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

Ankita Das*, Sarah Taylor, Alain Mir, Ishminder Mann, Nao Yasuyama, Magnolia Bostick, Jude Dunne, Andrew Farmer

Takara Bio USA, Inc., Mountain View, CA 94043, USA *Corresponding Author: ankita_das@takarabio.com

Abstract

Single-cell T-cell receptor (scTCR) clonotype analysis permits the determination of the specific TCR alpha-beta (α/β) 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 5′-RACE-like approach and SMART® technology, in conjunction with two novel next-generation sequencing (NGS) library preparation kits, using the same primer pairs, to capture full-length variable regions of TCR-α and -β chains.

Method 1, using the SMARTer® Human scTCR a/b Profiling Kit, permits NGS library preparation of FACS-sorted cells in 96-well plates. We present data showing α/β pairing from Jurkat, CCRF, PBMCs, and CD4+ T cells. In addition to the sensitivity of this method, the ability to pool the cDNA from 96 wells into 12 sequencing libraries adds to the ease of use. Consistent with immunology reports, unstimulated CCRF-CEM cells examined with this kit expressed a TCR-β but not a TCR-α chain.

Method 2 is an adaptation of the process above that scales to ~1,200 single cells using the SMARTer™ ICELL8® Single-Cell System, which enables single-cell isolation and nanoliter PCR in a nanowell chip. For proof-of-principle studies, Jurkat cells and CCRF-CEM cells were processed using an ICELL8 chip preprinted with barcoded oligos. Paired TCR α/β Jurkat clonotypes were detected in 77% and 87% cells in mixed and single cell populations, respectively.

The ability of the core biochemistry and PCR components of these kits to be used with either FACS-sorted cells in 96-well plates or >1,000 cells in novel ICELL8 chips (in development) points to the general utility and scalability of this approach in understanding paired scTCR clonotype diversity.

1 scTCR library preparation: 96-well plate workflow

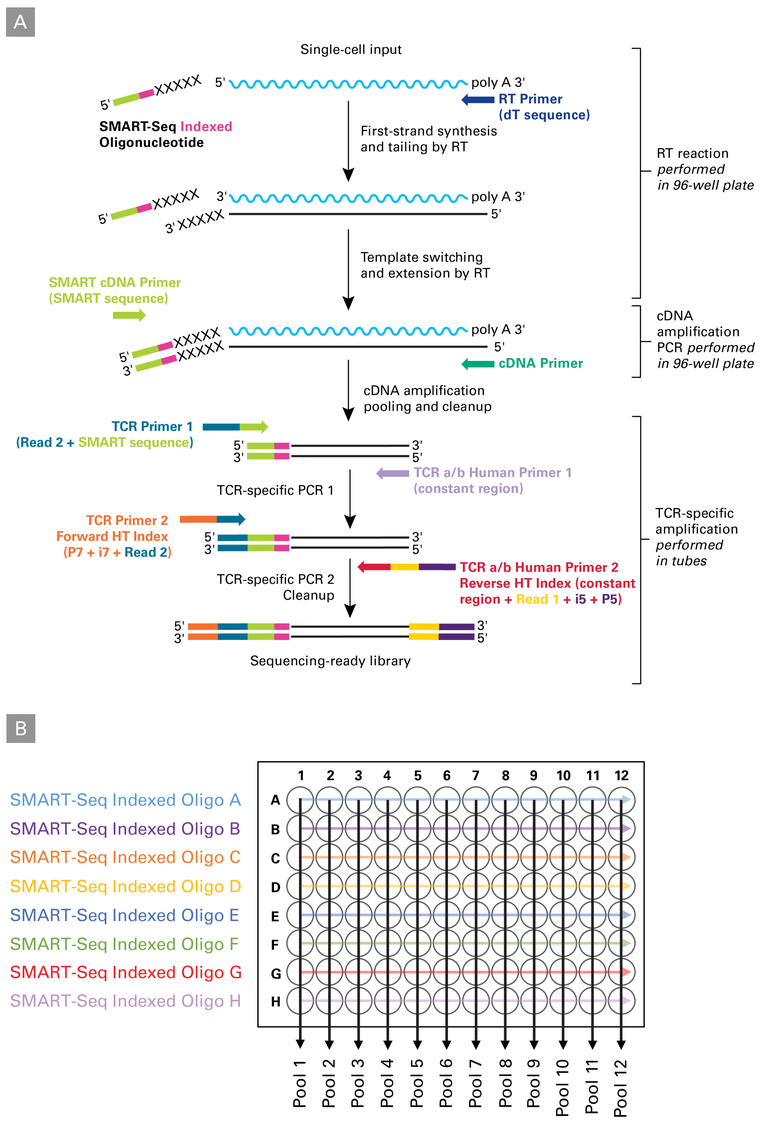

Figure 1. SMARTer Human scTCR a/b Profiling Kit workflow and pooling strategy. Panel A. First-strand cDNA synthesis is dT-primed (RT Primer) and performed by an MMLV-derived reverse transcriptase (RT), which adds nontemplated nucleotides to the 5′ end of each mRNA template. The SMART-Seq® Indexed Oligos anneal to these nontemplated nucleotides and serve as a template for the incorporation of an additional sequence of nucleotides into the first-strand cDNA by the RT (this is the template-switching step). Each of the eight different SMART-Seq Indexed Oligos provided in the kit contains a unique six-base in-line index that serves as a cell barcode to allow downstream cell identification after pooling. The additional sequence added to the cDNA by the RT—referred to as the "SMART sequence"—serves as a primer-annealing site for subsequent rounds of PCR, ensuring that only sequences from full-length cDNAs undergo amplification. After pooling (described in Panel B) and a cleanup step, two rounds of gene-specific PCR are performed in succession to amplify cDNA sequences corresponding to variable regions of TCRa and/or TCRb transcripts. The first gene-specific PCR uses the amplified double-stranded cDNA as a template and includes a forward primer with complementarity to the SMART sequence—which also incorporates the Illumina® Read 2 sequence (TCR Primer 1)—and reverse primers that are complementary to the constant (i.e., nonvariable) region of TCRa and TCRb (TCR a/b Human Primer 1). The second round of PCR takes the product from the first PCR as a template and uses a forward primer that binds to the Read 2 sequence added by the previous PCR step. The reverse primers bind in the constant region, internal to the PCR1 primers (TCR a/b Human Primer 2 Reverse HT Index), allowing amplification of the entire variable region and a portion of the constant region of TCRa and TCRb cDNA. The forward and reverse primers include adapter and index sequences that are compatible with the Illumina sequencing platform and allow for multiplexing of up to 96 samples in a single flow-cell lane. **Panel B.** Samples are pooled by column, such that each pool contains eight cells each with a differently indexed SMART-Seq Indexed Oligo. Different combinations of the Forward and Reverse HT indexes are used during PCR 2 to allow multiplexing of the samples into a single flow-cell lane.

2 Establishing thresholds for confident clonotype calls

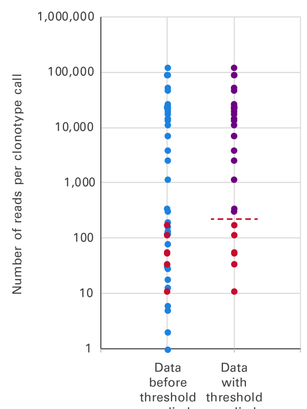

Figure 2. Establishing a threshold for clonotype calling using negative control wells. The number of reads mapping to the top TCRa and the top TCRb clonotype* identified for each cell before the established threshold was applied are shown on the left (blue dots). The clonotype calls that meet the threshold criteria are shown on the right (purple dots). Negative control wells are represented by red dots (negative control well clonotype read number mean = 74 reads, SD = 55 reads, mean + 3SD = 238 reads). The dashed line represents the established 238-read cutoff.

*Note: Throughout this poster, TCRa and TCRb are used to reference clonotypes, while TCR-α and TCR-β are used to refer to the expressed protein chain.

3 Cell identification and TCR α/β calls using the 96-well plate workflow

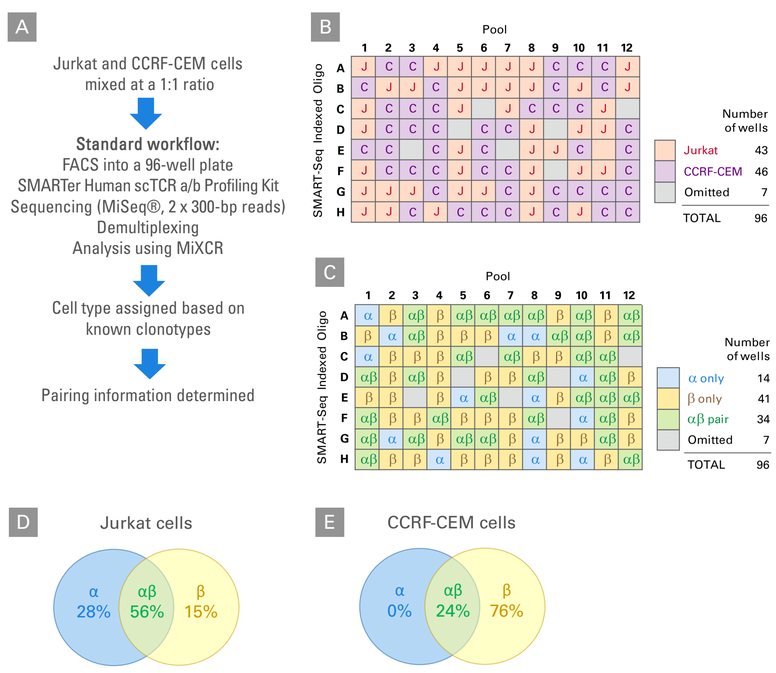

Figure 3. Analysis of a mixed cell population from a 96-well plate. Panel A. Workflow of the experimental setup to analyze a mixed population of Jurkat and CCRF-CEM cells for expressed clonotypes and α/β pairings. **Panel B.** Cell-type calling was based on the identified clonotypes for each well. The seven omitted cells did not have clonotype calls for either TCRa or TCRb with read numbers that were above the threshold. **Panel C.** Analysis of pairing information. Paired α/β chains were obtained for 34 cells in the plate. **Panels D and E.** Summary of the TCR-α and TCR-β information obtained from analyzing the 96-well plate. The alpha (α), beta (β), and alpha-beta (α/β) pairs are shown as a percent of cells analyzed. Omitted cells were not included in this analysis.

4 TCR α/β pairing obtained from stimulated PBMCs

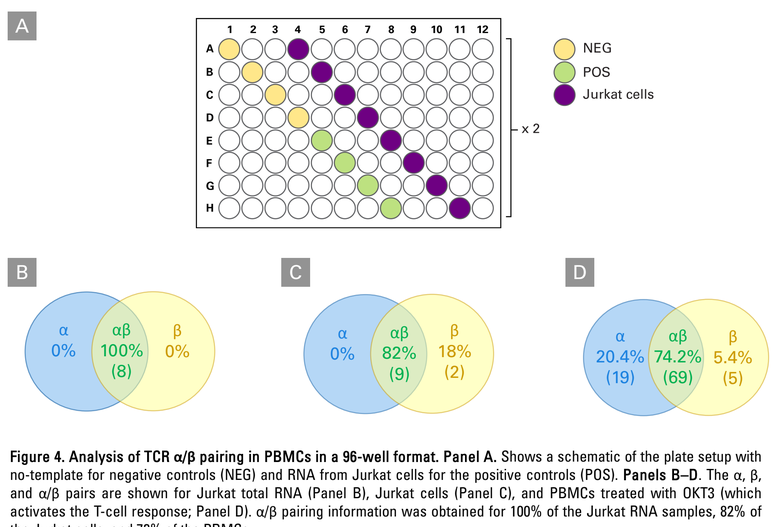

Figure 4. Analysis of TCR α/β pairing in PBMCs in a 96-well format. Panel A. Shows a schematic of the plate setup with no-template for negative controls (NEG) and RNA from Jurkat cells for the positive controls (POS). **Panels B–D.** The α, β, and α/β pairs are shown for Jurkat total RNA (Panel B), Jurkat cells (Panel C), and PBMCs treated with OKT3 (which activates the T-cell response; Panel D). α/β pairing information was obtained for 100% of the Jurkat RNA samples, 82% of the Jurkat cells, and 72% of the PBMCs.

5 TCR α/β pairing obtained from CD4+ T cells

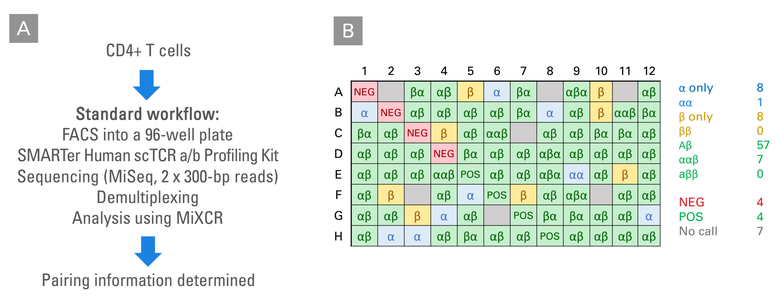

Figure 5. TCR α/β clonotype and pairing in CD4+ T cells. Panel A. Workflow of the experimental setup to obtain α/β pairing from CD4+ T cells sorted by FACS. Negative controls were performed as per the user manual recommendations, and the threshold for clonotype calling was determined based on the negative controls. **Panel B.** Summary of clonotype and pairing information: four wells were negative controls (NEG), and four wells were positive controls (POS). Cell information was obtained for 88 wells. At least one TCR chain sequence was identified in 81/88 wells (92%). α and β chains were identified in 64/88 wells (73%), and 7 of these wells contained two functional alpha sequences (α/α/β). A single α- and a single β-sequence pairing (α/β) was identified in 57/88 wells (65%).

6 Single-cell TCR α/β profiling with the SMARTer ICELL8 automation platform

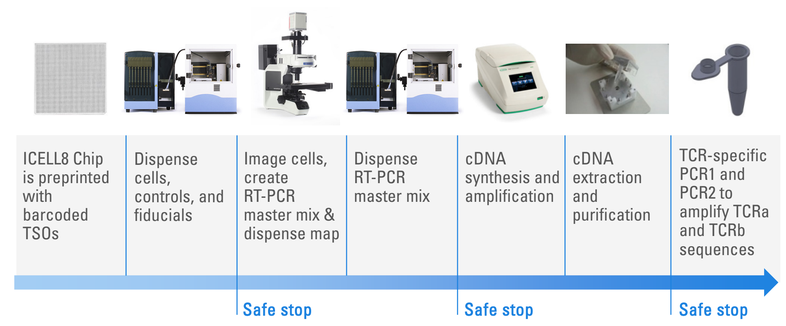

Figure 6. SMARTer ICELL8 workflow overview indicating safe process stopping points. 192 unique barcode-bearing template-switching oligos (TSOs) were printed onto an ICELL8 Chip in 24 replicates. Cultured cells (20 cells/µl) were dispensed into the wells of the ICELL8 Chip at an average of 1 cell/well. The CellSelect® software was used to identify and select single cells and control wells with unique barcodes. Cell lysis was followed by cDNA synthesis to barcode each cell's captured mRNA. cDNA was PCR-amplified via one-step RT-PCR. For each chip, amplified cDNA corresponding to 192 cells and controls was extracted to a single collection tube. The entire workflow time is two days with 2–3 hours of hands-on time.

7 Library preparation workflow for SMARTer ICELL8 platform

1. On chip: Cell dispense using ICELL8 Multisample Nanodispenser (1 cell/50 nl)
2. On chip: RT-PCR. *24 PCR cycles*
3. In tube: Cleanup using Macherey-Nagel Columns followed by a 0.6X SPRI bead cleanup
4. In tube: TCR-specific PCR1. *16 PCR cycles*
5. In tube: TCR-specific PCR2. *14 PCR cycles*
6. In tube: Double-sided SPRI bead cleanup
7. Sequencing-ready library

Figure 7. TCR-seq on-Chip workflow. cDNAs were synthesized via oligo-dT priming. Well-specific, barcoded template-switching oligos printed on the chip were used to define each single cell's transcriptome using single-primer RT-PCR. The TCRa and TCRb sequences of the TCR variable regions were selectively amplified from the pooled cDNA using primers targeting the constant region (Step 4, Figure 1A) of 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).

8 TCR α/β calls in different cell lines using the ICELL8 workflow

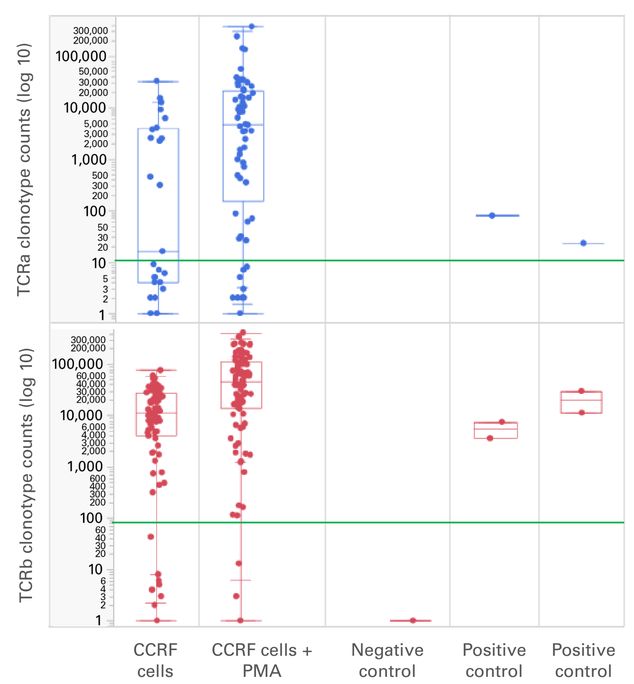

Figure 8. Threshold setting using clonotype count/barcode. The MiXCR output was filtered using Excel to define a read threshold for both TCRa and TCRb clonotypes (the solid line marks 80 read counts) greater than the 1X PBS negative controls. The data was analyzed using the JMP software and plotted into box plots where the lower bar represents 25th percentile and the upper bar the 75th percentile. Each dot represents TCRa and TCRb 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. The data generated from unstimulated and stimulated CCRF-CEM cells and Jurkat RNA (positive control) are shown. Induction of CCRF-CEM cells with phorbol myristate acetate (PMA) increases expression of the TCRa gene [Houghton, P. J. et al. Growth and differentiation of a human T-cell leukemia cell line, CCRF-CEM, grafted in mice. Cancer Res. 49, 7124–31 (1989)]. CCRF-CEM data was generated using cells treated with and without PMA. Treatment of CCRF-CEM cells with PMA increased the call rate by four-fold (from 14% to 52%) based on TCRa and TCRb clonotype data.

Conclusions

- The new SMARTer Human scTCR a/b Profiling Kit allows for generation of Illumina-ready libraries from FACS or manually sorted single cells and the determination of TCR α/β pairings.
- The workflow is optimized for the pooling of 96 cells into 12 libraries which can be further multiplexed for running in a single flow-cell lane.
- The SMARTer ICELL8 platform enables automated single-cell library prep using its ICELL8 MultiSample Nanodispenser for dispensing cells, and the CellSelect software for imaging and selection of viable cells for generating Illumina-ready libraries.
- When combined, the SMARTer ICELL8 platform and the SMARTer Human scTCR a/b Profiling Kit can be used to generate Illumina-sequencing libraries from thousands of single T cells for the determination of TCR α/β pairing information.

Takara Bio USA, Inc.
United States/Canada: +1.800.662.2566 • Asia Pacific: +1.650.919.7300 • Europe: +33.(0)1.3904.6880 • Japan: +81.(0)77.565.6999

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