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High-throughput single-cell T-cell receptor profiling with SMART technology

- Confident clonotype calling
  The ICELL8 workflow allows positive and negative controls for providing confidence in generated data
- Sensitive detection
  Full-length reads, with a majority of reads providing pairing information
- Useful for complex samples
  RACE-based approach allows for the detection of low-abundance TCR variants

Introduction

T-cell receptor (TCR) profiling of bulk samples has improved our understanding of TCR repertoire diversity, TCR-mediated antigen specificity, and mechanisms of adaptive immune response. However, while these studies can indicate which clonotypes are expressed and their relative frequencies in the bulk population, it is nearly impossible to determine the proper alpha-beta (αβ) pairing of specific receptor chains of the cells—except for some very rare cell populations. Single-cell T-cell receptor (scTCR) clonotype analysis permits the determination of the specific TCR αβ chain pairing expressed on each cell. This pairing information allows researchers to gain insight into T-cell heterogeneity and plasticity, determine the contribution of the pairing to antigen specificity of the individual TCR, and design therapeutic antibodies. Here we employ a novel next-generation sequencing (NGS) library preparation kit that utilizes a 5'-RACE-like approach and SMART technology, in conjunction with the ICELL8 Single-Cell System or the ICELL8 cx Single-Cell System, to capture full-length variable regions of TCRa and TCRb transcripts. (Note that throughout this tech note, TCRa and TCRb are used to reference nucleic acid transcripts, while TCR-α and TCR-β are used to refer to the expressed protein chain). Using the human TCR a/b profiling workflow together with automated ICELL8 platforms, which enable single-cell isolation and nanoliter-scale PCR in a nanowell chip, allows the clonotype analysis of >1,000 cells. This workflow can also be used to generate differential expression data by performing 5' end capture on the amplified cDNA.

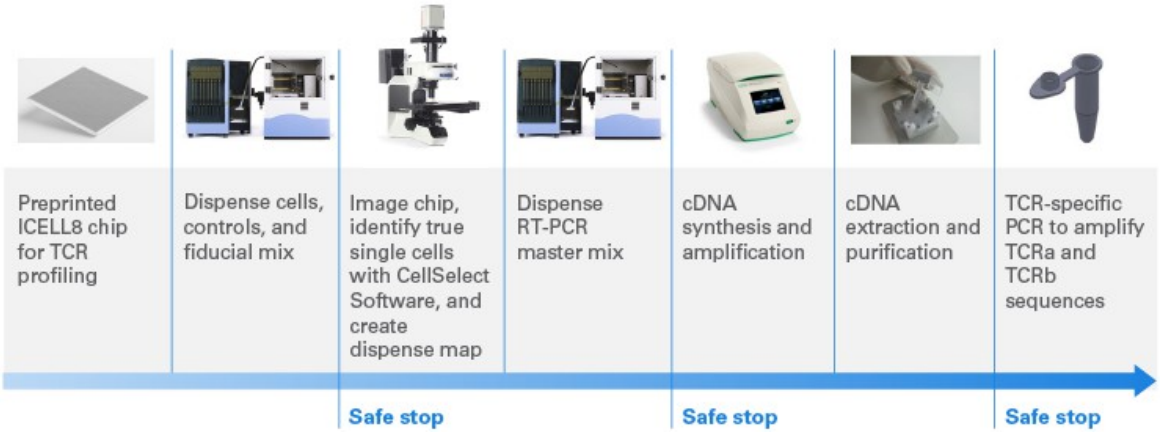

Figure 1. ICELL8 workflow overview with safe process stopping points indicated. 1,728 unique barcode-bearing template-switching oligos (TSOs) are printed onto a ICELL8 TCR Chip in triplicate. Cells are dispensed into the wells of the TCR chip at an average of one cell/well. The CellSelect Software is used to identify single cell-containing wells with unique barcodes. Cell lysis is followed by cDNA synthesis, via oligo-dT priming, to barcode each cell's captured mRNA. Well-specific, barcoded, template-switching oligos printed on the chip are used to define every individual cell's transcriptome using single-primer RT-PCR. Amplified cDNA is extracted to a single collection tube. The TCRa and TCRb sequences of the TCR variable regions are selectively amplified from the pooled cDNA using primers targeting the constant region of TCR subunits. Specific amplicons for the TCRa and TCRb subunits of the TCR complexes are generated using indexed nested PCR with gene-specific primers (GSP). The entire workflow time is two days with 2–3 hours of hands-on time.

Results

TCR clonotype calls in different cell lines using SMART chemistry and the ICELL8 workflow

For the experiments described below, the ICELL8 Human TCR a/b Profiling workflow was used to process T cells and peripheral blood

mononuclear cells (PBMCs) isolated on the ICELL8 Single-Cell System. The cDNA was generated via oligo-dT priming during RT-PCR performed on-chip (Figure 2, top). Template-switching oligos with well-specific barcodes printed on the chip were used to define every individual cell's transcriptome using single-primer amplification. Following extraction of the cDNA from the chip, the TCRa and TCRb sequences of the TCR variable regions were selectively amplified from the pooled cDNA using primers targeting the constant region of the TCR subunits. Specific amplicons for the TCRa and TCRb subunits of the TCR complexes were generated using indexed nested PCR with gene-specific primers (GSP; Figure 2, bottom).

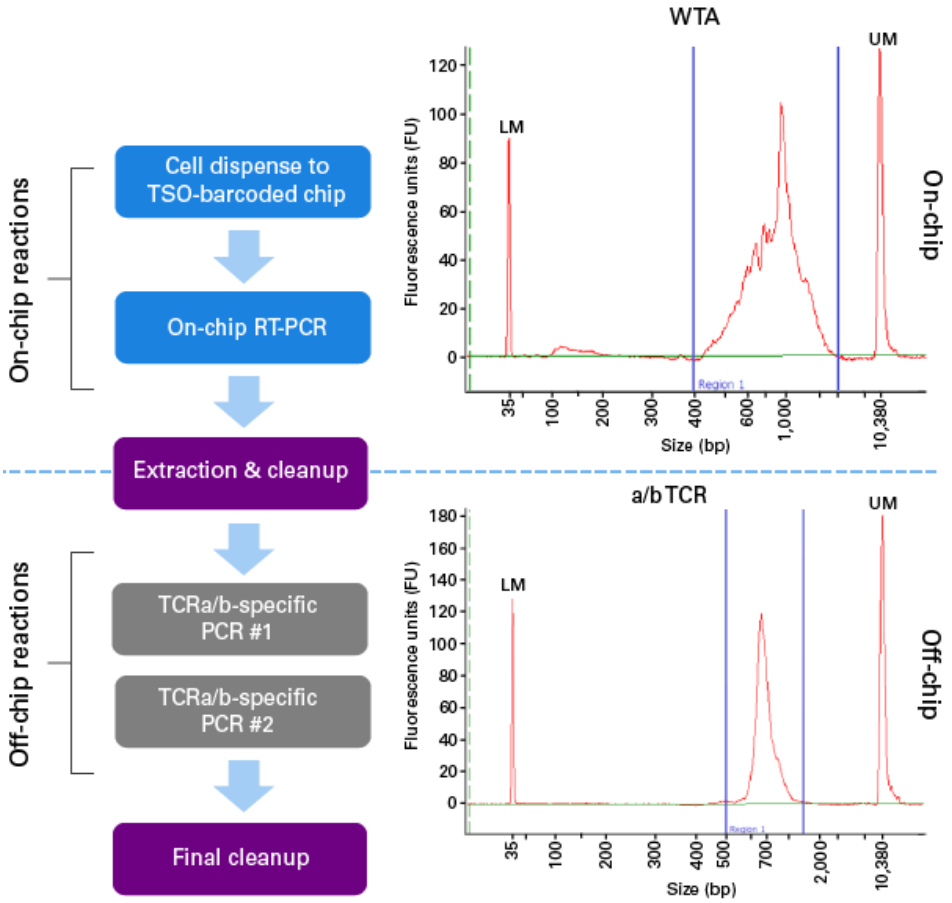

Figure 2. TCR-seq workflow. Top. After dispensing of samples (cells, positive control, and negative control) onto the ICELL8 TCR Chip, cDNAs were synthesized via oligo-dT priming on-chip. A Bioanalyzer trace for purified full-length cDNA shows an average cDNA amplicon length of 964 bp. Peaks labeled "LM" and "UM" correspond to DNA reference markers. **Bottom.** Following extraction of the cDNA from the chip, the TCRa and TCRb sequences of the TCR variable regions were selectively amplified. A Bioanalyzer trace of a library containing TCRa and TCRb sequences generated using the ICELL8 Human TCR a/b Profiling workflow shows broad peaks between ~650 to 1,150 bp and maximal peaks in the range of ~700 to 900 bp. Peaks labeled "LM" and "UM" correspond to DNA reference markers.

A benefit of the ICELL8 systems is that multiple controls can be set up to ensure confidence in the data. Negative controls, which contain all of the reaction components except the sample, can be used to set a threshold for confident clonotype calling. A total of 15 negative control wells were included in this experiment. To set the threshold for confident clonotype calling, the number of reads that were associated with the top clonotype in the negative control wells were identified. Since these wells are empty, any clonotype reads associated with negative control wells are background. We set the threshold for clonotype read counts at the mean clonotype reads for these empty wells plus three standard deviations. In this experiment, the threshold was set at eight clonotype reads (Figure 3, green cut-off line). After removing all clonotype calls with read counts below this threshold, amino acid sequences were examined and those that contained stop codons or frameshift mutations were removed. A positive control was set up with Jurkat total RNA. The clonotype calls for the Jurkat RNA-containing wells show only reads specific to the Jurkat TCRa and TCRb clonotypes, indicating a lack of barcode crosstalk between the wells.

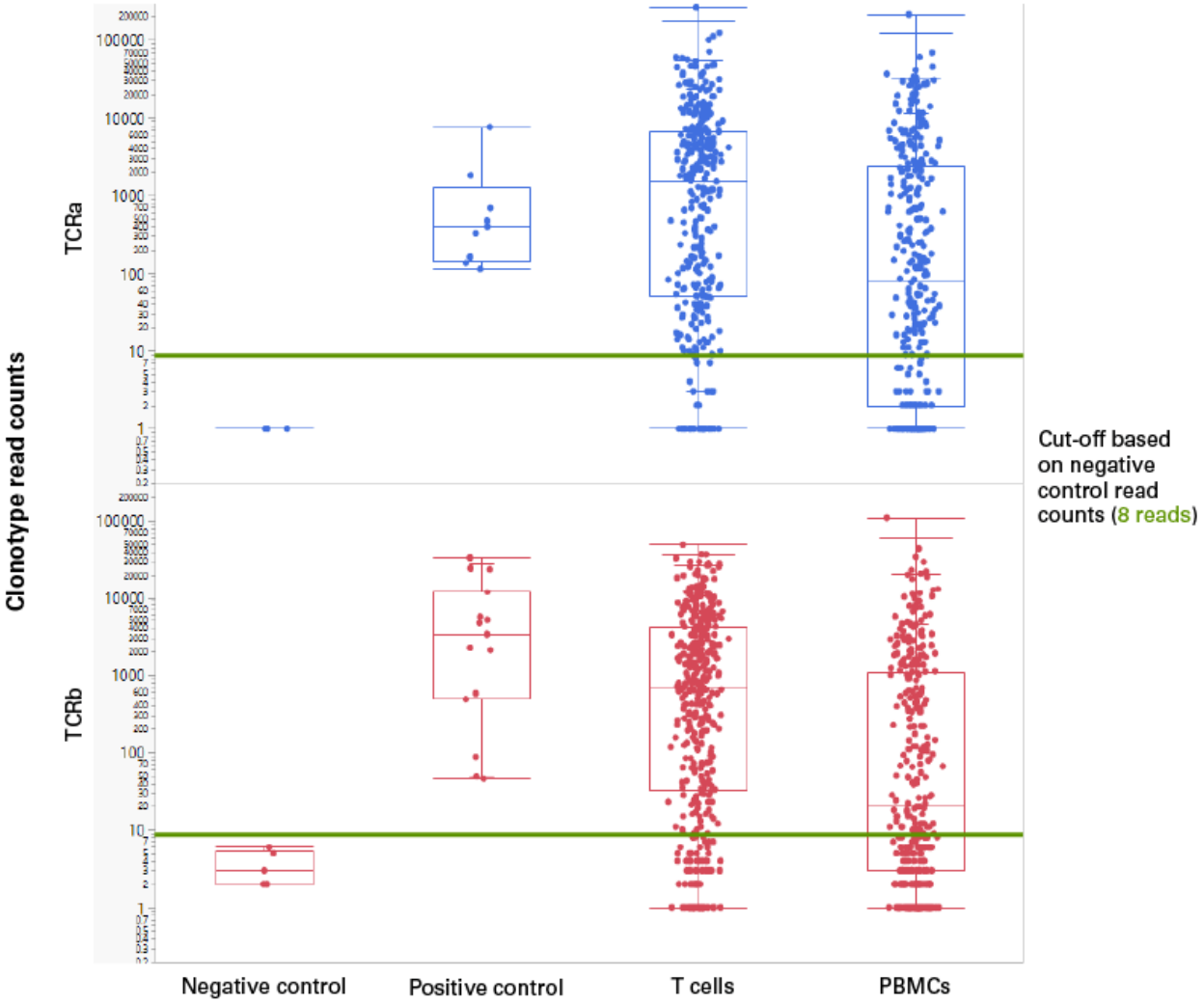

Figure 3. Clonotype read counts for T cells and PBMCs. The MiXCR output was filtered using Excel to define a read threshold for both TCRa and TCRb clonotypes (the solid line marks 8 read counts) greater than the 1X PBS negative controls. The data were analyzed using the JMP software and plotted into box plots. Each dot represents TCRa (blue) and TCRb (red) clonotypes in a cell. On-chip negative controls were used to set thresholds to enable the distinction between samples and NTC or "junk" low-read clonotypes.

Assessment of alpha-beta chain pairing

We next examined the data to determine cells where TCRa and TCRb sequences were detected (Table I). For the positive control samples, all wells contained clonotype calls for TCRa and/or TCRb. For the PBMC samples, not surprisingly, approximately 44% of cells had clonotype calls for TCRa and/or TCRb, since PBMCs are composed of more than just T cells. About 75% of the T cell samples included sequences for TCRa and/or TCRb. It is likely that some cells had no detectable TCR transcripts because negative selection during T cell preparation may have not been complete. Additionally, expression could have been too low to allow detection of the TCR clonotypes present in some cells.

Cell type	# of wells or cells	# of cells with clonotype calls	%of cells with clonotype calls
Neg control	15	0	0%
Pos control	15	15	100%
PBMC	590	261	44%
T cell	530	399	75%

Table I. Percentage of samples with clonotype calls

Examining the data further, a majority of PBMCs and T cells that contained clonotype calls had both alpha and beta transcripts, indicating the detection of an αß pairing (Figure 4). For PBMCs, of the 44% of samples where a TCR transcript was detected, 70% showed an αß pairing.

Meanwhile, 63% of the T cells with detected TCR transcripts contained an αß pairing. Interestingly, many of the pairing results were outside the αß-only pairing. For example, beta-beta (ßß), alpha-alpha (αα), alpha-alpha-beta (ααß), and other nonstandard combinations were observed. Similar reports have been made by other studies (Stubbington et al. 2016) and are not considered unusual.

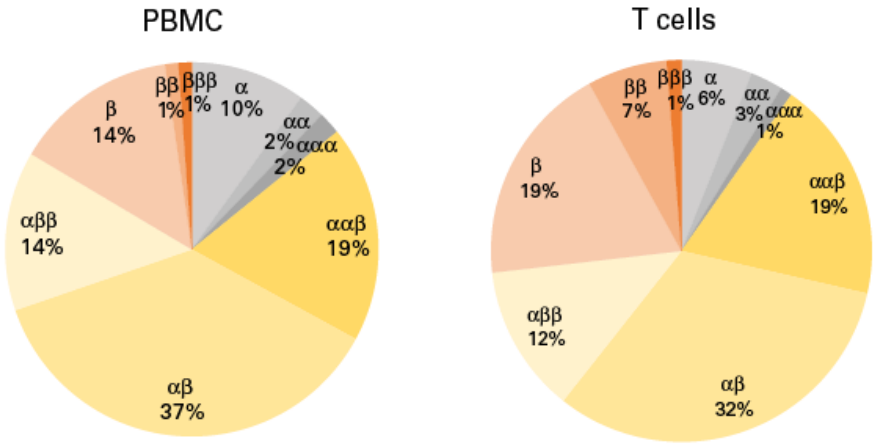

Figure 4. Alpha and beta chain pairs are called in 70% of PBMCs and 63% of T cells with identified clonotypes. Output files from our scTCR Analyzer pipeline report the top three clonotypes. Analysis revealed several pairing configurations including αα, ßß, αßß, ααß, ααα, and ßßß.

Clonotype distributions of TCRb transcripts

The distribution of TCR clonotypes identified from the sequencing data can also be depicted visually using chord diagrams (Figure 5). The chord diagrams represent the TCRb clonotype diversity observed in the PBMCs and T cells analyzed. In both samples, the same types of *TRBJ* gene segments were observed. However, in PBMCs the highest expressing clonotype was *TRBJ2-3* followed by *TRBJ2-7*, while in T cells *TRBJ2-7* was the most highly expressed followed by *TRBJ2-1*. Similarly, for the V gene segment the highly represented TRBV gene segments are different for the two cell types. In PBMCs, *TRBV5-1* showed the highest expression followed by *TRBV3-1*. In T cells, *TRBV20-1* was the highest expressing clonotype followed by *TRBV5-1*. We observed more clonotype diversity in T cells than PBMCs, which stems from having PBMC data from 261 cells compared to the T cell data which comes from 399 cells. Overall, the data represents the sensitivity of the chemistry in identifying various clonotype combinations, making this system an ideal choice for looking at complex samples.

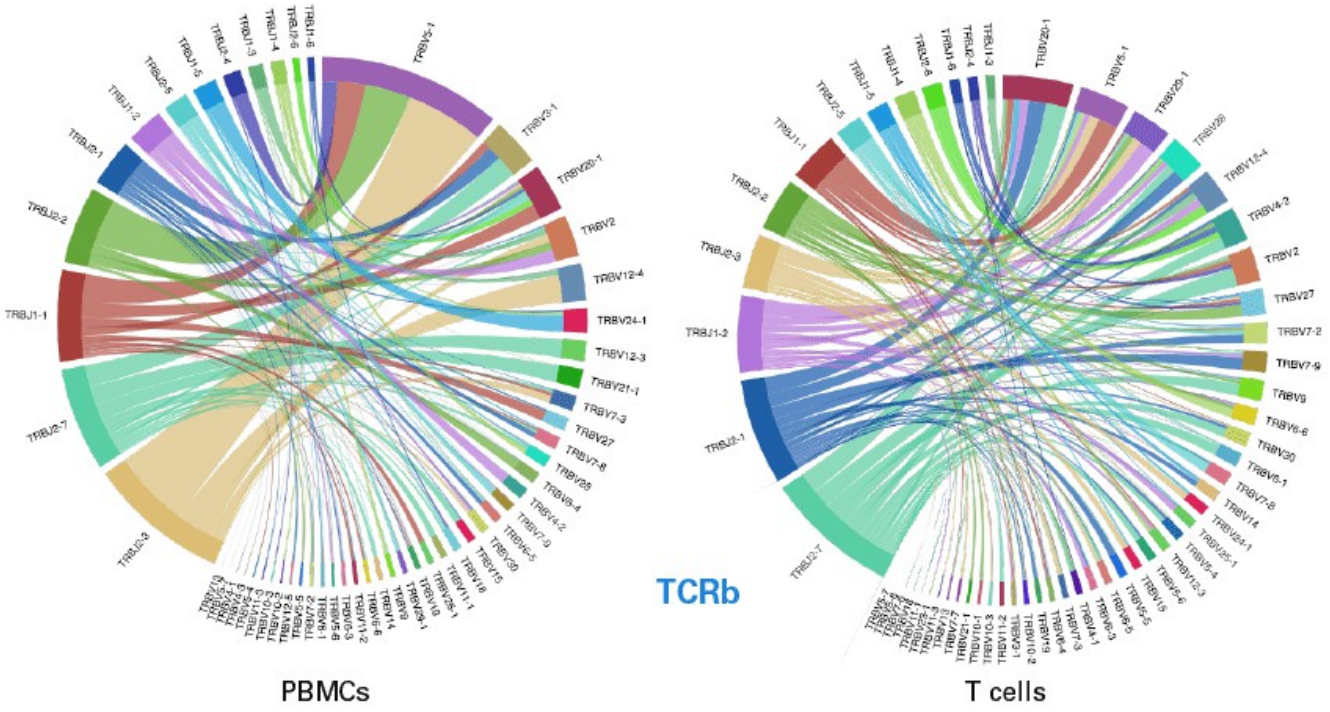

Figure 5. Chord diagrams reveal TCRb clonotype distributions. For each cell type (left: PBMCs; right: T cells), the chord diagram depicts the distribution of the indicated TCRb Variable-Joining (V-J) segment combinations. Each arc (on the periphery of each diagram) represents a V or J segment and is scaled

lengthwise according to the relative proportion at which the segment is represented in the dataset. Each chord (connecting the arcs) represents a set of clonotypes which include the indicated V-J combination and is weighted according to the relative abundance of that combination in the dataset.

Conclusions

The human TCR a/b profiling workflow for the ICELL8 systems can be used to generate Illumina sequencing libraries from thousands of single T cells for the determination of TCR αß pairing information. This highly sensitive approach to sequencing TCRs is achieved by using SMART cDNA synthesis and RACE-based gene-specific priming followed by TCR-specific PCR to fully capture and amplify TCRa and TCRb variable regions. The combination of this chemistry and the automated ICELL8 Single-Cell System or ICELL8 cx Single-Cell System enables profiling of >1,000 cells showcasing the general utility and scalability of this approach for studies investigating paired TCR clonotype diversity. Furthermore, the ability to set up positive and negative controls generates greater confidence in the data, especially when working with complex samples.

Methods

Libraries containing TCRa and TCRb sequences were generated using the ICELL8 Human TCR a/b Profiling workflow per the protocol in the user manual. For a positive control, Control Jurkat Total RNA (Takara Bio) was used. Isolated PBMCs (AllCells) were thawed in RPMI and washed once in media before staining the cells. T cells were isolated from whole blood using the EasySep Direct Human T Cell Isolation Kit (STEMCELL Technologies). Isolation was performed per manufacturer's recommendation. For sequencing, the final library was diluted to 13.5 pM, including a 5% PhiX Control v3 (Illumina) spike-in for sequencing. Sequencing was performed on an Illumina MiSeq® sequencer using the 600-cycle MiSeq Reagent Kit v3 (Illumina) with paired-end, 2 x 300 base pair reads.

After sequencing, the FASTQ files for each pool were demultiplexed using the ICELL8 scTCR Analyzer (available soon), and reads were assigned to each in-line index/sample well. Unless otherwise stated, demultiplexing was performed using exact match of the in-line index. Repertoire analysis for each sample well was performed using MiXCR 2.1.8 (Bolotin et al. 2015). Reads were aligned to reference V, D, J, and C genes of T-cell receptors then clonotype information for *CDR3* gene regions were extracted and reported to plain text files. Then a whole-chip summary report was generated collecting individual sample well information. A quality control tertiary analysis was later conducted to exclude clonotypes with few counts, based on negative controls (if any) included on-chip, excluding clonotypes with deletion and/or frameshift. Filtering statistics and clonotype statistics by cell type were reported as results.

References

Bolotin, D. A. *et al.* MiXCR: software for comprehensive adaptive immunity profiling. *Nat. Methods* **12,** 380–1 (2015).

Stubbington, M. J. T. *et al.* T cell fate and clonality inference from single-cell transcriptomes. *Nat. Methods* **13,** 329–32 (2016).

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