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BIOLOGY AT ITS BEST

Immunophenotyping Rare Gamma-Delta T (γδT) Cells and Memory T cells Using Laminar Wash™ AUTO System

I. INTRODUCTION

LAMINAR WASH AUTO SYSTEM

The Laminar Wash (LW) AUTO system consists of a wall-less plate and a laminar flow cell washer that enable automated washing of suspension cells at a fraction of the time and with higher viability of cells. The wall-less LW plate consists of an array of 96 hydrophilic spots separated and surrounded by a hydrophobic surface, which functions as a virtual wall. Each well has 2 microsatellite wells for the nozzles to rest, facilitating precisely controlled laminar flow wash. The Laminar Wash (LW) HT2000 washer is a 192-nozzled washing system which uses a laminar flow method to gently add up to 80 µL of wash buffer at a flow rate ranging from 5 µL/sec to 20 µL/sec to each well and aspirates to leave a remaining volume of 25 µL.

In the conventional flow cytometry protocol that utilizes the centrifugation method, immune cells undergo numerous washes that generate significant stress to cells. The laborious and time-consuming nature of centrifugation inevitably leads to inter-operator variation. With the Laminar Wash HT1000 washer, cells undergo a gentle laminar wash without added stress, allowing a higher retention of cells for acquisition and cell sorting. In addition, the expression of cell surface markers is unaltered while unbound antibodies are efficiently removed, resulting in more reliable and better interpretable data.

With the integration of Laminar Wash into Hamilton Microlab NIMBUS, Laminar Wash AUTO 1000 offers an enclosed environment to perform the staining and the washing of the cells with minimal manual handling, streamlining the entire workflow of immunostaining assay with fully trackable and reproducible protocols. One of the important features of the Laminar Wash AUTO 1000 system is a Peltier unit that allows the 96 well Laminar Wash plate to hold the samples in a temperature-controlled manner during the various steps of the immunostaining assay.

The Laminar Wash AUTO 1000 system integrated into a Hamilton Microlab NIMBUS unit: Numbus96 Extended enclosed: 53.5 in (L) X 27.9 in (W) X 35.0 in max (H)

γδT CELLS – A RARE T CELL POPULATION

Immunophenotyping of peripheral blood by flow cytometry has been a key method for immunosurveillance, particularly for the use in cancer therapy and due to its low cost and ease of blood sampling. Notably, gamma-delta T (γδT) cells are of increasing interest to cellular immunotherapy as they share innate and adaptive immune properties, enabling them to identify and target cancer-transformed cells faster than the more common alpha-beta T cells. However, γδT cells are rare as they account for less than 5% of peripheral T cell population, making it challenging to detect them by flow cytometry in a consistent and reproducible manner. In this application note, we demonstrate that Laminar Wash staining of γδT and memory T cell subsets result in higher cell retention, better staining and greater enu-meration of γδT cells. In addition, we show that the consistent staining of T cell antigens in turn leads to clearer segregation of memory T cell subsets.

II. REAGENTS

- 500 mL of 70% Ethanol with 1% Tween-20 (required for priming and shutdown of washer)
- 500 mL of Distilled Water with 1% Tween-20 (required for priming and shutdown of washer)
- 500mL of FACS Buffer (1% BSA in PBS)
- Surface antibody master mix preparation:
  - Pre-dilute the antibodies, as listed in Table 1, pre-titrated down to 0.2-0.4 of manufacturer's recommended concentration. They should be diluted to 25 µL in FACS buffer for each well.

Table 1: List of Surface Antibodies

Marker	Dye	Clone	Manufacturer	Vol per test (µL)	Cat. No.
Viability	Aqua		Invitrogen	0.5	L34957
CD3	APC	UCHT1	BioLegend	2	300412
CD4	BV570	RPA-T4	BioLegend	1	300534
VD2	BV711	B6	BioLegend	2	331412
CD27	BV650	O323	BioLegend	2	302828
CD45RA	APC-Fire750	HI100	BioLegend	2	304152

- Countbright Absolute Counting Bead (Invitrogen)

Table 1: Plate map for loading antibody master mix and buffer onto the Reagent Plate

		1	2	3	4	5	6	7	8	9	10	11	12
Plate 1 Reagents	A	Panel 1	Panel 2	Panel 3	Panel 4	Panel 5	Panel 6	Buffer 1	Buffer 2	Buffer 3	Buffer 4	Buffer for Sample Transfer	
	B												
	C												
	D												
Plate 2 Reagents	E	Panel 1	Panel 2	Panel 3	Panel 4	Panel 5	Panel 6	Buffer 1	Buffer 2	Buffer 3	Buffer 4		
	F												
	G												
	H												

With the Laminar Wash™ AUTO 1000 system, prepare the antibody master mixes and assay buffers in the Reagent Plate (96 V-bottom deep well plate). Rows A to D of the Reagent Plate are dedicated for reagents used for Plate 1, Rows E to H are dedicated for reagents used in Plate 2. Prepare sufficient antibody master mix for the samples by rounding the total number of wells to the higher multiple of 4 (e.g. 15 triplicates = 45 wells, round up to 48 wells). Add an additional 30 µL per deep well to account for dead volume in the Reagent Plate (refer to the equation below). Distribute the master mix equally to the 4 allocated wells of each panel on the Reagent Plate (Table 1).

Volume of mastermix per panel=
*(No. wells(round up to multiple of 4) * Reagent required/ well) + (30 L *4)*

- If one LW96-plate is loaded onto AUTO1000 for surface staining, aliquot surface antibody master mix into the wells A1, B1, C1 and D1 of the Reagent Plate. If two plates are loaded onto AUTO1000, load the surface antibody master mix for the second plate into the wells E1 to H1.

  Note: Current protocol in this application only requires loading of antibody master mix into column 1 of the Reagent Plate. For additional antibody panels, load into the subsequent columns 2 through 6.

- If an assay buffer (e.g. fix/perm buffer) is used, load the first buffer reagent into the wells A7 to D7. ***Note: For additional assay buffer reagents, load into the columns 8 through 10.***

PRIMING AND SHUTDOWN OF LW AUTO:

Start up and shutdown priming for the washer are mandatory maintenance of the instrument and will be carried out automatically at the start of AUTO1000, and as a shutdown step. Each priming step requires approximately 180mL of buffer, hence enough volume is required to be prepared.

Start-up priming sequence:

1. 180mL 70% Ethanol with 1% Tween-20
2. 180mL Distilled water with 1% Tween-20
3. 180mL assay wash buffer

Shutdown priming sequence:

1. 180mL Distilled water 1% Tween-20
2. 180mL 70% Ethanol with 1% Tween-20
3. Air

Buffer changes during assay sequence (e.g. perm/wash buffer) will require 180mL additional volume of new wash buffer for priming.

III. LAMINAR WASH™ AUTO1000 ASSAY WORKFLOW

Manual Handling | **AUTO1000**

- Collect whole blood in a tube containing EDTA.
- Lyse 100 µL of whole blood with 900 µL of 1X RBC Lysis Buffer for 5 mins in a 96-well deep well plate.

Centrifuge for 5 mins at 1500 rpm, decant supernatant.

- Repeat lysis by adding 900 µL of 1X RBC Lysis Buffer for 5 mins

Centrifuge for 5 mins at 1500 rpm, decant supernatant.

- Resuspend pellet in 25 µL of FACs buffer

Prepare surface antibody master mix for final volume of 25µL per sample (see reagent preparation section)

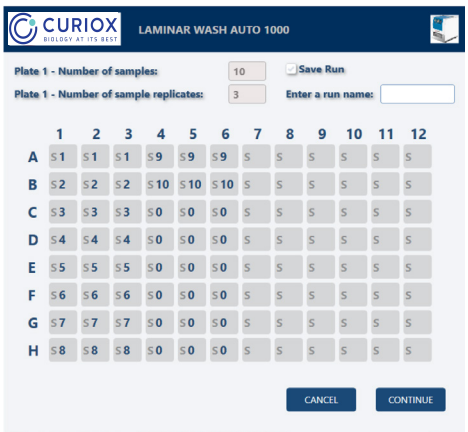
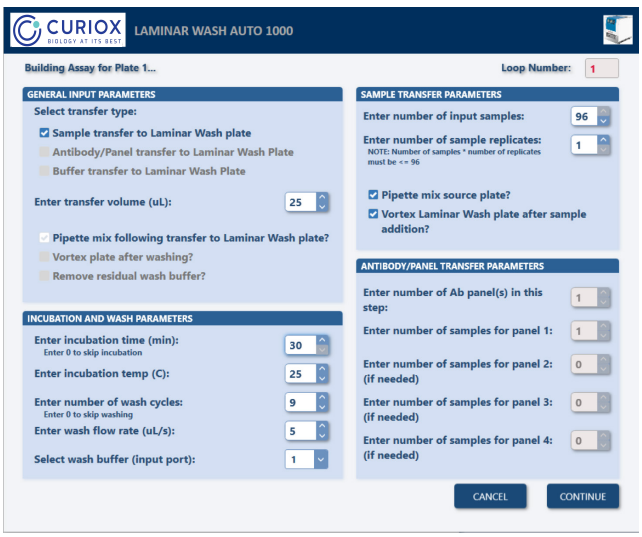


Plate map shows samples distributed in replicates as determined by user.

- Setup the parameters in AUTO1000 UI
- Load surface staining antibody master mix onto Reagent Plate
- Load the LW96 Plate, Sample Input Plate, Reagent Plate, Sample Output Plate and other consumables onto the AUTO1000 deck space as instructed in the interface



In Loop 1, the cells are being transferred to LW96-Plate, incubated for 30 minutes, washed 9 rounds at flow rate of 5 µL/s before moving to Loop 2.

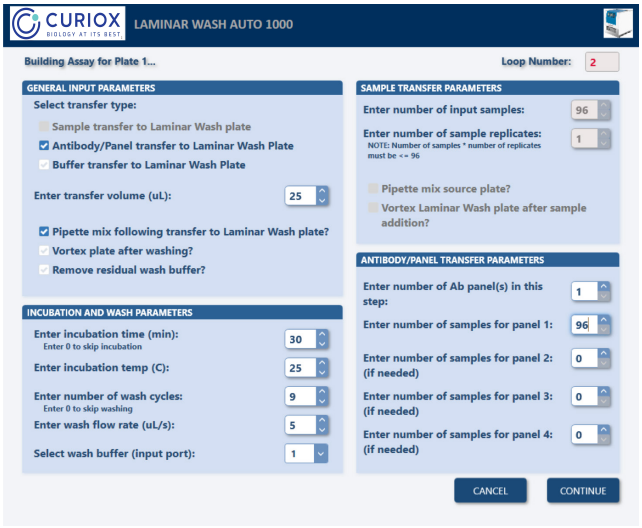
Initial cell wash

- Plate 25 µL of lysed blood sample to the LW96 plate
- Incubate for 30 mins at 25°C to allow cells to settle to the surface of the plate
- Prime HT1000 with FACS buffer
- Insert plate into HT1000
- Select no. of washes '9' at a flow rate of 5 µL/sec
- Start the wash[1]

There will be a residual volume of 25 µL in each well at the end of the wash.

Prepare surface antibody master mix for final volume of 25 µL per sample (see reagent preparation section).

III. LAMINAR WASH™ AUTO1000 ASSAY WORKFLOW *(cont.)*



In Loop 2, the cells are being stained with 25 µL of surface antibody, incubated for 30 minutes in dark, washed 9 rounds before moving to final step (transfer to output plate).

Surface Staining

- Add 25 µL of surface antibody master mix to the dispensing satellite well
- Mix gently 5 to 6 times
- Incubate plate for 30 mins at 25°C
- Insert plate into HT2000
- Select no. of washes '9' at a flow rate of 5 µL/sec
- Start the wash[1]

There will be a residual volume of 25 µL in each well at the end of the wash.

Collect Cells/Sample Transfer

- Add 25 µL FACS buffer to residual 25 µL and vortex for 10 secs to resuspend cells
- Collect all volume and transfer to conventional 96 well plate or microtubes
- Add 50 µL to each well to the final volume of 100 µL and vortex for 10s to resuspend cells
- Pipette around edges of well prior to collecting all volume.

Cells are now ready for acquisition.

1. *Each wash cycle dispenses up to 80 L of buffer into each well and aspirates 55 L, leaving a residual volume of 25 L in the well. For example, in a 9X wash cycle with an initial volume of '50¬ L', the 1st wash cycle will add an additional 30 L of buffer to the well to bring the volume to 80 L and aspirate 55 L, leaving a 25 L residual volume. The 2nd wash cycle onwards up to the 9th wash cycle, the washer dispenses 55 L of wash buffer into each well to bring the volume up to 80 L and aspirate 55 L to leave a residual volume of 25 L.*

IV. RESULTS

In the study of γδT cells, a main challenge is the rarity of this population in peripheral blood, particularly in healthy individuals where γδT cell frequencies are low. In addition, current whole blood processing methods involve lysis and multiple centrifugation steps, further causing mechanical stress to cells. As demonstrated below, fresh human whole blood was lysed in bulk and lymphocytes were stained with T cell markers using three different wash methods: Laminar Wash (LW), V-bottom microtitre plate (MTP) and polystyrene tube (PST). From 50 µL of whole blood lysed in bulk, Laminar Wash yielded higher live cell retention compared to the centrifuge wash methods using either MTP or PST (Figure 1a) based on bead counts. Significantly, LW retained 97% and 47% numerically more γδT cells (CD4- VD2+) than MTP and PST, respectively (Figure 1b).

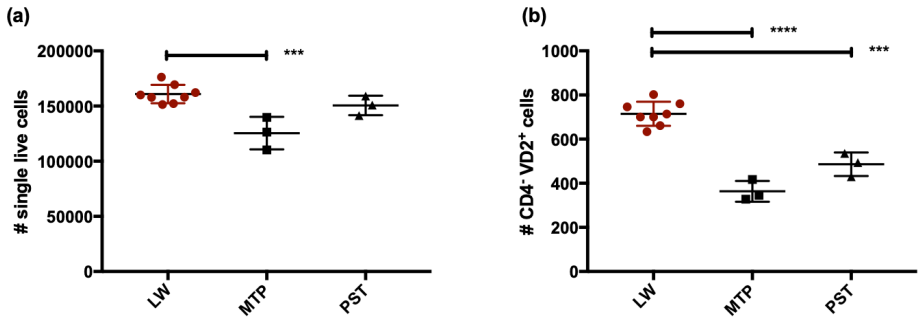


Figure 1. Comparison of (a) single live cells and (b) γδT cells acquired from LW (n=8), MTP (n=3) and PST (n=3) cell staining methods, from 50µL of whole blood, lysed.

The reason for the significant increase in γδT cells compared to total live cell retention in Figure 1 can be alluded to in the scatterplot of cells which were pre-gated on single, live cells for each of the three methods (Figure 2). In all four markers used to identify γδT cells (i.e. CD19, CD3, CD4, and VD2), apparent shifts in cell scattering towards higher fluorescence intensity was observed in Laminar Wash sample, compared to MTP or PST. Unlike the MTP or PST counterparts where stray cells could be observed outside of CD4+ gate, LW showed greater fidelity in antigen staining, resulting in a cleaner cell separation and higher frequency of the identifiable rare γδT population (Figure 2, bottom).

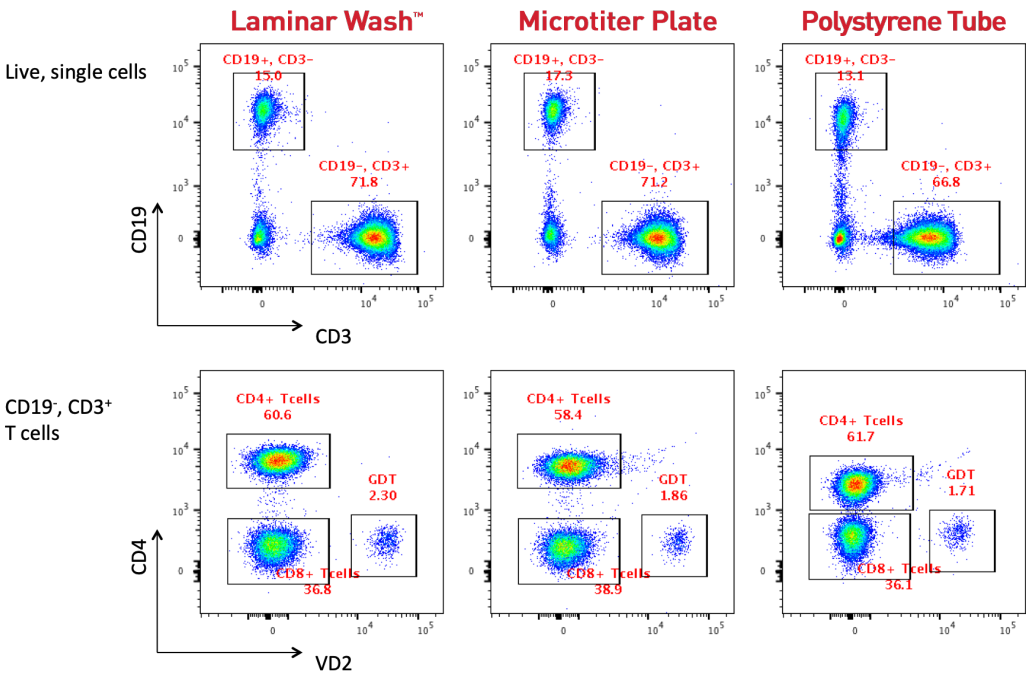


Figure 2. Scatterplots of live, single cells (top) and T cells (bottom) for LW, MTP and PST methods demonstrated higher staining with LW and clearer segregation with less stray cell scattering.

The MFI of positive and negative populations of the four markers were further enumerated in Figure 3, demonstrating higher sepa-ration of MFI between positive and negative populations in LW. MFI increased 3x in CD3 staining between LW and PST, and 1.7x in VD2 staining between LW and PST, showing the detrimental impact that centrifugation impinged on antigen-antibody binding. The greater magnitude of antibody binding to antigens on cells resulted in a larger percentage of identified CD4- VD2+ γδT cells in LW compared to MTP or PST.

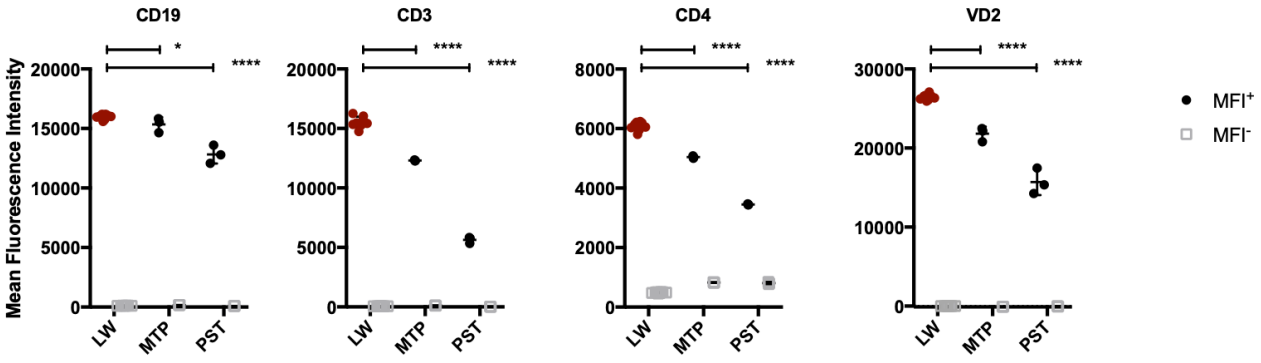


Figure 3. Enumeration of mean fluorescence intensities (MFI) of positive and negative populations of various markers stained by LW, MTP or PST.

An indicator of immunotherapy efficacy is the establishment of memory T cells. CD4+ and CD8+ memory T cell subsets were evaluated and compared by using either LW or centrifugation in PST. Interestingly, LW resulted in a clearer identification of distinct sub-populations of memory T cells, namely central memory (CD27+ CD45RA-), naïve (CD27+ CD45RA+), effector memory (CD27- CD45RA-) and effector memory RA (CD27- CD45RA+; CD8+ T cells only) compared to the PST samples. Such observations could be attributed to the gentle and effective laminar wash that preserved antigen-antibody binding while effectively removing background staining, as evidenced by the higher MFI in CD27+ population with LW samples. It is particularly important to determine memory T cell subsets in complex studies as memory population shifts are indicators of the efficacy of therapy and long-term establishment of immunity memory.

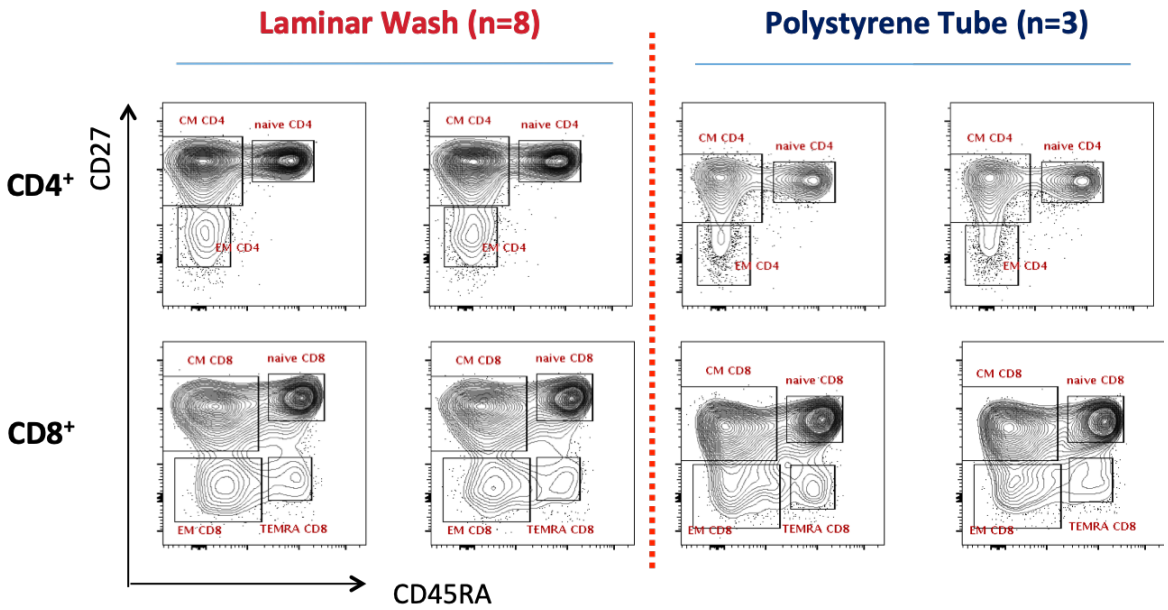


Figure 4. Contour plot showing segregation of me mory CD4+ and CD8+ T cell populations in cells stained by either LW or PST using centrifuge.

V. CONCLUSION

In conclusion, the data presented in this application note demonstrate that Laminar Wash™ is a more reliable method than the conventional centrifugation-based wash method in identifying γδT cells, a rare cell population in peripheral blood, yielding a better resolution of memory T cell subsets. With the Laminar Wash system, cells are kept away from mechanical stresses associated with repeated centrifugation and manual cell resuspension, thereby retaining improved overall viability and epitope preservation. The LW AUTO 1000 is a fully automated "walk-away" sample workstation with its traceability and time-saving benefit for cell-based immunoassays. The LW AUTO 1000 system offers a complete platform from initial sample preparation to endpoint data management with greater precision and consistency, further simplifying and accelerating the sample preparation process required especially for clinical and industrial use.

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