Takara Bio USA, Inc.

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

Cat. Nos. 634776, 634777, 634778
(111122)

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I. Introduction

SMART-Seq Human BCR (with UMIs) (Cat. Nos. 634776, 634777 & 634778) enables users to analyze B-cell receptor (BCR) repertoires from bulk total RNA samples. This kit can generate libraries from 10 ng–1 µg of total RNA from peripheral blood mononuclear cells (PBMCs) or 1–100 ng of total RNA from B cells. This kit may also be appropriate for other sample types, such as RNA extracted from human whole blood, but the protocol may need to be adjusted.

The workflow can be used to generate data for both heavy and light chains (kappa and lambda) of human immunoglobulin IgG, IgM, IgA, IgD and IgE (Figure 1). Using the Unique Dual Index Kits (Cat. Nos. 634752–634756, sold separately), the protocol results in indexed libraries that are ready for sequencing on Illumina® platforms. With sequencing output from Illumina sequencers as input to our free-to-use Cogent™ NGS Immune Profiler (CogentIP) and web-based Cogent NGS Immune Viewer bioinformatics software (Appendix D), we provide an end-to-end solution from samples to publication-ready data.

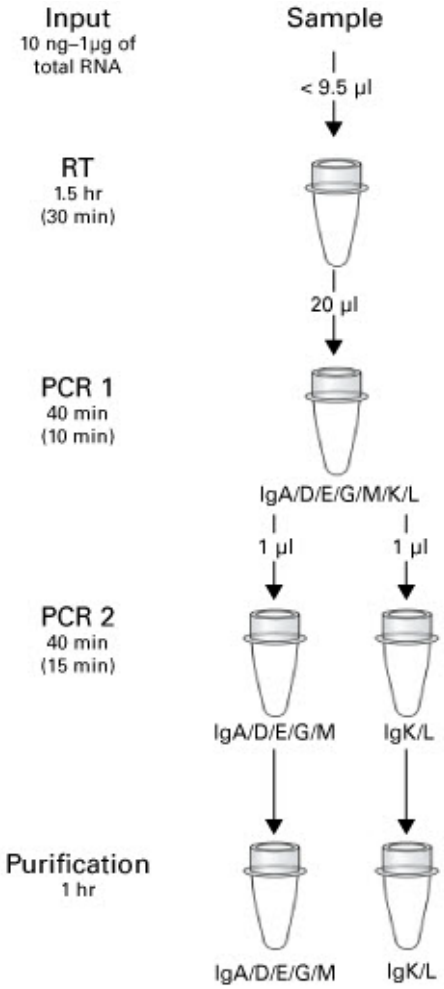

Figure 1. Protocol overview and timeline. Numbers between parentheses are estimated hands-on times. For each sample, after reverse transcription of mRNAs, the entire volume of the RT reaction (20 µl) is used as input in the first round of PCR (PCR 1), where all seven immunoglobulin chains are amplified. 1 µl of the PCR 1 product is used as input for a second round of PCR (PCR 2) which adds the same sequencing indexes to each amplified chain for a given sample but distinct indexes for each different sample. The heavy chains (IgA/D/E/G/M) and the light chains (IgK/L) are amplified separately. After PCR 2, the amplified libraries are purified, and we recommend analyzing and validating the results.

The SMART-Seq Human BCR (with UMIs) protocol 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 BCR transcripts (Figure 2). It also incorporates unique molecular identifiers (UMI) to facilitate PCR error correction and clonotype quantification during data analysis.

First-strand cDNA synthesis is oligo-dT primed and catalyzed by SMARTScribe™ Reverse Transcriptase (RT), which adds non-templated nucleotides at the 5′ end of each cDNA molecule. The SMART UMI Oligo anneals to these non-templated nucleotides, serves as a template for incorporation of a PCR handle into the first-strand cDNA, and uniquely tags each cDNA molecule with a UMI. Following reverse transcription, two rounds of PCR are performed to amplify BCR cDNAs. To capture the entire V(D)J region, primers in these PCRs anneal to sequences added by the SMART UMI Oligo at one end and the BCR 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 BCR variable region and a small portion of the constant region.

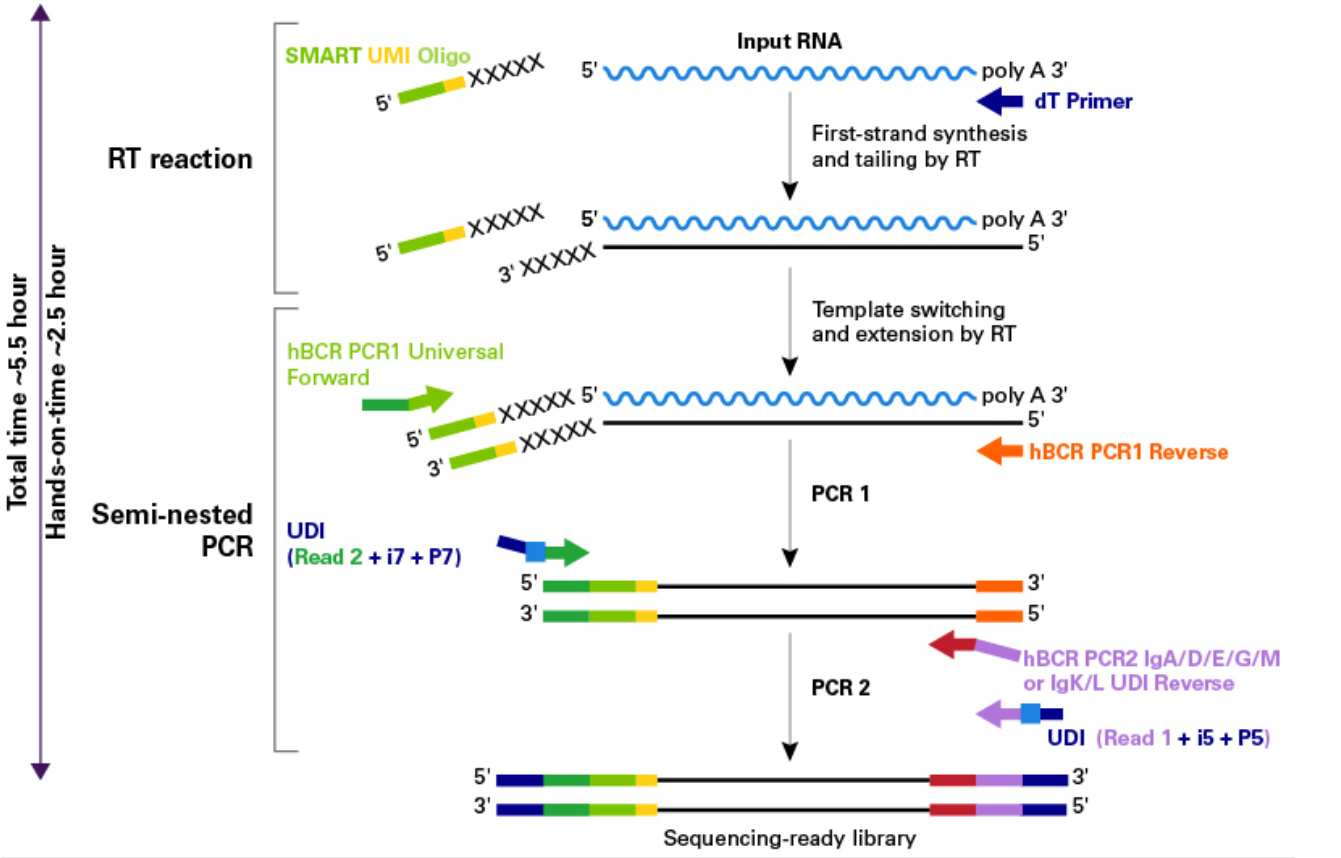

Figure 2. SMART-Seq Human BCR (with UMIs) technology. dT-primed, first-strand cDNA synthesis is followed by two rounds of successive PCR for amplification of cDNA sequences. After post-PCR purification, size selection, and quality analysis, the library is ready for sequencing.

II. List of Components

- The components of SMART-Seq Human BCR (with UMIs) have been designed to work together and are optimized for this 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 samples that can be put into RT reactions. There is enough material included in the kit to generate heavy chain (IgA, IgD, IgE, IgG, IgM), and light chain (IgK, and IgL) sequencing libraries from each RT reaction.

NOTES:

– **Cat. No. 634776** is a kit comprised of 4 units of Cat. No. 634778 (4 x 96 reactions).

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

SMART-Seq Human BCR (with UMIs)	Cap color	634777 (24 rxns)	634778 (96 rxns)	634776 (384 rxns)
Box 1 (Store at –70°C)				
Control Total RNA* (1 µg/µl)	–	5 µl	5 µl	4 x 5 µl
SMART UMI Oligo	Pink	24 µl	96 µl	4 x 96 µl
Box 2 (Store at –20°C)				
dT Primer	Light blue	48 µl	192 µl	4 x 192 µl
First-Strand Buffer (5X)	Purple	96 µl	384 µl	4 x 384 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	Purple	48 µl	192 µl	4 x 192 µl
Nuclease-Free Water†	–	5 ml	20 ml	4 x 20 ml
BCR Enhancer†	White	24 µl	96 µl	4 x 96 µl
RNase Inhibitor (40 U/µl)	White	24 µl	96 µl	4 x 96 µl
Elution Buffer‡ (10 mM Tris HCl, pH 8.5)	–	1 ml	4 x 1 ml	16 x 1 ml
hBCR PCR1 Universal Forward	Blue	24 µl	96 µl	4 x 96 µl
hBCR PCR1 Reverse	Red	24 µl	96 µl	4 x 96 µl
hBCR PCR2 HC Reverse	Yellow	24 µl	96 µl	4 x 96 µl
hBCR PCR2 LC Reverse	Orange	24 µl	96 µl	4 x 96 µl
PrimeSTAR® GXL Premix	Clear	2 x 1 ml	8 x 1 ml	32 x 1 ml

*Control RNA is human spleen Total RNA.

†Store Nuclease-Free Water and BCR Enhancer at –20°C. Once thawed, they can be stored at 4°C.

‡Store Elution Buffer at –20°C. Once thawed, the buffer can be stored at room temperature.

III. Additional Materials Required

The following reagents and materials are required but not supplied. The named products have been validated to work with this protocol.

- 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
  - 1.5 ml tubes
  - 0.2 ml tubes or strips

For PCR Amplification & Validation:

- Thermal cyclers
  - One dedicated for first-strand cDNA synthesis (Protocol V.A)
  - One dedicated for library amplification by PCR (Protocol V.B)
- For validation
  - Agilent 2100 Bioanalyzer: DNA 1000 Kit (Agilent, Cat. No. 5067-4401; Protocol V.D)
  - 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 (Protocol V.C):

- NucleoMag NGS Clean-up and Size Select (Takara Bio; 5-ml size: Cat. No. 744970.5; 50ml 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.

  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 minutes). 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 products Cat. No. 634778 (96 rxns) and Cat. No. 634776 (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 (Protocol 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 (Protocol V.B) and measure library concentration (Protocol V.D).
- All lab supplies related to cDNA synthesis should be stored in a closed, DNA-free cabinet. Reagents for cDNA synthesis should 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.
- If you are using this protocol for the first time, we strongly recommend that you perform negative and positive control reactions to verify that kit components are working properly.

B. Sample Recommendations

- This protocol has been optimized for 10 ng of total RNA extracted from peripheral blood mononuclear cells (PBMCs).
- RNA should be of high integrity (RIN>7) to enable oligo(dT)-priming
- The success of your experiment depends on the quality of your input RNA. Prior to cDNA synthesis, ensure your RNA is in nuclease-free water, intact, and free of contaminants. 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 type, input mass, and includes DNase treatment. We recommend NucleoSpin RNA XS kits (Takara Bio, Cat. No. 740902.10, 740902.50, or 740902.250).
- After RNA extraction, if your sample size is not limiting, we recommend evaluating total RNA quality using the Agilent RNA 6000 Pico Kit (Agilent, Cat. No. 5067-1513) or an equivalent platform. Refer to the manufacturer for instructions.

V. Protocols

A. Protocol: First-Strand cDNA Synthesis

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

For this protocol, you will need the following components:
First-Strand Buffer (5X), BCR Enhancer, SMART UMI Oligo, dT Primer, SMARTScribe Reverse Transcriptase, and RNase Inhibitor.

1. Thaw the First-Strand Buffer at room temperature. Do not store on ice.
2. Thaw BCR Enhancer, SMART UMI Oligo, and dT Primer on ice. Gently vortex each reagent to mix and centrifuge briefly. Store on ice until needed.
3. Preheat the thermal cycler to 72°C.

4. On ice, prepare samples and controls in nuclease-free, thin-wall PCR tubes, plates, or strips by adding the reagents in the order shown below.

Components	Sample	Negative control	Positive control
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
BCR Enhancer	1 µl	1 µl	1 µl
dT Primer	2 µl	2 µl	2 µl
Total Volume	12.5 µl	12.5 µl	12.5 µl

*The Control RNA is supplied at a concentration of 1 µg/µl. It should be thawed on ice and diluted serially in Nuclease-Free Water to obtain the appropriate concentration (10 ng–1 µg, your choice). We have tested this protocol extensively using the PCR cycling conditions below (Table 1) (16 cycles for PCR 1 and 20 cycles for PCR 2) with 10 ng of input Control RNA

5. Mix by gently vortexing and then centrifuge briefly.
6. Incubate the tubes or plates at 72°C in the preheated, heated-lid thermal cycler for 3 min. During this incubation, prepare the RT Master Mix.
7. At room temperature, prepare RT Master Mix by combining the following components in the order shown in the table below. Mix the RT Master Mix well by gently pipetting up and down then centrifuge briefly.

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

Components	1 rxn	12 rxns*	48 rxns*
First-Strand Buffer	4 µl	52 µl	212 µl
SMART UMI Oligo	1 µl	13 µl	53 µl
RNase Inhibitor	0.5 µl	6.5 µl	26.5 µl
SMARTScribe Reverse Transcriptase	2 µl	26 µl	106 µl
Total Volume	7.5 µl	97.5 µl	397.5 µl

*Includes ~10% overage

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

13. Place the tubes/plate in the preheated thermal cycler and run the following program:

42°C	90 min
70°C	10 min
4°C	forever

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

B. Protocol: BCR Amplification and Sequencing Library Generation

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

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

RNA source	Input amount	Number of PCR 1 cycles	Number of PCR 2 cycles*
PBMC	10 ng	16	21
PBMC	100 ng	16	18
PBMC	1 µg	16	16
B cell	1 ng	16	21
B cell	10 ng	16	18
B cell	100 ng	16	16
Whole blood	100 ng	16	25
Spleen	10 ng	16	20
Control RNA	10 ng	16	20
Control RNA	100 ng	16	18
Control RNA	1 µg	16	16

*If the number of cycles generates an insufficient library for sequencing, repeat PCR 2 with more cycles. See Appendix A for more details.

1. PCR 1

This PCR selectively amplifies full-length BCR V(D)J regions from first-strand cDNA generated with the previous protocol. The hBCR PCR1 universal forward primer anneals to the SMART UMI oligo sequence (incorporated during first-strand cDNA synthesis) and adds the Illumina Read 2 sequence. The hBCR PCR1 reverse primer—a mixture of reverse primers for IgA/D/E/G/M heavy chains and IgK/L light chains—anneals to sequences in the constant regions of hBCR heavy chain and light chain cDNA.

For this protocol, you will need the following components:

PrimeSTAR GXL Premix, hBCR PCR1 Universal Forward (blue cap), hBCR PCR1 Reverse (red cap), Nuclease-Free Water.

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

2. Prepare PCR1 Master Mix by combining the following components in the order shown in the table below. Gently vortex to mix then briefly centrifuge.

Components	1 rxn	12 rxns*	48 rxns*
Nuclease-Free Water	3 µl	40.5 µl	159 µl
hBCR PCR1 Universal Forward	1 µl	13.5 µl	53 µl
hBCR PCR1 Reverse	1 µl	13.5 µl	53 µl
PrimeSTAR GXL Premix	25 µl	337.5 µl	1,325 µl
Total Volume	**30 µl**	**405 µl**	**1,590 µl**

*Includes ~10% overage

3. Add 30 µl of the PCR1 Master Mix to each tube or well containing 20 µl of the first-strand cDNA produced from Section V.A. Mix well and briefly spin to collect the contents at the bottom of the tubes/wells.

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

PCR 1

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

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

2. PCR 2

This PCR reaction further amplifies sequences corresponding to the full-length BCR variable regions and adds Illumina sequencing adapters using a semi-nested approach. The Unique Dual Index primers are added during this step and anneal to a sequence added by hBCR PCR1 Universal Forward (from the previous step) and add Illumina P7-i7 index sequences. The nested hBCR PCR2 HC Reverse and hBCR PCR2 LC Reverse primers anneal to sequences in BCR constant regions that are internal to the sequences bound by the hBCR PCR1 Reverse primer and add both the Illumina Read 1 and P5-i5 index sequences.

Libraries can be pooled at this point of the protocol. For sample pooling and indexing recommendations, refer to Appendix B.

For this protocol, you will need the following components:

PrimeSTAR GXL Premix, hBCR PCR2 HC Reverse (yellow cap), hBCR PCR2 LC Reverse (orange cap), selected Unique Dual Index kit, Nuclease-Free Water.

NOTE: The heavy chains (IgG/M/A/D/E) and the light chains (IgK/L) are amplified separately in this PCR step. You will need to prepare two reactions if you want to amplify both Heavy and Light chains from a sample.

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

2. On ice, prepare a PCR 2 Master Mix by combining the following components in the order shown in the table. Gently vortex to mix and briefly centrifuge.

Components	1 rxn	12 rxns*	48 rxns*
Nuclease-Free Water	21 µl	283.5 µl	1,113 µl
hBCR PCR2 HC Reverse† -or- hBCR PCR2 LC Reverse†	1 µl	13.5 µl	53 µl
PrimeSTAR GXL Premix	25 µl	337.5 µl	1,325 µl
Total Volume	**47 µl**	**634.5 µl**	**2,491 µl**

*Includes +10% overage
†Each PCR primer is used in a separate PCR Master Mix. Use hBCR PCR2 HC Reverse when amplifying heavy chains; use hBCR PCR2 LC Reverse when preparing to amplify light chains.

NOTE: Alternatively, you can prepare the PCR 2 Master Mix with only the Nuclease-Free Water and PrimeStar GXL Premix, then plan to add 1 µl of each primer individually instead of including in the PCR2 Master Mix. This is particularly recommended if the number of samples is low.

3. For each reaction, add 47 µl of PCR 2 Master Mix to nuclease-free, thin-wall, 0.2 ml PCR tubes or plate wells.
4. Add 1 µl of appropriate PCR1 product to each corresponding PCR 2 tube/well.
5. Add 2 µl of the appropriate UDI (12.5 µM) to each tube/well. Gently vortex to mix and briefly spin to collect the contents at the bottom of the tubes/wells.
6. Place the tubes/plate in a preheated thermal cycler and run the following program:

PCR 2

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

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

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

C. Protocol: Purification of Amplified Libraries

PCR-amplified libraries are purified by immobilization on NucleoMag NGS Clean-up and Size Select beads. Purify the libraries again by immobilization on NucleoMag NGS Clean-up and Size Select beads. The beads are then washed with 80% ethanol and the libraries eluted with Nuclease-Free Water.

NOTES:

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

1. Vortex NucleoMag beads until evenly mixed, then add 25 µl of the NucleoMag beads to each sample.
2. Mix thoroughly by gently pipetting the entire volume up and down at least 10 times.

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

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

   NOTE: Ensure that the solution is completely clear, as any bead carryover will decrease the efficiency of size selection. There is no disadvantage to separating the samples for longer than 5 min.

5. While the reaction tubes are sitting on the magnetic separation device, use a pipette to transfer the supernatant (which contains your library) to clean PCR tubes. After transferring, remove the tubes containing the beads from the magnetic separation device and discard them.
6. Add 10 µl of NucleoMag beads to each tube containing supernatant.

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

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

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

8. Incubate at room temperature for 8 min to let the DNA bind to the beads.
9. Place the tubes on the magnetic separation device for ~10 min or until the solution is completely clear. The libraries are now bound to the beads.
10. While the tubes are sitting on the magnetic separation device, remove the supernatant with a pipette and discard it.
11. Keep the tubes on the magnetic separation device. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads, to wash away contaminants. Wait for 30 sec and use a pipette to carefully remove the supernatant containing contaminants. The library will remain bound to the beads during the washing process.
12. Repeat the ethanol wash (Step 11) once more.
13. Briefly spin the tubes (~2,000*g*) to collect the remaining liquid at the bottom of each tube. Place the tubes on the magnetic separation device for 30 sec, then remove all remaining liquid with a pipette.

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

    NOTE: Check the pellet frequently during this time and continue to Step 10 when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you'd like additional assistance with this determination.
    https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

    If the beads are overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads and recovery may be reduced.

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

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

16. Incubate at room temperature for at least 5 min to rehydrate.

17. Briefly spin the samples to collect the liquid from the side of the tube or sample well. Place the samples back on the magnetic separation device for 2 min or longer until the solution is completely clear.

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

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

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

D. Protocol: Library Validation

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

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

Please refer to the corresponding user manuals for detailed instructions.

1. **Qubit:** Use 1 µl of undiluted library for quantification using Qubit 2.0 Fluorometer (see manufacturer's instructions for more details).

2. **Bioanalyzer:** To validate libraries using DNA High Sensitivity Kit (Agilent, Cat. No. 5067-4401), dilute libraries to concentration of 0.2ng/µl and for Agilent DNA 1000 kit (Cat. No. 5067-1504), dilute libraries to 1ng/µl.

   Compare the results for your samples with Figure 3 (below) to verify whether each sample is suitable for further processing. High quality libraries should yield no product for negative control reactions and a broad peak spanning 500 bp–1,200 bp with a maximum between ~600 bp–900 bp for positive

controls and samples containing BCR RNA. The position and shape of electropherogram peaks will vary depending on which chain sequences are included in the library, the nature of the RNA sample, and the analysis method. In general, electropherogram peaks obtained with the Fragment Analyzer tend to be sharper than those obtained with the Bioanalyzer.

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

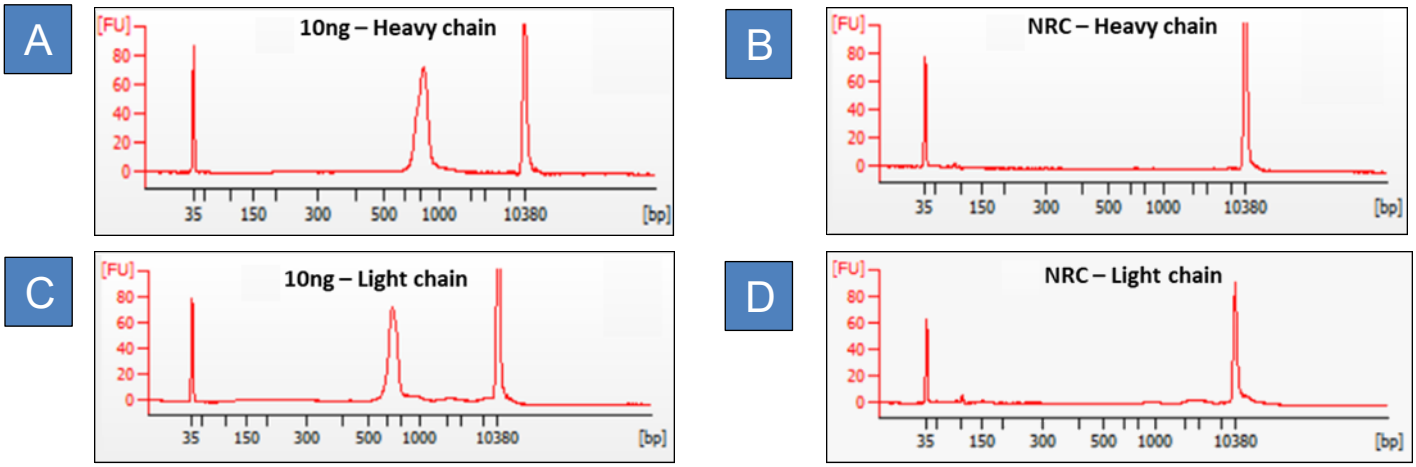

Figure 3. Example electropherogram results for BCR heavy and light (kappa or lambda) chain libraries validated using the Agilent 2100 Bioanalyzer High Sensitivity assay. Libraries containing BCR heavy- and light-chain sequences were generated using SMART-Seq Human BCR (with UMIs) and 10 ng of RNA obtained from a heterogeneous sample of PBMC RNA. Libraries were produced using 20 µl of first-strand cDNA as a template in a single PCR reaction for 16 cycles. 1 µl of the product from PCR1 was used as a template in a second, nested PCR reaction for 21 cycles. Following purification and size selection, libraries were analyzed on an Agilent 2100 Bioanalyzer. **(Panels A, C)** Broad peaks seen between ~500–1,200 bp and maximal peaks in the range of ~600–900 bp (typical results for a library generated from PBMC RNA), analyzed on the Bioanalyzer. **(Panels B, D)** No RNA Control (NRC) samples show no library produced and a flat Bioanalyzer profile within the range of 500–1,200 bp. NRC libraries often show dimer peaks in the 50–100 bp region.

VI. References

Chenchik, A., Zhu, Y., Diatchenko, L., Li., R., Hill, J. & Siebert, P. Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In *RT-PCR Methods for Gene Cloning and Analysis*. Eds. Siebert, P. & Larrick, J. (BioTechniques Books, MA), pp. 305–319 (1998).

Appendix A: Guidelines for PCR Cycling

The gene expression of each BCR chain can vary significantly from one B-cell population to another; it is therefore challenging to predict how many cycles of PCR will amplify sufficient material for downstream sequencing without over-amplification such that there are significant off-target PCR products. The guidelines provided in Table 1 are a good starting point for determining the number of cycles to perform in PCR 2 and have been determined by testing several varieties of sample types and input amounts.

It is possible that the suggested number of cycles will not generate enough material for downstream sequencing (see Appendix C). In these cases, we recommend increasing the number of cycles. The optimal range of library yield is between 3 ng/µl and 30 ng/µl (as determined by quantification with a Qubit dsDNA HS kit). If the library yield is lower than 3 ng/µl or over 30 ng/µl, we recommend increasing or reducing, respectively, the number of PCR 2 cycles by 1 to reach the optimal yield range.

Appendix B: Sample Pooling and Indexing Recommendations

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

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

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

Appendix C: 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, including the MiSeq instrument that we recommend for this kit.

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 20% 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.

NOTE: Follow Illumina guidelines on how to denature, dilute, and combine a PhiX control library with your own pool of libraries. Make sure to use a fresh and reliable stock of the PhiX control library.

To obtain the full length of V(D)J information from the BCR clones, 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. If using qPCR for quantification, we recommend diluting the pooled denatured libraries to a final concentration of 7.5 pM and using 20% PhiX control spike-in to achieve optimal cluster density. When relying on Qubit quantification, you may need to use a higher final concentration.

Alternatively, if only CDR3 region information is needed, use paired-end, 2 x 150 base reads and the following Illumina instrument and kit combinations (Table 2).

Table 2. Illumina instrument and reagent kit recommendations

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

The complexity of the human BCR repertoire varies from person to person. We generally recommend a minimum of 2,000,000 reads from an input of 10 ng PBMC RNA (or 1 ng B-Cell RNA) for heavy-chain and light-chain libraries. For libraries generated from >10 ng PBMC RNA, higher sequencing depth is recommended, as shown in Table 3 below.

However, the optimal conditions may vary for different sample types, sample masses, and sample complexities. We recommend trying a higher sequencing depth, then downsample to determine the minimum number of reads per library to determine the optimal sequencing depth.

Table 3. Recommended sequencing depth for BCR libraries prepared from human PBMC RNA or B-Cell RNA.

Input	Heavy chain	Light chain
10 ng PBMC RNA	2 million reads	2 million reads
100 ng PBMC RNA	~6 million reads	~6 million reads
1 µg PBMC RNA	~25 million reads	~25 million reads
1 ng B cell RNA	2 million reads	2 million reads
10 ng B-Cell RNA	~6 million reads	~6 million reads
100 ng B cell RNA	~25 million reads	~25 million reads

Appendix D: 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 BCR library (Figure 4). Analysis of UMIs provides higher accuracy for clonotype diversity and abundance measurements.

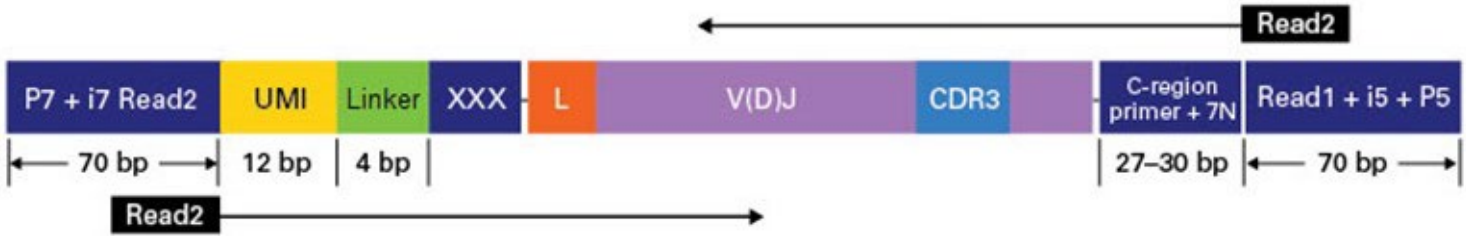

Figure 4. SMART-Seq Human BCR (with UMIs) library structure. First 19 nt from Read2 could be trimmed off if UMI analysis is not performed.

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