Takara Bio USA

SMART-Seq® Human TCR (with UMIs) User Manual

Cat. Nos. 634779, 634780, 634781
(110722)

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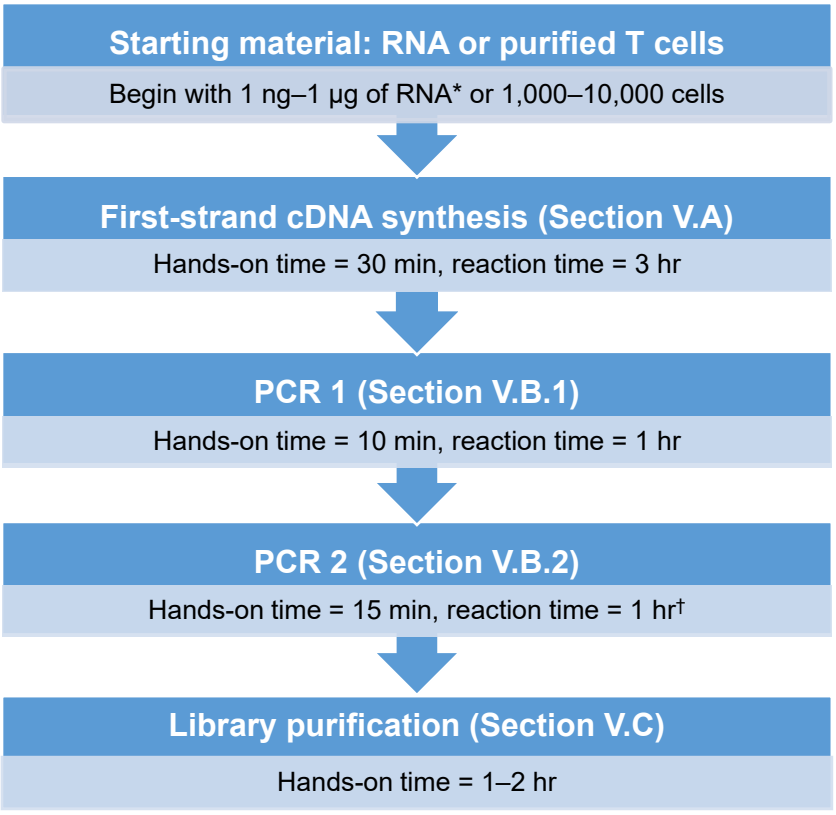
I. Introduction

SMART-Seq Human TCR (with UMIs) (Cat. Nos. 634779, 634780, 634781) enables users to analyze T-cell receptor (TCR) diversity from human RNA samples or directly from cells. The kit is designed to work with a range of RNA input amounts (RIN ≥7, depending on the sample type) and has been shown to yield high-quality sequencing libraries from as little as 10 ng–1 µg of total RNA obtained from peripheral blood leukocytes, 20 ng–200 ng of total RNA obtained from whole blood, 1 ng–100 ng of total RNA obtained from T cells, or from 1,000–10,000 purified, whole T cells.

The workflow can be used to generate data for both alpha- and beta-chain diversity, either in the same experiment or separately. Using the Unique Dual Index Kits (UDIs, Cat. Nos. 634752– 634756, sold separately), the protocol results in indexed libraries (up to 384) that are ready for sequencing on Illumina® platforms. With sequencing output from Illumina sequencers as input to our free-to-use Cogent™ NGS Immune Profiler and web-based Cogent NGS Immune Viewer bioinformatics software (Appendix C), we provide an end-to-end solution from samples to publication-ready data.



*depends on source of RNA (RIN ≥7)
†depends on number of cycles

Figure 1. Protocol overview and timeline.

SMART-Seq Human TCR (with UMIs) leverages the SMART® technology (**S**witching **M**echanism **a**t 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. It also incorporates unique molecular identifiers (UMIs) to facilitate PCR error correction and clonotype quantification during data analysis.

Input RNA or cells are oligo-dT primed followed by reverse transcription using SMARTScribe™ Reverse Transcriptase (RT), which adds non-templated nucleotides at the 5′ end of each cDNA molecule (Figure 2). The TCR SMART UMI Oligo anneals to these non-templated nucleotides, serves as a template for incorporation of a

PCR annealing site into the first-strand cDNA, and uniquely tags each cDNA molecule with a UMI. Following reverse transcription, two rounds of PCR are performed to amplify TCR cDNAs (Figure 2). To capture the entire V(D)J region, primers in these PCRs anneal to sequences added by the TCR SMART UMI Oligo at one end and the TCR constant region(s) at the other end. The second PCR takes the product from the first PCR as a template and uses semi-nested primers to amplify the entire TCR variable region and a small portion of the constant region (Figure 2).

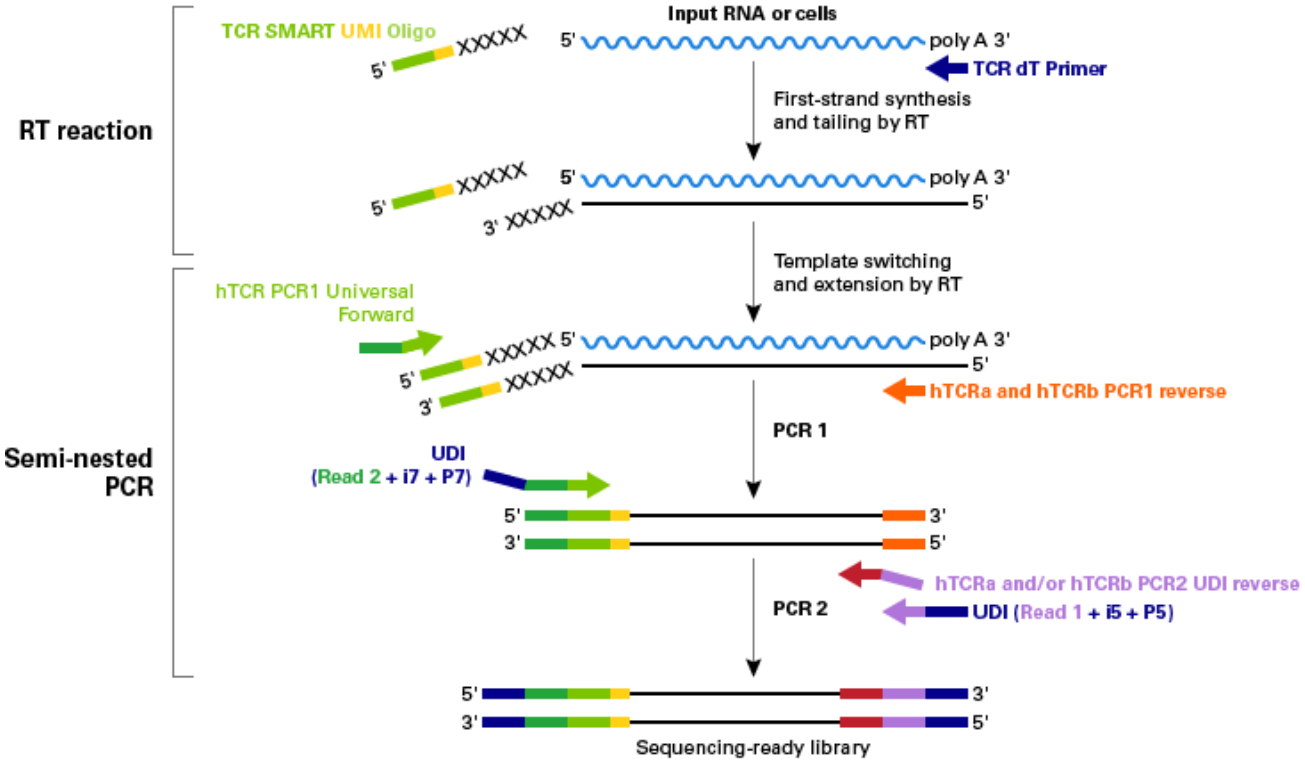


Figure 2. Schematic of technology and workflow for SMART-Seq Human TCR (with UMIs Input RNA or cells are oligo-dT primed using the TCR dT Primer (dark blue). Following oligo dT-priming, SmartScribe Reverse Transcriptase performs first strand cDNA synthesis on input RNA or cells and adds non-templated nucleotides to the 5' end of each cDNA molecule (black Xs). Upon reaching the 5' end of the RNA template, the TCR SMART UMI Oligo anneals to the non-templated nucleotides (XXXXX), incorporating UMI (yellow) and partial Illumina adapter (light green) complementary to the hTCR PCR1 Universal Forward primer. Following reverse transcription, semi-nested PCR is performed to amplify TCR cDNAs. In PCR 1, the hTCR PCR 1 Universal Forward primer anneals to the complementary sequence carried by the TCR SMART UMI Oligo (light green), incorporating Illumina Read2 sequence (dark green). hTCRa PCR1 Reverse and/or hTCRb PCR1 Reverse primers (orange) anneal to sequences in the constant regions of TCRα and TCRβ cDNA, respectively, to amplify the entire TCR V(D)J region. During the PCR 2, the nested TCRa and/or TCRb PCR2 UDI reverse primers anneal to sequences in TCR constant regions that are internal to the sequences bound by the hTCRa/b PCR1 Reverse primers and adds the Illumina Read 1 sequence (light purple). In the same PCR 2 reaction, Unique Dual Index Kit primers anneal to sequence added by hTCR PCR1 Universal Forward primer or the TCRa and/or TCRb PCR2 UDI reverse primes to add Illumina P7-i7 and P5-i5 index sequences (dark blue). The result is a sequencing-ready library that contains the entire TCR variable region with a small portion of the constant region.

II. List of Components

- The components of SMART-Seq Human TCR (with UMIs) have been specifically designed to work together and are optimized for this particular protocol. The substitution of reagents in the kit and/or modification of the protocol may lead to unexpected results.
- The reaction number for each kit specifies the number of libraries (TCRα alone, TCRβ alone, or TCRα and TCRβ combined) that can be generated with the indexing primers supplied.

NOTE: UDI Kits are not included and sold separately (Cat. Nos. 634752–634756). The user may select the appropriate size UDI kit depending on the number of libraries to be pooled; the UDI kits allow for preparing and pooling up to 384 Illumina-compatible libraries.

SMART-Seq Human TCR (with UMIs)	Cap color	634780 (24 rxns)	634481 (96 rxns)	634779 (384 rxns)
Box 1 (Store at –70°C.)				
Control RNA (1 µg/µl)	Clear	5 µl	5 µl	4 x 5 µl
TCR SMART UMI Oligo (48 µM)	Pink	30 µl	120 µl	4 x 120 µl
Box 2 (Store at –20°C)				
10X Lysis Buffer*	Clear	230 µl	920 µl	4 x 920 µl
5X Ultra® Low First-Strand Buffer	Red	96 µl	384 µl	4 x 384 µl
TCR dT Primer (12 µM)	Blue	48 µl	192 µl	4 x 192 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	Purple	48 µl	192 µl	4 x 192 µl
RNase Inhibitor (40 U/µl)	White	60 µl	240 µl	4 x 240 µl
Elution Buffer† (10 mM)	Clear	1.7 ml	4 x 1.7 ml	16 x 1.7 ml
Nuclease-Free Water	-	1.7 ml	4 x 1.7 ml	16 x 1.7 ml
PrimeSTAR® GXL DNA Polymerase (1.25 U/µl)	White	100 µl	400 µl	4 x 400 µl
5X PrimeSTAR GXL Buffer	White	1 ml	4 x 1 ml	16 x 1 ml
dNTP Mix (2.5 mM each)	White	400 µl	1.7 ml	4 x 1.7 ml
hTCR PCR1 Universal Forward (12 µM)	Green	24 µl	96 µl	4 x 96 µl
hTCRa PCR1 Reverse (12 µM)	Orange	48 µl	192 µl	4 x 192 µl
hTCRb PCR1 Reverse (12 µM)	Orange	24 µl	96 µl	4 x 96 µl
hTCRa PCR2 UDI Reverse (12 µM)	Green	24 µl	96 µl	4 x 96 µl
hTCRb PCR2 UDI Reverse (12 µM)	Green	24 µl	96 µl	4 x 96 µl

*Once thawed, store 10X Lysis Buffer at 4°C.
†Once thawed, store Elution Buffer at room temperature.

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:

- Unique Dual Index Kit
  - 96 indexes: Takara Bio, Cat. Nos. 634752, 634753, 634754, and/or 634755
  - 24 indexes: Takara Bio, Cat. No. 634756
- 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
- Minicentrifuge for 1.5 ml tubes
- Minicentrifuge for 0.2 ml tubes or strips

For PCR Amplification & Validation:

- Thermal cyclers

  - One dedicated for first-strand cDNA synthesis (Section V.A)
  - One dedicated for library amplification by PCR (Section V.B)
- For validation
  - Agilent 2100 Bioanalyzer: Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) or Agilent DNA 1000 Kit (Agilent, Cat. No. 5067-1504). The Agilent 4150/2200 TapeStation with High Sensitivity D5000 ScreenTape (Agilent, Cat. No. 5067-5592) or High Sensitivity D1000 ScreenTape (Agilent, Cat. No. 5067-5584) or an Agilent Fragment Analyzer with the HS NGS Fragment Kit (Agilent, Cat. No. DNF-474) may also be used.
  - Qubit dsDNA HS Kit (Thermo Fisher Scientific, Cat. No. Q32851)
- Nuclease-free thin-wall PCR tubes, 96 well plates, or strips (0.2 ml PCR 8-tube strip; USA Scientific, Item No.1402-4700 or similar)
- Nuclease-free low-adhesion 1.5 ml tubes (USA Scientific, Item No. 1415-2600), LoBind tubes (Eppendorf, Cat. No. 022431021), or similar

For Bead Purifications (Section V.C):

- NucleoMag NGS Clean-up and Size Select (Takara Bio; 5 ml size: Cat. No. 744970.5; 50 ml size: Cat. No. 744970.50; 500 ml size: Cat. No. 744970.500) If the NucleoMag product is not available, the AMPure XP PCR purification kit (Beckman Coulter; 5 ml size: Cat. No. A63880; 60 ml size: Cat. No. A63881) is an appropriate substitute and can be used in the same bead:sample ratio as NucleoMag NGS Clean-Up and Size Select.

  NOTES:
  - The kit has been specifically validated with the beads listed above. Substitutions may lead to unexpected results.
  - Beads need to come to room temperature before the container is opened. We strongly 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 min). Aliquoting is also instrumental in decreasing the chances of bead contamination.
- 100% ethanol (molecular biology grade)
- Magnetic separation device for small volumes—used to purify amplified libraries
  - For 8-tube strips: SMARTer-Seq® 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. General Recommendations

IMPORTANT! For Cat. No. 634781 (96 rxns) and Cat. No. 634779 (4 x 96 rxns), we recommend performing a **minimum** of 12 reactions per protocol run to ensure sufficient reagents to utilize 96 (or 384) reactions per kit.

- We recommend using two physically separated workstations to minimize contamination:

  - **A PCR Clean Workstation** for all pre-PCR experiments that require clean room conditions, e.g., first-strand cDNA synthesis (Section V.A). The PCR Clean Workstation should be in a clean room with positive air flow.
  - **A second workstation located in the general laboratory** where you will perform PCR (Section V.B) and measure library concentration (Section V.D).
- The assay is very sensitive to variations in pipette volume, etc. Please make sure all your pipettes are calibrated for reliable delivery and 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.
- If you are using this protocol for the first time, we strongly recommend that you perform negative and positive control reactions to verify that kit components are working properly.

B. Sample Recommendations

- This protocol has been optimized for 10 ng of total RNA from peripheral leukocytes in human blood (RIN ≥7. However, if your RNA sample is not limiting, we recommend that you start with more (up to 1 µg). The purified total RNA should be in nuclease-free water.
- This protocol has been used successfully to generate cDNA starting from 1,000–10,000 intact, cultured cells**.** For the removal of media prior to dilution, bulk cell suspensions should be washed and resuspended in Mg^{2+}- and Ca^{2+}-free PBS, as the presence of media can interfere with the first-strand synthesis. It is best to perform at least two washes with low-speed centrifugation to avoid cell damage. If necessary, test the effect of your media or FACS buffer on cDNA synthesis by performing a reaction with control RNA and the estimated amount of media that you expect to accompany your cell(s). **It cannot be used with cells that have undergone fixation.**
- Avoid using heparin for blood sample collection as it can inhibit downstream enzymatic steps such as cDNA synthesis and PCR. The success of your experiment depends on the quality of your input RNA. Prior to cDNA synthesis, please make sure that your RNA is intact and free of contaminants. Input RNA should be free from poly(A) carrier RNA that will interfere with oligo(dT)-primed cDNA synthesis
- The success of your experiment depends on the quality of your input RNA. Prior to cDNA synthesis, please make sure that your RNA is intact and free of contaminants. Input RNA should be free from poly(A) carrier RNA that will interfere with oligo(dT)-primed cDNA synthesis.
- RNA should be of high integrity (RIN>7) to ensure that the 3′ poly(A) tail is intact to enable oligo(dT)-priming and 5′ end of the RNA is intact to allow for generation of accurate clonotype information.
- When choosing a purification kit, ensure that it is appropriate for your sample amount. We recommend NucleoSpin RNA Plus, a mini kit for RNA purification with a DNA removal column (Takara Bio, Cat. No. 740984.50 or 740984.250).
- After RNA extraction, if your sample size is not limiting, we recommend evaluating total RNA Integrity Number (RIN) using the Agilent RNA 6000 Pico Kit (Cat. No. 5067-1513) or an equivalent

microfluidic device/ kit. Refer to the manufacturer's instructions about how to use the Agilent RNA 6000 Pico Kit.

V. Protocols

A. Protocol: First-Strand cDNA Synthesis

First-strand cDNA synthesis (from RNA) is primed by the TCR dT Primer and uses the TCR SMART UMI Oligo for template-switching at the 5′ end of the transcript.

For this protocol, you will need the following components:

10X Lysis Buffer, RNase Inhibitor, Nuclease-Free Water, Control RNA, TCR dT Primer, 5X Ultra Low First-Strand Buffer, TCR SMART UMI Oligo, and SMARTScribe Reverse Transcriptase.

1. Thaw the 5X Ultra Low First-Strand Buffer at room temperature. Do not store on ice.

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

2. Prepare a stock solution of Lysis/Inhibitor Mix by mixing the 10X Lysis Buffer with the RNase Inhibitor as indicated below (scale up as needed):

19 µl	10X Lysis Buffer
1 µl	RNase Inhibitor
20 µl	Total volume

   Mix briefly, then spin down.

   NOTE: The 10X Lysis Buffer contains a detergent. It is critical to avoid bubbles when mixing.

3. See Table 1 for guidelines on setting up reactions for your control and test samples. Prepare each reaction (10.5 µl total volume) in an individual 0.2 ml RNase-free PCR tube or an 8-well strip:

   - **Purified total RNA:** If you are working with purified total RNA, transfer 1–9.5 µl to a 0.2 ml RNase-free PCR tube. Add 1 µl of Lysis/Inhibitor Mix. Bring the volume to 10.5 µl with Nuclease-Free Water.
   - **Cells:** Bulk cell suspensions should be washed and resuspended in Mg^{2+} -and Ca^{2+}-free PBS. It is best to perform at least two washes with low-speed centrifugation to avoid cell damage. Transfer ≤ 5 µl of the cell suspension in PBS to a 0.2 ml RNase-free PCR tube containing the Lysis/Inhibitor mix from Step 2 and the appropriate volume of Nuclease-Free Water to bring the final reaction volume to 10.5 µl. Gently vortex or pipette to mix the sample. See Section IV.B for sample recommendations.

     NOTE Do not add Nuclease-Free Water directly to cells. Always combine Nuclease-Free Water and the Lysis/Inhibitor solution before adding to the cell suspension.

Components	Sample	Negative control	Positive control
Lysis/Inhibitor Mix (from Step 2)	1 µl	1 µl	1 µl
Sample	1–9.5 µl	–	–
Diluted Control RNA*	–	–	1–9.5 µl
Nuclease-Free Water	Up to 8.5 µl	9.5 µl	Up to 8.5 µl
Total volume	10.5 µl	10.5 µl	10.5 µl

*The Control RNA is supplied at a concentration of 1 µg/µl and should be diluted in Nuclease-Free Water with RNase Inhibitor (1 µl RNase Inhibitor in a final volume of 50 µl of water) to match the concentration of test sample. Perform serial dilutions on the Control RNA to obtain the appropriate concentration. We have tested this

protocol extensively using the PCR cycling conditions below (Table 1) (18 cycles for PCR 1 and 18 cycles for PCR2) with 10 ng of input Control RNA.

4. Place the samples on ice and add 2 µl of the TCR dT Primer (12 µM) to each sample. Mix well by gently vortexing and then spin the tube(s) briefly to collect the contents at the bottom of the tube.
5. Incubate the tubes at 72°C in a preheated hot-lid thermal cycler for 3 min, and incubate at 4°C for 2 min. During this incubation, prepare the RT Master Mix
6. At room temperature, prepare RT Master Mix by combining the following components in the order shown in the table below. Mix the RT Master Mix well by gently pipetting up and down then centrifuge briefly.

IMPORTANT!
- Remove the RNase Inhibitor from the freezer immediately before use, centrifuge briefly to mix, and store on ice.
- The Ultra Low First-Strand Buffer may form precipitates; vortex before using to ensure all components are completely in solution.
- Wait to add the SMARTScribe Reverse Transcriptase to the Master Mix until just prior to use (Step 11).

Components	1 rxn	12 rxns*	48 rxns*
5X Ultra Low First-Strand Buffer	4 µl	52 µl	212 µl
TCR SMART UMI Oligo	1 µl	13 µl	53 µl
RNase Inhibitor (40 U/µl)	0.5 µl	6.5 µl	26.5 µl
SMARTScribe Reverse Transcriptase	2 µl	26 µl	106 µl
Total volume	7.5 µl	97.5 µl	397.5 µl

*Includes ~10% overage

7. Immediately after the 3-min incubation at 72°C (Step 6), place the samples on ice for 2 min.
8. Remove the SMARTScribe Reverse Transcriptase from the freezer, centrifuge briefly, and store on ice.
9. Reduce the temperature of the thermal cycler to 42°C.
10. Add the SMARTScribe Reverse Transcriptase to the RT Master Mix according to the table above. Mix well by gently pipetting up and down.
11. Add 7.5 µl of the RT Master Mix (from Step 11) to each reaction tube or well. Mix the contents of each tube or well by pipetting gently and centrifuge briefly.
12. Place the tubes in a hot-lid thermal cycler preheated to 42°C. Run the following program:

    42°C 180 min
    70°C 10 min
    4°C forever

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

B. Protocol: TCR a/b Amplification and Sequencing Library Generation

Semi-nested PCR amplifies the entire V(D)J region and a portion of the constant region of TCRα/β cDNA and incorporates adapters and barcodes for Illumina sequencing platforms. Table 1 provides PCR-cycling recommendations, but optimal parameters may vary for different sample types, input amounts, and thermal cyclers. We recommend trying a range of cycle numbers to determine the minimum number necessary to obtain the desired yield.

Table 1. Cycling guidelines based on the amount of starting material

Input type	Input amount	Number of PCR1 cycles	Number of PCR2 cycles*
Control RNA	10 ng	18	18
PBL RNA	10 ng	18	20
	100 ng	18	16
	1,000 ng	18	14
Whole blood	20 ng	18	20
	200 ng	18	16
T-cell RNA	1 ng	18	24
	10 ng	18	20
	100 ng	18	16
	1,000 ng	18	14
Purified T cells	1,000 cells	18	24
	10,000 cells	18	20

*PCR cycles were adjusted with input RNA and the number of cells. Depending on your sample type and quality of RNA, you can modify by ± 2 cycles in PCR 2.

1. PCR 1

This PCR selectively amplifies TCR sequences from the first-strand cDNA generated with the previous protocol. The hTCR Universal Forward primer anneals to the SMART UMI oligo sequence (incorporated during first-strand cDNA synthesis) and adds the Illumina Read 2 sequence. hTCRa PCR1 Reverse and/or hTCRb PCR1 Reverse primers anneal to sequences in the constant regions of *TCRα* and *TCRβ* cDNA, respectively.

For this protocol, you will need the following components:

5X PrimeSTAR GXL Buffer, dNTP Mix, hTCR PCR1 Universal Forward, hTCRa Human PCR1 Reverse and/or hTCRb PCR1 Reverse, PrimeSTAR GXL DNA Polymerase, Nuclease-Free Water.

1. Thaw all the reagents needed for PCR (except the PrimeSTAR polymerase) on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
2. Prepare a mixture with 2 µl of hTCRa PCR1 Reverse and 1 µl of hTCRb PCR1 Reverse (per reaction; scale up as required) then use 1 µl of this mix in the PCR 1 Master Mix (below).

3. Prepare PCR1 Master Mix by combining the following components in the order shown in the table below. Remove the PrimeSTAR GXL DNA Polymerase from the freezer, gently mix the tube without vortexing, and add to the master mix just before use.

Components	1 rxn	12 rxns*	48 rxns*
Nuclease-Free Water	13 µl	175.5 µl	689 µl
5X PrimeSTAR GXL PCR Buffer	10 µl	135 µl	530 µl
dNTP Mix (2.5 mM each)	4 µl	54 µl	212 µl
hTCR PCR1 Universal Forward (12 µM)	1 µl	13.5 µl	53 µl
hTCRa/hTCRb PCR1 reverse primer mixture (12 µM)†	1 µl	13.5 µl	53 µl
PrimeSTAR GXL DNA Polymerase	1 µl	13.5 µl	53 µl
Total Volume	**30 µl**	**405 µl**	**1,590 µl**

*Includes ~10% overage

†See Step 2 for preparation of hTCRa/hTCRb PCR1 reverse primer mixture

Gently vortex to mix then briefly centrifuge.

4. Add 30 µl of PCR 1 Master Mix to each tube containing 20 µl of the first-strand cDNA product from Section V.A. Mix well and briefly spin to collect the contents at the bottom of the tubes/wells.
5. Place the tubes/plate in a preheated thermal cycler with a heated lid and run the following program:

PCR 1

95°C	1 min	
18 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. PCR 2

This PCR reaction further amplifies sequences corresponding to the full-length TCR variable regions and adds Illumina sequencing adapters using a semi-nested approach. The nested TCRa and/or TCRb PCR2 UDI reverse primers anneal to sequences in TCR constant regions that are internal to the sequences bound by the hTCRa/b PCR1 Reverse primers and adds the Illumina Read 1 sequence. In the same reaction, Unique Dual Index Kit primers anneal to a sequence added by hTCR PCR1 Universal Forward to add Illumina P7-i7 index sequences and to the Read 1 sequence added by the TCRb and/or TCRb PCR2 UDI reverse primers to add Illumina P5-i5 index sequences.

For this protocol, you will need the following components:

5X PrimeSTAR GXL Buffer, dNTP Mix, hTCRa/b PCR2 UDI Reverse, selected Unique Dual Index Kit, PrimeSTAR GXL DNA Polymerase, Nuclease-Free Water.

1. Thaw all the reagents needed for PCR (except the PrimeSTAR polymerase) on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.

2. Dilute the hTCRa and hTCRb PCR2 UDI Reverse primers: mix 2 µl of hTCRa PCR2 UDI Reverse, 2 µl of hTCRb PCR2 UDI Reverse, and 36 µl of Nuclease-Free Water. Mix well by vortexing gently and then spin the tubes briefly in a microcentrifuge.

   NOTE: The hTCRa and hTCRb PCR2 UDI reverse primers can also be used separately. In this case, we suggest diluting hTCRa and hTCRb primers at 1:20. Use 2 µl of the diluted primer of interest per reaction (scale up as required). If achieving equal coverage for each subunit is critical to your experiments, we recommend performing the reactions in PCR 2 separately. Consult Appendix B and Illumina literature for more information.

3. On ice, prepare a PCR 2 Master Mix by combining the following components in the order shown in the table. Remove the PrimeSTAR GXL DNA Polymerase from the freezer, gently mix the tube without vortexing, and add to the master mix just before use.

Components	1 rxn	12 rxns*	48 rxns*
Nuclease-Free Water	30 µl	405 µl	1,590 µl
5X PrimeSTAR GXL PCR Buffer	10 µl	135 µl	530 µl
dNTP Mix (2.5 mM each)	4 µl	54 µl	212 µl
hTCRa/b PCR UDI Reverse Primer mixture (1/20 diluted; Step 2)†	2 µl	27 µl	106 µl
PrimeSTAR GXL DNA Polymerase	1 µl	13.5 µl	53 µl
Total Volume	**47 µl**	**634.5 µl**	**2,491 µl**

*Includes ~10% overage

†If desired, only the TCRα or TCRβ chain can be amplified using the respective UDI Reverse Primer, instead of a mixture. Please refer to the note in Step 2.

   Gently vortex to mix then briefly centrifuge.

4. For each reaction, add 47 µl of PCR 2 Master Mix to a clean 0.2 ml tube.
5. Add 1 µl of the PCR 1 product (after Step 5 of the PCR 1 protocol) to each tube.
6. Add 2 µl of the appropriate UDI from the Unique Dual Index Kit (12.5 µM) to each sample. Mix well and briefly spin to collect the contents at the bottom of the tube(s).
7. Place the tubes/plate in a preheated thermal cycler with a heated lid and run the following program:

PCR 2

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

*Consult Table 1 (above) for PCR cycle number (*N*) guidelines.

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

C. Protocol: Purification of Amplified Libraries

TCR libraries are purified by two rounds of size selection using NucleoMag NGS Clean-up and Size Select. The beads are then washed with 80% ethanol and the libraries are eluted with Nuclease-Free Water.

NOTES:

- Before each use, bring bead aliquots to room temperature for at least 30 min. Immediately before use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous. Confirm that there is no remaining pellet of beads at the bottom of the tube. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.
- Bead:sample ratio is 0.7:1
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.
- Use a magnetic separation device for 0.2 ml tubes, strip tubes, or a 96-well plate.

1. Vortex NucleoMag beads until evenly mixed, then add 25 µl of the NucleoMag beads to each sample.
2. Mix thoroughly by gently 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 min 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 to 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. After transferring, remove the tubes containing the beads from the magnetic separation device and discard them.
6. Add 10 µl of NucleoMag beads to each tube containing supernatant.

   NOTE: Before use, ensure that the beads are fully resuspended. If the beads appear to have settled at the bottom of the tube, vortex to ensure that they are completely mixed before adding them to the tubes.

7. Mix thoroughly by gently pipetting the entire volume up and down at least 10 times. Do not vortex.
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. The libraries are now bound to the beads.
10. While the tubes are sitting on the magnetic separation device, remove the supernatant with a pipette and discard it.
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 sec 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 more.
13. Briefly spin the tubes (~2,000*g*) to collect the remaining liquid at the bottom of each tube. Place the tubes on the magnetic separation device for 30 sec, then remove all remaining liquid with a pipette.
14. Let the sample tubes rest open on the magnetic separation device at room temperature for ~2–2.5 min until the pellet appears dry and is no longer shiny. You may see a tiny crack in the pellet.

    NOTE: Check the pellet frequently during this time and continue to Step 15 when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you'd like additional assistance with this determination.
    https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

    If the beads are overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads and recovery may be reduced.
15. 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. 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 sides of the tube.
16. Incubate at room temperature for at least 5 min to rehydrate.
17. 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. Pipette these non-pelleted beads (without disrupting the existing pellet) up and down to resuspend them with the supernatant, and then pipette them towards the magnet where the rest of the beads have already pelleted. Continue the incubation until there are no beads le.ft in the supernatant.
18. Label new nuclease-free, low-adhesion tubes with sample information
19. Transfer clear supernatant containing purified TCR library into the tubes prepared in Step 18 and store at –20°C.

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

D. Protocol: Library Validation

To assess the success of library preparation, purification, and size selection, we recommend

- Analyzing and validating final libraries using Qubit 2.0 Fluorometer using a Qubit dsDNA HS kit (Thermo Fisher Scientific, Cat. No. Q32851) and
- Evaluating the libraries' size distributions with an Agilent 2100 Bioanalyzer and the DNA High Sensitivity Kit (Agilent, Cat. No. 5067-4401) or Agilent DNA 1000 kit (Agilent, Cat. No. 5067-1504).

Validating size distribution essential is to assess efficiency of library construction and determine the average library fragment size. Please refer to the corresponding user manuals for detailed instructions.

1. **Qubit:** Use 1 µl of undiluted library for quantification using Qubit 2.0 Fluorometer (see manufacturer's instructions for more details).
2. **Bioanalyzer:** To validate libraries using Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626), dilute libraries to concentration of 0.2 ng/µl. For Agilent DNA 1000 kit (Cat. No. 5067-1504), dilute libraries to 1 ng/µl. Molar concentration of the TCR library can be determined by integrating the region encompassing the major library peak.

   Compare the results for your samples with Figure 3 (below) to verify whether each sample is suitable for further processing. High quality libraries should yield no product for negative control reactions and a broad peak spanning 500 bp–1,200 bp with a maximum between ~600 bp–900 bp for positive controls and samples containing TCR RNA. The position and shape of electropherogram peaks will vary depending on which chain sequences are included in the library, the nature of the RNA sample, and the analysis method. In general, electropherogram peaks obtained with the Fragment Analyzer tend to be sharper than those obtained with the Bioanalyzer.

Following validation, libraries are ready for sequencing on Illumina platforms. See Appendix B for sequencing guidelines.

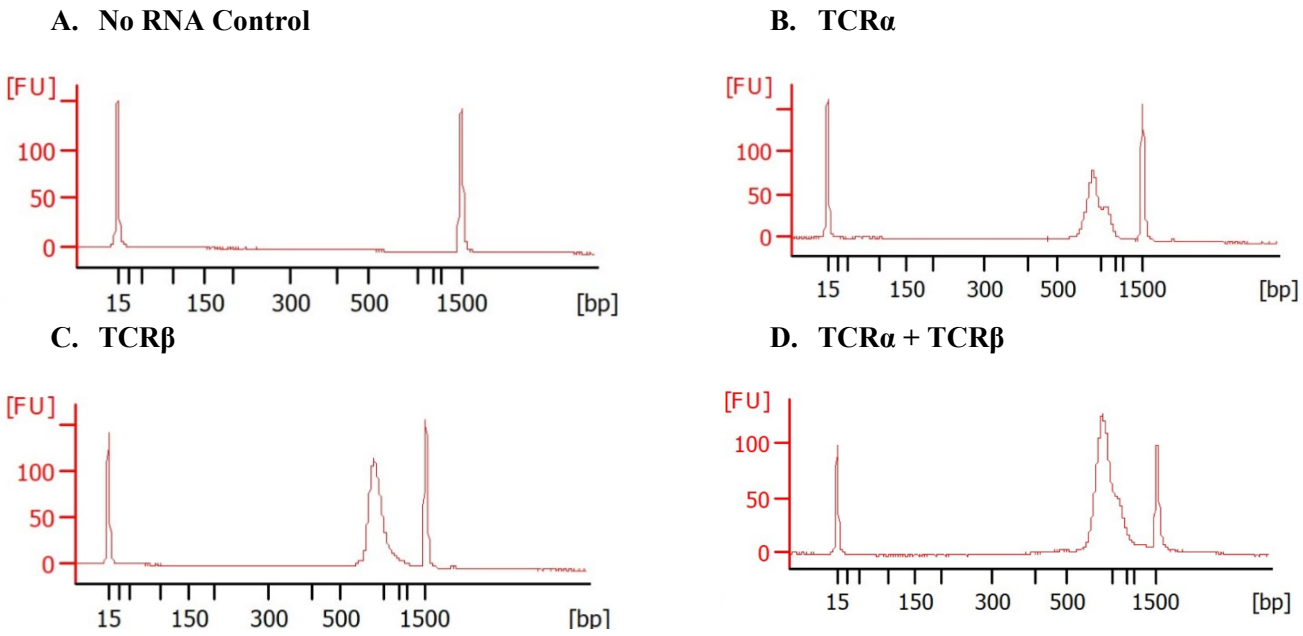


Figure 3. Example electropherogram results for TCRα + TCRβ libraries. Libraries containing *TRA* and *TRB* sequences were generated using SMART-Seq Human TCR (with UMIs) and 10 ng of Control RNA, respectively. Libraries were produced using 18 amplification cycles for PCR 1 and 18 amplification cycles for PCR 2. Following purification and size selection, libraries were analyzed on an Agilent 2100 Bioanalyzer using the DNA 1000 Kit. **Panel A**. The No RNA Control sample show no peaks between 15 and 1,500 bp. **Panels B, C**, and **D** show broad peaks between ~650–1,150 bp and maximal peaks in the range of ~600–800 bp (typical results for a library generated from peripheral blood leukocyte RNA).

VI. References

Bolotin, D. A. *et al.* MiXCR: software for comprehensive adaptive immunity profiling. *Nat. Methods* **12,** 380–381 (2015).

Shugay M. *et al.* MiGEC: Towards error-free profiling of immune repertoires. *Nat. Methods* **11**, 653–655 (2014).

Appendix A: Sample Pooling and Indexing Recommendations

Unique combinations of Illumina indexes are required to ensure enough diversity and allow for discrimination between samples when sequencing a pool of two or more libraries on a single flow cell. If you anticipate that the number of libraries will exceed the maximum index combinations provided in the UDI kits (384), the same library indexes could be used for each PCR 2 amplification of different chains originating from the same sample so that they are sequenced together as a single library. For example, the same index can be used for both heavy chain library and light chain library if they are from the same sample. Consult the Illumina literature (e.g., TruSeq® DNA Sample Preparation Guide) for appropriate pooling guidelines.

SMART-Seq Human TCR (with UMIs) requires use of the Unique Dual Index Kits (Cat. Nos. 634752–634756, sold separately). The indexes are 8-nt long and employ "IDT for Illumina TruSeq UD Indexes" i5 and i7 dual index sequences. In all versions of the UDI kits, the primers are provided in a 96-well plate format; the indexes in Unique Dual Index Kit (1–24) (Cat. No. 634756) are a subset of Unique Dual Index Kit (1–96) (Cat. No. 634752). Please consult the following resources for component information, best practices, pooling strategies, an index plate map, and index sequences.

- Unique Dual Index Kits Protocol-At-A-Glance (download)
- Indexes and plate maps (Excel files)
  - Unique Dual Index Kit (1-96) Indexes and Plate Map (download)
  - Unique Dual Index Kit (97-192) Indexes and Plate Map (download)
  - Unique Dual Index Kit (193-288) Indexes and Plate Map (download)
  - Unique Dual Index Kit (289-384) Indexes and Plate Map (download)
  - Unique Dual Index Kit (1-24) Indexes and Plate Map (download)

Appendix B: Guidelines for Library Sequencing

Following library validation by Qubit and Bioanalyzer, prepare the desired library pools for the sequencing run. Prior to pooling, libraries must be carefully quantified. We recommend quantification by qPCR using the Library Quantification Kit (Takara Bio, Cat. No. 638324). Alternatively, by combining the quantification obtained with the Qubit with the average library size determined by the Bioanalyzer, the concentration in ng/µl can be converted to nM. The following web tool is convenient for the conversion:

http://www.molbiol.ru/eng/scripts/01_07.html

Most Illumina sequencing library preparation protocols require libraries with a final concentration of 4 nM.

Prepare a pool of 4 nM as follows:

1. Dilute each library to 4 nM in Nuclease-Free Water. To avoid pipetting error, use at least 2 µl of each original library for dilution.
2. Pool the diluted libraries by combining an equal amount of each library in a low-bind 1.5 ml tube. Mix by vortexing at low speed or by pipetting up and down. Use at least 2 µl of each diluted library to avoid pipetting error.
3. Use a 5 µl aliquot of the 4 nM concentration pooled libraries. Follow the library denaturation protocol according to the latest edition of your Illumina sequencing instrument's documentation.

You should also plan to include a 10% PhiX control spike-in (PhiX Control v3, Illumina, Cat. No. FC-110-3001). The addition of the PhiX control is essential to increase the nucleotide diversity and achieve high-quality data generation. Make sure to use a fresh and reliable stock of the PhiX control library. Follow Illumina guidelines on how to denature, dilute, and combine a PhiX control library with your own pool of libraries. Spike-in guidelines for sequencing on the MiSeq® system and other Illumina sequencers are provided in Table 2.

Table 2. PhiX control spike-in guidelines for various Illumina sequencing instruments

Sequencing instrument	Loading concentration quantified by Qubit (pM)	Loading concentration quantified by qPCR (pM)	PhiX %
MiSeq – V2, V3	13	6.5	10%
MiniSeq™	1.1	0.55	30%
NextSeq® 500/550	1.8	0.9	20%
NovaSeq™ 6000	200*	100*	30%

*This loading concentration was optimized for XP workflow according to NovaSeq 6000 Denature and Dilute Libraries Guide.

To sequence the entire V(D)J region, sequencing should be performed on an Illumina MiSeq sequencer using the 600-cycle MiSeq Reagent Kit v3 with paired-end, 2 x 300 base pair reads. To cover the entire CDR3 region, it is possible to perform 1 x 150 single reads, but UMI information will not be captured (Figures 4, 5). To capture the UMI sequence, the 5′ end of the *TRA/B* variable region and the entire CDR3 region (Figure 4), use paired-end, 2 x 150 base reads and the following Illumina instrument and kit combinations (Table 3).

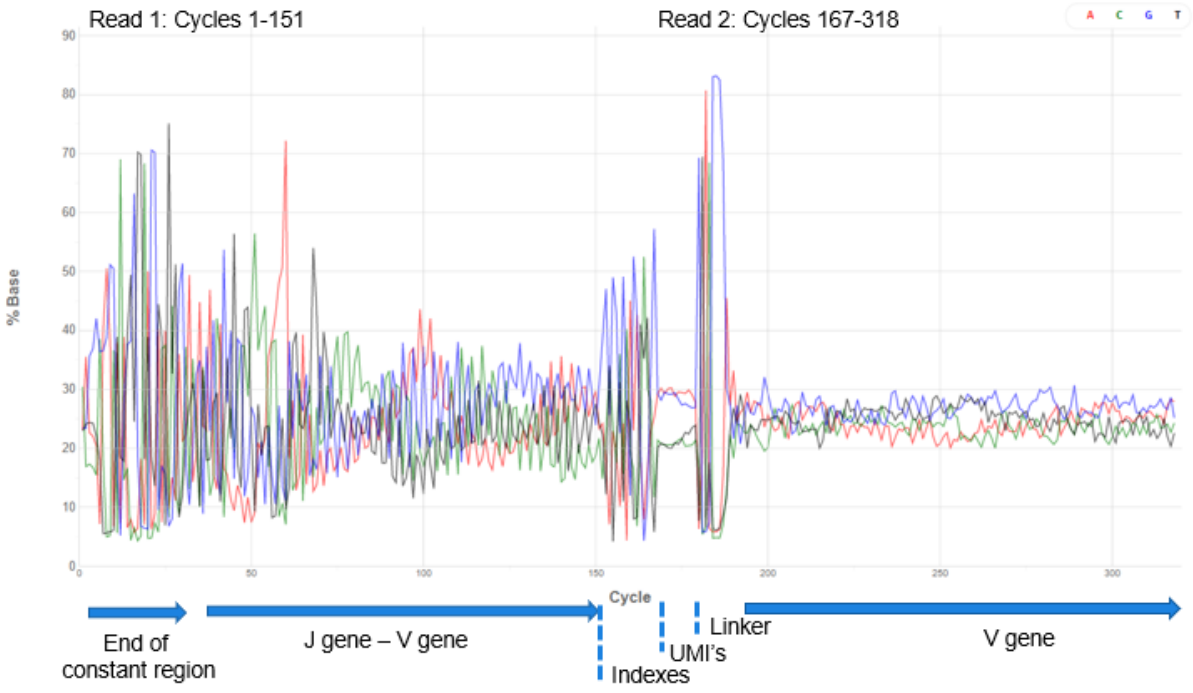


Figure 4. Percentage base calling from a typical NextSeq TCRα + TCRβ profiling run. Cycles 1–151 can cover entire CDR3 regions for the *TRA* and *TRB* genes, respectively. Cycles 152–167 are the index reads.

Table 3. Illumina instrument and reagent kit recommendations

Sequence	Kit	Cat. No.
MiniSeq	MiniSeq High Output reagent kit 300-cycle	Cat. No. FC-420-1003
	Mid Output reagent kit 300-cycle	Cat. No. FC-420-1004
NextSeq	NextSeq High Output kit v2.5 300-cycle	Cat. No. 20024908
	NextSeq Mid Output kit v2.5 300-cycle	Cat. No. 20024905
MiSeq	MiSeq reagent kit v2 300-cycle	Cat. No. MS-102-2002
	MiSeq reagent Micro kit v2 300-cycle	Cat. No. MS-103-1002
	MiSeq reagent Nano kit v2 300-cycle	Cat. No. MS-103-1001
	MiSeq reagent kit v3 600-cycle	Cat. No. MS-102-3003
NovaSeq	NovaSeq SP reagent kit 300-cycle	Cat. No. 20027465
	NovaSeq SP reagent kit 500-cycle	Cat. No. 20029137
	NovaSeq S4 reagent kit 300-cycle	Cat. No. 20012866

We generally recommend a minimum of 500,000 reads for TCRα and TCRβ libraries from an input of 10 ng PBMC, 20 ng whole blood RNA, or 10 ng T-cell RNA input. For libraries generated from >10 ng PBMC RNA or >20 ng whole blood RNA, higher sequencing depth is recommended, as shown in Table 4 below. However, the optimal conditions may vary for different sample types, sample masses, and sample complexities. We recommend trying a higher sequencing depth, then downsample to determine the minimum number of reads per library in order to determine the optimal sequencing depth.

Table 4. Recommended sequencing depth for TCR libraries prepared from human PBMC RNA, human whole blood RNA or T-cell RNA

Input	Mixed Library	TCRα	TCRβ
10 ng PBMC RNA **20 ng whole blood RNA**	~500K reads	~150K reads	~350K reads
100 ng PBMC RNA **200 ng whole blood RNA**	~5M reads	~1.5M reads	~3.5M reads
1 µg PBMC RNA	~25M reads	~7.5M reads	~17.5M reads
1 ng T-cell RNA	~50K reads	~15K reads	~35K reads
10 ng T-Cell RNA	~500K reads	~150K reads	~350K reads
100 ng T-cell RNA	~5M reads	~1.5M reads	~3.5M reads

Appendix C: Guidelines for Data Analysis

Upon completion of a sequencing run, data can be analyzed with our Cogent NGS Immune Profiler Software (CogentIP). To obtain CogentIP, please visit takarabio.com/ngs-immune-profiler. You can also generate tabulated outputs and publication-ready plots of CDR3 length distribution, V/D usage distribution and clonotype diversity using our Cogent NGS Immune Viewer at takarabio.com/ngs-immune-viewer.

CogentIP can also be used to remove duplicated sequences and correct errors from the PCR amplification process through analysis of the 12-nucleotide UMI contained within the TCR library (Figure 5). Analysis of UMIs provides higher accuracy for clonotype diversity and abundance measurements.

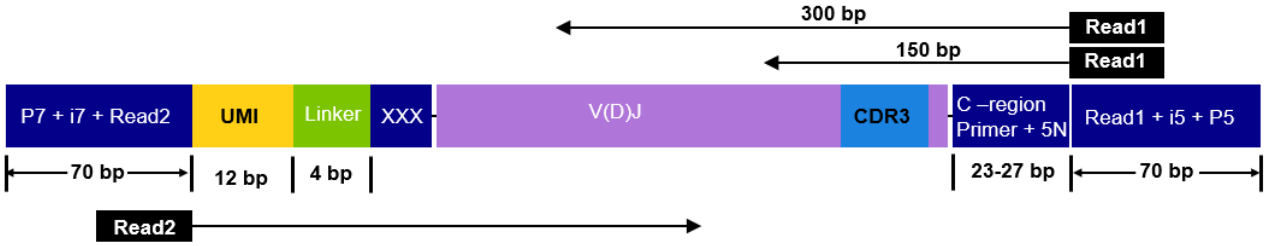


Figure 5. SMART-Seq Human TCR (with UMIs) library structure. The first 19 nt from Read 2 can be trimmed off if UMI analysis is not performed.

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