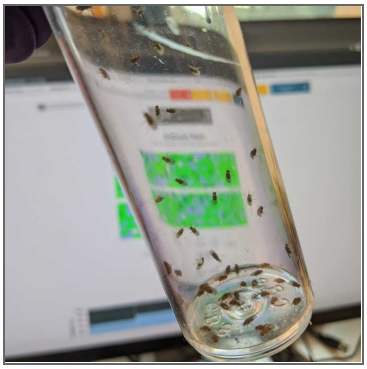

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MANUSCRIPT CITATION:

Protocol status: Working
We use this protocol and it's working

DNA extraction and Nanopore library prep from 15-30 whole flies- V.3.2

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Stanford University

ABSTRACT

This protocol is optimized for rapid and cost-effective (about $150) genome assembly of *Drosophila* species from laboratory lines using ONT PromethION sequencers. Following this protocol, a typical Drosophila Nanopore sequencing run should have read N50 of 20-40kbp. Sequencing is halted at about 40-60X depth of coverage (10-14 Gbp on MinKNOW for most species, assuming ~20% of data is removed by a quality filter).

GUIDELINES

This protocol is used to prepare ~1-2 ug of Nanopore library from a single reaction. The amount loaded onto the flow cell depends on the quality of the library. Larger amounts of longer libraries should be loaded to keep the molar concentration of adapted ends consistent. However, longer libraries tend to clog the flow cell more quickly, necessitating frequent DNase flushing and reloading and reducing throughput. Two libraries with the same N50 but where one has a larger number of >100kb fragments will sequence differently.

Ballpark estimates of R10.4.1 library loads maintaining good pore occupancy are:
Read N50 1kb: 10-15 ng library
Read N50 5kb: 25 ng library
Read N50 10kb: 50 ng library
Read N50 20kb: 100 ng library
Read N50 30kb: 200 ng library
Read N50 40kb+: 300 ng library

To maximize read lengths, one should not wait until all active pores have been depleted to flush and reload. A DNase flush should take place as soon as sequencing throughput starts to decrease, or about every 8 hours. A flow cell with loaded library can be stored at 4C overnight with no ill effects.

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PROTOCOL integer ID: 87457

Keywords: Drosophila, nanopore, ligation, bead-free, HMW, ultra-long

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MATERIALS

MATERIALS

- 10% SDS solution **Contributed by users**
- NEBNext Companion Module forOxford Nanopore Technologies Ligation Sequencing – 24 rxns **New England Biolabs Catalog #E7180S**
- Ligation sequencing kit 1D **Oxford Nanopore Technologies Catalog #SQK-LSK109**
- Chloroform **Millipore Sigma Catalog #CX1055-6**
- Phenol Chloroform Isoamyl Alcohol (25:24:1) Tris-saturated (pH 8.0) **Fisher Scientific Catalog #BP1752I-400**
- 3M sodium acetate **Contributed by users**
- Proteinase K Solution (20 mg/mL) RNA grade **Thermo Fisher Scientific Catalog #25530049**
- RNase A solution **Millipore Sigma Catalog #R6148**
- Tris-EDTA (TE) buffer pH 8.0 1X **Contributed by users**
- Homogenization Buffer (HB) [0.1M NaCl 30mM Tris-HCl pH 8.0 10 mM EDTA 0.5 Triton X-100] **Contributed by users**
- Lysis Buffer (LB) [0.1M Tris-HCl pH 8.0; 0.1M NaCl; 20mM EDTA] **Contributed by users**
- Hydration Buffer (STE) [400mM NaCl 20mM Tris-HCl pH 8.0 30mM EDTA] **Contributed by users**
- DNAse wash buffer (DWB) [300mM KCl 2mM CaCl2 10mM MgCl2 15 mM HEPES pH 8.0] **Contributed by users**
- Elution Buffer (EB) [10 mM Tris-HCl pH 8.0] **Contributed by users**
- Short Read Eliminator (SRE) **Circulomics Catalog #SS-100-101-01**

DNA extractions are performed in Phase lock gel tubes to minimize handling and to maximize yield. A cheaper alternative to the official phase lock gel tubes is to put ~200uL of Dow Corning High Vacuum Grease into a 2.0 mL LoBind tube with a small syringe. Care should be take with homebrew phase lock gel tubes as using too little grease will result in the phase lock layer collapsing during the chloroform extraction step.

Although less effective, a solution of [0.8M NaCl, 9% w/v PEG8000, 10mM Tris-Cl pH 8.0] can be substituted for the Short Read Eliminator. See John Tyson's "Rocky

Mountain" protocol for more details (https://www.protocols.io/view/rocky-mountain-adventures-in-genomic-dna-sample-pr-7euhjew). The SRE XS or XL versions can be used if DNA is short or sufficiently long. This may require a bit of trial and error to figure out.

Equipment

DNA LoBind tubes, 1.5 mL	NAME
Tubes	TYPE
Eppendorf	BRAND
022431021	SKU
https://online-shop.eppendorf.us/US-en/Laboratory-Consumables-44512/Tubes-44515/DNA-LoBind-Tubes-PF-56252.html	LINK
1.5 mL	SPECIFICATIONS

Equipment

DNA LoBind tubes, 2.0 mL	NAME
Tubes	TYPE
Eppendorf	BRAND
022431048	SKU
https://online-shop.eppendorf.us/US-en/Laboratory-Consumables-44512/Tubes-44515/DNA-LoBind-Tubes-PF-56252.html	LINK
2.0 mL	SPECIFICATIONS

Equipment

Large-orifice pipet tips, 200uL

NAME

Pipette tips

TYPE

Fisher

BRAND

02-707-134

SKU

https://www.fishersci.com/shop/products/fisherbrand-large-orifice-pipet-tips-1-200-l-packaging-hrs-10-x-96/02707134

LINK

200 uL

SPECIFICATIONS

Equipment

Dounce Homogenizer, 2mL

NAME

Tissue Grinder

TYPE

Kimble

BRAND

885300-0002

SKU

https://www.kimble-chase.com/advancedwebpage.aspx?cg=886&cd=4&SKUTYPE=202&SKUFLD=SKU&DM=1250&WEBID=6856

LINK

2 mL with Pestles A and B

SPECIFICATIONS

Equipment	
5PRIME Phase Lock Gel tube, light, 2mL	NAME
Quantabio	BRAND
2302830	SKU
https://www.quantabio.com/phase-lock-gel	LINK
Light	SPECIFICATIONS

PROTOCOL MATERIALS

Proteinase K Solution (20 mg/mL) RNA grade **Thermo Fisher Scientific Catalog #25530049**

Materials

10 mM Tris-HCL pH 8.0 **Contributed by users** Step 33

Elution Buffer (EB) [10 mM Tris-HCl pH 8.0] **Contributed by users**

In Materials and 3 steps

Short Read Eliminator (SRE) **Circulomics Catalog #SS-100-101-01**

In Materials and 2 steps

Homogenization Buffer (HB) [0.1M NaCl 30mM Tris-HCl pH 8.0 10 mM EDTA 0.5 Triton X-100] **Contributed by users**

Materials

Proteinase K **Thermo Fisher Scientific Catalog #100005393** Step 4.4

RNAse A **New England Biolabs Catalog #T30318-2** Step 4.4

Lysis Master Mix (LMM) **Contributed by users** In 2 steps

Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #SQK-LSK114**

Step 64

3M sodium acetate **Contributed by users** Materials

Chloroform **Merck MilliporeSigma (Sigma-Aldrich) Catalog #CX1055-6**

Materials, Step 13

DNA LoBind Tubes, 1.5 mL **Eppendorf Catalog #0030108051** In 2 steps

Flow Cell Priming Kit (EXP-FLP002) **Oxford Nanopore Technologies Catalog #EXP-FLP002**

Step 93

Lysis Buffer **Contributed by users** In 2 steps

Lysis Buffer (LB) [0.1M Tris-HCl pH 8.0; 0.1M NaCl; 20mM EDTA]**Contributed by users**

Materials

DNAse wash buffer (DWB) [300mM KCl 2mM CaCl2 10mM MgCl2 15 mM HEPES pH 8.0] **Contributed by users**

Materials

Tris-EDTA (TE) buffer pH 8.0 1X **Contributed by users** Materials

10% SDS solution **Contributed by users** Materials

Hydration Buffer (STE) [400mM NaCl 20mM Tris-HCl pH 8.0 30mM EDTA] **Contributed by users**

Materials, Step 2

RNase A solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R6148**

Materials

Large-Orifice Pipet Tips 200µL **Fisher Scientific Catalog #02-707-134** In 4 steps

Flowcell Wash Kit **Oxford Nanopore Technologies Catalog #EXP-WSH003**

Step 95

10% SDS **Contributed by users** In 2 steps

DNA Precipitation Buffer (PB) [0.8 M NaCl 9% w/v PEG 8000 10mM Tris-HCl pH 8.0] **Contributed by users**

Step 37

PCR Tubes, 0.2mL, flat cap, natural, PCR Tube; 0.2mL; Natural; w/flat cap; 1000/Pk. **Thermo Fisher Catalog #3412**

Step 48

NEBNext Companion Module forOxford Nanopore Technologies Ligation Sequencing – 24 rxns **New England Biolabs Catalog #E7180S**

In Materials and 2 steps

Ligation sequencing kit 1D **Oxford Nanopore Technologies Catalog #SQK-LSK109**

Materials, Step 93

Phenol Chloroform Isoamyl Alcohol (25:24:1) Tris-saturated (pH 8.0) **Fisher Scientific Catalog #BP1752I-400**

Materials, Step 10

3M Sodium Acetate **Contributed by users**

In 3 steps

Agencourt AmPure XP beads **Contributed by users Catalog #A63880**

Step 52

Nuclease-free water or water filtered using a Milli-Q filtering system **Ambion Catalog #AM9932**

Step 48

Oxford Nanopore Ligation Sequencing Kit **Oxford Nanopore Technologies Catalog #SQK-LSK110**

Step 64

ONT Flow Cell Wash Kit **Oxford Nanopore Technologies Catalog #EXP-WSH004**

Step 102

BEFORE START INSTRUCTIONS

This protocol is for DNA extraction from whole Drosophila. Before starting the protocol, 10-40 whole male flies should be starved for 1 day then quickly frozen in a -80 freezer. If flies must be transported but shipping on dry ice is an issue, preserve flies in 95% ethanol. We have sequenced flies shipped through the postal service (7 days in transit) without any major issues. While yield of the very long fragments (>100kb) will be smaller, it is not critical to have pristine samples for good Nanopore runs. Flies should ideally be preserved less than 6 months ago.

Initially, we utilized nuclear extractions thinking that it was critical to maximize the quality of extracted gDNA for Nanopore sequencing. However we realized that this required too many flies, making sample prep difficult and increasing haplotype diversity in the library. Additionally and somewhat counterintuitively, library prep will be less effective when DNA is too long. Therefore, some shearing of very high quality gDNA is essential. Given these issues, we now extract gDNA from a smaller number of whole flies and this is more than sufficient for the preparation of multiple Nanopore libraries and a short read prep.

The amount of starting material required can vary depending on the size of the fly and sample quality. For fresh, flash-frozen flies, we usually start with 30 *D. melanogaster*-sized flies for a yield of about 5ug of HMW gDNA. Half of that is used for library prep, so an effective library prep could certainly be done with 15 and possibly even fewer flies. For larger species like Hawaiian *Drosophila*, we have used as few as 6 flies. For these specimens, DNA yield is improved by clipping off the wings before running this protocol.

(Optional) Hydration of ethanol-fixed tissue

1h

1 Place flies on a sheet of filter paper and briefly dab with a Kimwipe to remove excess ethanol, then transfer the flies to a 1.5 mL tube.

2 Add 1.0 mL Buffer STE to the tube with the flies.

Hydration Buffer (STE) [400mM NaCl 20mM Tris-HCl pH 8.0 30mM EDTA] **Sigma Aldrich**

3 Incubate at room temperature for at least 00:15:00 .

15m

Tissue homogenization

15m

4 Prepare Lysis Buffer **Sigma Aldrich**, 10% SDS **Sigma Aldrich**, 3M Sodium Acetate **Sigma Aldrich**, and Lysis Master Mix (LMM) **Sigma Aldrich**

4.1 For Lysis Buffer **Sigma Aldrich**:

- 5 mL [M] 1 Molarity (M) Tris-HCl pH 8.0
- 2 mL [M] 0.5 Molarity (M) EDTA
- 0.292 g NaCl
- 43 mL DI H20

4.2 For 10% SDS **Sigma Aldrich**:

5 g SDS
50 mL Distilled H20

4.3 For 3M Sodium Acetate **Sigma Aldrich**:

- 2.461 g NaOAc
- 10 mL DI H20

4.4 Per 1 vial of flies (~25-50 flies), prepare Lysis Master Mix (LMM) **Sigma Aldrich**:

- 500 µL Lysis Buffer
- 15 µL of Proteinase K **Thermo Fisher Scientific Catalog #100005393** (fina

concentration of 561 µg/mL)

- 5 µL of RNAse A **New England Biolabs Catalog #T30318-2** (final concentration of 186 µg/mL)
- 15 µL 10% SDS (final concentration of 0.28% SDS)

5 Add 25-50 flies to a LoBind tube

Equipment

DNA LoBind tubes, 1.5 mL	NAME
Tubes	TYPE
Eppendorf	BRAND
022431021	SKU
https://online-shop.eppendorf.us/US-en/Laboratory-Consumables-44512/Tubes-44515/DNA-LoBind-Tubes-PF-56252.html	LINK
1.5 mL	SPECIFICATIONS

6 Homogenize flies with NEB pestle, working quickly to avoid endogenous nuclease digestion of DNA. Add 500 µL LMM to the LoBind tube and mix thoroughly using the pestle.

Equipment

Monarch Pestle Set	NAME
New England BioLabs	BRAND
T3000L	SKU
https://www.neb.com/products/t3000-monarch-pestle-set#Product%20Information	LINK

Lysis

4h

7 Incubate lysis tube at 50 °C for 03:00:00 to 04:00:00. Mix the tube with gentle rocking and inversion, until solution appears relatively homogeneous, at 00:45:00 intervals. 4h

Note

Sometimes a bit of vigorous shaking is needed, especially if there is a lot of materal. This is OK - the end product needs to be sheared for library prep anyway. Qualitative observations suggest that thorough mixing improves DNA yield and purity substantially.

Note

Incubation times as short as 1 hour at 55C have worked. We have not carefully tested how this affects yield.

Phenol chloroform extraction

1h

8 Spin down 1 phase lock gel tube per sample at 15000 x g for 00:00:30 .

Note

Although not essental, phase lock gel tubes help minimize shearing and loss of yield caused by repeated pipetting. Dow Corning High Vacuum Grease is compositionally identical to the light phase lock gel material. We buy the 5.3oz tube from Amazon and squeeze some into a 10mL BD syringe for dispensing. This size of tube/syringe fits well for minimial mess and hassle. Avoid overfilling and air bubbles. Autoclave but be warned this may cause a mess, so wrap the syringe in foil beforehand.

About 250 µL of grease is placed into a 2mL LoBind tube to make the homebrew phase lock gel tube.

IMPORTANT: If an insufficient amount of grease is applied, the phase lock layer will collapse during the chloroform extraction.

Reference: https://bitesizebio.com/18944/diy-phase-separating-gel-clean-and-cheap/

Safety information

WARNING: If you are using normal tubes in lieu of LoBinds, do not use polystyrene tubes for the phenol-chloroform extraction. They will melt and burst in the centrifuge. Polypropylene tubes do not melt.

Equipment

5PRIME Phase Lock Gel tube, light, 2mL	NAME
Quantabio	BRAND
2302820	SKU
https://www.quantabio.com/phase-lock-gel	LINK
Light	SPECIFICATIONS

9 Transfer the homogenate/lysis solution to the phase lock gel tube by pipetting with a wide-bore tip.

10 Add an equal volume (about 400 µL) of Tris-saturated phenol chloroform isoamyl alcohol (PCI) to the phase lock tube.

Safety information

This should be performed inside the fume hood.

Phenol Chloroform Isoamyl Alcohol (25:24:1) Tris-saturated (pH 8.0) **Sigma Aldrich Catalog #BP1752I-400**

11 Mix by placing tubes on a rocker at medium speed for 00:08:00 . 8m

Note

We use a rocking platform, so the tubes are placed on their sides horizontally to maximize the surface area. When solution is well mixed, aqueous (top) layer will be a cloudy milky color.

12 Centrifuge the phase lock tube at 10000 x g for 00:08:00. Phase lock layer should now separate aqueous and organic layers. 8m

13 Add an equal volume (usually 400 µL) of chloroform to the tube.

Safety information

This step should be performed inside the fume hood.

Chloroform **Merck MilliporeSigma (Sigma-Aldrich) Catalog #CX1055-6**

14 Mix by placing tubes on a rocker at medium speed for 00:08:00. 8m

15 Centrifuge the phase lock tube at 15000 x g for 00:08:00. Phase lock layer should now separate aqueous and organic layers. 8m

16 Quickly decant the aqueous (top) layer into a fresh 2.0 mL LoBind tube.

Note

Try to perform the decanting step in a few seconds, and don't tap/shake the phase lock tube to get the last drops out. Care must be taken as the chloform significantly weakens the phase lock gel layer. If the phase lock tube is inverted for too long during decanting, the layer will collapse and everything will pour out. It's best to leave a couple of drops behind but avoid the hassle of cleaning this up.

IMPORTANT: It is highly recommended to use LoBind tubes in this and subsequent steps. The coating will prevent DNA sticking to the tube. This is helpful for maximizing yield and minimizing shearing.

Safety information

This step should be performed inside the fume hood.

Equipment

DNA LoBind tubes, 2.0 mL	NAME
Tubes	TYPE
Eppendorf	BRAND
022431048	SKU
https://online-shop.eppendorf.us/US-en/Laboratory-Consumables-44512/Tubes-44515/DNA-LoBind-Tubes-PF-56252.html	LINK
2.0 mL	SPECIFICATIONS

DNA precipitation, wash, and resuspension

1h 30m

17 Chill 100% ethanol on ice and make 1 mL per sample of fresh 70% ethanol using nuclease-free water.

18 Add 0.1x volume (typically 50 µL) of 3M sodium acetate to the extract from Step 4.18. Gently swirl to mix.

3M Sodium Acetate **Contributed by users**

19 Add 2-2.5x volumes (typically 1100 µL) of cold 100% ethanol to the tube, and mix with careful swirling and gentle rocking.

Expected result

DNA should slowly precipitate into a single white stringy clump, and un-precipitated DNA should be visible as shimmering strands at the bottom of the tube that are attached to the white clump.

Note

If the extraction tube turns cloudy, it is likely salt precipitation because the solution is too nonpolar and not DNA. Add water dropwise with thorough mixing and the solution should clear up.

20 Using a P200 pipette and a wide-bore tip, transfer the stringy clump to a fresh 1.5 mL LoBind tube.

Note

This step can be somewhat tricky. The DNA clump can get stuck to your pipette tip and be very difficult to get off. Making sure that the DNA is fully precipitated (no un-precipitated strands present) and quick pipetting helps prevent this. Position the pipette tip right above the clump and aspirate quickly to bring the clump into the tip. Using a similar quick motion, dispense the DNA clump and liquid into the fresh 1.5 mL tube.

Equipment

DNA LoBind tubes, 1.5 mL	NAME
Tubes	TYPE
Eppendorf	BRAND
022431021	SKU
https://online-shop.eppendorf.us/US-en/Laboratory-Consumables-44512/Tubes-44515/DNA-LoBind-Tubes-PF-56252.html	LINK
1.5 mL	SPECIFICATIONS

21 Add 150 µL (or enough to cover the DNA) of 70% ethanol.

22 Remove and discard the ethanol. Be careful not to discard any DNA.

23 Add 150 µL of 70% ethanol to wash the DNA. Gently swirl to mix.

24 Centrifuge the tube at 2000 x g for 00:05:00. 5m

25 While being careful not to disturb the pellet, pipette off the ethanol.

26 Add 175 µL of 70% ethanol.

27 Spin at 10000 rcf for 00:01:00. 1m

28 Being careful not to disturb the DNA pellet, remove the ethanol.

29 Wash the pellet once more: go to step #26 and increase to 200uL 70% ethanol.

30 Spin at 10000 rcf for 00:01:00. 1m

31 Using a 10uL pipette, remove any excess ethanol.

32 Allow the DNA to air dry right until the moment it becomes translucent (usually 00:05:00).
Do not over-dry the pellet.

> **Note**
>
> Oftentimes the whole DNA pellet will not become translucent but the edges of the pellet will.

33 Depending on fly size, resuspend in 31-90uL of 10 mM Tris-HCL pH 8.0 **Sigma Aldrich** and incubate at 50 °C for at least 01:00:00. 1h

> **Note**
>
> Usually resuspend in 60 µL of 10 mM Tris-HCL per ~25 flies.

34 Briefly spin down tube to gather any condensation and store at 4 °C.

DNA resuspension 1w

35 Keep the DNA at 4C for at least 1 week to obtain proper resuspension. Every ~48 hours, mix the DNA gently with a P1000 or P200 pipette with a normal tip. This will encourage DNA to resuspend and make sure it is adequately sheared for library prep. We usually mix three times:

1. 10X with a P1000
2. 5X with a P1000
3. 5X with a P200

Note

While it is possible to over-shear the DNA, under-shearing makes for much worse Nanopore runs because it makes the sample too viscous. More shearing may be needed depending on how much the sample was mixed during incubation, or how fresh the flies were.

If there is a lot of DNA that doesn't seem to be resuspending, shearing with a blunt-end needle may be necessary. When using a blunt-end needle, be sure to 'unstick' the plunger before using it on DNA. Aspirate DNA into the needle (not the plunger) 3x times maximum.

36 Check sample concentration and quality of 1 µL aliquots using Qubit and Nanodrop.

Note

Ideally, this should Qubit at >75 ng/uL and have Nanodrop ratios of 260/280 >1.8 and 260/230 >2.0. If sample is above 150 ng/uL consider diluting with more 10mM tris.

Short Read Elimination 1

37 Using a cut-off P200 tip (a wide bore will be too small to fit in the PCR tube), gently transfer 30 µL of sample to a 1.5 mL DNA LoBind tube. Add 30 µL SRE XL buffer. Using a wide-bore P200 tip, quickly but gently mix the tube. The precipitation buffer described here can be used in place of the SRE buffer but is not as effective at removing small DNA fragments as SRE.

Note

Dilute sample down to 150 ng/uL as possible before mixing with buffer

Short Read Eliminator (SRE) **Circulomics Catalog #SS-100-101-01**

DNA LoBind Tubes, 1.5 mL **Eppendorf Catalog #0030108051**

DNA Precipitation Buffer (PB) [0.8 M NaCl 9% w/v PEG 8000 10mM Tris-HCl pH 8.0] **Contributed by users**

Large-Orifice Pipet Tips 200µL **Fisher Scientific Catalog #02-707-134**

38 Centrifuge the sample at 10000 x g for 00:30:00 or until DNA has pelleted and solution is no longer viscous. Meanwhile, prepare 500 µL fresh 70% ethanol with nuclease-free water.

39 Pipette off the supernatant, taking care not to disturb the DNA pellet.

> **Note**
>
> We have increased our yield by leaving 10-15 uL of supernatant in the bottom of the tube going into the first wash. This is particularly important if the pellet is invisible.

40 Add 150 µL of 70% ethanol. Pipette slowly, with the tip touching the front wall of the tube, so that the pellet is not disturbed.

41 Centrifuge at 10000 x g for 00:02:00 .

42 Pipette off the supernatant, taking care not to disturb the DNA pellet. Make sure all the supernatant is removed and only the pellet remains.

43 Repeat wash: go to step #40

Note

The second centrifuge (step 43) can be shorter, ~1 minute.

44 Briefly spin sample and use a P10 to remove any remaining ethanol.

45 Resuspend pellet in 48.5 µL EB.

Elution Buffer (EB) [10 mM Tris-HCl pH 8.0] **Sigma Aldrich**

46 Incubate the tube on the heat block at 50 °C for at least 01:00:00. Briefly spin down the tube to collect condensation. Incubate at least 48:00:00 and preferably for twice that time at 4 °C.

Note

Before proceeding to the next stage, we recommend 1 µL Qubit to ensure there is still DNA.

DNA repair and end-prep

47 Thaw NEBNext repair and DNA-tailing mixes and buffers from the Nanopore Companion Module. Vortex buffers and flick mixes after thawing. Spin down tubes and keep chilled on ice.

NEBNext Companion Module forOxford Nanopore Technologies Ligation Sequencing – 24 rxns **New England Biolabs Catalog #E7180S**

48 Add 3.5 µL of FFPE DNA Repair Buffer, 3.5 µL of End-Prep Reaction Buffer, 2 µL

of FFPE DNA Repair Mix, and 3 µL of End-Prep Reaction Mix to a PCR tube. Add remaining 47.5 µL of DNA sample to the PCR tube using a cut-off P200 tip. Mix tube with gentle flicking (or very gentle pipetting with the cut-off P200 tip), and then briefly spin down.

Note

To increase efficiency and decrease amount of pipette tips needed, prep PCR tubes with buffers and mixes and add the HMW DNA sample last.

Note

We have found that half-reaction volumes for this step do not decrease yield. To follow half-reaction volumes, see the Single Fly Forked Protocol for more information.

PCR Tubes, 0.2mL, flat cap, natural, PCR Tube; 0.2mL; Natural; w/flat cap; 1000/Pk.**Thermo Fisher Catalog #3412**

Nuclease-free water or water filtered using a Milli-Q filtering system **Ambion Catalog #AM9932**

49 In a thermal cycler, incubate at 20 °C for 01:00:00 then 65 °C for 00:30:00. 1h 30m After this, sample can be held at 4 °C temporarily until ready to proceed.

Bead Clean Up

50 Prepare 500 µL of 70% ethanol per sample.

51 Transfer sample from PCR tube to a LoBind tube using a cut-off P200 tip.

52 Add equal volume of AmPure XP beads (normally 59.5 µL) to the sample. Immediately use a P200 wide bore tip to mix 5x.

> **Note**
>
> This step must be performed quickly; otherwise, DNA will precipitate onto pipette tip and will result in sample loss.

> **Note**
>
> If needed, briefly spin down to ensure there are no bubbles or any sample on the wall of the LoBind tube.

Agencourt AmPure XP beads **Contributed by users Catalog #A63880**

53 Incubate at room temperature for 00:05:00 . 5m

54 Place the LoBind tube on a magnetic rack and wait until solution is clear and the beads are pelleted.

55 Remove the supernatant by placing pipette tip on the wall of the LoBind tube opposite of the beads. Pipette very slowly to ensure no DNA is pulled off.

> **Note**
>
> If DNA is pulled off, add supernatant back to tube and wait for solution to clear. Then try again.

56 Wash by adding 190 µL of 70% ethanol (enough to cover the beads on the wall of the LoBind tube).

Note

Work quickly to add the 70% ethanol at this step to avoid the beads drying out.

57 Remove and discard ethanol.

58 Wash again by adding 200 µL of 70% ethanol.

59 Remove and discard ethanol.

Note

Briefly spin and use a P10 pipette to remove any remaining excess of ethanol.

60 Resuspend sample in 32 µL of nuclease-free water.

61 Place tube on heat block at 50 °C until the pellet has dissolved.

Note

This step can take a long time. If there is concern about the DNA not resuspending off the beads, the tube can be stored at 4 °C overnight and then the sample removed from the beads the following morning.

62 Place LoBind tube on magnet rack until solution is clear.

63 Using a cut off P200 tip, remove the supernatant containing the aqueous DNA.

Note

Qubit 1 µL of sample to ensure DNA concentration before proceeding to next step.

Note

This is a safe stopping point. Sample can be stored at 4 °C .

Adapter ligation

64 Thaw AMXF, Quick T4 Ligase, LNB, and LFB from the NEBNext Nanopore Companion Module and the Nanopore LSK110 kit or LA, Quick T4 Ligase and LNB from Nanopore LSK114 hit . Mix AMXF or LA, Quick T4 ligase, and LFB by flicking. Mix LNB by pipetting. Briefly spin the tubes down and keep chilled on ice.

NEBNext Companion Module forOxford Nanopore Technologies Ligation Sequencing – 24 rxns **New England Biolabs Catalog #E7180S**

Oxford Nanopore Ligation Sequencing Kit **Oxford Nanopore Technologies Catalog #SQK-LSK110**

Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #SQK-LSK114**

65 Add 30 µL prepared DNA sample (the extra 1 µL can be used to Qubit), 2.5 µL AMXF or LA, and 5 µL Quick T4 ligase to a fresh 1.5 mL DNA LoBind tube. Gently flick the tube to mix.

66 Add 12.5 µL LNB to the sample. Working quickly, mix by gentle pipetting with a wide-bore tip. DNA precipitation is normal, but if the DNA precipitates before you finish mixing it will stick to your pipette tip and you will lose a significant amount of library.

Large-Orifice Pipet Tips 200µL **Sigma Aldrich Catalog #02-707-134**

67 Incubate the reaction mixture at room temperature for 00:20:00. 20m

68 Add 20 µL of AmPure XP beads and mix quickly with wide-bore tip.

69 Incubate at room temperature for 00:05:00. 5m

70 Place tubes on magnetic and wait for solution to clear.

71 On the magnet, use a cut-off P200 tip to pull the supernatant off the beads very slowly, then dispense the supernatant back onto the bead pellet slowly. Let the sample sit on the magnet for a few minutes.

72 Pipette off supernatant with a normal pipette tip, pipetting from the front of the tube away from the pellet.

73 Add 95 µL of LFB to the tube. SFB or a 1:1 dilution of PB can be used here.

> Note
>
> **DO NOT USE ETHANOL** TO WASH PREPARED LIBRARY. It will denature the motor protein.

Lightly tap the tube to encourage adapter on the beads to come off, but not necessarily for beads to resuspend.

74 Being careful not to disturb the pellet, pipette off all the supernatant.

75 Wash again using 105 µL of LFB. Pipette LFB on to the beads more quickly to get the pellet off the side of the tube. Lightly tap the tube to mix but not fully resuspend.

76 While on magnet remove LFB. Briefly spin and use a P10 pipette to remove any remaining excess of LFB.

77 Resuspend pellet in 21 µL EB for R9.4.1 sequencing or in 30 µL for R10.4.1 sequencing.

Elution Buffer (EB) [10 mM Tris-HCl pH 8.0] **Contributed by users**

78 Incubate library on the heat block at 34 °C for 01:00:00. Briefly spin down the tube to collect condensation then incubate for at least 48:00:00 before the next step. 2d 1h

79 Place sample on magnet wait until solution is clear. Use a cut off P200 tip to remove sample from beads and place in a new 1.5mL Lo Bind tube.

(Optional) Library size selection with SRE buffer

80 Quantify library concentration using 1 µL of the prepared library with Qubit. This step should not be performed unless library concentration is greater than 40 ng/uL. If the concentration is greater than 100ng/uL the library should be diluted to improve size selection performance.

81 Add an equal volume (20 µL) of SRE XL buffer to the library and gently pipette mix using a wide-bore tip.

Short Read Eliminator (SRE) **Circulomics Catalog #SS-100-101-01**

Large-Orifice Pipet Tips 200µL **Fisher Scientific Catalog #02-707-134**

82 Centrifuge at 10000 x g, 00:30:00 .

83 Pipette off the supernatant, being careful not to disturb the DNA pellet at the bottom of the tube.

> **Note**
>
> Similar to previous SRE step, leave 10-15uL of supernatant in the bottom of the tube for the first wash.

84 Add 100 uL of LFB, SFB, or 1:1 diluted PB (similar to step 46) to wash the pellet. It does not really matter which one is used.

> **Note**
>
> **DO NOT USE ETHANOL** TO WASH PREPARED LIBRARY. It will denature the motor protein.

85 Centrifuge tube at 10000 x g, Room temperature for 00:02:00 .

86 Being careful not to disturb the pellet, pipette off all the supernatant.

87 Repeat wash: go to step #84

88 Resuspend pellet in 21 µL EB for R9.4.1 sequencing or in 30 µL for R10.4.1 sequencing.

Elution Buffer (EB) [10 mM Tris-HCl pH 8.0] **Contributed by users**

89 Incubate the tube on the heat block at 37 °C for 01:00:00. Briefly spin down the tube to collect condensation, and incubate at least 48:00:00 at 4 °C before sequencing.

Tips for sequencing the library- R9.4.1

90 Thaw 1 tube SQB (SQK-LSK110), 2 tubes FB (EXP-FLP002), and 1 tube FLT (EXP-FLP002). Mix SQB and FB by flicking. Mix FLT with a pipette. Keep reagents on ice until ready to sequence.

Note

We recommend marking one tube of FB to use as dilution buffer for subsequent runs. Only one tube should be used to prepare the priming mix.

Safety information

The FB must be from the EXP-FLP002 kit. This will not work with version 1 of the kit.

91 Quantify the concentration of 1 µL library with Qubit. We usually end up with 1000 ng - 2000 ng of total library at this stage.

92 With a cut off P200 tip, transfer about 350 ng of prepared library to a fresh 1.5mL LoBind tube. This should not exceed 35 µL in volume.

DNA LoBind Tubes, 1.5 mL **Sigma Aldrich Catalog #0030108051**

Note

To maximize throughput and read length, it is critical to load enough library that flow cell pores will be occupied but not so much that they are oversaturated. The molar concentration of the library is a function of the fragment lengths so it is difficult to say exactly how much library to load. The average library prepared in this manner usually sequences well when 300 ng to 500 ng of DNA is loaded. Note that flow cells need to be flushed and reloaded so we usually aim to have at least 3 library loads.

93 Add an equal volume of SQB to the tube. Then, add FB from the marked tube (the one that we are not going to prepare the priming mix with) to a final volume of 70 µL.

For example, if 10 µL of [M] 35 ng/µL library was transferred in step 77, add 10 µL of SQB and 50 µL FB to the tube.

Ligation sequencing kit 1D **Sigma Aldrich Catalog #SQK-LSK109**

Flow Cell Priming Kit (EXP-FLP002) **Sigma Aldrich Catalog #EXP-FLP002**

94 Follow the official instructions to prime the flow cell, then add the prepared library to the flow cell. When loading the library, be sure to use a wide-bore pipette tip. Gently pipette mix the library before loading to ensure even distribution of the library across the flow cell membrane.

Large-Orifice Pipet Tips 200µL **Sigma Aldrich Catalog #02-707-134**

95 Over the course of a sequencing run, pores will get clogged and become inactive. It is essential to flush the flow cell at 10-14 hour intervals to make these pores available again. We recommend Nanopore's Flow Cell Wash Kit (EXP-WSH003).

Flowcell Wash Kit **Sigma Aldrich Catalog #EXP-WSH003**

Tips for sequencing the library-R10.4.1

30m

96 Thaw 1 tube SB (LSK110), 1 tube LIS, 1 tube of FCF per sample, and 1 tube FCT. Mix SQB and FB by flicking.

97 Warm the FCF at 37 °C for 00:30:00 30m

98 Add 30 µL FCT to FCF and pipette 10x to ensure thorough mixing

> **Note**
>
> We recommend marking the top of the FCF tube after FCT has been added.

99 Follow the official instructions to prime the flow cell.

100 While the flow cell is priming, prepare the library by adding 70 µL of LIS, Sample library (LIS and library should total to 100uL), and 100 µL SB. Lightly tap to mix until swirls disappear but wait to pipette mix until just before loading.

101 Pipette mix prepared library 2x times and then following official instructions to load the flow cell.

102 Over the course of a sequencing run, pores will get clogged and become inactive. It is essential to flush the flow cell at 10-14 hour intervals to make these pores available again. We recommend Nanopore's Flow Cell Wash Kit (EXP-WSH00).

ONT Flow Cell Wash Kit **Oxford Nanopore Technologies Catalog #EXP-WSH004**