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Analysis and Visualization of Mouse and Human Antibody Repertoires by Next-generation Sequencing

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Dr. Vineeta Bajaj
Review Editor
JoVE

Dear Editor:

I wish to submit a revised manuscript for publication in JoVE titled "Analysis and Visualization of Mouse and Human Antibody Repertoires by Next-generation Sequencing." The paper was coauthored by Lin Sun, Naoko Kono, Hiroyuki Toh, Hanbing Xue, Kaori Sano, Tadaki Suzuki, Akira Ainai, Yasuko Orba, Junya Yamagishi, Hideki Hasegawa, Yoshimasa Takahashi, and Shigeyuki Itamura.

We would like to thank the editorial comments to improve the quality of the manuscript. Our point-by-point responses are uploaded as rebuttal documents and all changes to the manuscript are tracked.

We also uploaded revised files of Table_1 and Table_of_Materials.

We also uploaded the file for copyright permission.

We hope that the revised version of this manuscript is now suitable for publication in JoVE.

We look forward to hearing from you at your earliest convenience.

Sincerely,

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1   **TITLE:**
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42   **KEYWORDS:**
43   Antibody Repertoire, Next-Generation Sequencing, NGS, RNA-seq, Immune Response,
44   Antigen Recognition, V-D-J Recombination, Immunoglobulin
45
46   **SUMMARY:**
47   Here, we describe protocols for the analysis and visualization of the structure and constitution

48 of whole antibody repertoires. This involves the acquisition of vast sequences of antibody
49 RNA using next-generation sequencing.
50
51 **LONG ABSTRACT:**

52 The immense adaptability of antigen recognition by antibodies is the basis of the acquired
53 immune system. Despite our understanding of the molecular mechanisms underlying the
54 production of the vast repertoire of antibodies by the acquired immune systems, it has not
55 yet been possible to arrive at a global view of a complete antibody repertoire. In particular, B
56 cell repertoires have been regarded as a black box because of their astronomical number of
57 antibody clones. However, next-generation sequencing technologies are enabling
58 breakthroughs to increase our understanding of the B cell repertoire. In this report, we
59 describe a simple and efficient method to visualize and analyze whole individual mouse and
60 human antibody repertoires. From the immune organs, representatively from spleen in mice
61 and peripheral blood mononuclear cells in humans, total RNA was prepared, reverse
62 transcribed, and amplified using the 5'-RACE method. Using a universal forward primer and
63 antisense primers for the antibody class-specific constant domains, antibody mRNAs were
64 uniformly amplified in proportions reflecting their frequencies in the antibody populations.
65 The amplicons were sequenced by next-generation sequencing (NGS), yielding more than 10^5
66 antibody sequences per immunological sample. We describe the protocols for antibody
67 sequence analyses including V(D)J-gene-segment annotation, a bird's-eye view of the
68 antibody repertoire, and our computational methods.
69
70 **INTRODUCTION:**
71 The antibody system is one of the fundamentals of the acquired immune system. It is highly
72 potent against invading pathogens due to its vast diversity, fine antigen recognition
73 specificity, and the clonal expansion of antigen-specific B cells. The repertoire of antibody-
74 producing B cells is estimated to be more than 10^{15} in a single individual[1]. This immense
75 diversity is generated with the help of VDJ gene recombination in the immunoglobulin genetic
76 loci[2]. Description of the entire B cell repertoires and their dynamic changes in response to
77 antigen-immunization is therefore challenging, but essential for a complete understanding of
78 the antibody response against invading pathogens.
79
80 Because of their astronomical diversity, B cell repertoires have been regarded as a black box;
81 however, the advent of NGS technology has enabled breakthroughs to an enhanced
82 understanding of their complexity[3,4]. Whole antibody repertoires have been successfully
83 analyzed, firstly in zebrafish[5], then mice[6], and humans[6,7]. Although NGS has now become a
84 powerful tool in the study of the adaptive immune response, basic analyses of the
85 commonalities and differences in antibody repertoires among individual animals are lacking.
86
87 In mice, it was reported that the IgM repertoires are almost identical between individuals,
88 whereas those of IgG1 and IgG2c are substantially different between individuals[8]. In addition
89 to V-gene usage profile, the observed frequency of VDJ-profile in naive peripheral B cells is
90 highly similar between individuals[8]. The analysis of the amino acid sequences of the VDJ-
91 region also showed the occurrence of the same junctional sequences in different mice much
92 more frequently than previously thought[8]. These results indicate that the mechanisms for the
93 antibody repertoire formation can be deterministic rather than stochastic[5,8,9]. The process of
94 antibody repertoire development in mice has also been successfully analyzed using NGS to

95 further highlight the potential of NGS to uncover the antibody immune system in detail[10].

97 In this report, we describe a simple and efficient method to visualize and analyze an antibody
98 repertoire at a global level.

100 **PROTOCOL:**

102 All animal experiments were performed according to institutional guidelines and with the
103 approval of the National Institute of Infectious Diseases Animal Care and Use Committee.
104 Sampling of PBMCs from healthy adult volunteers, used as the representative result in this
105 report, was performed with the approval of the Ethics Committee of the National Institute of
106 Infectious Diseases, Tokyo, Japan, and written informed consent was obtained from each
107 participant using an ethics committee-approved form.

109 **1. Primer Design**

111 1.1. Design a universal forward primer to cDNA to amplify the immunoglobulin mRNA without
112 bias from PCR primers, as used in the 5'-RACE[11,12] and SMART-PCR[13] techniques.

114 1.2. For the immunoglobulin VH gene amplification, design the immunoglobulin class-specific
115 sequences in the constant region as reverse primers[8,14] (**Figure 1A**).

117 **[Please insert Table 1 here]**

119 NOTE: Multiplex tag sequences can be added to any of these primers to label the library
120 molecules from different sample sources. Sequences for nested PCR can also be added,
121 according to the manual of the kit used[15].

123 **2. Nucleic Acid Isolation from Immune Cells and Tissues**

125 NOTE: The procedure given below is for extracting nucleic acids from the mouse spleen.
126 However, it is applicable to other immune tissues and human cells such as lymph nodes or
127 peripheral blood mononuclear cells (PBMCs) (**Figure 1B**).

129 2.1. Dissect the tissue, *e.g.*, spleen from an 8-week-old C57BL/6 mouse and pass it through a
130 stainless-steel mesh (200 to 400 μm) with 2 mL of PBS buffer to obtain dispersed cells.
131 Transfer the cell suspension to a 2.0 mL microcentrifuge tube, and centrifuge for 5 min at 600
132 $\times g$ and 4 ˚C. Discard the supernatant.

134 2.2. Add 800 μL of ACK lysing buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2)
135 to the pellet, and incubate on ice for 2 min to lyse red blood cells in the tissue.

137 2.3. Wash the tissue cells with 2 mL of PBS 3x, followed by centrifugation for 5 min at 600 × g
138 and 4 ˚C.

140 2.4. Add 800 μL of phenol/guanidine isothiocyanate reagent to the pellet, vortex thoroughly,
141 and incubate at about 25 ˚C for 5 min.

2.5. Add chloroform (200 µL), shake manually for 15 s, and then incubate for 2 min at about 25 ˚C.

2.6. Separate the phases by centrifugation for 15 min at 12,000 × *g* and 25 ˚C and transfer the upper aqueous phase to a fresh tube.

2.7. Add one volume of 70% ethanol, vortex briefly and apply it to the silica spin column.

2.8. Elute the RNA with 30–100 µL of water.

2.9. Quantitate initial RNA concentration using a fluorometer (**Table of Materials**).

2.10. Store the purified RNA at -80 ˚C.

3. cDNA Synthesis and PCR Amplification

NOTE: The method described below is based on the 5'-RACE[11,12] and SMART-PCR techniques[13]. The details and optimization of the reaction are described in the manual of the kit [15]. The starting materials for mouse immunoglobulin are the sample from step 2.10. The starting materials for human immunoglobulin are the sample from human tissues, ex. PBMC, treated as described in steps 2.3 to 2.10.

3.1 Synthesize the first-strand cDNA from 2 to 10 µg of total RNA template using 5´-RACE CDS primer (oligo-dT-containing) and SMART-PCR oligonucleotide (**Table of Materials**) according to the manufacturer's instructions[15].

3.2.1 For the mouse immunoglobulin, PCR-amplify cDNA with high-fidelity DNA Polymerase using the universal forward primer and immunoglobulin class-specific reverse primers (**Table 1**). Set the thermal cycling conditions as: 94 ˚C for 2 min, then 40 cycles of 94 ˚C for 30 s, 59 ˚C for 30 s, and 72 ˚C for 30 s, followed by a final extension step at 72 ˚C for 5 min.

NOTE: Typical experiments amplify IgM, IgG1, IgG2c, Igκ and Igλ immunoglobulin classes to look at the naive, Th1-dependent and Th2-dependent B cells (**Figure 3**).

3.2.2 For the human immunoglobulin, perform the 1st PCR using the universal forward primer and immunoglobulin class-specific reverse primers (**Table 1**) with tag sequences. Include the index sequences for each sample by 2nd PCR using index sequence primers. Use the following PCR conditions and the Taq polymerase: 94 ˚C for 2 min, 21 cycles (1st PCR) or 32 cycles (2nd PCR) at 94 ˚C for 30 s, 59 ˚C for 30 s, 72 ˚C for 30 s.

NOTE: Typical experiments amplify IgM, IgD, IgG (IgG1, IgG2, IgG3 and IgG4), IgA (IgA1 and IgA2), IgE, Igκ and Igλ immunoglobulin classes to look at all B cell populations (**Figure 4**).

3.3 Electrophorese the PCR products on an agarose gel and purify 600 to 800 bp fragments using a silica membrane spin-column.

189 3.3.1 Electrophorese the sample from 3.2.1 or 3.2.2 on 2% agarose gel.
190
191 3.3.2 Visualize the DNA bands on UV-transilluminator and excise the gel-slice containing the
192 broad band between 600 to 800 bp.
193
194 3.3.3 Add 10 µL of membrane binding solution per 10 mg of gel slice. Mix and incubate at 50–
195 65 °C until the gel slice is completely dissolved.
196
197 3.3.4 Transfer the gel solution on silica membrane spin-column. Wash once with washing
198 buffer and elute DNA with 50 µL of nuclease-free water (**Table of Materials**).
199
200 3.4 Quantify the purified amplicons with a fluorometer and pool amplicons from each
201 immunoglobulin class in equal amounts for NGS sequencing.
202
203 NOTE: Typically, 2-10 µg amplicon DNA was recovered for each immunoglobulin class. Mix
204 each sample solution equally in DNA amount to give rise 50 µL solution containing 10-20 ng
205 DNA/µL.
206
207 3.5 Determine the size and concentration of libraries using a micro-capillary based
208 electrophoresis with DNA sizing chip (**Table of Materials**). Store the libraries at - 20 °C.
209
210 **4. NGS Sequencing of Libraries**
211
212 4.1 Generate a SampleSheet.cvs for the sequencing run specifying sample name, index
213 information and instruct to obtain .fastq files only.
214
215 4.2 Thaw the reagent cartridge (**Table of Materials**) and the libraries.
216
217 4.3 Make 0.2 N NaOH and dilute the libraries to obtain the desired molar concentration.
218
219 4.4 Rinse and dry the flow cell. Add 600 µL of diluted and denatured library solution into the
220 well of the reagent cartridge.
221
222 4.5 Start the sequencing run.
223
224 **5. Quality Control of NGS Data**
225
226 5.1. Perform the quality control of FASTQ data using the "FASTX-Toolkit"[16].
227
228 NOTE: A basic example of the parameter settings used is as follows:
229   fastq_quality_trimmer -v -t 20 -l 200 -i [InFilename.fastq] -o [InFilename.fastq]
230   fastq_quality_filter -v -q 20 -p 80 -i [InFilename.fastq] -o [InFilename.fastq]
231   fastx_reverse_complement -v -i [InFilename.fastq] -o [InFilename.fastq]
232
233 5.2. Format the output files to "fasta nucleic acid (.fna)" by the following command:
234   fastq_to_fasta -v -n -i [InFilename.fastq] -o [InFilename.fna]
235

6. Extraction and Analysis of Immunoglobulin Sequences from .fna Data

NOTE: The example programs were implemented in a UNIX environment. Please use them as an example references because performance may depend on the operating system and hardware environment. The authors do not accept any liability for errors or omissions. The programming languages, Perl[17], R[18], and required modules need to be installed according to the instructions on the cited websites. the IgBLAST program need to be installed according to the instructions on the appropriate website[19,20].

6.1 Download the following examples of in-house programs for repertoire analyses from https://github.com/KzPipeLine/KzPipeLine:
03_PipeLine_Mouse.zip; A set of example programs for the analyses of mouse antibody sequences.
05_PipeLine_Human.zip; A set of example programs for the analyses of human antibody sequences.

6.2 Extract the antibody reads in the sequence data: Extract the immunoglobulin (Ig) sequences of each Ig-class from the data (.fna) by a Perl program that searches the signature sequences in each immunoglobulin constant region (**Table 2**).

6.2.1 For the mouse immunoglobulin heavy chain (IgH) genes, extract the reads by the following command:
$ perl 01_KzMFTIgCmgggaNtdVer3_Kz160607.pl [Input filename] [Output filename (suffix)]

6.2.2 For the mouse immunoglobulin light chain (IgL) genes extract the reads by the following command:
$ perl 01_KzMFTCkltNtdVer1_170810.pl [Input filename] [Output filename (suffix)]

6.2.3 For the human immunoglobulin heavy chain (IgH) genes, extract the reads by the following command:
$ perl 01_KzMfHuIgHCmgadeNtdVer1_Kz180312.pl [Input filename] [Output filename (suffix)]

6.2.4 For the human immunoglobulin light chain (IgL) genes, extract the reads by the following command:
$ perl 01_KzMfHuIgCkltNtd_180316.pl [Input filename] [Output filename (suffix)]

6.3 Annotate and check the productivity of V(D)J gene recombination:

NOTE: The method described below utilizes standalone IgBLAST[19] for the annotation of V(D)J gene segments in the sequence. Set the database for the V(D)J genes and the parameter settings for IgBLAST as described[20].

6.3.1 Annotate the mouse immunoglobulin heavy chain (IgH) genes by the following command:
$ igblastn -germline_db_V $IGDATA/ImtgMouseIghV_NtdDb.txt -germline_db_J $IGDATA/ImtgMouseIghJ_NtdDb.txt -germline_db_D $IGDATA/ImtgMouseIghD_NtdDb.txt -

283 organism   mouse   -domain_system   imgt   -query   ./$InFile   -auxiliary_data
284 $IGDATA/optional_file/mouse_gl.aux -show_translation -outfmt 7 >> ./$OutName
285
286 6.3.2 Annotate the mouse immunoglobulin light chain (IgL) genes by the following command:
287 $ igblastn   -germline_db_V $IGDATA/ImtgMouseIgkV_NtdDb.txt   -germline_db_J
288 $IGDATA/ImtgMouseIgkJ_NtdDb.txt -germline_db_D $IGDATA/ImtgMouseIghD_NtdDb.txt -
289 organism   mouse   -domain_system   imgt   -query   ./$InFile   -auxiliary_data
290 $IGDATA/optional_file/mouse_gl.aux -show_translation -outfmt 7 >> ./$OutName
291
292 6.3.3 Annotate the human immunoglobulin heavy chain (IgH) genes by the following
293 command:
294 $ igblastn   -germline_db_V $IGDATA/ImtgHumanIghV_NtdDb.txt   -germline_db_J
295 $IGDATA/ImtgHumanIghJ_NtdDb.txt -germline_db_D $IGDATA/ImtgHumanIghD_NtdDb.txt -
296 organism   human   -domain_system   imgt   -query   ./$InFile   -auxiliary_data
297 $IGDATA/optional_file/Human_gl.aux -show_translation -outfmt 7 >> ./$OutName
298
299 6.3.4 Annotate the human immunoglobulin light chain (IgL) genes by the following command:
300 $ igblastn   -germline_db_V $IGDATA/ImtgHumanIgkV_NtdDb.txt   -germline_db_J
301 $IGDATA/ImtgHumanIgkJ_NtdDb.txt -germline_db_D $IGDATA/ImtgHumanIghD_NtdDb.txt -
302 organism   human   -domain_system   imgt   -query   ./$InFile   -auxiliary_data
303 $IGDATA/optional_file/human_gl.aux -show_translation -outfmt 7 >> ./$OutName
304
305 6.4 Visualize the global feature of an antibody repertoire.
306
307 6.4.1 Visualize the mouse IgH repertoire by the following command:
308 $. 00a1_3DView_MoIgH_Kz180406.sh
309
310 NOTE: The input file is filename.fna (sequence data), preferably the output file from 6.2.1.
311 This file needs to be placed in a lower directory (folder) named "filename". In line 50 of the
312 shell script, assign a "filename" for Para_4.
313
314 6.4.2 Visualize the human IgH repertoire by the following command:
315 $. 00a1_3DView_HuIgH_Kz180411.sh
316
317 NOTE: The input file is filename.fna sequence data, preferably the output file of 6.2.3. This
318 file needs to be placed in the lower directory (folder) that name is "filename". In line 46 of
319 the shell script, assign a "filename" for Para_4.
320
321 6.4.3 Visualize the mouse IgL repertoire by the following command:
322 $. 00_2DViewS_MoIgL_Kz180406.sh
323
324 NOTE: With this pipeline, Igκ and Igλ are processed concomitantly. The input file is
325 filename.fna (sequence data), preferably the output file from 6.2.2. This file needs to be
326 placed in the lower directory (folder) named "filename". In line 53 of the shell script, assign a
327 "filename" for Para_4. The output file's name, ending with "_IgKlCount.txtDim2Rpm.txt"
328 gives the coordinates for a two-dimensional bar graph (**Figure 3**, IgL).
329

330   6.4.4 Visualize the human IgL repertoire by the following command:
331      $. 00_2DView_HuIgL_Kz180319.sh
332
333   NOTE: With this pipeline, Igκ and Igλ are processed concomitantly. The input file is
334   filename.fna sequence data, preferably the output file of 6.2.4. This file needs to be placed in
335   a lower directory (folder) named "filename". In line 53 of the shell script, assign "filename"
336   for Para_4. The output file name ending with "_IgKlCount.txtDim2Rpm.txt" gives the
337   coordinates for a two-dimensional bar graph (**Figure 4,** IgL).
338
339   **REPRESENTATIVE RESULTS:**
340
341   **Antibody repertoires of mouse**
342   A perspective of a murine antibody repertoire as a whole can be obtained from cells or tissues
343   such as the spleen, bone marrow, lymph node, or blood. **Figure 3** shows representative results
344   of IgM, IgG1, IgG2c, and immunoglobulin light chain (IgL) repertoires from a naïve mouse
345   spleen. The summary of the read numbers is shown in **Table 3**. For example, 166,175/475,144
346   reads contained IgM-specific signature sequence (**Table 2**) and 133,371/166,175 reads were
347   VDJ-productive inferred by IgBLAST[19].
348
349   **Figure 3** shows a repertoire profile of VDJ-rearrangement by 3D-VDJ-plot, in which the size of
350   each ball represents the relative number of reads; in other words, the number of antibody
351   mRNAs in whole B cells. The 3-D mesh consists of 110 IGHV, 12 IGHD, and 4 IGHJ, which are
352   aligned to reflect their order on the chromosome. In addition, the genes ambiguously
353   assigned by IgBLAST were collected separately in the last position for each IGHV, IGHD and
354   IGHJ line, giving rise to 7,215 nodes in the cuboid.
355
356   Also, shown in **Figure 3** is a 2D-VJ-plot showing the profile of VJ-rearrangement in the IgL
357   repertoire. The length of each bar on this plot represents the relative number of reads. The
358   x-axis represents 101 IGLVκ and 3 IGLVλ genes, and the y-axis represents 4 IGLJκ and 3 IGLJλ
359   genes. The unannotated V- and J-genes are represented on the right borderline.
360
361   The complementarity-determining region 3 (CDR3) sequences of these productive reads,
362   which give rise to the majority of antigen-binding specificity, are given in IgBLAST outputs.
363   The CDR3 sequences can be analyzed statistically, including biological or technical replicates,
364   as described previously[8,10].
365
366   **Human antibody repertoires**
367   A perspective of a human antibody repertoire as a whole can be analyzed from various tissues
368   including peripheral blood mononuclear cells (PBMCs) or pathological tissues. **Figure 4** shows
369   representative results of IgM, total IgG (IgG1, IgG2, IgG3, and IgG4), total IgA (IgA1 and IgA2),
370   IgD, IgE and IgL repertoires from normal PBMCs. A summary of the read numbers is shown in
371   **Table 3**. For example, 90,238/1,582,754 reads contained IgM-specific signature sequence and
372   67,896/90,238 reads were VDJ-productive.
373
374   The repertoire profile of VDJ rearrangement is shown on a 3D-VDJ-plot in which the size of
375   each ball represents the relative number of reads; in other words, the number of antibody
376   mRNAs from whole PBMCs (**Figure 4**). The 3-D mesh consists of 56 IGHV, 27 IGHD, and 6 IGHJ,

aligned in the order they appear on the chromosome. In addition, genes ambiguously assigned by IgBLAST are represented separately in the last position for each IGHV, IGHD and IGHJ line, giving rise to 11,172 nodes in the cuboid.

The profile of VJ-rearrangement in the IgL repertoire is depicted in a 2D-VJ-plot in which the length of each bar represents the relative number of reads (**Figure 4**). The x-axis represents 41 IGLVκ and 32 IGLVλ genes, and the y-axis represents 5 IGLJκ and 5 IGLJλ genes. The un-annotated V- and J-genes are represented on the right borderline.

The human CDR3 sequences are given in IgBLAST outputs and can be analyzed statistically as described previously[8,10].

FIGURE AND TABLE LEGENDS:

Table 1: Primer sequences for PCR-amplification of immunoglobulins

Table 2: Summary of the immunoglobulin signature sequences

Table 3: Summary of the read numbers in the experiments

Figure 1: Schematic representation of sequencing strategy for analyzing antibody repertoires in individual mice. (A) Total RNA from the immune cells or tissues was reverse-transcribed and PCR-amplified using the universal forward primer and immunoglobulin class-specific reverse primers. The amplicons from each immunoglobulin class were pooled and rendered for next-generation sequencing. (B) The biological replicates such as spleens from C57BL/6 mice were treated as follows: total RNAs were purified from spleen samples, and cDNAs were amplified by 5'-RACE using the universal primer and antibody class-specific primer. They were then rendered for next-generation sequencing with labeling primers for individual mice. Parts of the figure are adapted from[8] with permission.

Figure 2: Schematic of data-processing flowchart for analyzing antibody repertoires in individual mice. Amplicon reads obtained after next-generation sequencing were processed as follows: (1) read sequences were checked for the presence of antibody class-specific signature sequences; (2) sequences were examined for the V, D, and J gene fragments using IMGT/HighV-Quest and/or IgBLAST; (3) the sequences containing a productive VDJ junction were collected; and (4) these sequences were used for the analysis of overall repertoire features, CDR3, etc.

Figure 3: Global data visualization for mouse antibody repertoires. The overall repertoire profiles of each antibody class were visualized by 3D-VDJ-plot. The x-axis represents 110 IGHV genes ordered as on the chromosome. The y- and z-axis represents 12 IGHD and 4 IGHJ genes, respectively. The volume of spheres on each node represents the number of reads. Red spheres: un-annotated V, D, and J genes. The IgL read distributions are shown on a 2D-VJ-plot in which the length of each bar represents the relative number of reads. The x-axis represents 101 x IGLVκ and 3 x IGLVλ genes, and the y-axis represents 4 x IGLJκ and 3 x IGLJλ genes. The un-annotated V and J genes are represented on the right borderline.

424 **Figure 4: Global data visualization for human antibody repertoires.** The overall repertoire
425 profiles of each antibody class were visualized by 3D-VDJ-plot. The x-axis represents 56 IGHV
426 genes ordered as on the chromosome. The y- and z-axis represents 27 IGHD and 6 IGHJ genes,
427 respectively. The volume of spheres on each node represents the number of reads. Red
428 spheres: un-annotated V, D, and J genes. The IgL reads are arrayed on the 2D-VJ-plot in which
429 the length of each bar represents the relative number of the reads. The x-axis represents 41
430 x IGLVκ and 32 x IGLVλ genes, and the y-axis represents 5 x IGLJκ and 5 x IGLJλ genes. The un-
431 annotated V- and J-genes are represented on the right borderline.
432
433 **DISCUSSION:**
434 The method described here utilizes NGS for antibody RNA amplified using the 5'-RACE
435 method. In contrast to methods that use degenerate 5'-V_H gene primers, mRNAs of each
436 antibody class are amplified evenly using universal forward primers. In addition, the use of
437 antisense primers specific for the constant-region 1 (CH1) of the antibody gene enables
438 repertoire profiling of specific immunoglobulin classes. This is very beneficial for dissecting
439 the class-specific antibody response, as well as for comparing naive and immunized
440 repertoires[8,9].
441
442 A most likely pitfall of the method is a paucity of amplified immunoglobulin messages. The
443 depth of antibody repertoire obtained by this protocol substantially depends on the PCR
444 amplification described in steps 3.1 and 3.2. If the repertoire depth is not properly obtained,
445 changing the ratios of template cDNA and primers in steps 3.2.1 or 3.2.2 is strongly
446 recommended.
447
448 Generally, approximately 20% of the antibody reads produced by NGS are ambiguous
449 sequences[21]. Even with established "correction methods", 5-10% remain ambiguous[3]. We,
450 therefore, analyzed the sequence and filtered raw reads containing signature sequences
451 corresponding to immunoglobulin constant regions (CμH1, Cγ1H1, Cγ2cH1, etc.). Hence the
452 analysis of somatic hyper-mutations needs the careful examinations.
453
454 One of the limitations of this method is that immunoglobulin heavy and light chain pair is
455 unable to be inferred. Hence the repertoire view obtained by this method is not holistic.
456 However, it is possible to approximate the top-ranking pairs by statistical analysis of the
457 data[10]. Also, a novel method to sequence the immunoglobulin pairs was reported recently[3,4].
458
459 The immunoglobulin sequences in the output .fna data were extracted based on the presence
460 of immunoglobulin gene signature sequences. The V, D, and J gene segments were then
461 annotated and the productivity of V(D)J rearrangements were assessed. The
462 complementarity-determining region 3 (CDR3) sequences were also annotated. These
463 systematic examinations of immunoglobulin sequences in .fna data were usefully provided by
464 the IMGT/HighV-QUEST server[22-24]. However, building an automated processing pipeline has
465 the merit to analyze the big experimental data. The pipeline customized for each purpose is
466 possible to set up by using the standalone IgBLAST protocol[19]. This approach needs basic
467 programming literacy but is very useful for detailed analyses of the immunoglobulin system.
468 The pipelines described are the examples of the customized protocol (**Figure 2**).
469
470 The number of antibody reads is proportional to the amount of antibody RNAs in the sample,

471 reflecting the antibody constituents of the antibody system at given time points[5,8,25]. The
472 method described here gives a bird's eye view of the V(D)J constitution of an antibody
473 repertoire using R programs[8,18,26].
474
475 The global view of IgM antibody repertoires of individual naive mice revealed a highly
476 conserved VDJ-profile as compared to those of IgG1 or IgG2c[8]. It was reported that VDJ
477 combinations of immature zebrafish are highly stereotyped[9]. In contrast, human VDJ
478 combinations are reported to be highly skewed[6]. The highly conserved deterministic VDJ-
479 profiles in naive B cells are probably generated either by skewed VDJ-rearrangements or
480 negative selection with auto-antigens presented in the body. For example, IGHV11-2 is
481 expressed preferentially in the fetal IgM repertoire[27] and this predominance is attributed to
482 the autoreactivity of IGHV11-2 against senescent erythrocytes[27]. Interestingly, IGHV11-2 was
483 also the most common major repertoire in our previously published analysis of naive IgM[8].
484
485 The method described here is useful for deciphering antigen-responsive antibody repertoires
486 by inclusively analyzing the antibody-repertoire space generated in individual bodies,
487 avoiding inadvertent omission of key antibody repertoires[8,10]. This method also allows the
488 examination of detailed antibody network dynamism, which would facilitate accelerated
489 discovery of protective antibodies against newly emerging pathogens.
490
497
498 **DISCLOSURES:**
499 The authors have no conflicts of interest to disclose.
500
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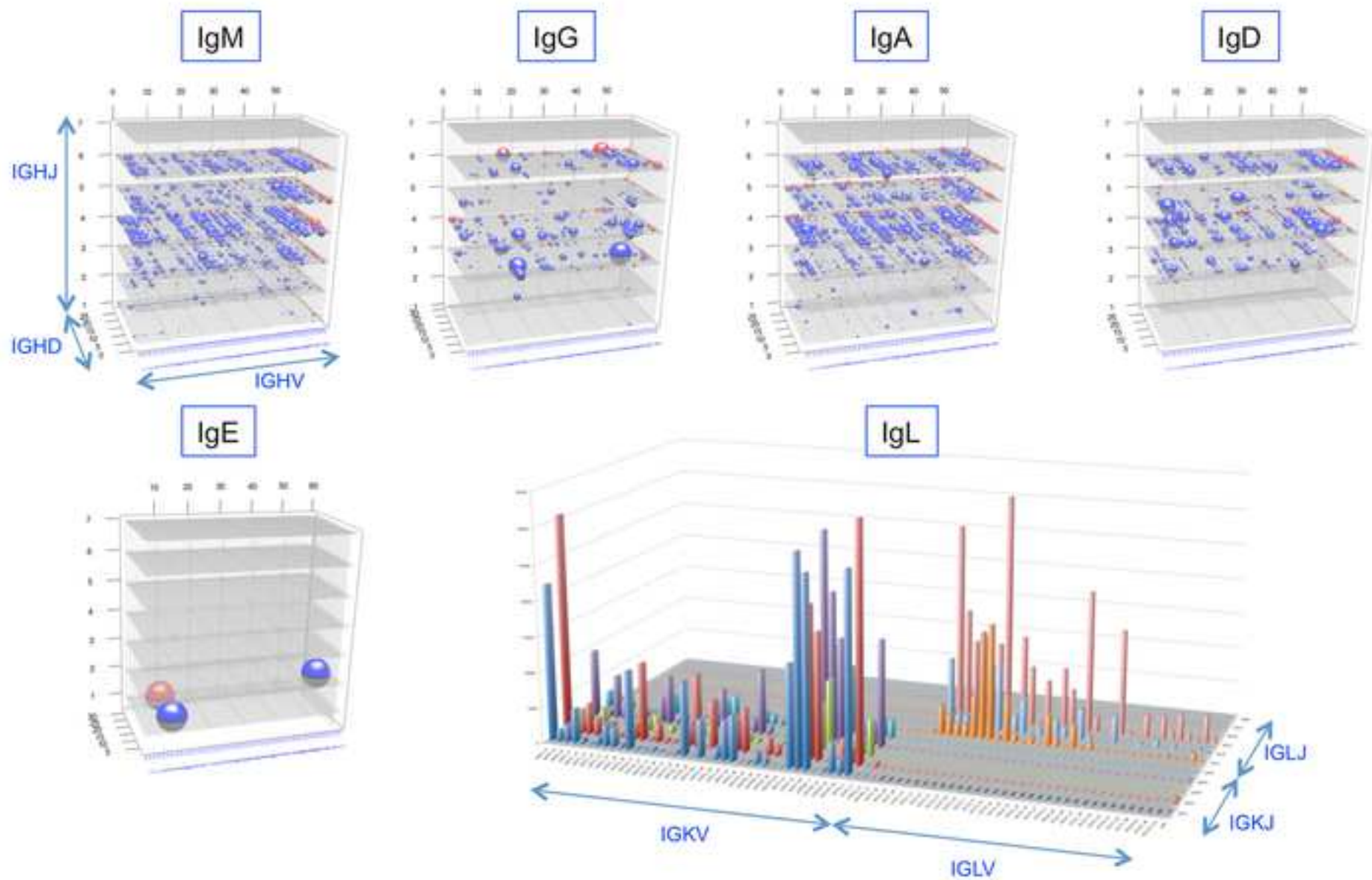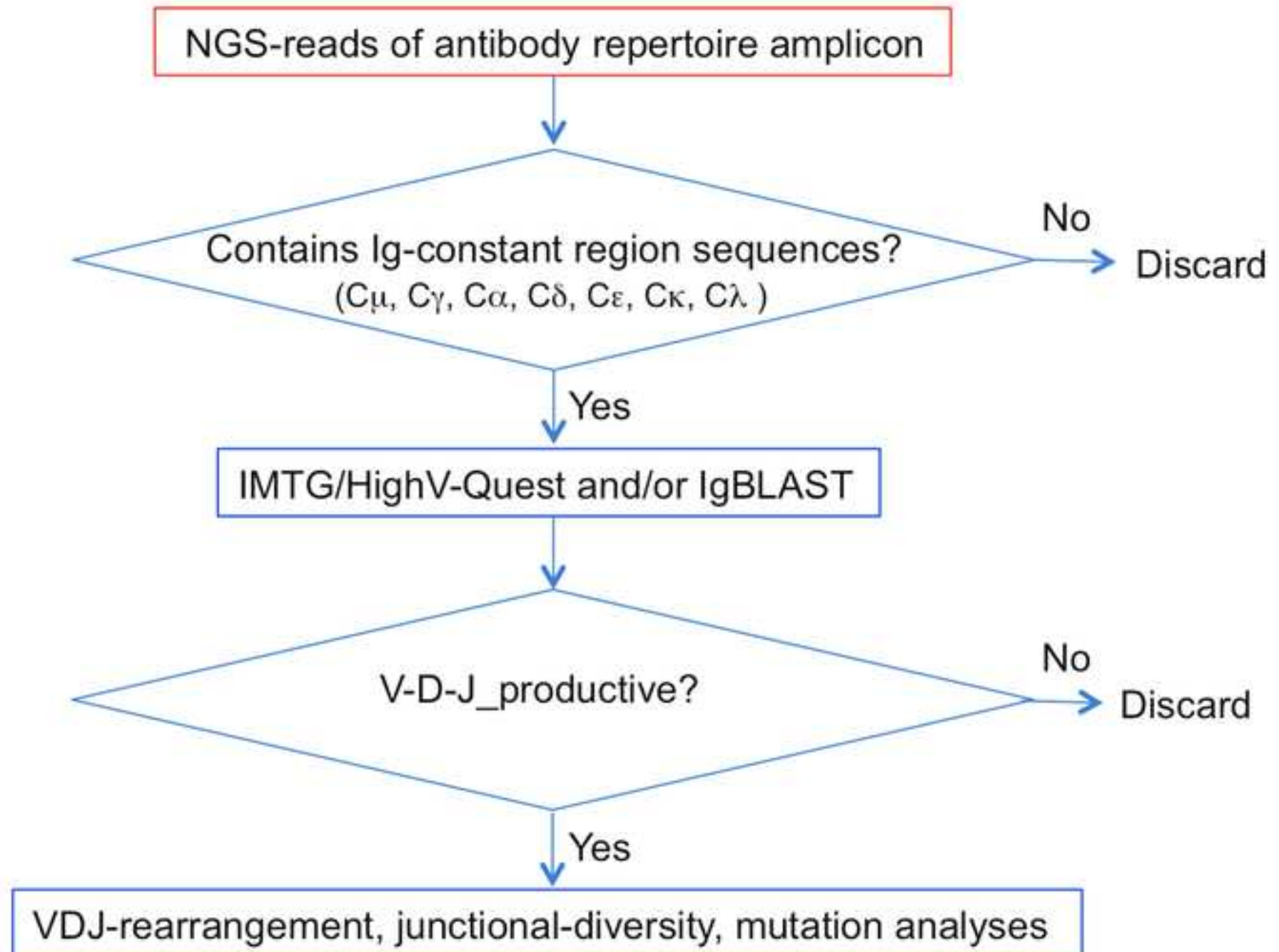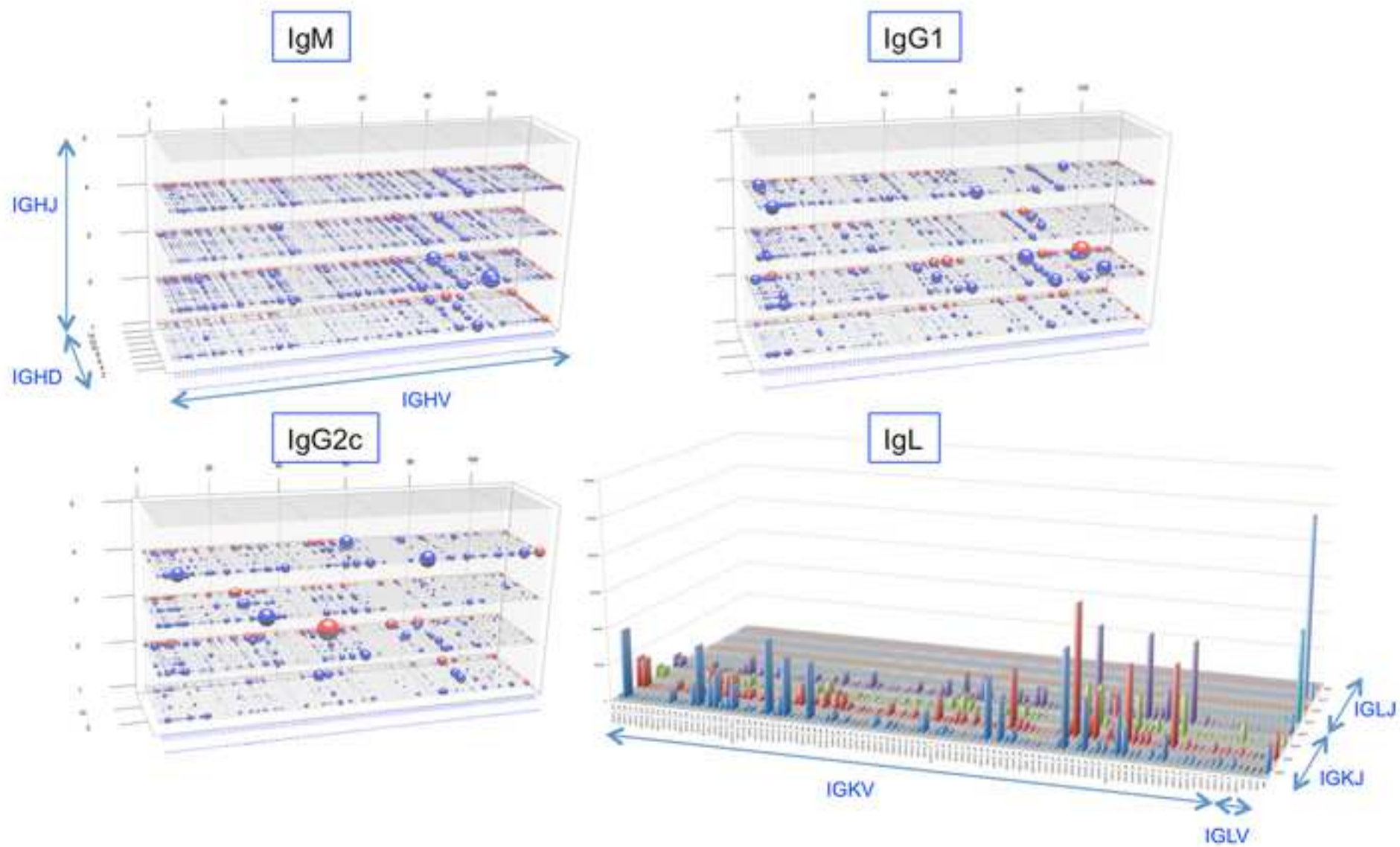567   (2004).
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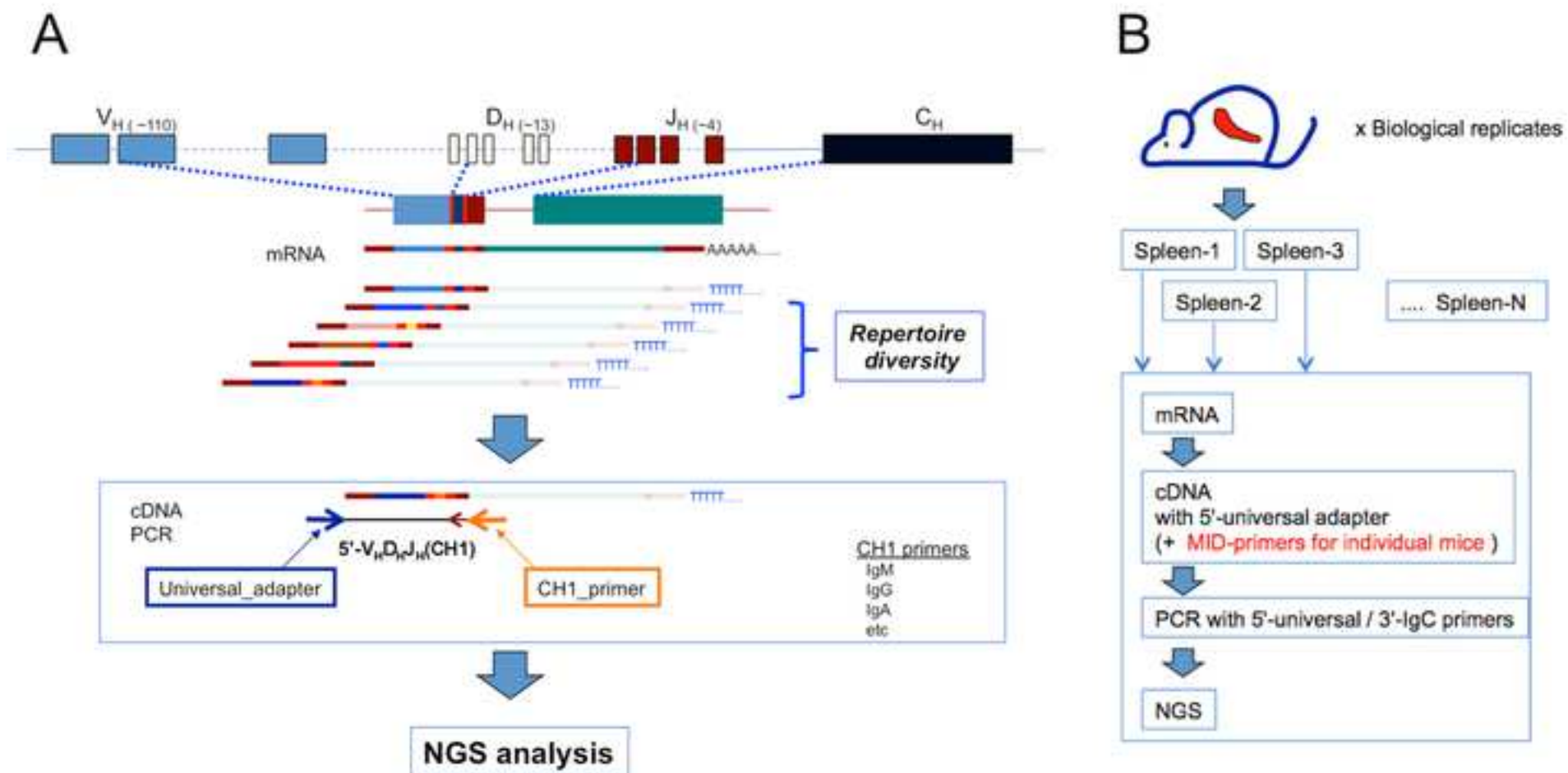
Figure 2

Figure 2

Figure 3

Figure 4

Table 1

Universal forward primer   5'- AAGCAGTGGTATCAACGCAGAGT-3'

Reverse primers for the mouse immunoglobulins (Ref.8)
IgM_CH1:   5'- CACCAGATTCTTATCAGACAGGGGGCTCTC -3'
IgG1_CH1:   5'- CATCCCAGGGTCACCATGGAGTTAGTTTGG -3'
IgG2c_CH1:   5'- GTACCTCCACACACAGGGGCCAGTGGATAG -3'
IgG3_CH1:   5'-ATGTGTCACTGCAGCCAGGGACCAAGGGA-3'
IgA_CH1:   5'-GAATCAGGCAGCCGATTATCACGGGATCAC-3'
Igκ_CH1:   5'- GCTCACTGGATGGTGGGAAGATGGATACAG -3'
Igλ_CH1:   5'- CTBGAGCTCYTCAGRGGAAGGTGGAAACA -3'

Reverse primers for the human immunoglobulins (Ref.14)
IgM_CH1:   5'- GGGAATTCTCACAGGAGACG -3'
IgG_CH1:   5'- AAGACCGATGGGCCCTTG -3'
IgD_CH1:   5'- GGGTGTCTGCACCCTGATA -3'
IgA_CH1:   5'- GAAGACCTTGGGGCTGGT -3'
IgE1_CH1:   5'- GAAGACGGATGGGCTCTGT -3'
IgE2_CH1:   5'- TTGCAGCAGCGGGTCAAGGG -3'
Igκ_CH1:   5'- TGCTCATCAGATGGCGGGAAGAT -3'
Igλ_CH1:   5'- AGAGGAGGGCGGGAACAGAGTGA -3'

Table 2

Immunoglobulin class	Sense	Antisense
Mouse immunoglobulin heavy chains (C57BL/6)		
IgM	AGTCAGTCCTTCCCAAATGTC	GACATTTGGGAAGGACTGACT
IgG1	AAAACGACACCCCCATCTGTC	GACAGATGGGGGTGTCGTTTT
(IgG1 variant)	AAAACAACACCCCCATCAGTC	GACTGATGGGGGTGTTGTTTT
IgG2c	AAAACAACAGCCCCATCGGTC	GACCGATGGGGCTGTTGTTTT
IgG3	GTGATCCCGTGATAATCGGCT	AGCCGATTATCACGGGATCAC
IgA	TCCCTTGGTCCCTGGCTGCAG	TCCCTTGGTCCCTGGCTGCAG
Mouse immunoglobulin light chains (C57BL/6)		
Igκ	CTGTATCCATCTTCCCACCATCCAGTGAGC	GCTCACTGGATGGTGGGAAGATGGATACAG
Igλ1	TGTTTCCACCTTCCTCTGAAGAGCTCGAG	CTCGAGCTCTTCAGAGGAAGGTGGAAACA
Igλ2	TGTTTCCACCTTCCTCTGAGGAGCTCAAG	CTTGAGCTCCTCAGAGGAAGGTGGAAACA
Igλ3	TGTTTCCACCTTCCCCTGAGGAGCTCCAG	CTGGAGCTCCTCAGGGGAAGGTGGAAACA
Igλ4	TGTTCCCACCTTCCTCTGAAGAGCTCAAG	CTTGAGCTCTTCAGAGGAAGGTGGGAACA
Human immunoglobulin heavy chains		
IgM	GGGAGTGCATCCGCCCCAAC	GTTGGGGCGGATGCACTCCC
IgG	GCTTCCACCAAGGGCCCATC	GATGGGCCCTTGGTGGAAGC
IgA	GCATCCCCGACCAGCCCCAA	GACCGATGGGGCTGTTGTTTT
IgD	GCACCCACCAAGGCTCCGGA	TCCGGAGCCTTGGTGGGTGC
IgE	GCCTCCACACAGAGCCCATC	GATGGGCTCTGTGTGGAGGC
Human immunoglobulin light chains		
Igκ	ACTGTGGCTGCACCATCTGC	GCAGATGGTGCAGCCACAGT
Igλ1,2,6	GTCACTCTGTTCCCGCCCTC	GAGGGCGGGAACAGAGTGAC
Igλ3,7	GTCACTCTGTTCCCACCCTC	GAGGGTGGGAACAGAGTGAC

Table 3

Mouse IgH	Total reads	IgM	IgG1	IgG2c		
Input	475,144					
IgC-containing		166,175	229,671	36,628		
VDJ-productive		133,371	196,583	31,446		

Mouse IgL	Total reads	IgKappa	IgLambda			
Input	527,668					
IgC-containing		178,948	21,446			
VJ-productive		160,924	16,988			

Human IgH	Total reads	IgM	IgG	IgA	IgD	IgE
Input	1,582,754					
IgC-containing		90,238	5,298	94,061	75,549	2,932
VDJ-productive		67,896	2,775	78,203	56,495	3

Human IgL	Total reads	IgKappa	IgLambda			
Input	1,582,754					
IgC-containing		120,316	64,148			
VJ-productive		97,169	52,324			

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.2 mL Strip Tubes	Thermo Fisher Scientific	AB0452	120 strips
100 bp DNA Ladder	TOYOBO	DNA-035	0.5 mL
2100 Bioanalyzer Systems	Agilent Technologies	G2939BA /2100	
Acetic Acid	Wako	017-00256	500 mL
Agarose, NuSieve GTG	Lonza	50084	
Ammonium Chloride	Wako	017-02995	500 g
Chloroform	Wako	038-02606	500 mL
Dulbecco's PBS (-)"Nissui"	NISSUI	08192	
Ethylenediamine-N,N,N',N'-tetraacetic Acid Disc	Wako	345-01865	500 g
Falcon 40 μm Cell Strainer	Falcon	352340	50/Case
ling lock tube 1.7 mL	BM EQUIPMENT	BM-15	
ling lock tube 2.0 mL	BM EQUIPMENT	BM-20	
MiSeq Reagent Kit v2	illumina	MS-102-2003	500 cycles
MiSeq System	illumina	SY-410-1003	
NanoDrop 2000c Spectrophotometer	Thermo Fisher Scientific		
Potassium Hydrogen Carbonate	Wako	166-03275	500 g
PureLink RNA Mini Kit	life technologies	12183018A	
Qubit 3.0 Fluorometer	Thermo Fisher Scientific	Q33216	
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32854	500 assays
SMARTer RACE 5'/3' Kit	Clontech	634858	
TaKaRa Ex Taq Hot Start Version	Takara Bio Inc.	RR006A	
Trizma base	Sigma	T6066	1 kg
TRIzol Reagent	AmbionThermo Fisher Scientific	15596026	100 mL
Ultra Clear qPCR Caps	Thermo Fisher Scientific	AB0866	120 strips
UltraPure Ethidium Bromide	Thermo Fisher Scientific	15585011	
Wizard SV Gel and PCR Clean-Up System	Promega	A9282	

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> Repertoires by Next-generation Sequencing

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Responses to the Editorial comments:

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

 # Response: We retained the format made in the manuscript.

2. Please address specific comments marked in the manuscript.

 # Response: We addressed the specific comments as follows:

Comment[1]: We agree with this comment.

Comment[2]: We agree with this comment.

Comment[3]: We agree with this comment.

Comment[4]: The word 'female' was removed. The word 'pass' was replaced by 'smash'.

Comment[5]: The word 'in the tissue' was added.

Comment[6]: The word 'tissue' was added.

Comment[7]: A step describing RNA quantitation was added as step 2.9.

Comment[8]: We explained about the human sample in Note, line 169-171.

Comment[9]: The words 'Illumina index primers' were changed to 'index sequence primers'.

Comment[10]: The step was expanded to explain this procedure (3.3.1 - 3.3.4).

Comment[11]: The number of immunoglobulin classes amplified and the yields were explained in the Notes added.

Comment[12]: The step was explained further.

Comment[13]: The explanation was expanded.

Comment[14]: The step was explained further.

Comment[15]: We understand this comment.

Comment[16]: Table 2 (signature sequences) was referred.

Comment[17]: IgBLAST was referred.

Comment[18]: Some troubleshooting and limitations were added.

3. We cannot film scripting steps. We can film steps involving graphical user interface,

button clicks, knob turns, discrete experimental steps only.

 # Response: We understand this and changed the highlighted part in the manuscript.

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 # Response: We obtained the explicit copyright permission for the Figure 1 and 2.

5. Please reword lines 444 and line 446-447 as it matches with the previously published literature.

 # Response: The lines 444 and line 446-447 were reworded.

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