EXPLORING CODING AND SIGNAL JOINTS OF NOVEL TCR BETA D-D REARRANGEMENTS FOR TRACKING T-CELL LEUKEMIA CLONALITY

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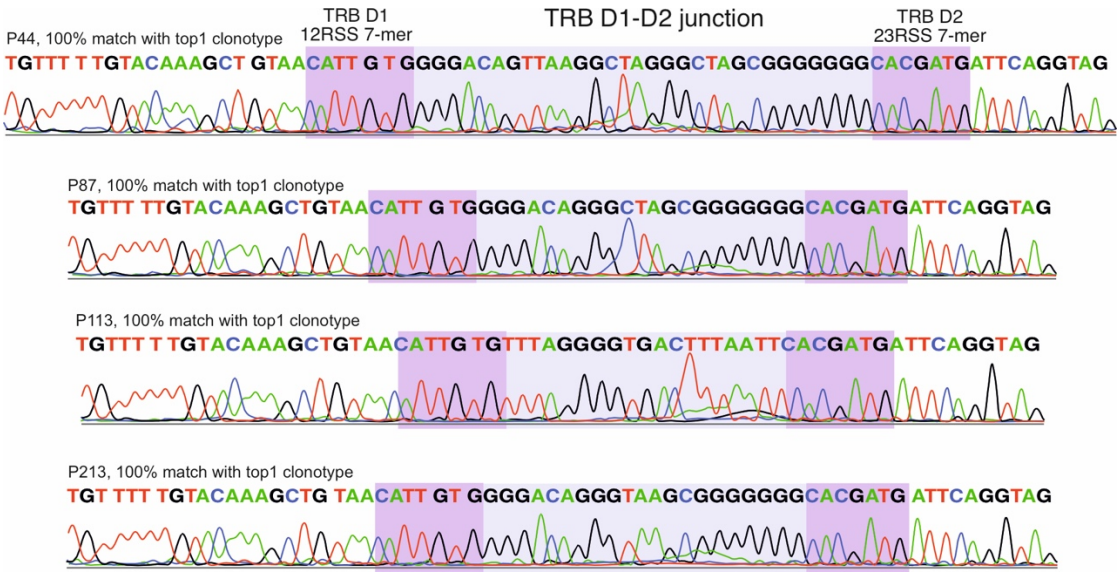
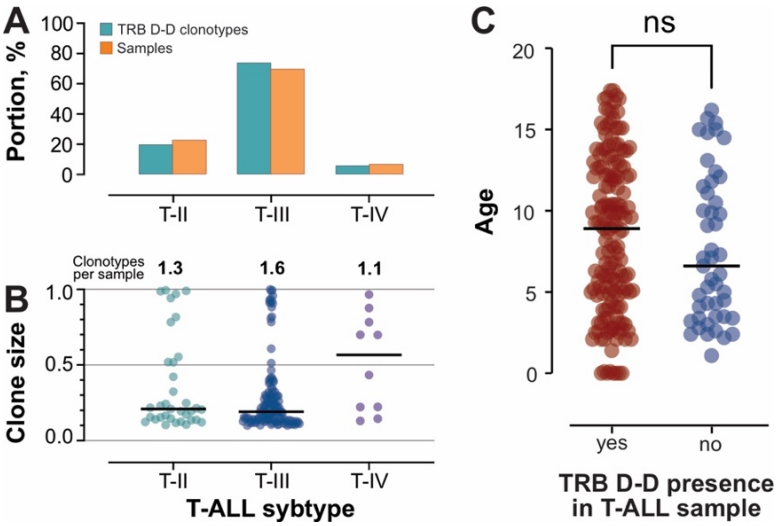
SUPPLEMENTARY

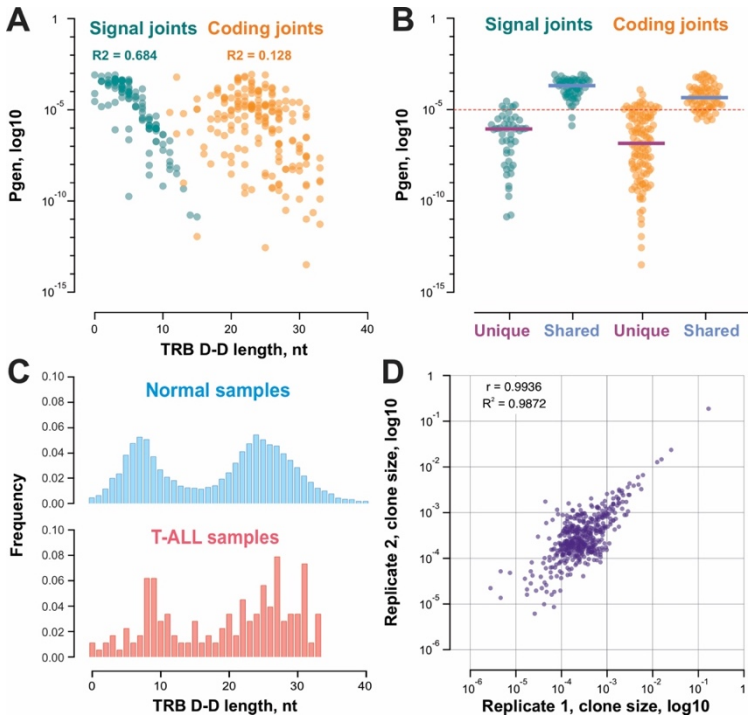
SUPPLEMENTARY FIGURES



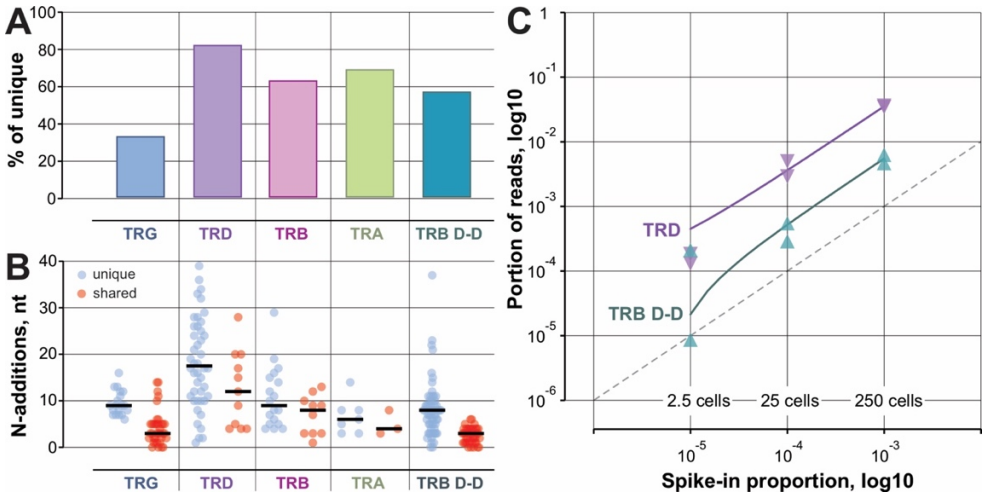
Supplementary Figure 1. *Validation of leukemia-related TRB D-D rearrangements by direct Sanger sequencing of TRB D-D amplicons.*



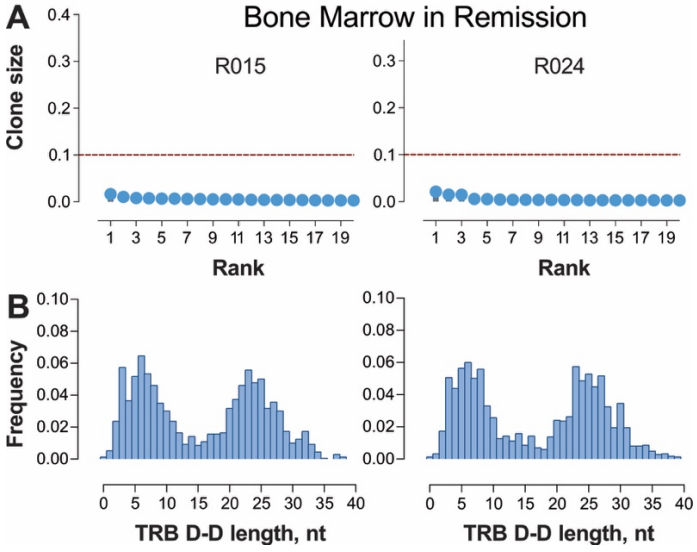
Supplementary Figure 2. *Distribution of TRB-DD markers across T-ALL subtypes and patient age.* ***A.*** *TRB D-D markers and BM samples distribution across T-ALL subtypes.* ***B.*** *Clone size distribution of leukemia-related TRB D-D markers across T-ALL subtypes.* ***C.*** *Distribution of TRB D-D positive and negative cases across patient ages.*



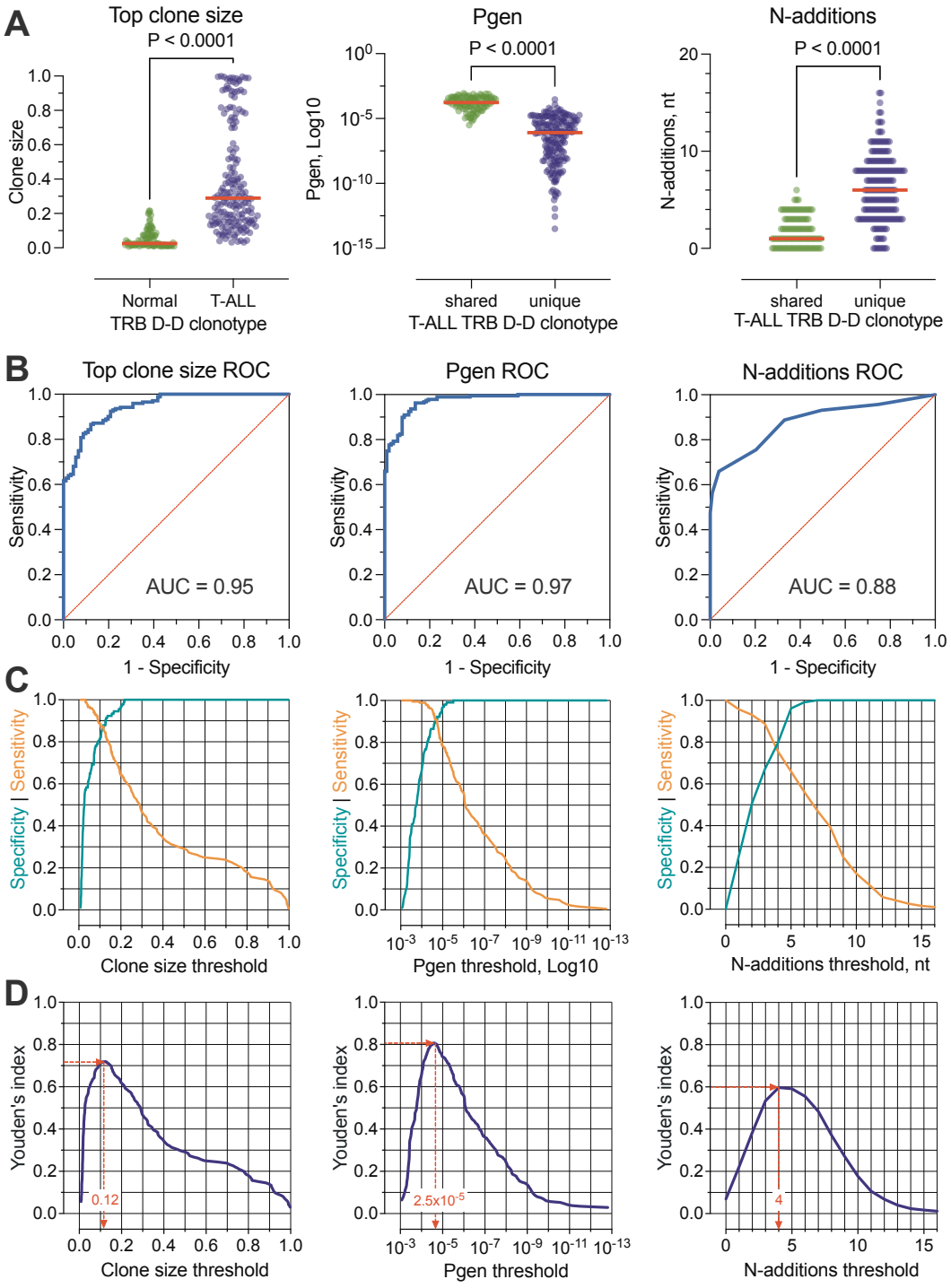
Supplementary Figure 3. *Properties of TRB D-D markers.* ***A.*** *Generation probability features of leukemia-related TRB D-D signal and coding joints.* ***B.*** *Comparison of generation probabilities of unique and shared leukemia-related TRB D-D. Solid Horizontal lines represent medians. The red dashed line represents the chosen threshold for MRD marker selection.* ***C.*** *Comparison of TRB D-D junction length (calculated excluding RSS) distributions in normal T cells and T-ALL.* ***D.*** *Precision of TRB D-D detection with the developed approach based on targeted NGS.*



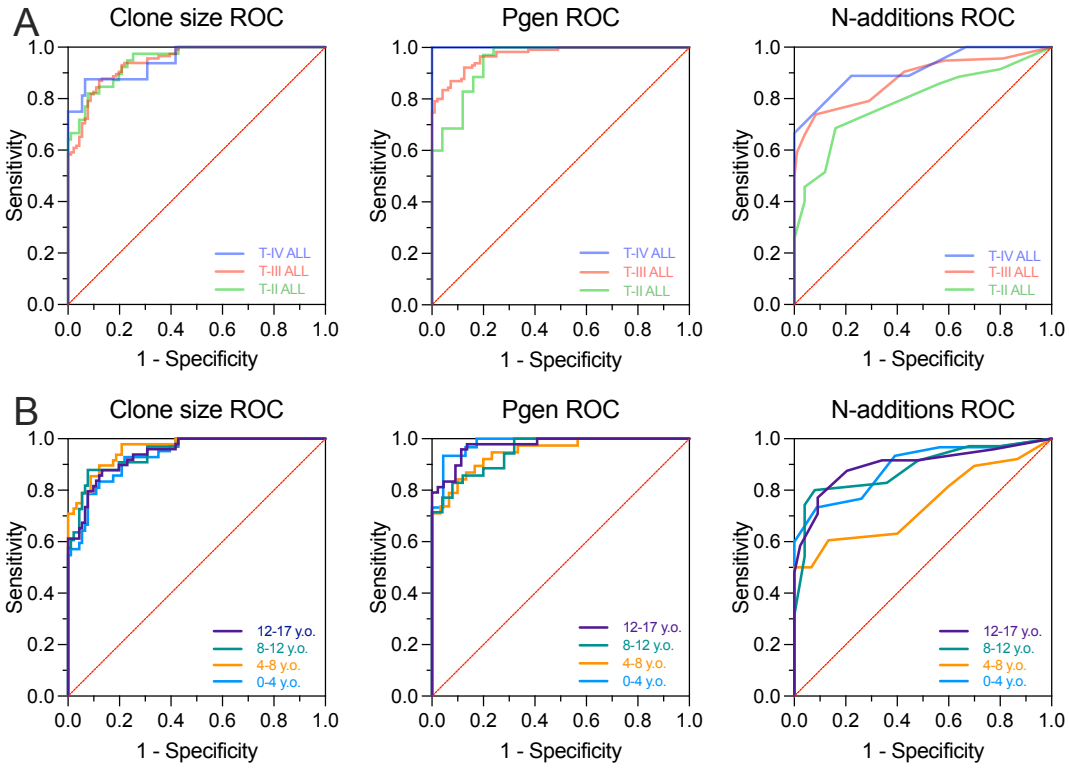
Supplementary Figure 4. *Comparison of TRB-DD and conventional TCR markers.* ***A.*** *Specificities of conventional TCR markers and TRB D-D (before unique clonotype selection). Leukemia-related clonotypes (clone size >10%) were considered as not-unique (shared) if they were found at least in one analysed normal sample.* ***B.*** *Differences of N-additions in unique and shared leukemia clonal markers.* ***C.*** *TRD and TRB D-D markers sensitivity comparison with spike-in serial dilutions. Each analysed sample contains mix of 1.6 μg DNA (250,000 nucleated cells) from regenerating bone marrow and spike-in DNA from T-ALL sample: 16 pg (2.5 cells), 160 pg (25 cells) and 1.6 ng (250 cells). Clonotype frequencies calculated as a ratio of sequencing reads covering target clonotype to the sum of reads covering corresponding TCR rearrangement types (TRD or TRB D-D). Both TRD and TRB D-D have detected the spiked-in leukemic clone at each tested concentration. While both markers' frequencies overestimate the expected spike-in content, TRB D-D shows values that are closer to the expected ones (dashed grey line).*



Supplementary Figure 5*. TRB D-D rearrangements in DNA from the Bone Marrow of leukemia patients in remission (R015 and R024 – remission sample IDs).* ***A.*** *Sizes of the top 20 TRB D-D clonotypes excluding "empty" TRB D-D.* ***B.*** *TRB D-D junction length distribution (calculated excluding RSS).*



Supplementary Figure 6. *Identification of optimal thresholds for TRB D-D clonotype parameters (clone size, Pgen, and N-additions) for selection of reliable leukemia-related TRB D-D markers.* ***A.*** *Comparison of TRB D-D clonotype parameter distributions.* ***B.*** *ROC curves for leukemia-related TRB D-D clonotype parameters.* ***C.*** *Specificity and sensitivity dependence on TRB D-D clonotype parameters thresholds.* ***D.*** *Identification of optimal thresholds for TRB D-D clonotype parameters using Youden's index (Sensitivity + Specificity - 1).*



***Supplementary Figure** 7. ROC curve analysis for clone size, Pgen, and N-additions as parameters of leukemia-related TRB D-D markers. **A.** T-ALL subtype-specific analysis. **B.** Age-stratified analysis.*

SUPPLEMENTARY TABLES

Supplementary Table 1. The variability of TRB D-D markers' sensitivity and specificity across T-ALL subtypes and age groups for selected clone size, Pgen, and N-additions thresholds.

		Clone size ≥ 0.1		Pgen ≤ 10^-5		N-additions > 4 nt	
		Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
T-ALL subtype	T-II	0.87	0.80	0.83	0.88	0.69	0.84
	T-III	0.90	0.81	0.87	0.89	0.74	0.92
	T-IV	0.88	0.80	1.00	1.00	0.67	1.00
Age group	0-4 years	0.86	0.80	0.93	0.87	0.73	0.91
	4-8 years	0.94	0.81	0.74	0.93	0.60	0.87
	8-12 years	0.88	0.81	0.86	0.84	0.80	0.92
	12-16 years	0.88	0.81	0.92	0.89	0.77	0.91

Supplementary Table 2. Co-occurrence of conventional TR markers and TRB D-D in T-ALL samples. Blue – unique clonotypes, Red – shared clonotypes. Orange rows - samples with no unique conventional TCR markers; mauve rows – samples with single unique conventional TCR markers. Clone size was calculated separately for each TCR family as PCR bias-corrected read counts portion.

Sample	TRG		TRD		TRB			TRA			TRB D-D		
P22			0.68	0.31							0.12	0.12	
P24	0.65		0.55	0.45	0.93						0.23	0.23	
P29	0.49	0.46	0.67	0.33	0.70						0.88		
P79	0.62	0.26	0.69	0.28	0.81						0.52		
P82	0.45										0.14	0.14	
P95	0.93		0.99		0.58	0.28					0.42		
P99	0.51	0.48			0.37	0.24	0.19	0.97			0.12		
P101	0.88				0.61						0.14		
P103			0.67	0.29	0.82						0.32	0.17	0.14
P105	0.52	0.46			0.44	0.31	0.21	0.98			0.70		
P107	0.48	0.46			0.66	0.21					0.15		
P109	0.56	0.42	1.00		0.97						0.29	0.27	
P116	0.66	0.31	0.61	0.39	0.64	0.22	0.13				0.94		
P117	0.45	0.45	0.57	0.42	0.58	0.28	0.12				0.30	0.19	
P123	0.54	0.33	0.25		0.73	0.22		0.86			0.78	0.17	
P132	0.66	0.30	0.73	0.27	0.54	0.10					0.32	0.17	
P133	0.93				0.90			0.68			0.22		
P135	0.95				0.52	0.43		0.38			0.78	0.13	
P139	0.55	0.31			0.36	0.35		0.89			0.96		
P140	0.64	0.32			0.44	0.31					0.30		
P146	0.96				0.58	0.38		0.63			0.25	0.21	
P148	0.54	0.42			0.49	0.21	0.20	0.72			0.22		
P149	0.57	0.35			0.63	0.22					0.10		
P150	0.59	0.30			0.63	0.20		0.78			0.19		
P154	0.81		0.33		0.68						0.14	0.10	
P163	0.80	0.12	0.81								0.15	0.14	
P164	0.91		0.68	0.31	0.81	0.12					0.24	0.20	0.16
P167	0.90				0.60	0.38		0.75			0.91		
P168	0.66	0.18	0.94		0.39	0.34		0.40	0.31	0.23	0.21	0.20	0.14
P169	0.41				0.63	0.24					0.16		
P171	0.55	0.29			0.57						0.99		
P182	0.65	0.32			0.91			0.75			0.94		
P192	0.93				0.83			0.56	0.43		0.24		
P193			1.00								0.99		
P194	0.50	0.43	0.85		0.81						0.99		
P204			0.69	0.30							0.13	0.11	0.10
P205	0.48	0.43			0.48	0.33					0.18	0.12	
P207	0.67		0.54	0.44							0.19	0.14	
P213	0.57	0.41			0.55	0.42		0.87	0.11		0.97		
P214	0.61	0.22			0.59	0.32		0.74	0.16		0.12		
P218	0.91				0.81			0.96			0.99		

Supplementary Table 3. TRB D-D markers, which are present in T-ALL onset and absent in relapse, in comparison with conventional TRB D-J and V-J markers. RP25 sample represents the emergence of a new TRB V-J clone in relapse.

Sample	V/D/J junction sequence	TRB V/D/J	N-additions	Clone size	
				Onset	Relapse
RP21	CGAAAGGT	D1-D2	4	0.26	0
	GGGACACGTCTGACGCTGACCATCGTAGCGGGAGGG	D1-D2	19	0.12	0
	GGGACAGGGACCACAATGAGCAGTTCTTCGGGCC	D1-J2-1	3	0.6	0
	GGGACAGGGTTGCTCTGGAAACACCATATATTTTGGAGA	D1-J1-3	2	0.35	0
RP25	GGGACAGGGGATATCGTGACTAGCGGGAGGG	D1-D2	4	0.87	0
	TGTGCCAGCAGTTTAGATGGAAAACAAGTCAATGGCTACACCTTC	V28-J1-2	16	0	0.7

SUPPLEMENTARY METHODS

Sample collection

The study was conducted according to the Helsinki Declaration and approved by a local ethical committee (Pirogov National Medical Research University). The anonymized DNA samples from T-ALL patient bone marrow aspirates (223 patients, ages 2-17) and peripheral blood of healthy volunteers (100 donors, ages 25-55; 2 donors, age 17) were collected at Rogachev National Medical Research Center and Pirogov National Medical Research University, respectively, during previous studies [1–3].

Supplementary Table 4. Distribution of T-ALL subtypes in the study cohort.

T-ALL subtype	male	female	male+female
T-II ALL	21.2%	6.0%	27.2%
T-III ALL	51.1%	13.6%	64.7%
T-IV ALL	6.5%	1.6%	8.2%
T-II + T-III + T-IV	78.8%	21.2%	100%

Library preparation and sequencing

The libraries for TRB D-D sequencing have been generated using target amplification with subsequent indexing PCR (Figure 1C) according to the previously described protocol [2], with 100 ng of DNA for leukemic samples and 800 ng of DNA for normal samples as templates. To avoid cross-contamination, all samples were individually indexed using Unique Dual Indices for Illumina (IDT, USA). The TRA, TRB, TRG, and TRD markers sequencing libraries have been generated using the Human DNA multiplex "7 Genes" kit (MiLaboratories, USA) according to the previously described protocol [3] for 60 randomly selected T-ALL samples. PCR amplification bias correction was performed according to previously published methodology [1]. OAR values for bias correction were calculated based on normal repertoires with default parameters (-min_outframe 15 r -z 1 -iter 1 -mt 1). Obtained OARs have been used for read counts correction in leukemic samples. The high-throughput sequencing (HTS) of the obtained libraries was performed with a 150 nt length in a single-end mode on the Illumina NextSeq550 instrument.

TRB D-D excision circles detection

TRB D-D excision circles have been analysed with targeted NGS. The library for sequencing has been generated using four aliquots of 1600 ng of genomic DNA from human thymus as a template. Excised signal joints (Amplicon1) PCR amplification was performed in four independent 100 μL reactions with D1rev and D2for (0.2 μM each) primers (Suppl.Table 4) and 1X Multiplex PCR Plus mix (Qiagen) with following profile: 95°C – 3 min (1 cycle); 95°C – 20s, 58°C – 20s, 72°C – 40s (25 cycles). All four reactions were pooled, and mix was purified with 1V AmPure XP beads (Beckman Coulter) and eluted in 50 μL of EB buffer (Qiagen).

Excised coding joints (Amplicon2) amplification was performed using 1 μL of purified Amplicon1 as a template and D1rev2 and D2for2 (0.2 μM each) primers (Suppl.Table 4) with PCR conditions described above.

The obtained Amplicon1 and Amplicon2 were used in subsequent indexing PCR to prepare Illumina sequencing libraries. Indexing reaction (25 μL) contained 1X Multiplex PCR Plus mix (Qiagen), 1 μL of UD index primers (Illumina) and 1 μL of diluted PCR product. Amplification profile: 95°C – 5 min (1 cycle); 95°C – 20 s, 56°C – 20 s, 72°C – 40s (20 cycles). The obtained amplicons were purified with 1V AmPure XP beads and eluted in 50 μL of EB buffer (Qiagen).

The HTS of the obtained libraries was performed with a 150 nt length in a paired-end mode on the Illumina NextSeq2000 instrument.

Supplementary Table 5. Oligonucleotide primers for TRB D-D excision circles detection.

Target	Primer	Nucleotide sequence
Excised signal joints	D1rev	5′-ACCCAGGAGGAAAGAAGAGGACT-3′
	D2for	5′-CTGCGCTTCCTGCCGCTGCCCAG-3′
Excised coding joints	D1rev2	5′-CCCGTAGAGTTGAATCATTGTGGC-3′
	D2for2	5′-TTGTATCATGGTGTAACATTGTGG-3′
5’ adapters for primers	Rev	5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′
	For	5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′

Spike-in libraries preparation

To imitate positive MRD samples we prepared the mixes of genomic DNA from regenerating bone marrow (BM) with serially diluted DNA extracted from T-ALL sample (spike-in). Three aliquots of 1600 ng (equivalent of 250,000 nucleated cells) BM DNA containing 16 pg (2.5 cells), 160 pg (25 cells) and 1.6 ng (250 cells) of spike-in DNA were prepared. Each aliquot was obtained in two replicates and was used as template for target PCR amplification. Each of six PCR reactions was performed in the volume of 100 μL, containing 1X Multiplex PCR Plus mix (Qiagen), TRD/TRB D-D primer mix (0.4 μM) and 1600 ng DNA. The amplification profile: 95°C – 3 min (1 cycle); 95°C – 20 s, 57°C – 90 s, 72°C – 40 s (10 cycles); 95°C – 20 s, 74°C – 70s (15 cycles). After amplification the PCR reaction was diluted by 200 μL of water and was used as a template for indexing PCR as described above. The HTS of the obtained libraries was performed with a 150 nt length in a paired-end mode on the Illumina NextSeq2000 instrument.

Data analysis

The obtained fastq files were preprocessed using FASTP software [4] to remove poor-quality reads and cut adapters. Target types of rearrangements were extracted from preprocessed

reads using MiXCR software (MiLaboratories, USA) [5] with additional references for TRB D-D extraction. The post-analysis, including clonotype frequency calculation, filtration, and the intersection, was performed using VDJtools software [6]. The number of N-additions was calculated as the nucleotide distance between the last nucleotide aligned to TRBD1 and the first nucleotide aligned to TRBD2. Statistical analysis and plotting were performed using GraphPad Prism 10 (https://www.graphpad.com) and Rstudio (https://posit.co).

The TRB D-D clonotype size was determined as the portion of sequencing reads after off-target removal. Unique clonotypes with a frequency ≥10% were classified as clonal leukemia-related TRB D-D markers using an established threshold (main text, Figure 1D) in line with standard practice [7]. Although several clonotypes with clone sizes ≥10% can be detected at the onset, it is not possible to predict which clones will survive therapy and which will not.

The TRB D-D generation probability model was inferred from normal TRB D-D repertoires using IGoR software [8], treating TRBD1 as a pseudo-V-gene and TRBD2 as a pseudo-J-gene.

Future direction

The next step for TRB D-D marker implementation in NGS-based MRD monitoring is extensive clinical validation against orthogonal methods, such as qPCR-based analysis of chimeric gene transcripts and/or flow cytometry-based MRD analysis. Here, we propose a protocol for the quantitative measurement of MRD in T-ALL samples using target Illumina sequencing of TRB D-D rearrangements. The entire pipeline requires at least two wet-lab rooms with separate ventilation systems and different pressures to minimize the risk of cross-contamination: an amplicon-free pre-PCR room with positive pressure for genomic DNA work, and a post-PCR room with negative pressure for amplicon work.

A) DNA isolation (Pre-PCR room)

DNA isolation from bone marrow (BM) samples is performed in a pre-PCR room using a column-based approach with the QIAamp DNA Blood Mini Kit, according to the manufacturer's protocol. Minimal input is 0.5 ml of BM aspirate for the onset sample and 3 ml of BM aspirate for the follow-up sample. DNA is eluted in deionized water, and the final concentration is adjusted to 40 ng/µL.

B) Onset BM sample sequencing for patient-specific marker identification

The NGS library preparation requires 2 subsequent PCR steps: target amplification (1st PCR) and indexing amplification (2nd PCR). All PCR steps are performed using 0.2 mL PCR tubes.

Target amplification (Step 1) for the onset sample.

The PCR components are mixed in the pre-PCR room according to Supplementary Table 6 and the PCR is run in the post-PCR room with an amplification profile summarised in Supplementary Table 7 (1st PCR section).

Supplementary Table 6. Components of the target PCR amplification of the onset sample.

Component	1rxn
Multiplex Mix, 2X	12.5 µL
Primer Mix, 2 µM each	2.5 µL
DNA 40 ng/µl	5 µL
Water	5 µL
Total volume	25 µL

Supplementary Table 7. PCR amplification profile for TRB D-D rearrangements detection.

1st PCR (Target amplification). Set lid temperature on 95°C.			
1 cycle	94°C	3 min	Ramp 4°C/s
10 cycles	94°C	20 s	Ramp 4°C/s
	57°C	1 min 10 s	Ramp 0.5°C/s
	72°C	40 s	Ramp 0.5°C/s
15 cycles	94°C	20 s	Ramp 4°C/s
	74°C	1 min 30 s	Ramp 0.5°C/s
Hold	4°C	∞	
2nd PCR (Indexing amplification). Set lid temperature on 95°C.			
1 cycle	94°C	3 min	Ramp 4°C/s
18 cycles	94°C	20 s	Ramp 4°C/s
	56°C	20 s	Ramp 4°C/s
	72°C	40 s	Ramp 4°C/s
Hold	4°C	∞	

Indexing PCR (Step 2) for the onset sample

After amplification, the 1st PCR reaction is diluted fivefold by adding 100 µL of deionized water. The diluted 1st PCR is used as a template for the indexing PCR.

The PCR components are mixed in the post-PCR room according to Supplementary Table 8 and the PCR is run in the post-PCR room with an amplification profile summarised in Supplementary Table 7 (2st PCR section).

Supplementary Table 8. Components of indexing PCR amplification.

Component	1rxn
Multiplex Mix, 2X	12.5 µL
UDP primer mix	1 µL
Diluted 1st PCR	2.5 µL
Water	9 µL
Total volume	25 µL

After amplification, the 2nd PCR amplicon is purified with 1V of AmPure XP beads according to the manufacturer's protocol, eluted into 50 µL of deionized water. Quantification is performed using a Qubit fluorometer with the HS dsDNA kit, according to the manufacturer's protocol. Quality control is performed using TapeStation electrophoresis with the High Sensitivity D1000 kit according to the manufacturer's protocol (pick size ~ 300 bp). The required sequencing coverage is 50,000 reads per onset sample. The minimum amount of PhiX control added to the sequencing run is 10%. The onset samples and follow-up samples cannot be sequenced in the same run.

C) Deep sequencing of follow-up bone marrow samples for MRD detection

The NGS library preparation requires 2 subsequent PCR steps: target amplification (1st PCR) and indexing amplification (2nd PCR). All PCR steps are performed using 0.2 mL PCR tubes.

The NGS libraries for MRD analysis are prepared in 8 aliquots, each containing 800 ng (~125,000 nucleated cells) of genomic DNA as input.

Target amplification (Step 1) for the follow-up sample.

The PCR components are mixed in the pre-PCR room according to Supplementary Table 9. The prepared master mix is distributed equally among eight 0.2 mL tubes (50 µL per tube). The PCR

is run in the post-PCR room with an amplification profile summarised in Supplementary Table 7 (1st PCR section).

Supplementary Table 9. Components of the target PCR amplification of the follow-up sample.

Component	8 rxn
Multiplex Mix, 2X	210 µL
Primer Mix, 10X	42 µL
DNA 40 ng/µL	168 µL
Total volume	420 µL

Indexing PCR (Step 2) for the follow-up sample.

After amplification, each aliquot of the 1st PCR reaction is diluted fivefold by adding 200 µL of deionized water. The diluted 1st PCR is used as a template for the indexing PCR. Each aliquot is indexed with a separate index pair. The PCR components are pre-mixed in the post-PCR room according to Supplementary Table 10, distributed across eight clean 0.2 mL tubes (20.5 µL per tube), and mixed with 2.5 µL from each diluted 1st PCR reaction and 2 µL of the UDP primers (a separate UDP pair for each aliquot). The PCR is run in the post-PCR room with an amplification profile summarised in Supplementary Table 7 (2nd PCR section).

Supplementary Table 10. Components of the target PCR amplification of the follow-up sample.

Component	8 rxn
Multiplex Mix, 2X	105 µL
Water	67 µL
Total volume	172 µL

After amplification, 20 µL of the 2nd PCR amplicons from each aliquot are mixed, purified with 1V (160 µL) of AmPure XP beads according to the manufacturer's protocol, and eluted into 50 µL of deionized water. Quantification is performed using a Qubit fluorometer with the HS dsDNA kit, according to the manufacturer's protocol. Quality control is performed using TapeStation electrophoresis with the High Sensitivity D1000 kit according to the manufacturer's protocol (pick size ~ 300 bp). The required sequencing coverage is 2 mln reads per follow-up sample. The minimum amount of PhiX control added to the sequencing run is 10%. The onset samples and follow-up samples cannot be sequenced in the same run.

D) Bioinformatic analysis

The extraction of TRB D-D rearrangements from sequencing data is performed with MiXCR V3 software (https://github.com/milaboratory/mixcr/) with the following JSON-formatted TRBDD library:

[{"taxonId": 9606, "speciesNames": ["hsa"],
"genes": [{"baseSequence": "nuccore://NG_001333.2",
"name": "TRBD1", "geneType": "V", "isFunctional": false, "chains": ["TRB"],
"anchorPoints": {"FR1Begin": 640052,
"CDR1Begin": 640127, "FR2Begin": 640147, "CDR2Begin": 640167, "FR3Begin": 640187,
"CDR3Begin": 640253, "VEnd": 640279}},
{"baseSequence": "nuccore://NG_001333.2",
"name": "TRBD2", "geneType": "J", "isFunctional": false, "chains": ["TRB"], "anchorPoints":
{"JBegin": 649759, "FR4Begin": 649779, "FR4End": 649869}}]}]

Command line script for alignment: java -jar mixcr.jar align -s hsa -b TRBDD_library.json SampleID.R1.fastq.gz SampleID.R2.fastq.gz SampleID.vdjca

Command line script for clonotype assembly: java -jar mixcr.jar assemble SampleID.vdjca SampleID.clns

Command line script for clonotype extraction: java -jar mixcr.jar exportClones SampleID.clns SampleID.txt

The resulting clonotypes table is converted to the format of VDJTOOLS (https://vdjtools-doc.readthedocs.io/en/master/).

Command line script: java -jar vdjtools.jar Convert -S mixcr SampleID.txt vdjtools.SampleID.txt

Calculate N-additions: java -jar vdjtools.jar Annotate vdjtools.SampleID.txt annotate.vdjtools.SampleID.txt

Clonotypes with clone size ≥0.1 and N-additions >4 are selected as MRD markers.

The MRD level up to 10^-5 is calculated as the number of aliquots of follow-up samples with detected MRD markers divided by the total number of analysed nucleated cells (800 ng DNA * 8 aliquots = 1 mln cells). Higher MRD levels are calculated as a proportion of reads that cover MRD markers.

Supplementary Table 11. Oligonucleotide primers for TRB D-D rearrangements detection.

Target	Primer	Nucleotide sequence
TRB D-D	D2Rev	5′-AAGCACCTCCTCTACCTGAATC-3′
	D1For	5′-GCTGCTCTGGTGGTCTCTCCC-3′
5’ adapters for primers	Rev	5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′
	For	5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′

Supplementary Table 12. Required equipment.

PCR box (pre-PCR and post-PCR rooms)	PCR cabinet (Biosan, UVT-B-AR)
Dry thermostat (pre-PCR room)	Thermo-Shaker for microtubes TS-100 (Biosan, TS-100)
Vortex and Microspin (pre-PCR and post-PCR rooms)	Minicentrifuge-Vortex Microspin (Biosan, FV-2400)
High-speed centrifuge (pre-PCR room)	High-speed Mini-centrifuge (Biosan, Microspin 12 Plus)
Thermocycler (post-PCR room)	T100 (BioRad, 1861096)
Magnetic rack (post-PCR room)	Magnetic Rack for Manual Nucleic Acid Extraction (Biosan, MagSorb-16)
Sequencer (post-PCR room or NGS room)	NextSeq 2000 Sequencing System (Illumina, 20038897) or MiSeq i100 Plus System (Illumina, 20115695)
Capillary Electrophoresis (post-PCR room)	4200 TapeStation System (Agilent, G2991BA)
DNA quantification (pre-PCR and post-PCR rooms)	Qubit 4 Fluorometer (Thermo Fisher Scientific, Q33226)
	NanoDrop Lite Plus Spectrophotometer (Thermo Fisher Scientific, NDLPLUSGL)
Pipettes (pre-PCR and post-PCR rooms)	Research plus, 1-channel, 10 µL (Eppendorf, 3123000020) Research plus, 1-channel, 20 µL (Eppendorf, 3123000039) Research plus, 1-channel, 200 µL (Eppendorf, 3123000055) Research plus, 1-channel, 1,000 µL (Eppendorf, 3123000063) Research plus, 8-channel, 10 µL (Eppendorf, 3125000010) Research plus, 8-channel, 100 µL (Eppendorf, 3125000036)
Refrigerator and Freezer (pre-PCR and post-PCR rooms)	Combined Refrigerator and Freezer (Biobase, BRF-25V450)

Supplementary Table 13. Required material.

Process	Unit
DNA isolation	QIAamp DNA Blood Mini Kit (Qiagen, 51104)
	Type I deionized water
	Ethanol
PCR amplification	Multiplex PCR *Plus* Kit (Qiagen, 206152)
Index Primers	IDT for Illumina PCR Unique Dual Indexes Set 1-4 (384 Indexes) (Illumina, 20043137)
Library purification	AmPure XP beads (Beckman Coulter, A63881)
Library quantification	Qubit dsDNA Quantification Assay Kit (Thermo Fisher Scientific, Q32851)
Library QC	High Sensitivity D1000 ScreenTape (Agilent, 5067-5584) High Sensitivity D1000 Reagents (Agilent, 5067-5585) Optical tube strips (8x Strip) (Agilent, 401428)
Illumina Sequencing	NextSeq 1000/2000 P1 XLEAP-SBS Reagent Kit (300 cycles) (Illumina, 20100982) or MiSeq i100 Plus 100M Reagent Kit (300 cycles) (Illumina, 20141599) PhiX Control v3 (Illumina, FC-110-3001)
Pipette Tips	10 µL Racked, Sterile, Filtered Pipette Tips (SSIbio, 4117NSF) 20 µL Racked, Sterile, Filtered Pipette Tips (SSIbio, 4237NAF) 200 µL Racked, Sterile, Filtered Pipette Tips (SSIbio, 4237NSF) 1000 µL Racked, Sterile, Filtered Pipette Tips (SSIbio, 4337NSF)
Plastic Tubes	2.0 mL Microcentrifuge Tubes (SSIbio, 1310-00) 0.2 mL 8-Strip PCR UltraFlux i Tubes, Flat Cap (SSIbio, 3247-00) Qubit Assay Tubes (Thermo Fisher Scientific, Q32856)

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