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

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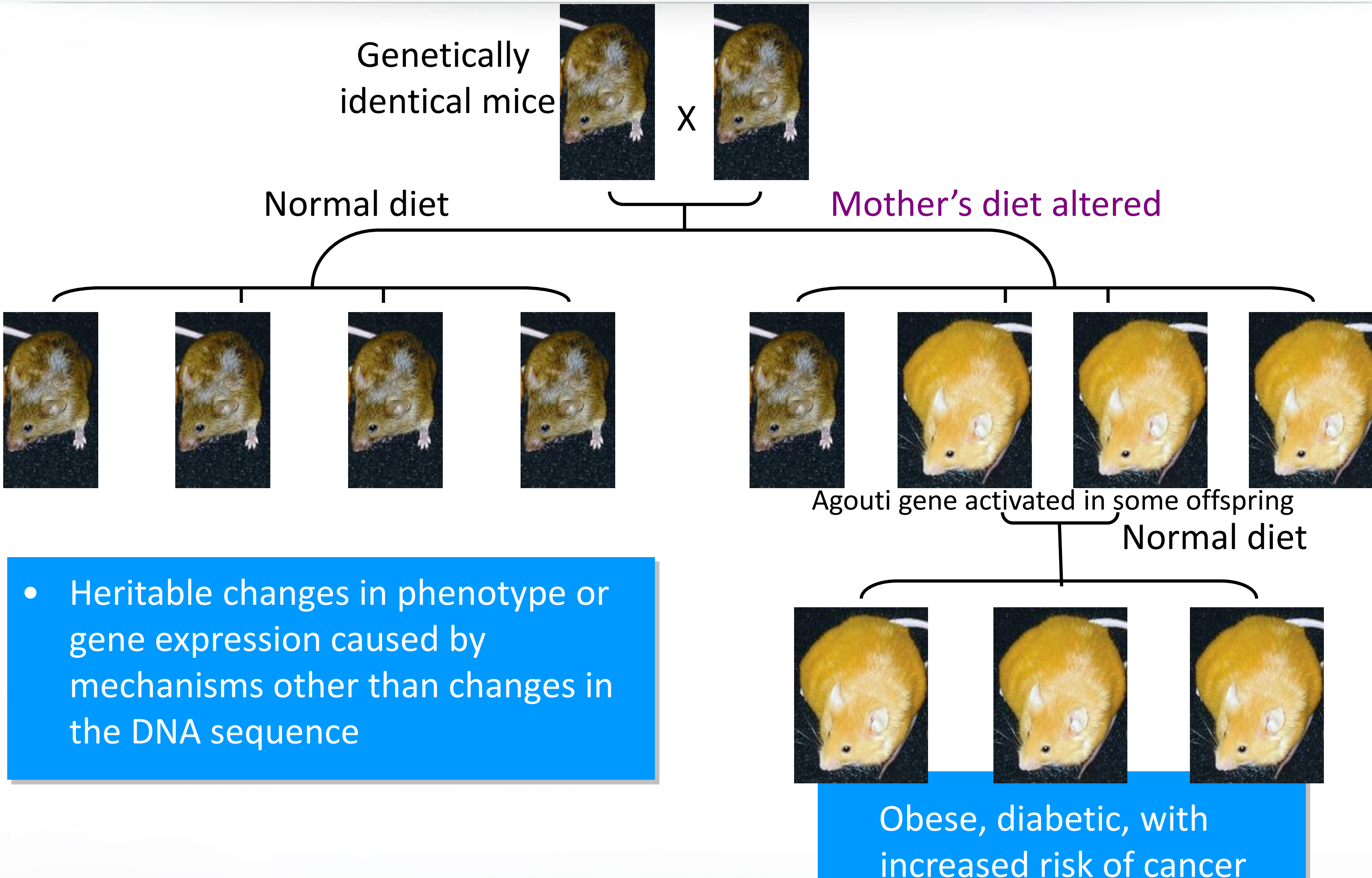
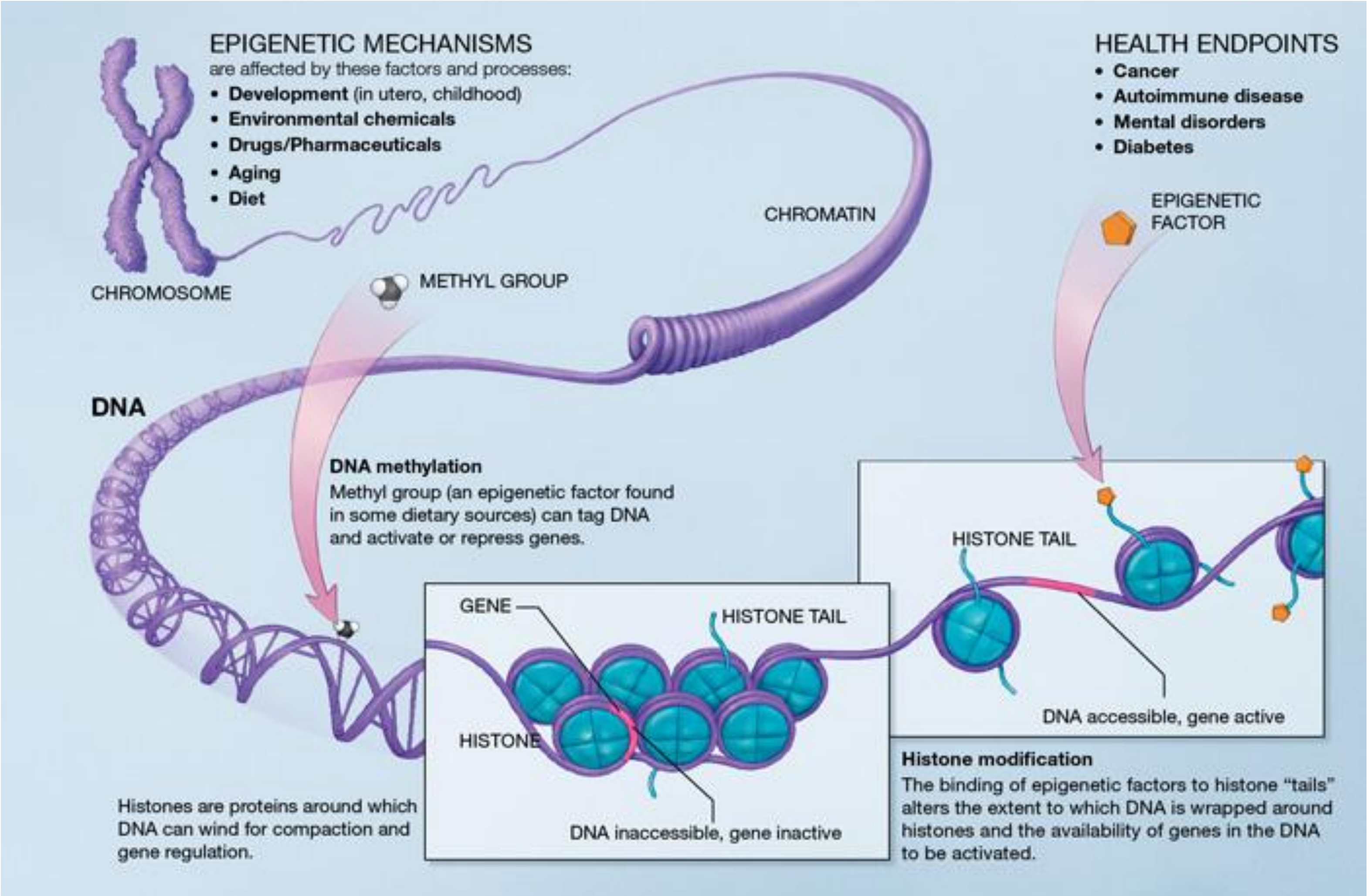
September 2012

Epigenetics in Action



- 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

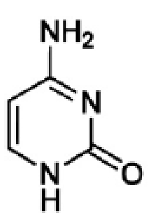
1. DNA methylation
2. Introduction of bisulfite conversion chemistry
3. Downstream analysis methods
4. Considerations for obtaining quality data

Overview

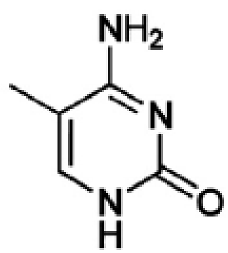
1. **DNA methylation**
2. Introduction of bisulfite conversion chemistry
3. Downstream analysis methods
4. Considerations for obtaining quality data

DNA Methylation
Primary Epigenetic Modification of DNA

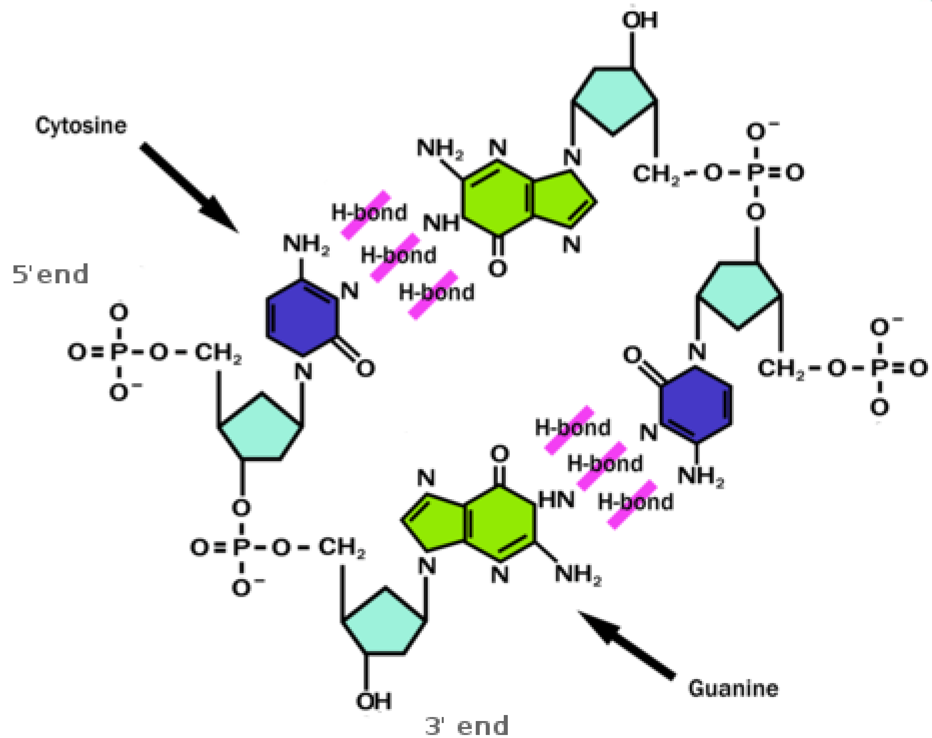
- The predominant epigenetic modification of DNA in mammalian genomes is methylation of cytosine nucleotides (5-MeC).
- The primary target sequence for DNA methylation in mammals is 5'-CpG-3' dinucleotides



Cytidine
C



5-methyl cytidine
5mC



DNA Methylation
Occurs Primarily at CpG Sites in Promoter Regions

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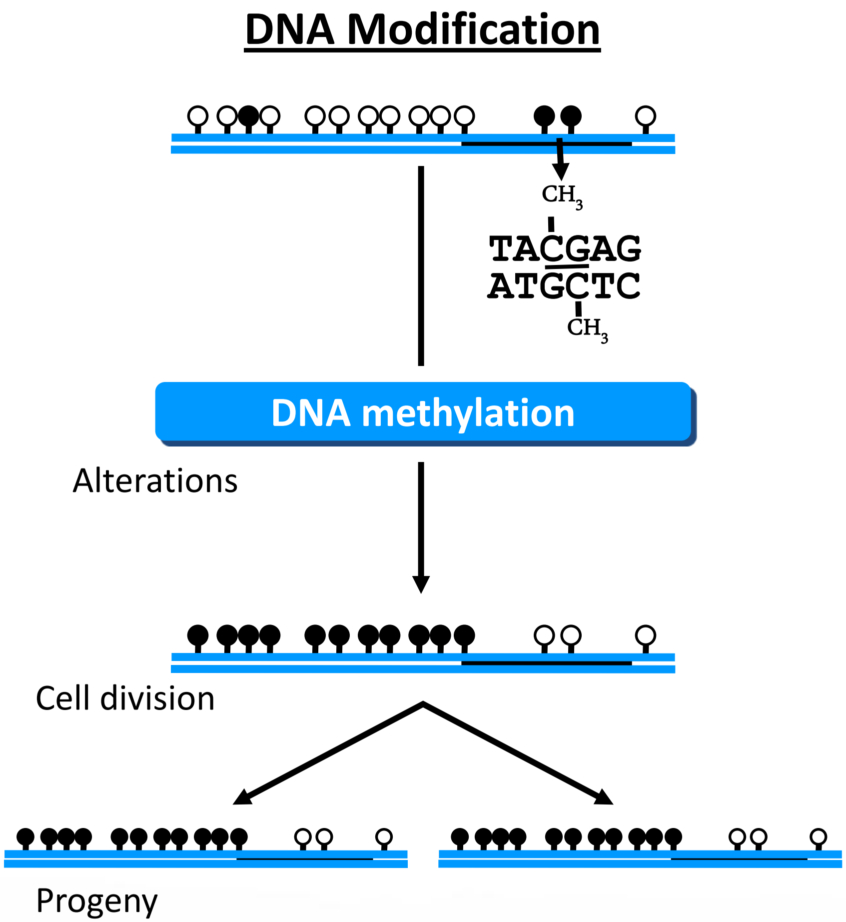
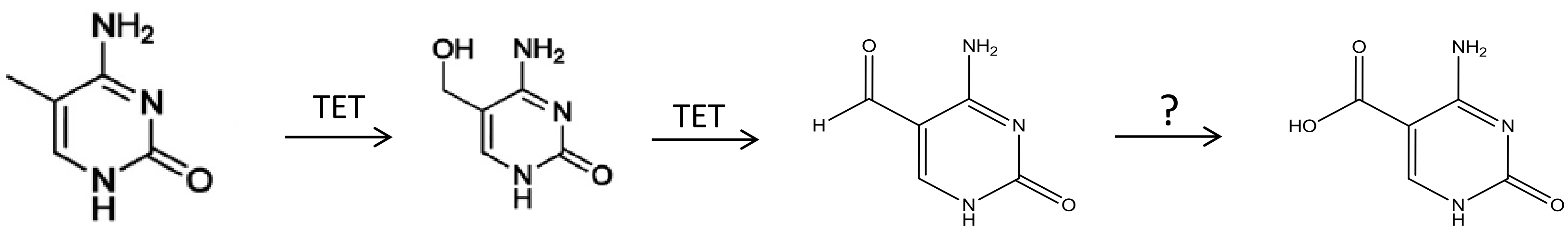
CpG islands

- 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



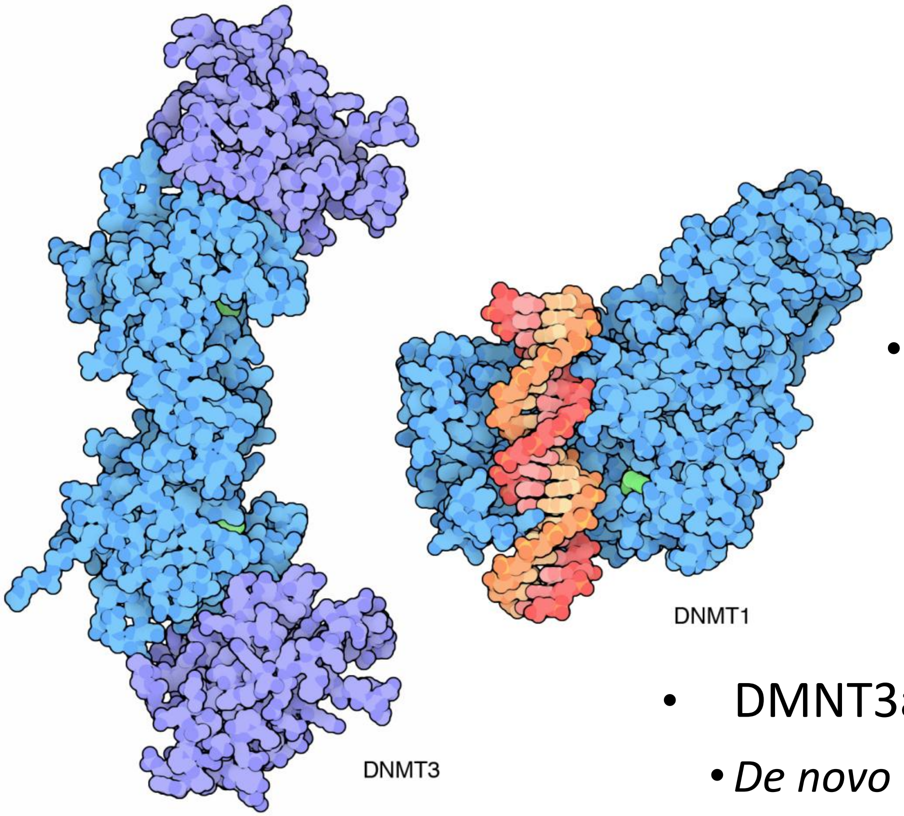
DNA Methylation
Potential Demethylation Pathway and Intermediates

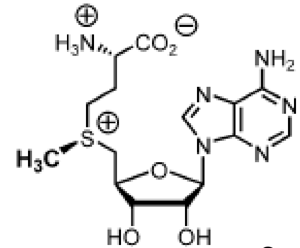
- Covalent addition of $-CH_3$ at the 5’ of the cytosine ring by methyltransferases.
- Mechanism for demethylation is unclear, but may involve TET proteins or oxidative pathways leading to intermediates.



5-methyl cytidine 5mC | 5-hydroxymethyl cytidine 5hmC | 5-formyl cytidine 5fC | 5-carboxyl cytidine 5caC

DNA Methylation
CpG Methyltransferases





SAM
S-adenolsyl-L-methionine

- DMNT1
  - Maintenance methylation
  - Looks for hemimethylated CpG and maintains methylation pattern following replication
- DMNT3a, DMNT3b
  - *De novo* methylation
- HCT116 – double knockout cell line
  - DNMT1 and DMNT3b inactive, low level methylation

Image credit: David S. Goodsell and RSCB PDB:
http://www.rcsb.org/pdb/101/motm.do?momID=139

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Studying DNA Methylation
Early Methods Lacked Sensitivity

Promega

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

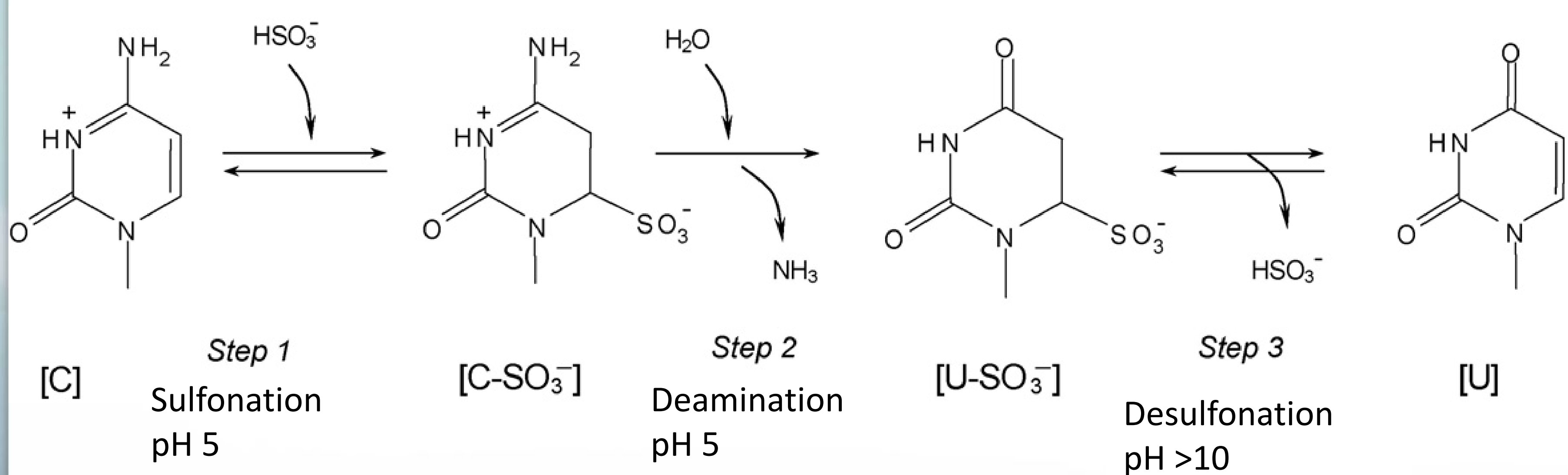
Bisulfite modification

- C’s are converted to U’s
- Methylated C’s are NOT converted

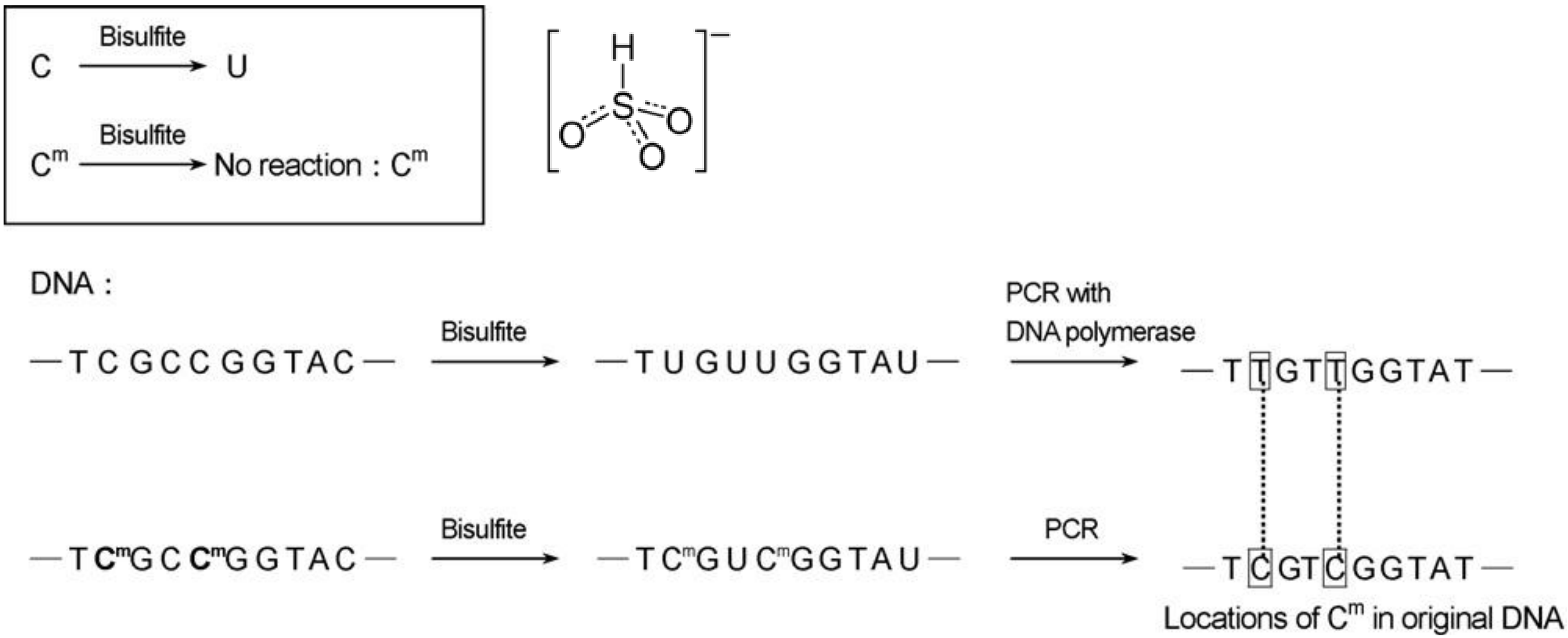
Introduction of chemistry:
Shapiro, et al. (1973) JBC vol 248, p4060
Application to DNA Methylation Detection
Frommer, et al. (1992) PNAS vol 89, p 1827

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)



Studying DNA Methylation
Bisulfite Conversion Converts Cytosines to Uracils



Bisulfite Conversion Chemistry
Harsh Reaction Conditions Degrade DNA

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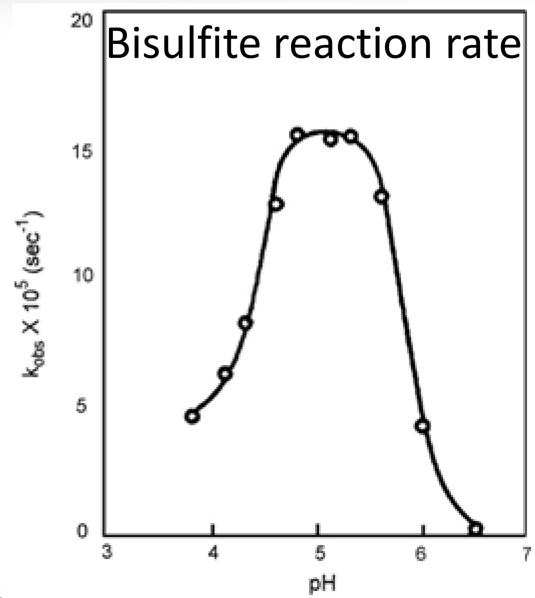
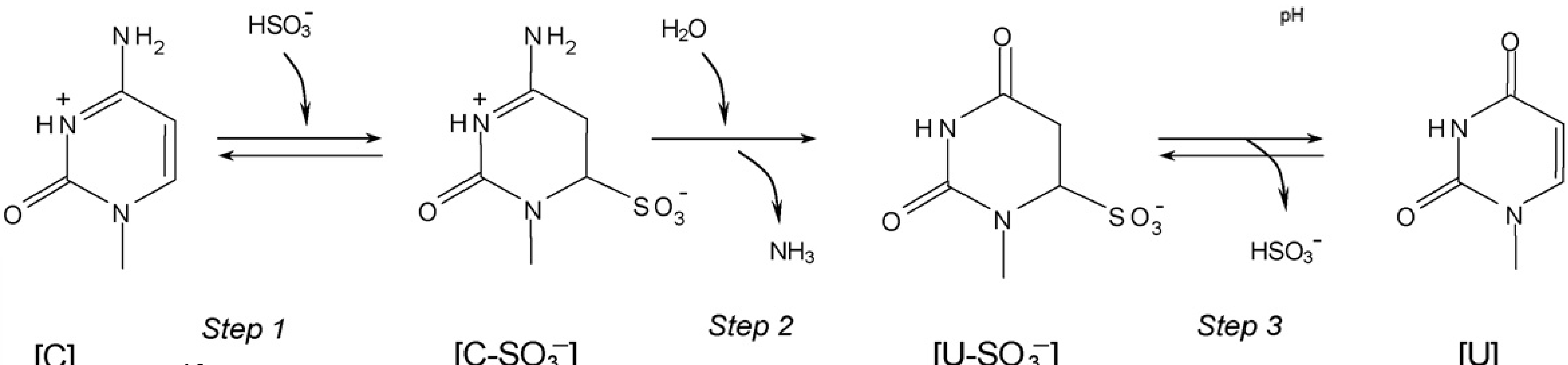
- Original conditions:
  - Denature with NaOH or high heat
- Incubate at pH 5 for 16-20 hours at ~55°C
  - Causes depurination, fragmentation
- Early protocols led to loss of >90 % starting material





Sulfonation
pH 5

Deamination
pH 5

Desulfonation
pH >10

Bisulfite Conversion Chemistry
Protocol Modifications Preserve DNA Quality

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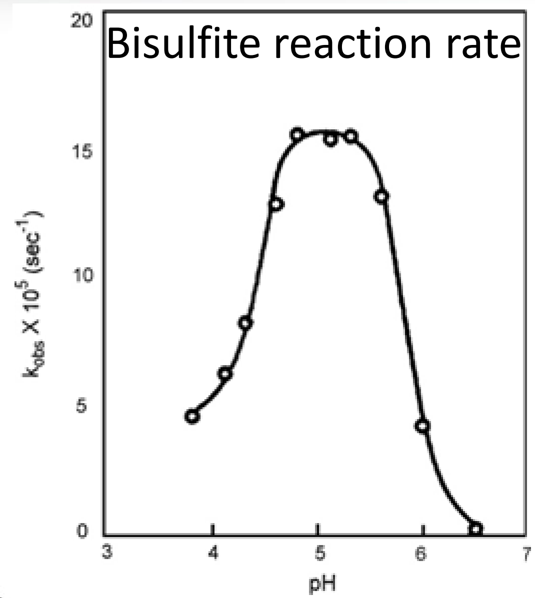
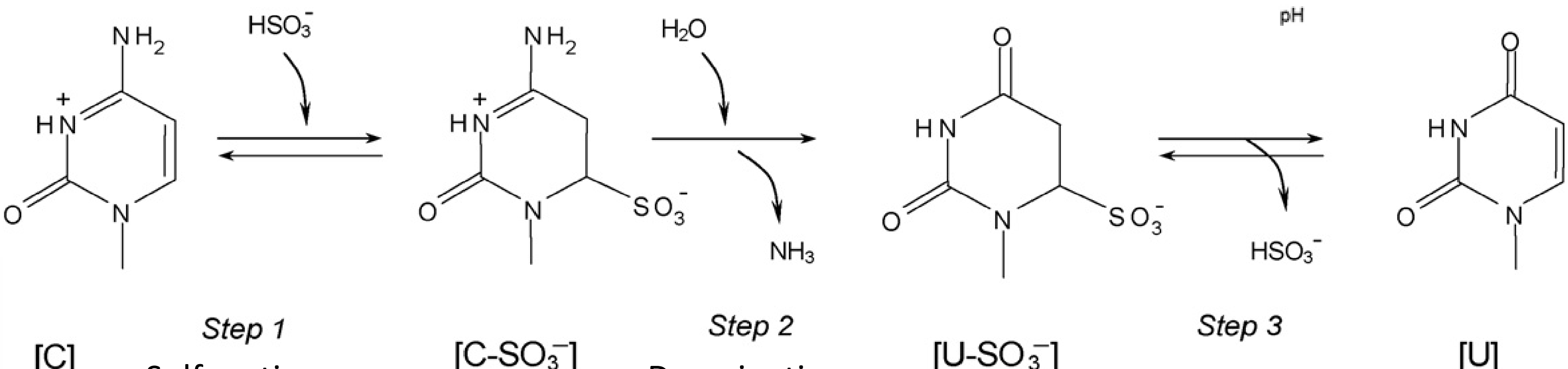
- Sodium bisulfite is most common, but formulations include K and NH_3 bisulfite
- Heat and incubation time are important considerations for preserving DNA quality which impact downstream analysis methods
- New commercial kits incubate for 30-60min





Sulfonation
pH 5

Deamination
pH 5

Desulfonation
pH >10

Bisulfite Conversion Chemistry
Protocol modifications Preserve DNA Quality

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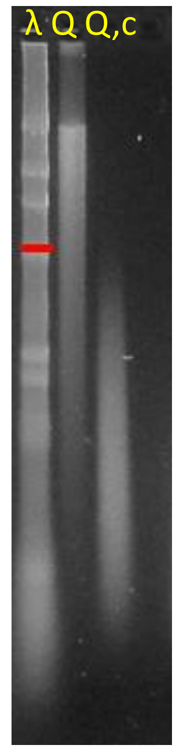
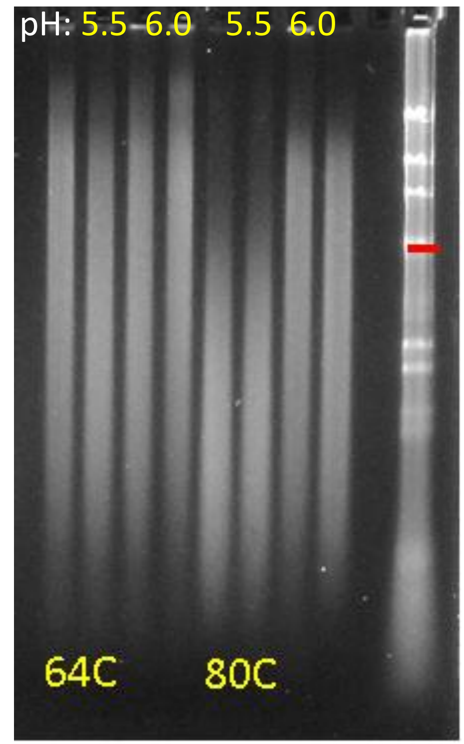
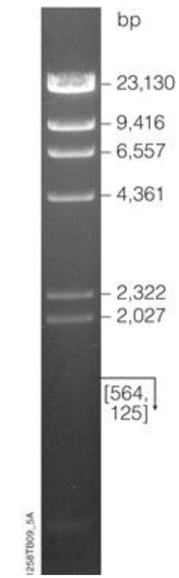
- λ/HindIII markers with commercially available DNA from Qiagen (Q = unconverted, Q,c = converted)
- Samples converted at either 64°C or 80°C at pH 5.5 or pH 6.0
  - Higher temperature improves denaturation, but increases fragmentation
  - Higher pH preserves DNA, but lowers conversion efficiency.







λ/HindIII Markers

Bisulfite Conversion Chemistry
Summary

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- 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

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1. DNA methylation
2. Introduction of bisulfite conversion chemistry
3. **Downstream analysis methods**
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Studying DNA Methylation
Downstream Analysis Methods

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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

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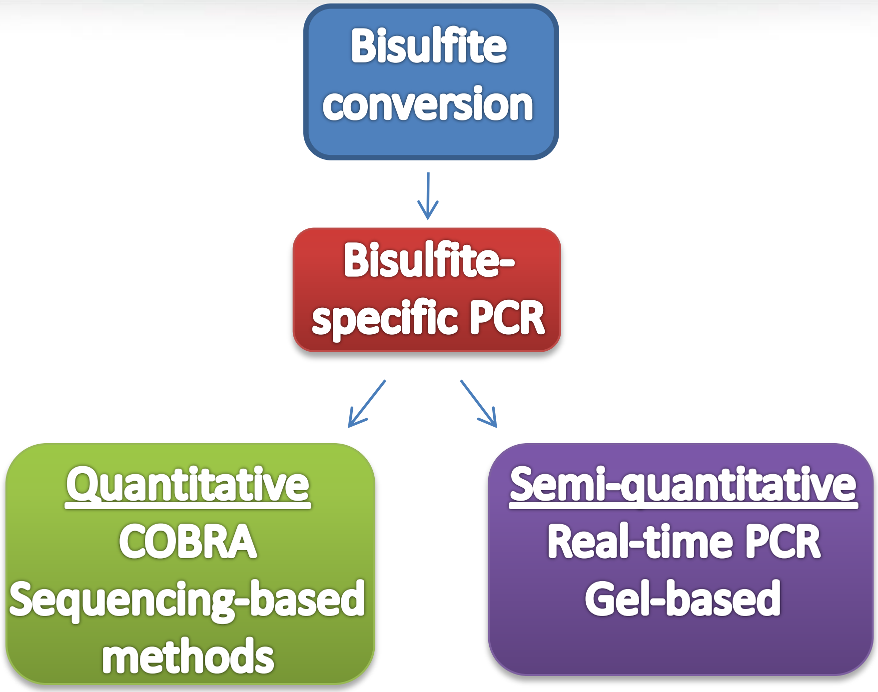

- COBRA – Combined Bisulfite Restriction Analysis
- Sequencing-based methods: pyrosequencing, CE-based sequencing, cloning and Sanger sequencing
- Semi-quantitative: Uses real-time or gel-based visualization to estimate percentages and general location of methylation

Bisulfite-specific PCR
Primers Must Be Specific to Converted Sequence

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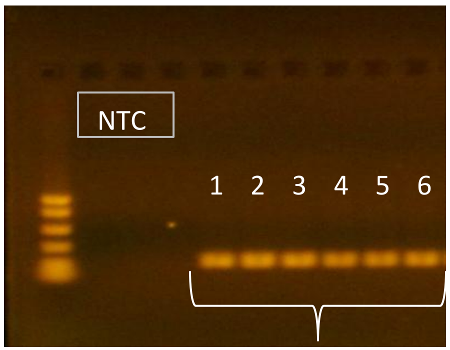
- Bisulfite-specific primer pair (blue): amplifies both bisulfite-converted methylated and bisulfite converted unmethylated DNA
- Wildtype-specific primer pair (orange): amplifies unconverted DNA, both methylated or unmethylated sequences
- Primers do not contain any CpG sites, but do contain non-CpG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

```
NON-CONVERTED FORWARD SEQUENCE (77 Cs, Tm = 98C)

AAAATGGGCTAGACACAAAGGACTCGGTGCTTGTCCCAGCCAGGCGCCCTCGGCGACGCGGGCAGCTGGG
AGGGGAATGGGCGCCCGGACCCAGCTGGGACCCCCGGGTGCGACTCCACCTACCTAGTCCGGCGCCAGGC
CGGGTCGACAGCTCCGGCAGCGCCAGCGCCGCGCCGTGTCCAGATGTCGCGTCAGAGGCGTGCAGCGGTT
TAGTTTAATTTCGCTTGTTTTCCAAATCTAGAAGAGG

BISULFITE CONVERTED FORWARD STRAND (UNMETHYLATED) (0 Cs, Tm = 84C)

AAAATGGGTTAGATATAAAGGATTTGGTGTTTGTTTTAGTTAGGTGTTTTTGGTGATGTGGGTAGTTGGG
AGGGGAATGGGTGTTTGGATTTAGTTGGGATTTTTGGGTGTGATTTTATTTATTTAGTTTGGTGTTAGGT
TGGGTTGATAGTTTTGGTAGTGTTAGTGTTGTGTTGTGTTTAGATGTTGTGTTAGAGGTGTGTAGTGGTT
TAGTTTAATTTTGTTTGTTTTTTAAATTTAGAAGAGG

BISULFITE CONVERTED FORWARD STRAND (METHYLATED) (25 Cs, Tm = 88C)

AAAATGGGTTAGATATAAAGGATTCGGTGTTTGTTTTAGTTAGGCGTTTTCGGCGACGCGGGTAGTTGGG
AGGGGAATGGGCGTTCGGATTTAGTTGGGATTTTCGGGTGCGATTTTATTTATTTAGTTCGGCGTTAGGT
CGGGTCGATAGTTTCGGTAGCGTTAGCGTCGCGTCGTGTTTAGATGTCGCGTTAGAGGCGTGTAGCGGTT
TAGTTTAATTTCGTTTGTTTTTTAAATTTAGAAGAGG
```

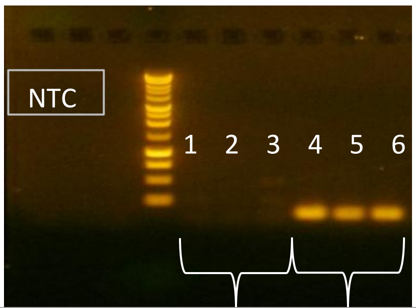
Bisulfite-specific PCR
Detecting Underconverted DNA

Bisulfite-specific primers: a band should be visible if DNA is converted



Band indicate bisulfite-converted DNA

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



No bands suggest complete conversion

Bands here shows unconverted DNA is present

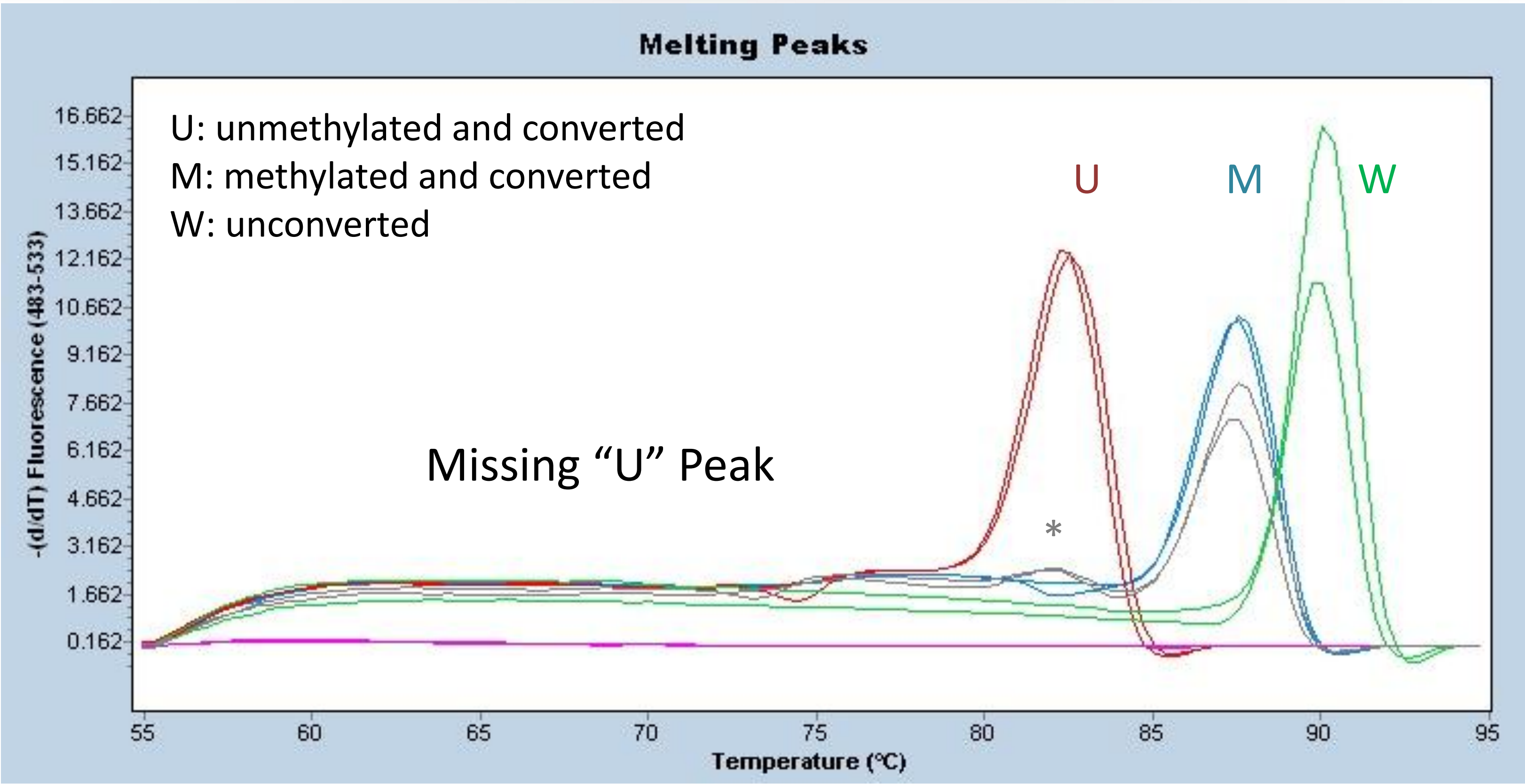
- Primers do not contain any CpG sites, but do contain non-CpG cytosines
- **Bisulfite-specific primer pair**: amplifies both bisulfite-converted methylated and bisulfite converted unmethylated DNA
- **Wildtype-specific primer pair**: amplifies unconverted DNA, both methylated or unmethylated sequences

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

Moskalev, et al. Nucleic Acids Research, 2011, Vol. 39, No. 11 e77

Bisulfite-specific PCR
Preferential Amplification of Methylated DNA



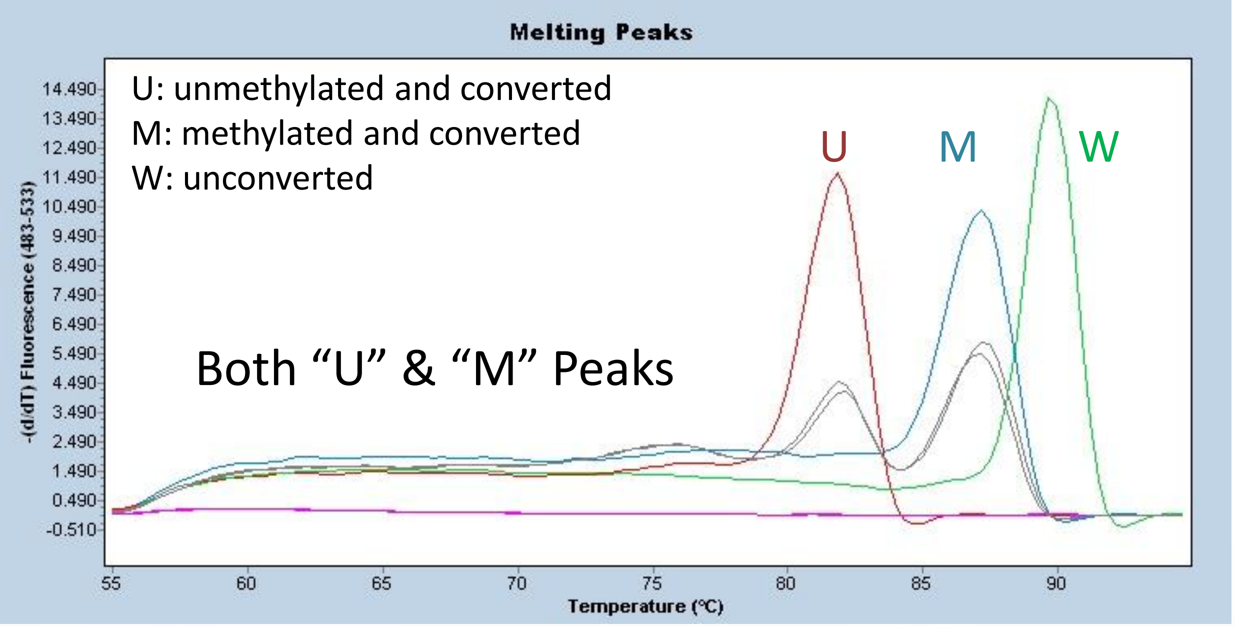
- Template DNA contains a mixture of fully methylated and converted, fully unmethylated and converted DNA.
- In this assay, methylated DNA is preferentially amplified over unmethylated DNA

Reece, et al. 2012. Manuscript in preparation.

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



Reece, et al. 2012. Manuscript in preparation.

Bisulfite-specific PCR
Designing Primers to Avoid PCR Bias

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Suggestions for bisulfite-specific primer design:

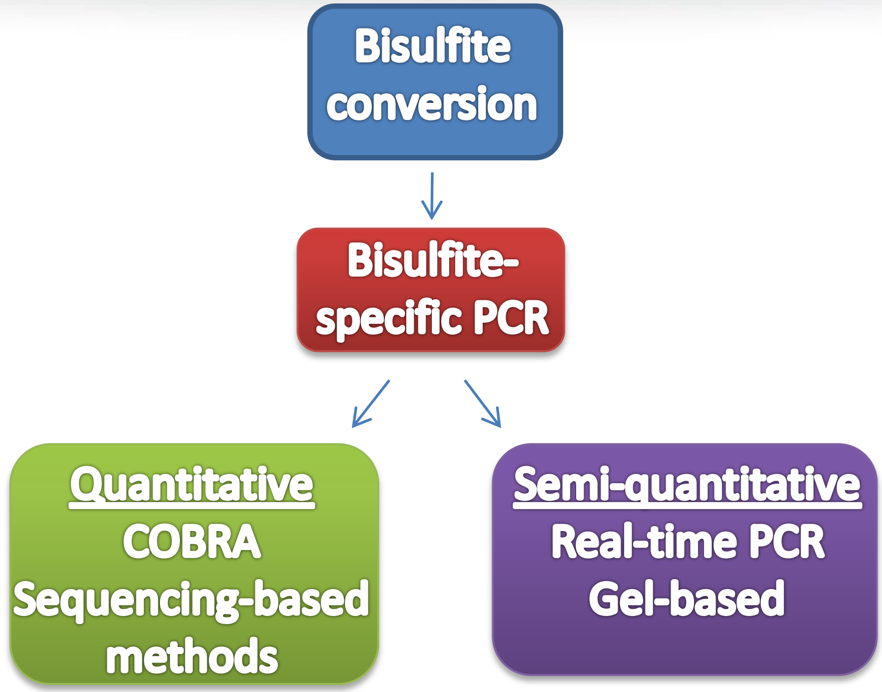
1. The fewer CpG sites, the better
2. When CpG sites are present, keep them away from the 3’ end of the primer
3. Salt-adjusted Tm of primer should be around 65°C to run PCR annealing step at 60°C
4. Include one or more T’s near 3’ end of primer (not originating from CpG site)
5. Evaluate primers for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

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- 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

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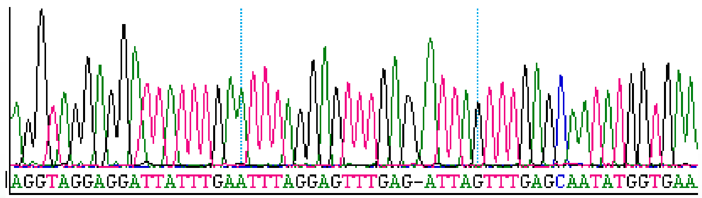
Purify DNA	Purify DNA from fresh/frozen/FFPE tissue, cells, etc e.g. Wizard® Genomic DNA Purification and ReliaPrep FFPE Miniprep Systems
Bisulfite Conversion*	**Coming soon!* MethylEdge™ Bisulfite Conversion System
Bisulfite-specific PCR end-point/ real-time	Amplify desired gene with end-point or real-time PCR e.g. GoTaq® Green Master Mix and GoTaq® qPCR Master Mix
Clean Up	Clean up salts, dNTPs, enzyme from amplicon e.g. Wizard® SV Gel and PCR Clean-Up System
Amplicon cloning	Clone amplicon using a T/A cloning system e.g. pGEM®-T Easy Vector Systems
Isolate Plasmid	Isolate the plasmid with miniprep e.g. PureYield™ Plasmid Miniprep System
Sequence	Sequence DNA!



MethylEdge™ Bisulfite Conversion System
Streamlined Protocol Complete in Under Two Hours

Promega

Prepare sample

- 100-500ng DNA in 20µl reactions

Add Conversion Reagent

- Add directly to sample
- Mix by pipetting

Program Thermocycler

- 98°C for 8 minutes
- 54°C for 60 minutes
- 4°C for up to 20 hours

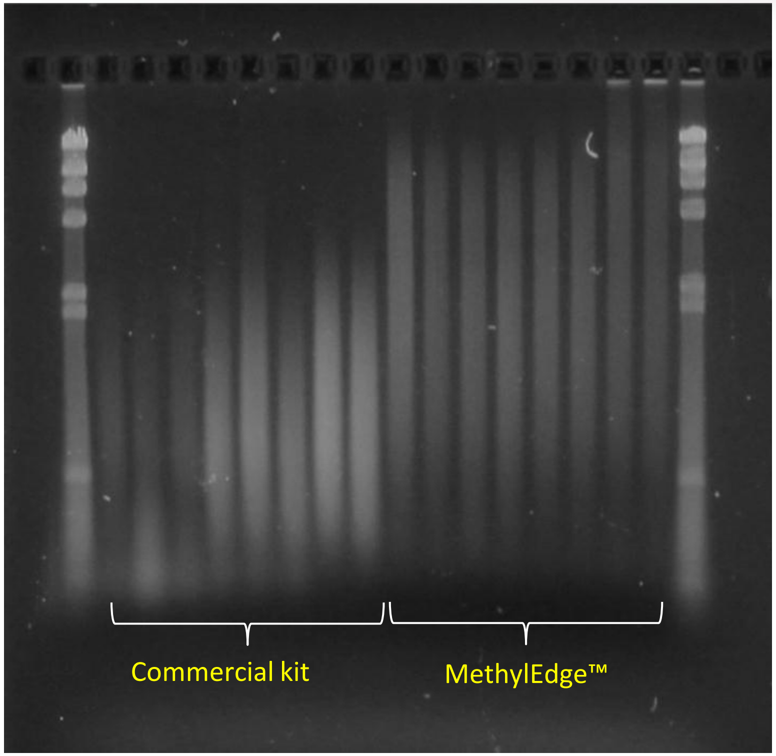
Desulfonated/ Clean-up

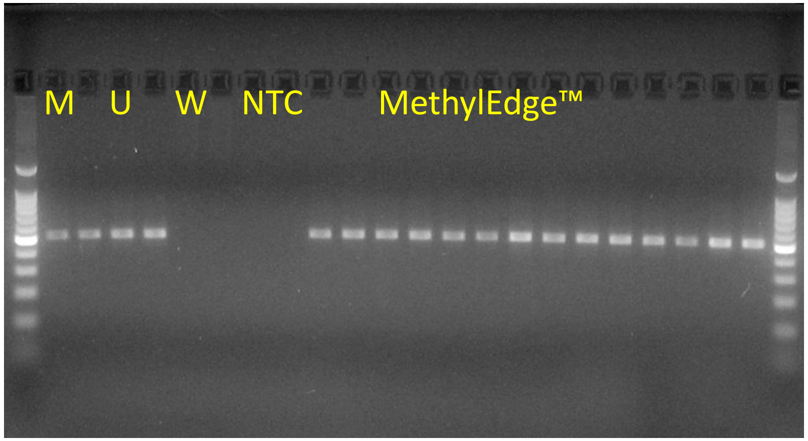
- All-in-One Spin column format

Downstream assays

- Bisulfite-specific PCR
- Sequencing
- Etc...

MethylEdge™ Bisulfite Conversion System
Maintaining DNA Integrity





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

- 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

Downstream Analysis Methods
Summary

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Genome-wide approaches

- Arrays and deep sequencing
- Facilitate epigenomic mapping

Gene-specific approaches

- Many rely on bisulfite-specific PCR prior to anaylsis
- COBRA, Bisufite 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

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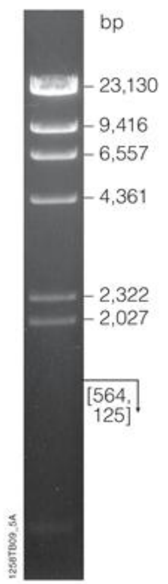
- Most commercial kits recommend converting 100-500ng per 20µl reaction for optimal results
  - Low end = 50pg
    - Issues: loss of sample, incomplete conversion due to bisulfite:DNA ratio
  - High end = 2µg
    - Issues: Incomplete conversion due to bisulfite:DNA ratio and possibly incomplete denaturation.
- 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

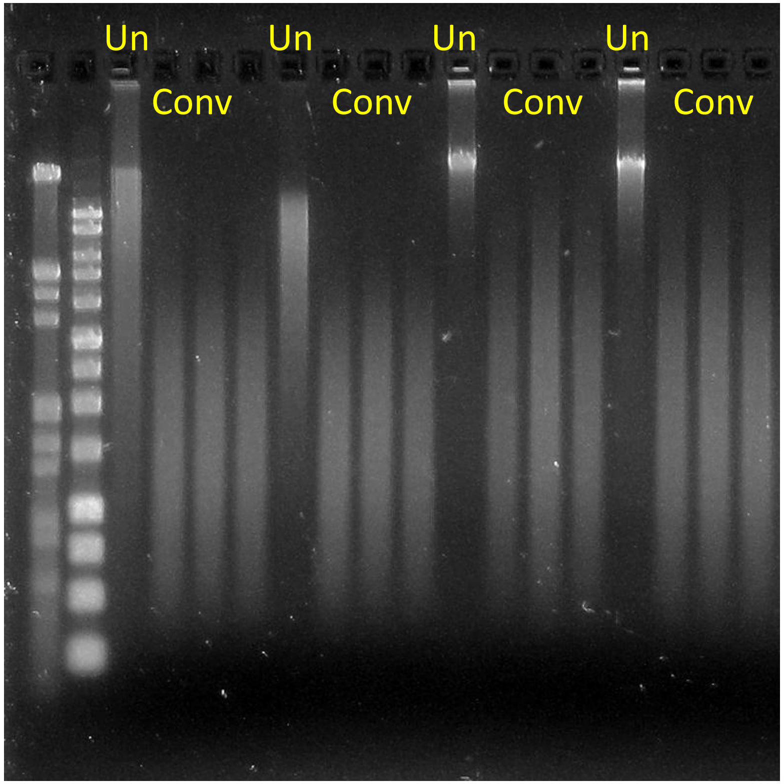
Improving Bisulfite-based Analysis and Data Quality
Sheared DNA Results in Smaller Fragment Size

Promega

Starting with DNA that is sheared will result in smaller fragments following bisulfite conversion.



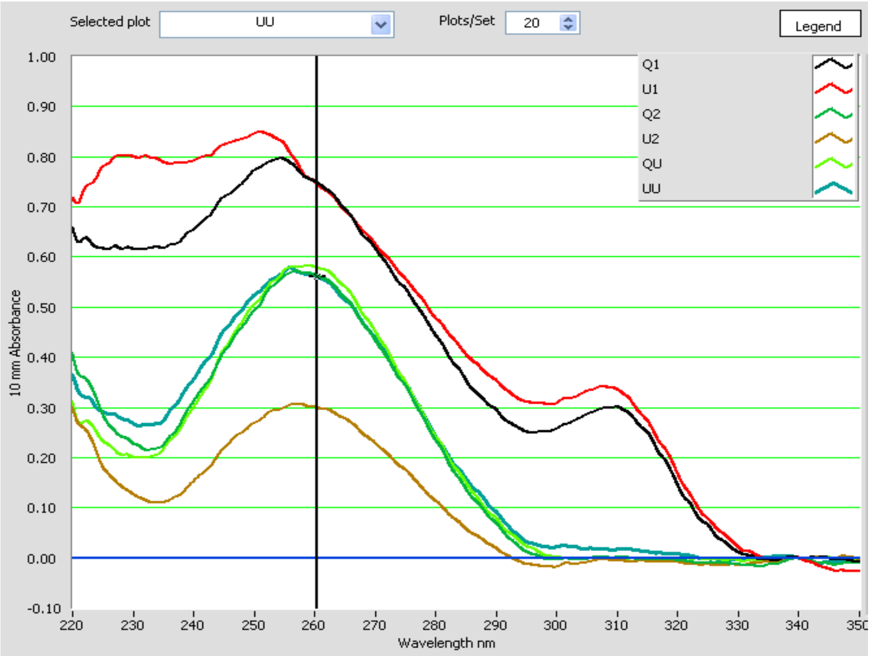
λ/HindIII
Markers

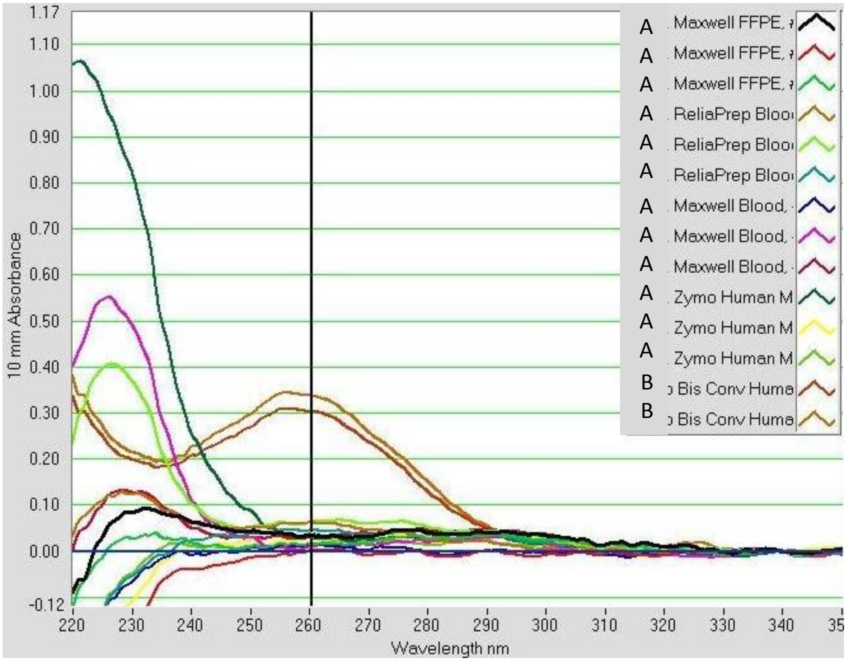


Un: unconverted DNA
Conv: bisulfite-converted DNA

Improving Bisulfite-based Analysis and Data Quality
Absorbance Scans Can Detect Impurities

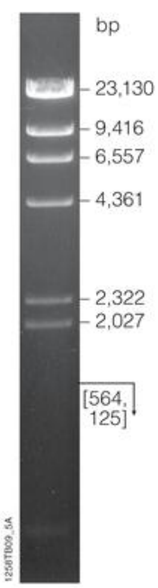
- Assess DNA quality following bisulfite conversion
  - Run absorbance scan to estimate concentration and look for impurities



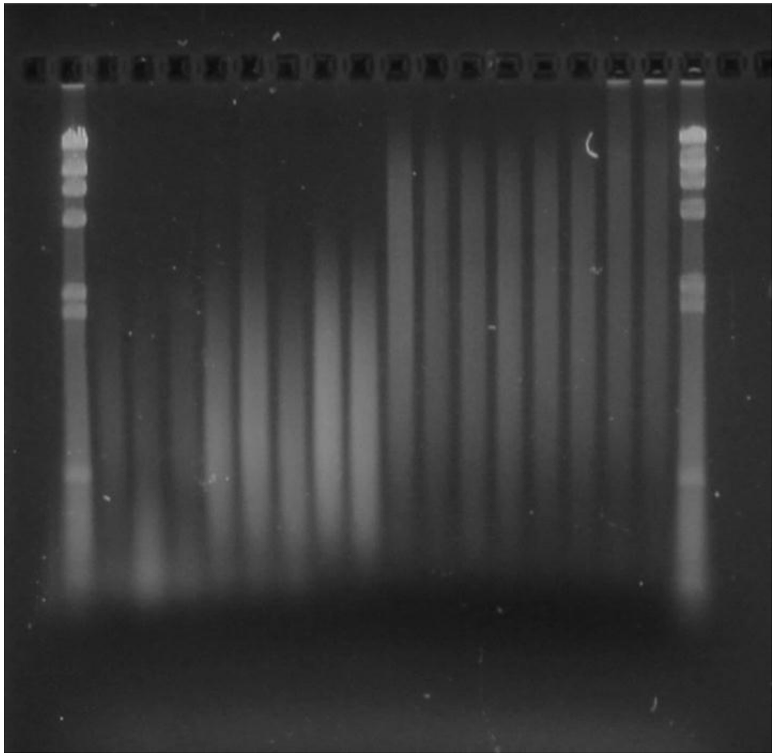


Improving Bisulfite-based Analysis and Data Quality
Visualizing DNA Provides Insight for Future Analysis

- Assess DNA quality following bisulfite conversion
  - Run samples on an agarose gel stained with a fluorescent total nucleic acid stain to visualize fragmentation



λ/HindIII Markers



Improving Bisulfite-based Analysis and Data Quality
Control Assays and DNA Sources Are Critical

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- 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

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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

<u>Interpret PCR Data</u>

- Ensure controls amplify as expected

<u>Direct Sequencing</u>

- Calculate conversion efficiency

Improving Bisulfite-based Analysis and Data Quality
Summary

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- 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

Promega

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

Questions?