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We use this protocol and it's working

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FLOUR-seq

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ABSTRACT

We presented three barcoded technologies (BD Rhapsody) that are accessible to most researchers: high-throughput single-cell ONT full-length RNA sequencing (FLOUR-seq). FLOUR-seq combines BD Rhapsody and nanopore sequencing to detect the RNA panorama (including nascent, mature, and degrading RNAs) in cells.The ultralong three barcodes can be discriminated using nanopore technology with 70% debarcoding efficiency and can detect the RNA panorama.

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61436

mouse testis cell dissociation

1d

1 The mouse testis were dissociated to single cells refer to Adult mouse testis cell dissociation (on ice). 1d

Priming and treating the BD Rhapsody Cartridge

20m

2 Prime and treat the BD Rhapsody Cartridge (Cat. No. 400000847). 20m

	A	B	C	D	E
	Step	Regent to load	Volume (µL)	P1200M pipette mode	Incubation at room temp.
	1	100% ethyl alcohol	700	Prime/Treat	-
	2	Air	700	Prime/Treat	-
	3	Room temp. Cartridge Wash Buffer 1 (Cat. No. 650000060)	700	Prime/Treat	1min
	4	Air	700	Prime/Treat	-
	5	Room temp. Cartridge Wash Buffer 1 (Cat. No. 650000060)	700	Prime/Treat	10min
	6	Air	700	Prime/Treat	-
	7	Room temp. Cartridge Wash Buffer 2 (Cat. No. 650000061)	700	Prime/Treat	<4h

Loading cells in cartridge

22m

3 Dilute 20000 cells with cold Sample Buffer (Cat. No. 650000062) to 650 µL. 5m

4 Load cartridge with materials listed using the P1200M pipette: 2m

	A	B	C
	Material to load	Volume (µL)	Pipette mode

	A	B	C
	Air	700	Prime/Treat
	Set P1200M pipette to Cell Load mode. Pipet-mix the cell suspension with a manual P1000 pipette.		
	Cell suspension	575	Cell Load

5 Incubate at room temperature (15°C to 25°C) for 15 minutes. During 15 minute incubation, prepare Cell Capture Beads**(Cat. No. 650000089)**. 15m

Preparing, loading and washing Cell Capture Beads

12m

6 Place Cell Capture Bead tube on magnet for 1 minute, and remove storage buffer. 2m
Remove tube from magnet, and pipet 650 µL cold Sample Buffer (Cat. No. 650000062) into tube.

7 Set P1200M pipette to P**rime/Treat**mode. Load cartridge with materials listed using the P1200M pipette: 1m

	A	B	C
	Material to load	Volume (µL)	Pipette mode
	Air	700	Prime/Treat
	Set P1200M pipette to Bead Load mode. Use a manual P1000 to gently pipet-mix beads in cold Sample Buffer (Cat. No. 650000062). Immediately load.		
	Cell Capture Beads	630	Bead Load

8 Incubate the cartridge at room temperature (15°C to 25°C) for 3 minutes. 3m

9 Place cartridge on the plate shaker plate adapter. Shake the cartridge at room temperature (15°C to 25°C) at 1,000 rpm for 15 seconds. 1m
Return cartridge to Express instrument, and wait 30 seconds.

10 Set P1200M pipette to Wash mode. Load cartridge with materials listed using the P1200M pipette: 5m

	A	B	C
	Material to load	Volume (µL)	Pipette modea
	Air	700	Wash
	Cold Sample Buffer (Cat. No. 650000062)	700	Wash
	Air	700	Wash
	Cold Sample Buffer (Cat. No. 650000062)	700	Wash

Lysing cells

7m

11 Add 75.0 µL 1 M DTT (Cat. No. 650000063) to one 15 mL Lysis Buffer bottle (Cat. No. 650000064). Briefly vortex lysis mix, place on ice. 2m

12 Move the left slider to **LYSIS** on Express instrument. Set P1200M pipette to **Lysis** mode. Set P1200M pipette to **Lysis** mode. 5m

	A	B	C
	Material to load	Volume (µL)	Pipette mode
	Lysis Buffer with DTT	550	Lysis

Incubate at room temperature (15°C to 25°C) for 2 minutes.

Retrieving Cell Capture Beads

9m

13 Place the 5 mL LoBind Tube in Express instrument drawer. Ensure P5000M pipette is set to **Retrieval** mode. Move the front slider to **BEADS** on Express instrument. 2m

14 Move the left slider to **RETRIEVAL**. Leave Retrieval magnet in down position for 30 seconds. 5m

Aspirate 5,000 µL Lysis Buffer with DTT with the P5000M pipette. Press down on P5000M pipette to seal against the gasket. Move the left slider to the middle position (**0**), andimmediatelyload 4,950 µL Lysis Buffer with DTT.

15 Remove pipette from gasket, and purge tip. Move the front slider to **OPEN**, and place the 5 mL LoBind Tube on large magnet with 15 mL tube adapter (V&P Scientific Cat. No. VP 772FB-1A) for 1 minute. 2m

Washing Cell Capture Beads 8m

16 After 1 minute incubation leaving the 5 mL tube containing retrieved Cell Capture Beads on large magnet, remove all but ~1 mL of supernatant without disturbing beads. 1m

17 Remove tube from magnet. Gently pipet-mix beads, and transfer them to a new 1.5 mL LoBind Tube. 1m

18 Place tube on magnet for ≤2 minutes, and remove supernatant. Avoid leaving Lysis Buffer or bubbles in tube. Lysis Buffer might cause the reverse transcription reaction to fail. 2m

19 Remove tube from magnet and pipet 1.0 mL of cold Bead Wash Buffer (Cat. No. 650000065) into tube. Pipet-mix. 1m

20 Place tube on 1.5 mL tube magnet for ≤2 minutes, and remove supernatant. 2m

21 Remove tube from magnet, and pipet 1.0 mL cold Bead Wash Buffer (Cat. No. 650000065) into tube. Pipet-mix, and place on ice. 1m

cDNA synthesis and template switching

1h 39m

22 In a new 1.5-mL LoBind tube, pipet the following reagents. 10m

cDNA/Template switching mix

	A	B
	Component	Volume (µL)
	RT Buffer	40
	dNTP	20
	RT 0.1 M DTT	10
	Bead RT/PCR Enhancer	12
	RNase Inhibitor	10
	Reverse Transcriptase	10
	Nuclease-free water	92
	Total	194

Gently vortex mix, briefly centrifuge, and place back on ice.

23 Place the tube of washed Cell Capture Beads on a 1.5-mL tube magnet for≥2 minutes. Remove the 5m
supernatant and pipet 194 µL of cDNA mix into the beads. Pipet-mix.

24 Incubate the bead suspension on the thermomixer at 1,200 rpm and 42°C for 30 minutes. 30m

25 Place tube on 1.5 mL tube magnet for ≤2 minutes, and pipet supernatant to a new tube. Remove tube from magnet, and pipet 200 µL the following reagents into tube. 5m

	A	B
	Component	Volume (µL)
	10x Exonuclease I reaction buffer (BD Express, Cat. No. 650000071)	20

	A	B
	Exonuclease I (BD Express, Cat. No. 650000072)	10
	Nuclease-free water	170

26 Place tube on 1.5 mL tube magnet for ≤2 minutes, and remove supernatant. Wash the beads twice with 1x RT buffer. 10m

27 Resuspend the beads with the saved supernatant in step 25, add 3 µL of template switch oligo (100 uM5' A AGC AGT GGT ATC AAC GCA GAG TAC rG rG +G 3') , and incubate on the thermomixer for 15 minutes at 1,200 rpm and 42°C. Add 2 µL of 1 M MgCl2 to the reaction mix, and incubate on the thermomixer for another 15 minutes at 1,200 rpm and 42°C. 35m

28 Briefly spin the tube with the bead suspension. Place the tube on the magnet for≤1 minute until clear. Remove the supernatant. 2m

29 Remove the tube from the magnet, and pipet 200 µL of cold Bead Resuspension Buffer into the tube. Pipet- mix. 2m

Performing PCR1

2h 14m

30 Place the tube beads in Bead Resuspension Buffer on a 1.5-mL magnet for≤1 minute. Remove the supernatant. Suspend the beads with following PCR reaction mix: 10m

	A	B	C
	Component	Volume (µl)	Final Conc
	KAPA HiFi HotStart 2x ReadyMix	100	1x
	Universal Oligo (BD Express, 650000074)	6	0.3 µM
	TSO PCR primer (10µM)	6	0.3µM
	Nuclease-free water	up to 200µl	

Universal Oligo: 5'-ACACGACGCTCTTCCGATCT-3'
TSO PCR primer: 5'-AAGCAGTGGTATCAACGCAGAGTAC-3'

31 Ensuring that the beads are fully resuspended, pipet 25 µL of PCR reaction mix with beads into each of 8 0.2-mL PCR tubes. 2m

32 Program the thermal cycler as follows. 1h 30m

	A	B	C	D
	Step	Cycles	Temperature	Time
	Hot start	1	95°C	3 min
	Denaturation	11	98°C	20 s
	Annealing		62°C	3 min
	Extension		72°C	4 min
	Final extension	1	72°C	2 min
	Hold	1	4°C	∞

33 Pipet-mix and combine the four reactions into a new 1.5-mL LoBind tube. Place the 1.5-mL tube on the magnet for≤1 minute. Pipet the supernatant into the new 1.5-mL LoBind tube without disturbing the beads. 2m

34 Purify the amplified products by 0.7x Agencourt AMPure XP Beads(Beckman Coulter). 30m

Performing PCR2

1h 10m

35 Prepare the PCR mix by combining and mixing the following components: 10m

	A	B	C
	Component	Volume (µl)	Final Conc
	KAPA HiFi HotStart 2x ReadyMix	500	1x

	A	B	C
	Library Forward Primer (BD Express,91-1085)	3	0.3 µM
	TSO lengthen primer (10µM)	3	0.3µM
	PCR1 product (50ng)		
	Nuclease-free water	up to 100µl	

TSO lengthen primer: 5'-CGACATGGCTACGATCCGACAAGCAGTGGTATCAACGCAGAG-3'
Library Forward primer: 5'-AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

36 Program the thermal cycler as follows. 30m

	A	B	C	D
	Step	Cycles	Temperature	Time
	Hot start	1	95°C	2 min
	Denaturation	5	98°C	20 s
	Annealing		62°C	20 s
	Extension		72°C	4 min
	Final extension	1	72°C	2 min
	Hold	1	4°C	∞

37 Purify the amplified products by 0.7x Agencourt AMPure XP Beads(Beckman Coulter). 30m

Nanopore sequencing 3d

38 Prepared sequencing using Oxford Nanopore Technologies SQK-LSK109 and sequenced on ONT PromethION platforms. 3d