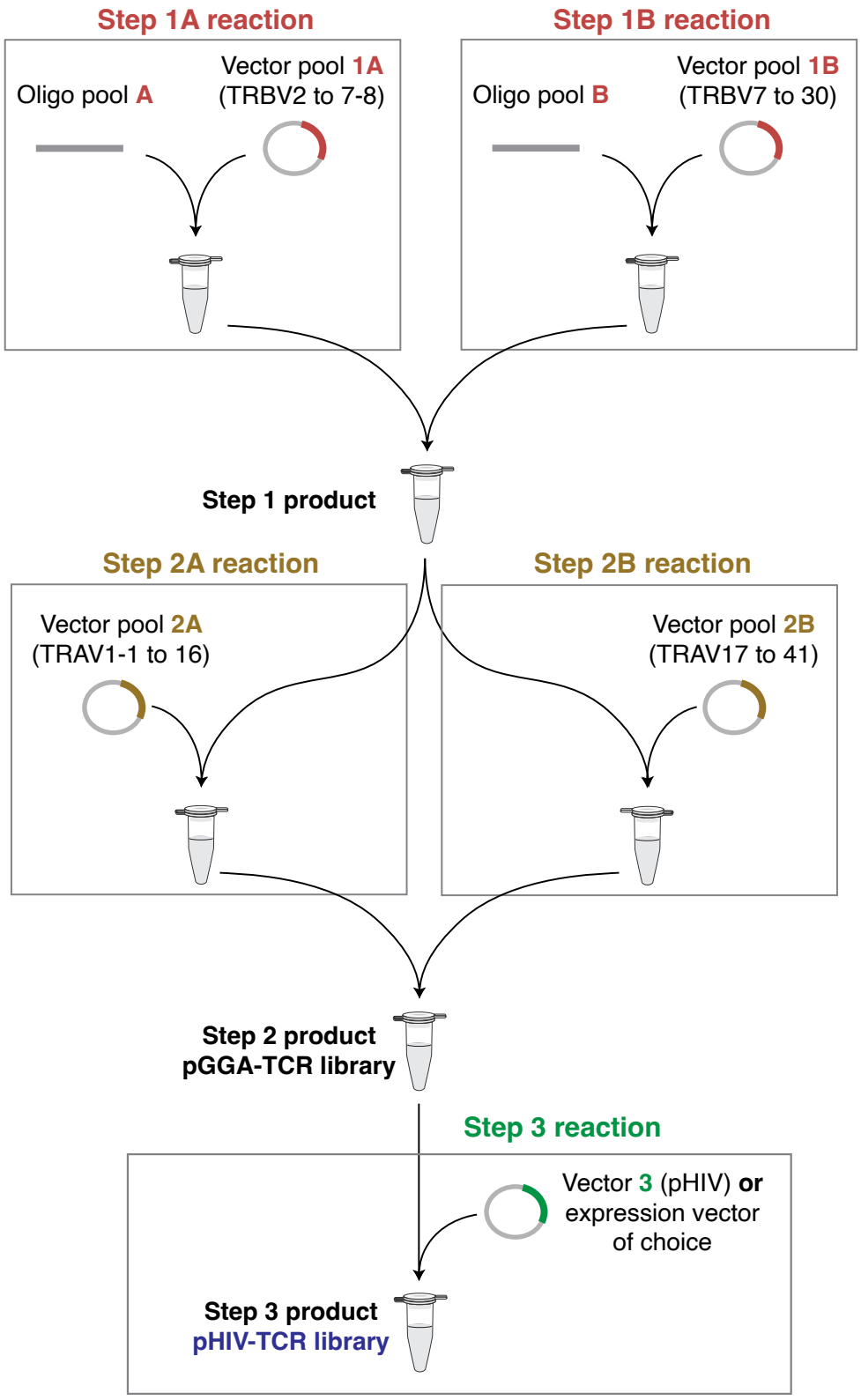
TCRAFT Overview



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 CDR3α and CDR3β amino acid sequences. See sample file on Github.

Output: oligos to order, split by length (≤ 300 bp and >300 bp). This enables separate orders depending on manufacturer (for example, Twist for ≤ 300 bp and IDT > 300 bp). In summary, the code output four .csv files with oligos to order: pool A ≤ 300 bp, pool A > 300 bp, pool B ≤ 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 vector **3**, a pHIV vector containing SapI sites and a LacZ insert. 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)

To execute this protocol, you require **~180 ng** of each vector pool and **~230 ng** of the final destination vector. To generate additional plasmid stock, transform at least **1 ng** of the 22-24 vector pools when transforming (yields >20,000 coverage) and ensure sufficient coverage.

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. Re-suspend 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. ≤ 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 x 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 *E. coli*	Thermo Fisher (Invitrogen)	18290015
MCE membrane filter, 0.025 um pore size	Millipore Sigma	VSWP02500
Chloramphenicol	Millipore Sigma	C0378
Electroporation cuvette (1 mm gap)	VWR	89047-206
1A and 1B vector pools		

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

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

- X uL Step 1 vector mix **1A** (to yield 0.1 pmol or 180 ng with 2929bp avg length)
- Y uL CDR3 oligo pool **A** (to yield 0.4 pmol, or 66ng with 268 bp avg length)
- 5 uL NEBridge Ligase Master Mix
- 1 uL BbsI-HF enzyme
- 9 – X – Y uL 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 15uL reaction with a 4:1 insert:vector ratio:

- X uL Step 1 vector mix **1B** (to yield 0.1 pmol or 180 ng with 2929bp avg length)
- Y uL CDR3 oligo pool **B** (to yield 0.4 pmol, or 66ng with 268 bp avg length)
- 5 uL NEBridge Ligase Master Mix
- 1 uL BbsI-HF enzyme
- 9 – X – Y uL 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 1uL BbsI-HF enzyme and 1uL rSAP to each tube
- Incubate at 37C for 1 hr, followed by 65C for 20 mins for final heat inactivation

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

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. 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 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.
3. 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.
4. Next day:
    i. Midiprep the cultures and count colonies to assess library coverage and reaction efficiency.
    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-CDR3βα-TRAC vectors. Step 2 inserts TRBC-P2A-TRAV fragments between the CDR3β and α. 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 midiprepped (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 (BsaI-HF-v2)	NEB	E1601
BsaI-HF-v2	NEB	R3733
BsmBI-v2	NEB	R0739
Nuclease-free water	Thermo Fisher (Invitrogen)	AM9937
Shrimp alkaline phosphatase (rSAP)	NEB	M0371
ElectroMAX DH10B *E. coli*	Thermo Fisher (Invitrogen)	18290015
MCE membrane filter, 0.025 um pore size	Millipore Sigma	VSWP02500
Chloramphenicol	Millipore Sigma	C0378
Electroporation cuvette (1 mm gap)	VWR	89047-206
2A and 2B vector pools		

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)
  - *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
- 17 – X – Y uL nuclease-free or Millipore water

Optional negative control: Replace step 1 final product with water.

B. Set up BsaI 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)
  - *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 **BsaI-v2** Master Mix
- 17 – X – Y uL nuclease-free or Millipore water

Optional negative control: Replace step 1 final product with water.

C. Cycling Conditions and Clean-up Cut

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

- **42C (BsmBI, 2A) or 37C (BsaI, 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 BsaI-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 (BsaI, 2B), followed by 80C for 20 mins for final heat inactivation

E. Dialyze and electroporate step 2 assembly product into DH10b *E. coli*, and midiprep the products, identically to step 1 (under chloramphenicol selection):

1. Drop dialyze product for 3 hours to remove inhibitory elements (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. 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 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.
3. 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.

4. Next day:
    i. Midiprep the cultures and count colonies to assess library coverage and reaction efficiency.
    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**aGAAGAGC – LacZ/filler – GCTCTTCt**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 *E. coli*	Thermo Fisher (Invitrogen)	18290015
MCE membrane filter, 0.025 um pore size	Millipore Sigma	VSWP02500
Electroporation cuvette (1 mm gap)	VWR	89047-206
Destination vector + antibiotic		

A. Set up and run SapI golden gate reaction:

1. Combine the following to create a 15uL reaction
   - X uL Destination (to yield 0.05pmol, use NEBioCalculator. pHIV-No EGFR 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
2. Incubate the reaction under the following cycling conditions
   - 37C, 5 min
   - 16C, 5 min
   - Repeat above for 60 cycles
   - 60C, 5 min

   Hold 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 and electroporate step 3 assembly product into DH10b *E. coli*, and midiprep the products, identically to step 1 and 2 (under destination vector antibiotic selection).

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.

TCRα amplicons (TRAV-CDR3α):

F	5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNGACGTTGAGGAAAACCCAGGAC-3'
R	5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNCAGACTTGTCACTGGATTTAGAGTCTCTCA-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 **14x (plasmid) / 23x (gDNA)**	
6	72°C	1 min
7	4°C	Hold

TCRβ amplicons (TRBV-CDR3β):

F	5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNACCGGCATGAGCCACC-3'
R	5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNGGTGTGGGAGATCTCTGCTTCTGA-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 **14x (plasmid) / 23x (gDNA)**	
6	72°C	1 min
7	4°C	Hold

To sequence the CDR3 oligos (CDR3β-CDR3α):

F	5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNACGCCAGATCCGGAAGAC-3'
R	5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNacggtctgagtcgGAAGAC-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 **12x**	
6	72°C	1 min