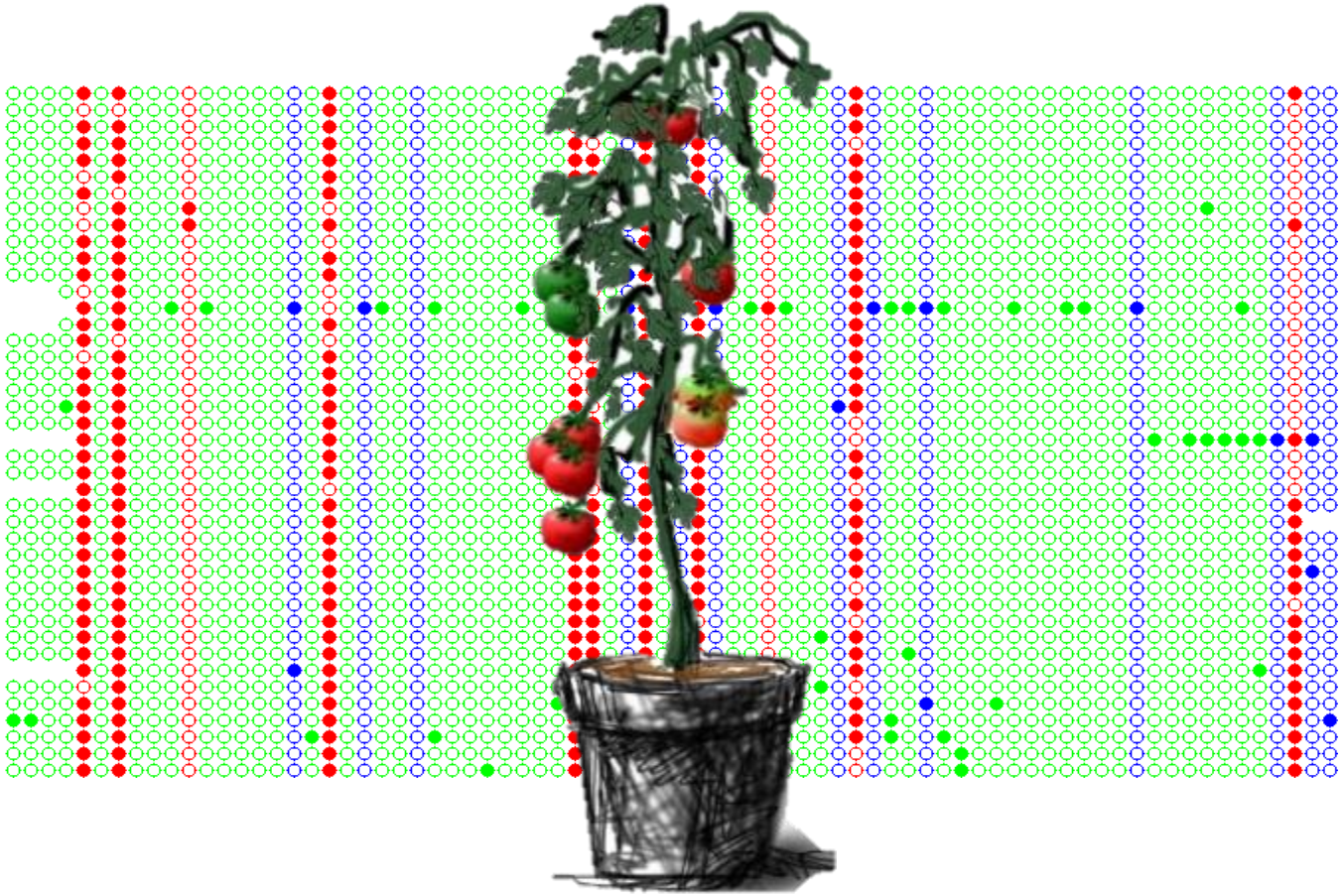
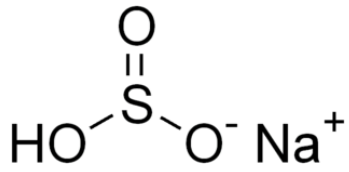
Bisulfite Sequencing for Site-Specific DNA Methylation Analysis



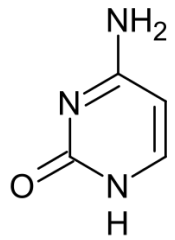
Outline

- Principles of Bisulfite Conversion
- Methodology of Bisulfite Sequencing
  - DNA extraction
  - Bisulfite treatment
  - Amplification
  - Cloning
  - Sequencing
- Summary

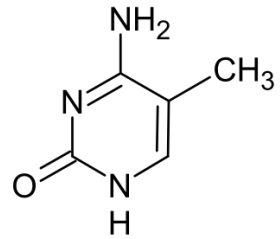
Principles of Bisulfite Conversion



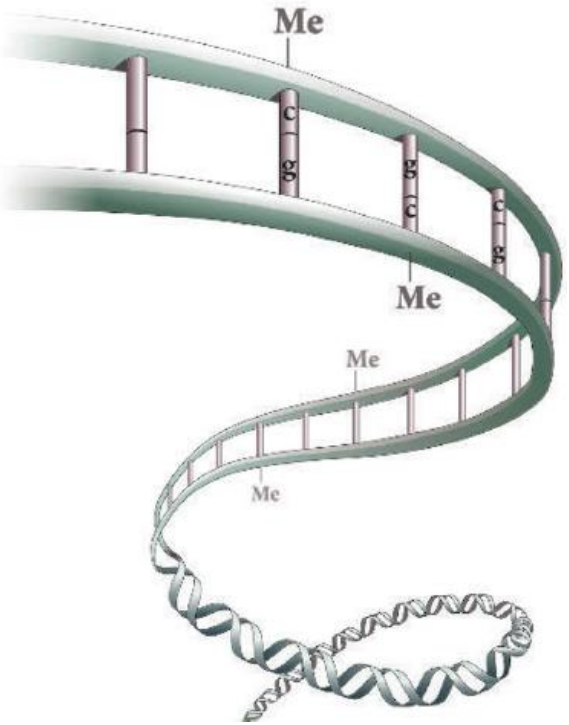
Sodium bisulfite



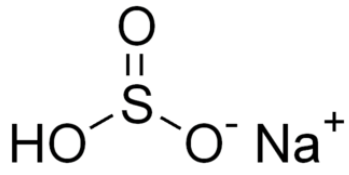
cytosine



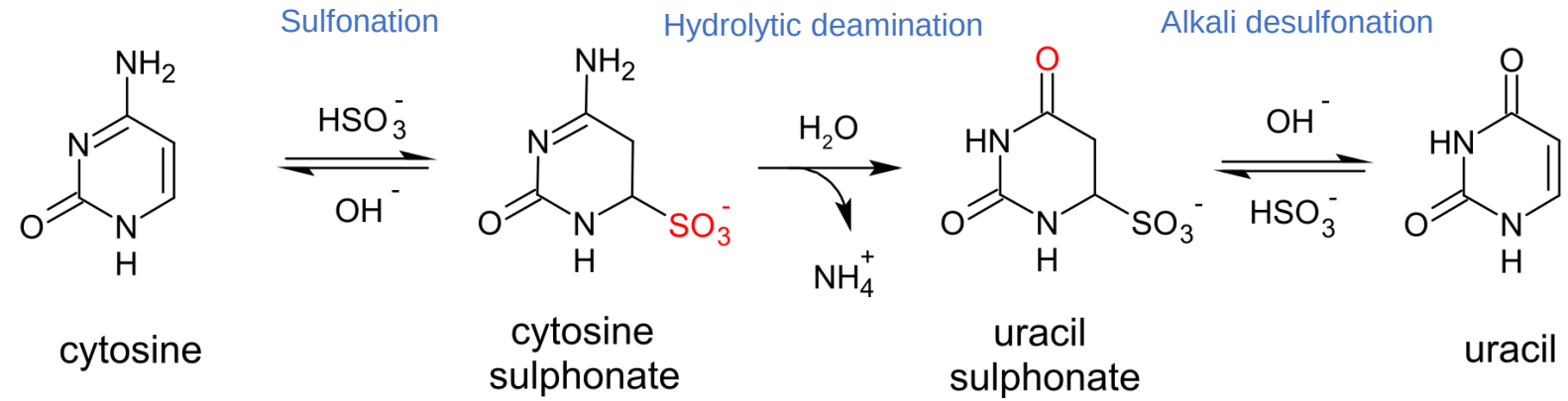
5-methylcytosine



Principles of Bisulfite Conversion



Sodium bisulfite

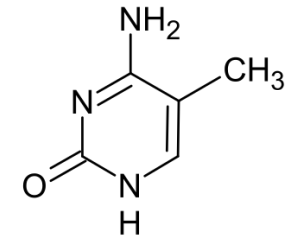


cytosine

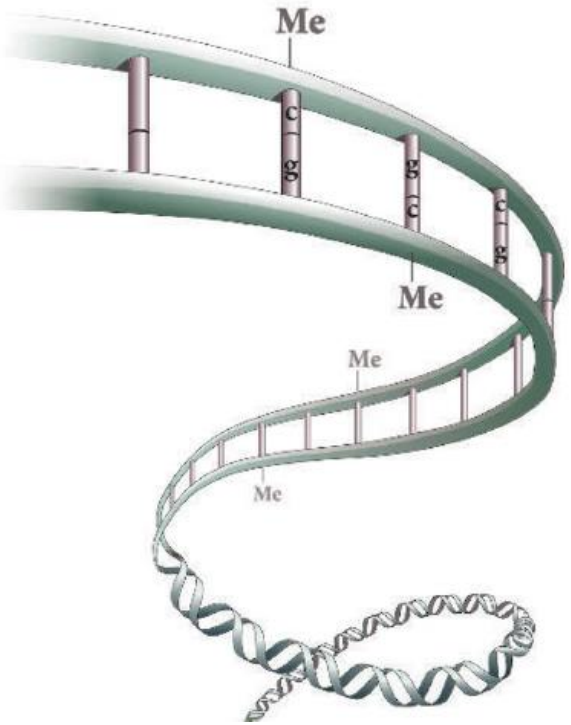
cytosine sulphonate

uracil sulphonate

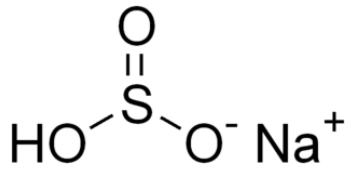
uracil



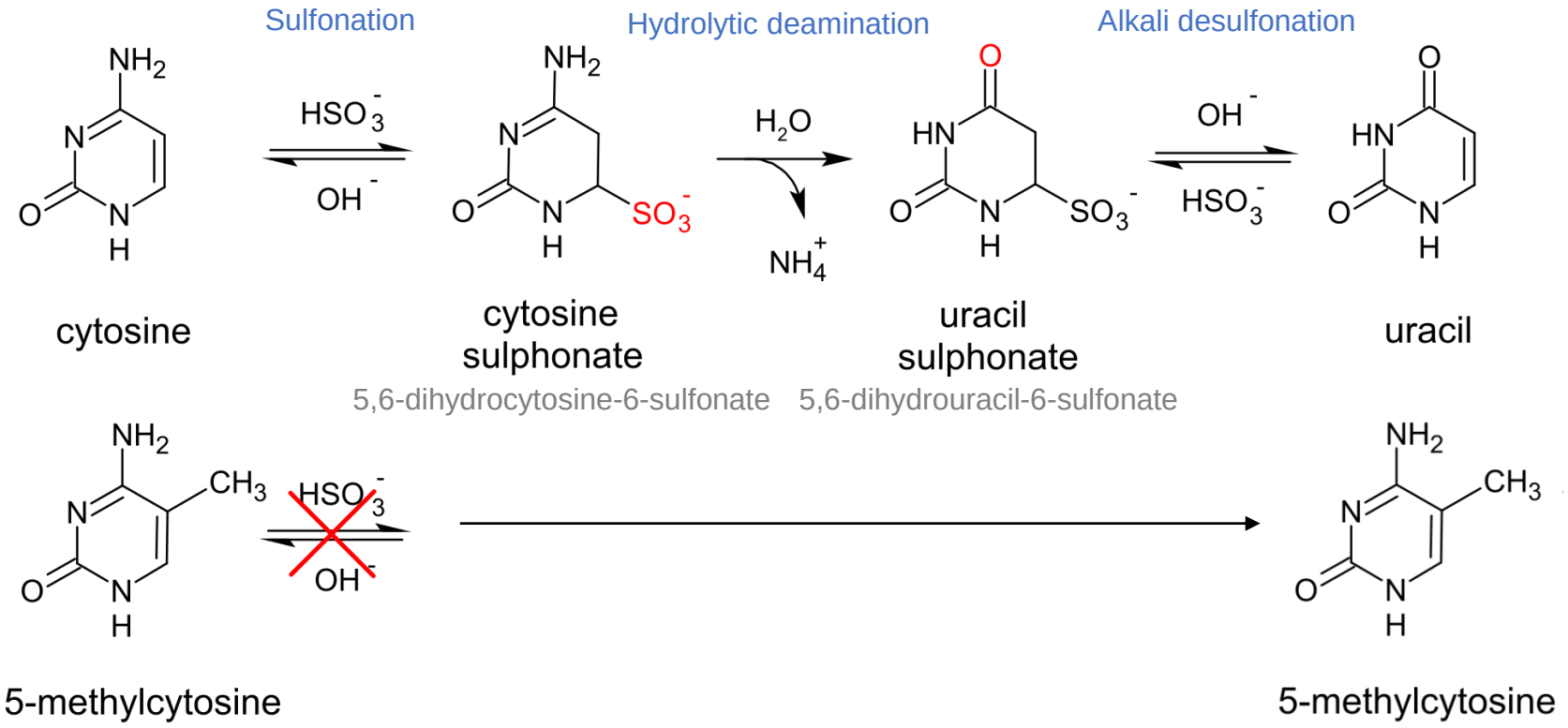
5-methylcytosine

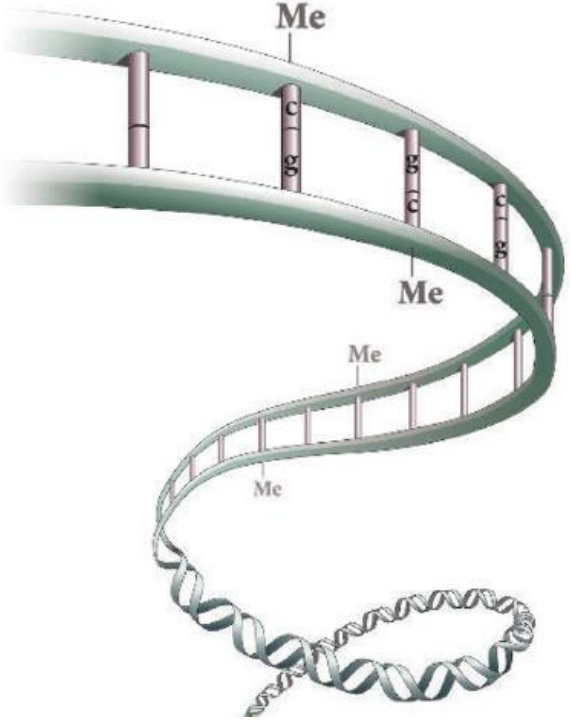


Principles of Bisulfite Conversion

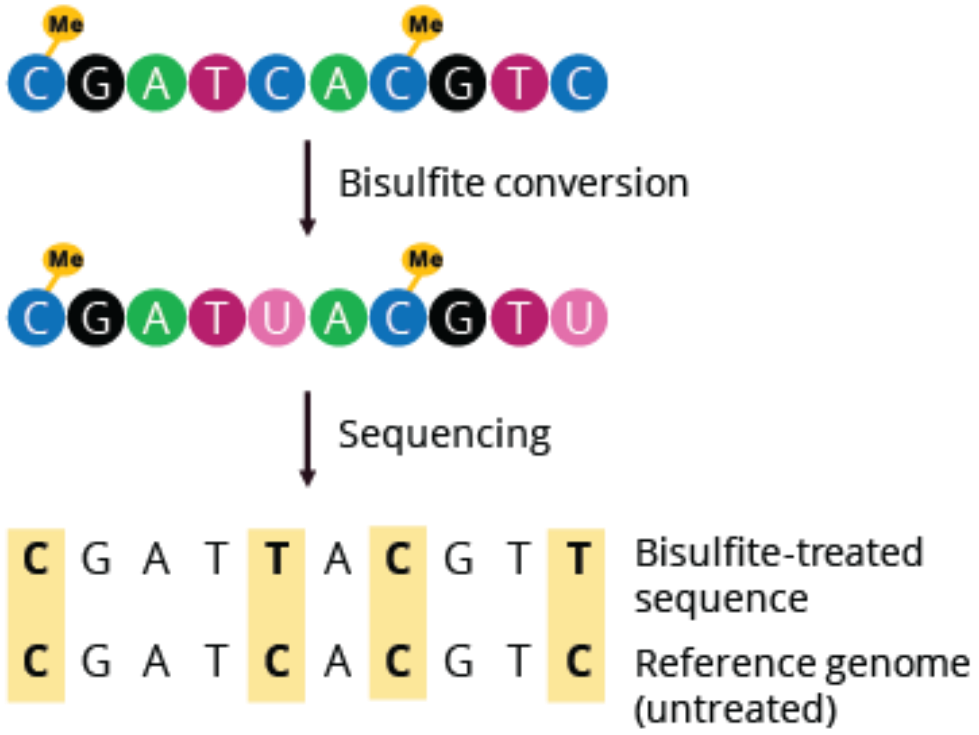


Sodium bisulfite





Principles of Bisulfite Conversion



Methodology of Bisulfite Sequencing

Methodology of Bisulfite Sequencing

- Plant genomic DNA extraction kit (recommended)
- Standard CTAB DNA extraction

Methodology of Bisulfite Sequencing

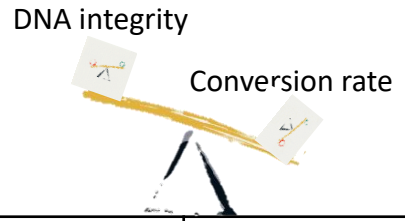
Genomic DNA extraction → **Bisulfite treatment** → Target amplification → Cloning & selection → Sequencing

Commercial kits available for **plant** DNA bisulfite conversion

Methodology of Bisulfite Sequencing

1. Mix preparation
2. DNA conversion →
3. DNA cleanup

Bisulfite treatment



Option 1

Step	Time (min)	Temperature
Denaturation	5	95°C
Incubation	25	60°C
Denaturation	5	95°C
Incubation	85	60°C
Denaturation	5	95°C
Incubation	175	60°C
Hold	∞	20°C

Duration: ~ 5 hrs

Option 2

Step	Time (min)	Temperature
Denaturation	2	95°C
Incubation	120	75°C
Denaturation	1	95°C
Hold	60	75°C
Hold	∞	10°C

X 8

Duration: ~ 18 hrs

Methodology of Bisulfite Sequencing

PCR amplification using the bisulfite-treated DNA

General considerations for primers design:

- Use a designated software (freely available on the web)
- Target a methylated region ≤ 500bp
- Design nested primers
- As few Cs as possible

Methodology of Bisulfite Sequencing

Freely available tools for primer design:

- Kismeth http://katahdin.mssm.edu/kismeth/primer_design.pl
- Methyl primer express

  https://www.thermofisher.com/order/catalog/product/4376041?SID=srch-srp-4376041
- Bisulfite Primer Seeker https://www.zymoresearch.com/pages/bisulfite-primer-seeker
- And more….

Bisulfite Primer Design

Use the form below to select the file containing your sequence in fasta format: ?

Sequence file: Choose File SITAB2.txt

Display name (optional): Example ?

Min. primer length	Max. primer length	Min. product length	Max. product length	Min. Tm
20	30	250	500	48

Number of primers 10

Primers for other strand ? ☑

Upload file and generate primers

http://katahdin.mssm.edu/kismeth/primer_design.pl

Ravi LAB Kismeth

Kismeth:Bisulfite Primer Design

Home

Bisulfite primers

File SITAB2.txt, size 687 bytes has uploaded successfully.

Input sequence (676 bp)
AAAAAAATAAAAAATAAAAAAAAAATTAGTCTCCCTTATCTTTTAAATAAAAAAATAAAAAATAAAAATAAATAATTTGGG
TTTGATGGCTTGTCTAAGCTGCAGTCTCCCTTTACTTCAAACTCATAAAGCGATTTCCAATTTCAGTGTATTCCCCTTTA
AATCAGTTGCAATATGTTTACCAAAATGTAAGGTGAAGTCTGTTCATATTCCATCAGTTTCTGAAGCTTCTTCTATTTCA
TCTACTAATTTGGAAGAAGAGGAGGACGACGATCCCACTGCTGAACTTGTTTATCTTGACCCTGAAATTGATCCTGAGAG
CTTATCTGAGTGGGAATTGGATTTTTGTTCAAGACCAATTCTTGATATTAGAGGGAAAAAATTATGGGAGCTTCTTGTTT
GTGATGATTCCCTCTCTCTTCAGTATACCAAATATTTTCCTAATAATCTTATCAATAGTATCACTTTGAAAGATGCTTTA
CTATCTATATCTAATGACTTAGGTATCCCATTACCCGATAAAATCAGATTCTTCAGGTACTCTTAACTTACATCCTCTCA
ACTGTTTTACATTTTCAATTCTCTCATCAATTGTTTTCCATTTTTAGGTCACAAATGCAAACTATTATTACAAGAGCTTG
CAACGAACTTGCCATCAAACCTGTTCCTAGCAAACG

Forward strand primers visualize

Count	forward primer	reverse primer	product length	for,rev pos	T1,T2(forward)	T1,T2(reverse)
1	AAATAAAAATAAATAATTTGGGTTTGATGG	CTCCCATAATTTTTTCCCTCTAATATCAA	273	58,361	50.6,50.6	54.4,54.4
2	TAATTTGGAAGAAGAGGAGGA	TACCTRAARAATCTRATTTTATC	250	245,516	48.5,48.5	42.7,48.1
3	AATATGTTTAYYAAAATGTAAGGTGAAGT	TAAARCATCTTTCAAARTRATACTATT	254	170,453	50.1,53	46.0,50.6
4	AAAATGTAAGGTGAAGTYTGTTYATATT	ATATARATARTAAARCATCTTTCAAA	254	182,464	49.6,52.6	43.7,48.5

T1 is the Tm calculated by replacing all C's with T's in the forward primer (and G's with A's in the reverse primer),
T2 is the Tm for the unmodified primer

Reverse strand primers visualize

Count	forward primer	reverse primer	product length	for,rev pos	T1,T2(forward)	T1,T2(reverse)
1	AGTTGAGAGGATGTAAGTTAAGAGTA	TCTCCCTTTACTTCAAACTCATAAA	408	537,104	53.2,53.2	51.1,51.1
2	TATTGATAAGATTATTAGGAAAATATTTGG	CTCCCTTATCTTTTAAATAAAAAAATAAAA	368	427,29	50.6,50.6	49.3,49.3
3	AATTGATGAGAGAATTGAAAATGTAAAA	AARCTTCTTCTATTTCATCTACTAATTT	313	564,223	49.6,49.6	49.6,51.1
4	GGAAAATATTTGGTATAYTGAAGAGAGAGG	AAAAATTARTCTCCCTTATCTTTTAAATAA	361	410,19	56.1,57.5	49.3,50.6
5	GAAAATGTAAAAYAGTTGAGAGGATGTAAG	TCCAATTTCARTRTATTCCCCTTTAAATCA	381	546,135	54.7,56.1	53.4,56.1
6	TGGTATAYTGAAGAGAGAGGGAAT	AAAAATAAAAAAAAAATTARTCTCCCTTATC	367	406,9	52.2,53.9	49.3,50.6
7	AAAAATGGAAAAYAATTGATGAGAGAATTG	CTTCAAACTCATAAARCRATTTCCAATTTC	431	575,114	52.0,53.4	53.4,56.1
8	AAAGTGATAYTATTGATAAGATTATTAGGA	CTRCARTCTCCCTTTACTTCA	318	437,98	50.6,52	48.5,52.4
9	TGATTTTATYGGGTAATGGGATA	TATTCCCCTTTAAATCARTTRCAATAT	328	503,148	48.1,49.9	49.1,52.1
10	TAAGTYATTAGATATAGATAGTAAAG	TCTRTTCATATTCCATCARTTTCT	253	475,198	46.9,48.5	47.1,50.5

T1 is the Tm calculated by replacing all C's with T's in the forward primer (and G's with A's in the reverse primer),
T2 is the Tm for the unmodified primer

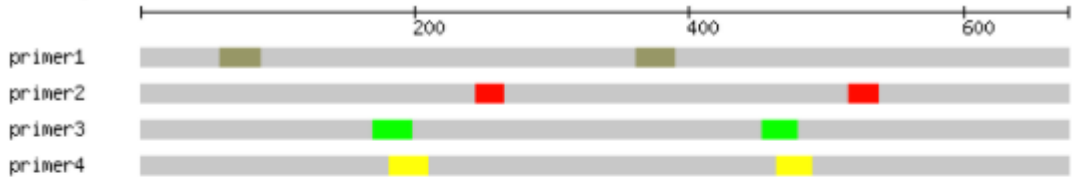
Please contact Ravi Sachidanandam (ravi.cshl.work[at]gmail.com) with any questions.

C -> Y (C or T) in Forward primer

G -> R (G or A) in Reverse primer

Visualization

Forward strand primers



highlighted regions correspond to features in track: primer4, primer3, primer2, primer1

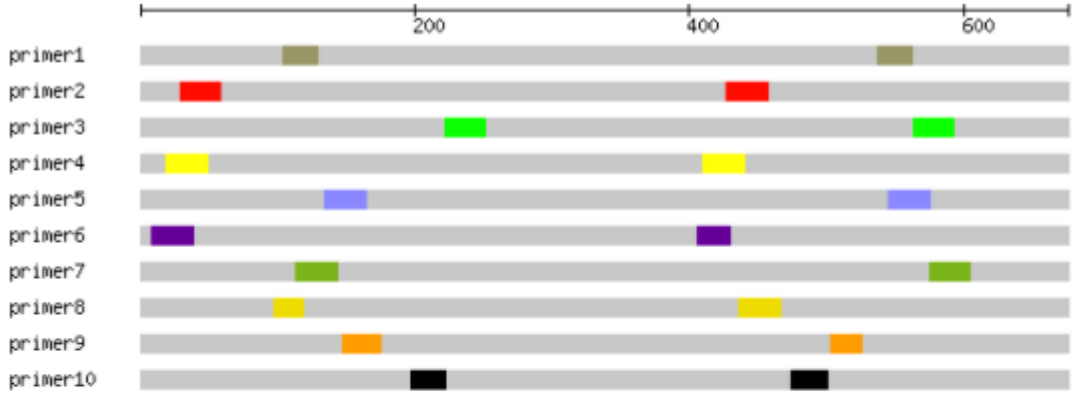
AAAAAAATAA AAAATAAAAA AAAATTAGTC TCCCTTATCT TTTAAATAAA AAAATAAAAA ATAAAAATAA ATAATTTGGG
TTTGATGGCT TGTCTAAGCT GCAGTCTCCC TTTACTTCAA ACTCATAAAG CGATTTCCAA TTTCAGTGTA TTCCCCTTTA
AATCAGTTGC AATATGTTTA CCAAAATGTA AGGTGAAGTC TGTTCATATT CCATCAGTTT CTGAAGCTTC TTCTATTTCA
TCTACTAATT TGGAAGAAGA GGAGGACGAC GATCCCACTG CTGAACTTGT TTATCTTGAC CCTGAAATTG ATCCTGAGAG
CTTATCTGAG TGGGAATTGG ATTTTTGTTC AAGACCAATT CTTGATATTA GAGGGAAAAA ATTATGGGAG CTTCTTGTTT
GTGATGATTC CCTCTCTCTT CAGTATACCA AATATTTTCC TAATAATCTT ATCAATAGTA TCACTTTGAA AGATGCTTTA
CTATCTATAT CTAATGACTT AGGTATCCCA TTACCCGATA AAATCAGATT CTTCAGGTAC TCTTAACTTA CATCCTCTCA
ACTGTTTTAC ATTTTCAATT CTCTCATCAA TTGTTTTCCA TTTTTAGGTC ACAAATGCAA ACTATTATTA CAAGAGCTTG
CAACGAACTT GCCATCAAAC CTGTTCCTAG CAAACG

⬇

No nesting option

Visualization

Reverse strand primers



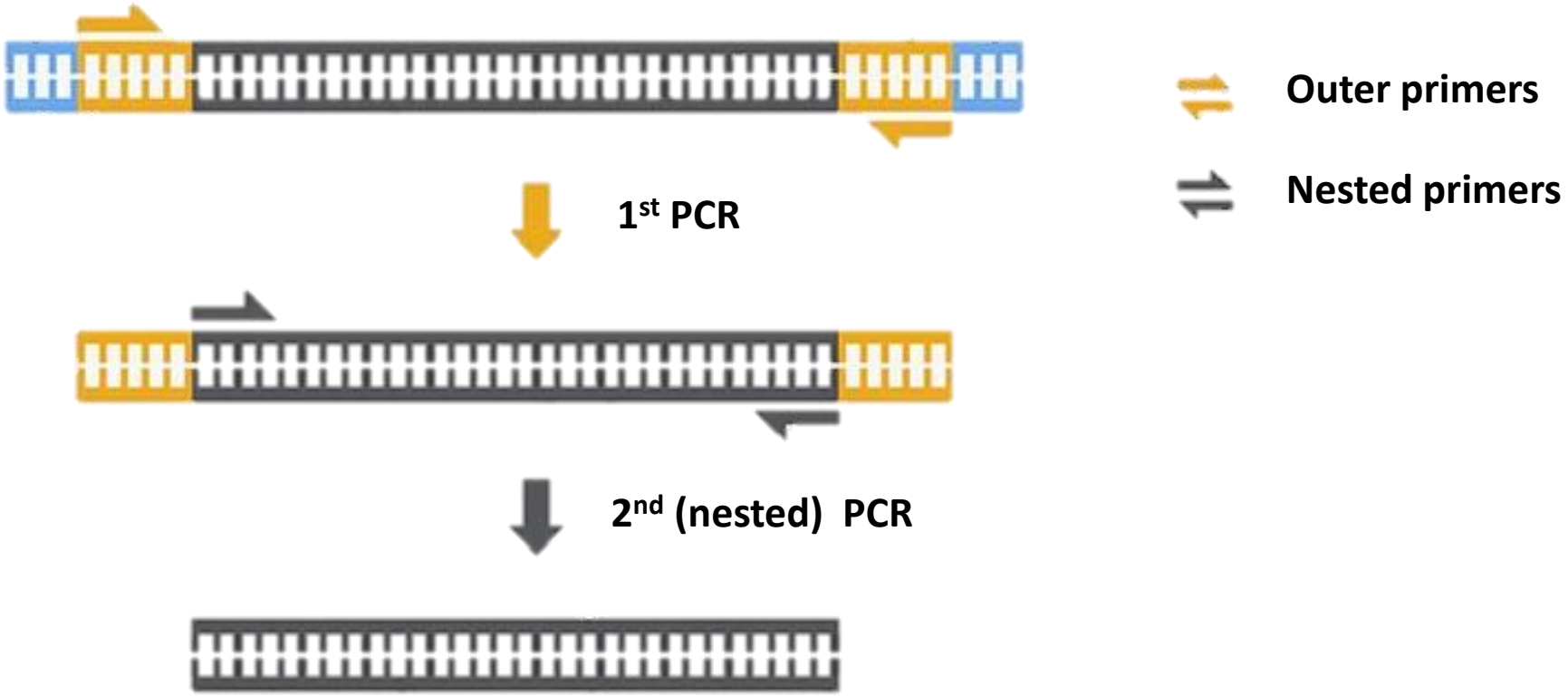
highlighted regions correspond to features in track: primer10, primer9, primer8, primer7, primer6, primer5, primer4, primer3, primer2, primer1

AAAAAAATAA AAAATAAAAA AAAATTAGTC TCCCTTATCT TTTAAATAAA AAAATAAAAA ATAAAAATAA ATAATTTGGG
TTTGATGGCT TGTCTAAGCT GCAGTCTCCC TTTACTTCAA ACTCATAAAG CGATTTCCAA TTTCAGTGTA TTCCCCTTTA
AATCAGTTGC AATATGTTTA CCAAAATGTA AGGTGAAGTC TGTTCATATT CCATCAGTTT CTGAAGCTTC TTCTATTTCA
TCTACTAATT TGGAAGAAGA GGAGGACGAC GATCCCACTG CTGAACTTGT TTATCTTGAC CCTGAAATTG ATCCTGAGAG
CTTATCTGAG TGGGAATTGG ATTTTTGTTC AAGACCAATT CTTGATATTA GAGGGAAAAA ATTATGGGAG CTTCTTGTTT
GTGATGATTC CCTCTCTCTT CAGTATACCA AATATTTTCC TAATAATCTT ATCAATAGTA TCACTTTGAA AGATGCTTTA
CTATCTATAT CTAATGACTT AGGTATCCCA TTACCCGATA AAATCAGATT CTTCAGGTAC TCTTAACTTA CATCCTCTCA
ACTGTTTTAC ATTTTCAATT CTCTCATCAA TTGTTTTCCA TTTTTAGGTC ACAAATGCAA ACTATTATTA CAAGAGCTTG
CAACGAACTT GCCATCAAAC CTGTTCCTAG CAAACG

Nested PCR primers:

- **primer7 + primer5**
- **Primer9 + primer 10**

Nested PCR for increased specificity and yield:



Methodology of Bisulfite Sequencing

Standard cloning procedure

- ligation into cloning vector
- Transformation into bacteria (*E.Coli*), colony selection (6-10 positive)
- Plasmid purification -> sequencing

Methodology of Bisulfite Sequencing

Sequence analysis

1) Clean vector sequences
2) Blast to validate identity
3) KISMETH analysis

Bisulfite Analysis

Display name: test ?

Select the files containing your sequences: ?

Formats **YES:** FASTA **NO:** WORD,PDF,EXCEL

Reference sequence file: Choose File AtMu1_plus_wt_leaf.fa

Comparison sequences file: Choose File AtMu1_plus_ddm1_leaf.fa

Parameters(rtfm before changing them)

Remove pcr duplicates: ☐

Min. fraction of positive matches: 0.8 Min. fraction of length: 0.5

Start of match: End of match:

Upload selected files and run analysis

Example data files from pilot studies

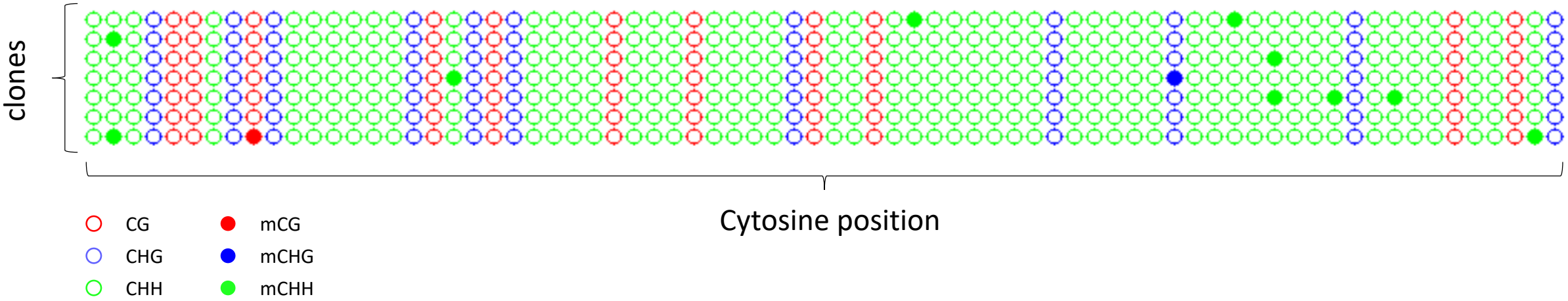
http://katahdin.mssm.edu/kismeth/revpage.pl

Bisulfite Analysis

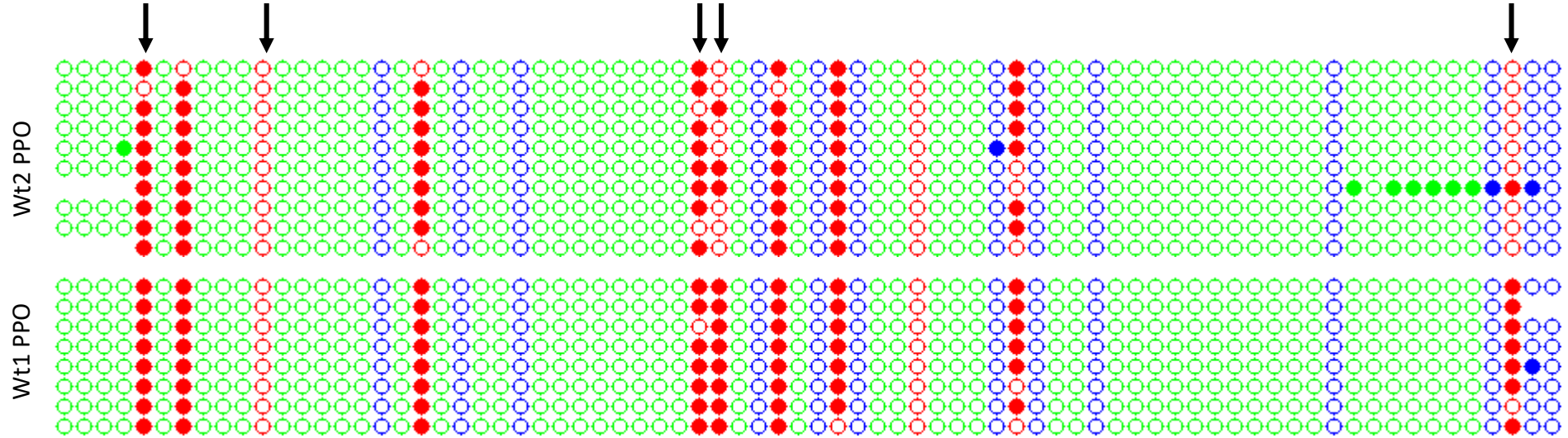
Overall statistic	Total methylation (%)
CG	1.29%
CHG	1.29%
CHH	2.74%
all	2.3126%

→ **~97.7 % conversion**

Unmethylated chloroplastic region

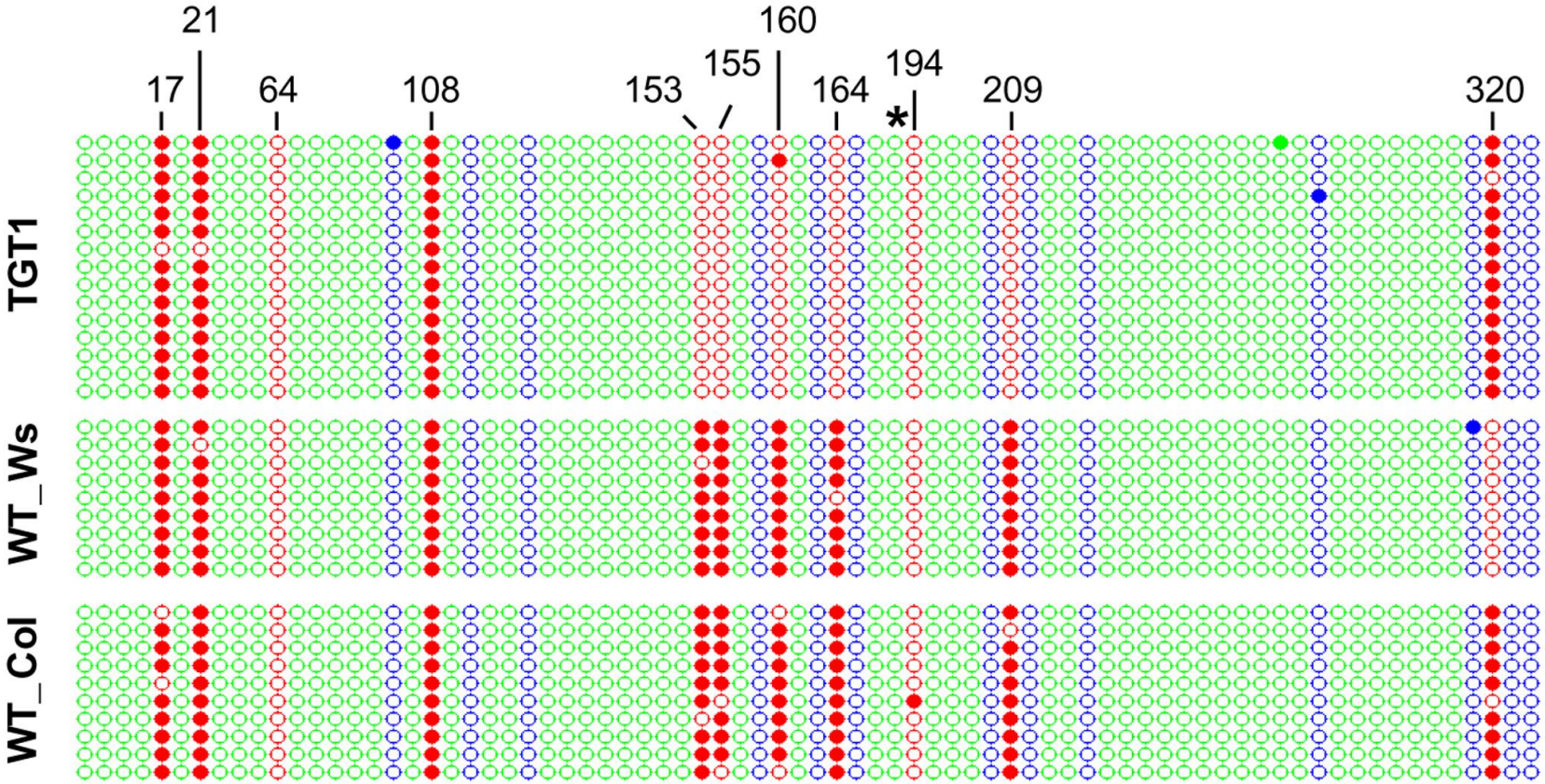


DNA bisulfite sequencing results – Example 1



○	CG	●	mCG
○	CHG	●	mCHG
○	CHH	●	mCHH

DNA bisulfite sequencing results – Example 2



Lieberman-Lazarovich M, Melamed-Bessudo C, de Pater S, Levy AA (2013) Epigenetic Alterations at Genomic Loci Modified by Gene Targeting in *Arabidopsis thaliana*. PLoS ONE 8(12): e85383. https://doi.org/10.1371/journal.pone.0085383

Summary

Bisulfite sequencing:

- Provides information on DNA methylation at a nucleotide level resolution, allowing the detection of the position of methylated cytosines and the level of methylation.
- Key factors for a successful assay: primer design, non-methylated control
- Can be used to detect methylation status
  - of a particular locus
  - genome wide