

Dec 22, 2024

Nanopore amplicon sequencing with R10.4.1 flow cells

DOI

<https://dx.doi.org/10.17504/protocols.io.5qpvo9owbv4o/v1>

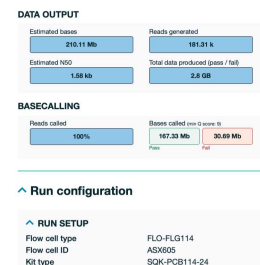
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Protocol Citation: Teng Li, Miao Wang, Zhengzheng Zhu, Allen Rodrigo 2024. Nanopore amplicon sequencing with R10.4.1 flow cells . protocols.io <https://dx.doi.org/10.17504/protocols.io.5qpvo9owbv4o/v1>

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Protocol status: Working

We use this protocol and it's working

Created: December 19, 2024

Last Modified: December 22, 2024

Protocol Integer ID: 116140

Keywords: Nanopore sequencing, long read, R10.4.1 flow cell, Flongle, amplicon sequencing, MinION, nanopore amplicon, method for nanopore amplicon, latest nanopore, efficiency of the latest nanopore, sequencing technology, nanopore, sequencing kit, microbiome profiling, oxford nanopore technology, including microbiome profiling, sequencing, latest version of flow cell, new flow cell, phylogenetic analysis, specifications of the new flow cell, flow cell, barcoded outer primer, pcr barcoding kit sqk, coi gene for animal

Funders Acknowledgements:

Teng Li

Grant ID: 9489-3727864-AB2J

Allen Rodrigo

Grant ID: 4020-12090

Abstract

This protocol describes an updated method for Nanopore amplicon sequencing with R10.4.1 flow cells, building upon the original protocol "**Nanopore amplicon sequencing**" by Yoshiyuki Matsuo (DOI: <https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4>). The two-step PCR approach enables sequencing with a user-defined inner primer set combined with barcoded outer primers, leveraging rapid adapter attachment chemistry. With the latest version of flow cells and sequencing kits, Nanopore sequencing can achieve Q20 accuracy. This method supports a wide range of applications, including microbiome profiling, taxonomy identification (e.g., COI gene for animals), phylogenetic analysis, and identifying genetic variations in targeted loci.

Since the **R9.4.1 flow cell** and the **PCR Barcoding Kit SQK-PBK004** used in the original protocol have been discontinued, and Oxford Nanopore Technologies has decided not to release a direct upgrade for the SQK-PBK004 kit, this protocol has been revised for compatibility with the latest **R10.4.1 flow cell** and the **cDNA-PCR Barcoding Kit SQK-PCB114.24**. The updates include modifications to reagent names and volumes, PCR cycle settings, and library preparation steps to align with the specifications of the new flow cell and kit. These changes ensure that the method remains robust and accessible while leveraging the enhanced accuracy and efficiency of the latest Nanopore sequencing technology. We also use the **Flongle flow cell FLO-FLG114** as an example, which is ideal for smaller-scale experiments or pilot studies, as well as Nanopore didn't provide a specific Flongle protocol for cDNA-PCR Barcoding Kit SQK-PCB114.24.

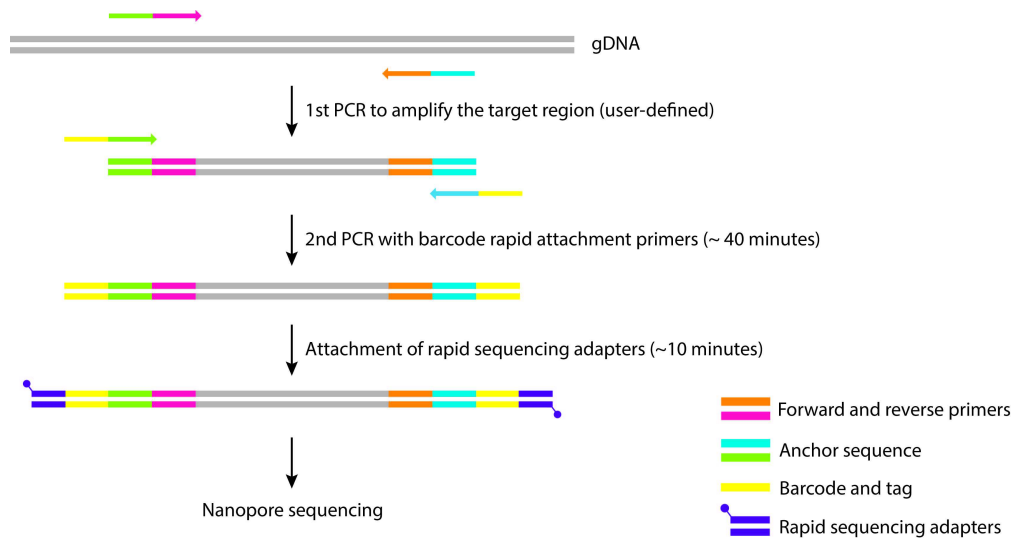


Materials

- KAPA2G Robust HotStart ReadyMix (2X) (Kapa Biosystems, KK5701)
- PCR-grade or nuclease-free water
- cDNA-PCR Barcoding Kit (Oxford Nanopore Technologies, SQK-PCB114.24)
- Agencourt AMPure XP (Beckman Coulter, A63880)
- Freshly prepared 70% ethanol
- Qubit 1X dsDNA High Sensitivity (HS) (Thermo-Fisher, Q33231)
- Flow cell R10.4.1 (Oxford Nanopore Technologies, FLO-FLG114)
- Flongle Sequencing Expansion (Oxford Nanopore Technologies, EXP-FSE002)
- [Optional] E-Gel EX Agarose Gels, 1% (Thermo Fisher Scientific, G402021)
- [Optional] E-Gel 1 Kb Plus Express DNA Ladder (Thermo Fisher Scientific, 10488091)

Workflow

1



Workflow for the two-step PCR approach in Nanopore amplicon sequencing library preparation.

For the first PCR amplification, target regions of interest are amplified using specific primers (user-defined) flanked by anchor sequences. In the second PCR reaction, rapid attachment barcode primers are employed to generate barcoded amplicons, which require approximately 40 minutes. Then, attach the rapid sequencing adapters to the PCR products, followed by priming and loading the flow cell. This figure was adapted from Yoshiyuki Matsuo (<https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4>).

The reason for choosing cDNA-PCR Barcoding Kit 24 V14 (SQK-PCB114.24)

2 Since the PCR Barcoding Kit (SQK-PBK004) has been discontinued, and Oxford Nanopore Technologies has decided not to release a direct upgrade, we explored alternative options by consulting with the Oxford Nanopore Technical Services team and looking through other related kits that are compatible with the R10 version flow cell. We find some alternative options:

- 1. Ligation Sequencing Kit V14 (SQK-LSK114) with PCR Barcoding Expansion Packs 1-12 (EXP-PBC001) or 1-96 (EXP-PBC096).** This combination maintains input consistency with SQK-PBK004 and allows for sequencing the full-length amplicons. However, it would require third-party reagents and involve ligation sequencing, which means that it will take more time and increase the cost.
- 2. cDNA-PCR Barcoding Kit 24 V14 (SQK-PCB114.24).** Identical workflow as SQK-PBK004, except for the upstream steps for cDNA preparation. Whilst this is a cDNA



kit, the barcodes supplied in the kit (BP01-24) are identical to those in SQK-PBK004 based on Nanopore's current [chemistry technical document](#) (see excerpt below):

Legacy PCR Barcoding Kit (SQK-PBK004): BP01-12

PCR-cDNA Barcoding Kit 24 V14 (SQK-PCB114.24): BP01-24

Barcode Flanking Sequences:

Top Strand: 5'-ATCGCCTACCGTGA-[barcode]-TTGCCTGTCGCTCTATCTTC-3'

Bottom Strand: 5'-ATCGCCTACCGTGA-[barcode]-TCTGTTGGTGCTGATATTGC-3'

The 3' ends of these outer primers match the 5' ends of the primers specified in the original protocol (matching sequences underlined) by Yoshiyuki Matsuo

(<https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4>)

Forward (FW) primer: 5'-TTTTCTGTTGGTGCTGATATTGC-[target-specific sequence]-3'

Reverse (RV) primer: 5'-ACTTGCCTGTCGCTCTATCTTC-[target-specific sequence]-3'

As such, these barcode primers can amplify the target amplicon in the second PCR reaction to generate barcoded amplicons. This would incorporate the rapid attachment chemistry to enable the addition of the sequencing adapters. For PCB114.24, we will use RA and ADB as per the [SQK-PCB114.24 protocol](#) (instead of RAP used with RBK004).

In sum, although the **Ligation Sequencing Kit V14 with PCR Barcoding Expansion Packs** can serve as an alternative to SQK-PBK004, it incurs higher costs and is more laborious. Conversely, the **cDNA-PCR Barcoding Kit 24 V14** offers a workflow nearly identical to SQK-PBK004, allowing for a transition with minimal changes, and making it a more efficient alternative option.

First-round PCR to amplify the target region

3

Note

The first-round PCR products with the following tailed primers are required:

Forward (FW) primer: 5'-TTTCTGTTGGTGCTGATATTGC-[target-specific sequence]-3'

Reverse (RV) primer: 5'-ACTTGCCTGTCGCTCTATCTTC-[target-specific sequence]-3'

Here, we use 16S rRNA gene-specific primers (in bold letters) to amplify the V1–V9 region of the 16S rRNA gene.

27F: 5'-TTTCTGTTGGTGCTGATATTGC **AGRGT**TYGATYMTGGCTCAG-3'

1492R: 5'-ACTTGCCTGTCGCTCTATCTTC **CGGYT**ACCTTGTTACGACTT-3'



Thaw the KAPA2G Robust HotStart 2X ReadyMix at Room temperature , spin down briefly, mix well by pipetting, and place On ice .

- 4 In each 0.2 ml thin-walled PCR tube, prepare the following mixture:

A	B	C
Reagent	Volume	Final concentration
Template DNA	x μ L	10 ng
10 μ M Forward primer	0.5 μ L	0.2 μ M
10 μ M Reverse primer	0.5 μ L	0.2 μ M
KAPA2G Robust HotStart 2X Ready Mix	12.5 μ L	1 X
PCR grade water	11.5 - x μ L	
Total	25 μL	

- 5 Mix gently by pipetting, and amplify using the following cycling conditions:

A	B	C	D
Cycle step	Temperature	Time	No. of cycles
Initial denaturation	95 °C	3 mins	1
Denaturation	95 °C	15 secs	25 – 35
Annealing	55 °C	15 secs	
Extension	72 °C	30 secs	
Hold	4 °C	∞	

[Optional] Analyze 2 μ L of the PCR products using gel electrophoresis to verify successful amplification.

PCR cleanup



- 6 Resuspend the AMPure XP Beads by vortexing.
- 7 Add a 0.5X volume ratio of resuspended AMPure XP Beads (circa  12 μL) to the sample and mix by pipetting.
- 8 Incubate on a Hula mixer (rotator mixer) for  00:05:00 at  Room temperature . In the meantime, prepare fresh 70% ethanol in nuclease-free water.

Note

If a Hula mixer (rotator mixer) is not available, invert gently by hand 25-30 times.

- 9 Briefly spin down the sample and pellet on a magnetic rack until supernatant is clear and colourless. Keep the tube on the magnetic rack (circa  00:02:00), and pipette off the supernatant.

Equipment

0.2 mL PCR 8 Strip Magnetic Separator

NAME

PERMAGEN

BRAND

MSRLV08

SKU

- 10 Keep the tubes on the magnet and wash the beads with  100 μL of freshly-prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
- 11 Repeat the previous step.

- 12 Spin down and place the tubes back on the magnet. Pipette off any residual ethanol. Allow to dry for ~  00:00:30 , but do not dry the pellets to the point of cracking.
- 13 Remove the tubes from the magnetic rack and resuspend each pellet in  10 µL of nuclease-free water by gently flicking the tube.
- 14 Incubate at  Room temperature for  00:05:00 , and spin down briefly.
- 15 Place the tube back to the magnet rack until the eluate is clear and colourless, for at least  00:01:00 .
- 16 Remove and retain  10 µL of eluate into a clean 0.2 ml thin-walled PCR tube per sample. Dispose of the pelleted beads
- 17 Quantify  1 µL of eluted sample using a Qubit fluorometer.

Second-round PCR with barcode rapid attachment primers

- 18 Thaw the Barcode Primers (BP01 - BP24) from the cDNA-PCR Barcoding Kit 24 V14 (SQK-PCB114.24) at  Room temperature , spin down briefly, mix well by pipetting, and place  On ice .
- 19 In each of the 0.2 ml PCR tubes, prepare the following reaction mixture:

A	B
Reagent	Volume
1st PCR products	1 – 5 µL (based on the concentration of the 1st PCR products)
Unique Barcode Primer (BP01-24)	0.75 µl
KAPA2G Robust HotStart 2X ReadyMix	12.5 µL

A	B
PCR grade water	6.75 – 10.75 µL
Total	25 µL

20 Amplify using the following cycling conditions:

A	B	C	D
Cycle step	Temperature	Time	No. of cycles
Initial denaturation	95 °C	3 mins	1
Denaturation	95 °C	15 secs	8 – 14
Annealing	62 °C	15 secs	
Extension	72 °C	30 secs	
Hold	4 °C	∞	

[Optional] Analyze 2 µL of the PCR products using gel electrophoresis to verify successful amplification.

Repeat PCR Cleanup

- 21 Resuspend the AMPure XP Beads by vortexing.
- 22 Add a 0.5X volume ratio of resuspended AMPure XP Beads (circa  12 µL) to the sample and mix by pipetting.
- 23 Incubate on a Hula mixer (rotator mixer) for  00:05:00 at  Room temperature . In the meantime, prepare fresh 70% ethanol in nuclease-free water.

Note

If a Hula mixer (rotator mixer) is not available, invert gently by hand 25-30 times.



- 24 Briefly spin down the sample and pellet on a magnetic rack until supernatant is clear and colourless. Keep the tube on the magnetic rack (circa  00:02:00), and pipette off the supernatant.

Equipment

0.2 mL PCR 8 Strip Magnetic Separator

NAME

PERMAGEN

BRAND

MSRLV08

SKU

- 25 Keep the tubes on the magnet and wash the beads with  100 μ L of freshly-prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
- 26 Repeat the previous step.
- 27 Spin down and place the tubes back on the magnet. Pipette off any residual ethanol. Allow to dry for ~  00:00:30 , but do not dry the pellets to the point of cracking.
- 28 Remove the tubes from the magnetic rack and resuspend each pellet in  10 μ L of nuclease-free water or Elution Buffer (EB) by gently flicking the tube.
- 29 Incubate at  Room temperature for  00:05:00 , and spin down briefly.
- 30 Place the tube back to the magnet rack until the eluate is clear and colourless, for at least  00:01:00 .
- 31 Remove and retain  10 μ L of eluate into a clean 0.2 ml thin-walled PCR tube per sample. Dispose of the pelleted beads.

- 32 Analyse  1 μL of the eluted sample for quantity using a Qubit fluorometer, and/or assess size and quality using an Agilent Bioanalyzer (or equivalent) for QC checks.
- 33 [Optional] Normalization may be required.

Adapter addition

- 34 Pool together equimolar samples of the barcoded amplicons to a total of  50 fmols into a clean 1.5 ml Eppendorf DNA LoBind tube. Make up the volume to  5.5 μL with Elution Buffer (EB).

Note

For the MinION flow cell, adjust this step to pool equimolar samples of the barcoded amplicons to a total of  50 fmols in  11 μL of Elution Buffer (EB).

Note

For example, full-length 16S rRNA gene amplicons (~ 1,600 bp),  50 fmols DNA equates to circa  50 ng .

For example, 12 barcoded samples can be pooled together in equal proportions to a total of 48 ng (approximately 50 fmols) in 5.5 μL of EB buffer, as specified in the table below:

A	B	C
Component	Volume	DNA
Sample 01 (10 ng/ μL)	0.4 μL	4 ng
Sample 02 (10 ng/ μL)	0.4 μL	4 ng

A	B	C
Sample 03 (10 ng/μL)	0.4 μL	4 ng
Sample 04 (10 ng/μL)	0.4 μL	4 ng
Sample 05 (10 ng/μL)	0.4 μL	4 ng
Sample 06 (10 ng/μL)	0.4 μL	4 ng
Sample 07 (10 ng/μL)	0.4 μL	4 ng
Sample 08 (10 ng/μL)	0.4 μL	4 ng
Sample 09 (10 ng/μL)	0.4 μL	4 ng
Sample 10 (10 ng/μL)	0.4 μL	4 ng
Sample 11 (10 ng/μL)	0.4 μL	4 ng
Sample 12 (10 ng/μL)	0.4 μL	4 ng
Elution Buffer (EB)	0.7 μL	-
Total	5.5 μL	48 ng

Note

If the quantity of amplicons is above 50 fmol, the remaining samples can be frozen at -20°C for a couple of months or freeze at 4°C for a couple of weeks. We recommend avoiding multiple freeze-thaw cycles to prevent DNA degradation.

35 Thaw the Rapid Adapter (RA) and Adapter Buffer (ADB) from the cDNA-PCR Barcoding Kit 24 V14 (SQK-PCB114.24) at  Room temperature , spin down briefly, mix well by pipetting, and place  On ice .

36 In a fresh 1.5 ml Eppendorf DNA LoBind tube, dilute the Rapid Adapter (RA) as follows and pipette mix:

A	B
Reagents	Volume



	A	B
	Rapid Adapter (RA)	1.5 µl
	Adapter Buffer (ADB)	3.5 µl
	Total	5 µl

- 37 Add  0.5 µL of the diluted Rapid Adapter (RA) to the pooled DNA sample, making the total volume  6 µL .

Note

For the MinION flow cell, adjust this step to add  1 µL of the diluted Rapid Adapter (RA) to the pooled DNA sample, making the total volume  12 µL .

- 38 Mix gently by **flicking the tube**, and spin down.
- 39 Incubate the reaction for  00:05:00 at  Room temperature . Spin down briefly.
- 40 Store the library  On ice until ready to load.

Loading the Flongle Flow Cell

- 41 Please check the number of pores in your flow cell prior to starting a sequencing experiment. To do the flow cell check, please follow the instructions in the Flow Cell Check document (<https://community.nanoporetech.com/protocols/flow-cell-check/>).

Note

We usually have ~60 pores in the Flongle flow cell, and the warranty covers a minimum of 50 active pores.

We got less than 5 pores in the Flongle flow cell for few times, so this step is highly recommended.

It should be noted that the warranty for the Flongle flow cells is quite short, only within four weeks from the date of purchase.

- 42 Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at  Room temperature before mixing by vortexing. Then spin down and store  On ice .
- 43 In a fresh 1.5 ml Eppendorf DNA LoBind tube, mix  117 μL of Flow Cell Flush (FCF) with  3 μL of Flow Cell Tether (FCT) and mix by pipetting.
- 44 Place the Flongle adapter into the MinION or one of the five GridION positions. Place the flow cell into the Flongle adapter, and press the flow cell down until you hear a click.
- 45 Peel back the seal tab from the Flongle flow cell, up to a point where the sample port is exposed.
- 46 To prime your flow cell with the mix of Flow Cell Flush (FCF) and Flow Cell Tether (FCT) that was prepared earlier, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 pipette tip inside the sample port and slowly dispense the  120 μL of priming fluid into the Flongle flow cell by slowly pipetting down. We also recommend twisting the pipette plunger down to avoid flushing the flow cell too vigorously.
- 47 Vortex the vial of Library Beads (LIB). Note that the beads settle quickly, so immediately prepare the Sequencing Mix in a fresh 1.5 ml Eppendorf DNA LoBind tube for loading the Flongle flow cell, as follows:

A	B
Reagents	Volume
Sequencing Buffer (SB)	15 µl
Library Beads (LIB)	10 µl
Pooled DNA library	5 µl
Total	30 µl

- 48 To add the  30 µL Sequencing Mix to the flow cell, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 tip inside the sample port and slowly dispense the Sequencing Mix into the flow cell by slowly pipetting down (i.e., twisting the pipette plunger down). Or, use a wide pore P200 tip to add the Sequencing Mix to the flow cell in a dropwise fashion and ensure each drop flows into the port before adding the next.
- 49 Seal the Flongle flow cell using the adhesive on the seal tab. Close the device lid and set up a sequencing run on MinKNOW.

Compare the results using old versus new flowcell and kit versions.

- 50 We compared the sequencing results obtained using the old version of flow cell and kit (R9.4.1 Flongle flow cell with the PCR Barcoding Kit SQK-PBK004) with the updated version of flow cell and kit (R10.4.1 Flongle flow cell with the cDNA-PCR Barcoding SQK-PCB114.24).

The results indicated that both approaches generate similar amounts of sequencing data, but the new version offers higher accuracy.

^ Run summary

DATA OUTPUT

Estimated bases

177.38 Mb

Reads generated

97.19 k

Estimated N50

1.6 kb

Total data produced (pass / fail)

1.97 GB

BASECALLING

Reads called

100%

Bases called (min Q score: 10)

67.79 Mb

75.59 Mb

Pass

Fail

^ Run configuration

^ RUN SETUP

Flow cell type	FLO-FLG001
Flow cell ID	ANE956
Kit type	SQK-PBK004
Flongle adapter ID	FA-05635

Full-length 16S rRNA gene sequencing result obtained using R9.4.1 Flongle flow cell (FLO-FLG001) with the PCR Barcoding Kit SQK-PBK004.

^ Run summary

DATA OUTPUT

Estimated bases	Reads generated
210.11 Mb	181.31 k
Estimated N50	Total data produced (pass / fail)
1.58 kb	2.8 GB

BASECALLING

Reads called	Bases called (min Q score: 9)	
100%	167.33 Mb	30.69 Mb
	Pass	Fail

^ Run configuration

^ RUN SETUP

Flow cell type	FLO-FLG114
Flow cell ID	ASX605
Kit type	SQK-PCB114-24
Flongle adapter ID	FA-07329

Full-length 16S rRNA gene sequencing result obtained using R10.4.1 Flongle flow cell (FLO-FLG114) with the cDNA-PCR Barcoding SQK-PCB114.24.

Acknowledgements

We thank Ying Jia and Rebecca from the Oxford Nanopore Technical Services team for their valuable suggestion.