Takara Bio USA, Inc.

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

Cat. Nos. 634422, 634423 & 634424
(022125)

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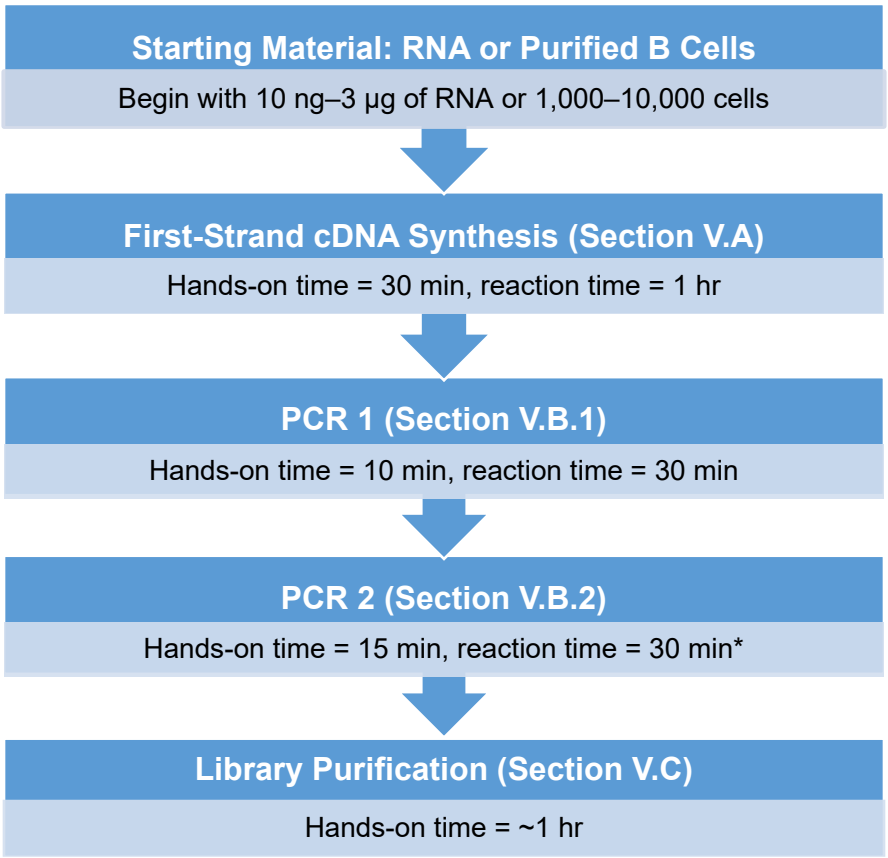
I. Introduction

B-Cell Receptor Profiling Using SMART® Technology

The **SMARTer Mouse BCR IgG H/K/L Profiling Kit** (Cat. Nos. 634422, 634423, 634424) enables users to analyze B-cell receptor (BCR) diversity from total RNA samples and whole cells. This kit is designed to work with a range of RNA input amounts (depending on the sample type), and has been shown to generate high-quality libraries from as little as 10 ng to 3 µg of total RNA from 1,000 to 10,000 purified B cells (e.g., hybridomas). Total RNA obtained from spleen, lymph node, PBMCs, and hybridomas have all been tested, and hybridoma cells have additionally been used directly in the kit without prior RNA isolation. As the name suggests, the kit can be used to generate data for both heavy (IgG only) and light chain diversity. The kit is not intended to identify the subclasses of IgG heavy chain that are expressed (i.e., IgG1, IgG2a, IgG2b, IgG2c, or IgG3). Included in the kit are primers that incorporate Illumina®-specific adaptor sequences during cDNA amplification. The protocol generates indexed libraries that are ready for sequencing on Illumina platforms.



*Depends on number of cycles

Figure 1. Protocol overview and timeline.

The SMARTer Mouse BCR IgG 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). First-strand cDNA synthesis is dT-primed (BCR dT Primer) and performed by the MMLV-derived SMARTScribe™ Reverse Transcriptase (RT), which adds non-templated nucleotides upon reaching the 5' end of each mRNA template. The BCR Oligonucleotide anneals to these non-templated nucleotides, and serves as a template for the incorporation of an additional sequence of nucleotides into the first-strand cDNA by the RT. (This is the template-switching step.) The BCR Oligonucleotide carries sequence from the Illumina Read Primer 2, serving as a primer-annealing site for subsequent rounds of PCR, and ensuring that only sequences from full-length cDNAs undergo amplification (Figure 2).

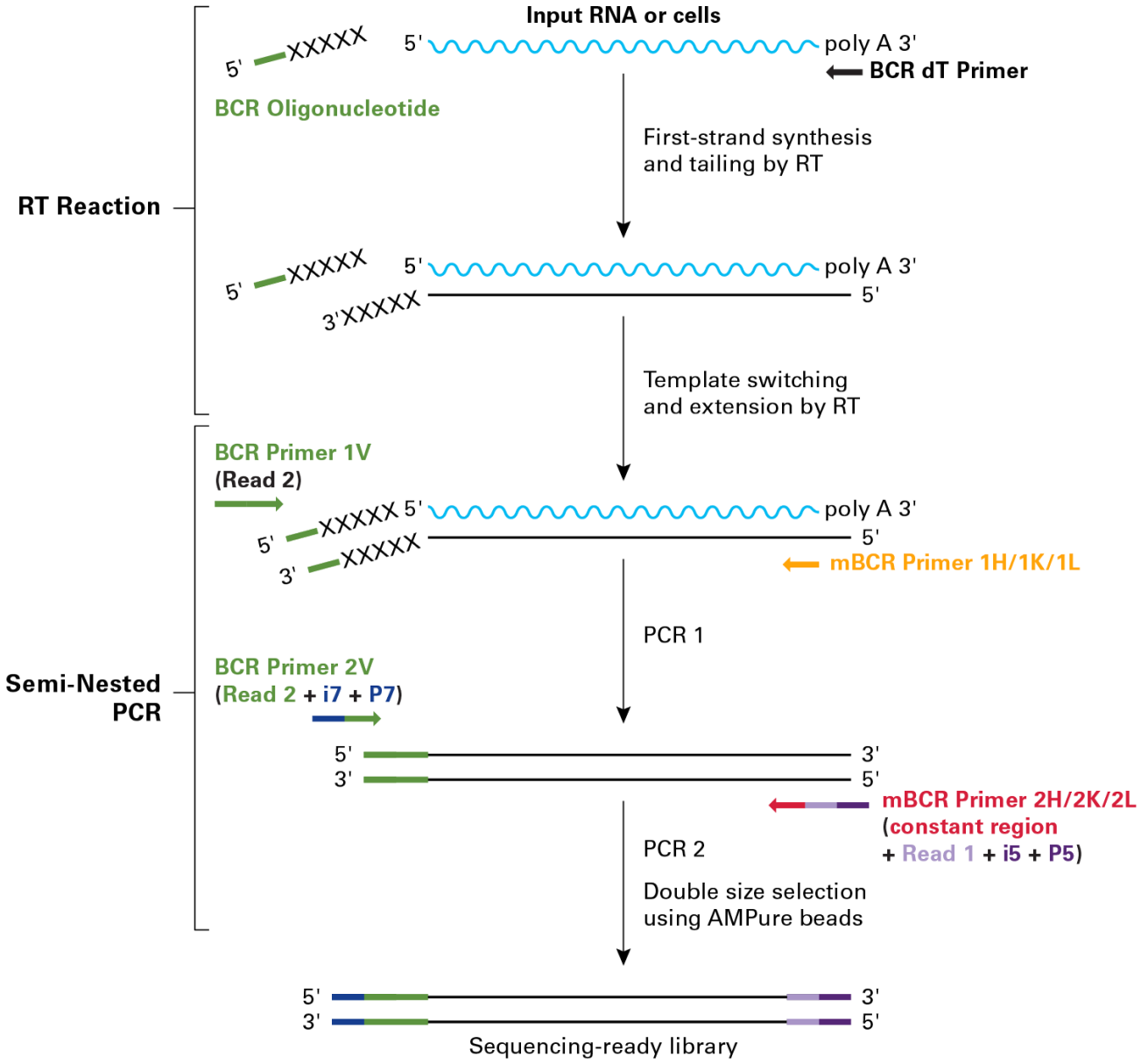


Figure 2. SMARTer Mouse BCR IgG H/K/L Profiling Kit workflow. dT-primed first-strand cDNA synthesis is followed by two rounds of successive PCR for amplification of cDNA sequences. After post-PCR purification, size selection, and quality analysis, the library is ready for sequencing.

Following reverse transcription, two rounds of PCR are performed in succession to amplify cDNA sequences corresponding to variable regions of BCR IgG heavy chain or BCR light chain (kappa or lambda) transcripts (Figure 3).

- The first PCR uses the first-strand cDNA as a template and includes a forward primer with complementarity to the Illumina Read Primer 2 sequence (BCR Primer 1V), and a reverse primer that is complementary to the constant (i.e., non-variable) region of BCR heavy or light chains (mBCR Primers 1H, 1K, or 1L). The chains are amplified in separate reactions. By priming from the Read Primer 2 sequence (added during template switching to the BCR Oligonucleotide) and the constant region, the first PCR specifically amplifies the entire variable region and a considerable portion of the constant region of BCR heavy or light chain cDNA.
- The second PCR takes the product from the first PCR as a template, and uses semi-nested primers (mBCR Primers 2H, 2K, or 2L) to amplify the entire variable region and a portion of the constant region of BCR heavy or light chain cDNA. As in PCR 1, the BCR subunit chains are amplified in separate reactions. The forward and reverse primers include adapter and index sequences that are compatible with the Illumina sequencing platform and allow for multiplexing of up to 96 samples in a single flow-cell lane.

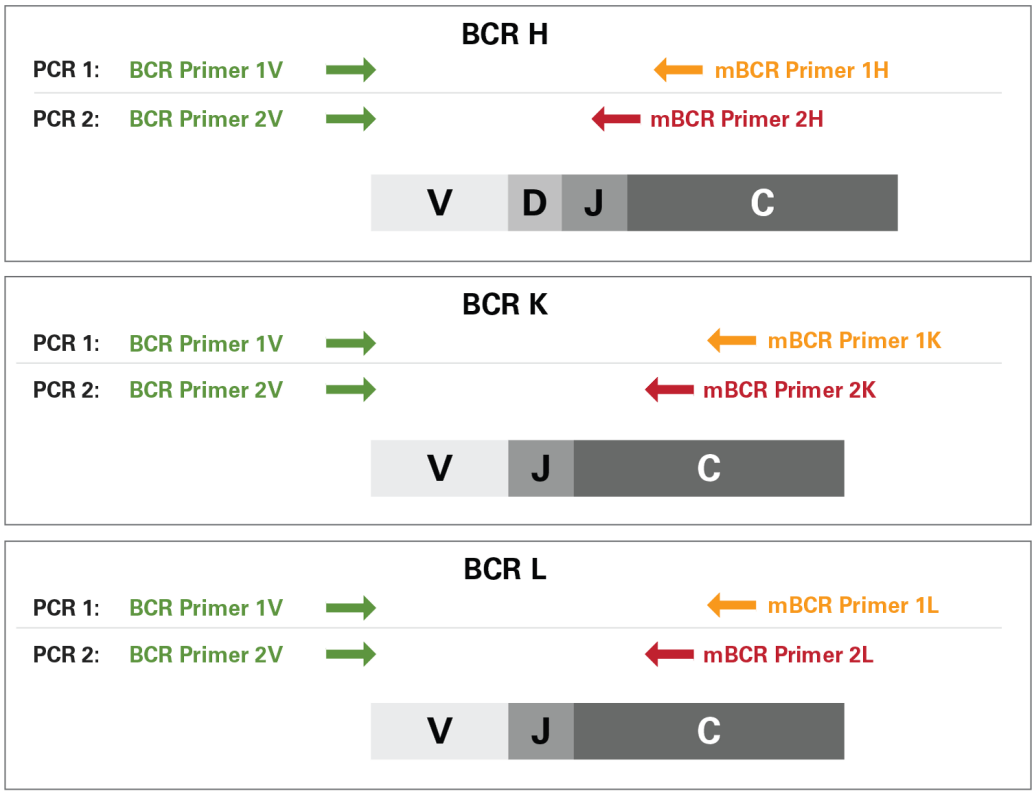


Figure 3. Two rounds of successive PCR for the amplification of cDNA sequences corresponding to variable regions of BCR IgG heavy chain or BCR light chain (kappa or lambda) transcripts. For each sample, after RT of all mRNAs in the sample, the user may specifically amplify 1–3 chains of IgG in two rounds of nested gene-specific PCR.

Following post-PCR purification, size selection, and quality analysis, the library is ready for sequencing (Figure 2).

II. List of Components

The SMARTer Mouse BCR IgG H/K/L Profiling Kit consists of the SMARTer Mouse BCR IgG H/K/L Profiling Kit Components (not sold separately) and the Mouse BCR H/K/L Indexing Primer Set HT for Illumina (not sold separately). **These components have been specifically designed to work together and are optimized for this particular protocol. Please do not make any substitutions.** The substitution of reagents in the kit and/or a modification of the protocol may lead to unexpected results. The reaction number for each kit specifies the number of libraries (BCR heavy chain, BCR kappa light chain and BCR lambda light chain combined) that can be generated with the indexing primers supplied.

SMARTer Mouse BCR IgG H/K/L Profiling Kit	**Cap color**	**634422 (12 rxn)**	**634423 (48 rxn)**	**634424 (96 rxn)**
SMARTer Mouse BCR IgG H/K/L Profiling Kit Components (Not sold separately. Storage conditions are listed below for Package 1 and Package 2.)				
Package 1 (Store at –70°C.)				
Control RNA (1 µg/µl)	–	5 µl	5 µl	5 µl
BCR Oligonucleotide (24 µM)	Pink	12 µl	48 µl	96 µl
Package 2 (Store at –20°C. Once thawed, store Nuclease-Free Water and 10X Lysis Buffer at 4°C and store Elution Buffer at room temperature. Continue to store all other reagents at –20°C.)				
BCR dT Primer (12 µM)	Blue	24 µl	96 µl	192 µl
5X Ultra® Low First-Strand Buffer	Red	48 µl	192 µl	384 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	Purple	24 µl	96 µl	192 µl
Nuclease-Free Water	–	5 ml	20 ml	2 x 20 ml
RNase Inhibitor (40 U/µl)	White	30 µl	60 µl	60 µl
10X Lysis Buffer	Clear/–*	230 µl	920 µl	1.85 ml
Elution Buffer (10 mM Tris-HCl, pH 8.5)	–	2 x 1.7 ml	8 ml	2 x 8 ml
mBCR Primer 1H (12 µM)	Light Blue	6 µl	24 µl	48 µl
mBCR Primer 1K (12 µM)	Khaki	6 µl	24 µl	48 µl
mBCR Primer 1L (12 µM)	Brown	6 µl	24 µl	48 µl
BCR Primer 1V (12 µM)	Green	18 µl	72 µl	144 µl
PrimeSTAR® GXL SP DNA Polymerase (1.25 U/µl)	White	200 µl	600 µl	2 x 600 µl
5X PrimeSTAR GXL SP Buffer	White/Green†	2 x 1 ml	6 ml	2 x 6 ml
dNTP Mixture (2.5 mM each)	White/Yellow†	800 µl	2.4 ml	4.8 ml

*Depending on the product size, these reagents are packaged in a tube with a clear cap or in a bottle.
†Depending on the product size, these reagents are packaged in a tube with a white cap or in a bottle with a green or yellow cap, as indicated.

SMARTer Mouse BCR IgG H/K/L Profiling Kit, continued	**Cap color**	**634422 (12 rxn)**	**634423 (48 rxn)**	**634424 (96 rxn)**
Mouse BCR H/K/L Indexing Primer Set HT for Illumina (Not sold separately. Store at –20°C. For details, see Appendix A)				
mBCR Primer 2H D501 (2H01; 12.5 µM)	Red	18 µl	18 µl	18 µl
mBCR Primer 2H D502 (2H02; 12.5 µM)	Red		18 µl	18 µl
mBCR Primer 2H D503 (2H03; 12.5 µM)	Red		18 µl	18 µl
mBCR Primer 2H D504 (2H04; 12.5 µM)	Red		18 µl	18 µl
mBCR Primer 2H D505 (2H05; 12.5 µM)	Red			18 µl
mBCR Primer 2H D506 (2H06; 12.5 µM)	Red			18 µl
mBCR Primer 2H D507 (2H07; 12.5 µM)	Red			18 µl
mBCR Primer 2H D508 (2H08; 12.5 µM)	Red			18 µl

SMARTer Mouse BCR IgG H/K/L Profiling Kit, continued	**Cap color**	**634422 (12 rxn)**	**634423 (48 rxn)**	**634424 (96 rxn)**
Mouse BCR H/K/L Indexing Primer Set HT for Illumina (Not sold separately. Store at –20°C. For details, see Appendix A)				
mBCR Primer 2K D501 (2K01; 12.5 µM)	Orange	18 µl	18 µl	18 µl
mBCR Primer 2K D502 (2K02; 12.5 µM)	Orange		18 µl	18 µl
mBCR Primer 2K D503 (2K03; 12.5 µM)	Orange		18 µl	18 µl
mBCR Primer 2K D504 (2K04; 12.5 µM)	Orange		18 µl	18 µl
mBCR Primer 2K D505 (2K05; 12.5 µM)	Orange			18 µl
mBCR Primer 2K D506 (2K06; 12.5 µM)	Orange			18 µl
mBCR Primer 2K D507 (2K07; 12.5 µM)	Orange			18 µl
mBCR Primer 2K D508 (2K08; 12.5 µM)	Orange			18 µl
mBCR Primer 2L D501 (2L01; 12.5 µM)	Green	18 µl	18 µl	18 µl
mBCR Primer 2L D502 (2L02; 12.5 µM)	Green		18 µl	18 µl
mBCR Primer 2L D503 (2L03; 12.5 µM)	Green		18 µl	18 µl
mBCR Primer 2L D504 (2L04; 12.5 µM)	Green		18 µl	18 µl
mBCR Primer 2L D505 (2L05; 12.5 µM)	Green			18 µl
mBCR Primer 2L D506 (2L06; 12.5 µM)	Green			18 µl
mBCR Primer 2L D507 (2L07; 12.5 µM)	Green			18 µl
mBCR Primer 2L D508 (2L08; 12.5 µM)	Green			18 µl
BCR Primer 2V D701 (2V01; 12.5 µM)	Blue	54 µl	54 µl	54 µl
BCR Primer 2V D702 (2V02; 12.5 µM)	Blue	54 µl	54 µl	54 µl
BCR Primer 2V D703 (2V03; 12.5 µM)	Blue	54 µl	54 µl	54 µl
BCR Primer 2V D704 (2V04; 12.5 µM)	Blue	54 µl	54 µl	54 µl
BCR Primer 2V D705 (2V05; 12.5 µM)	Blue	54 µl	54 µl	54 µl
BCR Primer 2V D706 (2V06; 12.5 µM)	Blue	54 µl	54 µl	54 µl
BCR Primer 2V D707 (2V07; 12.5 µM)	Blue	54 µl	54 µl	54 µl
BCR Primer 2V D708 (2V08; 12.5 µM)	Blue	54 µl	54 µl	54 µl
BCR Primer 2V D709 (2V09; 12.5 µM)	Blue	54 µl	54 µl	54 µl
BCR Primer 2V D710 (2V10; 12.5 µM)	Blue	54 µl	54 µl	54 µl
BCR Primer 2V D711 (2V11; 12.5 µM)	Blue	54 µl	54 µl	54 µl
BCR Primer 2V D712 (2V12; 12.5 µM)	Blue	54 µl	54 µl	54 µl

Storage Conditions

- Store Control RNA and BCR Oligonucleotide at –70°C.
- Store 10X Lysis Buffer 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. Additional Materials Required

The following reagents and materials are required but not supplied. They have been validated to work with this protocol. Please do not make any substitutions as you may not obtain the expected results:

- Single-channel pipettes: 10 µl, 20 µl, and 200 µl
- Eight-channel pipettes (recommended): 20 µl and 200 µl

- Filter pipette tips: 2 µl, 20 µl, and 200 µl
- Minicentrifuge for 1.5 ml tubes
- Minicentrifuge for 0.2 ml tubes or strips

For PCR Amplification & Validation:

- One dedicated thermal cycler used only for first-strand cDNA synthesis (Section V.A)
- One dedicated thermal cycler used only for library amplification by PCR (Section V.B)
- For validation using the Advanced Analytical Fragment Analyzer: High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical, Cat. No. DNF-474; Section V.D)
- For validation using the Agilent 2100 Bioanalyzer: DNA 1000 Kit (Agilent Technologies, Cat. No. 5067-1504; Section V.D)
- Nuclease-free thin-wall PCR tubes or strips (0.2 ml PCR 8-tube strip; USA Scientific, Item No. 1402-4700)
- Nuclease-free low-adhesion 1.5 ml tubes (USA Scientific, Item No. 1415-2600) or LoBind tubes (Eppendorf, Cat. No. 022431021)

For SPRI (Solid Phase Reversible Immobilization) Bead Purifications (Section V.C):

- Agencourt AMPure XP PCR purification kit—used to purify amplified libraries
  (5 ml size: Beckman Coulter Item No. A63880; 60 ml size: Beckman Coulter Item No. A63881)

  NOTE: SPRI beads need to come to room temperature before the container is opened. Therefore, we recommend aliquoting the beads into 1.5 ml tubes upon receipt and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 min). This will also decrease the chances of bead contamination. Mix well to disperse the beads before adding them to your reactions. The beads are viscous, so pipette slowly.

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

IV. General Considerations

A. Requirements for Preventing Contamination

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

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

  NOTES:
  - The PCR clean workstation must be located in a clean room with positive air flow, as contamination may occur very easily. Once contamination occurs it can be difficult to remove.
  - Strictly obey clean room operation rules.

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

B. General Requirements

- **The success of your experiment depends on the quality of your input RNA. Prior to cDNA synthesis, please make sure that your RNA is of high integrity and free of contaminants.**
- Avoid using heparin for blood sample collection, as it can inhibit downstream enzymatic steps such as cDNA synthesis and PCR.
- The assay is very sensitive to variations in pipette volume, etc. Please make sure all your pipettes are calibrated for reliable delivery, and make sure no reagent clings to the outside of the tips.
- All lab supplies related to cDNA synthesis need to be stored in a DNA-free, closed cabinet. Reagents for cDNA synthesis need to be stored in a freezer/refrigerator that has not previously been used to store PCR amplicons.
- Add enzymes to reaction mixtures last, and thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- Do not increase (or decrease) the amount or concentration of any reaction component. The amounts and concentrations have been carefully optimized for the included reagents and protocol.
- If you are using this protocol for the first time, we strongly recommend that you perform negative and positive control reactions to verify that kit components are working properly.

C. Sample Recommendations

1. Total RNA Extraction

- The performance of the SMARTer Mouse BCR IgG H/K/L Profiling Kit is dependent on the quality of the RNA starting material. Because this kit utilizes an oligo(dT) primer for first-strand cDNA synthesis, higher-quality RNA ensures selective and efficient results.

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

- When choosing a purification kit, ensure that it is appropriate for your sample amount. There are several commercially available kits designed to purify total RNA from extremely small samples (e.g., Takara Bio offers the NucleoSpin RNA XS kit for purification of RNA from ≥100 cells; Cat. No. 740902.10).

2. Evaluation of RNA Quality

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

D. Sample Requirements

1. Total RNA

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

2. Cells

This protocol has been used successfully to generate cDNA starting from 1,000–10,000 intact, cultured cells**. It cannot be used with cells that have undergone fixation.**

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

V. Protocols

A. Protocol: First-Strand cDNA Synthesis

(Perform in PCR clean workstation)
First-strand cDNA synthesis (from RNA) is primed by the BCR dT Primer and uses the BCR Oligonucleotide for template switching at the 5' end of the transcript.

For this protocol, you will need the following components:
10X Lysis Buffer (tube with a clear cap or a bottle, depending on product size), RNase Inhibitor (white cap), Nuclease-Free Water (bottle), Control RNA (clear cap), BCR dT Primer (blue cap), 5X Ultra Low First-Strand Buffer (red cap), BCR Oligonucleotide (pink cap), and SMARTScribe Reverse Transcriptase (purple cap).

1. Thaw the 5X Ultra Low First-Strand Buffer at room temperature. Thaw all the remaining reagents (except the enzyme) needed for first-strand cDNA synthesis on ice. Gently vortex each reagent to mix and spin down briefly. Store all but the 5X Ultra Low First-Strand Buffer on ice.

   NOTE: The 5X Ultra Low First-Strand Buffer may form precipitates. Thaw this buffer at room temperature and vortex before using to ensure all components are completely in solution.

2. Prepare a stock solution of 10X Reaction Buffer by mixing the 10X Lysis Buffer with the RNase Inhibitor as indicated below (scale up as needed):

19 µl	10X Lysis Buffer	(tube with a clear cap or a bottle, depending on product size)
1 µl	RNase Inhibitor	(white cap)
20 µl	**Total volume**	

   Mix briefly, then spin down.

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

3. See Table 1 below for guidelines on setting up reactions for your control and test samples. Prepare each sample (10.5 µl total volume) in individual 0.2 ml RNase-free PCR tubes or in an 8-well strip:

   - **Purified Total RNA:** If you are working with purified total RNA, transfer 1–9.5 µl to a 0.2 ml RNase-free PCR tube. Bring the volume to 9.5 µl with Nuclease-Free Water. Add 1 µl of 10X Reaction Buffer.
   - **Cells:** If you are working with cells, isolate cells in validated media, wash and resuspend in Mg^{2+}/Ca^{2+}-free PBS, and transfer to a 0.2 ml RNase-free PCR tube. Bring the volume to 9.5 µl with nuclease-free water. Add 1 µl of 10X Reaction Buffer. Gently vortex to mix the sample. It is critical to avoid bubbles when mixing. Incubate at room temperature for 5 min. See Section IV.D for more information on working with cells.

Table 1. Sample Preparation Guidelines

Components	Negative Control	Positive Control	Test Sample
10X Reaction Buffer (from Step 2)	1 µl	1 µl	1 µl
Nuclease-Free Water	9.5 µl	Up to 8.5 µl	Up to 8.5 µl
Diluted Control RNA*	–	1–9.5 µl	–
Sample	–	–	1–9.5 µl
Total volume	10.5 µl	10.5 µl	10.5 µl

*The Control RNA is supplied at a concentration of 1 µg/µl. It should be diluted in Nuclease-Free Water with RNase Inhibitor (1 µl RNase Inhibitor in a final volume of 50 µl of water) to match the concentration of your test sample. Perform serial dilutions on the Control RNA to obtain the appropriate concentration. We have tested this protocol extensively using the PCR cycling conditions below with 10 ng of input Control RNA.

4. Place the samples on ice and add 2 µl of the BCR dT Primer (blue cap) to each sample. Mix well by gently vortexing and then spin the tube(s) briefly to collect the contents at the bottom of the tube.
5. Preheat the thermal cycler to 72°C.
6. Incubate the tubes at 72°C in the preheated, hot-lid thermal cycler for 3 min.

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

7. Prepare enough Master Mix for all the reactions, plus 10% of the total reaction mix volume, by combining the following reagents in the order shown at room temperature.

Per reaction:

4 µl	5X Ultra Low First-Strand Buffer (red cap)*
1 µl	BCR Oligonucleotide (pink cap)
0.5 µl	RNase Inhibitor (white cap)
2 µl	SMARTScribe Reverse Transcriptase (purple cap)†
7.5 µl	**Total volume added per reaction**

*The 5X Ultra Low First-Strand Buffer should be thawed at room temperature and vortexed gently to remove any cloudiness in the buffer before using.

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

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

9. Immediately after the 3 min incubation at 72°C (Step 6), place the samples on ice for 2 min.
10. Reduce the temperature of the thermal cycler to 42°C.
11. Add 7.5 µl of the Master Mix to each reaction tube. Mix the contents of each tube by pipetting gently, and spin briefly to collect the contents at the bottom of each tube.
12. Place the tubes in a thermal cycler with a heated lid, preheated to 42°C. Run the following program:

42°C	45 min
70°C	10 min
4°C	forever

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

B. Protocol: BCR IgG H/K/L Amplification and Sequencing Library Generation by Semi-Nested PCR

(Perform Steps 1–3 in a PCR clean workstation)

IMPORTANT: This PCR protocol has been optimized based on an initial input of 10 ng of total RNA (isolated from mouse spleen) into the RT reaction described in the previous protocol. Optimal parameters may vary for different templates, cell types, thermal cyclers, and sample amounts. We **strongly** recommend trying a range of cycle numbers to determine the minimum number of cycles necessary to obtain the desired yield for your sample and conditions. Table 2 provides guidelines for optimization, depending on the amount of total RNA or the number of cells used for first-strand cDNA synthesis.

Table 2. Cycling Guidelines Based on Amount of Starting Material. Analysis of BCR heavy chain, BCR kappa light chain, or BCR lambda light chain using the corresponding primer set.

Input Type	Input Amount	Chain	Number of PCR 1 Cycles	Number of PCR 2 Cycles*
Control RNA	10–100 ng	H	18	15
		K	18	13
		L	18	15
Control RNA	100–1,000 ng	H	18	12
		K	18	10
		L	18	12
Control RNA	1 µg–3 µg	H	18	9
		K	18	7
		L	18	9
Purified B cells	1,000–10,000 cells	H	18	19
		K	18	19
		L	18	19

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

1. PCR 1

This PCR selectively amplifies BCR sequences from the first-strand cDNA generated with the previous protocol. A portion of the first-strand cDNA is used for amplification of each chain of interest. BCR Primer 1V anneals to the Illumina Read Primer 2 sequence (incorporated during first-strand cDNA synthesis). mBCR Primer 1H, mBCR Primer 1K, and mBCR Primer 1L anneal

to sequences in the constant regions of BCR heavy and light (kappa or lambda) chain cDNA, respectively.

For this protocol, you will need the following components:
5X PrimeSTAR GXL SP Buffer (tube with a white cap or a bottle with a green cap, depending on product size), dNTP Mixture (tube with a clear cap or a bottle with a yellow cap, depending on product size), BCR Primer 1V (green cap), mBCR Primer 1H (light blue cap), mBCR Primer 1K (khaki cap), and mBCR Primer 1L (brown cap), PrimeSTAR GXL SP DNA Polymerase (white cap), and Nuclease-Free Water (bottle).

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

10 µl	5X PrimeSTAR GXL SP Buffer (tube with a white cap or a bottle with a green cap)
4 µl	dNTP Mixture (tube with a white cap or a bottle with a yellow cap)
0.5 µl	BCR Primer 1V (green cap)
0.5 µl	mBCR Primer 1H, mBCR Primer 1K, or mBCR Primer 1L (light blue, khaki, or brown caps)*
1 µl	PrimeSTAR GXL SP DNA Polymerase (white cap)
29 µl	Nuclease-Free Water (bottle)
45 µl	**Total volume added per reaction**

*Each PCR primer is used in a separate PCR Master Mix.

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

3. Add 45 µl of PCR Master Mix to each tube containing 5 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 tube(s).

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

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

PCR 1

	95°C	1 min
18 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

2. PCR 2

This PCR further amplifies sequences corresponding to full-length BCR variable regions and adds Illumina HT sequencing adapters using a semi-nested approach. The BCR Primer 2V indexed primers anneal to a sequence added by BCR Primer 1V and add Illumina P7-i7 index sequences. The nested BCR heavy and BCR light (kappa or lambda) chain mBCR Primer 2 indexed primers anneal to sequences in BCR constant regions that are internal to the sequences bound by mBCR Primer 1H, mBCR Primer 1K, and mBCR Primer 1L, and add both the Illumina Read 1 sequence and P5-i5 index sequences.

IMPORTANT: Different combinations of BCR Primer 2V indexes (D701–D712) and mBCR Primer 2H/K/L indexes (D501–D508) must be used for each sample if samples are to be multiplexed on a single flow cell. See Appendix A for further details.

For this protocol, you will need the following components:
5X PrimeSTAR GXL SP Buffer (tube with a white cap or a bottle with a green cap), dNTP Mixture (tube with a white cap or a bottle with a yellow cap), BCR IgG H/K/L Mouse Indexing Primer Set HT for Illumina (blue, red, orange, and green caps), PrimeSTAR GXL SP DNA Polymerase (white cap), and Nuclease-Free Water (bottle).

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

10 µl	5X PrimeSTAR GXL SP Buffer (tube with a white cap or a bottle with a green cap)
4 µl	dNTP Mixture (tube with a white cap or a bottle with a yellow cap)
0.5 µl	mBCR 2H, mBCR 2K, or mBCR 2L (red, orange, or green caps)*
1 µl	PrimeSTAR GXL SP DNA Polymerase (white cap)
33 µl	Nuclease-Free Water (bottle)
48.5 µl	**Total volume added per reaction**

*Each PCR primer is used in a separate PCR Master Mix. For each set of up to 12 chain-specific (H, K, or L) reactions, use a chain-specific mBCR primer (H, K, or L) carrying the same i5 HT index (see Appendix A). For example, for 12 reactions prepare 3 chain-specific PCR Master Mixes: 1) mBCR Primer 2H D501, 2) mBCR Primer 2K D501, and 3) mBCR Primer 2L D501.

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

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

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

PCR 2 (similar to PCR 1, but with different cycle numbers)

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

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

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

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

The BCR sequencing library is size-selected and purified using AMPure XP beads. This approach involves two rounds of size selection, which together remove primers, primer dimers, and PCR products containing undersized or oversized inserts. In the first round, fragments larger than ~900 bp are immobilized on beads and removed from the supernatant. In the second round, the supernatant is added to fresh beads, which immobilize fragments within the desired size range of ~400–900 bp. The beads are then washed with 80% ethanol and fragments are eluted with Elution Buffer. This approach preserves library yield and complexity while maximizing the relevance and consistency of downstream sequencing results.

NOTES:

- Aliquot AMPure XP beads into 1.5 ml tubes upon receipt in the laboratory.
- Before each use, bring bead aliquots to room temperature for at least 30 min and mix well to disperse.
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.
- You will need a magnetic separation device for 0.2 ml tubes, strip tubes, or a 96-well plate.
- You will need a 200 µl and 20 µl multi-channel pipette to work quickly during the wash steps to avoid over-drying the beads.

For this protocol, you will need the following components:
Agencourt AMPure XP PCR Purification beads, 80% ethanol (made fresh), a magnetic separation device, and Elution Buffer (bottle).

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

   NOTE: The beads are viscous; pipette the entire volume and push it out slowly. **Mix thoroughly by pipetting or vortexing. It is critical to avoid generating bubbles when mixing, because bubbles make 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 in separating the samples for >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. Add 10 µl of AMPure XP beads to each tube containing supernatant.

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

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

   NOTE: The beads are viscous; pipette the entire volume and push it out slowly. **Mix thoroughly by pipetting or vortexing. It is critical to avoid generating bubbles when mixing, because bubbles make 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.
10. 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.)
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.
13. Cover the plate or strip tubes, then briefly spin (~2,000*g*) to collect any 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 ~1–2 min until the pellet appears dry and is no longer shiny. You may see a tiny crack in the pellet.

    NOTE: Be sure to dry the pellet only until it is just dry. The pellet will look matte with no shine.
    - If you under-dry the pellet, ethanol will remain in the sample wells. The ethanol will reduce your library recovery rate and ultimately your yield. Allow the plate to sit at room temperature until the pellet is dry.
    - If you over-dry the pellet, there will be cracks in the pellet. It will take longer than 2 min to rehydrate (Step 15) and may reduce library recovery and yield.
    - Visit takarabio.com/rna-seq-tips to view examples of moist, dry, and overly dry pellets.

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. Remove the samples from the magnetic separation device and mix thoroughly by pipetting up and down to ensure complete bead dispersion.

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

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 unpelleted beads up and down to resuspend them with the supernatant and then pipette them towards the magnet where the rest of the beads have already pelleted (without disrupting the existing pellet). Continue the incubation until there are no beads left in the supernatant.

18. Transfer clear supernatant containing the 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 samples may be stored at –20°C indefinitely.

D. Protocol: Library Validation

To determine whether library production, purification, and size selection were successful, we recommend analyzing and validating final libraries using an Advanced Analytical Fragment Analyzer and the High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical, Cat. No. DNF-474) **or** the Agilent 2100 Bioanalyzer and the DNA 1000 Kit (Agilent Technologies, Cat. No. 5067-1504). Please refer to the corresponding user manuals for detailed instructions.

1. Aliquot 1 µl of diluted library for validation using the Advanced Analytical Fragment Analyzer (Figure 4). (Libraries were diluted 1:5 for the examples shown below.) Alternatively, aliquot 1 µl of undiluted library for validation using the Agilent 2100 Bioanalyzer (Figure 5).
2. Compare the results for your samples with Figure 3 to verify whether each sample is suitable for further processing. Successful library production and purification should yield no product for negative control reactions, and a broad peak spanning 500 bp to 1,200 bp, with a maximum between ~700 bp and ~800 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.
3. Following validation, libraries are ready for sequencing on Illumina platforms. See Appendix C for sequencing guidelines.

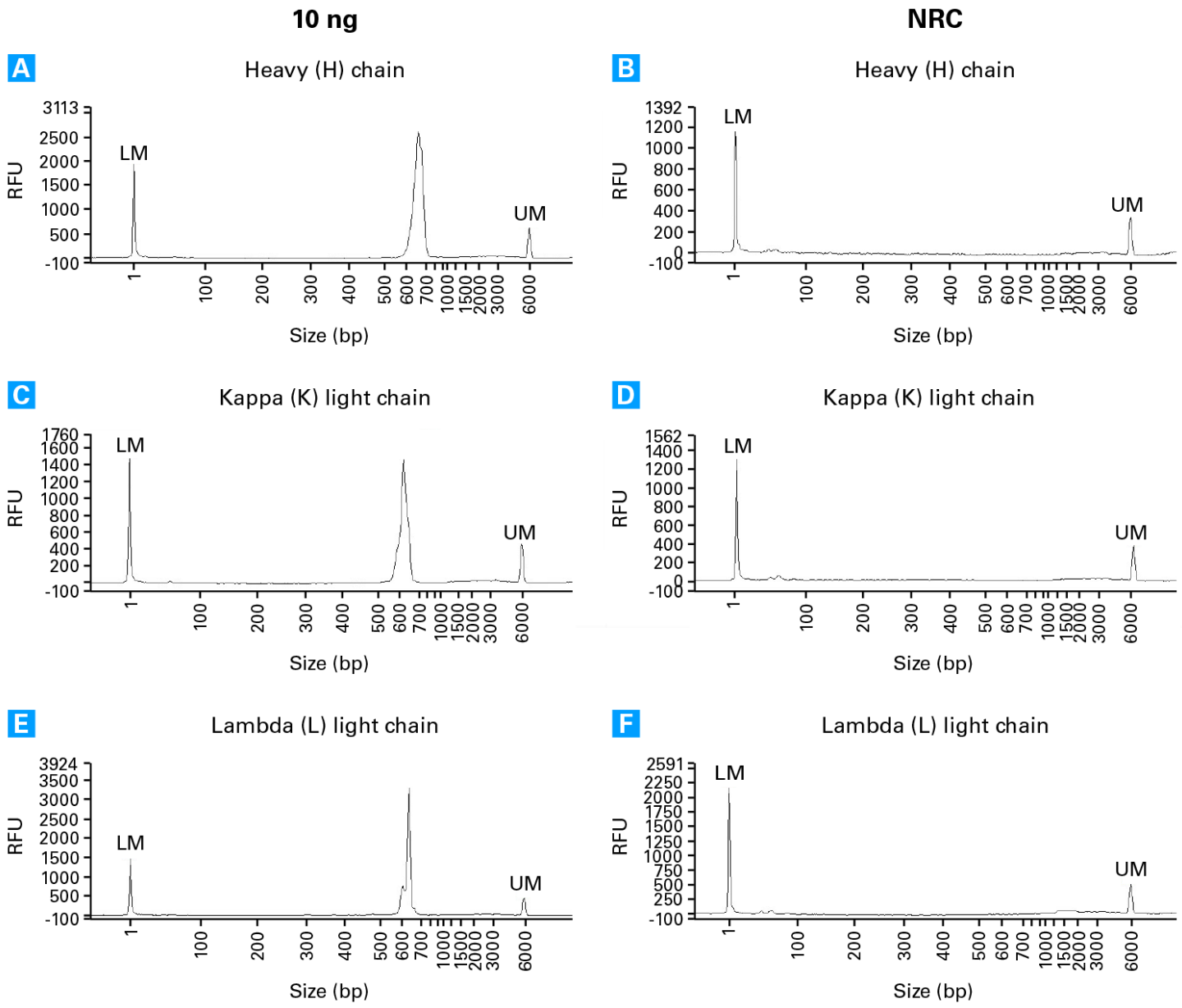


Figure 4. Example electropherogram results for BCR heavy and light (kappa or lambda) chain libraries validated using the Advanced Analytical Fragment Analyzer. Libraries containing BCR heavy and light chain sequences were generated using the SMARTer Mouse BCR IgG H/K/L Profiling Kit and 10 ng of RNA obtained from a heterogeneous sample of spleen RNA. Libraries were produced using 5 µl of first-strand cDNA as a template in 3 different PCRs for 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 19 cycles for heavy chain, 13 cycles for kappa chain, and 16 cycles for lambda chain. Following purification and size selection libraries were analyzed on an Advanced Analytical Fragment Analyzer (**Panels A–F**). Peaks labeled "LM" and "UM" correspond to DNA reference markers included in each analysis. **Panels A, C, and E** show relatively sharp peaks between ~500–800 bp and maximal peaks in the range of ~600-700 bp (typical results for a library generated from spleen RNA), analyzed on the Fragment Analyzer. No RNA Control (NRC) samples (**Panels B, D, and F**) show no library produced and a flat Fragment Analyzer profile within the range of 500–800 bp.

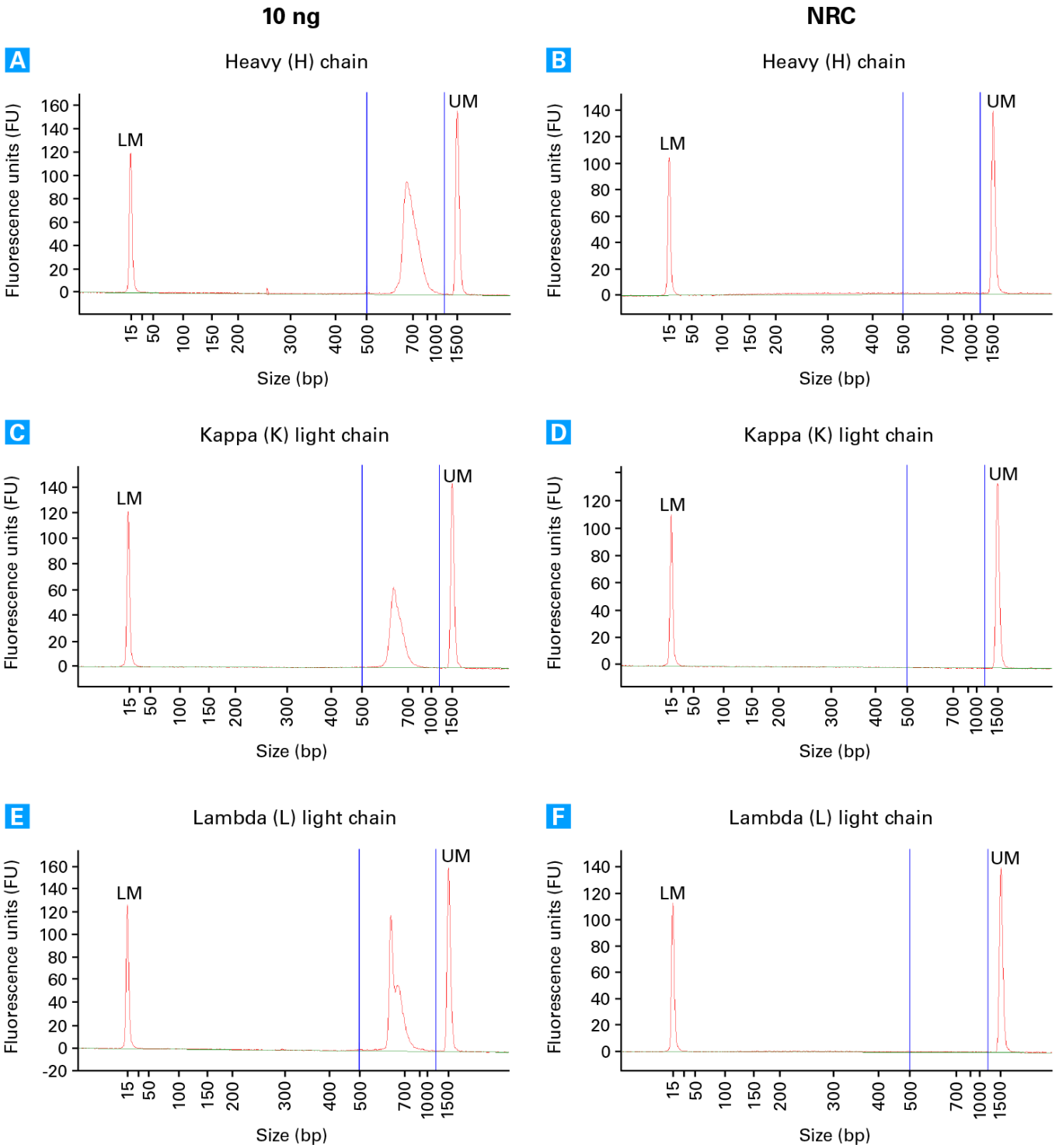


Figure 5. Example electropherogram results for BCR heavy and light (kappa or lambda) chain libraries validated using the Agilent 2100 Bioanalyzer. Libraries containing BCR heavy and light chain sequences were generated using the SMARTer Mouse BCR IgG H/K/L Profiling Kit and 10 ng of RNA obtained from a heterogeneous sample of spleen RNA. Libraries were produced using 5 µl of first-strand cDNA as a template in 3 different PCRs for 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 19 cycles for heavy chain, 13 cycles for kappa chain, and 16 cycles for lambda chain. Following purification and size selection libraries were analyzed on an Agilent 2100 Bioanalyzer (**Panels A–F**). Peaks labeled "LM" and "UM" correspond to DNA reference markers included in each analysis. **Panels A, C, and E** show broad peaks between ~500–1,200 bp and maximal peaks in the range of ~700–800 bp (typical results for a library generated from spleen RNA), analyzed on the Bioanalyzer. No RNA Control (NRC) samples (**Panels B, D, and F**) show no library produced and a flat Bioanalyzer profile within the range of 500–1,200 bp.

VI. References

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

Appendix A. Illumina HT Indexes

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. Consult the Illumina literature (such as the TruSeq® DNA Sample Preparation Guide) for appropriate pooling guidelines.

The BCR Primer 2V HT indexed primers contain the Read 2 sequence and i7 indexes. These primers are labeled sequentially ("2V01"–"2V12"), in correspondence with Illumina indexes D701–D712.

In addition to gene-specific sequences derived from the constant regions of Mouse BCR heavy chain and BCR light (kappa or lambda) chain, the mBCR Primer 2 HT indexed primers contain the Read 1 sequence and i5 indexes. Depending on the kit size, 1–8 different mBCR Primer 2H, mBCR Primer 2K, and mBCR Primer 2L HT indexed primers containing unique i5 indexes are included for amplification of BCR heavy chain and BCR light (kappa or lambda) chain, respectively. These primers are labeled sequentially ("Mouse 2H01"–"Mouse 2H08" or "Mouse 2K01"–"Mouse 2K08") or "Mouse 2L01"–"Mouse 2L08"), in correspondence with Illumina indexes D501–D508.

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

Table 3. BCR H/K/L Mouse Indexing Primer Set HT for Illumina Index Sequences.

BCR Primer 2V i7 HT Index			mBCR Primer 2H/K/L i5 HT Index		
Primer ID (cap label)	**Illumina ID**	**Index sequence**	**Primer ID (cap label)**	**Illumina ID**	**Index sequence**
2V01	D701	ATTACTCG	Mouse 2H01, 2K01, or 2L01	D501	TATAGCCT
2V02	D702	TCCGGAGA	Mouse 2H02, 2K02, or 2L02	D502	ATAGAGGC
2V03	D703	CGCTCATT	Mouse 2H03, 2K03, or 2L03	D503	CCTATCCT
2V04	D704	GAGATTCC	Mouse 2H04, 2K04, or 2L04	D504	GGCTCTGA
2V05	D705	ATTCAGAA	Mouse 2H05, 2K05, or 2L05	D505	AGGCGAAG
2V06	D706	GAATTCGT	Mouse 2H06, 2K06, or 2L06	D506	TAATCTTA
2V07	D707	CTGAAGCT	Mouse 2H07, 2K07, or 2L07	D507	CAGGACGT
2V08	D708	TAATGCGC	Mouse 2H08, 2K08, or 2L08	D508	GTACTGAC
2V09	D709	CGGCTATG			
2V10	D710	TCCGCGAA			
2V11	D711	TCTCGCGC			
2V12	D712	AGCGATAG			

Appendix B. Guidelines for PCR Cycling and Pooling

The gene expression of each of the B-cell receptor chains can vary significantly from one clonal or bulk 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, it is recommended that each chain of interest be amplified separately in each PCR step as shown in Figure 6. We do not recommend that the PCRs be multiplexed. The same library indexes should be used for each PCR2 amplification from the same sample so that they are sequenced together as a single library.

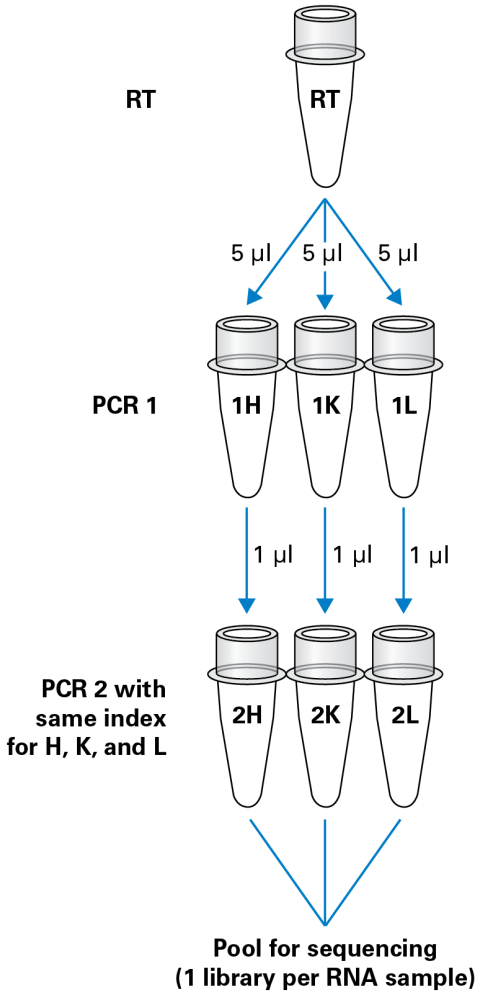


Figure 6. PCR cycling and pooling workflow. For each sample, after RT of all mRNAs in the sample, the user may specifically amplify 1–3 chains of IgG. Each amplification uses 5 µl of the RT. Following the first PCR, 1 µl of each PCR is used in a separate PCR to add the same sequencing indexes to each amplified chain for a given sample, but distinct indexes for each different sample. After this final amplification in PCR2, the user may validate each product on the Bioanalyzer or Fragment Analyzer. The user may then choose which amplified chain to pool for sequencing. The following is an example of how to index two samples: PCR1 = mBCR primer 1H, 1K, 1L, and 1V for all samples. PCR2 for Rxn 1 = mBCR primer 2H D501, 2K D501, 2L D501 + a 2V primer (e.g., 2V D701); Rxn 2 = mBCR primer 2H D501, 2K D501, 2L D501 + a 2V primer (e.g., 2V D702).

The guidelines provided in Table 2 and repeated below in Table 4 are a good starting point for determining the number of PCR cycles to perform in PCR2 and have been determined by testing several different hybridomas and bulk samples.

Table 4. Cycling Guidelines Based on Amount of Starting Material (repeated from Table 2).

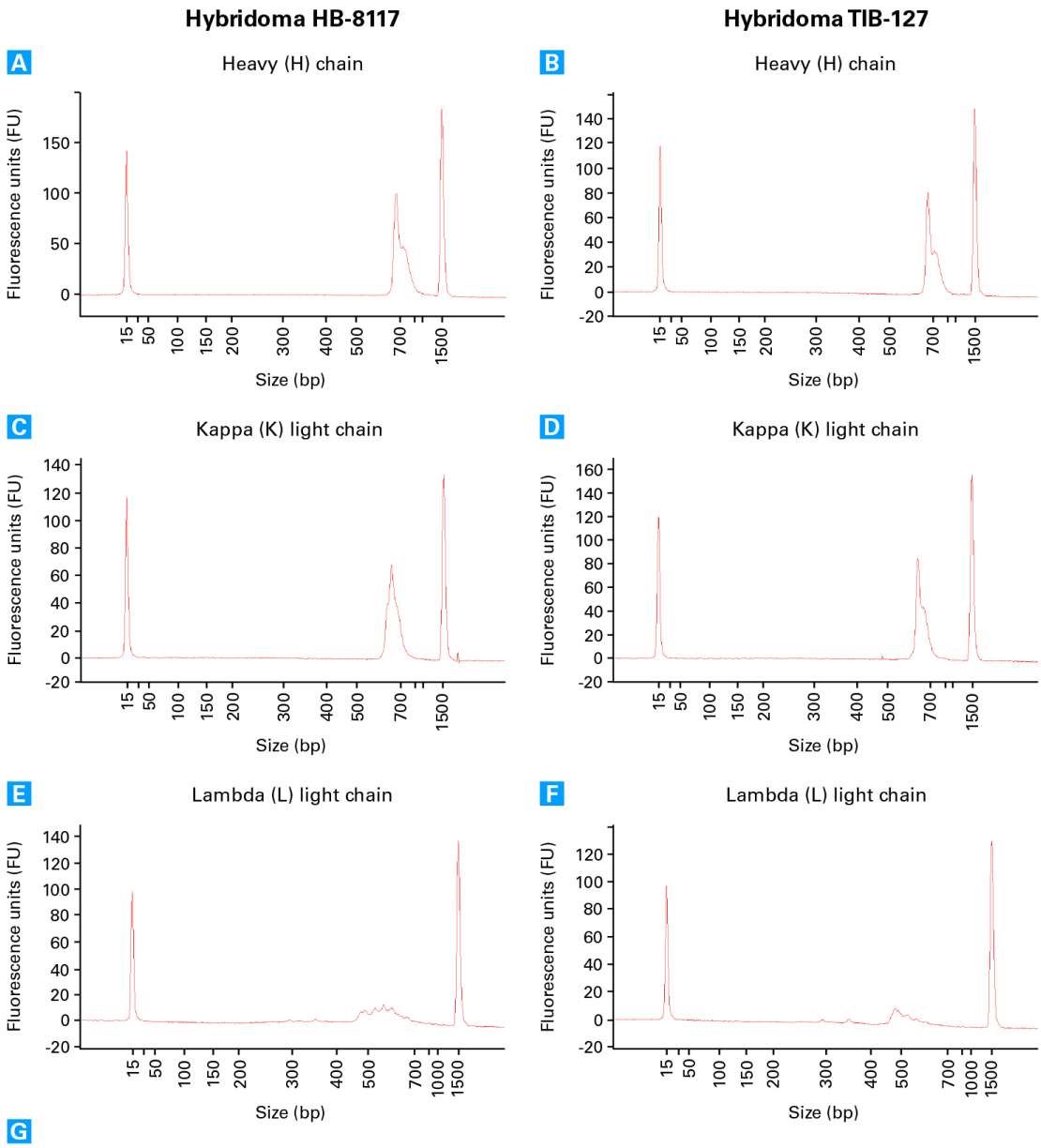
Input Type	Input Amount	Chain	Number of PCR 1 Cycles	Number of PCR 2 Cycles*
Control RNA	10–100 ng	H	18	15
		K	18	13
		L	18	15
Control RNA	100–1,000 ng	H	18	12
		K	18	10
		L	18	12
Control RNA	1 µg–3 µg	H	18	9
		K	18	7
		L	18	9
Purified B cells	1,000–10,000 cells	H	18	19
		K	18	19
		L	18	19

*If the number of cycles generates insufficient library for sequencing, repeat PCR 2 with four more cycles.

It is possible that the indicated number of cycles is not sufficient to provide enough material for downstream sequencing (see Appendix C). In these cases, it is recommended that the number of cycles be increased by 4 to generate sufficient material.

While starting with a higher number of cycles will generally produce sufficient material, the alignment rate to IgG sequences may suffer. This will not affect the determination of the predominant heavy and light chain sequences of hybridomas but may ultimately affect the number of clonotypes identified in bulk samples.

If samples are clonal B-cell populations or hybridomas, then they will likely express primarily (or exclusively) a kappa light chain or lambda light chain. In these cases, the light chain not being expressed will not give a characteristic Bioanalyzer or Fragment Analyzer electropherogram. (Examples of Bioanalyzer traces with a single discrete peak are shown in Figure 5, Panels A, C, E and Figure 7, Panels A–D.) Instead, the electropherogram will be broad with a lower sizing span and look more like the Bioanalyzer samples shown in Figure 7, Panels E and F. If the user requires a high alignment rate for each reaction, these PCR products may be excluded in the final sequencing library pool, as the alignment rate may be negatively affected. However, if only identification of primary variable heavy and light chain sequences is required, the PCR products may be included in the final sequencing pool without affecting identification or distribution of the primary clonotypes. Bioanalyzer examples are shown in Figure 7, Panel G.



G

	HB-8117		TIB-127	
Sequenced chains	HK	HKL	HK	HKL
Aligned to IG, %	98%	64%	93%	56%
Heavy chain CDR3 %	CARKSSYYGSTYVYFDVW 45%	CARKSSYYGSTYVYFDVW 44%	CARHDNSGW 40%	CARHDNSGW 39%
Kappa chain CDR3 %	CQQSRKVPSTF 27%	CQQSRKVPSTF 28%	CGQGYSYPYTF 50%	CGQGYSYPYTF 51%

Figure 7. Example electropherogram results for BCR heavy and light chain libraries from hybridomas (2Hx-2 [ATCC HB-8117] and CE9H9 [ATCC TIB-127]). Libraries containing BCR heavy and light chain sequences were generated using the SMARTer Mouse BCR IgG H/K/L Profiling Kit and 10 ng of RNA obtained from the indicated hybridomas. **Panels A–F.** The strong discrete peak in the electropherograms show that the kappa chain is being expressed in these hybridomas. In contrast, the lambda chain sequencing library is comparatively broad and a smaller size range than expected. If only heavy and kappa light chain PCR products are pooled and sequenced (HK in the table), 98% and 93% of the sequences align to IG reference sequences using MiXCR software (v1.8) for HB-8117 and TIB-127, respectively. If the lambda light chain PCR products are also pooled (HKL in **Panel G**), alignment drops to 64% and 56%, respectively. However, the majority heavy chain and kappa chain sequences are identical and in the same proportion for either sequencing sample, indicating the same information is obtained.

Appendix C. Guidelines for Library Sequencing and Data Analysis

Samples should be pooled to a final pool concentration of 4 nM for denaturation. We recommend diluting the pooled denatured libraries to a final concentration of 12.5 pM, including a 5–10% PhiX Control v3 (Illumina, Cat. No. FC-110-3001) spike-in for sequencing. While not essential, the addition of the PhiX control increases the nucleotide diversity and thus aids in high-quality data generation.

Sequencing should be performed on an Illumina MiSeq® sequencer using the 600-cycle MiSeq Reagent Kit v3 (Illumina, Cat. No. MS-102-3003) with paired-end, 2 x 300 base pair reads. Upon completion of a sequencing run, data can be analyzed on a variety of platforms, including MiXCR (Bolotin et al. 2015) or available software hosted on BaseSpace, Illumina's cloud computing environment for next-generation sequencing. MiXCR was specifically designed to process sequencing data obtained for T- and B-cell receptor repertoires, and can calculate clonotype frequencies from raw sequence inputs consisting of paired- or single-end reads. MiXCR also accounts for sequence quality, corrects PCR errors, and identifies germline hypermutations.

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