Study Phase Plan: MutaFlow^{BASIC} Pig-a Analysis Kit (P15MFv19)
Frozen Rodent Blood

An original signed Study Phase Plan document and Sample Submission Form are required for each study. If requesting GLP analysis, a copy of your protocol is also required prior to sample analysis.

A. Contact Information

Testing Facility Name and Address:

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_____

Testing Facility Study Director:

Name _____

Phone _____

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

☐ For analysis in compliance with FDA GLP regulations, initial here. Provide the agency name(s) that the data will be submitted to: _____. A copy of your protocol is required. Label samples with Sample ID, Study ID, Date Collected, Source (i.e., mouse or rat) and Type (i.e., blood).

☐ For Non-GLP analysis, initial here if a study phase 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 testing 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 MutaFlow® Study Phase Plan describes procedures for analyzing testing facility-submitted frozen mouse or rat blood samples and determining the frequencies of mutant phenotype erythrocytes (RBCs), mutant phenotype immature erythrocytes (reticulocytes, or RETs), and RETs using flow cytometry. The method is based on the endogenous *Pig-a* gene whose product is essential for the synthesis of glycosylphosphatidylinositol (GPI) anchors.

2. Introduction

Hematopoietic cells require GPI anchors to attach a host of proteins to their cell surface, for instance, CD24, CD59, and CD55. Importantly, of the genes required to form GPI anchors, only *Pig-a* is located on the X-chromosome. Mutations in the *Pig-a* gene can prevent functional anchors from being produced, resulting in cells lacking these proteins on their surface. Thus, cells without these cell surface markers represent a reliable phenotypic marker of *Pig-a* mutation. Flow cytometry is used for this analysis, as it provides a high-speed method for objective scoring of these very rare events. *Pig-a* mutation occurs spontaneously at a very low frequency, but mutagens cause an increase in the number of mutants relative to the background (spontaneous) level.

3. Proposed Study Dates

The experimental start and end dates will be documented in the raw data.

4. Experimental Procedures (performed at Testing Facility)

The testing 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. In addition to this Study Phase Plan, a Sample Submission Form should accompany each shipment of samples to Litron.

5. Flow Cytometric Analysis (performed at Test Site)

- **Sample Receipt, Labeling and Column Fractionation**

  Upon receipt at Litron, frozen blood samples will be either immediately thawed or stored in a -75 °C to -85 °C freezer and thawed at a later date. After thawing, samples are processed through Lympholyte®-Mammal Solution to remove the majority of leukocytes and platelets.

  Cells are then incubated with Anti-CD24-PE (mouse) or Anti-CD59-PE (rat) to label wild-type (wt) erythrocytes and Anti-CD61-PE (to label remaining platelets). Antibody-labeled samples are incubated with Anti-PE MicroBeads, which bind to these antibodies. A small fraction of each sample is stained with a nucleic acid dye (to differentiate leukocytes and RETs from mature RBCs). This dye solution also includes fluorescent Counting Beads and these “Pre-Column” samples are analyzed to capture Cell:Bead ratios.

  The remaining portion (majority) of the blood sample is applied to a column that has been suspended in a magnetic field. These columns selectively retain wt cells, whereas *Pig-a* mutant phenotype cells (lacking CD24 or CD59 on their surface) pass through the columns.

  Eluates are collected and centrifuged, stained with a nucleic acid dye (to differentiate leukocytes and RETs from mature RBCs). This dye solution also includes fluorescent Counting Beads and these “Post-Column” samples are analyzed on a flow cytometer to capture Mutant Cell:Bead ratios.

- **Flow Cytometer Calibration**

  An Instrument Calibration Standard sample 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.

6. Data Provided

For Pre-Column samples, a stop mode will be used based on the length of time needed to acquire at least 1,000 Counting Beads.

For Post-Column samples, a stop mode will be used based on the length of time needed to analyze nearly the entire volume of cells and Counting Beads.

From the Pre- and Post-Column analyses, the following values are calculated:

- Frequency of RETs, an index of bone marrow toxicity, expressed as percent of total RBCs
- Frequency of mutant-phenotype RBCs, expressed as number per 1,000,000 total RBCs
    - Note that upon acute mutagen exposure this index of genotoxicity is not expected to reach a maximal response until the entire cohort of circulating RBCs has turned over (approximately 30 days for mice, and approximately 60 days for rats).
- Frequency of mutant-phenotype RETs, expressed as number per 1,000,000 total RETs
    - Note that upon acute mutagen exposure this index of genotoxicity reaches a maximal value faster than mutant phenotype RBCs (often within approximately 2 weeks), since RETs are turned over at a much faster rate than the total RBC pool.

7. Evaluation and Interpretation of Results

No statistical analyses will be performed on the data, other than the calculations indicated above, and the testing facility will be responsible for the evaluation and interpretation of results.

8. Records Maintained

Unless indicated on page 1, the original study phase plan and study-specific records will be transferred to the testing facility at the completion of the study phase. Litron will maintain a copy of the study phase plan and copies of the paper and electronic records for two years following completion of the analysis. After the retention period, Litron will contact the testing facility and these items will either be discarded or sent to the testing facility-requested location.

9. References

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10. Effective Date: September 14, 2026