File S1

Methods for RAD-tags, paired-end contigs, and cDNA for RNA-seq

A. RAD-tags

1. Materials

1.1. DNA extraction and RNase A treatment

1. DNeasy Blood & Tissue Kit (Qiagen) (or similar)
2. RNaseA (Qiagen).

1.2. Restriction endonuclease digestion

1. Restriction enzyme (*Sbf*1-HF , New England Biolabs) : 20 U/µl
2. Clean, intact high-quality genomic DNA: >20 ng/µl.

1.3. P1 Adapter ligation

1. New England Biolabs (NEB) Buffer 2.
2. ATP (Epicentre): 100 m*M*.
3. P1 Adapter: 100 n*M.* A modified Solexa© adapter (2006 Illumina, Inc., all rights reserved). Prepare 100nM stocks of P1 in 1X NEB buffer #2 (or a buffer with a 50 mM final concentration of NaCl). For adapter annealing, prepare a 10 µ*M* stock of P1 adaptors, denature for 1 minute at 98C in PCR machine and decrease the temperature 1C per minute until 25C. Dilute to 100nM in 1X NEB #2 buffer and store in the freezer. Below, example barcoded *Sbf1* P1 adapter sequences. Asterisk denotes a phosphorothioate bond introduced to confer nuclease resistance to the double-stranded oligo, /5Phos/ denotes a phosphate group and "x" refers to barcode nucleotides.

Sbf1-P1 top oligo:

5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxxTGC*A-3'

Sbf1-P1 bottom oligo:

5'-/5Phos/xxxxxAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT-3'

4. T4 DNA Ligase (Epicentre): 10 U/µl.

1.4. Purification steps

1. DNA Clean & Concentrator-5 kit (ZYMO Research).

1.5. DNA shearing

1. Bioruptor, nebulizer or sonicator.

1.6. Size selection/agarose gel extraction

1. Agarose (Sigma or other manufacturer)
2. 0.5X TBE buffer
3. Loading Dye Solution (Fermentas or other manufacturer).
4. GeneRuler 100 bp DNA Ladder Plus (Fermentas or other manufacturer).
5. Razor blades.
6. MinElute Gel Purification Kit (Qiagen).

1.7. Perform end repair

1. End-It DNA End-Repair Kit (Epicentre).

1.8. 3´-dA overhang addition

1. NEB Buffer 2.
2. dATP (Fermentas): 10 m*M*
3. Exo-Minus Klenow DNA polymerase (Epicentre): 10 U/µl.

1.9. P2 Adapter ligation

1. New England Biolabs Buffer 2.
2. ATP: 100 m*M.*
3. P2 Adapter: 10 µ*M.* A modified Solexa© adapter (2006 Illumina, Inc., all rights reserved). Prepare 10 µ*M* stocks of PE (Paired End) adapter P2 in 1X NEB buffer #2 (or buffer with a 50 mM final concentration of NaCl). For adapter annealing, denature for 1 minute at 98C in PCR machine and decrease the temperature 1C per minute until 25C. Store in the freezer. Asterisk denotes a phosphorothioate bond and /5Phos/ denotes a phosphate group.

PE-P2- top oligo

5'-/5Phos/GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCAGAACAA-3'

PE-P2- bottom oligo

5'- CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T -3'

4. T4 DNA Ligase (Epicentre) 10 U/µl.

1.10. RAD tag Amplification/Enrichment

1. Phusion High-Fidelity DNA polymerase with HF Buffer (New England Biolabs).
2. dNTP 100 mM (Fermentas).
3. Modified Solexa© Amplification primer mix (2006 Illumina, Inc., all rights reserved): 10 µ*M.*

P5-forward primer: 5´- AATGATACGGCGACCACCGA -3´

P7-reverse primer: 3´- CAAGCAGAAGACGGCATACGA -3´

2. Methods

The protocol described below describes methods to prepare RAD-tag libraries for high-throughput Illumina sequencing (see also (Baird *et al.* 2008; Hohenlohe *et al.* 2010; Miller *et al.* 2007a; Miller *et al.* 2007b). The P1 adapter contains forward amplification and Illumina sequencing primer sites, as well as a barcode for sample identification. The second adapter (PE-P2), can be used for Paired-End sequencing. For genetic mapping, each individual sample has a unique barcode.

2.1. DNA extraction and RNase A treatment

1. Extract genomic DNA using the DNeasy Blood & Tissue Kit (Qiagen) or a similar product that produces very pure, high molecular weight, RNA-free DNA. High-quality DNA is extremely important. Quantify the DNA using a Qubit (Invitrogen) fluorometer to get the most accurate concentration readings.

2.2. Restriction endonuclease digestion

1. Digest 500ng-1µg genomic DNA for each individual sample with *Sbf*1-HF restriction enzyme in a 50 µl reaction volume, following the manufacturers instructions. In a microcentrifuge tube combine:

   5.0 µl 10X NEB Buffer 4
   0.5 µl *Sbf*1-HF (20 U/µl)
   500 ng-1µg genomic DNA
   H_2O to 50.0 µl.
   Incubate at 37C for 1h

2. Heat-inactivate the restriction enzyme for 30 minutes at 65C. Allow reaction to cool slowly to ambient temperature (30-60 min). If the enzyme cannot be heat-inactivated, purify with a ZYMO column following manufacturer's instructions prior to ligation.

2.3. P1 Adapter ligation

1. This step in the protocol ligates barcoded, Sbf1 restriction-site overhang P1 adapters onto Sbf1 compatible ends of the genomic DNA digested in the previous step
2. To each inactivated digest, add:

   6.0 µl 10X NEB Buffer 2
   3.0 µl Barcoded P1 Adapter (100 n*M*)
   0.6 µl ATP (100 m*M*)
   0.5 µl T4 DNA Ligase (10 U/µl)

   60.0 µl total volume.

   Add P1 adapters to the reaction before the ligase to avoid re-ligation of the genomic DNA. Incubate reaction at room temperature for 60 min or overnight in the refrigerator.

5. Heat-inactivate T4 DNA Ligase for 30 min at 65° C. Allow reaction to cool slowly to ambient temperature (30 min).

2.4. Sample multiplexing

2. Combine barcoded samples at desired ratio (about 16-24 samples per pool). Use a 100-300 µl aliquot containing 1-3 µg DNA total and freeze the rest at -20° C.

For genetic linkage maps, the best way to determine how many samples can be combined is to perform a pilot experiment. The size and polymorphism level of the genome under investigation will determine how deeply the samples need to be sequenced. Multiplex parents with several progeny and sequence them as a single sample. Use *Stacks* (CATCHEN *et al.* 2011) (http://creskolab.uoregon.edu/stacks/) to determine the total number of tags per individual plus the total number of polymorphic markers at the read length sequenced and use these data to determine the optimal read length and optimal number of progeny to pool. In general, aim for at least 25x coverage in the post-processed *SbfI* loci. For example, 50,000 loci would require 1 million retained reads per individual. If the organism being investigated has a low level of polymorphism, longer reads will provide more SNPs and hence more mappable markers. Higher coverage is better and results in fewer wrong genotypes, which occur mostly in under sequenced heterozygotes, but 35x coverage is generally sufficient. Run a single lane of progeny at the selected pooling complexity to determine if the number of reads is close to the expected number. A low read count in a lane may result in many missing genotypes in addition to many wrong genotypes.

2.5. DNA shearing

1. Shear DNA samples to an average size of 200-500 bp. The 300-600bp fraction seems to work just as well, so use a bigger or smaller size fraction if needed.
2. Dilute ligation reaction to 100 µl in water and shear in Bioruptor 10 times for 30 sec on high following manufacturer's instructions (may need some optimization in different organisms). For paired-end sequencing and building mini-contigs from the paired ends, make three replicas from the sample and shear one for 4 cycles, another for 7 cycles and another for 10 cycles and pool them together after shearing.
3. Clean up sheared DNA sample(s) using a Zymo-5 column following manufacturer's instructions. Elute in 20 µl EB (Elution Buffer).

2.6. Size selection/agarose gel extraction

1. Run the entire sheared sample on a 1.25% agarose, 0.5X TBE gel for 45-60 min at 100 V, next to a 100 bp DNA Ladder for size reference.
2. Cut a slice of the gel spanning 200-500 bp (for paired ends separately cut the 200-400 fraction, 400-600 and 600-900 fractions and proceed with each separately). Extract DNA using MinElute Gel Purification Kit (QIAGEN) by melting the agarose gel slices in the supplied buffer at room temperature (18-22° C) with agitation for 30 min. Elute twice with 18 µl EB (Elution Buffer) each time into the same eppendorf tube.

2.7. Perform end repair

To the eluate from the previous step, add:

5 µl 10x buffer

5 µl dNTP mix (1m*M*)

5 µl ATP

1.0 µl End-It Enzyme Mix.

Incubate at room temperature (RT) for 45 min.

3. Purify with ZYMO-5 column. Elute twice with 22 µl EB (Elution Buffer) into eppendorf tube.

2.8. 3´-dA overhang addition

To the eluate from the previous step, add:

5.0 µl 10X NEB Buffer 2

1.0 µl dATP (10m*M*)

1.5 µl Klenow (exo^{-}) (10U/µl, Epicentre).

Incubate at 37ºC for 30 min. Allow reaction to cool slowly to ambient temperature (15 min).

3. Purify with ZYMO-5 column. Elute twice with 22 µl EB (Elution Buffer) into eppendorf tube.

2.9. PE-P2 Adapter ligation

1. This step in the protocol ligates the PE-P2 adapter, a "Y" adapter with divergent ends that contains a 3´ dT overhang, onto the ends of blunt DNA fragments with 3´ dA overhangs from the previous step.
2. To the eluate from previous step, add:

   5.0 µl 10X NEB Buffer 2

   1.0 µl PE-P2 Adapter (10 µ*M*)

   1.0 µl ATP (100 m*M*)

   0.5 µl T4 DNA Ligase (10U/µl, Epicentre)

   Incubate reaction at room temperature for 60 min or overnight in the refrigerator

3. Purify with ZYMO-5 column. Elute twice with 26 µl EB (into same tube).

2.10. RAD tag Amplification/Enrichment

1. This step will perform high-fidelity PCR amplification on P1 and P2 adapter-ligated DNA fragments.
2. Quantify the DNA using a fluorometer (Qubit, Invitrogen) to get the most accurate concentration readings.

   Perform PCR amplification to determine library quality. In thin-walled PCR tube, combine:

   10 µl 5x HF buffer (NEB)

   1 µl dNTPs (10 mM)

   40-70 ng RAD library template (eluate from last step)

   2.0 µl Solexa primer mix (P5+P7 primers, 10 µ*M*)

   0.5 µl Phusion DNA polymerase (2 U/µl , NEB)

   H_2O to 50 µl

   Perform 12 cycles of amplification in thermal cycler:

   30 sec 98° C

   12x [10 sec 98° C, 30 sec 65° C, 30 sec 72° C],

   5 min 72° C

   hold 10° C.

Purify PCR reaction reaction with a ZYMO-5 column. Elute in 20 µl EB.

4. Load entire sample in 1X Orange Loading Dye on a 1.25% agarose, 0.5X TBE gel and run for 45 min at 100 V, next to 100 bp DNA size standard for size reference. Use a fresh razor blade to cut a slice of the gel spanning 250-550 bp (the amplified library will migrate at a slightly higher size range than the template). If using a smaller or larger size fraction, cut the amplified product corresponding to that size range. For paired ends cut the corresponding fraction according to the expected size range. Extract DNA using MinElute Gel Purification Kit following manufacturer's instructions. Melt agarose gel slices at room temperature in the supplied buffer. Elute in 20 µl EB.

5. Quantify the DNA using a Qubit (Invitrogen) fluorometer to get the most accurate concentration readings. Concentrations will range from 1-20 ng/µl. Determine the molar concentration of the library by examining the gel image and estimating the median size of the library smear. Use this number to calculate the molar concentration of the library.
6. For paired end sequencing, combine the libraries from the 200-400, 400-600 and 600-900 fractions at equal ratios.
7. Sequence libraries on Illumina Genome Analyzer following manufacturer's instructions.

B. Processing Illumina data *in silico* to recover *RAD-tag* loci and paired-end mini-contigs

The *Stacks* software package (Catchen *et al.* 2011) (http://creskolab.uoregon.edu/stacks/) can help to recover loci from map cross parents and progeny and to build paired-end mini-contigs associated with those loci. Briefly, *Stacks* identifies a locus in an individual by aligning sequenced Illumina reads adjacent to the restriction enzyme cut site. In the case of paired end reads, *Stacks* aligns the first end to form a locus and collates the paired end to create groups of reads associated with each locus. The collated sequences for each locus are fed into an assembler such as Velvet (Zerbino and Birney 2008) and the resulting assembled contigs are loaded into the Stacks database. *Stacks* can export the assembled paired-end mini-contigs associated with their upstream locus, and these sequences can be used to link loci to EST or genomic sequences from the same species or to identify orthologs in other species and perform conserved synteny analyses.

C. RNAseq Methods

1. Materials

1.1. Total RNA and mRNA isolation

1. RiboPure Kit (Ambion).
2. MicroPoly(A) Purist (Ambion).

1.2. 1st strand synthesis

1. Random Primers (hexamers) (3 µg/µl, Invitrogen).
2. High-quality mRNA: >10 ng/µl. (100 ng -1µg)
3. dNTP (Fermentas)
4. 5x First Strand Synthesis buffer (Invitrogen).
5. RNase inhibitor (Invitrogen or New England Biolabs).
6. Superscript III reverse transcriptase (Invitrogen).
7. RNase H (5 U/µl, New England Biolabs).

1.2. Second strand synthesis

1. Random Primers (hexamers) (3 µg/µl, Invitrogen).
2. New England Biolabs buffer #2 (as alternative use Klenow exo- buffer (Epicentre)
3. dNTP (Fermentas
4. Klenow exo- (10 U/µl, Epicentre) (alternatively use DNA polymerase I)

1.3. PE Adapter ligation

1. NEB Buffer 2.
2. ATP (Epicentre): 100 m*M*.
3. T-overhang PE (Paired End) Adapter: 10 µ*M.* A modified Solexa© adapter (2006 Illumina, Inc., all rights reserved). Prepare 10 µ*M* stocks of PE adapters in 1X NEB buffer #2 (or buffer with a 50 mM final concentration of NaCl). For adapter annealing, denature for 1 minute at 98C in PCR machine and decrease the temperature 1C per minute until 25C. Store in the freezer.

Below, example PE adapter sequences with no barcode (PEnoBC). Asterisk denotes a phosphorothioate bond introduced to confer nuclease resistance to the double-stranded oligo, "/5Phos/" denotes a phosphate group.

PEnoBC-top:

5´- ACACTCTTTCCCTACACGACGCTCTTCCGATC*T -3´

PEnoBC- bottom:

5´- /5Phos/GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG -3´

4. T4 DNA Ligase (Epicentre): 10 U/µl.

1.4. Purification steps

1. DNA Clean & Concentrator-5 kit (ZYMO Research).

1.5. DNA shearing

1. Bioruptor, nebulizer or Branson sonicator 450.

1.6. Size selection/agarose gel extraction

1. Agarose (Sigma or other manufacturer)
2. 1X TBE
3. 6X Orange Loading Dye Solution (Fermentas).
4. GeneRuler 100 bp DNA Ladder Plus (Fermentas).
5. Razor blades.
6. MinElute Gel Purification Kit (Qiagen).

1.7. End repair

1. End-It DNA End-Repair Kit (Epicentre).

1.8. 3´-dA overhang addition

1. NEB Buffer 2.
2. dATP (Fermentas): 10 m*M*
3. Klenow Fragment (3´ to 5´ exo^{-}, Epicentre): 10 U/µl.

1.9. Library Amplification

1. Phusion High-Fidelity DNA polymerase with HF Buffer (NEB).
2. Modified Solexa© Amplification primer mix (2006 Illumina, Inc., all rights reserved): 10 µ*M.*

PEprimer1

5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

PEprimer2

5'- CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-3'

2. RNA seq Methods

First strand synthesis

Combine:

1 µl Random Primers (3 µg/µl, Invitrogen)

1 µl 10 mM dNTPs (Fermentas)

11 µl purified mRNA or up to 1 µg (Ambion MicroPolyA-Purist)

Heat to 65˚C for 5 min., then ice

Collect contents at bottom of tube by brief centrifugation.

Add:

4 µl 5x First Strand Synthesis buffer (Invitrogen)

1 µl 0.1 mM DTT (Invitrogen)

1 µl RNase inhibitor (Invitrogen or New England Biolabs)

1 µl Superscript III reverse transcriptase (Invitrogen)

Mix by gentle aspiration

25˚C for 5 min.

Synthesis: Incubate at 50˚C for 1 hr.

Inactivation: 70˚C for 15 min.

Removal of RNA template: add 1 µl RNase H (5 U/µl, New England Biolabs)

Heat to 37˚C for 20 min.

Purify with Zymo-5 column – following manufacturer directions, using **7 volumes** (150 µl) of binding buffer (because it's single-stranded DNA), elute two times with 22 µl EB (Elution Buffer) (into same tube)

NOTE: Zymo-5 columns bind a maximum of 5 µg. If you expect larger yields, use more than one column and scale up second strand synthesis as necessary.

Second strand synthesis:

To previous eluate, add:

1.0 µl Random Primers (3 µg/µl, Invitrogen)

Heat to 95˚C for 2 min, cool down in ice for a couple of minutes and spin down for 20 seconds.

Add:

5.0 µl NEB buffer #2 (as alternative use Klenow exo- buffer (Epicentre))

1.0 µl 10 mM dNTPs

1.5 µl Klenow exo- (10 U/µl, Epicentre) (alternatively use DNA polymerase I)

Synthesis: 37˚C for 45 minutes.

Purify with Zymo-5 DNA column (see NOTE above) and:

Elute two times with 50 µl EB each time onto the same tube and proceed to shearing and adaptor ligation for EST building (or expression profiling) via Illumina sequencing.

Shearing:

Up to 2 µg DNA in 100 microliters in EB or TE (in 0.6 ml tubes, Axygen) – fill all other positions in Bioruptor holder with tubes containing 100 µl water. Before beginning, make sure Bioruptor tank water is 4˚C – bail out and replace with cold water if necessary and add a little crushed ice.

Set the controls to shear 30 sec. on, 30 sec. off for 15 cycles

Replace tank water with cold water and a little ice

Repeat shearing, 15 cycles.

(shearing can also be performed with a regular sonicator)

Zymo-5 column concentrate – elute 2 times with 17 µl EB.

End repair:

To eluate (approx. 34 µl), add Epicentre's End-It DNA Repair Kit reagents:

5 µl 10 X buffer

5 µl 1 mM dNTP mix

5 µl ATP

1 µl enzyme mix

Incubate 45 min at room temperature

Clean with Zymo-5 column, elute 2 times with 22 µl EB.

Addition of A overhangs:

To eluate (approx. 44 µl) add:

5 µl 10x Klenow exo- buffer (Epicentre)

1 µl dATP 10 mM

1.5 µl 10 U/µl Klenow Exo- (Epicentre)

Incubate 30 min at 37 C

Clean with Zymo-5 column, elute 2 times with 21.5 µl EB

Adapter Ligation:

Add to eluate from previous step (approx. 42 µl) add:

5 µl 10x NEB buffer 2

1 µl 25 mM ATP (Epicentre)

1 µl 10 µM T-overhang PE adapter (Illumina adapter)

0.5 µl T4 DNA ligase (10 U/µl, Epicentre)

Ligate 1 hour at room temperature or up to overnight in the refrigerator (prefered).

Alternative A. – Zymo column purify, elute 2 times with 6 µl elution buffer each time and proceed to amplification before size fractionation. Only do this if there is a small amount of material. It is generally better to size-fractionate before amplification.

Alternative B. – Zymo column purify, elute 2 times with 10 µl elution buffer and size fractionate (see below)

Size fractionation:

Run de DNA in a 2.5 % agarose gel with 100bp size standard.

Cut out and retain the 200 – 500bp fraction, carefully avoiding any unincorporated adapter. If desired, also cut the 500-700 bp fraction. These two fractions should constitute most of the sheared cDNA. The 200-500 bp size range may be less biased against short transcripts. Recover DNA with Qiagen gel extraction kit and MinElute columns. Dissolve gel at room temperature. Follow

manufacturer instructions, being sure to let wash buffer stand on column for at least 5 minutes before spinning through. Elute 2 times with 15 µl EB.

Library amplification

Quantify the DNA concentration using Qubit (Invitrogen) or some other high resolution fluorometer. Use 25-100 ng of template in a 12x cycle amplification using the Illumina PCR primers.

To DNA template add:

10 µl 5x PCR buffer HF (New England Biolabs)

1 µl 10 µM dNTP

1 µl 10 µM PCR primer mix (PE primer 1+2)

0.5 µl Phusion DNA polymerase (2 U/µl, NEB)

water to 50 µl

PCR conditions:

step1- 98C for 30 secs

step2- 98C for 10 secs

step3- 65C for 30 secs

step4- 72C for 30sec

step5- go to step 2 x12 times

step6- 72C for 5 minutes

step7- 10C hold

Clean with Zymo-5 column, elute 2 times with 12 µl EB

Size fractionation:

Run the PCR product in a 2 % agarose gel with 100bp size standards.

Cut the 300 – 600bp amplified fraction, carefully avoiding any unincorporated PCR primer. The PCR product will run at approximately 100bp higher size than the DNA template used in the PCR.

Gel purify using MinElute columns (QIAGEN) and elute in EB. The volume of EB will depend on the intensity of the DNA band. In general 20 µl will be sufficient. Add 2 µl of 1% Tween-20 before storing in the freezer.

Quantify the DNA concentration using Qubit (Invitrogen) or some other high resolution fluorometer and dilute a DNA sample to the recommended concentration for Illumina sequencing (5 or 10 nmolar).

PE Adapter

T-overhang PE Adapter: 10 µ*M.* A modified Solexa© adapter (2006 Illumina, Inc., all rights reserved). Prepare 10 µ*M* stocks of PE adapters in 1X NEB buffer #2 (or buffer with a 50mM final concentration of NaCl). Anneal adapters in PCR machine by going from 96C to 25C by decreasing the temperature 1C per minute.

Below, example PE adapter sequences with no barcode (PEnoBC). Asterisk denotes a phosphorothioate bond introduced to confer nuclease resistance to the double-stranded oligo, "/5Phos/" denotes a phosphate group.

PEnoBC-top:

5´- ACACTCTTTCCCTACACGACGCTCTTCCGATC*T -3´

PEnoBC- bottom:

5´- /5Phos/GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG -3´

Library Amplification

Modified Solexa© Amplification primer mix (2006 Illumina, Inc., all rights reserved): 10 µ*M.*

PEprimer1

5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

PEprimer2

5'-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-3'

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