EXPERIMENTAL PROTOCOL: Sequencing of peripheral blood T cell receptor beta chain (TCR-β)

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Introduction

The intended use of this protocol is the characterization of the T-cell repertoire, by using the Oncomine™ TCR Beta-SR Assay (RNA) (ThermoFisher; Cat. No. A39359), following the instructions of the manufacturer (MAN0017438, Revision C.0).
The Oncomine™ TCR Beta-SR Assay enables the characterization of the TCR-β chain by sequencing the complementarity-determining region 3 (CDR3), which allows studying the T-cell repertoire diversity and clonal expansion. Although this assay can be performed using both RNA or DNA, we use RNA as input.

Reagents

- Oncomine™ TCR Beta-SR Assay (RNA) (Ref. A39359), which includes:
  - Oncomine TCR BETA-SR Assay RNA Panel (Part No. A39163)
  - Ion AmpliSeq Library Kit Plus (Ref. 4488990)
- Ion Torrent™ Dual Barcode Kit 1-96 (Ref. A39360)
- Ion Library TaqMan™ Quantitation Kit (Ref. 4468802)
- SuperScript™ VILO™ Master Mix (Ref. 11756050)
- MagMAX mirVana Total RNA Isolation Kit (RNA only) (Ref. A27828)
- Agencourt™ AMPure™ XP Kit (Ref. A63880)
- Ion 530™ Chip kit (Ref. A27764)
- Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (Ref. A34461)
- Qubit™ RNA HS Assay Kit (Ref. Q32852)
- Agilent™ RNA 6000 Nano Kit (Ref. 5067-1511)
- Absolute ethanol
- Nuclease-free Water

Instruments and tools

- Centrifuge for blood tubes, 1.5mL tubes, and 96-well plates

- Thermal cycler, such as Veriti™ 96-Well Thermal Cycler
- Real-time PCR instrument, such as LightCycler® 480 System
- 384 well PCR plates
- 96 well PCR plates
- PCR plate cover
- Nonstick, RNase-free Microfuge Tubes, 1.5 mL
- DynaMag™-96 Side Magnet (Ref. 12331D)
- DynaMag™-2 Magnet (Ref. 12321D)
- Ion Chef™ (Ref. 4484177)
- Ion GeneStudio™ S5 Series Sequencer (Ref. A38194)
- QUBIT ASSAY TUBES (Ref. Q32856)
- Qubit™ 4 Fluorometer (Ref. Q33226)
- Agilent™ 2100 Bioanalyzer™ (Ref. G2939BA)

Before start

Peripheral blood was collected in EDTA tubes. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Paque Plus (GE Healthcare) and stored in RNAlater (Thermo Fisher Scientific) at -80ºC until further processing (see Ficoll protocol in this section).

Total RNA was isolated from PBMCs using the MagMAX mirVana Total RNA Isolation Kit (Thermo Fisher Scientific) and quantified through the Qubit RNA HS Assay Kit (Thermo Fisher Scientific).

As mentioned in the introduction, quality of the RNA should be measured prior to sequencing. The Agilent RNA 6000 Pico Assay (Agilent Technologies), was used to determine the RNA integrity number (RIN) in the Agilent 2100 Bioanalyzer. A RIN value greater or equal to 7 is recommended to follow subsequent TCR-β sequencing.

Of note, it is also recommended to do the *CD3 sample qualification assay*, while setting up this protocol, or for troubleshooting when needed (pages 132-136 of the manufacturer's manual, MAN0017438). This additional step consists of measuring the T-cell content of the samples, and is useful to adjust the RNA that should be used as input for sequencing.

It is also worth mentioning that in case two samples of the same patient (e.g. before and after treatment) are analyzed, it is recommended to sequence both of them in the same sequencing run.

There is also a similar assay, the Oncomine™ TCR Beta-LR Assay, which allows identification of variable gene allele-specific polymorphisms, by sequencing all complementarity-determining regions of the variable gene (CDR1, 2, and 3).

Nevertheless, it should be taken into account that while the Oncomine™ TCR Beta-SR Assay can be used with samples from peripheral blood of FFPE tissue, the Oncomine™ TCR Beta-LR Assay requires high quality RNA samples as input, as the amplicons generated by this assay consist of 330bp.

PROCEDURE

Brief description of the protocol: In this case, 25ng of RNA isolated from PBMCs were used as input. First of all RNA was retrotranscribed into cDNA. Afterwards, TCR-β libraries were prepared from 25 ng cDNA of each sample with the Oncomine TCR Beta-SR (RNA) Assays (Thermo Fisher Scientific) according to the manufacturer's instructions, which are specified below. The last step consisted of combining eight libraries diluted to 25 pM, which were introduced in the Ion Chef™ and sequenced on Ion GeneStudio™ S5 Series Sequencer using Ion 530™ Chips. Analyses were performed using Torrent Suite™ and Ion Reporter™ Softwares.

Procedure specified step by step:
Note: a longer version can be found in pages 33-40 of the manufacturer's manual, (MAN0017438).

1. **Reverse-transcribe RNA into cDNA**:
   i. First, add the following components into each well of a 96-well PCR plate:

Component	Volume
SuperScript™ IV VILO™ Master Mix	2 µL
Total RNA (25 ng to 2.5 µg)[1,2]	≤8 µL
Nuclease-free Water	to 10 µL
Total volume per well	**10 µL**

[1] Substitute an equal volume of nuclease-free water or Low TE to prepare a no-template control (NTC).
[2] To determine the minimum sample input see Appendix B, "Sample qualification and quantitation".

Note: Perform this step on ice.

   ii. Seal the plate, vortex thoroughly and briefly centrifuge.
   iii. Load the plate in a thermal cycler and run the following program:

Temperature	Time
25°C	10 minutes
50°C	10 minutes
85°C	5 minutes
10°C	Hold

   iv. Remove the plate from the thermal cycler and briefly centrifuge it.

Note: Samples can be stored at 10ºC for up to 16 hours in the thermal cycler, or -20ºC for long term-storage (upon transfer of the cDNA to RNase-free microcentrifuge tubes).

2. **Prepare target amplification reactions - TCRβ - SR – RNA:**

i. Remove the seal from the plate and add the following components (upon preparation a master mix according to the number of samples included) to each 10-µL cDNA synthesis reaction.

Note: Perform this step on ice.

Component	Volume
cDNA (from reverse transcription)	10 µL
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
5X Oncomine™ TCR Beta-SR Panel	4 µL
Nuclease-Free Water	2 µL
Total volume per well (includes 10 µL from cDNA synthesis)	**~20 µL**

ii. Seal the plate, vortex thoroughly and briefly centrifuge.

iii. Load the plate in a thermal cycler and run the following program:

Stage	Step	Temperature	Time
Hold	Activate the enzyme	95°C	2 minutes
20 cycles[1]	Denature	95°C	30 seconds
	Anneal	60°C	45 seconds
	Extend	72°C	45 seconds
Hold	Final extension	72°C	10 minutes
Hold	—	10°C	Hold

[1] Adjust cycle number according to the following table to accommodate variable sample input amounts. For more information, see Appendix D, "General sample input and sequencing depth guidelines".

Amount of cDNA starting material	Recommended adjustment to cycle number
2.5 ng	+3
25 ng to 2 µg	0

Note: Cycle number can be adjusted depending on the input material quality or quantity. Increase number by +3 when starting from FFPE samples. It should also be taken into account that input across all samples should be equivalent, so the cycle number is optimal for all the samples in the run.

iv. Remove the plate from the thermal cycler and briefly centrifuge it.

Note: Target amplification reactions can be stored at 10ºC overnight in the thermal cycler, or -20ºC for longer periods.

3. Partially digested amplicons:

Note: Perform this step on ice.

i. Remove the seal from the plate and add 2 µL of FuPa Reagent (which should also be kept in ice) to each amplified sample.

ii. Seal the plate, vortex thoroughly and briefly centrifuge.

iii. Load the plate in a thermal cycler and run the following program:

Temperature	Time
50°C	10 min
55°C	10 min
60°C	20 min
10°C	Hold (for up to 1 hour)

Note1: the final volume is 22µL.
Note2: after this step samples cannot be stored overnight, it is mandatory to proceed immediately to the next step.

iv. Remove the plate from the thermal cycler and briefly centrifuge it.

4. Ligate adapters to the amplicons and purify:

i. Briefly centrifuge the plate with the partially digested amplicons, as well as the plate of the dual barcode adapters to collect the contents.

ii. Remove the seal from the plate containing the partially digested amplicons and add the following components to each well in the order listed below:

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	Ion Torrent™ Dual Barcode Kit 1–96 adapters	2 µL
3	DNA Ligase (blue cap)	2 µL
—	**Total volume** (including ~22 µL of digested amplicon)	**~30 µL**

Note1: All the samples sequenced in the same chip should contain a unique barcode .Therefore, a different adapter must be ligated to each library.
Note 2: To prevent cross-contamination of the barcode adapters, only the foil seal from the wells that are going to be used should be removed, and if should be resealed after used.
Note3: There might be a precipitate in the Switch Solution tube, in this case, resuspend by vortexing or pipeting at room temperature.
Note 4: Add the DNA Ligase last. Do not combine DNA ligase and barcode adapters before adding to the digested amplicons.

iii. Seal the plate, vortex thoroughly and briefly centrifuge.

iv. Load the plate in a thermal cycler and run the following program:

Temperature	Time
22°C	30 minutes
68°C	5 minutes
72°C	5 minutes
10°C	Hold (for up to 1 hour)

Note: Samples can be stored at -20ºC for longer periods.

5. **Purify the library**:
   Note1: Bring Agencourt™ AMPure™ XP Reagent to room temperature (30 minutes before its use) and vortex thoroughly to disperse the beads before use.
   Note2: Prepare 70% ethanol (350 µL x # of samples) fresh daily.
   i. Briefly centrifuge the plate and remove the seal.
   ii. Add 45 µL (1.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
   Note: Ensure that the mixture in each well is homogeneous.
   iii. Incubate the mixture for 5 minutes at room temperature.
   iv. Place the plate in a magnetic rack such as the DynaMag™-96 Side Magnet, then incubate for 2 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.
   v. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet to wash the beads. Carefully remove, then discard the supernatant without disturbing the pellet.
   vi. Repeat step v for a second wash.
   vii. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes. Do not overdry.
   Note1: Do not overdry. When beads are wet, they shine. When beads are too dry, they start to crack.
   Note2: Residual ethanol inhibits library amplification. If needed, centrifuge the plate and remove remaining ethanol before air-drying the beads.
   Note3: Under conditions of low relative humidity, the beads air-dry rapidly. Do not overdry. In our case it takes a little longer.

6. **Elute the library**:
   i. Remove the plate with purified libraries from the plate magnet, then add 50 µL Low TE to the pellet to disperse the beads.
   ii. Seal the plate, vortex thoroughly and briefly centrifuge.
   iii. Incubate at room temperature for at least 2 minutes.
   iv. Place the plate on the magnet for at least 2 minutes.
   v. Transfer the supernatant (containing the purified libraries) to microtubes or to new wells on the same plate.
   Note: Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at –20°C. We recommend transferring the supernatant to an RNase-free microcentrifuge tube for long-term storage. Alternatively, seal the plate with a new MicroAmp™ Clear Adhesive Film for long-term storage.

7. **Quantify the library by qPCR and calculate the dilution factor**:
   i. Prepare a 100-fold dilution for quantification, by combining 2 µL of the library with 198 µL of Nuclease-free Water.
   ii. Prepare three 10-fold serial dilutions of the *E. coli* DH10B Ion Control Library (~68 pM, from the Ion Library TaqMan™ Quantitation Kit) at 6.8 pM, 0.68 pM, and 0.068 pM, which will be standards that should be indicated in the qPCR software.
   iii. Prepare a PCR reaction mix for duplicate reactions of each library sample, standard and NTC using the following table.
   Note: include a 5-10% overage to accommodate pipetting errors.

Component	Volume per reaction	
	96-well plate	384-well plate
Ion Library qPCR Master Mix, 2X	10 µL	5 µL
Ion Library TaqMan™ Quantitation Assay, 20X	1 µL	0.5 µL
Total	**11 µL**	**5.5 µL**

   iv. In a MicroAmp™ Optical Reaction Plate, set up duplicate PCR reactions for each sample, standard, and NTC. Add the following components to each well.

Component	Volume per reaction	
	96-well plate	384-well plate
PCR Reaction Mix	11 µL	5.5 µL
1:100 dilution of the sample[1]	9 µL	4.5 µL

[1] Substitute E. coli DH10B standards prepared in step 1 for standards. Substitute nuclease-free water for NTC.

   v. Seal the plate with a MicroAmp™ Optical Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
   vi. Run the reaction in the real-time instrument using the following programs:
   *Note: The fast cycling program was developed using the StepOnePlus™ Real- Time PCR System in **Fast** mode.*
      a. Enter the concentrations of the control library standards.
      b. Select ROX™ Reference Dye as the passive reference dye.
      c. Select a reaction volume of 20 µL.
      d. Select FAM™ dye/MGB as the TaqMan™ probe reporter/quencher.

IMPORTANT! We recommend using standard cycling conditions for the Oncomine™ Immune Repertoire Panels. Fast cycling can result in inaccurate quantification.

Reaction plate format	Run mode	Stage	Temperature	Time
96-well Standard OR 384-well Standard	Standard	Hold (UDG incubation)	50°C	2 min
		Hold (polymerase activation)	95°C	2 min
		Cycle (40 cycles)	95°C	15 sec
			60°C	1 min
48- / 96-well Fast OR 384-well Standard	Fast	Hold (UDG incubation)	50°C	2 min
		Hold (polymerase activation)	95°C	20 sec
		Cycle (40 cycles)	95°C	1 sec
			60°C	20 sec

vii. Following qPCR, calculate the average concentration of the undiluted library by multiplying the determined concentration × 100.

viii. Based on the calculated library concentration, determine the dilution that results in a concentration of ~25 pM for template preparation on the Ion Chef™ System.

Note1: Libraries typically have yields of 50–500 pM. However, yield is not indicative of library quality. After quantification, determine the dilution factor that results in a concentration of ~25 pM.

Note2: for libraries with low yield, usually from samples with low quality or quantity, a library amplification can be performed immediately following purification before elution, following steps on pages 38-40 of the manufacturer's manual (MAN0017438).

8. **Combine libraries before templating**:

Equal volumes of each library can be combined in order to sequence multiple barcoded libraries on a single chip, as indicated in the following table:

Ion sequencing chip	Suggested number of libraries (X)	
	SR libraries	LR libraries
Ion 520™ Chip	4	4
Ion 530™ Chip	8	8
Ion 540™ Chip	8–32[1]	Not recommended
Ion 550™ Chip	12–48	Not recommended

[1] Recommended for libraries prepared from 25 ng of PBL RNA. Libraries prepared from less material or DNA are often smaller and do not require as high sequencing depth. For more information, see page 154.

Note: Do NOT combine libraries that are prepared with different barcode adapter sets (for example, do not mix Ion Torrent™ Dual Barcode Kit 1–96 adapter libraries with Ion Select barcode adapter libraries).

i. Dilute all individual barcoded libraries to 25 pM concentration (50pM if using an Ion 550™ Chip).
ii. Combine 10 µL of each library in a single 1.5mL microtube.
iii. After adding all the libraries, mix by pipetting up and down 5 times and briefly centrifuge.
Note: Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at –20°C.

9. After library preparation, proceed to **template preparation and sequencing** using the following kits:

Chip	Template System	Sequencing System	Kit	User Guide
LR libraries				
Ion 520™ Chip Ion 530™ Chip	Ion Chef™ System	Ion GeneStudio™ S5 Series System	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (Cat. No. A34019)	*Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide* (Pub. No. MAN0016854)
SR libraries				
Ion 520™ Chip Ion 530™ Chip	Ion Chef™ System	Ion GeneStudio™ S5 Series System	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (Cat. No. A34461)	*Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide* (Pub. No. MAN0016854)
Ion 540™ Chip			Ion 540™ Kit – Chef (Cat. No. A27759)	*Ion 540™ Kit – Chef User Guide* (Pub. No. MAN0010851)
Ion 550™ Chip		Ion GeneStudio™ S5 Plus System or Ion GeneStudio™ S5 Prime System	Ion 550™ Kit – Chef (Cat. No. A34541)	*Ion 550™ Kit – Chef User Guide* (Pub. No. MAN0017275)

Note: In our case, we use Ion 520™ Chips or Ion 530™ Chips, with the Ion 510™ & Ion 520™ & Ion 530™ Kit-Chef, following instructions of the Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef USER GUIDE (MAN0016854).

i. Prepare a specific Run Plan, following instructions on pages 76-78 of the manufacturer's manual, (MAN0017438).
ii. Charge the Ion Chef™ templating, following instructions on pages 23-43 of the manufacturer's manual, (MAN0016854).
iii. Initialize the sequencer, following instructions on pages 46-48 of the manufacturer's manual, (MAN0016854).
iv. Sequence on the Ion S5 GeneStudio System. following instructions on pages 50-51 of the manufacturer's manual, (MAN0016854).
v. Analyze the data using Ion Reporter™ Software (following instructions on pages 79-104 of the manufacturer's manual, MAN0017438).