Takara Bio USA

SMARTer® Human scTCR a/b Profiling Kit User Manual

Cat. Nos. 634431, 634432
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I. Introduction

Single-Cell T-Cell Receptor Profiling Using SMART® Technology

The **SMARTer Human scTCR a/b Profiling Kit** (Cat. No. 634431 and 634432) enables users to analyze T-cell receptor (TCR) diversity from single T cells that have been sorted into a 96-well plate. As the name suggests, the workflow allows generation of libraries that provide information on both alpha- and beta-chain diversity. Included in the kit are primers that incorporate Illumina®-specific adaptor sequences during cDNA amplification. The protocol generates indexed libraries that are ready for sequencing on Illumina platforms.

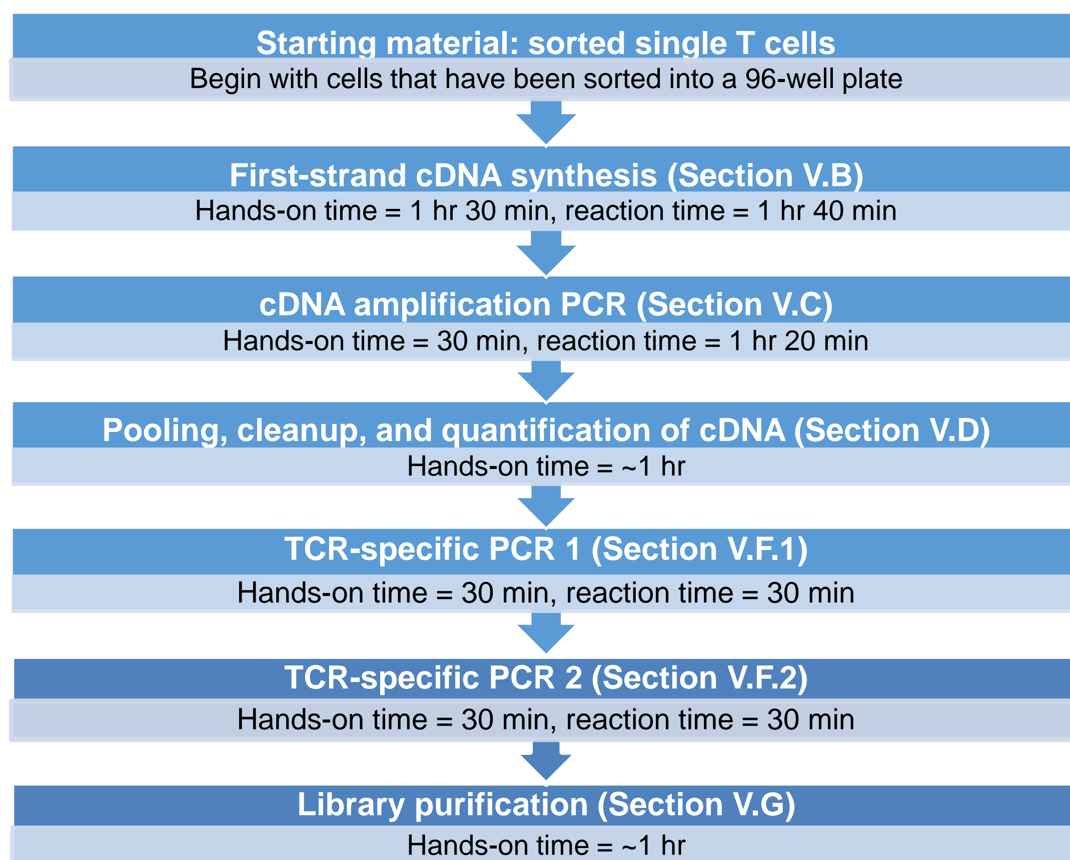

Figure 1. Protocol overview and timeline.

The SMARTer Human scTCR a/b Profiling Kit leverages SMART technology (**S**witching **M**echanism **at** 5' End of **R**NA **T**emplate) and employs a 5' RACE-like approach to capture complete V(D)J variable regions of TCR transcripts (Figure 2). First-strand cDNA synthesis is dT-primed (RT Primer) and performed by the MMLV-derived SMARTScribe™ Reverse Transcriptase (RT), which adds nontemplated nucleotides upon reaching 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). The eight different SMART-Seq Indexed Oligos provided in the kit each contain 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.

Following reverse transcription, amplification of the first-strand cDNA is performed to generate full-length double-stranded cDNA. After pooling and a cleanup step, two rounds of gene-specific PCR are performed in succession to amplify cDNA sequences corresponding to variable regions of TCR-α and/or TCR-β 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 TCR-α and TCR-β (TCR a/b Human Primer 1). By priming from the SMART sequence and the constant region, the first PCR specifically amplifies the entire variable region and a considerable portion of the constant region of TCR-α and TCR-β cDNA.
- The second gene-specific 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 TCR-α and TCR-β 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.

Following post-PCR purification, size selection, and quality analysis, the library is ready for sequencing.

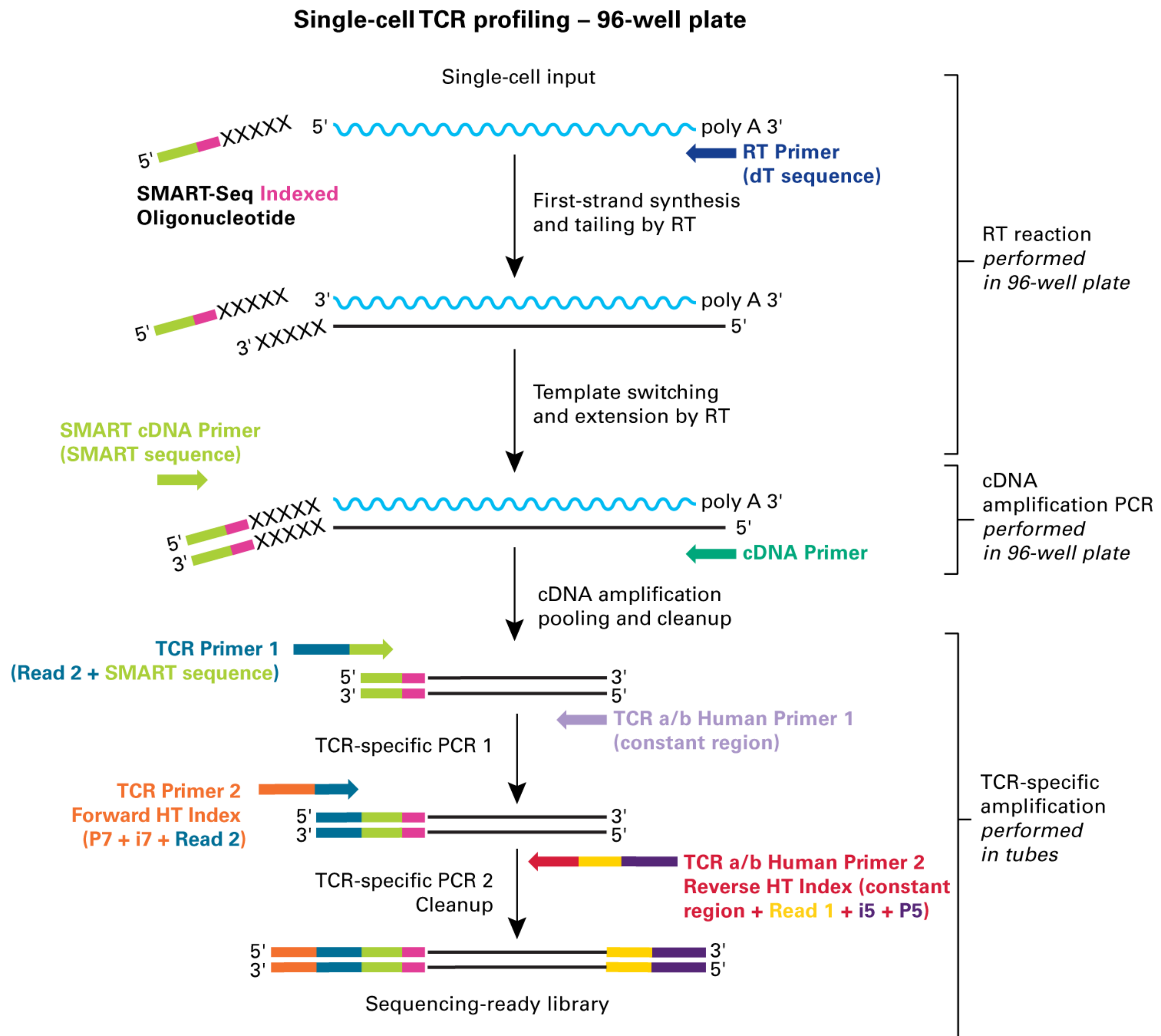

Figure 2. Schematic of technology and workflow for SMARTer Human scTCR a/b Profiling Kit.

II. List of Components

The SMARTer Human scTCR a/b Profiling Kit consists of the SMARTer Human scTCR a/b Profiling Kit Components (not sold separately) and the TCR a/b Human Indexing Primer Set HT for Illumina (not sold separately). **These components have been specifically designed to work together and are optimized for this particular protocol. Please do not make any substitutions.** The substitution of reagents in the kit and/or a modification of the protocol may lead to unexpected results. The reaction number for each kit specifies the number of cells that can be used to generate TCR-α/-β libraries with the pooling strategy and indexing primers supplied.

SMARTer Human scTCR a/b Profiling Kit	Cap color	634431 (96 cells + 2 control pools)	634432 (480 cells + 10 control pools)
SMARTer Human scTCR a/b Profiling Kit Components (Not sold separately. Store at –70°C).			
Control Jurkat Total RNA (1 µg/µl)	Yellow	5 µl	5 µl
SMART-Seq Indexed Oligo A (48 µM)	Pink	10 µl	50 µl
SMART-Seq Indexed Oligo B (48 µM)	Pink	10 µl	50 µl
SMART-Seq Indexed Oligo C (48 µM)	Pink	10 µl	50 µl
SMART-Seq Indexed Oligo D (48 µM)	Pink	10 µl	50 µl
SMART-Seq Indexed Oligo E (48 µM)	Pink	10 µl	50 µl
SMART-Seq Indexed Oligo F (48 µM)	Pink	10 µl	50 µl
SMART-Seq Indexed Oligo G (48 µM)	Pink	10 µl	50 µl
SMART-Seq Indexed Oligo H (48 µM)	Pink	10 µl	50 µl
SMARTer Human scTCR a/b Profiling Kit Components (Not sold separately. Store at –20°C).			
RT Primer (12 µM)	–	112 µl	560 µl
5X Ultra® Low First-Strand Buffer	Red	250 µl	1.25 ml
SMARTScribe Reverse Transcriptase (100 U/µl)	Purple	112 µl	560 µl
Nuclease-Free Water	–	2 ml	10 ml
RNase Inhibitor (40 U/µl)	White	250 µl	1.25 ml
10X Lysis Buffer	Neutral/–*	500 µl	2.5 ml
Elution Buffer (10 mM Tris-Cl, pH 8.5)	–	500 µl	2.5 ml
cDNA Primer (12 µM)	–	28 µl	140 µl
SMART cDNA Primer (12 µM)	Green	28 µl	140 µl
cDNA Amplification Polymerase	–	56 µl	280 µl
cDNA Amplification Buffer (2X)	–	1.4 ml	7 ml
TCRa Human Primer 1 (12 µM)	Orange	14 µl	70 µl
TCRb Human Primer 1 (12 µM)	Orange	14 µl	70 µl
TCR Primer 1 (12 µM)	Green	14 µl	70 µl
TCR Amplification Polymerase (1.25 U/µl)	Green	30 µl	150 µl
TCR Amplification Buffer (5X)	–	300 µl	1.5 ml
TCR Amplification dNTP Mixture (2.5 mM each)	White	120 µl	600 µl

*Depending on the product size, 10X Lysis Buffer is packaged in a 1.5-ml tube with a neutral cap (Cat. No. 634431) or in an 8-ml bottle (Cat. No. 634432)

SMARTer Human scTCR a/b Profiling Kit, continued	Cap color	634431 (12 pooled rxns from 96 cells)*	64432 (60 pooled rxns from 480 cells)*
TCR a/b Human Indexing Primer Set HT for Illumina (Not sold separately. Store at –20°C).			
TCRa Human Primer 2 Reverse HT Index 1 (aR1; 12.5 µM)	Red	12 µl	12 µl
TCRa Human Primer 2 Reverse HT Index 2 (aR2; 12.5 µM)	Red	-	12 µl
TCRa Human Primer 2 Reverse HT Index 3 (aR3; 12.5 µM)	Red	-	12 µl
TCRa Human Primer 2 Reverse HT Index 4 (aR4; 12.5 µM)	Red	-	12 µl
TCRa Human Primer 2 Reverse HT Index 5 (aR5; 12.5 µM)	Red	-	12 µl
TCRb Human Primer 2 Reverse HT Index 1 (bR1; 12.5 µM)	Red	12 µl	12 µl
TCRb Human Primer 2 Reverse HT Index 2 (bR2; 12.5 µM)	Red	-	12 µl
TCRb Human Primer 2 Reverse HT Index 3 (bR3; 12.5 µM)	Red	-	12 µl
TCRb Human Primer 2 Reverse HT Index 4 (bR4; 12.5 µM)	Red	-	12 µl
TCRb Human Primer 2 Reverse HT Index 5 (bR5; 12.5 µM)	Red	-	12 µl
TCR Primer 2 Forward HT Index 1 (F1; 12.5 µM)	Blue	12 µl	12 µl
TCR Primer 2 Forward HT Index 2 (F2; 12.5 µM)	Blue	12 µl	12 µl
TCR Primer 2 Forward HT Index 3 (F3; 12.5 µM)	Blue	12 µl	12 µl
TCR Primer 2 Forward HT Index 4 (F4; 12.5 µM)	Blue	12 µl	12 µl
TCR Primer 2 Forward HT Index 5 (F5; 12.5 µM)	Blue	12 µl	12 µl
TCR Primer 2 Forward HT Index 6 (F6; 12.5 µM)	Blue	12 µl	12 µl
TCR Primer 2 Forward HT Index 7 (F7; 12.5 µM)	Blue	12 µl	12 µl
TCR Primer 2 Forward HT Index 8 (F8; 12.5 µM)	Blue	12 µl	12 µl
TCR Primer 2 Forward HT Index 9 (F9; 12.5 µM)	Blue	12 µl	12 µl
TCR Primer 2 Forward HT Index 10 (F10; 12.5 µM)	Blue	12 µl	12 µl
TCR Primer 2 Forward HT Index 11 (F11; 12.5 µM)	Blue	12 µl	12 µl
TCR Primer 2 Forward HT Index 12 (F12; 12.5 µM)	Blue	12 µl	12 µl

*By this point in the protocol, the 96 cells have been condensed down into 12 pools.

TCR Primer 2 Forward HT Indexes 1–12 (F1–F12) correspond to Illumina TruSeq® HT indexes D701–D712. TCRa/TCRb Human Primer 2 Reverse HT Indexes 1–5 (aR1/bR1–aR5/bR5) correspond to Illumina TruSeq HT indexes D501–D505. For further details, see Appendix A.

Storage Conditions

- Store Control Jurkat Total RNA and SMART-Seq Indexed Oligos at –70°C.
- Store 10X Lysis Buffer at –20°C. Once thawed, the buffer can be stored at 4°C.
- Store Nuclease-Free Water at –20°C. Once thawed, the buffer can be stored at 4°C.
- Store Elution Buffer at –20°C. Once thawed, the buffer can be stored at room temperature.
- Store all other reagents at –20°C.

III. Additional Materials Required

The following reagents and materials are required but not supplied. They have been validated to work with this protocol. Please do not make any substitutions because you may not obtain the expected results:

- Single-channel pipettes: 10 µl, 20 µl, and 200 µl
- Eight-channel pipettes (recommended): 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, and 200 µl
- Microcentrifuge for 1.5-ml tubes
- Microcentrifuge for 0.2-ml tubes or strips
- Centrifuge for 96-well plates

For Cell Sorting

- 96-well polycarbonate PCR plates (USA Scientific Plastics, Cat No. 2796-3330)
- BD FACS Pre-Sort Buffer (BD, Cat No. 563503)
- Aluminum cooler block (prechilled/stored at –20C)
- Adhesive PCR Plate Foils (Thermo Fisher, Cat No. AB-0626)
- Dry ice in a suitable container for flash freezing cells

For PCR Amplification & Validation:

- One dedicated thermal cycler used only for first-strand cDNA synthesis (Section V.B)
- One dedicated thermal cycler used only for amplification by PCR (Section V.C)
- For quantification and validation using the Agilent 2100 Bioanalyzer (Sections V.E & V.H): High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) and the DNA 1000 Kit (Agilent, Cat. No. 5067-1504)
- For library quantification by qPCR: Library Quantification Kit (Takara Bio, Cat No. 638324, Section V.H)
- Nuclease-free thin-wall PCR tubes or strips (0.2-ml PCR 8-tube strip; USA Scientific, Item No. 1402-4700)
- Nuclease-free low-adhesion 1.5-ml tubes (USA Scientific, Item No. 1415-2600) or LoBind tubes (Eppendorf, Cat. No. 022431021)

For SPRI (Solid Phase Reversible Immobilization) Bead Purifications (Section X):

- Agencourt AMPure XP PCR purification kit—used to purify amplified cDNA and libraries (5-ml size: Beckman Coulter, Item No. A63880; 60-ml size: Beckman Coulter, Item No. A63881)

  NOTE: SPRI beads need to come to room temperature before the container is opened. Therefore, we recommend aliquoting the beads into 1.5-ml tubes upon receipt and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 minutes). This will also decrease the chances of bead contamination. Mix well to disperse the beads before adding them to your reactions. The beads are viscous, so pipette slowly.

- 100% ethanol (molecular biology-grade)
- Magnetic separation device for small volumes—used to purify amplified libraries
  - For 1.5-ml tubes: DynaMag-2 Magnet (Thermo Fisher, Cat. No.12321D)
  - For 8-tube strips: Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011)
  - For 96-well plates: Magnetic Stand-96 (Thermo Fisher, Cat. No. AM10027)

- Optional, depending on the choice of magnetic separation device (96-well format):
  - 96-well V-bottom Plate (500 µl; VWR, Cat. No. 47743-996)
  - MicroAmp Clean Adhesive Seal (Thermo Fisher, Cat. No. 4306311)
  - Low-speed benchtop centrifuge for a 96-well plate

IV. General Considerations

A. Requirements for Preventing Contamination

Before you set up the experiment, **make sure you have three physically separated workstations**:

- **A PCR clean workstation** for all pre-PCR experiments that require clean-room conditions, e.g., first-strand cDNA synthesis (Section V.B).

  GUIDELINES FOR PCR CLEAN WORKSTATION OPERATION:

  - The PCR clean workstation must reside in a clean room with positive air flow, as contamination may occur very easily. Once contamination occurs it can be difficult to remove.
  - Strictly obey clean room operation rules.
  - Only move materials/supplies from the PCR clean workstation to the general lab, NOT the other way around. Do not share any equipment/reagents between the PCR Clean Workstation and the general lab workstations.
  - Use a separate PCR thermal cycler (dedicated to first-strand cDNA synthesis) inside the PCR clean workstation for first-strand cDNA synthesis.
  - Wear gloves and sleeve covers throughout the procedure to protect your RNA samples from degradation by contaminants and nucleases. Be sure to change gloves and sleeve covers between each section of the protocol.
- **A second workstation in the general laboratory** for performing PCR (Sections V.C and V.F).
- **A third workstation in the general laboratory** for purification of the cDNA/library (Sections V.D and V.G) and measure the cDNA/library concentrations (Section V.E and V.H).

B. General Requirements

- **The success of your experiment depends on the quality of input cells. Prior to cDNA synthesis, ensure that your sorted cells are stored in the Reaction Buffer (which contains RNase inhibitor to prevent RNA degradation; Section V.A.1) at –80°C and are free of contaminants.**
- Avoid using heparin for blood sample collection, as it can inhibit downstream enzymatic steps such as cDNA synthesis and PCR.
- The assay is very sensitive to variations in pipette volume, etc. Please make sure all your pipettes are calibrated for reliable delivery, and make sure nothing is attached to the outside of the tips.
- All lab supplies related to cDNA synthesis need to be stored in a DNA-free, closed cabinet. Reagents for cDNA synthesis need to be stored in a freezer/refrigerator that has not previously been used to store PCR amplicons.
- Add enzymes to reaction mixtures last, and thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- Do not increase (or decrease) the amount or concentration of any reaction component. The amounts and concentrations have been carefully optimized for the included reagents and protocol.

- The in-plate controls recommended in the workflow are required during the analysis steps to establish a read number threshold for clonotype calling. **Do not omit them**.
- If you are using this protocol for the first time, we strongly recommend that you perform positive control reactions alongside the plate to verify that kit components are working properly.

C. Sample Requirements

- **Cells**
  This protocol has been used successfully to generate TCR sequencing libraries starting from single cells sorted into 96-well plates, either manually using a limited dilution approach or by fluorescence activated cell sorting (FACS).

IMPORTANT:

- Cells should be washed and then resuspended in PBS that is free of Mg^{2+} and Ca^{2+} ions before sorting or limited dilution to remove the culture media. The presence of media can interfere with the first-strand synthesis.
- To maintain cells in a single-cell suspension prior to FACS, we recommend that the cells are resuspended in BD FACS Pre-Sort Buffer (BD, Cat No. 563503).

V. Protocols

NOTE:

- Please read the entire protocol before starting. This protocol has been optimized for generation of sequencing-ready libraries for TCR repertoire profiling from single cells. Due to the sensitivity of the protocol, the input material needs to be collected and purified under clean-room conditions to avoid contamination. The whole cDNA synthesis process should also be carried out in a PCR clean workstation under clean-room conditions.
- Due to the low volumes and potential pipetting error, we recommend making an additional 15% of the master mixes for each 96-well plate to ensure there is sufficient volume for all wells.

A. Protocol: Cell Sorting

For this protocol, you will need the following components:
10X Lysis Buffer (tube with a neutral cap or bottle, depending on product size), RNase Inhibitor (white cap), Nuclease-Free Water

1. Prepare a stock solution of 10X Reaction Buffer by mixing the 10X Lysis Buffer with the RNase Inhibitor as indicated below (scale up as needed for multiple plates, 0.5 µl of 10X Reaction Buffer per well is required for step 2). For one 96-well plate 48 µl of 10X Reaction Buffer is required:

57 µl	10X Lysis Buffer	(tube with a neutral cap or bottle, depending on product size)
3 µl	RNase Inhibitor	(white cap)
60 µl	Total volume	

   Mix briefly, then spin down.

   NOTE: Lysis Buffer contains a detergent. It is critical to avoid creating bubbles when mixing.

2. Prepare a working solution of Reaction Buffer by mixing the 10X Reaction Buffer prepared in Step 1 with Nuclease-Free Water as indicated below:

   NOTE: This formulation is the volume needed per well. To have enough for all of the reactions, we recommend making an additional 15% of the total reaction mix volume.

   EXAMPLE: For a single 96-well plate, the total volume of working buffer needed is 552 µl. However, we recommend making an additional 82.8 µl (15% of the total volume required) for a total of 634.8 µl of Reaction Buffer.

Reagent	Volume per well
10X Reaction Buffer	0.5 µl
Nuclease-Free Water	5.25 µl
Total volume	5.75 µl

3. Dispense 5.75 µl of Reaction Buffer into each well of the 96-well plate that the cells will be sorted into. Briefly spin to ensure the Reaction Buffer is collected at the bottom of the well. Seal **firmly** with an adhesive film sealant.
   a. If using the plate immediately, place on prechilled aluminum cooler block to chill the Reaction Buffer. Go to Step 5.
   b. If preparing plates for future use, store plates at –80°C.

When ready to sort:

4. If using a plate that has been stored at –80°C:
   a. Take out the frozen 96-well PCR plate containing Reaction Buffer from –80°C and allow the Reaction Buffer to thaw. Perform a brief spin to collect the buffer at the bottom of the well.
   b. Place on prechilled aluminum cooler block until it is to be used for sorting.

   NOTES:
   - Ensure that the Reaction Buffer has completely thawed in each well prior to sorting.
   - If the cooler block was stored at –20°C for 25 minutes or more, it will maintain a temperature below 4°C for 45 minutes.
5. Remove the adhesive film and perform the cell sort into the prepared plate.
6. Place the plate with sorted cells in the prechilled cooler block and cover the plate with an adhesive sealing foil sheet (Adhesive PCR Plate Foils, Thermo Fisher, Cat No. AB-0626).

   IMPORTANT: Ensure the plate is sealed firmly to minimize any evaporation.
7. Immediately after sorting the cells and sealing the plate, spin the plate briefly to ensure the cells are collected at the bottom of each well in the Reaction Buffer.
8. Proceed to Step 1 in Section V.B.1 (Protocol: First-Strand cDNA Synthesis) or flash freeze the sorted cells by placing the plate on dry ice and then store the plates at –80°C until ready to use.

B. Protocol: First-Strand cDNA Synthesis (Perform in PCR clean workstation)

First-strand cDNA synthesis is primed by the RT Primer (which contains a poly dT sequence) and uses the SMART-Seq Indexed Oligos for template switching at the 5’ end of the transcript. Eight SMART-Seq Indexed Oligos (A–H) are supplied with the kit, each of which contains a unique six-base in-line index that allows cell identification after sequencing of the pooled cells.

For this protocol, you will need the following components:
10X Lysis Buffer (tube with a neutral cap or bottle, depending on product size), RNase Inhibitor (white cap), Nuclease-Free Water, Control Jurkat Total RNA, RT Primer, 5X Ultra Low First-Strand Buffer (red cap), SMART-Seq Indexed Oligos (A–H) (pink caps), SMARTScribe Reverse Transcriptase (purple cap)

NOTE: To process the plate and control pools, you will need two thermal cyclers.

Setting up the positive and negative control wells

The inclusion of the positive and negative control wells both alongside and within the plate are essential for library validation and downstream analysis, respectively. **Do not omit the controls from your experimental setup**. The kit includes sufficient reagents to generate libraries from 12 pools from a 96-well plate plus the two control pools (96 rxns, Cat. No. 634431) or libraries from 60 pools from five 96-well plates plus ten control pools (480 rxns, Cat. No. 634432).

The positive RNA control pool run alongside the plate allows confirmation of successful library generation. The negative RNA control pool run alongside the plate allows the background to be established. As these controls are for library validation, sequencing is not required.

The controls included within the plate are required for downstream analysis. The number of reads identified in the negative control wells can be used to set a threshold for the background allowing clonotype calls to be made with confidence. The positive control wells can be used to assess any potential cross-contamination between different wells; only clonotypes identified above the threshold, established by the negative control wells, in these positive control wells should be the Jurkat clonotype. For example, data on establishing and using thresholds set by the negative controls wells, see the technical note for this kit on our website. For an overview of the control set up see Figure 3.

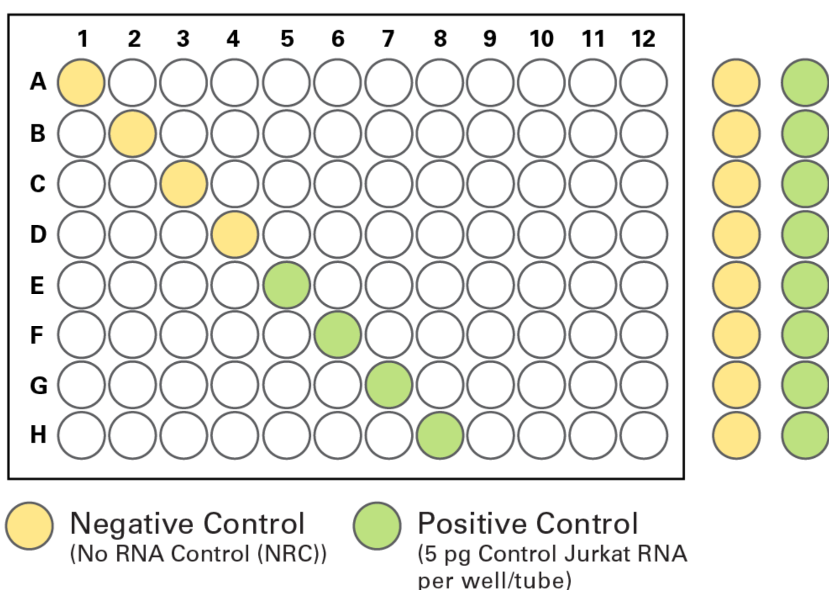

Figure 3. Setup of positive and negative controls.

If you are processing multiple plates at one time, ensure that the in-plate controls are included on each plate. One set of controls alongside is sufficient for each set of plates processed at a time.

NOTE: We recommend using 5 pg of Control Total Jurkat RNA in the positive control wells; we have found this amount to be comparable to cells we have tested. However, if you know that your test cells contain less RNA than this, reduce the amount of Control Total Jurkat RNA included in the in-plate controls to 2.5 pg to ensure that it is not preferentially amplified over your cell samples.

IMPORTANT: Due to the low volumes used in this protocol, when sealing plates with the adhesive film it is essential that the plate is sealed very firmly to avoid any evaporation.

1. Reagent prep

1. Thaw the 5X Ultra Low First-Strand Buffer at room temperature.

   NOTE: The 5X Ultra Low First-Strand Buffer may form precipitates. Thaw this buffer at room temperature and vortex before using to ensure all components are completely in solution.

2. Thaw all the remaining reagents needed for first-strand cDNA synthesis (except the enzyme) on ice. Gently vortex each reagent to mix and spin down briefly. Store on ice.

2. Preparing positive and negative controls

Diluting the Control Jurkat Total RNA

1. Mix 500 µl of Nuclease-Free Water with 10 µl of RNase Inhibitor.
2. Dilute Control Jurkat Total RNA for inclusion in positive controls:
   - Dilute Control Jurkat Total RNA to 20 ng/µl by mixing 98 µl of Nuclease-Free Water plus RNase Inhibitor with 2 µl of 1 µg/µl concentrated Control Jurkat Total RNA in a sterile 0.2-ml microcentrifuge tube.
   - Dilute Control Jurkat Total RNA to 200 pg/µl by mixing 198 µl of Nuclease-Free Water plus RNase Inhibitor with 2 µl of 20 ng/µl Control Jurkat Total RNA in a sterile 0.2-ml microcentrifuge tube.
   - Dilute Control Jurkat Total RNA to 5 pg/µl by mixing 78 µl of Nuclease-Free Water plus RNase Inhibitor with 2 µl of 200 pg/µl Control Jurkat Total RNA in a sterile 0.2-ml microcentrifuge tube.

   Keep all RNA dilutions on ice.

3. Prepare a stock solution of 10X Reaction Buffer by mixing the 10X Lysis Buffer with the RNase Inhibitor as indicated below (0.5 µl of 10X Reaction Buffer is required per RNA control in Step 6):

Volume	Reagent	Notes
19 µl	10X Lysis Buffer	(tube with a neutral cap or bottle, depending on product size)
1 µl	RNase Inhibitor	(white cap)
20 µl	Total volume	

Setting up the control reactions

4. To make the positive and negative controls mix the following for each control well (plus an additional 15% of the total reaction mix volume):

Table I. Control Sample Preparation Guidelines

Reagent	Positive control mix (5 pg Control Jurkat Total RNA)	Negative control mix (No RNA Control (NRC))
Control Jurkat Total RNA (5 pg/µl)	1 µl	–
Nuclease-Free Water	3.75 µl	4.75 µl
10X Reaction Buffer (from Step 3)	0.5 µl	0.5 µl
Total volume per well	5.25 µl	5.25 µl

5. Remove the plate of sorted cells from –80°C and keep chilled on a cooler block. Once thawed, gently vortex and briefly spin the plate to ensure the lysate is collected at the bottom of the well.
6. Using an eight-channel pipette set to 5.25 µl*, transfer the cells to a new PCR plate. Prior to transferring cell lysates ensure that all material has been collected at the bottom of the well. Pipette the well contents up and down several times and then transfer the lysates to a clean PCR plate, with the wells marked for controls left empty. Keep the plate cold by using the aluminum cold block.

   NOTE: *The cells were sorted into 5.75 µl of Reaction Buffer; transferring 5.25 µl allows for a small amount of evaporation.

7. Transfer 5.25 µl (5 pg) of the positive control mix (made in Step 4) to each of the positive control wells and each tube of the positive control PCR-strip tubes. Keep the plate cold by using the aluminum cold block and keep the tubes on ice.
8. Transfer 5.25 µl of the negative control mix (made in Step 4) to each of the negative control wells and each tube of the negative control PCR-strip tubes. Keep the plate cold by using the aluminum cold block, keep the tubes on ice.

3. First-strand cDNA synthesis (all samples)

1. Add 1 µl of the RT Primer (12 µM; blue cap) to each well/tube. Seal the plate firmly with PCR film and cap the tubes.
2. Preheat the thermal cycler to 72°C.
3. Incubate the tubes at 72°C in a preheated, hot-lid thermal cycler for 3 min.

   NOTE: Prepare your eight Master Mixes (each containing a differently indexed SMART-Seq Indexed Oligo, Step 4) while your tubes are incubating. The enzyme will be added just before use. Steps 4–8 below are critical for first-strand cDNA synthesis and should not be delayed after completing Step 3.

4. Prepare eight master mixes, each containing a different SMART-Seq Indexed Oligo (See Figure 4). You should prepare enough Master Mix for all the reactions by combining the following reagents in the order shown at room temperature.

   NOTE: This formulation is the volume needed per well; we recommend making an additional 15% of the total reaction mix volume.

2 µl	5X Ultra Low First-Strand Buffer (red cap)*
0.5 µl	SMART-Seq Indexed Oligo (48 µM; pink cap)**
0.25 µl	RNase Inhibitor (40 U/µl; white cap)
1 µl	SMARTScribe Reverse Transcriptase (100 U/µl; purple cap)***
3.75 µl	Total volume added per reaction

*The 5X Ultra Low First-Strand Buffer should be thawed at room temperature and vortexed gently to remove any cloudiness in the buffer before use.

**A different SMART-Seq Indexed Oligo (A–H) should be used for each row of the plate (see Figure 4).

***Add the reverse transcriptase to the Master Mix just prior to use, making sure to gently mix the reverse transcriptase tube **without** vortexing before adding it.

5. Mix the Master Mixes well by gently pipetting up and down and then spinning the tubes briefly in a microcentrifuge to collect the contents at the bottom of the tube.
6. Immediately after the 3-minute incubation at 72°C (Step 3), place the samples on ice for 2 minutes. Spin down briefly.
7. Reduce the temperature of the thermal cycler to 42°C.
8. Using an eight-channel pipette, add 3.75 µl of the Master Mixes to the corresponding row of the plate and tubes, as shown below. (i.e., all the wells in Row A will contain the master mix containing SMART-Seq Indexed Oligo A, all the wells in Row B will contain the master mix containing SMART-Seq Indexed Oligo B, etc.).

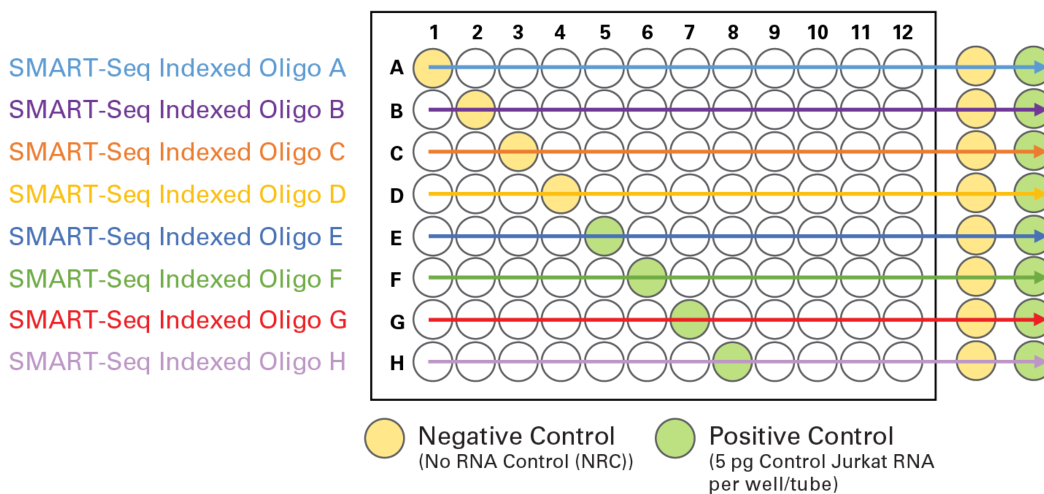

Figure 4. Loading SMART-Seq Indexed Oligo Master Mixes into the plate by row.

9. Seal the plate firmly with a PCR film and cap the tubes. Mix the contents of the plate by gently vortexing, and then spin briefly to collect the contents at the bottom of each well.
10. Place the tubes in a thermal cycler with a heated lid, preheated to 42°C. Run the following program:

42°C	90 min
70°C	10 min
4°C	forever

STOPPING POINT: The tubes can be stored at 4°C overnight.

C. Protocol: cDNA Amplification

Amplification of all first-strand cDNA is performed to generate sufficient material for the gene-specific amplification in the downstream PCR steps. The reaction is primed by the cDNA Primer (which contains complementarity to the sequence added by the RT primer) and the SMART cDNA Primer (which contains sequence complementarity to the SMART-Seq Oligos).

For this protocol, you will need the following components:
cDNA Amplification Buffer, cDNA Primer, SMART cDNA Primer, cDNA Amplification Polymerase, Nuclease-Free Water.

IMPORTANT! There are two polymerases included with this kit. Ensure you are using the correct one!

1. Thaw all the reagents needed for PCR (except enzyme) on ice. Gently vortex each to mix and spin down briefly. Store on ice.
2. Prepare a cDNA Amplification Master Mix for all wells. Combine the following reagents in the order shown. Remove the cDNA Amplification Polymerase from the freezer, gently tap the tube to mix, and add to the Master Mix just before use. Mix well by vortexing gently and spin the tube briefly in a microcentrifuge:

   NOTE: This formulation is the volume needed per well; we recommend making an additional 15% of the total reaction mix volume.

12.5 µl	cDNA Amplification Buffer (2X)
0.25 µl	cDNA Primer (12 µM)
0.25 µl	SMART cDNA Primer (12 µM)
0.5 µl	cDNA Amplification Polymerase
1.5 µl	Nuclease-Free Water
15 µl	Total volume added per well/reaction

3. Add 15 µl of PCR Master Mix to each well/tube containing first-strand cDNA from Section V.B.3, Step 10. Seal the plate firmly with PCR film.
4. Mix well and briefly spin down.
5. Place the plate/tubes in a preheated thermal cycler with a heated lid.
6. Commence thermal cycling using the following program:

   cDNA amplification PCR

	95°C	1 min
16 cycles:		
	98°C	10 sec
	65°C	30 sec
	68°C	3 min
	72°C	10 min
	4°C	forever

   STOPPING POINT: The tubes can be stored at 4°C overnight.

D. Protocol: Pooling and Purification of the cDNA Using Agencourt AMPure XP Beads

The amplified cDNA is pooled by column on the plate, such that each pool contains cDNA from eight cells, each generated with a different SMART-Seq Indexed Oligo (see Figure 5 under Step 1). The PCR-amplified cDNA pool is purified by immobilization on AMPure XP beads to remove primers and primer dimers. The beads are then washed with 80% ethanol, and the cDNA is eluted with Elution Buffer.

NOTES:

- Aliquot AMPure XP beads into 1.5-ml tubes upon receipt in the laboratory.
- Before each use, bring bead aliquots to room temperature for at least 30 minutes and mix well to disperse.
- You will need 1 ml of freshly prepared 80% ethanol per sample.

For this protocol, you will need the following components:

Agencourt AMPure XP PCR Purification beads (provided by user), 80% ethanol (provided by user; made fresh), a magnetic separation device for 1.5-ml tubes (provided by user), and Elution Buffer.

1. Pool the eight PCR products from each column into 1.5-ml tubes, as shown below:

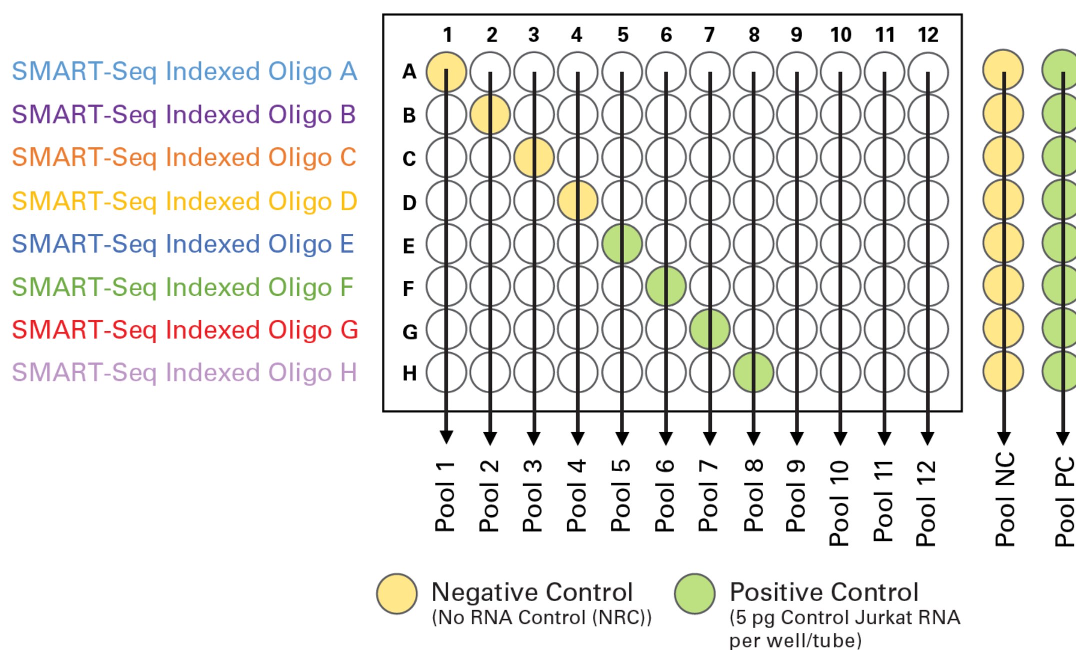

Figure 5. Strategy for combining 96 cells into 12 pools after cDNA amplification. Samples are pooled by column, such that each pool contains eight cells each with a differently indexed SMART-Seq Indexed Oligo. In addition to the 12 pools from each plate, there will also be an additional two pools from the positive (Pool PC) and negative (Pool NC) controls. The 480-rxn kit (Cat. No. 634432) provides a total of 60 pools from five plates (12 pools per plate) plus 10 pools for the positive and negative controls.

2. For the control pools, pool the eight NRC PCR products into a 1.5-ml tube and the eight positive control PCR products into a separate 1.5-ml tube, making two additional pools (Pool NC and Pool PC, respectively).
3. Vortex AMPure XP beads until evenly mixed, then add 160 µl of AMPure XP beads to each sample.

4. Mix thoroughly by pipetting the entire volume up and down at least 10 times.

   NOTE: The beads are viscous; pipette the entire volume, and push it out slowly. **Do not vortex. Vortexing will generate bubbles, making subsequent handling of the beads difficult.**

5. Incubate at room temperature for 8 minutes to let the DNA bind to the beads.
6. Briefly spin the samples to collect the liquid from the side of the tube. Place the samples on the 1.5-ml tube magnetic separation device for 10 min or longer, until the liquid appears completely clear and there are no beads left in the supernatant. The time required for the solution to clear will depend on the strength of the magnet.

   NOTE: Ensure that the solution is completely clear, as any bead carryover will decrease the efficiency of size selection. There is no disadvantage in separating the samples for more than 10 min.

7. While the reaction tubes are sitting on the magnetic separation device, remove the supernatant (the cDNA is bound to the beads).
8. Keep the tubes on the magnetic separation device. Add 500 µl of freshly made 80% ethanol to each sample, without disturbing the beads, to wash away contaminants. Wait for 30 seconds and carefully remove the supernatant. The cDNA will remain bound to the beads during the washing process.
9. Repeat the ethanol wash (Step 8) one more time.
10. Perform a brief spin of the tubes (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 seconds, then remove all the remaining ethanol with a pipette.
11. Let the sample tubes rest open on the magnetic separation device at room temperature until the pellet appears dry and is no longer shiny. You may see a tiny crack in the pellet. As the pellet is large, this may take up to 10 minutes.

    NOTE: Be sure to dry the pellet only until it is just dry. The pellet will look matte with no shine.

    - If you under-dry the pellet, ethanol will remain in the sample wells. The ethanol will reduce your cDNA recovery rate and ultimately your yield. Allow the tubes to sit at room temperature until the pellet is dry.
    - If you over-dry the pellet, there will be cracks in the pellet. It will take longer than 7 min to rehydrate (Step 13) and may reduce library recovery and yield.
    - Visit **takarabio.com/rna-seq-tips** to view examples of moist, dry, and overly dry pellets.

12. Once the bead pellet has dried, remove the tubes from the magnetic separation device and add 17 µl of Elution Buffer to cover the pellet. Remove the samples from the magnetic separation device and mix thoroughly by pipetting up and down to ensure complete bead dispersion.

    NOTE: Be sure that the beads are completely resuspended. The beads can sometimes stick to the insides of the tube.

13. Incubate at room temperature for at least 7 min to rehydrate.

14. Briefly spin the samples to collect the liquid from the side of the tube or sample well. Place the samples back on the magnetic separation device for 5 min or longer, until the solution is completely clear.

    NOTE: There may be a small population of beads that do not pellet against the magnet during incubation. Pipet these unpelleted beads up and down to resuspend them with the supernatant, and then pipet them towards the magnet where the rest of the beads have already pelleted (without disrupting the existing pellet). Continue the incubation until there are no beads left in the supernatant.

15. Transfer clear supernatant containing the purified cDNA from each tube to a nuclease-free, low-adhesion tube. Label each tube with sample information and store at –20°C if not performing the cDNA validation and quantification steps immediately.

E. Protocol: cDNA Validation and Quantification

To determine whether cDNA synthesis, purification, and size selection were successful, cDNA can be analyzed using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). Please refer to the user manual for detailed instructions.

For validation using the Agilent 2100 Bioanalyzer and the High Sensitivity Kit:

1. Aliquot 1 µl of the amplified cDNA for validation using the Agilent 2100 BioAnalyzer and the High Sensitivity DNA Chip from Agilent's High Sensitivity DNA Kit (Cat. No. 5067-4626).
2. Compare the results for your samples with Figure 6 (below) to verify whether each sample is suitable for further processing. Successful cDNA synthesis and purification should yield no product for negative control reactions, and a broad peak spanning ~400 bp to ~8,000 bp, for the positive control pool and pools samples containing cells.
3. To quantify the cDNA yield, set the region table to measure between 700 and 9,000 bp and obtain the yield in pg/µl.

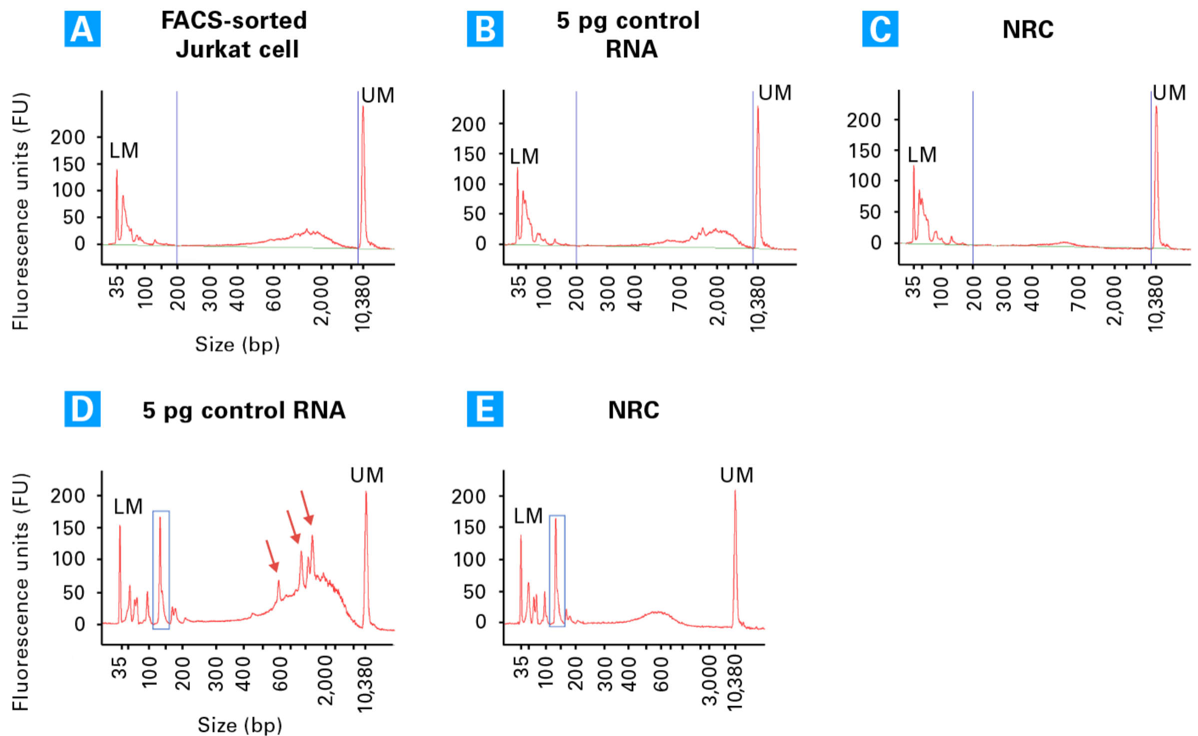

Figure 6. Example electropherogram profiles for cDNA quantification. cDNA profiles were generated using 16 cycles of amplification. Following purification and size selection the cDNA was analyzed using the Agilent 2100 Bioanalyzer and the DNA HS kit. The cDNA produced from a pool of sorted Jurkat cells or the 5 pg Control Jurkat Total RNA pools produces a broad peak spanning 400–9,000 bp. Peaks labeled "LM" and "UM" (Lower Marker and Upper Marker, respectively) correspond to DNA reference markers included in each analysis. **Panel A.** 8 x sorted Jurkat cell pooled sample, the cDNA concentration in the 700- to 9,000-bp range is 143.3 pg/µl. **Panel B.** 8 x 5 pg Control Jurkat Total RNA pooled sample. The positive control in this example shows a concentration of 156.2 pg/µl in the 700- to 9,000-bp range. **Panel C.** No RNA Control (NRC) pooled sample. The NRC shows no cDNA is produced. Note that the NRC may exhibit a small amount of background (indicated by minimal product in the 200- to 1,000-bp range), which is acceptable as long as the libraries from the RNA samples contain a significantly larger amount of material. **Panel D.** Additional example of 8 x 5 pg Control Jurkat Total RNA pooled sample with a high yield. The positive control in this example shows a concentration of 615.4 pg/µl in the 700- to 9,000-bp range. On occasion, an additional peak at ~133 bp (blue box) and multiple high molecular weight peaks within the broad cDNA peak (red arrows) may be observed. These peaks do not affect downstream library preparation. **Panel E.** No RNA Control (NRC) pooled sample with the additional peak at ~133 bp (blue box) and small amount of background in the 200- to 2,000-bp range. Again, these peaks do not affect downstream library preparation.

F. Protocol: TCR a/b Amplification and Sequencing Library Generation by Semi-Nested PCR

IMPORTANT:

- This PCR protocol has been optimized based on an initial input of eight cells per sample pool or 8 x 5 pg of Control Jurkat Total RNA per control pool into the RT reaction described in the previous protocol, and using 500 pg of cDNA template into TCR-specific PCR 1. Do not adjust the input amount or recommended PCR cycle numbers.
- **There are two polymerases included with this kit. Ensure you are using the correct one!**

1. TCR-specific PCR 1

This PCR selectively amplifies TCR sequences from the cDNA generated with the previous protocol. TCR Primer 1 anneals to the SMART sequence (incorporated during first-strand cDNA synthesis) and adds the Illumina Read 2 sequence. TCRa Human Primer 1 and TCRb Human Primer 1 anneal to sequences in the constant regions of TCR-α and TCR-β cDNA, respectively.

For this protocol, you will need the following components:
TCR Amplification Buffer (5X), TCR Amplification dNTP Mixture (2.5 mM each), TCR Primer 1 (green cap), TCRa Human Primer 1 (orange cap), TCRb Human Primer 1 (orange cap), TCR Amplification Polymerase, and Nuclease-Free Water.

1. Based on the quantification from the Bioanalyzer (Section V.E), transfer 500 pg of cDNA for each pooled sample into a clean PCR tube. Bring the volume up to 20 µl with Nuclease-Free Water. Store on ice.
2. Thaw all reagents needed for PCR (except the enzyme) on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
3. To make the TCRa + TCRb Human Primer 1 Mix, add the primers at 2:1 TCRa Human Primer 1: TCRb Human Primer 1 in a clean PCR tube. Mix well by vortexing gently and then spin the tube briefly in a microcentrifuge.

   EXAMPLE: For 14 pools, 14 µl of primer mix would be required so mix 12 µl of TCRa Human Primer 1 (orange cap) with 6 µl of TCRb Human Primer 1 (orange cap). The 18 µl recommended in this example includes additional reaction volume to ensure there is enough primer mix for all the samples.

4. Prepare enough PCR Master Mix for all the reactions, plus an additional 10% of the total reaction mix volume. Combine the following reagents (amounts are for 1 reaction, scale up as required) in the order shown:

10 µl	TCR Amplification Buffer (5X)
4 µl	TCR Amplification dNTP Mixture (2.5 mM each)
1 µl	TCR Primer 1 (12 µM; green cap)
1 µl	TCRa + TCRb Human Primer 1 Mix (from Step 3)
1 µl	TCR Amplification Polymerase
13 µl	Nuclease-Free Water
30 µl	Total volume added per reaction

   NOTE: Remove the TCR Amplification Polymerase from the freezer, gently mix the tube **without** vortexing, and add to the Master Mix just before use. Mix the Master Mix well by vortexing gently and then spin the tube briefly to collect the contents at the bottom of the tube.

5. Add 30 µl of PCR Master Mix to each tube containing 500 pg of cDNA in 20 µl from Step 1. Mix well and briefly spin to collect the contents at the bottom of the tubes.

6. Place the tubes in a preheated thermal cycler with a heated lid and run the following program:

TCR-specific PCR 1

	95°C	1 min	
16 cycles:			
	98°C	10 sec	
	60°C	15 sec	
	68°C	45 sec	
	4°C	forever	

STOPPING POINT: The tubes may be stored at 4°C overnight.

2. TCR-specific PCR 2

This PCR further amplifies sequences corresponding to full-length TCR variable regions and adds Illumina HT sequencing adapters using a semi-nested approach. The TCR Primer 2 Forward HT Index primers anneal to a sequence added by TCR Primer 1 and add Illumina P7-i7 index sequences. The nested TCRa and/or TCRb Human Primer 2 Reverse HT Index primers anneal to sequences in TCR constant regions that are internal to the sequences bound by TCRa Human Primer 1 and TCRb Human Primer 1 and add both the Illumina Read 1 sequence and P5-i5 index sequences.

IMPORTANT:

- Different combinations of Forward HT indexes (F1–F12) and Reverse HT indexes (aR1/bR1 for the 96-reaction kit, aR1-aR5/bR1-bR5 for the 480-reaction kit) must be used for each sample if samples are to be multiplexed on a single flow cell (See Appendix C).
- If using the 480-reaction kit, a different set of TCRa + TCRb Human Primer 2 Reverse HT Indexes is required for each plate. (e.g., Plate 1 uses Human aR1 and Human bR1, Plate 2 uses Human aR2 and Human bR2, etc.).
- The control pools alongside the plate will not be sequenced, and thus the indexes can be reused for these pools. See Appendix A for further details.

For this protocol, you will need the following components:
TCR Amplification Buffer (5X), TCR Amplification dNTP Mixture (2.5 mM each), TCR a/b Human Indexing Primer Set HT for Illumina (blue caps: forward primers; red caps: reverse primers), TCR Amplification Polymerase, and Nuclease-Free Water.

1. Thaw all reagents needed for PCR (except the enzyme which should be kept in the –20°C freezer until just before use) on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
2. To make the TCRa + TCRb Human Primer 2 Reverse HT Index Mix, add the primers at 2:1 TCRa Human Primer 2 Reverse HT Index: TCRb Human Primer 2 Reverse HT Index in a clean PCR tube. Mix well by vortexing gently and then spin the tube briefly in a microcentrifuge.

NOTE: For 14 pools, 14 µl of primer mix would be required; mix 12 µl of TCRa Human Primer 2 Reverse HT Index with 6 µl of TCRb Human Primer 2 Reverse HT Index (cap labels Human aR1 and Human bR1 in the 96-reaction kit. The 480-reaction kit includes Human aR1-aR5 and Human bR1-bR5).

3. Prepare enough PCR Master Mix for all of the reactions, plus 10% of the total reaction mix volume per reaction. Combine the following reagents in the order shown:

10 µl	TCR Amplification Buffer (5X)
4 µl	TCR Amplification dNTP Mixture (2.5 mM each)
1 µl	TCRa + TCRb Human Primer 2 Reverse HT Index Mix (from Step 2)
1 µl	TCR Amplification Polymerase (1.25 U/µl)
32 µl	Nuclease-Free Water
48 µl	Total volume added per reaction

NOTE: Remove the TCR Amplification Polymerase from the freezer, gently mix the tube **without** vortexing, and add to the Master Mix just before use. Mix the Master Mix well by vortexing gently and then spin the tube briefly to collect the contents at the bottom of the tube.

4. For each reaction, add 48 µl of PCR Master Mix to a clean 0.2-ml tube.
5. Add 1 µl of PCR product from PCR 1 (Section V. F.1) to each tube.
6. Add 1 µl of the appropriate TCR Primer 2 Forward HT Index primer (12.5 µM; blue cap; cap label F1-F12) to each sample (each sample requires a different forward index). Mix well and briefly spin to collect the contents at the bottom of the tube(s). Use indexes F1 and F2 again for the positive and negative control pools, respectively. Since these samples do not require sequencing, the same indexes can be repeated.
7. Place the tube(s) in a preheated thermal cycler with a heated lid and run the following program:

TCR-specific PCR 2

	95°C	1 min
14 cycles:		
	98°C	10 sec
	60°C	15 sec
	68°C	45 sec
	4°C	forever

STOPPING POINT: The tubes may be stored at 4°C overnight.

G. Protocol: Purification of Amplified Libraries Using Agencourt AMPure XP Beads

The TCR sequencing library is size-selected and purified using AMPure XP beads. This approach involves two rounds of size selection, which together remove primers, primer dimers, and PCR products containing undersized or oversized inserts. In the first round, fragments larger than ~900 bp are immobilized on beads and removed from the supernatant. In the second round, the supernatant is added to fresh beads, which immobilize fragments within the desired size range of ~400–900 bp. The beads are then washed with 80% ethanol and fragments are eluted with Elution Buffer. This approach preserves library yield and complexity while maximizing the relevance and consistency of downstream sequencing results.

NOTES:

- This is a second purification step and requires different volumes of reagents and incubation times than the protocol described in Section V.D. **DO NOT** interchange the protocols.
- Aliquot AMPure XP beads into 1.5-ml tubes upon receipt in the laboratory.
- Before each use, bring bead aliquots to room temperature for at least 30 minutes and mix well to disperse.
- You will need 400 µl of freshly prepared 80% ethanol per sample.

For this protocol, you will need the following components:
Agencourt AMPure XP PCR Purification beads (provided by the user), 80% ethanol (provided by the user; made fresh), a magnetic separation device for 0.2-ml tubes/tube strips or a 96-well plate (provided by the user), and Elution Buffer.

1. Vortex AMPure XP beads until evenly mixed, then add 22.5 µl of AMPure XP beads to each sample.
2. Mix thoroughly by pipetting the entire volume up and down at least 10 times.
   NOTE: The beads are viscous; pipette the entire volume, and push it out slowly. **Do not vortex. Vortexing will generate bubbles, making subsequent handling of the beads difficult.**
3. Incubate at room temperature for 8 minutes to let the DNA bind to the beads.
4. Briefly spin the samples to collect the liquid from the side of the tube or sample well. Place the samples on the magnetic separation device for ~5 min or longer, until the liquid appears completely clear and there are no beads left in the supernatant. The time required for the solution to clear will depend on the strength of the magnet.

   NOTE: Ensure that the solution is completely clear, as any bead carryover will decrease the efficiency of size selection. There is no disadvantage in separating the samples for longer than 5 min.
5. While the reaction tubes are sitting on the magnetic separation device, use a pipette to transfer the supernatant (which contains your library) to clean PCR tubes.
6. Remove the tubes containing the beads from the magnetic separation device and discard them. Add 10 µl of AMPure XP beads to each tube containing supernatant.
   NOTE: Ensure that the beads are fully resuspended before use. If the beads appear to have settled at the bottom of the tube, **gently** vortex to ensure that they are completely mixed.
7. Mix thoroughly by pipetting the entire volume up and down at least 10 times.
   NOTE: The beads are viscous; pipette the entire volume, and push it out slowly. **Do not vortex. Vortexing will generate bubbles, making subsequent handling of the beads difficult.**
8. Incubate at room temperature for 8 min to let the DNA bind to the beads.
9. Place the tubes on the magnetic separation device for ~10 min or until the solution is completely clear.
10. While the tubes are sitting on the magnetic separation device, remove the supernatant with a pipette and discard it (the library is now bound to the beads.)
11. Keep the tubes on the magnetic separation device. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads, to wash away contaminants. Wait for 30 seconds and use a pipette to carefully remove the supernatant containing contaminants. The library will remain bound to the beads during the washing process.
12. Repeat the ethanol wash (Step 11) once.

13. Seal the tubes and briefly spin down to collect the liquid at the bottom of the well.
14. Place the tubes on the magnetic stand for 30 seconds, then remove all the remaining ethanol by using the eight-channel pipette. Take care to ensure that the bead pellet is not disturbed while removing the ethanol.

NOTE: It is important to make sure all ethanol is removed so the beads elute well and recovery is efficient

15. Let the sample tubes rest open on the magnetic separation device at room temperature for ~2–2.5 minutes until the pellet appears dry and is no longer shiny. You may see a tiny crack in the pellet.

NOTES: Be sure to dry the pellet only until it is just dry. The pellet will look matte with no shine.
- If you under-dry the pellet, ethanol will remain in the sample wells. The ethanol will reduce your library recovery rate and ultimately your yield. Allow the plate to sit at room temperature until the pellet is dry.
- If you over-dry the pellet, there will be cracks in the pellet. It will take longer than 5 min to rehydrate (Step 17) and may reduce library recovery and yield.
- Visit takarabio.com/rna-seq-tips to view examples of moist, dry, and overly dry pellets.

16. Once the bead pellet has dried, remove the tubes from the magnetic separation device and add 17 µl of Elution Buffer to cover the pellet. Remove the samples from the magnetic separation device and mix thoroughly by pipetting up and down to ensure complete bead dispersion.

NOTE: Be sure that the beads are completely resuspended. The beads can sometimes stick to the insides of the tube.

17. Incubate at room temperature for at least 5 min to rehydrate.
18. Briefly spin the samples to collect the liquid from the side of the tube or sample well. Place the samples back on the magnetic separation device for 2 min or longer, until the solution is completely clear.

NOTE: There may be a small population of beads that do not pellet against the magnet during incubation. Pipet these unpelleted beads up and down to resuspend them with the supernatant, and then pipet them towards the magnet where the rest of the beads have already pelleted (without disrupting the existing pellet). Continue the incubation until there are no beads left in the supernatant.

19. Transfer clear supernatant containing the purified TCR library from each tube to a nuclease-free, low-adhesion tube. Label each tube with sample information and store at –20°C.

STOPPING POINT: The samples may be stored at –20°C.

H. Protocol: Library Validation and Quantification

To determine whether library production, purification, and size selection were successful, we recommend analyzing and quantifying the final libraries using the Agilent 2100 Bioanalyzer and the DNA 1000 Kit (Agilent, Cat. No. 5067-1504). Alternatively, the libraries can be quantified by qPCR using the NGS Library Quantification Kit (Takara Bio, Cat No. 638324). Please refer to the corresponding user manuals for detailed instructions.

1. Aliquot 1 µl of the amplified cDNA for validation using the Agilent 2100 BioAnalyzer and the DNA 1000 Kit (Agilent, Cat. No. 5067-1504).

2. Compare the results for your samples with Figure 7 (Panels A & B) to verify whether each sample is suitable for further processing. Successful library production and purification should yield no product for negative control reactions, and a broad peak spanning 550 bp to 1,200 bp, with a maximum between ~700 bp and ~900 bp for positive controls and samples generated from cells containing TCR libraries.
3. To quantify the libraries, set the region table to measure between 550 and 1,200 bp. After manual integration of the region between 550 and 1,200 bp, the pool of Jurkat cells in the example below (Panel A) shows a concentration of 12.8 nM, and the pool of 5 pg Control Jurkat Total RNA (Panel B) shows a concentration of 20.7 nM.
4. Following validation, libraries are ready for sequencing on Illumina platforms. See Appendix B for sequencing guidelines.

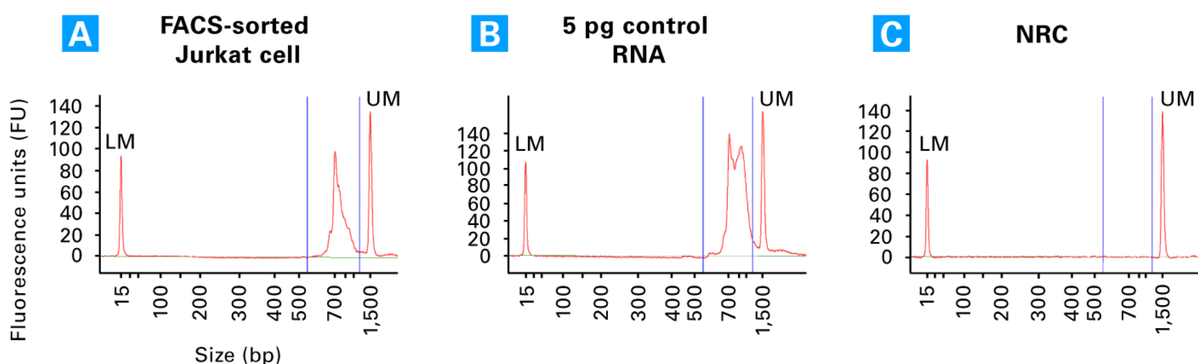

Figure 7. Example electropherogram results for TCR-α and TCR-β libraries. Libraries containing TCR-α and TCR-β sequences were generated using the SMARTer Human scTCR a/b Profiling Kit. All libraries were produced using 16 cycles of amplification for the cDNA PCR (profiles of cDNA for these libraries are shown in Figure 6), 16 amplification cycles for TCR PCR 1 and 14 amplification cycles for TCR PCR 2. Following purification and size selection, libraries were analyzed on an Agilent 2100 Bioanalyzer using the DNA 1000 Kit. Peaks labeled "LM" and "UM" correspond to DNA reference markers included in each analysis. The libraries produced from a pool of sorted Jurkat cells or the 5 pg Control Jurkat Total RNA pool show broad peaks between ~650- to 1,150-bp and maximal peaks in the range of ~700- to 900-bp (the NRC is flat within this range). Peaks labeled "LM" and "UM" correspond to DNA reference markers included in each analysis. **Panel A.** 8 x sorted Jurkat cell pooled sample, the library concentration in the 550- to 1,200-bp range is 12.8 nM. **Panel B.** 8 x 5 pg Control Jurkat Total RNA pooled sample. The positive control in this example shows a concentration of 20.7 nM in the 550- to 1,200-bp range. **Panel C.** NRC pooled sample. The no RNA control shows no library is produced. **Note:** It is possible to see some variability in the profile of the peaks, as shown by Panel A and Panel B. The distinct peaks seen in Panel B represent TCR-α (larger peak) and TCR-β (smaller peak) sequence fragments and can often be discerned. The differences in the Panel A and Panel B profiles correspond to the relative amounts of TCR-α and TCR-β present in the sample. Both profiles indicate successful library preparation.

Appendix A: Illumina HT Indexes

Unique combinations of Illumina indexes are required to discriminate between samples when sequencing a pool of two or more libraries on a single flow cell. Consult the Illumina literature (such as the TruSeq DNA Sample Preparation Guide) for appropriate pooling guidelines.

The TCR Primer 2 Forward HT Index primers contain the Read 2 sequence and i7 indexes. These primers are labeled sequentially ("F1"–"F12"), in correspondence with Illumina indexes D701–D712.

In addition to gene-specific sequences derived from the constant regions of Human TCR-α and TCR-β, the TCRa and TCRb Human Primer 2 Reverse HT Index primers contain the Read 1 sequence and i5 indexes. Depending on the kit size, one (96-reaction kit) or five (480-reaction kit) different TCRa and TCRb Human Primer 2 Reverse HT Index primers containing unique i5 indexes are included for amplification of TCRa and TCRb, respectively. These primers are labeled sequentially ("Human aR1"–"Human aR5" or "Human bR1"–"Human bR5"), in correspondence with Illumina indexes D501–D505.

In the 96-reaction kit (Cat No. 634431), we supply sufficient indexes for the 12 libraries that are generated by the protocol (12 forward and 1 reverse indexes). In the 480-reaction kit (Cat No. 634432), we supply sufficient indexes for the 60 libraries that are generated by the protocol (12 forward and 5 reverse indexes). The pairwise combination of 12 unique i7 indexes with 5 unique i5 indexes allows for multiplexing of up to 60 samples in a single flow cell lane.

Table II. TCR a/b Human Indexing Primer Set HT for Illumina index sequences

TCR Primer 2 Forward HT Index				TCR a/b Human Primer 2 Reverse HT Index			
Primer ID (cap label)	**Illumina ID**	**Index sequence**	**Cell Pool**	**Primer ID (cap label)**	**Illumina ID**	**Index sequence**	**Plate**
F1	D701	ATTACTCG	1	Human aR1/ Human bR1	D501	TATAGCCT	1
F2	D702	TCCGGAGA	2	Human aR2/ Human bR2	D502	ATAGAGGC	2
F3	D703	CGCTCATT	3	Human aR3/ Human bR3	D503	CCTATCCT	3
F4	D704	GAGATTCC	4	Human aR4/ Human bR4	D504	GGCTCTGA	4
F5	D705	ATTCAGAA	5	Human aR5/ Human bR5	D505	AGGCGAAG	5
F6	D706	GAATTCGT	6				
F7	D707	CTGAAGCT	7				
F8	D708	TAATGCGC	8				
F9	D709	CGGCTATG	9				
F10	D710	TCCGCGAA	10				
F11	D711	TCTCGCGC	11				
F12	D712	AGCGATAG	12				

Appendix B: Guidelines for Library Sequencing

Samples should be pooled to a final pool concentration of 4 nM. The control pool samples generated alongside your plate do not require sequencing and therefore should not be included in the final pool. This is because the controls contain the same indexes as two of your samples, making it impossible to distinguish the controls from the experimental samples during sample demultiplexing and analysis. We recommend diluting the pooled libraries to a final concentration of 13.5 pM, including a 5–10% PhiX Control v3 (Illumina, Cat. No. FC-110-3001) spike-in. While not essential, the addition of the PhiX control increases the nucleotide diversity and thus aids in high-quality data generation.

Sequencing should be performed on an Illumina MiSeq® sequencer using the 600-cycle MiSeq Reagent Kit v3 (Illumina, Cat. No. MS-102-3003) with paired-end, 2 x 300 base pair reads.

Appendix C: In-line Indexes

The SMART-seq Indexed Oligos contain in-line indexes that enable cell pooling; the in-line index begins at base 33 of Read 2. These primers are labeled sequentially, "SMART-Seq Indexed Oligo A"–"SMART-Seq Indexed Oligo H."

The pooled cell data can be demultiplexed using the in-line indexes. We have a simple piece of software called SMARTer Human scTCR Demultiplexer that can be used to demultiplex pooled samples. You can sign up to download the software on this page on our website.

The sequences of the in-line indexes are provided below, note that these are not required if you are using our demultiplexer as they are built into the program but are provided here for your convenience.

Table III. SMART-Seq Indexed Oligo in-line index sequences

Oligo name	Oligo ID (cap label)	Index Sequence
SMART-Seq Indexed Oligo A	A	GCCAAT
SMART-Seq Indexed Oligo B	B	ACAGTG
SMART-Seq Indexed Oligo C	C	CGATGT
SMART-Seq Indexed Oligo D	D	TGACCA
SMART-Seq Indexed Oligo E	E	CAGATC
SMART-Seq Indexed Oligo F	F	ACTTGA
SMART-Seq Indexed Oligo G	G	GGCTAC
SMART-Seq Indexed Oligo H	H	ATGTCA

Appendix D: Data Analysis

Data analysis can be performed on a variety of platforms, a detailed but nonexhaustive list of packages can be found at https://omictools.com/rep-seq-category.

While not required for analyses with many software packages, the initial 41 bases of Read 2 (which includes the SMART sequence and the in-line index) may need to be trimmed from raw sequence reads prior to downstream analyses (see Figure 8).

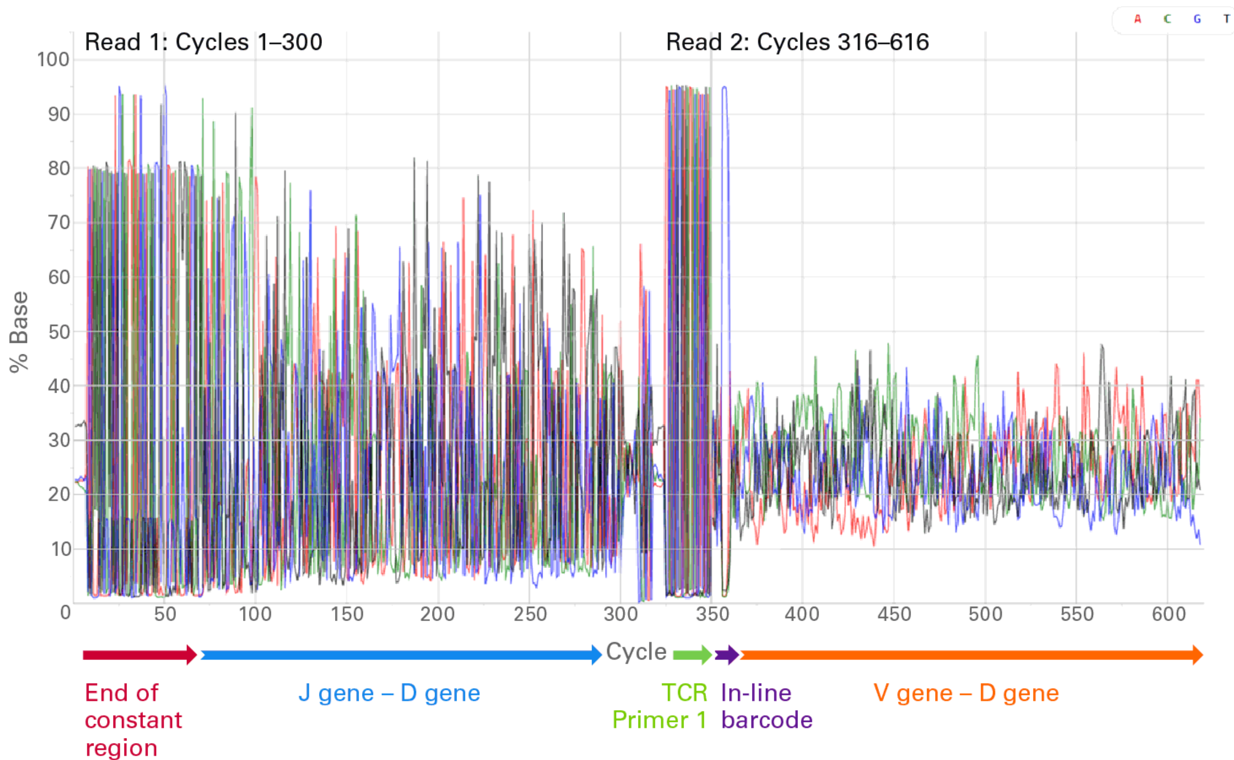

Figure 8. Percentage of bases called per cycle from a typical MiSeq scTCR-α and TCR-β profiling run. The TCR Primer 1 and in-line index sequences at the beginning of Read 2, which may need to be trimmed prior to data analysis. Cycles 301–315 contain the Illumina index reads.

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This document has been reviewed and approved by the Quality Department.