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ARTICLE

A linear-amplification VDJ-seq technique for quantification of immunoglobulin and T cell receptor diversity

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Abstract: The V(D)J recombination is essential for generating a highly diverse repertoire of antigen receptors expressed on T and B lymphocytes. Here, we developed a linear-amplification VDJ-seq technique for quantifying V(D)J recombination of antigen receptor genes. This technique takes advantage of linear amplification using in vitro transcription and reverse transcription to avoid bias generated by the PCR amplification of low copy number of target DNA. The unrearranged alleles are removed by in vitro cleavage with the CRISPR-Cas9 system. The linear-amplification VDJ-seq assay was applied in quantification of the Vκ-Jκ recombination of the mouse *Igκ* gene with Jκ capture primers. The Jκ genes were detected in 95.86% of clean reads with more than half containing the Vκ gene, indicating high specificity of capturing and amplification. We also applied this approach to quantify the usage of Jα within the Trav12 gene family of the *Tcra* gene.

Key words: immune repertoire, V(D)J recombination, antigen receptor, immunoglobulin κ gene, T cell receptor gene.

Résumé : La recombinaison V(D)J joue un rôle essentiel dans la génération d'un répertoire très diversifié de récepteurs d'antigènes exprimés chez les lymphocytes T et B. Dans ce travail, les auteurs décrivent une technique d'amplification linéaire VDJ-seq (laVDJ-seq) permettant de quantifier la recombinaison V(D)J au sein des gènes codant pour des récepteurs d'antigènes. Cette technique tire profit de l'amplification linéaire au moyen d'une transcription in vitro et d'une transcription inverse pour éviter les biais associés à l'amplification PCR d'un ADN cible à faible nombre de copies. Les allèles non-modifiés sont éliminés par clivage in vitro avec le système CRISPR-Cas9. L'analyse laVDJ-seq a été employée pour quantifier la recombinaison Vκ-Jκ au sein du gène *Igκ* de la souris à l'aide d'amorces de capture Jκ. Les gènes *Jκ* ont été détectés chez 95,86 % des séquences filtrées et plus de la moitié contenaient le gène *Vκ*, ce qui montre la grande spécificité de la capture et de l'amplification. Les auteurs ont également utilisé cette approche pour quantifier l'usage de Jα au sein de la famille Trav12 du gène *Tcra*. [Traduit par la Rédaction]

Mots-clés : répertoire immunitaire, recombinaison V(D)J, récepteur d'antigène, gène codant pour l'immunoglobuline κ, gène codant pour le récepteur de lymphocytes T.

Introduction

T and B lymphocytes recognize antigens of nearly all pathogen microbes and mutated cancer cells with highly diverse antigen receptors (Krangel 2016; Loguercio et al. 2018). The highly diverse repertoire of T-cell receptor (TCR) and B cell receptor (BCR) is generated through V(D)J recombination, in which V (Variable), D (Diverse), and J (Joining) gene segments of antigen receptor gene loci are rearranged in a nearly random manner (Carico et al. 2017; Chen et al. 2015). V(D)J recombination is initiated by the binding and cleavage of the Rag complex on recombination signal sequences (RSSs) flanking V, D, and J gene segments (Hao et al. 2015; Naik et al. 2019; Thwaites et al. 2019). Double strand breaks (DSBs) generated by the Rag protein are ligated together with DNA repair proteins to make assembled TCR and BCR (Kirkham et al. 2019). Many factors influence V(D)J recombination and change the repertoire of TCR and BCR (Chen and Krangel 2018; Ghraichy et al. 2018). Recent studies showed that changes of immune repertoire are associated with many types of diseases including infections, cancers, and autoimmune diseases (Cheng et al. 2019; Jayaraman et al. 2019;

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Table 1. Primers used in linear-amplification VDJ-seq.

Gene	Category	Sequence
Trav12	Capture primer	5′biotinCCCAGACAGAAGGCCTGGTCACT
	gRNA	TGCACATGAACCAAGGGTGCAGG
	RT primer	AGACGTGTGCTCTTCCGATCTACGCCACTCTCCATAAGAGCAGC
Jκ1	Capture primer	5′biotinGAAGCCACAGACATAGACAACGG
	gRNA	ACAGTGGAGTACTACCACTGTGG
	RT primer	AGACGTGTGCTCTTCCGATCTAAGAGACTTTGGATTCTACTTAC
Jκ2	Capture primer	5′biotinGAACAACTTAACAAGGTTAGACTTAGTG
	gRNA	TTTGTATGGGGGTTGAGTGAAGG
	RT primer	AGACGTGTGCTCTTCCGATCTCAAGAGTTGAGAAGACTACTTAC
Jκ4	Capture primer	5′biotinCCCAAACAGAACCAAAACGTCAC
	gRNA	TCAGGCAGGTTTTTGTAAAGGGG
	RT primer	AGACGTGTGCTCTTCCGATCTGTAAATGAGCAAAAGTCTACTTAC
Jκ5	Capture primer	5′biotinCCTAACATGAAAACCTGTGTCTTACAC
	gRNA	GCATGTCATAGTCCTCACTGTGG
	RT primer	AGACGTGTGCTCTTCCGATCTAAAGATGAGAAAAGTGTACTTACG

Miyasaka et al. 2019; Vordenbaumen et al. 2019; Wang et al. 2017). It is essential to detect V(D)J recombination of antigen receptor genes to understand adaptive immunity and diseases.

There are seven antigen receptor genes, including three immunoglobulin genes (*Igh*, *Igκ*, and *Igι*) and four TCR genes (*Tcra*, *Tcrb*, *Tcrg*, and *Tcrd*). They are located in six loci owing to *Tcra* and *Tcrd* sharing one locus (Chen et al. 2015; Ebert et al. 2013, 2015; Gebert et al. 2017; Proudhon et al. 2015; Seitan et al. 2011). Many studies on TCR repertoire applied multiplex PCR with Vβ- and Jβ-specific primers to evaluate *Tcrb* diversity, which may generate bias in detecting Vβ and Jβ usage because of the different efficiency of the primers (Goncalves et al. 2017; Scheinberg et al. 2007). The mouse *Igκ* contains 162 Vκ genes, 5 Jκ genes, and 1 Cκ gene, which spans 3.2 Mb on chromosome 6. Recently, a VDJ-seq technique was developed with biotin-labeled primer extension and capturing using streptavidin beads for quantification of *Igh* and *Igκ* diversity at the DNA level (Chovanec et al. 2018; Matheson et al. 2017).

The complexity of the *Tcra* locus is that not only does it share a locus with *Tcrd*, but it also has a large number of Vα and Jα genes. The strain C57BL/6 mouse *Tcra*/*Tcrd* locus contains 138 V gene segments distributed across 1.7 Mb while the strain 129 contains 104 V genes in 1.5 Mb (Carico et al. 2017; Hao and Krangel 2011; Proudhon et al. 2015). *Tcra* and *Tcrd* share a subset of V gene segments and only several V genes are *Tcrd* specific (Naik et al. 2015). The mouse *Tcra* contains 60 Jα genes and 1 Cα gene (Proudhon et al. 2015; Shih and Krangel 2010). Carico et al. (2017) applied 5′RACE combined with high-throughput sequencing to assess the pre-selection *Tcra* repertoire. But 5′RACE assay is based on mRNA of successfully rearranged *Tcra* gene, which may be deviated from the real rearrangement because of the difference in V gene promoter activity.

We modified the VDJ-seq technique with linear amplification using in vitro transcription and reverse-transcription after primer extension to avoid the bias generated in PCR amplification from low-copy-number template DNA. We also use the Cas9 in vitro cleavage to digest the unrearranged fragments instead of capturing with biotin-labeled primers. We applied the modified VDJ-seq in quantification of Vκ and Jκ usage of the mouse *Igκ* gene. We also quantified the usage of Jα within the Trav12 gene family of the *Tcra* gene. The linear-amplification VDJ-seq technique provides an efficient method for quantification of antigen receptor gene rearrangement.

Materials and methods

Primer design

Owing to the limited sequencing length and library size of 2 × 150 bp Illumina Hiseq, the outer biotinylated locus primer can be positioned up to 100 bp away from the J gene segment, whereas the RT primer (for reverse transcription) was placed within 20 bp of the J gene segment for optimal contiguous junction mapping across the J and V sequences. The length of primers ranged from 20 to 25 bp, with an optimal melting temperature around 58–60 °C for capture primer, and higher than 50 °C for specific RT primers. The primers used in this paper are listed in Table 1 and were synthesized by Sangon Biotech., China.

Sample requirements

The linear-amplification VDJ-seq assay can be applied on any cell population and tissue that can be obtained in sufficient numbers to yield 1–10 μg of high-quality, high-molecular-weight DNA. To get enough information, the starting DNA should be more than 1 μg. The B cells in our experiments were isolated from bone marrow cells with biotin-labeled anti-B220 antibody (Biolegend, 103204)

and streptavidin magnetic beads (eBioscience, 5-MSPB-6003-74). The thymocytes represent whole cells from the mouse thymus. The genomic DNA was extracted following the standard phenol/chloroform protocol after proteinase K (Invitrogen, 25530015) digestion overnight at 37 °C. Sonication (Qsonica sonicator Q800R2) was performed to obtain DNA fragments of 300–1000 bp in size.

Enrichment of target DNA

After end repair and dA-tailing, the DNA was added with T7 adapters (contain partial P5 sequence at downstream of T7) using T4 ligase (NEB). The ligation reaction (10 μg DNA, 0.4 nmol of annealed T7-SBS3 Adaptor, 2000 U of T4 ligase (NEB), 1× T4 ligase buffer in 50 μL) was performed at 16 °C for 12 h. T7-SBS Adaptor: PvG830-adaptor01s: GAATTTAATACGACTCACTATAGGGTACAC GACGCTCTTCCGATCTCGATGTT, PvG831-adaptor01as: ACATCGAGATCGGAAGAGCGTCGTGTACCCTATAGTGAG TCGTATTAAATTC (Sangon Biotech., China). The two oligos in 250 mM Tris-HCl (pH7.5) were annealed to form the double strained adaptor following the procedure of denature at 95 °C for 5 min and cooling down to room temperature slowly. Then the target DNA fragments were labeled by primer extension with outer reverse-specific biotin-labeled oligo and captured by streptavidin beads. The primer extension reaction (1 μg DNA, 10 pmol of Biotinylated capture primers (Sangon Biotech., China), 10 nmol of dNTP, 5 U of HotStart HiTaq DNA polymerase (Sangon Biotech., China), 1× PCR buffer in 50 μL) was performed following the program of 95 °C for 5 min, 56 °C for 5 min, and 72 °C for 15 min. The uncoupled primer was removed using a PCR purification kit (Qiagen). The capture reaction was performed with 20 μL of MyOne™ Streptavidin C1 magnetic beads (Thermo Fisher Scientific, 65001) in 1× binding buffer (5 mM Tris-HCl (PH 7.5), 0.5mM EDTA, 1M NaCl, 0.025% Tween-20). After extensive washing, the captured DNA was eluted with 200 μL of 2 mM biotin (Sigma, B4501) on a rotator for 3 h at room temperature.

In vitro Cas9 digestion and linear amplification

The Cas9 in vitro cleavage reaction mixture containing 30 nM sgRNA, 30 nM Cas9 Nuclease (NEB, M0386S) in 15 μL of 1× NEB buffer 3.1 was pre-incubated for 10 min at 25 °C, then added to15 μL of capture DNA in 1× NEB buffer 3.1 and incubated in 37 °C for 1 h to remove the unrearranged gene fragments. For linear amplification, first in vitro transcription was performed with the in vitro transcription kit (ThermoFisher Scientific, AM1354) following the protocol. The DNA was digested with DNase I at 37 °C for 15 min and the RNA was purified with Zymo RNA purification kit (Zymo, R1017). The eluted RNA was reverse-transcripted with SuperScript™ III First-Strand Synthesis System (ThermoFisher Scientific, 18080051) following the program of denaturation at 65 °C for 5 min, then reverse transcription at 50 °C for 4 h and inactivation at 85 °C for 5 min.

Library preparation

After purification with 1× AMPure XP beads (Beckman Coulter, A63881), the cDNA was amplified for Illumina library preparation. In total, 50 μL of PCR reaction, including 22 μL of cDNA, 1.5 pmol of P5 Multiplexing primer, 1.5 pmol of P7 Index primer, 25 μL of 2× Phanta Max Master Mix (Vazyme, P515), was used. The PCR program was 95 °C for 3 mins; 20–28 (depending on qPCR monitor) cycles of 98 °C for 20 s, 65 °C for 15 s, and 72 °C for 30 s; then 72 °C for 5 mins, 4 °C end. The PCR product was purified with PCR purification kit (Qiagen) for Illumina deep sequencing. The pair-end sequencing with 150 bp in size was performed to get three Gigabase sequencing data.

Bioinformatics analysis

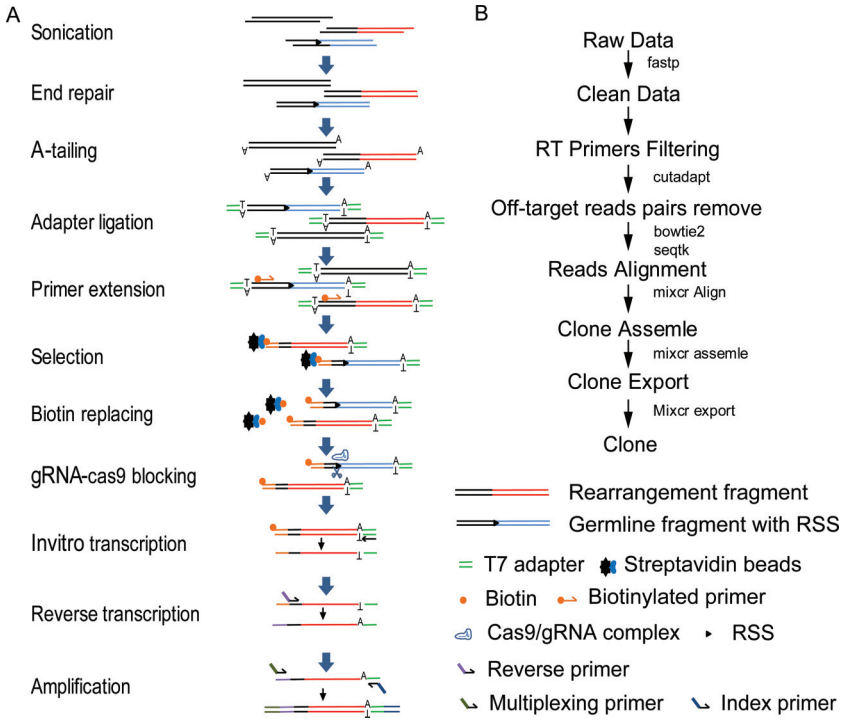
Unqualified reads were filtered through quality control using the software Fastp (Version 0.19.6) with default parameters. Read pairs without RT primer sequences were removed from the clean data by the software cutadapt (Version 1.18) (parameters: -g RT primer, -G primer, -m 50, –pair-filter=both, –discard-untrimmed). The reads after filtering were aligned to Jκ genes or *Trav12* genes using Bowtie2 (parameters: -x). The reads containing target sequences were aligned to the mouse IMGT TCR/IG library (Version repseqio.v1.5) using the software MiXCR (Version v2.1.10; parameter: -OvParameters.geneFeature ToAlign=VRegion,-OjParameters.geneFeatureToAlign= JRegion). Clones were assembled by mapped reads obtained with MiXCR align using the MiXCR assemble command. Clones were exported to a human-readable text file. Clones out of frame or containing stop codons were excluded with MiXCR export Clones command. Overviews of the analyses pipeline are shown in Fig. 1B.

Results and discussion

The main procedures of the linear-amplification VDJ-seq are fragmentation of genomic DNA, adaptor ligation, primer extension, capture, unrearranged gene segments cleavage, linear amplification, and PCR amplification (Fig. 1A). The adaptor used here contains a T7 promoter sequence for in vitro transcription in linear amplification. The linear amplification has two benefits: (1) it reduces the bias which is generated in the PCR process; (2) the sequence-specific RT-primer further reduces the non-specific DNA fragments from primer extension and improves the enrichment efficiency.

Cas9 nuclease is a sequence-specific double-strand DNA endonuclease that has been widely used for in vivo gene editing. It can also cleave double-stranded DNA in vitro with a sequence-specific guide RNA. To deplete unrearranged alleles, we designed guide RNAs targeted to the RSSs and performed in vitro Cas9 digestion. To test the cleavage efficiency of Cas9, a DNA fragment containing the Trav12 gene family RSS was amplified from thymocyte genomic DNA. A single guide RNA was prepared by in vitro transcription targeted to the Trav12 gene fam-

Fig. 1. A systematic overview of the linear-amplification VDJ-seq technique. (A) Experimental workflow to produce a library for sequencing. (B) Overview of the bioinformatics analysis pipeline.



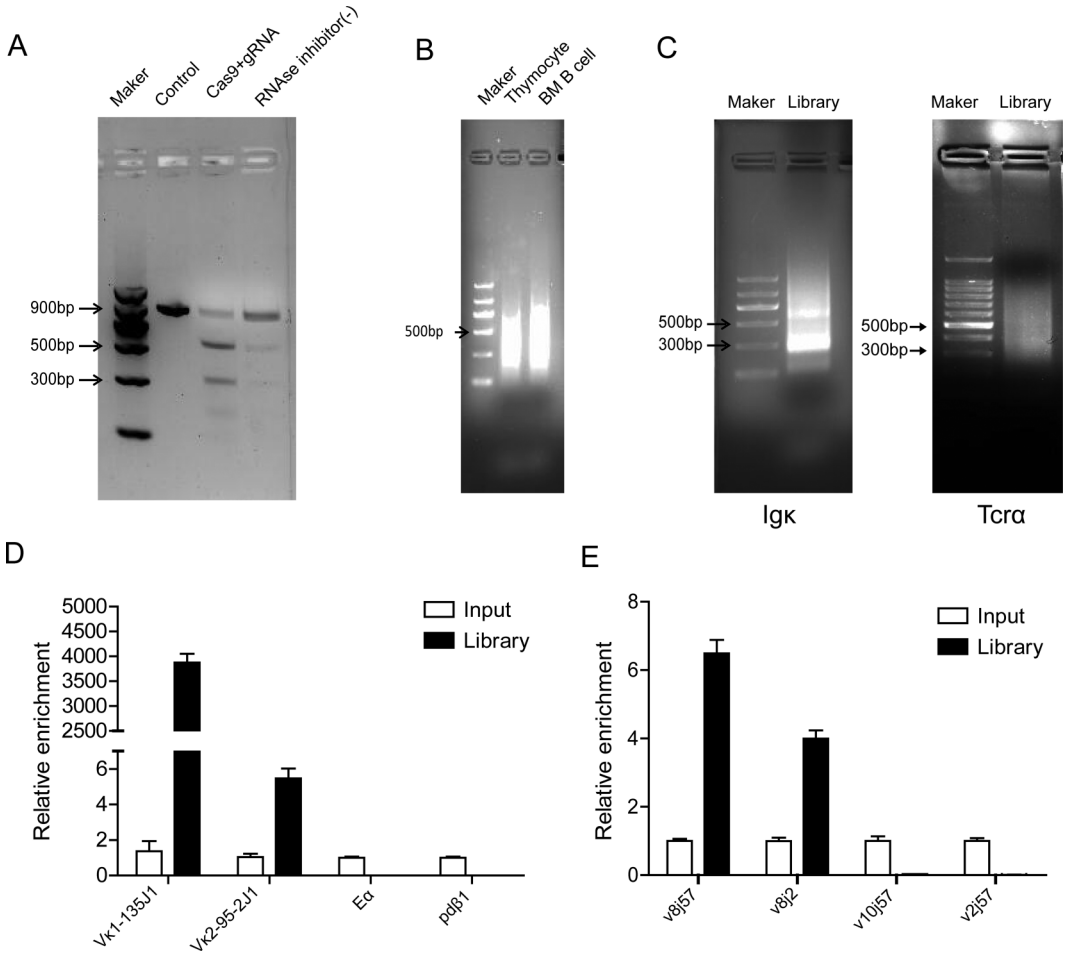
ily RSS. The Cas9 cleavage result showed that most of the DNA was cut into two fragments (Fig. 2A). The residue left by the undigested DNA might be from a variant in the RSS of the pseudogene *Trav12-4* of the Trav12 gene family, which gRNA cannot recognize.

We applied the linear-amplification VDJ-seq assay on the *Igκ* repertoire analysis of bone marrow B cells including pro-B, pre-B, and immature B cells. The genomic DNA was fragmentized by sonication to 300–700 bp in size (Fig. 2B). The sequencing library was made after adaptor ligation, primer extension and biotin capturing, Cas9 digestion, and linear amplification (Fig. 2C). Then we evaluated the enrichment of the rearranged *Vκ1-135Jκ1* in the library by qPCR. The *Vκ1-135Jκ1* was almost 4000 folds enriched in the library, while other regions like Eα and pDβ1 were undetectable (Fig. 2D). We also checked the enrichment of four Vα-Jα combination pairs including two targeted Vα8 and two non-targeted Vα genes (Fig. 2E).

We obtained 13 million clean reads, of which 95.86% contained the Jκ sequence. MiXCR was used for alignment of the Vκ gene and Jκ gene and separation of clonotypes with the same CDR3. Around 6 million reads (51.46% of the reads containing Jκ genes) were mapped to Vκ genes, indicating that this assay is sufficient to detect *Igκ* V(D)J recombination. There were 10 106 clonotypes and 43.87% are in-frame, which is higher than the expected 33% of in-frame rearrangements (Fig. 3A). This can be explained by the fact that most of the immature B cells contain one productive *Igκ* and the cells carrying non-productive on both alleles were deleted in the earlier stage. The Jκ gene mostly used was *Jκ1*, which was contained in 64% of all Vκ-Jκ recombined fragments (Fig. 3B). The Vκ usage varied widely across the locus, without any special distribution pattern (Figs. 3C and 3D). The recombination frequencies of Vκ genes from the same family varied more than 10-fold (Fig. 3E). Compared with other families, the recombination of *Vκ1* genes seems to occur more frequently. There was not a significant difference of usage between Vκ genes on the forward strand and on the reverse strand (Fig. 3F). This result is consistent with previous study (Matheson et al. 2017).

Previous studies have shown that Jα usage is an important feature of *Tcra* rearrangement. However, there is not a reasonable method to evaluate all Jα gene usage at the genomic DNA level due to the large number of Jα genes. We designed a capture primer in the Trav12 gene family to detect all Jα genes associated with *Trav12* genes. We obtained 16 million clean reads and 2.89% were mapped to *Trav12* genes, which may be due to the high similarity of the Vα genes to other regions of the mouse genome. In fact, the enrichment of the *Trav12* genes to other regions of the genome was more than 1000 folds. After MiXCR analysis, 23.4% of reads containing *Trav12* genes were mapped to Jα genes. There were 346 clonotypes of the reads containing Vα-Jα recombination, 41% of which are in-frame (Fig. 4A). We found that the usage of the members of the Trav12 gene family varies by over 10-fold (Fig. 4B). In addition, we noticed that the distribution of the Jα gene usage was similar to that of the Jα usage with the Vα genes between Trav11 and Trav5-4 of 5′RACE data in a previous study (Figs. 4C and 4D), which may be ex-

Fig. 2. Quality control of library preparation. (A) Analysis of CRISPR-Cas9 in vitro cleavage efficiency of the Trav12 gene family. The reactions with or without RNase inhibitor were run in agarose gel. Undigested control is about 900 bp, the two segments after cutting are about 300 and 600 bp, respectively. (B) Analyses of the sizes of DNA fragment after sonication by agarose gel electrophoresis. (C) Analysis of the sizes of library DNA size by agarose gel electrophoresis. (D) *Igκ* and (E) *Tcra* library enrichment test by qPCR. For the *Igκ* gene, *Vκ1-135* to *Jκ1* is one of the most common recombination pairs, *Vκ2-95-2* to *Jκ1* is one of the rarest pairs. The enhancer Eα of the *Tcra* gene and the promoter pDβ1 of the *Tcrb* gene were used as negative controls. For the *Tcra* gene, *Vα8-Jα57* and *Vα8-Jα2* pairs were detected by qPCR and *Vα10-Jα57* and *Vα2-Jα57* were used as negative controls.



plained by the location of the *Trav12* genes in the central Vα region (Carico et al. 2017). But our data provided the information of the pseudogenes like Jα61 and non-productive recombination, which truly reflects the V(D)J recombination of Jα genes.

Benefiting from the development of 5′RACE technology, great success has been achieved in the study of repertoire sequencing at the RNA level (Bernat et al. 2019; Carico et al. 2017; Leenen et al. 2016). However, the detection of repertoire at the DNA level still faces many difficulties. Unlike RNA-level repertoire, DNA-level repertoire contains the rearranged fragments that cannot be translated successfully, which more comprehensively reflects the rearrangement process. The most widely used genomic DNA-based repertoire detection technology at present is multiplex PCR technique (Robins et al. 2014; Rosati et al. 2017; Zeng et al. 2016). But its disadvantages are obvious. This method cannot detect new V allele variants owing to the fixed set of primers used. Multiplexing inevitably leads to dramatic bias in relative efficiency of amplification of different variable segments and thus to the loss of quantitative information (Okino et al. 2016; Peng et al. 2015). In view of this, researchers have developed some methods based on target enrichment (Bolland et al. 2016; Linnemann et al. 2013; Matheson et al. 2017). But these methods also have the problems of low capture efficiency and bias. We provided a linear-amplification VDJ-seq technique that can be used for quantification of V(D)J recombination at the genomic DNA level. This technique was successfully used for quantification of Vκ-Jκ recombination and Jα usage of *Tcra* rearrangement. The technique can also be used for quantification of V(D)J recombination of other antigen loci such as *Igh* and *Tcrb*.

Fig. 3. Distributions of Jκ, Vκ, and Vκ-Jκ combination of the *Igκ* gene in mouse pre-B and immature B cells. (A) Analyses of in-frame and out-of-frame clonotype of the *Igκ* repertoire, the red color represents the productive part and the blue color the non-productive part. Distribution of (B) Jκ and (C) Vκ genes including productive and non-productive clonotypes. Genes are arranged geographically from the 5′end of the locus (left) to the 3′end (J-proximal end; right). (D) Heatmap of the Vκ-Jκ combination of clonotypes of the *Igκ* gene. The log2-transformed recombination frequencies are used for each Vκ-Jκ combination. Rows represent Vκ genes and columns Jκ genes. (E) Recombination frequencies to *Jκ1* are shown for all genes in each Vκ family. (F) Recombination frequencies for genes on the forward and reverse strands.

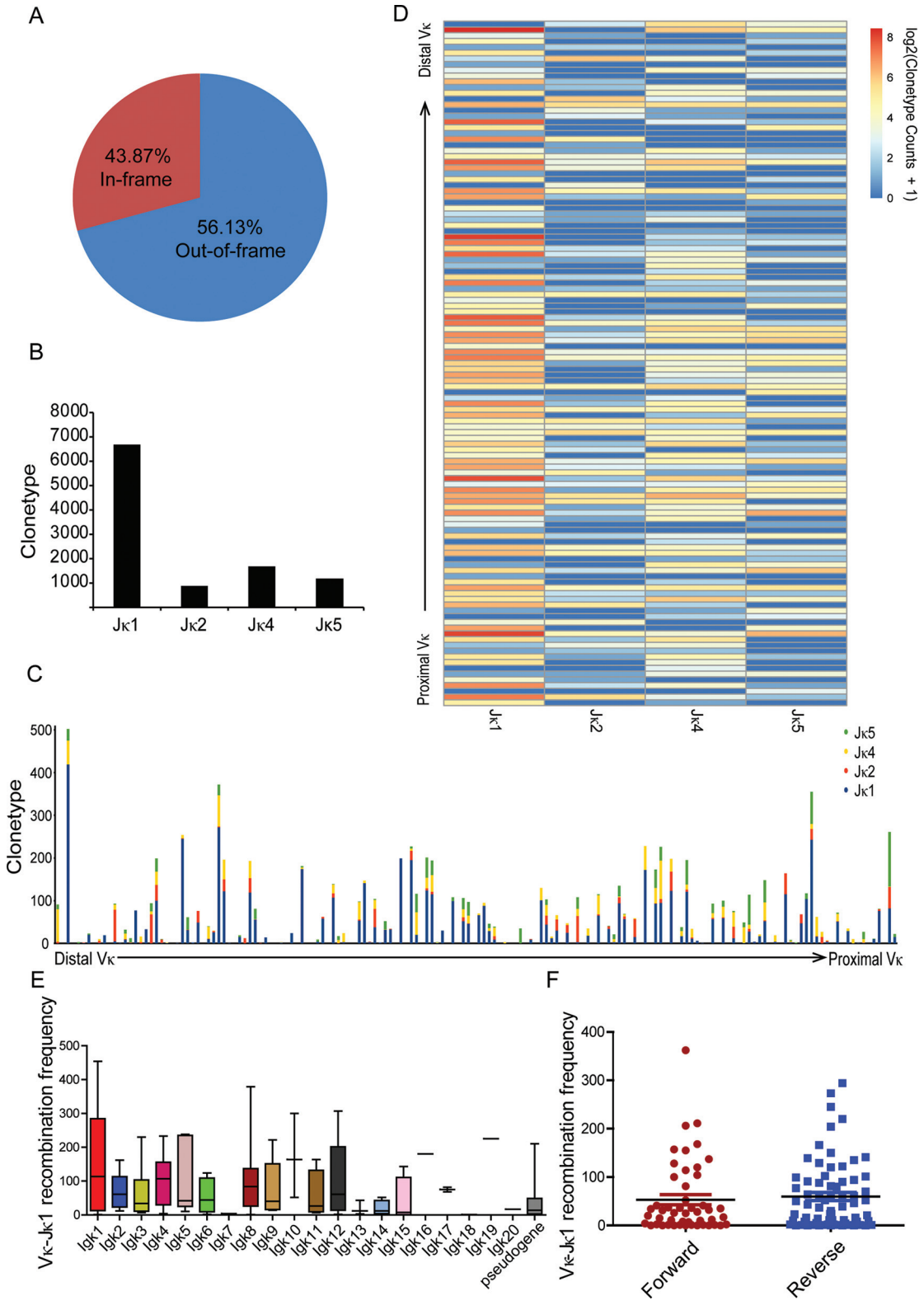
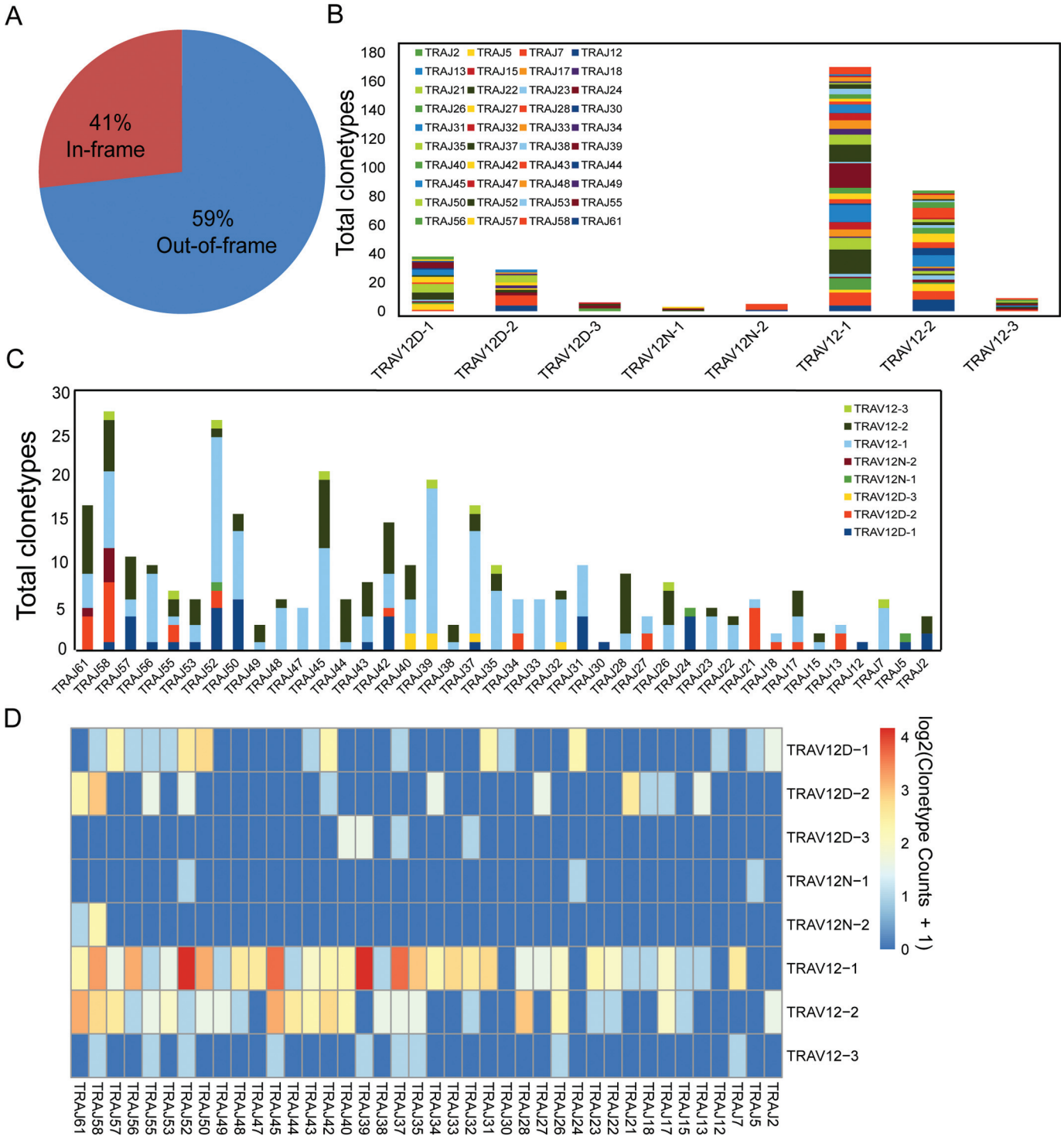


Fig. 4. Distributions of Jα, Vα, and Vα-Jα combination of the *Tcra* gene in mouse thymocytes. (A) Percentage of in-frame and out-of-frame clonotype of *Tcra* rearrangement, the red color represents the in-frame part and the blue color the out-of-frame part. The distributions of (B) Jα and (C) Trav12 gene family including all clonotypes. Genes are arranged geographically from the 5′end of the locus (left) to the 3′end (Eα-proximal end; right). (D) Heatmap of the Vα-Jα combination of clonotypes of the *Tcra* gene. The log2-transformed recombination frequencies are used for each Vα-Jα combination. Rows represent Vα genes and columns Jα genes.



To conclude, our method significantly improves capture efficiency by optimizing capture conditions and minimizing the bias through linear-amplification. This will contribute to further understanding of the antigen receptor rearrangement process.

Conflict of interest statement

The authors declare no conflict of interest.

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