MI
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HUMAN α/β TCR DNA KIT

User Manual v.3.0

Cat. # THABD-003
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TABLE OF CONTENTS

KIT OVERVIEW

The Human α/β TCR DNA Kit enables highly efficient amplification of rearranged T-cell receptor (TCR) alpha and beta genes using human genomic DNA as a template. The kit utilizes multiplex PCR with gene-specific primers targeting TCR joining (J) and variable (V) regions. All primers and reaction conditions are selected in a way that minimizes amplification bias and allows for accurate and sensitive TCR clonotypes diversity assessment.

The kit allows to start with DNA derived from 1,000 to 50,000 sorted/purified T cells, from peripheral blood leukocytes (PMBC), or from T cell-containing tissues (fresh-frozen or formalin-fixed paraffin-embedded).

The amplification of TCR alpha and TCR beta chains is performed simultaneously in the first PCR reaction. (see **Figure 1**). Second PCR amplification is used to introduce Illumina sample barcodes (Nextera Unique Dual Indexes) and oligonucleotides necessary for sequencing. The use of Unique Dual Indexes (UDI) significantly decreases cross-sample contamination resulting from “barcode hopping” effect. It is possible to use standard Nextera XT indexing primers however the level of cross-sample contamination will be high (up to 1%).

Obtained PCR products can be sequenced on any Illumina platform. Sequences allow reconstructing repertoire of alpha/beta T-cell receptors in a sample of interest.

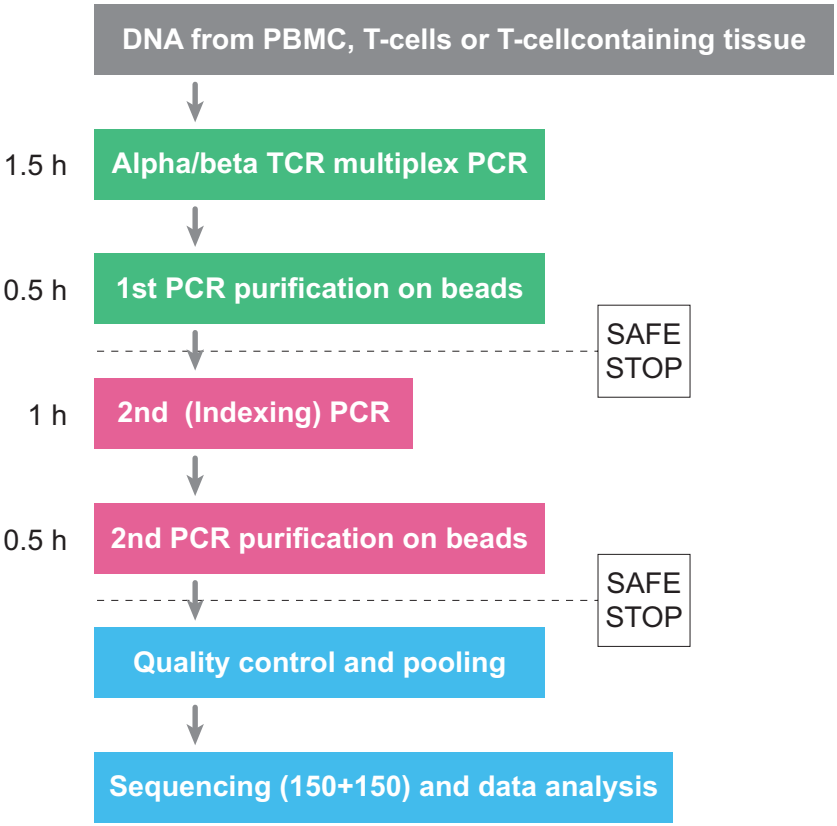

Figure 1. Human α/β TCR DNA kit pipeline.

KIT CONTENTS

The kit includes a set of reagents sufficient to prepare 96 TCR alpha/beta libraries starting from 96 DNA samples.

Name	Water volume to add (µl)
TR hum DNA PCR1	500
Control DNA	40
Nuclease free water	–

Store all components at –20°C

MATERIALS REQUIRED

- ☐ Thermal Cycler with heated lid and adjustable **ramp rate**
- ☐ Appropriate magnetic rack
- ☐ Low-speed benchtop Mini-centrifuge/vortex
- ☐ Agilent Bioanalyzer/Tape Station or agarose gel electrophoresis system
- ☐ Qubit® fluorometer with Qubit dsDNA High Sensitivity Assay Kit (TermoFischer, #Q32854)
- ☐ AMPure® XP Beads (Beckman Coulter, Inc. #A63881) or SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317)
- ☐ Freshly prepared 80% Ethanol
- ☐ EB (Elution Buffer, 10 mM Tris HCl, pH 8.0-8.5).
- ☐ dNTP mix (10mM each)
- ☐ Nuclease free water
- ☐ **Qiagen** HotStarTaq Plus DNA Polymerase (Cat# 203605) with 10x PCR Buffer
- ☐ 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)

GENERAL RECOMMENDATIONS TO PREVENT CONTAMINATION

General recommendations to lower the risk of DNA degradation and contamination should be implemented, such as using labcoats, gloves, tips with aerosol filters, certified DNA and DNAse free reagents.

NOTE: Performing non-template control reactions is strongly recommended for each experiment.

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, 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, DNA purification, and the 1st PCR preparation in the pre-PCR workspace (Steps 1-3). After the 1st PCR amplification transfer the tubes to the post-PCR workspace and perform all the other steps.

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

BEFORE YOU START

Prepare the first PCR primer mix. Add **500 µl** of nuclease free water (provided) to **TR hum DNA PCR1** and mix well by vortexing. Store primer mix at –20°C.

NOTE: Primer mix is stable under these conditions for at least one year. Repeated freeze-thaw cycles should be avoided since they may lead to primer degradation and decreased assay performance.

Add **40 µl** of nuclease free water (provided) to **Control DNA** and mix well by vortexing. Briefly spin the tube to drop down all the remaining liquid and incubate at 65°C for 15 minutes. Vortex well and spin down.

Store Control DNA at –20°C.

STARTING MATERIAL

Primers for TCR alpha and beta amplification are designed in a way that enables work even with partially degraded DNA templates. However, residual traces of proteins, salts or other contaminants (e.g. phenol, ethanol) may significantly decrease the efficiency of PCR reaction. Sample purity can be checked with spectrophotometer (e.g. Nanodrop). Perform additional sample purification if needed.

Minimal number of cells for the protocol is 1,000 sorted T-cells.

Amplification efficiency may be poor for tissues containing low number of T-cells. Do not put more than 300 ng of template DNA in one reaction. For comprehensive analysis of TCR repertoire of T-cell containing tissue sample perform multiple parallel reactions (containing 300 ng of template DNA each) with all isolated DNA.

Success of library preparation with DNA isolated from formalin-fixed paraffin-embedded (FFPE) samples depends on fixation protocol and quality of isolated DNA. Longer DNA will give more different TCRs.

PROTOCOL

NOTE: Perform steps 1–3 in pre-PCR box/room.

First PCR

1. In sterile reaction tube(s)/strip(s)/plate, mix the following reagents in a final reaction volume of 50 µl.

0–36,5 µl	Nuclease free water
5 µl	PCR Buffer (10x)
1 µl	dNTP (10mM each)
5 µl	TR hum DNA PCR1
2,5 µl	HotStarTaq Plus DNA Polymerase (5U/µl)
Up to 36,5 µl	DNA (5–300 ng)*
50 µl	Total

* For positive control reaction take 10 µl of Control DNA

2. Perform PCR with **Ramp set to a specific value for each step** using the following parameters:

94 °C	3 min (Ramp 4°C/s)	1
94 °C	20 s (Ramp 4°C/s)	8
57 °C	90 s (Ramp 0.5°C/s)	
72 °C	40 s (Ramp 0.5°C/s)	
94 °C	20 s (Ramp 4°C/s)	17
74 °C	80 s (Ramp 0.5°C/s)	

3. 15-20 minutes before the end of the 1st PCR reaction prepare the master mix for the second **indexing PCR** reaction.

16.2 µl	Nuclease free water
2 µl	PCR Buffer (10x)
0.4 µl	dNTP (10mM each)
0.4 µl	HotStarTaq Plus DNA Polymerase (5U/µl)
19 µl	Total

Keep the prepared master mix on ice or at +4°C

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

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

- Homogenize AMPure® XP Beads by vortexing.
- Transfer 10 µl of each of 1st PCR product to a new tube(s)/ strip(s)/plate.
- Add 10 µl of AMPure® XP Beads 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 1–5 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 centrifuge and put back on the magnetic rack. Remove all the residual ethanol using P10 pipette and tip.

NOTE: It is important to remove all the traces of ethanol as it could inhibit subsequent PCR reaction.

– Briefly dry the beads for 1 minute.

NOTE: It is important not to overdry the beads.

– Remove tubes from the magnetic rack. Add 19 µl of prepared master mix (from **step 3**) to each tube directly to the beads and mix well by pipetting. Ensure complete dissociation of the beads.

5a. 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 sample.

5b. 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 sample.

• Second (Indexing) PCR

6. Perform PCR using the following parameters (ramp for all stages set at maximum):

94 °C	3 min	1
94 °C	20 s	
55 °C	20 s	15–21*,**
72 °C	40 s	

* Depending on type and amount of starting material
** For control DNA we recommend 15 cycles

NOTE: The optimal number of 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) 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-DNA molecule. For example, the library, which started from 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 DNA molecules, and thus requires more sequencing reads to achieve a comparable coverage.

7a. Purify the obtained PCR products using AMPure® XP Beads (Beckman Coulter) according to the standard manufacturer protocol. Use 1:0.8 sample:AMPure® XP Beads ratio. Elute in 20 µl EB (10 mM Tris HCl, pH 8.0–8.5).

Determine the concentration of each library using Qubit fluorometer and Qubit dsDNA High Sensitivity Assay Kit. Verify the quality of the obtained PCR products by analyzing an aliquot of the sample alongside a DNA ladder on the agarose gel electrophoresis or Agilent Bioanalyzer/Tape Station. The library should have a size of approximately 400 bp. Pool libraries using equal volumes from each sample or equal number of nanograms.

7b. Recommended if starting from samples of similar nature/ cell amount.

Aliquot 5 µl of each of the second PCR product in a separate tube and mix well by vortexing. Take up to 100 µl of the mix and purify it using AMPure® XP Beads (Beckman Coulter) according to the standard manufacturer protocol. Use 1:0.8 sample:AMPure® XP Beads ratio. Elute in 20 µl EB (10 mM Tris HCl, pH 8.0–8.5).

Determine the concentration of the purified library pool using Qubit fluorometer and Qubit dsDNA High Sensitivity Assay Kit. Verify the quality of the obtained PCR product by analyzing an aliquot of the sample alongside a DNA ladder on the agarose gel electrophoresis or Agilent Bioanalyzer/Tape Station. The library should have a size of approximately 400 bp.

Sequencing recommendations

The obtained purified pooled library is ready for Illumina sequencing.

Use qPCR and Qubit to determine the library concentration according to the Illumina QC recommendations.

We recommend 150+150 nt paired end Illumina sequencing with standard Illumina® sequencing primers. Sequencing 150+150 nt is sufficient to cover the CDR3 region and a part of V gene that ensure correct V-gene identification. For accurate comparative analysis of the obtained TCR repertoire data, use the same sequencing platform and preferably use the same sequencing run for all the samples under comparison.

DATA ANALYSIS

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