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(54) Title: CLONING AND EXPRESSION SYSTEM FOR T-CELL RECEPTORS

(57) Abstract: The invention provides a method for rapid cloning of T-cell receptors (TCRs) (e.g., paired αβ and γδ TCR chains) and B-cell receptors (BCRs) (e.g., paired IgH or IgK or Igλ) from single cells by CDR3 substitution using single cell PCR products and Gibson Assembly techniques and a pre-generated TCR (or BCR) library in an expression vector.

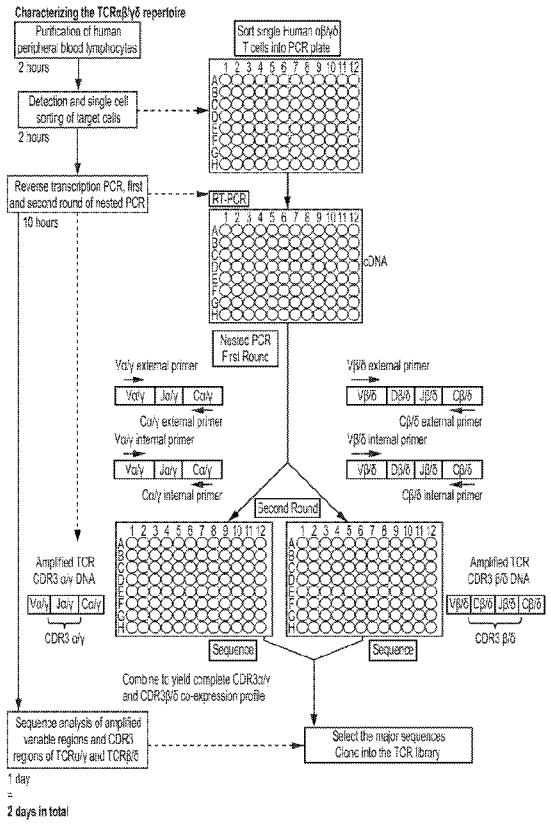

FIG. 1A

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CLONING AND EXPRESSION SYSTEM FOR T-CELL RECEPTORS

CROSS REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Patent Application Serial No. 62/263,318, filed on December 4, 2015, which is hereby incorporated by reference in its entirety.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

The United States Government has certain rights to this invention by virtue of funding reserved from Grant No. 5R01AI107625 awarded from the National Institute of Allergy and Infectious Diseases (NIAID) / National Institutes of Health (NIH).

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on November 30, 2016, is named 243734_000082_SL.TXT and is 76,261 bytes in size.

FIELD OF THE INVENTION

The invention is directed to methods for rapid cloning and expression of T-cell receptors (TCRs) and B-cell receptors (BCRs) and their use for drug screening, structural and functional studies and other applications. More particularly, the invention provides a method for rapid cloning of TCRs (*e.g.*, paired αβ and γδ TCR chains) or BCRs (*e.g.*, paired IgH or Igκ or Igλ) from single cells by CDR3 substitution using single cell PCR products and Gibson Assembly techniques and a pre-generated TCR (or BCR) library in an expression vector.

BACKGROUND OF THE INVENTION

T cells play a vital role in the control of viral infections and tumors. T cells are activated by antigen presenting cells via interactions between peptide-major histocompatibility complex (pMHC) and TCRs. This interaction can induce proliferation and the development of effector functions, including cytokine production and cytotoxic activity. T cells can also infiltrate infected or transformed tissues, *e.g.* as tumor-infiltrating lymphocytes (TILs), to perform these

effector functions [1,2]. However, in some chronic viral infections and tumors, responding effector T cells progressively get exhausted and become dysfunctional [3,4,5] . In addition, control of tumors and/or infection may require large numbers of highly reactive lymphocytes that cannot be achieved due to normal tolerance mechanisms. One effective method to overcome this barrier is the use of therapeutic adoptive transfer of lymphocytes [2,6,7].

Adoptive transfer of lymphocytes such as *in vitro* expanded or TCR-engineered antigen specific T cells has been successfully used to control viruses and tumors in patients[8,9,10,11,12]. *In vitro* expansion of viral or tumor-specific T cells require significant time to prepare and the targets are not usually fully characterized. Lymphocytes expressing engineered TCRs and chimeric antigen receptors (CAR) target specific antigens, with CARs recognizing surface antigens through immunoglobulin-type interactions[10,13] and TCRs recognizing tumor-associated pMHC complexes. CAR therapy directed against surface antigens requires a tumor-associated antigen that can be universally targeted (even on healthy, non-tumor tissue) without significant toxicity. Tumor-specific antigens that are targeted by TCRs represent an attractive alternative that can provide greater specificity and reduce non-tumor associated toxicities[14,15,16]. Additionally, engineered T cells expressing high-affinity antigen receptors can be conditioned to overcome immune tolerance, which has been a major limitation for immunotherapy[14,15,17]. Apart from the clinical applications, a robust system for the cloning and expression of TCRs is a valuable tool for the investigation of TCR structure and functions[18,19,20].

Techniques to rapidly profile and clone antigen-specific TCRs have improved and shortened the process of TCR-engineered immunotherapy[21,22]. These approaches are useful contributions to the field and are able to handle large cell inputs very effectively. However, for certain applications, the reported methods still have some limitations. First, approaches that rely on deep sequencing and cloning of bulk sorted cells can still be limited by target cell numbers. In contrast, single cell approaches can utilize input sizes starting with a single cell but are less efficient at dealing with high cell number inputs (greater than 10,000 cells). As a result, single cell methods are best directed at defined samples such as antigen-specific responses or tissue-associated infiltrating cells. Second, for bulk sorting, pairing of TCR chains requires algorithmic imputation, which can have difficulty dealing with cells expressing two distinct TCR chains of one type (*e.g.* two TCRα chains), which are quite common. A recently reported algorithm has addressed this concern by pairing bulk processed TCRs using barcoded pools of cells [23].

However, this method requires relatively large inputs to successfully pair and would likely not be appropriate for very small sample sizes as might be obtained from tissue biopsies or tetramer sorting of small populations.

Third, while currently reported methods are able to generate full length receptors either by synthesis or by 5' RACE-associated approaches at the single cell level, these methods require expansion of the isolated cells prior to TCR isolation, which likely causes bias in the TCR repertoire in the subsequent analyses and/or can reduce efficiency. Lastly, the majority of antiviral and antitumor adoptive therapy has focused on αβ T cell clones due to their exquisite antigen specificity. However, γδ T cells have also been shown to mediate antiviral and antitumor effects and are novel candidates for therapeutic development[24,25].

SUMMARY OF THE INVENTION

To date, there is little research about profiling and utilizing the TCRγδ repertoire for therapeutic purposes. Applying γδ T cells for immunotherapeutic applications may be a promising future approach in conjunction with traditional TCRαβ techniques. Therefore, it is important to establish a system to define the repertoire and functional activity for γδ T cells. Additionally, improving efficiencies for cloning αβ TCRs from single cells may have complementary uses in the lab and in the clinic.

There is a great need in the art to develop a rapid, efficient and accurate cloning and expression method and/or system for specific TCRs, and uses thereof for screening TCR-mediated therapeutics, as well as for other research and/or clinical applications. The present invention fulfils such needs, and provides such methods and platforms.

In one aspect, the invention provides a method for cloning a T cell receptor (TCR) from a single T cell, wherein said method comprises:

(a) performing RT-PCR with a primer mixture on a single T cell to obtain paired αβ or γδ TCR CDR3 DNA sequences comprising a partial variable (V) region, CDR3 region, and a partial constant (C) region,

(b) optionally sequencing the RT-PCR product obtained in step (a), and

(c) cloning the αβ or γδ TCR CDR3 DNA sequences obtained in step (a) into a corresponding TCRαβ or TCRγδ library.

In one embodiment, said T cell is a human or a mouse αβ or γδ T cell.

In one embodiment, the method comprises sorting of single T cells prior to step (a). In one specific embodiment, T cells are not stimulated prior to sorting.

In one embodiment, the primer mixture comprises sense primers comprising T-cell receptor gamma variable (TRGV) sequences and/or T cell receptor delta variable (TRDV) sequences and antisense primers comprising T-cell receptor gamma constant (TRGC) sequences and/or T-cell receptor delta constant (TRDC) sequences. In one specific embodiment, the primer mixture comprises 9 TRGV and 8 TRDV sense primers and single TRGC and TRDC antisense primers. In another specific embodiment, the primer mixture comprises 5 external and 5 internal TRGV and 13 external and 13 internal TRDV sense primers and single TRGC and TRDC antisense primers. In one embodiment, the TRGV and/or TRDV sense primers and the TRGC and/or TRDC antisense primers are selected from the primers listed in Table 1. In one embodiment, the TRGV and/or TRDV sense primers and the TRGC and/or TRDC antisense primers are selected from the primers listed in Table 6.

In one embodiment, the single cell RT-PCR of γδ or αβ TCR and sequencing are performed within not more than 2 days.

In one embodiment, the method further comprises cloning the resulting αβ or γδ TCR CDR3 DNA sequences into the TCRαβ and/or TCRγδ library constructed using the method described below.

In a related aspect, the invention provides a method for constructing a TCRαβ and/or TCRγδ library in an expression vector, comprising:

(a) synthesizing multiple pairs of TRGV and TRDV DNA fragments or TRAV and TRBV DNA fragments with a 15-25bp overlap to the vector sequence based on the amplified sequence of the TRGV/TRDV or TRAV/TRBV pairings, respectively, and

(b) performing a two- or three-way ligation with a linearized expression vector.

In one embodiment of the above library construction method, the expression vector is a retroviral or lentiviral expression vector. In one embodiment, the ligation in step (b) is performed using Gibson Assembly Cloning techniques. In one specific embodiment, Gibson Assembly Cloning techniques are optimized to clone synthesized TRGV/TRAV and TRDV/TRBV DNA fragments using g-blocks or other synthesized DNA fragments (e.g., long primers that are then annealed). In one embodiment, the TCRαβ and/or TCRγδ library is constructed after a single-cell amplification and synthesized paired TRGV/TRDV or

TRAV/TRBV receptors based on the sequence data. In one embodiment, the TCRαβ and/or TCRγδ library is constructed in 5 to 10 days. In one embodiment, the TCRαβ and/or TCRγδ library is used for drug screening or identification of TCRαβ- and/or TCRγδ-specific ligands.

In one embodiment of the above library construction, the method comprises substituting CDR3 regions of the existing clones in the TCRαβ and/or TCRγδ library with (i) products of RT-PCR performed using a primer mixture on a single T cell to obtain paired αβ or γδ TCR CDR3 DNA sequences comprising a partial variable (V) region, CDR3 region, and a partial constant (C) region and (ii) a linker DNA for overlap extension of PCR cloning. In one specific embodiment, said T cell is a human or a mouse αβ or γδ T cell. In one specific embodiment, the method comprises sorting of single T cells prior to RT-PCR. In one specific embodiment, T cells are not stimulated prior to sorting. In one specific embodiment, the primer mixture comprises sense primers comprising T-cell receptor gamma variable (TRGV) and/or T cell receptor delta variable (TRDV) and antisense primers comprising T-cell receptor gamma constant (TRGC) and/or T-cell receptor delta constant (TRDC) sequences. In one specific embodiment, the primer mixture comprises 9 TRGV and 8 TRDV sense primers and single TRGC and TRDC antisense primers. In one specific embodiment, the primer mixture comprises 5 external and 5 internal TRGV and 13 external and 13 internal TRDV sense primers and single TRGC and TRDC antisense primers. In one specific embodiment, the TRGV and/or TRDV sense primers and the TRGC and/or TRDC antisense primers are selected from the primers listed in Table 1. In one specific embodiment, the TRGV and/or TRDV sense primers and the TRGC and/or TRDC antisense primers are selected from the primers listed in Table 6. In one specific embodiment, said linkers are overlapping with the non-variant sequences of the TCRα/γ and TCRβ/δ single cell RT-PCR products. In one specific embodiment, said linker sequences are selected from those listed in Table 3. In one specific embodiment, the resulting TCRαβ and/or TCRγδ chains with CDR3 substitutions are used for T cell-mediated immunotherapy.

In a related aspect, the invention provides a TCRαβ and/or TCRγδ library constructed using any of the above methods. In another related aspect, the invention provides a host cell (e.g., a Nur-77-GFP Jurkat 76 cell or a Nur-77-Luciferase Jurkat 76 cell) comprising said TCRαβ and/or TCRγδ library construct.

In another aspect, the invention provides a method for cloning a B cell receptor (BCR) from a single B cell, wherein said method comprises:

(a) performing RT-PCR with a primer mixture on a single B cell to obtain paired IgH or Igκ or Igλ CDR3 DNA sequences comprising a partial variable (V) region, CDR3 region, and a partial constant (C) region,

(b) optionally sequencing the RT-PCR product obtained in step (a), and

(c) cloning the IgH or Igκ or Igλ CDR3 DNA sequences obtained in step (a) into a corresponding BCR library.

In one embodiment, said B cell is a human or a mouse B cell. In one embodiment, the method comprises sorting of single B cells prior to step (a).

These and other aspects of the present invention will be apparent to those of ordinary skill in the art in the following description, claims and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows a schematic of unbiased single-cell amplification of paired TCR CDR3 regions. Overview of the multiplex PCR protocol to amplify and sequence paired TCR CDR3 α/γ and CDR3 β/δ. After sorting single human αβ or γδ T cells into a 96-well plate, reverse transcription is performed to obtain single-cell cDNA. Taking human γδ T cells as an example, a first round of PCR is performed by using an external primer mixture of 9 TRGV and 8 TRDV sense and single TRGC and TRDC antisense primers following RT-PCR. The first-round PCR products are subjected to two separate second-round PCRs using a corresponding internal primers mix (9 sense TRGV, single antisense TRGC, and 8 sense TRDV, single antisense TRDC, respectively). The timeline of this process is shown on the left.

FIG. 1B shows an agarose gel electrophoresis image of TCR segments containing CDR3γ and CDR3δ is shown. Paired CDR3γ and CDR3δ products from the same cell were loaded in adjacent lanes. Negative control PCR reactions are shown in the boxed region and in the ladder lane, a 500bp label is shown.

FIG. 1C shows the determination of paired TRGV/TRDV usageby multiplex PCR and sequencing (n=14 human apheresis rings), with the percentage of different TRGV/TRDV usage in each sample assessed (mean±SEM).

FIG. 2A shows a schematic of rapid cloning and expression of human TCRαβ or TCRγδ in a retroviral vector. A schematic diagram of TCR cloning using gBlock® synthesized DNA

fragments and a linearized retroviral vector (pMICherry) is shown. Family specific TRGV and TRDV full length TCR chains were synthesized with a 15-20bp overlap sequence (light diagonal line shading) in the 2A region. Together with a linearized pMICherry expression vector, a three-way ligation is performed by using Gibson Assembly® Cloning. The timeline of this process is presented on the left.

FIG. 2B shows expression of TCR constructs in the Jurkat 76 TCR$\alpha^-\beta^-$ cell line. The vectors with human TRGV9/TRDV2 TCR genes and human influenza-specific TCR$\alpha\beta$ genes were co-transfected with the human CD3 construct into the Jurkat 76 TCR$\alpha^-\beta^-$ cell line. The flow cytometry results of transfected cells are shown.

FIG. 3A illustrates how *Nur77*-GFP Jurkat 76 TCR$\alpha^-\beta^-$ cells can report the TCR signaling activation. Following co-transfection of a murine K^bPB1_{703}-specific TCR $\alpha\beta$ derived from influenza-infected mice and a mouse CD3 construct, the $K^bPB1_{703}{}^+TCR\alpha\beta^+$ NJ76 cells (PB1-NJ76) were stimulated either with influenza $PB1_{703}$ peptide alone or $PB1_{703}$ peptide-pulsed splenotytes for 4 hrs. Anti-mouse CD3/α-human CD28 stimulation was also done as a positive control. The GFP expression was assessed by flow cytometry.

In FIG. 3B, the quantification of GFP expression in PB1-NJ76 cells is shown. Statistical differences were determined by One-way ANOVA.

In FIG. 3C, the transfected human TRGV9/TRDV2-NJ76 cells were pulsed with zoledronic acid (50ug/ml) for 3h at 37°C, and washed and incubated at 37°C for 12h. The GFP expression of stimulated NJ76 cells, non-stimulated TRGV9/TRDV2-NJ76 and stimulated TRGV9/TRDV2-NJ76 cells with zoledronic acid is shown in the top panel. Fold change of GFP expression in stimulated TRGV9/TRDV2-NJ76 cells with zoledronic acid compared to non-stimulated TRGV9/TRDV2-NJ76 is shown as a time course in the bottom panel. Statistical differences were determined by Two-way ANOVA; $p < 0.05$ was considered statistically significant. Data are mean ± SEM of two independent experiments. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n.s. non-significant.

FIG. 4 shows a schematic strategy of CDR3 substitution by overlap extension PCR. Based on the library of TCR$\alpha\beta$ and TCR$\gamma\delta$ established by the described cloning platform, the strategy for CDR3 substitution using multiplex single cell PCR products and linker DNA is shown. After the sequence analysis of single-cell PCR (shown in FIG. 1A), the target pairs of TCRs are chosen from the respective second round paired PCR plates, which include TCRα/γ_m

and TCRβ/δ_n (m represents a particular TRAV or TRGV subfamily; n represents a particular TRBV or TRDV subfamily). Beforehand, we generated a library of linker DNA by gBlock synthesis (IDT) (Table 3). The linker DNA consists of TRAC/TRGC-2A-TRBVn/TRDVn (n represents the TRB/DV subfamily) sequence. Using the single cell PCR products of α/γ and β/δ chains of the desired clonotypes and the relevant linker gBlock DNA, we carried out an overlap PCR with TRAm/GVm internal sense primer and TRB/DC internal antisense primer. The PCR products were visualized on an agarose gel, and subsequently purified to use as "mega-primer" for cloning into the existing construct from our TCR cloning library (pMIC- TCRα/γ_m- TCR β/δ_n) with the same TRGV and TRDV family usage but different CDR3s by overlap extension PCR. The timeline of the whole process is on the left.

FIGS. 5A and 5B depict the time lines of the two cloning platforms.

FIGS 6A-6C show co-transfection of human CD3 can improve the expression of human TCR constructs. FIGS. 6A and 6B show a copmarison of single transfection of human TCR constructs and co-transfection of human TCR constructs and human CD3. FIG. 6C shows quantification of mCherry/ametrine and TCR/CD3 expression is shown. Statistical differences were determined by One-way ANOVA; $p < 0.05$ was considered statistically significant. Data are mean ± SEM of two independent experiments. ***$p < 0.001$, ****$p < 0.0001$.

FIG. 7 shows a gating strategy of TCR-transfected-NJ76 cells in flow cytometry. The data of TCR-transfected-NJ76 cells after stimulation in FIGS. 3A-3C were analyzed by applying the gating strategy to all the samples. The gating is flowing "autofluorescence gate – lymphocytes gate – single cell gate – mCherry+Ametrine+ gate – TCR+CD3+ gate – GFP+ gate".

FIG. 8 shows a gating strategy of Human TCRγ/δ^+ $CD3^+$ cells single cell sorting. Single cells of human TCR$\gamma\delta^+$ $CD3^+$ cells from PBMC samples were sorted into 96-well plate by applying the gating strategy of FIG. 7, above. The gating is flowing "autofluorescence gate – lymphocytes gate – single cell gate – live/dead gate – dump gate (CD11b/14/19) – TCR$\gamma\delta^+$/$CD3^+$ gate".

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a rapid, efficient and accurate cloning and expression method and system for specific TCRs (e.g., paired $\alpha\beta$ and $\gamma\delta$ TCR chains from single cells)

which can be used for drug screening (e.g., for T cell-mediated anti-tumor or anti-infective immunotherapy), for structuring and functional analysis of TCRs, and other applications. The invention addresses the non-specific, labor-intensive and time-consuming issues of prior PCR-based cloning methods and provides a high-throughput, accurate and efficient method of TCR engineering for therapeutic and research applications.

In conjunction with single cell multiplex PCR techniques for TCRαβ or TCRγδ profiling [26,27], and Gibson Assembly® cloning of synthesized DNA, the invention provides rapid sequencing and cloning of specific TCRs in an expression vector (e.g., retroviral expression vector). By generating TCR libraries, the invention provides a cloning and expression method and system that is significantly accelerated by only requiring the substitution of the CDR3 region, resulting in TCR clones in appropriate expression vectors in as little as five days after cell isolation. The invention provides highly robust, inexpensive, efficient, and high-throughput means for TCR engineering for therapeutic and research applications.

In certain embodiments, the invention provides a method of single-cell amplification of paired TCR CDR3 α/γ and CDR3 β/δ regions comprising the steps of (a) sorting of single human αβ or γδ T cells; (b) performing RT-PCR to obtain a single-cell cDNA; and (c) amplifying the single-cell cDNA obtained in step (b) in a second round PCR with a primer mixture of TRGV and TRDV sense primers and TRGC and TRDC antisense primers. In one embodiment, the single-cell cDNAs are amplified with a primer mixture comprising nine (9) T-cell receptor gamma variable (TRGV) and/or eight (8) T-cell receptor delta variable (TRDV) sense primers and a single T-cell receptor gamma constant (TRGC) and/or T-cell receptor delta constant (TRDC) antisense primer. Non-limiting examples of external and internal sense primers targeting TRGV and TRDV and antisense TRGC and TRDC primers are listed in Table 1, below. Specific non-limiting examples of a method for sorting single cells, as well as conditions for RT-PCR and nested PCRs, are provided below (*see* Example 1, Materials & Methods).

In certain embodiments, the invention also provides a method of production of TCRαβ and TCRγδ library in an expression vector, comprising the steps of (a) synthesizing multiple pairs of TRGV and TRDV DNA fragments with a 15-20bp overlap in the sequence in the 2A region based on the TRGV/TRDV usage in human apheresis ring samples, and (b) performing a three-way ligation with a linearized expression vector (e.g., a retroviral vector, which is convenient for the future applications, like transduction of cell lines and TCR-transgenic mice)

using Gibson Assembly® Cloning or another type of ligation, including, e.g., conventional T4-mediated ligation. In certain embodiments, the TCRαβ and/or TCRγδ library is human library comprising the human TCRαβ and TCRγδ. In certain embodiments, the production of TCRαβ and TCRγδ library is performed after the single-cell amplification and paired TRGV/TRDV usage based on the sequence data. Exemplary primers targeting the 2A regions of human CD3δ, γ, ε and ζ genes are provided in Tables 2A & 2B.

The Gibson Assembly kit is an enzyme and buffer mix designed to optimize the overlap ligation of g-block gene fragments. In certain embodiments, Gibson Assembly protocol is optimized to synthesize the TRGV and TRDV DNA fragments or genes using g-block technique, IDT DNA). However, the ligation reaction can be performed using other suitable ligases known and available in the art. Using the methods of the present invention, a TCR library can be constructed in as fast as 10 days. As long as the library is established, it can be applied to substitute CDR3 regions from a new patient sample, which can dramatically reduce the cloning time from 10 days to 5 days. This could be beneficial for both adoptive transfer therapy and personalized therapy.

The invention provides a rapid cloning method based on the TCR library constructed, e.g., the TCRαβ and TCRγδ library, comprising the step of generating full-length paired αβ or γδ TCR chains by CDR3 substitution using multiplex PCR products and a linker DNA (overlap extension PCR). In certain embodiments, the linkers contribute to simultaneously substitute CDR3 regions of both TCRα/γ and TCRβ/δ. The linkers are overlapping with the single cell PCR products, therefore, no additional PCR steps are needed. In certain embodiments, the TCRα/γ and TCRβ/δ single cell PCR products and the linkers are mixed together and an overlap PCRs are performed, and then the PCR products generated from the overlap PCRs are used to substitute the CDR3 regions in the cloning library. Exemplary human TCRγδ linker sequences are provided in Table 3, below.

The methods of the present inventions improve the speed and specificity for cloning paired TCRs. With single cell analyses, the cloned paired TCRs of the present invention are more “specific” than examining T cell receptors in bulk because both chains of the receptor are from the same cell. The invention allows rapid cloning of TCRs that are responding to an infection or a tumor, which could be useful in developing targeted cell therapies, e.g., by reintroducing those receptors into the patient's cells to assist in mounting a more effective

response. The TCR sequences themselves can also be useful for understanding what targets the response is against. Although there is no simple way of decoding the target from the TCR sequence, databases and/or computer algorithms can be developed to evaluate the relationship on the antigens and the elicited T-cell responses via various TCRs. Furthermore, the invention provides an efficient and accurate TCR expression system for TCRs in a reporter cell line, which can be used for screening specific antigens directly. TCR sequencing can also be used for the detection of minimal residual disease in leukemia and lymphoma. Many tumors have rearranged their TCRs. In these cases, the specific TCR sequence in the tumor becomes a lineage "barcode" for the tumor itself. The methods of the invention can provide TCR sequencing information to determine the presence and/or quantity of tumor cells.

Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

"CDR3 region" or "the third complementarity determining region" is defined herein as the region from codon positions 105 to the end of the V-REGION in germline gDNA or cDNA, codon positions 105 to 117 in V-DOMAIN of rearranged gDNA or in cDNA (all the position numbers are according to the IMGT unique numbering; see Lefranc, M.-P., The Immunologist, 7, 132-136 (1999) and http://www.imgt.org/IMGTScientificChart/Nomenclature/IMGT-FRCDRdefinition.html).

As used herein the term "partial variable region" or "partial V region" is determined by the position of the internal forward primers targeting the amplified V-region of interest (e.g., α, β, γ, δ; see, e.g., Tables 8-11) until the codon position 105 (all the position numbers are according to the IMGT unique numbering; see Lefranc, M.-P., The Immunologist, 7, 132-136 (1999) and http://www.imgt.org/IMGTScientificChart/Nomenclature/IMGT-FRCDRdefinition.html).

As used herein the term "partial constant region" or "partial C region" refers to a region that includes the first codon of the C region until a position defined by the reverse internal primer used to amplify the C region of interest (e.g., α, β, γ, δ; see Tables 8-11).

The term "corresponding TCR library" means the same variable (V) family usage.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the content clearly dictates otherwise.

The terms “subject,” “individual,” and “patient,” are used interchangeably herein and refer to any mammalian subject for whom diagnosis, prognosis, treatment, or therapy is desired, particularly humans. Other subjects may include cattle, dogs, cats, guinea pigs, rabbits, rats, mice, horses, and so on. In some cases, the methods of the invention find use in experimental animals, in veterinary application, and in the development of animal models for disease, including, but not limited to, rodents including mice, rats, and hamsters; primates, and transgenic animals.

The term “about” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. For example, “about” can mean within an acceptable standard deviation, per the practice in the art. Alternatively, “about” can mean a range of up to ±20%, preferably up to ±10%, more preferably up to ±5%, and more preferably still up to ±1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated, the term “about” is implicit and in this context means within an acceptable error range for the particular value.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); DNA Cloning: A practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (MJ. Gait ed. 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds.(1985»; Transcription and Translation (B.D. Hames & S.J. Higgins, eds. (1984»; Animal Cell Culture (R.I. Freshney, ed. (1986»; Immobilized Cells and Enzymes (lRL Press, (1986»; B. Perbal, A practical Guide To Molecular Cloning (1984); F.M. Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994); among others.

Method and Platform of the Invention

The present invention provides several useful techniques for the analysis of TCR biology, including a single cell based protocol for γδ TCR amplification, a rapid protocol for TCR cloning

and expression, and a novel platform for functional characterization of TCR clones (see FIGS. 5A and 5B). The invention provides an accurate and efficient method to approach rapid TCR cloning at the single cell level, which can improve the development of multiple applications, including TCR-mediated immunotherapy.

The most prominent recent immunotherapy approaches involve T cell checkpoint blockade inhibitors[51]. However, these therapies depend on the presence of significant numbers of anti-tumor T cell responses. The *ex vivo* expansion of tumor infiltrating lymphocytes has also been successful, but is time consuming [52]. The method of the present invention significantly accelerates the amount of time needed to generate large numbers of anti-tumor T cells, by allowing the efficient transduction of identified anti-tumor receptors. The key to the application of the invention of directed T cell immunotherapy is the rapid and accurate isolation and cloning of paired TCRs. Thus far, various methods have been developed for the cloning of TCR genes by traditional PCR, but the acquisition and expression of TCRs is often labor-intensive, time-consuming, expensive, and non-specific. The present invention provides efficient acquisition of TCR gene products for cloning based on single cell isolation, with an amplification success rate of isolated paired single cell TCRγ and TCRδ CDR3 products of 71.25±18.75% based on total sorted single cells in each sample.

The invention also provides a platform for screening TCR activation after cloning. By inserting the Nur77 reporter into a Jurkat 76 TCRα-β- cell line, a useful system for monitoring specific TCR activation was generated, as demonstrated by stimulation of PB1-NJ76 by its cognate influenza-derived peptide and stimulation of TRGV9/TRDV2 T cells with, e.g., zoledronic acid.

In certain embodiments, the invention employs the insertion of Nur77-GFP BAC DNA into the Jurkat cell line as a reporter cell line. Prior approaches to detect T cell activation include CD69 expression on the cell surface[22], and detection of IFNγ, IL-2, and TNFα protein levels in cell culture supernatants by ELISA[21,22]. These conventional methods have some shortcomings. For instance, CD69 is common activation marker of T cells, so it cannot show the specific activation of T cells through TCR signaling - bystander activation can occur. Detection of IFNγ, IL-2 and TNFα by ELISA is time-consuming (2 days) and expensive, and Detection of IFNγ, IL-2 and TNFα by real-time is labor-intensive (requires RNA isolation), time-consuming (at least a day).

The Nur77-GFP system has been demonstrated to reflect specific TCR triggering after stimulation[40,41,42], instead of activation by other receptors on the cell surface, like TLRs, NKG2A/2D, or other inhibitory receptors. Furthermore, GFP is directly assayable by flow cytometry without any secondary processing. The Nur77-GFP system allows rapid and accurate detection of specific T cell stimulation in a high-throughput manner. In certain embodiments, the invention provides that zoledronic acid induces the GFP expression of TRGV9/TRDV2 Nur77-GFP cells, demonstrating that the invention platform is functional, and zoledronic acid can be used in combination with TRGV9/TRDV2-expressing cells as a positive control for the test platform and that peptides can be used in combination with their cognate TRBV/TRAV-expressing cells (as demonstrated in FIG. 3B) as a control for the test platform.

Therefore, the present invention provides a novel platform and/or system that can be used to test different molecules directly by stimulating the Nur77-GFP Jurkat cell line, and characterizing and quantifying the stimulation based on GFP expression by flow cytometry. The platform of the present invention is faster, easier and more inexpensive (no need to stain) to perform, and can be used to screen TCR-activating or modifying drugs in a high-throughput screening manner as compared to prior art platforms.

In other embodiments, a Nur77-Luciferase reporter, which has a lower signal to noise ratio, is included in the Nur77-GFP Jurkat cell line to decrease the background for certain types of drug screening. Insertion of Nur77-Luciferase BAC DNA into the Jurkat cell line instead of Nur77-GFP can be undertaken, followed by luciferase detection in various assays that include high-throughput screening platforms. The luciferase detection may be conducted with between 5 x 10^4 to 2.5 x 10^5 cells per well, or about 1 x 10^5 cells per well, for highly sensitive detection.

Another application of the Nur77-GFP system is to screen for compounds that inhibit T cell activation. The Nur77-GFP system of the invention can be used for drug screening, clinical applications, and basic immunology research.

Considering the variability of CDR3 sequences and TCR variable regions (approximately 10^{18} combinations in human TCR$\gamma\delta$ cells and 10^{16} combinations for TCR$\alpha\beta$ cells [53]) and the complexity of cloning all the different clones *de novo*, the invention provides a method using overlap extension PCR of a linker molecule with amplified single cell CDR3 products and a constructed $\gamma\delta$ (or $\alpha\beta$) TCR library to rapidly (less than 5 days) generate diverse TCR clones. Setting up a TCR clone library with all possible combination of TCRγ/TCRδ or TCRβ/TCRα

pairings and a DNA linker library allows the achievement of superior speed by overcoming the need to synthesize the hypervariable CDR3 portion of the DNA. In the method of the present invention, as soon as the sequence information of the TCR CDR3 region from the single-cell PCR and their family usage are known, the relevant clone can be picked up from the library with the required TRBV and TRAV families to use as a backbone for the final construct. The single-cell PCR products of TCRβ/γ and TCRα/δ can then be linked together by using two terminal primers and a DNA linker by overlapping PCR. Next, this target gene can be substituted directly into the existing clones and the irrelevant CDR3s can be replaced with the specific ones while preserving the family usage. In certain embodiments, new CDR3s of TRGV9-TRDV2 are put into the existing cloning of TRGV9-TRDV2 vector with different CDR3s by overlapping extension PCR. With the method of the present invention, cloning TCR genes can be achieved within 5 days.

As compared to prior art methods[21,22], important advantages of the methods of the present invention are as follows:

1. The method of the present invention provides a way to build a library of clones as a one-time necessity that can serve as a backbone to the second, rapid TCR cloning method.
2. The method of the present invention does not require stimulation of PBMCs (e.g., with PHA/IL-2), which saves at least 1 day (and potential variability and survival issues) and helps avoid bias in the TCR repertoire in the subsequent analyses (as stimulation of PBMCs may cause unnecessary selection of TCR repertoire due to expansion of a subset of T cells, as well as a high rate and/or non-physiological level of dual TCRα and dual TCRβ expression in a single cell due to clonal expansion under the stimulation).
3. The method of the present invention is substantially faster than the reported approaches and relies on more robust processes. Once the library is made, the invention platform only needs 5 days to clone TCRs. Prior approaches are more labor intensive because they clone the full-length TCR *de novo*. In contrast, the method of the present invention uses the single-cell PCR products to clone any pairs of TCRs, and the libraries constructed using these clones can be continuously used.
4. The method of the present invention can yield an average 71% of TCR pairs from human samples (compare with an average 34% yield reported in [22] or 147 T cells in samples from 61 patients identified in [21]).

5. While in prior methods[22] TCRα/γ and TCRβ/δ were inserted into different vectors to transfect cells, which may cause the biased expression of TCRs, in the method of the present invention TCRα/γ and TCRβ/δ are inserted under the same promoter separated by a 2A sequence to ensure their equal level of expression.
6. In certain embodiments, the method of the present invention uses Jurkat 76 cell line (the TCR-negative human T cell line) to express human TCR clones. Such human cell line likely more accurately mimics human T cells and is more amenable to adoptive transfer therapy than mouse cell lines used in prior art.
7. The single-cell technique used in the method of the present invention allows to examine small number of cells (as low as 1) which is important e.g., in solid tumors where the numbers of TILs are limiting.

Uses of the Methods of the Invention

The rapid TCR cloning method of the present invention is very useful for immunotherapy. Tumor-specific T cells have been characterized by broad non-specific surface phenotypes that can be used to isolate, clone, and express potential tumor-targeted clones[54]. The recent advancement of tumor sequencing has allowed for identification of tumor neoantigens and overexpressed self-antigens[55,56,57,58]. Combining these technologies will allow characterizion and tailoring of anti-tumor therapy.

T cell transfers have also been used for the treatment of opportunistic infections in immunosuppressed patients, particularly after hematopoietic stem cell transplant. The reactivation of herpes viruses like human cytomegalovirus and Epstein-Barr virus is a clinical dilemma that cannot always be addressed with antivirals[24,59,60]. Analogous to TIL therapies, *ex vivo* expansion of antiviral T cell specificities can be clinically useful, but suffers from similar workflow limitations. By generating a library of specific TCR constructs reactive against a range of viruses and HLA types, TCR-directed therapies could be used prophylactically or immediately at the earliest signs of reactivation.

In addition to these therapeutic applications, the method of the present invention significantly improves the workflow for cloning and expressing TCRs for study *in vitro*. This can include the characterization of biochemical features of the TCR-peptide-MHC interaction, or, in the case where ligands have not been identified transduced cell lines can be used for the screening of novel antigens. This is particularly useful in the context of γδ T cells, where very

few ligands have been identified and confirmed[18,61]. The GFP reporter line engineered can be used directly in high-throughput screening platforms; alternative reporters (such as, e.g., luciferase) can be easily substituted as well.

EXAMPLES

The present invention is also described and demonstrated by way of the following examples. However, the use of these and other examples anywhere in the specification is illustrative only and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to any particular preferred embodiments described here. Indeed, many modifications and variations of the invention may be apparent to those skilled in the art upon reading this specification, and such variations can be made without departing from the invention in spirit or in scope. The invention is therefore to be limited only by the terms of the appended claims along with the full scope of equivalents to which those claims are entitled.

EXAMPLE 1

Materials and Methods

Subjects and peripheral blood mononuclear cell (PBMC) samples. Samples were obtained using research protocols approved by St. Jude Children's Research Hospital's institutional review boards (IRB) (Memphis, TN). Peripheral whole blood was collected from heparinized apheresis rings from healthy immunocompetent individuals not taking immunomodulatory pharmaceutical agents. PBMCs were isolated via density gradient centrifugation (GE Healthcare Ficoll-Paque PLUS), and red blood cells (RBCs) were removed using RBC lysis buffer (8.3g NH_4Cl, 1g $KHCO_3$, and 1ml 0.1% Phenol Red in 1L distilled water). Isolated PBMCs were frozen in -80°C for future use. All PBMCs used in the paper were stored frozen. Compared to fresh PBMC data from healthy apheresis rings, frozen PBMCs did not have a significantly lower success rate for single-cell amplification.

Single cell sorting and staining. PBMCs were treated with human FcR blocking reagent (Miltenyi Biotec) on ice for 20 minutes. Human TCRγδ cells were isolated by staining with PE-conjugated anti-human TCRγ/δ (Biolegend, clone: B1), FITC-conjugated anti-human CD3 (Biolegend, clone: OKT3), a dump gate consisting of APC-conjugated anti-human CD11b/CD14/CD19 (Biolegend, CD11b clone: ICRF44; Biolegend, CD14 clone: HCD14; Biolegend, CD19 clone: HIB19) and Live/Dead Violet exclusion dye (Invitrogen, L34955) on

ice for 30 minutes. After staining, TCRγ/δ$^+$ CD3$^+$ cells were sorted directly into a 96-well PCR plate (Biorad) with a sorter (Model sy3200, Sony Biotech Synergy sorter) by the following gating strategy: autofluorescence gate - lymphocytes gate - single cell gate – live/dead gate – dump gate (CD11b/14/19) – TCRγδ/CD3 gate (FIG. 8). The last 2 columns of the plate were left empty for use as PCR negative controls. After sorting, plates were stored at −80°C until downstream processing. Human αβ T cells were also isolated using the same method[27], which consisted of APC-conjugated anti-human CD14/CD19/CD11b (Biolegend, CD14 clone: HCD14; Biolegend, CD19 clone: HIB19; Biolegend, CD11b clone: ICRF44), PE-conjugated anti-human TCRα/β (Biolegend, clone: IP26), FITC-conjugated anti-human CD3 (Biolegend, clone: OKT3) and Live/Dead Violet L34955 (Invitrogen). To increase PCR efficiency, the plates were pre-loaded with mixture of RT-PCR by SuperScript® VILO cDNA synthesis kit and single cells were sorted directly into these plates.

Reverse transcription, multiplex, nested single cell PCR, and sequencing. cDNA from TCRγδ and TCRαβ mRNA was reverse transcribed directly from the sorted and stored single cells in the PCR plate without any RNA extraction step using the iScript cDNA Synthesis Kit (Bio-Rad) in a 2.5μl reaction mix as per the method described previously[26]. The cDNA synthesis was carried out by incubating at 25°C for 5 min, 42°C for 30 min, and 80°C for 5 min (For the first round and second round of PCR). Alternatively, the SuperScript® VILO cDNA synthesis kit was used which produces a higher success rate for single cell PCR by incubating the reaction mixture at 25°C for 10 min, 42°C for 60 min, and 80°C for 5 min. The TCRαβ transcripts from each cell were amplified by a multiplex nested PCR strategy as described previously[26,27]. For amplification of TCRγδ transcripts, the overall strategy was similar to the published TCRαβ amplification (95°C for 2min, followed by 35 cycles of 95°C for 20s, 53°C for 20s, and 72°C for 45s, followed by final extension of 72°C for 7min.), except for the primers described in Table 1.

Table 1: Primers targeting human T cells receptor gamma (TRGV) and delta (TRDV) genes

TRGV gene(s) targeted by primer	External primer sequence	Internal primer sequence
HuTRGV3.5	5'TCTTCCAACTTGGAAGGG3' (SEQ ID NO:1)	5'GGTCATCTGCTGAAATCAC3' (SEQ ID NO:2)
HuTRGV7	5'TCTTCCAACTTGCAAGGG3' (SEQ ID NO:3)	5'GGTCATCTGCTGTAATCACTTG3' (SEQ ID NO:4)

TRGV gene(s) targeted by primer	External primer sequence	Internal primer sequence
HuTRGVA	5'GGGTCATCCTGTTTCCAG3' (SEQ ID NO:5)	5'TACCTAAGGACCTGTGTAGAGG3' (SEQ ID NO:6)
HuTRGVB	5'TGGCCTCCCAAAGTACTG3' (SEQ ID NO:7)	5'TCCTCTTTCTATGTCCCAGG3' (SEQ ID NO:8)
HuTRGV8	5'CCAACTTGGAAGGGAGAAC3' (SEQ ID NO:9)	5'AAAATGCCGTCTACACCC3' (SEQ ID NO:10)
HuTRGV9	5'CCAGGTCACCTAGAGCAAC3' (SEQ ID NO:11)	5'TGTCCATTTCATATGACGG3' (SEQ ID NO:12)
HuTRGV10	5'TTATCAAAAGTGGAGCAGTTC3' (SEQ ID NO:13)	5'CAGCTATCCATTTCCACGG3' (SEQ ID NO:14)
HuTRGV11	5'GAACAACCTGAAATATCTATTTCC3' (SEQ ID NO:15)	5'CATATCTTGGAAGGCATCC3' (SEQ ID NO:16)
HuTRGV1.2.4.6	5'GGGTCATCTGCTGAAATCAC3' (SEQ ID NO:17)	5'CCAGGAGGGGAAGGC3' (SEQ ID NO:18)
HuTRGC	5'GGTGTTCCCCTCCTGG3' (SEQ ID NO:19)	5'CCCAGAATCGTGTTGCT3' (SEQ ID NO:20)
HuTRDV1	5'GCCCAGAAGGTTACTCAAG3' (SEQ ID NO:21)	5'AGCAAAGAGATGATTTTCCTTA3' (SEQ ID NO:22)
HuTRDV2	5'ATTGAGTTGGTGCCTGAAC3' (SEQ ID NO:23)	5'TATATCAACTGGTACAGGAAGAC C3' (SEQ ID NO:24)
HuTRDV3	5'TGTGACAAAGTAACCCAGAGTTC3' (SEQ ID NO:25)	5'GGTACTGCTCTGCACTTACGAC3' (SEQ ID NO:26)
HuTRDV4/TRAV14	5'CAAACCCAACCAGGAATG3' (SEQ ID NO:27)	5'AGGAAAAGGAGGCTGTGAC3' (SEQ ID NO:28)
HuTRDV5/TRAV29	5'GCAAGTTAAGCAAAATTCACC3' (SEQ ID NO:29)	5'CTGCTGAAGGTCCTACATTC3' (SEQ ID NO:30)
HuTRDV6/TRAV23	5'TTGATAGTCCAGAAAGGAGG3' (SEQ ID NO:31)	5'CGTTTGACTACTTTCCATGG3' (SEQ ID NO:32)
HuTRDV7/TRAV36	5'GACAAGGTGGTACAAAGCC3' (SEQ ID NO:33)	5'ATCTCTGGTTGTCCACGAG3' (SEQ ID NO:34)
HuTRDV8/TRAV38-2	5'CAGTCACTCAGTCTCAACCAG3' (SEQ ID NO:35)	5'TCTGGTACAAGCAGCCTC3' (SEQ ID NO:36)
HuTRDC	5'CTTCATATTTACCAAGCTTGACAG3' (SEQ ID NO:37)	5'GATGACAATAGCAGGATCAAAC3' (SEQ ID NO:38)

Nine (9) TRGV external sense primers, nine (9) TRGV internal sense primers, eight (8) TRDV external sense primers and eight (8) TRDV internal sense primers targeted for individual TRGV and TRDV families were designed based on the sequences derived from the IMGT database[62]. For the antisense primer, single TRGC external, TRGC internal, TRDC external, and TRDC internal primers complementary to the published TRGC and TRDC sequences in IMGT were designed. Human TRAV14/DV4, TRAV23/DV6, TRAV29/DV5, TRAV36/DV7, and TRAV38-2/DV8 are shared primers in TRAV and TRDV primer sets.

The primers were synthesized by IDT and stored at -20°C at a stock concentration of 100μM in TE with low EDTA (pH8.0). The primers for each category (sense external, sense internal of TRGV and TRDV) were combined so that the final concentration of each primer in the mixture was 10μM. The antisense primers were diluted to 10μM. The PCR conditions for the TCRγδ nested PCR were 95°C for 2min, followed by 35 cycles of 95°C for 20s, 53°C for 20s, and 72°C for 45s, followed by final extension of 72°C for 7min. The PCR products were run on a 2% agarose gel to check for the success rate of the PCR as well as contamination following which the products were purified by a modified Exonuclease I - rShrimp alkaline phosphatase (ExoSAP-IT®) method[63] to eliminate unincorporated primers and dNTPs for high quality DNA sequencing. 1μl of the single cell PCR product was added into the mixture of 4.6μl of Tris-Cl (50mM, pH8.0), 0.2μl of Exonuclease I and 0.2μl of rShimp alkaline phosphatase and was incubated at 37°C for 15min and 80°C for 15min. The purified PCR products were sequenced using the relevant TRAC, TRBC, TRGC or TRDC primer. A schematic of the PCR strategy is shown in FIG. 1A.

gBlock® gene fragments, Gibson Assembly® and Transformation. The gBlock® gene fragments encoding the library of TRGVs and TRDVs were obtained from Integrated DNA Technologies (IDT). The expression vector pMICherry (10μg), which was modified from the parental pMIGII[64] by changing GFP to an mCherry reporter, was double digested by EcoR I (20units) and Xho I (20units) restriction enzymes (New England Biolabs) at 37°C for 3h as per manufacturer's instruction. Agarose gel purified-linearized pMICherry vector (100ng) and 2x TCR gBlock inserts were ligated in a three-way ligation, including the TCRγ gene, TCRδ gene, and linearized vector by using the Gibson Assembly® Cloning kit (New England Biolabs) per manufacturer's instructions. Two microliters of the ligation mixture was transformed into DH5α Competent *E. coli* (New England Biolabs) per manufacturer's instructions.

Generation of human CD3 constructs. Human CD3 δ, γ, ε and ζ genes were amplified from human PBMC cDNA using the primers in Table 2a. All the genes were linked together by overlap PCR with species-specific 2A regions inserted[35]. The types and amino acid sequences of the 2As used are shown in Table 2b. The CD3 gene complex was then cloned into an MSCV-based retroviral vector that contains an IRES[31,32,65] and ametrine as a reporter gene.

Table 2a: 2A Primers targeting human CD3δ, γ, ε and ζ genes

TRGV gene(s) targeted by primer	Primer sequence
CD3δ sense	5'CCCTCACTCCTTCTCTAGGCGCCGGAATTCGCCAGGATGGAACATAGCACG3' (SEQ ID NO:39)
CD3δ antisense	5'CCACGTCTCCCGCCAACTTGAGAAGGTCAAAATTCAAAGTCTGTTTCACCGGTCCCTTGTTCCGAGCC3' (SEQ ID NO:40)
CD3γ sense	5'GAATTTTGACCTTCTCAAGTTGGCGGGAGACGTGGAGTCCAACCCAGGGCCCATGGAACAGGGGAAG3' (SEQ ID NO:41)
CD3γ antisense	5'CCTCGACGTCACCGCATGTTAGCAGACTTCCTCTGCCCTCAGATCTTCTATTCCTCCTCAAC3' (SEQ ID NO:42)
CD3ε sense	5'CAGAGGAAGTCTGCTAACATGCGGTGACGTCGAGGAGAATCCTGGCCCAATGCAGTCGGGCACTC3' (SEQ ID NO:43)
CD3ε antisense	5'GTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCGGATCCTCCGATGCGTCTCTG3' (SEQ ID NO:44)
CD3ζ sense	5'CTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCCATGAAGTGGAAAGTG3' (SEQ ID NO:45)
CD3ζ antisense	5'GAGGGAGAGGGGCGGAATTGATCCTCGAGCAATTGTTAGCGAGGGGCCAG3' (SEQ ID NO:46)

Table 2b: Types and sequences of 2A regions

2A Type	2A amino acid sequence	Separation
F2A (foot-and-mouth disease virus)	VKQTLNFDLLKLAGDVESNPGP (SEQ ID NO:47)	CD3δ and CD3γ
T2A (Thosea asigna virus)	EGRGSLLTCGDVEENPGP (SEQ ID NO:48)	CD3γ and CD3ε
P2A (porcine teschovirus-1)	ATNFSLLKQAGDVEENPGP (SEQ ID NO:49)	CD3ε and CD3ζ

DNA Isolation, Cell Culture and Transfection. Recombinant pMICherry plasmids with full length TCRαβ or TCRγδ inserts were isolated in small scale by using a NucleoSpin® Plasmid kit (Clontech) and in large scale for transfection using a Plasmid Midi kit (Qiagen) per the manufacturer's instructions. The Neon® Transfection System was used to transfect 10μg TCRαβ or γδ DNA in the pMICherry vector into the human Jurkat 76 TCRα$^-$β$^-$ cell line (2×10^7 cells/mL, 100μl)[66], followed by three pulses with a voltage of 1,350V and a width of 10ms. The transfected cells were cultured for 48h before being assayed for TCRαβ or TCRγδ expression on the surface by FACS analysis. The human Jurkat 76 cells TCRα$^-$β$^-$ cell line was cultured in complete-RPMI

1640 medium, which is RPMI 1640 with 10% of fetal bovine serum, 1% Penicillin Streptomycin, and 1% L-glutamine at 37°C and 5% CO_2.

Immunofluorescent and flow cytometric analysis For surface staining, cells ($1\text{-}5\times10^5$) were harvested from culture and washed with FACS buffer (PBS with 1% of BSA and 0.1% sodium azide) prior to staining. The cells were treated with human FcR blocking reagent (Miltenyi Biotec) on ice for 20min, and cells were then treated with various fluorescent conjugated antibodies against cell surface markers in FACS buffer. Human γδ T cells were stained with APC-conjugated anti-human TCRγ/δ (Biolegend, clone: B1) or APC-conjugated anti-human TCRα/β (Biolegend, clone: IP26) and Pacific Blue-conjugated anti-human CD3 (Biolegend, clone: OKT3). For influenza-specific tetramer staining, cells ($1\text{-}5\times10^5$) were stained with APC-conjugated Influenza-M1 tetramer (Beckman Coulter, HLA-A*0201, GILGFVFTL (SEQ ID NO: 146)) in FACS buffer at room temperature for 1h prior to surface staining with the same staining antibodies described.

Modification of the CDR3 region by two-step overlap extension PCR cloning The substitution of the CDR3 was carried out by an overlap extension PCR cloning protocol[28]. A schematic diagram of the procedure is shown in FIG. 4. Briefly, a library of linker DNA was generated by gBlock synthesis at IDT (Table 3). The linker DNA consists of TRGC-2A-TRDVx (X represents the TRDV family) sequence. Using the single cell PCR products of γ and δ chains of the desired clonotype and the relevant linker gBock DNA we carried out an overlap PCR. The PCR reaction was set up and carried out as follows: 12.5μl 2×Phusion® high-fidelity DNA polymerase (New England Biolabs), 0.25μl of 100×DMSO, 1μl of 10μM TRGV internal sense primer, 1μl of TRDV internal antisense primer (Table 1), 1ng of linker DNA, and deionized H_2O up to 25μl. The PCR program was 98°C for 30s; 34 cycles of each at 98°C for 10s, 58°C for 30s, 72°C for 1min; then finally 72°C for 10min. The PCR products were visualized on a 1% agarose gel, and purified from the gel to use for cloning into the existing construct with the same TRGV and TRDV family usage. The reaction conditions used were as follows: 20ng of a TCR construct in pMICherry vector with identical TRGV and TRDV but an irrelevant CDR3γ and δ, with 50ng of the first step PCR products, 12.5μl of 2×Phusion® high-fidelity DNA polymerase, 0.25μl of 100×DMSO, and deionized H_2O up to 25μl. The PCR conditions used were 98°C for 30s; 17 cycles of each at 98°C for 10s, 65°C for 30s, 72°C for 4min; then finally 72°C for 10min. The PCR products were incubated with 1μl DpnI enzyme (New England Biolabs) at 37°C for 1h, and

2-3µl of the digested products transformed into NovaBlue Singles® competent cells (EMD Millipore).

Table 3 Human TCRγδ Linker DNA library
(lower case: TRGC sequence; bold: 2A sequence; *italic:* TRDVx sequence

Linker DNA Name	Sequence
HuLinkerDV1	5'catacctttgtcttcttgagaaatttttcccagatattattaagatacattggcaagaaaagaagagca acacgattctgggatcccaggaggggaacaccatgaagactaacgacacatacatgaaatttagctg gttaacggtgccagaagagtcactggacaaagaacacagatgtatcgtcagacatgagaataataaa aacggaattgatcaagaaattatctttcctccaataaagacagatgtcatcacaatggatcccaaagac aattggtcaaaagatgcaaatgatacactactgctgcagctcacaaacacctctgcatattacatgtac ctcctcctgctcctcaagagtgtggtctattttgccatcatcacctgctgtctgcttggaagaacggcttt ctgctgcaatggagagaaatca**GCCACGAACTTCTCTCTGTTAAAGCAA GCAGGAGACGTGGAAGAAAACCCCGGTCCC**ATGCTGTTCT CCAGCCTGCTGTGTGTATTTGTGGCCTTCAGCTACTCTGGATC AAGTGTGGCCCAGAAGGTTACTCAAGCCCAGTCATCAGTATC CATGCCAGTGAGGAAAGCAGTCACCCTGAACTGCCTGTATGA AACAAGTTGGTGGTCATATTATATTTTTTGGTACAAGCAACTTC CCAGCAAAGAGATGATTTTCCTTATTCGCC3' (SEQ ID NO:50)
HuLinkerDV2	5'catacctttgtcttcttgagaaatttttcccagatattattaagatacattggcaagaaaagaagagca acacgattctgggatcccaggaggggaacaccatgaagactaacgacacatacatgaaatttagctg gttaacggtgccagaagagtcactggacaaagaacacagatgtatcgtcagacatgagaataataaa aacggaattgatcaagaaattatctttcctccaataaagacagatgtcatcacaatggatcccaaagac aattggtcaaaagatgcaaatgatacactactgctgcagctcacaaacacctctgcatattacatgtac ctcctcctgctcctcaagagtgtggtctattttgccatcatcacctgctgtctgcttggaagaacggcttt ctgctgcaatggagagaaatca**GCCACGAACTTCTCTCTGTTAAAGCAA GCAGGAGACGTGGAAGAAAACCCCGGTCCC***ATGCAGAGGA TCTCCTCCCTCATCCATCTCTCTCTTCTGGGCAGGAGTCATGT CAGCCATTGAGTTGGTGCCTGAACACCAAACAGTGCCTGTGTCAA TAGGGGTCCCTGCCACCCTCAGGTGCTCCATGAAAGGAGAAGCG ATCGGTAACTACTATATCAACTGGTACAGGAAGACCCAAGG*3' (SEQ ID NO:51)
HuLinkerDV3	5'catacctttgtcttcttgagaaatttttcccagatattattaagatacattggcaagaaaagaagagca acacgattctgggatcccaggaggggaacaccatgaagactaacgacacatacatgaaatttagctg gttaacggtgccagaagagtcactggacaaagaacacagatgtatcgtcagacatgagaataataaa aacggaattgatcaagaaattatctttcctccaataaagacagatgtcatcacaatggatcccaaagac

	aattggtcaaaagatgcaaatgatacactactgctgcagctcacaaacacctctgcatattacatgtac ctcctcctgctcctcaagagtgtggtctattttgccatcatcacctgctgtctgcttggaagaacggcttt ctgctgcaatggagagaaatca**GCCACGAACTTCTCTCTGTTAAAGCAA GCAGGAGACGTGGAAGAAAACCCCGGTCCC***ATGATTCTTAC TGTGGGCTTTAGCTTTTTGTTTTTCTACAGGGGCACGCTGTGTGA CAAAGTAACCCAGAGTTCCCCGGACCAGACGGTGGCGAGTGGC AGTGAGGTGGTACTGCTCTGCACTTACGACACTG*3' (SEQ ID NO:52)
HuLinkerDV4	5'cataccttttgtcttcttgagaaatttttcccagatattattaagatacattggcaagaaaagaagagca acacgattctgggatcccaggaggggaacaccatgaagactaacgacacatacatgaaatttagctg gttaacggtgccagaagagtcactggacaaagaacacagatgtatcgtcagacatgagaataataaa aacggaattgatcaagaaattatctttcctccaataaagacagatgtcatcacaatggatcccaaagac aattggtcaaaagatgcaaatgatacactactgctgcagctcacaaacacctctgcatattacatgtac ctcctcctgctcctcaagagtgtggtctattttgccatcatcacctgctgtctgcttggaagaacggcttt ctgctgcaatggagagaaatca**GCCACGAACTTCTCTCTGTTAAAGCAA GCAGGAGACGTGGAAGAAAACCCCGGTCCC***ATGTCACTTTC TAGCCTGCTGAAGGTGGTCACAGCTTCACTGTGGCTAGGACCTG GCATTGCCCAGAAGATAACTCAAACCCAACCAGGAATGTTCGTGC AGGAAAAGGAGGCTGTGACTCTGG*3' (SEQ ID NO:53)
HuLinkerDV5	5'cataccttttgtcttcttgagaaatttttcccagatattattaagatacattggcaagaaaagaagagca acacgattctgggatcccaggaggggaacaccatgaagactaacgacacatacatgaaatttagctg gttaacggtgccagaagagtcactggacaaagaacacagatgtatcgtcagacatgagaataataaa aacggaattgatcaagaaattatctttcctccaataaagacagatgtcatcacaatggatcccaaagac aattggtcaaaagatgcaaatgatacactactgctgcagctcacaaacacctctgcatattacatgtac ctcctcctgctcctcaagagtgtggtctattttgccatcatcacctgctgtctgcttggaagaacggcttt ctgctgcaatggagagaaatca**GCCACGAACTTCTCTCTGTTAAAGCAA GCAGGAGACGTGGAAGAAAACCCCGGTCCC***ATGGCCATGC TCCTGGGGGCATCAGTGCTGATTCTGTGGCTTCAGCCAGACTGG GTAAACAGTCAACAGAAGAATGATGACCAGCAAGTTAAGCAAAATT CACCATCCCTGAGCGTCCAGGAAGGAAGAATTTCTATTCTGAACT GTGACTATACTAACAGCATGTTTGATTATTTCCTATGGTACAAAAAA TACCCTGCTGAAGGTCCTACATTCCTGATATC*3' (SEQ ID NO:54)
HuLinkerDV6	5'cataccttttgtcttcttgagaaatttttcccagatattattaagatacattggcaagaaaagaagagca acacgattctgggatcccaggaggggaacaccatgaagactaacgacacatacatgaaatttagctg gttaacggtgccagaagagtcactggacaaagaacacagatgtatcgtcagacatgagaataataaa aacggaattgatcaagaaattatctttcctccaataaagacagatgtcatcacaatggatcccaaagac aattggtcaaaagatgcaaatgatacactactgctgcagctcacaaacacctctgcatattacatgtac ctcctcctgctcctcaagagtgtggtctattttgccatcatcacctgctgtctgcttggaagaacggcttt

	ctgctgcaatggagagaaatca**GCCACGAACTTCTCTCTGTTAAAGCAA GCAGGAGACGTGGAAGAAAACCCCGGTCCC***ATGGACAAGA TCTTAGGAGCATCATTTTTAGTTCTGTGGCTTCAACTATGCTGGGT GAGTGGCCAACAGAAGGAGAAAAGTGACCAGCAGCAGGTGAAA CAAAGTCCTCAATCTTTGATAGTCCAGAAAGGAGGGATTTCAATTA TAAACTGTGCTTATGAGAACACTGCGTTTGACTACTTTCCATGGTA CC*3' (SEQ ID NO:55)
HuLinkerDV7	5'catacctttgtcttcttgagaaatttttcccagatattattaagatacattggcaagaaaagaagagca acacgattctgggatcccaggaggggaacaccatgaagactaacgacacatacatgaaatttagctg gttaacggtgccagaagagtcactggacaaagaacacagatgtatcgtcagacatgagaataataaa aacggaattgatcaagaaattatctttcctccaataaagacagatgtcatcacaatggatcccaaagac aattggtcaaaagatgcaaatgatacactactgctgcagctcacaaacacctctgcatattacatgtac ctcctcctgctcctcaagagtgtggtctattttgccatcatcacctgctgtctgcttggaagaacggcttt ctgctgcaatggagagaaatca**GCCACGAACTTCTCTCTGTTAAAGCAA GCAGGAGACGTGGAAGAAAACCCCGGTCCC***ATGATGAAGT GTCCACAGGCTTTACTAGCTATCTTTTGGCTTCTACTGAGCTGGGT GAGCAGTGAAGACAAGGTGGTACAAAGCCCTCTATCTCTGGTTGT CCACGAGGGAG*3' (SEQ ID NO:56)
HuLinkerDV8	5'catacctttgtcttcttgagaaatttttcccagatattattaagatacattggcaagaaaagaagagca acacgattctgggatcccaggaggggaacaccatgaagactaacgacacatacatgaaatttagctg gttaacggtgccagaagagtcactggacaaagaacacagatgtatcgtcagacatgagaataataaa aacggaattgatcaagaaattatctttcctccaataaagacagatgtcatcacaatggatcccaaagac aattggtcaaaagatgcaaatgatacactactgctgcagctcacaaacacctctgcatattacatgtac ctcctcctgctcctcaagagtgtggtctattttgccatcatcacctgctgtctgcttggaagaacggcttt ctgctgcaatggagagaaatca**GCCACGAACTTCTCTCTGTTAAAGCAA GCAGGAGACGTGGAAGAAAACCCCGGTCCC***ATGGCATGCC CTGGCTTCCTGTGGGCACTTGTGATCTCCACCTGTCTTGAATTTA GCATGGCTCAGACAGTCACTCAGTCTCAACCAGAGATGTCTGTG CAGGAGGCAGAGACCGTGACCCTGAGCTGCACATATGACACCAG TGAGAGTGATTATTATTTATTCTGGTACAAGCAGCCTCCCAG*3' (SEQ ID NO:57)

Nur77-GFP Jurkat 76 TCRα⁻β⁻ cell line. To characterize the functionality of TCRαβ or γδ clones, we established the *Nur77*-GFP Jurkat 76 TCRα⁻β⁻ cell line (NJ76 cells). After linearization of a *Nur77*-GFP BAC clone(constructed based on pTARBAC)[40] by mixing 10μg BAC DNA, 2μl 10× reaction buffer, 10units of PI-SceI restriction enzyme (New England Biolabs), and nuclease-free water to make the volume up to 20μl with incubation at 37°C for 3h

and inactivation at 65°C for 20min, 80μl of nuclease-free water, 15μl of sterile sodium acetate (3M, pH7.0), and 300μl of ethanol were added to the reaction mixture, which was then centrifuged at 12,000g for 30min at 4°C. The resulting DNA pellet was washed with 75% ethanol, dried in the air, and resuspended by Tris-EDTA buffer (pH 8.0). The Neon® Transfection System following the manufacturer's instruction was used to transfect the linearized BAC DNA into the human Jurkat 76 TCRα$^-$β$^-$ cell line (2×10^7 cells/mL, 100ul), and pulsed three times with a voltage of 1,350V and a width of 10ms. Cells were then cultured in complete-RPMI 1640 medium containing 500ug/ml Geneticin (Invitrogen) for selection.

Stimulation of $K^bPB1_{703}{}^+TCR\alpha\beta^+$ NJ76 cells (PB1-NJ76) by flu peptide PB1. NJ76 cell transfected with a murine K^bPB1_{703}-specific TCRαβ derived from influenza-infected mice and transfected cells were incubated with mouse splenocytes (cell number ratio of PB1-NJ76/splenocytes is 2:1), the influenza $PB1_{703\text{-}711}$ peptide (1μM/ml), mouse splenocytes and peptide, and mouse α-CD3 (2C11; 10μg/ml) and human α-CD28 (CD28.2; 10μg/ml) in c-RPMI 1640 medium at 37°C for 4h. The GFP expression in the mouse TCRαβ$^+$CD3$^+$ cell population was quantified by flow cytometry.

Stimulation of TRGV9/TRDV2-NJ76 cells by Zoledronic acid. NJ76 cells transfected with a TRGV9/TRDV2 clone were incubated with 50μg/ml zoledronic acid (Zometa, Novartis) in c-RPMI 1640 medium at 37°C for 3h, washed three times and incubated for 12h. The GFP expression in the TCRγδ$^+$CD3$^+$ cell population was quantified by flow cytometry.

EXAMPLE 2

Paired TCRγδ analysis of human PBMC samples at the single cell level

A strategy was developed to characterize the paired TCRγδ repertoire in humans. The primers were designed for all non-pseudogene TRGV and TRDV regions along with antisense primers for their respective constant regions. Two sets of primers (external and internal) were designed in order to perform a nested PCR (Table 1). The PCR products were examined by agarose gel electrophoresis before sequencing (FIG. 1B). The average success rate for obtaining paired CDR3γ and CDR3δ sequences at the single cell level from human PBMC samples by the present method is 71.25±18.75%. The TRGV/TRDV family usage was determined from the multiplex PCR products (FIG. 1C). On average, 20% of the sequences from the analysis of 14 human PBMCs were TRGV9/TRDV2. This technique along with the established mouse and

human αβ single cell multiplex PCR offers a rapid method (turnaround time ~3 days per 160 cells) for characterizing paired TCRγδ chains at the single cell level. The data of paired TRGV/TRDV family usage percentage in each human sample are shown in Table 4.

Table 4: TRGV/TRDV repertoire among 14 human samples. The percentage of paired TRGV/TRDV usage was analyzed from the sequencing results of each 14 human PBMC samples. Average values, standard deviation and standard error were reported.

	Mean	Std. Deviation	Std. Error
TRGV9/TRDV2	20.06	16.11	4.306
TRGV4/TRDV1	12.71	15	4.008
TRGV8/TRDV1	8.714	10.63	2.842
TRGV9/TRDV3	8.357	21.25	5.68
TRGV2/TRDV1	7.836	14.61	3.906
TRGV1/TRDV2	4.643	13.51	3.61
TRGV3/TRDV3	4.35	13.09	3.498
TRGV9/TRDV1	4.279	8.702	2.326
TRGV4/TRDV3	4.071	9.059	2.421
TRGV3/TRDV1	4.029	5.646	1.509
TRGV2/TRDV3	3.071	8.801	2.352
TRGV5/TRDV3	2.643	6.744	1.802
TRGV8/TRDV2	1.971	5.388	1.44
TRGV5/TRDV1	1.871	3.633	0.9711
TRGV2/TRDV2	1.871	3.633	0.9711

TRGV8/TRDV3	1.643	4.557	1.218
TRGV2/TRAV29/DV5	1.492	4.706	1.305
TRGV3/TRDV2	1.279	2.922	0.7808
TRGV10/TRDV1	1.071	2.31	0.6175
TRGV9/TRAV38-2/DV8	0.3571	1.336	0.3571
TRGV4/TRDV2	0.1857	0.6949	0.1857
TRGV9/TRAV29/DV5	0.1714	0.6414	0.1714

EXAMPLE 3

Establishment of human TCRαβ and TCRγδ retroviral expression clone library

Many of the downstream applications of paired TCRαβ or TCRγδ sequence analysis require cloning and expression of the antigen specific receptors for immunological studies such as structural and functional characterization, biochemical characterization, epitope identification, and gene therapy[18,29,30]. Thus, a rapid cloning method was developed to improve on conventional restriction enzyme-mediated ligation techniques, which can be cumbersome and time consuming. In addition, use of restriction enzymes for cloning becomes problematic because of the potential occurrences of restriction sites in some variable regions and the non-germline CDR3 sequences of the TCR chains. The vector used for TCR expression is pMICherry, which has been successfully used to construct TCR clones for the generation of retrogenic mice[31,32]. A schematic diagram of the cloned TCR chains in the pMICherry vector is shown in FIG. 2A.

To clone full length TCR chains, a TRGV9/TRDV2 clone was chosen to demonstrate the feasibility of the cloning system, since the TRGV9/TRDV2 clonotype is dominant in the TCRγδ repertoire analysis from human PBMCs (FIG. 1C). Similarly, a human TCRαβ pair was chosen derived from an influenza-specific CD8 T cell from an infected individual. Using the IMGT-

reported human TRGV, TRDV, TRGC, and TRDC sequences for TCRγδ or human TRAV, TRBV, TRAC and TRBC sequences for TCRαβ and the single cell CDR3γ and δ or CDR3α and β sequence data full length TCRγ and δ chains and TCRα and β chains were constructed and joined by the 2A "self-cleaving" site *in silico*. 2A oligopeptides can interact with the ribosomal exit tunnel to terminate sequence translation at the final codon (Pro) of the 2A sequence, and reinitiate translation of the following sequence [33]. Multi-cistronic 2A based retroviral vectors have been used for TCR:CD3 structural and functional studies[32,34–38]. The entire sequence of TCRγ-2A-TCRδ along with an 25bp overhang complementary to the ends of the linearized pMICherry vector were synthesized in two fragments of approximately 1kb each as gBlock® DNA fragments (IDT) with an internal 25bp overlap in the 2A segment. By using Gibson Assembly® Master Mix, the two gBlocks spanning the TCRγ-2A-TCRδ were ligated with the linearized vector in a three-way ligation. The process of cloning is shown in FIG. 2A. After this cloning procedure, an average of 70.9% of the colonies picked after transformation was entirely matched with target sequences. The others contained either point mutations resulting from the cloning process or no inserts. More than 30 different TCR constructs have been cloned by using this system, including, mouse and human TCR αβ and TCRγδ. The cloning system is highly reproducible.

To test the functionality of the TCR clones that were made following the method described in FIG. 2A, the human TRGV9/TRDV2 construct was transfected into the Jurkat 76 TCRα$^-$β$^-$ cell line and checked for the cell surface expression by anti-TCRγδ and anti-CD3 antibody staining and flow cytometry. Although Jurkat cells have endogenous CD3, the expression of TCRγδ was not robust. Since γδ T cells do not develop in CD3-deficient mice and patients [39] the human CD3 complex was cloned into an MSCV vector (pMIAmetrine) and co-transfected it with the human TCR constructs. mCherry and ametrine are the reporter genes in the pMICherry vector with human TCR genes and the pMIAmetrine vector with human CD3 genes, respectively. It is demonstrated that co-transfection of the human CD3 construct with the human TCRγδ and αβ chains can improve the surface expression level of TCR in a Jurkat cell line (FIG. 6). 3.76% of cells were double positive for mCherry and ametrine, and 19.5% of double-positive cells were TCRγδ and CD3 positive, which proved the functionality of the cloning and expression platform of the invention (*top panel*, FIG. 2B). The expression of the influenza virus-specific TCRαβ was analyzed by staining the transfected cells with APC-

conjugated influenza-M1 tetramer (HLA-A*0201, GILGFVFTL (SEQ ID NO: 146)) and CD3 antibody. The FACS plot shows 5.03% of transfected cells were double positive for mCherry and ametrine, 16.1% of which were positive for tetramer staining (*bottom panel*, FIG. 2B).

EXAMPLE 4

Effective TCR activation reporting by Nur77-GFP Jurkat 76 TCRα⁻β⁻ cells

An important application of TCR cloning and expression is to screen molecules that can activate or inhibit TCR signaling. Thus far, the common methods to detect TCR activation are using ELISA to detect cytokines (e.g. IFNγ) in the cell culture medium[21,22], intracellular staining to report cytokine production by flow cytometry, or qRT-PCR to quantify the mRNA expression of cytokines, which are time-consuming, labor-intensive, and expensive. In the present invention, a TCR activation reporter cell line, *Nur77*-GFP Jurkat 76 TCRα⁻β⁻ (NJ76 cells) was established by stably transducing *Nur77*-GFP BAC DNA into Jurkat 76 TCRα⁻β⁻ cells. The *Nur77*-GFP reporting system has been demonstrated to reflect specific TCR signal strength by GFP expression[40,41,42].

To test the functionality of NJ76 cells in reporting TCR activation, NJ76 cells were transfected with a murine K^bPB1_{703}-specific TCR αβ derived from influenza-infected mice along with a mouse CD3 construct. The $K^bPB1_{703}{}^+TCR\alpha\beta^+$ NJ76 cells (PB1-NJ76) were incubated with mouse splenocytes, the influenza $PB1_{703}$ peptide, splenotytes and peptide, or mouse α-CD3/human α-CD28 as a positive control for 4 hours and GFP expression in transfected NJ76 cells was detected by flow cytometry (FIG. 3A). The quantification of GFP levels is shown in FIG. 3B. The results show that PB1-NJ76 cells can robustly express GFP after specific peptide stimulation (PB1) with antigen-presenting cells. The gating strategy for GFP detection is shown in FIG. 7.

This TCR-activation reporting system has also been tested for TCRγδ signaling. Zoledronic acid (Zometa, Novartis) is an aminobisphosphonate that has demonstrated antitumor effects via inhibition of tumor growth and angiogenesis and induction of malignant cell apoptosis in humans[43,44,45]. In addition, zoledronic acid can specifically stimulate and expand human TRGV9/TRDV2 cells[46,47,48,49]. Since it can result in the accumulation of upstream metabolites in the mevalonate pathway , *e.g.* IPP, which induce the expansion of γδ T cells *in vitro* and *in vivo*, zoledronic acid pre-treatment can increase the cytolysis of some cancer cell lines by γδ T cells[50]. After transfection of the human TRGV9/TRDV2 vector and human CD3 vector into NJ76 cells,

the transfected TRGV9/TRDV2-NJ76 cells were pulsed with 50μg/ml of zoledronic acid for 3 hours, and washed and incubated the cells at 37°C for 12h. The GFP expression level was quantified in transfected TRGV9/TRDV2-NJ76 cells and control cells by flow cytometry. The *Nur77*-GFP expression level is shown in FIG. 3C (*top panel*). The transfected TRGV9/TRDV2-NJ76 cells showed a significantly higher level of GFP expression, which demonstrates that zoledronic acid can trigger γδ TCR signaling for the stimulation and expansion of γδ T cells. The fold change of GFP expression over time in stimulated TRGV9/TRDV2-NJ76 cells with zoledronic acid and non-stimulated TRGV9/TRDV2-NJ76 is shown in FIG. 3C (*bottom panel*).

EXAMPLE 5

Effective TCR activation reporting by Nur77-Luc Jurkat 76 TCRα⁻β⁻ cells

Nur77-GFP BAC DNA was modified by using recombineering to substitute the GFP with firefly luciferase. In particular, recombineering was undertaken to insert a cassette with Luciferase-SV40pA-PGK-Neo-bGHpA into the Nur77 gene. A clone with such insertion (*Nur77*-Luc BAC DNA) was isolated and sequenced. TCRα⁻β⁻ Jurkat 76 cells were transfected with the *Nur77*-Luc BAC DNA under a selectively complete RPMI medium with 500 μg/ml of Genetcin, yielding Nur77-Luciferase TCRα⁻β⁻ Jurkat cells.

To determine optimum conditions for luciferase assays, tests of various numbers of Nur77-Luciferase TCRα⁻β⁻ Jurkat cells per well were undertaken. In the tests, the cells were subjected to four hours of PMA/Ionomycin stimulation followed by measurement in a luciferase reader. These values are shown in Table 5 under the column "Stimulated". As a control, the luciferase reader was used to measure cells that were not stimulated. See Table 5, "Unstimulated" column. By comparison, the medium itself gave values of only 0 to 80 in the luciferase reader.

Table 5: Luciferase assay of various amounts of Nur77-Luciferase TCRα⁻β⁻ Jurkat cells

Cell number	Unstimulated	Stimulated
1×10^4	2640	9600
2.5×10^4	4400	19680
5×10^4	8640	37360
1×10^5	16400	62400

2.5×10^5	34560	97840
5×10^5	41520	49520
1×10^6	39440	32880

When assaying luciferase, using a cell number of 1×10^5 provides a four-fold rise in luciferase activity as compared with the unstimulated cells.

EXAMPLE 6

Rapid TCR cloning by CDR3 substitution using overlap extension PCR and TCR library

For TCRs, the only hypervariable regions are the CDR3 regions. Thus, cloning full length TCRs *de novo* for each application may expend unnecessary resources. To improve on this, a library was generated containing potential TRGV and TRDV "backbone" combinations that only require the swapping of individual CDR3 regions directly from PCR products. For example, in TRGV9/TRDV2 cells from PBMCs of healthy donors, the CDR3s of both γ and δ chains were found to be highly diverse (Table 4). To rapidly generate an array of TRGV9/TRDV2 clones with diverse CDR3γ and δ, a DNA linker, whose ends overlap with the TRGC and TRDV2 of the single cell PCR products, was designed. Similarly for other combinations of the TRGV/TRDV family several DNA linkers were designed. These DNA linkers contain the TRGC region, 2A and one of the TRDV regions, as is shown in FIG. 4. By overlap PCR with the single cell PCR products, DNA linkers, TRGV sense primers, and TRDC antisense primers, any pair of TCRγδ can be connected together. Next, the first-step PCR products were used as a mega primer with the appropriate clone from the library (e.g., TRGV9/TRDV2 with an irrelevant CDR3) as a template for the second-step overlap extension PCR. By using this substitution method, different γδ TCRs with matched CDR3s from the human single cell PCR products were successfully cloned. The same approach could be used with αβ TCRs, although the clone library would be larger. This CDR3 substitution approach can shorten the cloning process to within 5 days (FIG. 4).

Table 6: CDR3 amino acid sequences of paired human TRGV9/TRDV2 cells isolated from PBMCs (n=14)

Paired amino acid sequence in TRGV9-CDR3 region	Paired amino acid sequence in TRDV2-CDR3 region	Frequency

ALFIQELGKKIKV (SEQ ID NO:58)	ACDVLGDTEGRLI (SEQ ID NO:59)	2
ALWDGPYYKKL (SEQ ID NO:60)	ACDTVFTGGYSSWDTRQMF (SEQ ID NO:61)	2
ALWDIPPGQELGKKIKV (SEQ ID NO:62)	ACDTLGETSSWDTRQMF (SEQ ID NO:63)	2
ALWEAQELGKKIKV (SEQ ID NO:64)	ACDSGGYSSWDTRQMF (SEQ ID NO:65)	2
ALWEARQELGKKIKV (SEQ ID NO:66)	ACDTLFPGGSATDKLI (SEQ ID NO:67)	2
ALWEGTRGQELGKKIKV (SEQ ID NO:68)	ACDTVGAHTDKLI (SEQ ID NO:69)	2
ALWEVGDQELGKKIKV (SEQ ID NO:70)	ACDPLNTGGSFSLYTDKLI (SEQ ID NO:71)	2
ALWEVHSELGKKIKV (SEQ ID NO:72)	ACDTGGFRSSWDTRQMF (SEQ ID NO:73)	2
ALWEVHSELGKKIKV (SEQ ID NO:72)	ACDTGGFRSSWDTRQMF (SEQ ID NO:73)	2
ALWEVLELGKKIKV (SEQ ID NO:74)	ACDTVGMGIRLGDKLI (SEQ ID NO:75)	2
ALWEVLVGELGKKIKV (SEQ ID NO:76)	ACDILGINTDKLI (SEQ ID NO:77)	2
ALWEVPELGKKIKV (SEQ ID NO:78)	ACERLGDYVPDKLI (SEQ ID NO:79)	2
ALWEVQELGKKIKV (SEQ ID NO:80)	ACDRLLGDTDKLI (SEQ ID NO:81)	2
ALWEVQELGKKIKV (SEQ ID NO:80)	ACDTVAPRIGGLKYTDKLI (SEQ ID NO:82)	2
ALWEVQELGKKIKV (SEQ ID NO:80)	ACDTVGGPYTDKLI (SEQ ID NO:83)	2
ALWEVQELGKKIKV (SEQ ID NO:80)	ACDTVGGTAQ (SEQ ID NO:84)	2
ALWEVQELGKKIKV (SEQ ID NO:80)	ACDTVSGGSTPTWYTDKLI (SEQ ID NO:85)	2
ALWEVQELGKKIKV (SEQ ID NO:80)	ACDTVSIFTGDTTDKLI (SEQ ID NO:86)	2
ALWEVRELGKKIKV (SEQ ID NO:87)	ACDTILIFSPTGGDTDKLI (SEQ ID NO:88)	2
ALWEVRELGKKIKV (SEQ ID NO:87)	ACVPLGDWTDKLI (SEQ ID NO:89)	2
ALWEVRKQELGKKIKV (SEQ ID NO:90)	ACDTLGDDFDKLI (SEQ ID NO:91)	2
ALWEVTHNRQELGKKIKV (SEQ ID NO:92)	ACDTLLGTEAWDTRQMF (SEQ ID NO:93)	2
ALWGGAAGAYYKKL (SEQ ID NO:94)	ACDGKTTDTDKLI (SEQ ID NO:95)	2
ALWGGELGKKIKV (SEQ ID NO:96)	ACDLLGDTRYTDKLI (SEQ ID NO:97)	2
ALWVQELGKKIKV (SEQ ID NO:98)	ACVGITGDTDKLI (SEQ ID NO:99)	2
ALWEAHQELGKKIKV (SEQ ID NO:100)	ACDSLGDSVDKLI (SEQ ID NO:101)	1
ALWEANKKL (SEQ ID NO:102)	ACDLLRGAGGQIDKLI (SEQ ID NO:103)	1
ALWEAQELGKKIKV (SEQ ID NO:104)	ACDTVGGAFDTDKLI (SEQ ID NO:105)	1
ALWEATGLGKKIKV (SEQ ID NO:106)	ACDMGDTRSWDTRQMF (SEQ ID NO:107)	1
ALWEDLELGKKIKV (SEQ ID NO:108)	ACDTVSWGKNTDKLI (SEQ ID NO:109)	1
ALWEKEELGKKIKV (SEQ ID NO:110)	ACDTGDWGSSWDTRQMF (SEQ ID NO:111)	1
ALWEKELGKKIKV (SEQ ID NO:112)	ACDILDSTGGTDLTAQLF (SEQ ID NO:113)	1
ALWEMTQELGKKIKV (SEQ ID NO:114)	ACDTVRNTGGYAFAGIDKLI (SEQ ID NO:115)	1
ALWEPQELGKKIKV (SEQ ID NO:116)	ACDKVLGDSSWDTRQMF (SEQ ID NO:117)	1
ALWESKELGKKIKV (SEQ ID NO:118)	ACEGLGATQSSWDTRQMF (SEQ ID NO:119)	1
ALWEVGELGKKIKV (SEQ ID NO:120)	ACDKLLGDNELI (SEQ ID NO:121)	1
ALWEVHKLGKKIKV (SEQ ID NO:122)	ACDSLLGKGTDKLI (SEQ ID NO:123)	1
ALWEVKELGKKIKV (SEQ ID NO:124)	ACDTLRGSADKLI (SEQ ID NO:125)	1
ALWEVLQQELGKKIKV (SEQ ID NO:126)	ACDTVPARHTDKLI (SEQ ID NO:127)	1
ALWEVPVLGKKIKV (SEQ ID NO:128)	ACDTADRSSYTDKLI (SEQ ID NO:129)	1
ALWEVQELGKKIKV (SEQ ID NO:80)	ACDTLLGDPSSSWDTRQMF (SEQ ID NO:130)	1
ALWEVQELGKKIKV (SEQ ID NO:80)	ACDTLSGGYARTDKLI (SEQ ID NO:131)	1
ALWEVQELGKKIKV (SEQ ID NO:80)	ACDTVGILGDTGLGLI (SEQ ID NO:132)	1
ALWEVRELGKKIKV (SEQ ID NO:87)	ACDTIVSGYDGYDKLI (SEQ ID NO:133)	1
ALWEVRELGKKIKV (SEQ ID NO:87)	ACSILGDKTSDKLI (SEQ ID NO:134)	1
ALWEVRQELGKKIKV (SEQ ID NO:135)	ACDTVSQRGGYSDKLI (SEQ ID NO:136)	1
ALWEVRVQELGKKIKV (SEQ ID NO:137)	ACDPLERVGGPANTDKLI (SEQ ID NO:138)	1
ALWEVTELGKKIKV (SEQ ID NO:139)	ACDVLGDTGDDKLI (SEQ ID NO:140)	1
ALWGRELGKKIKV (SEQ ID NO:141)	ACDTVGSNTDKLI (SEQ ID NO:142)	1
ALWVQELGKKIKV (SEQ ID NO:98)	ACDVLGDTEADKLI (SEQ ID NO:143)	1
ALYGSPSGEELGKKNQG (SEQ ID NO:144)	ACDPLEGAGGHNTDKLI (SEQ ID NO:145)	1

Table 7: Sequences of primers to mouse sequences used in nested RT-PCR. (All primers are forward primers except two sequences indicated with "reverse" which represents reverse primers. External primers were used in the first round while internal ones were used in the second round of PCR.)

Primer name	External	SEQ ID	Internal	SEQ ID
TRGV1-3	GCAGCTGGAGCAAACTG	147	CTGAATTATCGGTCACCAG	148
TRGV4	CAAATATCCTGTAAAGTTTTCATC	149	GTTTAGAGTTTCTATTATATGTCCTTGCAAC	150
TRGV5	GATATCTCAGGATCAGCTCTCC	151	TACCCGAAGACCAAACAAGAC	152
TRGV6	TCACCTCTGGGGTCATATG	153	AGAGGAAAGGAAATACGGC	154
TRGV7	CAACTTGGAAGAAAGAATAATGTC	155	CACCAAGCTAGAGGGGTC	156
TRGC (reverse)	CTTTTCTTTCCAATACACCC	157	TCDGGAAAGAACTTTTCAAGG	158
TRDV1	ACCCAAATGTTGCATCAG	159	GTCTCTGACAATCCAAGAAGG	160
TRDV2	TCTGTGCAGGTGGCAG	161	CCCTGGACTGCACCTATG	162
TRDV4	TGTATATTTGGAACCAGTTGC	163	GATCCTGCCTCCTTCTACTG	164
TRDV5	CATCACGCTGACCCAG	165	GCTCCACTGACCAGACAG	166
TRDV6/TRAV15	CASCTTYTTAGTGGAGAGATGG	167	AYTCTGTAGTCTTCCAGAAATCAC	168
TRDV7/TRAV13	TCCTTGGTTCTGCAGG	169	TGCAGGAGGGGGAGA	170
TRDV8/TRAV14	GCAGCAGGTGAGACAAAG	171	CTCTGACAGTCTGGGAAGG	172
TRDV9/TRAV6-1/6-2	CAGATGCAAGGTCAAGTGAC	173	GGAGAAGGTCCACAGCTC	174
TRDV9/TRAV6-3/6-4	AAGGTCCACAGCTCCTTC	175	CAACTGCCAACAACAAGG	176
TRDV9/TRAV6-5/6-7	GTTCTGGTATGTGCAGTATCC	177	TCCTTCCACTTGCAGAAAG	178
TRDV10/TRAV4	TCTGSTCTGAGATGCAATTTT	179	GGITIMAGGAACAAAGGAGAAT	180
TRDV11/TRAV16	GTACAAGCAAACAGCAAGTG	181	ATTATTCTCTGAACTTTCAGAAGC	182
TRDV12/TRAV21	GTGCACTTGCCTTGTAGC	183	AATAGTATGGCTTTCCTGGC	184
TRDC (reverse)	TGAAAGAATTTTGCATATGGTTC	185	GAGATGACTATAGCAGGGTCG	186

Tables 8-11: "Partial" V and C regions amplified depending on the target sequence. (Column "Region" discloses nucleotide sequence positions based on the IMGT reference database, see Lefranc, M.-P., The Immunologist, 7, 132-136 (1999) and http://www.imgt.org/IMGTScientificChart/Nomenclature/IMGT-FRCDRdefinition.html.)

Table 8: Human γδ primer list with positions.

Primer name	SEQ ID NO	Primer Sequence	Region
HuTRDV4/TRAV14Ext	27	CAAACCCAACCAGGAATG	76..93
HuTRDV6/TRAV2Ext	31	TTGATAGTCCAGAAAGGAGG	115..134
HuTRDV/TRAV29Ext	29	GCAAGTTAAGCAAAATTCACC	84..104
HuTRDV7/TRAVExt	33	GACAAGGTGGTACAAAGCC	67..85
HuTRDV8/TRAVExt	35	CAGTCACTCAGTCTCAACCAG	68..88
HuTRDV1Ext	21	GCCCAGAAGGTTACTCAAG	61..79
HuTRDV2Ext	23	ATTGAGTTGGTGCCTGAAC	61..79
HuTRDV3Ext	25	TGTGACAAAGTAACCCAGAGTTC	52..74
HuTRDCExt	37	CTTCATATTTACCAAGCTTGACAG	196-173
HuTRDV4/TRAV14Int	28	AGGAAAAGGAGGCTGTGAC	101..119
HuTRDV6/TRAV2Int	32	CGTTTGACTACTTTCCATGG	170..189
HuTRDV/TRAV29Int	30	CTGCTGAAGGTCCTACATTC	197..216
HuTRDV7/TRAVInt	34	ATCTCTGGTTGTCCACGAG	90..108
HuTRDV8/TRAVInt	36	TCTGGTACAAGCAGCCTC	164..181
HuTRDV1Int	22	AGCAAAGAGATGATTTTCCTTA	184..205
HuTRDV2Int	24	TATATCAACTGGTACAGGAAGACC	157..180
HuTRDV3Int	26	GGTACTGCTCTGCACTTACGAC	108..129
HuTRDCInt	38	GATGACAATAGCAGGATCAAAC	150-129
HuTRGV10Ext	13	TTATCAAAAGTGGAGCAGTTC	52..72
HuTRGV11Ext	15	GAACAACCTGAAATATCTATTTCC	61..84
HuTRGV3.5Ext	1	TCTTCCAACTTGGAAGGG	55..72
HuTRGV8Ext	9	CCAACTTGGAAGGGAGAAC	59..77
HuTRGV1.2.4.6Ext	17	GGGTCATCTGCTGAAATCAC	100..119
HuTRGV9Ext	11	cCAGGTCACCTAGAGCAAC	61..79
HuTRGVAExt	5	GGGTCATCCTGTTTCCAG	26..43
HuTRGVBExt	7	TGGCCTCCCAAAGTACTG	18..35

HuTRGCExt	19	GGTGTTCCCCTCCTGG	186-171
HuTRGV10Int	14	CAGCTATCCATTTCCACGG	73..91
HuTRGV11Int	16	CATATCTTGGAAGGCATCC	108..126
HuTRGV3.5Int	2	GGTCATCTGCTGAAATCAC	101..119
HuTRGV1.2.4.6Int	18	CCAGGAGGGGAAGGC	168..182
HuTRGV8Int	10	AAAATGCCGTCTACACCC	83..100
HuTRGV9Int	12	TGTCCATTTCATATGACGG	209..227
HuTRGCInt	20	CCCAGAATCGTGTTGCT	167-151

Table 9: Mouse αβ primer list with positions (V region external and C region reverse primers and their positions).

Primer name	SEQ ID NO	Primer Sequence	Region
mTRBV1Ext	187	TACCACGTGGTCAAGCTG	101..118
mTRBV12Ext	188	GGGGTTGTCCAGTCTCC	94..110
mTRBV13Ext	189	GCTGCAGTCACCCAAAG	55..71
mTRBV16Ext	190	CCTAGgcACAAGGTGACAG	73..91
mTRBV14Ext	191	GCAGTCCTACAGGAAGGG	88..105
mTRBV15Ext	192	GAGTTACCCAGACACCCAG	65..83
mTRBV17Ext	193	GAAGCCAAACCAAGCAC	168..184
mTRBV19Ext	194	GATTGGTCAGGAAGGGC	99..115
mTRAV2Ext	195	cATcTACTGGTACCGACAGG	159..178
mTRBV2Ext	196	CAGTATCTAGGCCACAATGC	130..149
mTRBV20Ext	197	GGATGGAGTGTCAAGCTG	101..118
mTRBV23Ext	198	CTGCAGTTACACAGAAGCC	62..80
mTRBV24Ext	199	CAGACTCCACGATACCTGG	73..91
mTRBV29Ext	200	GCTGGAATGTGGACAGG	117..133
mTRBV3Ext	201	CCCAAAGTCTTACAGATCCC	61..80
mTRBV31Ext	202	CTAACCTCTACTGGTACTGGCAG	143..165
mTRBV4Ext	203	GACGGCTGTTTTCCAGAC	60..77
mTRBV5Ext	204	GGTATAAACAGAGCGCTGAG	155..174
Cba Rev Ext	205	CCAGAAGGTAGCAGAGACCC	252-233
mTRBV1Int	206	GTATCCCTGGATGAGCTG	150..167
mTRBV12Int	207	CCAGCAGATTCTCAGTCC	269..286

mTRBV13Int	208	GTACTGGTATCGGCAGGAC	147..165
mTRBV14Int	209	GGTATCAGCAGCCCAGAG	158..175
mTRBV15Int	210	GTGTGAGCCAGTTTCAGG	123..140
mTRBV16Int	211	GAAGCAACTCTGTGGTGTG	109..127
mTRBV17Int	212	GAACAGGGAAGCTGACAC	219..236
mTRBV19Int	213	GGTACCGACAGGATTCAG	167..184
mTRBV2Int	214	GGACAATCAGACTGCCTC	222..239
mTRBV20Int	215	GCTTGGTATCGTCAATCG	142..159
mTRBV23Int	216	GCCAGGAAGCAGAGATG	104..120
mTRBV26Int	217	GAggTGTATCCCTGAAAAGG	120..139
mTRBV24Int	218	GCACACTGCCTTTTACTGG	141..159
mTRBV29Int	219	GTACTGGTATCGACAAGACCC	153..173
mTRBV3Int	220	GATATGGGGCAGATGGTG	97..114
mTRBV31Int	221	CTGTTGGCCAGGTAGAGTC	206..224
mTRBV5Int	222	GCCAGAGCTCATGTTTCTC	180..198
Cbb Rev Int	223	GGGTAGCCTTTTGTTTGTTTG	88-68
mTRAV10.10aInt	224	CTACACTGAGTGTTCGAGAGG	89..109
mTRAV12Int	225	GGTTCCACGCCACTC	242..256
mTRAV13Int	170	TGCAGGAGGGGGAGA	98..112
mTRAV14Int	172	CTCTGACAGTCTGGGAAGG	113..131
mTRAV15Int	168	AYTCTGTAGTCTTCCAGAAATCAC	251..274
mTRAV16Int	182	ATTATTCTCTGAACTTTCAGAAGC	248..271
mTRAV17Int	226	TATGAAGGAGCCTCCCTG	97..114
mTRAV18Int	227	CAAGATTTCACCGCACG	103..119
mTRAV19Int	228	GCTGACTGTTCAAGAGGGA	108..126
mTRAV1Int	229	CTCCACATTCCTGAGCC	237..253
mTRAV21Int	184	AATAGTATGGCTTTCCTGGC	220..239
mTRAV2Int	230	ACTCTGAGCCTGCCCT	265..280
mTRAV4Int	180	GGiTiMAGGAACAAAGGAGAAT	210..231
mTRAV5-15-4Int	315	ATYCGTTCAAATATGGAAAGAAA	211..233
mTRAV6-16-2Int	174	GGAGAAGGTCCACAGCTC	178..195
mTRAV6-36-4Int	176	CAACtGCCAACAACAAGG	209..226
mTRAV6-36-4Int-1	176	CAACtGCCAACAACAAGG	209..226
mTRAV6-56-7Int	178	TCCTTCCACTTGCAGAAAG	271..289
mTRAV6-6Int	231	ACGGCTGGCCAGAAG	217..231
mTRAV8Int	232	AGAGCCACCCTTGACAC	244..260
mTRAV9Int	233	GCTTYGAGGCTGAGTTCAG	239..257
mTRAC Rev Int	234	GCACATTGATTTGGGAGTC	100-82
mTRAV1010aExt	235	AGAGAAGGTCGAGCAACAC	66..84
mTRAV11Ext	236	AAGACCCAAGTGGAGCAG	64..81
mTRAV12Ext	237	TGACCCAGACAGAAGGC	68..84
mTRAV13Ext	169	TCCTTGGTTCTGCAGG	88..103

mTRAV14Ext	171	GCAGCAGGTGAGACAAAG	87..104
mTRAV19Ext	238	gcAAGttAaAcAAAGCTCTCC	286..306
mTRBV31Ext	202	ctaACcTCtacTGGTACTGGCAG	176..198
mTRAV15Ext	167	CASCTTYTTAGTGGAGAGATGG	175..196
mTRAV16Ext	181	GTACAAGCAAACAGCAAGTG	168..187
mTRAV17Ext	239	CAGTCCGTGGACCAGC	61..76
mTRAV6-56-7Ext	177	gTTCTGGTAtGTGCAGTATCC	156..176
mTRAV18Ext	240	AACGGCTGGAGCAGAG	59..74
mTRAV2Ext	195	caTcTACTGGTACCGACAGG	147..166
mTRAV21Ext	183	GTGCACTTGCCTTGTAGC	103..120
mTRAV3Ext	241	GGCGAGCAGGTGGAG	64..78
mTRAV5-15-4Ext	242	GgcTACTTCcCtTGGTATAAGCAAGA	154..179
mTRAV4Ext	179	TcTGSTCTGAGATGCAATTTT	113..133
mTRAV6-36-4Ext	175	AAGGTCCACAGCTCCTTC	182..199
mTRAV6-36-4Ext-1	175	AAGGTCCACAGCTCCTTC	182..199
mTRAV6-6Ext	243	AGATTCCGTGACTCAAACAG	60..79
mTRAV7Ext	244	AGAAGGTRCAGCAGAGCCCAGAATC	65..89
mTRAV8Ext	245	GAGCRTCCASGAGGGTG	93..109
mTRAV9Ext	246	CCAGTGGTTCAAGGAGTG	217..234
mTRAC Rev Ext	247	GGCATCACAGGGAACG	276-261

Table 10: Human αβ primer list with positions (internal forward V region and reverse C region specific primers and their positions).

Primer name	SEQ ID NO	Primer Sequence	Region
huTRAV34int	248	aTcTCaccATAAACTGCACG	101..120
huTRAV1int	249	GCACCCACATTTCTKTCTTAC	175..195
huTRAV10int	250	GAAAGAACTGCACTCTTCAATG	110..131
huTRAV12-1	251	AAGATGGAAGGTTTACAGCAC	230..250
huTRAV13-1int	252	TCAGACAGTGCCTCAAACTAC	133..153
huTRAV13-2int	253	CAGTGAAACATCTCTCTCTGC	266..286
huTRAV14int	254	AGGCTGTGACTCTGGACTG	110..128
huTRAV16int	255	GTCCAGTACTCCAGACAACG	166..185
huTRAV17int	256	CCACCATGAACTGCAGTTAC	116..135
huTRAV18int	257	TGACAGTTCCTTCCACCTG	261..279
huTRAV19int	258	TGTGACCTTGGACTGTGTG	114..132
huTRAV2int	259	CACTCTGTGTCCAATGCTTAC	145..165
huTRAV20int	260	TCTGGTATAGGCAAGATCCTG	164..184

huTRAV21int	261	AACTTGGTTCTCAACTGCAG	109..128
huTRAV22int	262	CTGACTCTGTGAACAATTTGC	137..157
huTRBV3int	263	aATctTcaCAtCAATTCCCTG	185..205
huTRAV23int	264	TGCATTATTGATAGCCATACG	216..236
huTRAV24int	265	TGCCTTACACTGGTACAGATG	159..179
huTRAV25int	266	TATAAGCAAAGGCCTGGTG	157..175
huTRAV26-1int	267	CGACAGATTCACTCCCAG	160..177
huTRAV26-2int	268	TTCACTTGCCTTGTAACCAC	104..123
huTRAV27int	269	CTCACTGTGTACTGCAACTCC	109..129
huTRAV29int	30	CTGCTGAAGGTCCTACATTC	197..216
huTRAV3int	270	ATGCACCTATTCAGTCTCTGG	123..143
huTRAV8-24int	271	AGAGtgAAACCTCCTTCCAC	263..282
huTRAV30int	272	AGAAGCATGGTGAAGCAC	170..187
huTRAV35int	273	ACCTGGCTATGGTACAAGC	145..163
huTRAV36int	34	ATCTCTGGTTGTCCACGAG	90..108
huTRAV38int	274	CAGCAGGCAGATGATTCTC	183..201
huTRAV39int	275	TCAACCACTTCAGACAGACTG	130..150
huTRAV4int	276	ATTATATCACGTGGTACCAACAG	143..165
huTRAV40int	277	GGAGGCGGAAATATTAAAGAC	226..246
huTRAV41int	278	TTGTTTATGCTGAGCTCAGG	202..221
huTRAV5int	279	TACACAGACAGCTCCTCCAC	133..152
huTRAV6int	280	TGGTACCGACAAGATCCAG	163..181
huTRAV7int	281	TATGAGAAGCAGAAAGGAAGAC	226..247
huTRAV8-1int	282	GTCAACACCTTCAGCTTCTC	179..198
huTRAV8-7int	283	ATCAgaGGtTTTGAGGCTG	235..253
huTRAV8-6int	284	AACcAAGGACTCCAGCTTC	178..196
huTRAV8-3int	285	TTTGAGGCTGAATTTAAGAGG	244..264
huTRAV9-1	286	GAAACCACTTCTTTCCACTTG	262..282
hu TRAC Rev INT	287	TGTTGCTCTTGAAGTCCATAG	181-160

Primer name	SEQ ID NO	Primer Sequence	Region
huTRBV10-1int	288	TGGTATCGACAAGACCTGG	157..175
huTRBV10-2int	288	TGGTATCGACAAGACCTGG	157..175
huTRBV10-3int	289	GGAACACCAGTGACTCTGAG	103..122
huTRBV11int	290	GACTCCACTCTCAAGATCCA	277..296
huTRBV12int	291	CYACTCTgARGATCCAGCC	281..299
huTRBV13int	292	CATTCTGAACTGAACATGAGC	304..324
huTRBV5-1	293	CTTGGAGCTGGRSGACTC	327..344

huTRBV14int	294	ATTCTACTCTGAAGGTGCAGC	278..298
huTRBV15int	295	ATAACTTCCAATCCAGGAGG	242..261
huTRAV4int	276	aTTATaTcacgTGGTACCAACAG	146..168
huTRBV16int1	296	CTGTAGCCTTGAGATCCAGG	279..298
huTRBV17int	297	TGTTCACTGGTACCGACAG	150..168
huTRBV18int	298	CGATTTTCTGCTGAATTTCC	247..266
huTRBV19int	299	TTCCTCTCACTGTGACATCG	278..297
huTRBV2int	300	TTCACTCTGAAGATCCGGTC	280..299
huTRBV20int	301	ACTCTGACAGTGACCAGTGC	307..326
huTRBV23int	302	GCAATCCTGTCCTCAGAAC	289..307
huTRBV24int	303	GATGGATACAGTGTCTCTCGA	241..261
huTRAV6int	280	TGGTAcCgACAAGATCCAG	157..175
huTRBV25int	304	CAGAGAAGGGAGATCTTTCC	221..240
huTRBV2728int	305	TTCYCCCTGATYCTGGAGTC	277..296
huTRBV29int	306	TCTGACTGTGAGCAACATGAG	276..296
huTRBV3int	263	AATCTTCACATCAATTCCCTG	280..300
huTRBV30int	307	AGAATCTCTCAGCCTCCAGAC	236..256
huTRBV4int	308	CCTGCAGCCAGAAGACTC	297..314
huTRBV5-5	309	TCTGAGCTGAATGTGAACG	277..295
huTRBV6-1	310	GTGTRCCCAGGATATGAACC	123..142
huTRBV6-4int	311	TGGTTATAGTGTCTCCAGAGC	243..263
huTRBV7-1	312	TCYACTCTGAMGWTCCAGCG	280..299
huTRBV9int	313	GTACCAACAGAGCCTGGAC	159..177
huTRBC Rev Int	314	TTCTGATGGCTCAAACACAG	54-35

Table 11: Mouse γδ primer list with positions.

Primer name	SEQ ID NO:	External	SEQ ID NO:	Internal	Region
TRGV1-3	147	GCAGCTGGAGCAAACTG	148	CTGAATTATCGGTCACCAG	68-86
TRGV4	149	CAAATATCCTGTAAAGTTTTCATC	150	GTTTAGAGTTTCTATTATATGTCCTTGCAAC	251-281
TRGV5	151	GATATCTCAGGATCAGCTCTCC	152	TACCCGAAGACCAAACAAGAC	81-101
TRGV6	153	TCACCTCTGGGGTCATATG	154	AGAGGAAAGGAAATACGGC	137-155
TRGV7	155	CAACTTGGAAGAAAGAATAATGTC	156	CACCAAGCTAGAGGGGTC	87-104
TRGC (reverse)	157	CTTTTCTTTCCAATACACCC	158	TCDGGAAAGAACTTTTCAAGG	118-98
TRDV1	159	ACCCAAATGTTGCATCAG	160	GTCTCTGACAATCCAAGAAGG	87-107
TRDV2	161	TCTGTGCAGGTGGCAG	162	CCCTGGACTGCACCTATG	119-136
TRDV4	163	TGTATATTTGGAACCAGTTGC	164	GATCCTGCCTCCTTCTACTG	106-125
TRDV5	165	CATCACGCTGACCCAG	166	GCTCCACTGACCAGACAG	71-88

TRDV6/TRAV15	167	CASCTTYTTAGTGGAGAGATGG	168	AYTCTGTAGTCTTCCAGAAATCAC	251-274
TRDV7/TRAV13	169	TCCTTGGTTCTGCAGG	170	TGCAGGAGGGGGAGA	98-112
TRDV8/TRAV14	171	GCAGCAGGTGAGACAAAG	172	CTCTGACAGTCTGGGAAGG	113-131
TRDV9/TRAV6-1/6-2	173	CAGATGCAAGGTCAAGTGAC	174	GGAGAAGGTCCACAGCTC	178-195
TRDV9/TRAV6-3/6-4	175	AAGGTCCACAGCTCCTTC	176	CAACTGCCAACAACAAGG	209-226
TRDV9/TRAV6-5/6-7	177	GTTCTGGTATGTGCAGTATCC	178	TCCTTCCACTTGCAGAAAG	271-289
TRDV10/TRAV4	179	TCTGSTCTGAGATGCAATTTT	180	GGITIMAGGAACAAAGGAGAAT	210-231
TRDV11/TRAV16	181	GTACAAGCAAACAGCAAGTG	182	ATTATTCTCTGAACTTTCAGAAGC	248-271
TRDV12/TRAV21	183	GTGCACTTGCCTTGTAGC	184	AATAGTATGGCTTTCCTGGC	220-239
TRDC (reverse)	185	TGAAAGAATTTTGCATATGGTTC	186	GAGATGACTATAGCAGGGTCG	151-131

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The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

All patents, applications, publications, test methods, literature, and other materials cited herein are hereby incorporated by reference in their entirety as if physically present in this specification.

LIST OF SEQUENCES

HuTRGV3.5
External Primer: 5'TCTTCCAACTTGGAAGGG3' (SEQ ID NO:1)
Internal Primer: 5'GGTCATCTGCTGAAATCAC3' (SEQ ID NO:2)

HuTRGV7
External Primer: 5'TCTTCCAACTTGCAAGGG3' (SEQ ID NO:3)
Internal Primer: 5'GGTCATCTGCTGTAATCACTTG3' (SEQ ID NO:4)

HuTRGVA
External Primer: 5'GGGTCATCCTGTTTCCAG3' (SEQ ID NO:5)
Internal Primer: 5'TACCTAAGGACCTGTGTAGAGG3' (SEQ ID NO:6)

HuTRGVB
External Primer: 5'TGGCCTCCCAAAGTACTG3' (SEQ ID NO:7)
Internal Primer: 5'TCCTCTTTCTATGTCCCAGG3' (SEQ ID NO:8)

HuTRGV8
External Primer: 5'CCAACTTGGAAGGGAGAAC3' (SEQ ID NO:9)
Internal Primer: 5'AAAATGCCGTCTACACCC3' (SEQ ID NO:10)

HuTRGV9
External Primer: 5'CCAGGTCACCTAGAGCAAC3' (SEQ ID NO:11)
Internal Primer: 5'TGTCCATTTCATATGACGG3' (SEQ ID NO:12)

HuTRGV10
External Primer: 5'TTATCAAAAGTGGAGCAGTTC3' (SEQ ID NO:13)
Internal Primer: 5'CAGCTATCCATTTCCACGG3' (SEQ ID NO:14)

HuTRGV11
External Primer: 5'GAACAACCTGAAATATCTATTTCC3' (SEQ ID NO:15)
Internal Primer: 5'CATATCTTGGAAGGCATCC3' (SEQ ID NO:16)

HuTRGV1.2.4.6
External Primer: 5'GGGTCATCTGCTGAAATCAC3' (SEQ ID NO:17)
Internal Primer: 5'CCAGGAGGGGAAGGC3' (SEQ ID NO:18)

HuTRGC
External Primer: 5'GGTGTTCCCCTCCTGG3'(SEQ ID NO:19)

Internal Primer: 5'CCCAGAATCGTGTTGCT3' (SEQ ID NO:20)

HuTRDV1
External Primer: 5'GCCCAGAAGGTTACTCAAG3' (SEQ ID NO:21)
Internal Primer: 5'AGCAAAGAGATGATTTTCCTTA3' (SEQ ID NO:22)

HuTRDV2
External Primer: 5'ATTGAGTTGGTGCCTGAAC3' (SEQ ID NO:23)
Internal Primer: 5'TATATCAACTGGTACAGGAAGACC3' (SEQ ID NO:24)

HuTRDV3
External Primer: 5'TGTGACAAAGTAACCCAGAGTTC3' (SEQ ID NO:25)
Internal Primer: 5'GGTACTGCTCTGCACTTACGAC3' (SEQ ID NO:26)

HuTRDV4/TRAV14
External Primer: 5'CAAACCCAACCAGGAATG3' (SEQ ID NO:27)
Internal Primer: 5'AGGAAAAGGAGGCTGTGAC3' (SEQ ID NO:28)

HuTRDV5/TRAV29
External Primer: 5'GCAAGTTAAGCAAAATTCACC3' (SEQ ID NO:29)
Internal Primer: 5'CTGCTGAAGGTCCTACATTC3' (SEQ ID NO:30)

HuTRDV6/TRAV23
External Primer: 5'TTGATAGTCCAGAAAGGAGG3' (SEQ ID NO:31)
Internal Primer: 5'CGTTTGACTACTTTCCATGG3' (SEQ ID NO:32)

HuTRDV7/TRAV36
External Primer: 5'GACAAGGTGGTACAAAGCC3' (SEQ ID NO:33)
Internal Primer: 5'ATCTCTGGTTGTCCACGAG3'(SEQ ID NO:34)

HuTRDV8/TRAV38-2

External Primer: 5'CAGTCACTCAGTCTCAACCAG3' (SEQ ID NO:35)
Internal Primer: 5'TCTGGTACAAGCAGCCTC3' (SEQ ID NO:36)

HuTRDC
External Primer: 5'CTTCATATTTACCAAGCTTGACAG3' (SEQ ID NO:37)
Internal Primer: 5'GATGACAATAGCAGGATCAAAC3' (SEQ ID NO:38)

CD3δ sense
5'CCCTCACTCCTTCTCTAGGCGCCGGAATTCGCCAGGATGGAACATAGCACG3' (SEQ ID NO:39)
CD3δ antisense
5'CCACGTCTCCCGCCAACTTGAGAAGGTCAAAATTCAAAGTCTGTTTCACCGGTCCCTTGTTCCGAGCC3' (SEQ ID NO:40)

CD3γ sense
5'GAATTTTGACCTTCTCAAGTTGGCGGGAGACGTGGAGTCCAACCCAGGGCCCATGGAACAGGGGAAG3' (SEQ ID NO:41)
CD3γ antisense
5'CCTCGACGTCACCGCATGTTAGCAGACTTCCTCTGCCCTCAGATCTTCTATTCCTCCTCAAC3' (SEQ ID NO:42)
CD3ε sense
5'CAGAGGAAGTCTGCTAACATGCGGTGACGTCGAGGAGAATCCTGGCCCAATGCAGTCGGGCACTC3' (SEQ ID NO:43)
CD3ε antisense
5'GTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCGGATCCTCCGATGCGTCTCTG3' (SEQ ID NO:44)

CD3ζ sense
5'CTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCCATGAAGTGGAAAGTG3' (SEQ ID NO:45)
CD3ζ antisense
5'GAGGGAGAGGGGCGGAATTGATCCTCGAGCAATTGTTAGCGAGGGGCCAG3' (SEQ ID NO:46)

2A amino acid sequence F2A (foot-and-mouth disease virus)
VKQTLNFDLLKLAGDVESNPGP (SEQ ID NO:47)

2A amino acid sequence T2A (Thosea asigna virus)
EGRGSLLTCGDVEENPGP (SEQ ID NO:48)

2A amino acid sequence P2A (porcine teschovirus-1)
ATNFSLLKQAGDVEENPGP (SEQ ID NO:49)

HuLinkerDV1

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ATGATTTTCCTTATTCGCC3’ (SEQ ID NO:50)

HuLinkerDV2

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AGGAGAAGCGATCGGTAACTACTATATCAACTGGTACAGGAAGACCCAAGG3’ (SEQ
ID NO:51)

HuLinkerDV3

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gacactg3’ (SEQ ID NO:52)

HuLinkerDV4

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HuLinkerDV5

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HuLinkerDV6

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HuLinkerDV7

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HuLinkerDV8

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TGACACCAGTGAGAGTGATTATTATTTATTCTGGTACAAGCAGCCTCCCAG3' (SEQ ID
NO:57)

Paired amino acid sequence in TRGV9-CDR3 region

ALFIQELGKKIKV (SEQ ID NO:58
ALWDGPYYKKL (SEQ ID NO:60)
ALWDIPPGQELGKKIKV (SEQ ID NO:62)
ALWEAQELGKKIKV (SEQ ID NO:64)
ALWEARQELGKKIKV (SEQ ID NO:66)
ALWEGTRGQELGKKIKV (SEQ ID NO:68)
ALWEVGDQELGKKIKV (SEQ ID NO:70)
ALWEVHSELGKKIKV (SEQ ID NO:72)
ALWEVHSELGKKIKV (SEQ ID NO:72)
ALWEVLELGKKIKV (SEQ ID NO:74)
ALWEVLVGELGKKIKV (SEQ ID NO:76)
ALWEVPELGKKIKV (SEQ ID NO:78)
ALWEVQELGKKIKV (SEQ ID NO:80)
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ALWEVQELGKKIKV (SEQ ID NO:80)

ALWEVQELGKKIKV (SEQ ID NO:80)
ALWEVRELGKKIKV (SEQ ID NO:87)
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ALWEVRKQELGKKIKV (SEQ ID NO:90)
ALWEVTHNRQELGKKIKV (SEQ ID NO:92)
ALWGGAAGAYYKKL (SEQ ID NO:94)
ALWGGELGKKIKV (SEQ ID NO:96)
ALWVQELGKKIKV (SEQ ID NO:98)
ALWEAHQELGKKIKV (SEQ ID NO:100)
ALWEANKKL (SEQ ID NO:102)
ALWEAQELGKKIKV (SEQ ID NO:104)
ALWEATGLGKKIKV (SEQ ID NO:106)
ALWEDLELGKKIKV (SEQ ID NO:108)
ALWEKEELGKKIKV (SEQ ID NO:110)
ALWEKELGKKIKV (SEQ ID NO:112)
ALWEMTQELGKKIKV (SEQ ID NO:114)
ALWEPQELGKKIKV (SEQ ID NO:116)
ALWESKELGKKIKV (SEQ ID NO:118)
ALWEVGELGKKIKV (SEQ ID NO:120)
ALWEVHKLGKKIKV (SEQ ID NO:122)
ALWEVKELGKKIKV (SEQ ID NO:124)
ALWEVLQQELGKKIKV (SEQ ID NO:126)
ALWEVPVLGKKIKV (SEQ ID NO:128)
ALWEVQELGKKIKV (SEQ ID NO:80)
ALWEVQELGKKIKV (SEQ ID NO:80)
ALWEVQELGKKIKV (SEQ ID NO:80)
ALWEVRELGKKIKV (SEQ ID NO:87)
ALWEVRELGKKIKV (SEQ ID NO:87)
ALWEVRQELGKKIKV (SEQ ID NO:135)
ALWEVRVQELGKKIKV (SEQ ID NO:137)
ALWEVTELGKKIKV (SEQ ID NO:139)
ALWGRELGKKIKV (SEQ ID NO:141)

Paired amino acid sequence in TRDV2-CDR3 region

ACDVLGDTEGRLI (SEQ ID NO:59
ACDTVFTGGYSSWDTRQMF (SEQ ID NO:61)
ACDTLGETSSWDTRQMF (SEQ ID NO:63)

ACDSGGYSSWDTRQMF (SEQ ID NO:65)
ACDTLFPGGSATDKLI (SEQ ID NO:67)
ACDTVGAHTDKLI (SEQ ID NO:69)
ACDPLNTGGSFSLYTDKLI (SEQ ID NO:71)
ACDTGGFRSSWDTRQMF (SEQ ID NO:73)
ACDTGGFRSSWDTRQMF (SEQ ID NO:73)
ACDTVGMGIRLGDKLI (SEQ ID NO:75)
ACDILGINTDKLI (SEQ ID NO:77)
ACERLGDYVPDKLI (SEQ ID NO:79)
ACDRLLGDTDKLI (SEQ ID NO:81)
ACDTVAPRIGGLKYTDKLI (SEQ ID NO:82)
ACDTVGGPYTDKLI (SEQ ID NO:83)
ACDTVGGTAQ (SEQ ID NO:84)
ACDTVSGGSTPTWYTDKLI (SEQ ID NO:85)
ACDTVSIFTGDTTDKLI (SEQ ID NO:86)
ACDTILIFSPTGGDTDKLI (SEQ ID NO:88)
ACVPLGDWTDKLI (SEQ ID NO:89)
ACDTLGDDFDKLI (SEQ ID NO:91)
ACDTLLGTEAWDTRQMF (SEQ ID NO:93)
ACDGKTTDTDKLI (SEQ ID NO:95)
ACDLLGDTRYTDKLI (SEQ ID NO:97)
ACVGITGDTDKLI (SEQ ID NO:91)
ACDSLGDSVDKLI (SEQ ID NO:101)
ACDLLRGAGGQIDKLI (SEQ ID NO:103)
ACDTVGGAFDTDKLI (SEQ ID NO:105)
ACDMGDTRSWDTRQMF (SEQ ID NO:107)
ACDTVSWGKNTDKLI (SEQ ID NO:109)
ACDTGDWGSSWDTRQMF (SEQ ID NO:111)
ACDILDSTGGTDLTAQLF (SEQ ID NO:113)
ACDTVRNTGGYAFAGIDKLI (SEQ ID NO:115)
ACDKVLGDSSWDTRQMF (SEQ ID NO:117)
ACEGLGATQSSWDTRQMF (SEQ ID NO:119)
ACDKLLGDNELI (SEQ ID NO:121)
ACDSLLGKGTDKLI (SEQ ID NO:123)
ACDTLRGSADKLI (SEQ ID NO:125)
ACDTVPARHTDKLI (SEQ ID NO:127)
ACDTADRSSYTDKLI (SEQ ID NO:129)
ACDTLLGDPSSSWDTRQMF (SEQ ID NO:130)

ACDTLSGGYARTDKLI (SEQ ID NO:131)
ACDTVGILGDTGLGLI (SEQ ID NO:132)
ACDTIVSGYDGYDKLI (SEQ ID NO:133)
ACSILGDKTSDKLI (SEQ ID NO:134)
ACDTVSQRGGYSDKLI (SEQ ID NO:136)
ACDPLERVGGPANTDKLI (SEQ ID NO:138)
ACDVLGDTGDDKLI (SEQ ID NO:140)
ACDTVGSNTDKLI (SEQ ID NO:142)
ACDVLGDTEADKLI (SEQ ID NO:143)
ACDPLEGAGGHNTDKLI (SEQ ID NO:145)
GILGFVFTL (SEQ ID NO: 146)
TRGV1-3
External Primer: GCAGCTGGAGCAAACTG (SEQ ID NO: 147)
Internal Primer: CTGAATTATCGGTCACCAG (SEQ ID NO: 148)

TRGV4
External Primer: CAAATATCCTGTAAAGTTTTCATC (SEQ ID NO: 149)
Internal Primer: GTTTAGAGTTTCTATTATATGTCCTTGCAAC (SEQ ID NO: 150)

TRGV5
External Primer: GATATCTCAGGATCAGCTCTCC (SEQ ID NO: 151)
Internal Primer: TACCCGAAGACCAAACAAGAC (SEQ ID NO: 152)

TRGV6
External Primer: TCACCTCTGGGGTCATATG (SEQ ID NO: 153)
Internal Primer: AGAGGAAAGGAAATACGGC (SEQ ID NO: 154)

TRGV7
External Primer: CAACTTGGAAGAAAGAATAATGTC (SEQ ID NO: 155)
Internal Primer: CACCAAGCTAGAGGGGTC (SEQ ID NO: 156)

TRGC (reverse)
External Primer: CTTTTCTTTCCAATACACCC (SEQ ID NO: 157)
Internal Primer: TCDGGAAAGAACTTTTCAAGG (SEQ ID NO: 158)

TRDV1
External Primer: ACCCAAATGTTGCATCAG (SEQ ID NO: 159)
Internal Primer: GTCTCTGACAATCCAAGAAGG (SEQ ID NO: 160)

TRDV2
External Primer: TCTGTGCAGGTGGCAG (SEQ ID NO: 161)
Internal Primer: CCCTGGACTGCACCTATG (SEQ ID NO: 162)

TRDV4
External Primer: TGTATATTTGGAACCAGTTGC (SEQ ID NO: 163)
Internal Primer: GATCCTGCCTCCTTCTACTG (SEQ ID NO: 164)

TRDV5
External Primer: CATCACGCTGACCCAG (SEQ ID NO: 165)
Internal Primer: GCTCCACTGACCAGACAG (SEQ ID NO: 166)

TRDV6/TRAV15
External Primer: CASCTTYTTAGTGGAGAGATGG (SEQ ID NO: 167)
Internal Primer: AYTCTGTAGTCTTCCAGAAATCAC (SEQ ID NO: 168)

TRDV7/TRAV13
External Primer: TCCTTGGTTCTGCAGG (SEQ ID NO: 169)
Internal Primer: TGCAGGAGGGGGAGA (SEQ ID NO: 170)

TRDV8/TRAV14
External Primer: GCAGCAGGTGAGACAAAG (SEQ ID NO: 171)
Internal Primer: CTCTGACAGTCTGGGAAGG (SEQ ID NO: 172)

TRDV9/TRAV6-1/6-2
External Primer: CAGATGCAAGGTCAAGTGAC (SEQ ID NO: 173)
Internal Primer: GGAGAAGGTCCACAGCTC (SEQ ID NO: 174)

TRDV9/TRAV6-3/6-4
External Primer: AAGGTCCACAGCTCCTTC (SEQ ID NO: 175)
Internal Primer: CAACTGCCAACAACAAGG (SEQ ID NO: 176)

TRDV9/TRAV6-5/6-7
External Primer: GTTCTGGTATGTGCAGTATCC (SEQ ID NO: 177)
Internal Primer: TCCTTCCACTTGCAGAAAG (SEQ ID NO: 178)

TRDV10/TRAV4
External Primer: TCTGSTCTGAGATGCAATTTT (SEQ ID NO: 179)
Internal Primer: GGITIMAGGAACAAAGGAGAAT (SEQ ID NO: 180)

TRDV11/TRAV16
External Primer: GTACAAGCAAACAGCAAGTG (SEQ ID NO: 181)
Internal Primer: ATTATTCTCTGAACTTTCAGAAGC (SEQ ID NO: 182)

TRDV12/TRAV21
External Primer: GTGCACTTGCCTTGTAGC (SEQ ID NO: 183)
Internal Primer: AATAGTATGGCTTTCCTGGC (SEQ ID NO: 184)

TRDC (reverse)
External Primer: TGAAAGAATTTTGCATATGGTTC (SEQ ID NO: 185)
Internal Primer: GAGATGACTATAGCAGGGTCG (SEQ ID NO: 186)

WHAT IS CLAIMED IS:

1. A method for cloning a T cell receptor (TCR) from a single T cell, wherein said method comprises:
(a) performing RT-PCR with a primer mixture on a single T cell to obtain paired αβ or γδ TCR CDR3 DNA sequences comprising a partial variable (V) region, CDR3 region, and a partial constant (C) region,
(b) optionally sequencing the RT-PCR product obtained in step (a), and
(c) cloning the αβ or γδ TCR CDR3 DNA sequences obtained in step (a) into a corresponding TCRαβ or TCRγδ library.

2. The method of claim 1, wherein said T cell is a human or a mouse αβ or γδ T cell.

3. The method of claim 1 or claim 2, comprising sorting of single T cells prior to step (a).

4. The method of claim 3, wherein T cells are not stimulated prior to sorting.

5. The method of any one of claims 1-4, wherein the primer mixture comprises sense primers comprising T-cell receptor gamma variable (TRGV) sequences and/or T cell receptor delta variable (TRDV) sequences and antisense primers comprising T-cell receptor gamma constant (TRGC) sequences and/or T-cell receptor delta constant (TRDC) sequences.

6. The method of claim 5, wherein the primer mixture comprises 9 TRGV and 8 TRDV sense primers and single TRGC and TRDC antisense primers.

7. The method of claim 5, wherein the primer mixture comprises 5 external and 5 internal TRGV and 13 external and 13 internal TRDV sense primers and single TRGC and TRDC antisense primers.

8. The method of claim 5, wherein the TRGV and/or TRDV sense primers and the TRGC and/or TRDC antisense primers are selected from the primers listed in Table 1.

9. The method of claim 5, wherein the TRGV and/or TRDV sense primers and the TRGC and/or TRDC antisense primers are selected from the primers listed in Table 6.

10. The method of any one of claims 1-9, wherein the single cell RT-PCR of γδ or αβ TCR and sequencing are performed within not more than 2 days.

11. A method for constructing a TCRαβ and/or TCRγδ library in an expression vector, comprising:
(a) synthesizing multiple pairs of TRGV and TRDV DNA fragments or TRAV and TRBV DNA fragments with a 15-25bp overlap to the vector sequence based on the amplified sequence of the TRGV/TRDV or TRAV/TRBV pairings, respectively, and
(b) performing a two- or three-way ligation with a linearized expression vector.

12. The method of claim 11, wherein the expression vector is a retroviral or lentiviral expression vector.

13. The method of claim 11 or claim 12, wherein the ligation in step (b) is performed using Gibson Assembly Cloning techniques.

14. The method of any one of claims 11-13, wherein the TCRαβ and/or TCRγδ library is constructed after a single-cell amplification and synthesized paired TRGV/TRDV or TRAV/TRBV receptors based on the sequence data.

15. The method of claim 13, wherein said Gibson Assembly Cloning techniques are optimized to clone synthesized TRGV/TRAV and TRDV/TRBV DNA fragments using g-blocks or other synthesized DNA fragments.

16. The method of any one of clams 11-15, wherein the TCRαβ and/or TCRγδ library is constructed in 5 to 10 days.

17. The method of any one of claims 11-16, wherein the TCRαβ and/or TCRγδ library is used for drug screening or identification of TCRαβ- and/or TCRγδ-specific ligands.

18. The method of any one of claims 1-10, further comprising cloning the resulting αβ or γδ TCR CDR3 DNA sequences into the TCRαβ and/or TCRγδ library constructed using the method of any one of claims 11-17.

19. A method for rapid cloning of TCRαβ and/or TCRγδ chains using the TCRαβ and/or TCRγδ library constructed according to the method of any one of claims 11-17, said cloning method comprising substituting CDR3 regions of the existing clones in the TCRαβ and/or TCRγδ library with (i) products of RT-PCR performed using a primer mixture on a single T cell to obtain paired αβ or γδ TCR CDR3 DNA sequences comprising a partial variable (V) region, CDR3 region, and a partial constant (C) region and (ii) a linker DNA for overlap extension of PCR cloning.

20. The method of claim 19, wherein said T cell is a human or a mouse αβ or γδ T cell.

21. The method of claim 19 or claim 20, comprising sorting of single T cells prior to RT-PCR.

22. The method of claim 21, wherein T cells are not stimulated prior to sorting.

23. The method of any one of claims 19-22, wherein the primer mixture comprises sense primers comprising T-cell receptor gamma variable (TRGV) and/or T cell receptor delta variable (TRDV) and antisense primers comprising T-cell receptor gamma constant (TRGC) and/or T-cell receptor delta constant (TRDC) sequences.

24. The method of claim 23, wherein the primer mixture comprises 9 TRGV and 8 TRDV sense primers and single TRGC and TRDC antisense primers.

25. The method of claim 23, wherein the primer mixture comprises 5 external and 5 internal TRGV and 13 external and 13 internal TRDV sense primers and single TRGC and TRDC antisense primers.

26. The method of claim 23, wherein the TRGV and/or TRDV sense primers and the TRGC and/or TRDC antisense primers are selected from the primers listed in Table 1.

27. The method of claim 23, wherein the TRGV and/or TRDV sense primers and the TRGC and/or TRDC antisense primers are selected from the primers listed in Table 6.

28. The method of any one of claims 19-27, wherein said linkers are overlapping with the non-variant sequences of the TCRα/γ and TCRβ/δ single cell RT-PCR products.

29. The method of any one of claims 19-28, wherein said linker sequences are selected from those listed in Table 3.

30. The method of any one of claims 19-29, wherein the resulting TCRαβ and/or TCRγδ chains with CDR3 substitutions are used for T cell-mediated immunotherapy.

31. A TCRαβ and/or TCRγδ library constructed using the method of any one of claims 11-16.

32. A host cell comprising the TCRαβ and/or TCRγδ library construct of claim 31.

33. The host cell of claim 32 which is a Nur-77-GFP Jurkat 76 cell or a Nur-77-Luciferase Jurkat 76 cell.

34. A method for cloning a B cell receptor (BCR) from a single B cell, wherein said method comprises:
(a) performing RT-PCR with a primer mixture on a single B cell to obtain paired IgH or Igκ or Igλ CDR3 DNA sequences comprising a partial variable (V) region, CDR3 region, and a partial constant (C) region,
(b) optionally sequencing the RT-PCR product obtained in step (a), and
(c) cloning the IgH or Igκ or Igλ CDR3 DNA sequences obtained in step (a) into a corresponding BCR library.

35. The method of claim 34, wherein said B cell is a human or a mouse B cell.

36. The method of claim 34 or claim 35, comprising sorting of single B cells prior to step (a).

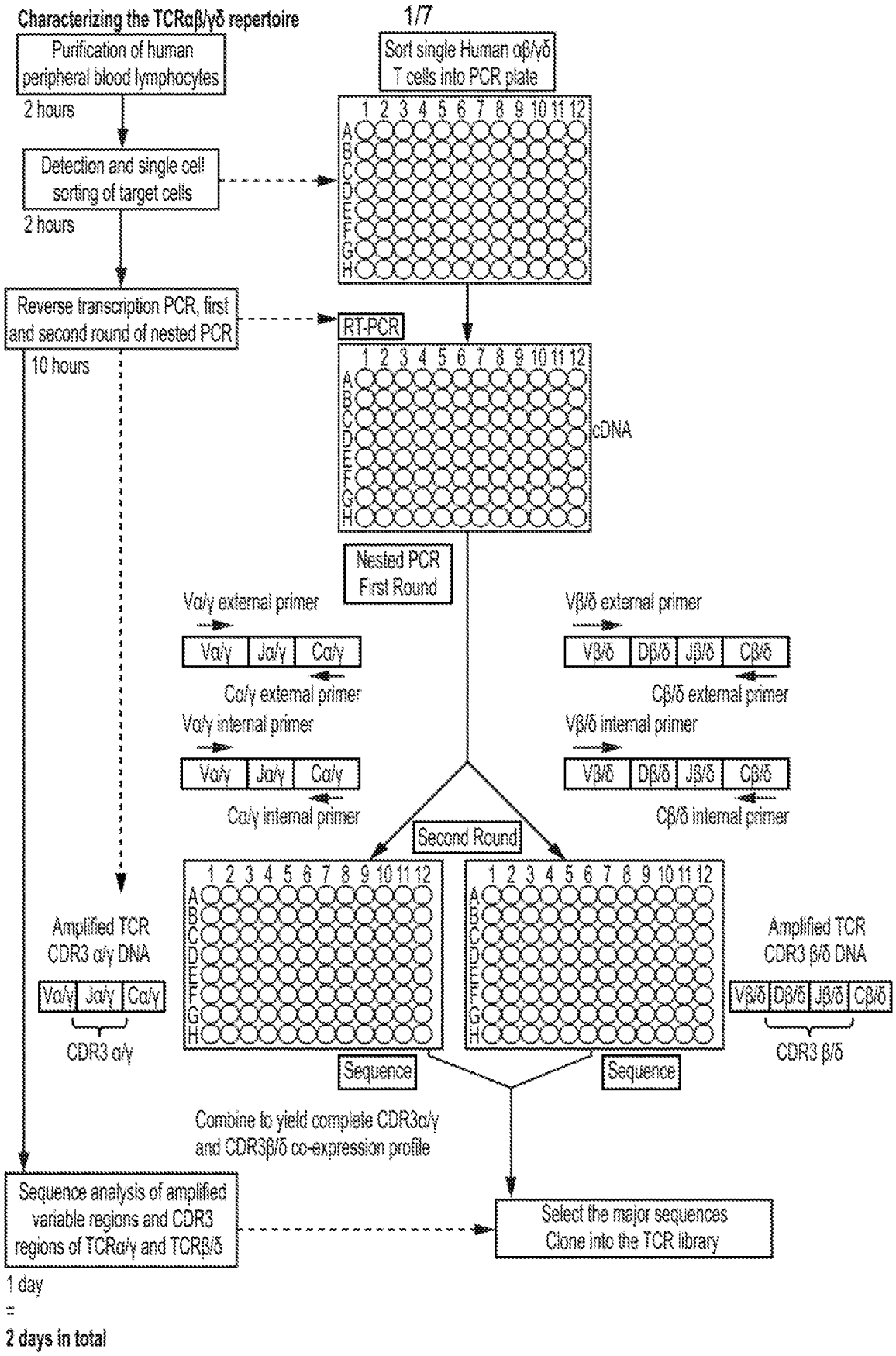
Characterizing the TCRαβ/γδ repertoire
1/7
Purification of human peripheral blood lymphocytes
2 hours
Detection and single cell sorting of target cells
2 hours
Sort single Human αβ/γδ T cells into PCR plate
Reverse transcription PCR, first and second round of nested PCR
10 hours
RT-PCR
cDNA
Nested PCR First Round
Vα/γ external primer
Vα/γ Jα/γ Cα/γ
Cα/γ external primer
Vα/γ internal primer
Vα/γ Jα/γ Cα/γ
Cα/γ internal primer
Vβ/δ external primer
Vβ/δ Dβ/δ Jβ/δ Cβ/δ
Cβ/δ external primer
Vβ/δ internal primer
Vβ/δ Dβ/δ Jβ/δ Cβ/δ
Cβ/δ internal primer
Second Round
Amplified TCR CDR3 α/γ DNA
Vα/γ Jα/γ Cα/γ
CDR3 α/γ
Amplified TCR CDR3 β/δ DNA
Vβ/δ Dβ/δ Jβ/δ Cβ/δ
CDR3 β/δ
Sequence
Sequence
Combine to yield complete CDR3α/γ and CDR3β/δ co-expression profile
Sequence analysis of amplified variable regions and CDR3 regions of TCRα/γ and TCRβ/δ
Select the major sequences Clone into the TCR library
1 day
=
2 days in total

FIG. 1A

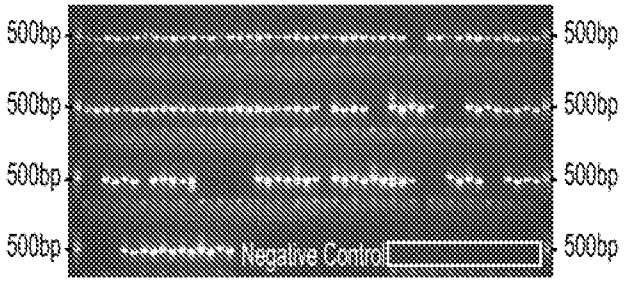
500bp
500bp
500bp
500bp
500bp
500bp
500bp
500bp
Negative Control

FIG. 1B

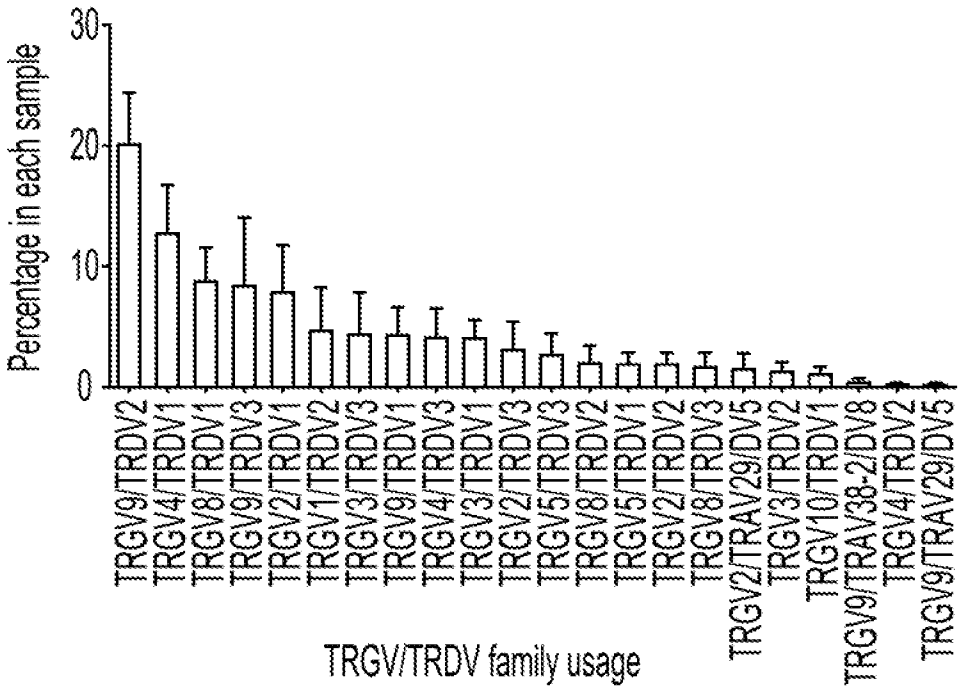
Percentage in each sample
30
20
10
0
TRGV9/TRDV2
TRGV4/TRDV1
TRGV8/TRDV1
TRGV9/TRDV3
TRGV2/TRDV1
TRGV1/TRDV2
TRGV3/TRDV3
TRGV9/TRDV1
TRGV4/TRDV3
TRGV3/TRDV1
TRGV2/TRDV3
TRGV5/TRDV3
TRGV8/TRDV2
TRGV5/TRDV1
TRGV2/TRDV2
TRGV8/TRDV3
TRGV2/TRAV29/DV5
TRGV3/TRDV2
TRGV10/TRDV1
TRGV9/TRAV38-2/DV8
TRGV4/TRDV2
TRGV9/TRAV29/DV5
TRGV/TRDV family usage

FIG. 1C

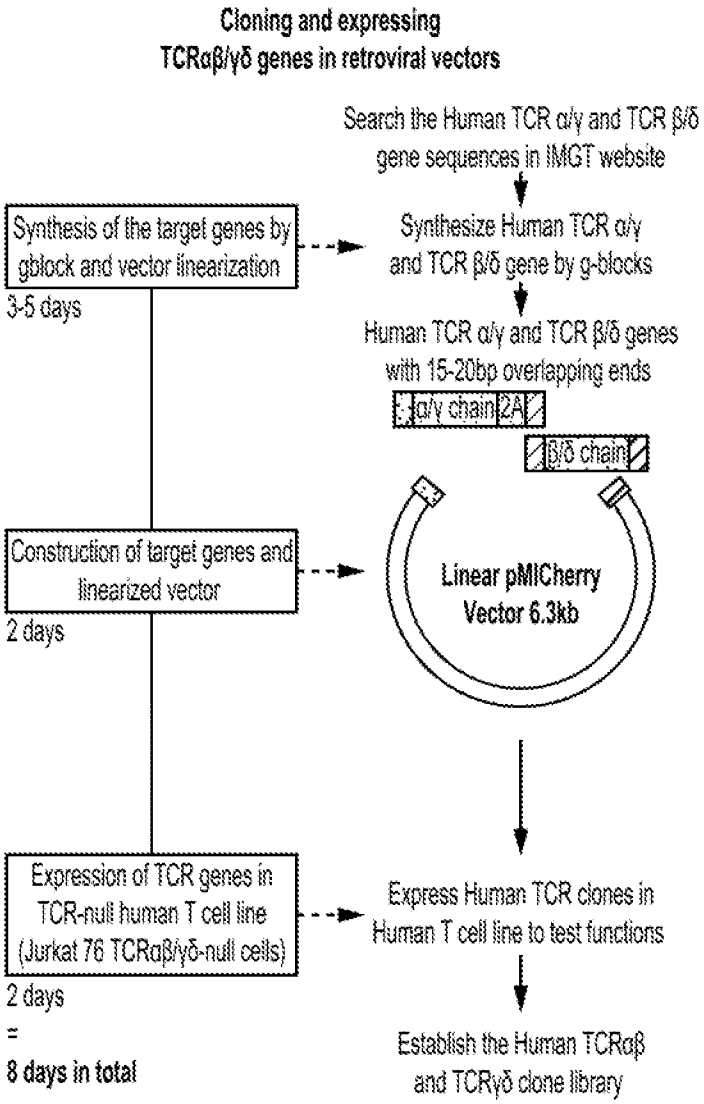
Cloning and expressing
TCRαβ/γδ genes in retroviral vectors
Search the Human TCR α/γ and TCR β/δ gene sequences in IMGT website
Synthesis of the target genes by gblock and vector linearization
3-5 days
Synthesize Human TCR α/γ and TCR β/δ gene by g-blocks
Human TCR α/γ and TCR β/δ genes with 15-20bp overlapping ends
α/γ chain
2A
β/δ chain
Construction of target genes and linearized vector
2 days
Linear pMICherry Vector 6.3kb
Expression of TCR genes in TCR-null human T cell line (Jurkat 76 TCRαβ/γδ-null cells)
2 days
=
8 days in total
Express Human TCR clones in Human T cell line to test functions
Establish the Human TCRαβ and TCRγδ clone library

FIG. 2A

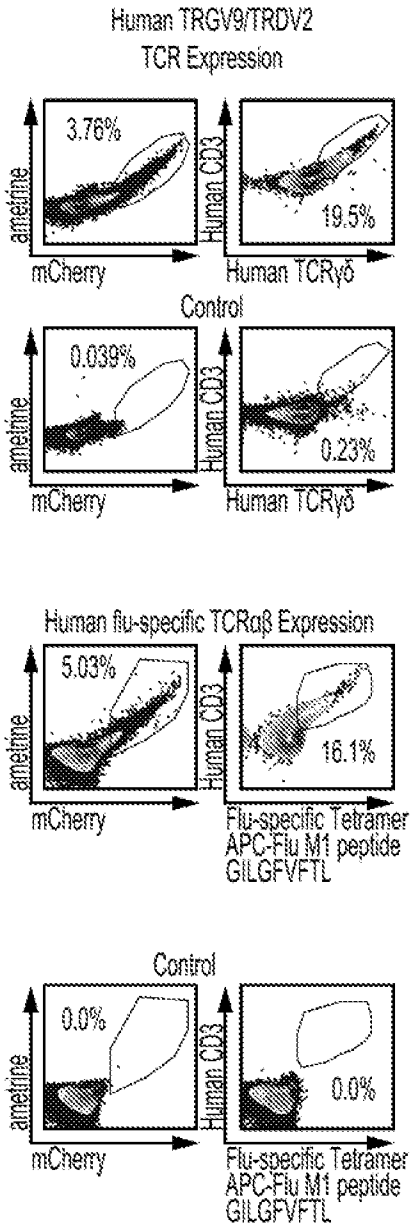
Human TRGV9/TRDV2
TCR Expression
3.76%
19.5%
ameinne
mCherry
Human CD3
Human TCRγδ
Control
0.039%
0.23%
Human flu-specific TCRαβ Expression
5.03%
16.1%
Flu-specific Tetramer
APC-Flu M1 peptide
GILGFVFTL
0.0%
0.0%

FIG. 2B

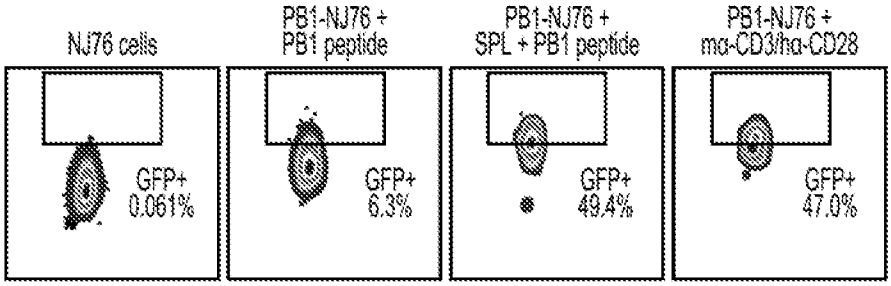

FIG. 3A

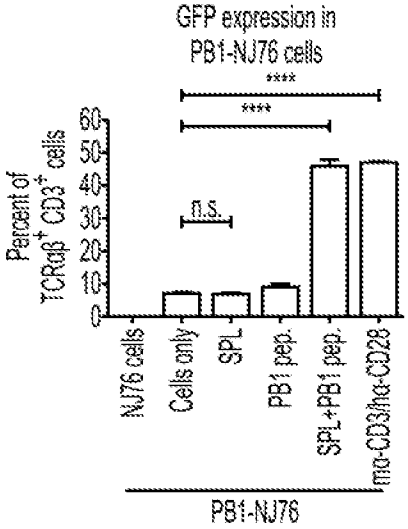

FIG. 3B

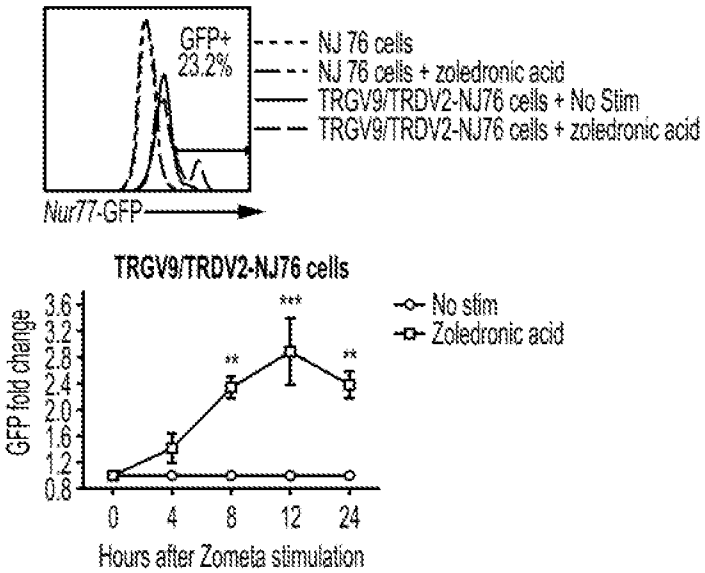

FIG. 3C

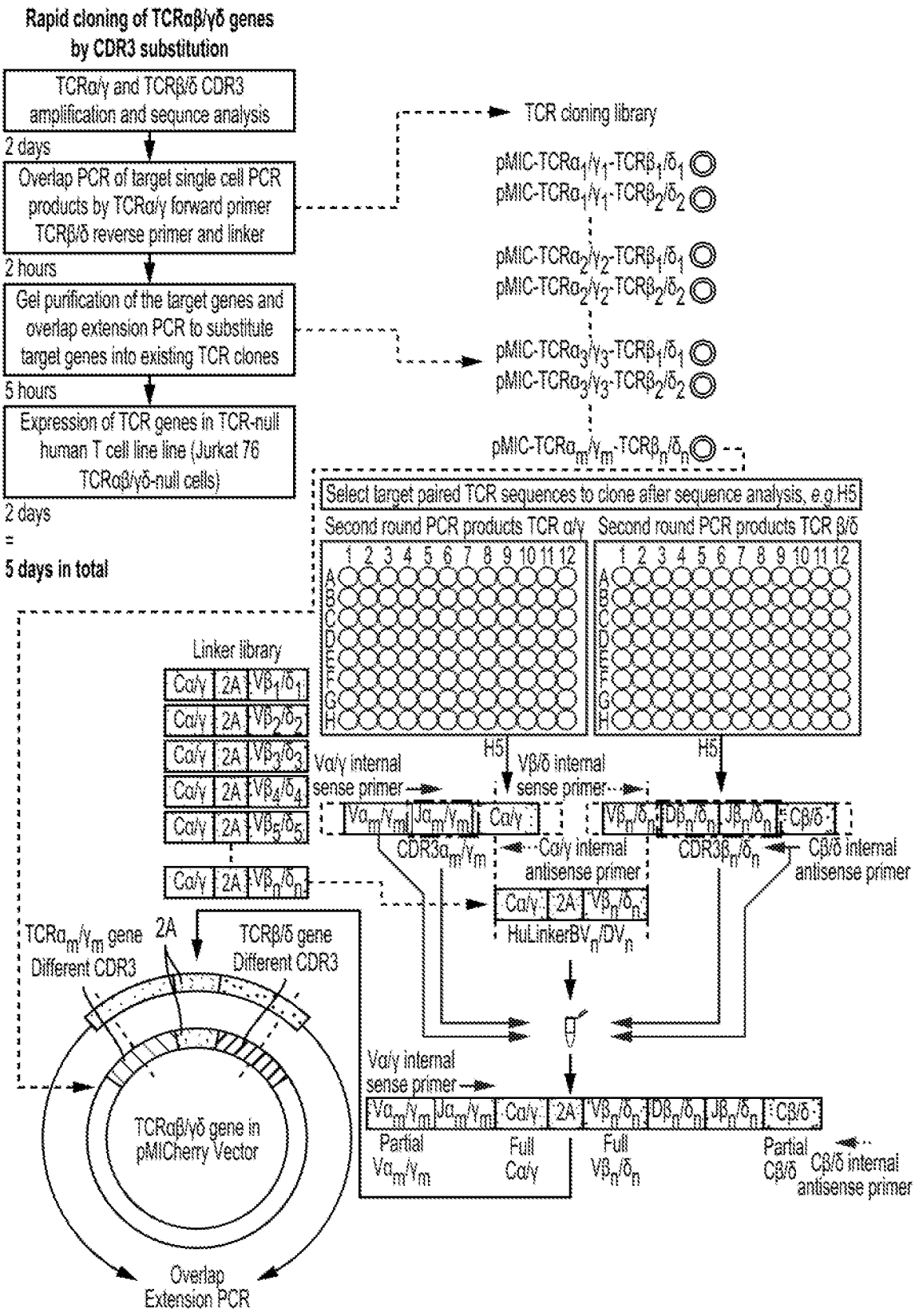
Rapid cloning of TCRαβ/γδ genes by CDR3 substitution
TCRα/γ and TCRβ/δ CDR3 amplification and sequnce analysis
2 days
Overlap PCR of target single cell PCR products by TCRα/γ forward primer TCRβ/δ reverse primer and linker
2 hours
Gel purification of the target genes and overlap extension PCR to substitute target genes into existing TCR clones
5 hours
Expression of TCR genes in TCR-null human T cell line line (Jurkat 76 TCRαβ/γδ-null cells)
2 days
=
5 days in total
TCR cloning library
pMIC-TCRα1/γ1-TCRβ1/δ1
pMIC-TCRα1/γ1-TCRβ2/δ2
pMIC-TCRα2/γ2-TCRβ1/δ1
pMIC-TCRα2/γ2-TCRβ2/δ2
pMIC-TCRα3/γ3-TCRβ1/δ1
pMIC-TCRα3/γ3-TCRβ2/δ2
pMIC-TCRαm/γm-TCRβn/δn
Select target paired TCR sequences to clone after sequence analysis, e.g.H5
Second round PCR products TCR α/γ
Second round PCR products TCR β/δ
Linker library
H5
Vα/γ internal sense primer
Vβ/δ internal sense primer
CDR3αm/γm
Cα/γ internal antisense primer
CDR3βn/δn
Cβ/δ internal antisense primer
HuLinkerBVn/DVn
TCRαm/γm gene Different CDR3
2A
TCRβ/δ gene Different CDR3
TCRαβ/γδ gene in pMICherry Vector
Overlap Extension PCR
Vα/γ internal sense primer
Partial Vαm/γm
Full Cα/γ
Full Vβn/δn
Partial Cβ/δ
Cβ/δ internal antisense primer

FIG. 4

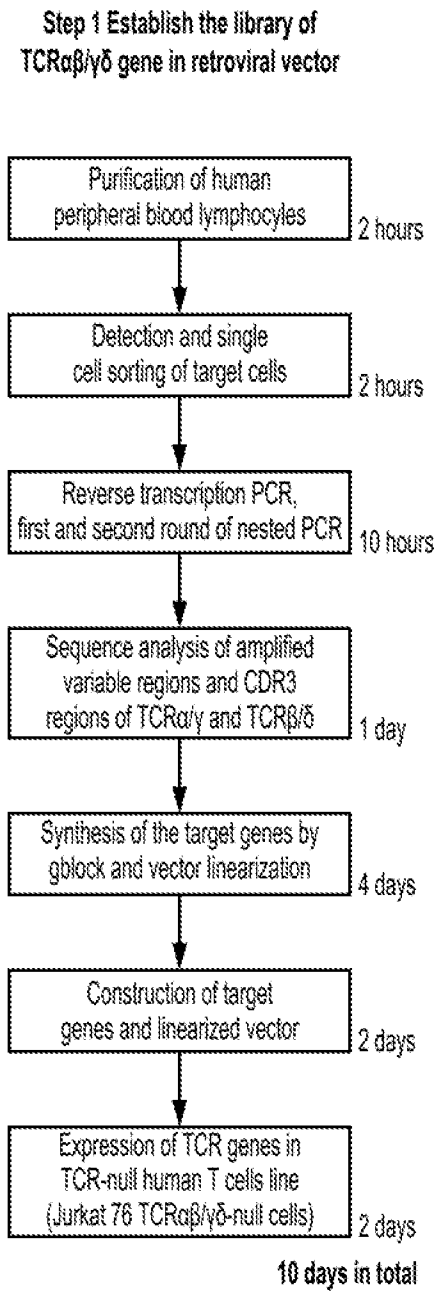
Step 1 Establish the library of TCRαβ/γδ gene in retroviral vector
Purification of human peripheral blood lymphocyles
2 hours
Detection and single cell sorting of target cells
2 hours
Reverse transcription PCR, first and second round of nested PCR
10 hours
Sequence analysis of amplified variable regions and CDR3 regions of TCRα/γ and TCRβ/δ
1 day
Synthesis of the target genes by gblock and vector linearization
4 days
Construction of target genes and linearized vector
2 days
Expression of TCR genes in TCR-null human T cells line (Jurkat 76 TCRαβ/γδ-null cells)
2 days
10 days in total

FIG. 5A

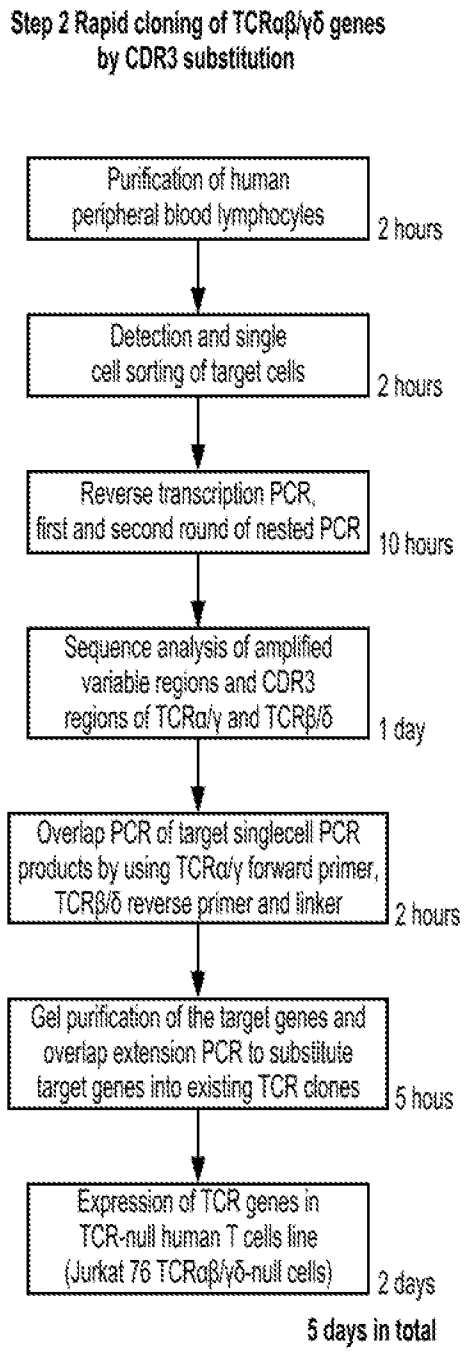
Step 2 Rapid cloning of TCRαβ/γδ genes by CDR3 substitution
Purification of human peripheral blood lymphocyles
2 hours
Detection and single cell sorting of target cells
2 hours
Reverse transcription PCR, first and second round of nested PCR
10 hours
Sequence analysis of amplified variable regions and CDR3 regions of TCRα/γ and TCRβ/δ
1 day
Overlap PCR of target singlecell PCR products by using TCRα/γ forward primer, TCRβ/δ reverse primer and linker
2 hours
Gel purification of the target genes and overlap extension PCR to substitute target genes into existing TCR clones
5 hous
Expression of TCR genes in TCR-null human T cells line (Jurkat 76 TCRαβ/γδ-null cells)
2 days
5 days in total

FIG. 5B

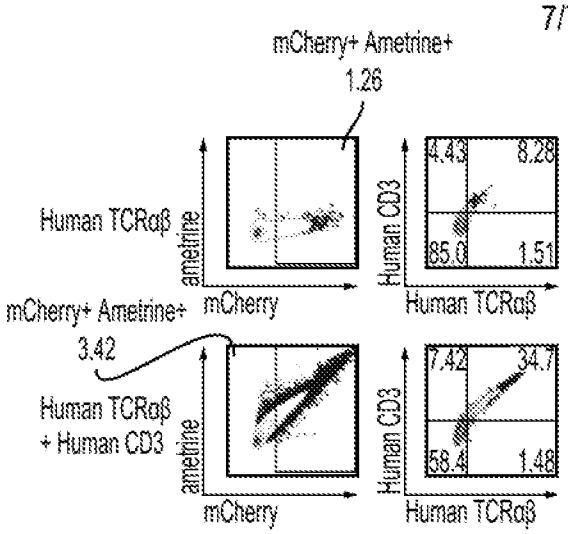

FIG. 6A

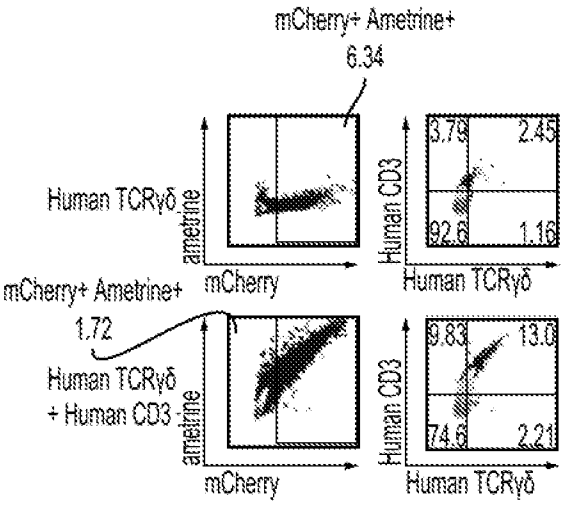

FIG. 6B

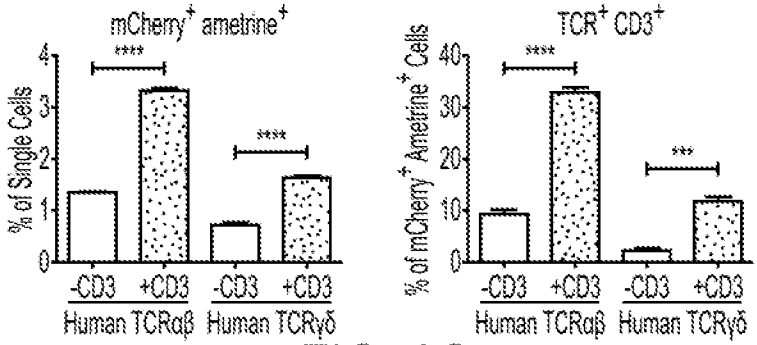

FIG. 6C

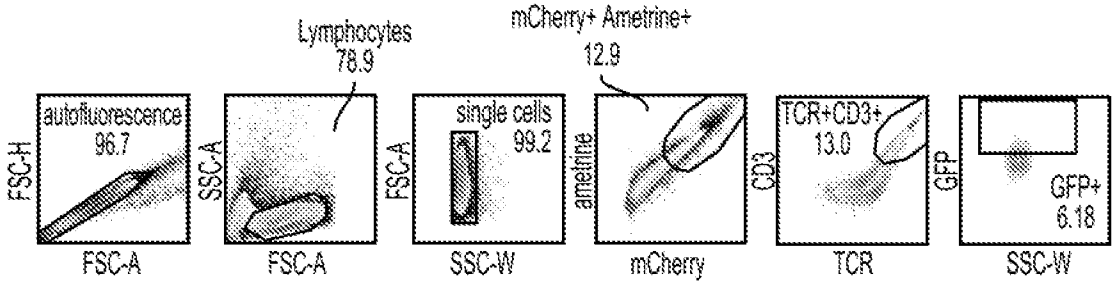

FIG. 7

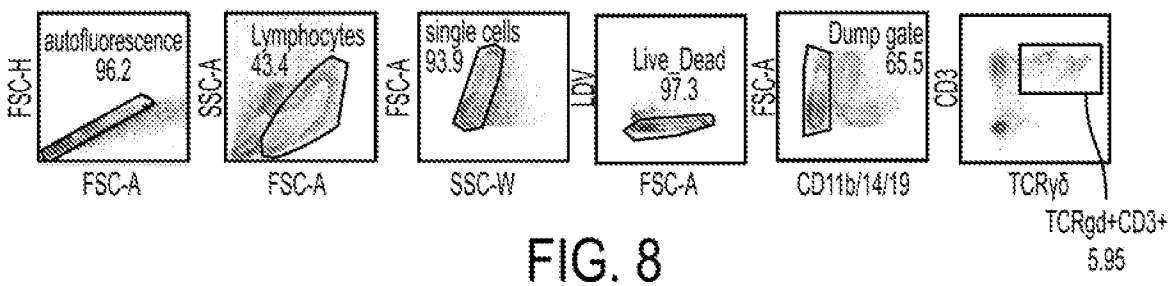

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/064735

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/064735

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-10, 14, 16-33
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/064735

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/17; C07K 14/705; C12N 15/10 (2017.01)
CPC - A61K 38/177; A61K 38/1774; C07K 14/7051; C12N 15/1082; C12N 15/1086; C12N 15/1096 (2017.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/133.1; 424/139.1; 435/6.11; 435/6.12; 506/18; 530/350 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007/0141048 A1 (OLEKSIEWICZ et al) 21 June 2007 (21.06.2007) entire document	34-36
Y	US 2011/0142842 A1 (OLWEUS et al) 16 June 2011 (16.06.2011) entire document	1-4, 11-13, 15
Y	US 2015/0337369 A1 (LELAND STANFORD JUNIOR UNIVERSITY) 26 November 2015 (26.11.2015) entire document	1-4
Y	DASH et al. "Paired analysis of TCRα and TCRβ chains at the single-cell level in mice," Journal of Clinical Investigation, 31 January 2011 (31.01.2011), Vol. 121, No. 1, Pgs. 288-295. entire document	11-13, 15
Y	VOGL et al. "Restriction site free cloning (RSFC) plasmid family for seamless, sequence independent cloning in Pichia pastoris," Microbial Cell Factories, 14 July 2015 (14.07.2015), Vol. 103, Pgs. 1-15. entire document	11-13, 15
A	KOBAYASHI et al. "A new cloning and expression system yields and validates TCRs from blood lymphocytes of patients with cancer within 10 days," Nature Medicine, 30 November 2013 (30.11.2013), Vol. 19, No. 11, Pgs. 1542-1546. entire document	1-4, 11-13, 15, 34-36
A	US 2015/0203886 A1 (UNIVERSITY OF TOYAMA) 23 July 2015 (23.07.2015) entire document	1-4, 11-13, 15, 34-36
A	WANG et al. "T-cell receptor αβ diversity inversely correlates with pathogen-specific antibody levels in human cytomegalovirus infection," Science Translational Medicine, 04 April 2012 (04.04.2012), Vol. 4, Issue 128, Pgs. 1-11. entire document	1-4, 11-13, 15, 34-36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 January 2017	08 FEB 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)