



MutaFlow[®]

Pig-a Mutation Analysis

MutaFlow^{BASIC} (rodent)

Instruction Manual

For research only. Not for use in diagnostic or therapeutic procedures.

Table Of Contents

1. Important Note	2
2. Materials Provided.....	2
3. Additional Materials Required	2
4. Ordering Information and Technical Services.....	2
5. License Agreement and Limited Product Warranty.....	2
6. First-Time Users	3
6.1. Study Design.....	3
7. Introduction	3
8. Overview of Method.....	3
9. Collect Whole Blood Samples	3
10. Ship Whole Blood Samples.....	4
11. Results.....	4
12. References.....	4
Appendix A: Blood Collection Advice Chart.....	5

1. IMPORTANT NOTE

PRIOR TO INITIATING ANY EXPERIMENT, YOU MUST CONTACT LITRON (585-442-0930 OR INFO@LITRONLABS.COM) TO DISCUSS SPECIFIC DATES AND TIMES FOR BLOOD SAMPLE SHIPMENT AND RECEIPT. BLOOD SAMPLES FOR PIG-A MUTATION ANALYSIS MUST BE PROCESSED AND ANALYZED AT LITRON WITHIN APPROXIMATELY 24 HRS OF RECEIPT, SO 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.

2. Materials Provided

Kit Component	Quantity ^a	Storage Condition
K ₂ EDTA Blood Collection tubes	25	Ambient
Anticoagulant/Diluent	10 ml	2 °C to 10 °C
Exakt-Pak Shipping Container	2	Ambient
Foam Cold Packs	4	-10 °C to -30 °C
Thin clear plastic bag for shipping required forms (Study Phase Plan and Sample Submission Form – see below)	1	Ambient

- a. Each kit provides sufficient materials for the analysis of up to 25 blood samples at Litron.

3. Additional Materials Required

- Refrigerator set at 2 °C to 8 °C
- -10 °C to -30 °C freezer for chilling the cold packs
- Shipping forms for overnight delivery service
- The Sample Submission Form and Study Phase Plan are available online (www.LitronLabs.com). Litron requires a Sample Submission Form and a signed Study Phase Plan. Analyses cannot be completed prior to receipt of an approved Protocol (for GLP studies).

4. Ordering Information and Technical Services

Litron Laboratories
 3500 Winton Place, Suite 1B
 Rochester, New York 14623
 Telephone: 585-442-0930
 Order Toll Free: 877-4-LITRON (877-454-8766)
 Fax: 585-442-0934
 email: info@LitronLabs.com
 World Wide Web: www.LitronLabs.com

5. License Agreement and Limited Product Warranty

By utilizing this kit, your company is agreeing to be bound by the terms of this License. This License allows the use of the MutaFlow[®] Kit for the analysis of 25 samples, either in-house (MutaFlow^{PLUS} Kit), or at Litron's facility (MutaFlow^{BASIC} Kit).

MutaFlow[®]. All rights reserved. MutaFlow[®] is a registered trademark of Litron Laboratories. Patent Nos. 7,824,874, 8,062,860, 8,187,826, and patents pending. Copyright 2003-2022, Litron Laboratories.

By accepting these products, you acknowledge that they will be used in accordance with their intended labeling (For in vitro research use only. Not for human or animal diagnostic or therapeutic use.). Uses other than the labeled intended use may be a violation of local laws.

This warranty limits our liability to replacement of this product. Litron shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

6. First-Time Users

We strongly recommend reading the entire instruction manual before performing these procedures.

Please do not deviate from the procedures described in this manual. It is important that these steps are followed exactly using the supplies and shipping materials supplied with this kit in order to achieve reliable results. If you have questions, please contact Litron Laboratories by calling (585) 442-0930, faxing us at (585) 442-0934, or sending an email to info@litronlabs.com.

6.1. Study Design

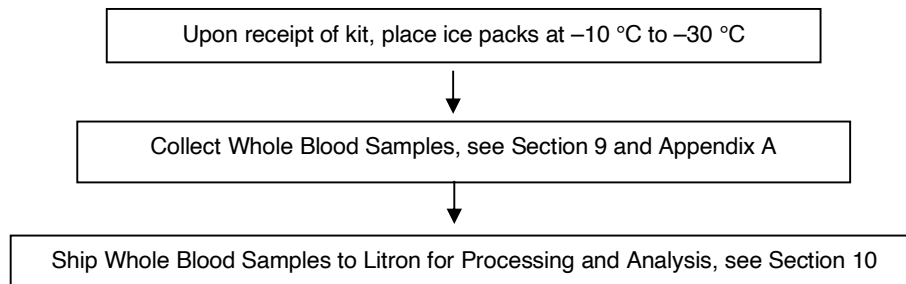
It is beyond the scope of this instruction manual to provide guidance about experimental designs. When considering the number of treatment groups, number of rodents per treatment group, treatment schedule, etc., please consult the OECD Test Guideline 470 “Mammalian Erythrocyte *Pig-a* Gene Mutation Assay” which can be found at [this link](#).

7. Introduction

This instruction manual describes procedures for collecting and shipping rodent blood that will be analyzed for the frequency of mutant phenotype erythrocytes (RBCs) and mutant phenotype immature erythrocytes (reticulocytes, or 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. 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.

8. Overview of Method

The following steps are performed when preparing whole blood samples for shipment to Litron using the MutaFlow^{BASIC} Whole Blood Kit.



9. Collect Whole Blood Samples

If whole blood samples are not received at Litron within 24 after collection, the resulting blood samples may be compromised and not compatible with flow cytometric analysis. We advise using the earliest “next day” shipping method available, e.g. FedEx FIRST OVERNIGHT. In addition, it is very important to follow the storage (and shipping) instructions provided in this manual.

IMPORTANT NOTE: For most rodent strains, it is important to evaluate at least several million reticulocytes for the mutant phenotype in order to avoid mutant-phenotype reticulocyte values of zero. For zero values to be an occasional rather than a common finding, collect between 120 μ L and 150 μ L of blood per rodent per time point. With these target volumes, it will be possible to process between 80 μ L and 120 μ L of blood per rodent per time point. Note that while we specify a range of blood volumes here, it is most ideal to collect and process consistent volumes within the same experiment whenever possible. If possible, collect enough blood to have backup samples in the event of any issues.

1. Collect free-flowing blood sample (see Appendix A). Required volumes are specific to the bleeding technique used, and are indicated in Appendix A.

- Repeat step 1 for each blood sample. Blood/Anticoagulant Solution can be maintained in K₂EDTA tubes at ambient temperature for up to 2 hrs. For longer periods of time, maintain K₂EDTA tubes at 2 °C to 8 °C.
- Place the samples in the plastic secondary container and maintain at 2 °C to 8 °C (not on ice) until shipment to Litron Laboratories (same day). Keep the blood cold, but not frozen. Blood should arrive at Litron within 24 hours after collection.

10. Ship Whole Blood Samples

Ship whole blood samples the same day they are collected for overnight delivery to Litron Laboratories. Trained personnel must follow the applicable guidelines and regulations regarding proper shipping and packaging of whole blood (USDOT, ICAO, IATA 650).

1. Complete Study Paperwork

Complete and sign the appropriate Sample Submission Form and Study Phase Plan and place them inside the thin clear plastic bag. These are necessary for sample analysis.

2. Package the Samples

Ensure that the ice packs are frozen. Place a frozen ice pack in the bottom of the box. Place the secondary container (screw-top wide-mouthed HDPE jar) housing the samples on top of the ice pack. Place the second ice pack on top of the container. See diagram at right.

3. Seal and Label the Box

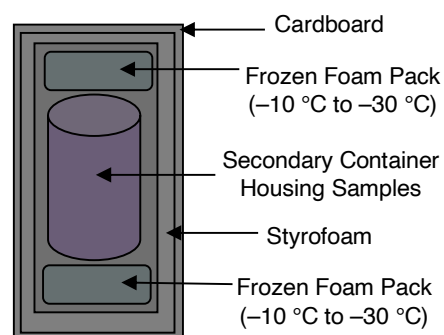
Place the thin clear plastic bag containing the applicable forms on top of the foam insert. Close the cardboard flaps of the outer box and use shipping tape to secure the middle seam of the box top. Please note, the nomenclature "Diagnostic Specimen" has been replaced by "Biological Substance, Category B". This wording, along with a UN 3373 label, must be visible on the outside of the box as well as on the air waybill in the "Nature and Quantity of Goods" box.

4. Ship Samples to Litron Laboratories at the following address:

Litron Laboratories
Attn: Processing Division
3500 Winton Place, Suite 1B
Rochester, New York 14623
585-442-0930

Immediately after shipping send an email to info@litronlabs.com including your name, telephone number, date of shipment, number of samples, shipping company, and the shipper's tracking number. You can also include the Study Phase Plan in the email.

Use the shipping box and cold packs that were provided by Litron. They have been specifically chosen for the purpose of maintaining proper temperatures during transit and to ensure that whole blood samples are received cold (2 °C to 8 °C), but not frozen. Ambient or frozen shipments are unacceptable.



Unexpected shipping delays may occur at any time. Therefore, it is best to ship samples on Monday or Tuesday and to avoid shipping during holidays.

11. Results

Results will be emailed after analysis and verification of data.

12. References

An updated list of journal articles utilizing this method can be found at www.LitronLabs.com/Resources/Publications/In-Vivo-MutaFlow-Kits.

OECD (2002) Test No. 470: Mammalian Erythrocyte Pig-a Gene Mutation Assay, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, <https://doi.org/10.1787/4faea90e-en>.

Appendix A: Blood Collection Advice Chart

Blood Collection Method*	Necessary Equipment	Blood Collection	Blood Storage	Miscellaneous Notes
Cardiac puncture	Appropriately sized needle (e.g., 20 gauge) and 1 cc syringe; equipment to deliver an overdose of CO ₂ or another anesthetizing agent	Coat a needle and syringe with kit-supplied Anticoagulant Solution. Expel the liquid. - For most needle and syringe combinations, this will leave approximately 50 to 60 μ L of Anticoagulant Solution in the so-called dead volume. - If using a fixed needle and syringe with considerably less dead volume, it will be necessary to leave approximately 50 to 60 μ L Anticoagulant Solution behind. Collect approximately 300 μ L blood per rodent per time point. It is important to open the caps on the K,EDTA Microtainer tubes (e.g., BD cat # 365974) as opposed to puncturing the septum with the needle to transfer the blood. Once blood is added, make sure the tube is tightly recapped for transport.	For blood that will be labeled and analyzed the day it is collected, immediately transfer 80 to 120 μ L blood per rodent per time point into a labeled microcentrifuge tube containing 100 μ L Anticoagulant Solution. Refrigerate blood samples in Anticoagulant Solution as soon as possible (they can remain at ambient temperature for up to 4 hours). For blood that will be stored overnight before labeling and analysis occurs, or for blood that will be shipped to another location, do <u>not</u> transfer to microcentrifuge tubes, rather maintain refrigerated in Microtainer tube until labeling or shipment occurs. Appendix D provides additional advice for these alternate procedures.	Animals should be anesthetized/overdosed with CO ₂ for this procedure, but the blood draw should occur while the rodent's heart is still beating
Venipuncture, jugular stick, or similar	Appropriately sized needle and 1 cc syringe; depending on the site of the vein or artery, equipment to deliver an overdose of CO ₂ or another anesthetizing agent may be necessary	Coat a needle and syringe with kit-supplied Anticoagulant Solution. Expel the liquid. - For most needle and syringe combinations, this will leave approximately 50 to 60 μ L of Anticoagulant Solution in the so-called dead volume. - If using a fixed needle and syringe with considerably less dead volume, it will be necessary to leave approximately 50 to 60 μ L Anticoagulant Solution behind. Collect approximately 300 μ L blood per rodent per time point. It is important to open the caps on the K,EDTA Microtainer tubes (e.g., BD cat # 365974) as opposed to puncturing the septum with the needle to transfer the blood. Once blood is added, make sure the tube is tightly recapped for transport.	For blood that will be labeled and analyzed the day it is collected, immediately transfer 80 to 120 μ L blood per rodent per time point into a labeled microcentrifuge tube containing 100 μ L Anticoagulant Solution. Refrigerate blood samples in Anticoagulant Solution as soon as possible (they can remain at ambient temperature for up to 4 hours). For blood that will be stored overnight before labeling and analysis occurs, or for blood that will be shipped to another location, do <u>not</u> transfer to microcentrifuge tubes, rather maintain refrigerated in Microtainer tube until labeling or shipment occurs. Appendix D provides additional advice for these alternate procedures.	Some animal use protocols allow rodents to be warmed prior to tail bleeding to promote blood vessel dilation; animals must be closely monitored during the period of heat exposure

Blood Collection Method*	Necessary Equipment	Blood Collection	Blood Storage	Miscellaneous Notes
Tail vein incision	Heat lamps and/or heat pads; animal restrainers; sterile surgical blades or razor bloods; heparin-coated capillary tubes	<p>Add 20 μL of kit-provided Anticoagulant Solution to each K₂EDTA Microtainer tube [we recommend BD cat # 365974 or comparable].</p> <ul style="list-style-type: none"> - One tube is needed for each animal. - Use two tubes if a backup (duplicate) sample is desired. <p>The goal of warming the animals and making an incision is to generate free-flowing blood. Once the blood starts flowing, use heparin-coated capillary tube(s) to collect between 120 to 150 μL of blood.</p> <ul style="list-style-type: none"> - Double the amount of blood if you wish to have a backup sample. <p>Immediately transfer 120 to 150 μL blood to each of one or two K₂EDTA tubes and gently pipette up and down 3 times to mix with the Anticoagulant Solution.</p>	<p>For blood that will be labeled and analyzed the day it is collected, immediately transfer 80 to 120 μL blood per rodent per time point into a labeled microcentrifuge tube containing 100 μL Anticoagulant Solution. Refrigerate blood samples in Anticoagulant Solution as soon as possible (they can remain at ambient temperature for up to 4 hours).</p> <p>For blood that will be stored overnight before labeling and analysis occurs, or for blood that will be shipped to another location, do <u>not</u> transfer to microcentrifuge tubes, rather maintain refrigerated in Microtainer tube until labeling or shipment occurs. Appendix D provides additional advice for these alternate procedures.</p>	<p>Tail vein incision will <u>not</u> provide sufficient blood volume unless the rodents are warmed to promote blood vessel dilation; animals must be closely monitored during the period of heat exposure and for a short time after to ensure bleeding has ceased</p>
Cheek puncture (i.e., submandibular bleed)	Appropriately sized lancets; heparin-coated capillary tube(s)	<p>Add 20 μL of kit-provided Anticoagulant Solution to each K₂EDTA Microtainer tube [we recommend BD cat # 365974 or comparable].</p> <ul style="list-style-type: none"> - One tube is needed for each animal. - Use two tubes if a backup (duplicate) sample is desired. <p>Once the blood starts flowing, use heparin-coated capillary tube(s) to collect between 120 to 150 μL of blood.</p> <ul style="list-style-type: none"> - Double the amount of blood if you wish to have a backup sample. <p>Immediately transfer 120 to 150 μL blood to each of one or two K₂EDTA tubes and gently pipette up and down 3 times to mix with the Anticoagulant Solution.</p>	<p>For blood that will be labeled and analyzed the day it is collected, immediately transfer 80 to 120 μL blood per rodent per time point into a labeled microcentrifuge tube containing 100 μL Anticoagulant Solution. Refrigerate blood samples in Anticoagulant Solution as soon as possible (they can remain at ambient temperature for up to 4 hours).</p> <p>For blood that will be stored overnight before labeling and analysis occurs, or for blood that will be shipped to another location, do <u>not</u> transfer to microcentrifuge tubes, rather maintain refrigerated in Microtainer tube until labeling or shipment occurs. Appendix D provides additional advice for these alternate procedures.</p>	<p>Some groups suggest this is most appropriate for mice, less so for rats; other groups have reported success using rats</p>

			Blood Storage	Miscellaneous Notes
Blood Collection Method* Retro-orbital bleed	Necessary Equipment Heparin-coated capillary tube(s)	Blood Collection Add 20 μ L of kit-provided Anticoagulant Solution to each K ₂ EDTA Microtainer tube [we recommend BD cat # 365974 or comparable]. - One tube is needed for each animal. - Use two tubes if a backup (duplicate) sample is desired. Once the blood starts flowing, use heparin-coated capillary tube(s) to collect between 120 to 150 μ L of blood. - Double the amount of blood if you wish to have a backup sample. Immediately transfer 120 to 150 μ L blood to each of one or two K ₂ EDTA tubes and gently pipette up and down 3 times to mix with the Anticoagulant Solution.	Blood Storage For blood that will be labeled and analyzed the day it is collected, immediately transfer 80 to 120 μ L blood per rodent per time point into a labeled microcentrifuge tube containing 100 μ L Anticoagulant Solution. Refrigerate blood samples in Anticoagulant Solution as soon as possible (they can remain at ambient temperature for up to 4 hours). For blood that will be stored overnight before labeling and analysis occurs, or for blood that will be shipped to another location, do <u>not</u> transfer to microcentrifuge tubes, rather maintain refrigerated in Microtainer tube until labeling or shipment occurs. Appendix D provides additional advice for these alternate procedures.	Miscellaneous Notes None

*Your chosen method should be in compliance with relevant local regulations, and all necessary animal use approvals you operate under. This takes precedence over any of the general advice supplied here.