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Nanopore genome sequencing with barcode

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We use this protocol and it's working

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Abstract

The modified version of official protocol using Kappa chemicals

Troubleshooting

Step1. End-prep & A-Tailing

1h

1 In a 200µl PCR tube, mix the following:

	Reagent	Volume	Cap color
	40 fmol DNA 250ng/ sample	25 µl	
	End Repair & A-Tailing Buffer (KAPA)	3.5 µl	Purple
	End Repair & A-Tailing Enzyme Mix (KAPA)	1.5 µl	Purple
	Total	30 µl	

1.1 Ensure the components are thoroughly mixed by pipetting, and spin down.

1.2 Using a thermal cycler, incubate at 20 °C for 00:30:00 and 65 °C for 00:30:00 . Hold at 4 °C

1h

Step2. Barcode Ligation

30m

2 Thaw LNB, Native Barcode at room temperature, spin down and mix by vortex.

Note

Place on ice immediately after thawing and mixing.

2.1 In a 200µl PCR tube, mix in the following order:

	Reagent	Volume	Color
	End repair and A-tailing reaction product	30µl	
	Native Barcode	2.5µl	White
	LNB (LSK109 Kit)	13.75µl	White

	Reagent	Volume	Color
	DNA Ligase (KAPA)	5µl	Yellow
	DI Water	3.75µl	
	Total	55µl	

2.2 Ensure the components are thoroughly mixed by pipetting, and spin down.

2.3 Using a thermal cycler, incubate at 20 °C 00:30:00 . 30m

Note

Prewarm HyperPure beads at RT before use.

Note

To achieve higher conversion rates and library yields, particularly for low-input samples, consider increasing the ligation time—to a maximum of 4 hrs at 20°C, then overnight at 2°C to 8°C. Please note that longer ligation times may lead to increased levels of adapter-dimer. Adapter concentrations may have to be optimized if ligation times are extended significantly.

Step3. Barcode Ligation Products clean-up 30m

3 Add 44 ul (0.8X) of resuspended HyperPure beads to the barcode-ligated reaction and mix by flicking the tube.

3.1 Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.

3.2 Prepare 500 ul of fresh 70% ethanol in Nuclease-free water.

3.3 Spin down the sample and pellet on a magnetic stand.

3.4 Keep the tube on the magnetic stand, and pipette off the supernatant.

3.5 Keep the tube on the magnetic stand and wash the beads with 200 ul of freshly-prepared 70% ethanol without disturbing the pellet.

3.6 Remove the ethanol using a pipette and discard.

3.7 Repeat the previous step to remove ethanol.

3.8 Spin down and place the tube back on the magnetic stand.

3.9 Pipette off any residual ethanol.

3.10 Allow to dry for ~30 seconds(1-3 min), but do not dry the pellet to the point of cracking.

3.11 Remove the tube from the magnetic stand and resuspend the pellet in 11 ul Nuclease-free water.(55 °C 1 min)

3.12 Pellet the beads on a magnetic stand until the eluate is clear and colourless.

3.13 Remove and retain 11 ul of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

3.14 Quantify 1 µl of eluted sample using a Quantus fluorometer.

Step4. Adapter Ligation (AMII) 30m

4 Pool samples to be totaly 100 fmol DNA mix.

4.1

	Reagent	Volume	Color
	~50 fmol pooled barcoded DNA in DDW	67.5 µl	
	Adapter Mix II (AMII)	5 µl	Green
	LNB(LSK109 Kit)	27.5 µl	White
	DNA Ligase (KAPA)	10 µl	Yellow
	Total volume	110 µl	

4.2 Using a thermal cycler, incubate at 20 °C 00:30:00 . 30m

Note

Prewarm HyperPure beads at RT before use.

Note

To achieve higher conversion rates and library yields, particularly for low-input samples, consider increasing the ligation time—to a maximum of 4 hrs at 20°C, or after 15 min incubation and then overnight at 2°C to 8°C. Please note that longer ligation times may lead to increased levels of adapter-dimer. Adapter concentrations may have to be optimized if ligation times are extended significantly.

Step5. DNA library clean-up 30m

5 Add 44 ul (0.4x beads) of resuspended HyperPuer beads to the adapter-ligated reaction and mix by pipetting.

5.1 Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.

5.2 Place on magnetic stand, allow beads to pellet and pipette off supernatant.

5.3 Wash the beads by adding either 250 µl Long Fragment Buffer (LFB) or 250 µl S Fragment Buffer (SFB)(<3k).

5.4 Flick the beads to resuspend, then return the tube to the magnetic stand and allow the beads to pellet.

5.5 Remove the supernatant using a pipette and discard.

5.6 Wash the beads by adding either 250 µl Long Fragment Buffer (LFB) or 250 µl S Fragment Buffer (SFB)(<3k).

5.7 Flick the beads to resuspend, then return the tube to the magnetic stand and allow the beads to pellet.

5.8 Remove the supernatant using a pipette and discard.

5.9 Spin down and place the tube back on the magnetic stand.

5.10 Pipette off any residual supernatant.

5.11 Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

5.12 Remove the tube from the magnetic stand and resuspend the pellet in 14 ul Elution Buffer.

5.13 Keep at RT for 10 min.

5.14 Pellet the beads on a magnetic stand until the eluate is clear and colourless.

5.15 Remove and retain 14 ul of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

5.16 Quantify 1 µl of eluted sample using a Quantus fluorometer.

Note

Loading 5–50 fmol (or less than 300ng) of final prepared library onto R9.4.1 flow cells.

Step6. Priming and loading

30m

6 During incubation, take out SQB, FLT, FLB from the frige 30 minutes earlier to thaw on ice.

6.1 Thaw the flow cell, Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature.

6.2 Open the GridION and slide the flow cell under the clip.

Note

Press down firmly on the flow cell to ensure correct thermal and electrical contact.

6.3 Check flow cell (Check the pore)

Note

This takes about 10 minutes.

6.4 Mix the Sequencing Buffer (SQB), Flush Tether (FLT) and Flush Buffer (FB) tubes by pipetting and spin down at room temperature.

6.5 Remove the flow cell from the machine and slide the priming port cover clockwise to open the priming port.

6.6 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µl):
1. Set a P1000 pipette to 200 µl.
2. Insert the tip into the priming port.

3. Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip.

Note

Visually check that there is continuous buffer from the priming port across the sensor array.

6.7 To prepare the flow cell priming mix, add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at room temperature.

	Reagent	Volume	Color
	Flush Tether (FLT)	30µl	Purple
	Flush Buffer (FB)	New one	Blue
	Total	1.2ml	

6.8 Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes. During this time, prepare the library for loading by following the steps below.

Note

Closed the priming port.

6.9 Thoroughly mix the contents of the Loading Beads (LB) by flick.

6.10 In a new tube, prepare the library for loading as follows:

	Reagent	Volume	Color
	Sequencing Buffer (SQB)	37.5µl	Red
	Loading Beads (LB),mixed immediately before use	25.5µl	Pink

	Reagent	Volume	Color
	DNA library	12µl	
	Total	75µl	

6.11 Complete the flow cell priming:
1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load 200 µl of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

Note

Load the library as soon as possible after this step.

6.12 Mix the prepared library gently by pipetting up and down just prior to loading.

6.13 Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

6.14 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the GridION.

Step7.Flow Cell Wash

30m

7 Place the tube of Wash Mix (WMX) on ice. Do not vortex the tube.

7.1 Thaw one tube of Wash Diluent (DIL) at room temperature.

7.2 Mix the contents of Wash Diluent (DIL) thoroughly by vortexing, spin down briefly and place on ice.

7.3 In a clean 1.5 ml Eppendorf DNA tube, prepare the following Flow Cell Wash Mix:

7.4

	Component	Volume	Color
	Wash Mix (WMX)	2µl	Brown
	Wash Diluent (DIL)	398µl	Brown
	Total	400µl	

7.5 Mix well by pipetting, and place on ice. Do not vortex the tube.

7.6 Stop or pause the sequencing experiment in MinKNOW, and leave the flow cell in the device.

7.7 Ensure that the priming port cover and SpotON sample port cover are in the positions indicated in the figure below.

7.8 Using a P1000, remove all fluid from the waste channel through Waste port 1.
As both the priming port and SpotON sample port are closed, no fluid should leave the sensor array area.

7.9 Rotate the flow cell priming port cover clockwise so that the priming port is visible.

7.10 Check for air between the priming port and the sensor array. If necessary, using a P1000 draw back a small volume to remove any air (a few µls):
1. Set a P1000 pipette to 200 µl
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer/liquid entering the pipette tip.
4. Visually check that there is continuous buffer from the priming port across the sensor array.

7.11 Load 400 µl of the prepared Flow Cell Wash Mix into the flow cell via the priming port, avoiding the introduction of air.

7.12 Close the priming port and wait for 30 minutes.

7.13 Ensure that the priming port cover and SpotON sample port cover are in the positions indicated in the figure below.

7.14 Using a P1000, remove all fluid from the waste channel through Waste port 1. As both the priming port and SpotON sample port are closed, no fluid should leave the sensor array area.

Note

It is vital that the flow cell priming port and SpotON sample port are closed to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.

Step8. Store the MinION/GridION flow cell for later use

30m

8 Thaw one tube of Storage Buffer (S) at room temperature.

8.1 Mix contents thoroughly by pipetting and spin down briefly.

8.2 Rotate the flow cell priming port cover clockwise so that the priming port is visible.

8.3 Check for air between the priming port and the sensor array. If necessary, using a P1000 draw back a small volume to remove any air (a few µls):
1. Set a P1000 pipette to 200 µl
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer/liquid entering the pipette tip.
4. Visually check that there is continuous buffer from the priming port across the sensor array.

8.4 Slowly add 500 µl of Storage Buffer (S) through the priming port of the flow cell.

8.5 Close the priming port.

8.6 Using a P1000, remove all fluid from the waste channel through Waste port 1. As both the priming port and SpotON sample port are closed, no fluid should leave the sensor array area.

Note

It is vital that the flow cell priming port and SpotON sample port are closed to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.

8.7 The flow cell can now be stored at 4-8°C.

8.8 When you wish to reuse the flow cell, remove the flow cell from storage, and allow it to warm to room temperature for ~5 minutes. You will need to perform a Flow Cell Check before loading the next library.

Note

We recommend storing libraries in Eppendorf DNA LoBind tubes at 4°C for short term storage or repeated use, for example, re-loading flow cells between washes.
For single use and long term storage of more than 3 months, we recommend storing libraries at -80°C in Eppendorf DNA LoBind tubes.
For further information, please refer to the DNA library stability Know-How document.