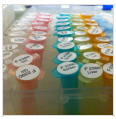

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Nsil1-Msp1 RAD-digest Detailed Protocol V.2

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ABSTRACT

This protocol comes with no guarantees, and is primarily used for the authors' purposes. This protocol is an adaptation from the work in the following publications:

Mascher M, Wu S, Amand PS, Stein N, Poland J (2013) Application of Genotyping-by-Sequencing on Semiconductor Sequencing Platforms: A Comparison of Genetic and Reference-Based Marker Ordering in Barley. PLoS ONE 8(10): e76925. https://doi.org/10.1371/journal.pone.0076925

Recknagel, H.,Jacobs, A.,Herzyk, P., &Elmer, K. R.(2015).Double-digest RAD sequencing using ion proton semiconductor platform (ddRADseq-ion) with nonmodel organisms.Molecular Ecology Resources,15,1316–1329.https://doi.org/10.1111/1755-0998.12406

MATERIALS

NAME	CATALOG #	VENDOR
1X Elution Buffer (EB)		
10X Adapter Buffer (AB) Stock		
1X Adapter Buffer (AB)		
Adapter 1		
Adapter 2		
1X TAE Buffer		
P1000 pipette and filter tips		
P200 pipette and filter tips		
P20 pipette and filter tips		
P10 pipette and filter tips		
P10 long barrel tips		
Aluminum tape sheets		
Kimwipes		
Gibco water		
Quibit Fluorometer and tubes		
PCR plates		
Strip Tubes		Axygen

NAME	CATALOG #	VENDOR
Agarose Powder		
SYBRsafe		
6X loading dye		
100bp ladder		
T4 Ligase		
T4 Ligase Buffer (contains ATP)		
2X Taq Master Mix (includes enzymes)		
Cutsmart Buffer		
Qubit dsDNA Broad Range Kit (100pg/µL-1000ng/µL)		
Qubit dsDNA High Sensitivity Kit (10pg/µL-100ng/µL)		
QIAquick PCR Purification Kit		
Pippin Prep Dye-Free Cassettes and Associated Solutions and Buffers		
NsiI1 (High Frequency)	R3127	New England Biolabs
Msp1	R0106	New England Biolabs

MATERIALS TEXT

Customized barcodes are required by this protocol and can be ordered from IDT (https://www.idtdna.com/pages)

Adapter Suspension, Dilution, Annealing and Working Stock

1 Suspend lyophilized single stranded adapters to final stock concentration of **100µM** in **1XEB**.

-Adapter 1 top and bottom will be combined in plate format. Each well corresponding to an individual adapter.
-Adapter 2 top and bottom will be combined in strip tubes.
-Final Working Stock will contain 0.02µM Adapter

2 Make 100µL of 10µM double stranded adapter.

70 µl gH20

10 µl 10X AB

10 µl Top adapter

10 µl Bottom adapter

_____

100 µl TOTAL

Repeat this step for both Adapter 1 and Adapter 2.

3 Place top and bottom Adapter 1 mixture onto the Thermocycler and run "RAD Anneal" program.

01:12:00 RAD Anneal

4 Check the annealed adapters with the Qubit.

5 Adapter 2 will remain at 10µM and can be stored at 4°C.

6 Dilute Adapter 1 from 10µM to 3µM.

- **3 µl Sample**
- **7 µl gH20**

_____

- **10 µl TOTAL**

7 Normalize Adapter 1 to 2.2ng/µL (0.1µM) and store at 4°C.

8 Create a100µLof "Working Adapter Stock".

- **20 µl Normalized (2.2ng/µL) Adapter 1**
- **30 µl 10µM Adapter 2**
- **50 µl 1X AB**

_____

- **100 µl TOTAL**

9 Seal plate, vortex and spin down before storing at 4°C until used.

DNA Normalization

10 Quantify genomic DNA to ensure samples contain between 20ng/µL and 150ng/µL.

Genomic DNA is selected based on gel electrophoresis and Qubit results.

11 Create a 30µL aliquot of 20ng/µL genomic DNA using AE Buffer as diluent.

12 Store at 4° unti used.

Restriction Digest

13 Create Restriction Master Mix:

Per reaction:

- **7.2 µl gH20**
- **0.4 µl Nsil1**
- **0.4 µl Msp1**
- **2 µl Cutsmart Buffer**

_____

10 µl **TOTAL**

14 Add 10µL of Restriction Master Mix to each well.

15 Add 10µL of 20ng/µL genomic DNA sample to the Restriction Master Mix.

16 Seal plate and run "RAD-digest" PCR program.

01:09:00 **RAD-digest program**

17 **Proceed directly to ligation step.**

Ligation

18 Create Ligation Master Mix:

Per reaction:

12.1 µl **gH20**

2.4 µl **T4 DNA Ligase Reaction BUffer**

0.5 µl **T4 DNA Ligase**

_____

15 µl **TOTAL**

19 Add 5µL of Adapter Working Stock to the 20µL restriction digest product.

20 Add 15µL of Ligation Master Mix to the 25µL of working adapter and restriction digest product.

40 µl **TOTAL**

21 Seal plate and run "RAD-ligation" PCR program.

02:25:00 **RAD-ligation Program**

22 Ligated DNA stored at 4°C until use.

Multiplex

23 Pool 5µL of each sample into a single tube.

24 Perform a column clean-up with QIAquick PCR Purification Kit.

1. Calculate the volume of pooled sample.
2. Add 5 volumes of PB to sample.
3. Transfer 600µL to filter column and spin at 17,900g for 1 minute.
4. Discard flow-through and add second 600µL aliquot to filter column before spinning at 17,900 for 1 minute.
5. Discard flow-through, add 750µL of PE and spin at 17,900g for 1 minute.

6. Discard flow-through and spin empty column at 17,900g for 1 minute.
7. Discard flow-through and transfer filter column to new (pre-labelled) epi tube.
8. Add 60µL of EB to the center of the membrane before spinning at 17,900g for 1 minute.
9. Keep flow-through and store at 4°C until size select.

Size Selection

25 Transfer 30µL of sample to a clean epi.

26 Add 10µL of Pippin Loading Solution (stored at 4°C)

27 Turn on the Pippin Prep

28 Perform and optical calibration.

29 Navigate to the main menu on the Pippin and select the most recent run from the drop down menu.

30 Create a new program by navigating to the Protocol Editor and altering the lane numbers.

31 Enter range values in the "BP Start" and "BP End" as 200-350bp respectively.

32 Select "USE INTERNAL STANDARDS" before saving the protocol under the current date.

33 Prepare the cassette by dislodging all bubbles behind the elution wells and filling any low reservoirs with electrophoresis buffer.

34 Remove 40µL of the buffer from the elution well and refill with fresh buffer before covering the wells with tape.

35 Perform continuity test.

36 Remove 40µL of electrophoresis buffer from the sample well and replace with 40µL of sample. Repeat for remaning samples.

37 Press "Start"

38 Once the run is complete, remove tape from elution wells and retreive 40µL of sample from the well. Repeat for all samples.

Remove the cassette from the optical nest. Cover all the wells with aluminum tape.

39

40 Fill rinse cassette with ddH20, place it in the cassette and close lid.

41 Allow probes to incubate for one minute. Discard the water and allow cassette to dry before storing.

Amplification

42 Prepare Amplification Master Mix:

Per reaction:

- **6.5 µl gH20**
- **12.5 µl Taq 2X Master Mix**
- **0.5 µl Ion Primer Forward**
- **0.5 µl Ion Primer Reverse**

_____

- **20 µl TOTAL**

-Each multiplexed sample are run in **quadruplicate.**

43 Add 5µL of size selected sample to 20µL aliquot of PCR Master Mix.

44 Run "RAD-amplification" program.

00:56:00 RAD-amplification program

45 Pool all four PCR library replicates.

46 Perform a column clean-up with QIAquick PCR Purification Kit

1. Calculate the volume of pooled sample.
2. Add 5 volumes of PB to sample.
3. Transfer 600µL to filter column and spin at 17,900g for 1 minute.
4. Discard flow-through, add 750µL of PE and spin at 17,900g for 1 minute.
5. Discard flow-through and spin empty column at 17,900g for 1 minute.
6. Discard flow-through and transfer filter column to new (pre-labelled) epi tube.
7. Add 50µL of EB to the center of the membrane before spinning at 17,900g for 1 minute.
8. Keep flow-through and store at 4°C until size select.

Sequencing

47 Create a 30µL aliquot to 200pM samole for processing on the Ion Chef.

48 Store all plates and sample pools at -20°C for long term storage.

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