Takara Bio USA, Inc.

SMARTer® Human TCR a/b Profiling Kit User Manual

Cat. Nos. 635014, 635015 & 635016
(021825)

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I. Introduction

T-cell receptor profiling using SMART® Technology

The **SMARTer Human TCR a/b Profiling Kit** (Cat. Nos. 635014, 635015, 635016) 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 (depending on the sample type) and has been shown to yield high-quality sequencing libraries from as little as 10 ng to 3 µg of total RNA obtained from peripheral blood leukocytes, or from 50 to 10,000 purified 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).

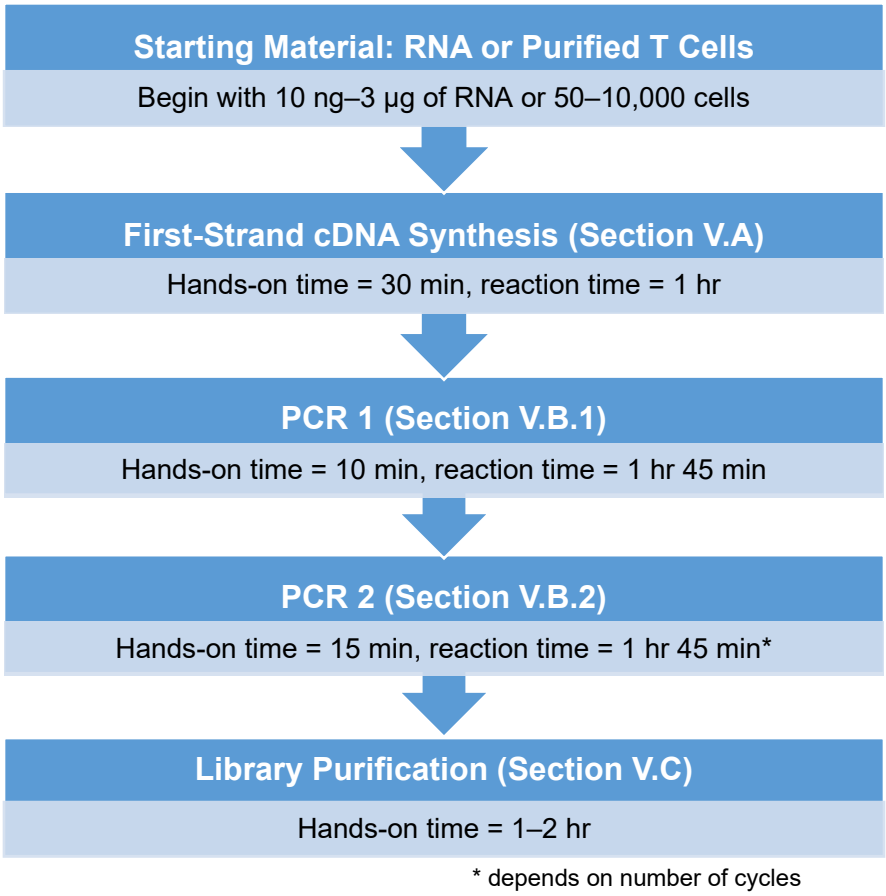

Figure 1. Protocol overview and timeline.

The SMARTer Human TCR a/b Profiling Kit 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. First-strand cDNA synthesis is dT-primed (TCR dT Primer) and performed by the MMLV-derived SMARTScribe™ Reverse Transcriptase (RT), which adds non-templated nucleotides upon reaching the 5' end of each mRNA template. The SMART-Seq® v4 Oligonucleotide anneals to these non-templated 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 "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, two rounds of PCR are performed in succession to amplify cDNA sequences corresponding to variable regions of TCR-α and/or TCR-β transcripts:

- The first PCR uses the first-strand cDNA as a template and includes a forward primer with complementarity to the SMART sequence (SMART Primer 1), and a reverse primer that is complementary to the constant (i.e. non-variable) region of TCR-α and/or TCR-β (TCRa/b Human Primer 1); both reverse primers may be included in a single reaction if analysis of both TCR subunit chains is desired. 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/or TCR-β cDNA.
- The second PCR takes the product from the first PCR as a template, and uses semi-nested primers (TCR Primer 2 and TCRa/b Human Primer 2) to amplify the entire variable region and a portion of the constant region of TCR-α and/or TCR-β cDNA. As in PCR 1, either or both TCR subunit chains may be amplified in a single reaction. The forward and reverse primers include adapter and index sequences which 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.

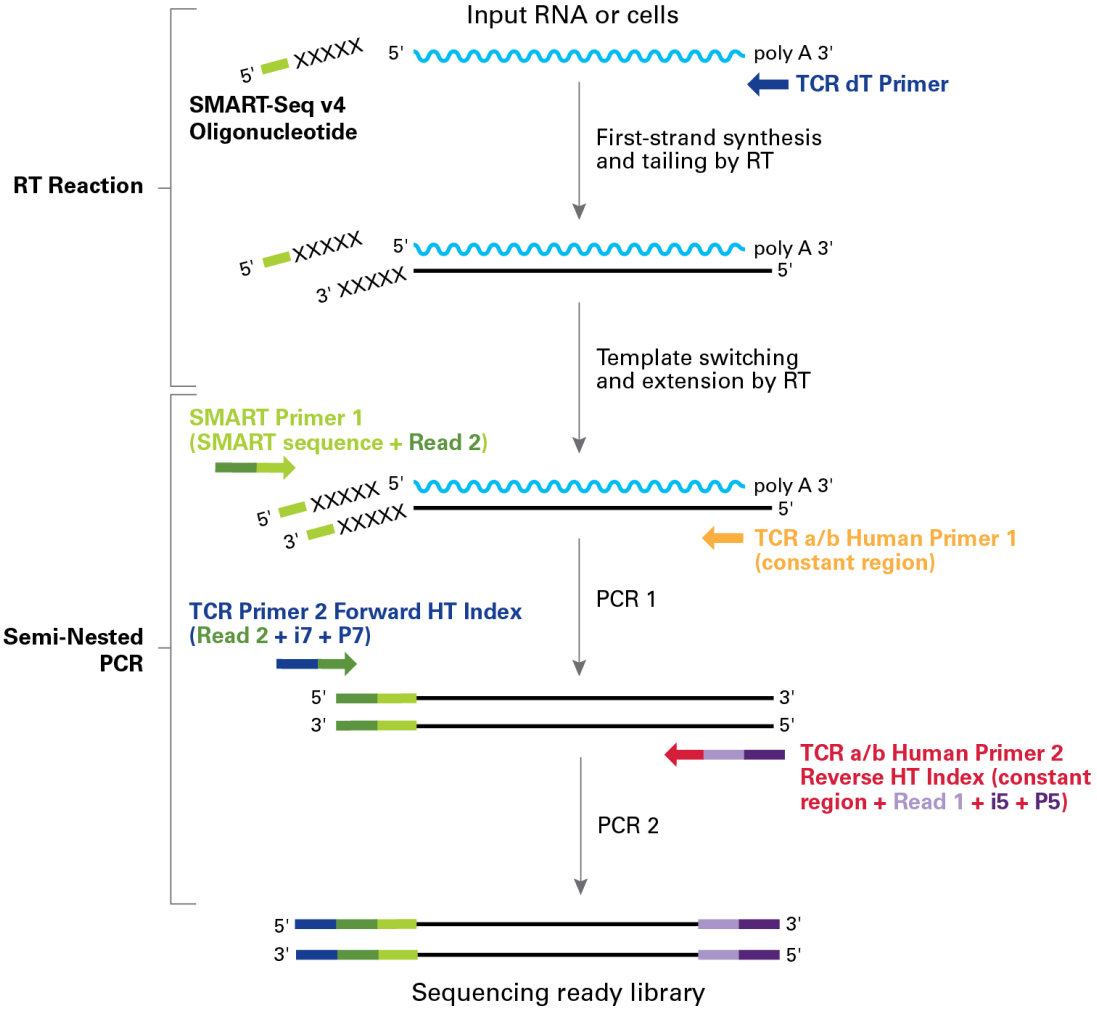

Figure 2. Schematic of technology and workflow for SMARTer Human TCRa/b Profiling Kit.

II. List of Components

The SMARTer Human TCR a/b Profiling Kit consists of the SMARTer Human TCR a/b Profiling Kit Components (not sold separately), the TCR a/b Human Indexing Primer Set HT for Illumina Components (not sold separately), and SeqAmp™ DNA Polymerase. **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 libraries (TCR-α alone, TCR-β alone, or TCR-α and TCR-β combined) that can be generated with the indexing primers supplied.

SMARTer Human TCR a/b Profiling Kit	**Cap color**	**635014 (12 rxns)**	**635015 (48 rxns)**	**635016 (96 rxns)**
SeqAmp DNA Polymerase (Store at –20°C.)				
SeqAmp DNA Polymerase	-	50 µl	200 µl	2 x 200 µl
SeqAmp PCR Buffer	-	1.25 ml	4 x 1.25 ml	8 x 1.25 ml
SMARTer Human TCR a/b Profiling Kit Components (Not sold separately. Storage conditions are listed below for Package 1 and Package 2.)				
Package 1 (Store at –70°C.)				
Control RNA (1 µg/µl)	Neutral	5 µl	5 µl	5 µl
Package 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.)				
SMART-Seq v4 Oligonucleotide (48 µM)	Pink	12 µl	48 µl	96 µl
TCR dT Primer (12 µM)	Blue	24 µl	96 µl	192 µl
5X Ultra® Low First-Strand Buffer	Red	48 µl	192 µl	384 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	Purple	24 µl	96 µl	192 µl
Nuclease-Free Water	-	1 ml	2 x 1 ml	4 ml
RNase Inhibitor (40 U/µl)	White	30 µl	120 µl	240 µl
10X Lysis Buffer	Neutral/-*	230 µl	920 µl	1.85 ml
Elution Buffer (10 mM Tris-Cl, pH 8.5)	-	1.7 ml	2 x 1.7 ml	4 x 1.7 ml
TCRa Human Primer 1 (12 µM)	Orange	12 µl	48 µl	96 µl
TCRb Human Primer 1 (12 µM)	Orange	12 µl	48 µl	96 µl
SMART Primer 1 (12 µM)	Green	12 µl	48 µl	96 µl

TCR a/b Human Indexing Primer Set HT for Illumina components are listed on the next page.

*Depending on the product size, 10X Lysis buffer is packaged in a 1.5 ml tube with a neutral cap (Cat. Nos. 635014 and 635015) or in an 8 ml bottle (Cat. No. 635016).

SMARTer Human TCR a/b Profiling Kit, continued	**Cap color**	**635014 (12 rxns)**	**635015 (48 rxns)**	**635016 (96 rxns)**
TCR a/b Human Indexing Primer Set HT for Illumina (Not sold separately. Store at –20°C. For details see Appendix A)				
TCRa Human Primer 2 Reverse HT Index 1 (aR1; 12.5 µM)	Red	12 µl	12 µl	12 µl
TCRa Human Primer 2 Reverse HT Index 2 (aR2; 12.5 µM)	Red	-	12 µl	12 µl
TCRa Human Primer 2 Reverse HT Index 3 (aR3; 12.5 µM)	Red	-	12 µl	12 µl
TCRa Human Primer 2 Reverse HT Index 4 (aR4; 12.5 µM)	Red	-	12 µl	12 µl
TCRa Human Primer 2 Reverse HT Index 5 (aR5; 12.5 µM)	Red	-	-	12 µl
TCRa Human Primer 2 Reverse HT Index 6 (aR6; 12.5 µM)	Red	-	-	12 µl
TCRa Human Primer 2 Reverse HT Index 7 (aR7; 12.5 µM)	Red	-	-	12 µl
TCRa Human Primer 2 Reverse HT Index 8 (aR8; 12.5 µM)	Red	-	-	12 µl
TCRb Human Primer 2 Reverse HT Index 1 (bR1; 12.5 µM)	Red	12 µl	12 µl	12 µl
TCRb Human Primer 2 Reverse HT Index 2 (bR2; 12.5 µM)	Red	-	12 µl	12 µl
TCRb Human Primer 2 Reverse HT Index 3 (bR3; 12.5 µM)	Red	-	12 µl	12 µl
TCRb Human Primer 2 Reverse HT Index 4 (bR4; 12.5 µM)	Red	-	12 µl	12 µl
TCRb Human Primer 2 Reverse HT Index 5 (bR5; 12.5 µM)	Red	-	-	12 µl
TCRb Human Primer 2 Reverse HT Index 6 (bR6; 12.5 µM)	Red	-	-	12 µl
TCRb Human Primer 2 Reverse HT Index 7 (bR7; 12.5 µM)	Red	-	-	12 µl
TCRb Human Primer 2 Reverse HT Index 8 (bR8; 12.5 µM)	Red	-	-	12 µl
TCR Primer 2 Forward HT Index 1 (F1; 12.5 µM)	Blue	12 µl	12 µl	12 µl
TCR Primer 2 Forward HT Index 2 (F2; 12.5 µM)	Blue	12 µl	12 µl	12 µl
TCR Primer 2 Forward HT Index 3 (F3; 12.5 µM)	Blue	12 µl	12 µl	12 µl
TCR Primer 2 Forward HT Index 4 (F4; 12.5 µM)	Blue	12 µl	12 µl	12 µl
TCR Primer 2 Forward HT Index 5 (F5; 12.5 µM)	Blue	12 µl	12 µl	12 µl
TCR Primer 2 Forward HT Index 6 (F6; 12.5 µM)	Blue	12 µl	12 µl	12 µl
TCR Primer 2 Forward HT Index 7 (F7; 12.5 µM)	Blue	12 µl	12 µl	12 µl
TCR Primer 2 Forward HT Index 8 (F8; 12.5 µM)	Blue	12 µl	12 µl	12 µl
TCR Primer 2 Forward HT Index 9 (F9; 12.5 µM)	Blue	12 µl	12 µl	12 µl
TCR Primer 2 Forward HT Index 10 (F10; 12.5 µM)	Blue	12 µl	12 µl	12 µl
TCR Primer 2 Forward HT Index 11 (F11; 12.5 µM)	Blue	12 µl	12 µl	12 µl
TCR Primer 2 Forward HT Index 12 (F12; 12.5 µM)	Blue	12 µl	12 µl	12 µl

Storage Conditions

- Store Control RNA at –70°C.
- Store 10X Lysis Buffer 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
- 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 first-strand cDNA synthesis (Protocol V.A)
- One dedicated thermal cycler used only for library amplification by PCR (Protocol V.B)
- For validation using the Advanced Analytical Fragment Analyzer: High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical, Cat. No. DNF-474; Protocol V.D)
- For validation using the Agilent 2100 Bioanalyzer: DNA 1000 Kit (Agilent, Cat. No. 5067-1504; Protocol V.D)
- 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 (Protocol V.C):

- Agencourt AMPure XP PCR purification kit—used to purify amplified 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 min). 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 USA, Inc., Cat. No. 635011)
    *Please visit takarabio.com/rna-seq-tips for a video tutorial on how to construct your own efficient magnetic separation device from rare earth bar magnets and a tip rack.
  - 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 clean room 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 clean room 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.**
- 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.
- 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**
  - The performance of the SMARTer Human TCR a/b Profiling Kit is dependent on the quality of the RNA starting material.

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

  - 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 from extremely small samples (e.g., we offer the NucleoSpin RNA XS kit for purification of RNA from ≥100 cells; Cat. No. 740902.10).

- **Evaluation of RNA Quality**
  After RNA extraction, if your sample size is not limiting, we recommend evaluating total RNA quality using the Agilent RNA 6000 Pico Kit (Cat. No. 5067-1513). Refer to the manufacturer's instructions about how to use the Agilent RNA 6000 Pico Kit.

D. Sample Requirements

1. Total RNA

This protocol has been optimized for cDNA synthesis starting from 10 ng of total RNA. However, if your RNA sample is not limiting, we recommend that you start with more total RNA (up to 3 µg). Purified total RNA should be in nuclease-free water.

2. Cells

This protocol has been used successfully to generate cDNA starting from 50–10,000 intact, cultured cells**. It cannot be used with cells that have undergone fixation.**

IMPORTANT: Cells should be washed and then resuspended in PBS that is free of Mg^{2+} and Ca^{2+} ions prior to lysis to remove the culture media. The presence of media can interfere with the first-strand synthesis.

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 10 ng of input RNA. 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 SMART-Seq v4 Oligonucleotide 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 (tube with a neutral cap or bottle, depending on product size), RNase Inhibitor (white cap), Nuclease-Free Water, Control RNA (neutral cap), TCR dT Primer (blue cap), 5X Ultra Low First-Strand Buffer (red cap), SMART-Seq v4 Oligonucleotide (pink cap), 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 enzyme) 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 10X Reaction Buffer by mixing the 10X Lysis Buffer with the RNase Inhibitor as indicated below (scale up as needed):

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**	

Mix briefly, then spin down.

NOTE: 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 sample (10.5 µl total volume) in individual 0.2 ml RNase-free PCR tubes or in 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. Bring the volume to 9.5 µl with nuclease-free water. Add 1 µl of 10X Reaction Buffer.
- **Cells:** If you are working with cells, isolate cells in validated media, wash and resuspend in PBS that is free of Mg^{2+} and Ca^{2+} ions, and transfer to a 0.2 ml RNase-free PCR tube. Bring the volume to 9.5 µl with nuclease-free water. Add 1 µl of 10X Reaction Buffer. Gently vortex or pipette to mix the sample. Incubate at room temperature for 5 min. See Section IV.D for more information on working with cells.

Table 1. Sample Preparation Guidelines

Components	Negative Control	Positive Control	Test Sample
10X Reaction Buffer (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 is supplied at a concentration of 1 µg/µl. It 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 your 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 (21 cycles for PCR 1 and 20 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.

NOTE: Prepare your Master Mix (Step 6) while your tubes are incubating. The enzyme will be added just before use (Step 9). Steps 10–11 below are critical for first-strand cDNA synthesis and should not be delayed after completing Step 7.

6. Prepare enough Master Mix for all the reactions, plus 10% of the total reaction mix volume, 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	SMART-Seq v4 Oligonucleotide (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 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 using.

   **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.

7. Mix the Master Mix well 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 3 min incubation at 72°C, 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 thermal cycler with a heated lid, preheated to 42°C. Run the following program:

42°C	45 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. We *strongly* recommend that you try a range of different cycle amounts to determine the minimum number of cycles necessary to obtain the desired yield for your sample and conditions. 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 Amount of Starting Material

Input Type	Input Amount	Number of PCR 1 cycles	Number of PCR 2 cycles
Control RNA	10–100 ng	21	18–20
Control RNA	100–1,000 ng	21	12–18
Control RNA	1,000–3,000 ng	18	15
Purified T cells	50–1,000 cells	21	18–20
Purified T cells	1,000–10,000 cells	21	15–18

1. PCR 1

This PCR selectively amplifies TCR sequences from the first-strand cDNA generated with the previous protocol. SMART 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/or TCRb Human Primer 1 anneal to sequences in the constant regions of TCRa and TCRb cDNA, respectively.

For this protocol you will need the following components:
2X SeqAmp PCR Buffer, SMART Primer 1 (green cap), TCRa Human Primer 1 (orange cap) and/or TCRb Human Primer 1 (orange cap), SeqAmp DNA Polymerase, Nuclease-Free Water.

1. Thaw all the reagents needed for PCR (except the enzyme) on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
2. Prepare enough PCR Master Mix for all the reactions, plus 10% of the total reaction mix volume. Combine the following reagents (per reaction) in the order shown:

25 µl	2X SeqAmp PCR Buffer	
0.5 µl	SMART Primer 1 (12 µM; green cap)	
0.5 µl	TCRa Human Primer 1 and/or TCRb Human Primer 1	(12 µM; orange cap)*
1 µl	SeqAmp DNA Polymerase	
3 µl	Nuclease-Free Water	
30 µl	**Total volume added per reaction**	

*TCRa Human Primer 1 and TCRb Human Primer 1 can be used separately or mixed together to yield information on both subunits. If using TCRa Human Primer 1 in combination with TCRb Human Primer 1, we suggest mixing 2 µl of TCRa Human Primer 1 with 1 µl of TCRb Human Primer 1 and using 0.5 µl of the mixture (scale up as required). However, if achieving equal coverage for each subunit is critical to your experiments we recommend performing the TCRa and TCRb reactions separately.

NOTE: Remove the SeqAmp DNA 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 spin the tube briefly to collect the contents at the bottom of the tube.

3. Add 30 µl of PCR 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.

4. Place the tube(s) in a preheated thermal cycler with a heated lid and run the following program:

PCR 1

95°C	1 min
18–21 cycles*:	
94°C	1 min
53°C	1 min
68°C	1 min
72°C	10 min
4°C	forever

*Consult Table 2 (above) for PCR cycle number guidelines

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

2. PCR 2

This PCR reaction 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 SMART 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 (R1–R8) must be used for each sample if samples are to be multiplexed on a single flow cell. See Appendix A for further details.

For this protocol you will need the following components:
2X SeqAmp PCR Buffer, TCR a/b Human Indexing Primer Set HT for Illumina (blue and red caps), SeqAmp DNA Polymerase, Nuclease-Free Water.

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

Per reaction

25 µl	2X SeqAmp PCR Buffer	
0.5 µl	TCRa and/or TCRb Human Primer 2 Reverse HT Index	(12 µM; red cap)*
1 µl	SeqAmp DNA Polymerase	
22 µl	Nuclease-Free Water	
48.5 µl	**Total volume added per reaction**	

*As for PCR 1, the TCRa and TCRb Human Primer 2 Reverse HT Index primers can be used separately or mixed together to yield information on both subunits. If using TCRa Human Primer 2 Reverse HT Index in combination with TCRb Human Primer 2 Reverse HT Index, we suggest mixing these primers at the same ratio used for PCR 1 and using 0.5 µl of the mixture (scale up as required). However, if achieving equal coverage for each subunit is critical to your experiments we recommend performing the TCRa and TCRb reactions separately. If all samples are to be pooled and sequenced in the same lane, each sample must have a unique combination of sequencing indexes. However, not all indexes can be pooled for multiplexing. Consult Appendix A and Illumina literature for more information.

NOTE: Remove the SeqAmp DNA 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 spin the tube briefly to collect the contents at the bottom of the tube.

3. For each reaction, add 48.5 µl of PCR Master Mix to a clean 0.2 ml tube.
4. Add 1 µl of PCR product from PCR 1 to each tube.
5. Add 0.5 µl of the appropriate TCR Primer 2 Forward HT Index primer (12.5 µM; blue cap) to each sample. Mix well and briefly spin to collect the contents at the bottom of the tube(s).

6. 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 reduced cycle numbers)

95°C	1 min
12–20 cycles*:	
94°C	1 min
53°C	1 min
68°C	1 min
72°C	10 min
4°C	forever

*Consult Table 2 (above) for PCR cycle number guidelines.

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

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

The TCR sequencing library is size-selected and purified using AMPure XP beads and either of two protocols:

- **Option 1 - Size Selection for Library Validation with a Fragment Analyzer (recommended)**
  This approach involves a double size selection, which removes primers, primer dimers, and PCR products containing undersized or oversized inserts. For the first selection, fragments larger than ~900 bp are immobilized on beads and removed from the supernatant. For the second selection, 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.
- **Option 2 – Size Selection for Library Validation with a Bioanalyzer (if necessary)**
  This approach involves an additional double size selection, as well as additional ethanol wash and elution steps. These additional steps are necessary for obtaining consistent library validation results using a Bioanalyzer.

Table 3. Alternative Volumes of AMPure XP Beads to Use for Library Size Selection and Purification.

Validation method	First bead volume (µl)	Second bead volume (µl)	Third bead volume (µl)	Fourth bead volume (µl)
1. Fragment Analyzer	25	10	-	-
2. Bioanalyzer	25	10	25	25

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 min 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:

Agencourt AMPure XP PCR Purification beads, 80% ethanol (made fresh), a magnetic separation device, Elution Buffer.

Option 1. Size-Selection Protocol for Library Validation with a Fragment Analyzer

1. Add 1 µl of 10X Lysis Buffer to each PCR product from Protocol V.B.
2. Vortex AMPure XP beads until evenly mixed, then add 25 µl of AMPure XP beads to each sample.
3. 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.**
4. Incubate at room temperature for 8 min to let the DNA bind to the beads.
5. 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.
6. 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.
7. 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.
8. 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.**
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 seconds 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.
14. OPTIONAL: 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 2 min to rehydrate (Step 15) 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 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 unpelleted beads 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 (without disrupting the existing pellet). 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.

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

Option 2. Size-Selection Protocol for Library Validation with a Bioanalyzer

1. Add 1 µl of 10X Lysis Buffer to each PCR product from Protocol V.B.
2. Vortex AMPure XP beads until evenly mixed, then add 25 µl of AMPure XP beads to each sample.
3. 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.**

4. Incubate at room temperature for 8 min to let the DNA bind to the beads.

5. 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.

6. 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.
7. 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.

8. 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.**

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 seconds 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.
14. OPTIONAL: 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 2 min to rehydrate (Step 16) 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 50 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 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 unpelleted beads 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 (without disrupting the existing pellet). Continue the incubation until there are no beads left in the supernatant.

19. Transfer clear supernatant (which contains your library) to clean PCR tubes.
20. Vortex AMPure XP beads until evenly mixed, then add 25 µl of AMPure XP beads to each sample.
21. 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.**

22. Incubate at room temperature for 8 min to let the DNA bind to the beads.
23. 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.

24. 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.
25. Remove the tubes containing the beads from the magnetic separation device and discard them. Add 25 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.

26. 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.**

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

29. 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).
30. 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.
31. Repeat the ethanol wash (Step 30) once.
32. OPTIONAL: 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.
33. 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 2 min to rehydrate (Step 34) and may reduce library recovery and yield.
34. 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 sides of the tube.
35. Incubate at room temperature for at least 5 min to rehydrate.
36. 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 unpelleted beads 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 (without disrupting the existing pellet). Continue the incubation until there are no beads left in the supernatant.
37. 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.

SAFE 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 an Advanced Analytical Fragment Analyzer and the High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical, Cat. No. DNF-474). If library validation must be carried out on an Agilent 2100 Bioanalyzer, perform two rounds of double size selection prior to

the analysis (Protocol V.C, Option 2) and use the DNA 1000 Kit (Agilent, Cat. No. 5067-1504). Please refer to the corresponding user manuals for detailed instructions.

1. Aliquot 1 µl of diluted library for validation using the Advanced Analytical Fragment Analyzer (libraries were diluted 1:5 for the examples shown on the next page). Alternatively, aliquot 1 µl of undiluted library for validation using the Agilent 2100 Bioanalyzer.
2. Compare the results for your samples with Figure 3 (on the next page) 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 650 bp to 1,150 bp, with a maximum between ~700 bp and ~800 bp for positive controls and samples containing TCR RNA. The position and shape of electropherogram peaks will vary depending on whether TCR-α and/or TCR-β sequence fragments are included in the library, the nature of the RNA sample, and the analysis method. Libraries consisting of either TCR-α or TCR-β sequence fragments yield electropherogram maxima that tend toward the upper and lower ends of the ~700–800 bp size spectrum, respectively. For some sample types (including RNA obtained from a single T-cell clonotype), analysis of libraries containing both TCR-α and TCR-β sequence fragments may yield two discernable peaks in the electropherogram results. In general, electropherogram peaks obtained with the Fragment Analyzer tend to be sharper than those obtained with the Bioanalyzer.
3. Following validation, libraries are ready for sequencing on Illumina platforms. See Appendix B for sequencing guidelines.

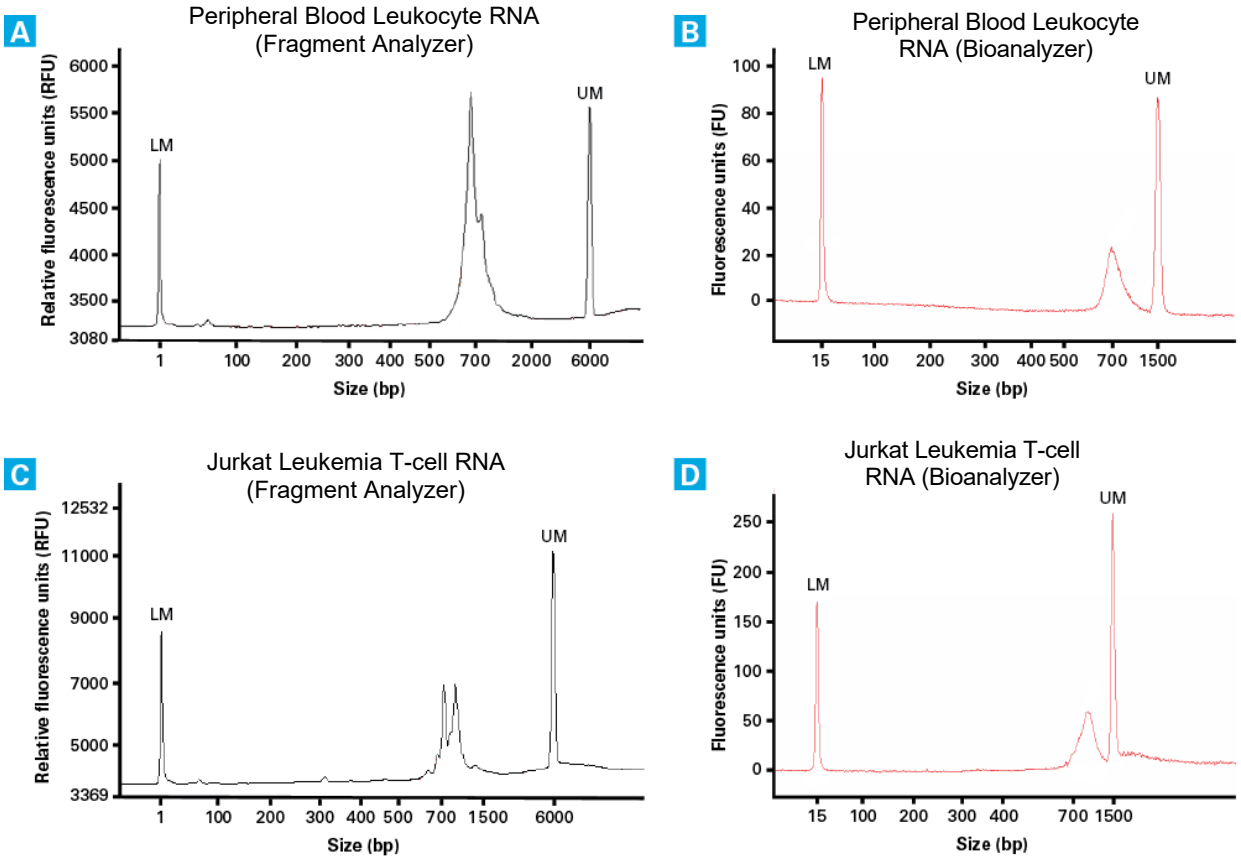

Figure 3. Example electropherogram results for TCR-α + TCR-β libraries. Libraries containing TCR-α and TCR-β sequences were generated using the SMARTer Human TCR a/b Profiling Kit and 10 ng of RNA obtained from either a heterogeneous population of peripheral blood leukocytes or a monoclonal line of Jurkat leukemia T cells, respectively. Both libraries were produced using 21 amplification cycles for PCR 1 and 20 amplification cycles for PCR 2. Following purification and size selection, both libraries were analyzed on an Advanced Analytical Fragment Analyzer (Panels A and C) and an Agilent 2100 Bioanalyzer (Panels B and D). Peaks labeled "LM" and "UM" correspond to DNA reference markers

included in each analysis. **Panel A and Panel B** show broad peaks between ~650–1150 bp and maximal peaks in the range of ~700–800 bp (typical results for a library generated from peripheral blood leukocyte RNA), analyzed on the Fragment Analyzer and Bioanalyzer, respectively. Distinct peaks representing TCR-α and TCR-β sequence fragments can be discerned when a library generated from Jurkat RNA is analyzed on the Fragment Analyzer (**Panel C**), but not when the same library is analyzed on the Bioanalyzer **(Panel D**).

Appendix A: Illumina HT Indexes

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. 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, respectively, the TCRa and TCRb Human Primer 2 Reverse HT Index primers contain the Read 1 sequence and i5 indexes. Depending on the kit size, 1–8 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 aR8" and "Human bR1"–"Human bR8", in correspondence with Illumina indexes D501–D508.

Pairwise combination of 12 unique i7 indexes with 8 unique i5 indexes allows for multiplexing of up to 96 samples in a single flow cell lane.

Table 4. 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**	**Primer ID (cap label)**	**Illumina ID**	**Index sequence**
F1	D701	ATTACTCG	Human aR1 or Human bR1	D501	TATAGCCT
F2	D702	TCCGGAGA	Human aR2 or Human bR2	D502	ATAGAGGC
F3	D703	CGCTCATT	Human aR3 or Human bR3	D503	CCTATCCT
F4	D704	GAGATTCC	Human aR4 or Human bR4	D504	GGCTCTGA
F5	D705	ATTCAGAA	Human aR5 or Human bR5	D505	AGGCGAAG
F6	D706	GAATTCGT	Human aR6 or Human bR6	D506	TAATCTTA
F7	D707	CTGAAGCT	Human aR7 or Human bR7	D507	CAGGACGT
F8	D708	TAATGCGC	Human aR8 or Human bR8	D508	GTACTGAC
F9	D709	CGGCTATG			
F10	D710	TCCGCGAA			
F11	D711	TCTCGCGC			
F12	D712	AGCGATAG			

Appendix B: Guidelines for Library Sequencing and Data Analysis

Samples should be pooled to a final pool concentration of 2–4 nM. 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. Upon completion of a sequencing run, data can be analyzed on a variety of platforms, including software hosted on BaseSpace, Illumina's cloud computing environment for next-generation sequencing.

Note that the initial 30 bases of read 2 (which include the SMART primer sequence) may need to be trimmed from raw sequence reads prior to downstream analyses (see Figure 4).

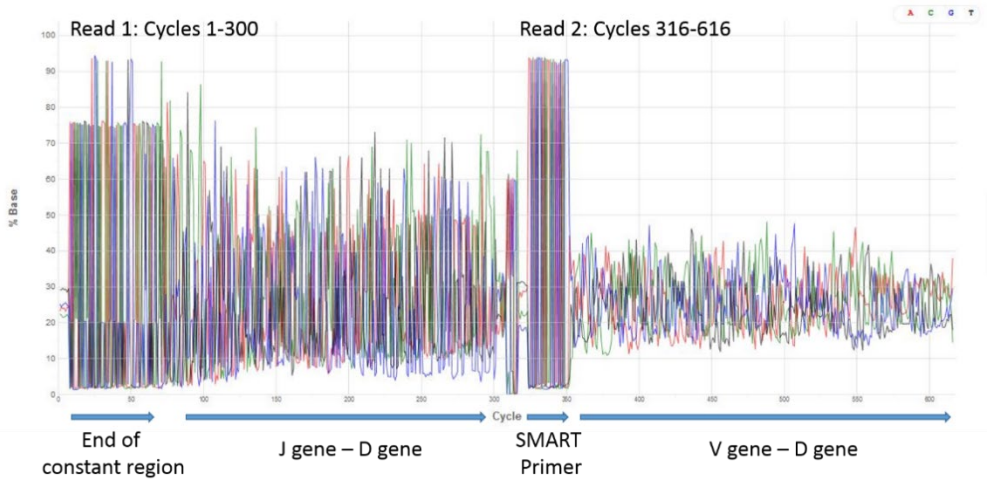

Figure 4. Percentage base calling from a typical MiSeq TCR-α + TCR-β profiling run. Note the SMART primer sequence at the beginning of read 2, which may need to be trimmed prior to data analysis. Cycles 301–315 are the index reads.

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