Takara Bio USA, Inc.

SMARTer® Human BCR IgG IgM H/K/L Profiling Kit User Manual

Cat. Nos. 634466 & 634467
(020625)

Takara Bio USA, Inc.
2560 Orchard Parkway, San Jose, CA 95131, USA
U.S. Technical Support: technical_support@takarabio.com

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

Table of Contents

Table of Figures

Table of Tables

I. Introduction

The **SMARTer Human BCR IgG IgM H/K/L Profiling Kit** (Cat. Nos. 634466 & 634467) enables users to analyze B-cell receptor (BCR) repertoires from total RNA samples. This kit can generate libraries from 10 ng to 1 µg of total RNA from peripheral blood mononuclear cells (PBMCs) or 1 to 100 ng of total RNA from B cells. This kit may be appropriate for other sample types, such as RNA extracted from human whole blood, but the protocol may need to be adjusted. The kit can be used to generate data for both heavy and light chains (kappa and lambda) of human immunoglobulin IgG and IgM (Figure 1). Included in the kit are primers that incorporate Illumina®-specific adapter sequences during cDNA amplification. The protocol generates indexed libraries that are ready for sequencing on Illumina platforms.

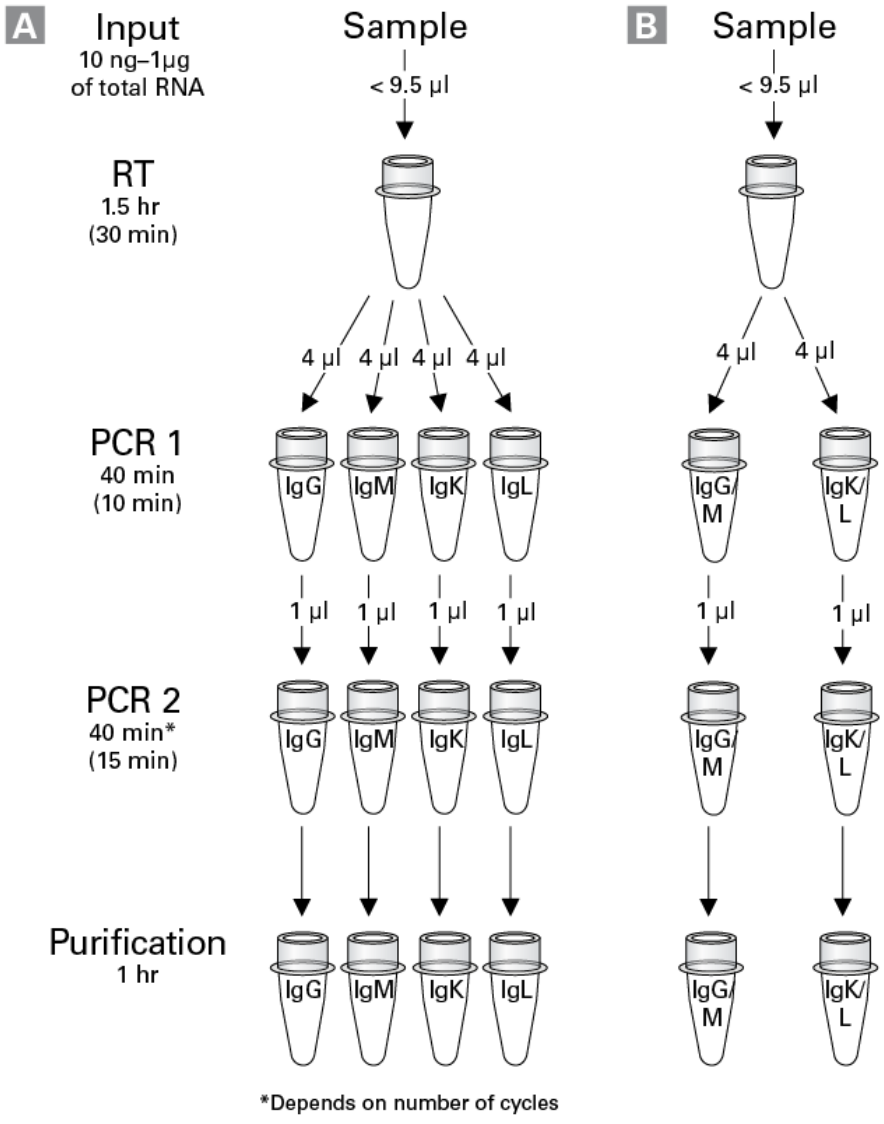

Figure 1. Protocol overview and timeline. **A**. For each sample, after reverse transcription of all mRNAs in the sample, the user may specifically amplify 1–4 chains of human BCR. Each amplification uses 4 µl of the RT. Following the first round of PCR (PCR 1), 1 µl of each PCR product is used in a separate PCR (PCR 2) to add the same sequencing indexes to each amplified chain for a given sample, but distinct indexes for each different sample. After this final amplification in PCR 2, the user may validate each product on the Bioanalyzer or Fragment Analyzer. The user may then choose which amplified chain to pool for sequencing. Numbers between parentheses are estimated hands-on times. **B.** To minimize hands-on time, it is also possible to combine the heavy chain primers (G+M) and light chain primers (K+L), resulting in only two PCR 2s per sample instead of four. Please see Appendix A for details.

The SMARTer Human BCR IgG IgM H/K/L 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 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 mRNA template. 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 sequence added by the SMART UMI Oligo at the 5′ end and the BCR constant region(s) at the 3′ 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.

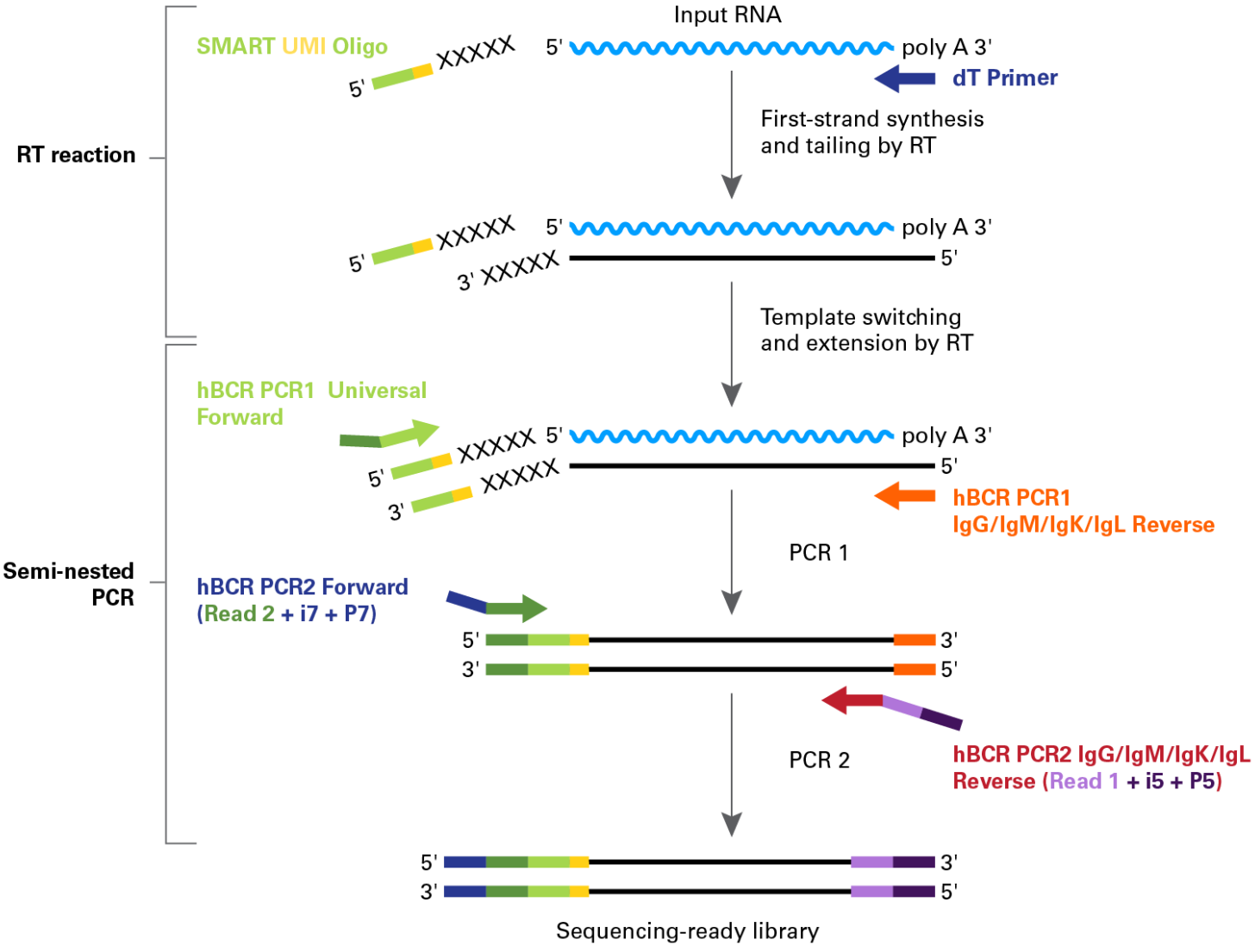

Figure 2. SMARTer Human BCR IgG IgM H/K/L Profiling Kit technology. dT-primed first-strand cDNA synthesis is followed by two rounds of successive PCR for amplification of cDNA sequences. After post-PCR purification, size selection, and quality analysis, the library is ready for sequencing.

II. List of Components

The components of SMARTer Human BCR IgG IgM H/K/L Profiling Kit 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 IgG, IgM, IgK, and IgL sequencing libraries from each RT reaction.

SMARTer Human BCR IgG IgM H/K/L Profiling Kit	Cap color	634466 (12 rxns)	634467 (48 rxns)
Package 1 (Store at –70°C.)			
Control RNA* (1 µg/µl)	–	5 µl	5 µl
SMART UMI Oligo	Pink	15 µl	60 µl
Package 2 (Store at –20°C. Once thawed, store Nuclease-Free Water and BCR Enhancer at 4°C and store Elution Buffer at room temperature. Continue to store all other reagents at –20°C.)			
dT Primer	Light blue	30 µl	120 µl
First-Strand Buffer (5X)	Purple	60 µl	230 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	Purple	30 µl	120 µl
Nuclease-Free Water	–	5 ml	20 ml
RNase Inhibitor (40 U/µl)	White	30 µl	120 µl
BCR Enhancer	White	300 µl	1 ml
Elution Buffer (10 mM Tris-HCl, pH 8.5)	–	1.7 ml	3 x 1.7 ml
hBCR PCR1 Universal Forward	Blue	60 µl	225 µl
hBCR PCR1 IgG Reverse	Red	15 µl	60 µl
hBCR PCR1 IgM Reverse	Yellow	15 µl	60 µl
hBCR PCR1 IgK Reverse	Orange	15 µl	60 µl
hBCR PCR1 IgL Reverse	Green	15 µl	60 µl
PrimeSTAR® GXL DNA Polymerase (1.25 U/µl)	White	200 µl	600 µl
5X PrimeSTAR GXL Buffer	White/–†	2 x 1 ml	6 ml
dNTP Mixture (2.5 mM each)	White/–†	800 µl	2.4 ml
Package 3 (Store at –20°C. For details, see Appendix B)			
hBCR PCR2 IgG Reverse 1	Red	15 µl	15 µl
hBCR PCR2 IgG Reverse 2	Red		15 µl
hBCR PCR2 IgG Reverse 3	Red		15 µl
hBCR PCR2 IgG Reverse 4	Red		15 µl
hBCR PCR2 IgM Reverse 1	Yellow	15 µl	15 µl
hBCR PCR2 IgM Reverse 2	Yellow		15 µl
hBCR PCR2 IgM Reverse 3	Yellow		15 µl
hBCR PCR2 IgM Reverse 4	Yellow		15 µl
hBCR PCR2 IgK Reverse 1	Orange	15 µl	15 µl
hBCR PCR2 IgK Reverse 2	Orange		15 µl
hBCR PCR2 IgK Reverse 3	Orange		15 µl
hBCR PCR2 IgK Reverse 4	Orange		15 µl
hBCR PCR2 IgL Reverse 1	Green	15 µl	15 µl
hBCR PCR2 IgL Reverse 2	Green		15 µl
hBCR PCR2 IgL Reverse 3	Green		15 µl
hBCR PCR2 IgL Reverse 4	Green		15 µl

SMARTer Human BCR IgG IgM H/K/L Profiling Kit	Cap color	634466 (12 rxns)	634467 (48 rxns)
hBCR PCR2 Forward 1	Blue	15 µl	60 µl
hBCR PCR2 Forward 2	Blue	15 µl	60 µl
hBCR PCR2 Forward 3	Blue	15 µl	60 µl
hBCR PCR2 Forward 4	Blue	15 µl	60 µl
hBCR PCR2 Forward 5	Blue	15 µl	60 µl
hBCR PCR2 Forward 6	Blue	15 µl	60 µl
hBCR PCR2 Forward 7	Blue	15 µl	60 µl
hBCR PCR2 Forward 8	Blue	15 µl	60 µl
hBCR PCR2 Forward 9	Blue	15 µl	60 µl
hBCR PCR2 Forward 10	Blue	15 µl	60 µl
hBCR PCR2 Forward 11	Blue	15 µl	60 µl
hBCR PCR2 Forward 12	Blue	15 µl	60 µl

*Control RNA: human spleen total RNA
†Depending on the product size, these reagents are packaged in a tube with a white cap or in a bottle, as indicated.

Storage conditions:

- Store Control RNA and SMART UMI Oligo at –70°C.
- Store BCR Enhancer at –20°C. Once thawed, the buffer can be stored at 4°C.
- Store Nuclease-Free Water at –20°C. Once thawed, the water 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. Human BCR Indexing Primer Set HT for Illumina Sequences

Illumina indexes are incorporated into human BCR profiling libraries through both forward and reverse PCR primers. The corresponding Illumina indexes are listed in Tables 1 and 2 below.

Table 1. BCR Indexing Forward Primer Set HT for Illumina Index Sequences.

BCR PCR2 Forward Primer i7 HT Index		
Primer Name	**Illumina ID**	**Index sequence**
hBCR PCR2 Universal Forward 1	D701	ATTACTCG
hBCR PCR2 Universal Forward 2	D702	TCCGGAGA
hBCR PCR2 Universal Forward 3	D703	CGCTCATT
hBCR PCR2 Universal Forward 4	D704	GAGATTCC
hBCR PCR2 Universal Forward 5	D705	ATTCAGAA
hBCR PCR2 Universal Forward 6	D706	GAATTCGT
hBCR PCR2 Universal Forward 7	D707	CTGAAGCT
hBCR PCR2 Universal Forward 8	D708	TAATGCGC
hBCR PCR2 Universal Forward 9	D709	CGGCTATG
hBCR PCR2 Universal Forward 10	D710	TCCGCGAA
hBCR PCR2 Universal Forward 11	D711	TCTCGCGC
hBCR PCR2 Universal Forward 12	D712	AGCGATAG

Table 2. BCR Indexing Reverse Primer Set HT for Illumina Index Sequences.

BCR PCR2 IgG/IgM/IgK/IgL Reverse Primer i5 HT Index					
IgG Primer Name	**IgM Primer Name**	**IgK Primer Name**	**IgL Primer Name**	**Illumina ID**	**Index sequence***
hBCR PCR2 IgG Reverse 1	hBCR PCR2 IgM Reverse 1	hBCR PCR2 IgK Reverse 1	hBCR PCR2 IgL Reverse 1	D501	TATAGCCT
hBCR PCR2 IgG Reverse 2	hBCR PCR2 IgM Reverse 2	hBCR PCR2 IgK Reverse 2	hBCR PCR2 IgL Reverse 2	D502	ATAGAGGC
hBCR PCR2 IgG Reverse 3	hBCR PCR2 IgM Reverse 3	hBCR PCR2 IgK Reverse 3	hBCR PCR2 IgL Reverse 3	D503	CCTATCCT
hBCR PCR2 IgG Reverse 4	hBCR PCR2 IgM Reverse 4	hBCR PCR2 IgK Reverse 4	hBCR PCR2 IgL Reverse 4	D504	GGCTCTGA

*Index sequence as read on a MiSeq® instrument.

IV. Additional Materials Required

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

- Pipettes: 10 µl, 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 VI.A)
  - One dedicated for library amplification by PCR (Protocol VI.B)
- For validation
  - Agilent 2100 Bioanalyzer: DNA 1000 Kit (Agilent, Cat. No. 5067-1504; Protocol VI.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 (available from Takara Bio; 5 ml size: Cat. No. 744970.5, 50 ml size: Cat. No. 744970.50, 500 ml size Takara Bio Cat. No. 744970.500)
  - If the above NuceloMag 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.

NOTE: The beads need to come to room temperature before the container is opened. Therefore, we recommend aliquoting the beads into 1.5 ml tubes upon receipt and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 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, 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 plater

V. General Considerations

A. General Recommendations

- We recommend using two physically separated work stations to minimize contamination:
  - **A PCR Clean Workstation** for all pre-PCR experiments that require clean room conditions, e.g., first-strand cDNA synthesis (Protocol VI.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 VI.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 kits.
- 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) or an equivalent platform. Refer to the manufacturer for instructions.

VI. Protocols

A. Protocol: First-Strand cDNA Synthesis

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

1. Thaw the First-Strand Buffer at room temperature. Thaw BCR Enhancer, SMART UMI Oligo, and dT Primer on ice. Gently vortex each reagent to mix and centrifuge briefly. Store all but the First-Strand Buffer on ice. Remove the SMARTScribe Reverse Transcriptase and RNase Inhibitor from the freezer immediately before use, centrifuge briefly and store on ice.

   NOTE: The 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. Preheat the thermal cycler to 72°C.

3. 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.

4. Mix by gently vortexing and then centrifuge briefly.

5. Incubate the tubes at 72°C in the preheated, heated-lid thermal cycler for 3 min. During this incubation, prepare the RT Master Mix.

6. At room temperature, prepare RT Master Mix by combining the following in the order shown. Wait to add the SMARTScribe Reverse Transcriptase to the Master Mix until just prior to use in Step 10.

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

*Ensure the First-Strand Buffer is completely in solution. Vortex gently to remove any cloudiness before use.
†Includes ~10% overage

7. Mix the RT Master Mix well by gently pipetting up and down, then centrifuge briefly.

8. Immediately after the 3-min incubation at 72°C (Step 5), place the samples on ice for 2 min.

9. Reduce the temperature of the thermal cycler to 42°C.

10. Add 7.5 µl of the RT Master Mix (Step 6) to each reaction tube. Mix the contents of each tube by pipetting gently and centrifuge briefly.

11. Place the tubes in a thermal cycler with a heated lid, preheated to 42°C. Run the following program:

42°C	90 min
70°C	10 min
4°C	forever

SAFE 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(s) and incorporate adapters and barcodes for Illumina sequencing platforms. Expression of different B-cell receptor chains can vary significantly among B-cell populations. Thus, we recommend separately amplifying each chain of interest (Figure 1A). See Appendix A for recommendations on multiplexing. Table 3 below provides PCR cycling recommendations, but optimal parameters may vary for different samples 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 B.

Table 3. 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	18	21
	100 ng	18	18
	1 µg	18	16
B cell	1 ng	18	21
	10 ng	18	18
	100 ng	18	16
Whole blood	100 ng	18	25
Spleen	10 ng	18	20
Bone Marrow	10 ng	18	20
Control RNA	10 ng	18	20
	100 ng	18	18
	1 µg	18	16

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

1. Protocol: Separate IgG/IgM/IgK/IgL Amplification

a) PCR 1

This PCR selectively amplifies full-length BCR V(D)J regions from first-strand cDNA. A portion of the first-strand cDNA is used for each amplification reaction (Figure 1). The hBCR PCR1 Universal Forward primer anneals to the 5′ end of transcripts via the SMART UMI Oligo sequence. The hBCR PCR1 IgG/IgM/IgK/IgL Reverse primers anneal to sequences in the constant regions of BCR heavy and light chain cDNAs.

NOTE: If multiplex PCR is preferred, in order to amplify all four chains at the same time, we recommend only combining heavy chain primers (G+M) and light chain primers (K+L) (Figure 1B). Please see Appendix A for protocol and recommended conditions.

1. Thaw 5X PrimeSTAR GXL Buffer, dNTP Mix, primers, and nuclease-free water on ice. Gently vortex each reagent to mix and centrifuge briefly. Store on ice. Remove the PrimeSTAR GXL DNA Polymerase from the freezer immediately before use, gently pipet to mix, centrifuge briefly, and store on ice.
2. Prepare a PCR1 Master Mix for each IgG/IgM/IgK/IgL chain of interest, by combining the following in the order shown, on ice. Gently vortex to mix and centrifuge briefly.

Components	1 rxn	12 rxns†	48 rxns†
Nuclease-Free Water	29 µl	391.5 µl	1,537 µl
5X PrimeSTAR GXL PCR Buffer	10 µl	135 µl	530 µl
dNTP Mixture	4 µl	54 µl	212 µl
hBCR PCR1 Universal Forward	1 µl	13.5 µl	53 µl
hBCR PCR1 IgG Reverse OR hBCR PCR1 IgM Reverse OR hBCR PCR1 IgK Reverse OR hBCR PCR1 IgL Reverse*	1 µl	13.5 µl	53 µl
PrimeSTAR GXL Polymerase	1 µl	13.5 µl	53 µl
Total Volume	**46 µl**	**621 µl**	**2,438 µl**

*Each PCR primer is used in a separate PCR Master Mix.
†Includes ~10% overage

NOTE: Alternatively, plan to add 1 µl of each primer individually instead of including them in the PCR1 Master Mix, particularly if the number of samples is low.

3. Add 46 µl of the appropriate IgG/IgM/IgK/IgL PCR1 Master Mix to nuclease-free, thin-wall 0.2 ml PCR plate/tube(s).
4. Add 4 µl of first-strand cDNA from Section VI.A. to the corresponding tube(s) containing PCR1 Master Mix. Gently vortex to mix and centrifuge briefly.
5. Place the plate/tube(s) in a preheated thermal cycler with a heated lid and run the following program:

PCR 1

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

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

b) PCR 2

PCR2 further amplifies the full-length BCR V(D)J regions and adds Illumina indexes using a semi-nested approach. The hBCR PCR2 Universal Forward 1–12 primers add P7/i7 index sequences. The hBCR PCR2 IgG/IgM/IgK/IgL Reverse 1–4 primers anneal to the constant region of the BCR sequence and add P5/i5 index sequences.

IMPORTANT: Different combinations of hBCR PCR2 Universal Forward 1–12 and hBCR PCR2 IgG/IgM/IgK/IgL Reverse 1–4 indexes must be used for each sample if samples are to be pooled and loaded on a single flow cell. See Appendix B for further details.

1. Thaw 5X PrimeSTAR GXL Buffer, dNTP Mix, primers, and nuclease-free water on ice. Gently vortex each reagent to mix and centrifuge briefly. Store on ice. Remove the PrimeSTAR GXL DNA Polymerase from the freezer immediately before use, gently pipet mix, centrifuge briefly, and store on ice.
2. For each IgG/IgM/IgK/IgL chain of interest, prepare a PCR2 Master Mix by combining the following in the order shown, on ice. Gently vortex to mix and centrifuge briefly.

Components	1 rxn	12 rxns†	48 rxns†
Nuclease-Free Water	32 µl	432 µl	1,696 µl
5X PrimeSTAR GXL PCR Buffer	10 µl	135 µl	530 µl
dNTP Mixture	4 µl	54 µl	212 µl
hBCR PCR2 IgG Reverse 1-4 OR hBCR PCR2 IgM Reverse 1-4 OR hBCR PCR2 IgK Reverse 1-4 OR hBCR PCR2 IgL Reverse 1–4*	1 µl	13.5 µl	53 µl
PrimeSTAR GXL Polymerase	1 µl	13.5 µl	53 µl
Total Volume	**48 µl**	**648 µl**	**2,544 µl**

*Each PCR primer is used in a separate PCR Master Mix.
†Includes ~10% overage

NOTE: Alternatively, plan to add 1 ul of each primer individually instead of including them in the PCR2 Master Mix, particularly if the number of samples is low. See Appendix B for guidelines on how to choose indexes.

3. For each reaction, add 48 µl of PCR2 Master Mix to nuclease-free, thin-wall, 0.2 ml PCR plate/tube(s).
4. Add 1 µl of appropriate PCR1 product to each corresponding PCR 2 tube.
5. Add 1 µl of the appropriate hBCR PCR2 Universal Forward 1–12 primer to each sample. Gently vortex to mix and centrifuge briefly.

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

PCR 2

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

*Consult Table 3 (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 NucleoMag NGS Clean-up and Size Select Beads

NOTES:
- Aliquot NucleoMag beads into 1.5 ml tubes upon receipt in the laboratory.
- Before each use, bring bead aliquots to room temperature for at least 30 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:
NucleoMag NGS Clean-up and Size Select beads, 80% ethanol (made fresh), a magnetic separation device, Elution Buffer.

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

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

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

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

5. While the reaction tubes are sitting on the magnetic separation device, use a pipette to transfer the supernatant (which contains your library) to clean PCR tubes.
6. Remove the tubes containing the beads from the magnetic separation device and discard them.

7. Add 10 µl of NucleoMag beads to each tube containing supernatant.

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

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

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

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

    NOTES:
    - Be sure to dry the pellet only until it is just dry. The pellet will look matte with no shine.
    - If you under-dry the pellet, ethanol will remain in the sample wells. The ethanol will reduce your library recovery rate and, ultimately, your yield. Allow the plate to sit at room temperature until the pellet is dry.
    - If you over-dry the pellet, there will be cracks in the pellet. It will take longer than 5 min to rehydrate (Step 16–17) and may reduce library recovery and yield.
    - Visit takarabio.com/rna-seq-tips to view examples of moist, dry, and overly dry pellets.

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

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

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

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

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

19. Transfer clear supernatant containing purified BCR 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 4°C overnight.

D. Protocol: Library Validation

To assess the success of library preparation, purification, and size selection, we recommend quantifying the libraries with a Qubit dsDNA HS kit (Thermo Fisher Scientific) and evaluating the libraries' size distributions with an Agilent 2100 Bioanalyzer and the DNA 1000 Kit (Agilent, Cat. No. 5067-1504).

1. 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 to 1,200 bp, with a maximum between ~600 bp and ~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.
2. Following validation, libraries are ready for sequencing on Illumina platforms. See Appendix C for sequencing guidelines.

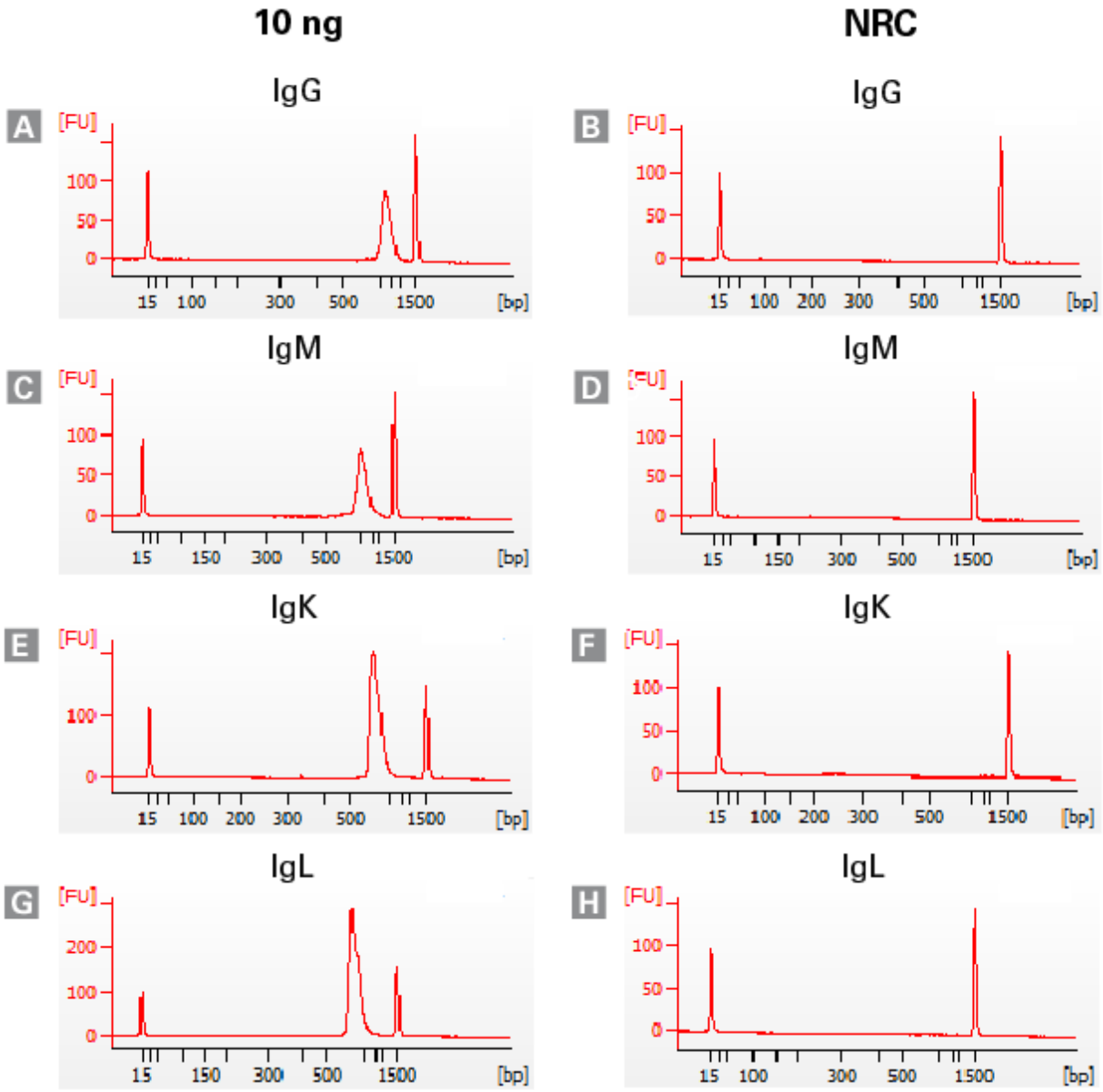

Figure 3. Example electropherogram results for BCR heavy and light (kappa or lambda) chain libraries validated using the Agilent 2100 Bioanalyzer. Libraries containing BCR heavy and light chain sequences were generated using the SMARTer Human BCR Profiling Kit and 10 ng of RNA obtained from a heterogeneous sample of PBMC RNA. Libraries were produced using 4 µl of first-strand cDNA as a template in 4 different PCRs for IgG and IgM heavy chain and kappa and lambda light chains, each for 18 cycles. 1 µl of the product from these PCRs was used as a template in a second, nested PCR reaction for 20 cycles for heavy chains. Following purification and size selection, libraries were analyzed on an Agilent 2100 Bioanalyzer (**Panels A–H**). **Panels A, C, E, and G** show broad peaks between ~500–1,200 bp and maximal peaks in the range of ~600–900 bp (typical results for a library generated from spleen RNA), analyzed on the Bioanalyzer. No RNA Control (NRC) samples (**Panels B, D, F, and H**) show no library produced and a flat Bioanalyzer profile within the range of 500–1,200 bp.

VII. 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 Multiplexing Heavy Chains (IgG + IgM) and Light Chains (IgK + IgL)

For optimal balance between the different chains, we recommend amplifying them separately. If multiplex PCR is desired despite the potential loss of PCR efficiency compared to single-tube PCRs, the heavy chain primers and light chain primers can also be pooled in the same PCR mix. We recommend only combining heavy chain primers (G and M) and light chain primers (K and L).

A. PCR 1

1. Thaw 5X PrimeSTAR GXL Buffer, dNTP Mix, primers, and Nuclease-Free Water on ice. Gently vortex each reagent to mix and centrifuge briefly. Store on ice. Remove the PrimeSTAR GXL DNA Polymerase from the freezer immediately before use, gently pipet mix, centrifuge briefly, and store on ice.
2. Prepare a PCR 1 Master Mix for each IgG/IgM/IgK/IgL chain of interest, by combining the following in the order shown on ice. 1 µl of IgG and 1 µl of IgM PCR1 Reverse primers (or IgK and IgL PCR1 Reverse primers) are added into the same PCR reaction to pair with 1 µl of Universal Forward primer. Gently vortex to mix and centrifuge briefly.

Components			1 rxn	12 rxns*	48 rxns*
Nuclease-Free Water			28 µl	378 µl	1,484 µl
5X PrimeSTAR GXL PCR Buffer			10 µl	135 µl	530 µl
dNTP Mixture			4 µl	54 µl	212 µl
hBCR PCR1 Universal Forward			1 µl	13.5 µl	53 µl
hBCR PCR1 IgG Reverse	**OR**	hBCR PCR1 IgK Reverse	1 µl	13.5 µl	53 µl
hBCR PCR1 IgM Reverse	**OR**	hBCR PCR1 IgL Reverse	1 µl	13.5 µl	53 µl
PrimeSTAR GXL Polymerase			1 µl	13.5 µl	53 µl
Total volume			**46 µl**	**621 µl**	**2,438 µl**

*Includes ~10% overage

3. Add 46 µl of the appropriate IgG/IgM/IgK/IgL PCR 1 Master Mix to nuclease-free thin-wall 0.2 ml PCR plate/tube(s).
4. Add 4 µl of first-strand cDNA from Section V.A. to the corresponding tube(s) containing PCR 1 Master Mix. Gently vortex to mix and centrifuge briefly.
5. Place the plate/tube(s) in a preheated thermal cycler with a heated lid and run the following program:

PCR 1

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

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

B. PCR 2

PCR 2 further amplifies the full-length BCR V(D)J regions and adds Illumina indexes using a semi-nested approach. The hBCR PCR2 Universal Forward 1–12 primers add P7/i7 index sequences. The hBCR PCR2 IgG/IgM/IgK/IgL Reverse 1–4 primers anneal to the constant region of the BCR sequence and adds P5/i5 index sequences.

1. Thaw 5X PrimeSTAR GXL Buffer, dNTP Mix, primers, and Nuclease-Free Water on ice. Gently vortex each reagent to mix and centrifuge briefly. Store on ice. Remove the PrimeSTAR GXL DNA Polymerase from the freezer immediately before use, gently pipet mix, centrifuge briefly, and store on ice.
2. For each IgG/IgM/IgK/IgL chain of interest, prepare a PCR 2 Master Mix by combining the following in the order shown on ice. 1 µl of IgG and 1 µl of IgM PCR2 Reverse primers (or IgK and IgL PCR2 Reverse primers) are added into the same PCR reaction to pair with 1 µl of PCR2 Forward primer. Gently vortex to mix and centrifuge briefly.

Components			1 rxn	12 rxns†	48 rxns†
Nuclease-Free Water			31 µl	418.5 µl	1,643 µl
5X PrimeSTAR GXL PCR Buffer			10 µl	135 µl	530 µl
dNTP Mixture			4 µl	54 µl	212 µl
hBCR PCR2 IgG Reverse*	OR	hBCR PCR2 IgK Reverse*	1 µl	13.5 µl	53 µl
hBCR PCR2 IgM Reverse *	OR	hBCR PCR2 IgL Reverse*	1 µl	13.5 µl	53 µl
PrimeSTAR GXL Polymerase			1 µl	13.5 µl	53 µl
Total Volume			**48 µl**	**648 µl**	**2,544 µl**

*Each PCR primer is used in a separate PCR Master Mix.
†Includes ~10% overage

3. For each reaction, add 48 µl of PCR 2 Master Mix to nuclease-free thin-wall 0.2 ml PCR plate/tube(s).
4. Add 1 µl of the appropriate PCR 1 product to each corresponding PCR 2 tube.
5. Add 1 µl of the appropriate hBCR PCR2 Universal Forward 1–12 primer to each sample. Gently vortex to mix and centrifuge briefly.
6. Place the plate/tube(s) in a preheated thermal cycler with a heated lid and run the following program:

PCR 2

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

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

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

Appendix B. Guidelines for PCR Cycling and Pooling

A. PCR Cycling

The gene expression of each of the B-cell receptor chains can vary significantly from one B-cell population to another. Therefore, it is 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. For this reason, we recommend that each chain of interest be amplified separately in each PCR step as shown in Figure 1A, with different library index combinations for each chain. The guidelines provided in Table 1 are a good starting point for determining the number of PCR cycles to perform in PCR 2 and have been determined by testing several a variety 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.

B. Sample Pooling and Illumina Indexing

Unique combinations of Illumina indexes are required to discriminate 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 kits, 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 all four chain libraries (IgG, IgM, IgK, and IgL) as long as they are from the same sample. Consult the Illumina literature (e.g., TruSeq® DNA Sample Preparation Guide) for appropriate pooling guidelines.

The hBCR PCR2 Universal Forward indexed primers contain the Read 2 sequence and i7 indexes. These primers are labeled sequentially and correspond to Illumina indexes D701–D712 (see Table 1). In addition to gene-specific sequences derived from the constant regions of human BCR IgG/IgM heavy chains and BCR light (kappa or lambda) chains, the hBCR PCR2 IgG/IgM/IgK/IgL Reverse indexed primers contain the Read 1 sequence and i5 indexes. Depending on the kit size, 1 or 4 different hBCR IgG/IgM/IgK/IgL R2 HT indexed primers containing unique i5 indexes are included for amplification of BCR heavy chains and BCR light (kappa or lambda) chains, respectively. These primers correspond with Illumina indexes D501–D504 (see Table 2). The pairwise combination of 12 unique i7 indexes with 4 unique i5 indexes allows for multiplexing of up to 48 samples in a single flow cell lane. A recommendation for pooling libraries with different index combinations for the 48-reaction kit is provided in Figure 4.

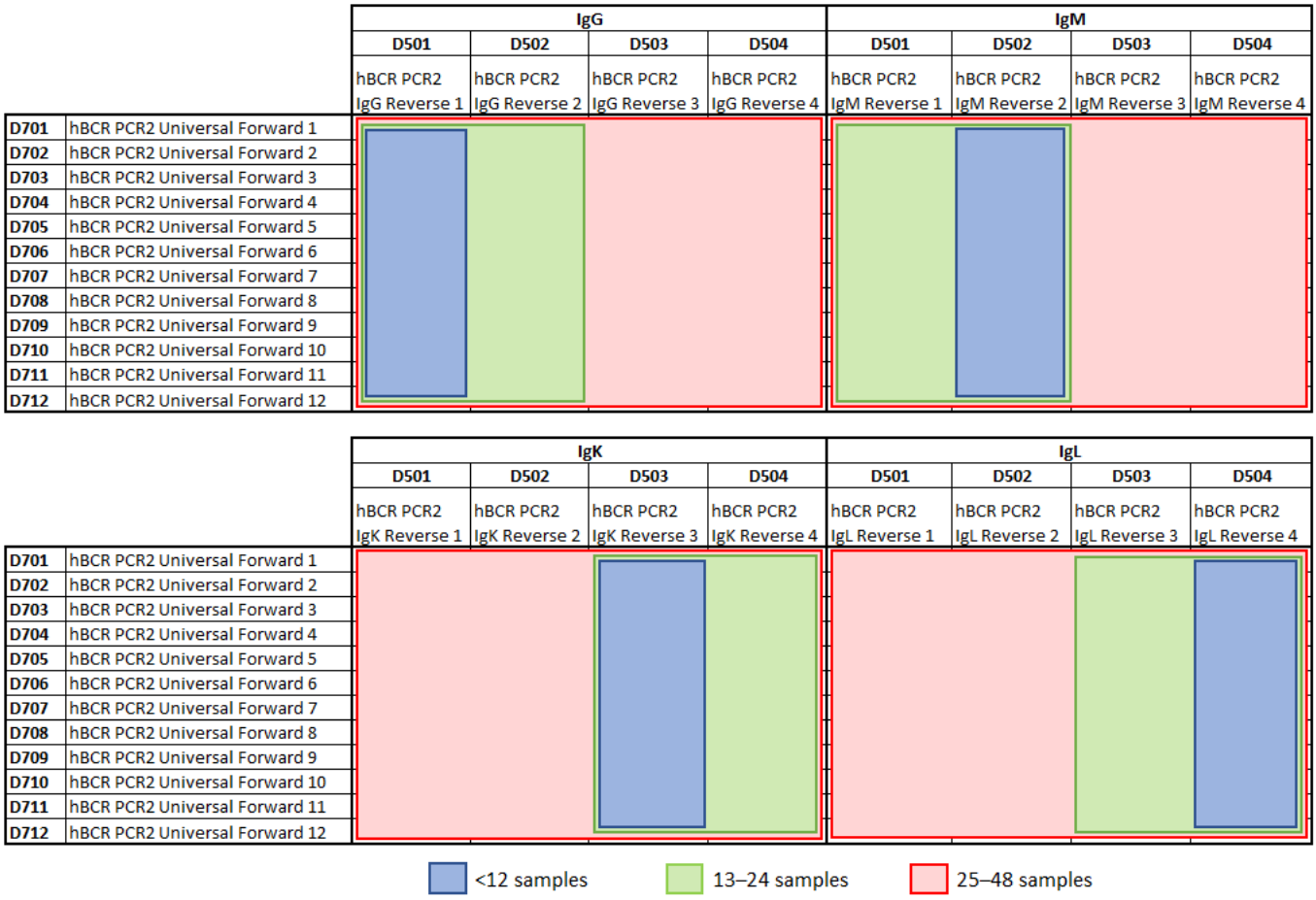

Figure 4. Pooling recommendations for libraries with different index combinations using the 48-reaction kit.

Appendix C. Guidelines for Library Sequencing and Data Analysis

Following library validation by Qubit and Bioanalyzer, prepare the desired library pools for the sequencing run. Prior to pooling, libraries must be carefully quantified. By combining the quantification obtained 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.edu.ru/eng/scripts/01_07.html. Alternatively, libraries can be quantified by qPCR using the Library Quantification Kit (Takara Bio, Cat. No. 638324).

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 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.

- 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.

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. When relying on Qubit quantification, we recommend diluting the pooled denatured libraries to a final concentration of 12.5 pM to achieve optimal cluster density. If using qPCR for quantification, you may need to use a lower final concentration.

The complexity of the human BCR repertoire varies from person to person. We generally recommend a minimum of 200,000 reads for heavy chain libraries (IgG and IgM) from an input of 10 ng PBMC RNA (or 1 ng B Cell RNA), and a minimum of 500,000 reads for light (IgK and IgL) chains from an input of 10 ng PBMC RNA (or 1 ng B cell RNA) input. For libraries generated from >10 ng PBMC RNA, higher sequencing depth is recommended, as shown in Table 4 below. However, the optimal conditions may vary for different samples types, sample masses, and sample complexities. We recommend trying a higher sequencing depth, then down sample to determine the minimum number of reads per library in order to determine the optimal sequencing depth.

Table 4. Recommended Sequencing Depth for BCR Libraries Prepared from Human PBMC RNA or B Cell RNA.

	10 ng PBMC RNA input	100 ng PBMC RNA input	1,000 ng PBMC RNA input	1 ng B cell RNA input	10 ng B cell RNA input	100 ng B cell RNA input
IgG, IgM	200k reads*	~1 x 10^6 reads	~5 x 10^6 reads	200K reads	~1 x 10^6 reads	~5 x 10^6 reads
IgK, IgL	500k reads	~2 x 10^6 reads	~10 x 10^6 reads	500K reads	~2 x 10^6 reads	~10 x 10^6 reads

*Reads per library. If using the multiplex PCR protocol (Appendix A), each PCR 2 product should be considered as two libraries.

Upon completion of a sequencing run, data can be analyzed with our Cogent NGS Immune Profiler Software. To obtain the Cogent NGS Immune Profiler Software, please visit takarabio.com/ngs-immune-profiler. As shown in Figure 5, a Human BCR Profiling library contains a 12-nucleotide UMI that could be used to remove duplicated sequences and correct errors from PCR amplification process.

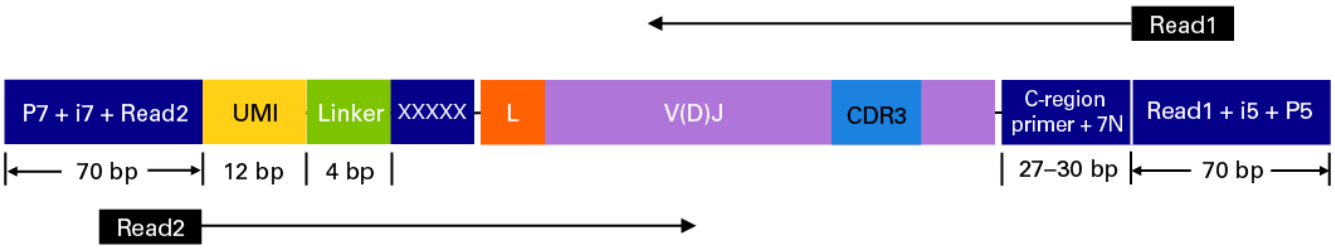

Figure 5. SMARTer Human BCR IgG IgM H/K/L Profiling library structure. First 19 nt from Read2 could be trimmed off if UMI analysis is not performed.

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

Notice to Purchaser

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

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

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