PstI-MspI GBS
Genotyping-by-sequencing Protocol PstI-MspI

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<u>Anneal Adapters</u>
1X Elution Buffer (EB) – 10mM Tris-Cl, pH 8.0-8.5
10X Adapter Buffer (AB) – 500mM NaCl, 100mM Tris-Cl

This is completed in plates for the barcoded adapters (Adapter1)

1. Suspend (dried) single-stranded adapter oligos to 100uM in 1X EB
2. Make 100ul of 10uM double stranded adapter:
   10ul – each single stranded oligo (@ 100uM)
   10ul – 10X AB
   70ul – H_20
   Heat to 95C and cool at 1C per/minute to 30C. Hold at 4C.
   (program "anneal")
3. Dilute adapters 3:10 to ~3uM and quantify using PicoGreen.
4. Normalize to 1.5 – 1.8 ng/ul (=0.1uM) depending on barcode length. *Note: These are the ng/ul concentrations for the short version of Adapter 1. See below. Full length adapters will need to be a higher ng/ul concentrations for the same molarity.*

bc length	ng/ul for 0.1uM
5	1.49
6	1.55
7	1.62
8	1.68
9	1.74
10	1.80

Make MspI common reverse Y-adapter (Adapter2) - single tubes
Common reverse adapters – follow same steps but leave Adapter2 at 10uM for working stock.

Working Adapter Stock
Each well in the working adapter plate will have 0.02uM (unique) barcode Adapter1 and 3uM (common) Adapter2.

In 96-well plate add:
20 ul – Barcoded Adapter1 @ 0.1uM
30 ul - MspI Adapter2 @ 10uM
50 ul – 1X AB
Mix well, spin down

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Normalize DNA

1. Quantify DNA using PicoGreen. Protocol "PicoGreenDNAQuant.doc"
   (DNA concentration must be between 20ng/ul and 150ng/ul for accurate pippetting. If too high, dilute before normalizing.)

Restriction (20ul)

10 ul – DNA (20ng/ul = 200ng total)
10ul – restriction master mix

PstI-HF – NEB #R3140 (20,000 units/ml)
MspI – NEB #R0106 (20,000 units/ml)

Restriction MasterMix:

Plate	Sample
220	2.0 ul – 10X NEB Buffer 4
44	0.4ul – PstI-HF (5 units)
44	0.4ul – MspI (5 units)
792	7.2ul – H_20

QIAgility Program: "PstI-MspI Normalize Digest"

Normalize 10ul DNA @ 20ng/ul into 96-well plate.
Add 10ul Restriction MasterMix to DNA and mix.

Ligation: 37C for 2 hour; 65C for 20 min; hold at 8C (program "reslig")

NOTE: Proceed directly to ligation.

Ligation (40ul)

20 ul - restriction digest
5 ul - Adapters (0.02uM Adapter1 = 0.1pmol, 3uM Adapter2 = 15pmol)
15 ul - Ligation Master Mix

Ligation MasterMix:

Plate	Sample
220	2.0 ul – NEB Buffer 4
44	0.4 ul - ATP @ 100mM (final concentration 1 mM)
55	0.5 ul – T4 DNA ligase (200 U)
1331	12.1 ul - H_20

QIAgility Protocol: "PstI-MspI Adapters Ligation"

To 20ul restriction digest add 5ul Adapters.
Add 15ul Ligation MasterMix and mix.

Incubate at 22C for 2h; 65C for 20 min; 8C forever (program "ligate-kill-hold")

NOTE: Completed ligation can be safely stored at -20C.

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Multiplex
Pool 5ul from each sample ligation to a single tube (Add 20ul to empty tube prior to running robot, total volume = 500ul)
QIAgility program: "Pool 96"

Clean-up on Qiagen column (QIAquick PCR Purification Kit)
For each library do two clean-ups.
1. combine 200ul of pooled Ligation DNA and 1000ul of buffer PB in a fresh tube,
2. add 600ul to column, spin down,
3. add second 600ul to column and spin down
Follow manufacturer's directions and re-suspend in 60ul. Combine the two clean-ups from that library.

Amplification (25ul **x** 8)
Make 8 PCR reactions for each library.

10 ul – DNA (digested library)
5 ul – 5x NEB MasterMix
2 ul – 10uM Illumina Primers PE (F & R) @ 10uM
8ul – H_20

PCR ("solexa")
95C (30 sec)
{ 95C (30 sec), 62 (20 sec), 68C (40 sec) } 17 cycles
72C (5 min)
4C (forever)

For each library, pool the 8 PCR reactions and clean-up on Qiagen column (QIAquick PCR Purification Kit). Follow manufacturer's directions and re-suspend in 30ul

Check library on 1% gel or BioRad Experion.

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ADAPTERS

ADAPTER 1

Barcoded adapters (“Adapter1”)
This is the adapter with variable length barcode on the end. An example of the AAGTGA barcode:

```
5’ CACGACGCTCTTCCGATCTXXXXXTGCA     GNNNNNNN 3’
3’ GTGCTGCGAGAAGGCTAGAXXXXX     TGCACNNNNNNN 5’
```

This is the short version of the barcoded (XXXXX) adapters
(Tm=57)

```
_bot  XXXXXagatcggaagagcgtcgtg
_top  cacgacgctcttccgatctXXXXXtgca
```

ADAPTER 2

Common adapters (“Adapter2”)
These adapters are designed respectively for the second enzyme used in the double digest. They are designed for the Paired-end sequencing. Adaptor2 is a Y-adaptor so the reverse primer (A2Rprimer) can only bind if the forward primer (A1Fprimer) has extended from other end (a PstI site with Adapter1)
Adapter2 has a 5’ CG overhang for MspI. (If an enzyme leaves a 3’ overhang (i.e. PstI) a different adapter combination must be designed)

Adapt2(PE) for MspI (C|CGG).

```
5’ nnnnnnnnC   CGAGATCGGAAGAGCGGGGACTTTAAGC
3’ nnnnnnnnGGC   TCTAGCCTTCTCGCCAAGTCGTCCTTACGGCTCTGGCTAG
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>Adpt2PE_top_cg
cgAGATCGGAAGAGCGGGGACTTTAAGC

>Adpt2PE_bot
GATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

AvaII (G|GWCC)
>Adpt2PE_top_gtc
gtcAGATCGGAAGAGCGGGGACTTTAAGC

>Adpt2PE_top_gac
gacAGATCGGAAGAGCGGGGACTTTAAGC

PRIMERS

The Illumina primers are identical to the oligos on the Illumina flow cells. They also have the complimentary sequence for the Adapter1 (Forward) and Adapter2 (Reverse) respectively.

>IlluminaF_PE (Tm=70) 58bp (Tm=57)
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

>IlluminaR_PE (Tm=70) 46bp (Tm=62)
CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAA

Adapter 1 + primer + barcode = 62-68bp
Adapter 2 + primer = 61bp
Min fragement size = ~125bp