Study Phase Plan: MicroFlow^{BASIC} Micronucleus Analysis Kit (M50MFv16) Rodent Whole Blood

An original signed Study Phase Plan document and Sample Submission Form are required for each study.

A. Contact Information

Test Facility Name and Address:

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_____

Test Facility Study Director:

Name _____

Phone _____

Fax _____

Email _____

Test Facility Lead QA Auditor:

Name _____

Phone _____

Fax _____

Email _____

Test Site Name and Address:
Litron Laboratories
3500 Winton Place, Suite 1B
Rochester, New York 14623
phone: 585-442-0930
fax: 585-442-0934
info@litronlabs.com
www.litronlabs.com

B. Study Information

Study ID: _____

Please Indicate Species: _____ Rat _____ Mouse Please Indicate Strain: _____

☐ 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., mouse or 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.

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

If applicable, please indicate any requested modifications to the Study Phase Plan:

_____

C. Study Phase Plan Approval

Study Director Signature: _____ **Date:** _____

For Litron use only

Principal Investigator _____ GLP Number: _____

Principal Investigator's Signature _____ Date

1. Objective

This MicroFlow® Study Phase Plan describes procedures for analyzing test facility-prepared mouse or rat blood samples for the presence of micronuclei (MN) using the MicroFlow procedure. Micronuclei will be analyzed in the CD71-positive immature erythrocyte (also called reticulocyte or RET) population to provide an indication of genotoxicity. The frequency of 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. 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. A Sample Submission Form and Sample Tracking Form should accompany each shipment of samples to Litron.

5. Flow Cytometric Analysis (performed at Test Site)

- **Sample Receipt**

  Upon receipt at Litron, whole blood samples will be diluted with anticoagulant and fixed into ultracold fixative. After that, the fixed blood samples will be stored in methanol or Long Term Storage Solution (LTSS) in a freezer (-85 °C ± 5 °C) until they are prepared 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 2 °C to 10 °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 2 °C to 10 °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* (for rat samples), or methanol-fixed blood from mice infected with *Plasmodium berghei* and methanol-fixed blood from rats (for mouse samples) will be used to configure and calibrate the flow cytometer before analysis.

- **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 for mouse blood samples) 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 per 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 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, study phase plan, and other 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.

9. References

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- Section 4 of the OECD Guidelines for the Testing of Chemicals: Mammalian Erythrocyte Micronucleus Test, Guideline 474 (Adopted 26th September 2014).
- 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 Harmonised Tripartite Guideline: Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use, S2(R1), current *Step 4* version dated 9 November 2011.

10. Effective Date: August 01, 2021