Preparing MAS-Seq libraries using MAS-Seq for 10x Single Cell 3’ kit

Procedure & checklist

Before you begin

This procedure describes the workflow for constructing single-cell MAS-Seq (Multiplexed Arrays Sequencing) libraries from 10x Chromium 3’ cDNA using the *MAS-Seq for 10x Single Cell 3’ kit* (102-659-600) for library prep and sequencing on PacBio systems.

This kit is intended for use with single-cell cDNA generated using the *10x Chromium Next GEM Single Cell 3’ kit* (v3.1), standard throughput. It has not been tested for use on low throughput (LT) or high throughput (HT) kits which are currently unsupported.

Overview	
Samples per Kit	8
Workflow time	2 days for up to 8 samples

cDNA input	
Quantity	15 ng per library or 60-75 ng per library For cDNA amount between 16-59 ng, normalize the input to 15 ng For cDNA amount >75 ng, normalize the input to 75 ng For cDNA amounts between 60-75ng, proceed without normalizing
Average segment lengths	500-1,000 bp
Average 16-segment array lengths	10-15 kb

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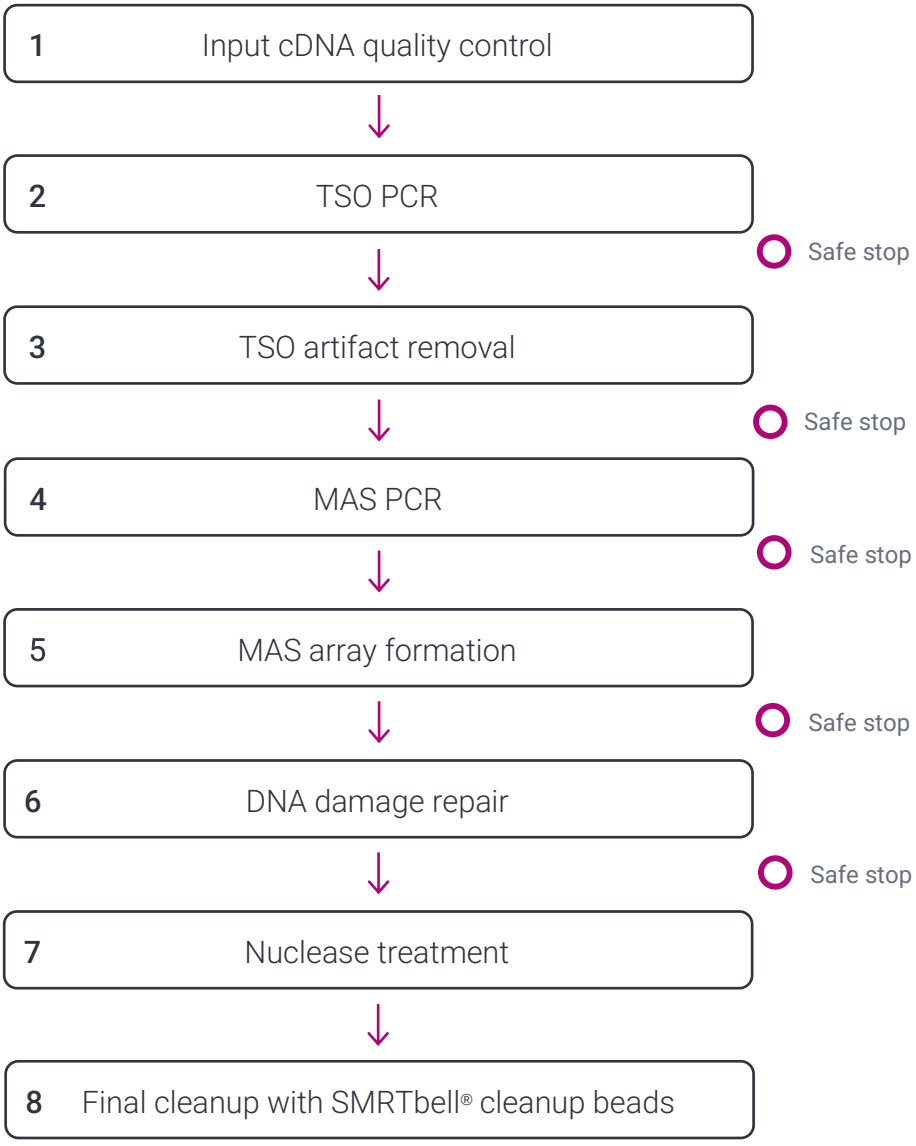
PN 102-678-600 REV02 NOV2022

Workflow



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Required materials and equipment

DNA sizing	
Femto Pulse system	Agilent Technologies, Inc. M5330AA
Femto Pulse gDNA 165 kb analysis kit	Agilent Technologies, Inc. FP-1002-0275
Agilent 2100 Bioanalyzer system	Agilent Technologies, Inc. G2939BA
Agilent High Sensitivity DNA Kit	Agilent Technologies, Inc. 5067-4627
DNA quantitation	
Qubit fluorometer	ThermoFisher Scientific Q33238
Qubit 1X dsDNA HS assay kit	ThermoFisher Scientific Q33230
SMRTbell® library preparation	
MAS-Seq for 10x 3' concatenation kit (Includes MAS PCR mix, MAS capture primers, MAS enzyme, MAS primers premix A-P, MAS adapters, MAS ligation additive, MAS ligase, MAS ligase buffer, DNA repair mix, Repair buffer, Nuclease mix, Nuclease buffer, and Elution buffer)	PacBio 102-407-900*
MAS capture beads kit (Includes MAS capture beads, MAS bead binding buffer, MAS bead washing buffer)	PacBio 102-428-400*
SMRTbell cleanup beads	PacBio 102-158-300*
200 Proof ethanol, molecular biology or ACS grade	Any major lab supplier (MLS)
Nuclease-free water, molecular biology grade	Any MLS
8-channel pipettes	Any MLS
Wide orifice tips (200µL)	Rainin 30389241
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Microcentrifuge	Any MLS
Magnetic separation rack compatible with 0.2 mL 8-tube strips	V&P Scientific VP 772F4-1
Magnetic bead rack	ThermoFisher Scientific 12321D
Thermocycler compatible with 0.2 mL 8-tube strips	Any MLS
Tube rotator	Any MLS
1.5 mL DNA LoBind tubes	Eppendorf 022431021

*sold as part of bundle *MAS-Seq for 10x Single Cell 3' kit* (102-659-600)

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General best practices

cDNA Input

Optimal range of 3,000-10,000 target cell recovery from the 10x Chromium 3' single cell workflow.
Follow the best practices in the 10x Chromium user guide.
Input cDNA quality control is highly recommended before proceeding to the MAS-Seq workflow.

Reagent and sample handling

Room temperature is defined as any temperature in the range of **18-23°C** for this protocol.
Thaw the repair buffer, nuclease buffer, MAS ligase buffer and elution buffer at room temperature.
Briefly vortex reagent buffers and MAS adapters prior to use. Enzyme mixes do not require vortexing.
Quick spin all reagents in a microcentrifuge to collect liquid at tube bottom prior to use.
Keep all temperature-sensitive reagents on ice.

Temperature-sensitive reagents		
Step used	**Tube**	**Reagent**
TSO PCR and MAS PCR	Green	MAS PCR mix 102-692-800
	Yellow	MAS capture primers (Fwd and Rev) 102-693-300 Fwd 102-693-900 Rev
	Orange	MAS primers premix (A-P) 102-694-000 A 102-694-100 B 102-694-200 C 102-694-300 D 102-694-400 E 102-694-500 F 102-694-600 G 102-694-700 H 102-694-800 I 102-694-900 J 102-695-000 K 102-695-100 L 102-695-300 M 102-695-500 N 102-695-600 O 102-695-700 P
MAS array formation	Light green	MAS enzyme 102-692-900
	Yellow	MAS ligase 102-693-000
	White	MAS ligase buffer 102-693-100
	Blue	MAS adapters 102-695-800 Fwd 102-695-900 Rev
DNA damage repair Nuclease treatment	Green	DNA repair mix 102-696-000
	Purple	Repair buffer 102-696-100
	Light green	Nuclease mix 102-696-200
	Light purple	Nuclease buffer 102-696-300

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Bring SMRTbell cleanup beads and Qubit 1X dsDNA HS reagents to room temperature for 30-60 minutes prior to use.

Pipette mix all bead binding and elution steps until beads are distributed evenly in solution.

Wide-bore pipette tips help to minimize foaming specifically when resuspending MAS capture beads.

Pipette mix all library prep reactions by pipetting up and down 10 times.

Samples can be stored at 4°C at all safe stopping points listed in the protocol.

1.5X SMRTbell cleanup is recommended before MAS array formation. If the cDNA contains smaller fragments <200bp, it is recommended to increase the SMRTbell cleanup ratio to 2X.

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Thermocycler programs

Program thermocycler(s) prior to beginning the protocol for the first time.

1. TSO PCR program (15 ng input)

Heated lid set at 105°C

Step	Time	Temperature	Cycles
1	3 min	98°C	1
2	20 sec	98°C	
3	30 sec	65°C	5
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

Or TSO PCR program (60-75 ng input)

Heated lid set at 105°C

Step	Time	Temperature	Cycles
1	3 min	98°C	1
2	20 sec	98°C	
3	30 sec	65°C	3
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

2. TSO artifact removal program

Heated lid set at 47°C

Step	Time	Temperature
1	30 min	37°C
2	Hold	4°C

3. MAS PCR program*

Heated lid set at 105°C

Step	Time	Temperature	Cycles
1	3 min	98°C	1
2	20 sec	98°C	
3	30 sec	68°C	9
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

* Note: If the total sample quantity is less than 50 ng, follow the table below for cycle number recommendations.

cDNA input amount	Cycle number
30 - 50 ng	9
12.5 - 29.9 ng	10

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4. MAS primer digestion program

Heated lid set at 47°C

Step	Time	Temperature
1	30 min	37°C
2	Hold	4°C

5. MAS array ligation program

Heated lid set at 52°C

Step	Time	Temperature
1	60 min	42°C
2	Hold	4°C

6. DNA damage repair program

Heated lid set at 47°C

Step	Time	Temperature
1	30 min	37°C
2	Hold	4°C

7. Nuclease treatment program

Heated lid set at 47°C

Step	Time	Temperature
1	60 min	37°C
2	Hold	4°C

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Workflow steps

1. Input cDNA quality control

This protocol requires at least 15 ng of 10x Chromium 3' single cell cDNA. Before you begin, evaluate the quantity and size distribution of input cDNA to determine whether it is suitable for the protocol (average size between 500-1500bp).

✔	Step	Instructions
	1.1	Bring the Qubit 1X dsDNA HS working solution and standards to **room temperature**.
	1.2	Pulse vortex or pipette mix each sample to homogenize the DNA in solution.
	1.3	Quick spin each sample to collect liquid.
	1.4	Take a **1 µL** aliquot from each sample.
	1.5	Measure DNA concentration with a Qubit fluorometer using the **1X dsDNA HS kit.**
	1.6	Dilute each sample to **1.0-1.5 ng/µL** in **elution buffer or water,** based on the Qubit reading.
	1.7	Measure DNA size distribution with a Bioanalyzer system using the **High Sensitivity DNA Kit.**
	1.8	Proceed to the next step of the protocol if sample quality is acceptable.

2. TSO PCR

This PCR step generates biotinylated DNA-fragments to enable removal of TSO priming artifacts generated during cDNA synthesis.

✔	Step	Instructions
		cDNA amplification with MAS capture primers
	2.1	Normalize cDNA sample input to 15 ng if it is between 15 ng and 60 ng using elution buffer. Normalize cDNA sample input to 75 ng if it is higher than 75 ng using elution buffer. For cDNA amounts between 60-75ng, proceed without normalizing. Set up the following PCR reaction on ice (**RM1**).

Reaction Mix 1 (RM1)

✔	Tube	Component	Volume
		Nuclease-free water	Make up volume
	Green	MAS PCR mix (2X) 102-692-800	25 µL
	Yellow	MAS capture primer Fwd 102-693-300	5 µL
	Yellow	MAS capture primer Rev 102-693-900	5 µL
		10x 3' cDNA library (1-5 ng/µL)	Up to 15 µL
		Total volume	**50 µL**

	2.2	Pipette mix **RM1.**
	2.3	Quick spin **RM1** in a microcentrifuge to collect liquid.
	2.4	Select the **TSO PCR program** based on cDNA input.
		Cleanup with 1.5X SMRTbell cleanup beads

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2.5	Add **1.5X** v/v (**75 µL**) of resuspended, room-temperature **SMRTbell cleanup beads** to each tube of amplified cDNA.
2.6	Pipette mix the beads until evenly distributed.
2.7	Quick spin the tube strip in a microcentrifuge to collect liquid.
2.8	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
2.9	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
2.10	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
2.11	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
2.12	Repeat the previous step.
2.13	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
2.14	Remove tube strip from the magnetic rack. **Immediately** add **42 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
2.15	Quick spin the tube strip in a microcentrifuge to collect liquid.
2.16	Leave at **room temperature** for **5 minutes** to elute DNA.
2.17	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
2.18	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
2.19	**Recommended**: Evaluate sample concentration. • Take a **1 µL** aliquot from each tube. • Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit.
2.20	Proceed to the next step of the protocol if sample quantity is acceptable (at least 150 ng) and not exceeding 1 µg. If the sample quantity is higher than 1 µg, only carry forward to **Step 3** with a maximum input of 1 µg.
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3. TSO artifact removal

In this step, removal of DNA fragments containing TSO artifacts is performed using MAS capture beads.

✔	Step	Instructions
	3.1	Bring **MAS capture beads kit** to room temperature. Resuspend the beads by vortexing.
	3.2	Transfer **10 µL resuspended MAS capture beads** to a PCR tube. Scale up the amount of beads if processing more than 4 samples (with 10% overage). If preparing more than 40 µL of beads, use a 1.5 mL Lo-bind tube instead of PCR tube.
	3.3	Place the tube on the magnet until beads separate fully from the solution.
	3.4	Carefully remove and discard the supernatant while the tube remains on the magnet. Avoid touching the bead pellet with the pipette tip.
	3.5	• Remove the tube from the magnet. • Add **40 µL MAS bead binding buffer** along the inside wall of the tube where the beads are collected and gently resuspend by pipetting using wide bore tips. **DO NOT VORTEX**. Note: the solution may be viscous. Highly recommend using wide bore tips to avoid foaming. When excess bubbles are present, lower cDNA recovery is expected. • Quick spin the tube in a microcentrifuge if needed. Note: Scale up the volume of MAS capture binding buffer accordingly, if preparing more than 40 µL of beads.
	3.6	Place the tube on the magnet until beads separate fully from the solution and remove the supernatant.
	3.7	• Resuspend the beads in **40 µL MAS bead binding buffer** by pipetting slowly using wide bore tips. **DO NOT VORTEX**. Note: the solution may be viscous. **Highly recommend using wide bore tips to avoid foaming. When excess bubbles are present, lower cDNA recovery is expected.** Note: Scale up the volume of MAS capture binding buffer accordingly, if preparing more than 40 µL of beads. • Distribute 40 µL of resuspended MAS capture beads into appropriate number of PCR tubes before proceeding to **Step 3.8**.
	3.8	Add **40 µL** of a solution containing the **biotinylated DNA-fragments** (from **Step 2.18**) to the resuspended beads. Mix carefully using wide bore tips to avoid foaming of the solution.
	3.9	Incubate the tube at **room temperature** for **15 minutes** on a rotator to keep the beads in suspension. Quick spin the tube in a microcentrifuge to collect liquid.
	3.10	Place the tube on the magnet until beads separate fully from the solution and remove the supernatant.
	3.11	Resuspend the MAS capture beads/DNA-complex in **80 µL MAS bead washing buffer** by pipette mixing until evenly distributed.
	3.12	Place the tube on the magnet until beads separate fully from the solution and remove the supernatant.
	3.13	Remove the tube from the magnet. Resuspend the MAS capture beads/DNA-complex in **80 µL MAS bead washing buffer** by pipette mixing until evenly distributed.
	3.14	Place the tube on the magnet until beads separate fully from the solution and remove the supernatant.
	3.15	Remove the tube from the magnet. Resuspend the MAS capture beads/DNA complex in **80 µL nuclease free water** by pipette mixing until evenly distributed.
	3.16	Place the tube on the magnet until beads separate fully from the solution and remove the supernatant.
	3.17	Resuspend the capture beads/DNA-complex in 40 µL of elution buffer by pipette mixing until evenly distributed.
	3.18	Add **2 µL MAS enzyme** to the sample with capture beads to cleave the captured DNA products from MAS capture beads.

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3.19	Pipette mix each sample and a very quick spin in a microcentrifuge to collect liquid.
3.20	Run the **TSO artifact removal program**.
3.21	Place the tube on the magnet for **1 minute** and move the **supernatant containing the library** to a fresh tube.
	Cleanup with 1.5X SMRTbell cleanup beads
3.22	Add **1.5X** v/v (**63 µL**) of resuspended, room-temperature **SMRTbell cleanup beads** to each sample.
3.23	Pipette mix the beads until evenly distributed.
3.24	Quick spin the tube strip in a microcentrifuge to collect liquid.
3.25	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
3.26	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
3.27	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
3.28	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
3.29	Repeat the previous step.
3.30	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
3.31	Remove tube strip from the magnetic rack. **Immediately** add **46 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
3.32	Quick spin the tube strip in a microcentrifuge to collect liquid.
3.33	Leave at **room temperature** for **5 minutes** to elute DNA.
3.34	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
3.35	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
3.36	**Recommended**: Evaluate sample concentration. • Take a 1 µL aliquot from each tube. • Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit.
3.37	Proceed to the next step of the protocol if sample quantity is acceptable (maximum 50 ng). • If cDNA amount is >50 ng, dilute the cDNA to 50 ng using elution buffer in a total volume of 45µL. Do not proceed with MAS PCR with cDNA amount >50ng as it might lead to PCR artifacts and chimera formation. Note: If the total sample quantity is less than 50 ng, follow the cycle number recommendations listed in **Step 4.8**.
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4. MAS PCR

Perform 16 parallel cDNA amplification reactions with MAS primers to generate DNA fragments containing orientation-specific MAS segmentation adapter sequences.

*** Note: All 16 PCR reactions are required, missing/incorrect primer pairs will result in no/low SMRTbell yield***

✔	Step	Instructions
		cDNA amplification with MAS primers (16 reactions per sample)
	4.1	Set up the following PCR reaction mix per sample **on ice** (**RM2**).

Reaction Mix 2 (RM2)

✔	Tube	Component	Volume
		Nuclease-free water	125 µL
	Green	MAS PCR mix (2X) 102-692-800	212.5 µL
		Purified cDNA from **Step 3.35**	45 µL
		Total volume	**382.5 µL**

	4.2	Pipette mix **RM2**.
	4.3	Quick spin **RM2** in a microcentrifuge to collect liquid.
	4.4	Add **22.5 µL** of **RM2** to a new PCR tube **on ice**. Repeat this step to prepare a total of 16 tubes per sample (each containing **22.5 µL** of **RM2**).
	4.5	Add **2.5 µL** of **MAS primers premix** into each of 16 PCR tubes **on ice** according to the table below.

1	MAS primers premix A
2	MAS primers premix B
3	MAS primers premix C
4	MAS primers premix D
5	MAS primers premix E
6	MAS primers premix F
7	MAS primers premix G
8	MAS primers premix H
9	MAS primers premix I
10	MAS primers premix J
11	MAS primers premix K
12	MAS primers premix L
13	MAS primers premix M
14	MAS primers premix N
15	MAS primers premix O
16	MAS primers premix P

	4.6	Pipette mix each sample. The total volume of each tube should be 25.0 µL.
	4.7	Quick spin the strip tubes in a microcentrifuge to collect liquid.
	4.8	Run the **MAS PCR program**. Reactions can be held overnight in the cycler. Note: **If the total sample quantity is less than 50 ng, follow the table below for cycle number recommendations.**

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cDNA input amount	Cycle number
35-50 ng	9
20-35 ng	10

Cleanup with 1.5X SMRTbell cleanup beads

4.9	Pool entire volume of all 16 reactions into a single 1.5 mL LoBind tube.
4.10	Add **1.5X** v/v (**600 µL)** of resuspended, room-temperature **SMRTbell cleanup beads** to the PCR pool.
4.11	Pipette mix the beads until evenly distributed.
4.12	Quick spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
4.13	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
4.14	Place 1.5mL LoBind tube in a magnetic separation rack until beads separate fully from the solution.
4.15	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
4.16	Slowly dispense **1 mL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
4.17	Repeat the previous step.
4.18	Remove residual 80% ethanol: • Remove LoBind tube from the magnetic separation rack. • Quick spin LoBind tube in a microcentrifuge. • Place LoBind tube back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
4.19	Remove LoBind tube from the magnetic rack. **Immediately** add **50 µL** of **elution buffer** to each tube and resuspend the beads.
4.20	Quick spin the LoBind tube in a microcentrifuge.
4.21	Incubate at room temperature for **5 minutes** to elute DNA.
4.22	Place the LoBind tube in a magnetic separation rack until beads separate fully from the solution.
4.23	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new LoBind tube**. Discard old tube with beads.
4.24	**Recommended**: Evaluate sample concentration. • Take a **1 µL** aliquot from each tube, dilute with **9 µL** of **elution buffer.** • Using **1 µL** of the dilution, measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit.
4.25	Proceed to the next step of the protocol if sample quantity is acceptable (required input, 10 µg). Do not proceed if less than 8 µg is available.

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5. MAS array formation

In this step, treat PCR amplified cDNA fragments with MAS enzyme to create single-stranded extensions to enable directional assembly of cDNA segments into a linear array.

✔	Step	Instructions
	5.1	In a 0.2 mL PCR tube, add **10 µg of sample** from **Step 4.23**, in **47 µL** of volume. Dilute with elution buffer going into this step if sample is too concentrated.
	5.2	Add **10 µL** of **MAS enzyme** to create single-stranded extensions on PCR amplified cDNA fragments to enable subsequent directional assembly of 16 PCR products**.**
	5.3	Pipette mix each sample.
	5.4	Run the **MAS primer digestion program**.
	5.5	Add **1.5 µL** of each **MAS adapter** (A Fwd and Q Rev) and **20µL** of **MAS ligation additive** to each sample.
	5.6	Pipette mix each sample.
	5.7	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample in the order and volume listed below.
	5.8	Pipette mix **RM3** with wide bore tips.
	5.9	Quick spin **RM3** in a microcentrifuge to collect liquid.
	5.10	Add **20 µL** of **RM3** to each sample.
	5.11	Pipette mix each sample with wide bore tips.
	5.12	Run the **MAS array ligation program**.
		Cleanup with 1.2X SMRTbell cleanup beads
	5.13	Add **1.2X** v/v (**120 µL**) of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	5.14	Pipette mix the beads with wide bore tips until evenly distributed.

✔	Tube	Component	Volume
	Blue	MAS adapter A Fwd 102-695-800	1.5 µL
	Blue	MAS adapter Q Rev 102-695-900	1.5 µL
	Red	MAS ligation additive 102-696-400	20 µL
		Total volume	**23 µL**

Reaction Mix 3 (RM3)

✔	Tube	Component	Volume
	White	MAS ligase buffer 102-693-100	10 µL
	Yellow	MAS ligase 102-693-000	10 µL
		Total volume	**20 µL**

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5.15	Quick spin the tube strip in a microcentrifuge to collect liquid.
5.16	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
5.17	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
5.18	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
5.19	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
5.20	Repeat the previous step.
5.21	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
5.22	Remove tube strip from the magnetic rack. **Immediately** add 43 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
5.23	Quick spin the tube strip in a microcentrifuge to collect liquid.
5.24	Leave at **room temperature** for **5 minutes** to elute DNA.
5.25	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
5.26	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
5.27	**Recommended**: Evaluate sample concentration. • Take a 1 µL aliquot from each tube, dilute with 4 µL of elution buffer • Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. The required amount of purified MAS array products to proceed with the DNA damage repair step = 5 µg.
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6. DNA damage repair

✔	Step	Instructions
	6.1	In a new PCR strip tube, add **5 µg** of MAS array (in **42 µL** of **elution buffer**) from **Step 5.26.**
	6.2	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample in the order and volume listed below.
	6.3	Pipette mix **RM4**.
	6.4	Quick spin **RM4** in a microcentrifuge to collect liquid.
	6.5	Add **8 µL** of **RM4** to each sample. Total volume should equal **50 µL.**
	6.6	Pipette mix each sample with wide bore tips.
	6.7	Quick spin the strip tube in a microcentrifuge to collect liquid.
	6.8	Run the **DNA damage repair program**.
		Cleanup with 1.2X SMRTbell cleanup beads
	6.9	Add **1.2X** v/v (**60 µL**) of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	6.10	Pipette mix the beads with wide bore tips until evenly distributed.
	6.11	Quick spin the tube strip in a microcentrifuge to collect liquid.
	6.12	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	6.13	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	6.14	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	6.15	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	6.16	Repeat the previous step.
	6.17	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	6.18	Remove tube strip from the magnetic rack. **Immediately** add **40 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	6.19	Quick spin the tube strip in a microcentrifuge to collect liquid.

Reaction Mix 4 (RM4)

✔	Tube	Component	Volume
	Purple	Repair buffer 102-696-100	6 µL
	Green	DNA repair mix 102-696-000	2 µL
		Total volume	**8 µL**

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	6.20	Leave at **room temperature** for **5 minutes** to elute DNA.
	6.21	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	6.22	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.

SAFE STOPPING POINT - Store at 4°C

7. Nuclease treatment

✔	Step	Instructions
	7.1	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below.

Reaction Mix 5 (RM5)

✔	Tube	Component	Volume
	Light purple	Nuclease buffer 102-696-300	5 µL
	Light green	Nuclease mix 102-696-200	5 µL
		Total volume	**10 µL**

	7.2	Pipette mix **RM5**.
	7.3	Quick spin **RM5** in a microcentrifuge to collect liquid.
	7.4	Add **10 µL** of **RM5** to each sample. Total volume should equal **50 µL.**
	7.5	Pipette mix each sample with wide bore tips.
	7.6	Quick spin the strip tube in a microcentrifuge to collect liquid.
	7.7	Run the nuclease treatment program.

8. Final cleanup with SMRTbell cleanup beads

✔	Step	Instructions for cleanup with 1.2X (v/v) SMRTbell cleanup beads
	8.1	Add **60 µL SMRTbell cleanup beads** to each sample from the previous step. Using wide bore tips, pipette mix the beads until evenly distributed.
	8.2	Quick spin the tube strip in a microcentrifuge to collect all liquid.
	8.3	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	8.4	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	8.5	Slowly pipette off the cleared supernatant without disturbing the beads. It is recommended to save the supernatant in another tube strip in case of poor DNA recovery.
	8.6	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	8.7	Repeat the previous step.

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8.8	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
8.9	Remove tube strip from the magnetic rack. **Immediately** add **20 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed with wide bore tips.
8.10	Quick spin the tube strip in a microcentrifuge to collect liquid.
8.11	Leave at **room temperature** for **5 minutes** to elute DNA.
8.12	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
8.13	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip** using wide bore tips. Discard old tube strip with beads.
8.14	Take a **1 µL** aliquot from each tube. Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Calculate the total mass. **Recommended**: Further dilute each aliquot to **250 pg/µL** with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system. If a Femto Pulse system is unavailable, a Bioanalyzer system may also be used for DNA sizing QC of the final SMRTbell library - but note that the sample electropherogram trace may partially overlap with the 17 kb upper marker.
8.15	Proceed to SMRT Link Sample Setup to prepare the SMRTbell library for sequencing.
8.16	Store SMRTbell libraries at 4°C if sequencing within the week. Long-term storage should be at -20°C. Minimize freeze-thaw cycles when handling SMRTbell libraries.

PROTOCOL COMPLETE

Revision history (description)	Version	Date
Clarified supported methods, added part numbers for tubes throughout.	02	November 2022

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