SeqCap Epi

Ultra-high complexity oligonucleotide probe pools for targeted enrichment-based bisulfite sequencing: A new technology for DNA methylation analysis

Daniel Burgess, Ph.D. Roche NimbleGen, Inc.
Technologies Converge in Sequence Capture - 2007
first demonstrated by Nimblegen and collaborators at BCM

1. Design oligo probes
2. Hybridize oligo probes to DNA
3. Sequence enriched DNA
4. Align sequence to reference to identify variants

Human Genome Sequence

Direct Selection (1995)

High-Density Oligonucleotide Microarrays
(2.1M features)

High-Throughput Next Generation Sequencing

Human Genome Sequence first demonstrated by Nimblegen and collaborators at BCM (1995)
There are more things in heaven and earth (and genomes)…

...than A, C, G, and T

DNA Methylation:

- Cytosine
- 5-Methylcytosine
- DNMTs
- AdoMet
- AdoHcy

Gene Expression
Dosage Compensation
Imprinting
Genome Stability
Development

DNA methylation
- Methyl marks added to certain DNA bases repress gene activity.

Histone modification
- A combination of different molecules can attach to the ‘tails’ of proteins called histones. These alter the activity of the DNA wrapped around them.

Nature 441, 143-145 (11 May 2006)
DNA Methylation is Very Important in Cancer

inactivation of tumor suppressors

Methylation in CpG islands is progressive (tends to spread)

Tumor Suppressor:

- Methylation goes **UP**
- Expression goes **DN**
- Rate of cell growth goes **UP**
- Fast growing cells take over population

*Jones and Baylin, 2002*
Growth of DNA Methylation Research reflected in growth of PubMed references

**Basic Methods**
- Restriction Enzyme Digestion
- Immunohistochemistry
- PCR
- Microarray analysis
- DNA Sequencing (Sanger, Next-Gen)

**Enabling Technologies**
- 5mC-specific antibodies
- Bisulfite mutagenesis

SEARCH DATE: 06 November 2013
Whole Genome Bisulfite Sequencing (WGBS)  
*powerful and high resolution, but innefficient*

<table>
<thead>
<tr>
<th></th>
<th>H1 human ES cells</th>
<th>IMR90 fetal lung fibroblasts</th>
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<tbody>
<tr>
<td>Genome Analyser II</td>
<td>1.16 billion</td>
<td>1.18 billion</td>
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<tr>
<td>Reads* (Illumina, 2x36):</td>
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<tr>
<td>Sequence yield:</td>
<td>87.5 Gb**</td>
<td>91.0 Gb</td>
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<tr>
<td>Average read depth</td>
<td>14.2×</td>
<td>14.8×</td>
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<tr>
<td>(per strand):</td>
<td></td>
<td></td>
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</table>

- >86% of both strands covered by at least one sequence read, covered 94% of the cytosines in the hg18 genome)
- ~25% of DNA methylation in H1 cells found in non-CG contexts, mCHG and mCHH (H = A, C or T),

* 376 total lanes; ** ~3 HiSeq 2500 lanes (2x100)*
Sequence Capture to Focus Bisulfite Sequencing

“SeqCap Epi”

<table>
<thead>
<tr>
<th>Target Regions</th>
<th>Prepare a MethylSeq Library</th>
<th>Bisulfite Conversion</th>
<th>Pre-Cap Amplify</th>
<th>Capture (probes target CG, CHG, CHH) and wash</th>
<th>Post-Cap Amplify</th>
<th>Sequence HiSeq or MiSeq</th>
</tr>
</thead>
</table>

- Genomic DNA
- Library Preparation with methylated adapters and uracil tolerant polymerase
- Chemical Mutagenesis (C>T conversion)
- Amplification
- Hybridization
- Capture and Washing
- Amplification and QC
- Sequencing
Sequence Capture to Focus Bisulfite Sequencing

workflow options: capture before or after bisulfite conversion?

**SeqCap Epi**
- MethylSeq Library Prep
- Bisulfite Conversion
- Pre-Capture Amplify Library
- Capture
- Post-Capture Amplify Library
- Sequence

**Alternative**
- MethylSeq Library Prep
- Capture
- Bisulfite Conversion
- Post-Capture Amplify Library
- Sequence
A Three-Hit Hypothesis

capture before bisulfite conversion leads to severe bottlenecking

- Higher output complexity
- Lower sample input required
+ Higher reproducibility
- Higher probe density required

SeqCap Epi

MethylSeq Library Prep

Bisulfite Conversion

Pre-Capture Amplify Library

Capture

Post-Capture Amplify Library

Sequence

Alternative

MethylSeq Library Prep

Capture

Bisulfite Conversion

Post-Capture Amplify Library

Sequence

100% input complexity

- lower output complexity
- higher sample input required
- lower reproducibility
+ lower probe density required
Bisulfite Conversion for Sequence Capture

Effectively Doubles Target Size

Cytosines 5’ to guanine may be methylated (m) in the genome

~90% of DNA destroyed by the bisulfite process

Un-methylated cytosine is converted to uracil (U), which is replaced by thymine (T) by DNA polymerase

After PCR amplification, strands are no longer complementary, and any differences in methylation state create different DNA molecules with C vs. T SNP
Intermediate CpG Methylation \textit{with} Bisulfite Conversion creates massive target diversity complicates probe design

- \textit{e.g.} for a short sequence with 3 possible methylation sites, there are 32 possible short fragments that could be produced:

<table>
<thead>
<tr>
<th>mC</th>
<th>Patterns</th>
<th>bs-treated fragments</th>
<th>New PCR strands</th>
<th>Total combinations</th>
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<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>12</td>
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<td>2</td>
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<td>12</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

- Consequence for probe design against a converted genome is probes must capture all possible C>T combinations
  - \(~30\text{M}\) methylate-able C in the human genome
  - typical capture design requires several MILLIONS of probes!
  - Solution? \textit{Innovate probe manufacture}
Maskless array synthesis (MAS)
Smart light to produce spatially ordered chemistry

1. Novel way to direct light
   - High info content (density)
   - Design speed and flexibility

2. Proprietary high-yield chemistry
   - Highest synthesis yields
   - Fast deprotection/coupling
   - High-fidelity long oligo probes
Maskless array synthesis (MAS)
Smart light to produce spatially ordered chemistry

1. Advancements in Feature Density
   2.1M per array
   4.2M per array

2. Multiple Arrays per Probe Pool

= photolabile protecting group
A C T G = nucleotides
Maskless array synthesis (MAS)

Smart light to produce spatially ordered chemistry

- Probes designed to fully-methylated targets
- Probes designed to partially-methylated targets
- Probes designed to fully un-methylated targets

SeqCap Epi Probe Pool
Early Protocol Refinement Using Human Cell lines

3.2Mb test design covering different 5mC configurations

Design targets regions of interest in hg19:

- ~500 gene promoters across a range of methylation occupancy (predicted via roadmap mC Seq from IMR90)
- >400 predicted bivalent domains
- 4 large contiguous imprinted regions (CDKN2A, H19-IGF2, XIST, ChrY region)

Fold Enrichment from Picard HsMetrics
Early Protocol Refinement Using Human Cell lines
3.2Mb design covering different 5mC configurations

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>gDNA INPUT (ug)</th>
<th>% MAPPED READS</th>
<th>% DUP</th>
<th>SAMPLED READS (million)</th>
<th>FOLD ENRICH</th>
<th>% ON TARGET READS</th>
<th>% ON TARGET BASES</th>
<th>MEAN DEPTH (X)</th>
<th>MEDIAN DEPTH (X)</th>
<th>% TARGET BASES &gt;1X</th>
<th>% TARGET BASES &gt;10X</th>
<th>% TARGET BASES &gt;20X</th>
<th>% TARGET BASES &gt;50X</th>
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Early Protocol Refinement Using Human Cell lines
3.2Mb design covering different 5mC configurations

- Input of 0.75 ug is sufficient for good performance (<1 ug of sample)
- With just 2.6M reads 83% of target space is >10x depth (high multiplexing feasible)
- Duplicate read rate is low with the workflow (<10% duplicates is achievable with this design)

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</tbody>
</table>
Benchmarking Technical Performance

general strategies

- **Assess biological variation**
  - Compare a “normal” cell line to a cancer cell line
    - Compare results from NA12762 (CEPH) and NA04671 (Burkitt’s Lymphoma)
    - Expect increase in 5mC at CpG in cancer

- **Assess reproducibility of measurement from samples**
  - Low technical variation from same sample compared means you can trust any differences observed between samples

- **Assess capacity of probe response across methylation range**
  - Utilize a hypomethylated human genome (HCT116 DKO DNMT1/DNMT3A)
  - Titrate 5mC per molecule in a time course of M.SssI treatment

- **Compare with Whole Genome Bisulfite Sequencing (“Gold Standard”)**
Reproducible DMR identification

**male CEPH (NA12762 =4) vs. Burkitt’s Lymphoma (NA04671=2)**

<table>
<thead>
<tr>
<th>Primary Target</th>
<th>Hyper Meth in Cancer</th>
<th>Hyper Meth in Cancer</th>
<th>No Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe Coverage</td>
<td>% G+C</td>
<td>% G+C</td>
<td>% G+C</td>
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<tr>
<td>NA12762 #1</td>
<td>Menzies et al.</td>
<td>Menzies et al.</td>
<td>Menzies et al.</td>
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<td>NA12762 #2</td>
<td>Menzies et al.</td>
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</table>
Low Technical Variation in Methylation Data
*Burkitt’s Lymphoma (NA04671=4)*

<table>
<thead>
<tr>
<th>Sample</th>
<th>PF Reads aligned</th>
<th>% Reads on target</th>
<th>Fold enrichment</th>
<th>% Dup reads</th>
</tr>
</thead>
<tbody>
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<td>4,427,673</td>
<td>53.1%</td>
<td>654.78</td>
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<tr>
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<td>4,732,482</td>
<td>47.3%</td>
<td>583.32</td>
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<td>632.84</td>
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<td>5,401,533</td>
<td>52.4%</td>
<td>646.29</td>
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</table>

- High fold enrichment
- Low duplicate read rate
- High correlation ($R^2$) between methylation data from same sample

<table>
<thead>
<tr>
<th>$R^2$</th>
<th>NA04671 #1</th>
<th>NA04671 #2</th>
<th>NA04671 #3</th>
<th>NA04671 #4</th>
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### Capacity of Probe Response Across Methylation Range

*many different patterns of 5mC are possible*

<table>
<thead>
<tr>
<th>Allelic/mosaic</th>
<th>Non-allelic/population-wide</th>
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</thead>
<tbody>
<tr>
<td>0.50</td>
<td>0.50</td>
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<tr>
<td><img src="image" alt="Allelic/mosaic pattern" /></td>
<td><img src="image" alt="Non-allelic/population-wide pattern" /></td>
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</table>
Capacity of Probe Response Across Methylation Range

experimental manipulation of 5mC in HCT116 DKO cells

- Capture DNA from a hypomethylated human genome: HCT116 DKO
  - Expect low 5mC (HCT116 is a Double Knock out of DNMT1/DNMT3A)

- *In vitro* methylation of HCT116 DKO DNA using M.Sssl (CG Methyltransferase) for a series of time points
  - Expect significant intermediate methylation added

- Make a 50:50 mix between 0 min and 60 min M. Sssl time points
  - Expect “allelic” methylation
Capacity of Probe Response Across Methylation Range

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Intermediate DNA methylation - unmethylated DNA captured  C and T shown
Capacity of Probe Response Across Methylation Range

experimental manipulation of 5mC in HCT116DKO cells

Intermediate DNA methylation - non-allelic methylation captured  C and T shown
Capacity of Probe Response Across Methylation Range

Experimental manipulation of 5mC in HCT116DKO cells

Intermediate DNA methylation - methylated DNA captured  C and T shown
Capacity of Probe Response Across Methylation Range

experimental manipulation of 5mC in HCT116DKO cells

Intermediate DNA methylation - allelic methylation captured  

C and T shown
SeqCap Epi vs. Whole Genome Bisulfite Shotgun

Comparison with published IMR90 methylome mC ratios

All Data: $R^2 = 0.79$

$>100X$: $R^2 = 0.85$

- Lister et al. used GAIIx 36 bp reads
- We used HiSeq 2000 100 bp reads
  - Resequencing IMR90 for better reference?

**SeqCap Epi vs. Whole Genome Bisulfite Shotgun**

3 HiSeq 2000 lanes (WGBS) vs. 1/3th MiSeq lane (SeqCap Epi)

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Which is the “Gold Standard?”

The Importance of Targeting Watson and Crick

example of a different technology targeting one strand resulting in a 470x strand coverage imbalance

The problem is:

- The most common type of SNP between any two individuals is a C->T transition. Many of these occur at CpG
- C->T transition at CpG is 6.7 fold over-represented from expectation
- If a sample has a SNP at a CpG (i.e. TpG), sequence analysis will assume the T is a bisulfite-converted C and report “unmethylated”
- The only way to resolve this with confidence is to read the sequence on the other strand (is it A or is it G?)

* 1 HiSeq lane per captured sample
The Importance of Targeting Watson and Crick

Nimblegen technology provides sufficient probes to target both strands

- Provides the capacity to identify the effect of sequence polymorphisms on 5mC calls
Plans for SeqCap Epi

- A design targeting Illumina 450K microarray CpGs
- Custom design kits targeting up to >200Mb
- Can be designed for species other than human
- Cell- and tissue-type-specific designs
- Further reduction in sample requirements
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