Flow Cytometric HLA Crossmatch Workflow with the Laminar Wash™ HT2000

INTRODUCTION

While complement-dependent cytotoxic crossmatch (CDCXM) and flow cytometric crossmatch (FXCM) are both common methods to predict donor-recipient compatibility in organ transplant facilities, FCXM has proven to be more sensitive and today is more widely used across human leukocyte antigen (HLA) labs. FCXM assays, however, typically use a legacy centrifugation method when preparing samples.

The Laminar Wash™ HT2000 provides a proven alternative to the comparatively inconsistent centrifugal process and its associated washing steps. The Laminar Wash™ provides reproducible, automated washes across operators and similar or improved cell retention and viability. It also enables a gentler handling of cells whilst eliminating plate flicking aerosolization and cross contamination. These benefits have been well documented by an increasing number of transplant laboratories that have adopted LW in their workflow. Laminar Wash HT2000 comes with a touchscreen for easy menu selection, as well as Buffer Exchanger for automated startup and shutdown sequence. The Laminar Wash™ is also available within a ready-to-use automation platform – the AUTO1000™ for a full walkaway solution.

The Laminar Wash AUTO1000 encloses HT2000 within a fully automated system for surface staining and intracellular staining protocols. Load the decks and leave the system to perform steps- 1-5 above and more, with flow cytometer loading a further option*. Pre-programmed with modifiable protocols that are easy for the user to immediately use. The platform is designed to produce the most quantitative and reproducible results for flow cytometry users. The Laminar Wash AUTO1000 System reduces user variability and day-to-day variation prevalent in flow cytometry. Unlike custom automation or centrifugation-based systems, the AUTO1000 provides easy, turnkey automation and exceptional flexibility. In addition, the AUTO1000 is much more compact, affordable and lower maintenance than automation systems built around centrifugation.

*with alternative deck model.

REAGENTS for HLA CROSSMATCH with the LAMINAR WASH™ HT2000

- 500 mL of Wash Buffer.
- 500 mL of 70% Ethanol with 1% Tween 20 (required for priming and shutdown of HT2000)
- 500 mL of Distilled Water with 1% Tween 20 (required for priming and shutdown of HT2000)
- PBMCs from donor
- Serum e.g., wash buffer for background, negative control serum, positive control serum, and patient/recipient serum.
- Antibody mastermix. Surface markers (Anti-CD3 and Anti-CD19) should be kept at the same volume as in regular protocol, whereas the secondary antibody (IgG-FITC) should be added a higher concentration (2X-10X higher). They should be diluted to 25 - 50 µL in Wash buffer for each well. For instance:

Preparation for Conventional method	
Reagent	**µL per sample**
CD3-APC	5
CD19-PE	10
IgG-FITC (***1:50 dilution***)	10
Total volume	25

Preparation for Laminar Wash™	
Reagent	**µL per sample**
CD3-APC	5
CD19-PE	10
IgG-FITC (***1:5 dilution***)	10
Total volume	25

INSTRUMENT SET UP

Priming of Laminar Wash™ HT2000 with Buffer Exchanger:

Priming for the washer is mandatory for startup and maintenance of the instrument. Each priming step requires approximately 150 – 200 ml of buffer. This is performed at the start of each day prior to the assay set-up. These steps take just 10 mins. The *Startup Prime* function on the HT2000 and buffer exchanger runs through 70% Ethanol with 1% Tween 20, Distilled Water with 1% Tween 20 and wash buffer. The switching of buffers is automated with the pre-programmed *Startup Prime* function.

1. Ensure Buffer Exchanger inlet ports are connected to respective priming and wash buffer vessels, while outlet port is connected to waste bottle.
2. On HT2000 touchscreen, select *Startup Prime* in Operation mode and select the wash buffer port. The pre-programmed function will prime HT2000 sequentially with the following buffers:
    a. 70% Ethanol with 1% Tween-20
    b. Distilled water with 1% Tween-20
    c. Selected wash buffer

Shutdown of Laminar Wash™ HT2000 with Buffer Exchanger:

Shutting down the instrument at the end of the day is an automated, mandatory process to ensure proper maintenance of the washer instrument.

1. On HT2000 touchscreen, select *Shutdown Maintenance* in Operation mode. The pre-programmed function will prime HT2000 sequentially with the following buffers:
    a. Distilled water with 1% Tween-20
    b. 70% Ethanol with 1% Tween-20
    c. Ambient air
2. The HT2000 is is now ready for power-down.

LAMINAR WASH™ ASSAY WORKFLOW with HT2000 and Buffer Exchanger.

CURIOX
BIOLOGY AT ITS BEST

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- Plate 250,000 PBMCs suspended in 25 µl of wash buffer to the Laminar Wash™ plate
- Add 30 µl of control or test serum [(1)] to the respective wells

Incubate plate at room temperature for 30 min.
Prime Laminar Wash™ HT2000 with wash buffer as described in Instrument Setup

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- Insert plate into Laminar Wash™ HT2000
- Set no. of washes at 19 [(2)] and initial volume at 55 µl
- Start the wash. Walk away and come back in 5-7 mins

There will be a residual volume of 25 µl in each well at the end of the wash
Prepare surface antibody mastermix[2] for the final volume of 25 µl per sample

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- Remove carefully 10 µl of residual volume from satellite well. [(3)]
- Vortex plate with residual volume.
- Add 25 µl of surface antibody mastermix [(1)] to all the wells
- Pipette mix gently 5-6 times.

Incubate plate at room temperature for 30 min. Protect from light

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- Insert plate into Laminar Wash™ HT2000
- Set no. of washes at 12 [(2)] and initial volume at 40 µl
- Start the wash. Walk away and come back in 5-7 mins

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

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Two methods for sample-acquisition on flow cytometer:

(A) **Prepare plate for direct acquisition from plate by high throughput sampler**

- Vortex plate to re-suspend cells.
- Add Wash buffer [(4)] to residual 25 µl in each well.

OR

(B) **Collect cells in a two-step method and transfer to tubes or a regular 96-well plate**

- Vortex plate to re-suspend cells.
- Add 30 µl of FACS buffer to residual 25 µl in each well. Vortex and transfer 45 µl to a FACS tube
- Add 45 µl of FACS buffer to the original well and vortex for 10s to re-suspend cells
- Pipette around edges of well and transfer the full amount to the same tube to Laminar Wash™ HT2000. Final volume is approximately 100 µl.

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Samples are now ready for acquisition

(1) See reagent preparation.
(2) Number of wash cycles on the Laminar Wash™ System depends on the sample type.
(3) Optional step to increase sensitivity.
(4) Complete the volume according to the needs of the flow cytometer used for acquisition.

RESULTS

CONSISTENCY TEST OF BACKGROUND (NEGATIVE CONTROL) AND POSITIVE CONTROL OF B and T CELLS IMPROVED BY LAMINAR WASH™

**Data adapted from the Curiox Biosystems webinar by Dr. Prabhakar Putheti, Assistant Director at Immunogenetics and Transplantation Laboratory, The Rogosin Institute, NY.*

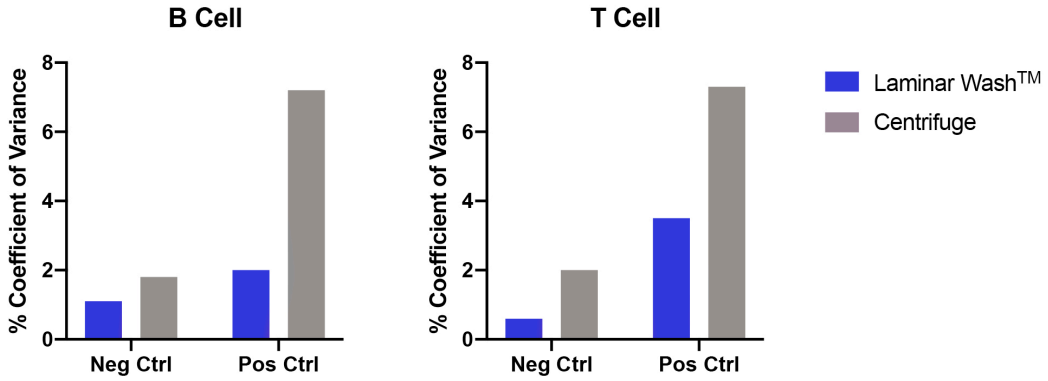

Figure 1. B-cells and T-cell %CV of MFI of negative and positive controls. Data obtained by 3 different operators running samples in duplicates. Laminar Wash improves user-to-user CV and reduces human error.

LAMINAR WASH™ DECREASED BACKGROUND in B CELL CROSSMATCH Without PRONASE TREATMENT

**Data adapted from the Curiox Biosystems webinar by Blanca Ponce-Ngo, Lab Manager at Transplant Immunology Lab, Montefiore Medical Center, NY.*

a.

		Pronase	MCF T Neg	MCF B Neg	ΔB = B-T (Background)	MCF T Pos	MCF B Pos	ΔB = B-T (Background)	CS T	CS B
Laminar Wash™	1	NO	261	264	3	599	672	73	338	408
	2	NO	259	259	0					
	3	YES	136	176	40	588	668	80	452	492
	4	YES	151	182	31					
Centrifugation	5	NO	258	374	116	589	708	119	331	334
	6	NO	265	371	106					
	7	YES	263	261	-2	685	779	94	422	518
	8	YES	269	268	-1					

b.

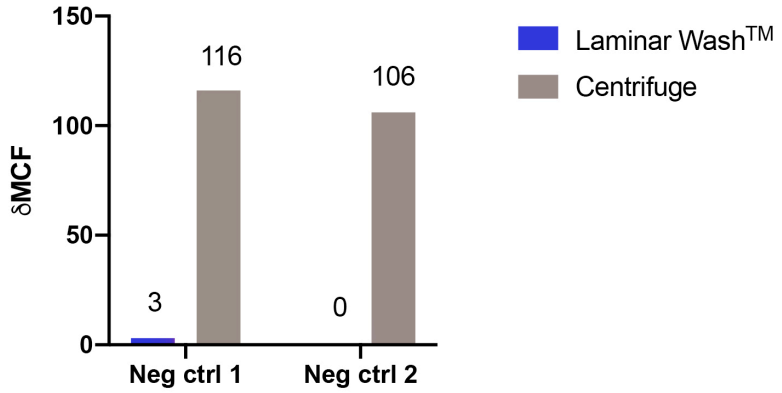

Figure 2. a. Decreased background in pronase-independent *B cell* crossmatching of PBL fresh cells with Laminar Wash™. **b.** ΔMCF was eliminated with Laminar Wash™ in non-pronase-treated B cells crossmatched with negative control serum.

CONCLUSION

In conclusion, the data obtained by different HLA labs demonstrated that the Laminar Wash™ technology improved consistency and reduced biohazard aerosolization by eliminating the need of centrifugation. Microplate or sample 'flicking' or the use of a pressurized pipette are also eliminated, reducing the risk of cross contamination and user variation. The Laminar Wash™ provided a gentle but extremely effective and consistent washing method as seen with the low B cell background in the absence of pronase-treatment. The results remained consistent despite variations in the cells being used – fresh or frozen, with or without Pronase, etc. The Laminar Wash™ method produced reproducible data across the replicates and increased user-to-user consistency, which is crucial when working with clinical samples.

- To see the Laminar Wash HT 2000™ in action **click here.**
- Email: **Sales@curiox.com** or visit **www.curiox.com/contact**

A summary of the Montefiore webinar can be found following this page.