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DNA extraction and Nanopore library prep from single wild-caught drosophilids V.2

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

Bernard Y Kim[1]

[1]Petrov Lab, Stanford University

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Bernard Kim
Petrov Lab, Stanford University

We have been assembling the genomes of many *Drosophila* species. This protocol fork allows for the sequencing of single wild-caught flies using Nanopore MinION sequencers, in contrast to our previous protocol that originally used gDNA extracted from tens or hundreds of flies from an inbred line.

While read lengths are not nearly close to what we were seeing before, libraries range from 2-10kb read N50 and typically produce genome assemblies with contig N50 >1 Mbp.

The protocol for ultra-long read sequencing from large pools of flies can be found here: dx.doi.org/10.17504/protocols.io.bdfqi3mw

For now, please cite the original genomes paper if you use this protocol: https://doi.org/10.7554/eLife.66405

An update that includes many single wild-caught fly genomes is in progress.

DOI

dx.doi.org/10.17504/protocols.io.dm6gpbdn8lzp/v2

Bernard Y Kim 2022. DNA extraction and Nanopore library prep from single wild-caught drosophilids. **protocols.io** https://dx.doi.org/10.17504/protocols.io.dm6gpbdn8lzp/v2 Bernard Kim

protocol

Kim, B. Y., Wang, J. R., Miller, D. E., Barmina, O., Delaney, E., Thompson, A., ... & Petrov, D. A. (2021). Highly contiguous assemblies of 101 drosophilid genomes. Elife, 10, e66405. https://doi.org/10.7554/eLife.66405

Some steps (tissue lysis) were accidentally deleted in the first version of the protocol. Those steps have been restored.

DNA extraction and Nanopore library prep from 15-30 whole flies, Bernard Kim

Drosophila, nanopore, ligation, HMW, single fly

protocol ,

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Flies were collected from the wild and stored in 95% ethanol at -20C. We have successfully assembled genomes from samples ranging from 4 months to 15 years old. Younger samples perform better, of course. Ideally the samples are no older than 5 years and stored properly (e.g. alcohol has not evaporated).

Apart from the gDNA extraction steps, this protocol mostly follows the standard LSK110 protocol. Slight modifications were made to improve library recovery and pore occupancy.

MATERIALS

10% SDS solution **Contributed by users** Step 8

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

Oxford Nanopore Ligation Sequencing Kit **Oxford Nanopore Technologies Catalog #SQK-LSK110** In 6 steps

Chloroform **Millipore Sigma Catalog #CX1055-6** Step 16

Phenol Chloroform Isoamyl Alcohol (25:24:1) Tris-saturated (pH 8.0) **Fisher Scientific Catalog #BP1752I-400** Step 12

3M sodium acetate **Contributed by users** Step 21

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

Monarch RNase A – 1 ml (2x0.5ml) **New England Biolabs Catalog #T3018L** Step 8

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** 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 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.

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

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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

(Optional) Hydration of ethanol-fixed tissue 45m

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

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

2 Add **500 µL** Buffer STE to the tube with the fly.

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 **00:15:00** at low to medium speed. 15m

4 Replace the solution with **500 µL** of fresh Buffer STE and incubate for another **00:15:00** on the rocker at low to medium speed. 15m

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

5 Use a pipette to remove as much of the liquid as possible, but do not dry the fly.

Tissue homogenization 5m

6 Add **283 µL** lysis buffer to the tube with the fly.

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

7 Using a pellet pestle, manually homogenize the fly tissue. While it's important to squish the fly enough so that soft tissue can be digested, it is okay for some bigger pieces of tissue, particularly those held together by tough cuticle like the legs, to remain.

Pellet Pestle

Kimble Kontes **K749520-1590**

1.5 mL Polypropylene w/o Microtubes RNAse DNase free

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TIP: Make sure fly bits aren't stuck on the pestle before discarding. Every bit of tissue counts!

Tissue lysis

8 Add **7.5 µL** **20 mg/mL** Proteinase K, **7.5 µL** 10% SDS, and **2 µL** **20 mg/mL** RNAse A to the tube. Gently flick and swirl to mix.

Monarch RNase A – 1 ml (2x0.5ml) **New England Biolabs Catalog #T3018L**

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

10% SDS solution **Contributed by users**

9 Incubate tube for **02:00:00** to **04:00:00** at **50 °C** . Gently mix tube every **00:45:00** . 4h

The tube should go from cloudy (due to undigested tissue bits) to clear as the digestion proceeds. Due to the pestle homogenization there will usually be bigger pieces of the fly, like the wings and legs, floating around. This is fine, just pipette as much liquid as you can get for the next step.

Phenol chloroform extraction 1h

10 Spin down 1 2mL phase lock gel tube 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 minimal mess and hassle. Avoid overfilling and air bubbles. We 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

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DNA LoBind Tubes 2.0 mL **Eppendorf Catalog #30108078**

11 Briefly spin down the homogenate/lysis solution, then transfer to the phase lock gel tube by decanting or pipetting with a wide-bore tip.

12 Add an equal volume (about **350 µL**) of Tris-saturated phenol chloroform isoamyl alcohol (PCI) to the phase lock tube.

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**

13 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.

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

15 Perform one more PCI extraction: **go to step #12** .

Use the same tube for the entire extraction, i.e. just add another 350uL of PCI/CHCl3 to the tube and proceed. The phase lock layer should hold through the extraction steps.

16 Add an equal volume (usually **350 µL**) of chloroform to the tube.

This should be performed inside the fume hood.

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

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

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

19 Quickly decant the aqueous (top) layer into a fresh 1.5 mL LoBind tube.

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

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mess.

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 chloroform 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 to 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.

This should be performed inside the fume hood.

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

DNA precipitation, wash, and resuspension 30m

20 Chill 100% ethanol on ice and make **500 µL** of fresh 70% ethanol using nuclease-free water.

21 Add 0.1 volume (typically **30 µL**) of 3M sodium acetate to the sample. Gently swirl to mix.

3M sodium acetate **Contributed by users**

22 Add 2-2.5 volumes (typically **675 µL**) of cold 100% ethanol to the tube, and mix with careful swirling and gentle rocking. It will be difficult to see precipitated DNA, but extractions from larger flies will usually have a few visible strands.

If the extraction tube turns cloudy, it is likely salt precipitation because the solution is too nonpolar. Add water dropwise with thorough mixing and the solution should clear up.

23 Centrifuge the tube at **5000 x g** for **00:05:00** to pellet the DNA. 5m

TIP: the pellet will often be invisible. Centrifuge the tubes with the hinge placed consistently towards the outside of the rotor so that you know where the pellet will be.

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

25 Add **200 µL** of 70% ethanol to wash the DNA. Gently swirl to mix.

26 Centrifuge the tube at **5000 x g** for **00:02:00** .

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27 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.

28 Wash the pellet once more: **go to step #25** .

29 Allow the tube to air dry no longer than **00:01:00** . **Do not over-dry**. 1m

30 Resuspend in **42 µL** 10mM Tris and incubate at **50 °C** for **00:30:00** . 30m

Carefully pipette the tris up and down the back of the tube to ensure you've resuspended all the DNA. Sometimes it will be spread out a bit along the back of the tube, rather than pelleted cleanly on the bottom.

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

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

DNA resuspension 15m

32 Pipette mix slowly 5X with a P200 tip to ensure proper resuspension.

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

Ideally, you'll have recovered >250ng DNA and absorbance ratios 260/280 >1.8 and 260/230 >2.0. We have successfully prepared Nanopore libraries with as little as 208ng of gDNA at this point.

Reserve DNA for short-read sequencing 15m

34 Reserve at least **20 ng** gDNA in **6 µL** Tris for Illumina library prep. Store at **-20 °C** until ready for library prep.

This is a conservative amount. As of April 2022, we use the Illumina kit with 1/5 reaction volumes to prepare libraries. We have successfully prepared Drosophila Illumina libraries with as little as 4-6 ng of gDNA depending on the kit and protocol. Your mileage may vary.

Although Illumina sequencing may no longer be necessary for genome assembly with the release of new flow cell chemistries, a short-read library will be useful for variant calling if this a wild-caught individual, so this is recommended irrespective of flow cell/kit chemistry.

DNA repair and end-prep

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35 Thaw NEBNext repair and dA-tailing mixes and buffers from the Nanopore Companion Module. Mix buffers by vortexing and enzyme 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**

36 Add all non-reserved DNA (up to **40 µL**) to a PCR tube. Dilute the HMW DNA with water to a final volume of **48.5 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**

37 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.

38 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** AMPure beads. Using a wide-bore P200 tip, quickly but gently mix the tube.

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

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

Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

39 Allow the sample to sit at least **00:05:00** at **Room temperature** to allow the beads to bind the DNA. Meanwhile, prepare **500 µL** fresh 70% ethanol with nuclease-free water. 5m

40 Pellet the beads by placing the tube onto a magnetic tube rack for **00:05:00** or until sample has completely cleared. 5m

Magnetic 1.5 mL tube rack
Any NA

41 Pipette off the supernatant, taking care not to disturb the bead pellet.

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42 Add **175 µL** of 70% ethanol. Pipette slowly, with the tip touching the front wall of the tube, so that the pellet is not disturbed.

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

44 Wash the pellet once more with 70% ethanol **go to step #42**

45 Briefly spin the sample tube down and place back onto the magnet. Remove any remaining drops on the bottom of the tube with a P10.

Magnetic 1.5 mL tube rack	
Any	NA

46 Immediately resuspend bead pellet in **31 µL** nuclease-free water.

47 Incubate the tube on the heat block at **50 °C** for **00:30:00** . Every 5 minutes, gently flick the tube to encourage any settled beads to resuspend. 30m

48 Briefly spin down the tube to collect condensation then place tube back on the magnetic rack. Allow tube to sit for **00:05:00** or until sample has completely cleared. 5m

49 With a cut-off P200 tip, transfer **31 µL** eluate to a fresh 1.5 mL LoBind tube.

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

50 Check for recovery with Qubit using **1 µL** of sample. Recovery should be at least **150 ng** DNA at this point.

Adapter ligation

51 Thaw AMXII, T4 ligase, LNB, and LFB from the NEBNext Nanopore Companion Module and the Nanopore LSK110 kit. Mix AMXII, 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**

52 Add **30 µL** prepared DNA sample, **2.5 µL** AMX, and **5 µL** T4 ligase to a fresh 1.5 mL DNA LoBind tube. Gently flick the tube to mix.

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These are 1/2 the standard prep volumes, hence elution in 31uL in Step 47.

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**

53 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.

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

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

54 Incubate the reaction mixture at **Room temperature** for **00:30:00** . 30m

55 Add **20 µL** AMPure beads and mix gently by flicking until uniform in color. Let sample sit for at least **00:05:00** , with occasional gentle mixing if necessary, so the DNA can bind to the beads. 5m

Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

56 Pellet the beads by placing the tube onto a magnetic tube rack for **00:05:00** or until sample has completely cleared. 5m

Magnetic 1.5 mL tube rack
Any **NA**

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

58 Remove tube from magnetic rack and add **100 µL** of LFB to the tube, flicking to mix.

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

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

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59 Pellet the beads by replacing the tube onto the magnetic tube rack for **00:05:00** or until sample has completely cleared. 5m

Magnetic 1.5 mL tube rack
Any NA

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

61 Remove the tube from the magnet and briefly spin down. Replace tube onto magnet and remove any remaining drops of supernatant with a P10.

62 Wash the pellet with LFB one more time. **go to step #58**

63 Resuspend beads in **26 µL** EB.

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

64 Incubate beads on the heat block at **37 °C** for **00:15:00** . Every 5 minutes, gently flick the tube to resuspend any settled beads. 15m

65 Briefly spin down the tube to collect condensation.

66 Place sample tube onto magnetic rack for at least **00:05:00** or until sample has cleared. 5m

Magnetic 1.5 mL tube rack
Any NA

67 Using a cut-off P200 tip, transfer **26 µL** eluate into a fresh 1.5 mL LoBind tube.

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

68 Quantify library concentration with Qubit using **1 µL** of the prepared library.

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Tips for sequencing the library

69 Thaw 1 tube sequencing buffer SBII (SQK-LSK110), 1 tube loading solution LS (SQK-LSK110), 1 tube flush buffer FB (EXP-FLP002), and 1 tube flush tether FLT (EXP-FLP002). Mix SBII, LS, and FB by flicking. Mix FLT with a pipette. Spin down and keep reagents on ice until ready to sequence.

Make sure the right versions of reagents from the ligation and/or auxiliary/expansion kits are being used.

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

70 With a cut off P200 tip, transfer at least **100 ng** of prepared library to a fresh 1.5mL LoBind tube. This should not exceed **25 µL** in volume (i.e. the volume of the eluate from Step 64).

These libraries tend to be pretty fragmented, with read N50s ranging from 2-10kbp. More than 100 ng library is needed for longer N50 libraries, but it often helps to have 2 library loads so that a flush & reload can be used to improve throughput. So this part is a bit of a guessing game that depends on library yield and sample quality.

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

71 Add the volume of LS that will result in a total volume of **25 µL** in the LoBind tube. For example, if **15 µL** of prepared library was initially added to the tube, add **10 µL** of LS for a total **25 µL** library + LS.

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

72 Add **25 µL** SBII to the tube for a total volume of **50 µL** .

50 uL is significantly lower than the standard preparation of 75 uL library+SQB+LS/LBII. We use this amount because 50 uL is enough to barely cover the flow cell membrane; this is more visibly apparent if using LBII instead of LS. This allows us to improve the concentration of library for very low yield samples.

THIS VOLUME HAS NOT BEEN TESTED ON PROMETHION FLOW CELLS. Validation is needed.

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

73 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**

74 Over the course of a sequencing run, pores will get clogged and become inactive. If you have enough library, we recommend to flush the flow cell at 10-14 hour intervals to make these pores available again.

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

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