HUMAN IG/TCR DNA MULTIPLEX 7GENES

Simultaneous profiling of human TRA, TRB, TRG, TRD, IGH, IGK, IGL repertoires on the DNA level in a single assay

User Manual v.1.7

Cat. # 7GHD-001

96 samples

MiLaboratories Inc

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KIT OVERVIEW

The **HUMAN IG/TCR DNA MULTIPLEX** kit (7GENES) is designed for the simultaneous repertoire profiling of all human adaptive immune receptor genes (immune adaptome), including TRA, TRB, TRG, TRD, IGH, IGK, and IGL, in a single assay, starting from DNA . The kit contains a multiplex primer set covering complete (all functional and rearrangeable non-functional) and incomplete V(D)J rearrangements of IG and TCR loci. The primer structures, concentrations, and other PCR conditions have been adjusted to minimize off-target amplification and quantitative bias, allowing accurate and sensitive IG and TCR clonotypes diversity assessment.

Table 1. The number of V, D, and J-segments detected in actual blood samples by 7GENES kit.

Locus	V-segments	D-segments (as part of incomplete rearrangements)	J-segments
TRA (14q11.2)	**47**(45/16)*	–	**59**(51/10)
TRB (7q34)	**56**(48/20)	**2**(2/0)	**13**(13/1)
TRG (7p14)	**12**(6/9)	–	**5**(5/0)
TRD (14q11.2)	**8**(8/0)	**2**(3/0)	**4**(4/0)
IGH (14q32.33)	**63**(57/105)	**27**(23/14)	**6**(6/3)
IGK (2p11.2)	**46**(41/37)	–	**5**(5/0)
IGL (22q11.2)	**44**(33/48)	–	**5**(5/6)

* The numbers in brackets indicate the numbers of functional/non-functional IG/TCR V, D, and J-segments in the genome according to the IMGT database.

DNA (or cDNA) obtained from any kind of source, including whole blood (venous and capillary), PBMC, separated B and T cell subsets, FFPE blocks (depending on DNA fragmentation), bone marrow, tissue biopsies, and tumor fragments can be used as input material. The 7GENES kit applies for lymphoid malignancy clonality detection and the conventional MRD (minimal residual disease) monitoring approach. The libraries obtained using the 7GENES kit can be sequenced on any Illumina instruments in PE150 or SE150 mode.

The kit is designed for 96 IG/TCR libraries starting from 96 DNA samples.

The kit allows to start with DNA derived from 25 to 25 000 sorted/purified T and B cells (per 25 µl assay) from peripheral blood leukocytes or T and B cell-containing tissues and

produces indexed ready-to-sequence-on-Illumina® libraries. Up to 96 samples can be processed in parallel.

IG and TCR loci target amplification is performed simultaneously in the first (multiplex) PCR. The second PCR amplification is used to introduce Illumina sample barcodes (Nextera Unique Dual Indexes) and oligonucleotides necessary for sequencing. Using Unique Dual Indexes (UDI) significantly decreases cross-sample contamination resulting from the “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%).

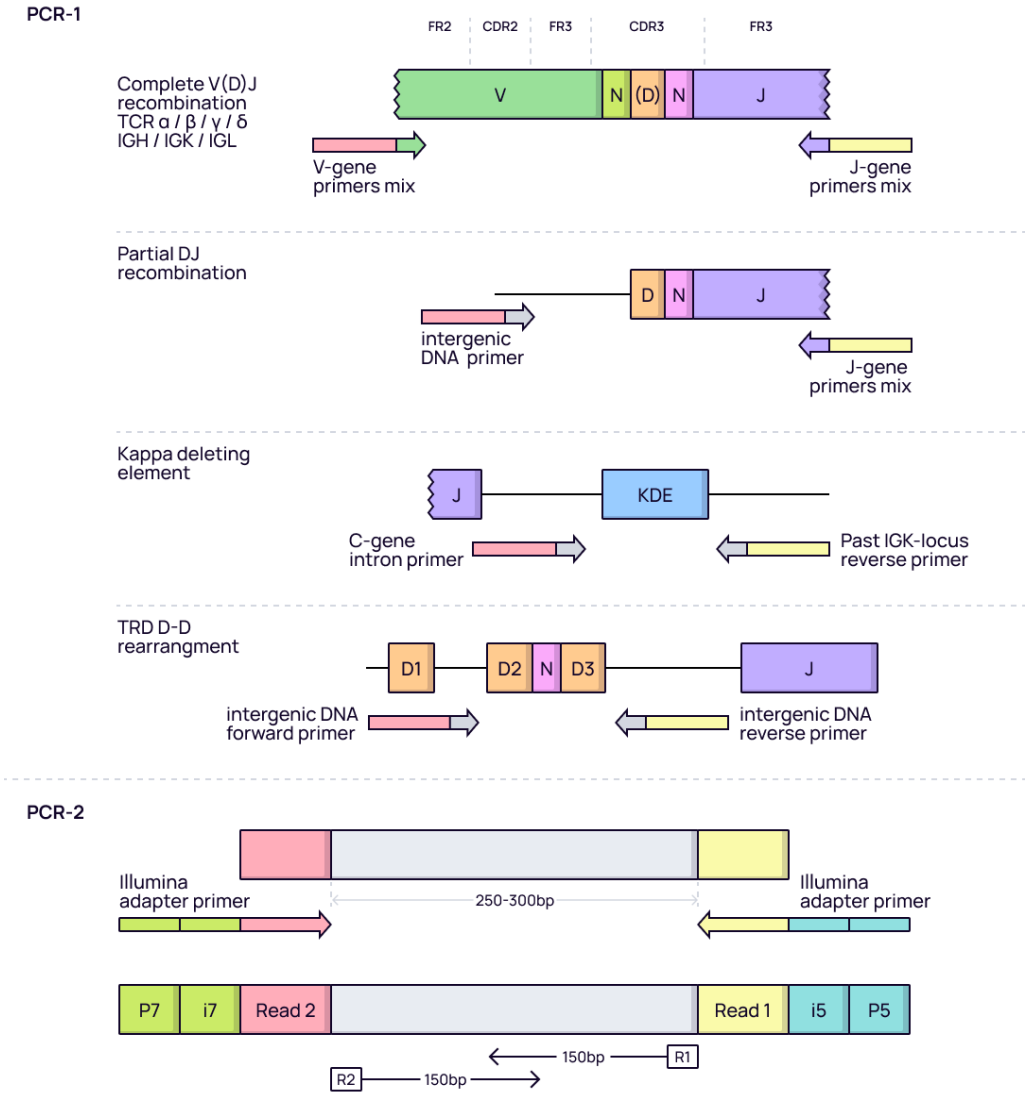

Figure 1. Scheme of library preparation for TCR/IG rearrangements sequencing. Besides the complete VD and VDJ rearrangements, 7GENES includes primers for the detection of a well-known spectrum of incomplete rearrangements: TRB DJ, IGH DJ, TRD VD, TRD DD, and TRD VJ. In addition, 7GENES contains primers for the identification of recombination events between Kappa deleting element located outside the IGK locus and intron between the last IGK J segment and IGK C segment. This rearrangement provides the C segment removal which leads to the inactivation of

the corresponding IGK allele. Kappa deleting element can be rearranged with IGK V genes and its products are also fully detectable by 7GENES.

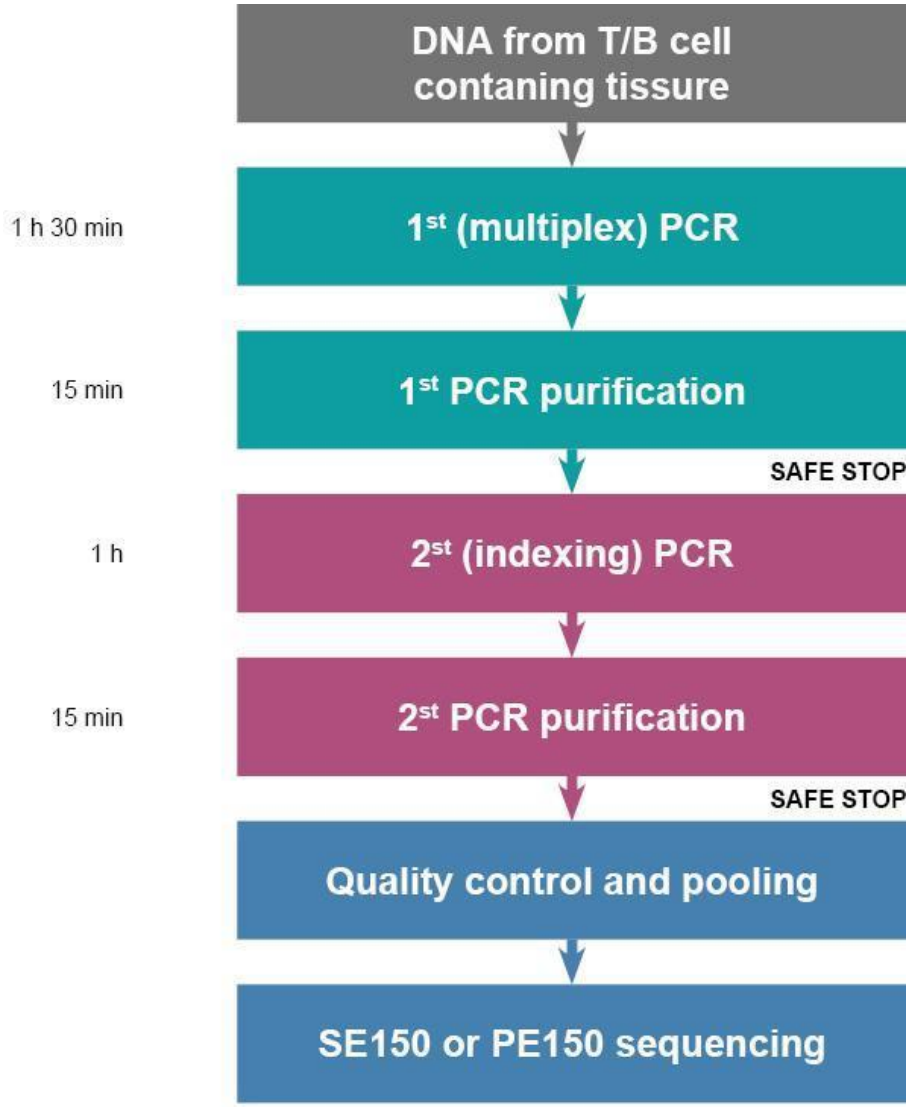

Figure 2. 7GENES kit pipeline.

KIT CONTENT

Table 2.

Component	Water volume to add (µl)	Description
IG/TCR multiplex primer mix	250	1st PCR primer mixes 10X after recovery
Control DNA	22	Control DNA final concentration 10 ng/µl

IG/TCR multiplex primer mix is shipped dry.

Transportation and storage until dissolved at RT.

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

MATERIALS REQUIRED BUT NOT INCLUDED

- HotStarTaq *Plus* DNA Polymerase (Qiagen, #203605, supplied with 10x PCR buffer and 25mM $MgCl_2$)
- dNTP mix (10mM each)
- AMPure® XP Beads (Beckman Coulter, Inc. #A63880) or SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317)
- Freshly prepared 80% Ethanol (400 µl per sample)
- Nuclease-free water
- Elution Buffer (EB, 10 mM Tris-HCl, pH 8.0-8.5) (Qiagen, #19086)
- 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)
- Thermal Cycler
- Agilent Bioanalyzer/TapeStation
- Appropriate Magnetic Rack
- Low-speed benchtop Mini-centrifuge/vortex
- Qubit® fluorimeter and Qubit dsDNA HS Assay Kit

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 DNAse-free reagents, and performing non-template control reactions.

To prevent cross-sample contamination, library preparation should be performed in two separate workspaces (PCR boxes) in different rooms: pre-PCR and post-PCR. The user should avoid transferring anything (reagents, pipets, racks, etc.) from the post-PCR to the 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 1st PCR reaction setup in the pre-PCR workspace. After the 1st PCR amplification, transfer the tubes to the post-PCR workspace and perform all other steps.

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

BEFORE YOU START

Add nuclease-free water to the tubes according to **Table 2** in **Kit Content**. Mix well by vortexing and spin down. Store diluted components at -20ºC.

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.

STARTING MATERIAL

Isolated genomic DNA or sorted cell nuclei can be used as a template for the application of the **HUMAN IG/TCR DNA MULTIPLEX Kit (7GENES).**

The confirmed minimum amount of material for the protocol is 25 T and/or B cell nuclei sorted directly into the tube for the 1st PCR reaction without DNA extraction (See **Appendix A** for our recommendations on nuclei extraction).

For genomic DNA isolation, use high quality kits

Optimal quantitative range is from 1 to 150 ng per reaction (1st PCR).

Residual traces of proteins, salts, or other contaminants (e.g., phenol, ethanol) can significantly reduce the efficiency of PCR reaction.

Do not use heparin-coated tubes for blood collection. Heparin dramatically decreases PCR efficiency.

High DNA quality is often critical for efficient library preparation.

Before starting work, determine the concentration in the isolated DNA. Use a Qubit® fluorimeter or another fluorescent-based method to determine DNA concentration. Dilute the DNA so that the concentration is about 15 ng/µl (admissible concentration from 0.1 to 15 ng/µl). Use DNase free water for dilution.

Do not put more than 150 ng of template DNA in one 25 µl reaction. To comprehensively analyze the IG/TCR repertoire of lymphocytes containing tissue samples, perform multiple parallel reactions (each containing 150 ng of template DNA) with all isolated DNA. To achieve a reliable clonality assessment of acute lymphoblastic leukemia, use 20-40 ng DNA from bone marrow aspirate and at least 10 000 reads per sample coverage during sequencing.

HUMAN IG/TCR DNA MULTIPLEX Kit (7GENES) allows amplification even with partially degraded DNA templates. For instance, DNA fragmentation with a peak at 500 bp makes it possible to extract up to 50% of IG/TCR rearrangements originally present in the sample.

The success of library preparation with DNA isolated from formalin-fixed paraffin-embedded (FFPE) samples depends on fixation protocol and the quality of isolated DNA. The average size of a fragment of fragmented DNA should be at least 300 bp.

PROTOCOL

NOTE: Perform steps 1-2 in a pre-PCR box/room.

See **Appendix B** for the Quick protocol for the large sample series and **Appendix C** for the Low DNA input protocol (< 3 ng). .

1st PCR (multiplex) amplification

1. In a sterile tube prepare Master Mix PCR1 for all samples based on 15 µl per reaction:

Component	Amount, µl
Nuclease-free water	7.5
10x PCR Buffer (Qiagen)	2.5
25mM MgCl2 (Qiagen)	1,0
10mM dNTP	0.5
10x IG/TCR multiplex primer mix	2.5
HotStarTaq Plus 5U/µl (Qiagen)	1,0
Total volume	**15 µl**

2. Add 3-150 ng DNA* dissolved in 10 µl into an empty sterile tube/strip/plate.

dissolved in 10 µl of the

* For control reaction, take 10 µl of control DNA (100 ng per reaction).

NOTE: Do not put more than 150 ng per reaction.

3. Add 15 µl of PCR1 Master Mix to each of the 10 µl DNA samples. Mix gently by pipetting.

4. Perform 1st PCR using the following parameters:

NOTE: Set the thermocycler's lid on **95ºC.** Avoid putting the reaction tubes into the edge positions of the thermocycler PCR performance zone.

Proceed in the post-PCR box/room.

94 ºC	**3 min (Ramp 4 ºC/s)**	**1 cycle**
94 ºC	**20 s (Ramp 4 ºC/s)**	**10 cycles**
57 ºC	**1 min 30 s (Ramp 0.5 ºC/s)**	
72 ºC	**40 s (Ramp 0.5 ºC/s)**	
94 ºC	**20 s (Ramp 4 ºC/s)**	**15 cycles**
74 ºC	**1 min 10 s (Ramp 0.5 ºC/s)**	

4 ºC	**Infinite hold**	

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

- Take 8 µl from each PCR product into empty sterile tube/strip.
- Homogenize beads by vortexing.
- Add 8 µl of beads to the tube with PCR product 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, without touching magnetic beads, 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 11 µl of nuclease-free water 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 10 µl of purified 1st PCR products into a new tube for PCR.

NOTE: Do not analyze the product after 1st PCR on the agarose gel or capillary electrophoresis. The DNA concentration at this stage is extremely low to minimize the possibility of cross-contamination.

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

2nd PCR (indexing) amplification

6. In a sterile tube prepare Master Mix PCR2 for all samples based on 14 µl per reaction:

Component	Amount, µl
Nuclease-free water	9.5
10x PCR Buffer (Qiagen)	2.5
25mM $MgCl_2$ (Qiagen)	1,0
10mM dNTP	0.5
HotStarTaq Plus 5U/µl (Qiagen)	0.5
Total volume	**14 µl**

7. Add the appropriate Master Mix (14 µl per reaction) to the purified PCR1 product (10 µl).

8. Add Index Primers:

8a. 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. The final reaction volume is 25 µl.

8b. If using **Nextera XT Indexing primers**: Add 1µl (5 pmol) of Nextera N70X and 1µl (5 pmol) of Nextera S50X primer to each reaction. Use unique Indexing primers combination for each reaction. The final reaction volume will be 26 µl, but it will not affect the efficiency of the reaction.

9. Perform PCR using the following parameters:

Set the thermocyclers lid on **95 ºC**

94 ºC	**3 min**	**1 cycle**
94 ºC	**20 s**	**14-24 cycles***
55 ºC	**20 s**	
72 ºC	**40 s**	
4 ºC	**Infinite hold**	

* Choose the number of cycles depending on the amount of DNA or T/B-cell count at the start (**Table 3**).

Table 3. The approximate number of PCR cycles and sequencing reads.

Estimated count of T/B cells in a sample	Amount of input DNA purified from sorted T/B cells	The approximate number of 2nd PCR cycles	Recommended sequencing read counts per sample
50 – 500	0.3 ng – 3 ng	24 – 20	2 000 – 20 000
500 – 5 000	3 ng – 30 ng	20 – 16	20 000 – 200 000
5 000 – 25 000	30 ng – 150 ng	16 – 14	200 000 – 1 000 000

NOTE: The optimal number of cycles may vary for different templates, thermal cyclers, and sample amounts. We recommend that you empirically determine the minimal number of PCR cycles required to obtain a sufficient amount (at least 30 ng of each library) for each experiment. It's important to note that the lower the number of cycles, the lower the quantitative PCR bias.

10. Verify quality of the obtained 2nd PCR product on the agarose gel (2.0%) next to the DNA ladder. The resulting indexed library should have a size at approximately 400-500 bp.

11. Pool the obtained indexed libraries by combining an equal volume portion from each individual sample from the 2nd PCR. The resulting amount of pooled PCR products should be at least 50 ng.

12. Purify 100 µl the obtained pooled DNA using standard AMPure® XP Beads protocol (Beckman Coulter) with 1:0.8 sample:beads ratio:

- Homogenize beads by vortexing.
- Add 80 µl of beads to 100µl pooled DNA 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 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 purified 2nd PCR libraries into a new tube.

13. Verify the quality of obtained pooled libraries by analyzing the aliquot by agarose gel or capillary electrophoresis (e.g., BIOANALYZER® or TapeStation®, Agilent Technologies). The library should have a peak at approximately 400-500 bp. (**Fig. 3**).

Quantify the obtained purified pools using Qubit fluorimeter and Qubit dsDNA High Sensitivity Assay Kit.

The resulting concentration of pooled DNA should be at least 1 ng/µl.

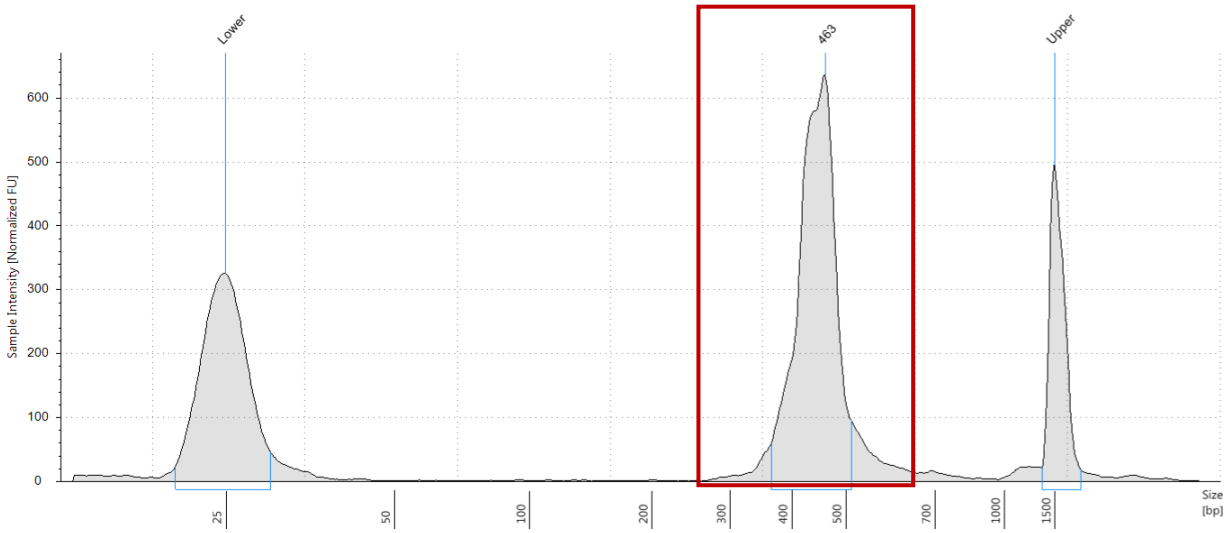

Figure 3. A typical library peak for IG/TCR repertoires. The shape and length distribution of the IG/TCR library may vary depending on the repertoire composition.

SAFE STOP POINT: Store purified libraries at -20ºC for 1-3 months.

SEQUENCING RECOMMENDATIONS

The obtained purified pooled library is ready for Illumina sequencing.

1 ng/µl of IG/TCR library corresponds to 4 nM molar concentration required for preparing loading library aliquot for Illumina instruments. Dilute the pooled library to 1 ng/µl concentration and proceed according to Illumina dilution/denaturation protocol.

The IG/TCR libraries prepared from normal blood samples and large pools (>100 samples) of poorly diverse libraries such as leukemia samples are not required for the PhiX spike-in for sequencing. In the other cases, adding to the IG/TCR library at least 10% of PhiX or another random library (e.g., RNA-Seq, Exome-Seq, etc.) is strongly recommended.

Analyze the resulting pooled library using 150 single ends or 150+150 paired-end Illumina sequencing with standard Illumina® sequencing primers. A single 150 bp read is enough to identify the hypervariable CDR3 region, J, V, and D-segments. The second 150 bp read can be used to decrease sequencing errors of the overlapping fragments in the proximity of CDR3, detect hypermutations in IG loci and increase the resolution of V-segments identification. Use the same sequencing platform for comparative analysis of the obtained IG/TCR repertoire data. Ideally, use the same sequencing run for the samples under comparison, or mix control and experimental samples to minimize batch effects.

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

Data analysis

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

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.

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: NUCLEI EXTRACTION FROM LYMPHOCYTES

Nuclei extraction buffer (1X NEB):

10 mM HEPES pH 7.5

10 mM KCl

4.5 mM MgCl2

All procedures should be performed on the ice or in the cooler at +4°C.

1. Add 10V of precooled (+4°C) 1X NEB to the tube with lymphocytes suspended in PBS and mix 10 times by pipetting.
2. Incubate for 5 min.
3. Centrifuge the tube at 1000g for 10 min.
4. Discard the supernatant
5. Suspend the nuclei pellet in 1X NEB buffer and use immediately for downstream applications (nuclei staining, sorting etc.)

Use obtained nuclei as a template for the 1st (multiplex) PCR.

APPENDIX B: QUICK PROTOCOL FOR THE LARGE SAMPLE SERIES

For the parallel processing of series of samples, we recommend an advanced protocol for experienced users.

NOTE: Perform steps 1-2 in a pre-PCR box/room.

1st PCR (multiplex) amplification

1. In a sterile tube prepare Master Mix PCR1 for all samples based on 15 µl per reaction:

Component	Amount, µl
Nuclease-free water	7.5
10x PCR Buffer (Qiagen)	2.5
25mM MgCl2 (Qiagen)	1,0
10mM dNTP	0.5
10x IG/TCR multiplex primer mix	2.5
HotStarTaq Plus 5U/µl (Qiagen)	1,0
Total volume	**15 µl**

2. Add 3-150 ng DNA dissolved in 10 µl into an empty sterile tube/strip/plate.

3. Add 15 µl of PCR1 Master Mix to each of the 10 µl DNA samples. Mix gently by pipetting.

4. Perform 1st PCR using the following parameters:

NOTE: Set the thermocycler's lid on **95ºC.** Avoid putting the reaction tubes into the edge positions of the thermocycler PCR performance zone.

Proceed in the post-PCR box/room.

94 ºC	**3 min (Ramp 4 ºC/s)**	**1 cycle**
94 ºC	**20 s (Ramp 4 ºC/s)**	**10 cycles**
57 ºC	**1 min 30 s (Ramp 0.5 ºC/s)**	
72 ºC	**40 s (Ramp 0.5 ºC/s)**	
94 ºC	**20 s (Ramp 4 ºC/s)**	**15 cycles**
74 ºC	**1 min 10 s (Ramp 0.5 ºC/s)**	
4 ºC	**Infinite hold**	

5. While the 1st PCR is going: In a sterile tube prepare Master Mix PCR2 for all samples based on 24 µl per reaction:

Component	Amount, µl
Nuclease-free water	19.5
10x PCR Buffer (Qiagen)	2.5
25mM $MgCl_2$ (Qiagen)	1,0
10mM dNTP	0.5
HotStarTaq Plus 5U/µl (Qiagen)	0.5
Total volume	**24 µl**

6. Purify the obtained 1st PCR products using AMPure® XP Beads (Beckman Coulter) with **1:1** sample:beads ratio:

- Homogenize beads by vortexing.
- Add 8 µl of beads to empty sterile tube/strip.
- Take 8 µl from each PCR product into a tube with 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 2–5 minutes).
- Keep tubes on the magnetic rack. Carefully, without touching the magnetic beads, remove and discard the cleared solution from the tubes.
- Add directly to the beads sediment 24 µl of prepared 2nd PCR mixture and pipet up and down 10 times.

NOTE: Magnetic beads present in reaction mix do not affect the PCR performance.

8. Add Index Primers:

8a. 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. The final reaction volume is 25 µl.

8b. If using **Nextera XT Indexing primers**: Add 1µl (5 pmol) of Nextera N70X and 1µl (5 pmol) of Nextera S50X primer to each reaction. Use unique Indexing primers combination for each reaction. The final reaction volume will be 26 µl, but it will not affect the efficiency of the reaction.

9. Perform the 2nd PCR using the following parameters:

Set the thermocyclers lid on **95 ºC**

94 ºC	**3 min**	**1 cycle**
94 ºC	**20 s**	**15 cycles**
55 ºC	**20 s**	
72 ºC	**40 s**	
4 ºC	**Infinite hold**	

10. Pool the obtained indexed libraries by combining 5 µl portions from each individual sample from the 2nd PCR. Mix the pooled library by vortexing.

11. Purify 100 µl of the obtained pooled DNA using standard AMPure® XP Beads protocol (Beckman Coulter) with 1:0.8 sample:beads ratio:

- Homogenize beads by vortexing.
- Add 80 µl of beads to 100 µl pooled DNA 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 21 µl of nuclease-free water for elution and mix well by pipetting.
- Incubate for 2 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 2nd PCR libraries into a new tube.

13. Verify the quality of the obtained pooled library by analyzing the aliquot by agarose gel or capillary electrophoresis (e.g., BIOANALYZER® or TapeStation®, Agilent Technologies). The library should have a peak at approximately 400-500 bp. (**Fig. 3**).

Quantify the obtained purified pools using Qubit fluorimeter and Qubit dsDNA High Sensitivity Assay Kit.

1 ng/µl of IG/TCR library corresponds to 4 nM molar concentration required for preparing loading library aliquot for Illumina instruments. Dilute the purified pooled library to 1 ng/µl concentration and take 5 µl to proceed with Illumina dilution/denaturation protocol.

Appendix C: Low DNA input protocol

This modified protocol is highly preferable for low (0.15 – 3 ng) DNA input libraries preparation.

NOTE: Perform steps 1-2 in a pre-PCR box/room.

1st PCR (multiplex) amplification

1. In a sterile tube prepare Master Mix PCR1 for all samples based on 24 µl per reaction:

Component	Amount, µl
Nuclease-free water	16.5
10x PCR Buffer (Qiagen)	2.5
25mM MgCl2 (Qiagen)	1,0
10mM dNTP	0.5
10x IG/TCR multiplex primer mix	2.5
HotStarTaq Plus 5U/µl (Qiagen)	1,0
Total volume	**24 µl**

2. Sort 25-500 lymphocyte nuclei or add 0.15-3 ng DNA dissolved in 1 µl into an empty sterile tube/strip/plate.

3. Add 24 µl of PCR1 Master Mix to each of the 10 µl DNA samples. Mix gently by pipetting.

4. Perform 1st PCR using the following parameters:

NOTE: Set the thermocycler's lid on **95ºC.** Avoid putting the reaction tubes into the edge positions of the thermocycler PCR performance zone.

Proceed in the post-PCR box/room.

94 ºC	**3 min (Ramp 4 ºC/s)**	**1 cycle**
94 ºC	**20 s (Ramp 4 ºC/s)**	**10 cycles**
57 ºC	**1 min 30 s (Ramp 0.5 ºC/s)**	
72 ºC	**40 s (Ramp 0.5 ºC/s)**	
94 ºC	**20 s (Ramp 4 ºC/s)**	**15 cycles**
74 ºC	**1 min 10 s (Ramp 0.5 ºC/s)**	
4 ºC	**Infinite hold**	

5. While the 1st PCR is going: In a sterile tube prepare Master Mix PCR2 for all samples based on 24 µl per reaction:

Component	Amount, µl
Nuclease-free water	19.5
10x PCR Buffer (Qiagen)	2.5
25mM $MgCl_2$ (Qiagen)	1,0
10mM dNTP	0.5
HotStarTaq Plus 5U/µl (Qiagen)	0.5
Total volume	**24 µl**

6. Purify the obtained 1st PCR products using AMPure® XP Beads (Beckman Coulter) with **1:1** sample:beads ratio:

- Homogenize beads by vortexing.
- Add 25 µl of beads to each tube with 25 µl 1st 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.
- Add to the beads sediment 10 µl of nuclease-free water, pipet up and down 10 times and add new 10 µl magnetic beads 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, without touching the magnetic beads, remove and discard the cleared solution from the tubes.
- Add directly to the beads sediment 24 µl of prepared 2nd PCR mixture and pipet up and down 10 times.

NOTE: Magnetic beads present in reaction mix do not affect the PCR performance.

8. Add Index Primers:

8a. 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. The final reaction volume is 25 µl.

8b. If using **Nextera XT Indexing primers**: Add 1µl (5 pmol) of Nextera N70X and 1µl (5 pmol) of Nextera S50X primer to each reaction. Use unique Indexing primers combination for each reaction. The final reaction volume will be 26 µl, but it will not affect the efficiency of the reaction.

9. Perform the 2nd PCR using the following parameters:

Set the thermocyclers lid on **95 ºC**

94 ºC	**3 min**	**1 cycle**
94 ºC	**20 s**	**22 cycles**
55 ºC	**20 s**	
72 ºC	**40 s**	
4 ºC	**Infinite hold**	

10. Pool the obtained indexed libraries by combining 5 µl portions from each individual sample from the 2ndPCR. Mix the pooled library by vortexing.

11. Purify 100 µl of the obtained pooled DNA using standard AMPure® XP Beads protocol (Beckman Coulter) with 1:0.8 sample:beads ratio:

- Homogenize beads by vortexing.
- Add 80 µl of beads to 100 µl pooled DNA 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, without touching the magnetic beads, 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 21 µl of nuclease-free water for elution and mix well by pipetting.
- Incubate for 2 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 2nd PCR libraries into a new tube.

13. Verify the quality of the obtained pooled library by analyzing the aliquot by agarose gel or capillary electrophoresis (e.g., BIOANALYZER® or TapeStation®, Agilent Technologies). The library should have a peak at approximately 400-500 bp. (**Fig. 3**). In case of the shorter peak presence, perform an additional step of purification with the same 1:0.8 sample:beads ratio.

Quantify the obtained purified pools using Qubit fluorimeter and Qubit dsDNA High Sensitivity Assay Kit.

1 ng/µl of IG/TCR library corresponds to 4 nM molar concentration required for preparing loading library aliquot for Illumina instruments. Dilute the purified pooled library to 1 ng/µl concentration and take 5 µl to proceed with Illumina dilution/denaturation protocol.

TROUBLESHOOTING

Problem	Possible reason	Possible Solution
Low 2nd PCR product yield or no 2nd PCR product	DNA contains impurities that inhibit PCR	In some cases, additional column-based purification of DNA can remove impurities. If this does not help, re-isolate the RNA
	DNA is heavily degraded	Re-isolate the DNA
	PCR undercycling	Repeat the 2nd PCR amplification, using two or three more PCR cycles.
Library bands and background smear are very intense	PCR overcycling	Repeat the 2nd PCR amplification, using two or three fewer PCR cycles
Library bands are present, but the background smear is intense, or alternative lower length fragments are visible	Low IG/TCR DNA content in the initial DNA sample	Add the second purification after the 1st PCR and repeat the 2nd PCR with the double purified 1st PCR amplicon.