Genotyping By Sequencing (GBS) Method Overview

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http://www.maizegenetics.net/
Topics Presented

- Background/Goals
- GBS lab protocol
- Illumina sequencing review
- GBS adapter system
- How GBS differs from RAD
- Modifying GBS for different species
- GBS Workflow
Background

Genotyping by sequencing (GBS) in any large genome species requires reduction of genome complexity.

I. Target enrichment

• Long range PCR of specific genes or genomic subsets

• Molecular inversion probes

• Sequence capture approaches hybridization-based (microarrays)

II. Restriction Enzymes (REs)

*Technically less challenging*

• Methylation sensitive REs filter out repetitive genomic fraction
QTL are often located in non-coding regions

*Vgt1, Tb, B* regulatory regions 60-150kb from gene

Map large numbers of genome-wide markers

Exon capture
Our goal is to create a public genotyping/informatics platform based on next-generation sequencing.

Create inexpensive, robust multiplex sequencing protocol → Low-coverage Sequencing Illumina GA → Informatics Pipeline → Impute missing data → Anchor markers across the genome → Combine genotypic & phenotypic data for GS and GWAS.
Open Source

• Method available for anyone to use / modify.
• Analysis pipeline details and code are public.
• Promote dataset compatibility.
• Method published in *PLoS ONE* to promote accessibility.
• Genotype calls publically available.
Overview of Genotyping by Sequencing (GBS)

- Focuses NextGen sequencing power to ends of restriction fragments
- Scores both SNPs and presence/absence markers
GBS is a simple, highly multiplexed system for constructing libraries for next-gen sequencing

- Reduced sample handling
- Few PCR & purification steps
- No DNA size fractionation
- Efficient barcoding system
- Simultaneous marker discovery & genotyping
- Scales very well
GBS 96-plex Protocol
(http://www.maizegenetics.net/gbs-overview)

Now at 384-plex

1. Plate DNA & adapter pair
GBS Adapters and Enzymes

Barcode Adapter

P1

Barcode (4-8 bp)

Illumina Sequencing Primer 1

“Sticky Ends”

Common Adapter

P2

Illumina Sequencing Primer 2

Restriction Enzymes

\[ApeK\] \[5' G \text{CWGC} 3'

\[Pst\] \[CTGCA G\]

\[EcoT22\] \[ATGCA T\]
GBS 96-plex Protocol
(http://www.maizegenetics.net/gbs-overview)

1. Plate DNA & adapter pair
2. Digest DNA with RE
3. Ligate adapters
4. Pool 96 samples
5. PCR
Pooled Digestion/Ligation Reactions (n=94)

PCR

GBS “Library”

PRC primers: P1 O1 P2 O2

Insert
GBS 96-plex Protocol
(http://www.maizegenetics.net/gbs-overview)

1. Plate DNA & adapter pair
2. Digest DNA with RE
3. Ligate adapters
4. Pool 96 DNA aliquots
5. PCR
6. Evaluate fragment sizes
Perform Titration to Minimize Adapter Dimers Before Sequencing

NOTE: Done once with a small number of samples. Adapter dimers constitute only 0.05% of raw sequence reads.
Small Fragments are Enriched in GBS Libraries

- **B73 RefGen v1**
- **IBM (B73 X Mo17) RILs**

The graph shows the distribution of ApeKI fragment size (bp) for B73 RefGen v1 and IBM (B73 X Mo17) RILs. The x-axis represents the ApeKI fragment size in base pairs (bp), while the y-axis represents the total fragments as a percentage. The bar graph indicates that small fragments are enriched in GBS libraries.
384-plex GBS Results for Maize

Mean read count per line = 528,000

C.V. = 0.22
Cluster Formation Amplifies Sequencing Signal

Denatured “Library” → Bridge Amplification → Flow cell with bound oligos → Linearization
Sequencing by Synthesis

HiSeq 2000

Flowcell

T

TG

TGC

TGCA

P1 primer

STOP

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"In Phase"

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"Out of Phase"

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GBS captures barcode and insert DNA sequence in single read
Variable Length GBS Barcodes Solves Sequence Phasing Issues

• First 12 nt used to calculate phasing.
• Algorithm assumes random nt distribution.
• Incorrect phasing causes incorrect base calls.

• Good design and modulating the RE cut site position with variable length barcodes produces even nt distribution.
Invariant GBS barcodes cause loss of signal intensity.
Successful GBS sequencing run.
GBS Adapter Design

a) Barcode adapter

DNA insert

Barcode sequence

Common adapter

5’-ACACTCTTTCCCTACACGAGCTTCGATCT-3’

3’-TGTAAGAAAGGGATGTCTGGAGAAGGCTTACGAGA-5’

b) PCR primer 1

Complement binds to flowcell oligo 1

Binds to 3’ strand of barcode adapter

5’-AATGATACGGCGACCACCGAGATCTACACTTCTCTTACACGAGCTCTTCCGATCT-5’

Paired-end sequencing primer 1

5’-ACACTCTTTCCCTACACGAGCTCTTCCGATCT-5’

c) PCR primer 2

Binds to 3’ strand of common adapter

Complement binds to flowcell oligo 2

TCTAGCCTTCTCAGGAATCGGTCTTTACGGCTCTGGCTAGACGATAACGCAAGA-5’

Paired-end sequencing primer 2

TCTAGCCTTCTCAGGAATCGGTCTTTACGGCTCTGGC-5’
Barcode Design Considerations

• Barcode sets are enzyme specific
  – Must not recreate the enzyme recognition site
  – Must have complementary overhangs
• Sets must be of variable length
• Bases must be well balanced at each position
• Must different enough from each other to avoid confusion if there is a sequencing error.
  – At least 3 bp differences among barcodes.
• Must not nest within other barcodes
• No mononucleotide runs of 3 or more bases

http://www.deenabio.com
Most significant GBS technical issue?

DNA quality
GBS does not use standard “Y” adapters
Same-ended Fragments Do Not Form Clusters

Denatured “library”

Flow Cell with Bound Oligos

Bridge Amplification

Cluster Formation & “Linearization”

Cleaved from surface

No P1 binding site
GBS vs. RAD

- Focuses NextGen sequencing power to ends of restriction fragments
- Scores both SNPs and presence/absence markers
Digest

Ligate adapters

Pool

Random shear

Size select

Ligate Y adapters

PCR

RAD

GBS

Reference

Davey et al. 2011
Modifying GBS

Considerations for using GBS with new species and / or different enzymes.
Why Modify the GBS Protocol?

- More markers
- Fewer markers (deeper sequence coverage per locus)
- Increase multiplexing
- More genome appropriate (avoid more repetitive DNA classes)
- Other novel applications (i.e., bisulfite sequencing).
Genome Sampling Strategies Vary by Species

Dependent on Factors that Affect Diversity:

- Mating System
  (Outcrosser, inbreeder, clonal?)
- Ploidy
  (Haploid, diploid, auto- or allopolyploid?)
- Geographical Distribution
  (Island population, cosmopolitan?)
Other Factors

• **Genome size**
  
  – The size of the genome has some bearing on the size of the fragment pool.
  
  – Amount of repetitive DNA directly correlated with genome size.

• **Genome composition**
  
  – The base composition of the genome can affect the frequency and distribution of the cut sites.
Sampling large genomes with methylation-sensitive restriction enzymes.

Methylated DNA → 5-base cutter → GBS Library → Sequenced Fragments

Unmethylated DNA → 6-base cutter → Sequenced Fragments
Optimizing GBS in New Species

Grape
Maize
Cacao
Barley
Rice
Sorghum
Shrub willow
Raspberry
Cassava
Deer Mouse
Vole
Giant squid
Solitary Bee
Scrub Jay
Grape
Maize
Cacao
Barley
Rice
Sorghum
Shrub willow
Raspberry
Cassava
Deer Mouse
Vole
Giant squid
Solitary Bee
Scrub Jay
Choosing Appropriate Restriction Enzymes

“Life is like a bowl of chocolates”.
Deer Mouse

ApeKI

Pstl
Giant Squid

Goose

ApeKI

PstI
Bovine GBS Libraries

**PstI**

**ApeKI**

**EcoT22I**
### Results for PstI and ApeKI libraries

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Proportion of sequence tags by copy number

- **PstI Library**:
  - Single copy: 93%
  - 2-5 copies: 9%
  - >5 copies: 8%

- **ApeKI Library**:
  - Single copy: 92%
  - 2-5 copies: 9%
  - >5 copies: 9%
Proportion of sequences by copy number

- **ApekI**
- **PstI**

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Bos taurus
PstI SNPs
Chromosome 1

Sorghum bicolor
ApeKI SNPs
Chromosome 1
### GBS SNP calls in *Sorghum bicolor*

**Lots of Missing Data**

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Missing Data Strategies

• **Impute**

• **Technical Options**
  – Reduce the multiplexing level
  – Sequence the same library multiple times

• **Molecular Options**
  – Choose less frequently cutting enzymes
GBS Workflow

DNA sample info entered to webform

Project approved? Samples shipped

GBS libraries made

DNA sequence data

HTS database

Analysis Pipelines

Reference genome

SNPs/Sample

Non-reference genome

States/Sample

Data File

Data File

Lab
GBS Team

Method Development
Rob Elshire
Ed Buckler
Sharon Mitchell

Laboratory/Production
Charlotte Acharya
Wenyan Zhu
Lisa Blanchard

Bioinformatics
Jeff Glaubitz
Qi Sun
James Harriman