Takara Bio USA

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

Cat. Nos. 634814, 634815 & 634816
(032724)

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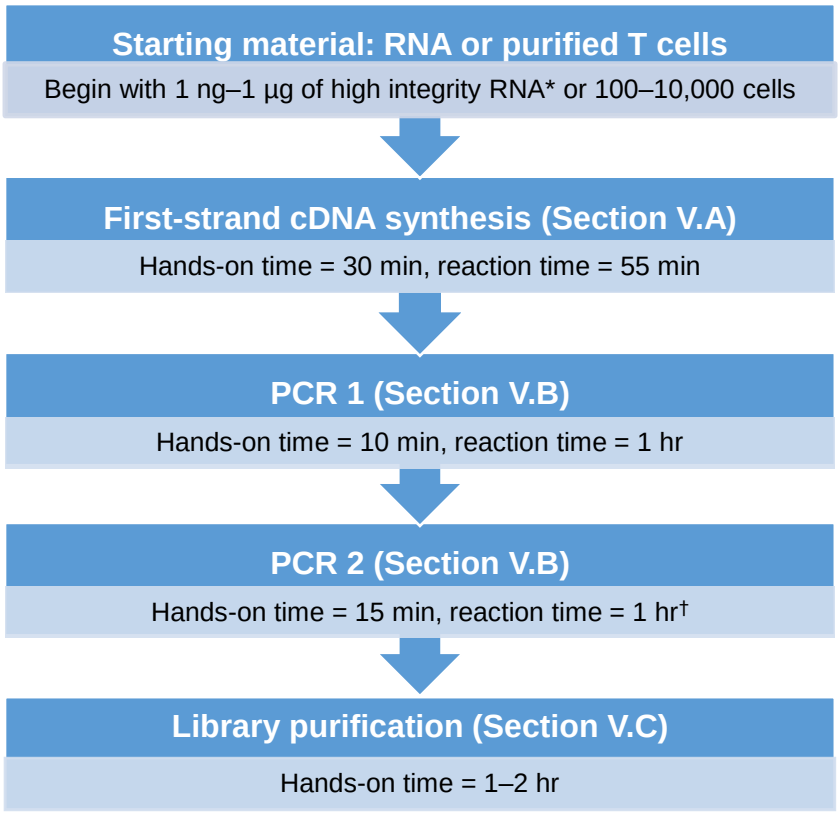
I. Introduction

SMART-Seq Mouse TCR (with UMIs) (Cat. Nos. 634814, 634815 & 634816) enables users to analyze T-cell receptor (TCR) diversity from mouse RNA samples or directly from cells. The kit is designed to work with high integrity total RNA (total RNA Integrity Number (RIN) >7) of various input quantity depending on the RNA source and has been shown to yield high-quality sequencing libraries from 1 ng–1 µg of total RNA obtained from mouse spleen or T cells; 10 ng–1 µg of total RNA obtained from mouse whole blood, bone marrow, or thymus; or from 100–10,000 purified mouse T cells (viability >70%).

The workflow can be used to generate data for both alpha- and beta-chain diversity, either in the same experiment or separately. Using the Unique Dual Index Kits (UDIs, Cat. Nos. 634752– 634756, sold separately), the protocol generates indexed libraries (up to 384 UDIs) that are ready for sequencing on Illumina® platforms. Sequencing reads of the SMART-Seq Mouse TCR (with UMIs) libraries can be analyzed using our free-of-charge Cogent™ NGS Immune Profiler (CogentIP) and web-based Cogent NGS Immune Viewer bioinformatics software (Appendix C). This provides an end-to-end solution from samples to publication-ready data.



*Depends on source of RNA (RIN > 7)
†Depends on number of cycles

Figure 1. SMART-Seq Mouse TCR (with UMIs) protocol overview and timeline.

SMART-Seq Mouse TCR (with UMIs) 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 mature TCR mRNA transcripts. It also incorporates unique molecular identifiers (UMIs) to correct PCR and sequencing errors, allowing greater confidence in clonotype identification.

Input RNA or cells are oligo-dT primed and followed by reverse transcription using SMARTScribe™ Reverse Transcriptase (RT). The RT adds nontemplated nucleotides at the 5′ end of each cDNA molecule (Figure 2). The TCR SMART UMI Oligo, carrying a partial Illumina adapter, anneals to the nontemplated nucleotides added by

the reverse transcriptase, promoting a template switch, and enabling the incorporation of UMIs as well as a partial Illumina adapter into the first-strand DNA. Following reverse transcription, two rounds of PCR are performed to amplify TCR cDNAs (Figure 2). To capture the entire V(D)J region, during PCR 1, primers anneal to sequences added by the TCR SMART UMI Oligo at one end and the TCR constant region(s) at the other end. The second PCR takes the product from the first PCR as a template and uses semi-nested primers to amplify the entire TCR variable region and a small portion of the constant region (Figure 2).

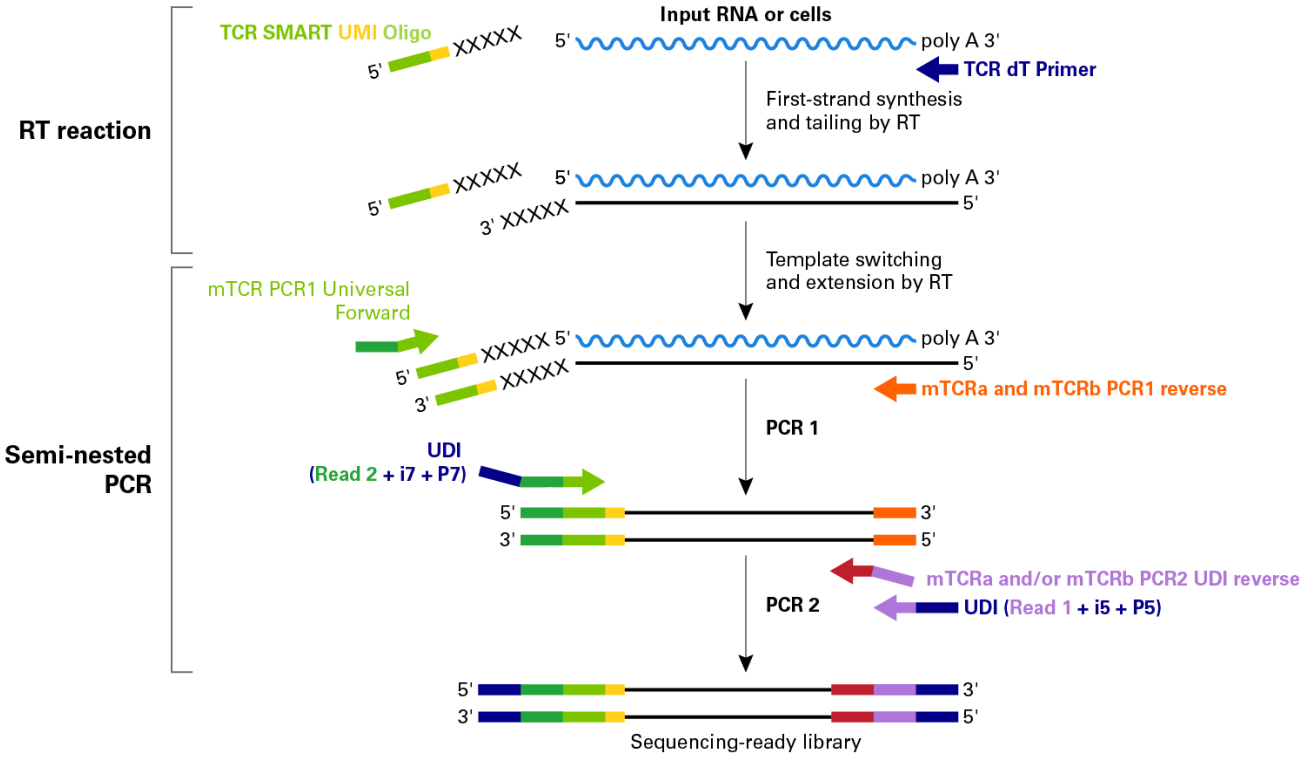


Figure 2. Schematic of the technology and workflow for SMART-Seq Mouse TCR (with UMIs). Input RNA or cells are oligo-dT primed using the TCR dT Primer (dark blue). Following oligo dT-priming, SmartScribe Reverse Transcriptase performs first strand cDNA synthesis on the sample and adds nontemplated nucleotides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

II. List of Components

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

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

Table 1. SMART-Seq Mouse TCR (with UMIs) components.

SMART-Seq Mouse TCR (with UMIs)	Cap color	634815 (24 rxns)	634816 (96 rxns)	634814 (384 rxns)
Box 1 (Store at –70°C)				
Control RNA (1 µg/µl)	-	5 µl	5 µl	4 x 5 µl
TCR SMART UMI Oligo (48 µM)	Pink	30 µl	120 µl	4 x 120 µl
Box 2 (Store at –20°C)				
10X Lysis Buffer*	Clear	230 µl	920 µl	4 x 920 µl
5X Ultra® Low First-Strand Buffer	Red	96 µl	384 µl	4 x 384 µl
TCR dT Primer (12 µM)	Blue	48 µl	192 µl	4 x 192 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	Purple	48 µl	192 µl	4 x 192 µl
RNase Inhibitor (40 U/µl)	White	60 µl	240 µl	4 x 240 µl
Elution Buffer†* (10 mM)	-	1.7 ml	4 x 1.7 ml	16 x 1.7 ml
Nuclease-Free Water	-	1.7 ml	4 x 1.7 ml	16 x 1.7 ml
PrimeSTAR® GXL Premix (2X)	White	2 x 1 ml	8 x 1 ml	32 x 1 ml
mTCR PCR1 Universal Forward (12 µM)	Green	24 µl	96 µl	4 x 96 µl
mTCRa PCR1 Reverse (12 µM)	Orange	24 µl	96 µl	4 x 96 µl
mTCRb PCR1 Reverse (12 µM)	Orange	24 µl	96 µl	4 x 96 µl
mTCRa PCR2 UDI Reverse (12 µM)	Green	24 µl	96 µl	4 x 96 µl
mTCRb PCR2 UDI Reverse (12 µM)	Green	24 µl	96 µl	4 x 96 µl

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

III. Additional Materials Required (Not Provided)

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 as you may not obtain the expected results:

- Unique Dual Index Kit
  - 96 indexes: Takara Bio, Cat. Nos. 634752, 634753, 634754, and/or 634755
  - 24 indexes: Takara Bio, Cat. No. 634756
- Single-channel pipettes: 10 µl, 20 µl, and 200 µl
- Eight-channel pipettes (recommended): 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, and 200 µl
- Minicentrifuge for 1.5 ml tubes
- Minicentrifuge for 0.2 ml tubes or strips

For PCR Amplification & Validation:

- Two thermal cyclers with heated lids
  - One thermal cycler used only for first-strand cDNA synthesis (Section V.A)
  - One thermal cycler used only for library amplification by PCR (Section V.B)

NOTE: The thermal cycler should always be used with the heated lid option turned on. If prompted to input a specific temperature, use 105°C.

Most thermal cyclers with heated lids will automatically adjust the lid temperature just above the highest block temperature within a cycling program. However, if your thermal cycler does not make this automatic adjustment, you may want to follow the manufacturer's instructions to choose a lower lid temperature for the reverse transcription step.

- For validation:
  - Agilent 2100 Bioanalyzer: Agilent High Sensitivity DNA Kit (Agilent Technologies, Cat. No. 5067-4626) or Agilent DNA 1000 Kit (Agilent Technologies, Cat. No. 5067-1504). The Agilent TapeStation (HSD1000/HSD5000) or Agilent Fragment Analyzer with the equivalent corresponding kits may also be used.
  - Qubit dsDNA HS Kit (Thermo Fisher Scientific, Cat. No. Q32851)
- Nuclease-free thin-wall PCR tubes, 96 well plates, or strips (0.2 ml PCR 8-tube strip; USA Scientific, Item No.1402-4700 or similar)
- Nuclease-free low-adhesion 1.5 ml tubes (USA Scientific, Item No. 1415-2600), LoBind tubes (Eppendorf, Cat. No. 022431021), or similar

For Bead Purifications (Section V.C):

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

NOTES:

– The kit has been specifically validated with the beads listed above. Substitutions may lead to unexpected results.

– Beads need to come to room temperature before the container is opened. We strongly recommend aliquoting the beads into 1.5 ml tubes upon receipt and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 min). Aliquoting is also instrumental in decreasing the chances of bead contamination.

- 100% ethanol (molecular biology grade)
- Magnetic separation device for small volumes—used to purify amplified libraries
  - For 8-tube strips: SMARTer-Seq™ Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011)
  - For 96-well plates: Magnetic Stand-96 (Thermo Fisher, Cat. No. AM10027)
- Optional, depending on the choice of magnetic separation device (96-well format):
  - 96-well V-bottom Plate (500 µl; VWR, Cat. No. 47743-996)
  - MicroAmp Clean Adhesive Seal (Thermo Fisher, Cat. No. 4306311)
- Low-speed benchtop centrifuge for a 96-well plate

IV. General Considerations

A. General Recommendations

IMPORTANT! For Cat. No. 634816 (96 rxns) and Cat. No. 634814 (4 x 96 rxns), we recommend performing a **minimum** of 12 reactions per protocol run to ensure sufficient reagents to utilize 96 (or 384) reactions per kit.

- We recommend using two physically separated workstations to minimize contamination:
  - **A PCR Clean Workstation** for all pre-PCR experiments that require clean room conditions, e.g., first strand cDNA synthesis (Section V.A). The PCR Clean Workstation should be in a clean room with positive air flow.
  - **A second workstation located in the general laboratory** where you will perform PCR (Section V.B) and measure library concentration (Section V.D).
- The assay is very sensitive to variations in pipette volume, etc. Please make sure all your pipettes are calibrated for reliable delivery and nothing is attached to the outside of the tips.
- All lab supplies related to cDNA synthesis need to be stored in a DNA-free, closed cabinet. Reagents for cDNA synthesis need to be stored in a freezer/refrigerator that has not previously been used to store PCR amplicons.
- Add enzymes to reaction mixtures last and thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- Do not increase (or decrease) the amount or concentration of any reaction component. The amounts and concentrations have been carefully optimized for the included reagents and protocol.
- If you are using this protocol for the first time, we strongly recommend that you perform negative and positive control reactions to verify that kit components are working properly.

B. Sample Recommendations

- This protocol has been optimized for 1 ng of total RNA from mouse spleen (RIN >7). However, if your RNA sample is not limiting, we recommend that you start with more (up to 1 µg). The purified total RNA should be in nuclease-free water.
- This protocol has been used successfully to generate cDNA starting from 100–10,000 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). **This kit cannot be used with cells that have undergone fixation.**
- Avoid using heparin for blood sample collection as it can inhibit downstream enzymatic steps such as cDNA synthesis and PCR.
- The success of your experiment depends on the quality of your input RNA. Prior to cDNA synthesis, please make sure that your RNA is intact and free of contaminants. Input RNA should be free from poly(A) carrier RNA that will interfere with oligo(dT)-primed cDNA synthesis.
- RNA should be of high integrity (RIN >7) to ensure that the 3′ poly(A) tail is intact to enable oligo(dT)-priming and that the 5′ end of the RNA is intact to allow for generation of accurate clonotype information.

After RNA extraction, if your sample size is not limiting, we recommend evaluating RIN using the Agilent High Sensitivity 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.

- When choosing a purification kit, ensure that it is appropriate for your sample amount. We recommend NucleoSpin RNA Plus, a mini kit for RNA purification with a DNA removal column (Takara Bio, Cat. No. 740984.50 or 740984.250).

V. Protocols

A. Protocol: First-Strand cDNA Synthesis

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

IMPORTANT: To avoid introducing contaminants into your samples, 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, RNase Inhibitor, Nuclease-Free Water, Control RNA, TCR dT Primer, 5X Ultra Low First-Strand Buffer, TCR SMART UMI Oligo, and SMARTScribe Reverse Transcriptase.

1. At room temperature, thaw the 5X Ultra Low First-Strand Buffer. Do not store on ice.
2. Thaw the remaining reagents needed for the first-strand cDNA synthesis (except the SMARTScribe Reverse Transcriptase) on ice. Gently vortex each reagent to mix and spin down briefly. Store on ice.
3. 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 for use in Step 4, plus 10% of the total reaction mix volume):

   Lysis/Inhibitor Mix:

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

   Mix briefly, then spin down.

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

4. See Table 2 (next page) for guidelines on setting up reactions for your control and test samples. Prepare each 10.5 µl total volume reaction in the wells of a nuclease-free 96-well PCR plate, individual nuclease-free 0.2 ml PCR tubes, or tubes of an 8-well strip:
   - **Purified total RNA:** If you are working with purified total RNA, transfer 1–9.5 µl to the plate well or tube. Add 1 µl of Lysis/Inhibitor Mix (from Step 3). Bring the volume to 10.5 µl with Nuclease-Free Water.
   - **Cells:** Bulk cell suspensions should be washed and resuspended in Mg 2^{+} -and $Ca2^{+}$-free PBS. It is best to perform at least two washes with low-speed centrifugation to avoid cell damage. Transfer ≤5 µl of the cell suspension in PBS to a plate well or tube containing the Lysis/Inhibitor mix from Step 3 and the appropriate volume of Nuclease-Free Water to bring the final reaction volume to 10.5 µl. Gently vortex or pipette to mix the sample. See Section IV.B for sample recommendations.

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

Table 2. Sample preparation guidelines.

Components	Sample	Negative control	Positive control
Lysis/Inhibitor Mix (from Step 3)	1 µl	1 µl	1 µl
Sample	1–9.5 µl	–	–
Diluted Control RNA*†	–	–	1–9.5 µl
Nuclease-Free Water	Up to 8.5 µl	9.5 µl	Up to 8.5 µl
Total volume	10.5 µl	10.5 µl	10.5 µl

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

†We have tested this protocol extensively using the PCR cycling conditions listed in Table 3 (21 cycles for PCR 1 and 18 cycles for PCR 2) with 10 ng of input Control RNA.

5. Place the samples on ice. Add 2 µl of the TCR dT Primer (12 µM) to each sample, mix well by gently vortexing, then spin the plate or tubes briefly to collect the contents at the bottom of the tube.
6. Incubate the tubes at 72°C in a preheated, hot-lid thermal cycler for 3 min.

NOTE: Prepare the RT Premix (Step 7) while your tubes are incubating. The SMARTScribe Reverse Transcriptase will be added just before use (Step 10). Steps 11 & 12 below are critical for first-strand cDNA synthesis and should not be delayed after completing Step 10.

7. In a tube, prepare enough RT Premix for all the reactions (scale up as needed), plus 10% of the total reaction mix volume, by combining the following reagents in the order shown at room temperature:

RT Premix:

4 µl	5X Ultra Low First-Strand Buffer*
1 µl	TCR SMART UMI Oligo
0.5 µl	RNase Inhibitor (40 U/µl)
5.5 µl	**Total volume per reaction**

*The Ultra Low First-Strand Buffer may form precipitates; vortex before using to ensure all components are completely in solution.

8. Immediately after the 3-min incubation at 72°C, place the samples on ice or at 4°C for at least 2 min (but no more than 10 min).
9. Preheat the thermal cycler to 42°C.
10. Remove the SMARTScribe Reverse Transcriptase from the freezer, centrifuge briefly. Calculate the total volume to add for all reactions based on 2 µl per reaction, plus 10% of the total volume, then add to the RT Premix (from Step 7) to make the RT Master Mix.

RT Master Mix:

5.5 µl	RT Premix
2 µl	SMARTScribe Reverse Transcriptase
7.5 µl	**Total volume per reaction**

Mix well by gently vortexing and then spin the tube briefly in a minicentrifuge to collect the contents at the bottom of the tube.

11. Add 7.5 µl of the RT Master Mix to each reaction tube or well. Mix the contents of each well or tube by pipetting gently and spin briefly.

12. Place the plate/tubes in the preheated thermal cycler. Run the following program:

42°C	45 min
70°C	10 min
4°C	forever

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

B. Protocol: TCRα/β Amplification and Sequencing Library Generation

Semi-nested PCR amplifies the entire V(D)J region and a portion of the constant region of TCRα/β cDNA and incorporates adapters and barcodes for Illumina sequencing platforms.

IMPORTANT: Table 3 provides PCR-cycling recommendations, but optimal parameters may vary for different sample types, input amounts, and thermal cyclers. We recommend trying a range of cycle numbers to determine the minimum number necessary to obtain the desired yield.

Table 3. Cycling guidelines based on the amount of starting material.

Input type	Input amount	Number of PCR1 cycles	Number of PCR2 cycles*
Control RNA	1 ng†	21	24
Spleen RNA	10 ng	21	18–20
	100 ng	21	16–18
	1,000 ng	21	14–16
Thymus RNA	10 ng	21	14–16
	100 ng	21	12–14
	1000 ng	21	12–14
Whole Blood RNA	10 ng	21	22–24
	100 ng	21	20–22
	1000 ng	21	18–20
Bone Marrow RNA	10 ng	21	22–24
	100 ng	21	20–22
	1000 ng	21	18–20
T-cell RNA	1 ng	21	24
	100 ng	21	16–18
	1000 ng	21	14–16
	1,000 ng	21	14
Purified T cells	100 cells	21	18–20
	10,000 cells	21	16–18

*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 to achieve appropriate yield.

†If higher Control RNA inputs are used, Spleen RNA cycling guidelines can be applied to Control RNA.

1. PCR 1

This PCR selectively amplifies TCR sequences from the first strand cDNA generated with the previous protocol. The mTCR Universal Forward primer anneals to the SMART UMI oligo sequence (incorporated during first strand cDNA synthesis) and adds the Illumina Read 2

sequence. mTCRa PCR1 Reverse and/or mTCRb PCR1 Reverse primers anneal to sequences in the constant regions of *TCRα* and *TCRβ* cDNA, respectively.

For this protocol, you will need the following components:

PrimeSTAR GXL Premix (2X), mTCR PCR1 Universal Forward, mTCRa PCR1 Reverse and/or mTCRb PCR1 Reverse, and Nuclease-Free Water.

1. Thaw all the reagents needed for PCR on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
2. Prepare a mTCRa/mTCRb PCR1 Reverse Mix for all reactions (scale up as needed), plus 10% of the total reaction mix volume.

   mTCRa/mTCRb PCR1 Reverse Mix:

1 µl	mTCRa PCR1 Reverse
1 µl	mTCRb PCR1 Reverse
2 µl	**Total volume per reaction**

   Gently vortex to mix then briefly centrifuge.
3. Prepare PCR1 Master Mix by combining the following components in the order shown in the table below (scale up as needed), plus 10% of the total reaction mix volume.

   PCR1 Master Mix:

4 µl	Nuclease-Free Water
0.5 µl	mTCR PCR1 Universal Forward (12 µM)
0.5 µl	mTCRa/mTCRb PCR1 Reverse Mix (from Step 2)
25 µl	PrimeSTAR GXL Premix (2X)
30.0 µl	**Total volume per reaction**

   Gently vortex to mix then briefly centrifuge.
4. Add 30 µl of PCR1 Master Mix to each well or tube containing 20 µl of the first-strand cDNA product from Section V.A. Mix well and briefly spin to collect the contents at the bottom of the tubes/wells.
5. Place the plate/tubes in a preheated thermal cycler with a heated lid and run the following program:

   PCR 1

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

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

2. PCR 2

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

For this protocol, you will need the following components:

PrimeSTAR GXL Premix (2X), mTCRa/b PCR2 UDI Reverse, selected Unique Dual Index Kit, and Nuclease-Free Water.

1. Thaw all the reagents needed for PCR [except PrimeSTAR GXL Premix (2X)] on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
2. Dilute the mTCRa and mTCRb PCR2 UDI Reverse primers using the following formula (scale up as needed), plus 10% of the total reaction mix volume.

IMPORTANT: Because of the small volumes, a minimum volume of Diluted mTCRa/b PCR UDI Reverse primer mixture should be created to ensure pipetting accuracy.

- For Cat. No. 634815 (24 rxns), a minimum volume should be created sufficient for 10 experimental reactions.
- For Cat. Nos. 634814 & 634816 (96 rxns), the minimum volume should be sufficient for 12 experimental reactions.

Diluted mTCRa/b PCR UDI Reverse primer mixture:

0.1 µl	mTCRa PCR2 UDI Reverse
0.1 µl	mTCRb PCR2 UDI Reverse
1.8 µl	Nuclease-Free Water
2.0 µl	**Total volume per reaction***

*Create a minimum volume sufficient for ten (Cat. No. 634815) or twelve (Cat. Nos. 634814 & 634816) reactions, per the note above.

Mix well by vortexing gently and then spin the plate or tubes briefly in a microcentrifuge.

NOTE: The mTCRa and mTCRb PCR2 UDI Reverse primers can also be used separately. In this case, we suggest diluting mTCRa and mTCRb primers at 1:10 (0.2 µl of mTCRa **-or-** mTCRb PCR2 UDI Reverse primer + 1.8 µl Nuclease-Free Water). Use 2 µl of the diluted primer of interest per reaction (scale up as required).

If achieving equal coverage for each subunit is critical to your experiments, we recommend performing the reactions in PCR 2 separately. Consult Appendix B and Illumina literature for more information.

3. On ice, prepare a PCR2 Master Mix by combining the following components in the order shown in the table (scale up as needed), plus 10% of the total reaction mix volume. Remove the PrimeSTAR GXL Premix (2x) from the freezer, gently mix the tube without vortexing, and add to the master mix just before use.

   PCR2 Master Mix:

20 µl	Nuclease-Free Water
2 µl	Diluted mTCRa/b PCR UDI Reverse primer mixture (from Step 2)*
25 µl	PrimeSTAR GXL Premix (2X)
47 µl	**Total volume per reaction**

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

   Gently vortex to mix, then briefly centrifuge.

4. For each reaction, add 47 µl of PCR2 Master Mix to a clean nuclease-free 96-well plate, 0.2 ml tube, or 8-tube strip.
5. Add 1 µl of the PCR1 product to each well/tube containing PCR2 Master Mix.
6. Add 2 µl of the appropriate UDI from the Unique Dual Index Kit (12.5 µM) to each well/tube.

   PCR2 reaction mixture:

47 µl	PCR2 Master Mix (Step 4)
1 µl	PCR1 product (Step 5)
2 µl	UDI
50 µl	**Total volume per reaction**

   Mix well and briefly spin to collect the contents at the bottom of the wells/tubes.

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

   PCR 2

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

   *Consult Table 3 (above) for PCR cycle number (*N*) guidelines.

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

C. Protocol: Purification of Amplified Libraries

TCR libraries are purified by two rounds of size selection using NucleoMag NGS Clean-up and Size Select (Takara Bio, Cat. No. 744970) beads. The beads are then washed with 80% ethanol and the libraries are eluted with Elution Buffer.

NOTES:

- Before each use, bring a 1.5 ml aliquot of NucleoMag NGS Clean-up and Size Select beads to room temperature for at least 30 min and mix well by vortexing. Use room-temperature Elution Buffer for this protocol.
- Bead:sample ratio is 0.45:1
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.
- Use a magnetic separation device for 0.2 ml tubes, strip tubes, or a 96-well plate.

1. Add 22.5 µl of the NucleoMag beads to each sample. 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.**

2. Incubate at room temperature for 8 min to let the DNA bind to the beads.
3. Briefly spin the samples to collect the liquid from the side sample well or tube. 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; any bead carryover will decrease the efficiency of size selection.

   NOTE: There is no disadvantage to separating the samples for longer than 5 min, if needed.

4. Keep the reaction plate or tubes sitting on the magnetic separation device. Use a pipette to transfer the supernatant (which contains your library) to a clean PCR plate or tubes. After transferring, remove the plate or tubes containing the beads from the magnetic separation device and discard.
5. Add 10 µl of NucleoMag beads to each well or tube containing supernatant.

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

6. Mix thoroughly by gently pipetting the entire volume up and down at least 10 times. Do not vortex.
7. Incubate at room temperature for 8 min to let the DNA bind to the beads.
8. Place the plate or tubes on the magnetic separation device for ~10 min or until the solution is completely clear. The libraries are now bound to the beads.
9. Keep the plate or tubes sitting on the magnetic separation device. Remove the supernatant carefully with a pipette and discard it.
10. 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.
11. Repeat the ethanol wash (Step 10) once more.
12. Briefly spin the plate or tubes (~2,000*g*) to collect the remaining liquid at the bottom of each well or tube.

13. Place the plate or tubes on the magnetic separation device for 30 sec and then remove all remaining liquid with a pipette.
14. Let the sample plate or tubes rest open on the magnetic separation device at room temperature for ~2–2.5 min until the pellets appears dry and are no longer shiny. You may see a tiny crack in the pellets.

    NOTE: Check the pellets frequently during this time and continue to Step 15 when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you'd like additional assistance with this determination.

    https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

    If the beads are overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads and recovery may be reduced.
15. Once the bead pellets have dried, remove the plate or tubes from the magnetic separation device and add 17 µl of Elution Buffer to cover each 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 well or tubes.
16. Incubate at room temperature for at least 5 min to rehydrate.
17. Briefly spin the samples to collect the liquid from the side of the sample well or tube. 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.
18. 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 tubes may be stored at –20°C indefinitely.

D. Protocol: Library Validation

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

- Analyzing and validating final libraries using Qubit 2.0 Fluorometer using a Qubit dsDNA HS kit (Thermo Fisher Scientific, Cat. No. Q32851) and
- Evaluating the libraries' size distributions with an Agilent 2100 Bioanalyzer and the High Sensitivity DNA Kit (Agilent Technologies, Cat. No. 5067-4626) or the Agilent DNA 1000 kit (Agilent Technologies, Cat. No. 5067-1504).

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

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

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

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

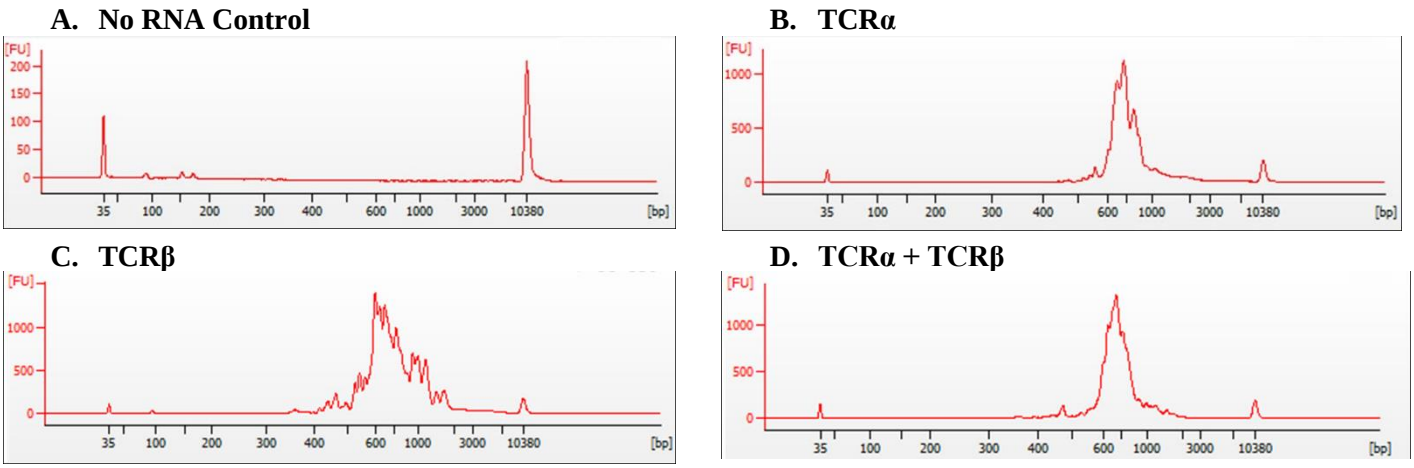


Figure 3. Example electropherogram results for TCRα + TCRβ libraries. Libraries containing *TRA* and *TRB* sequences were generated using SMART-Seq Mouse TCR (with UMIs) and 1 ng of Control RNA, respectively. Libraries were produced using 21 amplification cycles for PCR 1 and 24 amplification cycles for PCR 2. Following purification and size selection, libraries were analyzed on an Agilent 2100 Bioanalyzer using the High Sensitivity DNA Kit. **Panel A**. The No RNA Control sample show no peaks between 15–1,500 bp. **Panels B, C**, and **D** show broad peaks between ~650–1,150 bp and maximal peaks in the range of ~600–800 bp (typical results for a library generated from mouse spleen RNA).

VI. References

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

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

Appendix A: Sample Pooling and Indexing Recommendations

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

SMART-Seq Mouse TCR (with UMIs) requires use of the Unique Dual Index Kits (Cat. Nos, 634752–634756, sold separately). The indexes are 8 nucleotides long and employ “IDT for Illumina TruSeq UD Indexes” i5 and i7 dual index sequences. In all versions of the UDI kits, the primers are provided in a 96-well plate format; the

indexes in Unique Dual Index Kit (1–24) (Cat. No. 634756) are a subset of Unique Dual Index Kit (1–96) (Cat. No. 634752). Please consult the following resources for component information, best practices, pooling strategies, an index plate map, and index sequences.

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

Appendix B: Guidelines for Library Sequencing

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

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

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

Prepare a pool of 4 nM as follows:

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

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 (next page).

Table 4. Recommended loading concentrations and PhiX spike-in concentrations for Illumina sequencing instruments.

Sequencing instrument	Loading concentration quantified by Qubit (pM)	Loading concentration quantified by qPCR (pM)	PhiX %
MiniSeq™	1.1	0.55	30%
MiSeq–V2, V3	13	6.5	10%
NextSeq® 500/550	1.8	0.9	20%
NextSeq 1000/2000	650*	325*	10%
NovaSeq™ 6000	200†	100†	30%

*This loading concentration is recommended for the on-board denature/dilute protocol on the NextSeq 1000/2000.
†This loading concentration was optimized for XP workflow according to NovaSeq 6000 Denature and Dilute Libraries Guide.

To sequence the entire V(D)J region, sequencing should be performed on an Illumina MiSeq or NextSeq 1000/2000 sequencers with paired-end, 2 x 300 base pair reads. To cover the entire CDR3 region, it is possible to perform 1 x 150 single reads, but UMI information will not be captured (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 4) and the Illumina instrument and kit combinations in Table 5 (next page).

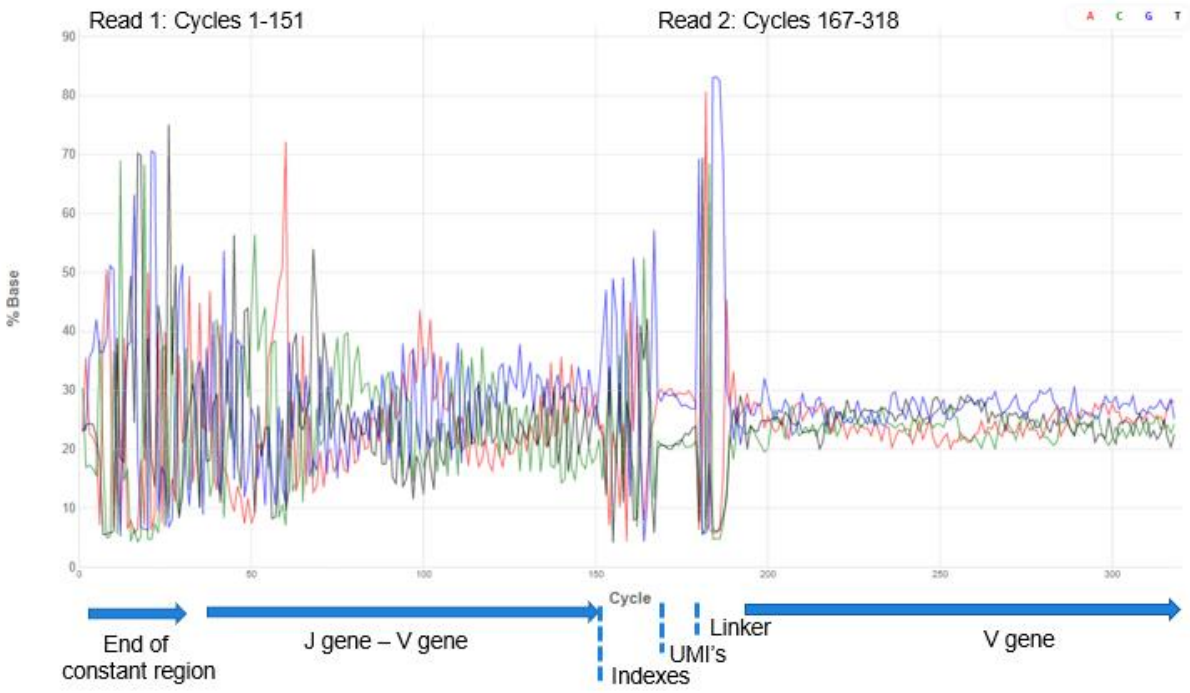


Figure 4. Percentage base calling from a typical NextSeq TCRα + TCRβ profiling run. Cycles 1–151 can cover entire CDR3 regions for the *TRA* and *TRB* genes, respectively. Cycles 152–167 are the index reads.

Table 5. Illumina instrument and reagent kit recommendations.

Sequence	Kit	Cat. No.
MiniSeq	MiniSeq High Output reagent kit 300-cycle	Cat. No. FC-420-1003
	Mid Output reagent kit 300-cycle	Cat. No. FC-420-1004
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
NextSeq 550	NextSeq High Output kit v2.5 300-cycle	Cat. No. 20024908
	NextSeq Mid Output kit v2.5 300-cycle	Cat. No. 20024905
NextSeq 1000/2000	NextSeq 1000/2000 P1 Reagents 300 Cycles	Cat. No. 20050264
	NextSeq 1000/2000 P1 Reagents 600 Cycles	Cat. No. 20075294
	NextSeq 1000/2000 P2 Reagents 300 Cycles	Cat. No. 20046813
	NextSeq 1000/2000 P2 Reagents 600 Cycles	Cat. No. 20075295
	NextSeq 2000 P3 Reagents 300 Cycles	Cat. No. 20040561
NovaSeq	NovaSeq SP reagent kit 300-cycle	Cat. No. 20027465
	NovaSeq SP reagent kit 500-cycle	Cat. No. 20029137
	NovaSeq S4 reagent kit 300-cycle	Cat. No. 20012866

We generally recommend a minimum of 1 x 10^6 reads (500,000 PE) for TCRα and TCRβ libraries from an input of 10 ng spleen RNA, 100 ng whole blood RNA, or 100 ng bone marrow RNA. For libraries generated from >10 ng spleen RNA or >100 ng whole blood RNA, higher sequencing depth is recommended, as shown in Table 6 (using spleen as an example).

NOTE: The optimal conditions may vary for different sample types, sample masses, and sample complexities. To determine the optimal sequencing depth, we recommend trying a higher sequencing depth then downsampling to determine the minimum number of reads per library. In general, inputs with higher T-cell content will require more reads to sufficiently sequence.

Table 6. Recommended sequencing depth for TCR libraries prepared using mouse RNA (from various sources).

Input	Mixed Library	TCRα	TCRβ
Spleen RNA (10 ng)	1 x 10^6 reads	1 x 10^6 reads	1 x 10^6 reads
Spleen RNA (100 ng)	5 x 10^6 reads	5 x 10^6 reads	5 x 10^6 reads
Spleen RNA (1 µg)	1.5 x 10^7 reads	5 x 10^6 reads	1 x 10^7 reads
Whole Blood RNA (100 ng)	1 x 10^6 reads	1 x 10^6 reads	1 x 10^6 reads
Bone Marrow RNA (100 ng)	1 x 10^6 reads	1 x 10^6 reads	1 x 10^6 reads

Appendix C: Guidelines for Data Analysis

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

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

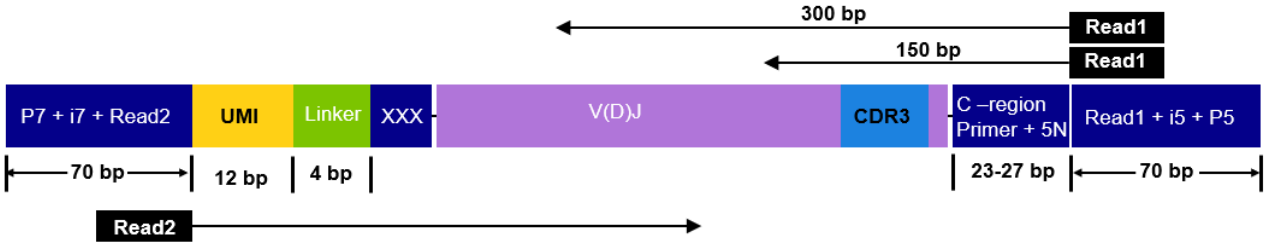


Figure 5. SMART-Seq Mouse TCR (with UMIs) library structure. The first 19 nucleotides from Read 2 can be trimmed off if UMI analysis is not performed.

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