Study Phase Plan: MutaFlow^{BASIC} Pig-a Analysis Kit (P10MFv16)
Rodent Whole 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.

PRIOR TO INITIATING ANY EXPERIMENT, YOU MUST CONTACT LITRON (INFO@LITRONLABS.COM OR 585-442-0930) TO DISCUSS SPECIFIC DATES AND TIMES FOR BLOOD SAMPLE SHIPMENT AND RECEIPT. BLOOD SAMPLES SUBMITTED SHOULD BE PROCESSED WITHIN APPROXIMATELY 24 HRS OF RECEIPT. THEREFORE, CAREFUL PLANNING AND COORDINATION WITH LITRON IN ADVANCE IS ESSENTIAL. WE CANNOT ENSURE PROPER RECEIPT AND ANALYSIS OF YOUR SAMPLES UNLESS THESE REQUIREMENTS ARE MET.

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

A. Contact Information

Test Facility Name and Address:

_____

_____

_____

_____

Test Facility Study Director:

Name _____

Phone _____

Fax _____

Email _____

B. Study Information

Study ID: _____ Indicate Species: _____ Rat _____ Mouse 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. 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 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 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 MutaFlow® Study Phase Plan describes procedures for analyzing test facility-submitted 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 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. 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, whole blood samples will be processed through Lympholyte®-Mammal Solution to remove the majority of leukocytes and platelets or placed into freezing solution and frozen for subsequent thawing and leukodepletion.

  Leukodepleted cells are then incubated with Anti-CD24-PE and Anti-CD45-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 preferably 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 test facility will be responsible for the evaluation and interpretation of results unless indicated in Section B.

8. Records Maintained

The original study phase plan and study-specific records will be transferred to the test 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 sponsor and these items will either be discarded or sent to the sponsor-requested facility.

9. References

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10. Effective Date: May 8, 2019