DNA Methylation Mechanisms and Analysis
Methods to Study this Key Epigenetic Control

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Epigenetics in Action

Genetically identical mice

Normal diet

Mother’s diet altered

- Agouti gene activated in some offspring

Normal diet

Obese, diabetic, with increased risk of cancer

- Heritable changes in phenotype or gene expression caused by mechanisms other than changes in the DNA sequence
Epigenetics Overview

http://commonfund.nih.gov/epigenomics/figure.aspx
Overview

1. DNA methylation
2. Introduction of bisulfite conversion chemistry
3. Downstream analysis methods
4. Considerations for obtaining quality data
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**DNA Methylation**

*Primary Epigenetic Modification of DNA*

- The predominant epigenetic modification of DNA in mammalian genomes is methylation of cytosine nucleotides (5-MeC).
- The primary target sequence for DNA methylation in mammals is 5'-CpG-3' dinucleotides.

DNA Methylation
Occurs Primarily at CpG Sites in Promoter Regions

CpG islands

• Definition
  • Small stretches of about 300-3000bp
  • >50% GC content, 5’ regulatory regions

• Methylation status
  • Generally non-methylated in coding regions allowing gene expression
  • Typically methylated in non-coding regions

• Genomic distribution
  • ~70% of promoter regions contain CpG islands
  • Only 1% of remaining genome contains CpG islands
DNA Methylation
Potential Demethylation Pathway and Intermediates

• Covalent addition of -CH$_3$ at the 5’ of the cytosine ring by methyltransferases.

• Mechanism for demethylation is unclear, but may involve TET proteins or oxidative pathways leading to intermediates.

5-methyl cytidine (5mC) → 5-hydroxymethyl cytidine (5hmC) → 5-formyl cytidine (5fC) → 5-carboxyl cytidine (5caC)
DNA Methylation
CpG Methyltransferases

- DMNT3a, DMNT3b
- De novo methylation

HCT116 – double knockout cell line
- DNMT1 and DMNT3b inactive, low level methylation

DMNT1
- Maintenance methylation
- Looks for hemimethylated CpG and maintains methylation pattern following replication

- DMNT3a, DMNT3b
- De novo methylation

SAM
S-adenosyl-L-methionine

Overview

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Studying DNA Methylation
Early Methods Lacked Sensitivity

Methylation-sensitive restriction enzymes combined with Southern blots
  • Requires 5µg DNA with a low percentage of sites methylated

Restriction digests combined with PCR
  • Requires complete digestion of methylated DNA to avoid false positives

Bisulfite modification
  • C’s are converted to U’s
  • Methylated C’s are NOT converted

Introduction of chemistry:
Shapiro, et al. (1973) JBC vol 248, p4060
Application to DNA Methylation Detection
Frommer, et al. (1992) PNAS vol 89, p 1827
Studying DNA Methylation
Bisulfite Conversion Converts Cytosines to Uracils

- Bisulfite Conversion is the most widely used technique for studying DNA methylation
  - Converts non-methylated cytosines to uracil
  - No distinction between 5-methyl cytosine (5mC) and 5-hydroxymethylcytosine (5hmC)

\[
\text{Step 1} \quad \text{Sulfonation pH 5} \quad \text{Step 2} \quad \text{Deamination pH 5} \quad \text{Step 3} \quad \text{Desulfonation pH >10}
\]
Studying DNA Methylation
Bisulfite Conversion Converts Cytosines to Uracils

DNA:

C → U

Cm → No reaction: Cm

PCR with DNA polymerase
Locations of Cm in original DNA
Bisulfite Conversion Chemistry
Harsh Reaction Conditions Degrade DNA

- Original conditions:
  - Denature with NaOH or high heat
  - Incubate at pH 5 for 16-20 hours at ~55°C
  - Causes depurination, fragmentation
  - Early protocols led to loss of >90% starting material

\[
\begin{align*}
\text{Step 1} &: \text{Sulfonation pH 5} & \text{[C]} &\rightarrow & \text{[C-SO}_3^-] \\
\text{Step 2} &: \text{Deamination pH 5} & \text{[C-SO}_3^-] &\rightarrow & \text{[U-SO}_3^-] \\
\text{Step 3} &: \text{Desulfonation pH >10} & \text{[U-SO}_3^-] &\rightarrow & \text{[U]}
\end{align*}
\]
Sodium bisulfite is most common, but formulations include K and NH₃ bisulfite.

Heat and incubation time are important considerations for preserving DNA quality which impact downstream analysis methods.

New commercial kits incubate for 30-60 min.
**Bisulfite Conversion Chemistry**

*Protocol modifications Preserve DNA Quality*

- $\lambda$/HindIII markers with commercially available DNA from Qiagen ($Q = \text{unconverted, } Q,c = \text{converted}$)
- Samples converted at either 64°C or 80°C at pH 5.5 or pH 6.0
  - Higher temperature improves denaturation, but increases fragmentation
  - Higher pH preserves DNA, but lowers conversion efficiency.
Bisulfite Conversion Chemistry
Summary

• Bisulfite Conversion is the most widely used technique for studying DNA methylation

• Harsh conditions (low pH/high temperature)
  • Variation in these parameters affect reaction rate and extent of DNA fragmentation

• Commercial kits and new protocols take only a few hours to complete and often yield less fragmented DNA compared to earlier methods
Overview

1. DNA methylation
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3. **Downstream analysis methods**
4. Considerations for obtaining quality data
Studying DNA Methylation
Downstream Analysis Methods

Wide range of techniques used to study DNA post-bisulfite conversion

- Methylation Specific Restriction Enzymes
- Sanger Sequencing or Pyrosequencing
- Microarrays
- PCR Techniques
  - Bisulfite Specific PCR (BSP)
  - COBRA—determination of methylation at specific RE sites within PCR amplicon
Studying DNA Methylation
Genome-wide Approaches

Sample Preparation Methods
- Bisulfite conversion
- Methylation-specific Restriction Digest
- Methylation-specific Immunoprecipitation

Arrays
- Affymetrix
- Agilent
- Illumina Bead

Deep Sequencing
- Roche/454
- ABI SOLiD
- Illumina SOLEXA

- Arrays: High-throughput, Hybridization/probe-based, Lower specificity
- Sequencing: High-throughput, polymerase or ligase-based, very complicated data analysis
Studying DNA Methylation
Gene-specific Approaches

- COBRA – Combined Bisulfite Restriction Analysis
- Sequencing-based methods: pyrosequencing, CE-based sequencing, cloning and Sanger sequencing
- Semi-quantitative: Uses real-time or gel-based visualization to estimate percentages and general location of methylation
**Bisulfite-specific PCR**

**Primers Must Be Specific to Converted Sequence**

- Bisulfite-specific primer pair (blue): amplifies both bisulfite-converted methylated and bisulfite converted unmethylated DNA
- Wildtype-specific primer pair (orange): amplifies unconverted DNA, both methylated or unmethylated sequences
- Primers do not contain any CpG sites, but do contain non-CpG cytosines

**Non-Converted Forward Sequence** (77 Cs, Tm = 98°C)

```
AAAATGGGCTAGACAAAGGACTGGTGTGTCCCCAGCCAGCGCTGGAGGCCGGCAGCGTGGG
AGGGGAATGGGCAGCCAACAGCTGGGACACCCCCCGGTGCGAGCTACCTACCTAGTCCGCCCGCAGGC
CGGTGCAACAGCTCGCCAGCCAGCCAGCAGGGCCGGGTGCTCCAGATGTGGGCTAGAGGGTGACAGGGT
TAGTTTAATTTGCTTGTTCCCAATCTTAGAAGAG
```

**Bisulfite Converted Forward Strand (Unmethylated)** (0 Cs, Tm = 84°C)

```
AAAATGGGTTGATATAAAAGGATTGGTTTGTGGTTTTAGTATTGGTGGTTTTGGTGTGGATTGGAAGGTG
AGGGGAATGGGTTTGGATTTTGGGTGGATTTTGGGTGGGTTTTGATTTTTTGATTGTTTGGG
TGGGTGTAGATTTTTGGGTAGTGGTGGGTTTTGGTGTGGGTTTTTAGGATGTGGGTAGTTG
TAGTTTAATTGTGGTTTGTATAATTTAGAAGAG
```

**Bisulfite Converted Forward Strand (Methylated)** (25 Cs, Tm = 88°C)

```
AAAATGGGTTGATATAAAAGGATTGGTTTGGTTTTAGTATTGGGCGGGCGGCGGCGGGTGGG
AGGGGAATGGGTTTGGATTTTGGGTGGATTTTGGGTGGGTTTTGATTTTTTGATTGTTTGGG
TGGGTGTAGATTTTTGGGTAGTGGGTTTTGGTGTGGGTTTTTAGGATGTGGGTAGTTG
TAGTTTAATTGTGGTTTGTATAATTTAGAAGAG
```
**Bisulfite-specific PCR**

**Detecting Underconverted DNA**

- Primers do not contain any CpG sites, but do contain non-CpG cytosines

- **Bisulfite-specific primer pair**: amplifies both bisulfite-converted methylated and bisulfite converted unmethylated DNA

- **Wildtype-specific primer pair**: amplifies unconverted DNA, both methylated or unmethylated sequences

Bands here shows unconverted DNA is present

No bands suggest complete conversion

Band indicate bisulfite-converted DNA

Wildtype-specific primers: No bands should be visible if conversion is complete

Bands here shows unconverted DNA is present

No bands suggest complete conversion

Band indicate bisulfite-converted DNA
**Bisulfite-specific PCR**

**Evidence of PCR Bias**

- Most literature examples show preferential amplification of bisulfite-converted unmethylated DNA over bisulfite-converted methylated DNA.
- Common techniques to overcome bias are based on destabilizing GC rich regions and secondary structure of methylated DNA.
- No universal approach has been reported to overcome problem.
- Correction of PCR bias by means of cubic polynomial regression\(^1\):
  - Involves running control samples varying in % methylation and calculating a regression curve.
  - Equation of the best-fitting curve is then used for correction of the data obtained from the samples of interest.

Bisulfite-specific PCR
Preferential Amplification of Methylated DNA

- Template DNA contains a mixture of fully methylated and converted, fully unmethylated and converted DNA.
- In this assay, methylated DNA is preferentially amplified over unmethylated DNA.

Bisulfite-specific PCR
Correction of PCR Bias by Modifying Cycling Protocol


- Tm differences of 2.3 - 5°C allowed selective amplification of unmethylated amplicons over corresponding methylated amplicons

**Melting Peaks**

U: unmethylated and converted
M: methylated and converted
W: unconverted

Both “U” & “M” Peaks

Bisulfite-specific PCR
Designing Primers to Avoid PCR Bias

Suggestions for bisulfite-specific primer design:

1. The fewer CpG sites, the better
2. When CpG sites are present, keep them away from the 3’ end of the primer
3. Salt-adjusted Tm of primer should be around 65°C to run PCR annealing step at 60°C
4. Include one or more T’s near 3’ end of primer (not originating from CpG site)
5. Evaluate primers for secondary structure or primer dimer complications

Wojdacz, et al., 2008, A new approach to primer design for the control of PCR bias in methylation studies, BMC Research Notes, 1:54
Bisulfite-specific PCR
Summary

• Important step for any gene-specific DNA methylation study

• Uses two primer pairs
  • To detect DNA (methylated or unmethylated) that has been bisulfite converted
  • To detect DNA that has not be converted

• PCR bias is a common problem
  • Follow published primer design recommendations
  • Qualify primers up front using validated control DNA sources
  • Can test bias by amplifying mixtures of fully methylated and unmethylated DNA in various ratios
  • If bias is still present, consider additives or experimental redesign
Studying DNA Methylation
Gene-specific Approaches

- COBRA – Combined Bisulfite Restriction Analysis Analysis
- Sequencing-based methods: pyrosequencing, CE-based sequencing, cloning and Sanger sequencing
- Semi-quantitative: Uses real-time or gel-based visualization to estimate percentages and general location of methylation
**Studying DNA Methylation**

**Sequencing Methods for Bisulfite-Converted DNA**

- **Pyrosequencing**
  - Detection of pyrophosphate upon nucleotide incorporation using ATP-coupled luciferase reaction
  - Light produced and measured only when complementary base is added to strand
  - No PCR reaction necessary
  - High-throughput, very expensive

- **Dye-terminator/Sanger Sequencing**
  - Each nucleotide is labeled with a different fluorophore
  - Sequence is read by chromatogram following capillary electrophoresis
  - Cloning and sequencing necessary
  - Low-throughput, more affordable
Bisulfite Sequencing
Direct Sequencing Workflow

- **Purify DNA**
  e.g. Wizard® Genomic DNA Purification and ReliaPrep FFPE Miniprep Systems

- **Bisulfite Conversion**
  *Coming soon!*
  MethylEdge™ Bisulfite Conversion System

- **Bisulfite-specific PCR**
  e.g. GoTaq® Green Master Mix and GoTaq® qPCR Master Mix

- **Clean Up**
  e.g. Wizard® SV Gel and PCR Clean-Up System

- **Amplicon cloning**
  e.g. pGEM®-T Easy Vector Systems

- **Isolate Plasmid**
  e.g. PureYield™ Plasmid Miniprep System

- **Sequence**
  Sequence DNA!
**MethylEdge™ Bisulfite Conversion System**

Streamlined Protocol Complete in Under Two Hours

1. **Prepare sample**
   - 100-500ng DNA in 20µl reactions

2. **Add Conversion Reagent**
   - Add directly to sample
   - Mix by pipetting

3. **Program Thermocycler**
   - 98°C for 8 minutes
   - 54°C for 60 minutes
   - 4°C for up to 20 hours

4. **Desulfonated/Clean-up**
   - All-in-One Spin column format

5. **Downstream assays**
   - Bisulfite-specific PCR
   - Sequencing
   - Etc...

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Promega

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MethylEdge™ Bisulfite Conversion System
Maintaining DNA Integrity

- MethylEdge™ Bisulfite Conversion System leaves DNA less fragmented than the leading commercial kit
- Bisulfite-specific PCR shows amplification of a 524bp fragment
- Direct sequencing indicates >99% conversion

U: unmethylated and converted
M: methylated and converted
W: unconverted
**Downstream Analysis Methods**

**Summary**

**Genome-wide approaches**
- Arrays and deep sequencing
- Facilitate epigenomic mapping

**Gene-specific approaches**
- Many rely on bisulfite-specific PCR prior to analysis
- COBRA, Bisulfite Sequencing, real-time and end-point PCR
- Elucidate epigenetic changes in genes important for specific processes and conditions
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Improving Bisulfite-based Analysis and Data Quality
DNA Concentration Can Affect Conversion Efficiency

**Most commercial kits recommend converting 100-500ng per 20µl reaction for optimal results**

- **Low end = 50pg**
  - Issues: loss of sample, incomplete conversion due to bisulfite:DNA ratio
- **High end = 2µg**
  - Issues: Incomplete conversion due to bisulfite:DNA ratio and possibly incomplete denaturation.

**Sheared vs unsheared DNA**

- Early protocols recommended extreme shearing
  - Advantage: better denaturation, avoid incomplete conversion
  - Disadvantage: starting with smaller fragments leads to even smaller fragments following conversion
- Recommendation: light shearing with 28G needle
Starting with DNA that is sheared will result in smaller fragments following bisulfite conversion.

*Un*: unconverted DNA  
*Conv*: bisulfite-converted DNA
Improving Bisulfite-based Analysis and Data Quality

Absorbance Scans Can Detect Impurities

- Assess DNA quality following bisulfite conversion
  - Run absorbance scan to estimate concentration and look for impurities
Improving Bisulfite-based Analysis and Data Quality
Visualizing DNA Provides Insight for Future Analysis

- Assess DNA quality following bisulfite conversion
  - Run samples on an agarose gel stained with a fluorescent total nucleic acid stain to visualize fragmentation
Improving Bisulfite-based Analysis and Data Quality
Control Assays and DNA Sources Are Critical

• Control Assays
  • Because the presence of a C following conversion is interpreted as a methylated site, conversion efficiency is critical!
  • Few published studies reference including control samples in bisulfite conversion, but studies looking at error rates indicate there are a number of factors that can influence conversion efficiency

• Always include a qualified DNA source in experiments
  • Qualified fully methylated or unmethylated DNA to run alongside unknowns during conversion
  • Qualified bisulfite-converted fully methylated or unmethylated DNA to run alongside bisulfite-converted unknowns in downstream assays.
Improving Bisulfite-based Analysis and Data Quality

Bisulfite Control Assay Workflow

- Methylated DNA
- Unmethylated DNA
- Unknown Sample

Bisulfite Conversion

Perform Quality Checks
- Absorbance Scan
- Total Nucleic Acid Gel Stain

Control PCR Assay
- End-point or Real-time

Interpret PCR Data
- Ensure controls amplify as expected

Direct Sequencing
- Calculate conversion efficiency
Improving Bisulfite-based Analysis and Data Quality

Summary

• Bisulfite:DNA ratio is important for conversion efficiency
• Most protocols are optimal at 100-500ng DNA/20uL reaction
• Denaturation is critical for full conversion
• Light shearing of source DNA is recommended
• Control assays are critical to ensure proper interpretation of data
• If sample is available, always assess purity and quality of bisulfite-converted DNA with an absorbance scan and agarose gel
DNA Methylation Mechanisms and Analysis Methods to Study this Key Epigenetic Control

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Questions?