Optimizing the DNA Methylation Analysis Workflow

Karen Reece, Ph.D.
Senior Research Scientist
Understanding the Challenges of DNA Methylation Analysis and Possible Solutions

Agenda

✓ Introduction to epigenetics
  • Overview
  • Mechanisms
  • Why study?

✓ Challenges/solutions at each step of DNA methylation analysis
  • gDNA purification
  • Quantitation
  • Bisulfite conversion

✓ Review analysis methods
  • Advantages and disadvantages of each
Epigenetics in Action
A Different Form of Gene Regulation

Genetically Identical Mice

Normal Diet

Obese, diabetic with increased risk of cancer

Epigenetic: Heritable changes in phenotype or gene expression caused by mechanisms other than changes in the DNA sequence.
Epigenetic Mechanisms
Changes in Chromatin Structure or DNA Methylation

Chromatin Structure
- Nucleosome positioning
- Histone variants
- Modified histones

DNA Modification (Methylation)
- 5-methyl-CpG
- Δ DNA methylation

Alterations
Cell division
Progeny
CpG Islands
Regions with High Concentrations of CpGs

CpG Islands
• Definition
  • ~200-3000bp regions
  • >50% GC content
• Genomic distribution
  • ~70% of promoter regions contain CpG islands
  • Only 1% of remaining genome contains CpG islands

Shores
• Regions flanking CpG islands
• Suggested to be where regulated methylation occurs
**Epigenetic Mechanisms**
**Regulation of Critical Biological Processes**

- **Chromatin structure**
- **DNA methylation**

Regulate Gene Expression

- Development
- Differentiation
- Cancer (other diseases)
Why Study Epigenetics?
To Understand Changes in Gene Expression

• To understand how epigenetic changes affect gene expression
  – Correlate gene expression with promoter DNA methylation status or factor binding
  – Identify epigenetic changes between samples (e.g. tumor vs. normal)
  – Discover epigenetic-based biomarkers of cancer and other diseases
DNA Methylation Analysis Workflow
From DNA Purification to Analysis

**Always use proper controls at each step!**
Genomic DNA Purification
Start with High Quality gDNA Free of Contaminants

Key Challenges

✓ Purifying sufficient DNA from:
  • Small samples
  • Difficult samples
  • Degraded samples

✓ Isolating high molecular weight DNA
  • Start large because bisulfite treatment fragments gDNA

✓ Isolating pure DNA free of downstream enzyme inhibitors

✓ Reducing time spent prepping DNA samples
Manual Genomic DNA Purification Options
ReliaPrep™ Blood gDNA Miniprep System

### Kit Specifications

<table>
<thead>
<tr>
<th>Specification</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Input</td>
<td>≤200µl fresh or frozen whole blood</td>
</tr>
<tr>
<td>Processing Time</td>
<td>&lt;1 hour</td>
</tr>
<tr>
<td>Elution Volume</td>
<td>50-200µl</td>
</tr>
<tr>
<td>Expected Yield</td>
<td>4-10µg per 200µl (depends on white blood cell count)</td>
</tr>
<tr>
<td>Expected Purity</td>
<td>$A_{260}/A_{280}$ ratios &gt;1.8</td>
</tr>
</tbody>
</table>

Ethanol-free purification of high quality gDNA

- **Ready-to-Use Convenience**: Complete kits
- **Fast Protocol**: Process 24 samples in <1 hour
- **Ethanol-free Protocol**: No ethanol carryover possible to inhibit downstream assays
- **High $A_{260}/A_{280}$ Ratios**: High quality gDNA supports successful downstream DNA methylation applications
Manual Genomic DNA Purification Options
ReliaPrep™ Blood gDNA Miniprep System

High Purity and Consistent Yields
Manual Genomic DNA Purification Options
ReliaPrep™ FFPE gDNA Miniprep System

**Kit Specifications**

<table>
<thead>
<tr>
<th>Sample Input:</th>
<th>5-50µm sections up to a total equivalent of ≤100µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Processing Time:</td>
<td>2.5 hours</td>
</tr>
<tr>
<td>Elution Volume:</td>
<td>30-50µl</td>
</tr>
<tr>
<td>Expected Yield:</td>
<td>Varies depending on the amount and quality of the FFPE tissue section</td>
</tr>
<tr>
<td>Sample Input:</td>
<td>5-50µm sections up to a total equivalent of ≤100µm</td>
</tr>
</tbody>
</table>

*Isolate More Intact, Amplifiable (Usable) gDNA*

- Improve your downstream assays by isolating more intact, amplifiable gDNA from difficult FFPE tissue samples.
- Optimize your sample processing workflow with a rapid, 2.5 hour purification protocol.
- Safely deparafinize your samples without the use of harsh organic solvents.
Improved Isolation of Intact, Amplifiable gDNA from FFPE tissue

Amplification of Fragments >400bp from DNA Isolated from FFPE Samples

Genomic DNA was purified from triplicate samples, and 10µl of the purified gDNA was used as template in a multiplex PCR reaction using GoTaq® Hot Start DNA polymerase. Fragments from 100-400bp were easily amplified and detected.
Manual Genomic DNA Purification Options
ReliaPrep™ FFPE gDNA Miniprep System

Earlier $C_q$ Values Indicate > Functional Yield Using the ReliaPrep™ System
Automated Genomic DNA Purification
Save Time & Reduce Errors with Maxwell® 16

Maxwell® 16 is a magnetic particle mover, not a liquid handler

Particle mover = fewer breakdowns, clogs and drips leading to cross-contamination.

- Sample Mixing
- Particle Capture
- DNA Binding
- Particle Washing
- DNA Elution

Magnets
Cartridges
Maxwell® 16 Instruments and Kits
Reduce Repeat Extractions and Save Time

✓ Decrease hands-on time with an easy-to-use automated system

✓ Consistently purify high quality gDNA reducing the number of failed downstream assays

✓ Streamline workflows with 30-45 minute instrument run times and process 1-16 samples per run

✓ Choose an optimized kit to match to your sample type(s)

- Cells
- Tissue
- FFPE tissue
- Mouse tails
- Blood
- Buccal swabs
Maxwell® 16 Cell LEV DNA Kit
Optimized for Limiting Cell Number Extractions

Isolation of gDNA from Limiting Numbers of Cells

- 28 minute instrument run time
- 50µl elution volume
Maxwell® 16 FFPE Plus LEV DNA Kit
Quickly Isolate More Amplifiable gDNA

Quick, Safe Protocol with High Yields of gDNA

Prep
(1-10) 5µm FFPE sections
Add Proteinase K Solution
Add Lysis Buffer & vortex
1 hr or overnight
70°C

Purify

Quantitate

Results from 1 Hour Pro K Digestion

<table>
<thead>
<tr>
<th>Sample</th>
<th>Yield (µg)</th>
<th>Conc. (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>3.6</td>
<td>72.4</td>
</tr>
<tr>
<td>Kidney</td>
<td>6.1</td>
<td>122.2</td>
</tr>
<tr>
<td>Brain</td>
<td>4.9</td>
<td>98.3</td>
</tr>
</tbody>
</table>

Successful qPCR Detection of TERT 164 Gene

- 28 minute instrument run time
- 50µl elution volume
**Genomic DNA Purification**

*Start with High Quality gDNA Free of Contaminants*

**Key Challenges**

- Purifying sufficient DNA from:
  - Small samples
  - Difficult samples
  - Degraded samples

- Isolating high molecular weight DNA
  - Start large because bisulfite treatment fragments gDNA

- Isolating pure DNA free of downstream enzyme inhibitors

- Reducing time spent prepping DNA samples

**Make sure you use a purification method appropriate for the type of tissue you are working with.**
DNA Methylation Analysis Workflow
From DNA Purification to Analysis

1. Purify
2. Quantify
3. Bisulfite Convert
4. Whole Genome Sequencing
5. Infinium Assay
6. Microarrays
7. Endpoint or qPCR
8. Sequencing
**DNA Quantification**
*An Important but Underappreciated Step*

**Key Challenges**

- Assessing:
  - Purity
  - Integrity
- Working with limiting samples - sensitivity
DNA Quantification by UV Absorbance
Quick, Easy but No Information on Integrity

UV Absorbance – the NanoDrop®

Advantages

• Provides purity information ($A_{260/280}$ & $A_{260/230}$ ratios)
• Low sample input (0.5-2µl)
• Wide dynamic range (2ng/µl – 15,000 ng/µl)
• No additional reagents required
• Quick

Disadvantages

• Overestimation of DNA concentration
  • Absorbance by other nucleic acids in the sample
  • Absorbance at 260nm by contaminating compounds
• No information on integrity
DNA Quantification with Fluorescent Dyes
Highly Sensitive Detection for Limiting Samples

QuantiFluor™ dsDNA Dye/Instrument

Advantages

• High sensitivity ($\geq 5\text{pg/assay @} \geq 0.05\text{pg/µl}$)

• Low sample input (measure accurately)

• Accurate quantification of dsDNA only
  • dsDNA-specific dye

• Flexible assay formats (single tubes to 96-well plates)

Disadvantages

• No information on integrity or purity

• Requires use of a standard

Easy Protocol: Add, Mix, Measure

504nm Excitation

Emits @ 531nm

Incubate at room temp for 5 minutes

Unbound dye

504nm

Quantify
DNA Quantification by Agarose Gel Electrophoresis
Assay Integrity but Non-quantitative

Agarose Gels + Fluorescent Dyes – Diamond™ Nucleic Acid Dye

Advantages

• Provides information on DNA integrity
• May show RNA contamination
• Low cost

Disadvantages

• Only provides an estimate of relative DNA amount
• No purity information on contaminating compounds
• Fairly slow
DNA Quantification with the Agilent Bioanalyzer
Provides Integrity and Quantitation Data

Agilent 2100 Bioanalyzer

Advantages

• Low volume sample input (1µl)
• Provides information on size and concentration
• Analyzes 12 samples in 30-40 minutes
• Electropherogram and gel-like image

Disadvantages

• High cost instrument and reagents
• Limited to gDNA <12kb in length
• No information on purity
Key Challenges

✓ Assessing:
  • Purity
  • Integrity

✓ Working with limiting samples - sensitivity

**Performing a quantification step will help you get the best results from your downstream methods and assist in troubleshooting any issues that arise, which will save you time in the long run.**
DNA Methylation Analysis Workflow
From DNA Purification to Analysis

1. Purify
2. Quantify
3. Bisulfite Convert
4. Perform Quality Checks
5. Endpoint or qPCR
6. Whole Genome Sequencing
7. Infinium Assay
8. Microarrays
9. Sequencing
Bisulfite Conversion
Converts Unmethylated Cytosines to Uracil

- Converts non-methylated cytosines to uracil
- 5-methylcytosine (5mC) & 5-hydroxymethylcytosine (5hmC) are resistant
- No information provided on its own
  - Must combine with downstream assay
Bisulfite Conversion
After Conversion, DNA Polymerase Converts dU to dT

Bisulfite Converts
**Unmethylated** Cytosines to Uracil

C → U

C → **Protected**

After Conversion, Polymerase Substitutes dT for dU

DNA:

\[ \text{T C G C C G G T A C} \] → \[ \text{T U G U U G G T A U} \] → \[ \text{T U G T G G T A T} \]

\[ \text{T C^{m} G C C G G T A C} \] → \[ \text{T C^{m} G U C G G T A U} \] → \[ \text{T C^{m} G T G G T A T} \]

Locations of C^{m} in original DNA
Key Challenges

✔ Fragmentation of gDNA into low molecular weight molecules
  • Temperature, pH, and time all affect degree of fragmentation

✔ Isolation of sufficient, large fragments suitable for amplification

✔ Incomplete conversion of unmethylated C’s
  ✔ All downstream assays/analysis assume 100% conversion of unmethylated C’s
  ✔ Incomplete conversion results in false positive calls of methylated C’s
Perform Quality Checks Before Proceeding with Downstream Assays

Quantify after bisulfite conversion

- Significant fragmentation may decrease recovery yield after conversion cleanup
- Converted DNA is highly single-stranded due to decreased complementarity
- Recommend using UV absorbance or ssDNA dye-based quantitation
  - Choose one and use it consistently

Assess level of fragmentation using gel analysis or Bioanalyzer

- Significant fragmentation may decrease recovery yield after conversion cleanup

Test conversion efficiency using bisulfite-specific PCR

- Amplify converted and unconverted DNA with a combination of WT- and converted-sequence specific PCR primer pairs including proper controls
**Bisulfite-specific PCR Testing**

**Design Primers Specific to WT or Converted Sequence**

- Chose primers with C’s but no CpG (no possible methylation)
- **WT-specific primers** only amplify unconverted DNA
- **BSP primers** amplify only converted DNA
- Test primers with pre-qualified converted and unconverted DNA to ensure specific amplification
**Bisulfite-specific PCR Testing**
*Use It to Assess Conversion Efficiency*

- **Bisulfite-specific Primers**
  - Bands indicate bisulfite-converted DNA present

- **Wild Type-specific Primers**
  - No bands suggest complete conversion
  - Bands indicate unconverted DNA is present

**Take Home:**
- Samples 1-3 = fully converted
- Samples 4-6 = partially converted
Bisulfite-specific PCR Testing
Beware 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 Testing
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

Rand, et al Epigenetics. 2006 Apr-Jun;1(2):94-100 used a differential denaturation in real time PCR to amplify unmethylated DNA in a methylated DNA background, after treatment with bisulfite.

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

Bisulfite-specific PCR Testing

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 been 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
Less DNA Fragmentation with the MethylEdge™ System Enables Improved Downstream Analysis

Bisulfite Conversion Kit Used

- = None
ME = Promega MethylEdge™ System
Zy = Zymo EZ DNA Methylation Gold Kit
Ne = NEB EpiMark™ Kit

**Larger fragment size allows amplification of longer target sequences, and higher quality DNA performs more reliably with downstream analysis methods.**
Less DNA Fragmentation Improves Analysis of Already Fragmented FFPE gDNA Samples

**Less Fragmented Converted gDNA Improves Detection Sensitivity**

- FFPE samples prepped with ReliaPrep™ FFPE gDNA System
- Bisulfite converted with MethylEdge™ (<2 hr) and competitor kit
- Analyzed with GoTaq® qPCR Master Mix with bisulfite-specific PCR
Higher Conversion Efficiency Improves DNA Methylation Analysis

Bisulfite Conversion Followed by BSP- & WT-specific Duplexed PCR

MethylEdge™ Converted DNA

Competitor System Converted DNA

Indicates incomplete conversion and loss of template

300bp band (unconverted)

250bp band (converted)
**MethylEdge™ Bisulfite Conversion System**
*Fast Protocol Saves Valuable Time*

**MethylEdge™ System**

1. **Input DNA** 8 min
2. **Denature DNA** 60 min
3. **Bisulfite Convert** 24 min
4. **Incubate**
5. **Column-based Desulfonation**
6. **Elute**

**EZ DNA Methylation-Gold™ Kit**

1. **Input DNA** 10 min
2. **Denature DNA** 150 min
3. **Bisulfite Convert**
4. **Incubate**
5. **Column-based Desulfonation**
6. **Elute**

**100 min Quicker!**
**MethylEdge™ Bisulfite Conversion System**

*Converted DNA Is Stable At -20°C Up To 13 Months*

- gDNA bisulfite-converted with MethylEdge™ and stored at -20°C
- Competitors recommend storing converted DNA for no more than one month
- Amplification of a 249bp fragment is unchanged after being stored for more than a year
Control DNA for Bisulfite Conversion Experiments
Essential For Preventing False Positives

**Methylated Human Control**
- Completely CpG Methylated DNA - male DNA enzymatically methylated with M.SssI methyltransferase and validated
- To be used during bisulfite conversion of experimental samples to verify that conversion has gone to completion

**Converted Methylated Human Control**
- Bisulfite Converted CpG Methylated DNA - above DNA bisulfite converted and validated
- To be run in parallel with experimental samples in downstream assays

**Unmethylated controls may be offered in the future.**
DNA Methylation Analysis Workflow
From DNA Purification to Analysis

Purify → Quantify → Bisulfite Convert → Endpoint or qPCR → Sequencing

Whole Genome Sequencing
Infinium Assay
Microarrays
Bisulfite Conversion and DNA Sequencing
Standard for Gene-specific Analysis

Advantages
- Uses well-established, accessible methodologies
- Single CpG level resolution
- Accessible bioinformatic analysis
- Low cost

Disadvantages
- Analysis limited to one locus per PCR product
- Requires sequencing of multiple clones per locus
**Bisulfite Conversion and Next-Gen Sequencing Standard for Whole Genome Analysis**

**Advantages**
- Whole genome analysis
- Single CpG level resolution

**Disadvantages**
- Requires:
  - Extensive bioinformatic support
  - Large data storage capabilities
- High cost
- Limited sample throughput
**Illumina Infinium Assay**

*Single CpG Analysis at Select Loci Across Genome*

**Advantages**
- Single CpG level resolution
- Easy to interpret Yes/No data
- Easy cross-lab comparisons

**Disadvantages**
- Possible missed calls
  - Hypermethylated regions across samples have different methylation patterns
  - Pick the wrong CpG and miss the call
- High cost
Microarrays (MeDIP-chip, CHARM, HELP)
Genome-wide Analysis at Lower Resolution

**Advantages**
- Assays thousands of genes/array
- Covers all CpGs within regions covered

**Disadvantages**
- Coverage limited to regions tiled on the arrays
- High cost
- Lower resolution analysis than sequencing based methods
- Difficult bioinformatic analysis to compare samples/identify differences

**Methylated DNA Immunoprecipitation-Chip**

- Fragment
- Denature
- IP
- Enrich
- Amplify
- Label
- Hybridize
- MeDIP
- Input

Promega
The quality of your DNA methylation analysis depends on careful execution of each step in the workflow.

- Isolation of clean, intact gDNA free of contaminants
- Accurate quantitation and quality assessment of gDNA
- Minimized DNA fragmentation when bisulfite converting your DNA
- Check bisulfite conversion efficiency before proceeding with analysis

Promega offers products to help overcome many of the major challenges associated with each step of DNA methylation analysis.

- ReliaPrep™ gDNA Purification Systems
- Maxwell® 16 Instrument & Purification Kits
- MethylEdge™ Bisulfite Conversion System
- QuantiFluor™ Instruments & dsDNA and ssDNA Dyes
- GoTaq® PCR and qPCR Reagents

**Always use proper controls at each step!**