Genotyping by Sequencing Protocol – Buckler Lab

Rob Elshire has developed this protocol and it has been published in PlosOne. http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0019379 Please feel free to use, and please cite the article. Our author list and title is: RJ Elshire, JC Glaubitz, Q Sun, JA Poland, K Kawamoto, ES Buckler, SE Mitchell. ***A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species.***

Please see the Buckler Lab website for updates and further information.

http://www.maizegenetics.net/

96 Plex Genotyping by Sequencing Protocol

Before You Start

Preparing Adapters

Resuspend lyophilized oligos in TE to 200uM. (Multiply the nmoles by 5 to get the volume of TE you need to use.)

Annealing Adapters:

- In a PCR plate bring together the barcoded adapters
- In a 0.2 ml tube bring together the common adapter
- Top strand DNA oligo (200 μM) 25ul
- Bottom strand DNA oligo (200 μM) 25ul
- TE 50ul

Total volume 100ul
Annealed Molarity: 50mM

In Thermocycler:

- 95 degrees for 2 minutes
- Ramp to 25 degrees by 0.1 degree per second.
- Hold at 25 degrees for 30 minutes.
- Hold at 4 degrees forever.

First Dilution:

Bring together barcoded adapters in 1.1 ml microdilution tubes:

- 5.6ul of annealed barcoded adapters
- 995ul of 1X TE
- Vortex and spin down

Total Volume: 1,000 ul.

Bring together common adapter in 1.5ml centrifuge tube:

- All of the annealed common adapter (100 ul)
- 900 ul of 1X TE
- Vortex and spin down

Total Volume: 1,000 ul.

Quantification:

Quantify the barcoded and common adapters using PicoGreen.

Concentrated Stock:

The adapters work in pairs for the PCR step in the protocol. You must have each of them in the ligation for it to proceed properly. The second (or common) adapter is the same in each ligation. You will mix the barcoded adapter with the common adapter to get the concentrated stock.

Bring together in a PCR plate:

- 300 ng barcoded adapter
- 300 ng common adapter
- Add 1x TE to make 200 ul
- Vortex, spin.
- The concentrated stock is at 3ng/ul.

Working Adapter Stock:

- Dilute the above stock mix 1:4 stock:water.
  - The working adapter stock is at 0.6ng/ul.
- The amount used in this protocol is optimized for maize. If using another species theoptimal amount must be determined experimentally.

Adapter Plate Setup

This protocol multiplexes 96 samples into one lane on the Illumina GA. We process 96 samples at a time with adapter layout as shown below (BC1 is bar-coded adapter 1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC1	BC2	BC3	BC4	BC5	BC6	BC7	BC8	BC9	BC10	BC11	BC12
B	BC13	BC14	BC15	BC16	BC17	BC18	BC19	BC20	BC21	BC22	BC23	BC24
C	BC25	BC26	BC27	BC28	BC29	BC30	BC31	BC32	BC33	BC34	BC35	BC36
D	BC37	BC38	BC39	BC40	BC41	BC42	BC43	BC44	BC45	BC46	BC47	BC48
E	BC49	BC50	BC51	BC52	BC53	BC54	BC55	BC56	BC57	BC58	BC59	BC60
F	BC61	BC62	BC63	BC64	BC65	BC66	BC67	BC68	BC69	BC70	BC71	BC72
G	BC73	BC74	BC75	BC76	BC77	BC78	BC79	BC80	BC81	BC82	BC83	BC84
H	BC85	BC86	BC87	BC88	BC89	BC90	BC91	BC92	BC93	BC94	BC95	BC96

Sample Prep Protocol

Plate out adapters

Make an adapter working stock plate of sufficient volume to make the number of adapter plates you require.

- Plate in array 6ul of each working adapter stock (or optimum amount as determined by titration) using the BioMek (See adapter protocol).
- Spin briefly.
- Cover with Airpore tape.
- Dry down using a SpeedVac Concentrator.

Plate out Sample DNA

- Plate out 10 ul of 10ng/ul sample DNA into 96 the well plate containing the dried down adapters using BioMek.
- Cover with Airpore tape.
- Spin Briefly.
- Dry down using a SpeedVac Concentrator.

Make a master mix for the Digest

Make a master mix for the number of reactions you need.
Bring together on ice:

Reagent	1 rxn	1 plate	4 plates
NEB Buffer 3	2	220	825
ApeKI (NEB R0643L)	1	100	375
H2O	17	1880	7,050
TOTAL	20	2,200	8,250

Get 2, 1.5 ml centrifuge tubes and pipette 1,100 ul of mix into each tube.

Digest

- Transfer 20 ul to each well using TECAN robot. (Add 1 ml of digest master mix to each centrifuge tube before starting the next plate.)
- Spin down.
- Cover with a rubber sealing mat (Micronic MP53012). (prevents loss)
- Incubate for 2 hours at 75 degrees C.
- Drop to 4 degrees C and hold.

Ligation

Master Mix

Make a master mix in for the number of reactions you need.
Bring together on ice:

Reagent	1 rxn	1 plate	4 plates
10x T4 DNA Ligase Reaction Buffer	5	550	2,063
T4 DNA Ligase (NEB, MK0202L)	1.6	176	660
H2O	23.4	2,574	9,653
TOTAL	30	3,300	12,376

Get 4, 1.5 ml centrifuge tubes and pipette 800 ul of mix into each tube.

Ligation Reaction

- Transfer 30ul of master mix into each well using TECAN robot. (Add 750 ul of ligation master mix to each centrifuge tube before starting the next plate.)
- Spin down.
- Ligate at 22 degrees C for 60 minutes.
- Heat at 65 for 30 minutes.
- Cool to 4 degrees C.

Pooling and cleanup

For this step, use a new tip for each well and rinse the tip in the PB buffer (provided in Qiagen PCR Cleanup Kit) to recover equal amounts of the product. If using the TECAN for this step, rinse the tips in the buffer and wash the tips after each transfer to prevent cross contamination.

- Take 5ul from each well and add to a 1.5mL eppendorf tube containing 1250ul of binding buffer (for 48 samples). Use two 1.5mL eppendorf tubes per plate.
- Cleanup with Qiagen PCR cleanup kit per kit instructions running both tubes from previous step through one column during binding.
- Elute in 50ul EB.

PCR Amplification

1. Prepare the following PCR reaction mix:

   DNA (2ul) -- this is the adapter modified dna fragments

   NEB 2x Taq Master Mix (NEB #M0270S) (25ul)

   PCR Primers 1 and 2 (2ul @ 25uM mix – 12.5uM of each primer)

   Water (21ul)

The total volume is 50ul.

2. Amplify using the following PCR protocol:

a. 5 minutes at 72deg C.

b. 30 seconds at 98deg C.

c. 18 cycles of:

10 seconds at 98deg C.

30 seconds at 65deg C.

30 seconds at 72deg C.

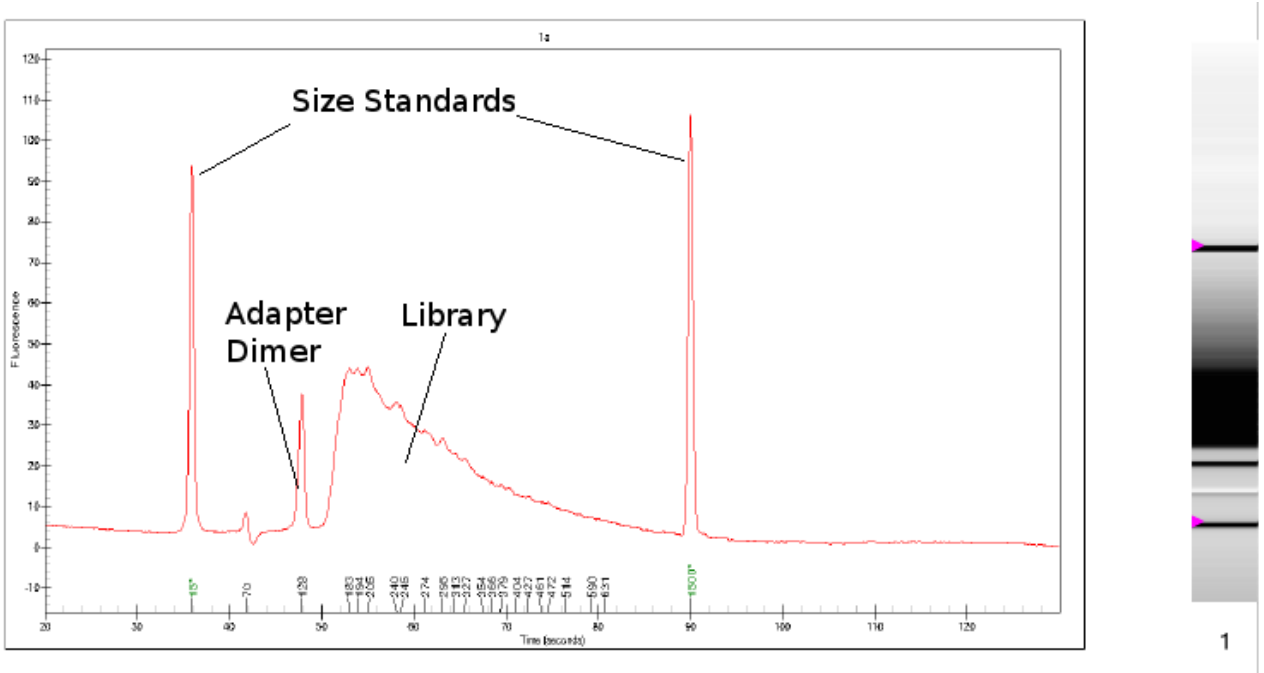
d. 5 minutes at 72deg C.

e. Hold at 4degC.

3. Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick column, eluting in 30ul of EB.

Validation on Experion

The following figure is an example of a library run on the BioRad Experion. The library seen in this figure is typical for *maize.* This example contains a large adapter dimer peak. These adapter dimers will generate clusters on a flow cell and will be sequenced – wasting cluster space and money. The above protocol was optimized for *maize* such that no adapter dimers are created. In order to optimize this for your material, start with our adapter and genomic DNA amounts and test more and less adapters on individual samples.



DNA Quantification and Quality

This protocol relies on the proper ratio of adapters to genomic DNA sticky ends. Factors that can affect this ratio include accurate DNA quantification and variation in DNA quality. Poor quality DNA samples will not digest completely. Differentially complete digestion across the samples in the 96 plex and/or differing amounts of sample DNA can cause varying ratios of adapters to sticky ends and therefore variation in the number of reads from any given sample.

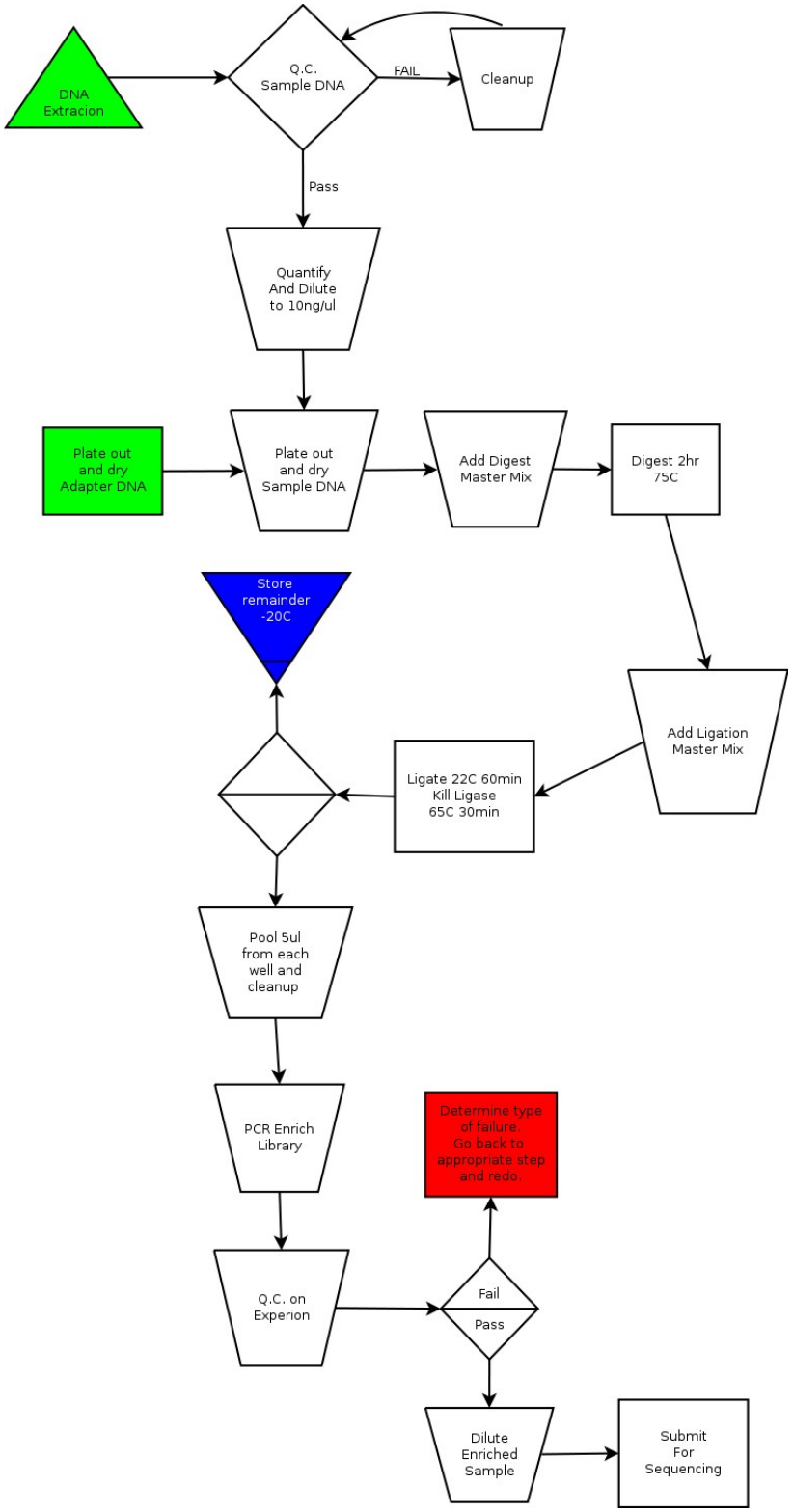
Quantification

We use the pico green method for genomic DNA quantification. Our tests with the Nanodrop have shown that it over estimates the amount of genomic DNA and does not give reproducible results.

Quality assessment

We do use spectroscopy to assess quality. Protein contamination is assessed by the 260/280 ratio. A 260/280 ratio greater than 1.7 indicates that the sample is free from protein contamination. Polysaccharide contamination is assessed by the 260/230 ratio. A 260/230 ratio greater than 1.7 indicates that the sample is free from polysaccharide contamination.

Workflow Diagram



Adapter Design Design and Ordering

The adapters are designed with the following considerations: ligate with sticky end (and middle base wobble) of the ApeKI cut site, the adapters are designed based on the paired end sequences (see:http://seqanswers.com/forums/showpost.php?p=1576&postcount=7) which works for either single read or paired end read, variable length (4-8bp) bar codes in a set for one end and a common adapter (sticky end and middle base wobble as above)for the other, some built in redundancy so sequencing errors would not result in wrong assignment of a sequence to a sample.

I ordered the adapters as single stranded oligos and ligated them as above. The oligos were synthesized by MWG Operon at a Scale: 10 nmol (60-mer limit) and Purification : HPSF (10-100 mers). Attached is the list of bar coded adapters as I ordered them. For example, the sequence of the barcoded adapters is: 5'-CWGxxxxAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT and 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTyyyy, where "xxxx" and "yyyy" denote the barcode complement and barcode sequences, respectively. A compatible set of 96 barcode sequences that have been used for multiplex sequencing is provided in the following table:

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CTCC	**TTCTC**	**TCGTT**	**CTATTA**	**AATATGC**	**TGCAAGGA**
TGCA	AGCCC	GGTTGT	GCCAGT	ACGTGTT	TGGTACGT
ACTA	GTATT	CCAGCT	GGAAGA	ATTAATT	TCTCAGTC
CAGA	CTGTA	TTCAGA	GTACTT	ATTGGAT	CCGGATAT
AACT	ACCGT	TAGGAA	GTTGAA	CATAAGT	CGCCTTAT
GCGT	GCTTA	GCTCTA	TAACGA	CGCTGAT	AACCGAGA
CGAT	GGTGT	CCACAA	TGGCTA	CGGTAGA	ACAGGGAA
GTAA	AGGAT	CTTCCA	TATTTTT	CTACGGA	ACGTGGTA
AGGC	ATTGA	GAGATA	CTTGCTT	GCGGAAT	CCATGGGT
GATC	CATCT	ATGCCT	ATGAAAC	TAGCGGA	CGCGGAGA
TCAC	CCTAC	AGTGGA	AAAAGTT	TCGAAGA	CGTGTGGT
TGCGA	GAGGA	ACCTAA	GAATTCA	TCTGTGA	GCTGTGGA
CGCTT	GGAAC	ATATGT	GAACTTC	TGCTGGA	GGATTGGT
TCACC	GTCAA	ATCGTA	GGACCTA	ACGACTAC	GTGAGGGT
CTAGC	TAATA	CATCGT	GTCGATT	TAGCATGC	TATCGGGA
ACAAA	TACAT	CGCGGT	AACGCCT	TAGGCCAT	TTCCTGGA

The common adapters 5' to 3' are:CWGAGATCGGAAGAGCGGTTCAG CAGGAATGCCGAG and CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT. The primers for the pcr step 5' to 3' are: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT and

CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT