**Single digest 150 bp RADtag protocol for *Ips typographus* (Granbarkborre)**

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Last updated 30 April 2020

This protocol is developed and optimised for RADtag libraries of *Ips typographus* in the MEEL Lab at Lund University. A total of 160 individual samples are pooled together using 40 P1 inline barcodes and 4 P2 index barcodes. This protocol has been modified from two other protocols, one used in the MEEL Lab for birds and *Ischnura elegans*, and one used in the Biodiversity lab for plants.

**Reagents and Materials (in order of use) Manufacturer Storage**

1X dsDNA HS Assay Kit (Invitrogen Q33231) 2-8 °C

SbfI-HF CCTGCA/GG (20,000 units/ml) (NEB R3642L) -20 °C

CutSmart Buffer (10X) (NEB B7204S) -20 °C

T4 DNA Ligase (2,000,000 units/ml) (NEB M0202M/T) -20 °C

rATP (100 mM) (Promega E6011) -30to-10 °C

NEBuffer 2 (10X) (NEB B7002S) -20 °C

Axygen PCR Tubes 0.5 ml (Axygen PCR-05-C) RT

MinElute Reaction Cleanup Kit (Qiagen 28206) Col. 2to8°C

SPRIselect (Beckman Coulter B23318) 15-30 °C

Quick Blunting Kit (NEB E1201S) -20 °C

Klenow Fragment (3’🡪 5’ exo-, 5,000 units/ml) (NEB M0212S) -20 °C

dATP (100 mM) (Thermo Scientific R0141) -25to-15 °C

Phusion Flash High-Fidelity PCR Master Mix (2x) (Thermo Scientific F548S) 25to-15 °C

**Always use filter tips when you prepare NGS-libraries in the lab. In all of the steps!**

**Primers** (TAG Copenhagen AS) -20 °C

P1-PCR-primer F (100pM/μl): 5’-AATGATACGGCGACCACCGA-3’

P2-PCR-primer R (100pM/μl): 5’-CAAGCAGAAGACGGCATACGA-3’

**P1 (i5) Adapters SbfI (8 bp inline barcode)** (TAG Copenhagen AS) -20 °C

P1 top:

5’-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxxxxxTGC\*A-3’

P1 bottom:

5’-Phos-xxxxxxxxAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T-3’

**P2 (i7) Adapters (8 bp index barcode)** (Eurofins Genomics AS) -20 °C

P2 top:

5’-Phos-GATCGGAAGAGCACACGTCTGAACTCCAGTCACxxxxxxxxATCAGAACAA-3’

P2 bottom:

5’-CAAGCAGAAGACGGCATACGAGATxxxxxxxxGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC\*T-3’

* Where xxxxxxx is replaced with unique bases to identify individuals (8 for P1 and 8 for P2).
* TGCA will ligate to SbfI digested DNA. Change for other enzymes.

**0. Preparing Adapters (only if required)**

**Reagents for preparing adapters**

1X Elution buffer (EB; 10 mM Tris-Cl, pH 8.5) (From MinElute Kit) 15 to 25 °C

10X NEBuffer 2 (NEB B7002S) -20 °C

10X Annealing Buffer (10X AB: 500 mM NaCl, 100 mM Tris-Cl, pH 7.5-8.0) RT

**Suspending adapters**

Prepare 100 µM stocks for each single stranded oligonucleotide by suspending it in 1X Elution buffer. See the delivery note for the volume to be used.

**P1 Adapters**

To prepare 200 μl (100 nM) of annealed P1 adapters, add reagents in the following order for each adapter:

ddH2O 16 μl

P1 Top (100 µM) 2 μl

P1 Bottom (100 µM) 2 μl

20 μl TOTAL (add 10-20% to each step to have some margin).

Mix by pipetting carefully up and down or by flicking the tube. Finish with a quick spin down.

Use a thermocycler: heat to 98 °C and cool at 1 °C/minute until 25 °C (73 cycles).

Dilute 2 μl of annealed adapters in 20 μl NEBuffer 2 (10X) and 178 μl ddH2O to 200 μl TOTAL as soon as the program is ready. The salt in the NEBuffer 2 is needed for the adapters to stay annealed. Store in freezer at -20 °C.

**P2 Adapters**

To prepare 100 μl (10 μM) of annealed P1 adapters, add reagents in the following order for each adapter:

ddH2O 70 μl

Annealing Buffer 10X 10 μl

P2 Top (100 µM) 10 μl

P2 Bottom (100 µM) 10 μl

100 μl TOTAL (Volume might have to be divided into two PCR tubes depending on thermocycler capacity).

Mix by pipetting carefully up and down or by flicking the tube. Finish with a quick spin down.

Use a thermocycler: heat to 98 °C and cool at 1 °C/minute until 25 °C (73 cycles).

Move annealed adapters to new tubes and store in freezer at -20 °C.

**1. Preparation before starting**

Extract DNA with the DNeasy Blood & Tissue Kit (optimised protocol to retrieve enough high-quality DNA from *Ips typographus*). Elute the samples in a minimum of 50 μl EB buffer.

Asses the quality and contamination of chemicals with the NanoDrop. The 280/260 ratio should be around 1.70-2.00, and the 230/260 ratio should be at least 1.00

Check the integrity of all the samples by running them on an agarose gel. The DNA should at least form a sharp band at the top, ideally without a smear and no RNA band in the lower range.

**EXTRA:** Quantify all samples with the Qubit, using the 1X dsDNA HS Assay Kit. Or measure only some samples and calculate an average ratio from the concentration from the NanoDrop.

**2. Restriction Digest**

Digest 500-1000 ng double stranded gDNA per sample. Keep the enzyme on ice when not in the freezer. Pipette the enzyme carefully as it is very viscous.

Use the same amount of DNA for all samples! Dilute according to the quantities measured with the NanoDrop or Qubit to a final volume of 50 μl. Add reagents in the following order:

ddH2O to 50 μl final volume

gDNA X μg

CutSmart Buffer 10X 5 μl

SbfI-HF (20 000 units/ml) 0.5 μl

50 μl TOTAL

Mix by pipetting carefully up and down or by flicking the tube. Finish with a quick spin down. Digest at 37 °C for 60 min. Heat inactivate at 80 °C for 20 minutes. Allow samples to cool slowly in room temperature for 30 minutes.

**3. Ligation of P1-Adapters**

The presence of the salt in the NEBuffer 2 is necessary for the P1-adapters to remain double stranded at room temperature. The concentration of NaCl should be 50 mM in the final volume.

Use different P1-adapters for samples going into the same pool! The designation of P1-adapters should have been thought of well in advance and outside of the lab before preforming this step.

Keep the enzyme on ice when not in the freezer. Pipette the enzyme carefully as it is very viscous. To the 50 μl digest, add the reagents in the following order:

NEBuffer 2 10X 6 μl

P1 adapter (100 nM) 3 μl

rATP (100 mM) 1 μl

T4 Ligase (2 000 000 units/ml) 0.5 μl

50+10.5=60.5 μl TOTAL

Mix by pipetting carefully up and down or by flicking the tube. Finish with a quick spin down. Incubate at room temperature for 60 min or overnight at 16 °C. Heat inactivate at 65 °C for 10 minutes. Allow samples to cool slowly in room temperature for 15 minutes.

**4. Pool Samples**

Only pool samples together with different P1 adapters!!!

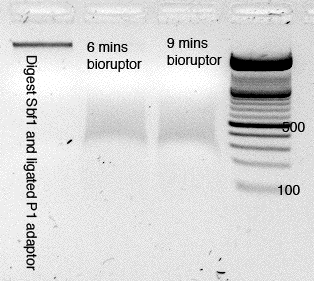
Pool such that you have 1-5 µg (max. 10 µg) to shear - so you have enough template DNA surviving until the end of the protocol. We used 20 µl from each sample, 40 samples per pool, creating 4 pools of 800 µl and 6666 ng each. Store the leftovers in the freezer.

**5. DNA Shearing**

Split each pool into 0.5 ml Axygen PCR tubes. There should be 100 μl of liquid in each tube. Add ddH2O if necessary. Use the Bioruptor to shear the DNA. Bubbles in the sample should be avoided. Make sure the starting temperature of the water is 0-3 °C without any ice. The container can be pre-cooled with water from the fridge.

Run the following program: 3 cycles of 30s on /60s off

**EXTRA:** Do a test sonication to optimise the shearing. Run each aliquot of the test pools for 1 cycle of 30s on /60s off, then remove one aliquot from each test pool, and continue for another round. Try several numbers of cycles. Run the test pools on a gel. Use the program where most of the material occurs within 230-700 bp and a peak around 400 bp.



Clean samples with the Qiagen MinElute Reaction Cleanup column. Elute all sub pools in EB buffer back together into one tube. Elute each tube with 13.5 μl. The end volume MUST be exactly 100 μl per pool in the next step. A small volume is however lost from the volume added, and some extra material should be stored for the Qubit and the BioAnalyzer. Add extra EB buffer if necessary.

**6. Size Selection with SPRI**

SPRI cleaning is done to remove unwanted fragments. We clean with SPRI 0.65x on the left and 0.55x on the right. This method relies heavily on proper concentration ratios between sample and SPRI. Therefore, the SPRI beads must be well mixed throughout the whole procedure.

First the SPRI beads must be resuspended by vortexing rigorously and making sure that no pellet is stuck in the bottom. Then the SPRI beads should be resuspended each time they are being added to a solution to make sure the concentration is even.

The volume of the sample should also be controlled with the pipette, and excess solution should be removed, or extra ddH2O added depending on the volume.

If in doubt of this procedure, it is useful to save the supernatant for each step in a separate tube. That way, your sample won’t be discarded by mistake.

**6a. Left side size selection** (to remove short fragments including excess adapters)

Vortex vessel with SPRI select to resuspend SPRI beads.

Add 0.65x ratio of beads to sample in 1.5 ml Eppendorf tubes (add 65 μl SPRI beads to a sample volume of 100 μl). Mix thoroughly by pipetting 10-15 times. Incubate for 1 min at room temperature.

Place tubes in the MagJet magnetic stand. Allow the beads to settle in a compact pellet. (The beads have now bound the desired fragments). Supernatant should now be clear. Remove the supernatant.

Wash pellet by carefully adding 180 μl 85% EtOH to each tube while tubes are still in the stand. Wait 30 sec. Remove and discard EtOH carefully. Remove last droplets of EtOH with a fine pipette and let the rest evaporate with the tubes open for a few minutes.

Remove tubes from stand and elute each pellet in 60 μl ddH2O. Pipette up and down 10-15 times to mix thoroughly. Incubate for 1 min at room temperature.

Place tubes in the MagJet stand and let the pellet aggregate into a distinct pellet.

Transfer each eluate to a new Eppendorf tube. The eluate now contains the wanted DNA.

**6b. Right side size selection** (to remove large fragments)

Vortex vessel with SPRI select to resuspend SPRI beads.

Add 0.55x ratio of beads to sample in 1.5 ml Eppendorf tubes (add 33 μl SPRI beads to a sample volume of 60 μl). Mix thoroughly by pipetting 10-15 times. Incubate 1 min at room temperature.

Place tubes in the MagJet magnetic stand. Allow beads to settle in a compact pellet. (The undesired large fragments are now bound to the beads.) The supernatant should be clear. Transfer the supernatant to a new Eppendorf tube.

During transfer of supernatant some of the volume will be lost. It therefore must be adjusted in the following step. Measure the volume for each pool with the pipette, then add a second round of SPRI select to the supernatant according to the following formula:

Sample volume μl × (1.8× – the initial ratio) × (volume of supernatant/93 μl) = volume of SPRI

Example: 60 μl × (1.8 – 0.55) × (volume of supernatant/93 μl) = X μl SPRI

Mix thoroughly by pipetting 10-15 times. Incubate 1 min at room temperature.

Place tubes in the MagJet magnetic stand. Allow beads to settle in a compact pellet. (The desired fragments are now bound to the beads.) The supernatant should be clear. Remove the supernatant.

Wash pellet by carefully adding 180 μl 85% EtOH to each tube while tubes are still in the stand. Wait 30 sec. Remove and discard EtOH carefully. Remove last droplets of EtOH with a fine pipette and let the rest evaporate with the tubes open for a few minutes.

Remove tubes from stand and elute each sample in 21 μl EB buffer. Pipette up and down 10-15 times to mix thoroughly, or vortex. Incubate for 1 min at room temperature.

Place tubes in the MagJet magnetic stand. Allow beads to settle in a compact pellet. Transfer the eluate to a clean Eppendorf tube. The eluate now contains the wanted DNA. Store some extra material for the Qubit and the BioAnalyzer, but make sure there is enough for the next step.

**7. Perform End Repair**

Use the Quick Blunting Kit to polish the ends of the DNA. Keep the enzyme on ice when not in the freezer. Pipette the enzyme carefully as it is very viscous. Add reagents in the following order:

DNA Eluate 19 μl

Blunting Buffer 10X 2.5 μl

dNTP Mix (1 mM) 2.5 μl

Blunt Enzyme Mix 1 μl

25 μl TOTAL

Mix by pipetting carefully up and down or by flicking the tube. Finish with a quick spin down.

Incubate at room temperature for 30 minutes. Continue with purification to stop reaction.

Purify with the Qiagen MinElute Reaction Cleanup Kit. Elute in 43 μl EB buffer by eluting into the same tube 3 times. First 10 μl, then 10 μl, then 23 μl.

**8. Add 3’-dA Overhang**

Add a 3’-dA overhang to the blunted ends. Keep the enzyme on ice when not in the freezer. Pipette the enzyme carefully as it is very viscous. Add reagents in the following order:

DNA Eluate 41 μl

NEBuffer 2 10X 5 μl

dATP 10 mM 1 μl

Klenow exo- (5000 units/ml) 3 μl

50 μl TOTAL

Mix by pipetting carefully up and down or by flicking the tube. Finish with a quick spin down.

Incubate at 37 °C for 30 min. Allow samples to cool slowly in room temperature for 15 minutes. Proceed instantly with purification.

Purify with Qiagen MinElute Reaction Cleanup Kit. Elute in 46 μl EB buffer by eluting into the same tube 3 times. First 10 μl, then 10 μl, then 26 μl.

**9. Ligation of P2 adapters**

First quantify all pools with the Qubit, using the 1X dsDNA HS Assay Kit and dilute all pools within the library to equal concentrations.

For each pool that share the same P1 adapters, make sure they get different P2 adapters in this stage. The salt in the NEBuffer 2 is important for the adapters in this stage as well. Keep the enzyme on ice when not in the freezer. Pipette the enzyme carefully as it is very viscous. Add reagents in the following order:

DNA Eluate 42.5 μl

NEBuffer 2 10X 5 μl

P2 Adapter (10 μM) 1 μl

rATP (100 mM) 1 μl

T4 Ligase (2 000 000 units/ml) 0.5 μl

50 μl TOTAL

Mix by pipetting carefully up and down or by flicking the tube. Finish with a quick spin down.

Incubate at room temperature for 60 min. Heat inactivate at 65 °C for 10 minutes to make absolutely sure that the reaction has stopped to avoid cross ligation of P2 adapters between pools. Allow samples to cool slowly in room temperature for 15 minutes. Proceed with purification.

Purify with the Qiagen MinElute Reaction Cleanup Kit. Elute DNA from all columns into one final Eppendorf tubes. Elute in 14 μl EB buffer. The end volume MUST be exactly 50 μl per tube in the next step. Add extra EB buffer if necessary.

**EXTRA:** The P2-adapters currently in use seem to vary in ligation efficiency. Based on previous library outcomes, it is possible to try to balance the pools accordingly to reduce variation in reads between pools. The following balance has been shown to work well when all P1-adapters are present in equal amounts in both pools:

**10. Left Side Size Selection with SPRI**

Clean with SPRI on the left side 0.6x to remove short fragments including excess adapters

Vortex vessel with SPRI select to resuspend SPRI beads.

Add 0.6x ratio of beads to samplein 1.5 ml Eppendorf tubes (add 30 μl SPRI beads to a sample volume of 50 μl). Mix thoroughly by pipetting 10-15 times. Incubate 1 min at room temperature.

Place tube in the MagJet magnetic stand. Allow beads to settle in a compact pellet. (The beads have now bound the desired fragments). Supernatant should now be clear. Remove the supernatant.

Wash pellet by carefully adding 180 μl 85% EtOH to the tube while the tube is still in the stand. Wait 30 sec. Remove and discard the EtOH carefully. Remove last droplets of EtOH with a fine pipette and let the rest evaporate with the tube open for a few minutes.

Remove the tube from stand and elute the pellet in 53 μl EB buffer. Pipette 10-15 times to mix thoroughly. Incubate for 1 min at room temperature.

Place the tube in the MagJet stand and let the pellet aggregate into a distinct pellet.  
Transfer the eluate to a new Eppendorf tube. The eluate now contains the wanted DNA. Store some extra material for the Qubit and the BioAnalyzer.

**11. PCR**

Quantify the library with the Qubit, using the 1X dsDNA HS Assay Kit. There should be 12.5-125 ng DNA in a 25 μl reaction (the more the better). Start by performing a test amplification. In a PCR tube, combine the following:

ddH2O to 25 μl final volume

Eluted DNA (RAD library template) X ng

Phusion PCR Master Mix (2x) 12.5

P1-PCR primer (10 μM) 1 μl

P2-PCR primer (10 μM) 1 μl

25 μl TOTAL

Also make a negative control with only EB buffer instead of template DNA. Run the following program in the thermal cycler:

98 ºC 30 seconds

then 18 cycles:

98 ºC 10 seconds

65 ºC 30 seconds

72 ºC 30 seconds

then

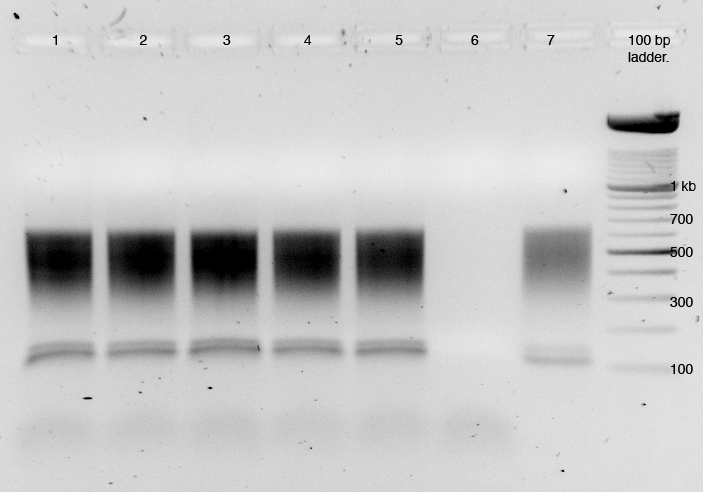
72 ºC 5 min

4 ºC hold

Run the sample, the negative control and some non-amplified template on an 1 % agarose gel. Use 5 μl of template and 1 μl from the library template and mix with 1 μl StopMix 1:1. Run for 45-60 min at 80 V.

If the amplification has worked, the product should be brighter than the non-amplified template. If it is at less than twice as bright, redo the test amplification with more DNA template. If it is at least twice as bright, continue with the real amplification. If amplification has worked well, it is also possible to lower the number of cycles to avoid to many PCR clones.

Divide your library template into several 50 μl reactions to generate enough template for sequencing. Dividing the amplification into larger reactions, with as much template as possible (but not more than described in the mastermix manual, 250 ng for 50 μl) will avoid some fragments taking over the amplification and skewing the library. Set up a negative control without any template. Run all samples on a new gel to make sure amplification has worked.



Lanes 1-5 are RAD PCR products (25 μl each, for 25 cycles). Lane 6 is the negative control. Lane 7 is a 25 μl PCR cleaned up with a Qiagen spin column (primer dimer remains)

**12. Final clean-up**

Purify with the Qiagen MinElute PCR Purification Kit. Elute DNA from all columns into Eppendorf tubes. Elute in ddH2O. The end volume MUST be exactly 50 μl per pool in the next step after saving 4 μl in total for the Qubit and the BioAnalyzer. Add extra EB buffer if necessary.

Clean with SPRI on the left only 0.6x as described in step 10 but elute in 50 μl EB buffer.

**Alternative:** It is also possible to run the full library on a gel, then cut it out with a sharp razor and purify it with the MinElute Gel extraction kit. Run 20 μl of library with 4 ul of 6X Gel Orange loading dye on a 1 % agarose gel for 60 min at 80 V. Use a suitable 1 kb ladder on each side of the library. The library should be cut above the 300 bp range to avoid sequencing into the adapter region of very short reads. The Aquatic group have the equipment required for this method.

**13. BioAnalyzer control**

Control the fragment distribution in the BioAnalyzer from the pre-PCR template, and both before and after clean-up of the post-PCR product. Quantify with the Qubit first, using 1 μl, and save 2-3 μl for the BioAnalyzer. The PCR by-products should have been mostly removed after the final clean-up. This is also a good opportunity to check material from other steps in the protocol, such as after the shearing and the first SPRI select.

**14. Sequence samples on the MiSeq**

**EXTRA:** Before sending the library for sequencing, it is possible to sequence it with a smaller platform at the department for a lower price. Depending on the outcome of the demultiplexing from the software *Stacks* of this smaller data set, it should be possible to interpret if the protocol has worked and went as expected. Control if any samples did not work properly and estimate the variation in read pairs between samples. Control why reads are being dropped during demultiplexing. The coverage will however probably be too low to estimate PCR-duplicates.

**15. Final sequencing**

When ordering sequencing, remember that the RAD-library is a low complexity library. The reason for this is the fact that the restriction cut site is expected at the same position on each of the forward reads. This means, that the machine will have trouble reading the low complexity region, as the signal will be too strong at each of the base positions. Therefore, the library needs to be diluted with at least 5 % PhiX for NovaSeq 6000 to add complexity to the library.

Some thought is also required when ordering depending on the barcodes used. Inline barcodes will be part of the sequence, but index barcodes require an extra step during the sequencing. Therefore, it is important to make sure that the sequencing facility is aware of on which of the adapters the index barcode is located (if not both) and the length in bases.