Sep 03, 2018

Working

hyRAD for birds

George Olah[1], Niccy Aitken[1], Tomasz Suchan[2], EcoGenomics Lab ANU[1]

[1]Australian National University, [2]Polish Academy of Sciences

dx.doi.org/10.17504/protocols.io.mt2c6qe

EBL_ANU

George Olah
Australian National University

ABSTRACT

This protocol builds on Suchan *et al.* (2016). It has been modified to work on fresh and museum samples of birds, specifically tested on Regent Honeyeater (*Anthochaera phrygia*).
The detailed protocol contains three main sections: (A) preparing ddRAD libraries as probes, (B) preparing whole genome libraries for samples of different quality, and (C) using probes to hybridise and isolate the same fragments from all samples.
Start with up to 600 ng whole genomic DNA per sample (our minimum was 14 ng), resuspended in 8 µl. Always include a negative control (containing no DNA, just buffer) in parallel to samples.

PROTOCOL STATUS

Working

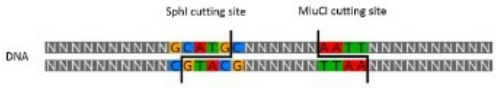
We use this protocol in our group and it is working

GUIDELINES

All purification was done using Solid Phase Reversible Immobilization (SPRI) beads with different bead : sample ratios at each step (specified in the protocol). AMPure beads are commonly used. However, we used Seramag throughout the protocol.
All quantitation was done by fluorescence using Qubit (Invitrogen). Fragment Analyzer, Tapestation, or LabChip GXII can also be used.
All visualization steps for size profiles were done using LabChip GXII (Caliper). Agarose gel, Fragment Analyzer, Tapestation, or Bioanalyzer can also be used.

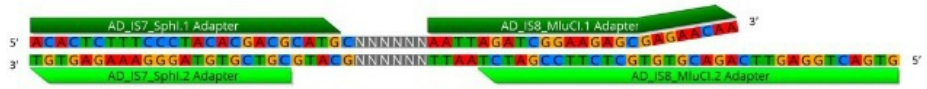
ddRAD libraries for probes

Total gDNA restriction digestion

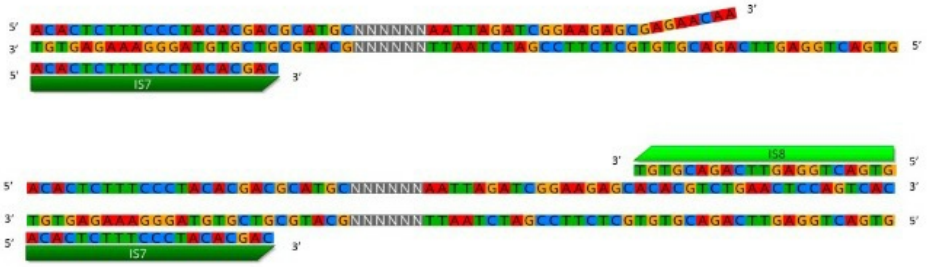


Adapter ligation

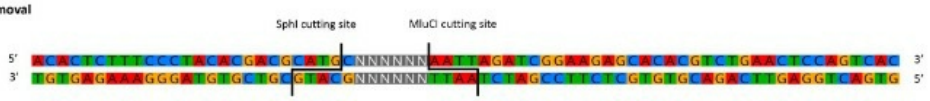
Size selection



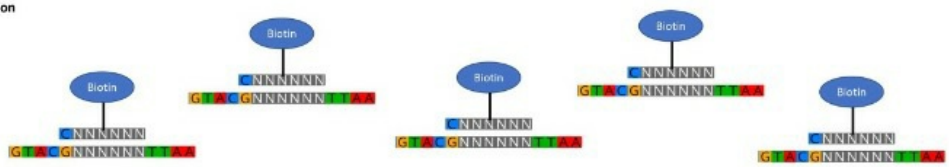
Amplification

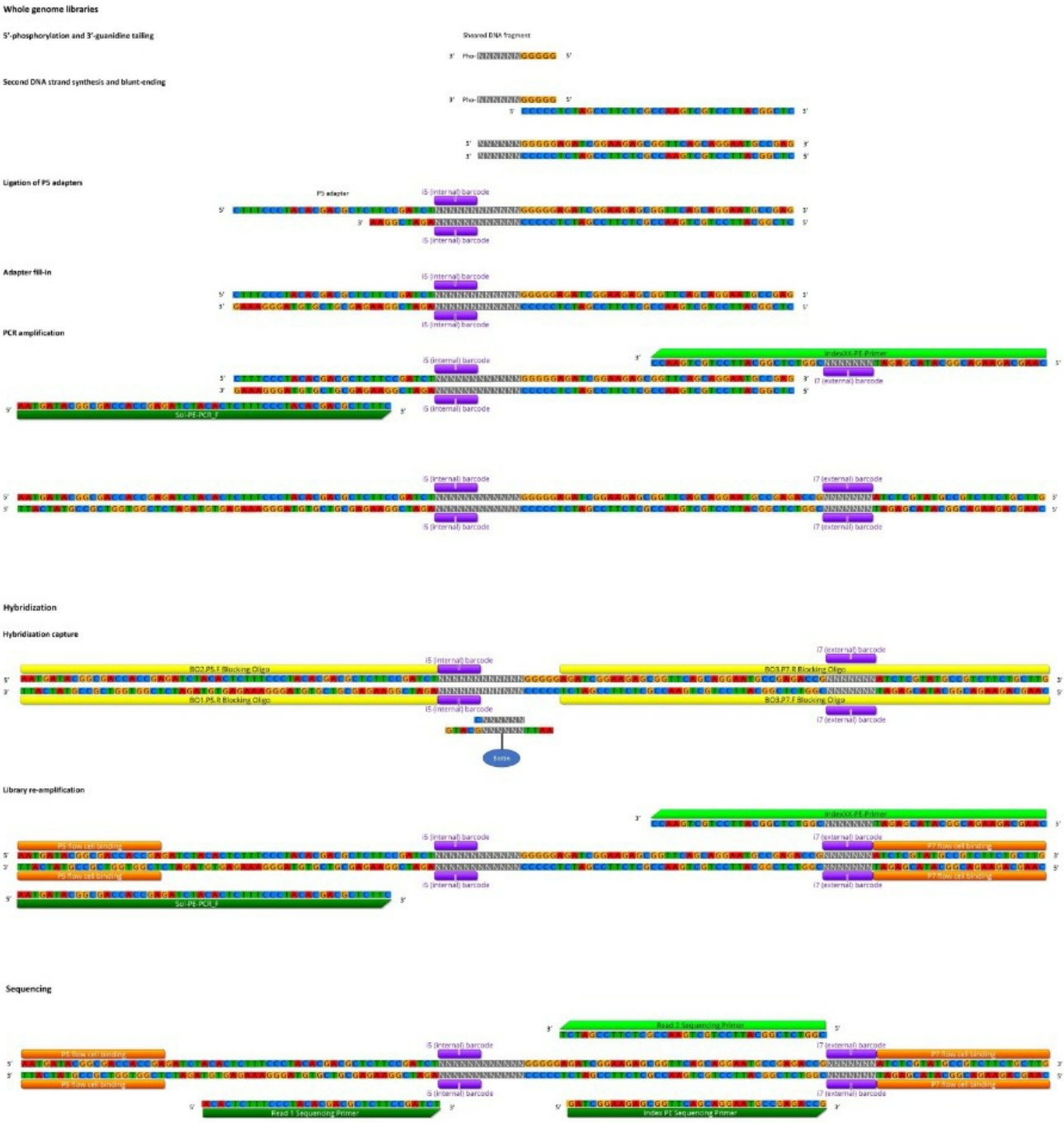


Adapter removal



Biotinylation





MATERIALS

NAME	CATALOG #	VENDOR
CutSmart Buffer - 5.0 ml	B7204S	New England Biolabs
ThermoPol II (Mg-free) Reaction Buffer Pack - 6.0 ml	B9005S	New England Biolabs
Bst DNA Polymerase Lg Frag - 1,600 units	M0275S	New England Biolabs
Terminal Transferase - 2,500 units	M0315L	New England Biolabs
NEBNext High-Fidelity 2X PCR Master Mix - 250 rxns	M0541L	New England Biolabs

NAME	CATALOG #	VENDOR
T4 Polynucleotide Kinase - 2,500 units	M0201L	New England Biolabs
T4 DNA Ligase - 100,000 units	M0202L	New England Biolabs
T4 DNA Polymerase - 150 units	M0203S	New England Biolabs
Klenow Fragment (3'-5' exo-) - 1,000 units	M0212L	New England Biolabs
MluCI - 1,000 units	R0538S	New England Biolabs
SphI-HF - 500 units	R3182S	New England Biolabs
EDTA		
Sterile water		
NEBuffer 4 - 5.0 ml	B7004S	New England Biolabs
BSA-Molecular Biology Grade - 12 mg	B9000S	New England Biolabs
Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles (Hydrophobic), 15 mL	65152105050250	Ge Healthcare
DecaLabel DNA Labeling Kit	K0651	Thermo Fisher Scientific
GTP Solution	R1461	Thermo Fisher Scientific
dNTP Mix (2.5 mM)	R72501	Thermo Fisher Scientific
SSC (20X)	AM9765	Thermo Fisher Scientific
Denhardts Solution (50X)	750018	Thermo Fisher Scientific
Cot1 - Chicken Hybloc DNA	CHB	
Dynabeads M-280 Streptavidin	11205D	Thermo Fisher Scientific

STEPS MATERIALS

NAME	CATALOG #	VENDOR
DecaLabel DNA Labeling Kit	K0651	Thermo Fisher Scientific

SAFETY WARNINGS

BEFORE STARTING

Prepare oligonucleotide working solutions and anneal adapters. Keep frozen until use.
All sequences are given in 5'-->3' direction.

AD_SphI adapter (IS7) - used in step #3

Name	Sequence
AD_IS7_SphI.1	ACACTCTTTCCCTACACGACGCAT*G
AD_IS7_SphI.2	Pho-CGTCGTGTAGGGAAAGAGTGT

AD_MluCI adapter (IS8) - used in step #3

Name	Sequence
AD_IS8_MluCI.1	Pho-AATTAGATCGGAAGAGCGAGAACAA
AD_IS8_MluCI.2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T

Pho = 5' Phosphorylation
* = 3' phosphorothioate bond

These oligos are HPLC purified.

To prepare 10 μM working solution, mix 10 μL of the RAD-P1.1 and RAD-P1.2 oligos (100 μM stock) with 80 μL of ultrapure water to obtain 100 μL of working solution. Anneal adapters as follows: heat to 95°C for 1 minute and slowly cool to 20°C at a ramp of 0.1°C/s.

ddRAD primers (10 μM) - used in step #6

Name	Sequence
IS7	ACACTCTTTCCCTACACGAC
IS8	GTGACTGGAGTTCAGACGTGT

PE-P7-CCCCC oligo (15 μM) - used in step #17
ctcggcattcctgctgaaccgctcttccgatctCCCCC

P5 indexed adapters - used in step #20

Name	Sequence
Index_P5-1	ctttccctacacgacgctcttccgatctGCAGCT
Index_P5-2	ctttccctacacgacgctcttccgatctTCCTCC
Index_P5-3	ctttccctacacgacgctcttccgatctGAACTA
Index_P5-4	ctttccctacacgacgctcttccgatctACAACC
Index_P5-5	ctttccctacacgacgctcttccgatctGGTAAC
Index_P5-6	ctttccctacacgacgctcttccgatctGTGGTC
Index_P5-7	ctttccctacacgacgctcttccgatctCCGCGT
Index_P5-8	ctttccctacacgacgctcttccgatctCTGACA
Index_P5-9	ctttccctacacgacgctcttccgatctCCGAAT
Index_P5-10	ctttccctacacgacgctcttccgatctAGCCGC
Index_P5-11	ctttccctacacgacgctcttccgatctTAGCGC
Index_P5-12	ctttccctacacgacgctcttccgatctTGACCT
Index_P5-13	ctttccctacacgacgctcttccgatctCTTATC
Index_P5-14	ctttccctacacgacgctcttccgatctGTAGCC
Index_P5-15	ctttccctacacgacgctcttccgatctCCATAG
Index_P5-16	ctttccctacacgacgctcttccgatctGAGGCA
Index_P5-17	ctttccctacacgacgctcttccgatctAATTGA
Index_P5-18	ctttccctacacgacgctcttccgatctACTCAC
Index_P5-19	ctttccctacacgacgctcttccgatctAAGTTG
Index_P5-20	ctttccctacacgacgctcttccgatctTACGAT
Index_P5-21	ctttccctacacgacgctcttccgatctCACCAC
Index_P5-22	ctttccctacacgacgctcttccgatctGCATTC
Index_P5-23	ctttccctacacgacgctcttccgatctTTCCAT
Index_P5-24	ctttccctacacgacgctcttccgatctAGGCGA
Index_P5-25	ctttccctacacgacgctcttccgatctCTCTCG
Index_P5-26	ctttccctacacgacgctcttccgatctGCCTTG
Index_P5-27	ctttccctacacgacgctcttccgatctAGGTTC
Index_P5-28	ctttccctacacgacgctcttccgatctTAGCAG
Index_P5-29	ctttccctacacgacgctcttccgatctCACTGC
Index_P5-30	ctttccctacacgacgctcttccgatctAGTGGC
Index_P5-31	ctttccctacacgacgctcttccgatctATGTAG
Index_P5-32	ctttccctacacgacgctcttccgatctGGTACG
Index_P5-33	ctttccctacacgacgctcttccgatctTAAGGT
Index_P5-34	ctttccctacacgacgctcttccgatctCCTGTA
Index_P5-35	ctttccctacacgacgctcttccgatctCCTCCA
Index_P5-36	ctttccctacacgacgctcttccgatctACAATA
Index_P5-37	ctttccctacacgacgctcttccgatctACTGAT
Index_P5-38	ctttccctacacgacgctcttccgatctATGTGT

Index_P5-39	ctttccctacacgacgctcttccgatctGTGTCC
Index_P5-40	ctttccctacacgacgctcttccgatctTAGAGT
Index_P5-41	ctttccctacacgacgctcttccgatctGCTAAG
Index_P5-42	ctttccctacacgacgctcttccgatctTGGACT
Index_P5-43	ctttccctacacgacgctcttccgatctCACTTA
Index_P5-44	ctttccctacacgacgctcttccgatctCTATTG
Index_P5-45	ctttccctacacgacgctcttccgatctAATACA
Index_P5-46	ctttccctacacgacgctcttccgatctATTCTT
Index_P5-47	ctttccctacacgacgctcttccgatctGGCATC
Index_P5-48	ctttccctacacgacgctcttccgatctGGTTGG
Index_P5-comp_1	AGCTGCagatcggaa
Index_P5-comp_2	GGAGGAagatcggaa
Index_P5-comp_3	TAGTTCagatcggaa
Index_P5-comp_4	GGTTGTagatcggaa
Index_P5-comp_5	GTTACCagatcggaa
Index_P5-comp_6	GACCACagatcggaa
Index_P5-comp_7	ACGCGGagatcggaa
Index_P5-comp_8	TGTCAGagatcggaa
Index_P5-comp_9	ATTCGGagatcggaa
Index_P5-comp_10	GCGGCTagatcggaa
Index_P5-comp_11	GCGCTAagatcggaa
Index_P5-comp_12	AGGTCAagatcggaa
Index_P5-comp_13	GATAAGagatcggaa
Index_P5-comp_14	GGCTACagatcggaa
Index_P5-comp_15	CTATGGagatcggaa
Index_P5-comp_16	TGCCTCagatcggaa
Index_P5-comp_17	TCAATTagatcggaa
Index_P5-comp_18	GTGAGTagatcggaa
Index_P5-comp_19	CAACTTagatcggaa
Index_P5-comp_20	ATCGTAagatcggaa
Index_P5-comp_21	GTGGTGagatcggaa
Index_P5-comp_22	GAATGCagatcggaa
Index_P5-comp_23	ATGGAAagatcggaa
Index_P5-comp_24	TCGCCTagatcggaa
Index_P5-comp_25	CGAGAGagatcggaa
Index_P5-comp_26	CAAGGCagatcggaa
Index_P5-comp_27	GAACCTagatcggaa
Index_P5-comp_28	CTGCTAagatcggaa
Index_P5-comp_29	GCAGTGagatcggaa
Index_P5-comp_30	GCCACTagatcggaa
Index_P5-comp_31	CTACATagatcggaa
Index_P5-comp_32	CGTACCagatcggaa
Index_P5-comp_33	ACCTTAagatcggaa
Index_P5-comp_34	TACAGGagatcggaa
Index_P5-comp_35	TGGAGGagatcggaa
Index_P5-comp_36	TATTGTagatcggaa
Index_P5-comp_37	ATCAGTagatcggaa
Index_P5-comp_38	ACACATagatcggaa
Index_P5-comp_39	GGACACagatcggaa
Index_P5-comp_40	ACTCTAagatcggaa
Index_P5-comp_41	CTTAGCagatcggaa
Index_P5-comp_42	AGTCCAagatcggaa
Index_P5-comp_43	TAAGTGagatcggaa

Index_P5-comp_44	CAATAGagatcggaa
Index_P5-comp_45	TGTATTagatcggaa
Index_P5-comp_46	AAGAATagatcggaa
Index_P5-comp_47	GATGCCagatcggaa
Index_P5-comp_48	CCAACCagatcggaa

Barcoded oligonucleotides are given in capital letters.
These adapters are barcoded in a similar way as RAD-P1 adapters. Prepare 25 μM working solution for as many PCR wells as P1 barcoded adapters are needed. Mix 25 μl of each oligo from the pair (100 μM stock) with 50 μL of ultrapure water to obtain 100 μL working solution.
Anneal adaptersas follows: heat to 95°C for 1 minute and bring to 20°C with a ramp of 0.1°C/s.
Keep them organized in PCR strips with individually capped tubes for convenient usage. Ensure no cross-contamination of barcoded adapters when pipetting.

Indexed primers - used in step #24

Name	Sequence
Sol-PE-PCR_F	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttc
Index01-PE-Primer	caagcagaagacggcatacgagatCCTGCGAcggtctcggcattcctgctgaacc
Index02-PE-Primer	caagcagaagacggcatacgagatTGCAGAGcggtctcggcattcctgctgaacc
Index03-PE-Primer	caagcagaagacggcatacgagatCGCATTAcggtctcggcattcctgctgaacc
Index04-PE-Primer	caagcagaagacggcatacgagatTTGATCCcggtctcggcattcctgctgaacc
Index05-PE-Primer	caagcagaagacggcatacgagatATCTTGCcggtctcggcattcctgctgaacc

Barcoded oligonucleotides are given in capital letters.
Each primer mix consists of the same forward (Sol-PE-PCR_F) and a plate indexed reverse primer (IndexXX-PE-Primer), each at 5 μM.
To prepare, mix 50 μl of the 100 μM stock primer Sol-PE-PCR_F and 50 μl primer Index01-PE-Primer (1-5) oligos with 900 μl of ultrapure water to obtain 1000 μl of a working solution.

Blocking oligos (100 μM) - used in step #26 and #29

Name	Sequence
BO1.P5.F	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT
BO2.P5.R	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
BO3.P7.F	AGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGIIIIIIIATCTCGTATGCCGTCTTCTGCTTG
BO4.P7.R	CAAGCAGAAGACGGCATACGAGATIIIIIIICGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

I = Inosine

TEN Buffer (100 ml, pH 7.5)
1 M NaCl (5.844 g NaCl)
10 mM Tris-Cl + 1 mM EDTA (10 ml 10x TE)
Ultrapure water up to 100 ml

10% SDS (100 ml)
10 g SDS (mw = 288.37)
Ultrapure water up to 100 ml

20x SSC Buffer

3 M NaCl (175.3 g)
300 mM Sodium Citrate (88.2 g)
pH to 7.0 with HCl
up to 1 L Ultrapure water

Oligo Hybridization Buffer (10x)
500 mM NaCl
10 mM Tris-Cl, pH 8.0
1 mM EDTA, pH 8.0

ddRAD libraries for probes

1 Total gDNA restriction digestion

Choose 5 fresh samples from different populations (if possible spanning the entire range of interest). Dilute 1,500 ng of DNA from each sample to the same concentration.
Digest DNA at ratio of 5-10 U restriction enzyme (RE) / 1,000 ng DNA.

Reagent	Volume (µl)	Final cc.
Ultrapure water	50-x	
CutSmart buffer (10x)	5	1x
SphI (20 000 U/ml)	0.75	15 U
MluCl (10 000 U/ml)	1.5	15 U
DNA	x	1,500 ng
Total	50	

For each sample mix ~1,500 ng DNA with master mix.

Incubate 10 hours at 37°C, hold at 4°C.

2 Purification

Perform SPRI bead purification with the bead : sample ratio 2:1 according to the manufacturer's instructions (see note for Seramag).

Seramag purification

Reagents and tools needed:
- freshly prepared 70% ethanol
- 10 mM Tris-HCl pH 8.5 or PCR-grad water for elution
- magnetic rack for PCR plates (or for 1.5 ml tubes if working on pooled samples)

The purification protocol:
1. Remove beads from the fridge, equilibrate to the room temperature, mix thoroughly by vortexing.
2. Add desired ratio of Sera-Mag beads to the purified sample and mix well by pipetting.
3. Incubate 5 minutes.
4. Place on the magnetic rack.
5. Let it stand for 5 minutes on the rack, aspirate and discard supernatant.
6. Add 200 µl of freshly prepared 70% EtOH, incubate 30 seconds, aspirate and discard EtOH.
7. Repeat the wash, aspirate and discard EtOH.
8. Wait until it dries out completely.
9. Add desired volume of Tris 10 mM or water (add 1 µl to the final volume to avoid pipetting out the beads), remove from rack.
10. Resuspend by pipetting or vortexing.
11. Incubate 5 minutes.
12. Place on the rack.

Let it stand for 5 minutes, pipette out and save supernatant. The eluted DNA is in the supernatant, do not discard it!

23 µl Ultrapure water for elution (1 µl for Qubit check)

3 Adapter ligation

Ligate short adapters containing only restriction site overhangs and primer binding sites for amplification.

Anneal adapters
Complementary single-stranded oligos need to be annealed before ligation (see Before Strarting).

Ligation

Reagent	Volume (µl)
AD_SphI adapter (10 µM)	6
AD_MluCI adapter (10 µM)	6
T4 ligase buffer (10x)	4
T4 DNA ligase (400 U/µl)	2
Total	18

Mix 22 µl of each digested DNA sample with the master mix (total volume = 40 µl).

Incubate 3 hours at 16°C, hold at 4°C.

Pool the ligation products together (200 µl total, in case of 5 samples).

4 Purification

Perform SPRI bead cleanup with the bead : sample ratio 2:1 according to the manufacturer's instructions.

31 µl Ultrapure water for elution (1 µl for Qubit check)

5 Size selection

Size-select the range of products from 200-300 bp using gel or alternative instrument.
We ran 3 lanes of a DNA300 chip (10 µl DNA in each lane) on LabChip XT (Caliper; 250 bp peak +/- 40 bp). Total eluate from all lanes was diluted to 100 µl total for subsequent PCR step.

Quantitate and confirm appropriate size profile of the eluate.

6 Amplification of ddRAD libraries

Amplify the ddRAD probes using primers IS7 and IS8. Perform PCRs in as many replicates as possible, given the available template (we did 20). Later re-amplify the PCR products if more products are needed. Biotinylation also increases the concentration if DecaLabel kit is used in step #11.

Do NOT vortex:

Reagent	Volume (µl)	Final cc.
Ultrapure water	5	
NEBNext HiFi PCR master mix (2x)	13	1x
IS7 (10 µM)	1	400nM
IS8 (10 µM)	1	400nM
Size-selected ddRAD library	5	
Total	25	

PCR program

initial denaturation	98°C	1 min	
denaturation	98°C	10 s	x38 cycles
annealing	50°C	20 s	x38 cycles
elongation	72°C	20 s	x38 cycles
final extension	72°C	10 min	

hold	4°C		

Pool the PCR products (e.g. 20 x 25 µl = 500 µl).

7 Purification

Perform SPRI bead cleanup with the bead : sample ratio 1:1 according to the manufacturer's instructions.

51 µl Ultrapure water for elution (1 µl for Qubit check)

8 Library quantification

Quantify the library concentration. You require around 1,000 ng of RAD library per capture (+ excess). If insufficient probe is obtained, the PCR product can be re-amplified.

9 Adapter removal

Remove adapters by digestionin as many reactions as required fordigestion of 5-10 µg pooled and purified RAD library in each 100 µl reaction.

Reagent	Volume (µl)	Final cc.
Ultrapure water	100-x	
CutSmart buffer (10x)	10	1x
SphI (20 000 U/ml)	3	60 U
MluCl (10 000 U/ml)	6	60 U
DNA	x	
Total	100	

Incubate 10 hours at 37°C, hold at 4°C.

10 Purification

Purify using SPRI, with the bead : sample ratio 1:1 according to the manufacturer's instructions in order to remove the digested adapters. Resuspend in 100 µl ultrapure water, plus an aliquot for quality check to verify that no digested adapters remain.

11 Biotinylation of the probes

Mix the following components into 1.5 ml microcentrifuge tube:

Reagent	Volume (µl)
DNA (max 1,000 ng)	10
Decanucleotide in 5X Reaction Buffer	10
Ultrapure water	24
Total	44

Vortex the tube and spin down in a microcentrifuge for 3-5 s.

Incubate the tube in a boiling water bath for 5-10 min and cool it on ice. Spin down quickly.

Mix the following:

Reagent	Volume (µl)
Biotin Labeling Mix	5
Klenow fragment, exo– (5 u)	1

Shake the tube and spin down in a microcentrifuge for 3-5 s.

Incubation at 37°C for up to 20 hours to increase the yield of labeled DNA.

Stop the reaction by the addition of 1 µl of 0.5 M EDTA, pH 8.0.

DecaLabel DNA Labeling Kit
by Thermo Fisher Scientific
Catalog #: K0651

12 Purification

Purify using SPRI, with the bead : sample ratio 1.5:1 according to the manufacturer's instructions.
Resuspend in 110 µl ultrapure water. Resuspension volume needed depends on the number of intended captures. Calculate 10 µl per capture plus an aliquot for checking the probes concentration.

Biotinylated probes can be stored at -20°C in 10 mM Tris for at least one year.

Whole genome libraries

13 DNA shearing

Shear high quality, high molecular weight DNA samples to approximately 200-300 bp length. We used the Bioruptor (Diagenode). Ancient, museum, or otherwise already degraded samples were not actively sheared.

Bioruptor settings:
Power – position High
Cycle condition (on/off) – 30s/90s
cycles – 7

14 5'-phosphorylation

DNA samples are first 5'-phosphorylated in order to allow subsequent adapter ligation in the next steps of the protocol.

Reagent	Volume (µl)
T4 PNK (10U/µl)	1
T4 ligase buffer	1
Total	2

Denature 8 µl of DNA by incubating for 5 min at 95°C and quickly chilling on ice.
Add 2 µl of master mix (total volume = 10 µl).

Incubate for 30 min at 37°C, heat-denature enzymes for 20 min at 65°C.

15 Purification

Perform SPRI cleanup with the bead : sample ratio 2:1 according to the manufacturer's instructions.

10 µl Ultrapure water for elution

16 3'-guanidine tailing

Assemble at room temperature, can precipitate when on ice:

Reagent	Volume (µl)
Ultrapure water	4.7
NEB buffer 4 (10x)	2
CoCl2 (2.5 mM)	2
GTP (100 mM)	0.8
TdT (20 U/µl)	0.5
Total	10

Denature DNA by incubating for 5 min at 95°C and quickly chilling on ice.
Add 10 µl of the master mix to the 10 µl DNA (total volume = 20 µl).

Incubate for 30 min at 37°C, heat-denature enzymes for 10 min at 70°C.

17 Second DNA strand synthesis

The second DNA strand is synthesized using Klenow Fragment (3' to 5' exo-), with a primer consisting of the Illumina P7 sequence and a poly-C sequence homologous to the poly-G tail added to the DNA strand in the previous reaction.

Reagent	Volume (µl)
NEB buffer 4 (10x)	1
dNTP mix (2.5 mM each)	6
PE-P7-CCCCC oligo (15 µM)	1
Klenow fragment (3'->5' exo-) (5 U/µl)	2
Total	10

Add 10 µl of the master mix to 20 µl of the DNA (total volume = 30 µl).

Incubate at room temperature (at 23°C) for 3 hours, heat-denature enzymes for 20 min at 75°C, hold at 4°C.

18 Blunt-ending

Reagent	Volume (µl)
Ultrapure water	3.95
NEB buffer 4 (10x)	0.5
BSA (10 mg/ml)	0.35
T4 DNA polymerase (3 U/µl)	0.2
Total	5

Add 5 µl of the master mix to 30 µl of the DNA (total volume = 35 µl). Keep on ice when assembling the reaction.

Incubate for 15 min at 12°C.

19 Purification

Perform SPRI cleanup with the bead : sample ratio 2:1 according to the manufacturer's instructions.

10 µl Ultrapure water for elution

20 Ligation of P5 adapters

Prepare barcoded P5 adapters to 25 µM (see Before Starting). Each sample should be mixed with a uniquely barcoded adapter! These adapters will be ligated to the 5'-phosphorylated end of the double-stranded product.

Reagent	Volume (µl)
Ultrapure water	6
T4 ligase buffer (10x)	2
T4 DNA ligase (400 U/µl)	1
Total	9

Add 1 µl of the barcoded adapter (25 µM) to 10 µl of the DNA. Add 9 µl of the master mix to each sample (total volume = 20 µl).

16 °C overnight incubation

21 Purification

Perform SPRI cleanup with the bead : sample ratio 1:1 according to the manufacturer's instructions.

10 µl Ultrapure water for elution

22 Adapter fill-in

Reagent	Volume (µl)
Ultrapure water	5.25
ThermoPol reaction buffer (10x)	2
dNTP mix (2.5 mM each)	2
BST polymerase, large fragment (8 U/µl)	0.75
Total	10

Add 10 µl of the master mix to 10 µl of the DNA (total volume = 20 µl).

Incubate for 20 min at 37°C.

23 Purification

Perform SPRI cleanup with the bead : sample ratio 2:1 according to the manufacturer's instructions.
Resuspend in 20 µl ultrapure water (or more, depending on the number of PCR replicates in the next step).

24 PCR amplification

Prepare indexed primer solutions (see Before Starting), which contain an uniquely barcoded primer for each 48 samples as external index.
Perform each PCR reaction in at least two replicates to account for the stochasticity of fragment amplification.

Reagent	Volume (µl)
NEB Next HiFi PCR master mix (2x)	12.5
Indexing primer mix (5 µM each)	2.5
Total	15

Add 15 µl of the master mix to 10 µl of the template (total volume = 25 µl).

PCR program

initial denaturation	98°C	30 s	
denaturation	98°C	10 s	x25 cycles
annealing	60°C	30 s	x25 cycles
elongation	72°C	30 s	x25 cycles
final extension	72°C	5 min	
hold	4°C		

Verify individual sample profiles and estimate concentrations. Pool equimolar the samples amplified using the same indexed primer.

25 Purification

After pooling, perform SPRI cleanup in order to remove primer-dimers, with the bead : sample ratio 0.7:1 according to the manufacturer's instructions.
Resuspend in 17 µl ultrapure water (10 µl for the capture, the rest for library quantification and profile validation).

Verify the probe profile and successful primer-dimer removal. Quantify the final library.

Hybridization

26 Hybridization capture

Libraries from different tissue types (fresh, historic, feathers) and age should be pooled separately (24 samples/capture reaction). Make sure to start this step only if you are able to carry out the next steps 48-72 hours after the incubation has started.

Library mix (LIB):

Reagent	Volume (µl)
Pooled gDNA library (500-1000 ng)	11.9

Cot-1 DNA (1 mg/ml)	0.5
BO.1 blocking oligo (100 µM)	0.8
BO.2 blocking oligo (100 µM)	0.8
BO.3 blocking oligo (100 µM)	0.8
BO.4 blocking oligo (100 µM)	0.8
Total	15.6

Hybridization mix (HYB):

Reagent	**Volume (µl)**
Biotinylated probes (500-1000 ng)	10
SSC (20x)	12
EDTA (500 mM)	0.4
SDS (10%)	0.4
Denhardt's solution (50x)	1.6
Total	24.4

On standard PCR machine (heated lid):

1. 95°C for 5 minutes – denature LIB (after denaturing LIB you bring the temperature down to 65°C)
2. 65°C for 5 minutes – heat LIB and HYB separately
3. Maintaining 65°C, mix LIB and HYB in the same tube and seal with parafilm
4. Incubate at 65°C for 48-72 hours. Mix twice each day.

27 Preparation of Streptavidin beads

Those library fragments hybridized with probes are then isolated on Streptavidin beads.

To prepare the beads:

1. Resuspend Dynabeads M-280 (10 mg/ml)
2. Dispense 50 µl of beads in a PCR tube
3. Wash:

- place on magnet, remove and discard supernatant
-

- resuspend beads in 200 µl of TEN (10 mM Tris-HCl 7.5, 1 mM EDTA, 1 M NaCl)
-

1. Perform previous step 3 times in total
2. Store in 70 µl of TEN at room temperature until use

NB: If more captures are expected, increase the initial amount of beads accordingly, transfer the final resuspension into an 1.5 ml eppendorf tube and add the appropriate volume of TEN.

28 Attaching hybridized probes to beads and washes

This is what separates out the hybridized DNA fragments from the free-floating non-target DNA. This may be one of the more sensitive parts of the protocol and deserves plenty of care.

1. Preheat the Streptavidin beads in the TEN buffer to **65°C.**
2. Add the **40ul** hybridization mix onto the Streptavidin beads and make sure all beads are resuspended. Mix by pipetting.
3. Incubate for **30min** at **23°C** (room-temp), flick the tube every 5 mins. Place the mix onto a magnetic plate and remove supernatant. (It may be a good idea to save this, just in case)
4. Add **200ul** of 1X SSC/0.1% SDS and incubate for **15min** at **65°C.** Place the mix onto a magnetic plate and remove supernatant.
5. Add **200ul** of 0.5X SSC/0.1% SDS and incubate for **15min** at **65°C.** Place the mix onto a magnetic plate and remove supernatant.
6. Add **200ul** of 0.1X SSC/0.1% SDS and incubate for **15min** at **65°C.** Place the mix onto a magnetic plate and remove supernatant.
7. Add **30ul** of ultrapure water and incubate in **80°C** for **10min**. Place the mix onto a magnetic plate immediately and transfer supernatant to a new tube after the beads have separated. This will be your captured libraries.

29 Library re-amplification

Prepare re-amplification master mix. The volumes are per captured library:

Reagent	**Volume (µl)**

NEB Next HiFi PCR master mix (2x)	25
Indexing primer mix (5 µM each)	5
Captured DNA library	15
Ultrapure water	5
Total	50

Use the same index as used in the indexing PCR of whole genome library preparation (in step #24)!

PCR program

initial denaturation	98°C	30 s	
denaturation	98°C	10 s	x15 cycles
annealing	60°C	30 s	x15 cycles
elongation	72°C	30 s	x15 cycles
final extension	72°C	5 min	
hold	4°C		

30 **Purification**

Perform SPRI clean-up using bead : sample ratio 1:1. Elute in 30 µl ultrapure water.

Verify the DNA size profile and molarity.

Libraries can now be pooled in equimolar ratios for submission to an Illumina Sequencer.