Automated noncontact washing of suspension cells using the C.WASH

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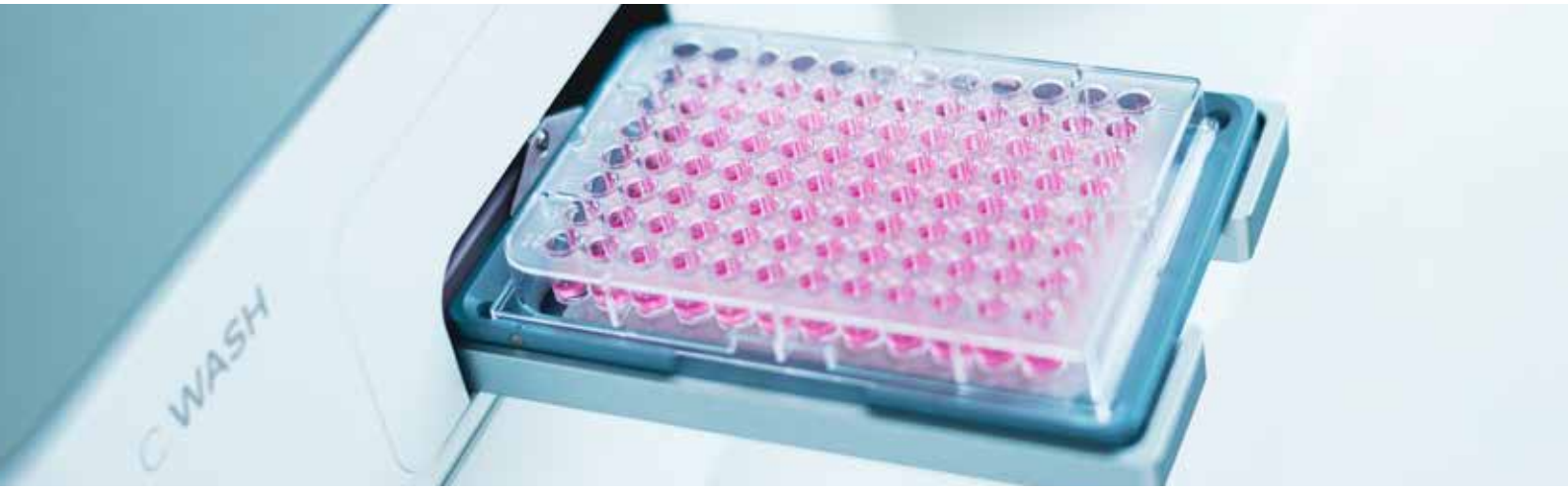

Abstract

A wide range of applications in immunology and hematology, including bioassay development, pharmacological profiling and compound screening, rely on the use of suspension cells. However, centrifugation prior to washing is essential, and removing supernatant from cell pellets remains prone to cell loss in some protocols. The C.WASH™ gently removes cell medium or wash buffer using centrifugal forces while retaining the previously pelleted cells. Combined with a contact-free dispensing system, the C.WASH allows efficient and reproducible washing of microtiter plates (MTP).

We evaluated the suitability of the C.WASH in washing suspension cells in 96-well MTP for phenotypic characterization by flow cytometry. We compared the performance of the C.WASH to manually washing cells under live, fixed and permeabilized states. For each protocol, cell loss was assessed following the washing steps. We demonstrated that the C.WASH can efficiently automate the washing of suspension cells, hence reducing hands-on processing time as well as the costs associated with pipette tip consumption.

Introduction

Drug discovery often uses phenotypic screening methods to identify lead compounds. Screening workflows rely on efficient methods that were developed and optimized for adherent cells, but can be cumbersome when applied to cells growing in suspension. With the rising number of compound libraries to be screened, as well as the increasing research toward engineering immune cells to fight cancer (e.g., CAR T-cells), it is essential for hematologists and immunologists to have access to systematic and automated ways to streamline downstream applications, such as phenotyping using flow cytometry. Sample preparation in multi-color flow cytometry often involves the use of flow cytometry tubes, U-bottom or V-bottom 96-well MTP. Cells in suspension are then incubated with a panel of fluorescently labelled antibodies or cell tracking dyes. Every excess of unbound antibody or labelling reagent is removed by performing several washing steps via centrifugation-based methods. Centrifugation is used to generate a cell pellet, and manual removal of the supernatant fraction away from the cell pellet requires processes that are challenging to scale, are prone to human error and can result in increased batch-to-batch variability.

As high-throughput screening requires automated removal of liquids from plates, scientists need to adapt manual protocols into high-throughput methods. Likewise, using pipette tips or other needle-based devices may induce cell loss. Moreover, buoyancy of cells is modified upon fixation and permeabilization. The automated cell washing should, therefore, be easily adapted to changes in cell properties.

In this Application Note, we demonstrate that the C.WASH is a simple and reproducible method to wash suspension cells. Following centrifugation of the plate to pellet cells, the liquid supernatant is evacuated using the C.WASH **(Figure 1)**. The automated liquid removal results in washes with consistent low residual volume and minimal cell loss across the plate. C.WASH automation hence minimizes the chance of human errors and saves both hands-on time and money in the screening process.

Materials and methods

Cell culture

Human Jurkat cells were maintained in RPMI-1640 medium (Thermo scientific, 21875034) supplemented with 10% Fetal Bovine Serum and at 37°C 5% CO_2. After counting, cells were seeded into 96-well V-bottom plates (Greiner, 651201) for testing.

Cell pelleting

Cell pelleting was performed in swinging bucket centrifuge (Heraeus, ThermoFisher). Live cells were spun 2 times for 5 minutes at 300g (600g for fixed and permeabilized cells).

Liquid evacuation

Manual washing was performed using an automated multichannel pipette (Sartorius, 300 µL), aspirating at the slowest speed on the edge of the well while tilting the plate to avoid disturbing the cell pellet. Automated washing was done using the C.WASH at a velocity of 2g for 5 seconds.

Cell fixation and permeabilization

Fixation was performed in a 96-well U-bottom plate by resuspending the cell pellet using 50 µL of 10% Formalin (Sigma / HT501128). Cells were incubated for 30 minutes at 4°C. Cells were washed twice with 100 µL Phosphate Buffered Saline (PBS) and were either used for testing or were permeabilized in 70% Methanol for 30 minutes at -20°C. Cells were washed twice with 100 µL PBS before analysis.

Cell counts

Live cells were lysed using CellTiter-Glo reagent (Promega) and transferred to a white opaque 96-well plate (Greiner 655073). Luminescence was measured using an EnSpire plate reader (PerkinElmer). Fixed and permeabilized cells were counted using a flow cytometer (Attune NxT, ThermoFisher). 20 µL was acquired from each well. Doublets and debris were excluded.

Results

Washing of live cells

To precisely determine cell numbers across the entire 96-well MTP, we used the CellTiter-Glo to measure the number of metabolically active cells in each well. The linearity between the cell seeding density and Relative Light Unit (RLU) was measured to ensure an accurate representation of the number of cells lost. Cell densities of Jurkat cells ranging from 12,500 to 100,000 cells per well were shown to have a linear relationship with RLU (R2=0.9973), indicating suitability for use in determining cell loss **(Figure 2)**.

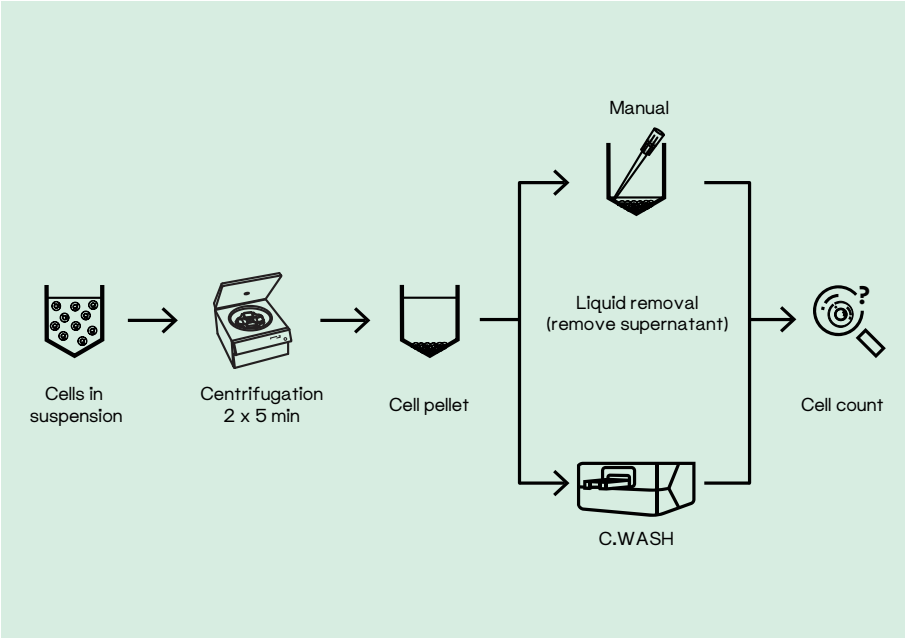

Figure 1. *A comparison between the C.WASH and a manual workflow. Suspension cells are spun prior to liquid removal using manual pipetting or the C.WASH. Cell count is performed thereafter to determine cell loss.*

The cell loss induced by manual washing was compared to automated washing using the C.WASH. Jurkat cells were seeded into 96-well V-bottom plates with 100,000 cells per well and subjected to 2 rounds of centrifugation for pelleting **(Figure 1)**. The supernatant was then removed manually using a multichannel pipette or by using the C.WASH at a velocity of 2g for 5 seconds.

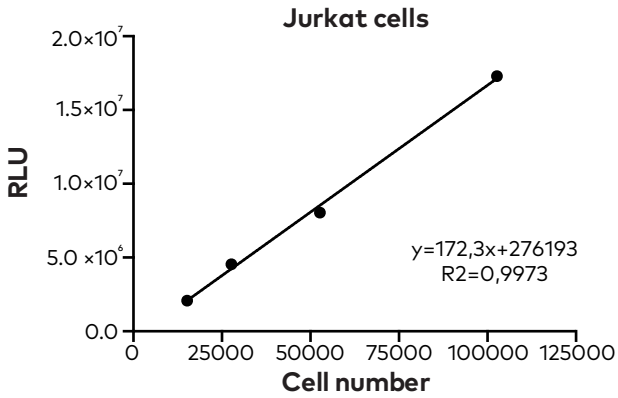

Figure 2. *Linearity of the CellTiter-Glo assay on Jurkat cells. Cells were seeded in quintuplicate at densities shown. The CellTiterGlo assay was performed and luminescence measured on an EnSpire plate reader. The results are expressed as RLU. Linearity was determined by linear regression analysis (line).*

Manual washing after pelleting via centrifugation resulted in cell loss across the entire plate. On average, 58% of cells were recovered across the plate **(Figure 3)**.

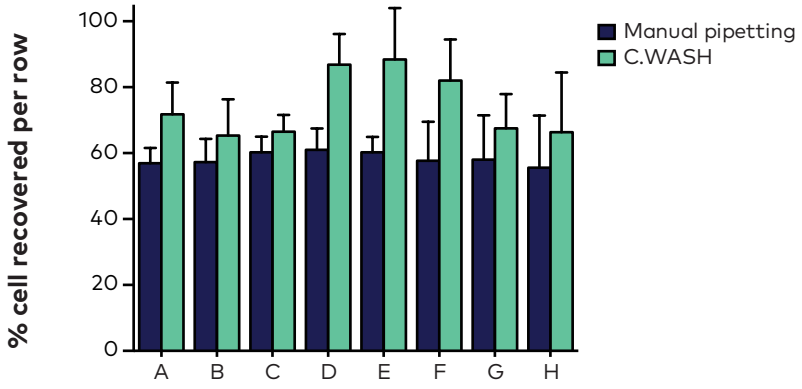

Figure 3. *Average cell recovery upon manual (blue) and C.WASH (green) washing. Each bar represents the mean and standard deviation of the 12 wells of the corresponding row.*

The C.WASH substantially reduced cell loss across the plate, as 74% of the cells were recovered **(Figure 3)**. Therefore, the C.WASH is suitable for washing suspension cells and has the advantage of drastically reducing the hands-on time required because liquid removal of the entire plate is performed in 5 seconds. Since the C.WASH is able to dispense up to 4 different liquid sources with its auto-priming function, it can facilitate and automate the washing of cells previously pelleted.

Washing of fixed and permeabilized cells

Fixation of cells after immunostaining is required in various protocols. It allows maintaining cells for days or weeks for later measurement, but is also a prerequisite to permeabilization and subsequent intracellular staining. For example, methanol is used as a permeabilization agent as it increases the reactivity of antibodies against nuclear antigens such as transcription factors. One should note that permeabilization induces changes in cells' physical properties (osmotic cell swelling), and that the cell pelleting procedure might therefore be affected.

We decided to compare the cell recovery of samples that were either fixed, or fixed and permeabilized, following a manual washing or an automated washing workflow using the C.WASH.

Since the CellTiter-Glo can only count live cells, we decided to estimate the cell number by flow cytometry (see **Figure 4** for gating strategy).

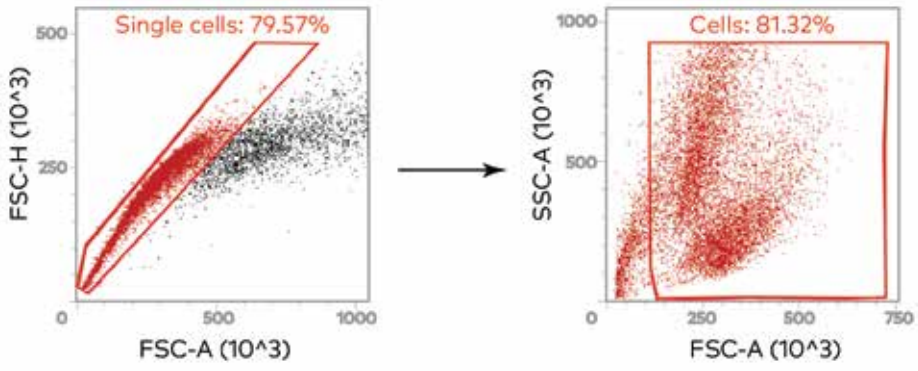

Figure 4. *Gating strategy to determine cell numbers after fixation and permeabilization (cell doublets and debris were excluded).*

Following cell seeding, cells were pelleted using centrifugation and resuspended in 50 µL formalin for fixation. Liquid evacuation during washing steps was either performed manually as described above or by using the C.WASH. After fixation, plates were washed with PBS before acquisition using a flow cytometer **(Figure 5)**. Two additional plates underwent a step of cold methanol permeabilization (70% v/v, 30 min. at –20°C) before washing with PBS and data acquisition **(Figure 5)**.

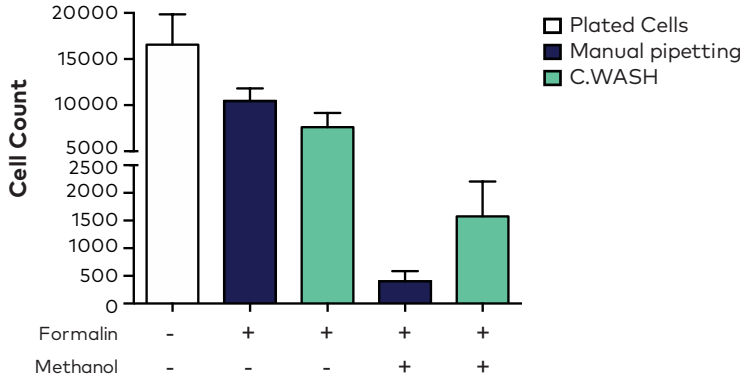

Figure 5. *Cell count after cell fixation or cell fixation and permeabilization. Liquid evacuation was performed manually (blue) or with the C.WASH (green). The protocol used for fixation (Formalin) and permeabilization (Methanol) is indicated below the x-axis. Each bar represents the average cell count (+ standard deviation) of the entire 96-well plate.*

Cell recovery following liquid evacuation after cell fixation is comparable between the manual and C.WASH workflow. However, the substantial cell loss observed after manual liquid removal of fixed and permeabilized cells is blunted if using the C.WASH. Therefore, the C.WASH enables greater cell recovery of suspension cells while reducing hands-on time and inconsistency caused by manual pipetting.

Conclusion

The C.WASH was tested to assess its suitability for washing suspension cells for downstream applications, such as cell preparation for flow cytometry on either live or fixed and/or permeabilized cells. A direct comparison between automated plate washing using the C.WASH and manual washing steps was performed.

The use of the C.WASH resulted in better cell recovery compared to the manual workflow. Overall, the benefit of using the C.WASH lies in the reduction of hands-on processing time and costs associated with pipette tip consumption. Moreover, the controlled velocity in which the liquid removal can be adjusted in the C.WASH allowed cells with lower buoyancy (e.g., methanol fixed) to be washed with minimal cell loss as compared to a manual workflow.

The Jurkat cells used in this study are from a widely used, immortalized cell line of human T lymphocyte cells. In recent years, cell therapy with modified T-cells has emerged as a promising treatment to fight tumor cells. CAR T-cell research is becoming increasingly popular and laboratories wishing to boost their throughput need systematic ways to automate their workflows. We have demonstrated that the C.WASH can reliably wash suspension cells in microtiter plates, opening the possibility for immunologists, hematologists and all flow cytometry experts to spend less time completing washing steps.

- The C.WASH can be used to gently wash suspension cells
- The C.WASH reduces both the time and cost required to perform washing steps compared to conventional manual methods
- Higher reproducibility and consistent data quality can be achieved by automating the washing steps

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CYTENA spun off from the University of Freiburg, Germany, in 2014 with its patented single-cell dispensing technology. Today, as part of BICO, the world's leading bioconvergence company, CYTENA continues building on that groundbreaking technology to develop high-precision instruments for isolating, dispensing, imaging and handling biological cells. Its award-winning devices are manufactured in Germany and used at prestigious academic and pharmaceutical labs around the world to automate workflows in numerous application areas, including stable cell line development, single-cell omics, high-throughput screening and drug discovery. CYTENA's breakthrough innovations for the lab combine advanced automation, state-of-the-art software engineering and the latest insights in cell biology to maximize efficiencies in the life sciences and create the future of health. Learn more at cytena.com.

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