MutaFlow Rodent Blood Freezing

Instruction Manual

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

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1. Materials Provided

Kit Component	Quantity[a]	Storage Condition[b]
Anticoagulant Solution	10 mL	2 °C to 8 °C
Blood Freezing Solution	30 mL	Ambient
Cryovials	50	Ambient
Cryovial Storage Boxes	2	Ambient

a. Sufficient materials are provided to collect 25 rodent blood samples and freeze in duplicate.
b. Please note that although kit components are shipped at ambient temperature, they must be stored at the temperatures indicated above upon receipt.

2. Additional Materials Required

- Blood collection supplies
- Depending on blood collection technique, heparin-coated capillary tubes, 115 µl capacity (e.g., RAM Scientific cat # 06 0005), requires 2 per rodent
- K_2EDTA Microtainer tubes (e.g., BD cat # 365974)
- Micropipettors (20 µL - 1000 µL) and tips
- 2 °C to 8 °C refrigerator
- Labels compatible with ultracold storage (Litron recommends Cryo-Tags® labels)
- –75 °C to –85 °C freezer
- Dry ice (if shipping samples to Litron or another site for analysis)

3. 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

4. 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® Rodent Blood Freezing Kit for the collection of 25 samples and for freezing in duplicate.

By accepting these products, you acknowledge that they will be used in accordance with their intended labeling (For i*n 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.

5. 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 reagents 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.

5.1. 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.

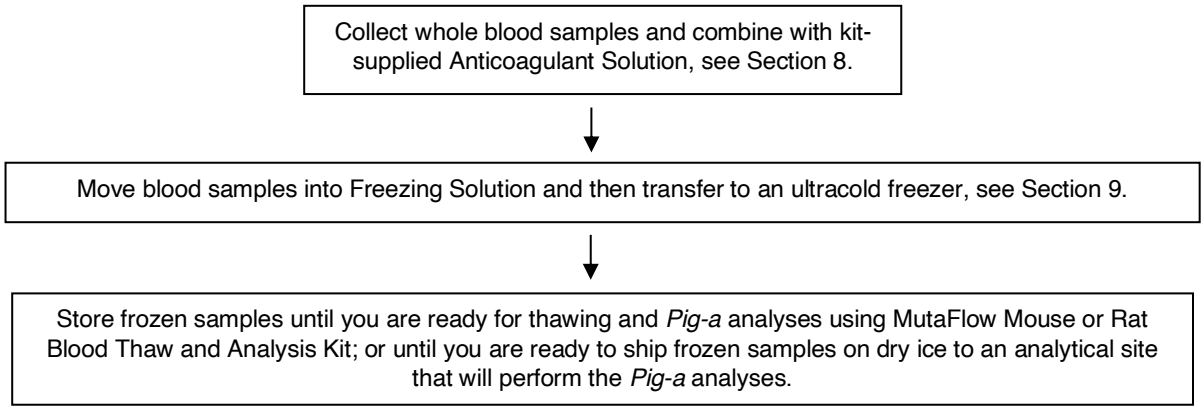
6. Introduction

This instruction manual describes procedures for collecting and freezing rodent blood that may subsequently be analyzed for the frequency of mutant phenotype erythrocytes (RBCs) and mutant phenotype immature erythrocytes (reticulocytes, or RETs) using flow cytometry.

The MutaFlow *Pig-a* analysis 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, CD55, and CD59). 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.

7. Overview of Method

The following steps are performed when preparing blood samples for storage using the MutaFlow Rodent Blood Freezing Kit.



8. Collect Whole Blood Samples

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 µL and 150 µL 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.

2. Repeat step 1 for each blood sample. Blood/Anticoagulant Solution can be maintained in K_2EDTA tubes at ambient temperature for up to 2 hrs. For longer periods of time, maintain K_2EDTA tubes at 2 °C to 8 °C.

9. Rodent Blood Freezing

1. Aliquot 500 µL of Rodent Blood Freezing Solution into pre-labeled cryovials, two per rodent, and maintain at ambient temperature.

Freezing in duplicate is strongly advised.

2. Gently resuspend cells by pipetting and transfer 100 µL to 150 µL of whole blood/Anticoagulant Solution mixture from the K_2EDTA tube into the Freezing Solution. Mix by gently pipetting 8 to 10 times. Repeat so that each sample is prepared in duplicate.

3. Repeat step 2 for additional samples in batches of up to 8 cryovials.

4. Incubate each batch of up to 8 cryovials in Freezing Solution at ambient temperature for 5 ± 2 minutes before transferring to a freezer set to –75 °C to –85 °C.

5. Samples should remain frozen for at least 48 hours before analysis or shipping. They can be stored frozen for at least 1 year. Frozen samples can be analyzed for *Pig-a* frequencies by purchasing a MutaFlow Mouse or rat Blood Thaw and analysis Kit. Alternatively, frozen samples can be shipped to an analytical site for *Pig-a* analyses. In this case it is important for the samples to remain frozen throughout transit using dry ice, replenishing dry ice as necessary.

10. 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/Freezing**	**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 µL 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 µL Anticoagulant Solution behind. Collect approximately 300 µL blood per rodent per time point. It is important to open the caps on the K_2EDTA 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.	The goal should be to freeze 100 µL to 150 µL of whole blood/Anticoagulant Solution mixture in duplicate. See Sections 8.2 and 9.	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 µL 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 µL Anticoagulant Solution behind. Collect approximately 300 µL blood per rodent per time point. It is important to open the caps on the K_2EDTA 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.	The goal should be to freeze 100 µL to 150 µL of whole blood/Anticoagulant Solution mixture in duplicate. See Sections 8.2 and 9.	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	Add 20 μL of kit-provided Anticoagulant Solution to each K_2EDTA 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. 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. - Double the amount of blood if you wish to have a back-up sample. Immediately transfer 120 to 150 μL blood to each of one or two K_2EDTA tubes and gently pipette up and down 3 times to mix with the Anticoagulant Solution.	The goal should be to freeze 100 μL to 150 μL of whole blood/Anticoagulant Solution mixture in duplicate. See Sections 8.2 and 9.	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
Cheek puncture (i.e., submandibular bleed)	Appropriately sized lancets; heparin-coated capillary tube(s)	Add 20 μL of kit-provided Anticoagulant Solution to each K_2EDTA 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 μL of blood. - Double the amount of blood if you wish to have a back-up sample. Immediately transfer 120 to 150 μL blood to each of one or two K_2EDTA tubes and gently pipette up and down 3 times to mix with the Anticoagulant Solution.	The goal should be to freeze 100 μL to 150 μL of whole blood/Anticoagulant Solution mixture in duplicate. See Sections 8.2 and 9.	Some groups suggest this is most appropriate for mice, less so for rats; other groups have reported success using rats

Blood Collection Method*	**Necessary Equipment**	**Blood Collection**	**Blood Storage**	**Miscellaneous Notes**
Retro-orbital bleed	Heparin-coated capillary tube(s)	Add 20 µL of kit-provided Anticoagulant Solution to each K_2EDTA 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 µL of blood. - Double the amount of blood if you wish to have a back-up sample. Immediately transfer 120 to 150 µL blood to each of one or two K_2EDTA tubes and gently pipette up and down 3 times to mix with the Anticoagulant Solution.	The goal should be to freeze 100 µL to 150 µL of whole blood/Anticoagulant Solution mixture in duplicate. See Sections 8.2 and 9.	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.