

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

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Anneal Adapters

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 – H₂OHeat 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 pipetting. 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:

<u>Plate</u>	<u>Sample</u>
220	2.0 ul - 10X NEB Buffer 4
44	0.4ul - <i>PstI</i> -HF (5 units)
44	0.4ul - <i>MspI</i> (5 units)
792	7.2ul - H ₂ O

QIAGEN 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:

<u>Plate</u>	<u>Sample</u>
176	2.0 ul - NEB Buffer 4
55	4 ul - ATP @ 10mM (final concentration 1 mM)
55	0.5 ul - T4 DNA ligase (200 U)
1034	8.5 ul - H ₂ O

QIAGEN 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 - H₂O

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 GNNNNNNNN 3'
3' GTGCTGCGAGAAGGCTAGAXXXXX TGCACNNNNNNNN 5'
```

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

```
_bot XXXXXagatcggagagcgtcgtg
_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
```

```
>Adpt2PE_top_cg
cgAGATCGGAAGAGCGGGGACTTTAAGC
```

```
>Adpt2PE_bot
GATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATCT
```

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)
CAAGCAGAAGACGGCATAACGAGATCGGTCTCGGCATTCTGCTGAA
```

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