Restriction-associated DNA (RAD) tags using IGA (Version 9)
Adapted by Erik Andersen and Justin Gerke (March 7, 2009)

1. Prepare genomic DNA by growing up three 10 cm NGMA plates of worms. Washing off the plates and letting settle by gravity. Genomic DNA is extracted using the Qiagen DNeasy Blood and Tissue Purification Kit standard protocol.
2. Quantify amount of DNA by Qubit fluorimeter using the Quant-it DNA BR kit (Invitrogen).
   A. Dilute component A (dye) with component B 1:200 A:B ratio.
   B. Add 10 μL standard #1 to 190 μL of AB mix in special Qubit tubes.
   C. Add 10 μL standard #2 to 190 μL of AB mix in special Qubit tubes.
   D. Add 1 μL of sample to 199 μL of AB mix in special Qubit tubes.
   E. Start the Qubit and use the BR calibration, follow prompts for standards and samples.
   F. Multiply concentration by 200 to get actual dsDNA concentration.
3. Anneal P1 and P2 primers by incubating in Buffer AB (50 mM NaCl, 10 mM Tris-Cl pH 8.0) for five minutes at 95ºC then cooling to room temp. P1 adapters (method 2) should be at 100 nM final concentration; P2 adapters (method 3) should be at 10 μM final concentration.

   P1 top: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxx-3'
   P1 bottom: 5'-/5Phos/AATTxxxxxAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-3'

   P2 top: 5'-/5Phos/CTCAGGCATCACTCGATTCCTCCGAGAACAA-3'
   P2 bottom: 5'- CAAGCAGAAGACGGCATACGACGGAGGAATCGAGTGATGCCTGAG*T-3'

   Forward amplification primer:
   5'AATGATACGGCGACCACCG*AGATCTACACTCTTTCCCTACACGACGCTCT-3'
   Reverse amplification primer: 5'-CAAGCAGAAGACGGCATACG*A-3'

   /5Phos/ is an added phosphate for ligation, and * denotes a thiol linker. [x] is the barcode.

4. Restrict 1 μg DNA for two hours at 37ºC in a 30 μL volume using 1.5 μL of NEB EcoRI HF 3 μL of NEB buffer 2. Do not exceed 2.5 μL of total enzyme (higher glycerol concentration is bad). Digest more than you need and check digestion by gel.
5. Inactivate restriction enzyme at 65ºC for 20 minutes.
6. Run 2 μL on a 2% TAE agarose to check for complete digestion. Re-digest with 1 μL of additional enzyme if not digested. After second digestion, check again by gel.
7. Ligate P1 adapter to each restriction sample.

Setup:
- 28 μL restriction sample (or whatever is left after checking by gel ~850 ng)
- 5 μL of 100 nM annealed P1 Adapter
- 2 μL NEB Buffer 2
- 0.5 μL of 100 mM rATP (Promega #E6011)
- 2 μL (1 U) T4 DNA ligase (Invitrogen, 1U/μL)
- Water to 50 μL final volume

8. Incubate at 25ºC for 20 minutes.
9. Heat inactivate at 65ºC for 20 minutes.
10. Pool samples at equimolar ratios or skip (5 μg maximum per pool). Six or more samples should be pooled because fewer leads to the sequencer failing when trying to read the cut site which is common to all sequences. Alternatively, the barcode could be five bp long, which helps to prevent some of the sequencer issues.
11. Add samples to 750 μL of shearing buffer (TE, pH 8.0 with 50% glycerol). Add a maximum of 250 μL of sample to 750 μL of shearing buffer. Pipette the samples into the nebulizer (Invitrogen #K7025-05)
12. Screw on blue cap finger-tight. Do not over tighten.
13. Keep the nebulizer on ice throughout the nebulization.
14. Shear the DNA by nebulizer to an average size of 400 bp for 6 minutes at 42 psi. Vapor != DNA loss.

15. Spin the DNA solution down in centrifuge in the microarray facility at 450 g for two minutes.
16. Add 3 mL of Qiagen buffer PB1 to the sample. Mix by pipetting. Use low-retention tips.
17. Concentrate the entire sample in a single Qiaquick spin column.
18. Add 750 μL Buffer PE and spin at 13,000 rpm for 30 seconds. Discard the flow-through.
19. Spin again for 30 seconds.
20. Put the column in a sterile 1.5 mL low-retention microfuge tube.
21. Add 30 μL Buffer EB and incubate at room temperature for at least one minute.
22. Spin at 13,000 rpm for 30 seconds.
23. Blunt the fragments using the Quick Blunting Kit (NEB #E1201L).

Setup:
30 μL eluted DNA
5 μL Blunting Buffer
5 μL 1 mM dNTP mix
2 μL Blunt enzyme mix
8 μL sterile water

24. Mix and incubate at 20ºC for 30 minutes.
25. Heat inactivate at 65ºC for 20 minutes.
26. Purify the samples using a QiaQuick Spin column (Qiagen), as above (5 volumes of Buffer PB, 250 μL). Elute into 30 μL Buffer EB.
27. Tail the samples.

Setup:
30 μL eluted DNA
5 μL of NEB Buffer 2
1 μL of 100 mM dATP (Fermentas #R0141)
3 μL (15 units) Klenow exo- (NEB #M0212L)
11 μL of water

28. Mix and incubate at 37ºC for 30 minutes to create 3' end overhangs.
29. Cool to room temperature slowly on the bench.
30. Purify the samples using a QiaQuick spin column (as above). Elute in 32 μL of Buffer EB.
31. Use 2 μL of eluted sample to calculate concentration using Quant-it DNA BR kit (Invitrogen).
32. Ligate buffer P2 at a 10:1 adaptor to DNA ends ratio. Calculate X using Promega's BioMath website (Micrograms to picomoles).

Setup:
30 μL eluted DNA
X μL of 10 μM annealed P2 adapter
10 μL Invitrogen 5X ligase buffer
2 μL T4 DNA ligase (Invitrogen, 1U/μL)
Water to 50 μL final volume

33. Run out the entire sample on a 2% TAE agarose gel.
34. Excise the gel from 200 bp to 400 bp into a 15 mL conical.
35. Dissolve the gel slice at room temperature in three volumes of Buffer QG. Use low-retention tips.
36. Once dissolved, add one gel volume of isopropanol.
37. Concentrate and clean-up using a QiaQuick spin column (as above). Elute in 40 μL of Buffer EB.
38. Amplify the fragments using Phusion Master Mix (NEB #F-531S) and the amplification primers. 18 cycles according to the Phusion Master Mix parameters, except 57ºC annealing temperature.

Setup:
5 μL of eluted DNA
5 μL of 10 μM amplification primer mix (0.5 μM final concentration of each primer)
25 μL Phusion High-Fidelity (HF) Master Mix (NEB #F-531L)
15 μL water

39. Elute the DNA using Agencourt Ampure XP beads.

A. Add 90 μL of beads for every 50 μL of PCR. Mix by pipetting 10 times. Let sit for 5 minutes.
B. Put on magnet for 2 minutes.
C. Remove supernatant.
D. Wash twice with 200 μL of 70% ethanol. Dispense over pellet while on magnet. Let sit for 30 seconds and then remove wash.
E. Dry for 5 minutes.
F. Elute into 40 μL EB by pipetting 10 times.
G. Put on magnet for 1 minute and move supernatant to a new tube.

40. Using the Quant-it DNA HS kit and 2-5 μL of purified sample, check the concentration on Qubit fluorimeter.
41. Calculate molarity using Promega's BioMath website (Micrograms to picomoles program).
42. Dilute to 10 nM. Remember 1 μM = 1 pmol/μL. Most samples are between 1-2 pmol in ~40 μL.
43. Submit for Illumina sequencing.