Takara Bio USA

SMARTer® Human TCR a/b Profiling Kit v2 User Manual

Cat. Nos. 634478, 634479
(051420)

Takara Bio USA, Inc.
1290 Terra Bella Avenue, Mountain View, CA 94043, USA
U.S. Technical Support: technical_support@takarabio.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

Table of Contents

Table of Figures

Table of Tables

I. Introduction

T-cell receptor profiling using SMART® Technology

The **SMARTer Human TCR a/b Profiling Kit v2** (Cat. Nos. 634478 & 634479) 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 ≥8, depending on the sample type) and has been shown to yield high-quality sequencing libraries from as little as 10 ng to 1 µg of total RNA obtained from peripheral blood leukocytes, 1 ng to 100 ng of total RNA obtained from T cells, or from 1,000 to 10,000 purified, whole T cells. As the name suggests, the kit can be used to generate data for both alpha- and beta-chain diversity, either in the same experiment or separately. Libraries produced with the kit are indexed and ready for sequencing on Illumina® platforms (see Appendix B for details).

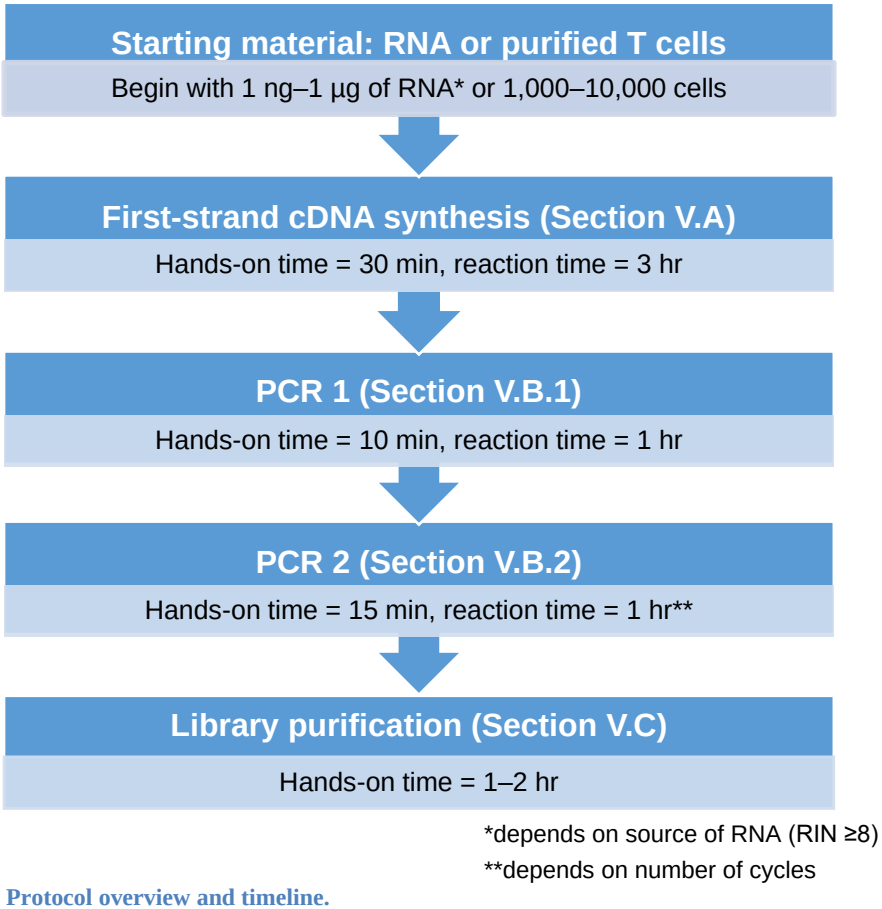

Figure 1. Protocol overview and timeline.

The SMARTer Human TCR a/b Profiling Kit v2 leverages 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 (UMI) to facilitate PCR error correction and clonotype quantification during data analysis. First-strand cDNA synthesis is dT-primed (TCR dT 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 TCR SMART UMI Oligo anneals to these nontemplated nucleotides and serves 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). This additional sequence—referred to as the "TCR Universal 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, two rounds of PCR are performed in succession to amplify cDNA sequences corresponding to variable regions of *TRA* and/or *TRB* transcripts:

- The first PCR uses the first-strand cDNA as a template and includes a forward primer with complementarity to the hTCR PCR1 Universal Forward and a reverse primer with complementarity to the constant (i.e., nonvariable) region of *TRA* and/or *TRB* gene (hTCRa/b PCR1 Reverse); both reverse primers are included in this reaction so analysis of both TCR subunit chains can be conducted if desired. By priming from the TCR universal forward sequence and the constant region, the first PCR specifically amplifies the entire variable region and a considerable portion of the constant region of *TCRa* and/or *TCRb* cDNA.
- The second PCR takes the product from the first PCR as a template. It uses semi-nested primers (SMARTer RNA unique dual indexes [UDIs] and hTCRa/b PCR2 reverse primers) to amplify the entire variable region and a portion of the constant region of the *TCRa* and/or *TCRb* cDNA. Both or either TCR subunit chains may be amplified in a single reaction. In addition to gene-specific sequences derived from the constant regions of human *TRA* and *TRB*, respectively, the hTCRa and hTCRb PCR2 UDI reverse primers contain the partial Read 1 sequence. SMARTer RNA UDIs contain unique dual-indexed PCR primers for the amplification of indexed Illumina-compatible NGS libraries. In combination with UDI primers, unique i5 indexes are included during the amplification of *TCRa* and *TCRb*, respectively. The SMARTer Human TCR a/b Profiling Kit v2 comes with a set of 24 unique dual indexes (Cat. No. 634451) supplied predispensed in 8-tube PCR strips (individually labeled U25 to U48). The forward and reverse primers include adapter and UDI sequences which are compatible with Illumina sequencing platforms and allow for multiplexing of up to 24 samples in a single flow-cell lane.
    - Users wishing to achieve higher levels of multiplexing should purchase SMARTer RNA Unique Dual Index Kit - 96U Set A and/or Set B (Cat. Nos. 634452 & 634457). The use of both 96 UDI kits (individually labeled U1 to U192) allows for multiplexing up to 192 samples. Please note that the 24 UDI kit (Cat. No. 634451) represents a subset of Cat. No. 634452 (96U Set A). Each dual index is provided in sufficient amounts for four uses.

Following post-PCR purification, size selection, and quality analysis, the library is ready for sequencing. This kit is designed for analysis with Takara Bio's Cogent™ NGS Immune Profiler Software, which removes PCR duplicates and errors based on unique molecular identifiers, allowing for more accurate and reliable clonotype calling and quantification. The TCR UMI Oligo contains 12 random nucleotides that are incorporated into the cDNA during the template-switching step.

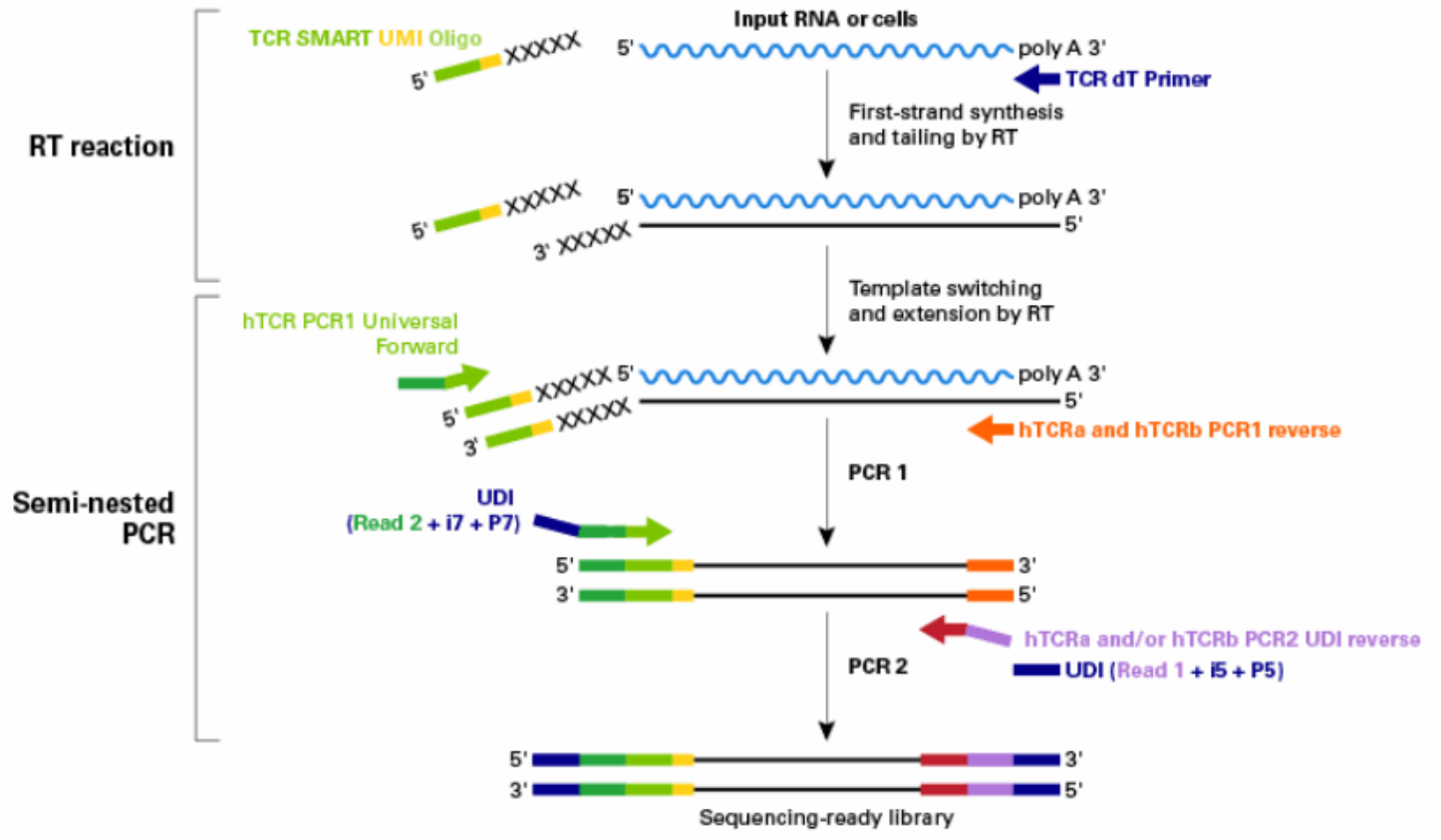

Figure 2. Schematic of technology and workflow for SMARTer Human TCR a/b Profiling Kit v2.

II. List of Components

The SMARTer Human TCR a/b Profiling Kit consists of the SMARTer Human TCR a/b Profiling Kit v2 Components (Cat. Nos. 634480 & 634481; not sold separately) and SMARTer RNA Unique Dual Index Kit – 24U (Cat. No 634451). **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 modification of the protocol may lead to unexpected results. The reaction number for each kit specifies the number of libraries (*TCRa* alone, *TCRb* alone, or *TCRa* and *TCRb* combined) that can be generated with the indexing primers supplied.

SMARTer Human TCR a/b Profiling Kit v2 Components	Cap color	634480 (12 rxns)	634481 (48 rxns)
(Not sold separately. Storage conditions are listed below for Package 1 and Package 2.)			
Box 1 (Store at –70°C.)			
Control RNA (1 µg/µl)	Clear	5 µl	5 µl
TCR SMART UMI Oligo (48 µM)	Pink	15 µl	60 µl
Box 2 (Store at –20°C. Once thawed, store 10X Lysis Buffer at 4°C and store Elution Buffer at room temperature. Continue to store all other reagents at –20°C.)			
10X Lysis Buffer	Clear	230 µl	920 µl
5X Ultra® Low First-Strand Buffer	Red	48 µl	192 µl
TCR dT Primer (12 µM)	Blue	24 µl	96 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	Purple	24 µl	96 µl
RNase Inhibitor (40 U/µl)	White	30 µl	120 µl
Elution Buffer (10 mM)	Clear	1.7 ml	2 x 1.7 ml
Nuclease-Free Water	-	1.7 ml	2 x 1.7 ml
PrimeSTAR® GXL DNA Polymerase (1.25 U/µl)	White	50 µl	200 µl
5X PrimeSTAR GXL Buffer	White	1 ml	2 x 1 ml
dNTP Mix (2.5 mM each)	White	800 µl	800 µl
hTCR PCR1 Universal Forward (12 µM)	Green	12 µl	48 µl
hTCRa PCR1 Reverse (12 µM)	Orange	24 µl	96 µl
hTCRb PCR1 Reverse (12 µM)	Orange	12 µl	48 µl
hTCRa PCR2 UDI Reverse (12 µM)	Green	12 µl	48 µl
hTCRb PCR2 UDI Reverse (12 µM)	Green	12 µl	48 µl

SMARTer RNA Unique Dual Index Kit – 24U (Cat. No 634451)	
(Store at –20°C.)	
U25 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U26 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U27 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U28 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U29 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U30 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U31 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U32 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U33 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U34 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U35 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U36 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U37 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U38 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U39 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U40 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U41 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl

(Contents continued on Page 8)

U42 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U43 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U44 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U45 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U46 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U47 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U48 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl

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
- Minicentrifuge for 1.5-ml tubes
- Minicentrifuge for 0.2-ml tubes or strips

For PCR Amplification & Validation:

- One dedicated thermal cycler used only for the first-strand cDNA synthesis (Protocol V.A)
- One dedicated thermal cycler used only for library amplification by PCR (Protocol V.B)
- (Optional) SMARTer RNA unique dual index kit – 96U set A and/or set B (Takara Bio Cat. Nos. 634452 & 634457; purchase if looking to multiplex >24 reactions)
- For validation using
  - Qubit: dsDNA HS Kit (Thermo Fisher Scientific, Cat. No. Q32851)
  - Agilent 2100 Bioanalyzer: DNA 1000 Kit (Agilent, Cat. No. 5067-1504; Protocol V.D)
  - qPCR: Library Quantification Kit (Takara Bio Cat. Nos. 638324)
  - Advanced Analytical Fragment Analyzer: High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical, Cat. No. DNF-474)
- 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 Bead Purifications (Protocol V.C):

- NucleoMag NGS Clean-up and Size Select (available from Takara Bio; 5-ml size: Cat. No. 744970.5, 50-ml size: Cat. No. 744970.50, 500-ml size Takara Bio Cat. No. 744970.500)

  NOTE: The 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 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. Requirements for Preventing Contamination

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

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

  NOTES:
  - The PCR clean workstation must be located 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 cleanroom operation rules.

- **A second workstation located in the general laboratory** where you will perform PCR (Protocol V.B) and measure library concentration (Protocol V.D).

B. General Requirements

- **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.**

  IMPORTANT: Input RNA should be free from poly(A) carrier RNA that will interfere with oligo(dT)-primed cDNA synthesis.

- 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 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.

C. Sample Recommendations

- **Total RNA Extraction**

  When choosing a purification kit, ensure that it is appropriate for your sample amount. There are several commercially available kits designed to purify total RNA (e.g., we offer the NucleoSpin RNA Plus, Mini kit for RNA purification with DNA removal column; Cat. No. 740984.50).

- **Evaluation of RNA Quality**

  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.

D. Sample Requirements

- **Total RNA**

  This protocol has been optimized for cDNA synthesis starting from 10 ng of total RNA (RIN ≥8). 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.

- **Cells**

  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.**

  IMPORTANT: A large volume of PBS can interfere with the first-strand synthesis**. We recommend preparing a concentrated cell suspension, and then preparing the sample as described in Step 3 of Section V.A with ≤ 5 µl of the resuspension to limit PBS carryover.**

V. Protocols

NOTE: Please read the entire protocol before starting. This protocol has been optimized for the generation of sequencing-ready libraries for TCR repertoire profiling from the input ranges specified in the introduction. Due to the sensitivity of the protocol, the input material (RNA or cells) needs to be collected and purified under clean-room conditions to avoid contamination. The whole process of cDNA synthesis should be carried out in a PCR clean workstation under clean-room conditions.

A. Protocol: First-Strand cDNA Synthesis (Perform in PCR Clean Workstation)

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.

IMPORTANT: To avoid introducing contaminants into your RNA sample, the first part of the cDNA synthesis protocol requires the use of a PCR clean workstation, ideally in a clean room.

For this protocol, you will need the following components:

10X Lysis Buffer (clear cap), RNase Inhibitor (white cap), Nuclease-Free Water, Control RNA (neutral cap), TCR dT Primer (blue cap), 5X Ultra Low First-Strand Buffer (red cap), TCR SMART UMI Oligo (pink cap), and SMARTScribe Reverse Transcriptase (purple cap).

1. Thaw the 5X Ultra Low First-Strand Buffer at room temperature. 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 all but the 5X Ultra Low First-Strand Buffer on ice.

   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. 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 (neutral cap)
1 µl	RNase Inhibitor (white cap)
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 to a 0.2-ml RNase-free PCR tube. Add 1 µl of Lysis/Inhibitor Mix and then bring the sample volume up to 10.5 µl with nuclease-free water. Gently vortex or pipette to mix the sample. See Section IV.D for more information on working with cells.

   Table 1. Sample preparation guidelines

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

   *The Control RNA (available from Takara Bio Cat. No. 636592, Lot# 1002007) 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 (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; blue cap) 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.

   NOTE: Prepare Master Mix (Step 6) while tubes incubate, adding enzyme just before Step 10. Steps 10–11 are critical for first-strand cDNA synthesis and should not be delayed after completing Step 5.

6. Prepare enough Master Mix for all the reactions, plus a 10% overage, by combining the following reagents in the order shown at room temperature.

   Per reaction:

4 µl	5X Ultra Low First-Strand Buffer (red cap)*
1 µl	TCRSMART UMI Oligo (48 µM; pink cap)
0.5 µl	RNase Inhibitor (40 U/µl; white cap)
2 µl	SMARTScribe Reverse Transcriptase (100 U/µl; purple cap)**
7.5 µl	Total volume

   *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.

   **Add the reverse transcriptase to the Master Mix just prior to Step 10, making sure to gently mix the reverse transcriptase tube without vortexing before adding it.

7. Mix the Master Mix thoroughly by gently pipetting up and down, and then spin the tube(s) briefly in a minicentrifuge to collect the contents at the bottom of the tube.

8. Immediately after the samples have completed the 5 min incubation (step 5), place the samples on ice for 2 min.

9. Preheat the thermal cycler to 42°C.

10. Add 7.5 µl of the Master Mix to each reaction tube. Mix the contents of each tube by pipetting gently and spin briefly to collect the contents at the bottom of each tube.

11. 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 by Semi-Nested PCR (Perform Steps 1–3 in a PCR Clean Workstation)

IMPORTANT: This PCR protocol has been optimized based on an initial input of 10 ng of total RNA (human blood, peripheral leukocytes) into the RT reaction described in the previous protocol. Optimal parameters may vary for different templates, cell types, thermal cyclers, and sample amounts. Table 2 provides rough guidelines for PCR optimization, depending on the amount of total RNA or the number of cells used for first-strand cDNA synthesis.

Table 2. 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
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 TCR 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 *TCRa* and *TCRb* cDNA, respectively.

For this protocol, you will need the following components:

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

1. Thaw all the reagents needed for PCR (except the polymerase) on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
2. Prepare 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.
3. Prepare enough PCR 1 Master Mix for all the reactions, plus a 10% overage. Combine the following reagents in the order shown.

Per reaction:

10 µl	5X PrimeSTAR GXL PCR Buffer
4µl	dNTP Mix
1 µl	hTCR PCR1 Universal Forward (12 µM; green cap)
1 µl	Mixture of hTCRa and hTCRb PCR1 reverse primers (12 µM; orange cap)*
1 µl	PrimeSTAR GXL DNA Polymerase
13 µl	Nuclease-Free Water
30 µl	Total volume

*This mixture provides PCR product with information for both *TCRa* and *TCRb*. Generation of *TCRa*-only or *TCRb*-only libraries can be obtained during PCR 2 by using only the desired PCR2 UDI reverse primer.

NOTE: Remove the PrimeSTAR GXL DNA polymerase from the freezer, gently mix the tube without vortexing, and add to the master mx just before use. Mix the PCR1 Master Mix thoroughly by vortexing gently and spin the tube briefly to collect the contents at the bottom of the tube.

4. Add 30 µl of PCR 1 Master Mix to each tube containing 20 µl of the first-strand cDNA product from Protocol V.A. Mix well and briefly spin to collect the contents at the bottom of the tube(s).

IMPORTANT: Transfer the samples from the PCR clean workstation to the general lab. All downstream processes should be performed in the general lab.

5. Place the tube(s) 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 SMARTer RNA unique dual index primers anneal to a sequence added by hTCR PCR1 Universal Forward and add Illumina P7-i7 index sequences. 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 add both the Illumina Read 1 and P5-i5 index sequences.

For this protocol, you will need the following components:

5X PrimeSTAR GXL Buffer, dNTP Mix, hTCRa/b PCR2 UDI Reverse (green caps), SMARTer RNA Unique Dual Index Kit – 24U primers, PrimeSTAR GXL DNA Polymerase, Nuclease-Free Water.

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

2. To 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 ul 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 PCR2 separately. Consult Appendix A and Illumina literature for more information.

3. Prepare enough PCR 2 Master Mix for all the reactions, plus a 10% overage. Combine the following reagents in the order shown.

   Per reaction:

10 µl	5X PrimeSTAR GXL Buffer
4 µl	dNTP Mix
2 µl	1/20 diluted hTCRa/b PCR2 UDI Reverse (12 µM; green cap)
1 µl	PrimeSTAR GXL DNA Polymerase
30 µl	Nuclease-Free Water
47 µl	Total volume added per reaction

   NOTE: Remove the PrimeSTAR GXL DNA Polymerase from the freezer, gently mix without vortexing, and add to the master mix just before use. Mix the PCR 2 Master Mix well by vortexing gently and spin the tube briefly to collect the contents at the bottom of the tube.

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 to each tube.
6. Add 2 µl of the appropriate SMARTer RNA unique dual index (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 tube(s) in a preheated thermal cycler with a heated lid and run the following program:

   PCR 2 (similar to PCR 1, but with variable cycle numbers depending on input)

95°C	1 min
14–24 cycles*	
98°C	10 sec
60°C	15 sec
68°C	45 sec
4°C	forever

   *Consult Table 2 (above; prior to the PCR 1 protocol) for cycle number guidelines.

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

C. Protocol: Purification of Amplified Libraries Using NucleoMag NGS Clean-up and Size Select Beads

NOTES:

- Aliquot NucleoMag 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.
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.
- You will need a magnetic separation device for 0.2-ml tubes, strip tubes, or a 96-well plate.

For this step, you will need the following components:
NucleoMag NGS Clean-up and Size Select beads, 80% ethanol (made fresh), a magnetic separation device, Elution Buffer.

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.
6. Remove the tubes containing the beads from the magnetic separation device and discard them.
7. Add 10 µl of NucleoMag 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, vortex to ensure that they are completely mixed.

8. 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.**

9. Incubate at room temperature for 8 min to let the DNA bind to the beads.
10. Place the tubes on the magnetic separation device for ~10 min or until the solution is completely clear.
11. 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).
12. 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.

13. Repeat the ethanol wash (Step 12) once more.
14. 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 seconds, then remove all remaining liquid with a pipette.
15. 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: 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 16–17) 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. 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.
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. Pipette these nonpelleted 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 left in the supernatant.
19. Transfer clear supernatant containing 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 indefinitely.

D. Protocol: Library Validation

To determine whether library production, purification, and size selection were successful, we recommend analyzing and validating final libraries using Qubit 2.0 Fluorometer using a Qubit dsDNA HS Assay Kit (Life Technologies, Cat. No. Q32851) and Agilent 2100 Bioanalyzer using the Agilent DNA 1000 Kit (Agilent, Cat. No. 5067-1504). If library validation must be carried out on an Advanced Analytical Fragment Analyzer, use the High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical, Cat. No. DNF-474). Please refer to the corresponding user manuals for detailed instructions.

1. Use 1 µl of undiluted library for quantitation using a Qubit 2.0 Fluorometer (see manufacturer's instructions for more details).
2. Use 1 µl of undiluted library for validation using the Agilent 2100 Bioanalyzer (see manufacturer's instructions for more details).
3. Following validation, libraries are ready for sequencing on Illumina platforms. See Appendix B for sequencing guidelines.

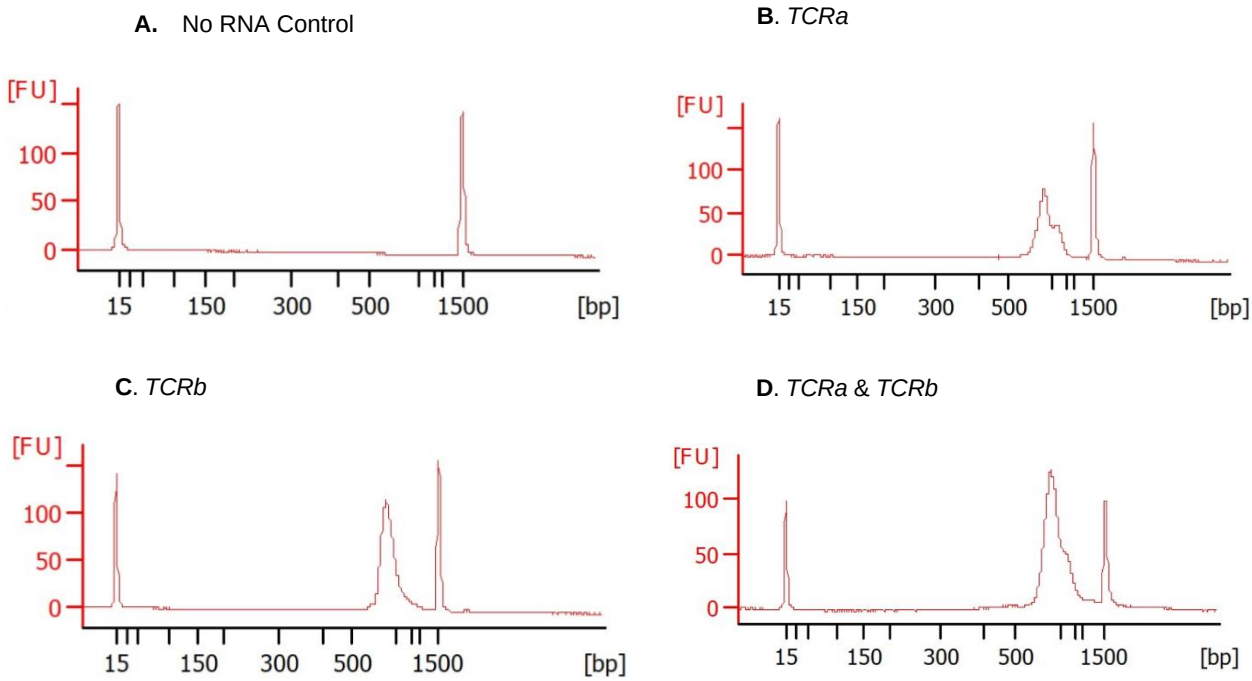

Figure 3. Example electropherogram results for *TCRa + TCRb* libraries. Libraries containing *TRA* and *TRB* sequences were generated using the SMARTer Human TCR a/b Profiling Kit v2 and 10 ng of Control RNA, respectively. Libraries were produced using 18 amplification cycles for PCR 1 and 18 amplification cycles for PCR 2. Following purification and size selection, libraries were analyzed on an Agilent 2100 Bioanalyzer (**Panels A–D**). **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: Illumina UDIs

Appropriate combinations of Illumina indexes are necessary to ensure enough nucleotide diversity and allow for discrimination between samples when sequencing a pool of two or more libraries on a single flow cell lane. Please consult the following resources for component information, best practices, pooling strategies, an index plate map, and index sequences.

- Unique Dual Index Kit Protocol-At-A-Glance
  (download at takarabio.com/resourcedoment/x102163)
- Unique Dual Index Sequence Information (Excel file)
  (download at takarabio.com/resourcedoment/x104651)

The SMARTer RNA Unique Dual Index Kit – 24U contains indexed PCR primers carrying the "IDT for Illumina UD" index sequences and offers up to 24 unique dual indexes (Table 3). Dual-index sequencing on a paired-end flow cell follows one of two workflows. Use i5 in the Table3 depending on the system. The indexed PCR primers are supplied predispensed in 8-tube PCR strips (individually labeled U25 to U48).

Table 3. SMARTer RNA unique dual index sequences

Index name	i7 bases for sample sheet	i5 bases for sample sheet (MiSeq®, NovaSeq™, HiSeq® 2000/2500)	i5 Bases for Sample Sheet (MiniSeq™, NextSeq®, HiSeq 3000/4000)
U25	ACTAAGAT	AACCGCGG	CCGCGGTT
U26	GTCGGAGC	GGTTATAA	TTATAACC
U27	CTTGGTAT	CCAAGTCC	GGACTTGG
U28	TCCAACGC	TTGGACTT	AAGTCCAA
U29	CCGTGAAG	CAGTGGAT	ATCCACTG
U30	TTACAGGA	TGACAAGC	GCTTGTCA
U31	GGCATTCT	CTAGCTTG	CAAGCTAG
U32	AATGCCTC	TCGATCCA	TGGATCGA
U33	TACCGAGG	CCTGAACT	AGTTCAGG
U34	CGTTAGAA	TTCAGGTC	GACCTGAA
U35	AGCCTCAT	AGTAGAGA	TCTCTACT
U36	GATTCTGC	GACGAGAG	CTCTCGTC
U37	TCGTAGTG	AGACTTGG	CCAAGTCT
U38	CTACGACA	GAGTCCAA	TTGGACTC
U39	TAAGTGGT	CTTAAGCC	GGCTTAAG
U40	CGGACAAC	TCCGGATT	AATCCGGA
U41	ATATGGAT	CTGTATTA	TAATACAG
U42	GCGCAAGC	TCACGCCG	CGGCGTGA
U43	AAGATACT	ACTTACAT	ATGTAAGT
U44	GGAGCGTC	GTCCGTGC	GCACGGAC
U45	ATGGCATG	AAGGTACC	GGTACCTT
U46	GCAATGCA	GGAACGTT	AACGTTCC
U47	GTTCCAAT	AATTCTGC	GCAGAATT
U48	ACCTTGGC	GGCCTCAT	ATGAGGCC

Appendix B: Guidelines for Library Sequencing and Data Analysis

A. Pooling Recommendations Following Library Validation by Qubit and Bioanalyzer

Prepare the desired library pools for the sequencing run. Prior to pooling, libraries must be carefully quantified. By combining the quantification obtained by the Qubit with the library size as determined by the Bioanalyzer, the concentration in ng/µl can be converted to nM. The following web tool is convenient for this conversion. Alternatively, libraries can be quantified by qPCR using the Library Quantification Kit (Cat. No. 638324), which was used to determine all loading concentrations in Table 4.

B. PhiX Control Spike-In Recommendations

Samples should be pooled to a final pool concentration of 4 nM for denaturation. We recommend using Qubit to determine each library concentration for pooling and using qPCR to determine accurate loading concentration for sequencing. 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 4.

Table 4. 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.

For full-length analysis, 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 (Figure 4). To cover the entire CDR3 region, it is possible to perform with single-read, 1 x 150 base reads but won't provide UMI information (Figure 4) To capture the UMI sequence, the 5′ end of the *TRA/B* variable region, and the entire CDR3 region, use paired-end, 2 x 150 base reads (Figure 5) and the following Illumina instrument and kit combinations (Table 5).

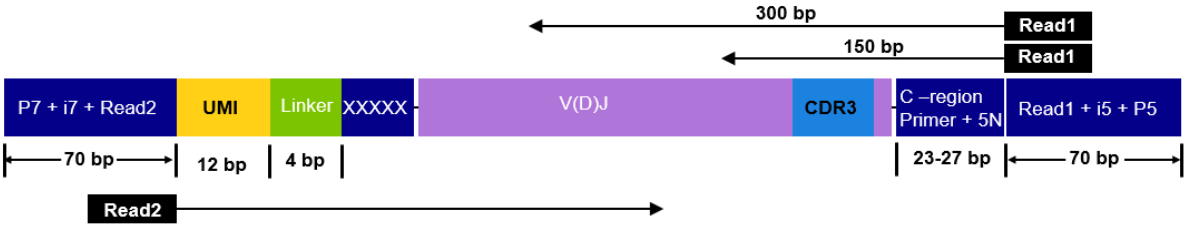

Figure 4. SMARTer human TCR profiling v2 library structure. The first 19 nt from Read 2 can be trimmed off if UMI analysis is not performed.

Table 5. Illumina instrument and reagent kit recommendations

Sequencer	Kit
MiniSeq	MiniSeq High Output reagent kit 300-cycle (Cat. No. FC-420-1003) MiniSeq 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)

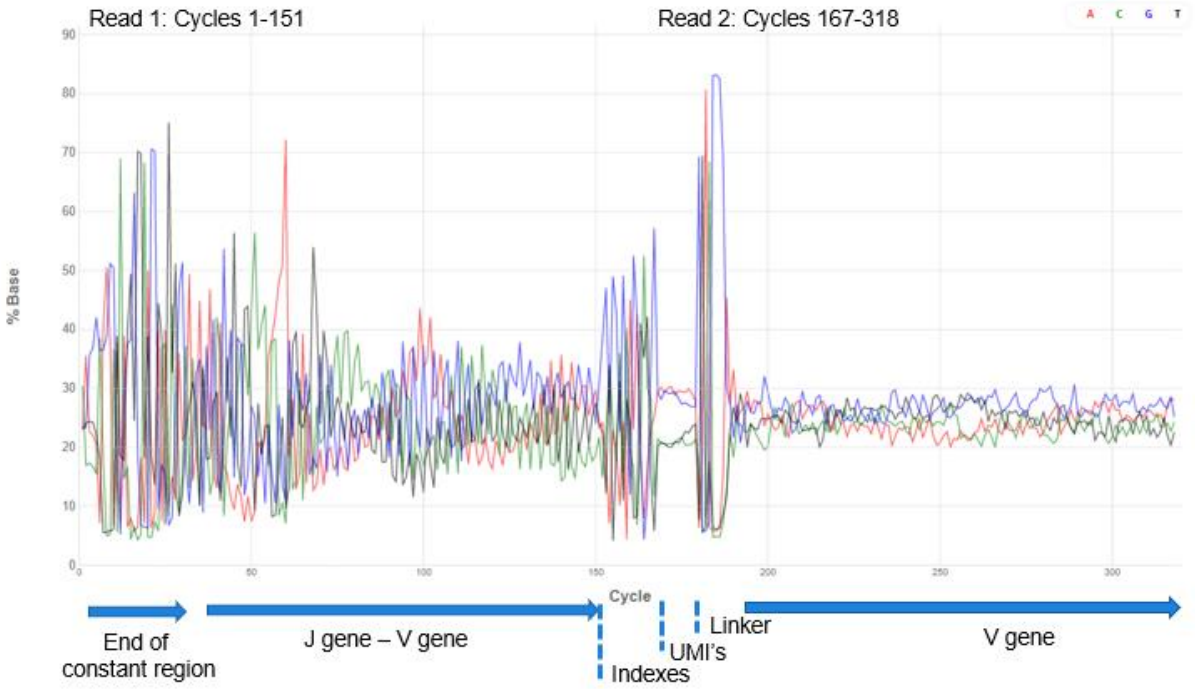

Figure 5. Percentage base calling from a typical NextSeq *TCRa + TCRb* profiling run. Cycles 1–151 can cover entire CDR3 regions for the *TRA* and *TRB* genes, respectively. Cycles 152–167 are the index reads.

We generally recommend a minimum of 500,000 reads for *TCRa* and *TCRb* libraries from an input of 10 ng PBMC RNA (or 10 ng T-cell RNA) input. For libraries generated from >10 ng PBMC RNA, higher sequencing depth is recommended, as shown in Table 6 below. However, the optimal conditions may vary for different samples 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 6. Recommended sequencing depth for TCR libraries prepared from human PBMC RNA or T-cell RNA

	10 ng PBMC RNA input	100 ng PBMC RNA input	1,000 ng PBMC RNA input	1 ng T-cell RNA input	10 ng T-cell RNA input	100 ng T-cell RNA input
***TCRa*, *TCRb* mixed library**	~500K reads	~5M reads	~25M reads	~50K reads	~500K reads	~5M reads
TCRa	~150K reads	~1.5M reads	~7.5M reads	~15K reads	~150K reads	~1.5M reads
TCRb	~350K reads	~3.5M reads	~17.5M reads	~35K reads	~350K reads	~3.5M reads

Note that the initial 12 bases of Read 2 include a random UMI sequence. These sequences will be available for UMI collapse when analyzed on the Cogent NGS Immune Profiler Software. To obtain the Cogent NGS Immune Profiler Software, please visit takarabio.com/ngs-immune-profiler.

Contact Us	
Customer Service/Ordering	**Technical Support**
tel: 800.662.2566 (toll-free)	tel: 800.662.2566 (toll-free)
fax: 800.424.1350 (toll-free)	fax: 800.424.1350 (toll-free)
web: http://www.takarabio.com/service	web: http://www.takarabio.com/support
e-mail: ordersUS@takarabio.com	e-mail: technical_support@takarabio.com

Notice to Purchaser

Our products are to be used for **Research Use Only**. They may not be used for any other purpose, including, but not limited to, use in humans, therapeutic or diagnostic use, or commercial use of any kind. Our products may not be transferred to third parties, resold, modified for resale, or used to manufacture commercial products or to provide a service to third parties without our prior written approval.

Your use of this product is also subject to compliance with any applicable licensing requirements described on the product's web page at takarabio.com. It is your responsibility to review, understand and adhere to any restrictions imposed by such statements

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