sample. In the event of bone marrow toxicity, the number analyzed may be reduced according to Litron's SOPs. The number of normochromatic erythrocytes (NCEs), MN-NCEs, RETs and MN-RETs are provided for each sample. The frequency of MN-RETs and MN-NCEs will be calculated as an indication of genotoxic potential. The % RET will be determined to provide an indication of bone marrow toxicity. Averages and standard deviations, per group and sex, will be provided (if known).

7. Evaluation and Interpretation of Results

No statistical analyses will be performed on the data, other than the calculations indicated above, and the test facility will be responsible for the evaluation and interpretation of results, unless the appropriate box in section B is initialed. If statistical analyses are requested, Litron's SOP for statistical evaluation will be followed.

8. Records Maintained

If requested in Section B, the original study phase plan, original MicroFlow report, and study-specific records (copies, if applicable) will be transferred to the test facility at the completion of the study phase. Litron will maintain copies of the report, protocol, and study phase plan, along with original study-specific records for two years following completion of the study. After the retention period, Litron will contact the sponsor and study-specific records will either be discarded or sent to the sponsor-requested facility. Electronic copies of some records will be stored off-site (ESL Federal Credit Union, Brighton Henrietta Branch, Rochester, NY) in addition to storage at Litron Laboratories.

9. References

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- Section 4 of the OECD Guidelines for the Testing of Chemicals: Mammalian Erythrocyte Micronucleus Test, Guideline 474 (Adopted 21st July 1997). This guideline states " . . . any appropriate mammalian species may be used provided it is a species in which the spleen does not remove micronucleated erythrocytes or a species which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations."
- Where applicable, GLP regulations for non-clinical laboratory studies as developed by the FDA (21 CFR 58). Please note that the computerized systems utilized for data acquisition, data analysis and report generation have undergone an internal validation guided by FDA GLP regulations. Litron is working towards 21 CFR part 11 compliance.
- Where applicable, Principles of GLP by OECD [C(97)186/FINAL].
- Where applicable, ISO 10993-3: Biological evaluation of medical devices – Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicology (2003-10-15).
- Where applicable, ICH Tripartite Guideline: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, S2A, adopted April 24, 1996.
- Where applicable, ICH Tripartite Guideline: Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals, S2B, adopted November 21, 1997.

10. Effective Date: January 1, 2010