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ARTIC LoCost Amplicon Sequencing Protocol (SQK-NBD114)

Forked from ARTIC SARS-CoV-2 sequencing protocol v4 (LSK114)

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We use this protocol and it's working

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Abstract

Library preparation and sequencing protocol for any ARTIC amplicon scheme. To check validated primer schemes, visit our open-source repository Primal Scheme Labs (https://labs.primalscheme.com/). To generate a new scheme, use Primal Scheme 3 (https://primalscheme.com).

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Changes in this version:
- Oxford Nanopore Technologies kit V14 reagents
- R10.4.1 MinION flow cells
- SFB wash volume reduced to 125 µL

Materials

Reagents:

	Component	Supplier	Part Number
	NEBNext® Ultra II End Repair/dA-tailing module	NEB	E7546
	Blunt/TA Ligase Master Mix	NEB	M0367
	Native Barcoding Kit V14 (24 or 96 barcodes)	ONT	SQK-NBD114.24 or SQK-NBD114.96
	NEBNext® Quick Ligation Module	NEB	NEB
	R10.4.1 flow cell	ONT	FLO-MIN114
	Qubit™ dsDNA HS Assay Kit	Invitrogen by Thermo Fisher Scientific	Q32854
	UltraPure™ BSA 50 mg/mL	Invitrogen by Thermo Fisher Scientific	AM2616
	100% ethanol (molecular biology grade)		
	Nuclease-free water		

Equipment:

- Magnetic rack
- Microcentrifuge
- Vortex mixer
- Thermal cycler
- P1000, P200, P20 and P2 pipette
- Ice bucket
- Timer
- Qubit fluorometer or equivalent

PCR Dilution (or Post-PCR Clean-up)

1 Label strip-tubes/plate and combine the following volumes of each PCR reaction:

	Component	Volume
	Pool 1 PCR reaction	2.5 µL
	Pool 2 PCR reaction	2.5 µL
	Nuclease-free water	45 µL
	Total	**50 µL**

Note

The PCR post-clean-up concentration is typically around [M] 100 ng/uL. This means we can pool them without quantification/normalisation to make a significant time saving. If you require even barcode representation perform clean-up and normalise to [M] 10 ng/uL then continue.

Note

Amplicons should be added in the **post-PCR** cabinet which should be cleaned with decontamination wipes and UV sterilised before and after use.

End Prep

2 In a new PCR strip-tube/plate set up the following reaction for each sample:

	Component	Volume
	PCR dilution from the previous step	3.3 µL
	Ultra II End Prep Reaction Buffer	1.2 µL
	Ultra II End Prep Enzyme Mix	0.5 µL

	Component	Volume
	Nuclease-free water	5 µL
	Total	**10 µL**

Note

Make a master mix of the end repair/dA-tailing reagents and nuclease-free water and aliquot
into strip-tube/plate to improve reproducibility.

3 Incubate the reaction as follows:
20 °C for 00:05:00
65 °C for 00:05:00
On ice for 00:01:00

Native Barcoding

4 In a new PCR strip-tube/plate set up the following barcode ligation reaction for each end-prepped sample:

	Component	Volume
	Blunt/TA Ligase Master Mix	5 µL
	End-preparation reaction mixture	0.75 µL
	Native barcode (01-96)	1.25 µL
	Nuclease-free water	3 µL
	Total	**10 µL**

Note

Use one native barcode from the SQK-NBD114.24 (1 - 24) or SQK-NBD114.96 (1 - 96) per sample.

Note

If processing <11 samples, increase quantities in the above reaction to allow for sufficient material for sequencing. For example, if processing 6 samples, double the component volumes for a final reaction volume of 20 µL for each sample.

5 Incubate the reactions as follows:
Room temperature for 00:20:00
65 °C for 00:10:00
On ice for 00:01:00

Note

The 65 °C incubation is to inactivate the DNA ligase to prevent barcode crossligation
when reactions are pooled in the next step. Alternatively, EDTA can be used to inactivate DNA ligase.

Pool and Clean-up

6 In a new 1.5 mL Eppendorf tube pool all barcoded samples together.

Note

If processing <24 samples pool the total volume from all barcodes. If processing 48 samples pool 5 µL from each native barcoding reaction. If processing 96 samples pool 2.5 µL from each native barcoding reaction to avoid exceeding a pool volume of 240 µL, which would make the clean-up volume too large.

7 Add 0.4X volume of AMPureXP (AXP) beads and mix gently by either flicking or pipetting. Incubate for 00:05:00 at Room temperature .

Note

Vortex the beads thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown colour.

Note

Incubate on a Hula mixer (rotator/orbital mixer) if available.

8 Place the tube on a magnetic rack and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Remove and discard the supernatant, being careful not to touch the pellet.

9 Add 125 µL of Short Fragment Buffer (SFB) and resuspend the bead pellet by pipette mixing. Pulse centrifuge to collect all liquid at the bottom of the tube and place on the magnet. Wait until the beads have pelleted to remove and discard the supernatant.

10 Repeat the previous step.

11 Wash the pellet with 200 µL of room-temperature freshly prepared 70% Ethanol. Remove and discard Ethanol, being careful not to touch the bead pellet.

12 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible.

13 With the tube lid open incubate for 00:00:30 or until the pellet loses its shine.

Note

This will allow any residual ethanol to evaporate.

Note

Do not let the pellet dry completely to the point of cracking.

14 Resuspend the pellet in 31 µL of Elution Buffer (EB). Mix gently by either flicking or pipetting. Incubate for 00:02:00 at Room temperature.

Note

10 mM Tris pH 8.0 can be used as an alternative to EB.

15 Place the tube on a magnet. Wait until the solution becomes clear/colourless and transfer the eluate into a fresh 1.5 mL Eppendorf DNA LoBind tube.

Note

Ensure that no beads are transferred into the fresh 1.5 mL Eppendorf DNA LoBind tube.

Note

You can now quantify your sample pool using a fluorometer, such as Qubit or Quantus. Concentration will vary depending on number and Ct of samples. This is to ensure that there was no significant DNA loss in the clean-up, and to check whether there is enough DNA to achieve an optimal run yield.

Adapter Ligation

16 Set up the adapter ligation reaction as follows:

	Component	Volume
	Pooled barcoded samples	30 µL
	Native Adapter (NA)	5 µL
	5X Quick Ligation Buffer	10 µL
	Quick T4 Ligase	5 µL

	Component	Volume
	Total	**50 µL**

17 Incubate the reaction for 00:02:00 at Room temperature .

Clean-up

18 Add 1X of the AMPureXP (AXP) beads to the sample tube and mix gently by either flicking or pipetting. Pulse centrifuge to collect all liquid at the bottom of the tube. Incubate for 00:05:00 at Room temperature .

Note

Vortex the beads thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown colour.

Note

Incubate on a Hula mixer (rotator/orbital mixer) if available.

19 Place on a magnetic rack and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Remove and discard the supernatant, being careful not to touch the bead pellet.

20 Add 125 µL of Short Fragment Buffer (SFB) and resuspend the bead pellet by pipette mixing. Pulse centrifuge to collect all liquid at the bottom of the tube and place on the magnet. Wait until the beads have pelleted to remove and discard the supernatant.

21 Repeat the previous step.

Note

Do not use ethanol in this clean-up to avoid damaging the adapter-protein complexes.

22 Pulse centrifuge and remove any residual Short Fragment Buffer (SFB).

Note

There is no need to air dry the pellet with SFB washes.

23 Resuspend the pellet in 16 µL of Elution Buffer (EB). Mix gently by either flicking or pipetting. Incubate for 00:02:00 at Room temperature .

Note

10 mM Tris pH 8.0 can be used as an alternative to EB.

24 Place the tube on a magnet. Wait until the solution becomes clear/colourless and transfer the eluate into a fresh 1.5 mL Eppendorf DNA LoBind tube.

Note

Ensure that no beads are transferred into the Eppendorf tube.

Note

Final library with the adapter can be stored in EB (or 10 mM Tris pH 8.0) in the fridge (~ 4 °C) for up to a week if needed.

DNA Quantification Using the Qubit Fluorometer

25 Prepare the Qubit working solution by diluting the Qubit dsDNA HS Reagent 1:200 in the Qubit dsDNA HS Buffer. The final volume in each tube must be 200 µL . Prepare

sufficient amount to accommodate all standards and samples. Each standard requires 190 µL of Qubit working solution, and each sample requires 199 µL.

Note

For optimal performance all solutions should be at room temperature.

Note

The Qubit standards should be stored at about 4 °C. Qubit dsDNA HS Buffer and Reagent can be stored at the room temperature. The Reagent should be protected from light.

26 Combine 190 µL of the Qubit working with 10 µL of each Qubit standard.

27 Combine 199 µL of the Qubit working solution with 1 µL library.

28 Vortex all Qubit tubes for 00:00:02 to 00:00:03, then spin them down. Allow all tubes to incubate at room temperature for 00:02:00.

Note

Be careful not to introduce bubbles.

29 On the Home screen of the Qubit Fluorometer select the dsDNA High Sensitivity as the assay type.

30 Calibrate the instrument by reading the standards. Insert the tube containing Standard #1 into the sample chamber, close the lid, then press Read standard. When the reading is complete, remove Standard #1. The
instrument displays the results on the Read standard screen.

31 Repeat the previous step but this time use the Standard #2.

32 Once calibrated, run the library sample. Select the sample volume and units by pressing the + or – buttons on the wheel to select the sample volume added to the assay tube (1 µL). Insert a sample tube into the sample chamber, close the lid, then press read tube. Once the reading is completed, remove the sample tube.

Note

The results show on the assay screen. The top value is the sample concentration. Bottom value is the dilution concentration.

Final Library

33 Prepare the final library in 12 µL of Elution Buffer (EB). Store the library on ice or at 4 °C until ready to load.

Note

The library loading amount depends on the fragment size. 100 fmol for < 1 kb fragments, 100 fmol for 1 - 10 kb or 300 ng for > 10 kb.

Note

If the library yields are below the input recommendations, load the entire library.

Note

Store DNA libraries in Eppendorf DNA LoBind tubes at 4 °C for short-term storage or repeated use. For single use and long-term storage of more than 3 months, store at -80 °C in Eppendorf DNA LoBind tubes.

Priming and Loading a MinION Flow Cell

34 Prepare the flow cell priming mix by combining the following components:

	Component	Volume
	Flow Cell Flush (FCF)	1,170 µL
	Flow Cell Tether (FCT)	30 µL
	Bovine Serum Albumin (BSA) at 50 mg/mL	5 µL
	Total	**1,205 µL**

Note

For optimal sequencing performance and improved output on R10.4.1 flow cells it is recommended to add Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/mL. Any other albumin types (e.g. recombinant human serum albumin) are not recommended.

Note

When using a tubes format, add 5 µL Bovine Serum Albumin (BSA) at 50 mg/mL and 30 µL Flow Cell Tether (FCT) directly to a tube of Flow Cell Flush (FCF).

35 Complete a flow cell check to assess the number of pores available before loading the library.

36 Slide the flow cell priming port cover clockwise to open the priming port.

37 Draw back a small volume to remove any bubbles:

- Set a P1000 pipette to 800 µL.
- Insert the tip into the priming port.
- Turn the wheel until the dial shows 820 µL to 830 µL, to draw back 20 µL to 30 µL or until you can see a small volume of the buffer entering the pipette tip.

38 Load 800 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles.

39 Close the priming port and wait for 00:05:00 .

40 During the waiting time, prepare the library for loading:

	Component	Volume
	Library	12 µL
	Library beads (LIB)	25.5 µL
	Sequencing buffer (SB)	37.5 µL
	Total	**75 µL**

Note

Vortex the library beads and add them to the final library immediately before use and they settle very quickly.

Note

The library beads are recommended for most sequencing experiments. However, they can be replaced by the Library Solution (LIS) when sequencing more viscous libraries.

41 Complete the flow cell priming - lift the SpotON sample port cover and open slide the flow cell priming port cover clockwise to open the priming port. Load 200 µL of the priming mix into the flow cell priming port.

42 Pipette mix the prepared library. Load dropwise onto the SpotON sample port.

43 Close the SpotON and priming ports.

44 Install the light shield on your flow cell.

MinION Sequencing

45 Start the sequencing run using MinKNOW. Select the FLO-MIN114 flow cell and SQK-NBD114 barcoding kit. Turn on dual barcoding (barcodes on both ends).

Protocol references

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