ddRADSeq in a Field Setting

Version 2

Mrinalini Watsa[1], Gideon Erkenswick[1], Dong Jin Matthew Park[2]

[1]University of Missouri - Saint Louis, [2]Grinnell College

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Mrinalini Watsa

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.
6. We stored blood on FTA cards and in Longmire's solution in our sampling program. We discovered that yields from FTA cards, regardless of including large numbers of hole punches per extraction, simply were too low for the sequencing goals of this project. Therefore, we include the protocol of a WGA amplification step for these low samples. Longmire's solution produced higher yields, but some times, multiple extractions might need to be pooled together with an SPRI bead cleanup to be fully effective.

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Buffer AE	19077	Qiagen
Buffer AE	19077	Qiagen

FTA Card Extractions

1 Take 1 to 3 (2-3 mm^3) 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.

2 Add **500 µl** of ddH2O to each tube.

3 Rinse each sample in ddH2O by vortexing three times for **00:00:05** each

4 Using a sterile tip, transfer each disk to a pre-labeled 0.8 mL PCR tube.

5 Add 45 µl to 50 µl of ddH2O so that it completely covers the disk

6 Spin down tubes and make sure disk(s) are submerged

7 Heat to 95 °C for 00:30:00 in a PCR machine

8 Remove tube and pulse vortex or gently tap 60 times.

9 Centrifuge for 00:00:30

10 Transfer all liquid into a pre-labelled tube – this will be Elution A

11 Add 30 µl of ddH_20 to the disk that remains in the PCR tube and repeat steps 5 - 10 to get Elution B

12 Store at 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 00:01:00

14 Add 10 µl of Proteinase K

15 Incubate overnightat 65 °C - 70 °C . The digest iscomplete when the colour of the supernatant has turned light orange

DNA Extractions from Blood Stored in Longmire Solution: Day 2

16 Add 500 µl of Binding Buffer to the tube containing 200 uL of Longmire's solution with blood.

17 Pulse vortex for 00:00:20

18 Incubate for 00:10:00 at 65 °C - 70 °C

19 Add 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 500 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 Add 720 µl 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.

25 Remove spin column and place into a labelled microcentrifuge tube.

26 Incubate tubes with caps off at 56 °C to evaporate residual ethanol for 00:15:00 .

27 During this incubation, place 2 ml or sufficient amount of elution buffer into the bath to heat it.

28 Add 20 µl of elution buffer directly onto the membrane of each spin column.

29 Incubate at roomtemperature for 00:10:00 .

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

32 Choose the OneDNA protocol on the Quantus fluorometer.

33 Calibrate the Quantus as follows: a. Fill a Qubit assay tube with 200 µl dye and click ‘Blank’ (under ‘Calibrate).

34 b. In a new Qubit assay tube, add 400 µl of dye and 2 µl of Lambda DNA, and click ‘Standard’. Then click 'Save'

35 Prepare a labeled Qubit assay tube for each sample to be quantified

36 Add 200 µl of dye to each Qubit assay tube.

NOTE
Critical Note: tubes containing dye need to be covered with aluminium foil to shield dye from light.

37 Vortex the samples to mix them well and quickly spin down.

38 Add 1 µl of each sample to each labeled Qubit assay tube.

39 Vortex and spin assay tubes, and incubate at 24 °C or rtp for 00:05:00 .

40 Measure the sample in the fluorometer and record value in ng/uL.

NOTE
We have reused Quantus assay tubes after a recycling program. Rinse each tube with 10% bleach, two washes in distilled water, and 70% ethanol. Then autoclave. These tubes should be measured once without the sample, for some of them will have a very small amount of residual DNA. Then, add the sample, incubate for 5 minutes, and re-measure. Subtract the original reading from this one for an accurate reading. This method allows us to recylce the plastic tubes without detriment to our accuracy.

Whole Genome Amplification

41 Prepare and label one PCR tube for each sample to be amplified using the (NAME KIT)

42 Add 9 µl of sample buffer (Green cap) to each tube.

43 Dilute template DNA to 10 ng/uL. Add 1 µl of this DNA to the PCR tube.

NOTE
If DNA concentration doesn't reach 10ng/uL, then add more. We added 3 µl of our template to each tube because our DNA concentration was too low.

44 Pulse vortex the PCR tubes.

45 Heat the samples at 95 °C for 00:03:00 and then cool to 4 °C .

NOTE
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 9 µl of Reaction Buffer (Blue cap) with 1 µl of Enzyme Buffer (Yellow cap).

SAFETY 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.

NOTE
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 10 µl of the Master Mix to each sample.

48 Incubate at 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 65 °C for 00:10:00 .

50 Pause point: Samples can be stored at 4° C.

Primer 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 In a thermocylcer heat the mixture at 80 °C for 00:01:00

54 Remove tube from the thermocylcer and place on a rack at 24 °C or rtp .

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 40 µl of annealed adapter and 360 µl 0.5X AE.

57 Store adapters at 4 °C or in freezer for long term storage.

Primer 1 Dilution

58 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. (You will need 20 tubes if using all the P1 adapters)

59 Add 19 µl of nuclease free water to each tube.

60 1Add 1 µl of P1 adapter to corresponding one tube above.

61 Repeat for all P1 adapters being used.

62 Seal tubes, vortex briefly to mix, centrifuge.

Primer 2 Adaptor Ligation

63 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_MspI and 50ul of P2R_MspI.

64 In a thermocylcer heat the mixture at 80 °C for 00:01:00

65 Remove tube from the thermocylcer and place on a rack at 24 °C or rtp

66 Allow to cool to 24 °C or rtp for at least 00:30:00 .

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

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

Barcoding and Pooling Strategy

69 Choosing how to pool your samples is dependent on the number of samples involved and how successful you have been in each case in extracting DNA from the samples. Additionally, the number of dual indices you create will inform the maximum number of samples that can be pooled together. As such, there are a few general guidelines to follow:
1. Make sure you combine samples in the same pool that have roughly the same DNA output. i.e. pool all samples with the same concentration (as per your fluorometer reading) together.
2. When choosing indices, follow the barcoding system utilised in Thrasher et al. 2018 if you plan to do Illumina-platform sequencing (i.e. the MiSeq or HiSeq). If you are utilising a MinION or other 3rd gen sequencer with a higher error rate, demultiplexing your samples becomes very difficult if you use barcodes as short as the ones in Thrasher et al. 2018. We strongly encourage you to extend the length of barcodes to more easily distinguish samples from each other bioinformatically post sequencing.

70 Once you have pooled your samples together, you will need 20uL of your pools, containing ~ 400 ng of DNA each. If you do not have this concentration, but you have this amount of DNA in total, consider doing an SPRI bead cleanup to concentrate your DNA so you are left with this amount in the end. If you simply do not have enough DNA, consider a second extraction combined with this one via a SPRI bead cleanup to reach these targets. Without this much DNA, you might have a lower yield of reads, although it is not impossible to achieve desired results. We recommend a bare minimum of 100 ng of DNA for any single sample.

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 SbfI-HF	0.75
20U/uL MspI	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 SbfI-HF	0.75
20U/uL MspI	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)

75 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:

37 °C for 00:30:00

20 °C for 01:00:00

4 °C hold

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.

83 Take and **label** photo of gel for future reference.

84

NOTE

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.

NOTE

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 10µl of solution per 10mg of agarose gel slice. Vortex the mixture

90 Incubate at **56 °C** for **00:10:00** or until the gel slice is completely dissolved.

NOTE

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

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 × g (14,000 rpm) for 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 Centrifuge the SV Minicolumn assembly for 00:01:00 at 16,000 × g (14,000rpm).

99 Empty the Collection Tube as before, and place the SV Minicolumn back in the Collection Tube.

100 Repeat the wash with 500 µl of Membrane Wash Solution, and centrifuge the SV Minicolumn assembly for 00:05:00 at 16,000 × g.

101 Remove the SV Minicolumn assembly from the centrifuge, being careful not to wet the bottom of the column with the flowthrough.

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.

103 Carefully transfer the SV Minicolumn to a clean 1.5ml microcentrifuge tube.

104 Apply 20 µl 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 Centrifuge for 00:01:00 at 16,000 × g (14,000 rpm).

106 Remove the eluted volume and place it back on the spin column.

107 Centrifuge for 00:01:00 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 4 °C or -20 °C .

EXPECTED RESULT

The volume of the eluted DNA should be approximately 18-19 µl.