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MAS-ISO-seq - from 10x Single Cell Gene Expression Libraries V.1

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We use this protocol and it's working

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Abstract

Method for MAS-ISO-seq from 10x Single Cell Gene Expression Libraries.

Materials

Reagents:
2x Kapa HiFi Uracil+ ReadyMix (Roche #7959079001)
SPRIselect (Beckman Coulter B23318)
Qubit (Thermo #Q32851)
Dynabeads™ kilobaseBINDER™ (Thermo #60101)
Low-EDTA TE (1X), pH 8.0 (VWR 10128-588)
USER® Enzyme (M5505S)
HiFi Taq DNA Ligase (M0647S)
Genomic DNA ScreenTape/Reagent

Primers:

	A	B	C
	cDNA_amp_primers		
	5' Libraries		
	Fwd_R1_10×5'_PT_MAS	CTACACGACGCTCTTCCGAT*C*T	AAO272
	Rev_10×5_dUbio_PT	/5Biosg/UAUAAGCAGTGGTATCAACGCAG*A*G	AAO273
	3' Libraries		
	dUbio_venus_PT	/5Biosg/UAUCTACACGACGCTCTTCCGAT*C*T	AAO365
	mars_PT	AAGCAGTGGTATCAACGCAG*A*G	AAO366

	A	B	C
	MAS-seq primer plate		
	Well Position	Name	Sequence
	A1	*A-Fwd_5'_10x*	AGCTTACTTGTGAAGATCTACACGACGCTCTTCCGATCT
	A2	B-Fwd_5'_10x	ACTTGTAAGCUGTCTAUCTACACGACGCTCTTCCGATCT
	A3	C-Fwd_5'_10x	ACTCTGUCAGGTCCGAUCTACACGACGCTCTTCCGATCT

	A	B	C
	A4	D-Fwd_5'_10x	ACCTCCTCCUCCAGAAUCTACACGACGCTCTTCCGATCT
	A5	*E*-Fwd_5'_10x	AACCGGACACACUTAGUCTACACGACGCTCTTCCGATCT
	A6	F-Fwd_5'_10x	AGAGTCCAAUTCGCAGUCTACACGACGCTCTTCCGATCT
	A7	G-Fwd_5'_10x	AATCAAGGCUTAACGGUCTACACGACGCTCTTCCGATCT
	A8	H-Fwd_5'_10x	ATGTTGAAUCCTAGCGUCTACACGACGCTCTTCCGATCT
	A9	I-Fwd_5'_10x	AGTGCGTUGCGAATTGUCTACACGACGCTCTTCCGATCT
	A10	J-Fwd_5'_10x	AATTGCGUAGTTGGCCUCTACACGACGCTCTTCCGATCT
	A11	K-Fwd_5'_10x	ACACTTGGUCGCAATCUCTACACGACGCTCTTCCGATCT
	A12	L-Fwd_5'_10x	AGTAAGCCUTCGTGTCUCTACACGACGCTCTTCCGATCT
	B1	M-Fwd_5'_10x	ACCTAGAUCAGAGCCTUCTACACGACGCTCTTCCGATCT
	B2	N-Fwd_5'_10x	AGGTAUGCCGGUTAAGUCTACACGACGCTCTTCCGATCT
	B3	O-Fwd_5'_10x	AAGUCACCGGCACCUTUCTACACGACGCTCTTCCGATCT
	C1	B-Rev_5'_10x	ATAGACAGCUTACAAGUAAGCAGTGGTATCAACGCAGAG
	C2	C-Rev_5'_10x	ATCGGACCUGACAGAGUAAGCAGTGGTATCAACGCAGAG
	C3	D-Rev_5'_10x	ATTCUGGAGGAGGAGGUAAGCAGTGGTATCAACGCAGAG
	C4	*E*-Rev_5'_10x	ACTAAGTGUGTCCGGTUAAGCAGTGGTATCAACGCAGAG
	C5	F-Rev_5'_10x	ACTGCGAAUTGGACTCUAAGCAGTGGTATCAACGCAGAG
	C6	G-Rev_5'_10x	ACCGTUAAGCCTTGATUAAGCAGTGGTATCAACGCAGAG
	C7	H-Rev_5'_10x	ACGCTAGGAUTCAACAUAAGCAGTGGTATCAACGCAGAG
	C8	I-Rev_5'_10x	ACAATUCGCAACGCACUAAGCAGTGGTATCAACGCAGAG
	C9	J-Rev_5'_10x	AGGCCAACUACGCAATUAAGCAGTGGTATCAACGCAGAG
	C10	K-Rev_5'_10x	AGATUGCGACCAAGTGUAAGCAGTGGTATCAACGCAGAG
	C11	L-Rev_5'_10x	AGACACGAAGGCUTACUAAGCAGTGGTATCAACGCAGAG
	C12	M-Rev_5'_10x	AAGGCTCUGATCTAGGUAAGCAGTGGTATCAACGCAGAG
	D1	N-Rev_5'_10x	ACTUAACCGGCAUACCUAAGCAGTGGTATCAACGCAGAG
	D2	O-Rev_5'_10x	AAAGGUGCCGGUGACTUAAGCAGTGGTATCAACGCAGAG
	D3	*P-Rev_5'_10x*	ATCTCGAGCCACTTCATAAGCAGTGGTATCAACGCAGAG

1 **Mix MAS primer pairs:**

Order primers reconstituted as 100µM

Mix equal volumes of primer pairs according to the table below to create a 50µM MAS primer mix plate.

Create a 5µM working MAS-primer plate by diluting 10-fold in low-TE.

	A	B	C
	MAS-seq primer mixing strategy		
		wells	adapter pairs
	1	A1 & C1	*A|B
	2	A2 & C2	B|C
	3	A3 & C3	C|D
	4	A4 & C4	D|E
	5	A5 & C5	E|F
	6	A6 & C6	F|G
	7	A7 & C7	G|H
	8	A8 & C8	H|I
	9	A9 & C9	I|J
	10	A10 & C10	J|K
	11	A11 & C11	K|L
	12	A12 & C12	L|M
	13	B1 & D1	M|N
	14	B2 & D2	N|O
	15	B3 & D3	O|P*

2 **WTA amplification:**

Set up the following reactions on ice

For 5' libraries:

	A	B	C
	Reagent	Reaction conc.	µL per. reaction
	Nuclease Free Water		35
	Kapa HiFi Uracil+ ReadyMix (2X)	1X	50
	Fwd_R1_10x 5'_PT_MAS AAO272(10uM)	0.5 µM	5
	Rev_10×5_dUbio_PT AAO273(10uM)	0.5 µM	5
	10× 5' cDNA library (whole transcriptome amplification); 2-5ng/µL		5
	Total		100 µL

For 3' libraries:

	A	B	C	D
	Reagent	Reaction conc.	µL per. reaction	
	Nuclease Free Water		35	
	Kapa HiFi Uracil+ ReadyMix (2X)	1X	50	
	Fwd_R1_10x 5'_PT_MAS	0.5 µM	5	

	A	B	C	D
	AAO365(10 uM)			
	Rev_10×5_d Ubio_PT AAO366(10 uM)	0.5 µM	5	
	10× 3' cDNA library (whole transcripto me amplificatio n); 2-5ng/µL		5	
	Total		100 µL	

	A	B	C	D	E
	Step	Temperatur e	Time	Cycles	
	Initial denaturatio n	98 °C	3 min	1x	
	Denaturatio n	98 °C	20 sec	5x	
	Annealing	65°C	30 sec		
	Elongation	72 °C	8 min		
	Final Elongation	72 °C	10 min	1x	
	Hold	4 °C	Hold		

3 Reaction cleanup and quantification

1. 0.8x SPRIselect cleanup - 80 µL beads in 100 µL PCR reaction from step 2.
2. Elute in 46 µL low-TE
3. Qubit quantification

4 TSO artifact removal

1. Transfer 10 µL (100 µg) resuspended Dynabeads™ kilobaseBINDER™ streptavidin beads to a PCR tube.
2. Place the tube on the magnet for 2 min.
3. Carefully remove and discard the supernatant while the tube remains on the magnet. Avoid touching the bead pellet with the pipette tip.
4. Remove the tube from the magnet. Add 40 µL Binding Solution along the inside wall of the tube where the beads are collected and gently resuspend by pipetting. Note: the solution may be viscous. Avoid foaming.
5. Place the tube on the magnet for 2 min and remove the supernatant.
6. Resuspend the beads in 40 µL Binding Solution.
7. Add 40 µL of a solution containing the biotinylated DNA-fragments to the resuspended beads. Mix carefully to avoid foaming of the solution.
8. Incubate the tube at room temperature for 3 hours on a roller to keep the beads in suspension.
9. Place the tube on the magnet and remove the supernatant as in step 3, above.
10. Wash the Dynabeads®/DNA-complex 2x in 80 µL Washing Solution and once in distilled water.
11. Resuspend the Dynabeads®/DNA-complex in 40ul Low-TE.
12. Add 2ul USER and incubate in a rotator at 37C for 2 hours.
13. Place the tube on the magnet and move the supernatant containing the library to a fresh tube.
14. Cleanup - 0.8x SPRI (32 µL beads in 40 µL library)
15. Elute in 46 µL low-TE.
16. Qubit quantification

5 MAS adapter PCR

Set up all reactions on ice

1. Create the following master mix:

	A	B	C	D	E	F
	Reagent	Reaction conc.	µL per. reaction			
	Nuclease Free Water		618.7			
	Kapa HiFi Uracil+ ReadyMix (2X)	1X	800			
	Purified cDNA from		21.3			

	A	B	C	D	E	F
	step 4					
	Total		1440 µL			

2. Distribute 90 µL of Master Mix into each of 15 PCR tubes
3. Distribute 10 µL 5 µM MAS-seq primer pair mix into each of 15 PCR tubes

Cycling conditions:

	A	B	C	D
	Step	Temperature	Time	Cycles
	Initial denaturation	98 °C	3 min	1x
	Denaturation	98 °C	20 sec	*n*x
	Annealing	65°C	30 sec	
	Elongation	72 °C	8 min	
	Final Elongation	72 °C	10 min	1x
	Hold	4 °C	Hold	

Note - optimal cycle number is a function of amount of input material after **TSO artifact removal**. See table below for cycle determination

	A	B
	cDNA concentration (ng/µL)	cycle number
	1 - 2.5	10
	2.5 - 5	9
	5 - 7.5	8

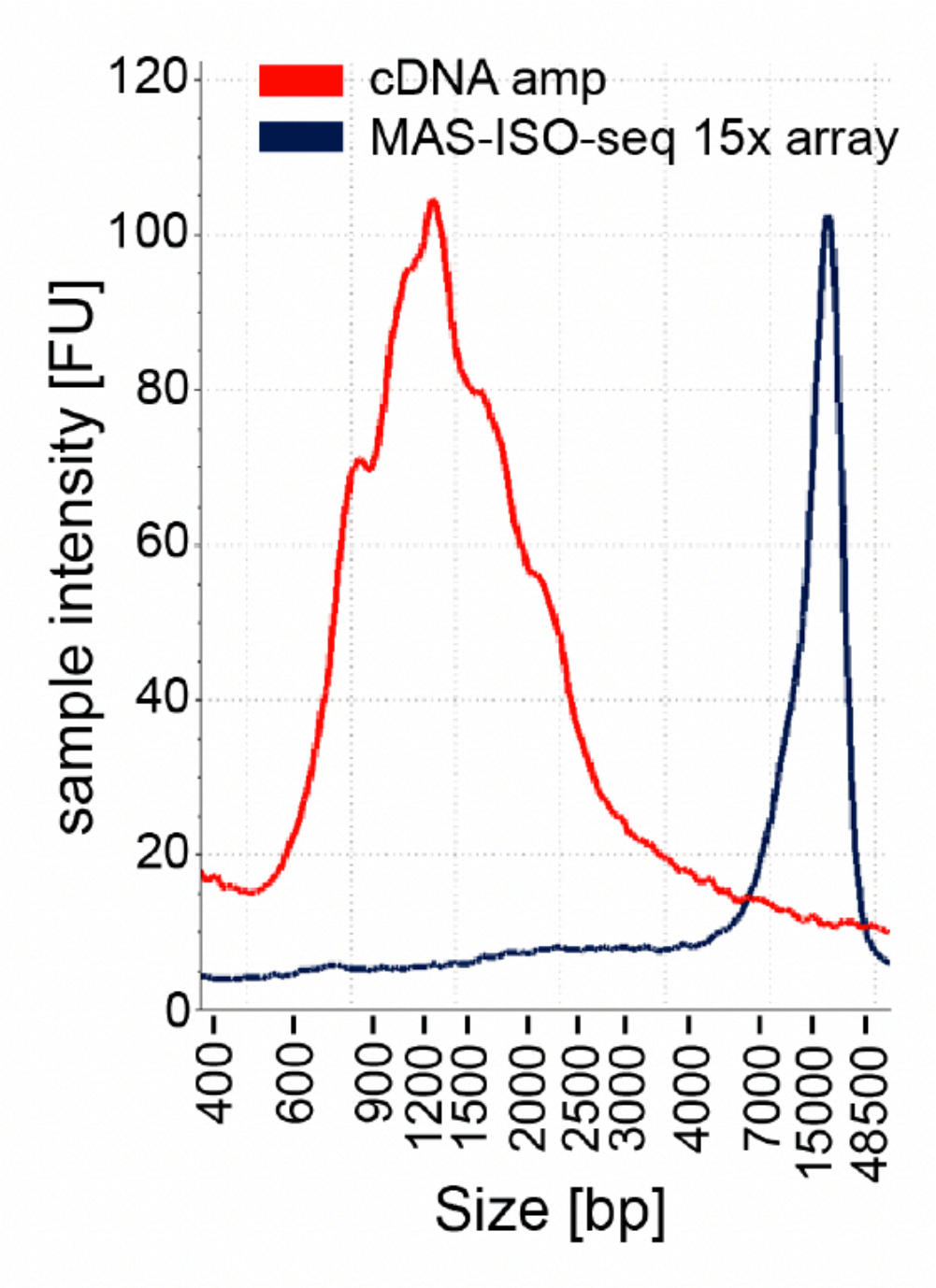
6 Reaction cleanup

1. Pool 15 × 95 µL reactions in 5ml tube. (Note* attaining equimolar amounts of each adapter PCR is key to efficient array assembly - be mindful to add the same amount of each adapter PCR to the pool. To account for handling, 95/100 µL of the PCR is advised to be pooled from each reaction.)
2. SPRIselect cleanup - 0.7x (997.5 µL beads in 1425 µL pooled **MAS adapter PCR**)
3. Elute in 450 µL low TE buffer.
4. Qubit quantify
5. Move 435 µL of the library to a 1.5 mL microtube tube and added 15 µL of USER enzyme. (Note* 10-15 µg is advised going into this step. Dilute going into this step if sample is too concentrated.)
6. Incubate reaction at 37 for 2.5 hours
7. Add 51 µL Hifi Ligase buffer and 15ul Hifi ligase to the USER reaction.
8. Distribute to 5x PCR tubes and set in thermocycler at 42C for 2 hours.
9. Pool reaction into a 1.5 mL microtube using a wide bore tip
10. SPRIselect cleanup - 0.7x (361.2 µL beads in 516 µL reaction), mix gently with wide bore tip. Set on rotator for 5 min.
11. Incubate at 37C for 10min - elute in 180 µL low TE.
12. Qubit quantify

7 **MAS library quantification**

Quantify MAS arrays with Genomic DNA ScreenTape or Femto Pulse.

See example below:



Optional Size Selection

SMRTbell Express Template Prep: Use this as input material for PacBio SMRTbell Express Template Prep Kit 2.0, starting at the " Remove ssDNA Overhangs" step (page 8: https://www.pacb.com/wp-content/uploads/Procedure-Checklist-Preparing-HiFi-SMRTbell-Libraries-using-SMRTbell-Express-Template-Prep-Kit-2.0.pdf)

Minimum starting material going into the PacBio SMRTbell Express Template Prep Kit 2.0 : **5µg**