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R2C2 protocol using 10x Single-Cell RNA-seq Assay cDNA

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We use this protocol and it's working

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Abstract

This protocol is based on the published Rolling Circle to Concatemeric Consensus (R2C2) method (1). The aim is to make elongated cDNA via rolling circle amplification using cDNA generated from the 10x Chromium 3' (or 5') single-cell RNA-seq assay to increase the accuracy of downstream sequencing on an Oxford Nanopore Technologies sequencing platform. The major modification from the original protocol is to add an additional cDNA re-amplification step (see section "10x cDNA re-amplification") to enrich for fragments with the proper sequence (poly(T) at 3' and TSO at 5') at both ends, which is essential in circularization with the 10x Splint fragment.

Image Attribution

Xian Adiconis

Materials

Oligos

	A	B	C
	Oligo Name	Vendor	Sequence
	10X_UMI_Splint_Forward	IDT	5'-AGATCGGAAGAGCGTCGTGTAGTGAGGCTGATGAGTTCCATANNNNTATATNNNNNATCACTACTTAGTTTTTTGATAGCTTCAAGCCAGAGTTGTCTTTTTCTCTTTGCTGGCAGTAAAAG -3'
	10X_UMI_Splint_Reverse	IDT	5'-CTCTGCGTTGATACCACTGCTTAAAGGGATATTTTCGATCGCNNNNNATATANNNNNTTAGTGCATTTGATCCTTTTACTCCTCCTAAAGAACAACCTGACCCAGCAAAAGGTACACAATACTTTTACTGCCAGCAAAGAG -3'
	PCR_primer1	IDT	5'- CTACACGACGCTCTTCCGATCT -3'
	PCR_primer2	IDT	5'- AAGCAGTGGTATCAACGCAGAGT -3'

Reagents

	A	B	C
	Reagents	Vendor	Part Number
	KAPA HiFi HotStart ReadyMix	KAPA Bioscience	KK2602
	NEBNext® High-Fidelity 2X PCR Master Mix	New England Biolabs	M0541S
	Select-a-Size DNA Clean & Concentrator Kit	Zymo Research	D4080
	ProNex® Size-Selective Purification System, 10 ml	Promega	NG2001
	NEBuilder® HiFi DNA Assembly Master Mix	New England Biolabs	E2621S
	NEB Buffer 2	New England Biolabs	B7002S
	Exonuclease I (20 U/µl)	New England Biolabs	M0293S
	Exonuclease III (100 U/µl)	New England Biolabs	M0206S
	Lambda Exonuclease (5 U/µl)	New England Biolabs	M0262S
	Agencourt AMPure® XP SPRI beads, 60 ml	Beckman Coulter Genomics	A63881
	phi29 DNA Polymerase (10 U/µl)	New England Biolabs	M0269S
	Deoxynucleotide (dNTP) Solution Mix (10mM)	New England Biolabs	N0447L
	Exo-Resistant Random Primer (10 µM)	Thermo Fisher Scientific	SO181
	T7 Endonuclease I (10 U/µl)	New England Biolabs	M0302S

Safety warnings

For hazard information and safety warnings, please refer to the MSDSs (Material Safety Data Sheets).

Splint generation

2h

1 Prepare the following on ice

10m

	A	B
		1X (µl)
	KAPA HiFi HotStart ReadyMix	25
	H2O	23
	10x_UMI_Splint_forward (100 µM)	1
	10x_UMI_Splint_reverse (100 µM)	1
	Total	50

2 Run the following program

11m

Thermocycler Conditions: (Lid@ 105 °C , 50 µl)

95 °C , 00:03:00

followed by 1 cycle of:

98 °C , 00:01:00

62 °C , 00:01:00

72 °C , 00:06:00

then

4 °C , 00:00:00 hold

3 Cleanup with Select-a-Size columns (Zymo) and a size cut-off of ~125 bp. Follow the Select-a-Size cleanup protocol for single-size selection, but in the buffer preparation add 85 µL of 100% ethanol to 500 µL of Select-a-Size DNA binding buffer. Elute in 15 µL DNA elution buffer (from the kit). QC with 1/5x dilution for Quant-it (Thermo Fisher Scientific) and 1/50x dilution for BioAnalyzer DNA HS assay (Agilent).

1h 30m

Label this product as "10x Splint", store at -20 °C for later use.

10x cDNA re-amplification

2h

4 Enrichment PCR

10m

Prepare the follow reaction mix on ice

	A	B
		1X (µl)
	NEBNext® High-Fidelity 2X PCR Master Mix	50
	12 µM PCR Primer 1	2
	12 µM PCR Primer 2	2
	20 ng 10x cDNA in H2O	46
	Total	100

5 Run the following program 9m 10s

Thermocycler Conditions: (Lid@ 105 °C, 100 µl)

98 °C, 00:00:45

followed by 10 cycle of:

98 °C, 00:00:10

62 °C, 00:00:15

72 °C, 00:03:00

then

72 °C, 00:05:00

4 °C, 00:00:00 hold

6 cDNA purification 1h 30m

6.1 Add 95 µL of resuspended, room-temperature ProNex beads to the PCR mix. Pipette mix 10 times. Perform a quick spin to collect all liquid from the sides of the tube.

6.2 Incubate sample on bench top for 00:05:00 at Room temperature. 5m

6.3 Place the tube on a magnetic stand to separate the beads from the supernatant. Use a P200 pipettor to remove the supernatant.

6.4 Wash 2 times with 200 µL of freshly prepared 80% ethanol. After removal of the second wash of 200 µL of ethanol, spin the tube strip briefly, return to magnetic stand and remove residual ethanol with a P20 pipettor. Do not let the beads dry out.

6.5 ▪ Remove the tube from the magnetic stand. Immediately add 15 µL of buffer EB (10 mM Tris-HCl, pH 8.5) and pipette mix to resuspend. Perform a quick spin to collect all liquid from the sides of the tube. Place at Room temperature for 00:05:00 to elute the DNA from the beads. 5m

6.6 Place the tube on a magnetic stand to separate the beads from the supernatant. Transfer the eluted cDNA samples to a new tube.

6.7 QC with 1/5x dilution for Quant-it and BioAnalyzer DNA HS assay. Store the remaining product at -20 °C for later use.

cDNA circularization 8h

7 Circularization 7h 20m

Prepare the following mix

	A	B
		1X (µl)
	10x Splint (200 ng)	2
	Re-amplified cDNA (200 ng)	8
	NEBuilder® HiFi DNA Assembly Master Mix	10
	Total	20

Incubate @ 50 °C for 01:00:00

Then add the following mix

	A	B
		1X (µl)
	NEB Buffer 2	5
	H2O	16
	Exonuclease I (20 U/µl)	3
	Exonuclease III (100 U/µl)	3
	Lambda Exonuclease (5 U/µl)	3
	Total	30

Incubate @ 37 °C for 06:00:00 , then @ 80 °C for 00:20:00

Cleanup the reaction with 40 µL SPRI beads (0.8x)

8 Circularization product purification 30m

8.1 Add 40 µL (0.8x) of resuspended, room-temperature AMPure XP SPRI beads to the reaction mix. Pipette mix 10 times. Perform a quick spin to collect all liquid from the sides of the tube.

8.2 Incubate sample on bench top for 00:05:00 at Room temperature . 5m

8.3 Place the tube on a magnetic stand to separate the beads from the supernatant. Use a P200 pipettor to remove the supernatant.

8.4 Wash 2 times with 200 µL of freshly prepared 80% ethanol. After removal of the second wash of 200 µL of ethanol, spin the tube strip briefly, return to magnetic stand and remove residual ethanol with a P20 pipettor. Do not let the beads dry out.

8.5 Remove the tube from the magnetic stand. Immediately add 30 µL of H_2O and pipette mix to resuspend. Perform a quick spin to collect all liquid from the sides of the tube. Place at Room temperature for 00:05:00 to elute the cDNA from the beads. 5m

8.6 Place the tube on a magnetic stand to separate the beads from the supernatant. Transfer the eluted cDNA samples to a new tube.

Rolling circle amplification 16h

9 Prepare the following mix, then split into 3 reaction wells of 50 µL each.

	A	B
		1X (µl)
	phi29 Buffer (10x)	15
	phi29 DNA Polymerase (10 U/µl)	3
	dNTP (10 mM)	7.5
	Exo-Resistant Random Primer (10 µM)	7.5
	Circularized cDNA	30
	H2O	87
	Total	150

Incubate in a thermocycler @ 30 °C for Overnight

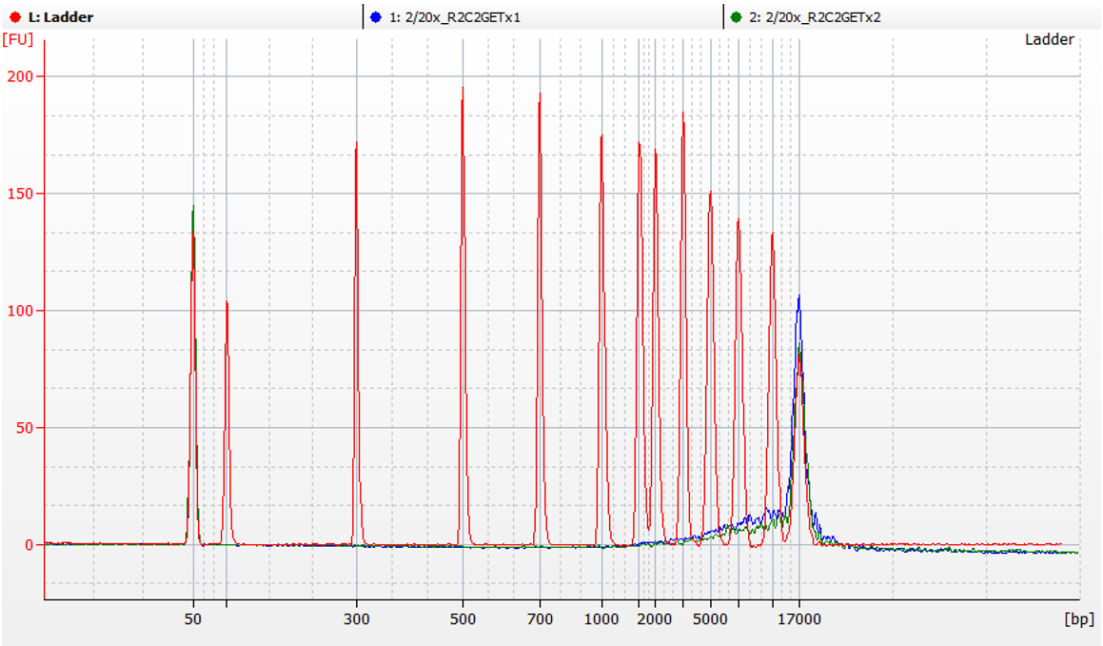
9.1 From this point on, be really careful with the DNA since it will shear pretty easily. Do not pipet up and down and try not to vortex.

10 SPRI clean and T7 endonuclease treatment 2h

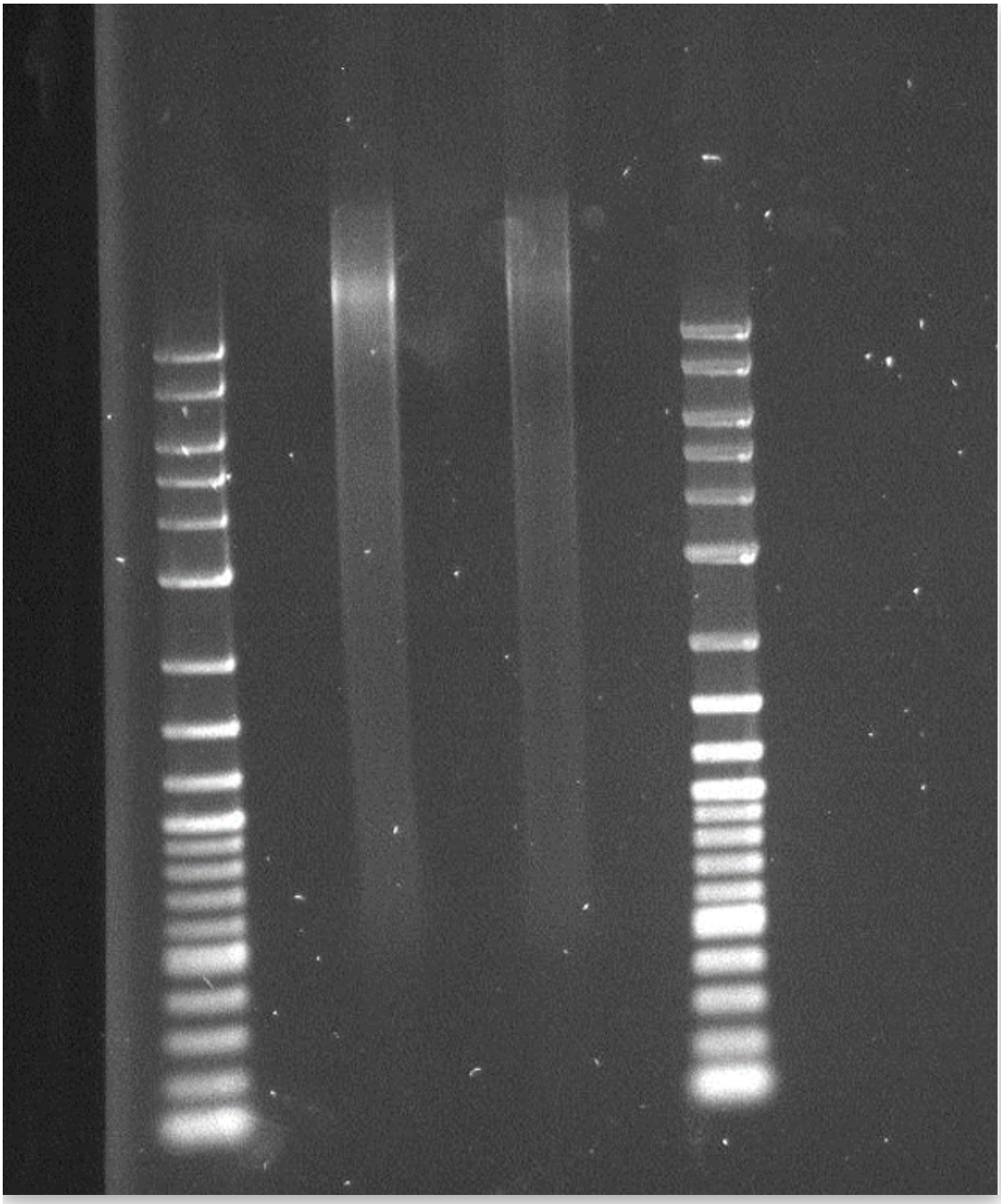
Pool all reactions and adjust volume to 300 µL with H_2O. Add 150 µL 150 µL SPRI beads (0.5x) to your DNA and leave on a rotator for 5 minutes to mix. If not homogeneous, flick the tube to mix.
Notes: After adding the AMPure XP SPRI beads and mixing by inverting the tubes, there's uneven distribution of the beads, mostly the sign of longer cDNA strand.
Place on a magnet stand until a complete pellet forms, wash twice with 500 µL 70% ethanol. After the ethanol removal, add the following mix to re-suspend the beads.

	A	B
		1X (µl)
	NEB Buffer 2	10
	T7 Endonuclease I (10 U/µl)	5
	H2O	90
	Total	105

Incubate on a thermal shaker at 37 °C at 1000 RPM for 02:00:00 . Agitate the tube occasionally to help the solution homogenize.
Place on a magnet stand to pellet the beads. Recover the supernatant that contains the DNA to a new tube. Purify the DNA with 0.5x volume of AMPure XP SPRI beads, wash twice with 500 µL 70% ethanol. Elute in 20 µL H_2O.
QC with Quant-it for concentration, run BioAnalyzer DNA 12000 assay (Agilent) or 1% agarose gel to visualize the size distribution. The majority of the fragments should be > 10kb.



Rolling circle amplified DNA trace with BioAnalyzer DNA 12000 assay



Rolling circle amplified DNA on a 1% agarose gel; the top band of the ladder is 10kb.

The elongated cDNA is now ready for downstream Oxford Nanopore Technologies (ONT) library prep.

Protocol references

1. Volden R, Vollmers C. Single-cell isoform analysis in human immune cells. Genome Biol. 2022;23(1):47.