HUMAN TCR RNA KIT v.2

TCR α and β Repertoires with UMI (5'RACE)

User Manual v.2.12

Cat. # THR-002
192 samples RNA

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TABLE OF CONTENTS

KIT OVERVIEW

The **Human TCR RNA Kit v.2** is designed for unbiased amplification of TCR alpha and beta cDNA libraries using 5'RACE with unique molecular identifiers (UMI) incorporated within template switch oligo. 5'RACE UMI technique allows for exact quantification of template cDNA molecules, control for input bottlenecks, and accurate normalization of samples for comparisons of repertoire diversity metrics (**Fig. 1**).

The kit includes a set of oligonucleotides sufficient to prepare 192 TCR alpha and 192 TCR beta cDNA libraries starting **from 192 RNA samples**.

The kit **does not** include enzymes, buffers and indexing primers for cDNA synthesis and PCR amplification.

The kit allows to start with RNA derived from 100 to 1 million sorted/purified T-cells, from peripheral blood leukocytes, or from T-cell containing tissues (see **Appendixes A** and **B** for recommended RNA isolation procedures), and produces indexed ready-to-use quence-on-Illumina® libraries. Up to 96 samples can be processed in parallel.

1st PCR amplification starts from the same cDNA sample and includes both TCR alpha and beta libraries in the same tube. 2nd PCR amplification starts from the product of the 1st PCR and is performed separately for TCR alpha and beta repertoires (**Fig. 2**).

Full-length TCR alpha and beta profiling requires 150+150 paired end Illumina sequencing with sufficient reads-per-UMI sequencing coverage.

The kit contains a limited amount of reagents for cDNA synthesis and the 1st PCR (192 reactions). For the 2nd PCR, reagents are provided in sufficient amounts to optimize the number of PCR cycles if necessary.

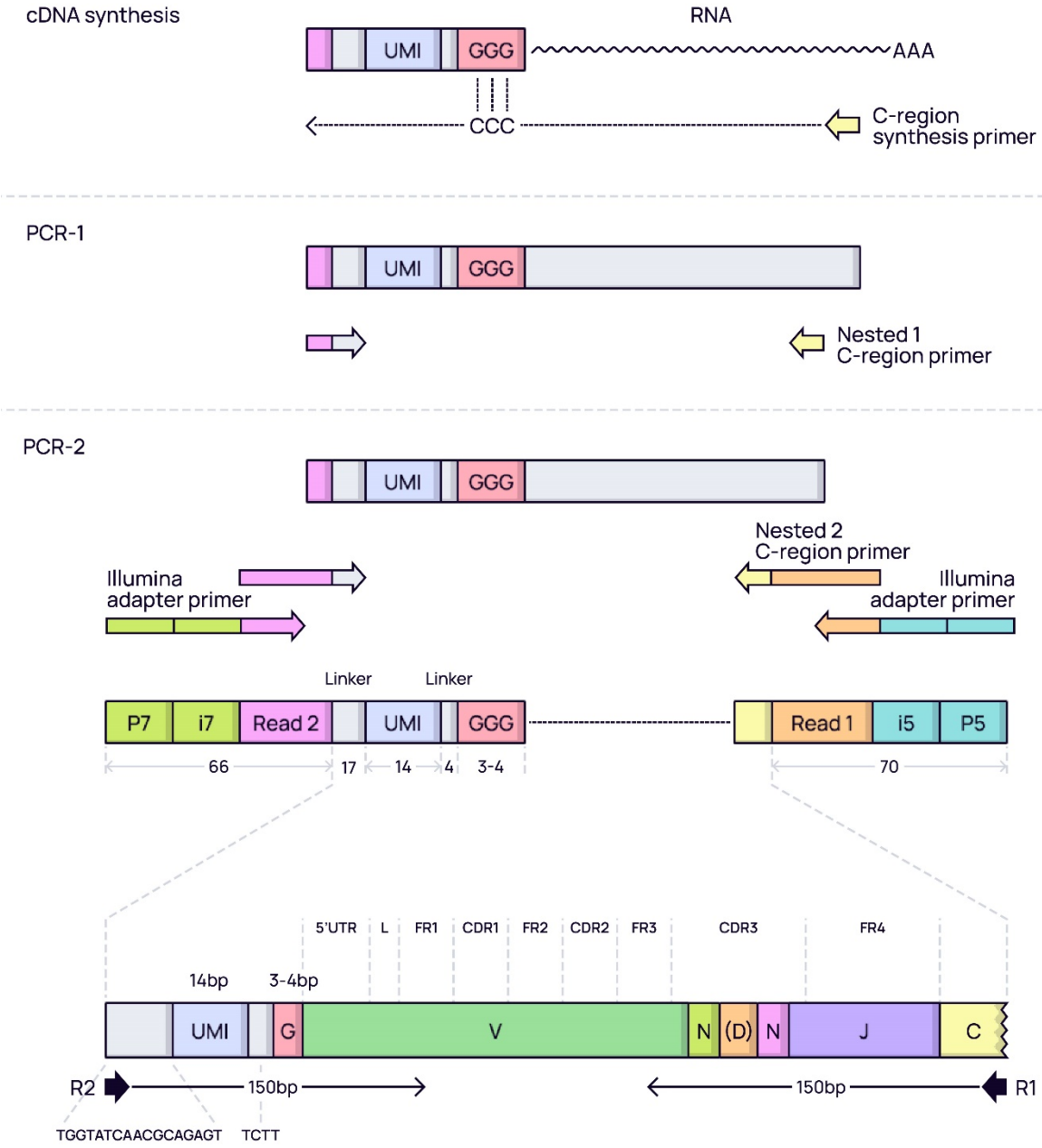

Figure 1. Scheme for TCR RACE libraries generation and sequencing.

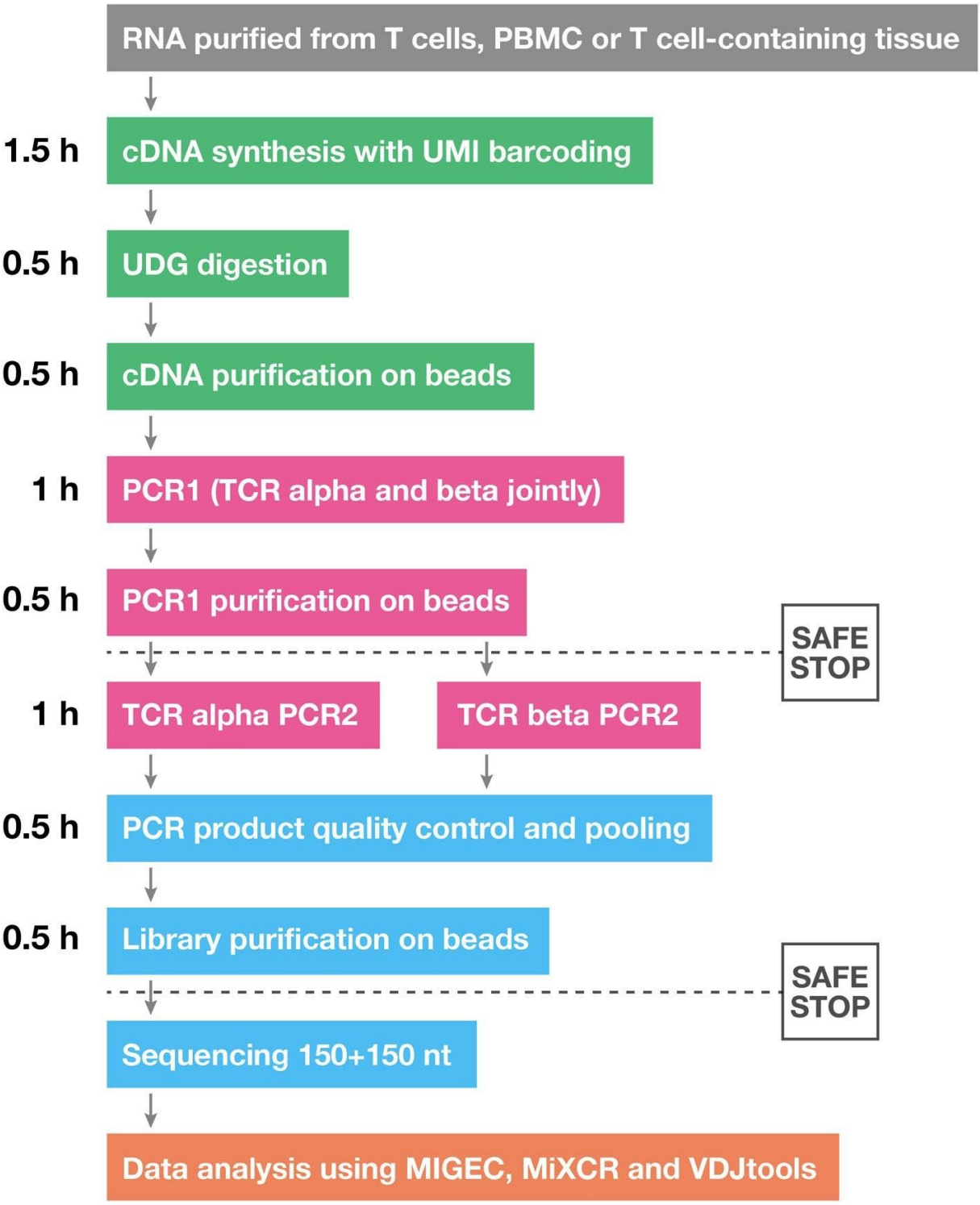

Figure 2. Human TCR RNA kit v.2 pipeline.

KIT CONTENTS

	Name	Volume (µl)	Description
1	NN oligo	460 (after recovery)	cDNA synthesis template switch oligo with UMI (15 X)
2	TR Hum Synt	460 (after recovery)	cDNA synthesis primers mix (15 X)
3	TR Hum PCR1	460 (after recovery)	1st PCR primers mix (25 X)
4	TR-A Hum PCR2	100 (after recovery)	2nd PCR primers mixes
5	TR-B Hum PCR2	100 (after recovery)	
6	Control RNA	120 (after recovery)	Control RNA final concentration 5 ng/µl
7	RNAse-free water	1500 X 2	Deionized nuclease-free water

Components 1-6 are shipped dry.

Transportation and storage until dissolved at RT.

Before use, add water and place components 1-5 in the freezer (at – 20ºC).

Add 120 µl of RNAse-free water to Control RNA. Incubate the tubes at room temperature for 10 min. Mix by vortexing and spin down. Make 10 aliquots of diluted control RNA 12µl each and store at -70ºC.

MATERIALS REQUIRED, BUT NOT INCLUDED

- SMARTScribe™ Reverse Transcriptase (Clontech, Takara, Inc. #639538, supplied with 5X First-Strand Buffe and DTT)
- RNAsin® (Promega, #N2515)
- dNTP mix (10mM each)
- Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs, #M0493L) with 5X reaction buffer
- Uracil-DNA Glycosylase (New England Biolabs, #M0280S)
- AMPure® XP Beads (Beckman Coulter, Inc. #A63881) or SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317)
- Freshly prepared 80% Ethanol (1200 **µl** per sample)
- Elution Buffer (EB, 10 mM Tris-HCl, pH 8.0-8.5)
- Nuclease free water
- IDT for Illumina Nextera DNA Unique Dual Indexes or IDT for Illumina — DNA/RNA UD Indexes, Tagmentation (any of #20027213, #20027214, #20027215 or #20027216) (recommended)

  or

  Nextera XT Index Kit (FC-131-1001) or Nextera XT Index Kit v2 (any of Set A, B, C and D, FC-131-2001, FC-131-2002, FC-131-2003, and FC-131-2004)
- Agarose gel electrophoresis system
- Agilent Bioanalyzer/Tape Station
- Thermal Cycler
- Appropriate Magnetic Rack
- Low-speed benchtop Mini-centrifuge/vortex
- Qubit® fluorometer with Qubit dsDNA HS and Qubit RNA HS Assay Kits

GENERAL RECOMMENDATIONS TO PREVENT CONTAMINATION

General recommendations to lower the risk of RNA degradation and contamination should be implemented, such as using lab coats, gloves, tips with aerosol filters, certified RNAse/DNAse free reagents, performing of non-template control reaction.

To prevent cross-sample contamination, the library preparation should be performed in two separate workspaces (PCR boxes) located in different rooms: pre-PCR and post-PCR. The user should avoid transferring anything (reagents, pipettes, racks etc.) from post-PCR to pre-PCR workspace. In the case of possible contamination, use decontaminating procedures with special reagents such as DNA-OFF™ (MP Biomedicals) and UV.

Perform cell isolation, RNA purification, cDNA synthesis, the 1st PCR reaction, and the 2nd PCR Master Mix setups in the pre-PCR workspace (**Steps 1–8** and **11–13**). After the 1st PCR amplification transfer the tubes to the post-PCR workspace and perform all other steps in the post-PCR workspace except for **Steps 11–13**.

Perform UV decontamination each time before making up a PCR master mix.

BEFORE YOU START

Add nuclease-free water to the tubes according to the **Table 1** in **Kit Content**. Incubate the tubes at room temperature for 10 min.

Mix by vortexing and spin down.

Make aliquots of NN-oligo depending on planned experimental setup. Each aliquot can be frozen/thawed no more than 3 times.

Store diluted components 1-5 at –20°C.

Add 120 µl of RNAse-free water to Control RNA. Incubate the tubes at room temperature for 10 min. Mix by vortexing and spin down. Make 10 aliquots of diluted control RNA 12µl each and store at -70ºC.

STARTING MATERIAL

See **Appendixes A** and **B** for our recommendations on RNA isolation. Use Qubit® fluorometer or other fluorescent based method to determine RNA concentration.

Verified minimal number of cells for the protocol is 100 T-cells sorted directly into Qiagen RLT® buffer, followed by RNA extraction with TRIzol® (**Appendix B**). B). Add co-precipitant to avoid losses (i.e. Carrier poly(A) RNA or Pellet Paint (Merck # 69049)).

High RNA quality is often critical for an efficient library preparation. Residual traces of proteins, salts or other contaminants may decrease the efficiency of the enzymatic activity necessary for optimal targeted enrichment.

Large amounts of gDNA may significantly affect cDNA synthesis (>200,000 cells) samples. It is recommended to include DNAse treatment in the RNA extraction procedure or perform independent DNAse digestion with additional purification of RNA before start.

Do not use heparin coated tubes for blood collection. Heparin dramatically decreases cDNA synthesis efficiency.

PROTOCOL

NOTE: Perform steps 1-8 and step 11-13 in a pre-PCR box/room.

cDNA synthesis with template switch

1. In sterile reaction tube(s)/strip(s)/plate, mix the following reagents in a final reaction volume of 12 µl:

Component	Amount, µl
RNA, up to 1000 ng per reaction*	1–10**
TR Hum Synt cDNA synthesis primer mix (15x)	2
RNAse free water	0–9
Total volume	**12 µl**

* For control reaction, take 10 µl of control RNA (50 ng per reaction).

** Do not add less than 1 µl of RNA to the reaction. If RNA solution is highly concentrated (more than 500 ng/µl), dilute it 2-4 times with RNase free water.

2. Place the reaction tube(s)/strip(s)/plate into a thermal cycler and incubate for 2 min at 70ºC, followed by 2 minutes at +4ºC.

3. While incubating, prepare Master Mix of the following components in a final volume of 18 µl per reaction:

Component	Amount, µl
5X First-Strand Buffer (Clontech)	6
DTT (20 mM)	3
NNoligo	2
dNTP solution (10 mM each)	3
RNAsine®, 40u/µl	1
SMARTScribe® Reverse Transcriptase (Clontech)	3
Total volume	**18 µl**

NOTE: Add SMARTScribe® reverse transcriptase to the Master Mix just before use and mix gently by pipetting.

4. Add 18 µl of Master Mix to each reaction, pipette gently, spin briefly, and incubate for 60 min at 42ºC.

5. Add 1 µl of Uracyl DNA glycosylase (5 U/µl, New England Biolabs), gently mix by pipetting, and incubate for 30 min at 37ºC.

NOTE: Uracyl DNA glycosylase treatment removes residual template switch oligo that is critical for the accurate labeling of starting cDNA molecules.

6. Purify obtained cDNA synthesis products using AMPure® XP Beads (Beckman Coulter). Use 1:1 sample:AMPure® XP Beads ratio.

- Homogenize beads by vortexing.
- Add 30 µl of beads to each reaction and mix well by pipetting up and down 10 times.
- Incubate for 5 minutes at RT and place on the magnetic rack.
- Wait for the solution to clear (which should take approximately 2–5 minutes).
- Keep tubes on the magnetic rack. Carefully remove and discard the cleared solution from the tubes.
- Keep tubes on the magnetic rack. Add 200 µl of freshly prepared 80% ethanol. Wait for 30 seconds and remove the ethanol.
- Repeat washing by 80% ethanol without taking off the magnetic rack and remove the ethanol.
- Remove tubes from the magnetic rack. Briefly spin down and place back on the magnetic rack. Remove all the residual ethanol.

  NOTE: It is important to remove all the traces of ethanol at this step as it could inhibit subsequent PCR reaction.
- Remove tubes from the magnetic rack. Add 13 µl of EB and mix well by pipetting.
- Incubate for 5 minutes at RT and place on the magnetic rack.
- Wait for the solution to clear.
- Keep tubes on the magnetic rack. Take 12 µl of cDNA solution into a new tube.

  NOTE: To reduce the drying time, get out the tubes from the magnetic separator after the second ethanol washing. Briefly spin the tubes to drop down all the remaining liquid. Place the tubes back on the magnetic separator, make sure there are no beads left in the supernatant and remove remaining liquid with a pipette.

During the elution step, AMPure beads clumping may significantly reduce the DNA yield, which is critical at the cDNA purification step. To maximize the cDNA yield, we recommend to break up the clumps using pipetting and heating at 50ºC for 10–15 min until complete dissolving.

SAFE STOP POINT: Purified product of the 1st PCR can be stored for 1–3 months at −20°C.

1st PCR amplification

NOTE: Both alpha and beta TCR chains are amplified in the same first round PCR reaction.

7. In a sterile tube, prepare PCR1 Master Mix in a final volume of 38 µl per reaction, plus 10% of the total reaction mix volume:

Component	Amount, µl
Nuclease free water	24.5
5X Q5® polymerase buffer (NEB)	10
dNTP mix (10 mM each)	1
TR Hum PCR1 primers mix (25 X)	2
Q5® Hot Start High-Fidelity DNA Polymerase(NEB)	0.5
Total volume	**38 µl**

8. Add 38 µl of PCR1 Master Mix to each of 12 µl cDNA samples. Mix gently by pipetting.

9. Perform PCR using the following parameters:

98ºC	30 s	
98ºC	10 s	
55ºC	20 s	18–21 cycles
72ºC	40 s	
72ºC	2 min	
4ºC	hold	

NOTE: Proceed in the post-PCR box/room.

10. Purify the obtained 1st PCR products using AMPure® XP Beads (Beckman Coulter).

Use **1:0.8** sample:AMPure® XP Beads ratio

- Homogenize beads by vortexing.
- Add 40 µl of beads to each reaction and mix well by pipetting up and down 10 times.
- Incubate for 5 minutes at RT and place on the magnetic rack.
- Wait for the solution to clear (which should take approximately 2–5 minutes).
- Keep tubes on the magnetic rack. Carefully remove and discard the cleared solution from the tubes.
- Keep tubes on the magnetic rack. Add 200 µl of freshly prepared 80% ethanol. Wait for 30 seconds and remove the ethanol.

- Repeat washing by 80% ethanol without taking off the magnetic rack and remove the ethanol.
- Remove tubes from the magnetic rack. Briefly spin down and place back on the magnetic rack. Remove all the residual ethanol.

  NOTE: It is important to remove all the traces of ethanol at this step as it could inhibit subsequent PCR reaction
- Remove tubes from the magnetic rack. Add 21 µl of EB and mix well by pipetting.
- Incubate for 5 minutes at RT and place on the magnetic rack.
- Wait for the solution to clear.
- Keep tubes on the magnetic rack. Take 20 µl of purified 1st PCR products into a new tube.

SAFE STOP POINT: Purified product of the 1st PCR can be stored for 1–3 months at −20°C.

2nd PCR amplification

NOTE: At this point, the samples must be split and processed separately for TCR alpha and beta libraries. Therefore, from each of the 1st PCR reactions, you will obtain two libraries in the 2nd PCR: one for TCR alpha and one for TCR beta.

11. Prepare working primer solutions for **PCR2-A** and **PCR2-B.**

For this, calculate the volume of PCR-2 primers required to process N number of samples based on 1 µl per sample. Dilute 250X stocks of **TR-A hum PCR2** and **TR-B hum PCR2** with nuclease-free water 1:9 according to the calculation, to obtain 25 X solutions. Discard diluted aliquots after use.

12. Prepare two separate PCR2 Master Mixes in a final volume of 22 µl per reaction without indexes (for several reactions, prepare a common premix):

For **TCR alpha**

Component	Amount, µl
Nuclease free water	15.25
5X Q5® polymerase buffer (NEB)	5
dNTP mix (10 mM each)	0.5
TR-A Hum PCR2 (25X)	1
Q5® Hot Start High-Fidelity DNA Polymerase (NEB)	0.25
Total volume	**22 µl**

For **TCR beta**

Component	Amount, µl
Nuclease free water	15.25
5X Q5® polymerase buffer (NEB)	5
dNTP mix (10 mM each)	0.5
TR-B Hum PCR2 (25 X)	1
Q5® Hot Start High-Fidelity DNA Polymerase (NEB)	0.25
Total volume	**22 µl**

13. For each reaction, add 22 µl of PCR2 Master Mix to a nuclease-free 0.2 ml tube/strip. Two separate reactions volumes are required for TCR alpha and TCR beta chain.

NOTE: Proceed in the post-PCR box/room.

14. Add 2 µl of purified 1st PCR product to each TCR alpha and TCR beta chain reaction.

15. Add Index Primers:

15a. If using **IDT for Illumina Nextera Unique Dual Indexing primers** (recommended): Add 1 µl of IDT for Illumina Nextera DNA UD Index to each reaction. Use a unique Index for each reaction.

15b. If using **Nextera XT Indexing primers**: Add 1 µl of Nextera N70X and 1 µl of Nextera S50X primer to each reaction. Use unique Indexing primers combination for each reaction. Final reaction volume will be 26 µl, but it will not affect the efficiency of the reaction.

16. Perform PCR using the following parameters:

98ºC	30 s	
98ºC	10 s	
55ºC	20 s	9–24 cycles*
72ºC	40 s	
72ºC	2 min	
4ºC	∞	

* Choose the number of cycles depending on the amount of RNA/T-cell numbers at the start (**Table 2**):

Table 2. Approximate number of PCR cycles for T-cells or RNA and sequencing reads

Estimated number of T-cells in a sample	Amount of template RNA purified from sorted T-cells/PBMC used at start	Recommended number of 1st PCR cycles	Approximate number of 2nd PCR cycles	Recommended number of sequencing reads
10^2– $5x10^2$	-	21	17–21	20 -50 reads per input T-cell
$5x10^2$ – $5x10^3$	-		15–19	
$5x10^3$ – $5x10^4$	5–50 ng		12–16	
$5x10^4$ – 10^5	50–100 ng		10–14	
10^5 – $5x10^5$	100–500 ng	18	14–18	
$5x10^5$ – 10^6	500–1000 ng		13–17	
-	10 **µl** control RNA		15–18 for TCRβ	

NOTE: The optimal number of 2nd PCR cycles may vary for different templates, cell types, thermal cyclers, and sample amounts. We recommend that you determine the minimal number of PCR cycles required to obtain a sufficient amount (at least 40 ng of each library) empirically for each experiment. Furthermore, we recommend that you generate parallel libraries of similar nature (e.g., ten samples of 2,000-20,000 sorted T-cells each, TCR alpha and beta chains) using the same number of PCR cycles, and mix the obtained libraries in equal volume proportion for sequencing. This allows obtaining an even coverage in terms of reads-per-cDNA. For example, the library, which started with 20,000 T-cells, may produce more PCR product than the one, which started with 2,000 T-cells, after the same number of PCR cycles. However, the former library would also carry proportionally more TCR cDNA molecules, and thus requires more sequencing reads to achieve a comparable coverage.

17. Verify the quality of the obtained PCR product on the agarose gel (1.2%), or by capillary electrophoresis (e.g. BIOANALYZER® or TapeStation®, Agilent Technologies).

The resulting library should have a peak at approximately 700 bp for TCR alpha and 670 bp for TCR beta chains (**Fig. 3**).

SAFE STOP POINT: Store purified libraries at −20°C.

18. Pool all the obtained 2nd PCR libraries (both TCR alpha and beta) by combining an equal volume portion of each individual sample from the 2nd PCR. The total resulting amount of pooled PCR products should be at least 100 ng.

TCR alpha and TCR beta pools can be mixed in equimolar ratio.

19. Purify the obtained pooled DNA (up to 100 **µl** of the pooled PCR product) using AMPure® XP Beads (Beckman Coulter) with **1:0.8** sample:beads ratio:

- Homogenize beads by vortexing.
- Add beads in a volume equal to 0.8 of the pooled 2nd PCR and mix well by pipetting up and down 10 times.
- Incubate for 5 minutes at RT and place on the magnetic rack
- Wait for the solution to clear (which should take approximately 2–5 minutes).
- Keep tubes on the magnetic rack. Carefully remove and discard the cleared solution from the tubes.
- Keep tubes on the magnetic rack. Add 200 µl of freshly prepared 80% ethanol. Wait for 30 seconds and remove the ethanol.
- Repeat washing by 80% ethanol without taking off the magnetic rack and remove the ethanol.
- Remove tubes from the magnetic rack. Briefly spin down and place back on the magnetic rack. Remove all the residual ethanol

  NOTE: It is important to remove all the traces of ethanol at this step as it could inhibit subsequent sequencing procedures.
- Remove tubes from the magnetic rack. Add 31 µl of EB and mix well by pipetting.
- Incubate for 5 minutes at RT and place on the magnetic rack.
- Wait for the solution to clear.
- Keep tubes on the magnetic rack. Take 30 µl of of purified 2nd PCR libraries into a new tube.

  NOTE: The elution volume can be changed to obtain a library with the desired concentration.

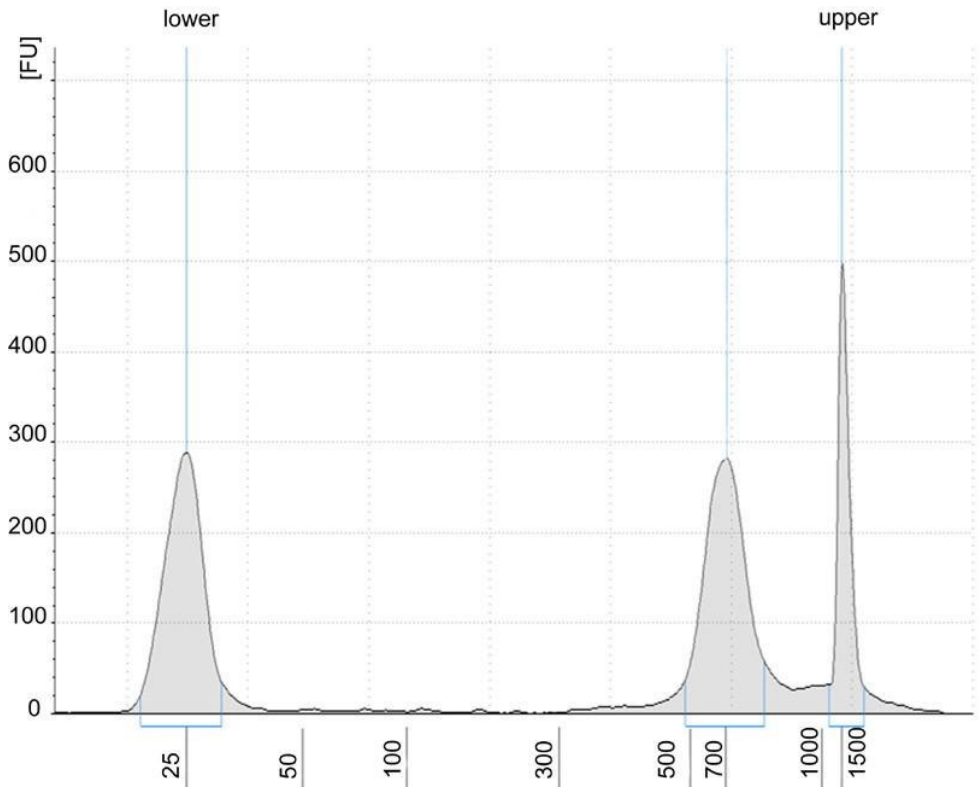

Figure 3. A typical al library peak for TCR alpha and beta library after cleaning. The shape and length distribution of a TCR library may vary depending on the repertoire composition.

20. Verify quality of obtained pooled libraries by analyzing the aliquot by agarose gel or capillary electrophoresis (e.g. BIOANALYZER® or TapeStation®, Agilent Technologies) (**Fig. 3**). Quantify the obtained purified pools using Qubit fluorimeter and Qubit dsDNA High Sensitivity Assay Kit.

If fragments shorter than 400 bp are detected repeat beads purification procedure using 1:0.7 sample:AMPure® XP Beads ratio

The resulting amount of pooled purified DNA should be at least 200 ng.

SAFE STOP POINT: Store purified libraries at −20°C.

SEQUENCING RECOMMENDATIONS

The obtained purified pooled library is ready for Illumina sequencing.

Determine library concentration according to the Illumina recommendations

Spike with 20% of PhiX or any other random library (e.g. RNA- Seq, Exome-Seq etc.).

Recommended coverage that allows for optimal UMI-based elimination of amplification biases and accumulated errors:

- 30–50 sequencing reads per input T-cell.

Analyze the resulting pooled library using at least 150+150 nt paired end Illumina MiSeq®, HiSeq® or NextSeq® sequencing, and the standard Illumina® sequencing primers. 150+150 nt length is sufficient to cover the CDR3 region. However, the longer the reads are, the more accurate the V gene segments identification is afterwards. For comparative analysis of the obtained TCR repertoire data, use the same sequencing length for all the samples of interest. Preferably, use the same sequencing machine. Ideally, use the same sequencing run for all the samples of comparison.

DATA ANALYSIS

Data analysis can be performed using **MiXCR™** software for UMI group assembly and clonotype extraction from raw sequencing reads.

> **NOTE**: **License is required to use MiXCR™.** Academic users can quickly get a license at https://licensing.milaboratories.com. Commercial license may be requested at https://licensing.milaboratories.com or by email: licensing@milaboratories.com.

Information regarding installing the software as well as the detailed pipeline describing how to process the data can be found at https://docs.milaboratories.com in sections “Getting Started” and “Guides”, respectively.

MiXCR™ software also provides a broad and rapidly growing range of repertoire postanalysis tools with table and graphical outputs. Please refer to documentation for more information.

APPENDIX A: PREPARING STARTING MATERIAL

TCR cDNA libraries can be generated starting from RNA isolated from sorted/isolated T-cells, PBMCs or any tissue containing T-cells. Control for the counts of T-cells of interest in a sample is desirable for the downstream data analysis.

When choosing a purification kit, ensure that it is appropriate for your sample type, input mass, and includes DNase treatment. The last point is critical because gDNA significantly affects cDNA synthesis and subsequent PCR amplification. We recommend RNeasy kits (Qiagen) or TRIzol reagent (Invitrogen). TRIzol reagent provides DNA-free RNA samples. RNeasy kits are compatible with gDNA removal via on-column DNAse treatment, gDNA eliminator columns or RNA cleanup after DNAse treatment in solution.

Lysed cells/tissues can be stored in RLT® buffer (QIAGEN) at –70°C for at least 6 months.

Large samples (>50,000 cells) can be also stored in TRIzol® at –70°C for up to 6 months.

For large samples (>50,000 cells): verify quantity and quality of the extracted RNA using Qubit and then Agilent Bioanalyzer or gel electrophoresis. RNA Integrity Number >7, or correct 28S rRNA:18S rRNA ratio (around 1.5–2.5:1) and a low number of shadow bands above and below 18S band are indicative of high quality RNA. However, it is often the case that medium quality RNA allows obtaining high quality TCR libraries.

For small samples (i.e. <50,000): it is not necessary to check quantity and quality of the extracted RNA.

Degraded RNA may indicate that the samples were stored too long before processing, isolated RNA was stored at an incorrect temperature or RNAse contamination.

Carrier poly(A) RNA can be used during the RNA extraction pro- cedure to increase the yield of isolated RNA. Carrier RNA will not interfere with primers for cDNA synthesis reaction.RNA can be stored in 75% ethanol for at least 1 year at –20°C, or at least 1 week at 4°C. For small RNA amounts (less than 100 ng), it is preferable to start cDNA synthesis immediately after RNA extraction.

Further recommendations should help to choose the appropriate strategy for RNA isolation in most situations.

PBMC

Do not use heparin coated tubes for blood collection. Heparin dramatically decreases cDNA synthesis efficiency.

Perform isolation of mononuclear cells from whole blood using Ficoll Paque density gradient centrifugation. Spin down the cells at 350 g for 15 min, remove the supernatant.

Add at least 50 **µl** (or 50 **µl** per $2x10^5$ cells) of RLT® buffer and mix by pipetting for:

- storage at -70°C for up to 6 months, or
- RNA purification using RNeasy Micro or Mini kit (QIAGEN), or
- RNA purification using 4 volumes of TRIzol® added per 1 volume of RLT® buffer (**Appendix B**).

Alternatively, place cells in at least 300 **µl** (or 300 **µl** per 3 x 10^6 cells) of TRIzol® and extract RNA using TRIzol® protocol (**Appendix B**).

Large amounts of gDNA may affect cDNA synthesis. For large numbers of cells (above 200,000 cells), when using a column-based RNA extraction method, DNAse treatment is recommended. gDNA eliminator columns (QIAGEN) can also be used for DNA removal.

From 100 to 50,000 sorted or purified T-cells

Sort/place directly in 50-300 **µl** RLT® buffer. The volume of RLT® buffer should not be diluted more than 20% during sorting. 50,000 sorted cells may carry the volume around 50 **µl** when using 70 µm nozzle for cell sorting. The cells are lysed immediately in the collection tube and mRNA is protected from degradation. Lysed cells can be stored in RLT® buffer at –70°C for at least 6 months.

Use RNeasy Micro or Mini kit (QIAGEN) for RNA purification. Do not use DNAse.

To obtain maximum number of cDNA molecules for very small samples (100-1000 cells). Add 4 volumes of TRIzol® to RLT® cellular lysate and extract RNA using TRIzol® protocol (**Appendix B**).

More than 50,000 sorted or purified T-cells

Sort/place cells into 300 **µl** of PBS, then spin down the cells at 350 g for 15 min, remove the supernatant.

Add at least 50 **µl** (or 50 **µl** per $2x10^5$ cells) of RLT® buffer and mix by pipetting for:

- storage at -70°C for up to 6 months, or
- RNA purification using RNeasy Micro or Mini kit (QIAGEN), or
- RNA purification using 4 volumes of TRIzol® (**Appendix B**).

Alternatively, place cells in at least 300 **µl** (or 300 **µl** per 3 x 10^6 cells) of TRIzol® and extract RNA using TRIzol® protocol (**Appendix B**).

Large amounts of gDNA may affect cDNA synthesis. For large numbers of cells (above 200,000 cells), when using a column-based RNA extraction method, DNAse treatment is recommended. gDNA eliminator columns (QIAGEN) can also be used for DNA removal.

T-cell containing tissue

Homogenize fresh tissue. Ideally, obtain a single cell suspension, for which incubation with DNAse and with proteases mixes (such as Liberase™ TL from Roche) can be recommended. Optionally, wash cells with PBS. Immediately proceed with RNeasy Micro kit (QIAGEN) using RLT® buffer or use TRIzol® (**Appendix B**) for RNA extraction.

APPENDIX B: RNA ISOLATION USING TRIZOL®

- Lyse cell pellet in TRIzol® reagent by repetitive pipetting. Use at least 300 µl of the reagent **per 100 – 3x10^6 cells**. For larger cell amounts increase the volume of the reagent according to the proportion of **1 ml per 10^7 cells**.
- If you have RNA in RLT buffer add 4 volumes of TRIzol® reagent and mix well by vortexing.
- Incubate the homogenized samples for 5 min at room temperature. Add 1/5 volume of chloroform and mix well by vortexing. Incubate tubes at room temperature for 3 min.
- Centrifuge the samples at >10,000 g for 10 min at 2 to 8°C, put on ice. After centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.
- Transfer the colorless aqueous phase without disturbing the in- terphase to a fresh tube. Add 1 **µl** of co-precipitant (Pellet Paint® #70748 Merck Millipore or analog) and mix well by pipetting.
- Precipitate the RNA from the aqueous phase by mixing with equal volume of isopropyl alcohol. Incubate samples at 15 to 30°C for 10 min and centrifuge at >10,000 x g for 10 min at 2 to 8°C. RNA with co-precipitant forms a visible pellet on the bottom of the tube.
- Remove the supernatant without disturbing the pellet.

If starting from <50,000 cells:

- Add 1 ml of freshly prepared 75% ethanol to the RNA pellet. Mix the sample by vortexing and centrifuge at >10,000 x g for 5 min at 2 to 8°C. Remove the supernatant.
- Dry the RNA pellet completely. It is important, however, not to overdry the pellet as this will decrease its solubility. Dissolve RNA in 10 **µl** of RNase-free water and **immediately** proceed to cDNA synthesis.

If starting from >50,000 cells:

- Add 1 ml of freshly prepared 75% ethanol to the RNA pellet. Mix the sample by vortexing and centrifuge at >10,000 x g for 5 min at 2 to 8°C. Remove the supernatant.
- Repeat this step.
- Dry the RNA pellet completely. It is important, however, not to overdry the pellet as this will decrease its solubility. Dissolve RNA in 10 **µl** of RNase-free water.
- Proceed to cDNA synthesis or store at –70°C for up to one week. For longer storage add 1/10 volume of sodium acetate (3M pH=5,5) and 3 volumes of 96% ethanol, mix well by vortexing.
- Store at –70°C.

TROUBLESHOOTING

Problem	Possible reason	Possible solution
Low product yield or no PCR product	RNA contains impurities that inhibit cDNA synthesis	In some cases, ethanol precipitation or additional column-based purification of RNA can remove impurities. If this does not help, reisolate the RNA.
	Excess of 1st strand cDNA in the first PCR reaction	Repeat the cDNA synthesis reaction. After purification (step 6) dilute 1st strand cDNA 1:5. Use 12 **µl** in 1st PCR reaction.
	RNA is heavily degraded	Re-isolate the RNA
	PCR under cycling	Repeat the 2nd PCR amplification, using two or three more PCR cycles.
2. Bands and back-ground smear are very intense	PCR over cycling	Repeat the 2nd PCR amplification, using two or three fewer PCR cycles.
3. Background smear is intense or short length fragments are visible	Low TCR RNA content in the initial RNA sample	Purify target library using AMPure XP beads or agarose gel purification.