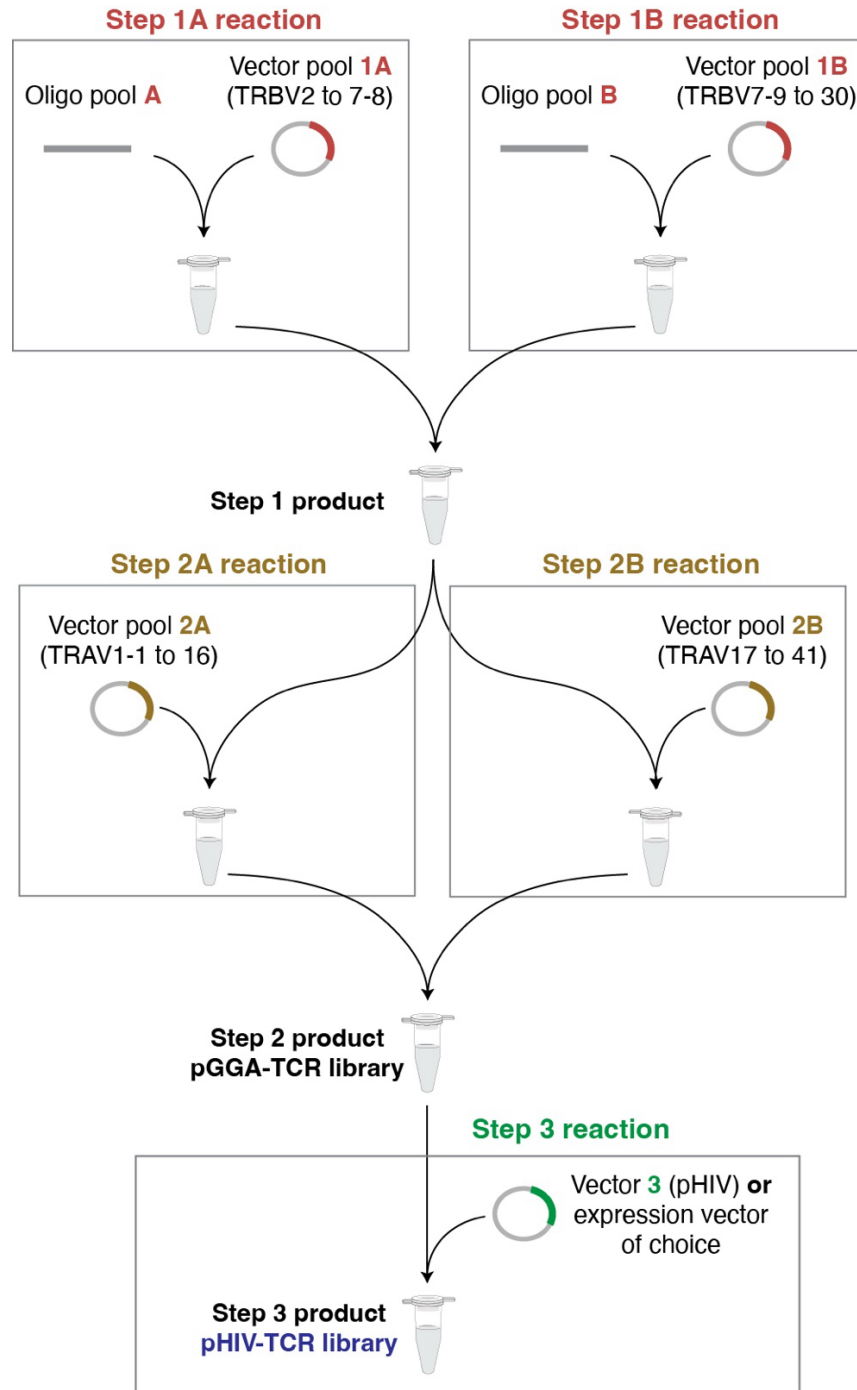


# TCRAFT Overview



## Step 0 – Design oligo pools

This protocol requires two oligo pools (A and B). Scripts to generate oligos can be found here: <https://github.com/birnbaumlab/TCRAFT/>.

**Input:** .csv file containing TRAV, TRBV, TRAJ, and TRBJ alleles as well as CDR3 $\alpha$  and CDR3 $\beta$  amino acid sequences. See sample file on Github (*Sample\_TCR\_List.csv*).

**Output:** oligo pools to order, split by length ( $\leq 300$  bp and  $>300$  bp). This enables separate orders with manufacturers offering different oligo lengths (for example, Twist for  $\leq 300$  bp and IDT  $> 300$  bp). In summary, the code generates four .csv files with oligos to order: pool A  $\leq 300$  bp, pool A  $> 300$  bp, pool B  $\leq 300$  bp, and pool B  $> 300$  bp.

In the oligo preparation section below, mix pools of different lengths proportionally to generate a single oligo pool A and single oligo pool B prior to amplifying.

Any manufacturer can be used but ensure pools A and B are ordered separately.

## Step 0 – Prepare vectors

This protocol requires four vector pools and a destination expression vector. The destination vector used in Gaglione *et al.* is a pHIV vector containing SapI sites and a LacZ insert (provided in Addgene kits). Users can design their own destination vector (see Step 3). Vectors are provided via Addgene as a pre-pooled kit and individually.

The following are the four vector pools with associated **antibiotic resistance**:

- **1A**, containing **TRBV-TRAC** vectors for TRBV2 to 7-8 (24 vectors) (**chloramphenicol**)
- **1B**, containing **TRBV-TRAC** vectors for TRBV7-9 to 30 (24 vectors) (**chloramphenicol**)
- **2A**, containing **TRBC-TRAV** vectors for TRAV1-1 to 16 (23 vectors) (**kanamycin**)
- **2B**, containing **TRBC-TRAV** vectors for TRAV17 to 41 (22 vectors) (**kanamycin**)

The TCRAFT protocol requires **~180 ng** of each vector pool and **~230 ng** of the final destination vector. To generate additional plasmid stocks, electroporate/transform at least **1 ng** of each vector pool and ensure sufficient transformation coverage (i.e.  $>100x$ ) for 22-24 vectors. For additional detail on amplifying vector pools, please see the provided **TCRAFT vector amplification protocol**. Individual vectors can be propagated in *E. coli* using standard methods.

## Step 0 – Amplify and prepare CDR3 oligo pools

We require two oligo pools, A and B, corresponding to step 1 vector pools 1A and 1B. Resuspend and amplify all oligo pools according to manufacturer instructions. The following is an example protocol. If you have multiple oligo pools for each of A and B (i.e.  $\leq 300$  bp and  $>300$  bp), we recommend mixing proportionally prior to amplifying (step B below).

### A. Resuspend Oligo Pool and Primers (Manufacturer Instructions)

1. Spin down oligo pool tubes at  $>6000 \times g$  for 5 mins.
2. Resuspend oligo pools with elution or TE buffer at a minimum of 5ng/uL total concentration.
  - Stock solution concentration (ng/uL) = total yield (ng) / resuspension volume (uL)
3. Resuspend amplification primers at 100 uM in elution buffer followed by a 1:10 dilution (10 uM) to generate a working solution.

*Amplification primer sequences:*

Forward primer	5'-ACGCCAGATCCGGAAGAC-3'
Reverse primer	5'-ACGGTCTGAGTCGGAAGAC-3'

- B. If you have multiple A and B oligo pools, combine all A pools and all B pools separately, maintaining molar proportionality.

### C. Set up PCRs to amplify each oligo pool:

1. Use KAPA HiFi HotStart PCR (Catalog #KK2502) or NEB Q5 High-Fidelity DNA polymerase kits (ex. NEB Catalog #M0544) according to manufacturer instructions.
2. Set up two 50 uL reactions per oligo pool. Use 20 ng of oligo pool as template per reaction. Amplify for 12-14 cycles.
3. Example cycling conditions for Q5 polymerase:
  - a. Initial denaturation: 98C for 30 s
  - b. 12 cycles:
    - i. Denaturation: 98C for 30 s
    - ii. Primer annealing: 68C for 30 s\*
    - iii. Extension: 72C for 30 s
  - c. Final extension: 72C for 5 min
  - d. Hold: 4C

\* Temperature determined using NEB calculator for Q5 polymerase. Use appropriate primer temperature for your selected polymerase and PCR setup.

### D. SPRI PCR Cleanup

1. Purify the PCR amplification products with SPRIselect (Beckman Coulter, Catalog #B23317) magnetic beads using a high bead-to-DNA ratio (1.8x). *Recommended:* Concentrate amplified oligo products by using the eluate from the SPRI cleanup for the first PCR to elute the duplicate PCR.

## Step 1 – Insert CDR3 oligos into TRBV-TRAC vectors

Step 1 inserts CDR3 oligo pool A into TRBV-TRAC vector pool A (**1A**) and CDR3 oligo pool B into TRBV-TRAC vector pool B (**1B**). The protocol requires a minimum of 3 days (day 1: reaction set up, overnight reaction incubation, day 2: electroporation, overnight culture, day 3: midiprep).

### Required reagents:

Reagent	Supplier	Catalog #
NEBridge Ligase Master Mix	NEB	M1100
BbsI-HF	NEB	R3539
Nuclease-free water	Thermo Fisher (Invitrogen)	AM9937
Shrimp alkaline phosphatase (rSAP)	NEB	M0371
ElectroMAX DH10B <i>E. coli</i>	Thermo Fisher (Invitrogen)	18290015
MCE membrane filter, 0.025 µm pore size	Millipore Sigma	VSWP02500
Chloramphenicol	Millipore Sigma	C0378
Electroporation cuvette (1 mm gap)	VWR	89047-206
<b>1A and 1B vector pools</b>		

#### A. Set up BbsI golden gate reaction (Pool 1A):

Combine the following to create a 15µL reaction with a 4:1 insert:vector ratio:

- X µL Step 1 vector mix **1A** (to yield 0.1 pmol or 180 ng with 2929bp avg length)
- Y µL CDR3 oligo pool **A** (to yield 0.4 pmol, or 66ng with 268 bp avg length)
- 5 µL NEBridge Ligase Master Mix
- 1 µL BbsI-HF enzyme
- 9 – X – Y µL nuclease-free or Millipore water

*Optional negative control:* Replace CDR3 oligo pool with water.

*Reminder:* mass of dsDNA (ng) = x pmol \* (length of dsDNA (bp) \* 615.96 g/mol/bp + 36.04 g/mol) / 1000

#### B. Set up BbsI golden gate reaction (Pool 1B)

Combine the following to create a 15µL reaction with a 4:1 insert:vector ratio:

- X µL Step 1 vector mix **1B** (to yield 0.1 pmol or 180 ng with 2929bp avg length)
- Y µL CDR3 oligo pool **B** (to yield 0.4 pmol, or 66ng with 268 bp avg length)
- 5 µL NEBridge Ligase Master Mix
- 1 µL BbsI-HF enzyme
- 9 – X – Y µL nuclease-free or Millipore water

*Optional negative control:* Replace CDR3 oligo pool with water.

#### C. Cycling Conditions and Clean-up Cut

Incubate in thermocycler under following conditions:

- 37C, 5 min
- 16C, 5 min
- Repeat above for 60 cycles
- 65C, 20 min (heat inactivate)
- Hold overnight at 4C

Next day - perform a clean-up cut:

- Add 1µL BbsI-HF enzyme and 1µL rSAP to each tube
- Incubate at 37C for 1 hr, followed by 65C for 20 mins for final heat inactivation

**D. Dialyze/SPRI clean and electroporate the step 1 assembly product into DH10b *E. coli*, and midiprep the products (under chloramphenicol selection):**

1. **Option 1:** Drop dialyze product for 3 hours to remove inhibitory components (i.e. salts) prior to electroporation. For each reaction product:
  - i. Fill a petri dish partially with Millipore water.
  - ii. Float an MCE membrane filter (0.025 pore size) on the water surface, shiny side up.
  - iii. Pipette the assembly product carefully onto the center of the filter as one droplet.
  - iv. Cover the petri dish and dialyze for 3 hours.
  - v. Carefully remove the droplet and place in a PCR or microcentrifuge tube. The assembly product is ready for electroporation.
2. **Option 2:** Purify the products with SPRIselect beads (Beckman Coulter, Catalog #B23317) using a 1.0x bead-to-DNA ratio.
  - i. Add SPRIselect beads at a 1.0x ratio to the assembly product in PCR tubes. Incubate for one minute.
  - ii. Place tubes on magnet and wait for beads to settle (> 1 min). Remove and discard supernatant.
  - iii. Add 180 uL of 85% ethanol and incubate for 30 seconds. Remove and discard supernatant.
  - iv. Remove tubes from magnet. Add 180 uL of 85% ethanol and resuspend beads. Transfer the beads to fresh PCR tubes and incubate for 30 seconds.
  - v. Place tubes on magnet and wait for beads to settle. Complete one more wash with 180 uL of 85% ethanol.
  - vi. Remove tubes from magnet and re-suspend beads well in 10-15 uL of nuclease-free water. Incubate for one minute before placing tubes back on magnet and removing the eluted assembly product. Be careful not to transfer beads.
3. Electroporate ElectroMAX DH10B *E. coli* bacteria according to manufacturer instructions (1 uL of assembly product per 25 uL bacteria). Aim for a minimum of ~1000x coverage. As a starting point to comfortably ensure >1000x coverage, we suggest one electroporation per 2000 TCRs. Anticipate 4e6 CFU/uL of assembly product).
  - i. Incubate cuvettes on ice prior to electroporation
  - ii. Combine DH10B *E. coli* and DNA on ice and mix gently:
    - 1 uL assembly product
    - 25 uL DH10B bacteria
  - iii. Incubate on ice for 10 minutes.
  - iv. Transfer bacteria to the center gap in cuvette, ensuring no air bubbles (suggested transfer of 22 uL to avoid air bubbles).
  - v. Electroporate using the following conditions:
    - 2000 V
    - 200 ohms
    - 25 uF
  - ii. Immediate add 1 mL of SOC media, mixing well.
  - iii. Incubate in shaker for 1 hour at 37C.
4. Plate 1:1000, 1:10,000, and 1:100,000 of 1 mL of the electroporation product on LB agar plates (chloramphenicol), and place the remainder into a ~200 mL (midiprep)

culture with chloramphenicol. In other words, generate serial dilutions with 1  $\mu$ L of the electroporation product as the top condition.

5. **Culture for 6-8 hours. Long/overnight cultures may be fine but can occasionally result in a skewed distribution where certain variable regions become overrepresented in the product:**
  - i. Midiprep the cultures and count colonies to assess library coverage and reaction efficiency. We conservatively recommend 1000x coverage to proceed. For example, if 1A contained 500 unique oligos, we need 500,000 total transformants for 1000x coverage. If we completed one electroporation, we need 500 colonies on the 1:1000 plate, 50 colonies on the 1:10,000 plate, and 5 colonies on the 1:100,000 plate.
  - ii. Mix the products (1A and 1B) proportionally by moles prior to proceeding to next step. We recommend using a small amount of each (1A and 1B) – **300 ng** is required for step 2. This is the **step 1 product**.

## Step 2 – Insert TRBC-TRAV fragment into Step 1 vectors

The Step 1 product consists of a pool of TRBV-CDR3 $\beta$  $\alpha$ -TRAC vectors. Step 2 inserts TRBC-P2A-TRAV fragments between the CDR3 $\beta$  and  $\alpha$ . The step 2 product is a complete TCR library in a pGGA vector (a cloning backbone). The protocol requires minimum of 3 days and can be started on the same day that the step 1 product is midprepped (day 1: reaction set up, overnight reaction incubation, day 2: electroporation, overnight culture, day 3: prep).

### Required Reagents

Reagent	Supplier	Catalog #
NEBridge Golden Gate Assembly Kit (BsmBI-v2)	NEB	E1602
NEBridge Golden Gate Assembly Kit (Bsal-HF-v2)	NEB	E1601
Bsal-HF-v2	NEB	R3733
BsmBI-v2	NEB	R0739
Nuclease-free water	Thermo Fisher (Invitrogen)	AM9937
Shrimp alkaline phosphatase (rSAP)	NEB	M0371
ElectroMAX DH10B <i>E. coli</i>	Thermo Fisher (Invitrogen)	18290015
MCE membrane filter, 0.025 $\mu$ m pore size	Millipore Sigma	VSWP02500
Chloramphenicol	Millipore Sigma	C0378
Electroporation cuvette (1 mm gap)	VWR	89047-206
<b>2A and 2B vector pools</b>		

#### A. Set up **BsmBI** golden gate reaction (Pool **2A**):

Combine the following to create a 20uL reaction:

- X uL Step 1 final product (150ng)
- Y uL Step **2A** vector mix (to yield a 2:1 vector mix:Step 1 product molar ratio)
  - o *Example calculation:* The average vector mix length is 3209 bp. For step 1 product with an average length of 3078 bp, use 313 ng of **2A** vector mix.
- 2 uL T4 Ligase Buffer
- 1 uL **NEBridge BsmBI-HFv2 Master Mix (from NEBridge Golden Gate Assembly Kit, NOT BsmBI-HF-v2 enzyme only)**
- 17 – X – Y uL nuclease-free or Millipore water

*Optional negative control:* Replace step 2A vector mix with water.

#### B. Set up **Bsal** golden gate reaction (Pool **2B**)

Combine the following to create a 20uL reaction:

- X uL Step 1 final product (150ng)
- Y uL Step **2B** vector mix (to yield a 2:1 vector mix:Step 1 product molar ratio)
  - o *Example calculation:* The average vector mix length is 3209 bp. For step 1 product with an average length of 3078 bp, use 313 ng of **2B** vector mix.
- 2 uL T4 Ligase Buffer
- 1 uL **NEBridge Bsal-v2 Master Mix (from NEBridge Golden Gate Assembly Kit, NOT Bsal-HF-v2 enzyme only)**
- 17 – X – Y uL nuclease-free or Millipore water

*Optional negative control:* Replace step 2A vector mix with water.

### C. Cycling Conditions and Clean-up Cut

Unlike Step 1, incubate Bsal and BsmBI reactions in separate thermocyclers.

- **42C (BsmBI, 2A) or 37C (Bsal, 2B), 5 min**
- 16C, 5 min
- Repeat above for 60 cycles
- 60C, 5 min
- 80C, 20 min (heat inactivate)
- Hold overnight at 4C

Next day - perform a clean-up cut:

- Add **1uL BsmBI-v2 (Pool 2A) or 1uL Bsal-HF-v2 (Pool 2B)** enzyme (**NOT Golden Gate Master Mix**) and 1uL rSAP to tube
- Incubate at **55C for 1 hr (BsmBI, 2A) or 37C for 1 hr (Bsal, 2B)**, followed by 80C for 20 mins for final heat inactivation

### E. Dialyze/SPRI clean and electroporate the step 1 assembly product into DH10b *E. coli*, and midiprep the products (under chloramphenicol selection):

1. **Option 1:** Drop dialyze product for 3 hours to remove inhibitory components (i.e. salts) prior to electroporation. For each reaction product:
  - i. Fill a petri dish partially with Millipore water.
  - ii. Float an MCE membrane filter (0.025 pore size) on the water surface, shiny side up.
  - iii. Pipette the assembly product carefully onto the center of the filter as one droplet.
  - iv. Cover the petri dish and dialyze for 3 hours.
  - v. Carefully remove the droplet and place in a PCR or microcentrifuge tube. The assembly product is ready for electroporation.
2. **Option 2:** Purify the products with SPRIselect beads (Beckman Coulter, Catalog #B23317) using a 1.0x bead-to-DNA ratio.
  - i. Add SPRIselect beads at a 1.0x ratio to the assembly product in PCR tubes. Incubate for one minute.
  - ii. Place tubes on magnet and wait for beads to settle (> 1 min). Remove and discard supernatant.
  - iii. Add 180 uL of 85% ethanol and incubate for 30 seconds. Remove and discard supernatant.
  - iv. Remove tubes from magnet. Add 180 uL of 85% ethanol and resuspend beads. Transfer the beads to fresh PCR tubes and incubate for 30 seconds.
  - v. Place tubes on magnet and wait for beads to settle. Complete one more wash with 180 uL of 85% ethanol.
  - vi. Remove tubes from magnet and re-suspend beads well in 10-15 uL of nuclease-free water. Incubate for one minute before placing tubes back on magnet and removing the eluted assembly product. Be careful not to transfer beads.
3. Electroporate ElectroMAX DH10B *E. coli* bacteria according to manufacturer instructions (1 uL of assembly product per 25 uL bacteria). Aim for a minimum of ~1000x coverage. As a starting point to comfortably ensure >1000x coverage, we suggest one electroporation per 2000 TCRs. Anticipate 2e6 CFU/uL of assembly product).
  - i. Incubate cuvettes on ice prior to electroporation
  - ii. Combine DH10B *E. coli* and DNA on ice and mix gently:
    - 1 uL assembly product

- 25 uL DH10B bacteria
  - iii. Incubate on ice for 10 minutes.
  - iv. Transfer bacteria to the center gap in cuvette, ensuring no air bubbles (suggested transfer of 22 uL to avoid air bubbles).
  - v. Electroporate using the following conditions:
    - 2000 V
    - 200 ohms
    - 25 uF
  - iv. Immediate add 1 mL of SOC media, mixing well.
  - v. Incubate in shaker for 1 hour at 37C.
4. Plate 1:1000, 1:10,000, and 1:100,000 of 1 mL of the electroporation product on LB agar plates (chloramphenicol) and place the remainder into a ~200 mL (midiprep) culture with chloramphenicol.

- 5. Culture for 6-8 hours. Long/overnight cultures are usually fine but can occasionally result in a skewed distribution where certain variable regions are overrepresented in the product:**
- i. Midiprep the cultures and count colonies to assess library coverage and reaction efficiency. We conservatively recommend 1000x coverage to proceed.
  - ii. Mix the products (2A and 2B) proportionally by moles prior to proceeding to next step. We recommend using a small amount of each (2A and 2B) – **244 ng** is required for the next step. This is the **step 2 product**.

## Step 3 – Transfer TCR to destination vector

The step 2 product consists of a complete TCR library in the pGGA backbone vector. To enable expression of TCRs in cells, users can transfer this complete TCR library to a destination vector of choice. We have constructed a simple pHIV vector containing LacZ allowing for easy transfer of step 2 products with the 3-bp SapI type IIS enzyme. We suggest using Golden Gate assembly with SapI to facilitate efficient transfer of the TCR library from the cloning vector to a destination expression vector. If users wish to design new or modify existing expression vectors, we suggest adding the following to the desired destination vector, where SapI sites are underlined and the 3-bp overhang sites matching the TCR library are **bolded**. Ensure that there are **no SapI sites elsewhere in the vector**. If not interested in using SapI, we have included several unique and dual cut sites on either side of the TCR to facilitate transfer (see map).

5' - **GCA**GAAGAGC – LacZ/filler – GCTCTTC**ATC** - 3'

The following protocol transitions the TCR library from the pGGA vector to a destination vector; in this example, the destination vector is a pHIV vector.

### Required reagents:

Reagent	Supplier	Catalog #
NEBridge Ligase Master Mix	NEB	M1100
SapI	NEB	R0569
Nuclease-free water	Thermo Fisher (Invitrogen)	AM9937
Shrimp alkaline phosphatase (rSAP)	NEB	M0371
ElectroMAX DH10B <i>E. coli</i>	Thermo Fisher (Invitrogen)	18290015
MCE membrane filter, 0.025 µm pore size	Millipore Sigma	VSWP02500
Electroporation cuvette (1 mm gap)	VWR	89047-206
<b>Destination vector + antibiotic</b>		

#### A. Set up and run SapI golden gate reaction:

- Combine the following to create a 15uL reaction
  - X uL Destination (to yield 0.05pmol, use NEBioCalculator. For example, our pHIV vector is 7,440bp, so 0.05 pmol is 229.1ng).
  - Y uL Step 2 product (to yield 244ng, or 0.10pmol assuming 3950bp)
  - 5 uL NEBridge Ligase Master Mix
  - 1 uL SapI enzyme
  - 9 – X – Y uL Millipore water
- Incubate the reaction under the following cycling conditions
  - 37C, 5 min
  - 16C, 5 min
  - Repeat above for 60 cycles
  - 60C, 5 minHold overnight at 4C, then run final digest and heat inactivation step below:
  - 60C, 5 min
  - 65C, 20 min
  - Hold 4C

*Note: In contrast with steps 1 and 2, there is no clean-up cut for step 3.*

- #### B. Dialyze or SPRI clean and electroporate step 3 assembly product into DH10b *E. coli*, and midiprep the products, identically to steps 1/2 (under destination vector antibiotic selection). We recommend a shorter 6-8 hour culture to minimize skew.

# PCR amplification for NGS

Examples of primer sequences to add partial Illumina adapters are listed below. Cycling conditions are provided for NEBNext Ultra II Q5 Master Mix (NEB, # M0544) as a starting point for optimization with 1 µg of genomic DNA and 50 ng of plasmid/DNA template.

TCR $\alpha$  amplicons (TRAV-CDR3 $\alpha$ ):

F	5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNGACGTTGAGGAAAACCCAGGAC-3'
R	5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNCCAGACTTGTCACTGGATTTAGAGTCTCTCA-3'

Step	Temperature	Time
1	98°C	30 sec
2	98°C	30 sec
3	70°C	30 sec
4	72°C	45 sec
5	Go to step 2, repeat <b>14x (plasmid) / 23x (gDNA)</b>	
6	72°C	1 min
7	4°C	Hold

TCR $\beta$  amplicons (TRBV-CDR3 $\beta$ ):

F	5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNACCGGCATGAGCCACC-3'
R	5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNGTGTGGGAGATCTCTGCTTCTGA-3'

Step	Temperature	Time
1	98°C	30 sec
2	98°C	30 sec
3	70°C	30 sec
4	72°C	45 sec
5	Go to step 2, repeat <b>14x (plasmid) / 23x (gDNA)</b>	
6	72°C	1 min
7	4°C	Hold

To sequence the CDR3 oligos (CDR3 $\beta$ -CDR3 $\alpha$ ):

F	5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNACGCCAGATCCGGAAGAC-3'
R	5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNacggtctgagtcgGAAGAC-3'

Step	Temperature	Time
1	98°C	30 sec
2	98°C	30 sec
3	70°C	30 sec
4	72°C	30 sec
5	Go to step 2, repeat <b>12x</b>	
6	72°C	1 min