SCHNABLE LAB
Plant Genomics

2035C Roy J. Carver Co-Laboratory
Plant Sciences Institute
Iowa State University
Ames, Iowa 50011-3650
515 294-7209
FAX 515 294-5256

Genotyping by Multiplexing Amplicon Sequencing

(Last revised 6 July 2015 – Wei Wu, Sanzhen Liu, and Patrick Schnable -- Unpublished)

1. Primer mix

a) Order primers with normalized dry condition (10 nM) 25 nM scale
b) Hydrate primers with (100 ul) PCR grade water and dilute to 100 uM final concentration
c) Mix primers

The primer design may divide the total number of primers into several plexes (groups), on average 30 SNPs/plex. For each plex, take 5 ul of each primer (including 1st and 2nd , 5ul each), and combine them in one sterile reservoir. Add water to make up to 1000ul. For example,

30 primers x 5 ul= 150 ul x 2 = 300ul + 700 ul water = 1 ml

If you want to increase the plexing level, you can combine two plexes into one plex, so 60 primers x 2 x 5ul = 600ul + 400ul water = 1ml. After combing 60 pairs of primers, add 400 ul of water to add up to 1000 ul.

2. Aliquote samples onto 384 plates.

Each reaction requires 2 ul of gDNA at a concentration from 10ng/ul -50ng/ul. Spin the DNA samples down after aliquot.

The following table is an example of layout. In this example, there are total 96 samples and 4 plexes (~120 SNPs). Each sample is duplicated four times on the plate (2 ul each well).

SCHNABLE LAB
Plant Genomics

2035C Roy J. Carver Co-Laboratory
Plant Sciences Institute
Iowa State University
Ames, Iowa 50011-3650
515 294-7209
FAX 515 294-5256

Aliquot DNA samples in 384-well plate: (total 96 samples x 4 PCR reaction)

384-well	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24	Plex #
A B C D	AB CD EF GH	1
E F G H	AB CD EF GH	2
I J K L	AB CD EF GH	3
M N O P	AB CD EF GH	4

3. PCR mix set up

	final conc. in 5ul	5 ul vol (1x)(uL)	96 wells + 25%
Water	-	0.85	102
Qiagen Hotstar PCR Buff. (10x) (15mM Mg^{2+})	1.25 X	0.625	75
MgCl2 (25 mM)	1.625 mM	0.325	39.6
dNTPs (25 mM ea.)*	500 uM	0.1	12
Primer Mix (500 nM ea.)	100 nM	1	120
KBio science Klear Taq 5U/ul	1 U/rxn	0.2	24
	Total	**3**	**372**

Dispense 3 ul of the PCR cocktail into each well on the 384 plate with samples.
PCR program: Wei Wu> Touchdown PCR cycles
Steps:

SCHNABLE LAB
Plant Genomics

2035C Roy J. Carver Co-Laboratory
Plant Sciences Institute
Iowa State University
Ames, Iowa 50011-3650
515 294-7209
FAX 515 294-5256

1) 94°C 10 min
2) 94°C 20 sec
3) Touchdown over 64-56 1 min
   10 cycles of step 2 and 3 (0.8°C decrease per cycle)
4) 94°C 20 sec
5) 57°C 60 sec
6) 68°C 30 sec
   20 cycles of step 4 to 6
7) 72°C 3 min
8) 4°C forever
   Quick spin before the next step.

4. Dilute the 1st PCR plate and pool all amplicons from one sample.

- Add 15 ul PCR water into each well
- Seal plate with films, vortex, and then quick spin
- Take 5 ul out into a new plate, and put all reactions with the same sample together into one well. For example: A1, E1, I1 and M1 are combined together in one well (on a new PCR plate). These four wells have the same DNA samples with different PCR primer plexes.
- Mix the plate well after sealing, and then spin down

5. Barcoding PCR amplicons

- Take 2 ul of DNA from each mixed amplicon pool into a new PCR plate
- Add 1 ul 100nM barcode primer into each well separately
- Make PCR mix according the chart below

	final conc	5 ul vol (1x)(uL)	96 wells + 25%
water	-	0.85	102
Qiagen Hotstar PCR Buff. (10x) (15mM Mg^{2+})	1.25 X	0.625	75
MgCl2 (25 mM)	1.625 mM	0.33	39.6
dNTPs (25 mM ea.)*	500 uM	0.1	12
Primer Mix (500 nM ea.)	100 nM	0	0
KBio science Klear Taq 5U/ul	1 U/rxn	0.2	24
Aliquot master mix	**Total**	**2**	**253**

SCHNABLE LAB
Plant Genomics

2035C Roy J. Carver Co-Laboratory
Plant Sciences Institute
Iowa State University
Ames, Iowa 50011-3650
515 294-7209
FAX 515 294-5256

- Dispense 2 ul of PCR cocktail mix into each well with samples and primers.
  PCR program: Wei Wu> Touchdown PCR cycles
  Steps:

  1) 94°C 10 min
  2) 94°C 20 sec
  3) Touchdown over 64-56 1 min
     10 cycles of step 2 and 3 (0.8°C decrease per cycle)
  4) 94°C 20 sec
  5) 57°C 60 sec
  6) 68°C 30 sec
     20 cycles of step 4 to 6
  7) 72°C 3 min
  8) 4°C forever
     Quick spin before the next step.

6. Pool each population/library
- Add 15 ul of water into each well
- Take out 5 ul from each well and combine them into a clean reservoir.

7. AMPure Purification (>100 bp selection)
1) Shake AMPure beads to fully resuspend the magnetic beads before using.
   Note: Equilibrate AMPure beads to room temperature before using.
2) Add 1.8x mixed AMPure beads to PCR reaction.
3) Mix by pipetting five times and incubate for 5 min at room temp.
4) Place the tube on magnetic stand for ~2 min to let beads separate from solution.
5) Remove supernatant and discard.
6) Rinse beads twice with 200 ul of 70% ethanol, incubating 30 sec before discarding ethanol.
7) Dry beads for ~5-15 min to remove traces of ethanol. Do not overdry (lots of cracking).
8) Add 40 ul elution buffer.
9) Place tubes or plate on magnetic plate for ~1 min.
10) Transfer eluent to new tube or plate.

8. nanodrop or DNA analyzer to quantify DNA.

9. The purified PCR pool is ready for NGS library construction.