Chapter 3

One-Step Next-Generation Sequencing of Immunoglobulin and T-Cell Receptor Gene Recombinations for MRD Marker Identification in Acute Lymphoblastic Leukemia

Patrick Villarese, Chrystelle Abdo, Matthieu Bertrand, Florian Thonier, Mathieu Giraud, Mikaël Salson, and Elizabeth Macintyre

Abstract

Within the EuroClonality-NGS group, immune repertoire analysis for target identification in lymphoid malignancies was initially developed using two-stage amplicon approaches, essentially as a progressive modification of preceding methods developed for Sanger sequencing. This approach has, however, limitations with respect to sample handling, adaptation to automation, and risk of contamination by amplicon products. We therefore developed one-step PCR amplicon methods with individual barcoding for batched analysis for IGH, IGK, TRD, TRG, and TRB rearrangements, followed by Vidjil-based data analysis.

Key words Next-generation sequencing, One step, T cell receptor, B cell receptor

1 Introduction

Recombination of the V (D) J genes of immunoglobulin (IG) and T cell receptor (TR) loci is an essential step in the differentiation of B and T cells, allowing the production of a unique antigen receptor which is present in all clonal progeny. As such, acute lymphoblastic leukemias (ALLs) are characterized by clonal, homogeneous IG/TR rearrangement patterns that are widely used for clonal tracking during evaluation of response to treatment, commonly referred to as quantification of minimal (or measurable) residual disease (MRD) [1]. The EuroMRD group has played a seminal role in developing, standardizing, and accompanying optimized use of IG/TR clonal markers in lymphoid malignancies, essentially using CDR3 clone-specific quantitation by PCR. Initial IG/TR target identification was based predominantly on EuroClonality/BIOMED-2 multiplex PCR-based protocols for IG/TR targets combined with heteroduplex analysis or fragment length (GeneScan) analysis, followed by Sanger sequencing and design of

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CDR3-specific PCR primers [2–4]. With the development of NGS immunogenetics [5–9], the EuroClonality-NGS working group developed a standardized two-step multiplex amplicon approach to IG/TR target identification in ALL that enabled switching of sequencing adaptors and a reduction of the total number of primers required for individual sample identification in mixed libraries [10].

Two-step PCR approaches, however, have several limitations, particularly in MRD laboratories, where contamination by PCR products can be a risk of false-positive results. These include more extensive sample handling with consequent increased overall cost and risk of contamination and reduced suitability for automation. We therefore developed a single-step PCR approach to screening for IG/TR rearrangements in lymphoid malignancies, as described here.

2 Materials

2.1 Sample Preparation

1. 15 mL polypropylene tubes.
2. Phosphate-buffered saline (1xPBS) without Ca2+ and Mg2+ pH 7.4 (Invitrogen).
3. Sysmex XE 2100.
4. Maxwell RSC instrument (Promega).
5. Maxwell RSC Buffy Coat DNA kit (Promega).
6. Nanodrop ND2000 (Thermo Fisher Scientific).
7. Centrifuge (1000 × *g*).
8. 2 mL tubes (Eppendorf).

2.2 PCR Amplification

1. UltraPure Distilled Water DNase-/RNase-Free (Invitrogen).
2. SafeSeal Microcentrifuge Tubes (Sorenson).
3. 0.2 mL Thin-walled Tubes with Flat Caps (Thermo Fisher Scientific).
4. Kit FastStart High Fidelity PCR System, dntPack (Roche Diagnostic).
5. Thermocycler BioRad T100 or Applied Biosystem Veriti 96.

2.3 Sample Purification

1. Agencourt AMPure XP (Beckman Coulter).
2. 0.8 mL 96-well storage plate (Thermo Fisher Scientific).
3. TE buffer pH 8 (Invitrogen).
4. MicroAmp Optical Adhesive Film (Thermo Fisher Scientific).
5. DynaMag-96 Side Skirted Magnet (Thermo Fisher Scientific).

2.4 Sample Assay

1. Assay plate, 96 well (Costar).
2. Kit QuantiFluor ONE dsDNA System (Promega).
3. GLOMAX (Promega).
4. Qubit 4 fluorometer (Thermo Fisher Scientific).
5. Qubit assay tubes (Thermo Fisher Scientific).
6. 2100 Bioanalyzer Instrument (Agilent Technologies).
7. Agilent High Sensitivity DNA Kit (Agilent Technologies).

2.5 Pool Sample (2 nM)

1. TE buffer (Invitrogen).
2. DNA low bind tubes 1.5 mL (Eppendorf).

2.6 Denaturation Step Before Sequencing

1. Sodium Hydroxide solution, 10 M in H_2O (Sigma Aldrich).
2. PhiX Control v3 (Illumina).
3. UltraPure Distilled Water DNase-/RNase-Free (Invitrogen).

2.7 Sequencing

1. UltraPure Distilled Water DNase-/RNase-Free (Invitrogen).
2. Tween 20 (Euromedex).
3. Precision wipes (KIMTECH Science).
4. MiSeq Reagent Kit V2 2x250pb (Illumina).
5. MiSeq System (Illumina).

2.8 Bioinformatic Analysis

Access to a Vidjil server allowing hosting of patient data [11] (see http://www.vidjil.org/doc/healthcare/).

3 Methods

3.1 Sample Preparation

1. Use blood or bone marrow cells.
2. Enumerate white blood cells, e.g., with theSysmex XE2100 system.
3. Extract DNA from ten million white blood cells with the Maxwell RSC Buffy Coat DNA kit.
4. After extraction, quantify DNA by Nanodrop.
5. If necessary, adjust DNA concentration to 100 ng/μL with TE buffer pH 8.

3.2 PCR Amplification

3.2.1 Prepare a Mix of Primers for each Target of Interest (See Notes Below)

1. Prepare the primer mix for IGH VDJ FR2 (*see* **Note 1**).
2. Prepare the primer mix for IGH DHJH (*see* **Note 2**).
3. Prepare the primer mix for IGK (*see* **Note 3**).
4. Prepare the primer mix for TRG (*see* **Note 4**).
5. Prepare the primer mix for TRD (*see* **Note 5**).

6. Prepare the primer mix for TRB DJ (*see* **Note 6**).
7. Prepare the primer mix for TRB VDJ (*see* **Note 7**).

Importantly, each primermix should be prepared with the same index.

3.2.2 PCR Amplification

1. Prepare the PCR mix for each reaction on ice (*see* Table 1).
2. First, mix H_20, buffer, and $MgCl_2$ on ice.
3. Then prepare a 0.1× dilution of Taq polymerase with H_2O.
4. Add primer indexes to the mix.
5. Lastly, add 100 ng of patient DNA to each PCR (or 250 ng DNA for the TRG reaction).
6. Run amplification protocol in a thermocycler (*see* Table 2).

3.3 Sample Purification

Remark: TRG samples do not need to be purified, but other targets must be purified with double purification ratio 0.6×/0.25×. Take out the AMPure XP Kit at least 30 min before use.

1. Take a storage plate and add 28.8 μL of Agencourt beads per well.
2. Add 48 μL of sample to the beads per well.
3. Cover with an adhesive film.
4. Centrifuge at 280 × *g* for 1 min.
5. Put the plate on a microplate shaker at 200 × *g* for 2 min.
6. Incubate the plate for 5 min at room temperature.
7. Centrifuge at 280 × *g* for 1 min.
8. Put the plate to the side skirted magnet for 5 min.
9. Transfer 76 μL of supernatant to a new storage plate.
10. Add to the new wells 19 μL beads for the IGH VDJ/IGK/TRD/TRB DJ/TRB VDJ reactions and 15.2 μL for the IGH-DJ reaction.
11. Cover with adhesive film.
12. Centrifuge at 280 × *g* for 1 min.
13. Put the plate to the side skirted magnet for 5 min.
14. Discard the supernatant and wash the beads twice with 190 μL 70% ethanol.
15. Shift the plate on the side skirted magnet and wait 1 min.
16. Discard all supernatant and wait 1 min.
17. Leave the plate on the side skirted magnet and add 10 μL TE.
18. Centrifuge at 280 × *g* for 1 min.
19. Put the plate to the side skirted magnet for 5 min.
20. Collect 8.5 μL of each sample.

Table 1
PCR reaction mixes for different IG and TR targets

IGH V-J FR2			TRG			TRB V-J		
	Final conc.	*μL/1×*		*Final conc.*	*μL/1×*		*Final conc.*	*μL/1×*
H_2O	n.a.	20	H_2O	n.a.		H_2O	n.a.	30.75
10× buffer	1×	5	10× buffer	1×	5	10× buffer	1×	5
$MgCl_2$ 25 mM	2 mM	4	$MgCl_2$ 25 mM	1.5 mM	3	$MgCl_2$ 25 mM	4 mM	8
dNTPs 10 mM	0.2 mM	1	dNTPs 10 mM	0.2 mM	1	dNTPs 10 mM	0.2 mM	1
Primer mix FR2V – 10 μM pool A	0.1 μM	11	Primer mix A 10 μM	0.1 μM	2.5	Primer mix 100 μM VB	Variable	0.5
Primer mix FR2V – 10 μM pool B	0.2 μM	5	Primer mix B 10 μM	0.05 μM	0.5	Primer mix 10 μM JB	0.025 μM each	1.75
Primer mix T7JH – 10 μM pool C	0.1 μM	1	Primer mix TRGV11 10 μM	0.2 μM	1			
			Primer mix C 20 μM	0.2 μM	2			
Taq high Fidelity; 5 U/μL; dil. 1:10	1 U	2	Taq high Fidelity; 5 U/μL; dil. 1:10	2.5 U	5	Taq high Fidelity; 5 U/μL; dil. 1:10	1 U	2
IGH D-J			**TRD**					
H_2O	n.a.	33	H_2O	n.a.	37.25			
10× buffer with 18 mM $MgCl_2$	n.a.	5	10× buffer with 18 mM $MgCl_2$	n.a.	5			
dNTPs 10 mM	0.2 mM	1	dNTPs 10 mM	0.2 mM	1			
Primer mix DH 10 μM	0.1 μM	4	Primer mix V DD2	0.1 μM	0.45			
Primer mix JH 10 μM	0.2 μM	1	Primer mix J DD	0.1 μM	0.3			

(continued)

Table 1
(continued)

Taq high Fidelity; 5 U/μL; dil. 1:10	2.5 U	5	Taq high Fidelity; 5 U/μL; dil. 1:10	2.5 U	5
IGK			**TRB D-J**		
H_2O	n.a.	22	H_2O	n.a.	30.25
10× buffer with 18 mM $MgCl_2$	n.a.	8	10× buffer	1×	5
dNTPs 10 mM	0.4 mM	2	$MgCl_2$ 25 mM	4 mM	8
Primer mix VK 10 μM	0.2 μM	13	dNTPs 10 mM	0.2 mM	1
Primer mix intron new 10 μM	0.2 μM	1	Primer mix 10 μM DB	0.1 μM each	1
Primer mix Kde 10 μM	0.2 μM	1	Primer mix 10 μM JB	0.025 μM each	1.75
Taq high Fidelity; 5 U/μL; dil. 1:10	1 U	2	Taq high Fidelity; 5 U/μL; dil. 1:10	1 U	2

Table 2
Amplification protocols for different IG and TR targets

IGH V-J FR2 (35 cycles)	IGH D-J (35 cycles)	IGK (30 cycles)	TRD (35 cycles)	TRG (35 cycles)	TRB V-J (35 cycles)	TRB D-J (35 cycles)
94 °C 10′	94 °C 10′	94 °C 10′	94 °C 8′	94 °C 8′	94 °C 10′	94 °C 10′
94 °C 1′	94 °C 1′	92 °C 30″	94 °C 45″	94 °C 45″	94 °C 1′	94 °C 1′
63 °C 1′	63 °C 1′	61 °C 40″	62 °C 1′	57 °C 1′	65 °C 1′	63 °C 1′
72 °C 30″	72 °C 30″	72 °C 40″	72 °C 1′30″	72 °C 1′30″	72 °C 30″	72 °C 30″
72 °C 30′	72 °C 30′	72 °C 30′	72 °C 10′	72 °C 10′	72 °C 30′	72 °C 30′
15 °C final	15 °C final	15 °C final	15 °C final	15 °C final	15 °C final	15 °C final

3.4 Sample Assay Quantification

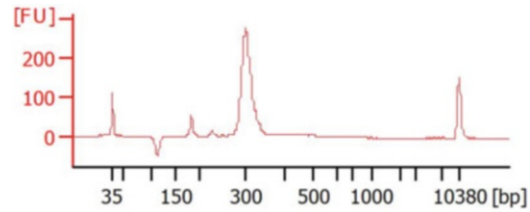
1. Take an assay plate.
2. Prepare a dilution of 199 μL ds DNA Dye buffer +1 μL purified library.
3. Measure the concentration in ng/μL of samples at GLOMAX.
4. Transform ng/μL into nM with this formula:

$$= (\text{conc ng}/\mu\text{L} \times 10^6)/(\text{size of library in base pairs} \times 660).$$

Option: One can verify the size of each library by electrophoresis on a Bioanalyzer 2100. Analyze 1 μL sample with the DNA High Sensitivity Agilent kit.

After migration, profiles and sizes should be as illustrated below (example is shown for TRB VDJ).

Locus	IGH R2	IGH DJ	IGK	TRG	TRD	TRB DJ	TRB VDJ
Median size (pb)	430	250	300	260	300	300	300



3.5 Pool Preparation (2 nM)

1. Make a dilution at 2 nM of each sample with TE.
2. Make an equimolar pool at 2 nM with 5 μL of each sample.
3. Measure the concentration of the pool by Qubit.

4. Transform ng/μL into nM with this formula:

$$= \left(\text{conc ng}/\mu\text{L} \times 10^6\right)/(\text{size of library in base pair} \times 660).$$

3.6 Denaturation Step

1. Normalize the library pool to 2 nM in Resuspension Buffer.
2. Prepare 0.1 N NaOH: 5 μL 2 N NaOH +95 μL H_2O (or 1 μL 10 N NaOH +99 μL H_2O).
3. Vortex.
4. Put the HT1 tube in ice.
5. Add in an Eppendorf tube: 5 μL library pool 2 nM + 5 μL 0.1 N NaOH.
6. Vortex.
7. Centrifuge quickly.
8. Incubate for 5 min at room temperature (DNA denaturation).
9. Add 823 μL ice-cold HT1 to prepare a 12 pM denatured library pool.
10. Vortex.
11. Centrifuge quickly.
12. Place the Eppendorf tube on ice until it settles in the cartridge.
13. Add in another Eppendorf tube 120 μL 20 pM denatured PhiX library +80 μL ice-cold HT1 to prepare a 12pM denatured PhiX library.
14. Vortex.
15. Centrifuge quickly.
16. Place the Eppendorf tube on ice.

Adding 10% of PHIX control in pool library:

(a) In 2 mL low bind tube: 540 μL 12pM denatured library pool +60 μL 12pM denatured PhiX library.
(b) Vortex.
(c) Centrifuge quickly.
(d) Place the Eppendorf tube in ice.
(e) Load 600 μL into the "load sample" well of the MiSeq cartridge V2.

3.7 Bioinformatic Analysis with the Vidjil Platform

1. Copy each of the FASTQ files in the folder MiSeq Output.
2. Connect to the Vidjil server [11] with a personal login and password.
3. Create a "run" and as many "patients" as necessary (*see* Fig. 1).

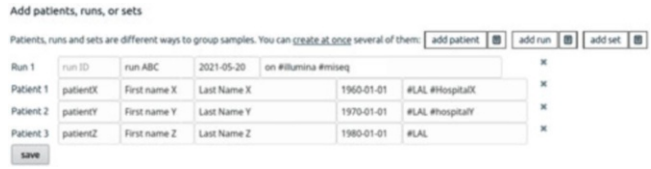


Fig. 1 Adding patients and runs in Vidjil

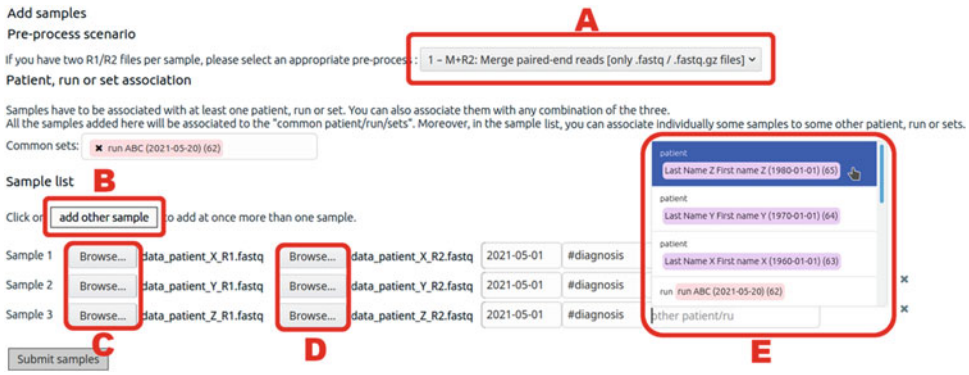


Fig. 2 Adding samples in Vidjil. The rectangles refer to the different steps described in the main text

(a) Click on *runs* and then on *new runs*.

(b) Fill information on the run (date, metadata on the sample using tags prefixed with a #). Afterwards, the samples can be searched by tags.

(c) Add as many *patients* as required and specify a first and last name for each case.

4. Open the created run and click on the *Add samples* button.

(a) Select the pre-process *M + R2: Merge paired-end reads* (A in Fig. 2).

(b) Click on *Add other sample* to have as many sample lines as required (B in Fig. 2).

(c) Add each sample one by one.

- Select the FASTQ file for the R1 reads in the first field (C in Fig. 2).
- Select the FASTQ file for the R2 reads in the second field (D in Fig. 2).
- Enter the sampling date.
- In the last field, type the last name of the patient and select the corresponding one in the list that appears (E in Fig. 2). This will associate the sample to the patient, which will then be available from *run* or *patient*.

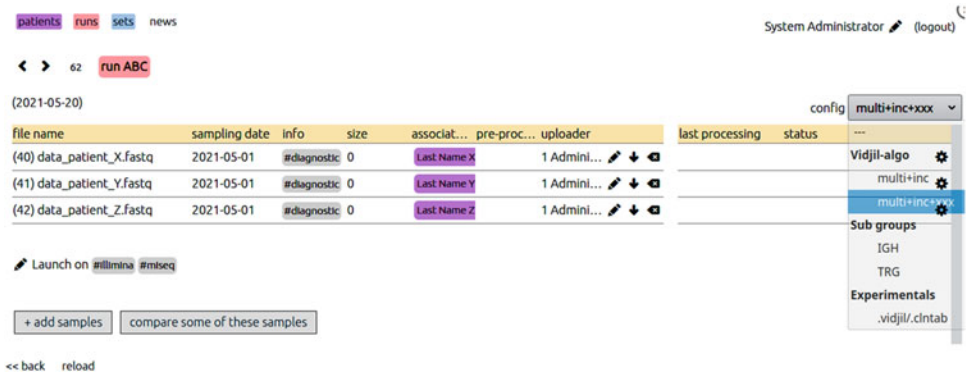


Fig. 3 Selecting configuration and launching Vidjil-algo processes

5. Submit the samples.
6. Choose the configuration of the algorithm: "multi+inc+xxx." This is the advised configuration for target identification as it will detect both complete and incomplete recombinations (Fig. 3).
7. Launch the analysis with the selected configuration for each sample.
8. Click on *reload*, at the bottom left, to see the job status going through the different steps: QUEUED → ASSIGNED → RUNNING → COMPLETED. It is possible to launch several processes at the same time (some will wait in the QUEUED/ASSIGNED states).
9. Once the jobs are completed, return to the patient list to visualize the results by clicking on the configuration name.
10. Analyze the sample to determine the markers of interest (Fig. 4).
    (a) The percentage of analyzed reads should normally be above 90%; otherwise the sequencing run may be of poor quality (A in Fig. 4).
        - In case this percentage is too low, investigate the reason why by clicking on the info button in the upper left panel (B in Fig. 4).
        - Specifically, check the percentage of reads that are classified as:
            - UNSEG only V/5′ (reads only matching V genes).
            - UNSEG only J/3′ (reads only matching J genes).
            - UNSEG too few V/J (reads matching no V or J gene).

Fig. 4 Analyzing the clonotypes in the Vidjil client. Clonotypes are viewed at the same time in a Genescan-like view, a grid view (depending on V/J genes) and in a list. Moreover, the sequences of the selected clonotypes appear at the bottom

(b) Identify the loci of interest, with at least 10,000 reads (C in Fig. 4).

11. Study each clonotype of interest one by one.
12. Switch in order to each of those loci.
13. Cluster all sub-clonotypes linked to the clonotype being studied.
    (a) Select all the clonotypes with the same V and J genes as the studied clonotype.
    (b) Align the sequences (D in Fig. 4).
    (c) Remove the sequences that do not align properly with the studied clonotype.
    (d) Realign the sequences.
    (e) Restart steps c and d until all the sequences align with only few differences.
    (f) Cluster the aligned sequences (button *cluster*, E in Fig. 4).
14. Send the clonotypes to IMGT/V-QUEST [12–15], by clicking on the IMGT button (F in Fig. 4). Next the V, D, and J genes as computed by IMGT/V-QUEST are underlined. This must be taken into account for the design of the patient-specific primer in case of MRD analysis by qPCR.
15. Save the analysis by going to the menu at the top left corner and click on *save*.

4 Notes

1. Primer mix for IGH VDJ FR2. Each primer should be mixed with the same index; mixing of primers needs to be repeated for each unique index. Prepare a 1.5 mL low bind tube for primer mixes A, B, and C:
   (a) Tube A: combine primer for index D502.
   - Add 2 μL of each primer at 100 μM + 396 μL H_2O; each primer is at 10 μM.

IGHVFR2-5	IGHVFR2-21	IGHVFR2-33	IGHVFR2-44
IGHVFR2-10	IGHVFR2-22	IGHVFR2-38	F93
IGHVFR2-11	IGHVFR2-23	IGHVFR2-39	F83
IGHVFR2-12	IGHVFR2-27	IGHVFR2-41	F88
IGHVFR2-13	IGHVFR2-28	IGHVFR2-42	F75
	IGHVFR2-32	IGHVFR2-43	

   (b) Tube B: combine primer for index D502.
   - Add 2 μL of each primer at 100 μM + 90 μL H_2O; each primer is at 10 μM.

IGHVFR2-18	IGHVFR2-36
IGHVFR2-19	IGHVFR2-45
IGHVFR2-20	

   (c) Tube C: combine primer for index D701.
   - Add 2 μL of each primer at 100 μM + 36 μL H_2O; each primer is at 10 μM.

T7-JH consensus
T7-IGJH-137(faham)

2. Primer mix for IGH DHJH. Each primer should be mixed with the same index; mixing of primers needs to be repeated for each unique index. Prepare a 1.5 mL low bind tube for primer mixes of DH primers and JH primers:
   (a) Tube DH primer: combine primer for index D502.
   - Add 2 μL of each primer at **10 μM**; each primer is at 10 μM.

DH1	DH4
DH2	DH5a
DH3a	DH5b
DH3b	DH6

   (b) Tube JH primer: combine primer for index D701.

- Add 2 μL of each primer at 100 μM + 36 μL H_2O; each primer is at 10 μM.

T7-JH consensus
T7-IGJH-137(faham)

3. Primer mix for IGK. Each primer should be mixed with the same index; mixing of primers needs to be repeated for each unique index. Prepare a 1.5 mL low bind tube for primer mix Vkappa, Intron, Jkappa, and Kde:
   (a) Tube Vkappa: combine primer for index D502.
   - Add 5 μL of each primer at 100 μM + 585 μL H_2O; each primer is at 10 μM.

VK1-A	VK5
VK1-D	VK7
VK1-E	VK2-A
VK1-F	VK2-Bdef
VK6-D	VK2-D
VK4	VK3-B
	VK3-C

   (b) Tube Intron: primer for index D502.
   - Dilute 5 μL of each primer at 100 μM + 45 μL H_2O.

   (c) Tube Jkappa: combine primer for index D701.
   - Add 4 μL of each primer at 100 μM + 108 μL H_2O; each primer is at 10 μM.

JK1-4
JK5
JK3

   (d) Tube Kde: primer for index D701.
   - Dilute 5 μL of each primer at 100 μM + 45 μL H_2O.

4. Primer mix for TRG. Each primer should be mixed with the same index; mixing of primers needs to be repeated for each unique index. Prepare a 1.5 mL low bind tube for primer mix A TCRGV, mix B TCRGV, and mix C TCRGJ, TCRGV11:
   (a) Tube mix A TCRGV: combine primer for index D502.
   - Add 2 μL of each primer at 100 μM + 90 μL H_2O; each primer is at 10 μM.

TRGV2	TRGV3/5
TRGV4	TRGV10
TRGV8	

(b) Tube mix B TCRGV: combine primer for index D502.

- Add 2 μL of each primer at 100 μM + 36 μL H_2O; each primer is at 10 μM.

TRGV7
TRGV9

(c) Tube TCRGV11: primer for index D502.

- Dilute 1 μL of each primer at 100 μM + 36 μL H_2O.

(d) Tube mix C TCRGJ: primer for index D701.

- Mix 7 μL of each primer at 20 μM.

TRGJ1/2	TRGJP1
TRGJP01	TRGJP2

5. Primer Mix for TRD. Each primer should be mixed with the same index; mixing of primers needs to be repeated for each unique index. Prepare a 1.5 mL low bind tube for primer mix VDD2 and mix JDD:

(a) Tube mix VDD2: combine primer for index D502.

- Mix 5 μL of each primer at 100 μM.

VD1	VD5
VD2	VD6
VD3	VD8
VD4	VD7
	DD2-5'

(b) Tube mix JDD: combine primer for index D701.

- Mix 5 μL of each primer at 100 μM.

DD3-3'	JD4
JD1	Jalpha29
JD2	
JD3	

6. Primer mix for TRB DJ. Each primer should be mixed with the same index; mixing of primers needs to be repeated for each unique index. Prepare a 1.5 mL low bind tube for primer mix TRB DB and mix TRB JB:

(a) Tube mix TRB DB: combine primer for index D502.

- Mix 2 μL of each primer at 10 μM + 36 μL H_2O.

TRBDB1
TRBDB2

(b) Tube mix TRB JB: combine primer for index D701.

- Mix 2 μL of each primer at 10 μM + 252 μL H_2O.

TRBJ1.1	TRBJ2.1
TRBJ1.2	TRBJ2.2
TRBJ1.3	TRBJ2.3
TRBJ1.4	TRBJ2.4
TRBJ1.5	TRBJ2.5
TRBJ1.6	TRBJ2-6_1
	TRBJ2-6_2
	TRBJ2.7

7. Primer mix for TRB VDJ. Each primer should be mixed with the same index; mixing of primers needs to be repeated for each unique index. Prepare a 1.5 mL low bind tube for primer mix TRB VB and mix TRB JB:

(a) Tube mix TRB VB: combine primer for index D502.

- Mix each primer at 100 μM with the volume below:

primer	volume (μl)	primer	volume (μl)	primer	volume (μl)
TRBV2	2	TRBV3-1	4	TRBV5-6	8
TRBV4	2	TRBV5-1univ	4	TRBV10-3	8
TRBV5-5	2	TRBV6-4	4	TRBV11-1	8
TRBV5-3	2	TRBV7-7	4	TRBV12-3	8
TRBV5-4	2	TRBV28	4	TRBV19	8
TRBV5-8	2	TRBV30	4	TRBV20-1	8
TRBV6-2	2			TRBV23-1	8
TRBV6-6	2				
TRBV6-7	2				
TRBV7-3	2				
TRBV7-5	2				
TRBV7-8	2				
TRBV9	2				
TRBV10-2	2				
TRBV12-5	2				
TRBV13	2				
TRBV14	2				
TRBV15	2				
TRBV16	2				
TRBV18	2				
TRBV21-1	2				
TRBV24-1	2				
TRBV25-1	2				
TRBV27	2				
TRBV29-1	2				

(b) Tube mix TRB JB: combine primer for index D701.

- Mix 2 μL of each primer at 10 μM + 252 μL H_2O.

TRBJ1.1	TRBJ2.1
TRBJ1.2	TRBJ2.2
TRBJ1.3	TRBJ2.3
TRBJ1.4	TRBJ2.4
TRBJ1.5	TRBJ2.5
TRBJ1.6	TRBJ2-6_1
	TRBJ2-6_2
	TRBJ2.7

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