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🌐 Nsil1-Msp1 RAD-digest Detailed Protocol V.2

📖 Evolutionary Applications

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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>

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We use this protocol and it's working

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PROTOCOL integer ID:
19485

Keywords: RAD-seq, ddRAD-seq, genotyping-by-sequencing, genome sequencing, PCR, semiconductor sequencing

MATERIALS

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- ☒ 1X Elution Buffer (EB) **Contributed by users**
- ☒ 10X Adapter Buffer (AB) Stock **Contributed by users**
- ☒ 1X Adapter Buffer (AB) **Contributed by users**
- ☒ Adapter 1 **Contributed by users**
- ☒ Adapter 2 **Contributed by users**
- ☒ 1X TAE Buffer **Contributed by users**
- ☒ P1000 pipette and filter tips **Contributed by users**
- ☒ P200 pipette and filter tips **Contributed by users**
- ☒ P20 pipette and filter tips **Contributed by users**
- ☒ P10 pipette and filter tips **Contributed by users**
- ☒ P10 long barrel tips **Contributed by users**
- ☒ Aluminum tape sheets **Contributed by users**
- ☒ Kimwipes **Contributed by users**
- ☒ Gibco water **Contributed by users**
- ☒ Qubit Fluorometer and tubes **Contributed by users**
- ☒ PCR plates **Contributed by users**
- ☒ Strip Tubes **Axygen**
- ☒ Agarose Powder **Contributed by users**
- ☒ SYBRsafe **Contributed by users**
- ☒ 6X loading dye **Contributed by users**
- ☒ 100bp ladder **Contributed by users**
- ☒ T4 Ligase **Contributed by users**
- ☒ T4 Ligase Buffer (contains ATP) **Contributed by users**
- ☒ 2X Taq Master Mix (includes enzymes) **Contributed by users**
- ☒ Cutsmart Buffer **Contributed by users**
- ☒ Qubit dsDNA Broad Range Kit (100pg/μL-1000ng/μL) **Contributed by users**
- ☒ Qubit dsDNA High Sensitivity Kit (10pg/μL-100ng/μL) **Contributed by users**
- ☒ QIAquick PCR Purification Kit **Contributed by users**
- ☒ Pippin Prep Dye-Free Cassettes and Associated Solutions and Buffers **Contributed by users**
- ☒ NsiI (High Frequency) **New England Biolabs Catalog #R3127**
- ☒ MspI **New England Biolabs Catalog #R0106**

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**.

Note

- 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 gH2O

🧴 10 μL 10X AB

🧴 10 μL Top adapter

🧴 10 μL Bottom adapter

🧴 100 μL TOTAL

Note

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 gH₂O

🧪 10 μL TOTAL

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

8 Create a 100 μL of "Working Adapter Stock".

🧪 20 μL Normalized (2.2ng/ μL) Adapter 1

🧪 30 μL 10 μM 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 .

Note

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

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

12 Store at 4°C until used.

Restriction Digest

13 Create Restriction Master Mix:

Per reaction:

🧴 7.2 μ L gH₂O

🧴 0.4 μ L NsiI

🧴 0.4 μ L MspI

🧴 2 μ L Cutsmart Buffer

🧴 10 μ L TOTAL

14 Add 10 μ L of Restriction Master Mix to each well.

15 Add 10 μ L of 20ng/ μ L 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 μ L gH₂O

🧪 2.4 μ L T4 DNA Ligase Reaction Buffer

🧪 0.5 μ L T4 DNA Ligase

🧪 15 μ L TOTAL

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

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

🧪 40 μ L 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 5 μ L 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 600 μ L to filter column and spin at 17,900g for 1 minute.
4. Discard flow-through and add second 600 μ L aliquot to filter column before spinning at 17,900 for 1 minute.
5. Discard flow-through, add 750 μ L 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 remaining samples.
- 37** Press "Start"
- 38** Once the run is complete, remove tape from elution wells and retrieve 40µL of sample from the well. Repeat for all samples.
- 39** Remove the cassette from the optical nest. Cover all the wells with aluminum tape.
- 40** Fill rinse cassette with ddH₂O, 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 μ L gH2O

🧪 12.5 μ L Taq 2X Master Mix

🧪 0.5 μ L Ion Primer Forward

🧪 0.5 μ L Ion Primer Reverse

🧪 20 μ L TOTAL

Note

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

- 43 Add 5 μ L of size selected sample to 20 μ L 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 sample for processing on the Ion Chef.

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