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FLEXIR-seq - Full-Length targEted capture using eXon probes for IsofoRms

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We use this protocol and it's working

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Abstract

This protocol outlines the procedure for Target enrichment of cDNA using Twist probes for sequencing on ONT PromethION. It is used to investigate full-length transcript isoforms including transcripts that are lowly-expressed, and which are cell-type specific. It provides a powerful method for deep, quantitative, characterisation of transcript isoforms.

Materials

Consumables

Beckman Coulter Agencourt RNAClean XP beads (A63987)
Beckman Coulter Agencourt AMPure XP beads (A63881)
Eppendorf 1.5 ml DNA LoBind tubes (E0030108051)
Invitrogen Dynabeads M-270 Streptavidin (65305)
Life Technologies RNaseOUT 40 U/µl (10777019)
NEB NEBNext Quick Ligation Reaction Buffer (B6058)
NEB T4 DNA Ligase 2M U/ml (M0202M)
NEB Lambda Exonuclease (M0262L)
NEB Thermolabile Exonuclease I (M0568)
NEB USER (Uracil-Specific Excision Reagent) Enzyme (M5505L)
NEB 10 mM dNTP solution (N0447)
NEB LongAmp Hot Start Taq 2X Master Mix (M0533S)
ONT cDNA-PCR Barcoding Kit 24 v14 (SQK-PCB114.24)
ONT Ligation Sequencing Kit v14 (SQK-LSK114)
Sigma KOD Xtreme Hot Start DNA polymerase (PN 71975-3)
ThermoFisher Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (EP0752)
Twist Mechanical Fragmentation Library Preparation Kit 1 (101280)
Twist Library Preperation Kit 2 (100401)
Twist Universal Adapter System (101307)
Twist Standard Hyb and Wash Kit v2 (104445)
Twist Universal Blockers (100856)
Twist Custom Long-Read Panel special request (designed on your capture requirements) (107171)

Equipment

Thermocycler (Eppendorf Mastercycler M50 or similar)
Centrifuge (Sigma 1-14 or similar)
Magnet for tubes (Life Technologies DynaMag-2 or similar)
Magnet for plates (Invitrogen Dynal MPC-96 or similar)
ONT PromethION sequencing platform

Section 1: Make long stranded cDNA from total RNA (using ONT cDNA-PCR Barcoding Kit 24 v14 #SQK-PCB114.24)

1 For each sample, prepare up to 2000ng of total RNA in 10µl nuclease-free water, in 0.2 ml PCR tubes.

2 Mix the sample by flicking the tube to avoid unwanted shearing. Spin down briefly in a microfuge.

3 Prepare the following mix per sample:

	A	B
	Reagent	**Volume (µl)**
	RNA	10
	cDNA RT Adapter (CRTA)	1
	Annealing Buffer (AB)	1
	Total Volume	**12**

4 Mix gently by flicking the tubes, and spin down. Incubate the reactions in the thermal cycler at 60°C for 5 mins, then cool for 5 minutes at room temperature.

5 After the incubation, to each of the 0.2 ml PCR tubes containing the RNA sample, add the following (ensure the components are thoroughly mixed by flicking the tube and spin down):

	A	B
	Reagent	**Volume (µl)**
	RNA sample (from previous step)	12

	A	B
	NEBNext Quick Ligation Reaction Buffer	3.6
	T4 DNA Ligase 2M U/ml	1.4
	RNaseOUT	1
	Total	**18**

6 Incubate for 10 minutes at room temperature.

7 After the 10min incubation, to each of the 0.2 ml PCR tubes, add the following (ensure the components are thoroughly mixed by flicking the tube and spin down):

	A	B
	Reagent	**Volume (µl)**
	RNA sample (from previous step)	18
	Lambda Exonuclease	1
	NEBnext USER (Uracil-Specific Excision Reagent)	1
	Total	**20**

8 Incubate for 5 minutes at 37°C in the thermal cycler.

9 Resuspend the RNase-free XP beads by vortexing. Add 36 µl of resuspended RNase-free XP beads to each reaction and mix gently by flicking the tubes.

10 Incubate for 5 minutes at room temperature. Flick the tubes every 45 sec.

11 Spin down the samples and pellet on a magnet.

12 Keep the tubes on the magnet, and pipette off the supernatant.

13 Keep the tubes on the magnet and wash the beads with 100 µl of Short Fragment Buffer (SFB) as follows:
a- Wash the beads with 100 µl of Short Fragment Buffer (SFB).
b-Keeping the magnetic rack on the benchtop, rotate the tube by 180°. Wait for the beads to migrate towards the magnet and to form a pellet.
c-Rotate the tube 180° again (back to the starting position), and wait for the beads to pellet again.
d-Without disturbing the pellet, remove the Short Fragment Buffer (SFB) using a pipette and discard. Repeat the previous step. Spin down and place the tubes back on the magnet.

14 Pipette off any residual buffer.

15 Briefly allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

16 Remove the tubes from the magnetic rack and resuspend each pellet in 12 µl of nuclease-free water.

17 Incubate at room temperature for 10 minutes.

18 Pellet the beads on a magnet until the eluate is clear and colourless.

19 Remove and retain 12 µl of eluate into a clean 0.2 ml thin-walled PCR tube per sample.

20 To each of the 0.2 ml PCR tubes, add the following:
Ensure the components are thoroughly mixed by flicking the tubes and spin down.

	A	B
	Reagent	**Volume (µl)**
	Eluted sample (from previous step)	12
	RT Primer (RTP)	1
	dNTPs (10 mM)	1
	Total	**14**

21 Incubate the reaction for 5 minutes at room temperature.

22 To each of the 0.2 ml PCR tubes, add the following:
Ensure the components are thoroughly mixed by flicking the tubes and spin down.

	A	B
	Reagent	**Volme (µl)**
	RT primed sample (from previous step)	14
	Maxima H Minus 5x RT Buffer	4.5
	RNaseOUT	1
	Strand Switching Primer II (SSPII)	2
	Total	**21.5**

23 Incubate at 42°C for 2 minutes in the thermal cycler.

24 Add 1 µl of Maxima H Minus Reverse Transcriptase to each tube. The total volume will be 22.5 µl per tube. Mix gently by flicking the tubes, and spin down.

25 Incubate using the following protocol using a thermal cycler:

	A	B	C	D
	Cycle step	**Temperature**	**Time**	**No. of cycles**
	Reverse transcription and strand-switching	42°C	30 mins	1
	Heat inactivation	85°C	5 mins	1
	Hold	4°C		

26 The cDNA can be stored at -20C overnight.

Section 2: Twist Long Read Library preparation RNA (using Twist Mechanical Fragmentation Library Preparation Kit #101280 and Twist Universal Adapter System #101307)

27 Normalise the cDNA to 500ng input. Dilute the sample in Nuclease Free water (NFW) for a total volume if 35µl. Pipette into a 0.2-ml thin-walled PCR striptube and place on ice.

28 To each of the 0.2 ml PCR tubes, add the following on ice:

	A	B
	Reagent	**Volume (µl)**
	10x ERA Buffer	5
	ERA Enzyme Mix	10
	Total	**15**

Mix the diluted gDNA sample and reagents by flicking with a finger, then pulse-spin to ensure all of the solution is at the bottom of the tube.

29 Incubate using the following protocol using a thermal cycler: Start the program to pre-chill the thermal cycler

	A	B
	Temperature	**Time**
	4°C	Hold
	20°C	30 mins
	65°C	30 mins
	4°C	Hold

When the thermal cycler program is complete and the sample block has returned to 4°C, remove the samples from the block and place them on ice.

30 Add 5 µl Universal Adapters into each sample well. Mix gently by pipetting and keep on ice. Prepare a ligation mix in a 1.5-ml microcentrifuge tube on ice as indicated below.

	A	B
	Reagent	**Volume (µl)**
	Water (chilled)	15
	DNA Ligation Buffer	20
	DNA Ligation Mix	10
	Total	**45**

31 Add 45 µl ligation Mix well by flicking the tube. Cap the tubes and pulse-spin to ensure all solution is at the bottom of the tube.

32 Incubate the ligation reaction at 20°C for 1 hour in the thermal cycler, IMPORTANT: Turn off the heated lid or set to the minimum temperature

33 Then move the samples to the bench top. Proceed to the sample purification.

34 Vortex the pre-equilibrated DNA Purification Beads until well mixed.

35 Add 80 µl of homogenized DNA Purification Beads to each ligation sample. Mix well by flicking the tubes.

36 Incubate the samples for 5 minutes at room temperature.

37 Place the samples on a magnetic plate for 10 minutes.

38 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the tubes from the magnetic plate, remove and discard the supernatant.

39 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet).

40 Incubate for 1 minute at room temperature, then remove and discard the ethanol.

41 Repeat the wash once, for a total of two washes, while keeping the samples on the magnetic plate.

42 Carefully remove all remaining ethanol with a 10-µl pipette, making sure not to disturb the bead pellet.

43 Air-dry the bead pellet on the magnetic plate for 5 minutes. IMPORTANT: Do not overdry the beads. This may result in lower recovery of DNA.

44 Remove the tubes from the magnetic plate and add 37 µl of 0.1x TE buffer to each sample.

45 Mix by flicking until homogenized. Incubate at room temperature for 2 minutes.

46 Place the tubes on a magnetic plate and let stand for 3 minutes.

47 Transfer 35 µl of the clear supernatant containing the ligated and indexed libraries to a clean 0.2-ml thin-walled PCR strip-tube making sure not to disturb the bead pellet.

48 Prepare a PCR mix in a 1.5-ml microcentrifuge tube on ice as indicated below. Mix well by gentle pipetting.

	A	B
	Reagent	**Volume (µl)**
	Water (chilled)	10
	2X XtremeBuffer	100
	dNTPs (2mM)	40
	KOD Xtreme Hot Start DNA Polymerase	4
	Total	**154**

49 Add 10 µl Twist UDI Primers from the provided 96-well plate to each of the ligated libraries and mix well by gentle flicking.

50 Add 154 µl PCR master mix to each sample. Pulse-spin the sample tube.

51 Transfer 100 µl of 200 µl reaction to another tube and immediately transfer it to the thermal cycler.

52 Incubate using the following protocol. Set the temperature of the heated lid to 105°C.

	A	B	C	D
	Step	**Temperature**	**Time**	**Number of cycles**
	Initialization	94°C	2 minutes	1
	Denaturation	98°C	10 seconds	8
	Annealing	58.8°C	30 seconds	

	A	B	C	D
	Extension	68°C	10 minutes	
	Final Extension	68°C	10 minutes	1
	Final hold	4°C	Hold	-

53 Remove the sample from the block when the thermal cycler program is complete.

54 Vortex the pre-equilibrated DNA Purification Beads until mixed.

55 Add 50 µl (0.5X) homogenized DNA Purification Beads to the 1st and 2nd PCR reactions. Mix well by flicking.

56 Incubate the samples for 5 minutes at room temperature.

57 Place the samples on a magnetic plate for 1 minute.

58 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the tubes from the magnetic plate, remove and discard the supernatant.

59 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet), incubate for 1 minute at room temperature, then remove and discard the ethanol.

60 Repeat this wash once, for a total of two washes, while keeping the samples on the magnetic plate.

61 Carefully remove all remaining ethanol with a 10-µl pipette, making sure not to disturb the bead pellet.

62 Air-dry the bead pellet on the magnetic plate for 5 minutes. Do not overdry the bead pellet.

63 Remove the plate or tubes from the magnetic plate and add 22 µl water to the 1st PCR reaction. Mix by flicking until homogenized. NOTE: The 2nd PCR reaction will be eluted using the elution from the 1st PCR reaction

64 Incubate at room temperature for 2 minutes.

65 Place the tubes on a magnetic plate and let stand for 3 minutes.

66 Transfer 22 µl of the supernatant to the 2nd PCR reaction and repeat **steps 38-39** for the 2nd PCR reaction.

67 Transfer 20 µl of the clear supernatant containing the amplified, indexed libraries from both PCR reactions to a clean 0.2-ml thin-walled PCR strip-tube, making sure not to disturb the bead pellet.

68 Do a DNA Broad range Qubit and run on the Fragment analyser with the standard DNA kit to check for the quantity and size of the library fragments.

STOPPING POINT: If not proceeding immediately to a Twist Target Enrichment System, store the amplified indexed libraries at –20°C

Section 3: Twist Target Enrichment System (using Twist Standard Hyb and Wash Kit v2 with the custom Panel #104446 and Twist Universal Blockers #100578).

69 In order to multiplex 8 libraries, pipette 500ng of each library into a 0.2-ml thin-walled PCR tube. This is done by using the concentration of each amplified, indexed library to calculate the volume (in µl) of each library needed for hybridization. If the amount of library is insufficient, a smaller amount can be use but is may result in decreased library complexity and a low capture yield.

70 Dry the indexed library pool using a vacuum concentrator using low or no heat. Once the pool is completely dried down, cap the tube and set aside at room temperature.

71 Heat the Hybridization Mix at 65°C in the heat block for 10 minutes then cool to room temperature on the benchtop for 5 minutes.

72 Prepare a probe solution in a clean thin-walled PCR 0.2-ml strip-tube. Mix by pipetting up and down 10 times.

	A	B
	Reagent	**Volume (µl)**
	Hybridizatio n Mix	20
	Custom Panel	4
	Water	4
	Total	**28**

73 Resuspend the dried indexed library pool (from Step 2) by adding the reagents described below. Mix by pipetting up and down 10 times.

	A	B
	Reagent	**Volume (µl)**
	Dried Indexed Library Pool	-
	Blocker Solution	5
	Universal Blockers	7
	Total	**12**

74 Heat the probe solution to 95°C for 2 minutes in a thermal cycler with the lid at 105°C, then immediately cool on ice for 5 minutes.

75 While the probe solution is cooling on ice, heat the tube containing the resuspended indexed library pool at 95°C for 5 minutes in a thermal cycler with the lid at 105°C

76 Equilibrate both the probe solution and resuspended indexed library pool to room temperature on the benchtop for 5 minutes.

77 Vortex and spin down the probe solution, then transfer the entire volume to the resuspended indexed library pool. Mix well by vortexing.

78 Pulse-spin the tube to ensure all solution is at the bottom of the tube.

79 Add 30 µl Hybridization Enhancer to the top of the entire capture reaction.

80 Pulse-spin the tube to ensure there are no bubbles present. **IMPORTANT:** Seal the tube tightly to prevent excess evaporation over the 16-hour incubation.

81 Incubate the hybridization reaction at 70°C for 16 hours overnight in a thermal cycler with the lid at 85°C. NOTE: Halting hybridization between 15–17 hours will not affect downstream capture quality.

82 **Before starting step 82. Do the following:**

- Preheat the following tubes at 48°C until any precipitate is dissolved: Binding Buffer, Standard Wash Buffer 1 and Wash Buffer 2
- For each hybridization reaction:
  - Equilibrate 800 µl Binding Buffer to room temperature
  - Equilibrate 225 µl Std Wash Buffer 1 to 68ºC
  - Leave 700 µl Wash Buffer 2 at 48°C
- Equilibrate the Dynabeads M-270 Streptavidin Beads to room temperature for at least 30 minutes
- Make fresh 0.2 N NaOH using 100 µL of 2 N NaOH into 900 µL of molecular biology grade water

83 Vortex the pre-equilibrated Streptavidin Binding Beads until mixed.

84 Add 100 µl Streptavidin Binding Beads to a 1.5-ml microcentrifuge tube. Prepare one tube for each hybridization reaction.

85 Add 200 µl Binding Buffer to the tube and mix by pipetting.

86 Place the tube on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand

87 Repeat the wash **(Steps 85 and 86)** two more times for a total of three washes.

88 After removing the clear supernatant from the third wash, add a final 200 µl Binding Buffer and resuspend the beads by vortexing until homogenized.

89 Heat the resuspended beads at 68°C for at least 10 min.

90 After the hybridization of the library is complete, open the thermal cycler lid and directly transfer the volume of each preheated Streptavidin Binding Beads from **Step 89** into the corresponding tube of hybridization reaction. Mix by pipetting and flicking.

IMPORTANT: Rapid transfer directly from the thermal cycler at 70°C is a critical step for minimizing off-target binding. Do not remove the tube of hybridization reaction from the thermal cycler or otherwise allow it to cool to less than 70°C before transferring the solution to the washed Streptavidin Binding Beads. Allowing to cool to room temperature for less than 5 minutes will result in as much as 10–20% increase in off-target binding.

91 Incubate the tube of the hybridization reaction with the Streptavidin Binding Beads for 5 minutes at 68°C, agitation is not required. NOTE: Do not vortex. Aggressive mixing is not required.

92 Remove the tube containing the hybridization reaction with Streptavidin Binding Beads from the mixer and pulse-spin to ensure all solution is at the bottom of the tube.

93 Place the tube on a magnetic stand for 1 minute.

94 Remove and discard the clear supernatant including the Hybridization Enhancer. Do not disturb the bead pellet.

95 Remove the tube from the magnetic stand and add 200 µl 68°C Standard Wash Buffer 1. Mix by flicking.

96 Incubate the tube for 5 minutes at 68°C.

97 Pulse-spin to ensure all solution is at the bottom of the tube(s) and transfer the entire volume from Step 96 (~200 µl) into a new 1.5-ml microcentrifuge tube, one per hybridization reaction. Place the tube on a magnetic stand for 1 minute.

IMPORTANT: This step reduces background from non-specific binding to the surface of the tube

98 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

99 Remove the tube from the magnetic stand and add 200 µl of 48°C Wash Buffer 2. Mix by flicking, then pulse-spin to ensure all solution is at the bottom of the tube.

100 Incubate the tube for 5 minutes at 48°C.

101 Place the tube on a magnetic stand for 1 minute.

102 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

103 Repeat the wash (**Steps 99-102**) two more times, for a total of three washes.

104 After the final wash, use a 10 µl pipette to remove all traces of supernatant. Proceed immediately to the next step. Do not allow the beads to dry.

105 Remove the tube from the magnetic stand and add 12 µl water. Mix by flicking until homogenized, then place this solution, hereafter referred to as the Streptavidin Binding Bead slurry, on ice.

106 Add 12 µl 0.2 N NaOH (freshly diluted from 2 N NaOH) to the Streptavidin Binding Bead slurry. Vortex and spin down. Incubate at room temperature for 5 minutes.

107 Add 24 µl 200 mM Tris-HCl pH 8 to the mixture from **step 37**. Vortex and spin down.

108 Place the mixture on a magnetic rack and transfer the supernatant into a clean thin-walled PCR 0.2-ml strip-tube. Discard the beads.

109 Prepare a PCR mixture by adding the following reagents to the tube containing the supernatant. Mix by pipetting. Split 200 µl reaction into two 100 µl tubes.

	A	B
	Reagent	**Volume (µl)**
	Supernatant from Step 108	50
	Amplification Primers	6
	2X XtremeBuffer	100

	A	B
	dNTPs (2mM)	40
	KOD Xtreme Hot Start DNA Polymerase	4
	Total	**200**

110 Pulse-spin the tube, transfer them to the thermal cycler. Incubate using the following protocol. Set the temperature of the heated lid to 105°C.

	A	B	C	D
	Step	**Temperature**	**Time**	**Number of cycles**
	Initialization	94°C	2 mins	1
	Denaturation	98°C	10 secs	17
	Annealing	58.8°C	30 secs	
	Extension	68°C	10 mins	
	Final Extension	68°C	10 mins	1
	Final hold	4°C	Hold	-

111 Vortex the pre-equilibrated DNA Purification Beads until well mixed.

112 Combine the two separated 100 µl reactions into one 1.5-ml tube.

113 Add 100 µl (0.5X) DNA Purification Beads to the 1.5-ml tube. Mix well by flicking.

114 Incubate for 5 minutes at room temperature.

115 Place the tube on a magnetic plate for 1 minute or until the supernatant is clear.

116 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tube from the magnetic plate, remove and discard the clear supernatant.

117 Wash the bead pellet by gently adding 500 µl freshly prepared 80% ethanol (do not disturb the pellet).

118 Incubate for 1 minute at room temperature, then remove and discard the ethanol.

119 Repeat this wash once more, for a total of two washes, while keeping the tube on the magnetic plate.

120 Carefully remove all remaining ethanol using a 10 µl pipette, making sure to not disturb the bead pellet.

121 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

122 Remove the tube from the magnetic plate and add 42 µl water to each capture reaction. Mix by flicking until homogenized.

123 Incubate at room temperature for 2 minutes.

124 Place the plate or tube on a magnetic plate and let stand for 3 minutes.

125 Transfer 40 µl of the clear supernatant containing the enriched library to a clean thin-walled PCR 0.2-ml tube making sure to not disturb the bead pellet.

126 Validate and quantify each enriched library using the Agilent bioanalyser with the High sensitivity assay and a Qubit dsDNA High Sensitivity Assay.

Section 4: ONT Ligation (using ONT Ligation Kit V14 #SQK-LSK114). The aim is to ligate ONT adapters to the enriched libraries for sequencing on the PromethION.

127 Add 10 ul of NFW to the 40ul of enriched library

128 Add the following to the 50ul of library. Thoroughly mix the reaction by gently flicking and briefly spinning down

	A	B
	Reagent	**Volume (µl)**
	NEBnext Ultra II End-prep Reaction Buffer	7
	NEBnext Ultra II End-prep Enzyme Mix	3
	Total	**10**

129 Using a thermal cycler, incubate using the following protocol

	A	B
	Temperature	**Time**
	20°C	5 mins
	65°C	5mins

130 Resuspend the AMPure XP Beads (AXP) by vortexing.

131 Add 60 µl of resuspended the AMPure XP Beads (AXP) to the end-prep reaction and mix by flicking the tube.

132 Incubate for 5 minutes at room temperature. Every 1 minute gently flick the tube.

133 Prepare 500 µl of fresh 80% ethanol in nuclease-free water.

134 Spin down the sample and pellet on a magnet until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant.

135 Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

136 Repeat step 135

137 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 30 seconds, but do not dry the pellet to the point of cracking.

138 Remove the tube from the magnetic rack and resuspend the pellet in 61 µl nuclease free water. Incubate for 2 minutes at room temperature.

139 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

140 Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

141 Spin down the Ligation Adapter (LA) and T4 DNA Ligase, and place on ice.

142 Thaw Ligation Buffer (LNB) at room temperature, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.

143 Thaw the Elution Buffer (EB) at room temperature and mix by vortexing.

144 Thaw either Short Fragment Buffer (SFB) at room temperature and mix by vortexing. Then spin down and place on ice.

145 In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order. Thoroughly mix the reaction by gently flicking and briefly spinning down.

	A	B
	Reagent	**Volume (µl)**
	DNA sample from the previous step	60
	Ligation Adapter (LA)	5

	A	B
	Ligation Buffer (LNB)	25
	T4 DNA Ligase	10
	Total	**100**

146 Thoroughly mix the reaction by gently flicking and briefly spinning down. Incubate the reaction for 10 minutes at room temperature.

147 Resuspend the AMPure XP Beads (AXP) by vortexing.

148 Add 40 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by flicking the tube.

149 Incubate for 5 minutes at room temperature. Every 1 minute gently flick the tube.

150 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless.

151 Wash the beads by adding either 250 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.

152 Repeat step 151.

153 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for 30 seconds, but do not dry the pellet to the point of cracking.

154 Remove the tube from the magnetic rack and resuspend the pellet in 25 µl Elution Buffer (EB).

155 Spin down and incubate for 10 minutes at room temperature.

156 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

157 Remove and retain 32 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

158 The prepared library is used for loading into the PromethION flow cell. Store the library ice or at 4°C until ready to load. 50 fmol is required for loading onto the flow cell.

159 Quantify the library using the Qubit DNA high sensitivity assay.

Protocol references

Oxford Nanopore Technologies cDNA-PCR Barcoding Kit 24 V14 protocol (pcr-cdna-sequencing-v14-barcoding-sqk-pcb114-24-PCB_9201_v114_revC_06Dec2023-minion)

Oxford Nanopore Technologies Ligation sequencing amplicons V14 (SQK-LSK114 (VACDE_9163_v114_revU_29Jun2022)

Twist Long Read Library Preparation and Standard Hyb v2 Enrichment (DOC-001320 REV 2.0)