



MutaFlow[®]

Pig-a Mutation Analysis

In Vitro MutaFlow 150

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 Conditions ^b
1X Buffer Solution	1 L	Unopened: Ambient Opened: 2 °C to 8 °C
Anti-CD45-FITC Antibody ^c	65 µL	2 °C to 8 °C
Anti-CD90.2-PE Antibody ^c	25 µL	2 °C to 8 °C
Isotype Control Antibody ^c	25 µL	2 °C to 8 °C

- Sufficient materials are provided to analyze 150 samples.
- Please note that although some kit components are shipped at ambient temperature, they must be stored at the temperatures indicated above upon receipt.
- Warning! Contains sodium azide. Irritant. See SDS (available on website).

2. Additional Materials Required

- Cell line L5178Y/*Tk*^{+/−} 3.7.2C and appropriate growth medium
- CO₂-regulated, 37 °C incubator
- Cell culture supplies such as T25 flasks, etc.
- 20 °C freezer
- 4 °C refrigerator
- Centrifuge
- Vortex mixer
- Heat-inactivated fetal bovine serum (FBS)
- Filter-sterilized deionized water (dH₂O)
- Disposable pipettes sized 5, 10, 25, and 50 mL
- Polypropylene centrifuge tubes (e.g., 15 mL)
- Micropipettors (20 µL - 1000 µL) and tips
- Flow cytometry tubes
- Flow cytometer equipped with 488 nm excitation source. Optional: red laser line, e.g. 633 nm
- Optional: DRAQ7™ Solution, Cell Signaling Technologies Catalog No. 7406

3. Technical Support

Litron Laboratories
 3500 Winton Place, Suite 1B
 Rochester, New York 14623
 Telephone: 585-442-0930
 Order Toll Free: 877-4-LITRON (877-454-8766)
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4. Introduction

This instruction manual describes procedures for the flow cytometric enumeration of mutant phenotype cells using the mouse lymphoblastoid cell line L5178Y/*Tk*^{-/-} 3.7.2C (hereafter abbreviated L5178Y). The method is based on the endogenous *Pig-a* gene whose product is essential for the synthesis of glycosylphosphatidylinositol (GPI) anchors.

Lymphoblastoid cells require GPI anchors to attach a host of proteins to their cell surface, for instance, CD90.2. 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* gene mutation.

5. Recommended Reading for First-Time Users

Please read the entire instruction manual before performing these procedures. Deviating from the procedures described in this manual may adversely affect the results of your assay. Substitution of kit components, changes in cell culture conditions, incubation times, reagent volumes, etc., is not advisable. If you have questions, please contact Litron by calling (585) 442-0930, faxing us at (585) 442-0934, or sending an email to info@litronlabs.com.

5.1. Cell Lines

This instruction manual and accompanying reagents were designed for use with the mouse lymphoblastoid cell line L5178Y. As the fluorescent antibodies in this kit are specific for mouse antigens, the ability to apply these kit reagents to other cell lines is unknown.

5.2. Fluorescent Reagents

Anti-CD90.2-PE Antibody binds to the CD90.2 protein which is expressed at high levels on the cell surface of non-mutant "wildtype" L5178Y cells. Loss of cell surface CD90.2-PE-associated fluorescence is used in this assay as a phenotypic reporter of *Pig-a* gene mutation.

Anti-CD45-FITC Antibody serves to identify a pan-leukocyte marker that is expressed at high levels on healthy L5178Y cells. As CD45 is not anchored to the cell membrane of L5178Y cells using GPI anchors, by restricting *Pig-a* gene mutant cell frequency determinations to CD45-positive cells, users can ensure that i) all cells came into sufficient contact with the antibody-containing solution, and ii) cells evaluated for the *Pig-a* mutant phenotype do not have global dysfunction(s) with membrane receptor expression that could be mistaken as *Pig-a* mutation.

For users with flow cytometers that are equipped with a red diode laser, optional DNA stain DRAQ7™ is used as a membrane-integrity dye. It allows users to gate out cells with compromised membranes (DRAQ7™-positive cells), which results in *Pig-a* gene mutant cell frequency determinations that are not greatly influenced by the presence of dead or dying cells.

5.3. Chemical Treatment

L5178Y cells are commonly treated for 3 to 4 hours or for 24 hours. Although any number of treatment periods should be compatible with this assay, the majority of supporting data generated to date were developed with 24 continuous hours of exposure.

5.4. Negative and Positive Controls

Solvent control-treated cultures should be included in each experiment. It is a good practice to avoid exceeding 1 % v/v concentration when organic solvents such as DMSO are used.

Ideally, concurrent positive control-treated cultures should also be included in each experiment. The majority of supporting data generated to date have used ethyl methanesulfonate as a concurrent positive control. For 24 hour continuous treatment, we found that 250 μ M to 500 μ M ethyl methanesulfonate elicits a robust and reproducible *Pig-a* mutant response.

5.5. Cytotoxicity

It is important to consider cytotoxicity while conducting genotoxicity assays, and most assays are understood to have a cytotoxicity limit beyond which data are not considered meaningful or reliable. There are numerous approaches for studying chemically-induced cytotoxicity. The majority of data generated to date with this assay were developed with cytotoxicity measurement made 24 hours after the beginning of treatment. At this 24 hour time point, cell counts were made and expressed relative to the mean value of solvent control cultures. These “relative cell counts” were used to guide top concentration evaluated for induction of *Pig-a* mutants.

At Litron we limited the highest concentration tested to one that causes approximately 50 % reduction to Relative Cell Count (RCC). It should be noted that this value, the manner in which cytotoxicity is determined, and the time at which cytotoxicity is evaluated should be considered provisional and in need of further study.

5.6. Mutant Manifestation Time

Inactivating mutation(s) to the *Pig-a* gene require several days before they fully manifest as CD90.2-negative L5178Y cells; this is known as mutant manifestation time. In our experience, cells should be analyzed for the *Pig-a* mutant phenotype approximately one week after initiation of treatment. At this point mutagen-induced frequencies have stabilized and are at their highest point.

5.7. Isotype Control

Optimal fluorescent resolution is critical when scoring rare events. Therefore, an Isotype Control sample is crucial for consistently setting appropriate PMT voltages and compensation on a day-to-day basis, and for establishing the demarcation line that defines mutant and wild-type cells. In other words, Isotype Control Antibody-labeled cells may be thought of as mutant mimics, and the use of these specimens are described in greater detail later in this manual.

5.8. Cell Passage

It is advisable to maintain L5178Y cells in log-phase growth and at a cell density less than 1×10^6 per mL. This cell density should not be exceeded during routine passage, or the approximately 1 week of mutant manifestation time. Especially during the mutant manifestation time, we recommend determining cell densities of each culture and splitting cells into fresh growth media on a daily basis, or at most every other day.

During mutant manifestation time, our current best advice is to transfer at least 1×10^6 total cells per culture into fresh media every time cells are passed. For groups that have spontaneous mutant cell frequencies lower than 20 mutant cells per 10^6 wildtype cells, higher numbers of cells may have to be considered. By passing a minimum number of cells (and thereby numbers of mutant cells), it is possible to reduce the likelihood of causing large changes to mutant cell frequency by passing too few cells.

6. Cell Treatment Protocol

6.1. Exposure

1. Aliquot low passage, healthy, log-phase L5178Y cells into appropriate vessels. As a general rule, each treated culture should consist of greater than or equal to 1×10^6 total cells. For example, T25 flasks that contain 10 mL of growth medium with cells at 2×10^5 /mL suits this purpose.
2. Treat cells with test chemical(s) of interest.
3. Return treated cells to a 37 °C incubator for treatment time of either 3 to 4 hours, or for 24 hours.

6.2. Wash (Short Term Treatment)

1. After a 3 to 4 hour treatment time, resuspend the cultures by pipetting and transfer the entire volume of each sample into a separate, labeled conical centrifuge tubes.
2. Centrifuge the tubes at $340 \times g$ for 5 minutes.
3. Aseptically aspirate to remove supernatants.

4. Resuspend cells to a consistent volume with fresh growth media. This can be the original volume or less, for instance $\frac{1}{2}$ the original volume. The key considerations are that it should be a consistent volume, and it should result in cell densities that are amenable to scoring with your cell counting device/method of choice.
5. Pipette to form homogeneous suspensions and perform cell counts on each culture to determine cell density.
6. Pass at a density that ensures the cells do not exceed 1×10^6 per mL over the next 20 to 21 hours, and pass a minimum of 1×10^6 total cells per culture. We routinely utilize 10 mL in T25 flasks.
7. Incubate cultures for 20 to 21 hours.
8. At approximately 24 hours from initial treatment, pipette to form homogeneous suspensions and perform cell counts on each culture to determine cell density. Calculate % RCC for each treated culture based on the average cell density of solvent control cultures.
9. Determine the highest test chemical concentration based on a pre-defined cytotoxicity limit. For example, at Litron we pass only those concentrations that approached but did not exceed 50 % reduction to RCC.
10. Pass cells based on step 8 cell counts. Pass at a density that ensures the cells do not exceed 1×10^6 per mL over the next 24 hours, and pass a minimum of 1×10^6 total cells per culture. We routinely utilize 10 mL in T25 flasks.

6.3. Wash (Long Term Treatment)

1. After a 24 hour treatment time, resuspend the cultures by pipetting and transfer the entire volume of each sample into a separate, labeled conical centrifuge tubes.
2. Centrifuge the tubes at $340 \times g$ for 5 minutes.
3. Aseptically aspirate to remove supernatants.
4. Resuspend cells to a consistent volume with fresh growth media. This can be the original volume or less, for instance $\frac{1}{2}$ the original volume. The key considerations are that it should be a consistent volume, and it should result in cell densities that are amenable to scoring with your cell counting device/method of choice.
5. Pipette to form homogeneous suspensions and perform cell counts on each culture to determine cell density. Calculate % RCC for each treated culture based on the average cell density of solvent control cultures,.
6. Determine the highest test chemical concentration based on a pre-defined cytotoxicity limit. For example, at Litron we pass only those concentrations that approached but did not exceed 50 % reduction to RCC.
7. Pass cells based on step 5 cell counts. Pass at a density that ensures the cells do not exceed 1×10^6 per mL over the next 24 hours, and pass a minimum of 1×10^6 total cells per culture. We routinely utilize 10 mL in T25 flasks.

6.4. Passage

Over the course of the mutant manifestation time (approximately 1 week), perform cell counts to determine cell density as described above (6.2, step 5). Pass cells so that they do not grow over 1×10^6 /mL, and pass a minimum of 1×10^6 total cells per culture.

Ideally, repeat passage at approximately 24 hour intervals. Note that for the supporting data collected to date, cells were passed over one weekend at no lower than 1.5×10^5 total cells. In our experience this was not found to overly influence mutant cell frequencies if performed infrequently and if all other passages transferred a minimum of 1×10^6 total cells per culture. However, this may be a lab-specific variable and dependent on the spontaneous frequency of mutant cells. This advice should therefore be considered provisional and something that requires lab-specific determination.

7. Reagent Preparation

Solutions should be made fresh on the day of analysis. It is best to prepare 10 % extra to accommodate transfer loss.

7.1. Buffer Solution + 1 % FBS

Number of cultures	Volume of 1X Buffer Solution	Volume of FBS
1	4.95 mL	0.05 mL

1. Use the chart above to determine the volume of Buffer Solution + 1 % FBS required. Scale up as necessary.
2. Add the required volumes of 1X Buffer Solution and FBS to a clean vessel. Filter sterilize and store on ice or refrigerate until use.

7.2. Incomplete Antibody Solution

Number of cultures	Volume of Buffer Solution + 1 % FBS	Volume of Anti-CD45-FITC Antibody
1	100 μ L	0.3 μ L

1. Use the chart above to determine the volume of Incomplete Antibody Solution required. Scale up as necessary. Prepare an extra 1.0 mL for the Working Isotype Control Solution (Section 7.4, below). For example, for 20 cultures prepare 2.0 mL + 0.2 mL extra + 1.0 mL for the Working Isotype Control Solution, for a total of 3.2 mL.
2. Combine the required volumes of Buffer Solution + 1 % FBS and Anti-CD45-FITC Antibody in a clean vessel and mix gently. Protect from light and store on ice or refrigerate until use.

7.3. Complete Antibody Solution

Number of cultures	Volume of Incomplete Antibody Solution	Volume of Diluted Anti-CD90.2-PE Antibody
1	100 μ L	0.25 μ L

1. Use the chart above to determine the volume of Complete Antibody Solution required. Scale up as necessary.
2. Dilute the Anti-CD90.2-PE Antibody by adding 2 μ L of the antibody to 18 μ L of Buffer Solution + 1 % FBS (scale up if needed). Mix gently.
3. Combine the required volumes of Incomplete Antibody Solution and diluted Anti-CD90.2-PE Antibody in a clean vessel and mix gently. Protect from light and store on ice or refrigerate until use.

7.4. Working Isotype Control

Volume of Incomplete Antibody Solution	Volume of Diluted Isotype Control Antibody
1.0 mL	2.5 μ L

1. Dilute the Isotype Control Antibody by adding 2 μ L of the antibody to 18 μ L of Buffer Solution + 1 % FBS. Mix gently.
2. Combine the required volumes of Incomplete Antibody Solution and diluted Isotype Control Antibody in a clean vessel and mix gently. Protect from light and store on ice or refrigerate until use.

7.5. Working DRAQ7™ Solution (Optional)

Number of cultures	Volume of Buffer Solution + 1 % FBS	Volume of DRAQ7™
1	1 mL	0.5 μ L

1. Use the chart above to determine the volume of Working DRAQ7™ Solution required. Scale up as necessary.
2. Combine the required volumes of Buffer Solution + 1 % FBS and DRAQ7™ in a clean vessel and mix gently. Protect from light and store on ice or refrigerate until use.

8. Preparing Cells for Analysis

1. Resuspend cultures and perform cell counts to determine cell density.
2. Centrifuge 2×10^6 total cells from each culture. Centrifuge another 2×10^6 total cells from one additional solvent (or untreated) control culture that will be used with the Working Isotype Control Antibody Solution as the Isotype Control sample. Centrifugation should be performed in conical 15 mL centrifuge tubes at $340 \times g$ for 5 minutes.
3. Aspirate supernatants and tap pellets loose.
4. Add 100 μ L Complete Antibody Solution to the first tube. Use gentle pipetting to mix and immediately transfer entire tube contents to a well of a 96-well plate. Repeat for each culture.
5. To the one tube that was designated for the Isotype Control sample, add 100 μ L Working Isotype Control Antibody Solution. Use gentle pipetting to mix and immediately transfer entire tube contents to a well of the 96-well plate.
6. Incubate the 96-well plate in a 4 °C refrigerator, covered in foil, for 20 minutes.
7. Aliquot 3 mL cold Buffer Solution + 1 % FBS to a conical 15 mL centrifuge tube for each well, including the Isotype Control sample. Appropriately label tubes and keep cold until needed.
8. After incubation, resuspend cells with gentle pipetting and transfer the entire volume from each well into the appropriately conical 15 mL centrifuge tube containing 3 mL cold HBSS + 1 % FBS (from step 7, above). Centrifuge tubes at $340 \times g$ for 5 minutes.
9. Aspirate supernatant and tap pellets loose.
10. If using DRAQ7™, resuspend samples in 1 mL Working DRAQ7™ Solution. Otherwise, resuspend samples in 1 mL Buffer Solution + 1 % FBS.
11. If using DRAQ7™, incubate tubes in a 4 °C refrigerator for 10 minutes. Otherwise, proceed to the next step.
12. Transfer tubes to ice and protect from light until ready for flow cytometric analysis. Analyze within 3 hours.

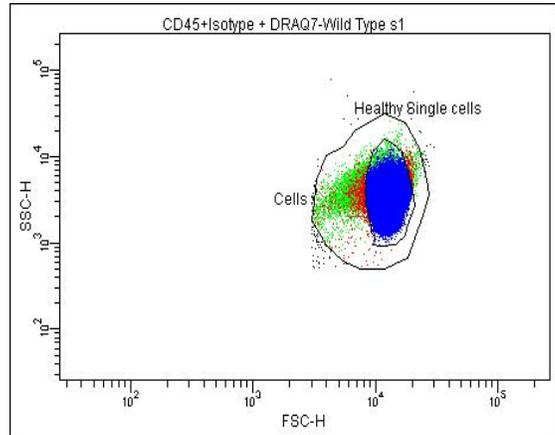
9. Flow Cytometric Setup and Data Acquisition

Important Notes:

- The following setup and compensation instructions are specific for FACSDiva™ software, but should be useful with other software packages.
 - Prior to analyzing experimental samples, it is recommended that you analyze at least one solvent control and one positive control sample first to verify that the template and instrument settings are appropriate.
1. Before analyzing samples, ensure that the flow cytometer is working properly. Follow the manufacturer's instructions for the appropriate setup and quality control procedures. Open the template file or create one following the guidance in Appendix A.
 - If using FACSDiva™ software, perform the following steps:
 - a. Locate the desired FACSDiva™ template (.xml file).
 - b. Open the following folders on your computer: My computer > New Volume(D) > BDEExport > Templates > Experiment > General.
 - c. Drag the template into the General folder.
 - d. Close this window and start the FACSDiva™ software.
 - e. Click on "Experiment" in the menu bar and create a new folder. Select the new folder and click the "New Experiment" button on the Browser toolbar. The Experiment Template dialog appears. Click the "General Tab" and select your template.

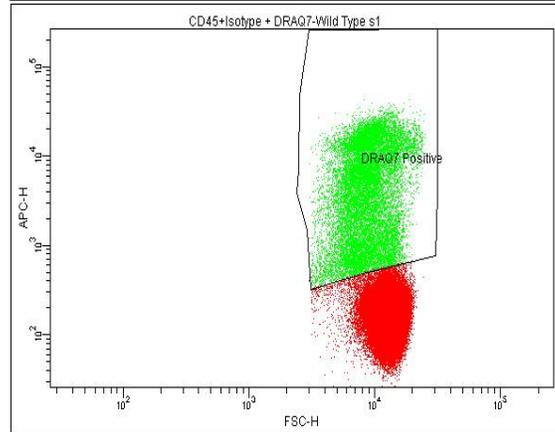
- Place the Isotype Control sample on the flow cytometer and begin collecting events. While viewing Plot A, adjust instrument settings and the “Healthy Single Cells” region so that healthy cells fall within it. Adjust the larger “Cells” region so that it also includes dead/dying cells that are moderately lower in Forward Scatter, as shown to the right.

Cells that fall within the more restrictive “Healthy Single Cells” region will be analyzed for mutant cell frequency. Cells that fall within the larger “Cells” region will be used to evaluate the frequency of dead or dying cells (i.e., DRAQ7™-positive).



- If using Working DRAQ7™ Solution, while viewing Plot B, adjust the voltages of the DRAQ7™-associated fluorescence so that the DRAQ7™-negative population is positioned in the lower decades but not in the origin, and so that the DRAQ7™-positive events are in the upper decades of log fluorescence, without falling off scale, as shown in the image to the right.

Adjust the “DRAQ7™-Positive” region so that it encompasses the cells with higher APC fluorescence.

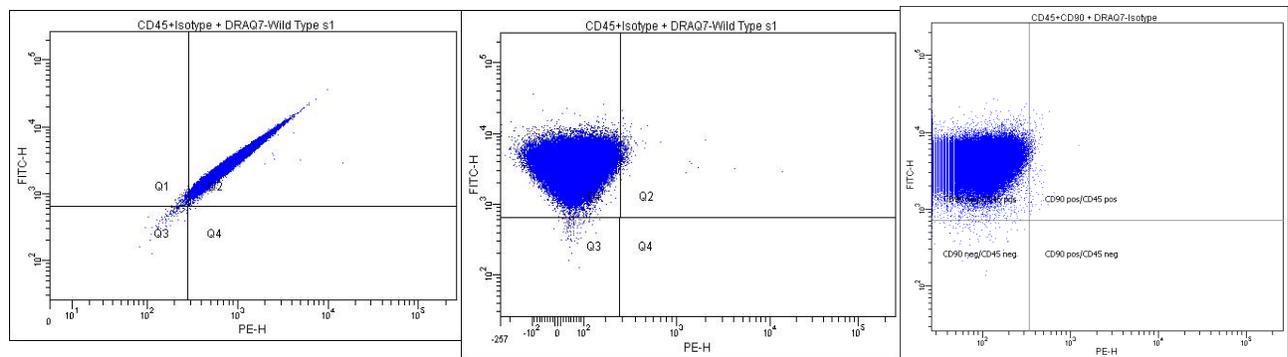


If not using Working DRAQ7™ Solution, ensure no cells are included in the “DRAQ7™-Positive” region, as this will exclude them from analysis.

- While viewing Plot D, adjust the voltages of the FITC- and PE-associated fluorescence detectors so that events appear approximately within the first to second decade of PE fluorescence and within the third to fourth decade of FITC fluorescence. It should look like the plot below, on the left (before compensation).

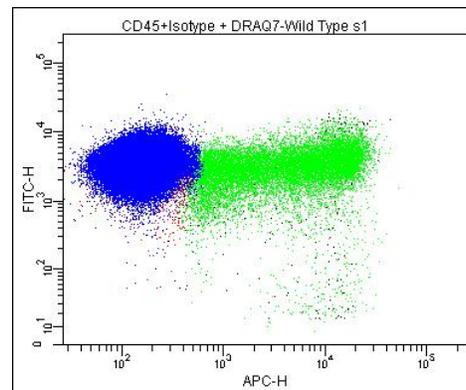
Adjust compensation to remove the spillover of FITC-associated fluorescence from the PE channel. The majority of cells should now be located in upper left quadrant of Plot D. By using bi-exponential scaling, the plot will have a triangle-shaped look, as seen in the plot below, in the middle (after compensation). A version of the plot after compensation with log scaling can be seen below, on the right.

It’s important to locate as many of the Isotype Control cells in the upper left quadrant, as this is where the mutant cells will appear. However, it is best to ensure the vertical quadrant line is close to, and partially inside, this population. This prevents subtle variations in staining from causing overestimations of mutant frequencies.

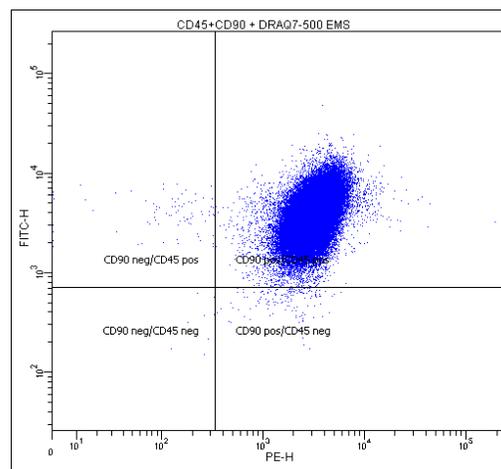
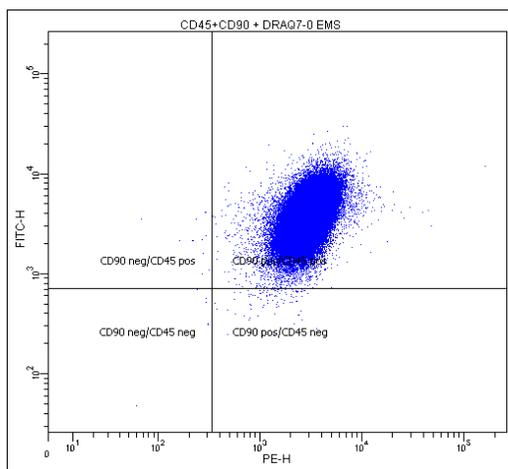


- If using Working DRAQ7™ Solution, while viewing Plot C, the DRAQ7™-positive cells should be positioned to the right of the DRAQ7™-negative cells. No compensation is needed for this plot. It should look like the plot to the right.

- Remove the Isotype Control sample from the flow cytometer and run water to flush mutant cells out of lines for 1 to 2 minutes. Place a solvent control sample on the flow cytometer. Maintain the compensation settings from the Isotype Control sample.
- Confirm that the positioning of the gates appropriately captures the desired populations. The vast majority of the wild type cells should be positioned in the upper right quadrant of Plot D. These are cells with CD90.2 on their surface, but also CD45. Pig-a mutant cells should appear in the upper left quadrant of Plot D, as they are positive for CD45 (indicating they do not have a global membrane receptor expression malfunction), yet do not have CD90.2 on their surface.



An example Plot D from a solvent control can be seen below, on the left, and an example Plot D from a positive control can be seen below, on the right.



- Run samples with a flow rate that achieves approximately 3,500 to 4,000 events per second until 1×10^6 single, healthy, cells are collected. (If you used DRAQ7 stain, acquire 1×10^6 DRAQ7-negative cells.)
- After each sample, run water to flush mutant cells out of lines for 1 to 2 minutes.
- Among cells within the “Healthy Single Cells” region, divide the number of cells that are both CD90-negative and CD45-positive by the number of total CD45-positive cells. Multiple by 1,000,000 in order to express the mutation frequency as number of mutant phenotype cells per one million cells.

10. References

- Bemis JC, Avlasevich SA, Labash, C, McKinzie P, Revollo J, Dobrovolsky V, Dertinger S (2018) Environmental & Molecular Mutagenesis 59, 18 - 29.
- Bemis JC, Heflich RH (2019) Mutation Research 847, 403028.

11. 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 *In Vitro* MutaFlow® Kit for the preparation and analysis of 200 samples.

MutaFlow® All rights reserved. *μ*icroFlow® is a trademark of Litron Laboratories. U.S. Patent Nos. 7,445,910 and 7,645,593. Copyright 2014-2022, Litron.

DRAQ7™ nucleic acid stain is a trademark of is a trademark of Biostatus Limited. FACSDiva™ is a trademark of BD Biosciences Inc.

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. This product is for research purposes only and is not intended for clinical or diagnostic use.

Appendix A – Template Preparation and Representative Plots

Data acquisition template files are available from Litron’s website: www.LitronLabs.com (or email pigatechsupport@litronlabs.com) but are specific to FACSDiva™ software. The next pages show actual screen images of the FACSDiva™ template graphs. Flow cytometry operators who are not using FACSDiva™ software should find these pages valuable for constructing their own data acquisition and analysis template.

We recommend that if you are using FACSDiva™ software, set the fluorescence parameter to “Height” rather than “Area”. The Isotype Control sample may be run using Single-Color Compensation controls and auto-compensation if available with your software package.

1. Defining Gates:

- G1 = R1 = “Healthy Single Cells”
- G2 = R2 = “Cells”
- G3 = R3 = “DRAQ7-positive”
- G4 = R4 = “Healthy Single Cells” AND NOT “DRAQ7-positive”

2. Gate and parameters for each Plot:

Plot A	No Gate	SSC-H vs. FSC-H
Plot B	G2	FL4-H vs FSC-H
Plot C	G2	FL1-H vs FL4-H
Plot D	G4	FL1-H vs FL2-A

3. Quadrant Key for Plot D:

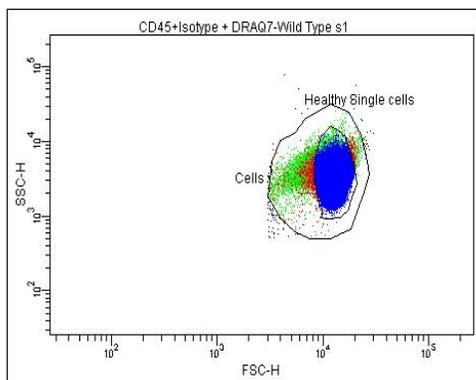
- UL = CD45-positive, CD90.2-negative cells
- UR = CD45-positive, CD90.2-positive cells
- LL = CD45-negative, CD90.2-negative cells
- LR = CD45-negative, CD90.2-positive cells

4. Alternate names for detectors:

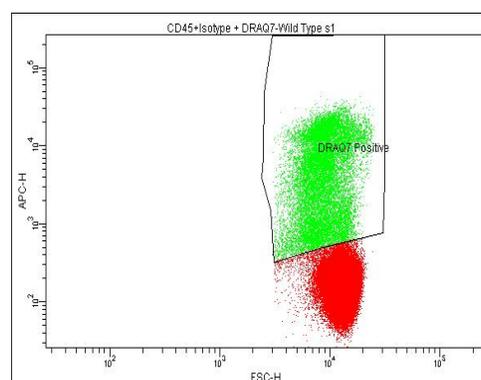
Green	FL1	FITC
Orange	FL2	PE
Far red	FL4	APC

5. Save the template file. This template file should be suitable for all analyses. To ensure consistency of data, it is preferable that no changes be made to the location and size of the regions between samples.

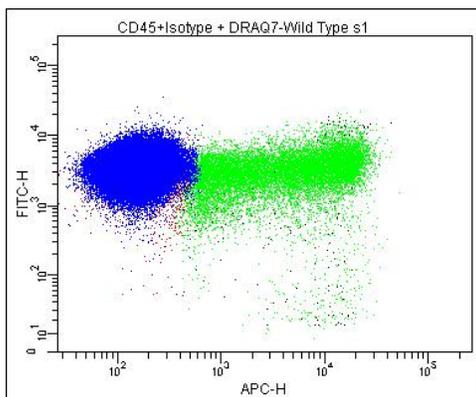
Plot A



Plot B



Plot C



Plot D

