Chapter 14

Gene Profiling and T Cell Receptor Sequencing from Antigen-Specific CD4 T Cells

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Abstract

The paucity of pathogenic T cells in circulating blood limits the information delivered by bulk analysis. Toward diagnosis and monitoring of treatments of autoimmune diseases, we have devised single-cell analysis approaches capable of identifying and characterizing rare circulating CD4 T cells.

Key words CD4 T cells, Single-cell analysis, TCR sequences, Autoimmunity, Peripheral blood, Microfluidics

1 Introduction

One of the hallmarks of the immune system is the heterogeneity of each of the cell populations it is composed of. The classical way of addressing this heterogeneity has been to subset each population with a series of cell surface markers amenable to fluorescence-activated cell sorting techniques (FACS). Counterintuitively, FACS analysis, a technique based on single-cell isolation principles, is usually followed by population analysis and the ignorance of heterogeneity within the studied population. Like many of our colleagues, we have been questioning what heterogeneity might mean in physiology and diseases. When studying T cells for instance, we have known for decades that plasticity allows interconversion of effector functions when environmental cues change. The extent of this variability and the specificity of each effector program in the context of antigen-driven immune responses must be studied to devise better strategies for vaccination and better understanding of autoimmunity. Attempts at studying gene expression or T cell receptor (TCR) usage of single T cells are not new but have met very limited success until recently [1, 2]. The development of microfluidic technologies and better molecular biology reagents

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have revolutionized the field over the past 5 years. We have moved from days when the efficiency of paired sequencing of α and β TCR chains was exceptional [3], to a situation where this technique is routine and whole exome sequencing from single cells is the new frontier [4]. Like for FACS analysis, a small number of platforms are now available for the genomic interrogation of single cells. Droplet-based microfluidic is one system [5], whereas purely microfluidic system is the other [6]. We used the latter, mainly because it entered the field first and we committed to this approach early on. In any case, we have used this approach to study antigen-specific $CD4^+$ T cells in the context of vaccines and autoimmunity. For efficiency, and in order to collect the most information from a single cell, we have developed a workflow that allows the profiling of 96 genes by quantitative PCR (qPCR), as well as the sequencing of paired α-β TCR chains from the same original reverse transcription of pMHC-FACS sorted T cells. The technique has proven robustness, high reproducibility, and can be used for routine analysis of antigen-specific T cells [7]. For our purpose, we have been using a Fluidigm Biomark HD system and its two main components, the Dynamic Array for qPCR, and the Access Array for the making of libraries for next-generation sequencing.

2 Materials

2.1 Cell Sorting Components

1. Allegra X-14R centrifuge (Beckman Coulter, Inc., Brea, CA, USA).
2. PBS: Dulbecco's Phosphate-Buffered Saline, 1× (Corning, Corning, NY, USA).
3. Cell strainer, 70 μm (ThermoFisher Scientific, Waltham, MA, USA).
4. FACS buffer: 2% FCS, 2 mM EDTA in PBS, sterile filtered.
5. RBC Lysis Buffer: 0.165 M NH_4Cl in deionized water, sterile filtered.
6. Fc block: 20 μg/mL in FACS buffer.
7. Tetramers: In a molar ratio of 1:5, biotinylated MHC molecules expressed with peptides of interest are mixed in sterile PBS with PE-labeled streptavidin (Life Technologies, Carlsbad, CA, USA) (*see* **Note 1**). The reaction is incubated overnight, in darkness, at room temperature.
8. Antibodies: For the samples the following antibodies are mixed together 1:200 in FACS buffer: FITC anti-mouse CD3ε, APC/Cy7 anti-mouse CD4, APC anti-mouse/human CD11b, APC anti-mouse/human CD45R/B220, APC anti-mouse CD49b, and APC anti-mouse CD8a (all from BioLegend, San Diego, CA, USA). For the compensations the following antibodies are

mixed individually 1:200 in FACS buffer: FITC anti-mouse CD3ε, APC/Cy7 anti-mouse CD4, APC anti-mouse/human CD45R/B220, and PE anti-mouse CD4 (BioLegend, San Diego, CA, USA).

9. Hard-Shell 96-Well Semi-Skirted PCR plates, High-Profile (BioRad, Hercules, CA, USA).
10. RT Mix Solution 1 (master mix for one plate): 144 μL 5×VILO reaction mix (SuperScript VILO cDNA Synthesis Kit, ThermoFisher Scientific), 36 μL 20 U/μL SUPERase-In (ThermoFisher Scientific), 30 μL 10% NP40 (ThermoFisher Scientific), and 390 μL Nuclease-free water (TEKnova, Hollister, CA, USA).
11. FACSAria I cell sorter (BD Biosciences, San Jose, CA, USA).

2.2 Reverse Transcription and Pre-amplification Components

1. T100 Thermal Cycler (BioRad).
2. RT Mix Solution 2 (master mix for one plate): 18.0 μL 10× SuperScript Enzyme Mix (SuperScript VILO cDNA Synthesis Kit, ThermoFisher Scientific), 14.4 μL T4 Gene 32 Protein (New England Biolaps, Ipswich, MA, USA), and 87.6 μL Nuclease-free water (TEKnova).
3. 10× STA (Specific Target preAmplication) Primer Mix: pool all primer pairs (100 μM) (*see* **Notes 2** and **3**) in equal volumes, and dilute in 1× DNA Suspension Buffer (TEKnova) giving a final concentration of 500 nM of each primer. Make several aliquots for one time use, and store at −20°C.
4. STA Reaction Mix (master mix for one plate): 780 μL TaqMan PreAmp MasterMix (Life Technologies), 156 μL 10× STA Primer Mix, and 7.8 μL 0.5 M EDTA, pH 8.0 (Life Technologies).
5. Exonuclease (master mix for one plate): 72 μL Exonuclease I Reaction Buffer (10×), 144 μL Exonuclease I (20 U/μL) (New England Biolabs), and 504 μL Nuclease-free water (TEKnova).

2.3 qPCR Components

1. IFC Controller HX (Fluidigm, San Francisco, CA, USA).
2. 96.96 Dynamic Array IFC (Fluidigm).
3. Control line fluid (150 μL) (Fluidigm).
4. Sample Pre-Mix Solution (master mix for one plate): 480 μL 2× Sso Fast EvaGreen Supermix With Low ROX (BioRad) and 48 μL 20× DNA Binding Dye Sample Loading Reagent (Fluidigm).
5. qPCR Primer Plate: 96 primer pairs (100 μM) (DELTAgene Assays, Fluidigm (*see* Table 1)) are separated in a 96-well master plate. Aliquot into working plates for use up to five times and store at −20°C (*see* **Note 4**).

6. Assay Mix Solution (master mix for one plate): 540.0 μL 2× Assay Loading Reagent (Fluidigm) and 486.0 μL 1× DNA Suspension Buffer (TEKnova).
7. BioMark HD instrument (Fluidigm).

2.4 Access Array Components

1. IFC controller AX (Fluidigm) (*see* **Note 5**).
2. 48.48 Access Array IFC (Fluidigm).
3. Control line fluid (300 μL) (Fluidigm).
4. 1× Access Array Harvest solution (Fluidigm).
5. 1× Access Array Hydration Reagent v2 (Fluidigm).
6. Internal TCR 5′ Primer Plate (20×): In half of a 96-well plate add to each well 5 μL 20× Access Array Loading Reagent (Fluidigm) and 87 μL Nuclease-free water (TEKnova). Add 8 μL of each TCR specific internal 5′ primer (50 μM) (IDT, Coralville, IA, USA) (*see* Table 3) to the individual wells, giving a final concentration of each primer of 4 μM (*see* **Notes 3** and **6**). Aliquot into working plates for use to up to five times and store at −20°C.
7. Sample Pre-Mix Solution (master mix for one sample plate): 60 μL 10× FastStart High Fidelity Reaction Buffer Without $MgCl_2$ (Roche, Basel, Switzerland), 108 μL 25 mM $MgCl_2$ (Roche), 30 μL DMSO (Roche), 12 μL 10 mM PCR Grade Nucleotide Mix (Roche), 6 μL 5 U/μL FastStart High Fidelity Enzyme Blend (Roche), 30 μL 20× Access Array Loading Reagent (Fluidigm), 186 μL Nuclease-free water (TEKnova).
8. Barcoded Internal TCR 3′ Primer Plate: In half of a 96-well plate make 48 pairs of 3′ primers with matching barcodes (IDT) binding the constant regions of TCRα and TCRβ (TRAC and TRBC) respectively (*see* Table 4 and **Note 3**). Combine equal volumes of each primer (100 μM) giving a final concentration of 50 μM. Aliquot into working plates for use to up to five times and store at −20 °C.

2.5 Library Construction Components for Illumina Sequencing

1. AMPure XP magnetic beads (Beckman Coulter, Carlsbad, CA, USA).
2. Magnetic Separator.
3. 2100 Bioanalyzer instrument and High-Sensitivity DNA chip (Agilent, Santa Clara, CA, USA).
4. 80% ethanol.
5. Nuclease-Free water.
6. Qubit –Fluorometer and ds DNA high sensitivity assay (ThermoFisher Scientific, Waltham, MA, USA) (similar fluorescent DNA quantitative detection methods can be substituted).
7. NEBNext Ultra DNA Library Prep Kit for Illumina.

Table 1
96 primer pairs for pre-amplification and qPCR

Target	Forward	Reverse	Target	Forward	Reverse
Bcl6	GGGGAAACCCAGTCAG AGTA	CTCAGAGAAACGGCAGT CAC	**Aim2**	CTGCTACAGAAGTCTGTCCTCA	TAGCTTTCAGCACC GTGACA
Ccr2	TGAGGCTCATCTTTGCC ATCA	GGATTCCTGGAAGGT GGT CAA	**Bcl2**	ATGTGTGTGGAGAGCGTCAA	GATGCCGGTTCAGGT ACTCA
Ccr3	CTGGACTCATAAAGG ACTTAGCA	GTGGTGCCCACTCATAT TCA	**Ccr1**	TCCTCAAAGGCCCAGAAACA	GCTGAGGAACTGGT CAGGAA
Ccr4	GACTGTCCTCAGGATC ACTTTCA	CCTGGGTGGTGTCTGT GAC	**Ccr7**	GTGGTGGCTCTCCTTGTCA	GGTATTCTCGC CGATGTAGTCA
Ccr5	GGAGGTGAGACATCC GTTCC	GGTCGGAACTGACCCTT GAAA	**Pdl-1**	CAGCCTGCTGTCACTTGCTA	GACGTTGCTGCCATA CTCCA
Ccr6	AAGGCACATATGCGG TCAAC	CCTGGACGATGGCAATG TAC	**Cd44**	TTCCTTCGATGGACCGGTTA	TACTCGCCCTTCTT GCTGTA
Cd28	CTGCTGTTCTTGGCTC TCAAC	GGGCGACTGCTTTACCA AAA	**Ceacam1**	GCGACTGTGCGATTTCATGTA	AGGTCAGGGTCACA GAGTCTA
Cd3e	TGCTACACACCAGCC TCAAA	AGGTCCACCTCCACACA GTA	**Cxcl10**	GGGCCATAGGGAAGCTTGAA	GGATTCAGACATCT CTGCTC ATCA
Cd4	AAGGGACACTGCATC AGGAA	CCCATCACCTCACAGGTC AA	**Cxcr3**	ACCAGCCAAGCCATGTACC	GGGAGAGGTGCTG TTTTCCA
Cd40	CTATGGGGCTGCTTG TTGAC	TCGTGGAGGTACTGTTT GTCA	**Cxcr4**	GGTAACCACCACGGCTGTA	CAGGGTTCCTTGTTG GAGTCA

(continued)

Table 1
(continued)

Target	Forward	Reverse	Target	Forward	Reverse
Cd80	AGTCGTCGTCATCGTT GTCA	GTTTGTTTCTCTGCT TGCCTCA	**Fyn**	TGGCTCGGTTGATTGAAG ACA	GGGCTGTCCACTTA ATGGGAA
Cd86	CATGGGCTTGGCAAT CCTTA	CATTGAAATAAGCTTGCGT CTCC	**Icos**	TGACCCACCTCCTTTTCAA GAA	TACGGGTAGCCAGA GCTTCA
Cd8a	CAGCAAGGAAAACGAAGG CTAC	GCAGCACTGGCTTGGTA GTA	**Ifi44**	TCTTGGTGGGCTGTGAT GAA	TCATCCTTGGCCT TGATGGAA
Ctla4	GGACTTGGCCTTTTGT AGCC	CTGAAGGTTGGGTCACCT GTA	**Ifi44l**	GCAAACATGACAGAAACTG TGAC	CAACCTTCGCTCT GAAGCATAA
Foxp3	CCCACACCTCTTCTTCC TTGAA	GACGGTGCCACCATGA CTA	**Ifit1**	GCTACCACCTTTACAGC AACC	AGTGACATCTCAGC TGAAGCA
Gapdh	CAAGGTCATCCCAGAG CTGAA	CAGATCCACGACGGAC ACA	**Ifit3**	TTTTCCTGGCACCATGA ACC	TCCACAGCACATC TGTCTCA
Gata4	GTAATGCCTGCGGCC TCTA	TGGTTTGAATCCCCTCC TTCC	**Ifngr1**	CTGGGAATACCAGAACATG TCAC	TGCAGGAATCAG TCCAGGAA
Gsk3a	GAACTGGTGGCCATC AAGAA	ATTGCAGTGGTCCAGCT TAC	**Il12rb**	GCGTTGAGAAGACATCGT TCC	TGGAAACCCTGT AGCAACTCA
Gsk3b	GCAGCCTTCAGCTTT TGGTA	GGAGTTGCCACTACTGTG GTTA	**Il18r1**	AAGAGGACAGCTCAGACC CTAA	GAAGCATGCAGT TTGCCTTCA
Hprt	CAGTACAGCCCCAAA ATGGTTA	AGTCTGGCCTGTATCCA ACA	**Il27r**	GGTCCCAACCTTTCACTT CAC	AAACCCCACAGG GACAGAAA

Icam1	AGGGCTGGCATTGTT CTCTA	TGTCGAGCTTTGGGAT GGTA	**Irf1**	TACCTGGGTCAGGACTTG GATA	TCAGAGAGACT GCTGCTGAC
Ifng	CCACGGCACAGTCA TTGAAA	GCCAGTTCCTCCAGATAT CCAA	**Irf2**	GTGGCTGGAGGAGCAGAT AAA	GCATCCAGGGG ATCTGGAAAA
Il10	AAAGGACCAGCTGGAC AACA	TAAGGCTTGGCAACCCAA GTA	**Irf4**	TCCCCATTGAGCCAAGC ATA	CGAGGATGTCCC GGTAATACA
Il12b	ATCGTTTTGCTGGTG TCTCC	GGAGTCCAGTCCACCT CTAC	**Irf7**	GATCCGCATAAGGTGTAC GAAC	TGCTGAGGCTCA CTTCTTCC
Il17A	CAGACTACCTCAACCG TTCCA	CACTGAGCTTCCCAGATC ACA	**Isg15**	GGACGGTCTTACCCTTT CCA	TCGCTGCAGTTCT GTACCA
Il1r2	AGTGCAGCAAGACTC TGGTA	AGACCTTGAGTTCCACAG ACA	**Jak1**	TGGCCCGTTCATCAAGC TTA	CAGGGGATTCGCT CTATGCA
Il2	CCCAGGATGCTCACCTT CAAA	CCGCAGAGGTCCAAGT TCA	**Jak2**	TGCCGCAGGACAAAGAAT ACb	TGCTCTCCGTCAA GGATTCA
Il-21	GATCCTGAACTTCTAT CAGCTCCA	GGCCTTCTGAAAACAGG CAAA	**Ly6e**	GTTTCATGCCAGGAGAAA GACC	AGGGTGTAGCCAA GGTTGAC
Il25	CTCTCTCAGAAGGCC TGTCA	CCCACGATCATTGCCA AGAA	**Map2k6**	ATGCCGGTTGCAAACCA TAC	TGTCAGACTTCAC ACTGTA CCC
Il27	GGCCAGGTGACAGGAGAC	CAGGAACCAGCTTGTAC CAGAA	**Mapk8**	GGGAGAAATGGTTTGCCA CAA	AGGTGTTCCGAGC TGTTCAA

(continued)

Table 1
(continued)

Target	Forward	Reverse	Target	Forward	Reverse
Il2ra	TGCGTTGCTTAGGAA ACTCC	CTGGTGTTCAAGTTGAGC TGTA	**Mx1**	GAGATGACCCAGCACCT GAA	GGATAATCAGAGG GATCTG TCTCC
Il3	TCGTGGAAAGCCAAG GAGAA	AGATGTAGGCAGGCAAC AGTTA	**Nur77**	CAATGCTTCGTGTCAGC ACTA	TGTTTGCCAGGCA GATGTAC
Il4	ACGGAGATGGATGT GCCAAA	GAAGCACCTTGGAAGCC CTA	**Oas1b**	GTGCTGCCAGCCTATGA TTTA	CGATAACTTGCCCT CCTTCC
Il4ra	AACATCTCCAGAGAGGA CAACC	CTCAGCCTGGGTTCCTT GTA	**Oas2**	CTGTACTCTCCCAGCCT GAA	GTGGCTTGGAGTG ACGAAAA
Il5	GATGAGGCTTCCTGT CCCTA	TTCAGTATGTCTAGCCCC TGAA	**Oasl1**	GTCATCGAGGCCTGTG TCA	TCTGCTGGGTCCA GGATGATA
Il5ra	CTGCTGAACTCAAAG CTCCA	AGTGGGTGTGGCTACTT ACA	**Pd1**	CTGGAAGCAAGGACGA CAC	CTGGAAGTCCAGC TCCTCATA
Il6	CGATGATGCACTTGC AGAAA	ACTCCAGAAGACCAGAG GAA	**Rsad2**	GAGCAATGGCAGCCTTA TCC	TGTCGCAGGAGAT AGCAAGAA
Il7	GCTGCAGTCCCAGT CATCA	AGGCAGCAGAACAAGGA TCA	**Socs3**	AAGGCCGGAGATTTCG CTTC	TGGCTGCAGCTG CTTCG
Il7r	AAAGCATGATGTGGC CTACC	GGGATTGTTGTTCTTGTG TGGAA	**Stat1**	GCAGGTGTTGTCAGATC GAAC	ATGCACGGCTGT CGTTCTA
Nfkb1	ACCGTATGAGCCTGT GTTCA	GTAGCCTCGTGTCTTCT GTCA	**Stat3**	TGGGCATCAATCCTGTG GTA	CCAATTGGCGGC TTAGTGAA
Ppara	AGGGTACCACTACGG AGTTCA	ACACCAGCTTCAGCCGA ATA	**Stat4**	CCCAAGGAGATGAAGTG CAGTA	ATGGAATGCA ACTCCTCTGTCA

Pparg	ACCCAATGGTTGCTG ATTACA	AGGTGGAGATGCAGGT TCTA	**Stat5**	AGCCAGGACCACAATG CTA	CCTTGTCAGGCAC AGCAAA
Ppargc1a	AAACCACACCCACA GGATCA	GCTCTTCGCTTTATTGCT CCA	**Tgfbr2**	TCTGTGAGAAGCCGCAT GAA	GGCAAACCGT CTCCAGAGTAA
Pten	GAGACATTATGACACC GCCAAA	AAGTTCTAGCTGTGGT GGGTTA	**Tnfaip3**	CACTTTGTACCCCTGGT GAC	CCTACCCCGGTC TCTGTTAA
Tbx21	CAAGTTCAACCAGCA CCAGAC	CCACGGTGAAGGACAG GAA	**Traf2**	TCTGTCCCAATGATGGAT GCA	GCAGGAATGGGCA AAGTCC
Tnf	CAAATGGCCTCCCTCT CATCA	GCTACAGGCTTGTCACTC GAA	**Vav1**	CCTCTGCAGCGATTCC TTAA	GTGGGTATGCACA GAGAACA
Tnfrsf1a	AGCTTGTGTCCCCAA GGAAA	CGGACAGTCACTCACCA AGTA	**Zap70**	GTGTCCTCCTGAGATGTA TGCA	GTTCCGCATACGT TGTTCCA
Tnfrsf1b	AGTGCATGAGGCTGA GCAA	GCCTTGCATAGCACATT TCCA	**Zeb2**	GGCAAGGCCTTCAAGTA CAA	TGCAGTTTGGGCAT TCGTAA

8. 2× KAPPA HiFi HotStart ReadyMix PCR kit (KAPA Biosystems, Wilmington, MA, USA) (similar High Fidelity PCR ready mixes can be substituted).
9. E-Gel® 2%EX Gel (ThermoFisher Scientific, Waltham, MA, USA).
10. DNA Clean & Concentrator™-5 kit (Zymo Research, Irvine, CA, USA).
11. MiSeq Sequencing System (Illumina, San Diego, CA, USA).

3 Methods

3.1 Sample Preparation

General measures are taken to avoid RNA/DNA contamination and degradation of samples. This includes using only sterile, RNAase- and DNAase-free tips, tubes, and plates, frequent changing of gloves, and keeping a clean work environment. Unless otherwise noted samples are kept at 4 °C in between reactions.

3.1.1 Single-Cell Sorting

1. Harvest organs of interest from a mouse and produce single-cell suspensions using a 70 μm cell strainer and the piston of a 3 mL sterile syringe. Harvest and wash in sterile PBS. Spin cells down (1200 rpm (350×*g*), 5 min, 20 °C) (*see* **Note 7**).
2. Wash samples once in FACS buffer and lyse red blood cells (RBC) by incubating for 5 min in 5 mL RBC lysis buffer, followed by another wash in FACS buffer (*see* **Note 8**).
3. Incubate cells in Fc-block for 15 min at room temperature. Meanwhile, distribute the cells into a conical bottom 96-well plate. Extra wells are included for compensation of each color and a blank, in addition to a negative control (*see* **Note 9**).
4. Wash once in FACS buffer and stain samples with PE-labeled tetramers for 1 h at room temperature in darkness. The compensations and blank samples are incubated in FACS buffer only.
5. Wash the cells three times. Samples are stained with the following mix of antibodies: FITC anti-mouse CD3ε, APC/Cy7 anti-mouse CD4, APC anti-mouse/human CD11b, APC anti-mouse/human CD45R/B220, APC anti-mouse CD49b, and APC anti-mouse CD8a. The compensations are stained with FITC anti-mouse CD3ε, APC/Cy7 anti-mouse CD4, APC anti-mouse/human, CD45R/B220, and PE anti-mouse CD4, respectively. The blank is resuspended in FACS buffer only. Incubate the cells for 30 min at 4 °C, in darkness.
6. Wash the cells twice in FACS buffer, and transfer each well to 500 μL FACS buffer in 5 mL polystyrene tubes.

7. Sort single cells directly into a 96-well PCR plate containing 5 μL per well of RT Mix Solution 1 (*see* **Notes 10** and **11**). Cells are sorted by gating an APC-negative and FITC-, APC/Cy7-, and PE-positive population. Seal the plates immediately after sorting with adhesive plate seals, and briefly spin on a prechilled centrifuge. Plates can be frozen on dry ice and stored at −80°C.

3.1.2 Reverse Transcription and Pre-amplification

The following protocols are based on Fluidigm's user guide "Real-Time PCR Analysis". All the reactions are performed in a Thermal Cycler and the sample plate briefly spun before and after each reaction. Due to the small volumes and repetitive pipetting, only electronic pipettes are used.

1. Thaw the sample plate on ice and denature the RNA by incubating for 90 s at 65 °C, followed by immediate chilling on ice for 5 min.
2. Add 1 μL of RT Mix Solution 2 to each well and do reverse transcription under the following conditions: 25 °C, 5 min; 50 °C, 30 min; 55 °C, 25 min; 60 °C, 5 min; 70 °C, 10 min; final hold at 4 °C.
3. Pre-amplify the target cDNA by adding 9 μL STA Reaction Mix to each well and run the following reaction: 95 °C, 10 min; 96 °C, 5 s and 60 °C, 4 min (20 cycles); final hold at 4 °C (*see* **Note 12**).
4. Remove unincorporated primers by adding 6 μL exonuclease to each well and vortex the plate for 20 s before spinning and placing it in the Thermal Cycler: 37 °C, 30 min; 80 °C, 15 min; final hold on 4 °C.
5. Dilute the final products by adding 54 μL 1× DNA suspension buffer (fivefold dilution of the STA reaction), and store the STA pre-amplified sample plate at −20 °C.

3.2 qPCR

All Fluidigm products must be warmed to room temperature before usage.

1. Prime a 96.96 Dynamic Array IFC by injecting 150 μL control line fluid into each of the two accumulators and place the chip in an IFC Controller HX. Run the script "Prime (136×)" (*see* **Note 13**).
2. Prepare a sample plate by distributing 4.4 μL freshly made Sample Pre-Mix Solution into each well of a 96-well plate. Using a multi-channel pipette transfer 3.6 μL from each well of the STA pre-amplified sample plate into the Sample Pre-Mix (*see* **Note 14**).
3. Similarly, prepare an assay plate by distributing 9.5 μL freshly made Assay Mix Solution into each well of a 96-well plate.

Using a multi-channel pipette transfer 0.5 μL from each well of the qPCR Primer Plate into the Assay Mix Solutions.

4. Place adhesive seals and vortex each plate for 20 s and centrifuge for 30 s.
5. Carefully load 5 μL of each sample- and assay well into the respective inlets in the primed IFC. Place in the IFC Controller HX and run the script "Load Mix (136×)" (*see* **Note 15**).
6. Place the loaded IFC in the BioMark HD. Using the BioMark Data Collection software, select the protocol "GE 96×96 Fast PCR + Melt v2.pcl" to run a thermal mix and qPCR under the following conditions: 70 °C, 40 min; 60 °C, 30 s; 95 °C, 1 min; 96 °C, 5 s and 60 °C, 20 s (30 cycles); 60 °C, 3 s; 60–95 °C, 1°C/3 s (*see* **Note 16**).
7. Analyze the data using Real-Time PCR Analysis 4.0.1 software, and visualize results using SINGuLAR Analysis Toolset 3.0.

3.3 TCR Sequencing

3.3.1 Access Array

1. Prime a 48.48 Access Array IFC by injecting 300 μL control line fluid into each of the two accumulators, and add 500 μL 1× Access Array Harvest solution to wells H1–H3, and 500 μL 1× Access Array Hydration Reagent v2 to well H4. Place the chip in an IFC Controller AX (pre-PCR) and run the script "Prime (151×)" (*see* **Note 13**).
2. Make a sample plate in half of a 96-well plate by distributing 7.2 μL freshly made Sample Pre-Mix Solution per well. With a multi-channeled pipette, transfer 2 μL sample from each well of one half of the STA pre-amplified sample plate, and 0.8 μL from the Barcoded Internal TCR 3′ Primer Plate (*see* **Notes 14** and **17**).
3. Place adhesive seals and vortex the sample plate and the Internal TCR 5′ Primer Plate (20×) for 20 s and centrifuge for 30 s.
4. Carefully load 4 μL from each well of the sample plate and the 5′ Primer Plate (20×) into the respective inlets of the primed IFC. Place the chip in the same IFC Controller AX as used for priming and run the script "Load Mix (151×)" (*see* **Note 15**).
5. Transfer the IFC to the BioMark HD, and using the BioMark Data Collection software, start the protocol "AA No I 48x48 Standard v1" to run a thermal mix and PCR under the following conditions: 50 °C, 2 min; 70 °C, 20 min; 95 °C, 10 min; 95 °C, 15 s, 60 °C, 30 s, and 72 °C, 60 s (10 cycles); 95 °C, 15 s, 80 °C, 30 s, 60 °C, 30 s, and 72 °C, 60 s (2 cycles); 95 °C, 15 s, 60 °C, 30 s, and 72 °C, 60 s (8 cycles); 95 °C, 15 s, 80 °C, 30 s, 60 °C, 30 s, and 72 °C, 60 s (2 cycles); 95 °C, 15 s, 60 °C, 30 s, and 72 °C, 60 s (8 cycles); 95 °C, 15 s, 80 °C, 30 s, 60 °C, 30 s, and 72 °C, 60 s (5 cycles); 72 °C, 3 min; final hold at 10 °C.

6. After the PCR is finished, remove the remaining fluid from wells H1–H4 and replace with 600 μL 1× Access Array Harvest Solution. Add 2 μL 1× Access Array Harvest Solution into each of the sample inlets.
7. Place the IFC in a different IFC Controller AX (post-PCR) and harvest the amplification products back into the sample inlets by using the script "Harvest v5 (151×)."
8. Transfer the products from each sample inlet and combine into an Eppendorf tube and store at −20 °C.

3.3.2 Library Construction and Illumina Sequencing

1. Clean up the pooled DNA amplicons by using AMPure XP magnetic bead isolation kit. Add 1.8× volume of the magnetic bead solution (based on volume of sample pool), allow nucleic acid amplicons to bind to the magnetic beads for 10 min, then place tube in a magnetic separator and wash the beads with 80% ethanol. Remove ethanol from the tube and repeat wash step, for a total of two washes. Allow magnetic beads to dry with the lid of the tube open at room temperature for 10 min. Remove the tube from the magnetic separator and add 30 μL of nuclease-free water, wait 2 min for the amplicon products to elute off the beads, and then place the tube in the magnetic separator and remove the 30 μL nuclease-free water containing the DNA libraries and transfer it to a fresh microfuge tube.
2. Analyze cleaned up DNA amplicon products for length distribution using an Agilent 2100 Bioanalyzer instrument and High Sensitivity dsDNA chip.
3. Quantitate cleaned up DNA amplicon products with a Qubit fluorometer and dsDNA high sensitivity assay.
4. Using quantitation values from the Qubit, take 10 ng of the DNA amplicons (*from* **step 1**), into the NEBNext Ultra DNA Library Prep Kit for Illumina; follow the manufacturer's guidelines for library preparation (during the protocol, no magnetic bead size-selection is performed and PCR amplification is done with 15 cycles).
5. After PCR amplification, the DNA libraries are cleaned up with AmpureXP magnetic beads (*see* **step 1** above) and the cleaned up products are purified using a 2% E-Gel EX agarose gel. DNA products in the range of 270–490 bp (*see* **Note 18**) are excised and isolated from the gel using Zymo Agarose Dissolving Buffer (ADB) and DNA Clean & Concentrator™-5 kit (*see* **Note 19**). Elute the DNA library products in 20 μL nuclease-free water.
6. The DNA libraries are diluted to the appropriate concentrations, and loaded onto the MiSeq system following the manufacturer's guidelines (*see* **Note 20**).

Table 2
External primers for pre-amplification (TCR sequencing)

Target	Forward (TRAV)	Target	Forward (TRBV)
TRAV1	GGTTATCCTGGTACCAGCA	TRBV1	TACCACGTGGTCAAGCTG
TRAV2	CATCTACTGGTACCGACAGG	TRBV2	CAGTATCTAGGCCACAATGC
TRAV3	GGCGAGCAGGTGGAG	TRBV3	CCCAAAGTCTTACAGATCCC
TRAV4	TCTGSTCTGAGATGCAATTTT	TRBV4	GACGGCTGTTTTCCAGAC
TRAV5-1/5-4(D)	GGCTACTTCCCTTGGTATAAGCAAGA	TRBV5	GGTATAAACAGAGCGCTGAG
TRAV6-1/6-2	CAGATGCAAGGTCAAGTGAC	TRBV12	GGGGTTGTCCAGTCTCC
TRAV6-3/6-4(D)	AAGGTCCACAGCTCCTTC	TRBV13	GCTGCAGTCACCCAAAG
TRAV6-5/6-7(D)	GTTCTGGTATGTGCAGTATCC	TRBV14	GCAGTCCTACAGGAAGGG
TRAV6-6	AGATTCCGTGACTCAAACAG	TRBV15	GAGTTACCCAGACACCCAG
TRAV7	AGAAGGTRCAGCAGAGCCCAGAATC	TRBV16	CCTAGGCACAAGGTGACAG
TRAV8	GAGCRTCCASGAGGGTG	TRBV17	GAAGCCAAACCAAGCAC
TRAV9	CCAGTGGTTCAAGGAGTG	TRBV19	GATTGGTCAGGAAGGGC
TRAV10/10a(D)	AGAGAAGGTCGAGCAACAC	TRBV20	GGATGGAGTGTCAAGCTG
TRAV11	AAGACCCAAGTGGAGCAG	TRBV23	CTGCAGTTACACAGAAGCC
TRAV12	TGACCCAGACAGAAGGC	TRBV24	CAGACTCCACGATACCTGG
TRAV13	TCCTTGGTTCTGCAGG	TRBV26	GGTGAAAGGGCAAGGAC

TRAV14	GCAGCAGGTGAGACAAAG	TRBV29	GCTGGAATGTGGACAGG
TRAV15	CASCTTYTTAGTGGAGAGATGG	TRBV30	CCTCCTCTACCAAAAGCC
TRAV16	GTACAAGCAAACAGCAAGTG	TRBV31	CTAACCTCTACTGGTACTGGCAG
TRAV17	CAGTCCGTGGACCAGC		
TRAV18	AACGGCTGGAGCAGAG		
TRAV19	GCAAGTTAAACAAAGCTCTCC		
TRAV21	GTGCACTTGCCTTGTAGC		
	Reverse (TRAC)		Reverse (TRBC)
TRAC-rev.	GGCATCACAGGGAACG	TRBC-rev.	CCAGAAGGTAGCAGAGACCC

Table 3
Internal 5′ primers for access array (TCR sequencing)

Target	Forward (TRAV)	Target	Forward (TRBV)
TRAV1	CTCCACATTCCTGAGCC	TRBV1	GTATCCCTGGATGAGCTG
TRAV2	ACTCTGAGCCTGCCCT	TRBV2	GGACAATCAGACTGCCTC
TRAV3	GCCCTCCTCACCTGAG	TRBV3	GATATGGGGCAGATGGTG
TRAV4	GGITIMAGGAACAAAGGAGAAT	TRBV4	CAGGTGGGAAATGAAGTG
TRAV5-1/5-4(D)	ATYCGTTCAAATATGGAAAGAAA	TRBV5	GCCAGAGCTCATGTTTCTC
TRAV6-1/6-2	GGAGAAGGTCCACAGCTC	TRBV12	CCAGCAGATTCTCAGTCC
TRAV6-3/6-4(D)	CAACTGCCAACAACAAGG	TRBV13	GTACTGGTATCGGCAGGAC
TRAV6-5/6-7(D)	TCCTTCCACTTGCAGAAAG	TRBV14	GGTATCAGCAGCCCAGAG
TRAV6-6	ACGGCTGGCCAGAAG	TRBV15	GTGTGAGCCAGTTTCAGG
TRAV7	CAKGRCYTCYYTCAACTGCAC	TRBV16	GAAGCAACTCTGTGGTGTG
TRAV8	AGAGCCACCCTTGACAC	TRBV17	GAACAGGGAAGCTGACAC
TRAV9	GCTTYGAGGCTGAGTTCAG	TRBV19	GGTACCGACAGGATTCAG
TRAV10/10a(D)	CTACACTGAGTGTTCGAGAGG	TRBV20	GCTTGGTATCGTCAATCG
TRAV11	AACAGGACACAGGCAAAG	TRBV23	GCCAGGAAGCAGAGATG
TRAV12	GGTTCCACGCCACTC	TRBV24	GCACACTGCCTTTTACTGG
TRAV13	TGCAGGAGGGGGAGA	TRBV26	GAGGTGTATCCCTGAAAAGG

TRAV14	CTCTGACAGTCTGGGAAGG	TRBV29	GTACTGGTATCGACAAGACCC
TRAV15	AYTCTGTAGTCTTCCAGAAATCAC	TRBV30	GGACATCTGTCAAAGTGGC
TRAV16	ATTATTCTCTGAACTTTCAGAAGC	TRBV31	CTGTTGGCCAGGTAGAGTC
TRAV17	TATGAAGGAGCCTCCCTG		
TRAV18	CAAGATTTCACCGCACG		
TRAV19	GCTGACTGTTCAAGAGGGA		
TRAV21	AATAGTATGGCTTTCCTGGC		

Table 4
Internal barcoded 3′ primers for access array (TCR sequencing)

Name	Barcoded Reverse (TRAC)	Name	Barcoded Reverse (TRBC)
BC1TRAC	ATCACGGCACATTGATTTGGGAGTC	BC1TRBC	ATCACGGGGTAGCCTTTTGTTTGTTTG
BC2TRAC	CGATGTGCACATTGATTTGGGAGTC	BC2TRBC	CGATGTGGGTAGCCTTTTGTTTGTTTG
BC3TRAC	TTAGGCGCACATTGATTTGGGAGTC	BC3TRBC	TTAGGCGGGTAGCCTTTTGTTTGTTTG
BC4TRAC	TGACCAGCACATTGATTTGGGAGTC	BC4TRBC	TGACCAGGGTAGCCTTTTGTTTGTTTG
BC5TRAC	ACAGTGGCACATTGATTTGGGAGTC	BC5TRBC	ACAGTGGGGTAGCCTTTTGTTTGTTTG
BC6TRAC	GCCAATGCACATTGATTTGGGAGTC	BC6TRBC	GCCAATGGGTAGCCTTTTGTTTGTTTG
BC7TRAC	CAGATCGCACATTGATTTGGGAGTC	BC7TRBC	CAGATCGGGTAGCCTTTTGTTTGTTTG
BC8TRAC	ACTTGAGCACATTGATTTGGGAGTC	BC8TRBC	ACTTGAGGGTAGCCTTTTGTTTGTTTG
BC9TRAC	GATCAGGCACATTGATTTGGGAGTC	BC9TRBC	GATCAGGGGTAGCCTTTTGTTTGTTTG
BC10TRAC	TAGCTTGCACATTGATTTGGGAGTC	BC10TRBC	TAGCTTGGGTAGCCTTTTGTTTGTTTG
BC11TRAC	GGCTACGCACATTGATTTGGGAGTC	BC11TRBC	GGCTACGGGTAGCCTTTTGTTTGTTTG
BC12TRAC	CTTGTAGCACATTGATTTGGGAGTC	BC12TRBC	CTTGTAGGGTAGCCTTTTGTTTGTTTG
BC13TRAC	AGTCAAGCACATTGATTTGGGAGTC	BC13TRBC	AGTCAAGGGTAGCCTTTTGTTTGTTTG
BC14TRAC	AGTTCCGCACATTGATTTGGGAGTC	BC14TRBC	AGTTCCGGGTAGCCTTTTGTTTGTTTG
BC15TRAC	ATGTCAGCACATTGATTTGGGAGTC	BC15TRBC	ATGTCAGGGTAGCCTTTTGTTTGTTTG
BC16TRAC	CCGTCCGCACATTGATTTGGGAGTC	BC16TRBC	CCGTCCGGGTAGCCTTTTGTTTGTTTG
BC17TRAC	GTAGAGGCACATTGATTTGGGAGTC	BC17TRBC	GTAGAGGGGTAGCCTTTTGTTTGTTTG
BC18TRAC	GTCCGCGCACATTGATTTGGGAGTC	BC18TRBC	GTCCGCGGGTAGCCTTTTGTTTGTTTG
BC19TRAC	GTGAAAGCACATTGATTTGGGAGTC	BC19TRBC	GTGAAAGGGTAGCCTTTTGTTTGTTTG
BC20TRAC	GTGGCCGCACATTGATTTGGGAGTC	BC20TRBC	GTGGCCGGGTAGCCTTTTGTTTGTTTG

BC21TRAC	GTTTCGGCACATTGATTTGGGAGTC	BC21TRBC	GTTTCGGGGTAGCCTTTTGTTTGTTTG
BC22TRAC	CGTACGGCACATTGATTTGGGAGTC	BC22TRBC	CGTACGGGGTAGCCTTTTGTTTGTTTG
BC23TRAC	GAGTGGGCACATTGATTTGGGAGTC	BC23TRBC	GAGTGGGGGTAGCCTTTTGTTTGTTTG
BC24TRAC	GGTAGCGCACATTGATTTGGGAGTC	BC24TRBC	GGTAGCGGGTAGCCTTTTGTTTGTTTG
BC25TRAC	ACTGATGCACATTGATTTGGGAGTC	BC25TRBC	ACTGATGGGTAGCCTTTTGTTTGTTTG
BC26TRAC	ATGAGCGCACATTGATTTGGGAGTC	BC26TRBC	ATGAGCGGGTAGCCTTTTGTTTGTTTG
BC27TRAC	ATTCCTGCACATTGATTTGGGAGTC	BC27TRBC	ATTCCTGGGTAGCCTTTTGTTTGTTTG
BC28TRAC	CAAAAGGCACATTGATTTGGGAGTC	BC28TRBC	CAAAAGGGGTAGCCTTTTGTTTGTTTG
BC29TRAC	CAACTAGCACATTGATTTGGGAGTC	BC29TRBC	CAACTAGGGTAGCCTTTTGTTTGTTTG
BC30TRAC	CACCGGGCACATTGATTTGGGAGTC	BC30TRBC	CACCGGGGGTAGCCTTTTGTTTGTTTG
BC31TRAC	CACGATGCACATTGATTTGGGAGTC	BC31TRBC	CACGATGGGTAGCCTTTTGTTTGTTTG
BC32TRAC	CACTCAGCACATTGATTTGGGAGTC	BC32TRBC	CACTCAGGGTAGCCTTTTGTTTGTTTG
BC33TRAC	CAGGCGGCACATTGATTTGGGAGTC	BC33TRBC	CAGGCGGGGTAGCCTTTTGTTTGTTTG
BC34TRAC	CATGGCGCACATTGATTTGGGAGTC	BC34TRBC	CATGGCGGGTAGCCTTTTGTTTGTTTG
BC35TRAC	CATTTTGCACATTGATTTGGGAGTC	BC35TRBC	CATTTTGGGTAGCCTTTTGTTTGTTTG
BC36TRAC	CCAACAGCACATTGATTTGGGAGTC	BC36TRBC	CCAACAGGGTAGCCTTTTGTTTGTTTG
BC37TRAC	CGGAATGCACATTGATTTGGGAGTC	BC37TRBC	CGGAATGGGTAGCCTTTTGTTTGTTTG
BC38TRAC	CTAGCTGCACATTGATTTGGGAGTC	BC38TRBC	CTAGCTGGGTAGCCTTTTGTTTGTTTG
BC39TRAC	CTATACGCACATTGATTTGGGAGTC	BC39TRBC	CTATACGGGTAGCCTTTTGTTTGTTTG
BC40TRAC	CTCAGAGCACATTGATTTGGGAGTC	BC40TRBC	CTCAGAGGGTAGCCTTTTGTTTGTTTG

(continued)

Table 4
(continued)

Name	Barcoded Reverse (TRAC)	Name	Barcoded Reverse (TRBC)
BC41TRAC	GACGACGCACATTGATTTGGGAGTC	BC41TRBC	GACGACGGGTAGCCTTTTGTTTGTTTG
BC42TRAC	TAATCGGCACATTGATTTGGGAGTC	BC42TRBC	TAATCGGGGTAGCCTTTTGTTTGTTTG
BC43TRAC	TACAGCGCACATTGATTTGGGAGTC	BC43TRBC	TACAGCGGGTAGCCTTTTGTTTGTTTG
BC44TRAC	TATAATGCACATTGATTTGGGAGTC	BC44TRBC	TATAATGGGTAGCCTTTTGTTTGTTTG
BC45TRAC	TCATTCGCACATTGATTTGGGAGTC	BC45TRBC	TCATTCGGGTAGCCTTTTGTTTGTTTG
BC46TRAC	TCCCGAGCACATTGATTTGGGAGTC	BC46TRBC	TCCCGAGGGTAGCCTTTTGTTTGTTTG
BC47TRAC	TCGAAGGCACATTGATTTGGGAGTC	BC47TRBC	TCGAAGGGGTAGCCTTTTGTTTGTTTG
BC48TRAC	TCGGCAGCACATTGATTTGGGAGTC	BC48TRBC	TCGGCAGGGTAGCCTTTTGTTTGTTTG

3.4 Data Analysis

1. TCR sequencing analysis is always started by the identification of bar codes.
2. TCR chain identity is determined using the IMGT database (http://www.imgt.org).

4 Notes

1. Biotinylated MHC molecules can be produced in house (our case) or obtained from the NIH tetramer core facility.
2. Include primer pairs for all targets of both qPCR and TCR α and β sequencing. We combine the same 96 target-specific primer pairs as used during the qPCR (*see* Table 1), with the 42 external primer pairs for TCR sequencing (*see* Table 2), the latter being part of a nested PCR.
3. The sequences of primers for TCR sequencing were published by Thomas et al. [8].
4. The gene expression profiling can be carried out for any gene of interest. Our particular panel includes seven categories: cell surface receptors, chemokine receptors, cytokines, transcription factors, interferon response, metabolism, and signaling molecules. Primers pairs were selected from the Fluidigm catalog of validated reagents (DELTAgene Assays) (*see* Table 1).
5. Two separate IFCs are needed, one for pre- and one for post-PCR.
6. If less than 48 5′ primers: use 1× DNA Suspension Buffer (TEKnova) in the remaining wells. If more than 48 5′ primers: combine multiple primers per well.
7. The timing of tissue harvesting ➔ cell staining ➔ cell sorting ➔ reverse transcription should be as tight as possible to avoid cell death and poor quality cDNA synthesis.
8. Lysis of RBCs is done for splenocytes only.
9. For compensations and the negative control we use splenocytes, as these are the most abundant of our samples. Before distribution, 10 μL is removed from each sample to calculate total cell numbers for records.
10. qPCR experiments should include a minimum of four blank wells and, if possible, wells with 5, 10, and 50 cells.
11. We sort into a hard-shelled, deep welled PCR plate to avoid cross contamination in the downstream processing.
12. If the sample plate is to be used only for access array and not qPCR, it can be an advantage to increase to 25 cycles.
13. The primed chip must be loaded within an hour of finishing priming.

14. A minimum overage of 3 μL/well is helpful to avoid loading air bubbles.
15. The loaded chip must immediately be transferred to the Biomark upon finishing.
16. Turn on the Biomark at least 20 min prior to use to allow the camera to cool to 4 °C. Use scotch tape to gently remove potential dust from the chip surface.
17. Pairing of TCR α and β chains is made possible by using internal barcoded 3′ primers binding TRAC and TRBC (*see* Table 4). As access array allows for up to 48 samples, we designed 48 6 bp barcodes that were added to the TRAC and TRBC 3′ primers. By loading the barcode-based 3′ primer pairs together with the samples (instead of with the 5′ primers as suggested in Fluidigm's protocol for Access Array), the TCR α and β sequences from the same cell will share the same barcode.
18. Sizes based on primers from Tables 3 and 4.
19. 4× volume of ADB should be used to dissolve the gel and the mixture should be incubated at 37 °C for 5–10 min, until the gel is dissolved.
20. A 2× 300 sequencing run is performed based on estimated 5 M reads per sample.

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