HUMAN TCR RNA MULTIPLEX

TCR α and β repertoires with UMI

User Manual v.1.6

Cat. # THRM-001

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Kit overview

The **HUMAN TCR RNA MULTIPLEX** kit is designed for the amplification of TCR alpha and beta cDNA libraries using the combination of highly sensitive multiplex PCR and Unique Molecular Identifiers (UMIs). UMIs are introduced along with 1st strand cDNA synthesis and allow for advanced PCR and sequencing error correction, elimination of PCR biases, exact quantification of template cDNA molecules, and accurate normalization of samples for comparison of repertoire diversity metrics.

The kit includes a set of reagents sufficient to prepare 24 TCR alpha and 24 TCR beta cDNA libraries starting from 24 RNA samples.

The kit allows to start with RNA derived from 100 to 0.5 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-sequence-on-Illumina® libraries. Up to 24 samples can be processed in parallel.

1st PCR amplification starts from the same cDNA sample and is performed separately for TCR alpha and TCR beta chain repertoires. Sample barcodes are introduced in course of 2nd (indexing) PCR amplification that starts from the product of the 1st PCR (**Fig. 1**).

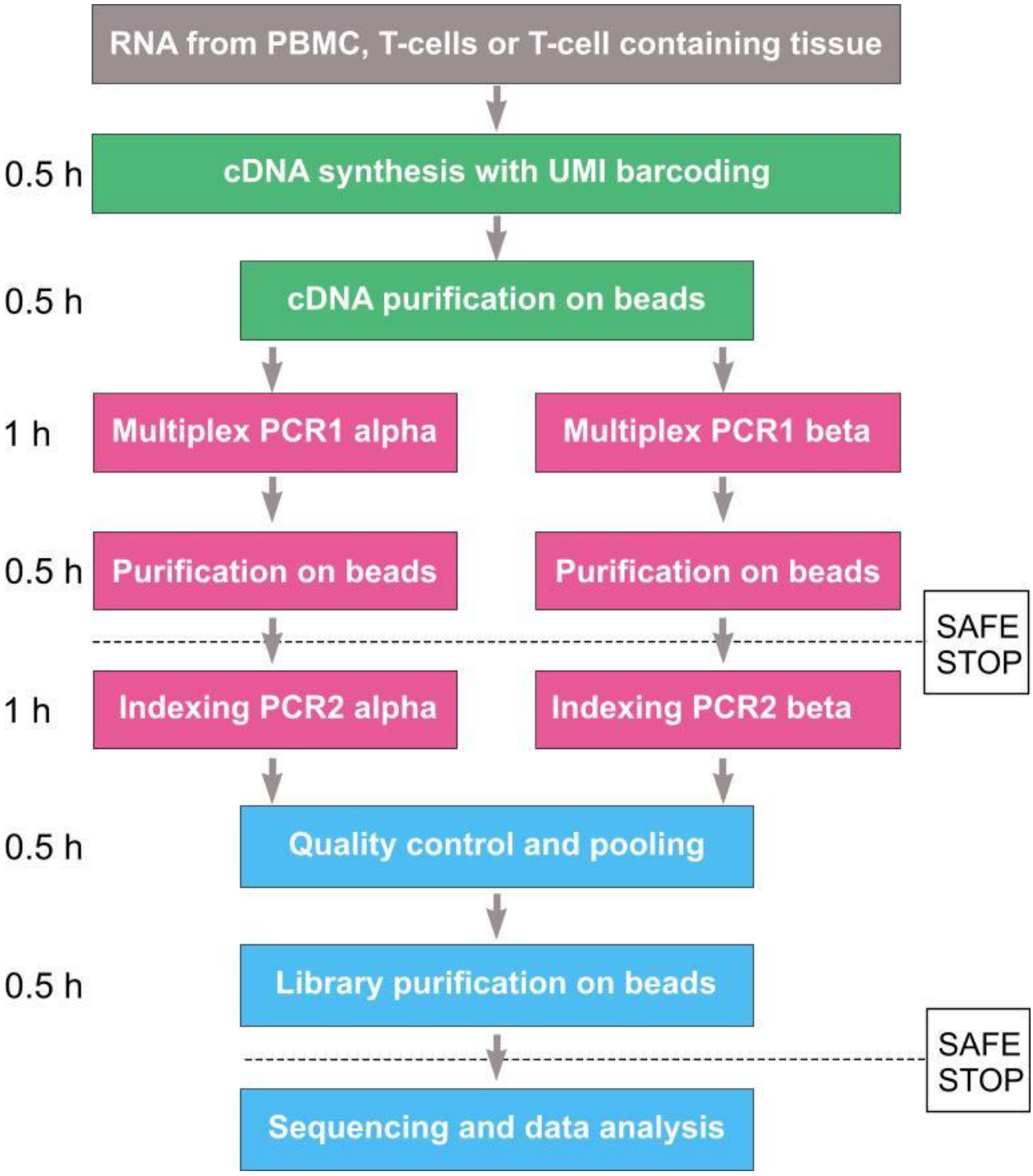

Figure 1. HUMAN TCR RNA MULTIPLEX kit pipeline.

Kit content

Table 1.

	Component	Volume (µl)	Description
1	TR Hum synt M	60 (after recovery)	Synthesis primer mix
2	TR-A Hum PCR1	60 (after recovery)	1st PCR primer mixes
3	TR-B Hum PCR1	60 (after recovery)	
4	TR-A Hum PCR2	30 (after recovery)	2nd PCR primer mixes
5	TR-B Hum PCR2	30 (after recovery)	
6	Control RNA	40 (after recovery)	Control RNA final concentration 5 ng/µl
7	Water	1 000 X 2	Deionized nuclease-free water
8	Binding Buffer	1 000	DNA resorption buffer on beads

Components 1-6 are shipped dried.

Transportation and storage until dissolved at RT.

Before use, add water and place the kit in the freezer (at –20°C).

Materials required but not Included

- SuperScript III™ (ThermoFisher Scientific, Invitrogen™, #18080044, supplied with reaction buffer and DTT)
- RNAsin® 40 U/µl (Promega, #N2515)
- dNTP mix (10mM each)
- Qiagen Multiplex PCR Plus kit (Qiagen, #206152)
- AMPure® XP Beads (Beckman Coulter, Inc. #A63880) or SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317)
- Freshly prepared 80% Ethanol (1200 µl per sample)
- Nuclease free water
- Elution Buffer (EB, 10 mM Tris-HCl, pH 8.0-8.5)
- 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 and 50 bp DNA Ladder (NEB, #N3236)
- Thermal Cycler
- Agilent Bioanalyzer/Tape Station
- Appropriate Magnetic Rack
- Low-speed benchtop Mini-centrifuge/vortex
- Qubit® fluorometer and Qubit dsDNA HS Assay Kit

General recommendations to prevent contamination

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

To prevent cross-sample contamination 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, pipets, 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 and the 1st PCR reaction setup in the pre-PCR workspace (Steps 1-8 and 10-12). After the 1st PCR amplification transfer the tubes to the post-PCR workspace and perform all other steps (9-19) except for steps 10-12.

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.

Store diluted primers at -20°C. RNA solution recommended to store at -70°C.

Starting material

See **Appendixes A** and **B** for our recommendations on RNA isolation.

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**).

High RNA quality is often critical for the efficient library preparation.

Large amounts of gDNA may significantly affect cDNA synthesis when starting from large (>200,000 cells) samples. It is recommended to perform DNAse treatment and additional purification of RNA before start.

Use Qubit® fluorometer or other fluorescent based method to determine RNA concentration.

For a small amount of starting material (100-50,000 T-cells), preferably, do not use DNAse and take all the RNA extracted from the sample of interest for cDNA synthesis.

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

Protocol

NOTE: Perform steps 1-8 and 10-11 in a pre-PCR box/room.

cDNA synthesis

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

Component	**Volume, µl**
RNA, up to 500 ng per reaction*	1-10**
TR Hum Synt	2
RNAse free water	0-9
Total volume	12 µl

***** For the control reaction, take 5 µl of control RNA (25 ng per reaction).

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

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

3. While incubating, prepare Master Mix of the fgollowing components in a final volume of 8 µl per reaction:

Component	**Volume, µl**
5X First-Strand Buffer (Thermo Fisher)	4
DTT (100 mM, Thermo Fisher)	1
dNTPs (10 mM each)	1
RNAsine®, (40 U/µl, Promega)	1
SuperScript III® Reverse Transcriptase (200 U/µl, Thermo Fisher)	1
Total volume	8 µl

NOTE: Add SuperScript III Reverse Transcriptase to the Master Mix just before use and mix gently by pipetting.

4. Add 8 µl of Master Mix to each reaction, pipette gently, spin briefly, and incubate for **30 minutes at 55°C**.

5. Purify the obtained cDNA synthesis products using **modified** AMPure® XP Beads protocol (Beckman Coulter) with **Binding Buffer** included in the kit, as follows:

- 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.
- Remove tubes from the magnetic rack. Add 10 µl of EB (10 mM Tris-HCl, pH 8.0-8.5) and mix well by pipetting, incubate for 1 minute at RT.
- Add 15 µl of **Binding Buffer** and mix well by pipetting.
- 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.
- 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 for elution 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 cDNA solution into a new tube.

  NOTE: During the elution steps, beads clumping may significantly reduce the cDNA 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 50°C for 10-30 minutes until complete dissolving.

1st PCR amplification

NOTE: At this point, the cDNA samples must be split and processed separately for TCR alpha and TCR beta libraries.

6. In a sterile tube, prepare two separate PCR1 Master Mixes in a final volume of 40 µl per reaction:

For **TCR alpha**

Component	**Amount, µl**
Nuclease free water	13
2x Multiplex PCR Master Mix (Qiagen)	25
TR-A hum PCR1	2
Total volume	40 µl

For **TCR beta**

Component	**Amount, µl**
Nuclease free water	13
2x Multiplex PCR Master Mix (Qiagen)	25
TR-B hum PCR1	2
Total volume	40 µl

7. Add 40 µl of PCR1 alpha or PCR1 beta Master Mix to each of 10 µl cDNA samples. Mix gently by pipetting.

8. Perform PCR using the following parameters:

95°C for 5 min

94°C for 30 s
60°C for 90 s
72°C for 30 s } **18-24 cycles ***

68°C for 4 min
4°C hold

* Choose the number of cycles depending on the amount of RNA/T-cell count at start (see **Table 2** below).

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

9. Purify the obtained PCR products using standard AMPure® XP Beads protocol (Beckman Coulter) with 1:1.5 sample:beads ratio:

- Homogenize beads by vortexing.
- Add 75 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 for elution 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 cDNA solution into a new tube.

SAFE STOP POINT: Purified product of the 1st PCR can be stored up to 1 month at −20°C.

2nd PCR amplification

10. 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 stocks PCR2-A and PCR2-B with nuclease-free water 1:9 according to the calculation. Discard diluted aliquots after use.

11. Prepare two separate PCR2 Master Mixes in a final volume of 22 µl per reaction without indexes:

For **TCR alpha**

Component	**Amount, µl**
Nuclease free water	8.5
Multiplex PCR Plus Mix (Qiagen)	12.5
TR-A hum PCR2 (diluted 1:9)	1
Total volume	22 µl

For **TCR beta**

Component	**Amount, µl**
Nuclease free water	8.5
Multiplex PCR Plus Mix (Qiagen)	12.5
TR-B hum PCR2 (diluted 1:9)	1
Total volume	22 µl

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

12. 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.

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

14. Add Index Primers:

14a. 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 unique Index for each reaction.

14b. 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.

15. Perform PCR using the following parameters:

95°C for 5 min

94°C for 30 s
55°C for 90 s
72°C for 30 s } **13-23 cycles ***

68°C for 4 min
4°C hold

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

Table 2. Approximate number of PCR cycles and sequencing reads.

Estimated count 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	0.1 ng – 0.5 ng	24	18-23	50-100 reads per input T cell*
5x10^2 -5x10^3	0.5 ng – 5 ng	24	14-20	
5x10^3 – 5x10^4	5 ng – 50 ng	21	13-16	
5x10^4 - 5x10^5	50 ng – 500 ng	18	13-16	

*Recommended coverage that allows for UMI-based elimination of amplification biases and accumulated errors. For activated T cells, preferably use 100 reads per input T cell.

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 30 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 proportions for sequencing. This allows obtaining an even coverage in terms of reads-per-cDNA (reads-per-UMI). For example, the library, which started from 50,000 T-cells may produce more PCR product than one which started with 20,000 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.

16. Verify quality of the obtained PCR product on the agarose gel (1.5%) next to the DNA ladder, or by capillary electrophoresis (e.g. BIOANALYZER® or TapeStation®, Agilent Technologies). The library should have a peak at approximately 330-370 bp and 360-400 bp for TCR alpha and beta chains, respectively (**Fig. 2**).

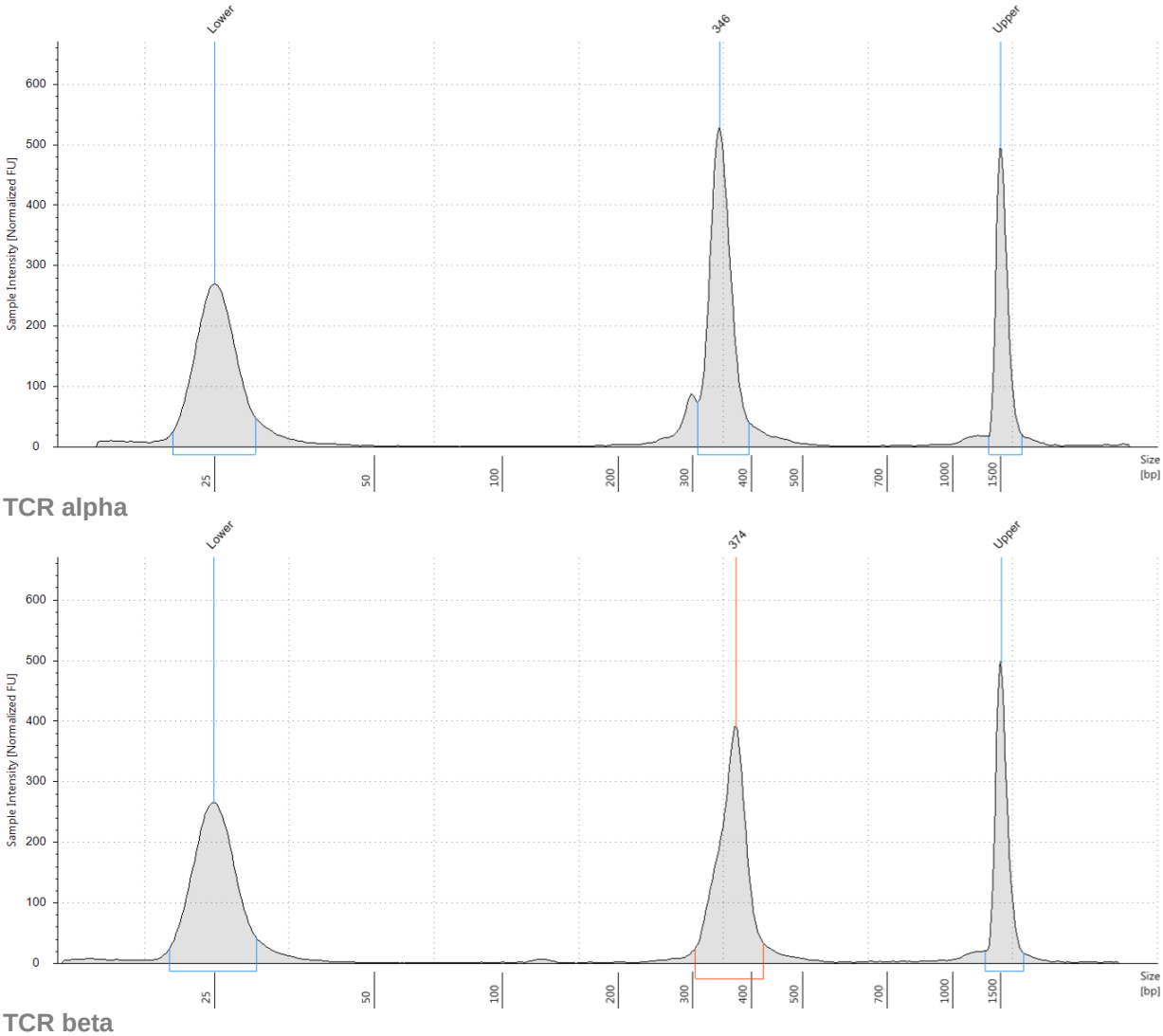

Fig 2. A typical library peak for TCR alpha and beta chains. The shape and length distribution of a TCR library may vary depending on the repertoire composition.

17. Pool the obtained libraries separately for TCR alpha and TCR beta. To do this, combine equal volume portions from each individual sample after the 2nd PCR.

If 1-4 libraries are prepared at the same time, the whole reaction volumes of the 2nd PCRs can be pooled. If there are many samples, pool 5-10 µl from each 2nd PCR.

The total amount of pooled PCR products should be at least 50 ng.

18. Purify the obtained PCR products using standard AMPure® XP Beads protocol (Beckman Coulter) with 1:1 sample:beads ratio:

- Homogenize beads by vortexing.
- Add volume of beads equal to pooled DNA 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 for elution 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 cDNA solution into a new tube.

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

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

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

SAFE STOP POINT: Store purified libraries at -20ºC.

Sequencing recommendations

The obtained purified pooled library is ready for Illumina sequencing.

Determine library concentration according to the Illumina recommendations.

Spike with at least 15% of PhiX or another random library (e.g. RNA-Seq, Exome-Seq etc).

Recommended coverage that allows for optimal UMI-based elimination of amplification biases and accumulated errors: 50-100 sequencing reads per input T cell.

Analyze the resulting pooled library using at least 150+150 paired end Illumina sequencing with standard sequencing primers. For comparative analysis of the obtained TCR repertoire data, use the same sequencing platform. Ideally, use the same sequencing run for the samples under comparison, or mix control and experimental samples in order to minimize batch effects.

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

Data analysis

20. Extract repertoires using MiXCR™ software, a universal tool for fast and accurate analysis of T- and B- cell receptor repertoire sequencing data.

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.

21. 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.

It is preferable to use freshly isolated cell sample. In the case of frozen samples, culture thawed cells overnight in RPMI-1640 supplemented with 10% human serum.

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 extracted RNA, using QuBit and 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 cells) it is not necessary to check quantity and quality of 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 procedure to increase the yield of isolated RNA. Carrier RNA will not interfere with primers for cDNA synthesis reaction.

Purified RNA can be stored in water or in 75% ethanol at -70°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:

1. PBMC

Perform isolation of mononuclear cells from whole blood using Ficoll Paque density gradient centrifugation. Spin down the cells at 350g for 15 minutes, 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 number of cells (> 200,000), when using a column-based RNA extraction method, DNAse treatment is recommended. gDNA eliminator columns (QIAGEN) can also be used for DNA removal.

2. 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 small samples (100-10,000 cells), add 4 volumes of TRIzol® to RLT® cellular lysate and extract RNA using TRIzol® protocol (**Appendix B**).

3. 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 minutes, 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 (> 200,000), when using a column-based RNA extraction method, DNAse treatment is recommended. gDNA eliminator columns (QIAGEN) can also be used for DNA removal.

4. T cell-containing tissue

Homogenize fresh tissue. Ideally, obtain a single cell suspension using incubation with DNAse and proteases mixes (such as Liberase™ TL from Roche). 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 minutes at room temperature. Add 1/5 volume of chloroform and mix well by vortexing. Incubate tubes at room temperature for 3 minutes.
- Centrifuge the samples at > 10,000 g for 10 minutes 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 interphase 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 minutes and centrifuge at > 10,000 x g for 10 minutes 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.

I. 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 minutes 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.

II. 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 minutes 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 24% ethanol, mix well by vortexing. Store at -70°C.

Troubleshooting

Problem	Possible reason	Possible solution
Low TR-A/TR-B PCR product yield or no TR-A/TR-B 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, re-isolate the RNA
	RNA is heavily degraded	Re-isolate the RNA
	PCR undercycling	Repeat the 2nd PCR amplification, using two or three more PCR cycles. If this does not help, repeat the cDNA synthesis reaction and the 1st PCR reaction using three more PCR cycles.
TR-A/TR-B bands and background smear are very intense	PCR overcycling	Repeat the 2nd PCR amplification, using two or three fewer PCR cycles
TR-A/TR-B bands are present but background smear is intense or alternative length fragments are visible	Low TCR RNA content in the initial RNA sample	Purify target library using AMPure XP beads and size selection protocol or agarose gel purification