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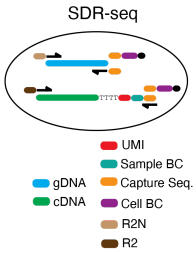
scDNA-scRNA-seq (SDR-seq) V.1



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Protocols

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We use this protocol and it's working

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Abstract

scDNA-scRNA-seq (SDR-seq) is a method that enables a targeted readout of both genomic and transcriptomic targets in thousands of single cells per experiment. It combines an *in-situ* reverse transcription (RT) step of fixed cells with a multiplexed PCR in an emulsion-based droplets using the microfluidic Tapestri device from MissionBio. A single-cell suspension is fixed with glyoxal, permeabilized and used to perform *in-situ* reverse transcription. This converts RNA to cDNA while adding unique molecular identifiers (UMIs), a sample barcode (sample BC) and a capture sequence to the cDNA. These cells containing cDNA and gDNA are used as an input for the Tapestri machine where cells are lysed, treated with proteinase K and mixed with reverse primers for each gDNA or RNA target in a first droplet. This droplet is then fused into a second droplet that contains PCR reagents, forward primers containing a capture sequence (CS) overhang and a cell barcoding bead with distinct cell barcode oligos and a matching CS. The multiplexed PCR amplifies gDNA and RNA targets simultaneously in each droplet while cell barcoding is ensured by the complementary CS in the forward primers and the cell barcode oligos. After breaking emulsions, separate NGS libraries for both gDNA and RNA can be generated by having distinct overhangs on the reverse primers (R2N Nextera for gDNA; R2 TruSeq for RNA). This enables to sequence each library at different sequencing depth and length.

Materials

Glyoxal – Sigma #128465
Acetic acid (Glacial) – Sigma #695092
RNasin® Ribonuclease Inhibitor – Promega #N2615
Enzymatics Rnase Inhibitor - Enzymatics #280520
IGEPAL CA-630 – Sigma #I8896
Digitonin – ThermoFisher Scientific #BN2006
dNTP-Mix - ThermoFisher Scientific #R0192
Maxima H Minus Reverse Transcriptase - ThermoFisher Scientific #EP0751
Tapestri Single-Cell DNA CoreAmbient Kit v2 - MB51-0007
Tapestri Single-Cell DNA Core -20 Kit v2 - MB51-0010
Tapestri Single-Cell DNA Bead Kit - MB51-0009

Troubleshooting

Design and order oligos

1 **In-situ RT primers**

The following oligos need to be ordered to perform the *in-situ* RT reactions. We recommend ordering at least 8 RT primers for optimal performance of SDR-seq. More RT primers can be ordered in case more samples are required or to improve removal of contaminating ambient RNA reads during analysis.

	A	B
	Name	**Sequence**
	RT_primer_1	GTACTCGCAGTAGTCGACACGTCTCGCCTTANNNNNNNNTTTTTTTTTTTTTTTTVN
	RT_primer_2	GTACTCGCAGTAGTCGACACGTCCTAGTACGNNNNNNNNTTTTTTTTTTTTTTTTVN
	RT_primer_3	GTACTCGCAGTAGTCGACACGTCTTCTGCCTNNNNNNNNTTTTTTTTTTTTTTTTVN
	RT_primer_4	GTACTCGCAGTAGTCGACACGTCGCTCAGGANNNNNNNNTTTTTTTTTTTTTTTTVN
	RT_primer_5	GTACTCGCAGTAGTCGACACGTCAGGAGTCCNNNNNNNNTTTTTTTTTTTTTTTTVN
	RT_primer_6	GTACTCGCAGTAGTCGACACGTCCATGCCTANNNNNNNNTTTTTTTTTTTTTTTTVN
	RT_primer_7	GTACTCGCAGTAGTCGACACGTCGTAGAGAGNNNNNNNNTTTTTTTTTTTTTTTTVN
	RT_primer_8	GTACTCGCAGTAGTCGACACGTCCCTCTCTGNNNNNNNNTTTTTTTTTTTTTTTTVN

Overview of 8 RT primers used for in-situ RT reaction. CS: GTACTCGCAGTAGTC; CS extension for universal primer: GACACGTC. Sample barcodes: N701-706

2 **Targeted primer design for gDNA and RNA primers**

Targeted gDNA primers were designed using the Tapestri Designer online tool (https://designer.missionbio.com). Targeted RNA primers were selected by taking the "inner primer" sequences designed using the TAP-seq primer prediction tool with at targeted Tm of 60 ° C (min 58 ° C and max 62 ° C) and a product size range from 150-300 bp (https://github.com/argschwind/TAPseq). Two target-specific primers are needed for each gDNA target. Only one target-specific primer is needed for RNA targets, because all RNA targets are amplified by a universal primer binding to the CS + extension introduced via the *in-situ* RT. Primers designed by the respective design tools require overhangs as described in the table below.

	A	B
	Name	**Sequence**
	Droplet_gDNA_fwd	GTACTCGCAGTAGTC-gDNA_specific_sequence
	Droplet_gDNA_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-gDNA_specific_sequence
	Droplet_RNA_fwd	GTACTCGCAGTAGTCGACACGTC
	Droplet_RNA _rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-cDNA_specfic_sequence

3 **Primers for separate library generation**

After breaking emulsions following the multiplexed PCR in emulsion-based droplets library primers with the respective overhangs are required to give specificity for gDNA (R2N) and RNA (R2) targets. gDNA targets will be amplified with library primers containing R1N and R2N binding sequences. RNA targets will be amplified with library primers containing R1N and R2 binding sequences.

	A	B
	Name	**Sequence**
	R1N_i5_S502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC

	A	B
	R1N_i5_S503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTC
	R1N_i5_S505	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC
	R1N_i5_S506	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC
	R1N_i5_S507	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTC
	R1N_i5_S508	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGTC
	R2N_i7_N701	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG
	R2N_i7_N702	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG
	R2N_i7_N703	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG
	R2N_i7_N704	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG
	R2N_i7_N705	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG
	R2N_i7_N706	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG
	R2_i7_N701	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTGACTGGAGTTCA

	A	B
		GACGTGT
	R2_i7_N702	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTGACTGGAGTTCA GACGTGT
	R2_i7_N703	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTGACTGGAGTTCA GACGTGT
	R2_i7_N704	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTGACTGGAGTTCA GACGTGT
	R2_i7_N705	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTGACTGGAGTTCA GACGTGT
	R2_i7_N706	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTGACTGGAGTTCA GACGTGT

Prepare primer pools for targeted gDNA and RNA amplification

4 **Prepare oligo pools**

4.1 Forward and reverse oligo pools containing the targeted gDNA and RNA primers were ordered as oPools from IDT (50 pmol/oligo).

4.2 Reverse gDNA and RNA oligo pools were diluted to a concentration of 120 µM in H2O each and then mixed 1:1 for usage in Step 2.6 of the **Tapestri V2 User Guide**.

4.3 Forward gDNA oligo pools were diluted to 20 µM. The universal Droplet_RNA_fwd oligo that binds at all RNA targets was also diluted to 20 µM. The forward gDNA oligo pools and the universal Droplet_RNA_fwd oligo were mixed 1:1 for usage in Step 4.8 of the **Tapestri V2 User Guide**.

Prepare Buffers

5 All Buffer reactions are calculated for fixation of two sampels. Adjust volumes according to sample number.

Glyoxal fixation buffer pH 0

	A	B	C	D	E
	Reagent	**Stock**	**Final (1x)**	**Volume (µl)**	**Comment**
	EtOH (%)	100	20	400	
	Glyoxal (%)	40	3	150	
	Acetic acid (Glacial) (%)	100	0.75	15	
	NaCl (mM)	5000	120	48	
	H2O			1362	
	NaOH (1M)			25	to pH 4 (adjust volume)
	Final Volume			2000	

Wash Buffer 1

	A	B	C	D
	Reagent	**Stock**	**Final (1x)**	**Volume (µl)**
	DTT (mM)	100	1	45
	BSA (%)	10	2	900
	RNasin Ribonuclease Inhibitor (U/µl)	20	0.04	9
	PBS (1x)			3546

	A	B	C	D
	Final Volume			4500

Wash Buffer 2

	A	B	C	D
	Reagent	**Stock**	**Final (1x)**	**Volume (µl)**
	TRIS pH7.5 (mM)	1000	10	40
	NaCl (mM)	5000	10	8
	MgCl2 (mM)	1000	3	12
	Tween20 (%)	10	0.1	40
	RNasin Ribonuclease Inhibitor (U/µl)	20	0.1	20
	DTT (mM)	100	1	40
	BSA (%)	10	2	800
	H2O			3040
	Final Volume			4000

Permebalization Buffer

	A	B	C	D	E
	Reagent	**Stock**	**Final (1x)**	**Volume (µl)**	**Comment**
	IGEPAL CA-630 (%)	10	0.1	6	
	Digitonin (%)	5	0.01	1.2	preheat to 65 C for 10 min

	A	B	C	D	E
	Wash Buffer 2			592.8	
	Final Volume			600	

Resuspension buffer

	A	B	C	D
	Reagent	**Stock**	**Final (1x)**	**Volume (µl)**
	DTT(mM)	100	1	10
	BSA (%)	10	2	200
	RNasin Ribonuclease Inhibitor (U/µl)	20	0.3	15
	1x PBS			775
	Final Volume			1000

Dissociation - Fixation - Permeablization

6 **Dissociation**

6.1 Prepare single cell suspension according to cell line or tissue requirements as for other single cell technologies. Filter cell suspension through a 40 µm cell strainer.

6.2 We recommend using 1.5×10^6 cells as input for fixation. We tested performance of SDR-seq with a minimum of 0.35×10^6 cells as input for fixation. Resuspend cells in 1 ml DPBS -/-. To proceed with sampels viability should be >85 %.

Example of cells before fixation using Countess II

7 Fixation

7.1 We recommend using 15 ml Polypropylene centrifuge tubes and swinging bucket rotors in the appropriate centrifuges to minimize cell loss during this process.

7.2 Spin cells at 300g for 5 min and remove supernatant.

7.3 Resuspend cells in 200 µl of glyoxal fixation buffer (pH adjusted) and incubate at room temperature for 7 min.

7.4 Add 1 ml of ice-cold wash buffer 1 and gently invert the tube 2-3 times to mix.

7.5 Spin at 400 g for 5 min at 4 °C and carefully take off supernatant.

7.6 Repeat step 7.4 and 7.5 for a total of 2x washes.

7.7 Resuspend cells in 175 µl ice-cold permeabilization buffer and incubate on ice for 4 min.

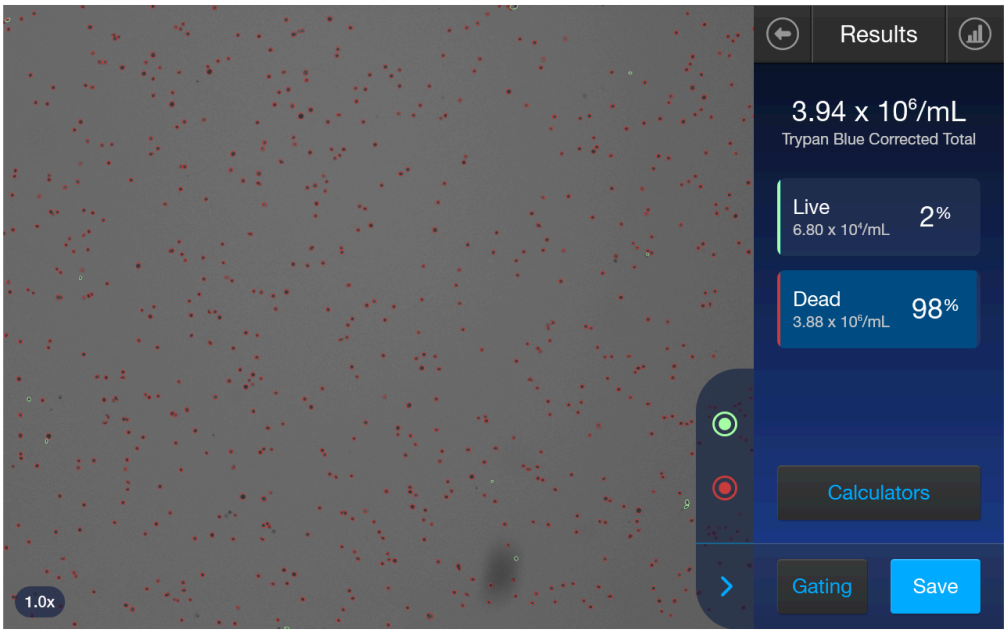
7.8 Add 1 ml of ice-cold wash buffer 2 and gently invert the tube 2-3 times to mix.

7.9 Spin at 400 g for 5 min at 4 °C and carefully take off supernatant.

7.10 Resuspend cells in 200 µl resuspension buffer.

7.11 Filter cell suspension through a 40 µm cell strainer.

7.12 Count cells.



Example of cells after fixation using Countess II

7.13 Dilute cell suspension to 1.4×10^6 cells/ml

In-situ reverse transcription

8 We recommend to use a total of 48 wells of a 96-well PCR plate with 10,000 cells/well per experiment during in-situ reverse transcription (RT). This is calculated to have enough surplus for the Tapestri microfluidic device (optimal input ranges from 1.05-2×10^5 cells). Multiple samples can be multiplexed per Tapestri lane by using distinct sample barcodes. We recommend at using at least 8 RT sample barcode primers per SDR-seq experiment to improve removal of ambient RNA during analysis.

8.1 Prepare the following RT MM on ice. Mix by pipetting up and down for 10x times.

	A	B	C	D	E
	Reagent	**Stock**	**Final**	**1x (µl)**	**52x (µl)**
	5x Maxima H Minus RT Buffer	5	1	4	208
	Enzymatics Rnase Inhibitor (U/µl)	40	0.25	0.125	6.5
	RNasin Ribonuclease Inhibitor (U/µl)	20	0.25	0.25	13
	dNTPs (µM per base)	10000	500	1	52
	Maxima H Minus RT (U/µl)	200	20	2	104
	H2O	NA	NA	0.625	32.5
	Total volume per reaction			8	416
	Final reaction volume			20	1040

8.2 If using 8 sample barcodes as we recommend prepare the following master mix per sample barcode on ice. Otherwise adjust accordingly.

	A	B	C
		1x (µl)	**6.25x (µl)**
	MM RT	8	50
	RT Primer (12.5 µM)	4	25
	Fixed cells (1.4 M/ml)	8	50

8.3 Add cells last, mix by pipetting up and down 10x.

8.4 Aliquot 20 µl of the master mix containing RT primers and fixed cells into the appropriate number of wells into a 96-well plate. In the above example this would be 6 wells per sample barcode.

8.5 Put the RT-plate into a Thermocycler that is preheated to 50 °C and run the following program to perform the *in-situ* RT:

	A	B	C
	Tempearture °C	**Time**	**Cycle**
	50	10 min	
	8	12 sec	3x
	15	45 sec	
	20	45 sec	
	30	30 sec	
	42	2 min	
	50	3 min	
	50	5 min	

	A	B	C
	4	-	

8.6 Put the RT plate on ice.

8.7 Pool all RT reactions into a 15 ml Polypropylene centrifuge tube containing 10 ml DPBS -/- with 1 % BSA. Make sure to pipet up and down in the RT plate 2-3 times without introducing bubbles to resuspend cells in the RT plate before pooling. Wash each well with 200 µl DPBS -/- with 1 % BSA to reduce cell loss during this step.

8.8 Spin cells at 500 g for 10 min at 4 °C and carefully take off supernatant.

8.9 Resuspend in 35-50 µl ice-cold Cell Buffer provided by MissionBio.

8.10 Count and dilute cell suspension to 4,500 cells/µl.

Encapsulation, Lysis, Digestion, Barcoding, Targeted PCR and CleanUp

9 We then followed the **Tapestri V2 User Guide** Step2 (Encapsulate Cells), Step 3 (Lyse and Digest Cells), Step 4 (Barcode Cells), Step 5 (UV Treatment and Targeted PCR Amplification) and Step 6 (Cleanup PCR Products). gDNA and RNA targets combined were treated as the total amplicon panel size and the specifications of the multiplexed PCR was adjusted according to the recommendations of the **Tapestri V2 User Guide**.

Library PCR

10 Library PCR primers were used to amplify separate gDNA (R1N + R2N) and RNA (R1N +R2) NGS libraries.

10.1 Set up the following PCR reactions:

PCR – gDNA library

	A	B
	Reagent	**µl**
	MisssionBio Library PCR MM (2x)	25
	Cleaned up sample	15
	Primer R1N – i5 (2.5 µM)	5
	Primer R2N – i7 (2.5 µM)	5

PCR – RNA library

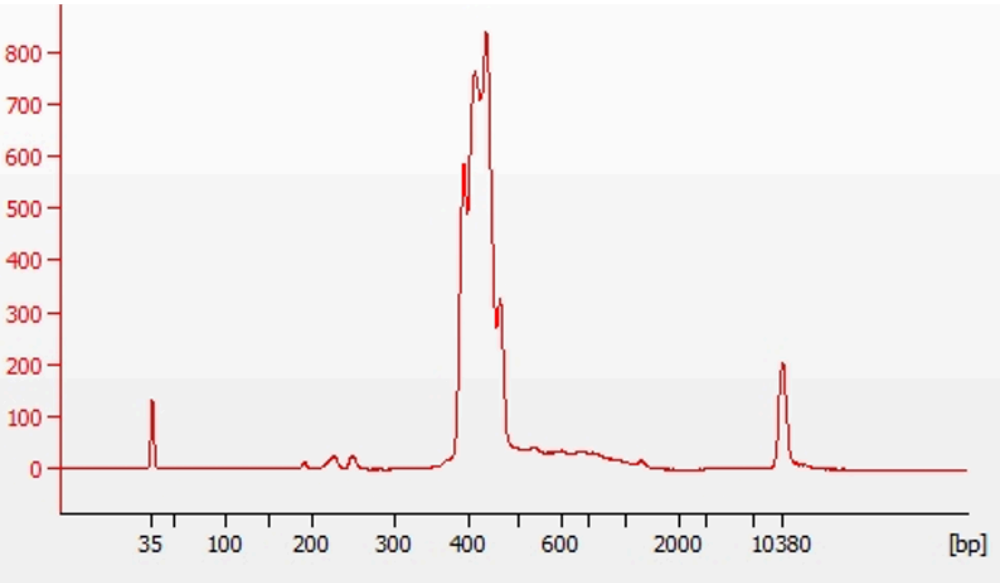
	A	B
	Reagent	**µl**
	MisssionBio Library PCR MM (2x)	25
	Cleaned up sample	15
	Primer R1N – i5 (2.5 µM)	5
	Primer R2N – i7 (2.5 µM)	5

10.2 Run the following PCR cycling program for both libraries on a thermocycler.

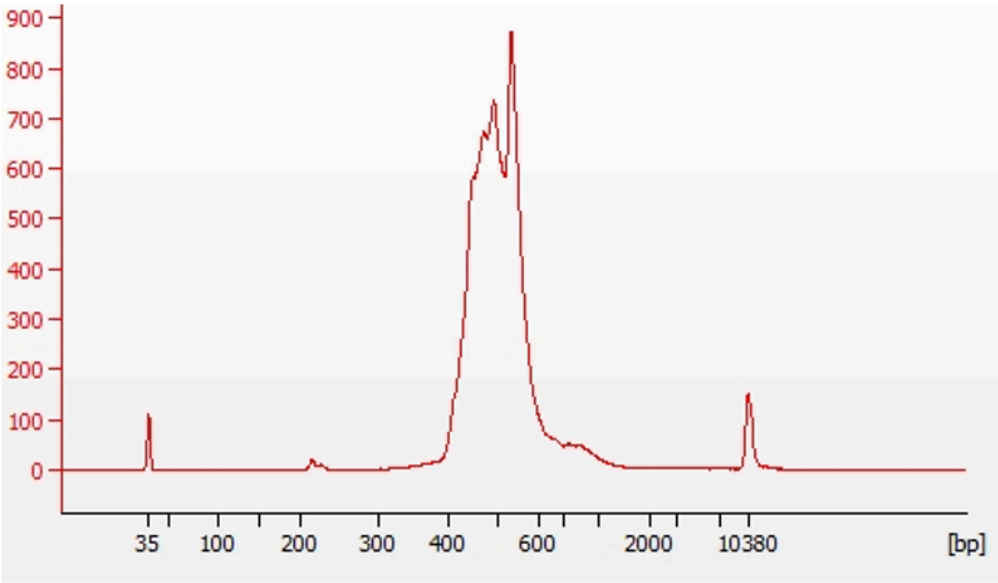
	A	B	C
	Tempearture °C	**Time**	**Cycle**
	95	3 min	
	98	20 sec	10x
	62	20 sec	

	A	B	C
	72	45 sec	
	72	2 min	
	4	-	

10.3 Follow the library cleanup described in Step 7.7 and the subsequent recommendations in Step 8 (Quantify and Normalize Sequencing Library) in the **Tapestri V2 User Guide**.



Example Bioanalyzer track of gDNA library.



Example Bioanalyzer track of RNA library.

Sequencing

11 The separate gDNA and RNA libraries are sequenced with the following read length specifications on Illumina sequencers. Read depth can be adjusted according to experimental needs. We recommend a read depth of at least 10000 reads/cell per 100 amplicons for each library.

gDNA library

	A	B	C
	Read	**Cycles**	**Type**
	R1N	161	Cell barcode + gDNA amplicon
	R2N	161	gDNA amplicon
	i5	8	Library PCR sample BC
	i7	8	Library PCR sample BC

RNA library

	A	B	C
	Read	**Cycles**	**Type**
	R1N	76	Cell barcode + *in-situ* RT sample BC +UMI
	R2	46	RNA amplicon
	i5	8	LibraryPCR sample BC
	i7	8	Library PCR sample BC

Analysis

12 To generate count matrices for gDNA and RNA libraries please use the package **SDRranger**. We recommend selecting for high-quality cells first followed by variant calling using the GATK HaplotypeCaller (v4.2.3.0) in each cell separately.

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