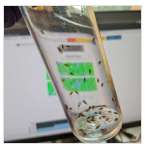

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# DNA extraction and Nanopore library prep from 15-30 whole flies

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ABSTRACT

We have been assembling the genomes of many *Drosophila* species. With that in mind, we developed this protocol to keep the cost of sequencing down to <$500 per assembly while maintaining a decent number of very long reads. Using these guidelines, a typical Drosophila Nanopore sequencing run should have read N50 of 20-40kbp with 5-15% of data in reads >100kbp. Sequencing is halted at about 40-50X depth of coverage (8-10 Gbp for most species). This of course depends on the quality of the sample, quality and quantity of the prepared library, and the frequency at which the flow cell is flushed and reloaded. We typically run 3-4 species per 2 flow cells, usually for ~14-18 Gbp of data per flow cell.

This protocol borrows several elements from John Tyson's "Rocky Mountain" protocol and we thank him for several insightful discussions.
https://www.protocols.io/view/rocky-mountain-adventures-in-genomic-dna-sample-pr-7euhjew

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KEYWORDS

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



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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 (e.g. phenol-chloroform vs. Circulomics kit preps) will sequence differently.

Ballpark estimates of library size and throughput from a single load onto a relatively fresh flow cell are:
Read N50 10kb: 150 ng library, 10Gb+
Read N50 20kb: 250 ng library, 8Gb
Read N50 30kb: 350 ng library, 4Gb
Read N50 40kb+: 500 ng library, 3Gb

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.

MATERIALS TEXT

MATERIALS

10% SDS solution **Contributed by users** Step 11

NEBNext Companion Module forOxford Nanopore Technologies Ligation Sequencing – 24 rxns **New England Biolabs Catalog #E7180S** In 3 steps

Ligation sequencing kit 1D **Oxford Nanopore Technologies Catalog #SQK-LSK109** In 6 steps

Chloroform **Millipore Sigma Catalog #CX1055-6** Step 21

Phenol Chloroform Isoamyl Alcohol (25:24:1) Tris-saturated (pH 8.0) **Fisher Scientific Catalog #BP1752I-400** Step 17

3M sodium acetate **Contributed by users** Step 26

Proteinase K Solution (20 mg/mL) RNA grade **Thermo Fisher Scientific Catalog #25530049** Step 11

RNase A solution **Millipore Sigma Catalog #R6148** Step 11

Tris-EDTA (TE) buffer pH 8.0 1X **Contributed by users** Step 36

Homogenization Buffer (HB) [0.1M NaCl 30mM Tris-HCl pH 8.0 10 mM EDTA 0.5% Triton X-100] **Contributed by users** Step 6

Lysis Buffer (LB) [0.1M Tris-HCl pH 8.0; 0.1M NaCl; 20mM EDTA] **Contributed by users** Step 6

Hydration Buffer (STE) [400mM NaCl 20mM Tris-HCl pH 8.0 30mM EDTA] **Contributed by users** In 2 steps

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** In 3 steps

Short Read Eliminator (SRE) **Circulomics Catalog #SS-100-101-01** In 2 steps

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.



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DNA LoBind tubes, 1.5 mL
Tubes
**Eppendorf** **022431021**
1.5 mL

DNA LoBind tubes, 2.0 mL
Tubes
**Eppendorf** **022431048**
2.0 mL

Large-orifice pipet tips, 200uL
Pipette tips
**Fisher** **02-707-134**
200 uL

Dounce Homogenizer, 2mL
Tissue Grinder
**Kimble** **885300-0002**
2 mL with Pestles A and B

5PRIME Phase Lock Gel tube, light, 2mL
**Quantabio** **2302830**
Light

BEFORE STARTING

This protocol is for DNA extraction from whole Drosophila. Before starting the protocol, 10-40 whole male flies should be starved for 2 days 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



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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] **Contributed by users**

3 Incubate on a platform rocker or rotator for **00:30:00** at low to medium speed.

4 Replace the solution with **1.0 mL** of fresh Buffer STE and incubate for another **00:30:00** on the rocker at low to medium speed.

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

5 Transfer the tissue to a new sheet of filter paper and wick off excess liquid.

## Tissue homogenization 15m

6 Chill Dounce homogenizer, homogenization buffer (HB), and lysis buffer (LB) on ice.

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**

7 Place flies into Dounce homogenizer with **1 mL** of HB. Dounce 10 times with looser fitting pestle A, then 10 times with tighter fitting pestle B.

**WARNING**: It is easy to break the glass homogenizer if used improperly.

Wrap the base of the homogenizer in a bit of paper towel in case of breakage, then brace it against your hand or another soft surface. Do not brace it against a hard surface like a lab bench. To homogenize, slowly move pestle up and down vertically, without twisting or trying force the pestle down if it feels stuck. When the sample is fully homogenized, you should be able to dounce with minimal resistance from fly chunks. Suction from douncing will help mix the pellet that initially gathers at the bottom.

Move the pestly slowly to minimize foaming.



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Dounce Homogenizer, 2mL
Tissue Grinder

**Kimble** **885300-0002**

2 mL with Pestles A and B

8 Use a P200 with a wide-bore tip to transfer the homogenate into a new 1.5 mL tube.

Large-orifice pipet tips, 200uL
Pipette tips

**Fisher** **02-707-134**

200 uL

9 Rinse the homogenizer with **500 µl** of HB, and use a wide-bore tip to transfer to the tube with the rest of the homogenate.

10 Pellet fly material at **2000 x g** for **00:05:00** , preferably at **4 °C** .

11 Meanwhile, prepare a solution of **380 µl** LB, **10 µl** Proteinase K, **10 µl** 10% SDS, and **2 µl** RNase A in a 1.5 mL LoBind tube.

DNA LoBind tubes, 1.5 mL
Tubes

**Eppendorf** **022431021**

1.5 mL

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

10% SDS solution **Contributed by users**

RNase A solution **Millipore Sigma Catalog #R6148**

12 After homogenate has been pelleted, carefully discard the supernatant by decanting. While still inverted, wipe up white lipid smear near lip of tube with a Kimwipe so that it doesn't a chance to drip down onto the pellet.



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13 Resuspend pellet in 100uL of HB using vigorous mixing with a wide-bore tip. Transfer homogenate into tube with LB and again mix thoroughly with a wide-bore tip.

Lysis 4h

14 Incubate lysis tube at **50 °C** for **02:00:00** to **04:00:00** . Mix the tube with gentle rocking and inversion, until solution appears relatively homogeneous, at **01:00:00** intervals.

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.

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

Phenol chloroform extraction 1h

15 Spin down 2 phase lock gel tubes per sample at **15000 x g** for **00:00:30** .

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/

**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.

5PRIME Phase Lock Gel tube, light, 2mL

**Quantabio** 2302830

Light

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

17 Add an equal volume (about **600 µl** ) of Tris-saturated phenol chloroform isoamyl alcohol (PCI) to the phase lock tube.



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This should be performed inside the fume hood.

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

18 Mix by placing tubes on a rocker at medium speed for **00:08:00** .

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.

19 Centrifuge the phase lock tube at **16000 x g** for **00:08:00** . Phase lock layer should now separate aqueous and organic layers.

20 Perform one more PCI extraction: **go to step #17** .

Use the same tube for the second extraction, i.e. just add another 600uL of PCI to the tube and proceed. The phase lock layer should hold through the extraction steps.

21 Decant aqueous (top) layer into a fresh phase lock tube. Add an equal volume (usually **600 µl** ) of chloroform to the tube.

We find decanting more effective than pipetting. If the pipette tip touches the phase lock gel, it makes a huge mess.

This should be performed inside the fume hood.

Chloroform **Millipore Sigma Catalog #CX1055-6**

22 Mix by placing tubes on a rocker at medium speed for **00:08:00** .

23 Centrifuge the phase lock tube at **16000 x g** for **00:08:00** . Phase lock layer should now separate aqueous and organic layers.

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

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



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prevent DNA sticking to the tube. This is helpful for maximizing yield and minimizing shearing.

This should be performed inside the fume hood.

DNA LoBind tubes, 2.0 mL
Tubes
Eppendorf 022431048
2.0 mL

DNA precipitation, wash, and resuspension 1h 30m

25 Chill 100% ethanol on ice and make **1 mL** of fresh 70% ethanol using nuclease-free water.

26 Add 0.1 volume (typically **50 µl** ) of 3M sodium acetate to the extract from Step 24. Gently swirl to mix.

3M sodium acetate **Contributed by users**

27 Add 2-2.5 volumes (typically **1200 µl** ) of cold 100% ethanol to the tube, and mix with careful swirling and gentle rocking. 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.

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.

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

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. PosResuspending DNAition 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.

DNA LoBind tubes, 1.5 mL
Tubes
Eppendorf 022431021
1.5 mL

29 Centrifuge the tube at **2000 x g** for **00:02:00** to pellet the DNA.



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

31 Add **200 µl** of 70% ethanol to wash the DNA. Gently swirl to mix.

32 Centrifuge the tube at **2000 x g** for **00:02:00** .

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

I usually spin the tube down briefly (~2 sec) and use a P10/P20 to grab the last bit of ethanol in the tube.

34 Wash the pellet once more: **go to step #31** .

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

36 Resuspend in **65 µl** 1X TE buffer and incubate at **50 °C** for **01:00:00** .

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

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

DNA resuspension 1w

38 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

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.

39 Check sample concentration and quality of **1 µl** aliquots using Qubit and Nanodrop.

Ideally, this should Qubit at >75 ng/uL and have Nanodrop ratios of 260/280 >1.8 and 260/230 >2.0.

DNA repair and end-prep



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40 Thaw NEBNext repair and dA-tailing mixes and buffers from the Nanopore Companion Module. Mix buffers by vortexing and mixes by flicking. Spin down tubes and keep chilled on ice.

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

41 Add 3000-4000 ng of HMW DNA (typically **30 µl** ) to a PCR tube. Dilute the HMW DNA with water to a final volume of **48 µl** . 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 the tube. Mix tube with gentle flicking (or very gentle pipetting with a cut-off P200 tip).

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**

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

43 Using a cut-off P200 tip (a wide bore will be too small to fit in the PCR tube), gently transfer sample to a 1.5 mL DNA LoBind tube. Add **60 µl** SRE 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 effect at removing small DNA fragments as SRE.

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**

44 Centrifuge the sample at **10000 x g, Room temperature** 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, or **500 µl** of PB diluted 1:1 with nuclease-free water.

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

46 Add **150 µl** of 70% ethanol or diluted PB solution. Pipette slowly, with the tip touching the front wall of the tube, so that the pellet is not disturbed.



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47 Centrifuge at **10000 x g, Room temperature** for **00:02:00** .

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

49 Repeat steps 46-48 once more.

50 Resuspend pellet in **31 µl** EB.

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

51 Incubate the tube on the heat block at **50 °C** for **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** .

Adapter ligation

52 Thaw AMX, T4 ligase, LNB, and LFB from the NEBNext Nanopore Companion Module and the Nanopore LSK109 kit. Mix AMX, 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**

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

53 Add **30 µl** prepared DNA sample (the extra **1 µl** can be used to Qubit), **2.5 µl** AMX, and **5 µl** T4 ligase to a fresh 1.5 mL DNA LoBind tube. Gently flick the tube to mix.

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

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**

54 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.

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



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

55 Incubate the reaction mixture at **Room temperature** for **01:00:00** .

56 Centrifuge the tube at **10000 x g, Room temperature** for **00:30:00** to pellet the DNA.

57 Pipette off the supernatant, being careful not to disturb the DNA pellet.

58 Add **100 µl** of LFB to the tube. SFB or a 1:1 dilution of PB can be used here (similar to step 46).

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

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

59 Centrifuge the sample at **10000 x g, Room temperature** for **00:02:00** .

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

61 Repeat steps 58-60 to wash the pellet once more.

62 Resuspend pellet in **31 µl** EB.

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

63 Incubate library on the heat block at **40 °C** for **00:00:00** . Briefly spin down the tube to collect condensation then incubate for at least **48:00:00** before the next step.

(Optional) Library size selection with SRE buffer

64 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/µL** . If the concentration is greater than **100 ng/µL** the library should be diluted to improve size selection performance.

65 Add **30 µl** of prepared library to a fresh 1.5 mL DNA LoBind tube.



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

66 Add an equal volume ( **30 µl** ) of SRE 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**

67 Centrifuge at **10000 x g, Room temperature , 00:30:00** .

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

69 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.

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

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

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

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

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

72 Repeat steps 69-71 to wash the pellet once more.

73 Resuspend the pellet in **31 µl** EB.

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

74 Incubate the tube on the heat block at **40 °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

75 Thaw 1 tube SQB (SQK-LSK109), 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.



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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.

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

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

77 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 **Eppendorf Catalog #0030108051**

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.

78 Add an equal volume of SQB from the LSK109 kit 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 **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 **Oxford Nanopore Technologies Catalog #SQK-LSK109**

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

79 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 **Fisher Scientific Catalog #02-707-134**

80 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 **Oxford Nanopore Technologies Catalog #EXP-WSH003**



**Citation:** Bernard Y Kim, Danny E. Miller, Jeremy Wang (07/16/2021). DNA extraction and Nanopore library prep from 15-30 whole flies. https://dx.doi.org/10.17504/protocols.io.bdfqi3mw

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