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Version 2

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

 Evolutionary Applications

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**Protocol status:** Working

**We use this protocol and it's working**

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**Keywords:** RAD-seq, ddRAD-seq, genotyping-by-sequencing, genome sequencing, PCR, semiconductor sequencing, marker ordering in barley, semiconductor sequencing platform, sequencing, digest detailed protocol, application of genotyping, barley, digest detailed protocol this protocol, genotyping, using ion proton semiconductor platform, ion proton semiconductor platform

## 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

### MATERIALS

- ✕ 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**
- ✕ 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) **New England Biolabs Catalog #R3127**

 Msp1 **New England Biolabs Catalog #R0106**

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

## Troubleshooting



## Adapter Suspension, Dilution, Annealing and Working Stock

- 1 Suspend lyophilized single stranded adapters to final stock concentration of **100 $\mu$ M** 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 0.02 $\mu$ M Adapter

- 2 Make 100 $\mu$ L of 10 $\mu$ M double stranded adapter.

🧪 70  $\mu$ L gH<sub>2</sub>O

🧪 10  $\mu$ L 10X AB

🧪 10  $\mu$ L Top adapter

🧪 10  $\mu$ L Bottom adapter

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🧪 100  $\mu$ L 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 10 $\mu$ M and can be stored at 4°C.

- 6 Dilute Adapter 1 from 10 $\mu$ M to 3 $\mu$ M.

🧪 3  $\mu$ L Sample


🧪 7  $\mu$ L gH<sub>2</sub>O


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🧪 10  $\mu$ L TOTAL




- 7 Normalize Adapter 1 to 2.2ng/ $\mu$ L (0.1 $\mu$ M) and store at 4°C.
- 8 Create a 100 $\mu$ L of "Working Adapter Stock".

 20  $\mu$ L Normalized (2.2ng/ $\mu$ L) Adapter 1

 30  $\mu$ L 10 $\mu$ M Adapter 2

 50  $\mu$ L 1X AB

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 100  $\mu$ L 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/ $\mu$ L and 150ng/ $\mu$ L.

### Note


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


- 11 Create a 30 $\mu$ L aliquot of 20ng/ $\mu$ L 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  $\mu$ L gH<sub>2</sub>O

 0.4  $\mu$ L NsiI

 0.4  $\mu$ L MspI

 2  $\mu$ L Cutsmart Buffer

---



🧪 10  $\mu$ L TOTAL

- 14 Add 10 $\mu$ L of Restriction Master Mix to each well.
- 15 Add 10 $\mu$ L of 20ng/ $\mu$ 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  $\mu$ L gH2O
- 🧪 2.4  $\mu$ L T4 DNA Ligase Reaction Buffer
- 🧪 0.5  $\mu$ L T4 DNA Ligase
- 
- 🧪 15  $\mu$ L TOTAL
- 19 Add 5 $\mu$ L of Adapter Working Stock to the 20 $\mu$ L restriction digest product.
- 20 Add 15 $\mu$ L of Ligation Master Mix to the 25 $\mu$ L of working adapter and restriction digest product.  
🧪 40  $\mu$ 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 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 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 ddH<sub>2</sub>O, 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 gH<sub>2</sub>O

🧪 12.5 µL Taq 2X Master Mix

🧪 0.5 µL Ion Primer Forward



🧪 0.5  $\mu$ L Ion Primer Reverse

🧪 20  $\mu$ L TOTAL

#### Note

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

- 43 Add 5 $\mu$ L of size selected sample to 20 $\mu$ 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 600 $\mu$ L to filter column and spin at 17,900g for 1 minute.
  4. Discard flow-through, add 750 $\mu$ L 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 50 $\mu$ L 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 30 $\mu$ L aliquot to 200pM samole for processing on the Ion Chef.
- 48 Store all plates and sample pools at -20°C for long term storage.