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Profiling mouse B-cell receptors with SMART technology

- 5' RACE-like approach captures complete V(D)J variable regions of BCR transcripts »
- Specific, accurate amplification of BCR heavy and light chains »
- PCR pooling strategy for highly sensitive sequencing of different chains from the same sample »
- Flexibility in PCR pooling for different experimental requirements regarding alignment and identification of primary chain sequences »

Introduction

B cells are an essential part of the adaptive immune response, functioning via B-cell receptors (BCRs) expressed on their surface. Each B cell expresses a different BCR that allows it to recognize molecular patterns in pathogens. Development of BCRs (Figure 1) is a multistep process in which the progenitor cell undergoes V(D)J recombination in the germline and additional somatic hypermutations (SHM), resulting in a final product with a specific CDR3 (complementarity determining region 3) sequence in the hypervariable region of the immunoglobulin. The unique CDR3 sequence in BCRs is critical for dictating antigen specificity. Taken together, the molecular events described above facilitate receptor diversity and the generation of heavy (H) chain isotypes. BCR diversity enables B cells to recognize and respond to a wide range of pathogens. Upon exposure to a stimulus or stimuli, the lambda (L) and kappa (K) light chain genes of the BCR undergo rearrangements to generate specific gene segments. This further development results in different light chain isotypes being generated from the same B-cell clone.

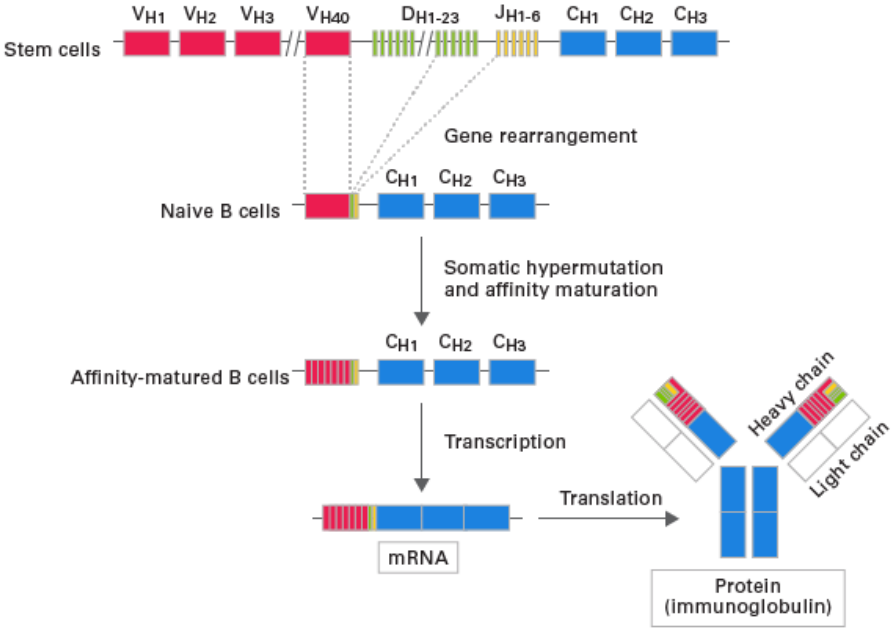


Figure 1. BCR development. The progenitor cell undergoes recombination of V, D, and J segments in the germline, which generates two identical heavy chains. Recombination of V and J segments generates two identical light chains. Random nucleotide additions or deletions at the junctions of the V, D, and J segments provide additional diversity. Furthermore, B cells activated by immune responses undergo somatic hypermutation (SHM), in which additional point mutations are introduced.

Understanding the profiles of BCRs, (i.e., sequencing the full-length CDR3 regions to determine the diversity of receptors and the clonotypes, defined by expression of specific H, K, and L gene segments) can not only aid in gaining insights into the adaptive immune response in healthy individuals, but also in those with a wide range of conditions, including infectious diseases, allergies, autoimmune disorders, cancers, and aging (Yaari & Kleinstein, 2015). Accurate determination of the clonotypes and isotypes expressed by the immune system will aid in a complete picture of the B-cell repertoire.

Recently, next generation sequencing (NGS) approaches for profiling B-cell repertoires have provided valuable insights into the adaptive immune response and antibody engineering. There are two major approaches used in profiling B-cell repertoires—multiplex PCR and 5' RACE combined with NGS. While multiplexing allows you to amplify multiple BCR genes in one reaction, it may prove challenging with regard to sensitivity, specificity, and biases in amplification of certain sequences, all of which can lead to difficulties in accurate identification of isotypes. On the other

hand, the 5'-RACE method reduces variability and allows for priming from the constant region of BCR heavy or light chains. However, the burden of designing optimized primers would still be left to the individual user in this case. The new SMARTer Mouse BCR IgG H/K/L Profiling Kit (SMARTer mouse BCR kit) solves this problem by combining the benefits of 5' RACE with gene-specific amplification (Figure 2) to provide a highly sensitive and reproducible method for profiling B-cell repertoires, by capturing complete V(D)J variable regions of BCR transcripts. The high sensitivity of the kit allows for accurate identification of top clonotypes and reliable assignment of isotype in a majority of cases, based on the sequencing of the H, K, and L chains.

Features of the kit:

- Starts with 10 ng–3 µg of total RNA from spleen, lymph node, PBMCs, and hybridomas
- 5' RACE-based approach combined with gene-specific primer amplification of cDNA libraries is optimized for sensitive and specific clonotype detection
- Optimized library generation workflow for amplification of light chains for mapping and identification of clonotypes
- Accurate amplification of mouse IgG subclasses and identification via sequencing in a majority of cases

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. First-strand cDNA synthesis is dT-primed, and the template-switching activity ensures that only sequences from full-length cDNAs undergo PCR amplification (Figure 2). Two rounds of PCR are then performed in succession to amplify cDNA sequences corresponding to the variable regions of BCR IgG heavy chain or BCR light chain (kappa or lambda) transcripts (Figure 2, Panel B). The first PCR uses the first-strand cDNA as a template, and heavy or light chains are amplified in separate reactions. This PCR specifically amplifies the entire variable region and a portion of the constant region of BCR heavy or light chain cDNA. The second PCR uses the first PCR product as a template and uses semi-nested primers to amplify the entire variable region and a portion of the constant region of heavy or light chain cDNA. Following post-PCR purification, size selection, and quality analysis, the library is ready for sequencing on Illumina platforms.

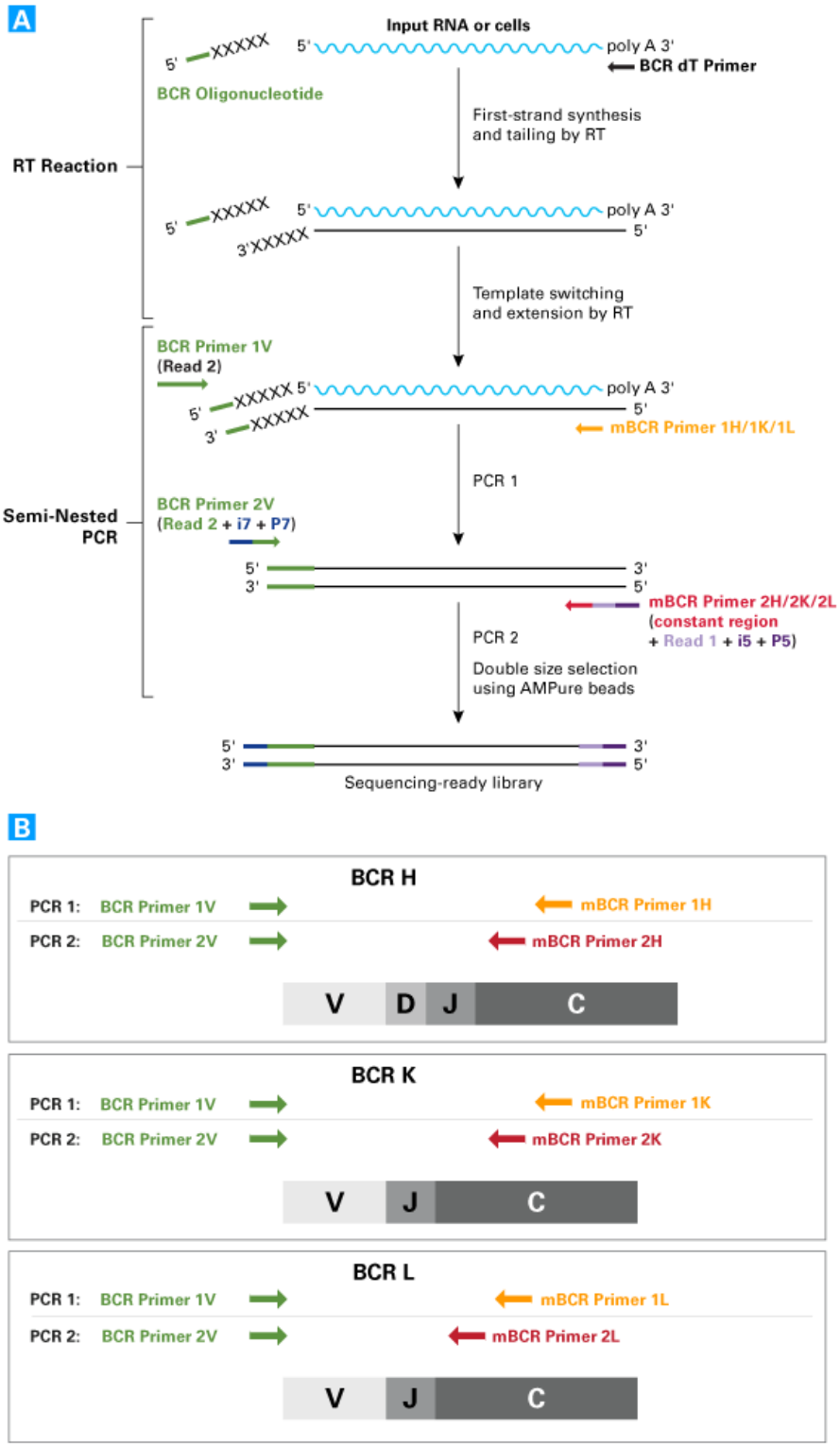


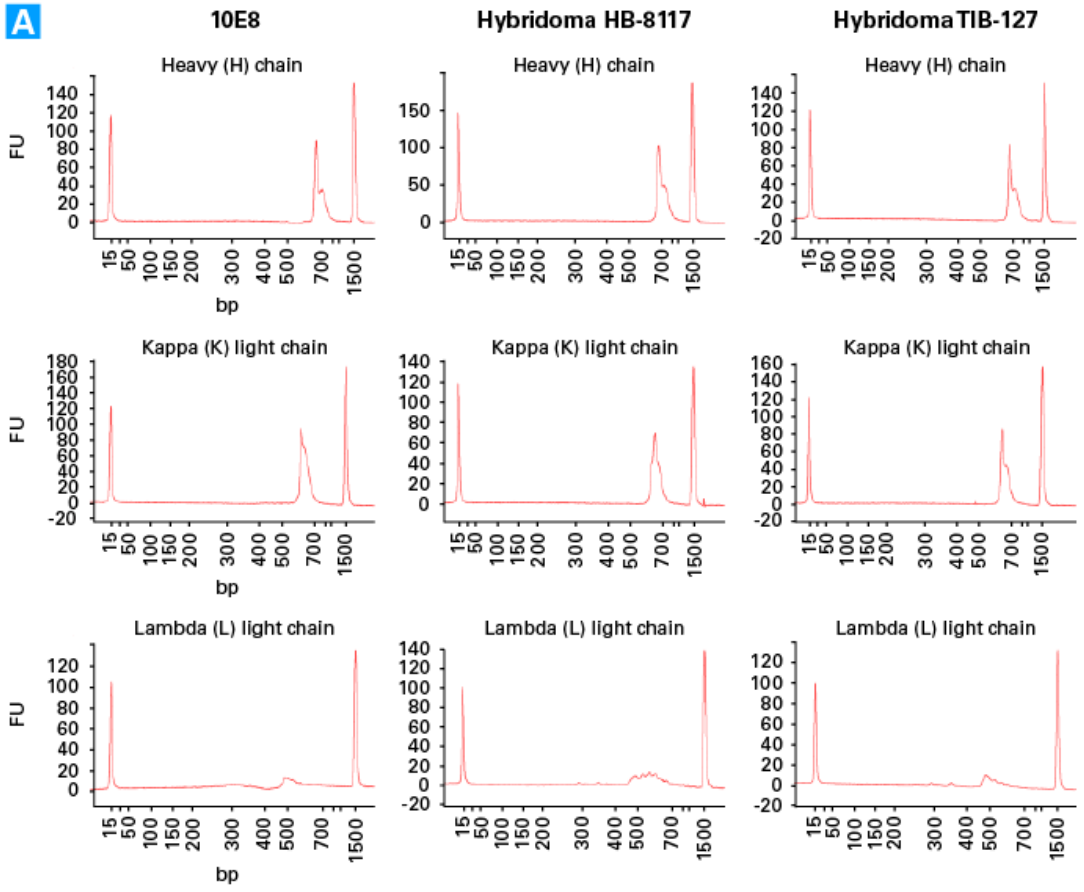
Figure 2. SMARTer Mouse BCR IgG H/K/L Profiling Kit workflow. Panel A. First-strand cDNA synthesis is dT-primed (BCR dT Primer) and performed by the MMLV-derived SMARTScribe Reverse Transcriptase (RT), which adds nontemplated nucleotides upon reaching the 5' end of each mRNA template. The BCR Oligonucleotide anneals to these nontemplated 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 contains 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. **Panel B.** 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., nonvariable) 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 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.

Results

Specific amplification of IgG subclasses and kappa and lambda light chains

In order to determine if the BCR-specific primers amplified the expected regions of the BCR loci, RNA was isolated from several hybridoma samples with different IgG subtypes. 10 ng of RNA was used for each sample as input for the SMARTer mouse BCR kit. Bioanalyzer results for three hybridoma samples (10E8, HB-8117, and TIB-127; Figure 3, Panel A) show the expected peaks for each heavy and light chain, demonstrating the correct amplification of each IgG subclass. Mapping metrics for each sample were determined, with alignment against all immunoglobulin (IG) sequences (Figure 3, Panel B). Alignments of the amplified libraries against IG sequences were over 90% on-target for these samples. In general, we consistently see over 70% on-target alignment. The top two clones representing the heavy chain and primary light chain (kappa). In most cases, these top clones make up >70% of the clonotype fraction combined. Overlapping was also high (65% or better), indicating that the same bases were read from each sequencing primer. This attribute could help identify somatic hypermutations. As expected for hybridoma samples, only a small number of clones were identified for each.



B

RNA source			10E8	HB-8117	TIB-127
Expected isotype and subclass			IgG1	IgG2a	IgG2c
Total sequencing reads			870,000	870,000	870,000
% successfully aligned to IG sequences			98%	98%	93%
% overlapped			80%	79%	77%
% overlapped and aligned			79%	78%	72%
Clone fraction ≥ 0.0001			3	4	6
Distribution of top 2 clones			50%, 46%	45%, 27%	50%, 40%
Top 2 clones	Top clone	V	IGHV4-2, IGHV4-1	IGHV9-3, IGHV9-2, IGHV9-1	IGKV6-20, IGKV6-29
		D	IGHD2-4	IGHD1-1	
		J	IGHJ3	IGHJ1	IGKJ2
		Subclass	IGHG1 (IgG1)	IGHG2C (IgG2c)	IGKC (Kappa)
	2nd clone	V	IGKV3-4	IGKV3-1	IGHV5-9, IGHV5-17, IGHV5-6, IGHV5-12
		D			IGHD5-6, IGHD2-3, IGHD5-4
		J	IGKJ5	IGKJ1	IGHJ3, IGHJ1
		Subclass	IGKC (Kappa)	IGKC (Kappa)	IGHG3 (IgG3)

Figure 3: Accurate amplification of all five subclasses of IgG. Libraries containing BCR heavy and light chain (kappa) sequences were generated using the SMARTer Mouse BCR IgG H/K/L Profiling kit, starting with 10 ng of RNA isolated from an in-house hybridoma (10E8) and two ATCC hybridoma samples (HB-8117 and TIB-127) with different IgG subtypes. **Panel A.** Bioanalyzer traces show gene-specific amplification of heavy or light chains of each hybridoma. In each sample, the expected peak between 700–900 bp is observed. The strong discrete peaks 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 of a smaller size range than expected. Peaks labeled "LM" and "UM" correspond to DNA reference markers included in each analysis. **Panel B.** Mapping metrics determined using MiXCR software (version 1.8), aligned against all IG sequences. Output from the MiXCR software for the top two clones shows the V, D, and J alleles and the isotype and subclass as identified by the software.

Optimized workflow for clonotype detection

In order to optimize sequencing, detect all expressed heavy and light chain genes, and obtain accurate clonotype identification, this kit utilizes a unique pooling strategy for library amplification (Figure 4). Following reverse transcription of all mRNAs in a sample, the user may specifically amplify 1–3 chains of IgG in separate reactions. The second PCR adds the same sequencing indexes to each amplified chain for a given sample, but distinct indexes for each sample. After sample validation, a particular amplified chain can be pooled for sequencing. In generating libraries specific to heavy or light chains, clonotypes can be accurately assigned. This key strategy allows highly sensitive sequencing of different chains from the same sample even with a very small amount of starting material.

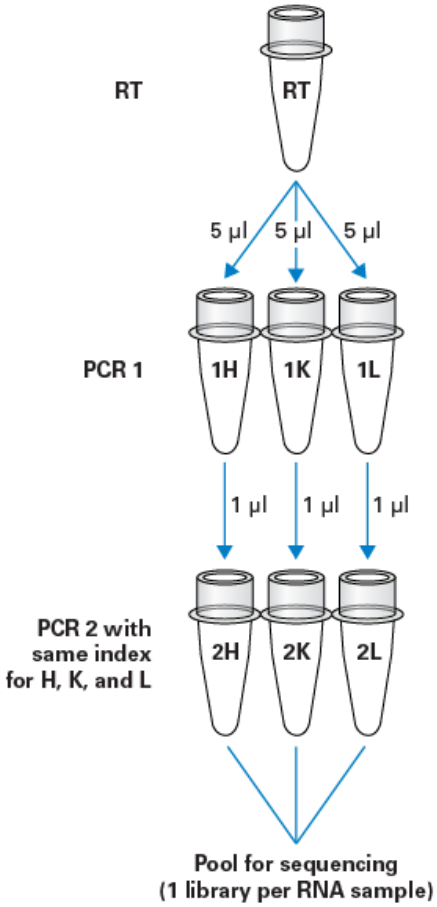


Figure 4. PCR cycling and pooling workflow. For each sample, after RT of all mRNAs in the sample, the user may specifically amplify 1–3 chains of IgG. Each amplification uses 5 µl of the RT. Following the first PCR, 1 µl of each PCR is used in a separate PCR to add the same sequencing indexes to each 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.

Decisions made in PCR pooling affect alignment and on-target percentages (Figure 5). If samples are clonal B-cell populations or hybridomas, they will likely express primarily (or exclusively) a kappa light chain or lambda light chain. In these cases, the light chain not expressed will not give a characteristic Bioanalyzer electropherogram (Figure 3, Panel A). For these samples, if the PCR-amplified lambda light chain is included in sequencing, on-target rates are expected to drop. However, the proper identification of the hybridoma clonotype will not be adversely affected. Sequencing results for samples where only heavy and kappa light chain PCR products were pooled ("HK" in the table below) had over 70% of the sequences align to IG reference sequences. When the lambda light chain products were also pooled ("HKL" in the table below), this alignment dropped significantly. Even so, the majority of heavy chain and kappa light chain sequences are identical in the same proportion for each sequencing sample, indicating that the same information is obtained. This leaves the user with a certain amount of flexibility. If higher alignment rates are required, the PCR products of the nonexpressed light chain may be excluded in the final sequencing library pool. If only identification of primary variable heavy and light chain sequences is needed, all PCR products can be included without affecting identification or distribution of primary clonotypes.

%successful alignment									
Hybridoma	**10E8**			**HB-8117**			**TIB-127**		
Chains sequenced together	HK	HKL	L	HK	HKL	L	HK	HKL	L
% aligned to IG	98%	37%	0.34%	98%	64%	0.46%	93%	56%	0.49%

	10E8		HB-8117		TIB-127	
Sequenced chains	HK	HKL	HK	HKL	HK	HKL
Heavy chain CDR3 %	CARAYDY DRAWFG YW 50%	CARAYDY DRAWFG YW 51%	CARKSSY YGSTYVY FDVW 45%	CARKSSY YGSTYVY FDVW 44%	CARHDNS GW 40%	CARHDNS GW 39%
Kappa chain CDR3 %	CQQSND NPLTF 46%	CQQSND NPLTF 44%	CQQSRK VPSTF 27%	CQQSRK VPSTF 28%	CGQGYS YPYTF 50%	CGQGYS YPYTF 51%

Figure 5. PCR pooling affects alignment and on-target percentages. Libraries containing BCR heavy and light chain sequences were generated using the SMART Mouse BCR kit as described in Figure 3 above. If only heavy and kappa light chain PCR products are pooled and sequenced ("HK" in the table), a high percentage of the sequences align to IG reference sequences (using MiXCR software version 1.8) for each sample. If the lambda light chain PCR products are also pooled ("HKL" in the table), alignment percentages drop. However, the majority heavy chain and kappa chain sequences (top two clones) are identical and in the same proportion for each sequencing sample. CDR3 amino acid sequence, which defines the affinity of the antibody, is given for each clonotype shown. For full clonotype information, see Figure 3.

Conclusions

The SMARTer Mouse BCR IgG H/K/L Profiling Kit is a powerful tool for profiling mouse B-cell receptors. By leveraging SMART technology and combining a 5' RACE-like approach with gene-specific primer amplification, this workflow captures complete V(D)J variable regions of BCRs and is optimized for highly sensitive and specific clonotype detection. With primers that incorporate Illumina-specific adaptor sequences during cDNA amplification, the protocol generates indexed libraries, ready for sequencing on Illumina platforms. An additional advantage is the unique PCR cycling and pooling workflow which reduces sequencing cost while enabling accurate clonotype identification. By avoiding multiplex PCR, this kit also avoids the pitfalls of biases in amplification of certain sequences, helping to provide a complete and accurate view of mouse BCR repertoires.

Methods

Libraries containing BCR heavy and light chain sequences were generated using the SMARTer Mouse BCR IgG H/K/L Profiling Kit as per the protocol given in the user manual. 10 ng of RNA was obtained as starting material from the indicated hybridomas (10E8, HB-8117, and TIB-127) with different IgG subtypes. 10E8 is an in-house hybridoma; the others are ATCC lines. ATCC determined the expected isotype for their lines; 10E8 was determined in-house. Hybridomas were cultured according to established methods. Libraries were produced using the first-strand cDNA as a template in three different PCRs for heavy chain and kappa and lambda light chains. The product of these PCRs was used as template in a set of nested PCRs, one for each chain. Following purification and size selection, libraries were validated using the Agilent 2100 Bioanalyzer.

Mapping metrics were determined using MiXCR software (version 1.8) and aligned against IG (all immunoglobulin sequences). A threshold was set to a clone fraction of at least 0.01%.

References

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

Yaari, G. and Kleinstein, S.H. Practical guidelines for B-cell receptor repertoire sequencing analysis. *Genome Med.* **7**:121 (2015).

Related Products

Cat. #	Product	Size	License	Quantity	Details
634422	SMARTer® Mouse BCR IgG H/K/L Profiling Kit	12 Rxns		*	

The SMARTer Mouse BCR IgG H/K/L Profiling Kit 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 inputs, from 10 ng to 3 µg of total RNA obtained from 1,000 to 10,000 purified B cells. This 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).

This kit leverages SMART technology and employs a 5' RACE-like approach to capture complete V(D)J variable regions of BCR transcripts. Included in the kit are primers that incorporate Illumina-specific adaptor sequences during cDNA amplification. **This kit supports up to 12 rxns.**

Documents	Components	Image Data

634423	SMARTer® Mouse BCR IgG H/K/L Profiling Kit	48 Rxns		*	
634424	SMARTer® Mouse BCR IgG H/K/L Profiling Kit	96 Rxns		*	

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