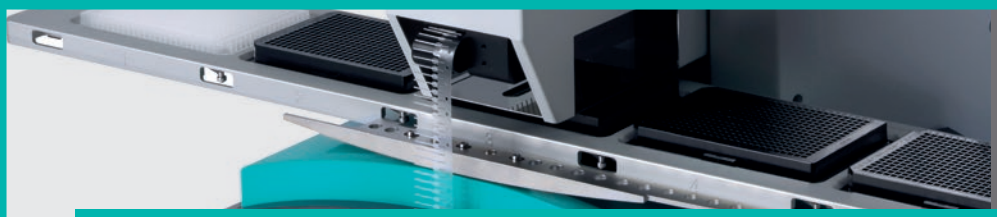


mosquito® HTS
application note

low-volume, single-cell RNA-seq library prep: use of mosquito® liquid handlers in Nextera XT library preparation and magnetic bead clean up

introduction

Single-cell sequencing involves isolating a single cell and examining its sequence information using next-generation sequencing technologies, to fully characterise cells and provide powerful insights into cellular differences. The field of single-cell genomics is advancing rapidly, yet researchers face multiple challenges, including reproducibility, sensitivity, scalability and cost, particularly when large numbers of cells are analysed. However, automation and miniaturisation has been shown to address these limitations.

Nextera XT sample prep kits (Illumina, Inc., USA) are commonly used to prepare DNA libraries based on enzymatic fragmentation of DNA samples using only very small amounts of input DNA. To ensure high accuracy and precision, most library preparation protocols recommend volumes that are within the range of manual pipettes or large volume liquid handlers. However, only a fraction of each of the preparations is then required for sequencing. By using a liquid handler that can handle low volumes of solutions with different viscosities, smaller reaction volumes could be prepared, saving on reagent cost and sample input.

This application note presents a high-throughput, miniaturised workflow, in which differentiated pancreatic stems cells were studied using single-cell RNA-seq (scRNA-seq), in Dr. Louise Laurent's lab at University of California, San Diego, USA. Miniaturisation of the library prep volumes was achieved using TTP Labtech's mosquito HTS and HV liquid handlers, which accurately dispense volumes of between 25 nL and 1.2 µL and 0.5 to 5 µL, respectively. These instruments use true positive-displacement pipetting technology, which means they can work at the same setting across liquids with different viscosities, such as alcohols, buffers, enzymes and low to high concentrations of gDNA (200-300 ng/µL).

The resulting single-cell RNA-seq data were analysed to determine the reproducibility of this system and its ability to distinguish not only between cells at different stages of differentiation but also between individual cells within each stage.

For more detailed results and discussion please refer to this journal publication: J Lab Autom. 2016 Aug; 21(4):557-67.

case study: single-cell analysis of differentiated human pancreatic stem cells methods

Pancreatic progenitor cells were differentiated from human embryonic stem cells (WA09) then dissociated into a cell suspension. An average cell concentration of 2.5 x 105 cells/mL was loaded into the C1 Single-Cell Auto Prep System (Fluidigm, San Francisco, USA), which generated and amplified cDNA from cells at two differentiation stages. Two cells from stage 1 and two from stage 2 were selected for single-cell analysis. The resulting cDNA was diluted to a final concentration of 0.1 ng/µL and then converted to Illumina sequencing libraries using the Nextera XT kit (Illumina, San Diego, USA) and the mosquito HTS liquid handler (Fig 1).

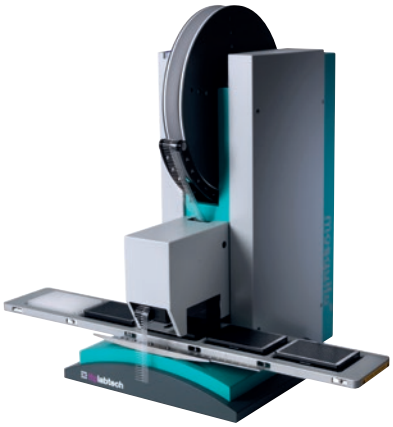
Libraries were generated in three different final reaction volumes of 2 µL, 4 µL and 8 µL in quadruplicate in 384-well PCR plates using as low as 20, 40 and 80 pg of cDNA per reaction, respectively. Illumina recommended volumes and sample input are 50 µL and 1 ng, respectively.

Excess primer dimers, nucleotides, salts and enzymes were removed using magnetic bead clean up. Low-volume and high-throughput bead clean-up was performed on the TTP Labtech mosquito HV liquid handler using a 384 well magnet (TTP Labtech, SZZ00136) and Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, USA). 2 µL of each single-cell library and 1.8 µL of beads were mixed and the libraries were cleaned up using the standard protocol to pull down and wash the beads, and then elute the libraries off the beads. The resulting purified libraries were analysed on the BioAnalyser (Agilent Technologies, Santa Clara, USA), normalised to 0.1 ng/µL, pooled and loaded onto a sequencer (HiSeq 2500, Illumina). The 48 pooled libraries were sequenced at an average total read depth of 5.6 million reads per sample. The resulting sequencing data were analysed, and correlations between the three different reaction volumes and biological and technical replicates were assessed for quality and reproducibility.

key benefits

mosquito liquid handlers for low-volume single-cell RNA-seq library preps:

- **reduce cost through miniaturisation of reagent volumes**
- **provide reliable pipetting of solutions with low to high viscosity**
- **increase reproducibility and data quality with accurate pipetting at low volumes**



ttplabtech

results

miniaturisation does not affect the technical replicates

Fluidigm has validated the Nextera XT kit final reaction volume down to 10 µL, with 125-375 pg of sample input. Here using mosquito HTS, the reaction volume and sample input were reduced to 2 µL and 20 pg, respectively.

After DEseq normalisation of the data set, correlations between replicates, reaction volumes and cell types were calculated using Pearson's correlation. In this analysis, the mean correlation coefficient was greater than 0.936 between each technical replicate for each cell at each reaction volume, both with and without down-sampling. We also noted that the correlation coefficients between different reaction volumes for a given cell were all greater than 0.918.

To further determine whether the reaction volume affected the reproducibility of the library preparation, the coefficient of variation (CV) for each library was calculated, using DESeq normalised data. The CVs were between 2.9 and 3.8 for all reaction volumes and all cells. When the mean CV for each cell irrespective of reaction volume was calculated, there were no significant differences between the overall CVs and the CVs from each reaction volume separately (Fig 2).

miniaturisation does not affect the biological replicates

Using two different clustering methods, 2D principal component analysis (Fig 3a), and hierarchical clustering (Fig 3b), a clear separation was observed between the libraries from each of the four cells. Importantly, the libraries did not cluster according to reaction volume, even within a single cell.

miniaturisation does not affect library complexity

A potential concern with decreasing the reaction volume for library preparation is that we could introduce sampling error, which could result in decreased detection of transcripts expressed at low levels, thus decreasing the complexity of the libraries. First the union of overlapping transcripts among the four replicates for each reaction volume for each cell were taken, and then the overlaps between the 2-µL, 4-µL, and 8-µL libraries were inspected (Fig 4, top); secondly, the intersect of overlapping transcripts among the four replicates for each reaction volume for each cell were taken and then the overlaps between the 2-µL, 4-µL, and 8-µL libraries were inspected (Fig 4, bottom). Overall, the overlaps were very similar across volume range, confirming the miniaturisation does not affect the library complexity.

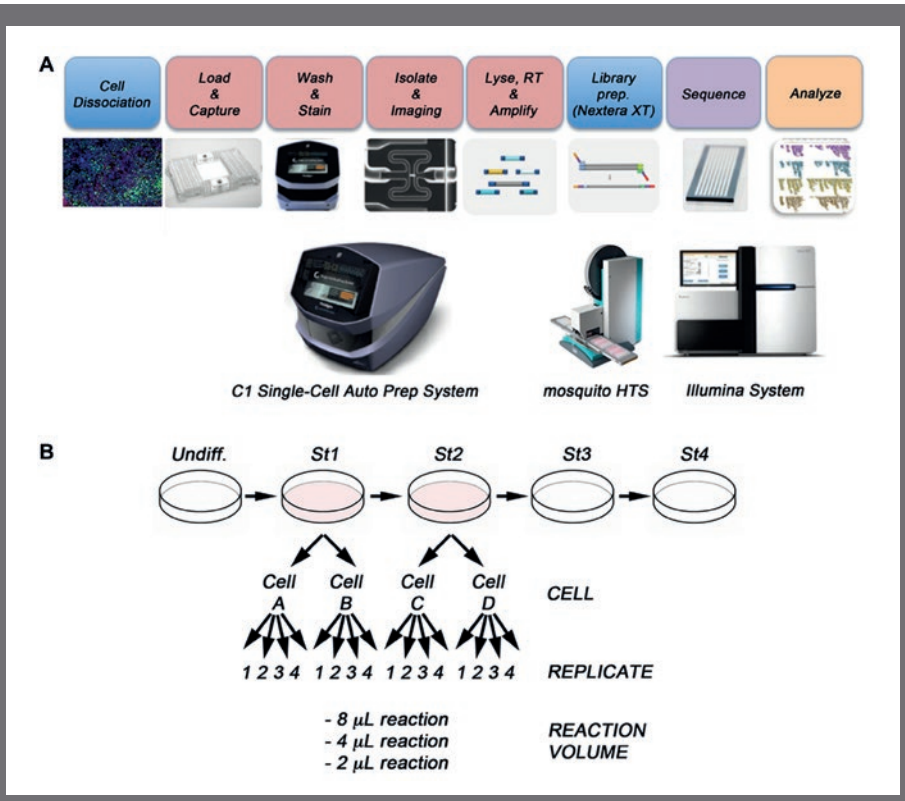


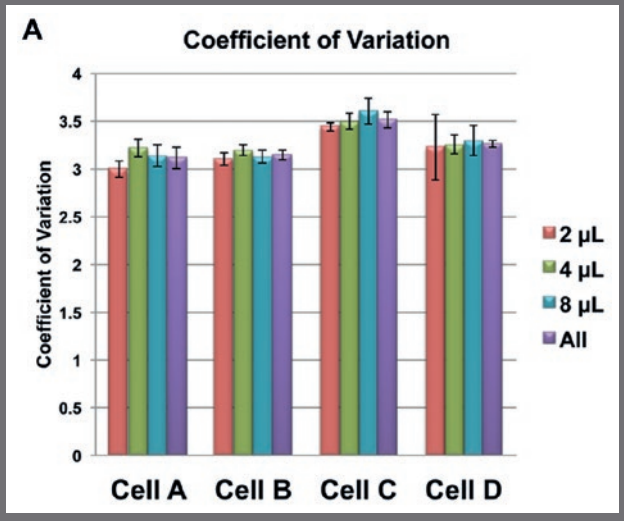
Fig 1. (a) Schematic of workflow for the single-cell sequencing of differentiated pancreatic stem cells. After differentiation, the cell cultures are dissociated to single cell suspensions and loaded onto a Fluidigm C1 Single-Cell Auto Prep Array for mRNA-Seq where the mRNA is reverse transcribed and the cDNA amplified. Libraries are prepared using the Nextera XT kit and mosquito HTS (TTP). Finally, libraries are pooled and sequenced on an Illumina HiSeq 2500. (b) WA09 human embryonic stem cells were differentiated in vitro to the pancreatic lineage. Two independent cells from stage 1 (cell A and cell B) and two cells from stage 2 (cell C and cell D) were selected for single-cell analysis. For library preparation, 2 µL, 4 µL, and 8 µL final volume reactions were tested, with four technical replicates per reaction volume.

reagent volume (nL)	reaction volumes (µL)		
	2	4	8
Atm enzyme mix	200	400	800
TD buffer	400	800	1,600*
cDNA (0.1 ng/L)	200	400	800
NT buffer	200	400	800
NPM enzyme mix	600	1,200*	2,400*
Double Index	200 200	400 400	800 800

* total volume was made up of multiple pipetting of smaller volumes

Table 1. Volumes (nl) of reagents and cDNA pipetted by mosquito HTS to obtain total reaction volumes of 2, 4 or 8 µL.

Fig 2. Mean coefficients of variation (CVs) for each reaction volume for each cell calculated from DESeq normalised data. The purple bars are the mean CVs for each cell, irrespective of reaction volume.



conclusions

The resulting single-cell RNA-seq data demonstrated that miniaturisation using mosquito low-volume liquid handlers does not affect the reproducibility or complexity of the library prepared whilst providing significant cost savings through miniaturisation of reaction volumes. Even at low reaction volumes it was possible to distinguish between cells at different stages of differentiation and also between individual cells within each stage.

This technical advance will significantly decrease both the cost and labour required for single-cell transcriptome studies, making analysis of hundreds to thousands of single cells feasible.

acknowledgements

We would like to thank Dr. Louise Laurent's group at Sanford Consortium of Regenerative Medicine, University of California, San Diego (UCSD), USA for their collaboration.

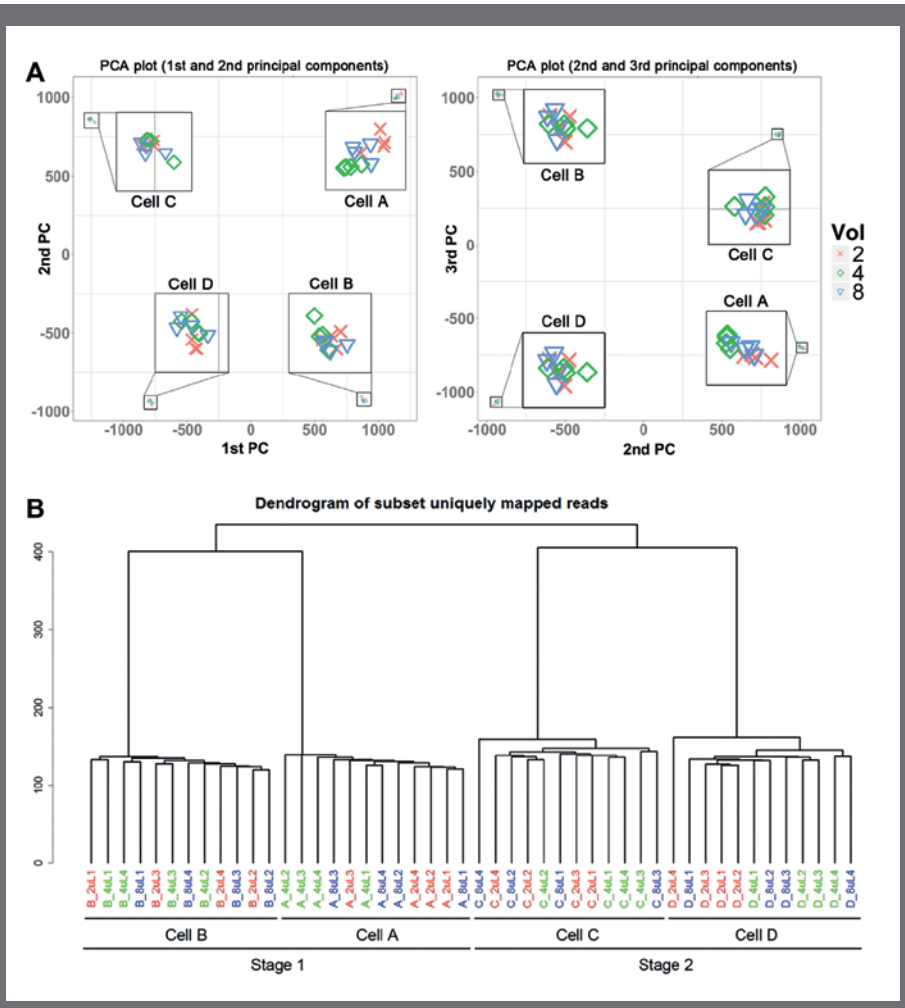


Fig 3. Clustering analysis. (a) Principal component analysis (PCA) for libraries. (b) Hierarchical clustering of all

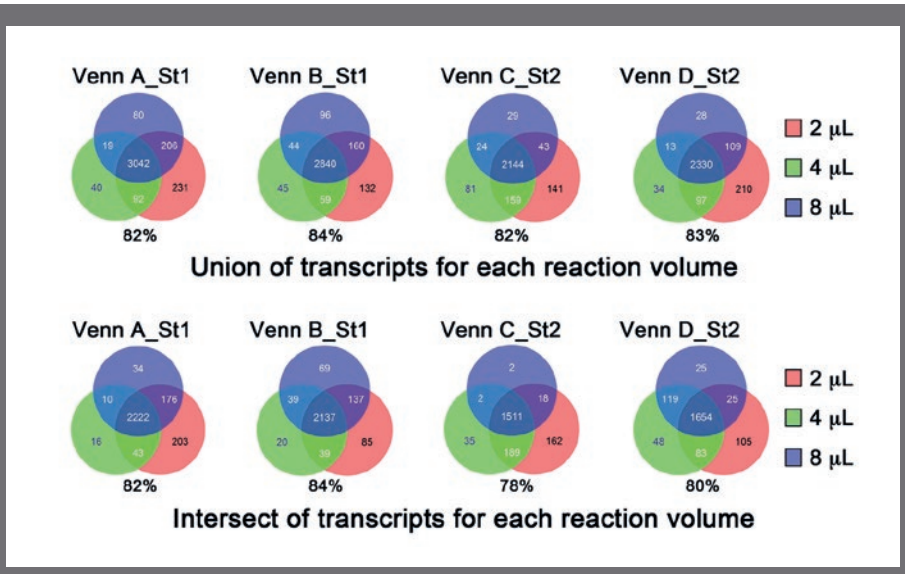


Fig 4. Venn diagrams displaying the overlap in detected transcripts among the different reaction volumes, using the union (top) or intersect (bottom) of detectable genes in the four replicates. The percentage of transcripts in the common region of intersection (i.e., 2 µL ∩ 4 µL ∩ 8 µL) compared with all transcripts (i.e., 2 µL ∪ 4 µL ∪ 8 µL) is shown below each Venn diagram.

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