Supporting Information for online publication

1. **Table S1.** Characteristics of twelve microsatellite markers used for parentage analyses.
2. **Supplementary Protocol**

Table S1. Characteristics of twelve microsatellite markers used for parentage analyses from 769 variegated fairy-wrens sampled from 2012 to 2016 at Lake Samsonvale, Queensland, Australia.

Locus*	Repeat motif	Forward (F) and Reverse (R) primer sequences	No. of alleles	Size range (bp)
MaLa02	AGAT	F- AAGCAGCCAAGATGTTAGTTTCC	32	172-316
		R- AATGTGGATCGGCTAAAGAATGC		
MaLa03	AATG	F- TTGTCTATGTATGACAACGTGGC	8	264-316
		R- GACAGACTGCAATGTTCCTGTAC		
MaLa04	AATG	F- ACGGACTGAAGTAATACAAGCAC	8	302-346
		R- TGAGCCTTTGAATGTACTCTCAC		
MaLa05	AAGG	F- ACTTGGAAGGGTGAGGATTTATTC	20	165-275
		R- TGTTCTTCCTTGATGTAACCAACC		
MaLa06	ATCC	F- ATCCATCTCCACAGTACCCTTAC	11	152-212
		R- ATCAGGGTGTCACTATGTCCTTG		
MaLa07	AAGT	F- TATGACCGGTGAGATTCCTGTAG	7	257-289
		R- GCAGTTCTGCTAAACACATATGC		
MaLa08	ACT	F- AAAGGTCAAATCCATGGGTTGAG	13	200-248
		R- AACATACTCCCTTATCCCATTCAG		
MaLa10	AATAG	F- AAATCCACCATTGTTTAGGAGGC	26	258-330
		R- TCTTAACAGAGGAATGGGTTTGC		
MaLa13	ACAG	F- ATTACCACATTTGTCTGCCTGTC	23	290-374
		R- TGACCTGTTTGTAAAGGTTCACC		
MaLa14	AGAT	F- GTGGCACTGATAAATTTCTATGGC	21	199-309
		R- TCAGTGATGATTTCTTTGGCTGG		
MaLa16	AAGG	F- AAAGGACAGGGAAAGGGAAGTAG	14	167-223
		R- CTGTGAGTAAAGCCATGGTCAAG		
MaLa18	AACC	F- AGCAGCAAACAGATATCCAAGTG	13	234-282
		R- TTTCATCTGCATGGTTCCACTG		

* Microsatellites were multiplexed at an annealing temperature of 60°C (*MaLa02*, *MaLa03*, *MaLa07*, and *MaLa18*; *MaLa04*, *MaLa06*, *MaLa08*, and *MaLa10; MaLa05*, *MaLa13*, *MaLa14*, and *MaLa16*). A pigtailed sequence (GTT TCT) was added to the 5' end of all reverse primers to reduce genotyping error, by adding stability to the PCR reaction. No loci deviated from Hardy-Weinberg equilibrium or were in linkage disequilibrium.

Supplementary Protocol

DNA extraction

There are many methods of DNA extraction that would be suitable. Highly quality DNA (i.e. with little degradation) and DNA concentrations around 20-50ng/ul are preferred.

We extract DNA using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturers protocol with the following changes. To reduce the costs of DNA extraction we use bulk reagents from Qiagen and UPrep® Spin Columns (LPS, catalog # M858143). Approximately 100ul of blood (stored in Queens lysis buffer at RT) is added to 150ul 1X Phosphate Buffered Saline (PBS) and digested with 200ul Buffer AL (Qiagen) and 20ul of 20mg/ml Proteinase K (VWR) overnight at 64 degC. In the final step DNA is eluted in warmed nuclease-free water (Qiagen). Two elutions of 80ul (E1) and 150ul (E2) are performed into separate 1.5ml tubes.

Check the quality of the DNA by running 1-2ul of the extracted DNA on a 1% agarose gel. The DNA should run as one high molecular weight band with little degradation (seen as smearing or the presence of low molecular weight products on the gel).

Quantify and normalize genomic DNA

Two microliters of DNA is used to determine the DNA concentration of the first elutions (E1) using the Qubit dsDNA BR Assay Kit and the Qubit® Fluorometer (Life Technologies) following the manufacturers protocol. Those samples with a concentration higher than 30ng/ul are diluted to approximately 25ng/ul with nuclease-free water. For those samples with concentrations lower than 10ng/ul, both elutions are pooled and the DNA concentrated by evaporation using an Eppendorf Vacufuge. Concentrations of these concentrated samples are determined as above and if necessary further concentration or dilution performed.

Order and prepare adapters and index primers.

See table 2 below for a list of oligos to order. We order these from IDT (idtdna.com) at a scale of 100nmole with standard desalting.

Resuspend oligos to 100uM in 0.5X AE Buffer (Qiagen). (add buffer at 10X nmoles of primer (in ul). E.g. for a primer with 95.4 nmoles add 954ul 0.5XAE).

For the P1 and P2 adapters you need to anneal the complementary oligos together.

For P1 adapters (Final concentration =5uM):

1. In a PCR tube mix equal volumes of each oligo together to get a final concentration of 50uM of adapter. E.g. 20ul of P1F_X and 20ul of P1R_X
2. In a thermocylcer heat the mixture at 80 degC for 1 min

3. Remove tube from the thermocylcer and place on a rack at RT.
4. Allow to cool to RT for at least 30min.
5. Further dilute the adapter to 5uM with 0.5 X AE in 1.5ml tube. E.g Add 40ul of annealed adapter and 360ul 0.5X AE.
6. Store adapters at 4 degC or in freezer for long term storage.

For P2 adapter (Final concentration =25uM): Note: you will need to make several batches of P2 adapter to have enough for each experiment (you need approximately 250ul per 80 samples)

1. In a PCR tube mix equal volumes of each oligo together to get a final concentration of 50uM of adapter. E.g. 50ul of P2F_MspI and 50ul of P2R_MspI.
2. In a thermocylcer heat the mixture at 80 degC for 1 min
3. Remove tube from the thermocylcer and place on a rack at RT.
4. Allow to cool to RT for at least 30min.
5. Further dilute the adapter to 25uM with 0.5 X AE in 1.5ml tube. E.g Add 100ul of annealed adapter and 100ul 0.5X AE.
6. Store adapter at 4 degC or in freezer for long term storage.

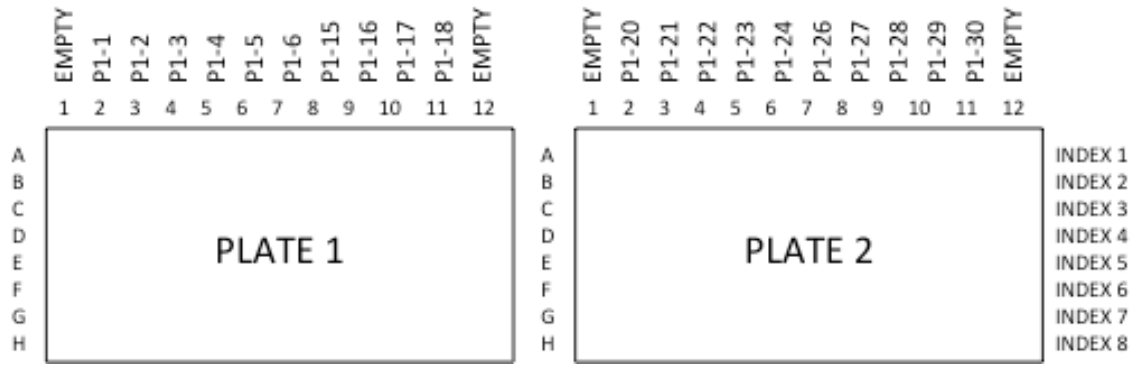
For the PCR primers (P1_PCR_for and P-indexX) you will need to dilute these primers to 5uM.
This is a 1:20 dilution. Eg. 10ul of oligo + 190ul 0.5X AE.

Digest Samples with *SbfI* and *MspI* and ligate P1 and P2 adapters

Set up arrangement of samples in 96 well plate:

- Samples should be grouped in groups of 20 (each sample will carry a unique barcode which is part of the P1 adapter) that will be pooled and have unique index sequences added (during PCR enrichment). We have 20 P1 adapters and 12 Index primers, making a total of 240 DNA samples that can be run in one lane.
- If you have samples with large differences in DNA concentration, group samples with similar DNA concentrations together in one index group.
- Prepare a spreadsheet, recording the P1 adapter, index group and plate location for each sample

Example of our plate layout for 160 samples (8 index groups)



Consumables:
96 well PCR plate
Adhesive sealing sheets (ThermoFisher Scientific, AB0558)
strip tubes or single PCR tubes
caps for strip tubes
2ml tubes
1.5ml tubes
10X CutSmart Buffer (NEB) (provided with Enzymes)
20U/ul SbfI-HF (NEB, R3642L)
20U/ul MspI (NEB, R0106S)
10mM ATP (NEB, P0756L)
400U/ul T4 DNA ligase (NEB, M0202L)
gDNA samples (normalized, usually approx. 10-30ng/ul in a total volume of at least 25ul)
5uM P1 adapters (see below)
25uM P2 adapter (see below)
Nuclease-free water (Qiagen, 129115)
(1% agarose gel, 6X loading dye, 1XTAE, 1kb ladder)

Prepare 1:20 dilutions of P1 adapters:

1. This can be done in a strip of PCR tubes to allow you to use the multichannel pipette to dispense the P1 adapters. (You will need 20 tubes if using all the P1 adapters)
2. Label tubes.
3. Add 19ul of nuclease free water to each tube.
4. Add 1ul of P1 adapter to corresponding one tube above. Repeat for all P1 adapters being used.
5. Seal tubes, vortex briefly to mix, centrifuge.

Setup digestion-ligation reaction:

1. A standard digestion-ligation reaction has the following:

Reagents	**Volume (ul) for one reaction**

Nuclease Free water	Up to total volume
10X CutSmart Buffer	Dilute to 1X (vol depends on total vol)
25uM P2 adapter	2.5
10mM ATP	1mM (volume depends on total vol)
20U/ul SbfI-HF	0.75
20U/ul MspI	0.75
400U/ul T4 DNA ligase	0.75
Normalized DNA	Approx. 200-500ng (vol depends on conc.)
TOTAL	20-40ul

The remainder of this protocol is an example where our reaction volume is 40ul and we add 20ul of DNA at approximately 20ng/ul.

2. Make a master mix of everything except the P1 adapters and DNA in a 2ml tube.

   Make the master mix for a few extra reactions so that you do not run out. We usually do our reactions in plates with 80 samples per plate.

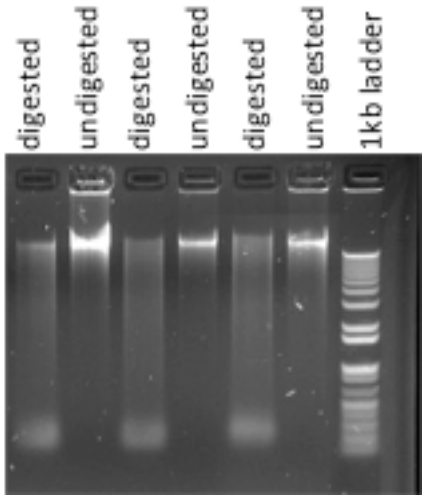
Reagents	Volume (ul) for one reaction	Volumes (ul) for Master mix for 80 samples (90 rxns)
Nuclease Free water	6.25	562.5
10X CutSmart Buffer	4	360
25uM P2 adapter	2.5	225
10mM ATP	4	360
20U/ul SbfI-HF	0.75	67.5
20U/ul MspI	0.75	67.5
400U/ul T4 DNA ligase	0.75	67.5
TOTAL	19	1710

3. Vortex briefly to mix and centrifuge
4. Aliquot 19ul of master mix to each well of a 96 well PCR plate. (note: we do 80 samples per plate and therefore leave column 1 and column 12 empty)

   If you would prefer to use a multichannel pipette to aliquot the master mix: First divide your master mix into a strip of 8 PCR tubes (210ul per tube), and then use this strip of 8 tubes as the reservoir to aliquot 19ul per well using the 8-channel multichannel pipette.
5. Add 1ul of P1 adapter to the corresponding well of the PCR plate (make sure that each group of 20 digestion-ligation reactions receives a unique P1 adapter). If you set the P1 adapters up in a strip of PCR tubes then you can

use a multichannel pipette to add the adapters. In our case each column of the PCR plate has a different P1 adapter.

6. Add 20ul of gDNA to each well. (**Note:** every well in the plate will have a different DNA sample).
7. Use a multichannel pipette, set to 30ul, and gently pipette up and down a few times to mix each reaction.
8. Seal the PCR plate well using adhesive seal.
9. Place on the thermocylcer and run the following program:
   Choose the preheated lid option and set to 100 degC
   37 degC for 30min
   20 degC for 60min
   hold at 4 degC
10. If desired, select three samples from each plate and check for digestion on a gel:
    -randomly select three wells from your PCR plate and remove 2ul of the reaction into a tube containing 2ul of gel loading dye.
    -for comparison take 1ul of the corresponding undigested gDNA and add 2ul of gel loading dye.
    -Run these on a 1% agarose gel along with a marker. You want to see a smear for the digested sample compared to the mostly intact undigested DNA.



<u>Pool reactions within Index groups and clean up digested DNA.</u>

<u>Consumables:</u>

SPRI beads (Agencourt AMPure XP, Beckman Coulter, A63880) or home made MagNA (see protocol at end of methods)
Freshly prepared 70% Ethanol
1.5ml and 2ml tubes
screw-capped tube required by core facility for sample submission
AE Buffer (Qiagen)
Qubit dsDNA BR assay kit (ThermoFisher Scientific, Q32853)

Equipment:
Magnetic separation rack for 1.5ml tubes (e.g. NEB, S1509S)
Qubit Fluorometer (ThermoFisher Scientific)
*BluePippin (Sage Science)

* *In our case these are services provided by the Biotechnology Resource Center Genomics Facility at Cornell University (http://www.biotech.cornell.edu/brc/genomics-facility)*

Pool reactions within index groups and clean up DNA.

1. Remove SPRI beads from refrigerator and allow to warm to room temperature.
2. Combine the digestion-ligation reactions from one index group (20 samples with unique adapters) in 2ml tube:
   In our example above: all the digestion-ligation reactions from row A in both plate 1 and plate 2 are combined for Index 1
   We will have a total of 800ul in the tube (40ul X20).
   This will be repeated with row B (index 2), row C, (index 3), etc.
   You will have up to 12 tubes each with 800ul of digested-ligated samples.
3. Mix SPRI beads well
4. Add 1.5X volumes of SPRI beads to the 2ml tube with the combined digested-ligated samples (mix well each time you pipette, to ensure that beads are even distributed in the mixture).
   In our case we will add 1.2ml SPRI beads to each tube containing 800ul digested-ligated sample.
5. Mix gently by pipetting up and down or turning tube over.
6. Incubate at room temperature for 5min
7. Place tube on the magnetic rack to capture the beads. This may take many minutes. When the beads are captured to the side of the tube and the supernatant is clear you are ready to proceed.
8. Remove and discard the supernatant (keeping the tube on the magnetic rack)
9. Wash the beads by pipetting 2ml 70% Ethanol into the tubes. Keep the tubes on the magnetic rack.
10. Incubate for 1min and then remove and discard the ethanol
11. Repeat the Ethanol wash (steps 9 and 10)
12. Remove as much ethanol as possible and allow the beads to air-dry for 5-10 mins (keep tubes in the magnetic rack).
13. Remove the tube from the magnetic rack and resuspend the beads in 36ul AE buffer. Pipette repeatedly over the side of the tube to resuspend all beads and wash the beads down into the suspension.
14. Incubate at room temperature for 2min.
15. Return the tubes back to the magnetic rack and incubate until the beads are captured on the side of the tube and the supernatant is clear.
16. Transfer 34ul of the supernatant to a new tube (screw capped tube for submission to core facility).

Determine concentration after clean-up
Determine the concentration of your DNA using the Qubit dsDNA BR Assay kit and 2ul of cleaned up DNA (from above). (Concentration is usually around 100ng/ul).

Submit to core facility for size selection.
Request size selection in the range of 450-600bp using BluePippin. Note this is a narrow window and to ensure that the same loci are recovered in all index groups it is important to be careful to have the same size range selected for each index group.

Low cycle PCR to add index groups.

Consumables
Qubit dsDNA HS assay kit (ThermoFisher Scientific, Q32851)
Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB, M0531L)
P1_PCR_For primer (5uM)
Index primers (5uM)
PCR tubes and caps

Determine concentration of DNA after size selection.
Determine the concentration of the DNA post pippin size selection using 2ul of the size selected DNA and the Qubit dsDNA HS Assay kit. (The concentration is usually around 1ng/ul or less).

Low-cycle PCR
Set up multiple PCR reactions for each index group (the number will depend on the volume of size selected DNA received back after pippin). We aim for approximately 10ng of DNA per PCR reaction. Therefore at around 1ng/ul if you get approximately 30ul total volume you can do 3 PCR reactions. These reactions can be prepared as a master mix with all reagents and then divided between 3 (or more) PCR tubes each with 25ul.

Reagent	Volume (ul) for 1 reaction	Volume (ul) for 3 reactions
Adapter ligated and size selected DNA	10	30
Phusion Master mix (2X)	12.5	37.5
Primer P1_PCR_for (5uM)	1.25	3.75
Index primer (*P_Index"X") (5uM)	1.25	3.75

* use a different Index primer for each group of samples.

Place on the thermocylcer and run the following program:
Choose the preheat option and set to 100 degC

- 98 degC for 30 sec

- 11 cycles of:
  98 degC for 5 sec
  60 degC for 25 sec
  72 degC for 10 sec
- 72 degC for 5min
- Hold at 10 degC

Pool reactions within Index groups and clean up digested DNA.

Consumables:
SPRI beads (Agencourt AMPure XP, Beckman Coulter, A63880) or home made MagNA (see protocol at end of methods)
Freshly prepared 70% Ethanol
1.5ml tubes
screw-capped tube required by core facility for sample submission
AE Buffer (Qiagen)
Qubit dsDNA HS assay kit (ThermoFisher Scientific, Q32853)
1 or 2% agarose gel, loading dye, 1kb ladder

Equipment:
Magnetic separation rack for 1.5ml tubes (e.g. NEB, S1509S)
Qubit Fluorometer (ThermoFisher Scientific)
*BluePippin (Sage Science)
*Fragment Analyzer (Advanced Analytical)

* *In our case these are services provided by the Biotechnology Resource Center Genomics Facility at Cornell University (http://www.biotech.cornell.edu/brc/genomics-facility)*

Pool the PCR reactions
Pool the PCR reactions from the same group in 1.5ml tube and run 5ul on a 1 or 2% agarose gel to check for enrichment. There should be a band visible around 500-600bp, but it may be faint.

Clean up DNA post PCR.
1. Remove SPRI beads from refrigerator and allow to warm to room temperature.
2. Mix SPRI beads well
3. Add 0.7X volumes of SPRI beads to the 1.5ml tube with the pooled PCR reactions (mix well each time you pipette, to ensure that beads are even distributed in the mixture).
   In our case we will add 52.5ul SPRI beads to each tube containing 75ul PCR product.
4. Mix gently by pipetting up and down or turning tube over.

5. Incubate at room temperature for 5min
6. Place tube on the magnetic rack to capture the beads. This may take many minutes. When the beads are captured to the side of the tube and the supernatant is clear you are ready to proceed.
7. Remove and discard the supernatant (keeping the tube on the magnetic rack)
8. Wash the beads by pipetting 500ul 70% Ethanol into the tubes. Keep the tubes on the magnetic rack.
9. Incubate for 1min and then remove and discard the ethanol
10. Repeat the Ethanol wash (steps 9 and 10)
11. Remove as much ethanol as possible and allow the beads to air-dry for 5-10 mins (keep tubes in the magnetic rack).
12. Remove the tube from the magnetic rack and resuspend the beads in 36ul AE buffer. Pipette repeatedly over the side of the tube to resuspend all beads and wash the beads down into the suspension.
13. Incubate at room temperature for 2min.
14. Return the tubes back to the magnetic rack and incubate until the beads are captured on the side of the tube and the supernatant is clear.
15. Transfer 34ul of the supernatant to a new 1.5ml tube.

Determine concentration of DNA after cleanup.
Determine the concentration of the DNA post PCR using 2ul of the cleaned up DNA and the Qubit dsDNA HS Assay kit. (The concentration may still be low, but you should see an increase in total mass compared to the pre-PCR post-pippin readings).

Submit 4ul DNA from each index group for fragment analysis:
Perform fragment analysis on the DNA post PCR to determine average size of the fragments within each pool. This information will be used to pool the index groups in the final library. We submit our samples to the Biotechnology Resource Center Genomics Facility at Cornell University.

Pool and Dilute samples to create final library

Consumables
O.5X AE + 0.1% Tween: 500ul AE buffer (Qiagen, 19077) + 500ul nuclease free water (Qiagen, 129115) + 1ul Tween 20 (Fisher Scientific, BP337-100)
Screw capped tubes for library submission to the core facilty

Using the concentrations determined by Qubit and the average size (provided by the fragment analysis), dilute and pool the index groups to create one library for Illumina sequencing.

Follow the calculations shown in the table below calculate the current molarity of each index group. Then dilute each index group to 2nM (this can be done in any volume, but 20ul is usually sufficient) with 0.5XAE containing 0.1%Tween.

For an example see “index 1” entry in the table below.

Finally combine equal volumes of each normalized index together in one screw capped tube. (e.g. add 20ul of each normalized index (2nM). If combining 12 index groups you will have a total of 240ul)

Submit for Illumina sequencing

Instrument: HiSEQ 2500
Run Length: 100 single end
These ddRAD experiments have a low 5' complexity, therefore make sure to request that the library is run with the addition of the Illumina PhiX control (>5%). Our libraries are usually run with the addition of 15% PhiX.

Table 1: Example of calculations required to normalize individual index groups

	Enter data		Calculations				dilute to 2nM		
	Size (bp)	**conc (ng/ul)**	**MW (g/mol)**	**convert conc to g/L**	**Molarity of index (M, mol/L)**	**Molarity of Index (nM, nmol/L)**	**vol (ul) of index**	**0.5X AE/0.1% Tween to add (ul)**	**total volume (ul)**
	Avg bp from frag analysis output	*Conc. in ng/ul from Qubit*	*= Size (bp) X 660Da (g/mol)*	*= conc (ng/ul) ÷ 1000*	*= conc (g/L) ÷ MW*	*= M X 10^9*	*= 2nM (desired molarity) X total Vol ÷ current molarity (nM)of index*	*=total volume - volume of index to be added*	
index 1	590	1.63	389400.00	1.63E-03	4.19E-09	4.19	9.6	10.4	20.0
index 2									
index 3									

Table 2: Oligos for ddRAD-seq

Sequence Name	Sequence	barcode length	Bases
P1F_1	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT ATC ACG TGC* A	6	43
P1F_2	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CGA TGT TGC* A	6	43
P1F_3	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TTA GGC TGC* A	6	43
P1F_4	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TGA CCA TGC* A	6	43
P1F_5	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT ACA GTG TGC* A	6	43
P1F_6	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GCC AAT TGC* A	6	43
P1F_15	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CTT GAT GC*A	5	42
P1F_16	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TCA CCT GC*A	5	42
P1F_17	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CTA GCT GC*A	5	42
P1F_18	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT ACA AAT GC*A	5	42
P1F_20	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT AGC CCT GC*A	5	42
P1F_21	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GTA TTT GC*A	5	42
P1F_22	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CTG TAT GC*A	5	42
P1F_23	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT AGC ATT GC*A	5	42
P1F_24	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT ACT ATT GC*A	5	42
P1F_26	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CTTGCTT TGC* A	7	44
P1F_27	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT ATGAAAC TGC* A	7	44
P1F_28	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT AAAAGTT TGC* A	7	44
P1F_29	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GAATTCA TGC* A	7	44
P1F_30	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GGACCTA TGC* A	7	44
P1R_1	/5Phos/CGT GAT AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AA*A		42
P1R_2	/5Phos/ACA TCG AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AA*A		42
P1R_3	/5Phos/GCC TAA AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AA*A		42
P1R_4	/5Phos/TGG TCA AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AA*A		42
P1R_5	/5Phos/CAC TGT AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AA*A		42
P1R_6	/5Phos/ATT GGC AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AA*A		42
P1R_15	/5Phos/TCA AGA GAT CGG AAG AGC GTC GTG TAG GGA AAG AGT GTA A*A		41
P1R_16	/5Phos/GGT GAA GAT CGG AAG AGC GTC GTG TAG GGA AAG AGT GTA A*A		41
P1R_17	/5Phos/GCT AGA GAT CGG AAG AGC GTC GTG TAG GGA AAG AGT GTA A*A		41
P1R_18	/5Phos/TTT GTA GAT CGG AAG AGC GTC GTG TAG GGA AAG AGT GTA A*A		41
P1R_20	/5Phos/GGG CTA GAT CGG AAG AGC GTC GTG TAG GGA AAG AGT GTA A*A		41
P1R_21	/5Phos/AAT ACA GAT CGG AAG AGC GTC GTG TAG GGA AAG AGT GTA A*A		41
P1R_22	/5Phos/TAC AGA GAT CGG AAG AGC GTC GTG TAG GGA AAG AGT GTA A*A		41
P1R_23	/5Phos/ATG CTA GAT CGG AAG AGC GTC GTG TAG GGA AAG AGT GTA A*A		41
P1R_24	/5Phos/ATA GTA GAT CGG AAG AGC GTC GTG TAG GGA AAG AGT GTA A*A		41
P1R_26	/5Phos/AAGCAAG AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AA*A		43
P1R_27	/5Phos/GTTTCAT AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AA*A		43
P1R_28	/5Phos/AACTTTT AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AA*A		43

P1R_29	/5Phos/TGAATTC AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AA*A		43
P1R_30	/5Phos/TAGGTCC AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AA*A		43
P2F_MspI	/5Phos/CGA GAT CGG AAG AGC GAG AAC AA		34
P2R_MspI	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T		34
P2F_EcoRI [#]	/5phos/AAT TAG ATC GGA AGA GCG AGA ACA A		25
P1_PCR_for	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC* T		58
P_index1	CAA GCA GAA GAC GGC ATA CGA GAT CGT GAT GTG ACT GGA GTT CAG ACG TGT G*C		53
P_index2	CAA GCA GAA GAC GGC ATA CGA GAT ACA TCG GTG ACT GGA GTT CAG ACG TGT G*C		53
P_index3	CAA GCA GAA GAC GGC ATA CGA GAT GCC TAA GTG ACT GGA GTT CAG ACG TGT G*C		53
P_index4	CAA GCA GAA GAC GGC ATA CGA GAT TGG TCA GTG ACT GGA GTT CAG ACG TGT G*C		53
P_index5	CAA GCA GAA GAC GGC ATA CGA GAT CAC TGT GTG ACT GGA GTT CAG ACG TGT G*C		53
P_index6	CAA GCA GAA GAC GGC ATA CGA GAT ATT GGC GTG ACT GGA GTT CAG ACG TGT G*C		53
P_index7	CAA GCA GAA GAC GGC ATA CGA GAT GAT CTG GTG ACT GGA GTT CAG ACG TGT G*C		53
P_index8	CAA GCA GAA GAC GGC ATA CGA GAT TCA AGT GTG ACT GGA GTT CAG ACG TGT G*C		53
P_index9	CAA GCA GAA GAC GGC ATA CGA GAT CTG ATC GTG ACT GGA GTT CAG ACG TGT G*C		53
P_index10	CAA GCA GAA GAC GGC ATA CGA GAT AAG CTA GTG ACT GGA GTT CAG ACG TGT G*C		53
P_index11	CAA GCA GAA GAC GGC ATA CGA GAT GTA GCC GTG ACT GGA GTT CAG ACG TGT G*C		53
P_index12	CAA GCA GAA GAC GGC ATA CGA GAT TAC AAG GTG ACT GGA GTT CAG ACG TGT G*C		53

* = Phosphorothioate Bond, inhibits exonuclease degradation
/5Phos/ = 5' Phosphorylation, inhibits degradation by 3' exonulceases and blocks extension by DNA polymerases

[#]Note: P2F_EcoRI is combined with P2R_MspI to create a P2 adapter for use with EcoRI digested DNA. It is only needed if you are going to use EcoRI instead of MspI in the digest.

Homemade MagNA:

This protocol is taken from Rohland, N. and Reich, D. (2012) Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture.Genome Res. May;22(5):939-46. doi: 10.1101/gr.128124.111. and a detailed protocol can be found: https://ethanomics.files.wordpress.com/2012/08/serapure_v2-2.pdf

Consumables:

Sera-Mag SpeedBeads Carboxyl Magnetic Beads Hydrophobic, GE Healthcare (VWR, 10204-670)
PEG-8000 (VWR, 0159-500G)
0.5M EDTA, pH 8.0 (Fisher Scientific, BP2482-1)
1.0M Tris, pH 8.0 (Fisher Scientific, BP1758-500)
Tween 20 (Fisher Scientific, BP337-100)
5M NaCl (Sigma, 59222C-500ML)
GeneRuler Ultra Low Range DNA Ladder (Thermo Scientific, SM1211)
Nuclease free water

Prepare 1XTE (In a 50ml conical using sterile solutions add the following):

- 500 ul 1M Tris, pH8
- 100 ul 0.5M EDTA, pH 8
- fill to 50ml with nuclease free water

Wash Sera-Mag Beads:

1. Mix the Sera-Mag beads well and transfer 1ml to a 1.5ml tube
2. Place the tube on the magnet stand and wait a couple of minutes until all the beads are collected on the magnet and the supernatant is clear.
3. Remove the supernatant and discard.
4. Remove the tube from the magnet, add 1 ml 1XTE, mix and return tibe to magnet.
5. Repeat the wash the 1XTE.
6. Finally remove the tube from the magnet and resuspend the beads in 1 ml 1XTE.

Prepare the MagNA beads:

7. Add 9g PEG-8000 to a new 50 ml conical tube.
8. Add 10ml 5M NaCl
9. Add 500 ul 1M Tris, pH 8
10. Add 100 ul 0.5M EDTA, pH 8
11. Fill conical to approximately 49ml with nuclease free water
12. Mix for about 3-5 min until the solution becomes clear (you will need to stop shaking and wait for the bubbles to clear)
13. Add 27.5 ul Tween 20
14. Mix the 1 ml SeraPure + TE solution from above and add to the conical.
15. Make up to 50 ml with nuclease free water and gently mix until evenly distributed.
16. Test using aliquots of ladder (you can compare with commercial Ampure XP).
17. Wrap 50 ml tube in foil and store at 4 degC.
18. If storing for an extended period of time – retest monthly.

To test the MagNA:

1. Mix 6ul of GeneRuler with 54 ul nuclease free water
2. Aliquot 20ul into three 1.5ml tubes.
   Mix the MagNA well to ensure that the beads are evenly distributed.
3. To the first tube add 18 ul MagNA (0.9X vol), to the second tube add 30 ul MagNA (1.5X vol), keep the remaining GeneRuler for the control.
4. Incubate the tubes with beads for 5min at RT
5. Place the tubes with beads on a magnet stand and wait until beads have collected at the magnet and the supernatant is clear.
6. Remove the supernatant.
7. Wash the beads twice with 500 ul freshly prepared 70% Ethanol. Keep the tubes on the magnet, add the ethanol, allow to stand for 1 min and then remove the ethanol and repeat.
8. Remove as much ethanol as possible and air dry the beads for approximately 5 min.
9. Remove the tube from the magnet stand and resupend the beads in 20 ul nuclease free water.
10. Allow to stand for 2 min
11. Place tubes on magnet stand and remove supernatant to a new 1.5ml tube.
12. Add 4ul 6X laoding dye to all the tubes (incl. the 20ul control) and run on a 1.5% or 2% agarose gel.
13. For 0.9X only the top band (300bp) is retained and for 1.5X the 100bp band is lost, but the 150bp, 200bp and 300bp bands are retained.