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snASE - MAS-Seq protocol

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We use this protocol and it's working

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Abstract

Modified version of PacBio's MAS-seq protocol for snASE samples.

Quality Control

1 1 Bring the Qubit 1X dsDNA HS working solution and standards to room temperature.

2 2 Pulse vortex or pipette mix each sample to homogenize the DNA in solution.

3 3 Quick spin each sample to collect liquid.

4 4 Take a 1 µL aliquot from each sample.

5 5 Measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit.

6 6 Dilute each sample to 1.0-1.5 ng/µL in elution buffer or water, based on the Qubit reading.

7 7 MeasureDNA size distribution with a Bioanalyzer system using the High Sensitivity DNA Kit.

8 8 Proceed to the next step of the protocol if sample quality is acceptable.

TSO PCR

9 Prepare PCR mix per sample:

	A	B
	Component	**Volume**
	Nuclease-free water	Make up volume
	MAS PCR mix	25 µL
	MAS 5’ capture primer mix	10 µL
	10x 3’ cDNA library	Up to 15 µL
	Total Volume	50 µL

10 Pipette-mix RM1.

11 Quick spin RM1 in a microcentrifuge to collect liquid.

12 Select the TSO PCR program based on cDNA input.

13 Cleanup with 1.5X SMRTbell cleanup beads

14 Add 1.5X v/v (75 µL) of resuspended, room-temperature SMRTbell cleanup beads to each tube of amplified cDNA.

15 Pipette mix the beads until evenly distributed.

16 Quick spin the tube strip in a microcentrifuge to collect liquid.

17 Leave at room temperature for 10 minutes to allow DNA to bind beads.

18 Place tube strip in a magnetic separation rack until beads separate fully from the solution.

19 Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.

20 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.

21 Repeat the previous step.

22 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.

23 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.

24 Quick spin the tube strip in a microcentrifuge to collect liquid.

25 Leave at room temperature for 5 minutes to elute DNA.

26 Place tube strip in a magnetic separation rack until beads separate fully from the solution.

27 Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip. Discard old tube strip with beads.

28 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.

29 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

TSO artifact removal

30 Bring MAS capture beads kit to room temperature. Resuspend the beads by vortexing.

31 Transfer 10 µL resuspended MAS capture beads per sample 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 LoBind tube instead of PCR tube.

32 Place the tube on the magnet until the beads separate fully from the solution.

33 Carefully remove and discard the supernatant while the tube remains on the magnet. Avoid touching the bead pellet with the pipette tip.

34 Remove the tube from the magnet.
Add 40 µL MAS bead binding buffer along the insidewall 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.

35 Place the tube on the magnet until the beads separate fully from the solution and remove the supernatant.

36 Resuspendthe 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 µLof resuspended MAScapture beads into the appropriate number of PCR tubes before proceeding to Step 3.8.

37 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.

38 Incubate the tube at room temperature for 15 minutes on a rotatorto keep the beads in suspension. Quick-spin the tube in a microcentrifuge to collect liquid.

39 Place the tube on the magnet until the beads separate fully from the solution and remove the supernatant.

40 Resuspend the MAS capture beads/DNA-complex in 80 µL MAS bead washing buffer by pipette mixing until evenly distributed.

41 Place the tube on the magnet until the beads separate fully from the solution and remove the supernatant.

42 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.

43 Place the tube on the magnet until the beads separate fully from the solution and remove the supernatant.

44 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.

45 Place the tube on the magnet until the beads separate fully from the solution and remove the supernatant.

46 Resuspend the capture beads/DNA-complex in 40 µL of elution buffer by pipette mixing until evenly distributed.

47 Add 2 µL MAS enzyme to the sample with capture beads to cleave the captured DNA products from MAS capture beads.

48 Pipette-mix each sample and a very quick spin in a microcentrifuge to collect liquid.

49 Run the TSOartifact removal program. Heated lid set at 47°C

	A	B	C
	Step	Time	Temperature
	1	30 minutes	37°C
	2	Hold	4°C

50 Place the tube on the magnet for 1 minute and move the supernatant containing the library to a fresh tube.

51 Cleanup with 1.5X SMRTbell cleanup beads.

52 Add 1.5X v/v (63 µL) of resuspended, room-temperature SMRTbell cleanup beads to each sample.

53 Pipette-mix the beads until evenly distributed.

54 Quick-spin the tube strip in a microcentrifuge to collect liquid.

55 Leave at room temperature for 10 minutes to allow DNA to bind beads.

56 Place tube strip in a magnetic separation rack until the beads separate fully from the solution.

57 Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.

58 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.

59 Repeat the previous step.

60 Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until the beads separate fully from the solution. Pipette off residual 80% ethanol and discard.

61 Remove the 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.

62 Quick-spin the tube strip in a microcentrifuge to collect liquid.

63 Leave at room temperature for 5 minutes to elute DNA.

64 Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

65 Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new 0.5 mL LoBind tube. Discard the old tube strip with beads.

66 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.

67 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

MAS PCR

68 Set up the following PCR reaction mix per sample on ice.
Reaction Mix 2 (RM2):

	A	B
	Master mix components	**Volume for 16X concatenation***
	PCR Grade Water	176−X µL
	MAS PCR mix	220 µL
	55 ng of amplified cDNA from Step 67	X µL
	Total volume	396 µL

69 Quick-spin RM2 in a microcentrifuge to collect liquid.

70 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)

71 Add 2.5 µL of MAS primers premix A-PQ into each of 16 PCR tubes on ice, with each tube getting only one primer. Tube 1= Primer mix A, Tube 2=Primer mix B,... Tube 16= Primer mix PQ. DO NOT ADD MORE THAN ONE PRIMER PREMIX TO A TUBE

72 Pipette-mix each sample. The total volume of each tube should be 25.0 µL.

73 Quick-spin the strip tubes in a microcentrifuge to collect liquid.

74 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.

	A	B
	cDNA Input amount	Cycle Number
	30−50 ng	9
	12.5−29.9 ng	10

75 Clean up with 1.5X SMRTbell cleanup beads.

76 Pool entire volume of all 16 reactions into a single 1.5 mL LoBind tube.

77 Add 1.5X v/v (600 µL) ofresuspended, room-temperature SMRTbell cleanup beads to the PCR pool.

78 Pipette-mix the beads until evenly distributed.

79 Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.

80 Leave at room temperature for 10 minutes to allow DNA to bind beads.

81 Place 1.5mL LoBind tube in a magnetic separation rack until beads separate fully from the solution.

82 Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.

83 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.

84 Repeat the previous step.

85 Remove residual 80% ethanol: • Remove the LoBind tube from the magnetic separation rack. • Quick-spin the LoBind tube in a microcentrifuge. • Place the LoBind tube back in a magnetic separation rack until beads separate fully from the solution. Pipette off residual 80% ethanol and discard.

86 Remove the LoBind tube from the magnetic rack. Immediately add 50 µL of elution bufferto each tube and resuspend the beads.

87 Quick-spin the LoBind tube in a microcentrifuge.

88 Incubate at room temperature for 5 minutes to elute DNA.

89 Place the LoBind tube in a magnetic separation rack until beads separate fully from the solution.

90 Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new LoBind tube. Discard old tube with beads.

91 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.

92 Proceed to the next step of the protocol if sample quantity is acceptable (required input: 10 µg). Do not proceed if less than 6 µg is available.

MAS Array Formation

93 In a 0.2 mL PCR tube, add 10 µg of sample from Step 4.22, in 47 µL of volume. Dilute with elution buffer going into this step if sample is too concentrated.

94 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.

95 Pipette-mix each sample.

96 Run the MAS primer digestion program.

97 Add 3 µL of MAS adapter bc01–04 (use a single barcode per sample) and 20 µL of MAS ligation additive to each sample for a total volume of 80 µL.

98 Pipette-mix each sample.

99 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.
Reaction Mix 3 (RM3):

	A	B
	Component	**Volume**

	A	B
	MAS Ligase B uffer	10 µL
	MAS Ligase	10 µL
	TOTAL volum e	**20 µL**

100 Pipette-mix RM3 with wide bore tips.

101 Quick-spinRM3 in a microcentrifuge to collect liquid.

102 Add 20 µL of RM3 to each sample.

103 Pipette-mix each sample with wide bore tips

104 Run the MAS array ligation program. Heated lid set at 47°C

	A	B	C
	Step	Time	Temperature
	1	30 Minutes	37°C
	2	Hold	4°C

105 Cleanup with 1.2X SMRTbell cleanup beads

106 Add 1.2X v/v (120 µL) of resuspended, room-temperature SMRTbell cleanup beads to each sample.

107 Pipette-mix the beads with wide bore tips until evenly distributed.

108 Quick-spin the tube strip in a microcentrifuge to collect liquid.

109 Leave at room temperature for 10 minutes to allow DNA to bind beads.

110 Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

111 Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.

112 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.

113 Repeat the previous step.

114 Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack.

115 Add 1.2X v/v (120 µL) of resuspended, room-temperature SMRTbell cleanup beads to each sample.

116 Pipette-mix the beads with wide bore tips until evenly distributed.

117 Quick-spin the tube strip in a microcentrifuge to collect liquid.

118 Leave at room temperature for 10 minutes to allow DNA to bind beads.

119 Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

120 Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.

121 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.

122 Repeat the previous step.

123 Remove residual 80% ethanol:
Remove the tube strip from the magnetic separation rack.
Quick-spin the tube strip in a microcentrifuge.
Place the tube strip back in a magnetic separation rack until beads separate fully from the solution.
Pipette off residual 80% ethanol and discard.

124 Remove the tube strip from the magnetic rack. Using a wide bore pipette tip, immediately add 43 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.

125 Quick-spin the tube strip in a microcentrifuge to collect liquid.

126 Leave at room temperature for 5 minutes to elute DNA.

127 Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

128 Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new 0.5 mL LoBind tube. Discard old tube strip with beads.

129 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 is 5µg

DNA Damage repair

130 In a 0.2 mL PCR tube, add 5 µg of sample from Step 5.26, in 42 µL of volume. Dilute with elution buffer
going into this step if sample is too concentrated

131 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.
Reaction Mix 4 (RM4):

	A	B
	Component	Volume
	Repair buffer	6 µL

	A	B
	DNA Damage repair mix	2 µL

132 Pipette-mix RM4.

133 Quick-spin RM4 in a microcentrifuge to collect liquid.

134 Add 8 µL of RM4 to each sample. Total volume should equal 50 µL.

135 Pipette-mix each sample with wide bore tips.

136 Quick-spin the strip tube in a microcentrifuge to collect liquid.

137 Run the DNA damage repair program. Heated Lid set to 47°C

	A	B	C
	Step	Time	Temperature
	1	30 Minutes	37°C
	2	Hold	4°C

138 Clean up with 1.2X SMRTbell cleanup beads

139 Add 1.2X v/v (60 µL) of resuspended, room-temperature SMRTbell cleanup beads to each sample.

140 Pipette-mix the beads with wide bore tips until evenly distributed.

141 Quick-spin the tube strip in a microcentrifuge to collect liquid.

142 Leave at room temperature for 10 minutes to allow DNA to bind beads

143 Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

144 Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.

145 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.

146 Repeat the previous step.

147 Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.

148 Remove the tube strip from the magnetic rack. Using a wide bore pipette tip, immediately add 40 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.

149 Quick-spin the tube strip in a microcentrifuge to collect liquid.

150 Leave at room temperature for 5 minutes to elute DNA.

151 Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

152 Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip. Discard old tube strip with bead

Nuclease Treatment

153 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):

	A	B
	Component	Volume
	Nuclease Buffer	5 µL
	Nuclease Mix	5 µL
	Total Volume	10 µL

154 Pipette-mix RM5.

155 Quick-spin RM5 in a microcentrifuge to collect liquid.

156 Add 10 µL of RM5 to each sample. Total volume should equal 50 µL.

157 Pipette-mix each sample with wide bore tips.

158 Quick-spin the strip tube in a microcentrifuge to collect liquid.

159 Run the nuclease treatment program.

	A	B	C
	Step	Time	Temperature
	1	60 Minutes	37°C
	2	Hold	4°C

Final cleanup with SMRTbell cleanup beads

160 Add 60 µL SMRTbell cleanup beads to each sample from the previous step. Using wide bore tips, pipette-mix the beads until evenly distributed.

161 Quick-spin the tube strip in a microcentrifuge to collect all liquid.

162 Leave at room temperature for 10 minutes to allow DNA to bind beads.

163 Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

164 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.

165 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.

166 Repeat the previous step.

167 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.

168 Remove the tube strip from the magnetic rack. Immediately add 20 µL of elution bufferto each tube and resuspend the beads by pipetting 10 times or until evenly distributed with wide bore tips.

169 Quick-spin the tube strip in a microcentrifuge to collect liquid.

170 Leave at room temperature for 5 minutes to elute DNA.

171 Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

172 Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new 0.5 mL LoBind tube. Discard old tube strip with beads.

173 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.

174 Proceed to SMRT Link Sample Setup to prepare the SMRTbell library for sequencing.

175 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