UPDATED: 14 July 2016
GBS protocol for 144 Samples using CviAII (NEB R0604)
Before starting
DNA concentration: Put 100ng of DNA for each genotype in a PCR plate and lyophilize in the speed vac. Remember to cover with the airpore tape (QIAGEN). This PCR plate will be used for library preparation (See pg. 2).
N.B. You can also use an internal control (the genotype of the reference genome or another species) and a blank for each library.

Barcode preparation
Resuspend oligos at 200uM in TE buffer (Multiply the nmoles by 5 to get the volume of TE to use). Keep oligos in plates and transfer with a multichannel pipette to another PCR plate for annealing.

Annealing adapters
- Put everything in a 96 well PCR plate
- 12.5 ul fwd oligo
- 12.5 ul rev oligo
- TE 25 ul

Total volume 50ul

In thermocycler
- 95 C for 2 min
- Ramp to 25 C by 0.1 degree x sec
- hold at 25 for 30 min
- Hold at 4 C

Dilution: dilute 50ul annealed adapter with 950ul TE 1X. Vortex and spin. Quantify with QUBIT high sensitivity kit.

Working Adapter Stock: Mix 150ng barcoded adapter with 150 ng of common adapter. Add TE 1X to 200ul. The stock solution is at 1.5 ng/ul. For each reaction we need to add 4.5 ng of working adapter (3ul for each reaction).

Sample Prep Protocol (GBS 144 multiplexed) UPDATED 14 June 2016

Digestion

In a 96 well plate with 100ng dry DNA (pg. 1) plate **3ul** of Working adapter stock and **17ul** of digestion mix.

Digestion mix (20 ul total final volume)

- **2ul** CutSmart buffer
- 5U/reaction of CviAII. If Using the NEB R0640L (10 U/ul), use **0.5ul/reaction**
- **14.5ul** H_2O

For all the 144 reaction prepare a mix for 156 reaction

Mix for 156 reaction (use the boxes and the multichannel pipette)

- 312ul CutSmart buffer
- 78ul CviaII (NEB R0640L, 10 U/ul)
- 2262ul H_2O

V tot 2652ul
Aliquot 17ul in each well
Spin down the plate.
Incubate for **2h at 25 C**, hold at 4 degree. Program **CVICUT**

Ligation

Ligase Mix (30ul of total volume)

- 3ul T4 Ligase buffer (10x)
- 1.6ul T4 DNA ligase (NEB M0202L)
- 25.4ul H_2O

Mix for 155 reaction (use the boxes and the multichannel pipette)

- 465ul T4 Ligase buffer (10x)
- 248ul T4 DNA ligase (NEB M0202L)
- 3937ul H_2O

V tot 4650ul
Aliquot 30ul in each well (final volume 50ul per well)
Spin down the plate. Ligate a 22° for 60 min, heat 65°C for 30 minutes, Cool to 4° and hold. Program **LIGATION**

Pooling and Clean-up

- Take 7ul from each well with a multichannel pipette, and transfer to a petri dish. When finished to transfer mix everything. Transfer 360ul of mix in 2 different 1.5ml eppendorf tubes.
- Clean with the GeneJET purification kit (use additional step with isopropanol binding for fragments <500bp) **running both tubes from previous step in the same column (add up to 800ul until all the mix is finished)**. Centrifuge at 15000g
- Elute in 50ul

PCR amplification
Prepare 4 different reaction and mix them after PCR, before clean-up step
PCR mix (50ul total)

- 2ul DNA
- 25ul NEB2x Taq master mix
- 2ul mix primer A & B (mix at 12.5uM for each primer)*
- 20.4ul H_2O

*100uM stock, mix at final concentration of 12.5uM for each primer (divide by 8, 2.5ul of each stock primer with 15ul H_2O in 20ul)

Mix for 5 Reaction

- 10ul DNA
- 125ul NEB2x Taq master mix
- 10ul mix primer A & B (mix at 12.5uM for each primer)*
- 102ul H_2O

Aliquot 50ul in 4 different PCR tubes

Amplification:

- 5 min 72°C
- 30 sec at 98°C
- 18 cycle: 98°C for 30s, 65°C for 30s 72°C for 30s
- 5 min 75°C
- hold 4°C

Program **ENRICH**

Final clean up (see 1st clean up for details)

- Mix the different PCR reactions and clean-up with the GeneJET purification kit (use additional step with isopropanol binding for fragments <500bp)
- Elute in 30ul
- Quantify on QUBIT and check with experion/bioanalyzer