



ddRADSeq in a Field Setting

Version 1

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ABSTRACT

This protocol begins with blood stored in Longmire's solution and has as its goal, the sequencing of genomic DNA from several individuals onto a single MinION sequencing run. It is based on a protocol outlined by Thrasher et al. (2018) for the same process on a large number of warbler samples, but it differs from this original protocol in a few key ways:

- 1. Since we ultimately were targeting sequencing by a MinION sequencer, the ultimate processing steps reflect library preparation for runs on this device, instead of Illumina sequencers.
- 2. For the same reason, we also size selected larger fragments than can be sequenced on a MiSeq platform.
- 3. This protocol is also meant to only analysed a few individuals (20 or less) since the MinION read coverage is lower than that of a MiSeq.
- 4. The entire protocol was carried out in a field laboratory in the southeastern Peruvian Amazon, and therefore many of the luxuries a institutional laboratory can afford were not present to us
- 5. We used gel-based size selection in the absence of methods such as Blue Pippin.

PROTOCOL STATUS

In development

We are still developing and optimizing this protocol.

STEPS MATERIALS

| NAME V | CATALOG # | VENDOR ~ |
|-----------|-----------|----------|
| Buffer AE | 19077 | Qiagen |
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FTA Card Extractions

- Take 1 to 3 (2-3 mm³) punches from an FTA card using a fine scalpel or a hole punch, while maintaining sterility. Transfer it into a 1.5 mL microcentrifuge tube.
- **□**500 µl of ddH20 to each tube.
- Rinse each sample in ddH2O by vortexing three times for **(4) 00:00:05**
- Using a sterile tip, transfer each disk to a pre-labeled 0.8 mL PCR tube.
- □50 μl of ddH2O so that it completely covers the disk

Spin down tubes and make sure disk(s) are submerged **© 00:30:00 ₹ 95 °C** for Remove tube and pulse vortex or gently tap 60 times. 8 9 **© 00:00:30** Centrifuge for Transfer all liquid into a pre-labelled tube - this will be Elution A 10 11 **□**30 μl of ddH₂0 to the disk that remains in the PCR tube and repeat steps 5 - 10 to get Elution B 12 § 4 °C if using it soon, or store at § -20 °C for longterm storage. DNA Extractions from Blood Stored in Longmire Solution: Day 1 13 Vortex sample for **(900:01:00** 14 of Proteinase K **□**10 µl 15 8 70 °C ₫ 65 °C . The digest iscomplete when the colour of the supernatant has turned light orange Incubate overnightat DNA Extractions from Blood Stored in Longmire Solution: Day 2 16 of Binding Buffer to the tube containing 200 uL of Longmire's solution with blood. 17 **©** 00:00:20 Pulse vortex for 18 Incubate for **© 00:10:00**

19 □ 500 μl of 96% ethanol and pulse vortex 5 times 20 Centrifuge at 16000 G for **© 00:02:00** 21 Carefully transfer as much supernatant as possible to the spin column, loading | 300 µl | at a time, centrifuge at 6000 G for (00:02:00 and discard the liquid. 22 Centrifuge at 6000 G for (00:02:00 and discard the liquid. 23 of Wash Buffer, centrifuge at 6000 G for **© 00:02:00** and discard the liquid. 24 Centrifuge again at 10,000 G for © 00:04:00 and discard the liquid. Remove spin column and place into a labelled microcentrifuge tube. 25 26 Incubate tubes with caps off at **8 56 °C** to evaporate residual ethanol for 27 During this incubation, place 2 ml or sufficient amount of elution buffer into the bath to heat it. 28 of elution buffer directly onto the membrane of each spin column. 29 **© 00:10:00** Incubate at roomtemperature for 30 Centrifuge at 10,000 G for (00:05:00 to collect eluted DNA. 31 Repeat steps 28 to 30 with **□**30 μl of elution buffer for a second elution B. If you like, you can also elute this directly into Elution A for one tube at the end. **Quantify DNA**

Choose the OneDNA protocol on the Quantus fluorometer.

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| 44 | Pulse vortex the PCR tubes. |
|-------|---|
| 45 | Heat the samples at 8 95 °C for © 00:03:00 and then cool to 8 4 °C. |
| | Heating DNA for longer than 3 minutes at higher temperatures can cause damage to the DNA. |
| 46 | While waiting for samples to cool, prepare a Master Mix: For each amplification reaction, combine $\boxed{9~\mu I}$ of Reaction Buffer (Blue cap) with $\boxed{1~\mu I}$ of Enzyme Buffer (Yellow cap). |
| | ASAFETY INFORMATION Prepare the Master Mix right before you use it and keep in on ice. If there is any left over after WGA, do not reuse it. |
| | Our enzyme:buffer concentration was 1:5.6, rather than 1:9 (by accident). This did not appear to have an effect on the amplification. |
| 47 | Transfer $\boxed{10~\mu l}$ of the Master Mix to each sample. |
| 48 | Incubate at 8 30 °C for © 01:30:00 . If you had low concentration to start with, this can be extended for 30 more minutes. |
| 49 | Inactivate samples at 8 65 °C for © 00:10:00 . |
| 50 | Pause point: Samples can be stored at 4° C. |
| Prime | r 1 Adaptor Ligation |
| 51 | Resuspend oligos to 100uM in 0.5X AE Buffer (Qiagen), using supplier's instructions if needed. |
| 52 | In a PCR tube mix equal volumes of each oligo together to get a final concentration of 50uM of adapter. E.g. 20ul of P1F_X and 20ul of P1R_X 11. |

53 **© 00:01:00** In a thermocylcer heat the mixture at 80 °C for 54 Remove tube from the thermocylcer and place on a rack at 55 Allow to cool to § 24 °C or rtp for at least **© 00:30:00** 56 Further dilute the adapter to 5uM with 0.5 X AE in 1.5ml tube. E.g Add of annealed adapter and 57 or in freezer for long term storage. Store adapters at 8 4 °C **Primer 1 Dilution** Label tubes. These dilutions can be done in a strip of PCR tubes to allow you to use the multichannel pipette to dispense the P1 adapters. 58 (You will need 20 tubes if using all the P1 adapters) 59 **□**19 μl of nuclease free water to each tube. 60 of P1 adapter to corresponding one tube above. Repeat for all P1 adapters being used. 61 Seal tubes, vortex briefly to mix, centrifuge. 62 **Primer 2 Adaptor Ligation** In a PCR tube mix equal volumes of each oligo together to get a final concentration of 50uM of adapter. E.g. 50ul of P2F_Mspl and 50ul of 63 64 In a thermocylcer heat the mixture at § 80 °C 65 Remove tube from the thermocylcer and place on a rack at 3 24 °C or rtp 66 for at least | **© 00:30:00** Allow to cool to § 24 °C or rtp

 $Further \ dilute \ the \ adapter \ to \ 25 uM \ with \ 0.5 \ X \ AE \ in \ 1.5 ml \ tube. \ E.g \ Add \ 100 ul \ of \ annealed \ adapter \ and \ 100 ul \ 0.5 X \ AE.$

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Store adapter at 8 4 °C or in a freezer for long term storage.

Barcoding and Pooling Strategy

- 69 Enter rest of the barcoding and pooling steps here, one by one, do not paste a list!
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Double Restriction Enzyme Digest

71 Make a master mix of everything except the P1 adapters and DNA in a 2ml tube. Make the master mix for a few extra reactions so that you do not run out. Use the ligation mix table below. If you do not have those exact concentrations, then use the table from the next step instead:

| Reagents | Volume (uL) for one reaction |
|----------------------|------------------------------|
| Nuclease free water | 6.25 |
| 10X CutSmart Buffer | 4 |
| 25uM P2 adaptor | 2.5 |
| 10mM ATP | 4 |
| 20U/uL Sbfl-HF | 0.75 |
| 20U/uL Mspl | 0.75 |
| 400U/uL T4DNA ligase | 0.75 |
| Total | 19 |

72 If you do not have those exact concentrations, then use the table below:

| Reagents | Volume (uL) for one reaction |
|----------------------|------------------------------|
| Nuclease free water | Up to total volume |
| 10X CutSmart Buffer | Dilute to 1X |
| 25uM P2 adaptor | 2.5 |
| 10mM ATP | Dilute to 1mM |
| 20U/uL Sbfl-HF | 0.75 |
| 20U/uL Mspl | 0.75 |
| 400U/uL T4DNA ligase | 0.75 |
| Normalized DNA | Approx. 200-500ng |
| Total | 19 |

Alternative Master Mix Calculator for DNA concentrations that are different.

- 73 Vortex briefly to mix and centrifuge.
- 74 Aliquot 19 ul of master mix to each well of a PCR strip tube or plate (depending on the size of your reaction)
- Add 1ul of P1 adapter to the corresponding well of the PCR plate (make sure that each pair of 2 digestion-ligation reactions receives a unique P1 adapter).
- 76 Add 20ul of gDNA to each well. Note: every well will have a different DNA sample.

- 77 Use a pipette set to 30ul and gently pipette up and down a few times to mix each reaction.
- 78 Seal each PCR strip tube well, making sure the snap caps are down and that the contents of the tube are settled at the bottom.
- 79 Incubate at the following temperatures and times:

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§ 37 °C for © 00:30:00

§ 20 °C for © 01:00:00

§ 4 °C hold
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Gel-based Size Selection and Purification

80 1. Set a thick 1% agarose gel. For the MiniOne PCR system, we used ~19 mL agarose / gel + 1.5 uL dye, and the 6-toothed comb. Our gels could hold up to 35uL of product

81

NOTE

Critical Note: Multiple samples can be run on the same gel, but to prevent contamination across lanes, only run samples that will be pooled into the same pool on the same gel.

We additionally reused 1x TB buffer for gels containing samples from a common pool.

Load your samples into wells along with a 100bp ladder. Samples may have to be loaded into multiple wells.

82

NOTE

We ran gels for ~25 minutes until the 1000 bp band could be clearly distinguished from the 1500 bp band on the ladder.

Run until the desired size can be clearly distinguished from the ladder.

73 Take and label photo of gel for future reference.

84



The following steps are based on the gel-extraction kit you use. We used this kit



Wizard SV Gel and PCR Clean-Up System by Promega

Catalog #: A9281

| 85 | Prepare an empty 1.5 mL microcentrifuge tube and record its weight. |
|----|--|
| 86 | Using one gel excision tip, cut out the region surrounding desired size as cleanly as possible for each lane and combine all gel slices in the microcentrifuge tube. |
| | Take and label photo of excised gel for future reference. For all samples, we excised the region surrounding the 500 bp and the 1000 bp band. |
| 87 | Re-weigh microcentrifuge tube and record. |
| 88 | ☼ go to step #85 Repeat for all samples in your dataset |
| 89 | Add Membrane Binding Solution at a ratio of $10\mu l$ of solution per $10mg$ of agarose gel slice. Vortex the mixture |
| 90 | Incubate at 8 56 °C for © 00:10:00 or until the gel slice is completely dissolved. |
| | Note: Vortex the tube every few minutes to increase the rate of agarose gel melting. |
| 91 | Centrifuge the tube briefly at room temperature to ensure the contents are at the bottom of the tube. |
| | Note: Once the agarose gel is melted, the gel will not resolidify at room temperature. |
| 92 | Place one SV Minicolumn in a Collection Tube for each dissolved gel slice. |
| 93 | Transfer the dissolved gel mixture to the SV Minicolumn assembly and incubate for © 00:01:00 at room temperature. |
| 94 | Centrifuge the SV Minicolumn assembly in a microcentrifuge at 16,000 \times g (14,000 rpm) for \bigcirc 00:01:00 . |
| 95 | Remove the SV Minicolumn from the Spin Column assembly, and discard the liquid in the Collection Tube. |
| 96 | Return the SV Minicolumn to the Collection Tube. |

- 97 Wash the column by adding **□**700 μl of Membrane Wash Solution to the SV Minicolumn. 98 (900:01:00 Centrifuge the SV Minicolumn assembly for at 16,000 × g (14,000rpm). Empty the Collection Tube as before, and place the SV Minicolumn back in the Collection Tube. 99 100 ⊒500 µl of Membrane Wash Solution, and centrifuge the SV Minicolumn assembly for (9 00:05:00 Repeat the wash with 16,000 × g. Remove the SV Minicolumn assembly from the centrifuge, being careful not to wet the bottom of the column with the flowthrough. 101 102 Empty the Collection Tube, and recentrifuge the column assembly for © 00:01:00 with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol. Carefully transfer the SV Minicolumn to a clean 1.5ml microcentrifuge tube. 103 104 of nuclease-free water directly to the center of the column without touching the membrane with the pipette tip. NOTE Alternatives: You can use an elution buffer of your choice here instea of nuclease-free water, depending on your downstream applications 105 **© 00:01:00** Centrifuge for at 16,000 × g (14,000 rpm). Remove the eluted volume and place it back on the spin column. 106 107 **© 00:01:00** Centrifuge for at 16,000 × g (14,000 rpm) to concentrate your DNA. 108 Discard the SV Minicolumn, and store the microcentrifuge tube containing the eluted DNA at **⋈EXPECTED RESULT** The volume of the eluted DNA should be approximately $18-19 \mu l$. This is an open access protocol distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited
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