A SMARTer Approach to Profiling the Human T-Cell Receptor Repertoire Using the ICELL8 Single-Cell System

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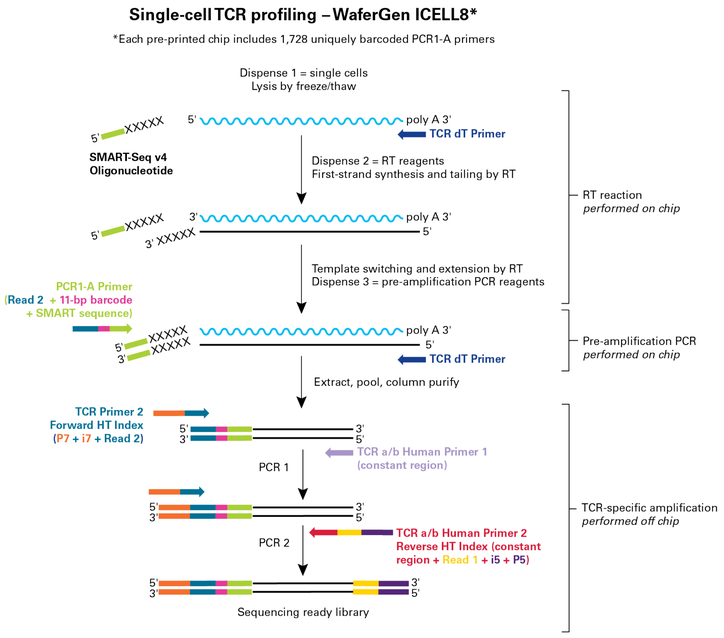
Introduction

Profiling T-cell receptor (TCR) diversity is critical for understanding the adaptive immune system and can provide valuable insights in studies involving immuno-oncology, immune deficiency, autoimmunity, and vaccine response. While low-throughput approaches have yielded important insights concerning TCR repertoire dynamics, the development of next-generation sequencing (NGS) technologies has dramatically expanded the prospects for this research area. The development of a SMARTer® NGS library-preparation workflow for the WaferGen ICELL8™ Single-Cell System provides researchers with a high-throughput approach for capturing full-length VDJ sequence information for single-cell TCR profiling. This approach, in combination with phenotyping, enables researchers to identify specific pairings of alpha and beta chains that comprise functional receptors in individual cells, and provides a starting point for classifying these individual T-cells on the basis of function, maturity, and other complex parameters such as the timing of cytokine secretion.

Using SMART® technology, we have developed an NGS library-preparation kit for TCR profiling that employs a 5′ RACE-like approach to capture full-length variable regions of TCR-α and/or TCR-β subunits. While approaches that utilize genomic DNA as input material require multiplexed PCR strategies, amplification of TCR sequences derived from RNA can be accomplished using single primer sets for each subunit. This enables the user to obtain sequencing-ready TCR libraries from RNA in ~2.5 hours of hands-on time. This approach was originally developed for RNA or bulk-cell inputs, requiring a minimum of 10 ng of RNA or 50 cells. Starting with RNA obtained from human peripheral blood, this method yields libraries containing TCR-α and/or TCR-β sequences. When analyzed on an Illumina® MiSeq® using 300-bp paired-end reads, >70% of sequencing reads map to TCR variable regions, and the most highly represented clonotypes remain consistent across a range of input amounts.

In this poster, we present a modified library preparation protocol adapted for the WaferGen ICELL8 Single-Cell System that allows for high-throughput analysis of TCR sequence information from single cells. In preliminary studies performed in-tube on Jurkat cells, an average of 92% of sequencing reads mapped to TCR sequences, and 90% of reads could be used for clonotype identification. With this platform, we can generate libraries for ~1,000 single cells at a time, which can be pooled together and sequenced in a single MiSeq run. Analysis of individual Jurkat cells from pooled libraries generated in this manner identified >60% of reads mapping to TCR sequences in most cells, with ~70–80% of these reads being used in clonotype identification.

Workflow for TCR α/β sequencing library preparation



Library preparation workflow and PCR strategy for single-cell TCR profiling using the ICELL8 System with the SMARTer method. On-chip reactions: Dispense #1 (single-cell solution): T cells, such as Jurkat cells, are dispensed using MSND+ into WaferGen 72 x 72 chips with (or without) pre-printed, barcoded PCR primers, using methods designed to maximize the single-cell yield as dictated by Poisson statistics. Automated imaging of the cells is performed using CellSelect™ software. Single cell-containing wells are down-selected so each barcode is used only once. There are three copies of each barcode (n=1,728) on the chip that provide well-specific addresses. Cells are lysed on-chip by freeze-thaw and immediately processed. **Dispense #2 (RT mix)**: First-strand cDNA synthesis is primed by the TCR dT Primer and performed by an MMLV-derived reverse transcriptase (RT) in the presence of cell lysis buffer. Upon reaching the 5′ end of each mRNA molecule, the RT adds non-templated nucleotides to the first-strand cDNA. The SMART-Seq® v4 Oligonucleotide contains a sequence that is complementary to the non-templated nucleotides added by the RT, and hybridizes to the first-strand cDNA. In the template-switching step, the RT uses the remainder of the SMART-Seq v4 template-switching oligo as a template for the incorporation of an additional sequence on the end of the first-strand cDNA. **Dispense #3 (pre-amp mix):** Ten cycles of pre-amplification are performed to incorporate the pre-printed, PCR1-A Primer (used in concert with the TCR dT Primer). The contents of the chip are extracted by centrifugation using a fixture, followed by column purification. **In-tube PCR amplification reactions:** Full-length variable regions of TCR cDNA are selectively amplified by PCR using indexed primers that are complementary to the oligonucleotide-templated sequence (TCR primer 2 Forward HT Index), and the constant region(s) of TCR-α and/or TCR-β subunits (TCR a/b Human Primer 1). A subsequent round of PCR is performed to further amplify variable regions of TCR-α and/or TCR-β subunits and incorporate adapter sequences, using TCR Primer 2 Forward HT Index and TCR a/b Human Primer 2 Reverse HT Index. Included in the primers are adapter and index sequences (Read 2 + i7 + P7 and Read 1 + i5 + P5, respectively) that are compatible with the Illumina sequencing platform. Following purification, size selection, and quality analysis, TCR cDNA libraries are sequenced on the Illumina platform using 300 bp paired-end reads.

Bolotin, D. A., Poslavsky, S., Mitrophanov, I., Shugay, M., Mamedov, I. Z., Putintseva, E. V., & Chudakov, D. M. (2015) MiXCR: software for comprehensive adaptive immunity profiling. *Nat. Methods* **12**(5):380–381.

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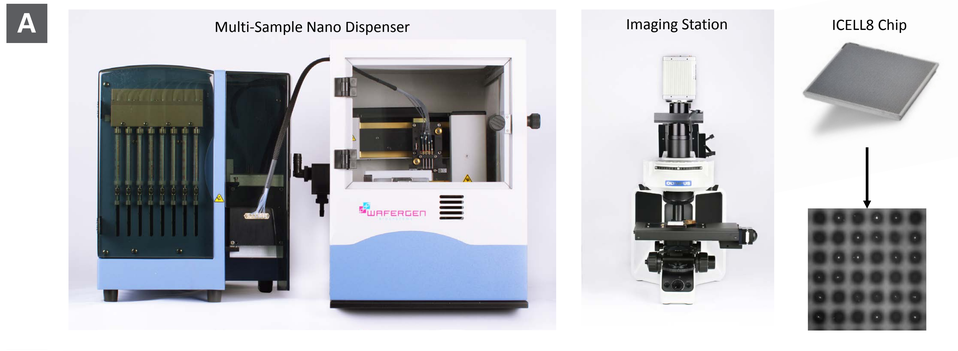
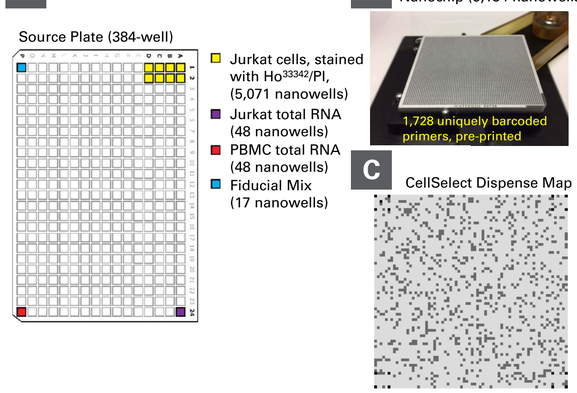
Single-cell genomics workflow: the WaferGen ICELL8 System



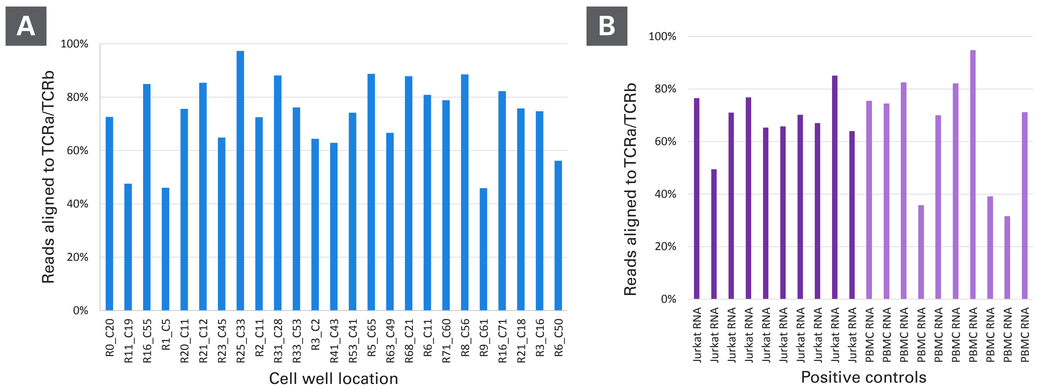
Overview of the WaferGen ICELL8 System: ICELL8 System Components – Multisample Nanodispenser (MSND) dispenses cells and reagents in nanoliter volumes into the wells of the ICELL8 chip, Imaging Station captures images of fluorescently labeled cells in the ICELL8 chip, 36 wells at a time (representative image shown), and CellSelect software analyzes images and identifies single cell-containing wells for further processing. **B:** Single-cell TCR sequencing workflow

Experimental design



Experimental design for generating single-cell TCR sequencing libraries from Jurkat cells dispensed into a nanochip. Panel A. The 384-well source plate was seeded with dual-stained Jurkat cells, positive control RNAs, and a fluorescent Fiducial Mix (FM). **Panel B.** Inputs (cells, controls, or FM) were dispensed into a pre-printed nanochip using the MSND+ robotic nanodispenser. **Panel C.** Following automated image acquisition and analysis, a dispense map for the subsequent dispenses was generated using CellSelect software. A total of 1,519 single cells were identified, and 1,179 single cells were selected as live candidates. These candidates were further down-selected to a total of 824 Jurkat cells (plus 20 control RNA samples) for further processing, library preparation, and sequencing.

Sequencing read mapping to TCR subunits for libraries generated directly from RNA controls and single Jurkat cells on the ICELL8 System



Alignment of sequencing reads from cells and RNA controls. To evaluate the performance of the SMARTer workflow on the ICELL8 system, the protocol was performed on Jurkat cells. The resulting cDNA libraries were sequenced and then analyzed using MiXCR (Bolotin et al., 2015). **Panel A.** The percentages of sequencing reads that map to CDR3 regions in TCR-α or TCR-β from 25 randomly selected cells, which were part of a library prepared from 824 Jurkat cells ("R" and "C" refer to row and column positions, respectively, for each cell). In the majority of cells (21/25), >60% of reads mapped to TCR-α or TCR-β sequences. The correct Jurkat clonotype (TRAV8-4,TRAJ3/TRBV12-3,TRBJ1-2) was identified in all cells. **Panel B.** The percentages of sequencing reads that map to CDR3 regions in TCR-α or TCR-β from positive controls (5 pg Jurkat RNA or 5 pg PBMC RNA). Again, in the majority of controls (16/20), >60% of reads mapped to TCR-α or TCR-β sequences. The correct Jurkat clonotype (TRAV8-4,TRAJ3/TRBV12-3,TRBJ1-2) was identified in all wells containing Jurkat positive control RNA.

Sequencing metrics and clonotype calling

	Single barcode (1)	Single barcode (2)	1,728 barcodes
Number of single-cell wells (Jurkat cells)	1,471	1,471	824
Number of control wells (RNA)	6	48	10 + 10
Total sequencing reads	734,613	1,130,907	4,168,990
Successfully aligned reads	614,886	778,300	2,790,532
Successfully aligned reads (percent)	**83.7%**	**68.8%**	**66.9%**
Number of reads used in clonotype calling	594,173	716,531	2,663,900
Reads used (percent of total)	**80.9%**	**63.4%**	**63.9%**
High-quality reads (percent of reads used)	69.9%	72.0%	61.8%
Low-quality reads (percent of reads used)	30.1%	28.0%	38.2%
Reads mapping to Jurkat clonotype:			
TRAV8-4, TRAJ3	50.3%	80.9%	35.2%
TRBV12-3, TRBJ1-2	49.7%	13.7%	64.6%
TOTAL	**99.9%**	**94.6%**	**99.8%**

Sequencing data analysis for three independent library preparation runs using the ICELL8 System. To evaluate the performance of the SMARTer workflow on the ICELL8, the protocol was initially performed on Jurkat cells and libraries were generated using a single barcode ((1) and (2)). Validation was then performed on pre-printed chips containing 1,728 barcodes. The resulting cDNA libraries were sequenced and then analyzed using MiXCR (Bolotin et al., 2015).

In our validation experiments where a single barcode was used, 1,471 Jurkat cells and either 6 or 48 PBMC RNA control wells were included in the final library. Both of these experiments gave good numbers of reads mapping to CDR3 regions in TCR-α or TCR-β (~84% and ~69% for run 1 (1) and run 2 (2), respectively), with the vast majority of these reads being used for clonotype calling. In (1), over 99% of the reads used for clonotype calling identified the correct Jurkat clonotype (TRAV8-4,TRAJ3/TRBV12-3,TRBJ1-2). In (2), where more PBMC RNA control samples were used, this number was ~95% (the remaining ~5% of reads identified alternative clonotypes present in the PBMC RNA controls).

We then tested the workflow on ICELL8 chips pre-printed with 1,728 barcodes. In this experiment, 824 Jurkat cells, 10 Jurkat RNA controls, and 10 PBMC controls were included in the final library. In this case, ~67% of reads mapped to CDR3 regions in TCR-α or TCR-β. Over 99% of the reads used for clonotype calling identified the correct Jurkat clonotype (TRAV8-4,TRAJ3/TRBV12-3,TRBJ1-2), with good representation of both the alpha and beta chains.

Conclusions

- Our SMARTer method provides a streamlined workflow for the generation of Illumina-ready TCR sequencing libraries that minimizes the likelihood of amplification biases by avoiding multiplex PCR.
- The 5′-barcoding system employed in this approach is a new and flexible addition to the WaferGen platform. It could potentially be utilized in combination with other gene-specific, Illlumina-indexed PCR primer sets to characterize the 5′-ends of other genes of interest as well as for performing 5′-tag counting.
- The combinatorial complexity enabled by uniquely indexing the TCR α/β amplicons created from each chip (SMARTer method), coupled with the capability of well-specific barcoding of the libraries generated from each cell (WaferGen ICELL8 system), provides a powerful approach for large-scale studies of T-cell receptor genomics.
- Paired identification of TCR alpha and beta chains in single-cells, and matching of these pairings with their cognate antigens (neoantigens) is an important analytical step in guiding immunotherapy strategies.
- The SMARTer workflow can be applied effectively on the WaferGen ICELL8 system, allowing for high-throughput single-cell isolation and experimentation, and yielding highly reproducible results.

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