Supplementary Protocols—Supple et al. 2013

Supple MA, Hines HM, Dasmahapatra KK, Lewis JJ, Nielsen DM, Lavoie C, Ray DA, Salazar C, McMillan WO, and Counterman BA. 2013. Genomic architecture of adaptive color pattern divergence and convergence in *Heliconius* butterflies. *Genome Research*. doi: 10.1101/gr.150615.112

RNA-Seq Illumina Paired-End Sequencing Sample Preparation Protocol

This protocol is derived from two protocols. The steps from mRNA isolation to cDNA synthesis were modified from protocol RNASeq_v2.2.doc from the Yoav Gilad Lab. The subsequent steps were based on the protocol "Preparing Samples for Sequencing ChIP-seq DNA – Illumina GA II" from the HTSF UNC at Chapel Hill, Version 1.3 by Piotr Mieczkowski.

I. Total RNA isolation and QC

1. Store dissected tissues in RNAlater at -20°C.
2. Remove wings from RNAlater with forceps, removing as much liquid as possible, and weigh in a pre-weighed 1.5 mL tube.
3. Follow RNAqueous kit (Invitrogen) extraction protocol: the RNAqueous micro kit for 5th instar wings and the RNAqueous Kit for pupal wings.

LYSIS: Add the appropriate volume of lysis buffer given tissue weight. Homogenize tissue using handheld Tissueruptor for 10-15 seconds. Wash the tip of the homogenizer between samples by running it briefly through 5 washes: 1. water + soap, 2. ethanol + water, 3. distilled water, 4. RNase Zap (Ambion), 5. distilled water.

ELUTION: 40µL first wash (50µL for Day 3 or larger wings), 10µL second wash.

4. Run TURBO DNA-free (Invitrogen) protocol. 1µL TURBO DNAase for smaller samples (5th and Day 1) and 2µL for larger samples (>Day 3).
5. Measure RNA concentration (typically 200-1000 ng/µL) using Nanodrop and assess quality using Bioanalyzer.

II. mRNA isolation

Isolation with Dynabeads mRNA purification kit (Invitrogen) with adjustments to accommodate smaller RNA concentration. This kit should remove all RNA without a long poly-A region, thus isolating mostly mRNA from other types of RNA.

1. Dilute 10µg of total RNA with nuclease-free H_2O to 50µL in a 1.5 mL RNase-free non-stick tube.
2. Heat the sample at 65°C for 5 minutes and place the tube on ice.
3. Aliquot 100µL of Dynal oligo(dT) beads (Invitrogen) into a 1.5mL RNase-free non-stick tube.
4. Wash the beads twice with 100µL of Binding Buffer and remove the supernatant.
5. Resuspend the beads in 50µL of Binding Buffer, and add the 50µL of total RNA; rotate the tube at room temperature for 5 minutes, and remove the supernatant.
6. Wash the beads once with 100µL of Washing Buffer B and a second time in 30µL, remove supernatant.
7. Aliquoting 80µL of Binding Buffer to a new 1.5mL RNase-free non-sticky Eppendorf tube.
8. Add 20µL of 10mM Tris-HCl to the tube with beads and heat the beads at 80°C for 2 minutes to elute mRNA. Immediately put the beads on the magnet stand and transfer the supernatant (mRNA) to the tube from step 7.
9. Heat the mRNA sample from step 8 at 65°C for 5 minutes.

10. Wash the beads from step 8 twice with 30µL of Washing Buffer B, and remove the supernatant.
11. Add 100µL of mRNA sample from step 9 to the beads; rotate the tube at room temperature for 5 minutes.
12. Remove the supernatant and wash the beads once with 100µL of Washing Buffer B and a second time with 30µL.
13. Remove the supernatant from the beads, add 10µL of 10mM Tris-HCl and heat the beads at 80°C for 2 minutes to elute mRNA. Put the beads on the magnet stand and transfer the supernatant (mRNA) to a fresh 200µL thin wall PCR tube.

III. mRNA fragmentation

1. Add 1µL 10x Fragmentation Buffer (Ambion) to 9µL mRNA at 100ng.
2. Incubate at 70°C for 5 minutes.
3. Add 1µL of Stop Buffer included with the fragmentation buffer kit and put the tube on ice.
4. Transfer the solution to a 1.5 ml microcentrifuge tube. Add 1µL of 3M NaOAC, pH 5.2, 2µL of glycogen (5ug/µL), and 30µL of 100% EtOH. Incubate the tube at -80°C for 30 minutes.
5. Spin the tube at 14000 rpm for 25 minutes at 4°C in a microcentrifuge
6. Wash the pellet with 70% EtOH and air-dry the pellet (20 min).
7. Resuspend the RNA in 5.5µL of RNase free water.

IV. cDNA synthesis – 1st strand

1. In a 200ml thin wall PCR tube put 6µL random hexamer primer (500ng/µL, Promega) with 5.5µL RNA.
2. Incubate at 65°C for 5 min., then put tube on ice.
3. Mix for each sample:

5X first strand buffer (Invitrogen)	4 µL
100mM DTT (Invitrogen)	2 µL
dNTP mix 10mM	1 µL
RNase OUT (20U/uL) (Invitrogen)	1 µL

4. Add 7.5µL of the mix to each tube, vortex, and heat at 25°C for 2 min in thermocycler.
5. Add 1uL Superscript II (Invitrogen) to each sample then put in thermocycler at:

25°C	10min
42°C	50min
70°C	15min
4°C	HOLD

Put tubes on ice.

V. cDNA synthesis – 2nd strand

1. Add 61µL water to each sample.
2. To each sample add 10X second strand buffer (500mM Tris-HCl pH7.8, 50mM MgCl2, 10mM DTT) + 3µL dNTP mix 10mM.
3. Mix and incubate on ice for 5 min.
4. Add 1µL RNaseH 2U/µL (Invitrogen) and 5 uL DNAPol I 10U/µL (Invitrogen).
5. Mix and incubate at 16°C for 2.5 hrs.
6. Purify the DNA with Qiaquick PCR purification kit (Qiagen) and elute in 31µL elution buffer.

VI. End Repair

1. Prepare the following reaction mix, in order:

Eluted DNA	30µL
H_2O	10µL
T4 DNA ligase buffer with 10mM ATP (NEB)	5µL
dNTP mix (10mM)	2µL
T4 DNA polymerase (3U/µL) (NEB)	1.2µL
Klenow DNA polymerase (5U/µL) (NEB), diluted 1:4 with water	0.8µL
T4 Polynucleotide kinase (10U/µL) (NEB)	1µL

2. Mix well using pipettor.
3. Incubate the sample at 20°C for 30 min.
4. Purify with a QIAquick PCR spin column (Qiagen), and elute in 35µL of EB.

VII. Adding A's to DNA

1. Prepare the following reaction mix:

Eluted DNA	34µL
Klenow buffer (NEB buffer 2)	5µL
dATP (1 mM)	10µL
Klenow 3' to 5' exo- (5U/µL) (NEB)	1.2µL

2. Mix well using pipettor.
3. Incubate at 37°C in for 30 min.
4. Purify with a QIAquick MinElute column (Qiagen) and elute in 11µL of EB.

VIII. Ligating Adaptors

1. Prepare the following reaction mix (Total 30ul) :

Eluted DNA	10µL
H_2O	to 30 uL
2x Quick Ligation buffer (NEB, Quick Ligation Kit)	15µL
Adaptor oligo mix	XµL
Quick DNA Ligase (NEB)	1.5µL

Adaptor: Add 1µL of 1:9 µL adaptor dilution for <100ng. Typically we had 150-200ng so added 2µL.

2. Mix well using pipettor.
3. Incubate the sample at room temperature for 15min.
4. Purify the DNA with QIAquick MinElute column (Qiagen) and elute in 10µL of EB.

IX. Purify Ligation Products

1. Prepare a 60mL gel – 2% with GenePure HiRes Agarose + 1X TAE. After cooling add 2.5uL EtBr to gel (10mg/ml).
2. Pour gel, add 3ul 6X orange loading dye to each 10uL sample.
3. Add 8 uL of 0'GeneRuler Ladder.
4. Run at 120V for 60 min.
5. Cut bands from 150-275 bp.
6. Perform DNA purification using Qiagen Gel Extraction Kit using 6X volume QG to 1X volume gel. Incubate at room temperature and add 2 gel volumes isopropanol after gel has dissolved.

X. PCR enrichment

1. Set up PCR master mix:

DNA	36ul
5 × Phusion Buffer HF	10µL
PCR primer 1.1	1µL
PCR primer 2.1	1µL
10 mM dNTP mix	1.5µL
Phusion polymerase	0.5µL
Total volume 50ul	

2. Run following PCR cycle:

98°C	30 sec
18 cycles of:	98°C 10 sec; 65°C 30 sec; 72°C 30 sec
72°C	5 min
4°C	hold

3. Purify with QIAquick MinElute column (Qiagen) and elute in 14µL of EB.

Paired End DNA oligonucleotide sequences

PE Adapters

5' /Phos/-GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT

PE PCR Primers

5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
5'CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

Whole Genome Illumina Paired-End Sequencing Sample Preparation Protocol

I. SHEAR GENOMIC DNA

In 100uL volume, shear 1ug of genomic DNA to 300-500bp using covaris machine.

- 10% Duty cycle
- Intensity → 4
- Cycle burst → 200

} 50sec

Make a 1.2% agarose gel to check fragment size.
Purify using 1.8X volume of **Agencourt AMPure beads**. Elute with 50uL EB.

II. XP BEADS DNA PURIFICATION

1. Add appropriate amount (1.0X or 1.8X) of AgentCourt AMPure XP beads to each DNA sample.
2. Vortex. Incubate at room temperature for 10 minutes on a rotator.
3. Quick spin. Place in magnetic separation stand.
4. After solution clears, carefully remove the supernatant without disturbing the pellet (be careful not to remove any beads).
5. Without disturbing the pellet, add 500uL (or enough to cover the beads) of freshly made 70% Ethanol. Wait for 2 or 3 minutes.
6. Carefully remove ethanol without disturbing the pellet or taking any beads.
   NOTE: Do not remove tubes from the magnetic separation stand while doing the last two steps.
7. Repeat 70% ethanol wash. Try to remove ethanol as much as possible in one pipetting.
8. Remove from magnetic separation stand and let dry on a thermomixer at 37°C for 2 or 3 minutes.
9. Elute the DNA from beads with EB buffer. The amount of EB buffer is dependent on your downstream reaction but usually more than 30uL.
10. Vortex. Allow DNA to elute for a few minutes.
11. Quick spin. Place in magnetic separation stand.
12. After the solution clears, collect supernatant in a new tube.

III. LARGE SCALE SOLEXA LIBRARY.

1. End repair

Reagents	uL
DNA (>500ng)	50
10X T4 DNA ligation buffer (NEB)	10
dNTP mix (10mM)	4
ATP (100mM)	1
T4 DNA Polymerase (3u/uL)	5
Klenow DNA Polymerase (5u/uL)	1
T4 PNK (10u/uL)	5
H_20 (NF)	24
TOTAL	**100**

Incubate at 20°C for 30 min. on a thermomixer.
Purify using 1.8X volume of Agencourt AMPure beads. Elute with 32uL EB.

2. 3' Adenylation (Poly A tail)

Reagents	uL
NEB buffer 2 (≈ Klenow buffer)	5
dATP (1mM)	10
DNA	32
Klenow 3'-5' Exo minus (5u/uL)	3
TOTAL	**50**

Incubate at 37°C for 30 min. on a thermomixer.
Purify using 1.8X volume of Agencourt AMPure beads. Elute with 35uL EB.

3. Ligation of adaptors to DNA fragments

Reagents	uL
DNA	35
Adaptor mix (15uM)	5
2X Quick ligase reaction buffer	45
Quick ligase	5
TOTAL	**90**

Incubate at room temperature for 30 min.
Purify using 1.0X volume of AgentCourt AMPure beads. Elute with 30uL EB.

4. Enrich the adaptor-modified DNA fragment by PCR

Here we use Phusion High-Fidelity DNA Polymerase kit by New England BioLab.

Reagents	**uL**
DNA	3
5X HF buffer	10
PCR primer 1.1 (25uM)	0.5
PCR primer 2.1 (25uM)	0.5
dNTP mix (10mM)	1
Phusion enzyme	0.5
H_20 (NF)	34.5
TOTAL	**50**

Cycle

- 98°C → 30 sec
- 8 cycles
  - 98°C → 10 sec
  - 65°C → 30 sec
  - 72°C → 30 sec
- 72°C → 5 min
- Hold at 4°

PCR primer 2.1 is different for each sample and must be added separately.
Purify using 1.0X volume of AgentCourt AMPure beads. Elute with 30uL EB.