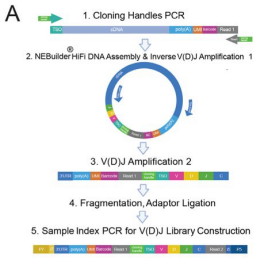
Feb 19, 2026 Version 1

Generation of cirVDJseq libraries from 3′-barcoded cDNA V.1



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We use this protocol and it's working

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Abstract

This protocol describes circVDJ-seq, a method for simplified and cost-efficient TCR VDJ profiling from 3′-barcoded cDNA generated in single-cell or single-nucleus nucleus RNA-seq, RNA+ATAC multi-omics, or spatial transcriptomics workflows. 3′-barcoded cDNA is modified with Gibson assembly overhangs, circularized, depleted of remaining linear DNA, and subjected to nested PCR and 10x Genomics VDJ library construction to generate sequencing-ready TCR libraries.
The expected outcome is a discrete, high-quality VDJ library peak on TapeStation High Sensitivity DNA assays with sufficient yield for Illumina sequencing using custom primers. When applied to human tissues, circVDJ-seq enables robust recovery of TCRα/β VDJ sequences and clonal repertoires, allowing characterization of T cell clonality and immune microenvironments across diverse clinical samples.

Attachments

PDF
2025.09.16.675546v1....
5.9MB

XLSX
Plumbom and Obermaye...
25KB

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Guidelines

- This protocol is optimized for TCR V(D)J enrichment from **3′-barcoded cDNA** derived from single-cell or single-nucleus RNA-seq, RNA+ATAC, or spatial transcriptomics workflows. Using other cDNA types may require re-optimization of input amount and cycle numbers.
- Whenever possible, start with **high-quality 3′-barcoded cDNA** that has already passed QC (TapeStation/Fragment Analyzer and Qubit). Degraded or very low-complexity input will reduce clonotype recovery.
- Maintain accurate **AMPure XP/SPRIselect bead ratios** (0.8X–0.9X as indicated). Deviations in bead volume critically affect size selection and yield. Mix beads thoroughly before use.
- Avoid **over-drying** bead pellets. Over-dried beads are difficult to resuspend and can cause large losses of DNA. Pellets should appear matte but not cracked.
- Use **freshly prepared 80% ethanol** for washes. Residual salts or lower ethanol concentration will impair cleanup efficiency.
- Adjust the number of PCR cycles (especially **V(D)J Amplification 1**) according to sample type and expected T-cell content. Over-amplification can introduce bias and increase PCR artifacts.
- Perform **QC after each major stage** (post-PCR#1, post-circularization, post-V(D)J enrichment, final libraries) using Qubit and TapeStation to monitor yield and fragment size and to identify issues early.
- For library construction, follow the **Chromium Next GEM Single Cell 5′ v2 (Dual Index) V(D)J Library Construction** instructions closely, using the custom V(D)J cDNA as input and the custom sequencing primers listed in the protocol.
- Include appropriate **controls** where possible (e.g. T-cell–rich reference sample or a previously validated sample) to benchmark clonotype recovery and library performance between runs.

Materials

Chemicals/kits

	Chemicals/Kits	Additional Description	Manufacturer
	Ethanol	Catalog number: 11096.02	SERVA Electrophoresis
	Elution Buffer (EB)	Catalog number: 19086	QIAGEN
	Agencourt AMPure XP	REF: A63881	Beckman Coulter GmbH
	KAPA HiFi HotStart ReadyMixPCR Kit	REF: 07958927001	KAPA Biosystems
	QubitTM dsDNA HS Assay Kit	Quantitation Range: 0.2-100 ng	Invitrogen
	High Sensitivity D1000 ScreenTape Kit	Sizing Range: 35 – 1000 bp	Agilent Technologies
	High Sensitivity D5000 ScreenTape Kit	Sizing Range: 100 – 5000 bp	Agilent Technologies
	Water	Bioperformance certified, Lot No. RNBJ7736	Sigma-Aldrich
	NEBuilder HiFi DNA Assembly Master Mix	Catalog number: E261S	New England BioLabs
	CutSmart Buffer	Catalog number: B7204S	New England BioLabs
	Lambda Exonuclease	Catalog number: M0262S, 5,000 units/mL	New England BioLabs
	Library Construction Kit	Lot: 160026, PN: 1000190	10X Genomics
	KAPA Library Quantification (Illumina)	Lot:0000121161	Roche

	Chemicals/Kits	Additional Description	Manufacturer
	Primers & LightCycler 480 qPCRMix		
	KAPA Library Quantification (Illumina) DNA Standards 1-6	Lot: 0000120225	Roche
	Tween-20	Lot: P9416	Sigma-Aldrich

Table 1.1: Overview of chemicals/kits

Devices and Software

	Device/Software	Model/Designation	Manufacturer
	Vortex Mixer	Vortex-Genie 2	Scientific Industries
	PCR Thermal Cyclers	Mastercycler X50s	eppendorf
	Thermomixer	with thermoblocks for 24 reaction vessels 1.5 mL, 2 mL	eppendorf
	Magnetic Stand 0,2	to 8 purifications in parallel	Thermo Fisher Scientific
	Thermoblock	ThermoStat plus	eppendorf
	Fluorometer	Qubit 3 Fluorometer	Thermo Fisher Scientific
	Mini-centrifuge	Rotilabo‰-mini-centrifuge "Uni-fuge"	Carl Roth
	Centrifuge	Centrifuge 5427 R	eppendorf
	PCR Plate Spinner	max. capacity: 2 plates	VWR International
	Repetitive pipette	Multipette‰ E3x	eppendorf

	Device/Software	Model/Designation	Manufacturer
	Charging Stand	Charging Stand 2 for one electronic Multipette	eppendorf
	Dispenser tips	Eppendorf Combitips advanced (0,1 ml, 0,5 ml, 2,5 ml)	eppendorf
	Pipette Tips	xTip 4, low retention manual filter pipette tip (20 µL, 200 µL)	Biotix
	DNA LoBind Tubes	1,5 mL, 0,5 mL	eppendorf
	Thin-walled Tubes with Flat Caps	0.5 mL	Thermo Fisher Scientific
	Pipette tips	SafeSeal-Tips Professional Line (10µL, 20 µL, 200µL, 1000µL)	Biozym Scientific
	Pipettes	Eppendorf Research plus pipette (0.1–2.5µL, 0.5–10 µL, 2–20µL, 10–100 µL, 20–200µL, 100–1,000 µL)	eppendorf
	Multichannel pipettes	Pipet-Life XLS, 8-Channels (0.5-10 µL, 2-20 µL, 20-200 µL)	RAININ
	PCR 8er-SoftStrips	0.2 ml	Biozym Scientific
	PCR Tubes	PCR-02-L-C, 0.2mL maximum recovery, thin wall, clear	Axygen
	Pipettor	Pipetboy acu 2	INTEGRA Biosciences
	Serological pipettes	5 mL, 10 mL	Sarstedt
	Conical Centrifuge Tubes	FalconTM, 15mL, 50mL	Fisher Scientific

	Device/Software	Model/Designation	Manufacturer
	PCR plates for Roche LightCycler 480	Catalog number: 732-1462, No. Of wells: 96	VWR International
	PCR Seal Sheets	Optically clear film, adhesive seal. 140×77mm	4titude
	LightCycler 480 Sealing Foil	REF: 04729757001	Roche Molecular System
	Optical Film Compression Pad	4TI-0563	4titude
	Benchtop Cooler	StrataCooler LP	Agilent Technologies
	Vortex Mixer	IKA MS3 Vortexer	IKA
	LightCycler 480 II System	Serial number: 27785	Roche
	TapeStation	Agilent 4200 TapeStation System	Agilent Technologies
	TapeStation Analysis Software	-	Agilent Technologies

Table 1.2: Devices and softwares

Primer

	A	B	C	D
	Oligo name	Sequence (5′ → 3′)	Comment	Manufacturer
	TCRGOT_1	GAGCAAGTATGTACCGTTCCAAGCAGTGGTATCAACGCAGAG		IDT

	A	B	C	D
	TCRGOT_2	GGAACGGTACATACTTGCTCCTACACGACGCTCTTCCGATCT		IDT
	TRAC_3UTR_1	/5BiotinTEG/GTCTGGGCGTGTTGTATGTC	5′-BiTEG modification	IDT
	TRAC_3UTR_2	/5BiotinTEG/GTGTTGTATGTCCTGCTGCC	5′-BiTEG modification	IDT
	TRBC1_3UTR	/5BiotinTEG/CACACTCACGGCTGAAATCT	5′-BiTEG modification	IDT
	TRBC2_3UTR	/5BiotinTEG/CCCTGAAGATTGAGCTCCCA	5′-BiTEG modification	IDT
	HTCR_o_alpha	/5BiotinTEG/TGAAGGCGTTTGCACATGCA	5′-BiTEG modification	IDT
	HTCR_o_beta	/5BiotinTEG/TCAGGCAGTATCTGGAGTCATTGAG	5′-BiTEG modification	IDT
	HTCR_i_alpha	/5BiotinTEG/AGTCTCTCAGCTGGTACACG	5′-BiTEG modification	IDT
	HTCR_i_beta	/5BiotinTEG/TCTGATGGCTCAAACACAGC	5′-BiTEG modification	IDT
	P5_SI_TT	AATGATACGGCGACCACCGAGATCTACACNNNNNNNNNGTGACTGGAGTTCAGACGTG*T		IDT

	A	B	C	D
	P5_SI-TT-A1	AATGATACGGCGACCACCGAGATCTACACGTAACATGCGGTGACTGGAGTTCAGACGTG*T		IDT
	P5_SI-TT-A2	AATGATACGGCGACCACCGAGATCTACACGTGGATCAAAGTGACTGGAGTTCAGACGTG*T		IDT
	P5_SI-TT-A3	AATGATACGGCGACCACCGAGATCTACACCACTACGAAAGTGACTGGAGTTCAGACGTG*T		IDT
	P5_SI-TT-A4	AATGATACGGCGACCACCGAGATCTACACCTCTAGCGAGGTGACTGGAGTTCAGACGTG*T		IDT
	P5_SI-TT-A5	AATGATACGGCGACCACCGAGATCTACACGTAGCCCTGTGTGACTGGAGTTCAGACGTG*T		IDT
	P5_SI-TT-A6	AATGATACGGCGACCACCGAGATCTACACTAACGCGTGAGTGACTGGAGTTCAGACGTG*T		IDT

	A	B	C	D
	P5_SI-TT-A7	AATGATACGGCGACCACCGAGATCTACACTCCCAAGGGTGTGACTGGAGTTCAGACGTG*T		IDT
	P5_SI-TT-A8	AATGATACGGCGACCACCGAGATCTACACCGAAGTATACGTGACTGGAGTTCAGACGTG*T		IDT
	P5_SI-TT-A9	AATGATACGGCGACCACCGAGATCTACACAAGTGGAGAGGTGACTGGAGTTCAGACGTG*T		IDT
	P5_SI-TT-A10	AATGATACGGCGACCACCGAGATCTACACCGTGACATGCGTGACTGGAGTTCAGACGTG*T		IDT
	P7_TRAC_3UTR_f3_i7	CAAGCAGAAGACGGCATACGAGATCTGAGTCAGTAGCGNNNNNNNNNGTATGTCCTGCTGCCGATGC*C		IDT
	P7_TRBC1_3UTR_f_i7	CAAGCAGAAGACGGCATACGAGATCTGAGTCAGTAGCGNNNNNNNNNACGGCTGAAATCTCC		IDT

	A	B	C	D
		CTAACCCA*G		
	P7_TRBC2_3UTR_f_i7	CAAGCAGAAGACGGCATACGAGATCTGAGTCAGTAGCGNNNNNNNNNGATTGAGCTCCCAACCCCCAA*G		IDT
	P7_TRAC_3UTR_f3_i7_A1	CAAGCAGAAGACGGCATACGAGATCTGAGTCAGTAGCGAGTGTTACCTGTATGTCCTGCTGCCGATGC*C		IDT
	P7_TRBC1_3UTR_f_i7_A1	CAAGCAGAAGACGGCATACGAGATCTGAGTCAGTAGCGAGTGTTACCTACGGCTGAAATCTCCCTAACCCA*G		IDT
	P7_TRBC2_3UTR_f_i7_A1	CAAGCAGAAGACGGCATACGAGATCTGAGTCAGTAGCGAGTGTTACCTGATTGAGCTCCCAACCCCCAA*G		IDT
	P7_TRAC_3UTR_f3_i7_A2	CAAGCAGAAGACGGCATACGAGATCTGAGTCAGTAGCGGCCAACCCTGGTATGTCCTGCTGCCGATGC*C		IDT

	A	B	C	D
	P7_TRBC1_3 UTR_f_i7_A2	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG GCCAACCC TG ACGGCTGA AATCTCCC TAACCCA* G		IDT
	P7_TRBC2_3 UTR_f_i7_A2	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG GCCAACCC TG GATTGAGC TCCCAACC CCCAA*G		IDT
	P7_TRAC_3U TR_f3_i7_A3	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG TTAGACTG AT GTATGTCC TGCTGCCG ATGC*C		IDT
	P7_TRBC1_3 UTR_f_i7_A3	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG TTAGACTG AT ACGGCTGA AATCTCCC TAACCCA* G		IDT
	P7_TRBC2_3 UTR_f_i7_A3	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG TTAGACTG AT GATTGAGC TCCCAACC CCCAA*G		IDT
	P7_TRAC_3U TR_f3_i7_A4	CAAGCAGA AGACGGCA TACGAGAT		IDT

	A	B	C	D
		CTGAGTCA GTAGCG TATCTTCAT C GTATGTCC TGCTGCCG ATGC*C		
	P7_TRBC1_3 UTR_f_i7_A4	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG TATCTTCAT C ACGGCTGA AATCTCCC TAACCCA* G		IDT
	P7_TRBC2_3 UTR_f_i7_A4	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG TATCTTCAT C GATTGAGC TCCCAACC CCCAA*G		IDT
	P7_TRAC_3U TR_f3_i7_A5	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG GAGCATCT AT GTATGTCC TGCTGCCG ATGC*C		IDT
	P7_TRBC1_3 UTR_f_i7_A5	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG GAGCATCT AT ACGGCTGA AATCTCCC TAACCCA* G		IDT
	P7_TRBC2_3 UTR_f_i7_A5	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG GAGCATCT		IDT

	A	B	C	D
		AT GATTGAGC TCCCAACC CCCAA*G		
	P7_TRAC_3UTR_f3_i7_A6	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG CCCTAACT TC GTATGTCC TGCTGCCG ATGC*C		IDT
	P7_TRBC1_3UTR_f_i7_A6	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG CCCTAACT TC ACGGCTGA AATCTCCC TAACCCA* G		IDT
	P7_TRBC2_3UTR_f_i7_A6	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG CCCTAACT TC GATTGAGC TCCCAACC CCCAA*G		IDT
	P7_TRAC_3UTR_f3_i7_A7	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG TACTACCTT T GTATGTCC TGCTGCCG ATGC*C		IDT
	P7_TRBC1_3UTR_f_i7_A7	CAAGCAGA AGACGGCA TACGAGAT CTGAGTCA GTAGCG TACTACCTT T ACGGCTGA AATCTCCC		IDT

	A	B	C	D
		TAACCCA*G		
	P7_TRBC2_3UTR_f_i7_A7	CAAGCAGAAGACGGCATACGAGATCTGAGTCAGTAGCGTACTACCTTTGATTGAGCTCCCAACCCCCAA*G		IDT
	Sequencing primers:		readout:	
	spTCR_Read1 (90)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	VDJ sequence	IDT
	spTCR_Read2 (28)	CATACTTGCTCCTACACGACGCTCTTCCGATCT	UMI and cell barcode	IDT
	spTCR_Read3 (10)	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC	i7 Index	IDT
	spTCR_Read4 (10)	CAAGCAGAAGACGGCATACGAGATCTGAGTCAGTAGC*G	i5 Index	IDT

Table 1.3: Overview of the primers

Before start

- Ensure that **3′-barcoded cDNA** from the upstream single-cell / single-nucleus / spatial workflow is available, quantified, and stored at −20 °C or −80 °C.
- Order and resuspend all **primers and oligos** used in the protocol (TCRGOT primers, TRAC/TRBC primers, outer/inner TCR primers, indexing primers, custom sequencing primers). Verify sequences and working concentrations.
- Thaw and prepare all **buffers and enzymes** (KAPA HiFi HotStart ReadyMix, NEBuilder HiFi DNA Assembly Master Mix, Lambda exonuclease + buffer, AMPure XP beads, SPRIselect, 10x Genomics V(D)J reagents). Mix thoroughly and keep enzymes on ice.
- Bring AMPure XP and SPRIselect beads to **room temperature for at least 30 minutes** and vortex well to ensure homogeneous suspension before use.
- Pre-program thermal cyclers with all required PCR and incubation profiles (PCR#1, circularization, Lambda exonuclease digestion, V(D)J Amplification 1 & 2, fragmentation/A-tailing, adapter ligation, indexing PCR).

- Verify access to all required **equipment**:

- Magnetic rack compatible with the tube/plate format used
- Qubit (or equivalent) with dsDNA HS assay kit
- TapeStation (or equivalent) with HS D5000 and D1000 ScreenTapes
- Refrigerated microcentrifuge
- Thermomixer or heating block with shaking option
- Illumina **NextSeq 550** (or access to a facility that provides runs with the 90–28–10–10 configuration).

- Prepare a clean **PCR work area** with filtered pipette tips, dedicated pipettes.
- Plan sample layout, indices, and multiplexing strategy in advance to ensure **non-overlapping sample indices** in the sequencing run.

PCR #1 – amplification of 3′-barcoded cDNA

1 This step amplifies the 3′-barcoded cDNA and adds the Gibson assembly overhangs required for circularization.

Input: 1–15 ng of 3′-barcoded cDNA

Prepare PCR #1 reaction mix on ice

For each 25 µL reaction:

	Component	25 µL reaction	Final conc.
	PCR-grade water	Up to 25 µL	N/A
	2X KAPA HiFi HotStart ReadyMix	12.5 µL	1X
	10 µM TCRGOT_1 primer	0.75 µL	0.3 µM
	10 µM TCRGOT_2 primer	0.75 µL	0.3 µM
	3′-barcoded cDNA template	1-15 ng	1-15 ng

PCR#1 reaction mix

- Assemble all components on ice.
- Mix gently by pipetting and briefly spin down to collect liquid at the bottom.

2 **PCR cycling**

Place the tubes in a pre-cooled thermal cycler and run the following program (6 cycles of steps 3-5):

	A	B	C	D
	Step	Temperature	Duration	Cycles
	Initial denaturation	95 °C	3 min	1
	Denaturation	98 °C	20 s	6
	Annealing	65 °C	30 s	
	Extension	72 °C	2 min	
	Final extension	72 °C	5 min	1

PCR#1 programm design

- Hold at 4 °C until proceeding to cleanup.

3

PCR #1 Cleanup

This cleanup removes primers, enzymes, and small fragments using AMPure XP beads.

1. Bring the AMPure XP beads to the room temperature for at least 30 minutes and thoroughly vortex the to fully resuspend.
2. Add **20 µL AMPure XP beads (0.9X)** to each 25 µL PCR reaction.
3. Mix by pipetting up and down ~15 times, or vortex.
4. Incubate for **5 min at room temperature** to allow DNA to bind to the beads.
5. Place the tubes on a magnetic rack (high) for 5minutes or until the solution becomes clear and the beads are fully pelleted.
6. Carefully remove and discard the supernatant without disturbing the bead pellet.
7. Add **200 µL of freshly prepared 80% ethanol** to the pellet and incubate for ~30 s.
8. Remove the ethanol carefully.
9. Repeat the ethanol wash (steps 7–8) for a total of **2 washes**.
10. Air-dry the bead pellet for **2-3 min** (do not overdry; pellets should appear matte but not cracked).
11. Remove the tubes from the magnet and add **20.5 µL EB buffer**.
12. Resuspend the beads thoroughly by pipetting up and down ~15 times, or vortex.
13. Incubate for **2 min at room temperature** to elute DNA.
14. Place the tubes back on the magnet (low) until the solution clears.

15. Transfer **20 µL** of the clear supernatant to new tubes. This is your cleaned PCR #1 product.

4 **PCR #1 Quality Control**

Assess yield and fragment size distribution:

- **Qubit dsDNA HS assay** – to quantify DNA concentration.
- **Agilent TapeStation HS D5000** – to confirm expected fragment size and absence of primer-dimers.

Circularization of cDNA

5 In this step, Gibson assembly is used to circularize the amplified cDNA.

Prepare circularization reaction

For each sample:

	Component	Volume
	cDNA template	15–20 ng
	1X CutSmart buffer	to 170 µL total
	2X NEBuilder HiFi DNA Assembly Master Mix	10 µL

circularization reaction mix

- Combine cDNA, CutSmart buffer, and NEBuilder HiFi DNA Assembly Master Mix in a total reaction volume of **200 µL**.
- Mix thoroughly by pipetting and briefly spin down.
- Incubate at **50 °C for 1 hour** in a thermomixer or thermal cycler (optional gentle shaking).
- After incubation, proceed immediately to the next step.

6 Circularization Cleanup

Purify the circularized cDNA using AMPure XP beads.

1. Vortex (brought to the room temperature for at least 30min) AMPure XP beads to completely resuspend.
2. Add **0.9X volume AMPure XP beads** to each circularization reaction.
3. Mix by pipetting 15 times.
4. Incubate **5 min at room temperature**.
5. Place tubes on the magnet (high) until the solution is clear and beads are pelleted.
6. Carefully remove and discard the supernatant.
7. Add **200 µL 80% ethanol** to the pellet and incubate ~30 s.
8. Remove the ethanol carefully.
9. Repeat the ethanol wash (steps 7–8) for a total of **2 washes**.
10. Air-dry the pellet for **2-3 min** (avoid overdrying).
11. Remove tubes from the magnet and add **20.5 µL EB buffer**.
12. Resuspend the beads thoroughly (pipette up and down ~15 times).
13. Incubate **5 min at room temperature**.
14. Place tubes on the magnet (low) until the solution clears.
15. Transfer **20 µL** of the eluate to new tubes. This is the circularized cDNA.

7 Lambda Exonuclease Digestion

This step removes residual linear DNA, enriching for circular molecules.

Prepare the following mix for each sample:

	Component	Volume
	Circularized DNA	20 µL
	10X Lambda Exonuclease Buffer	5 µL
	Lambda Exonuclease Enzyme	1 µL
	Nuclease-free H_2O	24 µL
	Total	50 µL

Lambda Exonuclease Digestion Reaction Mix

a. Incubation

- Incubate at **37 °C for 30 min** to digest remaining linear DNA.

b. Enzyme inactivation

- Stop reaction by adding EDTA to 10mM.
- Heat-inactivate at **75 °C for 10 min**.
- Cool samples on ice or at 4 °C before proceeding to cleanup.

8 **Post-digestion Cleanup**

Purify the circularized, exonuclease-treated DNA using AMPure XP beads.

1. Bring the AMPure XP beads to the room temperature for at least 30 minutes and thoroughly vortex the to fully resuspend.
2. Add **0.8X volume AMPure XP beads** to each 50 µL digestion reaction.
3. Mix by pipetting ~15 times.
4. Incubate **5 min at room temperature**.
5. Place tubes on the magnet until the solution is clear (~5 min).
6. Remove the supernatant carefully.
7. Add **80% ethanol** to wash the beads (originally written as 2 mL; please confirm – other washes use 200 µL).
8. Wait **30 s**, then remove the ethanol.
9. Repeat the ethanol wash for a total of **2 washes**.
10. Air-dry the pellet for **2-3 min**.
11. Remove tubes from the magnet and add **25.5 µL EB + 0.05% Tween-20**.
12. Resuspend beads thoroughly by pipetting ~15 times.
13. Incubate **5 min at room temperature**.
14. Place tubes on the magnet (low) until the solution clears.
15. Transfer **25 µL** of eluate to new tubes. This is your circularized cDNA ready for V(D)J amplification.

9 **Circularization QC**

Evaluate DNA yield and fragment size:

- **Qubit dsDNA HS assay**
- **Agilent TapeStation HS D5000**

V(D)J Amplification 1 (outer PCR)

10 This nested PCR enriches TCR α and β V(D)J regions from circularized cDNA.

a. Prepare V(D)J 1 primer mix on ice

	Primer	Volume
	100 µM TRAC_3UTR_1	1 µL
	100 µM HTCR_o_alpha	1 µL
	100 µM TRBC1_3UTR	1 µL
	100 µM TRBC2_3UTR	1 µL
	100 µM HTCR_o_beta	1 µL
	H_2O	5 µL
	Total	10 µL

V(D)J primer mix

b. Prepare V(D)J Amplification 1 reaction mix on ice

	Component	Volume	Final conc.
	cDNA template	22.5 µL	—
	2X KAPA HiFi HotStart ReadyMix	25.6 µL	1X
	V(D)J 1 Primer Mix	3.1 µL	0.3 µM
	Total	51.2 µL	

V(D)J amplification 1 reaction mix

- Mix gently and briefly spin down.

11 **PCR cycling**

Run the following program:

	Step	Temperature	Duration	Cycles
	Initial denaturation	95 °C	3 min	1
	Denaturation	98 °C	20 s	12–15*

	Step	Temperature	Duration	Cycles
	Annealing	62 °C	30 s	
	Extension	72 °C	1 min	
	Final extension	72 °C	1 min	1

PCR programm design

∗Use **12 cycles** as default; increase up to **15 cycles** for low T cell content or low DNA yield.

12 **V(D)J Amplification 1 Cleanup**

Purify the first V(D)J amplification using AMPure XP beads.

1. Vortex AMPure XP beads thoroughly.
2. Add **46.1 µL AMPure XP beads (0.8X)** to each PCR reaction.
3. Mix by pipetting 15 times.
4. Incubate **5 min at room temperature**.
5. Place tubes on the magnet (high) until the solution clears.
6. Carefully remove and discard the supernatant.
7. Add **200 µL 80% ethanol** to the pellet and incubate ~30 s.
8. Remove ethanol.
9. Repeat the ethanol wash for a total of **2 washes**.
10. Air-dry the pellet for **2-3 min**.
11. Remove from the magnet and add **10 µL EB buffer**.
12. Resuspend thoroughly by pipetting ~15 times.
13. Incubate **5 min at room temperature**.
14. Place on the magnet (low) until the solution clears.
15. Transfer **10 µL** to new tubes. This is the template for V(D)J Amplification 2.

V(D)J Amplification 2 (inner PCR)

13 This second nested PCR further enriches TCR V(D)J sequences and introduces inner primers.

a. Prepare V(D)J 2 primer mix on ice

	Primer	Volume
	100 µM TRAC_3UTR_2	1 µL
	100 µM HTCR_i_alpha	1 µL

	Primer	Volume
	100 µM TRBC1_3UTR	1 µL
	100 µM TRBC2_3UTR	1 µL
	100 µM HTCR_i_beta	1 µL
	H_2O	5 µL
	Total	10 µL

V(D)J 2 primer mix

b. Prepare V(D)J Amplification 2 reaction mix on ice (25 µL reaction)

	Component	25 µL reaction	Final conc.
	cDNA template (from step 10)	10 µL	—
	2X KAPA HiFi HotStart ReadyMix	12.5 µL	1X
	V(D)J 2 Primer Mix	1.5 µL	0.3 µM
	H_2O	1 µL	N/A

V(D)J 2 amplification mix

- Mix gently, spin down briefly.

14 **PCR cycling**

	Step	Temperature	Duration	Cycles
	Initial denaturation	95 °C	3 min	1
	Denaturation	98 °C	20 s	10
	Annealing	62 °C	30 s	
	Extension	72 °C	1 min	
	Final extension	72 °C	1 min	1

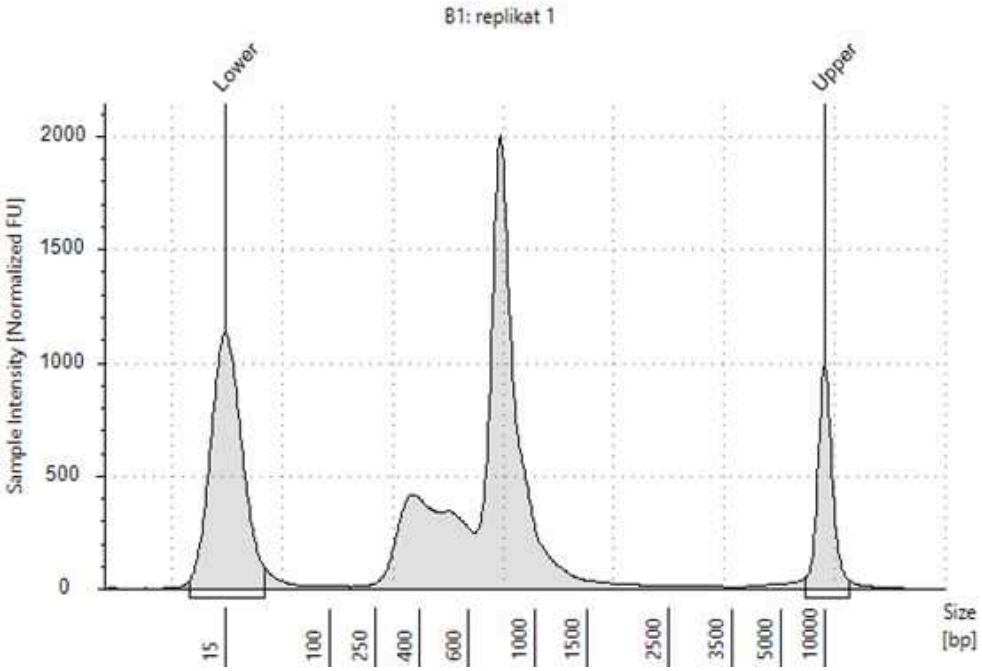
PCR programm design

15 **V(D)J Amplification 2 Cleanup**

1. Vortex AMPure XP beads thoroughly.
2. Add **22.5 µL AMPure XP beads (0.9X)** to each PCR reaction.
3. Mix by pipetting 15 times.
4. Incubate **5 min at room temperature**.
5. Place tubes on the magnet (high) until the solution clears.
6. Remove and discard the supernatant.
7. Add **200 µL 80% ethanol** to the pellet; incubate ~30 s.
8. Remove ethanol.
9. Repeat the ethanol wash for a total of **2 washes**.
10. Air-dry the pellet for **2-3 min**.
11. Remove from magnet and add **20 µL EB buffer**.
12. Resuspend thoroughly (15× pipetting).
13. Incubate **5 min at room temperature**.
14. Place on the magnet (low) until the solution clears.
15. Transfer **20 µL** to new tubes. This is your enriched V(D)J cDNA for library construction.

16 **V(D)J Amplification QC**

- **Qubit dsDNA HS assay** – quantify DNA.
- **Agilent TapeStation HS D5000** – confirm size distribution and enrichment of V(D)J products.



Amplified TCR cDNA library from human PBMCs. Initial cDNA was generated with the 3'GEX v3.1 assay (10X Genomics)

V(D)J Library Construction

17 Proceed to "V(D)J Library Construction" from Chromium Next GEM Single Cell 5' v2 (Dual Index)

Input: 2-3 ng

Fragmentation, End Repair & A-tailing

a. Determine the volume for **25% concentration** of sample. Dispense the sample volume in a tube on ice. If the volume required is less than 20 µL, adjust the total volume of each sample to 20 µL with nuclease-free water.
b. Vortex Fragmentation Buffer. Verify there is no precipitate.
c. Prepare Fragmentation Mix on ice. Pipette mix and centrifuge briefly.

Component	PN	1X (µL)	4X + 10% (µL)
Nuclease-free Water	-	15	66

	Fragmentation Buffer	2000091	5	22
	Fragmentation Enzyme	2000090/2000104	10	44
	Total	-	30	132

Fragmentation Mix

d. Add 30 µl Fragmentation Mix into each tube containing 20 µl sample.
e. Pipette mix 15x (pipette set to 30 µl) on ice. Centrifuge briefly.
f. Incubate (Lid Temperature: 65 °C):

	Step	Temperature	Duration
	Fragmentation	32 °C	2 min
	End repair & A-tailing	65 °C	30 min
	Hold	4 °C	hold

Fragmentation thermal programm

18 **Adaptor Ligation**

a. Prepare adapter ligation mix
For each sample:

	Component	PN	1X (µL)	4X + 10% (µL)
	Ligation Buffer	2000092	20	88
	DNA Ligase	220110/220131	10	44
	Adapter Oligos	2000094	20	88
	Total	—	50	220

Adaptor Ligation Mix

Mix well by pipetting and spin down briefly.

b. Ligation reaction
1. Remove samples from the thermal cycler.
2. Add **50 µL Adapter Ligation Mix** to each **50 µL** fragmented sample (total 100 µL).

3. Mix by pipetting 15× (pipette set to 90 µL).
4. Spin down briefly.

c. Incubation
Incubate in a thermal cycler (lid at 30 °C):

	Step	Temperature	Duration
	1	20 °C	15 min
	2	4 °C	hold

Adaptor Ligation programm

19 **Post Adaptor Ligation Cleanup - SPRIselect**

1. Vortex SPRIselect reagent thoroughly.
2. Add 0.8X µL SPRIselect (80 µL) to each 100 µL ligation reaction.
3. Mix by pipetting 15× (pipette set to 150 µL).
4. Incubate 5 min at room temperature.
5. Place tubes on the magnet (high) until the solution is clear.
6. Remove and discard the supernatant.
7. Add 200 µL 80% ethanol to the pellet; wait 30 s.
8. Remove ethanol.
9. Repeat the ethanol wash for a total of 2 washes.
10. Briefly centrifuge and return the tubes to the magnet (low).
11. Remove any residual ethanol and air-dry the pellet for 2 min.
12. Remove tubes from the magnet and add **30.5 µL EB buffer**.
13. Resuspend beads thoroughly by pipetting 15× (continue mixing if beads appear clumpy).
14. Incubate 5 **min at room temperature**.
15. Place on the magnet (low) until the solution clears.
16. Transfer **30 µL** of the eluate to a new tube strip. This is the adapter-ligated DNA for indexing PCR.

20 **Sample Index PCR**

This step adds sample indices and completes library amplification.

a. Choose sample indices
Select appropriate sample index primers such that no indices overlap between samples in a multiplexed run.

b. Prepare Sample Index PCR reaction mix (example for sample 1)

	Component	Volume
	Amp Mix (PN-2000047/2000103)	50 µL
	P7_TRAC_3UTR_f3_i7_A1 (10 µM)	3 µL
	P7_TRBC1_3UTR_f_i7_A1 (10 µM)	3 µL
	P7_TRBC2_3UTR_f_i7_A1 (10 µM)	3 µL
	P5_SI-TT-A1 (10 uM)	3 µL
	H_2O	8 µL
	Total	70 µL

Sample Index PCR mix

(For additional samples, use the corresponding index primer pairs A2, A3, A4, etc.)

c. PCR setup

1. Add **70 µL Sample Index PCR Reaction Mix** to **30 µL** of adapter-ligated DNA (total 100 µL).
2. Mix gently and spin down.

d. PCR cycling

Run the following program (lid at 105 °C):

	Step	Temperature	Duration	Cycles
	Initial denaturation	98 °C	45 s	1
	Denaturation	98 °C	20 s	8
	Annealing	54 °C	30 s	
	Extension	72 °C	20 s	

	Step	Temperature	Duration	Cycles
	Final extension	72 °C	1 min	1

Sample Index PCR thermal programm

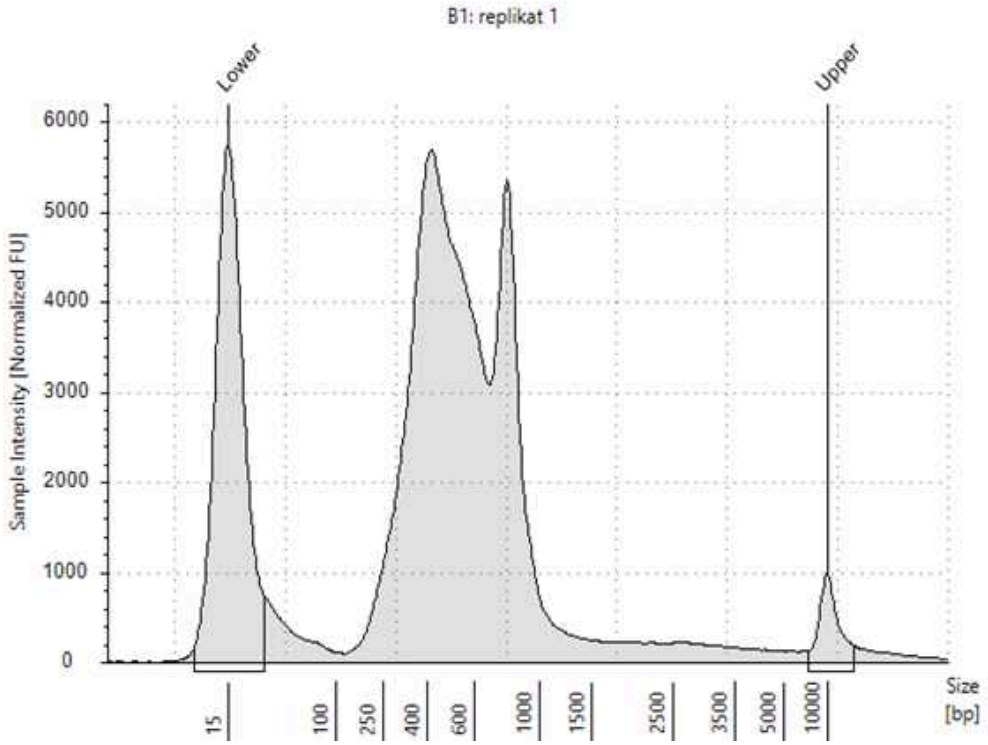
21 **Post Sample Index PCR Cleanup – SPRIselect**

1. Vortex SPRIselect reagent thoroughly.
2. Add 0.9X **SPRIselect (90 µL)** to each 100 µL PCR reaction.
3. Mix by pipetting 15× (pipette set to 150 µL).
4. Incubate **5 min at room temperature**.
5. Place on the magnet (high) until the solution clears.
6. Remove the supernatant carefully.
7. Add **200 µL 80% ethanol**; wait **30 s**.
8. Remove ethanol.
9. Repeat the ethanol wash for a total of **2 washes**.
10. Briefly centrifuge and place back on the magnet (low).
11. Remove any residual ethanol and air-dry the pellet for **2 min**.
12. Remove from the magnet and add **25.5 µL EB buffer**.
13. Resuspend beads by pipetting 15×.
14. Incubate 5 **min at room temperature**.
15. Place on the magnet (low) until the solution clears.
16. Transfer **25 µL** of the eluate to a new tube strip.
17. Store libraries at **4 °C for up to 72 h** or at **−20 °C** for long-term storage.

22 Post Sample Index PCR QC

Perform final library QC:

- **Qubit dsDNA HS assay** – for accurate concentration.
- **Agilent TapeStation HS D1000** – to verify the expected library size distribution and absence of major contaminants.



TCR short-read sequencing library

Library Quantification and Sequencing

23 **qPCR quantification**

- Quantify final circVDJ-seq libraries using a suitable qPCR-based library quantification kit (e.g. for Illumina platforms), following the manufacturer's instructions.
- Normalize libraries to the desired loading concentration for NextSeq 550 Mid-Output runs.

Sequencing run setup

- Sequence libraries on an **Illumina System** with the following **custom read configuration: 90–28–10–10 (note that in contrast to the standard Illumina workflow, the library indices are sequenced in the last two sequencing cycles).**
- Use the following **custom sequencing primers**:

	Primer name	Read (cycles)	Sequence (5' → 3')
	spTCR_Read 1 (90)	VDJ sequence (90)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
	spTCR_Read 2 (28)	Cell barcode + UMI (28)	CATACTTGCTCCTACACGACGCTCTTCCGATCT
	spTCR_Read 3 (10)	i5 Index (10)	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC
	spTCR_Read 4 (10)	i7 Index (10)	CAAGCAGAAGACGGCATACGAGATCTGAGTCAGTAGC*G

Custom Sequencing Primers

Protocol references

https://www.biorxiv.org/content/10.1101/2025.09.16.675546v1.full