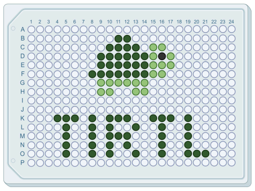
May 20, 2025 Version 1

TIRTL-seq 96-well V.1



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We use this protocol and it's working

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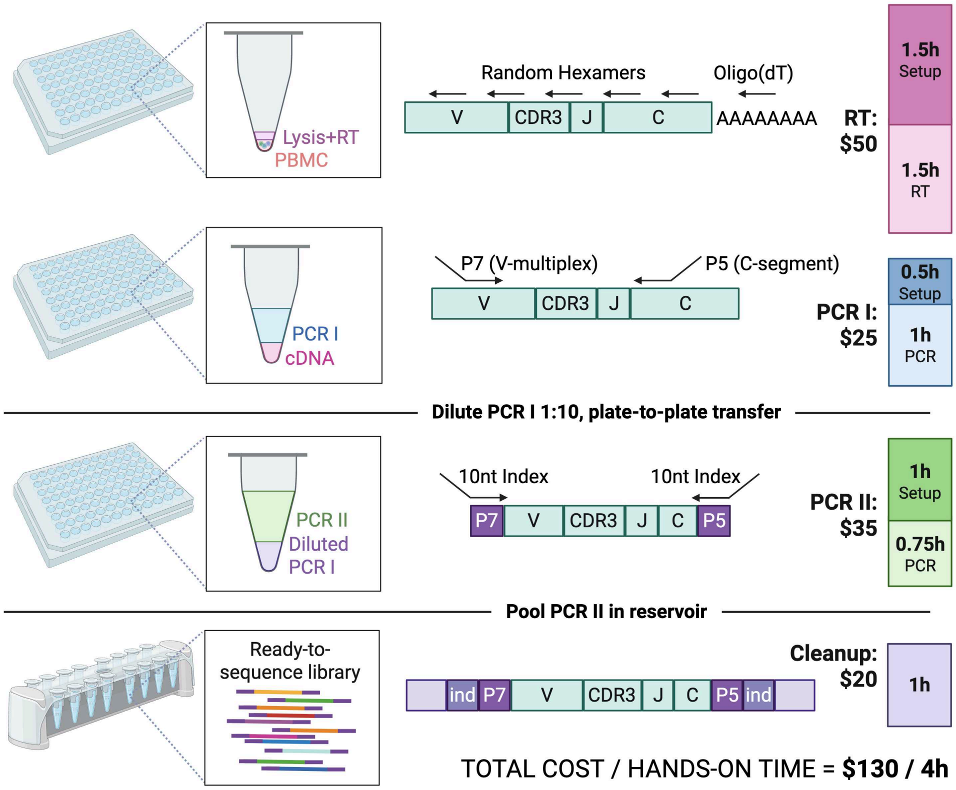
Disclaimer

Abstract

TIRTL-seq is a method to obtain paired TCR-sequencing information from millions of human T cells. It is based on splitting cells into 96-384 wells, preparing and sequencing TCRalpha and TCRbeta libraries from each well in miniaturized reactions, and then pairing alpha and beta chains based on co-occurrence patterns or relative frequency variations between wells. For more details about the method, please see our preprint (**https://pubmed.ncbi.nlm.nih.gov/39345544/**). While the 384-well protocol requires some automation, this protocol describes a modified 96-well plate version that can be performed using only standard multichannel pipettes. The 96-well protocol has increased volumes for easier pipetting, can accommodate similar cell numbers to the 384-well protocol, and has a similar cost and preparation time, but also has lower resolution.



Schematic of TIRTL-seq protocol. Briefly, a cell suspension is distributed into 96-well plates containing RT/lysis mastermix using multichannel pipettes. After the RT reaction, PCR I mastermix with V-segment and C-segment primers is dispensed into the same plate. The PCR I product is then diluted and transferred to the PCR II plate for indexing PCR with well-specific unique dual indices. The PCR II products are pooled by centrifugation, purified, size-selected using magnetic beads, and sequenced on an Illumina platform. Total library preparation cost is listed for one 96-well plate.

Guidelines

Input material:

1. We found the TIRTL-seq protocol to be robust and perform well on both fresh and cryopreserved PBMCs, as well as on T cells enriched by magnetic beads or sorted using a cell sorter. Sample viability should be >50%. If dealing with low viability (<50%) or low T cell content tissue, we recommend enriching for T cells (i.e. CD4/CD8 positive isolation with Dynabeads with Detach or sorting) prior to TIRTL-seq. **Note that this version of the protocol works only for human T cells.**
2. Increasing the number of T cells per well increases the yield of paired TCRs without affecting the cost, with best results achieved at ~10 million PBMCs/purified T cells per 96-well plate (or 100K cells/well). If you have even more T cells available, it is recommended to do multiple plates per sample instead of scaling up volumes or loading more cells per well.
3. **Note that TIRTL-seq is not able to pair clones found in <4 cells in your sample.** If you have very few T cells, consider single cell/well sorting modification of the protocol (see Appendix I; almost all individual cells will be paired in this modification) or doing antigen-independent T cell expansion before running the TIRTL-seq to expand small clones to >3 cells/clone level.

Materials

TIRTL_seq_primers_v1.xls

Primers Primers (see Excel file above for sequences)

1. hTCRab_Vmix_P7 (Forward V multiplex with P7 adapters)
2. hTCRab_Cmix_IDXX_P5 (Reverse C-segment primers with P5 adapters and plate barcodes)
3. Well Index Primers (Nextera P5 and P7 with 10nt indices from cite SS3xpress protocol with minor modifications)

Reagents

Cell culture

- RPMI-1640 **Thermo Fisher Scientific Catalog #**22400089
- Fetal Bovine Serum **Thermo Fisher Scientific Catalog #**16140071
- L-Glutamine (200 mM) **Thermo Fisher Catalog #**25030081
- Penicillin-Streptomycin (10,000 U/mL) **Thermo Fisher Scientific Catalog #**15140122
- Gibco™ DPBS no calcium no magnesium **Thermo Fisher Scientific Catalog #**14190144

Molecular Biology

- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**X100-100ML
- Nuclease-Free Water (not DEPC-Treated) **Thermo Fisher Scientific Catalog #**AM9937
- dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10mM) **Thermo Fisher Scientific Catalog #**R0192
- Random Hexamer **Thermo Fisher Scientific Catalog #**#SO142
- Oligo(dT)18 **Integrated DNA Technologies, Inc. (IDT) Catalog #**custom order
- Dithiothreitol (DTT) **Thermo Fisher Scientific Catalog #**707265ML
- Recombinant RNase Inhibitor **Takara Bio Inc. Catalog #**2313B
- Maxima™ H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog #**EP0753
- 2X KAPA2G Fast Multiplex Kit **Roche Diagnostics Catalog #**7961430001
- Q5 Hot Start High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #**M0493L
- Ampure XP beads **Beckman Coulter Catalog #**A63881
- Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q32854
- D1000 ScreenTape **Agilent Technologies Catalog #**5067-5582
- D1000 Reagents **Agilent Technologies Catalog #**5067-5583
- D1000 Ladder **Agilent Technologies Catalog #**5067-5586

Plastics and Other Consumables

Eppendorf twin.tec® PCR 96-well plate, skirted **Eppendorf Catalog** #951020401

V-bottom 200 mL reservoir **ClickBio, Inc. Catalog** #CBVBLOK200-1

Sealing films Polyester **VWR International (Avantor) Catalog** #60941-062 (For steps where a loose seal is desired, e.g. transferring plates between pre- and post-PCR I zones)

MicroAmp Clear Adhesive Film **Applied Biosystems (ThermoFisher Scientific) Catalog** #4306311 (For sealing plates for PCR and storage)

Optional (see Recommended Equipment)

12.5 µL GRIPTIPS (Sterile, Filter, XYZ Racks of 384 Tips) **Integra Biosciences Catalog** #6455

Recommended Equipment

This protocol can be carried out exclusively using standard multichannel pipettes (minimally, a 1-10 uL multichannel and a 20-200 uL multichannel) and standard single channel pipettes.

96-well plate thermocyclers: We use Bio Rad C1000 Touch/S1000, but almost any other thermocycler should work.

Plate centrifuges with bucket rotors: We use a Sorvall ST8R/ST8 with an M10 rotor. One is in the pre-PCR zone, one is in the post-PCR zone (for pooling by centrifugation). **NOTE:** for pooling by centrifugation with ClickBio reservoir, make sure it fits into the bucket (for Thermo Fisher M10 rotor it fits into unsealed bucket, cat. 75005723).

Thermo Fisher Qubit Fluorometer: Used to quantify final library.

Agilent Tapestation: Used to determine library size and quality.

We recommend the following other instruments:

While not required, an Integra Mini 96 (12.5 uL head) or Integra ViaFlo (96-channel, 12.5 uL head) is also helpful to ensure rapid and accurate transfer of Well Index primers in the PCR II step. If you choose to use one of these liquid handlers, you will also need the Integra 12.5 uL GRIPTIPS listed under Plastics and Other Consumables above. These are sold in racks of 384 tips, so each tip box can be used for four 96-well plates.

Troubleshooting

Safety warnings

To avoid contamination with PCR products it is crucial to separate pre-PCR and post-PCR zones. Reagents, equipment, and samples should never move from post-PCR zones to pre-PCR zones. We recommend doing all work in PCR workstations or biosafety cabinets and using UV to decontaminate the workspace after each procedure. We also recommend that you work in gloves, use filtered tips, and put away primers, beads, and other reagents before opening any PCR product.

Steps performed in the pre-PCR zone:
PBMC preparation, RT reaction setup, PCR I reaction setup, primer dilution, reagent/primer storage. PCR I/PCR II plates are never opened or stored in this zone.

Steps performed in the post-PCR zone: PCR I reaction dilution, transfer of PCR I product to PCR II reaction. PCR II pooling, PCR II pool size-selection, and QC. In our lab, PCR II pooling, size-selection, and QC (the stage with highest PCR product concentration) is also done in a third separate room (post PCR II zone).

Ethics statement

Please note that this protocol is designed for human samples and prior approval from your Institutional Review Board (IRB) or equivalent ethics committee is required before commencing any work. Please make sure that processing of human tissues is performed in accordance with your local biosafety regulations, including appropriate containment and personal protective equipment.

Before start

Primer Preparations

PCR I - Forward primer mix (hTCRab_Vmix_P7 2.27uM each):

We use a downsized subset of forward V-segment specific primers from Howie et al (Sci Trans Med, 2015: https://www.science.org/doi/full/10.1126/scitranslmed.aac5624) modified with P7 adapter sequences and excluding pseudogene-specific primers. 88 primers are ordered from IDT in a 96-well plate format at 400 uM each in IDTE buffer. See the attached excel file for list of primer sequences.

1. Mix equal volume of each primer with multichannel pipette in a reservoir.
2. Aliquot 400 uL of primer mix into 1.5 ml tubes with 400 uL of nuclease free water to get 2.27uM each working dilution.
3. Store tubes at -20 °C.

PCR I - Reverse primer mixes with plate-specific barcodes (hTCRab_Cmix_IDXX_P5 10uM each):

We included 7 or 10nt long custom inline index sequences for pooling plates together for sequencing; these indices are distinct from well-specific barcodes in PCR II primers (see below). We chose the index sequences to reduce amplification bias among the different C segment genes. Reverse primers with plate indices were ordered from IDT in tube format as 100 uM LabReady solution in IDTE buffer. See the attached excel file for list of primer sequences.

1. Mix 50 uL of TCRalpha + 50 uL of TCRbeta + 400 uL of nuclease free water to get 10 uM each working dilution. Note the plate index, as this will be crucial for the demultiplex pipeline. We advise to mix TCRalpha_ID01 with TCRbeta_ID01, TCRalpha_ID02 with TCRbeta_ID02 and so on (other combinations are likely to work, but this has not been tested).
2. Store tubes with mixed plate index reverse primers at -20 °C.

PCR II (indexing with well-specific barcodes):

We use primers from Hagemann-Jensen et al (Smart-seq3xpress - Nature Biotech, 2022: https://www.nature.com/articles/s41587-022-01311-4) with small modifications to a few underperforming barcodes. The primers were ordered from IDT in 384-well plate format 200 uM concentration (one plate of 384 i5 forward primers and one plate of 384 i7 reverse primers). See the attached excel file for list of primer sequences. We use the same primers for 96-well TIRTL-seq as 384-well TIRTL-seq and simply use one “quadrant” of primers for 96-well plates—i.e. odd-numbered rows and columns beginning with A1 or B1 or even numbered rows and columns beginning with A2 or B2. Alternatively, plates could be ordered with only 96 i5 forward primers and 96 i7 reverse primers; any 96 unique primers from the given spreadsheet will work.

To minimize potential contamination and freeze-thaw cycles we recommend making as many mixed primer plates as possible and using a serial dilution strategy to optimize storage.

To make 20X stock (20uM each):

1. Dispense 24 uL of nuclease-free water into 384 well plates and briefly centrifuge the plates.
2. Using Integra Viaflo with 384-channel head: Dispense **3 uL** of source forward primers (IDT 384 well plate, 200 uM each) from i5 IDT plate to the 384 well plates containing 24 uL of nuclease-free water using repeat dispense program (if making multiple plates). Eject tips once done.

3. Dispense 3 uL of source reverse primers (IDT 384 well plate, 200 uM each) from i7 IDT plate to the 384 well plates containing 27 uL of diluted i5 primer using repeat dispense program.
4. Seal the plates, making sure all the corners are sealed. Label the plates. (**20 uM each of forward and reverse primers in 30 uL**).
5. Store the plates in -20 °C.

To make 1X working dilution primer plate (1 uM each) from 20X stock:

1. Dispense **23.8 uL** of nuclease-free water into 384 well plates using Integra Welljet liquid dispenser and briefly centrifuge the plates.
2. Briefly centrifuge the Stock (20X) PCR II Primer plate (thaw first and centrifuge if frozen), carefully remove the seal.
3. Mix the contents using the mix program in the Integra Viaflo. Discard the tips if there are some extra droplets outside or some air bubbles trapped.
4. Stamp 1.25 uL from Stock (20X) PCR II Primer plate to the 384 well plates containing 23.8 uL of nuclease-free water using repeat dispense program (if making multiple plates).
5. Seal the plates, making sure all the corners are sealed. Label the plates. (**1 uM each of forward and reverse primers in 25 uL**)
6. Store the plates in -20 °C.

1. Cell Preparation

1 ***NOTE:*** *This section describes processing for cryopreserved PBMCs/T cells. If starting from fresh/sorted/magnetically enriched PBMCs/T cells, go to* ***step 3****.*
Thaw cryopreserved cells in a 37 °C water bath until only a small ice crystal remains, then add suspension drop-wise to 10 mL complete RPMI (cRPMI) pre-warmed to 37 °C.

- Complete RPMI = RPMI 1640, 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin

2 Centrifuge 500xg for 5 minutes.

3 Aspirate supernatant and resuspend cells in 5 mL DPBS.

4 Count cells. We recommend counting cells with live/dead dyes (AO/PI, trypan blue, etc.) to determine cell viability.

5 Centrifuge 500xg for 5 minutes.

6 Aspirate supernatant and resuspend cells in 500 uL DPBS. Transfer immediately to a 1.5 mL tube, being careful to collect the entire volume.

7 Centrifuge 500xg for 5 minutes.

8 Aspirate supernatant carefully and resuspend to a final volume of **exactly** 110 uL DPBS. ⚠

CRITICAL: Do not alter this cell resuspension volume; always use 110 uL for a full 96-well plate. Using only part of a 96-well plate is not recommended because doing so will greatly reduce TCR pairing efficiency.

2. Reverse Transcription

9 **In pre-PCR zone:**
Prepare Lysis+RT master mix as follows:

	A	B	C
	Component	1X Volume (1 well)	150X Volume (1X 96-well plate)
	H2O	0.6 uL	90 uL
	Triton X-100 (1% v/v solution)	0.16 uL	24 uL
	dNTPs (10 mM each)	0.1 uL	15 uL
	Random Hexamer (200 ng/uL)	0.025 uL	3.75 uL
	Oligo(dT) (50 uM)	0.1 uL	15 uL
	5X MaximaH RT Buffer	0.4 uL	60 uL
	DTT (100 mM)	0.1 uL	15 uL
	RNase Inhibitor (40 U/uL)	0.08 uL	12 uL
	Maxima H Enzyme (200 U/uL)	0.04 uL	6 uL
	TOTAL	1.6 uL	240 uL

10 Distribute 30 uL Lysis+RT master mix to each tube of a 0.2 mL tube strip.

11 Load 1.6 uL/well Lysis+RT master mix from the tube strip to each well of an empty 96-well plate using a multichannel pipette.

12 Distribute 13.75 uL cells to each tube of a new 0.2 mL tube strip.

13 Load 1 uL/well cells from the tube strip to the 96-well plate using a multichannel pipette and mix.
NOTE: Cells can also be sorted directly into 96-well plates—see Appendix I.

14 Seal plate and pulse centrifuge 1000xg for 10 seconds.

15 Incubate in a thermocycler with the following protocol:

	Temperature	Time

42 °C	5 minutes
25 °C	10 minutes
50 °C	60 minutes
94 °C	5 minutes
4 °C	hold

SAFE STOP POINT: The RT plate can safely be stored at -80 °C for several days before proceeding to PCR I.

3. PCR I

16 **In pre-PCR zone:**
Prepare PCR I master mix as follows:

A	B	C
Component	1X Volume (1 well)	120X Volume (1X 96-well plate)
hTCRab_Vmix (2.27 uM each)	0.9 uL	108 uL
hTCRab_Cmix_IDXX_P5 (10 uM each)	0.8 uL	96 uL
2X KAPA2G Fast Multiplex	5 uL	600 uL
H2O	1.3 uL	156 uL
TOTAL	8 uL	960 uL

CRITICAL: If multiple plates will be pooled and sequenced together in the same run, each plate MUST use a unique Plate Index Reverse primer. Note the Plate Index primer used for each plate, and prepare the master mix for each plate separately.

17 Distribute 120 uL PCR I master mix to each tube of a 0.2 mL tube strip.

18 Dispense 8 uL/well PCR I master mix from the tube strip to the RT 96-well plate using a multichannel pipette and mix.

19 Seal the plate and pulse centrifuge 1000xg for 10 seconds.

20 Incubate in a thermocycler with the following protocol:

	A	B	C
	Temperature	Time	Cycles
	95 °C	3 minutes	
	95 °C	15 seconds	20 cycles
	59 °C	30 seconds	
	72 °C	1 minute	
	72 °C	5 minutes	
	4 °C	hold	

SAFE STOP POINT: The PCR I plate can safely be stored at -20 °C for days to weeks before proceeding to PCR II.

4. PCR II

21 **In pre-PCR zone:**
Prepare Q5 master mix as follows:

	A	B	C
	Component	1X Volume (1 well)	120X Volume (1X 96-well plate)
	5X Q5 Buffer	2 uL	240 uL
	dNTPs (10 mM each)	0.2 uL	24 uL
	Q5 Hot Start DNA Polymerase	0.1 uL	12 uL
	H2O	3.7 uL	444 uL
	TOTAL	6 uL	720 uL

22 Distribute 90 uL Q5 master mix to each tube of a 0.2 mL tube strip.

23 Load 6 uL/well Q5 master mix from the tube strip in an empty 96-well plate using a multichannel pipette.

24 Loosely seal plate and pulse centrifuge 1000xg for 10 seconds.

25 Transfer plate to post-PCR I zone.

CRITICAL: To prevent contamination between experiments, it is critical to not open the PCR I plate in the pre-PCR I zone; it should only be opened in the post-PCR I zone. Similarly, DO NOT return to the pre-PCR zone on the same day after proceeding past this step.

26 **In post-PCR I zone:**

CRITICAL: To prevent contamination of primer plates, open the primer plate in the PCR workspace first, and reseal and store the primer plate BEFORE opening any PCR I product plates. DO NOT simultaneously have the primer plate and diluted PCR I products opened in the PCR workspace.

27 Add 2 uL/well Well Indexing primers from 1X primer plate to Q5 plate using a multichannel pipette or Integra Viaflo or Integra Mini.

28 Reseal 1X primer plate and return to -20 °C storage.

29 Dilute PCR I product ~10x by adding 90 uL H2O to PCR I plate using a multichannel pipette and mix well.

30 Add 2 uL diluted PCR I product to Q5+Primer plate using a multichannel pipette and mix.

31 Seal plate and pulse centrifuge 1000xg for 10 seconds.

32 Incubate in a thermocycler with the following protocol:

1.

	A	B	C
	Temperature	Time	Cycles
	98 °C	30 seconds	
	98 °C	10 seconds	15-18 cycles
	58 °C	10 seconds	
	72 °C	50 seconds	
	72 °C	2 minutes	
	4 °C	hold	

CRITICAL: Choose the number of PCR cycles used based on the number of cells loaded in the experiment. For experiments with >2000 cells/well, 15 cycles is recommended. For experiments with 1-2000 cells/well, up to 18 cycles is recommended.

33 Proceed to pooling and cleanup.

CRITICAL: Proceed immediately to pooling and cleanup. PCR II products cannot be safely stored for more than a few hours without cleanup.

5. PCR II Pooling and Cleanup

34 Pool PCR II products by carefully inverting the PCR II plate into a ClickBio V-bottom 200 mL reservoir. Ensure the plate fits snugly in the reservoir.

35 Centrifuge the plate+reservoir assembly at 100xg for 30 seconds, such that centrifugation will empty all wells into the reservoir. After centrifugation, ensure all wells in the plate are empty.

36 Collect 100 uL pooled PCR II product and transfer to a 1.5 mL tube.

37 Purify the PCR II library using AMPure beads at ratio of beads to sample indicated below:

- For most samples, we recommend using a 0.9:1 bead:sample ratio.

- If using high numbers of cells (>10,000 cells/well or 4 million cells/plate), using a 0.8:1 bead:sample ratio may yield a cleaner Tapestation trace.

- If performing TIRTL-seq on single cells directly sorted into plates, use a 0.7:1 bead:sample ratio and perform the full cleanup (using the same ratio of beads:sample) twice.

38 Mix beads and PCR II product well and incubate for 10 minutes at room temperature.

39 Place beads on a magnet and wait for the solution to clear (~3 minutes).

40 Carefully remove supernatant without disturbing beads.

41 Wash beads with 200 uL **freshly prepared** 80% ethanol. Wait for 30 seconds, then discard the ethanol.

42 Repeat the above wash step for 2 washes total.

43 Briefly spin the tube, remove residual ethanol and air dry beads for 1-3 minutes.

44 Elute final library by adding 45 uL Nuclease-free water to beads and mixing well. Incubate for 5 minutes at room temperature.

45 Place beads on a magnet and wait for the solution to clear (~3 minutes).

46 Carefully remove 45 uL purified library and proceed to final QC.

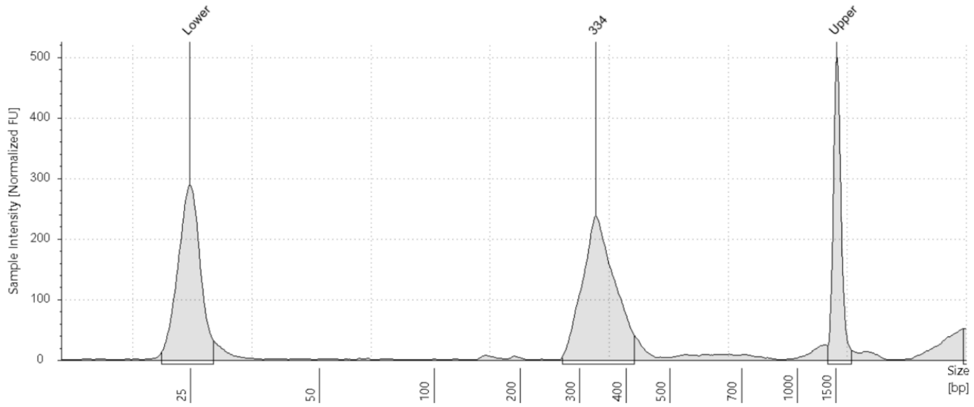
47 **SAFE STOP POINT:** Purified libraries can be stored at -20 °C for many days and weeks before sequencing.

6. Final Library QC

48 **In post-PCR II zone:**
Quantify the final library using a Qubit fluorometer and the Qubit dsDNA HS assay according to manufacturer instructions. Expected concentration after purifying 100uL of PCRII products eluted in 45uL is >10 ng/uL, most likely ~30-40 ng/uL for samples with good viability and high number of cells/well.

49 Run the final library on an Agilent Tapestation using D1000 reagents (or similar) to inspect quality and confirm the library size. The final library size should be ~340nt, and no short (<250nt) products should be present.

A representative Tapestation D1000 trace is below.



7. Sequencing

50 Final purified libraries are ready to sequence on any Illumina sequencer (preferred read length 2×150nt).

51 The target read number is approximately 100 million reads per 10 million cells.

52 If libraries are prepared from multiple plates that have been uniquely indexed in PCR I, and all libraries are of similar size and quality, they can be pooled (usually in equal proportion by mass) for sequencing in a single run in the same lane. Scale up the number of reads requested accordingly. Note that wells within plates are barcoded on both sides, while plates are only barcoded on C-segment side, so there is a possibility of low-level barcode exchange (index hopping) across matching wells between plates sequenced on one lane.

8. Troubleshooting

53

	A	B
	Problem	Potential Solution(s)
	Short peaks (<300bp) are present in the QC	Repeat library clean up with lower bead:sample ratio (e.g. 0.8:1).
	Faint band/low yield	Repeat PCR II with higher number of cycles
	Some wells seem empty or generate unexpectedly low numbers of unique clones after sequencing	If there is a clear geometric pattern, make sure the non-contact liquid dispenser is well calibrated (dispensing expected volume of liquid exactly in the center of the well) using food coloring.
		If only the last wells are empty, check if you run out of liquid early during the dispense and increase cell suspension volume.
		If last wells are low/empty and first wells have higher amounts of cells this indicates settling of cells before the dispense. Resuspend cells immediately before dispensing.

9. Data Analysis

54 See https://github.com/pogorely/TIRTL for guidance on data analysis

10. Appendix I: Single-cell TIRTL-seq

55 In certain cases you might be interested in sequencing a very low number of cells or while simultaneously measuring surface expression of markers of interest on sequenced cells. In this case you can perform the protocol after index sorting a single cell/well into a 96-well plate pre-loaded with Lysis+RT master mix with the following modifications:

1. Increase the number of PCR II cycles to 18.
2. Perform two successive cleanups 0.7:1 Ampure XP:sample ratio to exclude short primer-dimer band.
3. Sequence at 0.5-1 million 2×150nt reads per plate on any Illumina sequencer.
4. Do not run combinatorial TIRTL-seq analysis on resulting data: resulting mixcr cloneset usually have just one (or two for double alphas) major clones per well with additional low frequency clonotypes resulting from sequencing errors. Just filtering out low frequency erroneous clones (e.g. <50 reads or <10% readFraction) is sufficient to call paired TCR for each well.