



## **RNase H-dependent PCR-enabled T-cell receptor sequencing for highly specific and efficient targeted sequencing of T-cell receptor mRNA for single-cell and repertoire analysis**

**Li, Shuqiang; Sun, Jing; Allesøe, Rosa Lundbye; Datta, Krishnalekha; Bao, Yun; Oliveira, Giacomo; Forman, Juliet; Jin, Roger; Olsen, Lars Rønn; Keskin, Derin B.**

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## RNase H-dependent PCR-enabled T Cell Receptor sequencing (rhTCRseq) for Highly Specific and Efficient Targeted Sequencing of T Cell Receptor mRNA for Single-Cell and Repertoire Analysis

Shuqiang Li<sup>1,2,3</sup>, Jing Sun<sup>2</sup>, Rosa Allesøe<sup>2,4</sup>, Krishnalekha Datta<sup>5</sup>, Yun Bao<sup>5</sup>, Giacomo Oliveira<sup>2</sup>, Juliet Forman<sup>1,2,3</sup>, Roger Jin<sup>6</sup>, Lars Rønn Olsen<sup>4</sup>, Derin B. Keskin<sup>2,3</sup>, Sachet A. Shukla<sup>2,3</sup>, Catherine J. Wu<sup>1,2</sup>, Kenneth J. Livak<sup>2,3</sup>

<sup>1</sup>Broad Institute of MIT and Harvard, Cambridge, MA, USA

<sup>2</sup>Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

<sup>3</sup>Translational Immunogenomics Lab, Dana-Farber Cancer Institute, Boston, MA, USA

<sup>4</sup>Department of Bio- and Health Informatics, Technical University of Denmark, Kongens Lyngby, Denmark

<sup>5</sup>Integrated DNA Technologies, Redwood City, CA, USA

<sup>6</sup>Massachusetts Institute of Technology, Cambridge, MA, USA

### Abstract

rhTCRseq (RNase H-dependent PCR-enabled T cell receptor sequencing) is a procedure that can be used to determine paired alpha/beta T cell receptor (TCR) clonotypes in single cells or perform both alpha and beta TCR repertoire analysis in bulk RNA samples. Relying on the enhanced

Corresponding Author: Kenneth J. Livak, kennethj\_livak@dfci.harvard.edu, Phone 617-582-9694, Mobile 408-375-8104, Fax 617-632-6380.

#### Author contributions

S.L. and K.J.L. devised the rhTCRseq scheme. S.L. developed and performed rhTCRseq experimentation. J.S., R.A., J.F., R.J., L.R.O., and S.A.S. devised and wrote the computational pipeline and performed data analysis. K.D. and Y.B. developed rhPCR protocols and provided rhPCR primers. G.O. and D.B.K. provided single cells sorted into plates for the development of the rhTCRseq protocol. G.O. performed the single-cell preparation and sorting of Supplementary Fig. 3. C.J.W. and K.J.L. directed the overall study design. S.L., J.F., C.J.W. and K.J.L. wrote the manuscript with comments and editing by all authors.

#### Competing interests

J.S. is currently an employee of Moderna Therapeutics. K.D. is an employee of IDT; Y.B. was an employee of IDT during the time of this work; K.J.L. was a paid consultant of IDT during a portion of this work; D.B.K. has previously advised Neon Therapeutics, and owns equity in Aduro Biotech, Agenesis Inc., Ampliphi BioSciences Corp., Biomarin Pharmaceutical Inc., Bristol Myers Squibb Com., Celldex Therapeutics Inc., Editas Medicine Inc., Exelixis Inc., Gilead Sciences Inc., IMV Inc., Lexicon Pharmaceuticals Inc., Sangamo Therapeutics, and Stemline Therapeutics Inc.; C.J.W. is a founder of Neon Therapeutics and a member of its scientific advisory board. The remaining authors declare no competing financial interests. C.J.W. is subject to a conflict of interest management plan for the reported studies because of her competing financial interests in Neon Therapeutics. Under this plan, C.J.W. may not access identifiable human subjects data nor otherwise participate directly in the IRB-approved protocol reported herein. C.J.W.'s contributions to the overall program strategy and data analyses occurred on a de-identified basis.

**TWEET** rhTCRseq for Highly Specific and Efficient Targeted Sequencing of T Cell Receptor mRNA for Single-Cell and Repertoire Analysis

#### Related links

Key reference using this protocol

1. Keskin, D. B. *et al.* Neoantigen vaccine generates intratumoral T cell responses in phase Ib glioblastoma trial. *Nature* **565**, 234–239 (2019). doi: 10.1038/s41586-018-0792-9.

specificity of RNase H-dependent PCR (rhPCR), it achieves TCR-specific amplification and addition of dual index barcodes in a single PCR step. For single cells, the stages of the protocol are sorting single cells into 96- or 384-well plates, generation of full-length cDNA libraries, the single TCR-specific amplification step, a second PCR on a pooled sample to generate a sequencing library, and sequencing on the MiSeq platform. In the bulk RNA method, the sorting and cDNA library steps are replaced with a reverse transcriptase reaction that add a Unique Molecular Identifier (UMI) to each cDNA molecule in order to improve the accuracy of repertoire frequency measurements. Compared to other multiplex PCR methods for TCR sequencing, rhTCRseq has a streamlined workflow and the ability to analyze single cells in 384-well plates. Compared to TCR reconstruction from single-cell transcriptome sequencing data, it improves the success rate for obtaining paired alpha/beta information and ensures recovery of complete CDR3 sequences, which is a prerequisite for the cloning and expression of discovered TCRs. Although it does not have the throughput of droplet-based methods, rhTCRseq is well-suited to the analysis of small sorted populations, especially cases where analysis of 96 or 384 single cells is sufficient to identify predominant T cell clones. For single cells, sorting typically requires two to four hours and can be performed days, or even months, before library processing. The remainder of the single cell protocol takes on the order of four days, including data processing. For bulk RNA, the overall time is about three days, including data processing.

## EDITORIAL SUMMARY

Here the authors describe rhTCRseq, RNase H-dependent PCR-enabled TCR sequencing, for repertoire analysis from bulk RNA samples or single-cell profiling.

## Keywords

T cell receptor repertoire; T cell receptor; paired TCR $\alpha\beta$  single-cell sequencing; RNase H-dependent PCR; immunotherapy; neoantigen vaccination; rhPCR-enabled TCR sequencing; rhTCRseq

## Introduction

Characterization of the immune response to pathogens, cancer, and therapeutic interventions has long used T cell receptor (TCR) diversity/heterogeneity as a measure of clonal diversity and immune response because T cells are primary effector cells of antigen-specific immunity. A TCR interacts with a peptide antigen bound to a major histocompatibility complex (MHC) molecule mainly through the paired alpha- and beta-complementarity determining regions 3 (CDR3). Thus, characterizing the antigen specificity of a T cell involves determining the sequence of the CDR3 segments.

Recent single-cell sequencing technologies have facilitated the identification of which TCR interacts with a particular antigen through establishing the exact pairing of TCR-alpha and TCR-beta chains. The first methods described used multiplex PCR with a mixture of *TRAV*- and *TRBV*-specific forward primers for targeted TCR amplification from single-cell cDNA followed by sequencing of these low complexity libraries<sup>1-5</sup>. A second approach reconstructs TCR sequences from global single-cell RNA sequencing (scRNA-seq) data<sup>6-9</sup>.

Because these computational methods require full-length sequence information, they cannot be applied to the end-counting scRNA-seq data generated by droplet-based procedures. Recently, 10x Genomics has commercialized a protocol that does enable determination of single-cell TCR sequences from their droplet-generated libraries and this product has begun generating published results<sup>10,11</sup>. Finally, pairSEQ is not a single-cell method, but it does generate paired alpha/beta information by computational imputation from TCR sequence data acquired by dividing a bulk sample into 96 subsets<sup>12</sup>.

## Overview of the protocol

Here we detail the rhTCRseq (rhPCR-enabled TCR sequencing) protocol that was used in Keskin *et al.*<sup>13</sup> to analyze TCR clonotypes in glioblastoma patients who were part of a neoantigen vaccination clinical trial. rhTCRseq uses highly specific PCR to reliably achieve targeted amplification of alpha and beta CDR3 segments from TCR transcripts in a streamlined workflow. In order to develop a method for amplifying TCR segments that is compatible with next-generation sequencing (NGS) library generation, rhTCRseq takes advantage of RNase H-dependent PCR (rhPCR), a technique that greatly enhances the specificity of PCR<sup>14</sup>. As diagrammed in Fig. 1a, rhPCR uses 3'-blocked oligonucleotides with a single ribo residue located approximately five nucleotides from the 3' end. By including thermostable RNase H in the amplification reaction, these blocked oligonucleotides are cleaved at the RNA base if, and only if, the oligonucleotide is hybridized to an appropriate target. Cleavage generates a free 3'-hydroxyl that is extended by *Taq* DNA polymerase. Thus, functional primers are generated *in situ* during the PCR and accurate hybridization of the proto-primers is required during every round of PCR in order to achieve exponential amplification. This technique is very specific because the absence of free primers not hybridized to target essentially eliminates primer dimer formation, and the requirement of RNase H for high-fidelity base pairing severely reduces off-target amplification.

For the analysis of single cells, Fig. 1b diagrams the scheme for using rhPCR to re-amplify CDR3 segments from whole transcript single-cell cDNA libraries, such as those generated as an intermediate step in Smart-seq<sup>215,16</sup>. To amplify CDR3-containing segments, 69 rhPCR primers (38 alpha and 31 beta) were designed to hybridize to all functional alpha and beta TCR variable genes (Supplementary Table 1). These primers include a Rd1 (Read 1) sequence needed for Illumina-based sequencing. The two rhPCR primers specific for *TRAC* and *TRBC* (the constant segments of TCR alpha and beta loci, respectively) have an Illumina Rd2 (Read 2) sequence. In addition to the TCR-specific primers, the amplification reaction contains flanking rhPCR primers (Supplementary Table 2) that incorporate index sequences into the final amplification products and append the P5 and P7 sequences needed for Illumina-based sequencing. By using distinct index sequences for different single-cell libraries, a single PCR step specifically amplifies CDR3 segments and introduces barcodes for each sample. The 768 primers listed in Supplementary Table 2 enable dual indexing for pools of up to 384 samples. Dual indexing is used to minimize the complications of data interpretation caused by index switching<sup>17,18</sup>. After the first PCR step shown in Fig. 1b, samples are pooled for further processing and sequencing.

We observed the procedure of Fig. 1b to demonstrate high fidelity in determining TCR sequences in the Keskin *et al.* study<sup>13</sup>. In this case, TCR sequences in single tumor-infiltrating lymphocytes (TILs) from Patient 7 were determined both using TraCeR<sup>6</sup> reconstruction from Smart-seq2<sup>15</sup> scRNA-seq data and using rhTCRseq on the same Smart-seq2 cDNA libraries. For the 277 cells from Patient 7 with paired alpha/beta-chain data by rhTCRseq, the predominant CDR3 sequences identified by TraCeR were identical to the most frequent CDR3 sequences detected by rhTCRseq. In addition, the *TRAV* and *TRBV* (the variable segments of TCR alpha and beta loci, respectively) gene assignments and the *TRAJ* and *TRBJ* (the joining segments of TCR alpha and beta loci, respectively) sequences matched between the two methods. There was no case of TraCeR analysis identifying a *TRAV* or *TRBV* allele that was not also detected by rhTCRseq, but it is difficult to assess the breadth of coverage achieved with the rhTCRseq primer set analyzing cells one at a time. Therefore, the rhTCRseq protocol was adapted for amplifying CDR3 segments in bulk RNA samples (Fig. 1c). In the first step, *TRAC*- and *TRBC*-specific primers are used in a reverse transcriptase (RT) reaction to synthesize TCR cDNA. These primers include a Rd2 sequence needed for Illumina-based sequencing and a Unique Molecular Identifier (UMI)<sup>19,20</sup>. There is an exonuclease treatment between the RT and PCR steps to digest the UMI-containing primers so that UMIs are only incorporated during the RT step. Use of the UMI enables counting of the original number of cDNA molecules prior to PCR, which improves the accuracy of repertoire frequency measurements<sup>21–26</sup>. The PCR1 step is the same as in the single-cell protocol except the rhPCR primers specific for *TRAC* and *TRBC* are not included. The steps after this point are the same for the two protocols. As detailed in the Supplementary Note under “Analysis of bulk RNA from five normal individuals,” determination of the TCR repertoire in peripheral blood mononuclear cell (PBMC) RNA samples (S. L., R. A., J. F., R. J., D. B. K., K. J. L., unpublished data) shows that the rhTCRseq primer set does have breadth of coverage across the functional alpha and beta TCR variable genes (Supplementary Fig. 1, Supplementary Table 3). Supplementary Fig. 2 and Supplementary Table 4 show the reproducibility of rhTCRseq when applied to the five normal bulk RNA samples.

### Single cell applications of rhTCRseq

A key finding of Keskin *et al.*<sup>13</sup> is the evidence provided by single-cell TCR analysis that neoantigen-specific T cells generated in response to peptide vaccination can migrate from the peripheral blood to a glioblastoma in the brain. For Patient 7, TraCeR reconstruction identified paired alpha and beta information for 160 of 384 cells (41.7%), but predominantly only partial CDR3 sequences were recovered. Using rhTCRseq on the Smart-seq2 cDNA libraries in 96-well plates, the yield of paired alpha/beta sequences was improved to 277 of 384 cells (72.1%) and, for all 277 cells, complete CDR3 sequences were obtained. Having complete CDR3 sequences proved to be critical for the cloning and expression of discovered TCRs<sup>27</sup>, and the subsequent testing to demonstrate neoantigen specificity of individual TCRs.

As detailed in the Supplementary Note under “Single-cell analysis of tumor-specific T cells in 384-well format,” rhTCRseq has been applied to single T cells sorted into a 384-well plate (Supplementary Figure 3) and used to determine the clonality of peripheral T cells that

are specific for tumor tissue (S. L., R. A., G. O., J. F., R. J., K. J. L., unpublished data). The paired alpha/beta clonotype for each single cell are reported in Supplementary Table 5. As shown in Supplementary Fig. 4, 49 putative tumor-specific clones were detected, ranging in size from 2–30 cells per clone. For this experiment, a workflow was developed for processing single cells in 384-well plates, starting with preparation of full-length cDNA libraries followed by re-amplification of CDR3 segments as depicted in Fig. 1b. Compared to the Smart-seq2 procedure for preparing full-length cDNA libraries, streamlining and reduced cost were achieved by minimizing the number of purifications and lowering reaction volumes.

The pairing of TCR sequence information and RNA expression data is vital for understanding how a particular clonotype relates to the functional state of the T cell. rhTCRseq amplifies TCR segments from single-cell cDNA libraries, which can be further processed to generate single-cell sequencing libraries to obtain whole-transcript scRNA-seq data. By following this strategy, TCR clonotype can be linked to the most complete method currently available for determining single-cell function. Expense precludes very deep sequencing of single-cell libraries which means it can be difficult to detect specific mutations (or splice variants) in scRNA-seq data. However, because the cDNA libraries are whole-transcript libraries, rhPCR primers can be designed that flank known alterations and adding these primers to the initial PCR step of rhTCRseq should enable very sensitive detection of known mutations and splice variants.

### Bulk RNA applications of rhTCRseq

For Patient 8 in the Keskin *et al.* study<sup>13</sup>, single TILs were not available for analysis, so a different strategy was adopted to associate TCR clonotypes found in the peripheral blood with those in the tumor. T cells from a post-vaccination peripheral blood sample were stimulated with individual neoantigen peptides used in the vaccine. Single-cell TCR sequencing of these stimulated T cells using rhTCRseq in 96-well plates identified putative peptide-specific clonotypes. For this analysis, single-cell cDNA libraries were made as described in this protocol. Peripheral blood and tumor RNA were then analyzed using the rhTCRseq protocol for bulk RNA. Peptide-specific alpha- and beta-chain sequences were detected in a post-vaccination blood sample and in a post-vaccination tumor sample obtained when the patient relapsed. These sequences were not detected in pre-vaccination blood or tumor samples.

Related to cancer, studies<sup>28–30</sup> have investigated how changes in TCR-beta chain diversity and clonality in peripheral blood are associated with response to treatment with immune checkpoint inhibitors. These analyses would have been enhanced by using methods, such as rhTCRseq and others<sup>23,31</sup>, that add information about TCR-alpha chain usage and provide improved UMI-based frequency measurement. These methods would provide the same advantages to the TCR analysis of bulk tumor RNA samples. Li *et al.*<sup>32</sup> were able to extract beta CDR3 information from bulk RNA-seq data deposited in The Cancer Genome Atlas (TCGA). From the subsequent analysis of 9,142 RNA-seq samples across 29 cancer types, they obtained an average of 65 clonotypes per tumor sample. In comparison, targeted methods like rhTCRseq can identify many more TCR clonotypes in a tumor RNA sample.



As reported in Supplementary Table 10 of Keskin *et al.*<sup>13</sup>, rhTCRseq identified 1716–2559 unique TCR-alpha clonotypes and 2197–4084 unique TCR-beta clonotypes in two glioblastoma samples. Although interpretation can be confounded by variability in tumor purity, studies<sup>33–36</sup> have used targeted TCR sequencing of bulk tumor RNA samples to show that differences in TCR-beta chain diversity are associated with response to immunotherapy. Beausang *et al.*<sup>37</sup> used TCR sequencing of bulk DNA to show that TILs in breast cancer have a higher TCR-beta clonality compared to adjacent normal breast tissue.

### Strengths and limitations of rhTCRseq

Many protocols have been devised that employ multiplex PCR with a mixture of *TRAV*- and *TRBV*-specific forward primers for targeted TCR amplification from bulk genomic DNA or bulk transcript RNA<sup>38–43</sup>. This approach has been extended to the determination of paired TCR alpha/beta clonotypes in single cells<sup>1–5</sup>. These existing methods use nested primers to achieve specific amplification of CDR3 segments. As described here, the major advantage of rhTCRseq is its high level of specificity, which enables specific TCR amplification and addition of barcodes in a single PCR step. After this barcoding, samples can be pooled so there is only one sample for the subsequent steps of library generation. This greatly simplifies the workflow and reduces cost compared to previous multiplex PCR methods for targeted TCR amplification.

For repertoire analysis, rhTCRseq uses transcript RNA as a substrate. This has pluses and minuses compared to using genomic DNA. Because each cell has multiple TCR transcripts compared to just one genomic DNA copy, RNA analysis can be performed with limited amounts of input material. This also means that clonotype frequencies derived from RNA data are skewed by differential TCR expression levels. This emphasis on more highly expressed TCRs can be advantageous for certain analyses, but makes it difficult, if not impossible, to quantify clonal expansions on a cell count basis. The dependence on expression levels also means that direct comparison of RNA- and DNA-based frequency values is problematic.

One limitation of rhTCRseq is that it does not have the throughput of droplet-based methods. To date, though, droplet-based methods can only generate end-counting scRNA-seq data. Thus, rhTCRseq coupled with whole-transcript scRNA-seq balances the moderate throughput of 384 cells per processing run with the sensitive detection of paired complete CDR3 segments and with the information gained from whole-transcript, single-cell analysis. rhTCRseq is well-suited to the analysis of small sorted populations where the economics of droplet-based methods are less favorable. It has the added flexibility of decoupling isolating single cells from library processing, enabling archiving of single cell plates.

### Experimental Design

**Cell sorting and lysis**—For sorting (Steps 1A(i-iii)), we have found that we can dry sort single cells into 384-well plates. This simplifies the sorting process somewhat because it obviates the need to dispense lysis buffer into the wells prior to sorting. Thorough lysis is important for maximizing the yield of cDNA in single-cell protocols. Trombetta *et al.*<sup>15</sup> used a guanidine thiocyanate buffer to ensure complete lysis, but this required a bead cleanup of

the RNA to remove the guanidine thiocyanate prior to the RT reaction. Performing this bead cleanup, especially in a 384-well plate, is cumbersome and can lead to loss of RNA. The rhTCRseq protocol achieves robust liberation of cellular RNA by incubating the cells with Proteinase K at 50 °C (Steps 1A(iv-ix)). Inactivation of the Proteinase K is achieved by a combination of heat inactivation at 72 °C and the inclusion of a protease inhibitor in the RT reaction. Thus, there is no need for a post-lysis purification step.

**Single-cell cDNA library construction**—This protocol generates single-cell cDNA libraries in 96- or 384-well plates using reagents from the NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module (New England BioLabs E6421L). Steps 1A(x-xvii) are essentially the same as for Smart-seq<sup>2</sup><sup>15,16</sup>: a RT reaction with an oligo dT primer and a template-switching oligo followed by PCR amplification of full-length cDNA using a single primer. In order to make the volumes compatible with a 384-well plate and to reduce cost, reaction volumes have been reduced by a factor of ten compared to those recommended in the kit manual. For 96-well plates, the reduction factor is five. Following PCR, the individual single-cell cDNA libraries are purified using bead cleanup (Steps 1A(xviii-xxv)) and normalized to equal concentrations (Steps 1A(xxvi-xxviii)). The normalized libraries are ready for TCR-specific amplification as described below. The cDNA libraries can also be used for making sequencing libraries to perform whole-transcript scRNA-seq. Library construction can be done using the Nextera XT system<sup>15</sup> or the more conventional route of fragmentation followed by ligation of adaptors with Illumina sequences<sup>44-46</sup>.

**TCR-specific amplification of single-cell cDNA libraries**—Targeted TCR amplification (Steps 1A(xxix-xxxv)) is performed by first transferring an aliquot of each normalized single-cell cDNA library to a 96- or 384-well plate. Then, a pair of barcode rhPCR primers with the structures P5.IDTxxx.Rd1x and P7.IDTyyy.Rd2x are added to each well. P5 and P7 refer to the Illumina sequences required for attachment to the flow cell; IDTxxx and IDTyyy refer to 8-nucleotide index sequences; and Rd1x and Rd2x refer to the 5' portions of the Illumina TruSeq Read1 and Read 2 primers. The designation IDTxxx and IDTyyy indicates that the two index sequences added to any particular well are not the same sequence. Using the pipetting scheme outlined in Table 1 enables transferring barcode primers stored in 96-well plates to a 384-well plate such that each well gets a unique dual index. Next, a PCR mix containing rhPCR primers specific for TCR is added to each well. The structures of these primers are Rd1.AVxx, Rd1.BVxx, Rd2.AC, and Rd2.BC. Rd1 and Rd2 refer to the sequences corresponding to the Illumina TruSeq Read1 and Read 2 primers; AVxx refer to sequences specific for all functional *TRAV* genes; BVxx refer to sequences specific for all functional *TRBV* genes; and AC and BC refer to *TRAC*- and *TRBC*-specific sequences, respectively. In the PCR, the concentrations of the TCR-specific primers are low (50 nM) and the barcode primers are high (2 μM). This is done to favor the formation of full-length P5/P7-containing amplicons, but this strategy is not 100% effective. Therefore, after pooling and purification (Steps 2–16) of the 96 or 384 reactions from the TCR-specific amplification, a second PCR is performed using the generic primers P5 and P7 (Steps 17–18). The products of this second PCR are purified and qualified for sequencing by BioAnalyzer analysis (Steps 19–29). Standard paired-end MiSeq sequencing is performed



using the 300-cycle kit and reads of 248 nt read 1, 48 nt read 2, 8 nt index 1, and 8 nt index 2 (Steps 30–35).

**Adaptation of single cell protocol for bulk RNA**—For bulk RNA, the *TRAC*- and *TRBC*-specific primers are included as part of the RT reaction. To do this, conventional primers (not rhPCR primers) with structure Rd2.UM7.AC and Rd2.UM7.BC are mixed with total RNA, NP-40, and dNTPs, then incubated at 65 °C to relax RNA secondary structure and allow the primers to hybridize to the RNA (Steps 1B(i–v)). Rd2 refers to the sequences corresponding to the Illumina TruSeq Read 2 primer; UM7 is a 7-nucleotide random sequence that is the UMI; and AC and BC refer to *TRAC*- and *TRBC*-specific sequences, respectively. A standard RT reaction is performed (Steps 1B(vi–ix)) without a template-switching oligo. Then, an exonuclease reaction is performed (Steps 1B(x–xiii)) to digest the RT primers. This prevents the UMI-containing primers from being incorporated during subsequent PCR steps. TCR-specific PCR amplification (Steps 1B(xiv–xix)) is similar to the single cell protocol except there are no *TRAC*- and *TRBC*-specific primers in the reaction. Following pooling and purification of the PCR products (Steps 2–16), a second PCR is performed using the generic primers P5 and P7 (Steps 17–18). After this, the bulk RNA protocol is the same as the single cell protocol (Steps 19–35). For tumor samples, RNA isolated from fresh frozen tissue always works better than formalin-fixed paraffin-embedded (FFPE) material. RNA should have an RNA Integrity Number (RIN) of at least 4.

**Data analysis**—After preparing the fastq files from the sequencing run and installing the required software (Step 36), a pipeline written in Python and Bash shell scripts takes sequencing data from single cell or bulk RNA sequencing and analyzes it to report information about the TCR alpha and beta clonotypes present in each sample (Steps 37–39). As detailed in Box 1, the pipeline first determines which TCR locus each sequencing read came from and separates the reads by gene (*TRA* locus or *TRB* locus). Then the program MiXCR<sup>47</sup> aligns reads to reference variants of V, D, J, and C regions and assembles clonotypes. For single cell data, clonotypes are filtered and collapsed based on sequence similarity. For bulk RNA data, clonotypes are filtered and collapsed based on UMIs, first within each well and then between replicates. The single cell outputs (Step 39) are used to determine the paired alpha/beta TCR clonotype of each single cell and to identify any clones in the collection of single cells analyzed. The bulk RNA outputs (Step 39) report the frequencies of alpha and beta clonotypes separately. These frequencies can be used to calculate a variety of parameters to characterize TCR repertoire diversity.

**Controls**—Negative controls with no cells or RNA added should be included with each plate run. Typically, positive controls are not used. For sequencing, the spike-in library PhiX Control v3 is included at 5% as a quality control to ensure robust cluster identification.

## Materials

- T cells from sample of interest for single cell protocol ! **CAUTION** For work with human subjects, informed consent and appropriate approvals from user's institution are required. Informed consent was obtained from all study subjects as part of DF/HCC IRB approved protocols.

- Total RNA from blood or tumor for bulk RNA protocol ! **CAUTION** For work with human subjects, informed consent and appropriate approvals from user's institution are required. Informed consent was obtained from all study subjects as part of DF/HCC IRB approved protocols.

## Reagents

- NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module (New England BioLabs, cat. no. E6421L). This kit contains NEBNext Cell Lysis Buffer (10×), Murine RNase Inhibitor, NEBNext Single Cell RT Primer Mix, NEBNext Single Cell RT Buffer (4×), NEBNext Template Switching Oligo, NEBNext Single Cell RT Enzyme Mix, NEBNext Single Cell cDNA PCR Master Mix (2×), and NEBNext Single Cell cDNA PCR Primer.
- Proteinase K (800 units/mL, New England BioLabs, cat. no. P8107S)
- Prionex (MilliporeSigma, cat. no. G0411–100ML)
- Elastase Inhibitor III (AAPV, MilliporeSigma, cat. no. 324745–5MG)

**▲CRITICAL** This is the only inhibitor that has been tested in the rhTCRseq protocol. Use and concentration of a different Proteinase K inhibitor would need to be validated.

- ProNex Size-Selective Purification System (125 mL, Promega, cat. no. NG2002)
- Quant-iT High-Sensitivity dsDNA Assay Kit (Thermo Fisher, cat. no. Q33120)
- Water, PCR certified (Teknova, cat. no. W3331)
- TE Buffer (10 mM Tris, pH 8.0; 1 mM EDTA, Teknova, cat. no. T0224)
- DNA Suspension Buffer (10 mM Tris, pH 8.0; 0.1 mM EDTA, Teknova, cat. no. T0221)
- P5.IDTxxx.Rd1x.x1 and P7.IDTyyy.Rd2x.x1 oligonucleotides (Supplementary Table 2, Integrated DNA Technologies, custom product)

**▲CRITICAL** All oligonucleotides are listed in the 5' to 3' direction.

- RNase H2 Enzyme Kit (includes RNase H2 Enzyme at 2 units/μL and RNase H2 Dilution Buffer, Integrated DNA Technologies, cat. no. 11–02-12–01)
- 1 M Tris-HCl, pH 8.4 (Teknova, cat. no. T1084)
- 1 M KCl (Teknova, cat. no. P0325)
- 1 M MgCl<sub>2</sub> (Teknova, cat. no. M0302)
- dNTP Mix, 25 mM each (Thermo Fisher, cat. no. FERR1121)
- Rd1.AVxx.x1 and Rd1.BVxx.x1 oligonucleotides (Supplementary Table 1, Integrated DNA Technologies, custom product)
- Rd2.AC.x1 and Rd2.BC.x1 oligonucleotides (Supplementary Table 1, Integrated DNA Technologies, custom product)
- Hot Start Taq DNA Polymerase (New England BioLabs, cat. no. M0495L)

- AMPure XP beads (Beckman Coulter, cat. no. A63880)
- 200 Proof Ethanol (Decon Labs, cat. no. 2716) ! CAUTION Ethanol is flammable, keep away from open flame.
- Beads Buffer: 20% polyethylene glycol (PEG 8000), 2.5M sodium chloride (Teknova, cat. no. P4137)
- P5 primer, AATGATACGGCGACCACCGAGATCTACAC (Integrated DNA Technologies, custom product)
- P7 primer, CAAGCAGAAGACGGCATACGAGAT (Integrated DNA Technologies, custom product)
- Q5 Hot Start HiFi PCR Master Mix (New England BioLabs, cat. no. M0543S)
- 1 N NaOH (MilliporeSigma, cat. no. 109137) ! CAUTION Causes severe skin burns and eye damage.
- PhiX spike-in library (Control v3, Illumina, cat. no. FC-110–3001)
- MiSeq 300-cycle Reagent Kit v2 (Illumina, cat. no. MS-102–2002)
- 10% NP-40 (Thermo Fisher, cat. no. 28324)
- Rd2.UM7.AC primer,  
gtgactggagttcagacgtgtgctctccgatctNHNNNHVTCAGCTGGTACACGGCA  
(Integrated DNA Technologies, custom product)
- Rd2.UM7.BC primer,  
gtgactggagttcagacgtgtgctctccgatctNHNNNHVTCTCTGCTTCTGATGGCTCA  
A (Integrated DNA Technologies, custom product)
- 50% glycerol (Teknova, cat. no. G1796)
- 1 M dithiothreitol (Teknova, cat. no. D9750) ! CAUTION Dithiothreitol is harmful if swallowed. It causes skin irritation and serious eye irritation.
- RNaseOUT (40 units/μL, Thermo Fisher, cat. no. 10777019)
- SuperScript II Reverse Transcriptase (200 units/μL, Thermo Fisher, cat. no. 18064014)
- Exonuclease I (20 units/μL, New England BioLabs, cat. no. M0293S)
- RNase H2 Dilution Buffer (Integrated DNA Technologies, cat. no. 11–01-02–12)
- NEBNext Ultra II FS DNA Library Prep Kit (New England BioLabs, cat. no. E7805L). The single-cell cDNA libraries prepared using the rhTCRseq protocol can be processed to make sequencing libraries using this kit. E6421L for cDNA library construction and E7805L for sequencing library construction can be purchased together as the NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina (New England BioLabs, cat. no. E6420L)

## Equipment

- Twin.tec PCR plate (384 wells, LoBind, skirted, PCR clean, colorless, Eppendorf, cat. no. 0030129547)
- Twin.tec PCR plate (96 wells, LoBind, skirted, PCR clean, colorless, Eppendorf, cat. no. 0030129512)
- 0.2-mL PCR 8-tube FLEX-FREE strip (attached clear flat caps, natural, USA Scientific, cat. no. 1402–4700)
- 20- $\mu$ L 8-channel pipette (Pipet-Lite Multi Pipette L8–20XLS+, Rainin, cat. no. 17013803)
- 200- $\mu$ L 8-channel pipette (Pipet-Lite Multi Pipette L8–200XLS+, Rainin, cat. no. 17013805)
- 2- $\mu$ L pipette (Pipet-Lite LTS Pipette L-2XLS+, Rainin, cat. no. 17014393)
- 20- $\mu$ L pipette (Pipet-Lite LTS Pipette L-20XLS+, Rainin, cat. no. 17014392)
- 200- $\mu$ L pipette (Pipet-Lite LTS Pipette L-200XLS+, Rainin, cat. no. 17014391)
- 1000- $\mu$ L pipette (Pipet-Lite LTS Pipette L-1000XLS+, Rainin, cat. no. 17014382)
- 20- $\mu$ L pipette tips (RT-LTS-A-10 $\mu$ L-/F/L-960/10, Rainin, cat. no. 30389226)
- 200- $\mu$ L pipette tips (RT-LTS-A-200 $\mu$ L-/F/L-960/10, Rainin, cat. no. 30389240)
- 1000- $\mu$ L pipette tips (RT-LTS-A-1000 $\mu$ L-/F/L-768/8, Rainin, cat. no. 30389213)
- MagnaBot 384 magnetic separation stand (Promega, cat. no. V8241)
- 10x magnetic separation stand for 8-tube strip (10x Genomics, cat. no. 230003)
- DynaMag-96 side skirted magnetic separation stand (Thermo Fisher, cat. no. 12027)
- MagneSphere magnetic separation stand (12-hole, 1.5 mL vial, Promega, cat. no. Z5342)
- CoolRack XT cooling block for 384-well PCR plate (Corning, cat. no. 432055) for keeping plate cool on the deck of the Mantis liquid dispenser. When not in use, block is stored at  $-20^{\circ}\text{C}$ .
- Cooling block for 96-well PCR plate (Eppendorf, cat. no. 022510509) for keeping plate cool on the deck of the Mantis liquid dispenser. When not in use, block is stored at  $-20^{\circ}\text{C}$ .
- DNA LoBind microcentrifuge tubes (1.5 mL, PCR clean, colorless, Eppendorf, cat. no. 022431021)
- LSE vortex mixer (Corning, cat. no. 6775)
- 3-inch head for vortex mixer (Corning, cat. no. 480100)
- Mantis liquid dispenser (Formulatrix, cat. no. MANTV3.2)

- 1250- $\mu$ L pipette tips (Sterile Non-Filtered Extra Long Pipet Tip, Thomas Scientific, cat. no. 1158U40). For use as reservoir on Mantis liquid dispenser.
- 1000- $\mu$ L pipette (PIPETMAN Classic P1000, Gilson, cat. no. F123602). Required for using the 1250- $\mu$ L pipette tips.
- C1000 Touch Thermal Cycler (with 96 Deep Well Reaction Module, Bio-Rad, cat. no. 1851197)
- C1000 TOUCH 384 Well Reaction Module (Bio-Rad, cat. no. 1840138)
- PX1 PCR Plate Sealer (Bio-Rad, cat. no. 1814000)
- Peelable foil heat seal (Bio-Rad, cat. no. 1814045)
- 5430 microcentrifuge (with 2-place microplate swing bucket rotor, Eppendorf, cat. no. 022620572)
- Synergy H1 hybrid multi-mode microplate reader (BioTek, cat. no. H1MF)
- 2100 Bioanalyzer (Agilent, cat. no. G2939BA)
- High Sensitivity DNA Kit (Agilent, cat. no. 5067–4626)
- MiSeq system (Illumina, cat. no. SY-410–1003)

## Software

- BLAST<sup>48</sup> version NCBI-BLAST-2.2.30+ (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/2.2.30/>)
- MiXCR<sup>47</sup> version 2.1.5 (<https://github.com/milaboratory/mixcr/releases/tag/v2.1.5>)
- Java JDK version 8 (<https://www.oracle.com/technetwork/java/javase/downloads/jdk8-downloads-2133151.html>)
- GNU parallel version 20180722 (<http://git.savannah.gnu.org/cgit/parallel.git/>) [Tange, O. *GNU Parallel 2018* at <[https://zenodo.org/record/1146014#.W\\_1SJhNKjmV](https://zenodo.org/record/1146014#.W_1SJhNKjmV)>]
- Scripts and reference files from the Github repository at <https://github.com/julietforman/rhTCRseq>

## Reagent setup

**25 mM AAPV Proteinase K inhibitor**—Dissolve 5 mg Elastase Inhibitor III in 400  $\mu$ L DMSO, dispense 20  $\mu$ L aliquots, and store at  $-80^{\circ}\text{C}$  for up to six months.

**P5.IDTxxx.Rd1x.x1 / P7.IDTyyy.Rd2x.x1 barcode primer plates**—There are 768 barcode primers in Supplementary Table 2. These were obtained in eight 96-well plates with each primer at a concentration of 200  $\mu$ M. As shown in Supplementary Table 2, these plates are designated: P5.1, P5.2, P5.3, P5.4, P7.1, P7.2, P7.3, and P7.4. The table includes the well position of each primer and its corresponding barcode, with the barcodes designated IDT001-IDT384. The 8-nucleotide barcode is underlined in each of the primer sequences.

Four Quadrant Plates at concentration 6  $\mu$ M each primer were made by mixing 3  $\mu$ L 200  $\mu$ M P5 barcode primer, 3  $\mu$ L 200  $\mu$ M P7 barcode primer, and 94  $\mu$ L TE Buffer following this scheme:

Quadrant Plate A: P5.1 + P7.2

Quadrant Plate B: P5.2 + P7.3

Quadrant Plate C: P5.3 + P7.4

Quadrant Plate D: P5.4 + P7.1

Table 1 shows the pipetting pattern used to transfer primers from the Quadrant Plates to a 384-well plate. The last tab in Supplementary Table 2 is a MiSeq sample sheet showing the correspondence of index sequences to individual samples. The Sample\_ID column in the sample sheet shows the well position in the 384-well plate. These barcode primer plates are stored at  $-20^{\circ}\text{C}$  for up to one year.

**Rd1.AV.x1 / Rd1.BV.x1 primer mix**—Combine 69 rhPCR primers at a concentration of 5  $\mu$ M each by mixing 5  $\mu$ L 500  $\mu$ M each primer with 155  $\mu$ L TE Buffer. These primers are specific for the V segments of the human alpha and beta TCR genes and are designed to amplify all productive alpha and beta alleles. The sequences are in Supplementary Table 1. Store at  $-20^{\circ}\text{C}$  for up to one year.

**Rd2.AC.x1 / Rd2.BC.x1 primer mix**—Combine the two rhPCR primers at a concentration of 5  $\mu$ M each prepared by mixing 5  $\mu$ L 500  $\mu$ M each primer with 490  $\mu$ L TE Buffer. These primers are specific for the C segments of the human alpha and beta TCR genes. The sequences are in Supplementary Table 1. Store at  $-20^{\circ}\text{C}$  for up to one year.

**Ethanol, 80% (vol/vol)**—Prepare 80% (vol/vol) ethanol just before use by mixing 4 mL 200 proof ethanol with 1 mL water.

**5 $\times$  RT Buffer (75 mM Tris-HCl, pH 8.4; 375 mM KCl; 50 mM MgCl<sub>2</sub>; 25% glycerol)**—Mix 15  $\mu$ L 1 M Tris-HCl, pH 8.4, 75  $\mu$ L 1 M KCl, 10  $\mu$ L 1 M MgCl<sub>2</sub>, 100  $\mu$ L 50% glycerol. Store at room temperature for up to one year.

**NaOH, 0.2 N**—Prepare just before use by mixing 4  $\mu$ L 1 N NaOH with 16  $\mu$ L water.

## Procedure

CRITICAL: The workflow of this protocol is diagrammed in Fig. 2.

1. Choose Option A for processing single cells and Option B for processing bulk RNA.



## Option A: rhTCRseq for single cells

### Single cell sorting ● Timing 30–60 min sorter set-up, 10–45 min per plate

- i. Dry sort single cells into a 96- or 384-well PCR plate. In order to have a negative control, do not sort cells into 2–4 wells.

▲**CRITICAL** Ensure the sorter is properly aligned so that each cell is deposited to the center of its well.

### ? TROUBLESHOOTING

- ii. Immediately centrifuge the plate at 800× g for 1 min at 4 °C and place on dry ice.
- iii. Store the plate at –80 °C.

■**PAUSE POINT** Single-cell plates can be stored for at least one year at –80 °C.

### cDNA library construction ● Timing 8 h

- iv. Dilute Proteinase K to 80 units/mL by mixing 5 µL 800 units/mL Proteinase K with 45 µL Prionex and keep on ice.
- v. Prepare the following lysis mix and keep on ice:

Component	Volume for 96-well plate with overage (µL)	Volume for 384-well plate with overage (µL)	Final concentration
NEBNext Cell Lysis Buffer (10×)	12	20.8	0.5×
Murine RNase Inhibitor	6	10.4	-
Proteinase K (80 units/mL)	24	41.5	8 units/mL
NEBNext Single Cell RT Primer Mix	24	41.5	-
Water	174	301	-
<b>Total volume</b>	<b>240</b>	<b>415.2</b>	

- vi. Remove single-cell plate from –80 °C freezer (Step 1Aiii), place on ice for 2 min, centrifuge briefly, and place plate in cooling block on the deck of the Mantis.
- vii. Use Mantis to dispense 1 µL of lysis mix to each of the wells if using a 384-well plate, or 2 µL per well for a 96-well plate.
- viii. Cover the plate with foil and heat seal using plate sealer at 168 °C for 3 s.
- ix. Centrifuge briefly, then transfer plate to thermal cycler and run the protocol: 50 °C for 30 min, 72 °C for 5 min, hold at 4 °C.
- x. Prepare the following reverse transcriptase (RT) mix and keep on ice:

Component	Volume for 96-well plate with overage (μL)	Volume for 384-well plate with overage (μL)	Final concentration in RT reaction
NEBNext Single Cell RT Buffer (4×)	120	207.5	1×
AAPV Proteinase K inhibitor (25 mM)	9.6	16.6	0.5 mM
NEBNext Template Switching Oligo	24	41.5	-
NEBNext Single Cell RT Enzyme Mix	48	83	-
Water	38.4	66.4	-
<b>Total volume</b>	<b>240</b>	<b>415</b>	

- xi.** With the plate in cooling block on the deck of the Mantis, use Mantis to dispense 1 μL of RT mix to each of the wells if using a 384-well plate, or 2 μL per well for a 96-well plate.
- xii.** Cover the plate with foil and heat seal using plate sealer at 168 °C for 3 s.
- xiii.** Vortex and centrifuge briefly, then transfer plate to thermal cycler and run the protocol: 42 °C for 90 min, 70 °C for 10 min, hold at 4 °C.
- xiv.** Prepare the following PCR mix and keep on ice:

Component	Volume for 96-well plate with overage (μL)	Volume for 384-well plate with overage (μL)	Final concentration in PCR reaction
NEBNext Single Cell cDNA PCR Master Mix (2×)	1200	2065	1×
NEBNext Single Cell cDNA PCR Primer	48	82.6	-
Water	672	1156.4	-
<b>Total volume</b>	<b>1920</b>	<b>3304</b>	

- xv.** With the sample plate in cooling block on the deck of the Mantis, use Mantis to dispense 8 μL of PCR mix to each of the wells if using a 384-well plate, or 16 μL per well for a 96-well plate, using a 1250-μL pipette tip.
- xvi.** Cover the plate with foil and heat seal using plate sealer at 168 °C for 3 s.
- xvii.** Vortex and centrifuge briefly. Transfer plate to thermal cycler and run the following PCR protocol:

Cycle number	Denature	Anneal	Extend
<b>1</b>	98 °C, 45 s		
<b>2–23</b>	98 °C, 10 s	62 °C, 15 s	72 °C, 3 min

Cycle number	Denature	Anneal	Extend
24			72 °C, 5 min

- xxiii.** Warm ProNex beads to room temperature.
- xix.** With the sample plate at room temperature, use Mantis to dispense 11  $\mu$ L of ProNex beads to each of the wells if using a 384-well plate, or 21  $\mu$ L per well for a 96-well plate, using a 1250- $\mu$ L pipette tip.
- xx.** Vortex briefly and incubate at room temperature for 10 min.
- xxi.** Place the plate on a magnetic stand, wait until solution clears (approximately 2 min for beads to collect), then discard supernatants using 8-channel pipette.
- xxii.** With plate remaining in the magnetic stand, wash the beads by adding 30  $\mu$ L of Promega Wash Buffer to each of the wells if using a 384-well plate, or 100  $\mu$ L per well for a 96-well plate, using an 8-channel pipette, waiting 30 s, and discarding the supernatants.
- xxiii.** Repeat Step 1Axxii one more time. Remove as much residual Wash Buffer as possible.
- xxiv.** After air drying the beads for 10 min, use Mantis to add 17  $\mu$ L Promega Elution Buffer to each well, using a 1250- $\mu$ L pipette tip.
- xxv.** Vortex briefly, incubate at room temperature for 5 min, place the plate on the magnetic stand, wait for solution to clear (approximately 2 min for beads to collect), then transfer 15  $\mu$ L supernatant from each well to a fresh plate.

■ **PAUSE POINT** Amplified libraries can be stored for at least one year at  $-20^{\circ}\text{C}$ .

- xxvi.** Determine the concentrations of the cDNA libraries using 1- $\mu$ L aliquots and the Quant-iT High-Sensitivity dsDNA Assay Kit. Measure fluorescence on the Synergy H1MF microplate reader.
- xxvii.** Normalize DNA concentration to 0.2 ng/ $\mu$ L by combining 1.5  $\mu$ L of each library with the appropriate volume of TE in wells of a fresh plate. If concentration is less than 0.2 ng/ $\mu$ L, combine 1.5  $\mu$ L of library with 0.6  $\mu$ L TE. Dispense variable volumes of TE using the Mantis.
- xxviii.** Store both the original and normalized cDNA library plates at  $-20^{\circ}\text{C}$ .

■ **PAUSE POINT** Original and normalized cDNA library plates can be stored for at least one year at  $-20^{\circ}\text{C}$ .

### TCR-specific amplification ● Timing 3 h

- xxix.** Transfer 2  $\mu$ L (400 pg) of each normalized cDNA library to a fresh 96- or 384-well plate on ice. **CRITICAL STEP:** cDNA libraries prepared by another method can alternatively be used at this point as long as the other method uses a template-switch reverse transcriptase step and full-length cDNA amplification. For example, for Patient 7 in Keskin *et al.*<sup>13</sup>, cDNA libraries prepared using a modified version of Smart-seq2<sup>15</sup> were used as starting material for Step 1Axxix.

- xxx.** From the four Quadrant Plates A-D of barcode primers, use an 8-channel pipette to add 2  $\mu$ L 6  $\mu$ M each P5.IDTxxx.Rd1x.x1 / P7.IDTyyy.Rd2x.x1 to each of the wells following the pipetting scheme in Table 1. **CRITICAL STEP:** For a 96-well plate, only one of the Quadrant Plates is used.
- xxxi.** Dilute RNase H2 to 20 mU/ $\mu$ L by mixing 1  $\mu$ L 2 units/ $\mu$ L RNase H2 Enzyme with 99  $\mu$ L RNase H2 Dilution Buffer and keep on ice.
- xxxii.** Prepare the following PCR mix (PCR1) and keep on ice:

Component	Volume for 96-well plate with overage ( $\mu$ L)	Volume for 384-well plate with overage ( $\mu$ L)	Final concentration in PCR reaction
Tris-HCl, pH 8.4 (1 M)	10.8	36.9	15 mM
KCl (1 M)	18	61.5	25 mM
MgCl <sub>2</sub> (1 M)	2.9	9.84	4 mM
dNTPs (25 mM each)	11.5	39.36	0.4 mM each
Rd1.AV.x1 / Rd1.BV.x1 primer mix (5 $\mu$ M each)	7.2	24.6	50 nM each
Rd2.AC.x1 / Rd2.BC.x1 primer mix (5 $\mu$ M each)	7.2	24.6	50 nM each
RNase H2 (20 mU/ $\mu$ L)	18	61.5	0.5 mU/ $\mu$ L
Hot Start Taq DNA Polymerase (5 units/ $\mu$ L)	28.8	98.4	0.2 units/ $\mu$ L
Water	135.6	463.3	-
<b>Total volume</b>	<b>240</b>	<b>820</b>	

- xxxiii.** With sample plate in cooling block on the deck of the Mantis, use Mantis to dispense 2  $\mu$ L PCR mix to each of the wells.
- xxxiv.** Cover the plate with foil and heat seal using plate sealer at 168 °C for 3 s.
- xxxv.** Transfer plate to thermal cycler and run the following protocol:

Cycle number	Denature	Anneal	Extend
<b>1</b>	95 °C, 5 min		
<b>2–19</b>	96 °C, 20 s	60 °C, 6 min	

**Option B: rhTCRseq for bulk RNA—CRITICAL:** For bulk RNA rhTCRseq, we recommend using 1–50 ng total RNA per reaction. For any particular study, RNA samples should be diluted to the same concentration. RNA can be isolated by any of the standard methods for purifying total RNA. For each sample, we recommend running four to eight replicates. The protocol is written for running 96 reactions. This could be 24 samples with 4 replicates, 12 samples with 8 replicates, or other combinations. Twelve samples with 8 replicates will be used as an example here. One of the samples should be a negative control with no RNA added.

**cDNA synthesis ● Timing 3 h**

- i. Prepare the following mix and keep on ice:

Component	Volume per tube (μL)	Volume for 12 tubes with overage (μL)	Final concentration in RT reaction
NP-40 (10%)	1	15	0.25%
dNTPs (25 mM each)	0.8	12	0.5 mM
Rd2.UM7.AC primer (10 μM)	0.84	12.6	210 nM
Rd2.UM7.BC primer (10 μM)	0.28	4.2	70 nM
Water	7.08	106.2	-
<b>Total volume</b>	<b>10</b>	<b>150</b>	

- ii. Dispense 10 μL of this mix to each of 12 1.5-mL microcentrifuge tubes.
- iii. Add 10 μL 1–50 ng/μL RNA to each tube.
- iv. From each tube, transfer 2 μL to each of 8 wells in a 96-well PCR plate.
- v. Transfer plate to thermal cycler and run the protocol: 65 °C for 5 min, hold at 4 °C.
- vi. Prepare the following RT mix and keep on ice:

Component	Volume per reaction (μL)	Volume for 96 reactions with overage (μL)	Final concentration in RT reaction
RT Buffer (5×)	0.8	96	1×
Dithiothreitol (1 M)	0.04	4.8	10 mM
RNaseOUT (40 units/μL)	0.08	9.6	0.8 units/μL
Superscript II Reverse Transcriptase (200 units/μL)	0.02	2.4	1 units/μL
Water	1.06	127.2	-
<b>Total volume</b>	<b>2</b>	<b>240</b>	

- vii. With a 96-well sample plate on the cooling block on the deck of the Mantis, use Mantis to dispense 2 μL RT mix to each of the 96 wells.
- viii. Cover the plate with foil and heat seal using plate sealer at 168 °C for 3 s.
- ix. Transfer plate to thermal cycler and run the protocol: 42 °C for 90 min, 85 °C for 5 min, hold at 4 °C.
- x. Prepare diluted exonuclease as follows and keep on ice:

Component	Volume per reaction (μL)	Volume for 96 reactions with overage (μL)	Final concentration in exonuclease reaction
Exonuclease I (20 units/μL)	0.025	3	0.083 units/μL
RNase H2 Dilution Buffer	1.975	237	-
<b>Total volume</b>	<b>2</b>	<b>240</b>	

- xi.** With the 96-well sample plate on the cooling block on the deck of the Mantis, use Mantis to dispense 2 μL diluted exonuclease to each of the 96 wells.
- xii.** Cover the plate with foil and heat seal using plate sealer at 168 °C for 3 s.
- xiii.** Transfer the plate to the thermal cycler and run the protocol: 37 °C for 15 min, 85 °C for 15 min, hold at 4 °C.

### TCR-specific amplification ● Timing 3 h

- xiv.** From one of the four Quadrant Plates A-D of barcode primers, use an 8-channel pipette to add 4 μL 6 μM each P5.IDTxxx.Rd1x.x1 / P7.IDTyyy.Rd2x.x1 to each of the 96 wells.
- xv.** Dilute RNase H2 to 20 mU/μL by mixing 1 μL 2 units/μL RNase H2 Enzyme with 99 μL RNase H2 Dilution Buffer and keep on ice.
- xvi.** Prepare the following PCR mix (PCR1) and keep on ice:

Component	Volume per reaction (μL)	Volume for 96 reactions with overage (μL)	Final concentration in PCR reaction
Tris-HCl, pH 8.4 (1 M)	0.18	21.6	15 mM
KCl (1 M)	0.3	36	25 mM
MgCl <sub>2</sub> (1 M)	0.048	5.76	4 mM
dNTPs (25 mM each)	0.192	23.04	0.4 mM each
Rd1.AV.x1 / Rd1.BV.x1 primer mix (5 μM each)	0.12	14.4	50 nM each
RNase H2 (20 mU/μL)	0.3	36	0.5 mU/μL
Hot Start Taq DNA Polymerase (5 units/μL)	0.48	57.6	0.2 units/μL
Water	0.38	45.6	-
<b>Total volume</b>	<b>2</b>	<b>240</b>	

- xvii.** With the 96-well sample plate on the cooling block on the deck of the Mantis, use Mantis to dispense 2 μL PCR mix to each of the 96 wells.
- xviii.** Cover the plate with foil and heat seal using plate sealer at 168 °C for 3 s.
- xix.** Transfer plate to thermal cycler and run the protocol:



Cycle number	Denature	Anneal	Extend
1	95 °C, 5 min		
2–21	96 °C, 20 s	60 °C, 6 min	

### Pooling, purification, and final PCR. ● Timing 3.5 h

2. Pool all the reactions by mixing 2 µL of each sample. Pooling is done by using an 8-channel pipette to transfer samples from all wells of each column repeatedly into the same wells of one column of a new 96-well PCR plate. This requires 12 transfers for a 96-well plate and 48 transfers for a 384-well plate. The eight pooled samples are then combined by transferring to a 1.5-mL microcentrifuge tube to generate a 192-µL pooled sample for a 96-well plate and a 768-µL pooled sample for a 384-well plate. Store remainder of unpooled samples at –20 °C for up to six months.
3. Warm AMPure XP beads to room temperature.
4. To the pooled sample, add:

Component	Volume for pool from 96-well plate (µL)	Volume for pool from 384-well plate (µL)
AMPure XP beads	19.2	76.8
Beads Buffer	96	384

5. Pipet up and down, then incubate at room temperature for 5 min.
6. Place the tube on the magnetic stand and wait 5 min or until solution clears for beads to collect.
7. Transfer supernatant to fresh tube and add:

Component	Volume for pool from 96-well plate (µL)	Volume for pool from 384-well plate (µL)
AMPure XP beads	6.4	25.6
Beads Buffer	32	128

8. Pipet up and down, then incubate at room temperature for 5 min.
9. Place the tube on the magnetic stand, wait until solution clears (approximately 5 min for beads to collect), and discard supernatant.
10. With tube remaining on the magnetic stand, wash the beads by adding 1 mL freshly prepared 80% ethanol, waiting 30 s, and discarding the supernatant.
11. Repeat Step 10 one more time. Remove as much residual 80% ethanol as possible.
12. After air drying the beads for 5 min, add 21 µL DNA Suspension Buffer.

13. Pipet up and down, place the tube on the magnetic stand, wait 2 min for beads to collect, then transfer 20  $\mu$ L supernatant to one of the unused wells in the 96-well PCR plate used to pool samples (Step 2).
14. Add 16  $\mu$ L AMPure XP beads.
15. Repeat Steps 8–13 using the 96-well magnetic stand and 100  $\mu$ L 80% ethanol for Steps 10 and 11, then transferring 20  $\mu$ L supernatant to another unused well in the 96-well PCR plate.
16. In an adjacent unused well in the 96-well PCR plate, prepare 1:10 dilution by mixing 2  $\mu$ L purified pooled sample and 18  $\mu$ L DNA Suspension Buffer.

■PAUSE POINT Samples can be stored for up to one week at  $-20^{\circ}\text{C}$ .

17. In 8-tube PCR strip, prepare two PCR reactions (PCR2) on ice. Each reaction contains:

Component	Volume per sample ( $\mu$ L)	Final concentration in PCR reaction
Purified pooled sample from Step 15 (original) or Step 16 (1:10 dilution)	5	-
P5 primer (10 $\mu$ M)	2.5	0.5 $\mu$ M
P7 primer (10 $\mu$ M)	2.5	0.5 $\mu$ M
Q5 Hot Start HiFi PCR Master Mix (2 $\times$ )	25	1 $\times$
Water	15	-
Total volume	50	

18. Transfer strip to thermal cycler and run one of these protocols:

- i. Protocol for single cell procedure (Option A):

Cycle number	Denature	Anneal	Extend
1	98 $^{\circ}\text{C}$ , 30 s		
2–13	98 $^{\circ}\text{C}$ , 10 s	62 $^{\circ}\text{C}$ , 2 min	
14			75 $^{\circ}\text{C}$ , 2 min

- ii. Protocol for bulk RNA procedure (Option B):

Cycle number	Denature	Anneal	Extend
1	98 $^{\circ}\text{C}$ , 30 s		
2–18	98 $^{\circ}\text{C}$ , 10 s	62 $^{\circ}\text{C}$ , 2 min	
19			75 $^{\circ}\text{C}$ , 2 min

19. Warm ProNex beads to room temperature.

20. To each of the two PCR samples from Step 18, add 55  $\mu$ L ProNex beads.
21. Pipet up and down, then incubate at room temperature for 10 min.
22. Place the strip on the 10x magnetic stand in the High orientation, wait until solution clears (approximately 2 min for beads to collect), then transfer supernatants to two unused tubes in the strip.
23. Add 15  $\mu$ L ProNex beads to each, pipet up and down, and incubate at room temperature for 10 min.
24. Place the strip on the 10x magnetic stand in the High orientation, wait until solution clears (approximately 2 min for beads to collect), then discard supernatants.
25. With strip remaining in the magnetic stand, wash the beads by adding 100  $\mu$ L Promega Wash Buffer per well, waiting 30 s, and discarding the supernatants.
26. Repeat wash step 25 one time. Remove as much residual Wash Buffer as possible.
27. After air drying the beads for 10 min, add 20  $\mu$ L Promega Elution Buffer to each sample.
28. Pipet up and down, place in the 10x magnetic stand in the Low orientation, wait until solution clears (approximately 2 min for beads to collect), then transfer the two supernatants to unused wells in the 96-well plate used to store the samples after purification of the pooled TCR-specific PCR (Step16).
29. Use 1  $\mu$ L of each of the two final PCR products from Step 28 to measure the fragment size distribution and estimate concentration using the 2100 BioAnalyzer and High Sensitivity DNA Kit. Fragment size should be a set of peaks predominantly in the range 260–400 bp (see Fig. 3b,c). Concentration is determined setting the size range to 200–1000 bp. Of the two libraries, the one with the flattest baseline is used for sequencing.

■PAUSE POINT Amplified libraries can be stored for at least one year at  $-20^{\circ}\text{C}$ .

### Sequencing ● Timing 28 h

30. Perform sequencing using the 300-cycle Reagent Kit v2 on the Illumina MiSeq according to the manufacturer's protocol. As part of the set-up, upload a sample sheet like the one found in the last tab in Supplementary Table 2 to the instrument. This sample sheet specifies 248 nt read 1, 48 nt read 2, 8 nt index 1, and 8 nt index 2. We have found that the sequencing depth using the MiSeq has been adequate for analyzing 384 single cells or 96 bulk RNA reactions at 50 ng RNA per reaction.

### ? TROUBLESHOOTING

31. Dilute the selected (see Step 29) final library from Step 28 to 4 nM using water.

32. Mix 4.5  $\mu$ L 4 nM library, 0.5  $\mu$ L PhiX spike-in, and 5  $\mu$ L freshly prepared 0.2 N NaOH, then incubate at room temperature for 5 min.
33. Add 990  $\mu$ L Hyb Buffer (from MiSeq kit), mix by inversion, and place on ice. The library concentration is now 20 pM.
34. To prepare final loading library at 8 pM, mix 400  $\mu$ L 20 pM library with 600  $\mu$ L Hyb Buffer.
35. Load 600  $\mu$ L 8 pM library into MiSeq cartridge then follow the Illumina instructions to run the sequencer.

**Data analysis ● Timing 8 h—CRITICAL:** The required input for the rhTCRseq pipeline includes the sequencing reads in fastq.gz files separated by well, with four files per well. Each filename for each well should begin with the fastq\_basename prefix for that well, and the four filenames should end in \_L001\_R1\_001, \_L001\_R2\_001, \_L001\_I1\_001, and \_L001\_I2\_001. Also required is the SampleSheet.csv file for the run.

36. Install MiXCR-2.1.5, NCBI Blast, Java, and GNU Parallel.
37. Set up directory structure as follows:
  - choose a ROOT\_DIR in which all requirements for the pipeline will go
  - create a directory <ROOT\_DIR>/scripts (add to this folder all of the files from the Github repository folder rhTCRseq/scripts)
  - create a directory <ROOT\_DIR>/data
  - create a directory <ROOT\_DIR>/out
  - create a directory <ROOT\_DIR>/blast\_database (add to this folder all of the blast database files from the Github repository folder rhTCRseq/blast\_database)
38. Prepare to analyze a particular run:
  - open the config file <ROOT\_DIR>/scripts/config.py and edit the variables in the file to match your run
  - create a directory in <ROOT\_DIR>/data/ with the same name as RUN\_NAME in config.py (place the fastq.gz files from the sequencing run in this folder)
  - create a directory in <ROOT\_DIR>/out/ with the same name as RUN\_NAME in config.py (place SampleSheet.csv for the run into this folder)

After these steps are complete, the directory structure should look like this:

```
ROOT_DIR
blast_database
  TRV_primer.fasta
  TRV_primer.fasta.nhr
  TRV_primer.fasta.nin
```

TRV\_primer.fasta.nog  
TRV\_primer.fasta.nsd  
TRV\_primer.fasta.nsi  
TRV\_primer.fasta.nsq  
TRC\_primer.fasta  
TRC\_primer.fasta.nhr  
TRC\_primer.fasta.nin  
TRC\_primer.fasta.nog  
TRC\_primer.fasta.nsd  
TRC\_primer.fasta.nsi  
TRC\_primer.fasta.nsq  
target\_gene\_primer\_forward.fasta  
target\_gene\_primer\_forward.fasta.nhr  
target\_gene\_primer\_forward.fasta.nin  
target\_gene\_primer\_forward.fasta.nog  
target\_gene\_primer\_forward.fasta.nsd  
target\_gene\_primer\_forward.fasta.nsi  
target\_gene\_primer\_forward.fasta.nsq  
target\_gene\_primer\_reverse.fasta  
target\_gene\_primer\_reverse.fasta.nhr  
target\_gene\_primer\_reverse.fasta.nin  
target\_gene\_primer\_reverse.fasta.nog  
target\_gene\_primer\_reverse.fasta.nsd  
target\_gene\_primer\_reverse.fasta.nsi  
target\_gene\_primer\_reverse.fasta.nsq  
target\_gene\_reverse.fasta  
target\_gene\_reverse.fasta.nhr  
target\_gene\_reverse.fasta.nin  
target\_gene\_reverse.fasta.nog  
target\_gene\_reverse.fasta.nsd  
target\_gene\_reverse.fasta.nsi  
target\_gene\_reverse.fasta.nsq  
target\_gene\_forward.fasta  
target\_gene\_forward.fasta.nhr  
target\_gene\_forward.fasta.nin  
target\_gene\_forward.fasta.nog  
target\_gene\_forward.fasta.nsd  
target\_gene\_forward.fasta.nsi  
target\_gene\_forward.fasta.nsq  
data  
    <RUN\_NAME>  
    <fastq.gz files>  
out  
    <RUN\_NAME>

```

    SampleSheet.csv
scripts
    blast.sh
    count_umi.py
    separate_fastq.py
    collapse_rules.txt
    parse_blast_results.py
    analyze_tcr.py
    compare_clonotype.py
    merge_TRBV.py
    mixcr.sh
    get_parallel_range.py
    print_description.py
    config.py
    run_pipeline.sh
    make_index_list.py

```

## 6. Run the pipeline:

```

cd <ROOT_DIR>/scripts
./run_pipeline.sh

```

The steps in the pipeline are detailed in Box 1. The output files are shown in Table 2 for the single cell protocol and in Table 3 for the bulk RNA protocol.

**Troubleshooting**—Troubleshooting advice can be found in Table 4.

## Timing

**Sorting for single cell protocol:** Steps 1Ai-1Aiii, single cell sorting: 40 min to 2 h.

Cells can be sorted when the cells are ready and sorting time is available. Plates with sorted cells can be stored at  $-80^{\circ}\text{C}$  for at least one year and the rhTCRseq protocol can be commenced at Step 1Aiv whenever it is convenient.

**Day 1:** Steps 1Aiv-ix, lysis mix preparation and cell lysis: 50 min

Steps 1Bi-v, RNA sample preparation and primer annealing: 15 min

Steps 1Ax-xiii or 1Bvi-ix, RT mix preparation and RT reaction: 2 h

Steps 1Axiv-xvii, cDNA PCR mix preparation, Mantis dispense, and cDNA PCR: 2 h 30 min

Steps 1Axviii-xxv, cDNA PCR product purification: 1 h

Steps 1Axxvi-xxviii, cDNA quantification and normalization: 1 h 30 min



Steps 1Bx-xiii, Exonuclease I treatment: 45 min

Steps 1Bxiv-xix, PCR1 mix preparation and PCR1: 3 h

**Day 2:** Steps 1Axxix-xxxv, PCR1 mix preparation and PCR1: 3 h

Steps 2–16, PCR1 pooling and purification: 1 h (Day 1 for Option B)

Steps 17–18, PCR2 amplification: 45 min (started on Day 1 for Option B)

Steps 19–28, PCR2 purification: 45 min

Step 29, library quality control: 1 h

**Day 3:** Steps 30–35, sequencing: 28 h (Day 2 for Option B)

**Day 4:** Steps 36–39, data processing: 8 h (Day 3 for Option B)

**Anticipated results**—Fig. 3a shows the Bioanalyzer trace for the cDNA library from one cell. We have observed the average concentration per run of single-cell cDNA libraries to be in the range 0.5 to 6.1 ng/μL. Fig. 3b shows the expected Bioanalyzer trace of the final rhTCRseq library from a pool of 384 single cells. For single-cell TCR sequencing, the range of average reads per cell has been 5760–13919 reads for *TRA* and 6767–17619 reads for *TRB*. Fig 3c shows the expected Bioanalyzer trace of the final rhTCRseq library for the bulk RNA protocol. For both single cells and bulk RNA, predominantly fragments in the range 260–400 nt should be observed. The difference is that the pattern for single-cell libraries tends to be spikier than for bulk RNA libraries.

For single cells, clonotypes are assembled based on the CDR3 sequence results in output file `mixcr_clonotype_per_well.list` (Table 2) and are classified as containing: (1) one alpha and one beta; (2) two alpha and one beta; (3) one alpha and two beta; (4) one or two alpha only; (5) one or two beta only (6) multiple alpha and beta; and (7) lacking a productive alpha and beta. Table 5 shows the distribution of these classes for the single-cell rhTCRseq experiment described in the Supplementary Note (384 single cells). These results indicate a success rate of 90% (344/384) in obtaining useful paired alpha/beta information (categories 1–3). The success rate depends predominantly on the quality of the input cells and the precision of sorting. For high quality cells, we have observed success rates of 65–90% of single cells analyzed per plate. For bulk RNA results, the number of unique clonotypes observed depends entirely on the amount of RNA analyzed and the percent T cells in the original sample.

**Code availability statement:** Code and reference files used for data analysis are publicly available from the Github repository at <https://github.com/julietforman/rhTCRseq>

**Data availability statement:** All TCR clonotype data for the results presented in Supplementary Figures 1, 2, and 4 are provided in Supplementary Tables 3 and 5. All other data are available from the corresponding author upon reasonable request. Example data

used for data analysis are publicly available from the Github repository at <https://github.com/julietforman/rhTCRseq>

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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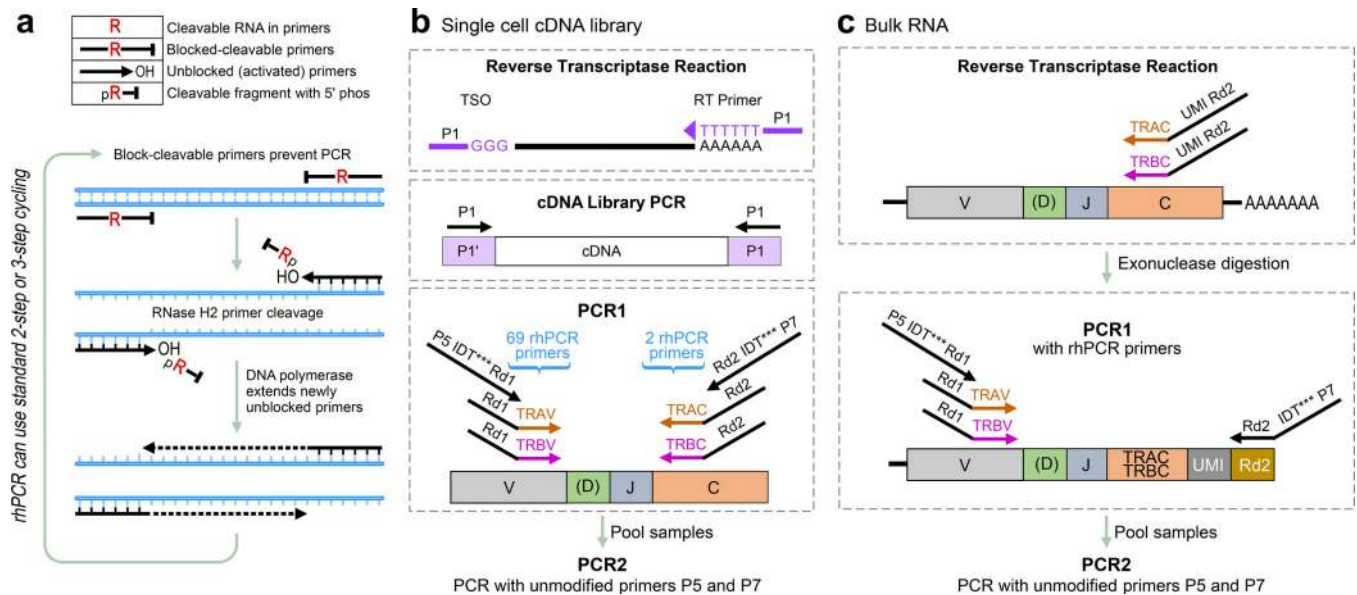
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**Box 1 |****Steps in the pipeline used to analyze rhTCRseq data**

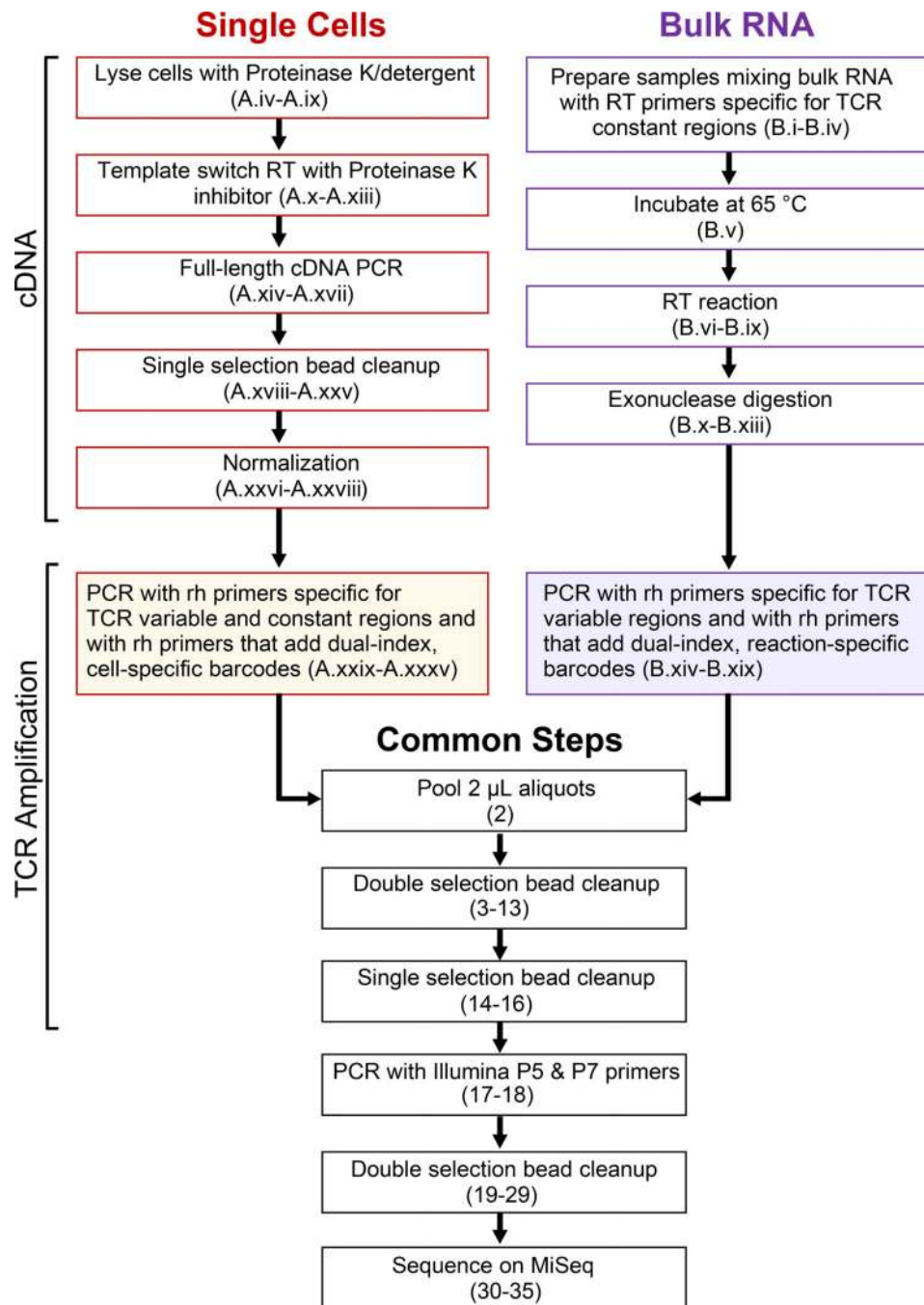
1. Validate demultiplexing results of the fastq files from the Illumina MiSeq and count the total number of reads per well for initial quality assessment.
2. Separate reads into alpha- and beta-chain files by using BLAST<sup>48</sup> to align to the chain-specific primers used in the amplification.
3. Assemble and annotate the reads using MiXCR<sup>47</sup> with default parameters for alignment to known V, D and J regions, assembly of each TCR, and identification of the CDR3 region.
4. Merge *TRBV* genes that are indistinguishable based on the locations of the primers for those genes.
5. Additional steps were performed to adjust for overestimations of clonotypes and UMIs introduced by errors in the PCR or sequencing steps:
  - i. For single cell results, collapse clonotypes if they share identical V- and J-segments and the CDR3 nucleotide sequences have 95% or greater similarity. The clonotype with higher frequency is retained, and the read counts of the subsumed clonotype are merged with the higher frequency clonotype.
  - ii. For bulk RNA results, delete UMIs that are supported by only one read, then delete clonotypes that have no remaining UMIs.
  - iii. For bulk RNA results, collapse clonotypes to the majority clonotype if they share identical V- and J-segments, the CDR3 nucleotide sequences have 95% or greater similarity, and the CDR3 amino acid sequences have the same length.
  - iv. For bulk RNA results, collect clonotypes from all the replicates of a given sample, sort in groups with the same J segment and CDR3 sequence, and collapse to the clonotype within each group that has the highest UMI count.
  - v. For bulk RNA results, remove nonproductive clonotypes.



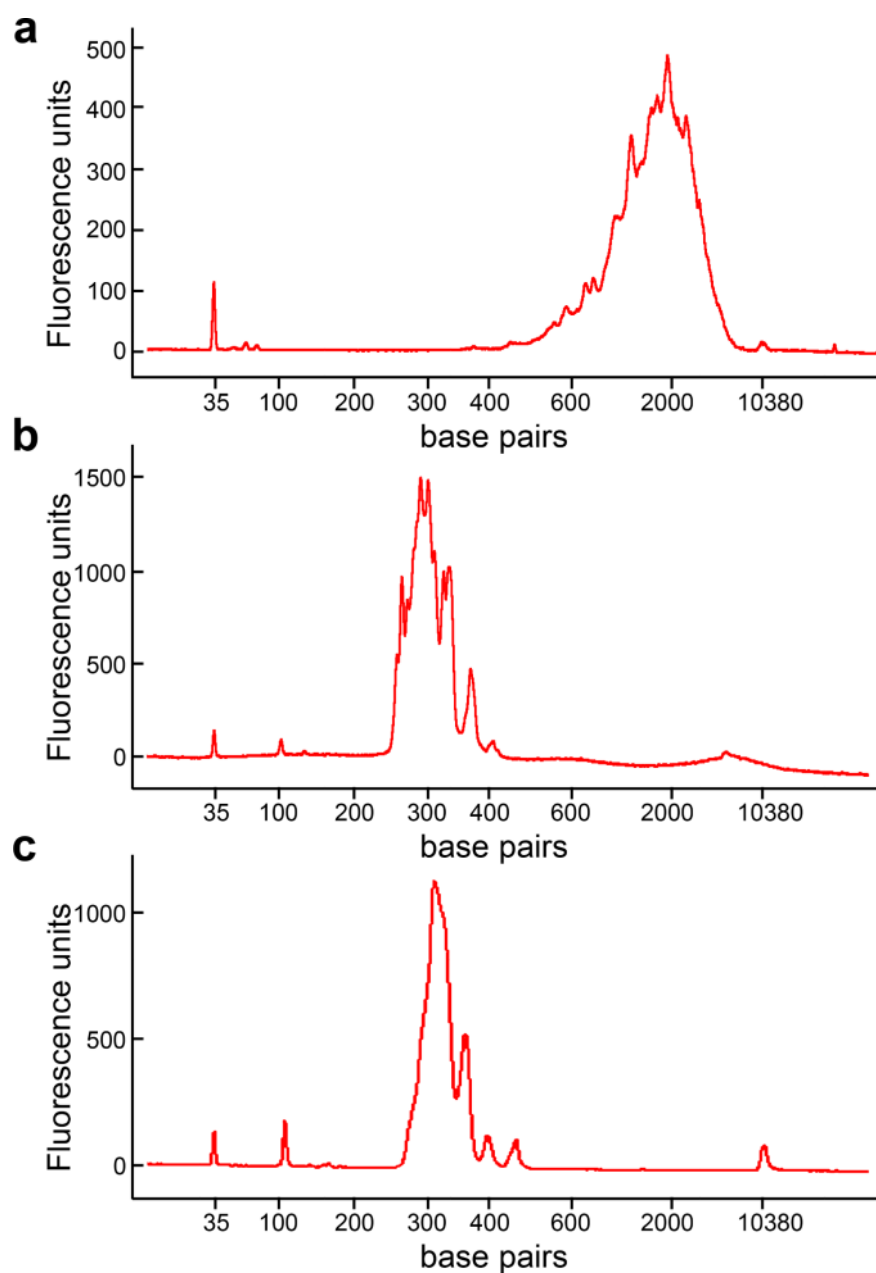
**Fig. 1 |. Critical features and schemes of rhTCRseq.**

**a**, Mechanism for the enhanced specificity of rhPCR. Instead of conventional primers, rhPCR uses 3'-blocked oligonucleotides each containing a single ribo base. Upon high fidelity hybridization to its target, each oligonucleotide is cleaved at the ribo base by thermostable RNase H2 to generate a primer with a 3'-hydroxyl that can be extended by DNA polymerase. **b,c**, Scheme for TCR-specific amplification from single cell cDNA libraries (**b**) and from bulk RNA (**c**). V, (D), J, and C indicate segments of the TCR transcript. Arrowheads indicate the 3' end of primers. TSO refers to the NEBNext Template Switching Oligo. P1 refers to the NEBNext Single Cell cDNA PCR Primer. Segments specific for the variable (*TRAV*, *TRBV*) and constant (*TRAC*, *TRBC*) regions of alpha and beta TCR transcripts, and for the Illumina read 1 and read 2 sequences (Rd1, Rd2) are labelled. There are 69 rhPCR primers specific for *TRAV* and *TRBV*, 1 rhPCR primer specific for *TRAC*, and 1 rhPCR primer specific for *TRBC*. IDT\*\*\* indicates 8-nucleotide index sequence. P5 and P7 refer to the Illumina sequences required for flow cell attachment. P5.IDT\*\*\*.Rd1 and P7.IDT\*\*\*.Rd2 are rhPCR primers. UMI refers to Unique Molecular Identifier.





**Fig. 2 |. Schema of the single cell and bulk RNA rhTCRseq workflows.**  
Diagram outlining the parts of the protocol that are described in detail in steps 1–35.



**Fig. 3 |. Expected results for library constructions using the rhTCRseq protocol for single cells.** **a**, Bioanalyzer trace for single-cell, full-length cDNA library from Step 1Axxv. The distribution of fragments looks very similar to that reported in Figure 3 of Trombetta *et al.*<sup>15</sup> for a Smart-Seq2 single-cell cDNA library. **b,c**, Bioanalyzer trace for final rhTCRseq libraries prepared from 384 single cells (b) and bulk RNA (c) from Step 29.

Table 1 |

Scheme for pipetting from four 96-well plates to one 384-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	A	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
B	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C
C	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C
E	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
F	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C
G	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
H	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C
I	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
J	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C
K	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
L	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C
M	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
N	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C
O	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
P	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C

The numbers in the first row and the letters in the leftmost column indicate the coordinates of a 384-well plate. The remaining letters A, B, C, and D refer to four 96-well plates. Using an 8-channel pipette, primers in each column of 96-well plate A are pipetted to a column with wells marked A in this diagram of a 384-well plate. This requires 12 transfers. The process is repeated for 96-well plates B, C, and D. The overall operation requires 48 transfers, with 8 wells being pipetted per transfer.

**Table 2 |**

Data analysis output files for single cell protocol

Results folder	Content
clone_stat_all.txt	The final number of clonotypes and the percentage collapsed for each locus in each well
collapse_stat_all.txt	Information about clonotypes that were collapsed
mixcr_clonotype_per_well.list	Information about the top clonotypes in each well
mixcr_clonotype_recurrence.list	Information about which clonotypes are found in which wells
mixcr_clonotype_TRA_across_well.table	With one row per well, records the clone fraction of the top TRA clonotype in that well and its occurrence in the other wells
mixcr_clonotype_TRB_across_well.table	With one row per well, records the clone fraction of the top TRB clonotype in that well and its occurrence in the other wells

Table 3 |

Data analysis output files for bulk RNA protocol

Results folder	Content
[well]_clonotype_count_TRA.csv	For each well, reports all unique TRA clonotypes with V identity, J identity, CDR3 nucleic acid sequence, CDR3 amino acid sequence, read count, and UMI count
[well]_clonotype_count_TRB.csv	For each well, reports all unique TRB clonotypes with V identity, J identity, CDR3 nucleic acid sequence, CDR3 amino acid sequence, read count, and UMI count
[sample]_clones_count.csv	Read counts for combined replicates
[sample]_clones_umi_count.csv	UMI counts for combined replicates
[sample]_observation_across_replicates.csv	Number of replicates in which each clonotype appears
[sample]_clonotype_count.csv	Number of clonotypes with each V gene for combined replicates
[sample]_clonotype_count.png	Bar plot of clonotypes per V gene for combined replicates

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**Table 4 |**

## Troubleshooting table

Step	Problem	Possible reason	Solution
1Ai	>25% of cells have no productive clonotype	Misalignment of the sorter so that cells are not being sorted to the center of the wells	Ensure the sorter is properly aligned and that the plate is put squarely in place. Alignment can be checked using fluorescent beads or by placing parafilm across a plate and seeing that sorted droplets are in the center of each well. Rodrigues and Monard <sup>49</sup> describe a method using microspheres that can be used to validate sorter alignment.
1Ai	>25% of cells have no productive clonotype plus >25% have alpha only or beta only	Dormant or dying cells	If thawing frozen cells, let cells recover longer before sorting. Be sure to include a viability marker in the sorting.
1A(vii-xxxiv)	>10% of cells have multiple clonotypes	Cross-well contamination prior to pooling	Scrupulous attention to detail during pipetting. A particularly vulnerable point is the washing of the beads during purification of the cDNA libraries. Cross-contamination of barcode primer plates will also produce multiple clonotypes. In this case, the contaminated P5.IDTxxx.Rd1x.x1 / P7.IDTyty.Rd2x.x1 barcode primer plate needs to be re-made from stocks.
1Biii or 30	For the bulk RNA protocol, a low number of clonotypes are detected. A possible cutoff is <500 clonotypes (alpha + beta) detected per sample.	This could indicate the fraction of T cells is low in the starting material used to prepare RNA	The read depth can be increased by using a platform other than MiSeq or the amount of input RNA can be increased.
		This could be due to poor RNA quality	Re-extract RNA to obtain a sample with a RIN of at least 4

**Table 5 |**

Enumeration of TCR-alpha and -beta TCR clonotypes detected in 384 putative tumor-specific T cells.

	<b>1 Alpha + 1 Beta</b>	<b>2 Alpha + 1 Beta</b>	<b>1 Alpha + 2 Beta</b>	<b>1 or 2 Alpha Only</b>	<b>1 or 2 Beta Only</b>	<b>Multiple Alpha and Beta</b>	<b>No Alpha and Beta</b>
<b>Number of cells</b>	289	49	6	5	17	1	17
<b>Total cells</b>	344			40			

In each single cell, and separately for alpha and beta, detected clonotypes are ranked by number of CDR3 reads. Nonproductive CDR3 sequences with stop codon or frame shift are ignored. If less than 100 CDR3 reads are counted for the predominant sequence, then no alpha/beta clonotype is reported for that cell. If the CDR3 reads for the predominant sequence are 20x or greater than the second sequence, one alpha/beta clonotype is reported for that cell. If the sum of the CDR3 reads for the first and second sequences is 20x or greater than the third sequence, two alpha/beta clonotypes are reported for that cell. Any remaining cells are reported as multiple. If a cell has two alpha clonotypes and two beta clonotypes, it is also reported as multiple.