C.WASH allows fast, tip-free bead-based DNA purification for single cell RNA-sequencing

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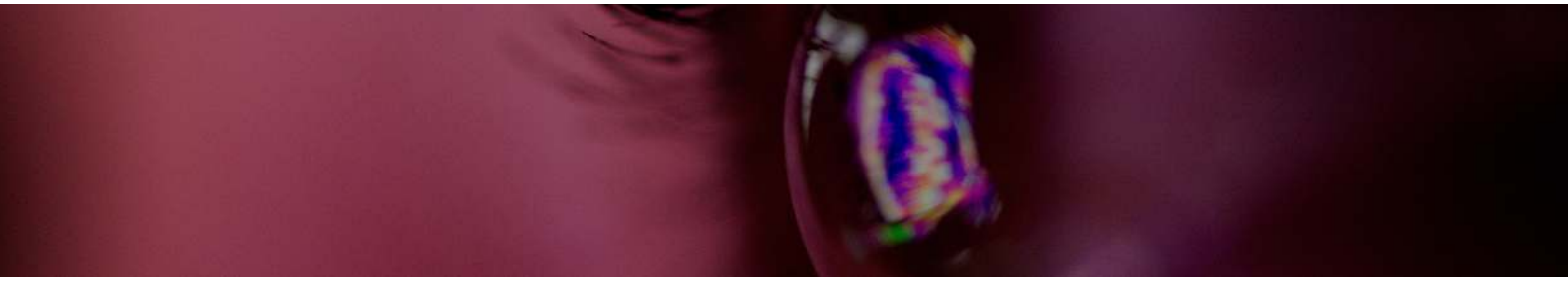

Introduction

The development of next-generation sequencing (NGS) technology has greatly promoted our understanding of complex biological processes. In recent years, transcriptomics analysis (RNA sequencing or RNA-seq) has benefited from major technological advances that allow characterization of gene expression at the single-cell level (scRNA-seq). In contrast to the traditional bulk RNA-seq, scRNA-seq greatly improves the resolution to assess transcriptional similarities and differences within a population of cells and can reveal complex and rare cell populations. In the last decade, scRNA-seq has benefited from a continuously growing interest among the scientific community. Such interest has benefitted from development of more sensitive plate-based methods (i.e., higher number of genes per cell and more even coverage across transcripts) as well as lower costs for technology and tools needed to conduct scRNA-seq studies. The process of NGS library preparation is also becoming increasingly automated through the use of liquid handlers, such as the I.DOT.

However, magnetic bead cleanup is still often performed manually, creating a time-consuming bottleneck in the library preparation workflow. While automated liquid handlers can reduce hands-on time, they are often expensive and require extensive calibration. In both cases, bead cleanup relies heavily on the quality of the pipetting, and inconsistencies can lead to variation between samples.

The multiple steps involved often require using multiple boxes of pipette tips, which increases the cost of libraries considerably. In contrast, automated magnetic bead cleanup has the potential to streamline the NGS library prep workflow, ensuring consistent results, as published in (1).

The C.WASH non-contact plate washer and liquid dispenser helps simplify and standardize magnetic bead cleanup. It is specifically designed for cellular assays and bead-based DNA purification. All C.WASH applications ensure there is no well-to-well cross-contamination during liquid removal or reagent dispensing.

In this experiment, we evaluated the implementation of the C.WASH to conduct an automated bead-based DNA purification during scRNA-seq library preparations. Importantly, we designed the experiment to detect any potential cross-sample contamination when using the C.WASH. The instrument quickly and gently removes liquids from PCR plates using centrifugal forces, while magnetic plate carriers maintain the magnetic beads at the bottom of the plates. The integrated non-contact dispenser quickly and uniformly adds reagents, such as ethanol, during the washing steps. Non-contact washing saves several hundred pipette tips per plate in 96-well plates, and over 1,000 in 384-well plates.

In this scRNA-seq experiment, we deposited alternating rows of sorted human and mouse single cells into 384-well plates. After cDNA synthesis using the Smart-seq3 protocol and automated bead clean-up using C.WASH, we demonstrated that there is no cross-species contamination from samples of any adjacent wells or other samples.

The C.WASH enables an automated bead-based DNA purification that simplifies and accelerates NGS library preparation workflows and saves on pipette tips, while safeguarding cross-sample contamination of PCR amplified cDNA fragments as effectively as other automated liquid handlers.

Materials and methods

Cell sorting and library preparation

Human (HEK293FT, Invitrogen) and mouse (NIH3T3, ATCC) cells were sorted by Fluorescence-activated cell sorting (FACS) in 384-well plates containing 0.3 uL Smart-seq3 lysis buffer (2). The low volume of lysis was protected from evaporation using 3 uL of VaporLock (Qiagen) (3). We sorted mouse and human cells in alternating rows of a 384-well plate (Thermo Fisher) using a BD FACSMelody (100 um nozzle, BD Biosciences). Row H in the plate did not receive any cells as a negative control. cDNA was synthesized and amplified using Smart-seq3 protocol at 1/10 scale with 20 cycles for cDNA amplification PCR (2 & 3). PCR products (volume 1 uL) were diluted using 9 uL H_2O.

DNA purification

DNA purification was performed using 6 uL home-made beads (22% PEG; ratio of 1:0.6 sample:beads) and performed with either a G5563AA Bravo Automated Liquid Handling Platform equipped with the G5056 384 Channel ST Disposable Tip Head (Agilent Genomics) or C.WASH as previously published (4). In both plates, beads were added using the Bravo with 20 mixing cycles to ensure a homogenous suspension. Briefly, after settling of beads (3 min at room temperature) on a magnet (Alpaqua Engineering), the supernatant was removed, and the beads were washed twice with 80% ethanol and eluted in water. The workflow for both liquid handler is shown in **Table 1**.

Table 1. *Beads clean-up workflow on both liquid handlers.*

	BRAVO	**C.WASH**
Removal of supernatant	3 pipetting steps at 0.5 mm, 0.2 mm, 0.1 mm height	50 g for 10 sec. Acceleration: 1,000 rpm Deceleration: 750 rpm
1st wash Ethanol 80%	12 µl	15 µl
Removal of Ethanol	2 pipetting steps at 0.3 mm, 0.1 mm	50 g for 10 sec. Acceleration: 1,000 rpm Deceleration: 750 rpm
2nd wash Ethanol 80%	not performed	15 µl
Removal of Ethanol	not performed	50 g for 10 sec. Acceleration: 1,000 rpm Deceleration: 750 rpm
Elution in H_2O	10 µl 10 mixing cycles	10 µl
Total Process Time	8 min	2 min

In the case of the C.WASH sample, the beads were resuspended in the H_2O elution by shaking at 2300 rpm for 1 min using a ThermoMixer C (Eppendorf).

cDNA concentrations were measured using the QuantiFluor dsDNA HighSensitivity assay in a Fluostar Omega plate reader (BMG Labtech), and all samples were subsequently normalized for cDNA concentration. Next, 0.1 ng of cDNA were tagmented as described in (2). Libraries were then sequenced on the DNBSEQ G400RS platform (MGI) in SE100 configuration.

Read alignments and gene expression estimation

Raw non-demultiplexed fastq files were processed using zUMIs (version 2.9.7) with STAR (v2.7.3a) to generate expression profiles for both the 5′ ends containing UMIs as well as combined full-length and UMI data. To extract and identify the UMI-containing reads in zUMIs, find_pattern: ATTGCGCAATG was specified for file1 as well as base_ definition: cDNA (23–100; single end), and UMI (12–19) in the YAML file. UMIs were collapsed using a Hamming distance of 1.

All cells were mapped to the combined human (hg38) and mouse genomes (mm10) and supplemented with additional STAR parameter '--clip3pAdapterSeq CTGTCTCTTATACACATCT' to trim adapters. Gene expression was quantified with gene annotations from Ensembl GRCh38.91 (human) and GRCm38.91 (mouse).

Results and discussion

We used Smart-seq3, the most sensitive scRNA-protocol to date, for detecting potential cross contamination while automating the library clean-up with C.WASH. We sorted HEK293FT (human) and NIH3T3 (mouse) cells in alternating rows of a 384-well plates, at the exception of the middle row (H), to be used as a negative control. After synthesizing and amplifying cDNA with the Smart-seq3 protocol, the bead clean-up was performed with either a Bravo liquid handler or the C.WASH. In both plates, we eluted the beads in 10 µL of nuclease-free H2O and determined the cDNA concentrations using a fluorimetric assay.

Tagmented libraries were sequenced and the resulting data processed using zUMIs (5). We then mapped all reads from all wells against both the human and mouse genome, without the known information of the species to avoid any biases. After applying commonly used quality control filtering (min. 100,000 sequenced reads, >= 50% of reads in exons), we analyzed the specificity of read mappings with regard to the species of origin. Clearly, nearly all wells could be classified unambiguously into the human and mouse cell lines (>= 95% of reads specific). We overlay the inferred species identity to the position in the 384-well plates, which recapitulated the experimental design during FACS sorting precisely (**Figure 1**). Moreover, row (H), that was left without any cells, does not show any reads, confirming the expected performance of C.WASH.

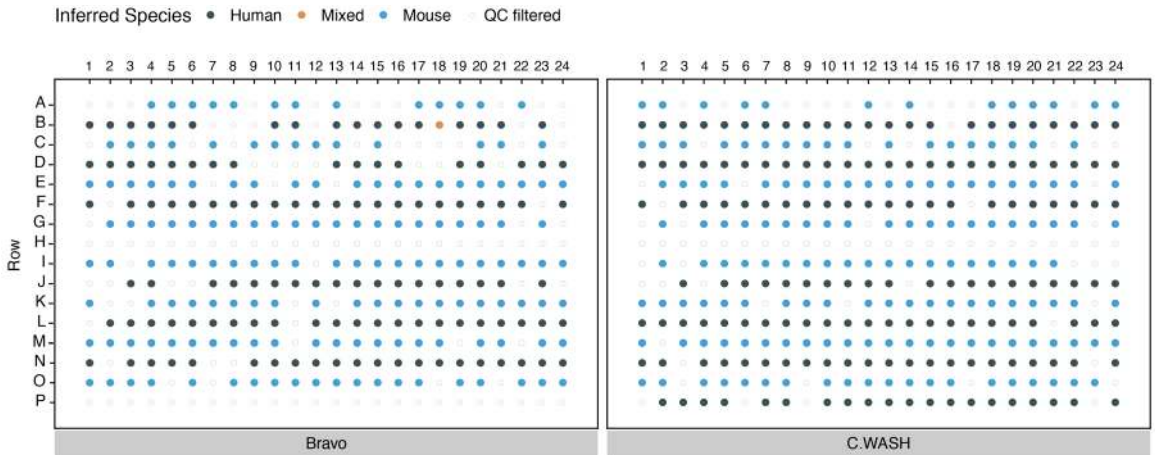

Figure 1. *Visualization of species calling of libraries clean-up with either Bravo or C.WASH. Inferred species are depicted. HEK293FT were sorted in rows A, C, E, G, I, K, M, O, and NIH3T3 were sorted in rows B, D, F, J, L, N, P. Row H did not receive any cells.*

Moving to a more quantitative analysis, we showed the number of reads mapping to the respective genomes for each of the cells (**Figure 2**). These results show the automated bead clean up did not lead to sample cross-contamination of amplified cDNA libraries.

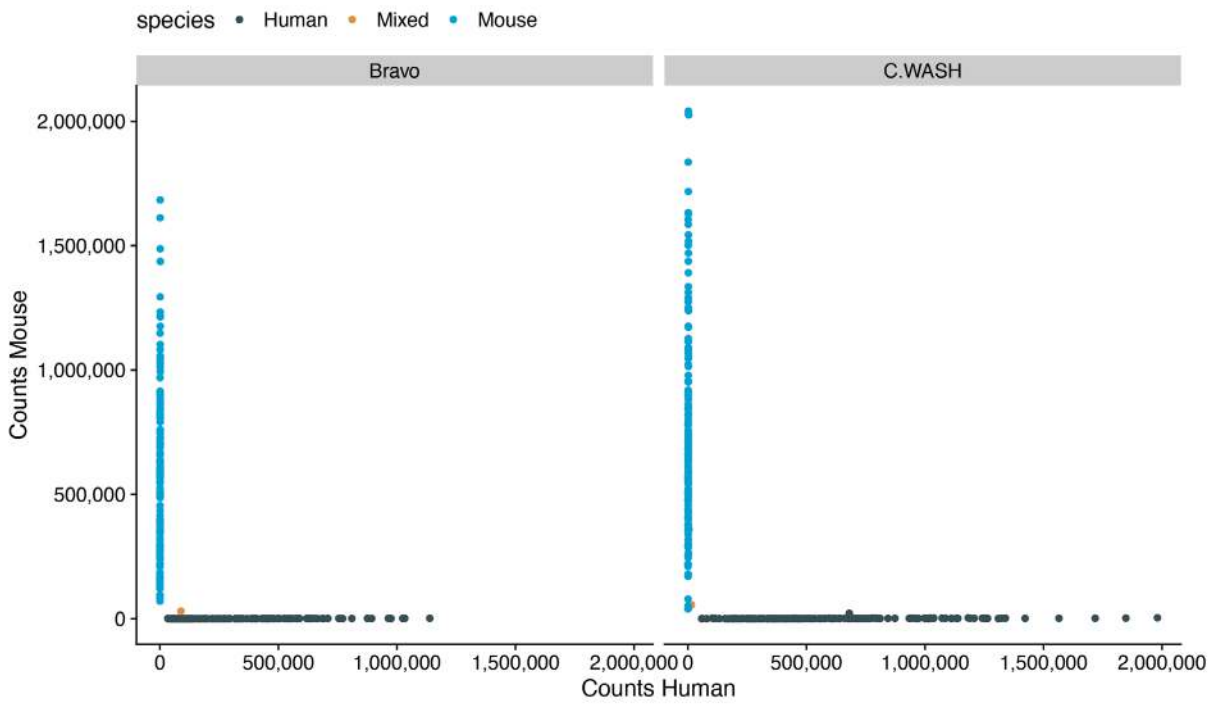

Figure 2. *Numbers of reads mapping to the respective genomes.*

Therefore, the bead clean-up workflow using C.WASH is a safe and reliable 1-to-1 replacement for conventional manual methods, while reducing both the time and cost required to perform this assay. Indeed, the full workflow from the first bead supernatant removal takes only 2 minutes. As C.WASH provides standalone operation, along with seamless integration into automated high-throughput workflows, it would considerably increase the number of samples and plates processed per day.

Plastic consumables have become a driving cost for plate-based assay protocols. Performing as many steps as possible without pipette-tips drastically drives down the cost of every assay. The non-contact nature of the dispensing steps, but also for liquid removal of the C.WASH, is a key to save consumable costs and make workflows more environmentally friendly. Moreover, the recent COVID-19 crisis has shown that some regions have suffered due to major delays in the supply chain, which led to considerable setbacks in obtaining results (6). With these results, we demonstrate that the C.WASH can be an essential liquid handling equipment for many NGS laboratories.

References

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