

ABRF
DNA Sequencing
Research Group
February 11, 2006

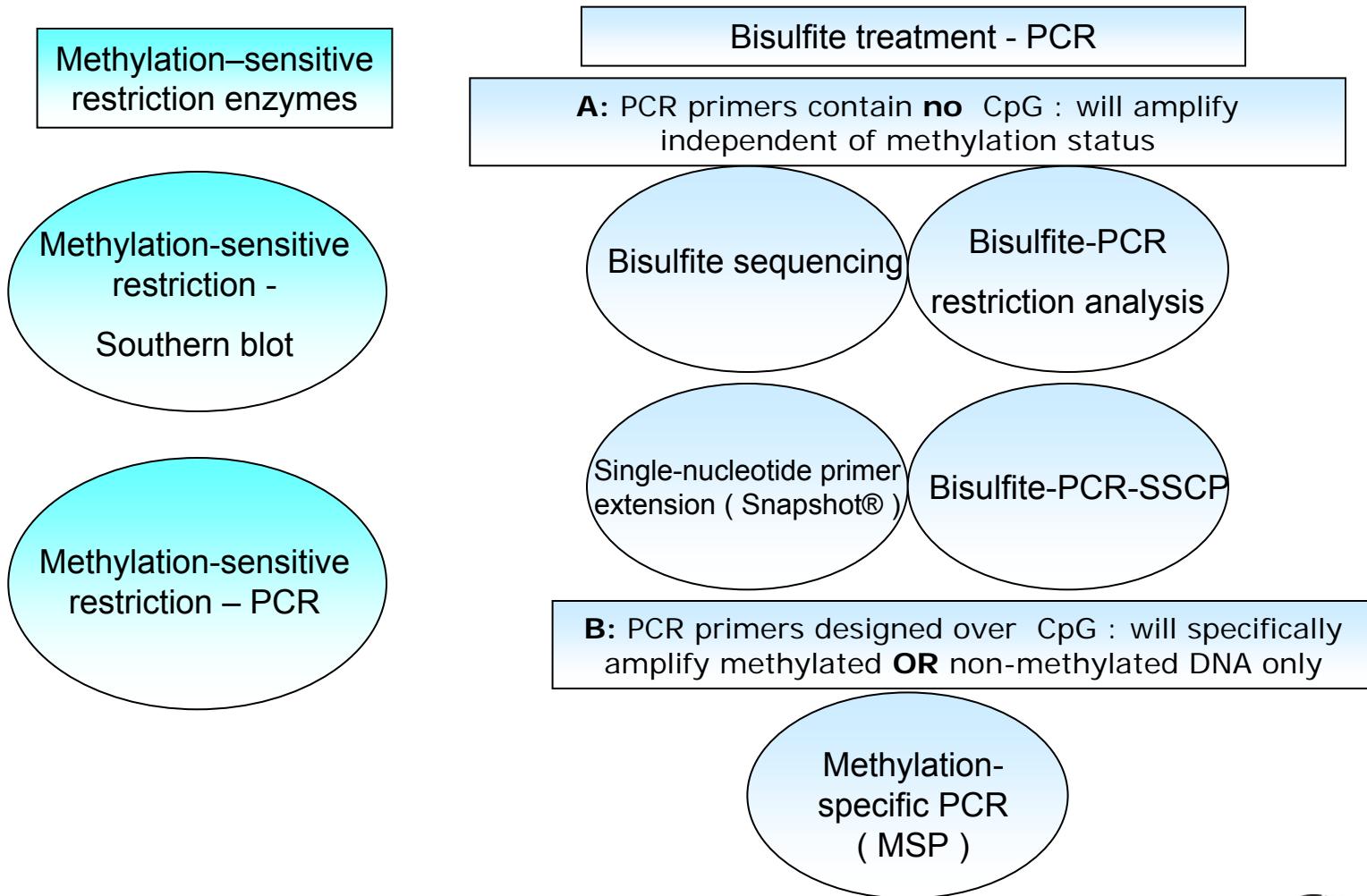
Improved Protocols for Bisulfite Sequencing and Fragment Analysis of Methylated gDNA

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AB Molecular Biology – Genetic Analysis

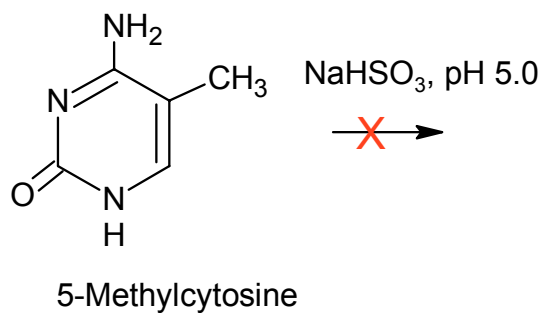
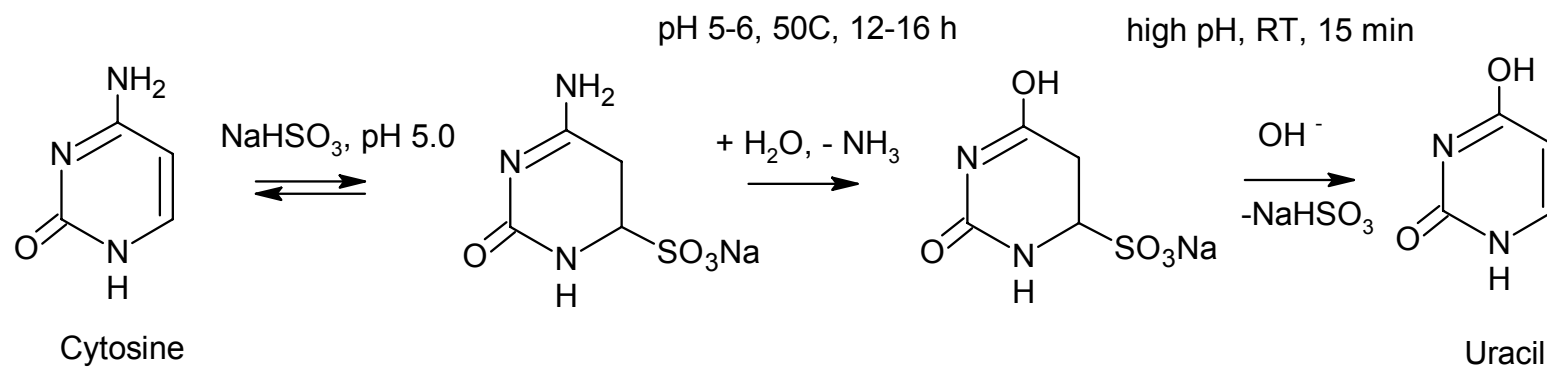
Epigenetic modification in human genome by DNA-methylation

- Methylation occurs on the cytosine residue of CpG dinucleotide
- CpG is underrepresented in the genome and is mostly methylated (X-chromosome silencing, Alu repeats, genomic imprinting)
- CpG islands are 1 kb stretches with high CpG density
- CpG islands in promotor regions are typically **not** methylated, allowing transcription factors to bind → gene activity
- There are about 27,000 CpG islands in human genome
- Age- and disease (cancer) – related aberrant methylation in promotor CpG islands causes gene silencing and decrease of gene expression
- A list of genes positively identified as methylated in cancer currently includes 66 genes (www.mdanderson.org: methylation in cancer)

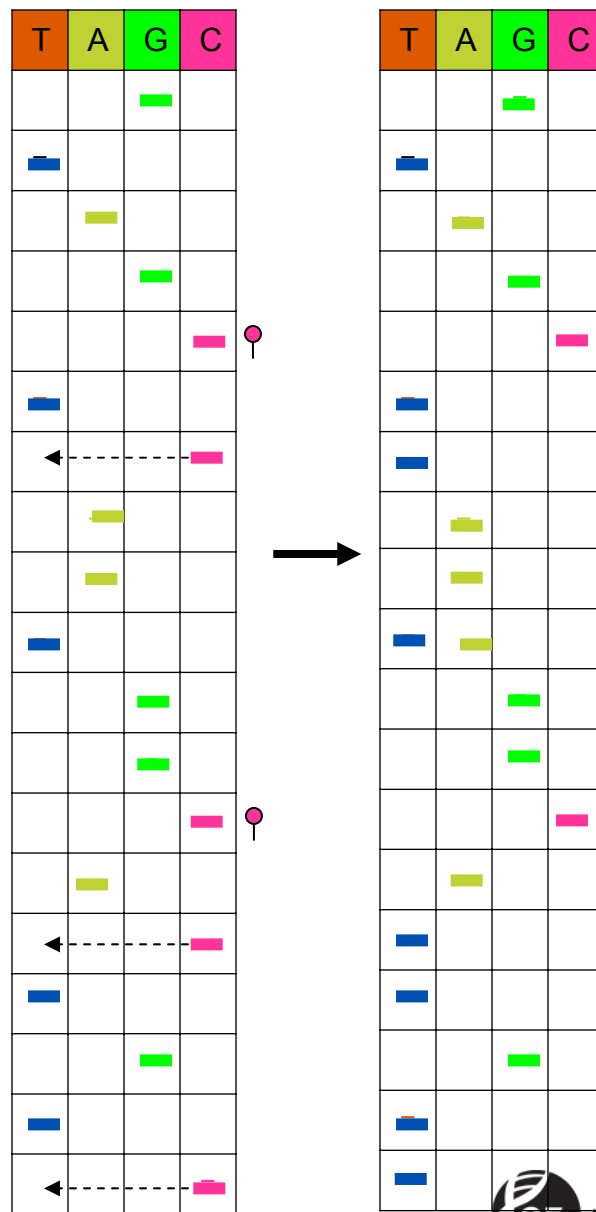
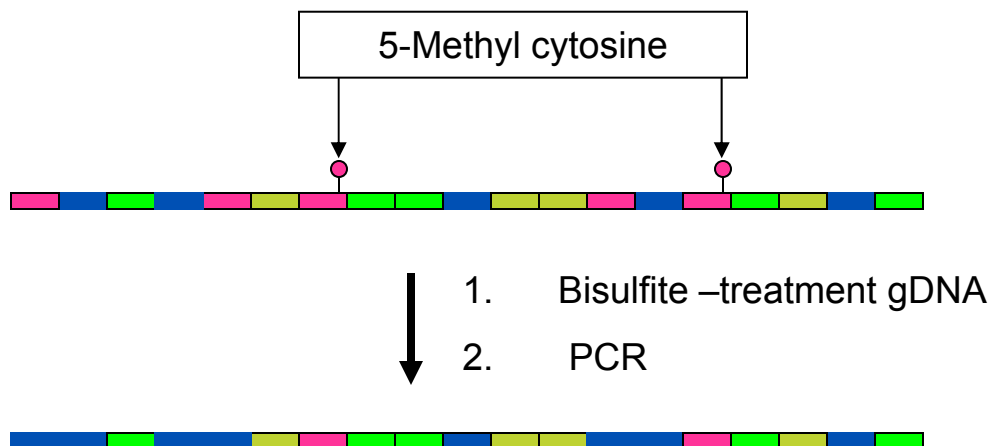
Methods to analyse methylation status



Chemistry of bisulfite treatment



Effect of bisulfite treatment on DNA sequence



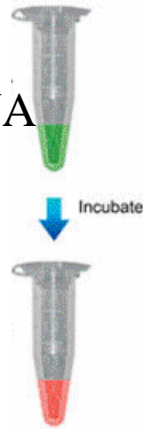
Bisulfite / PCR / Sequencing workflow

Stage	Processing step	Tools
Database target sequence containing CpG	PCR primer design	MethPrimer or Methyl Primer Express® software
gDNA		
↓	1. Bisulfite treatment	Steps 1-to-4: use commercially available bisulfite-modification kits
↓	2. DNA cleanup	
↓	3. De-sulfonation	
↓	4. DNA cleanup	
↓	5. PCR	
↓	6. Exo/SAP or DNA cleanup	
↓	7. DNA sequencing	BDT v. 1.1 chemistry, 3100/3730 electrophoresis, KB™ basecaller
↓	8. Dye terminator cleanup	
↓	9. Electrophoresis	
DNA sequence		
↓	Data evaluation	SeqScape® software
Positional CpG –genotype		

Bisulfite treatment workflow

STEP 1

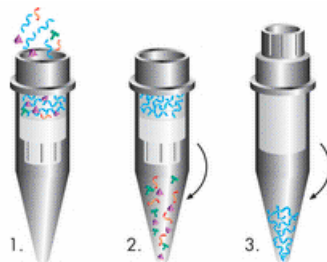
Denature gDNA



Add bisulfite reagent and incubate 15 h @ 50 C

STEP 2

Purify with Microcon 100



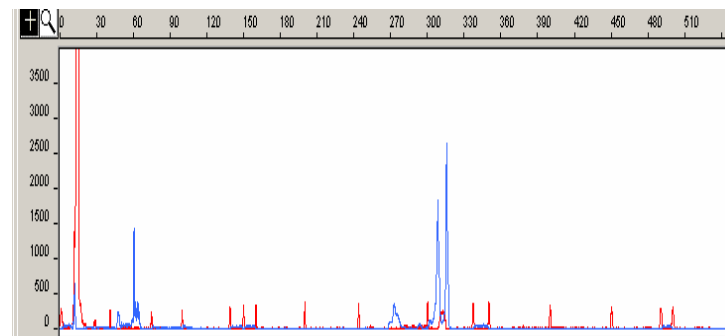
STEP 3

PCR

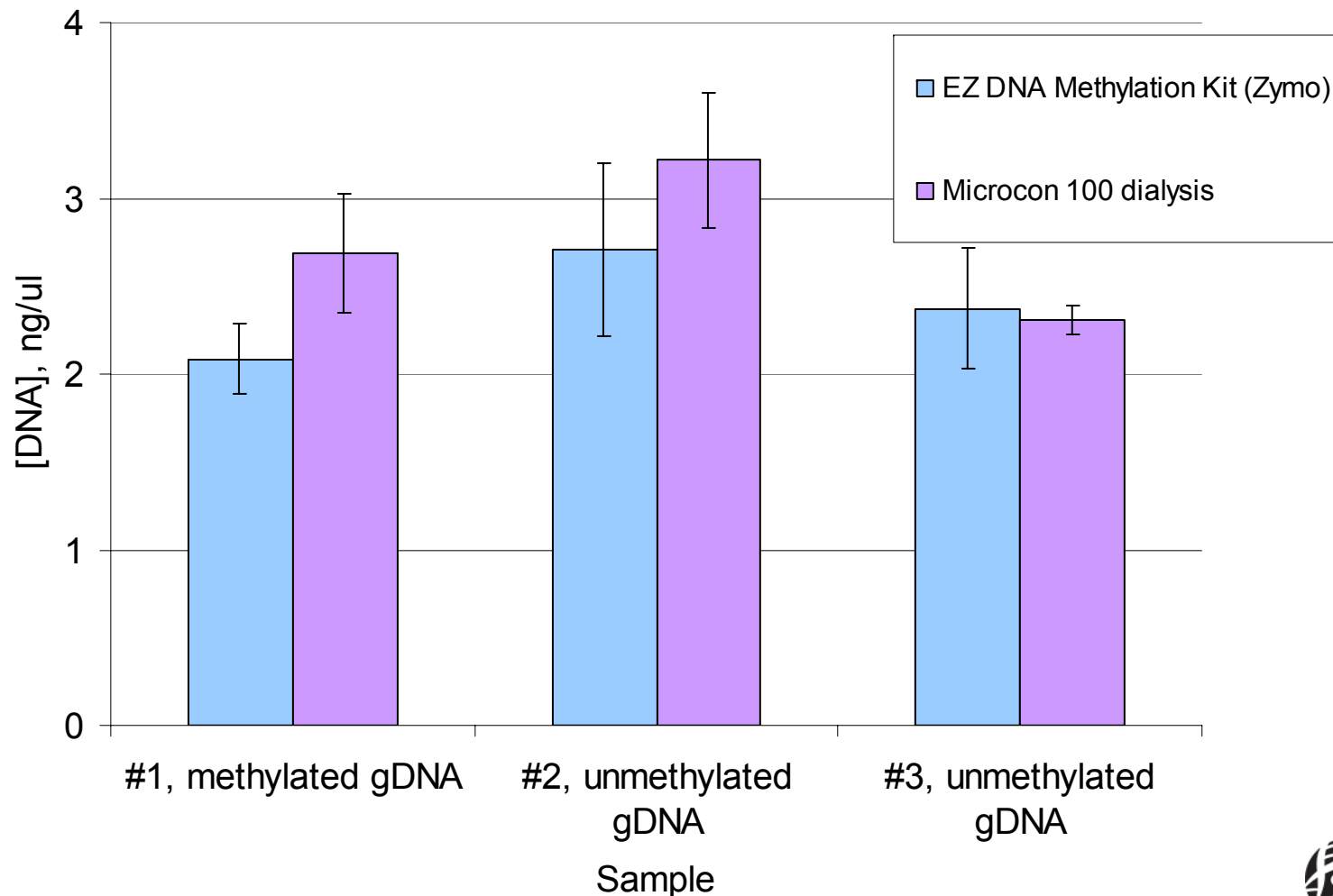


STEP 4

Fragment Analysis



DNA recovery: modified 'EZ DNA methylation kit' using Microcon 100 spin dialysis device



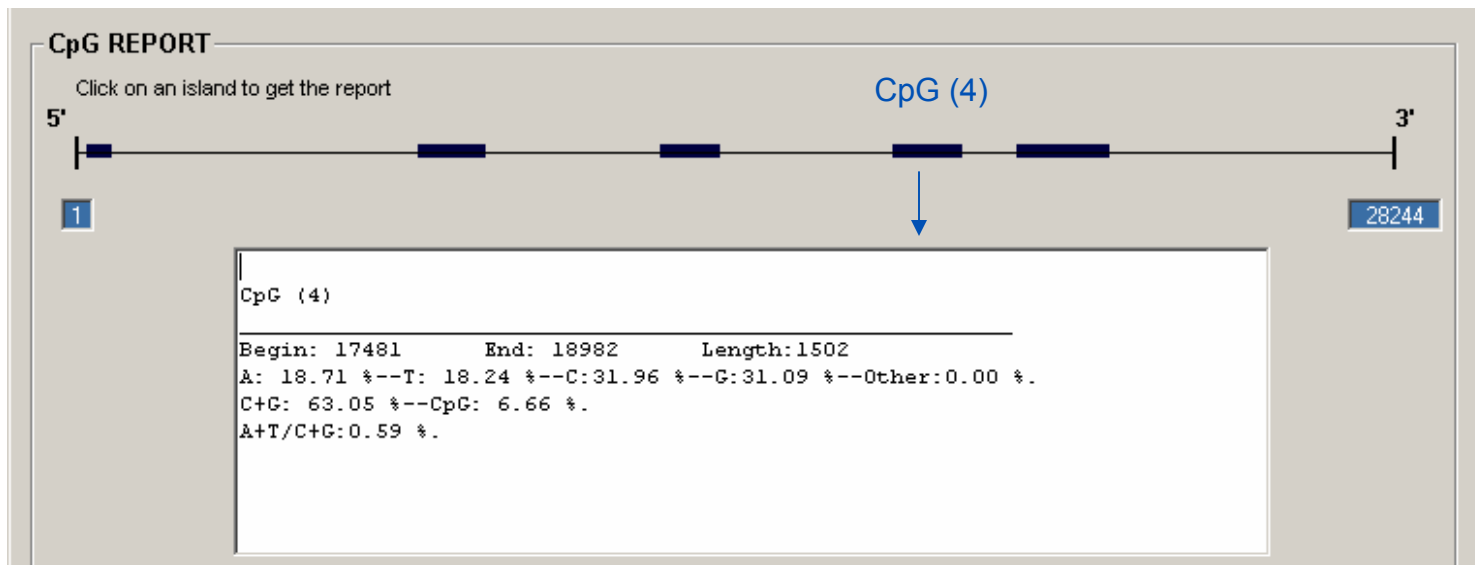
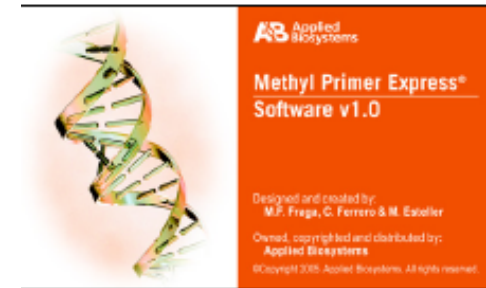
PCR and cycle sequencing protocols

PCR of bisulfite-converted gDNA:		PCR conditions for tailed primers:
AmpliTaq Gold® 10X buffer	1.0 µl	95°C /5 min 5x (95°C /30 s; 60°C /2:00 min; 72°C/ 3:00 min) 30x (95°C /30 s; 65°C /1:00 min; 72°C/ 3:00 min) 60°C /85 min, 4°C Hold
dNTP 2.5 mM each	0.8 µl	
MgCl ₂ 25 mM	0.8 µl	
AmpliTaq Gold® (5 U/ul)	0.2 µl	
Fwd and Rev Primer mix (2.5 µM each)	0.5 µl	
Bisulfite-gDNA template (5-10ng/ul)	0.5 µl	
Water	6.2 µl	
Total	10.0 µl	

DNA sequencing of bisulfide/PCR product:		Sequencing conditions for universal primers:
Bisulfite/PCR product (1-5 ng/ul *)	1 µl	96°C /1 min 25x (96°C /10 s; 50°C / 4:00 min) 4 °C Hold
BigDye® Terminator v1.1 Ready Reaction Mix	8 µl	
Primer (M13 Forward or Reverse, 3.2 uM)	1 µl	
Water	10 µl	
Total volume	20 µl	

* may require dilution

Detection of CpG islands in gDNA (28 kb GenBank file) using Methyl Primer Express® software (AB)

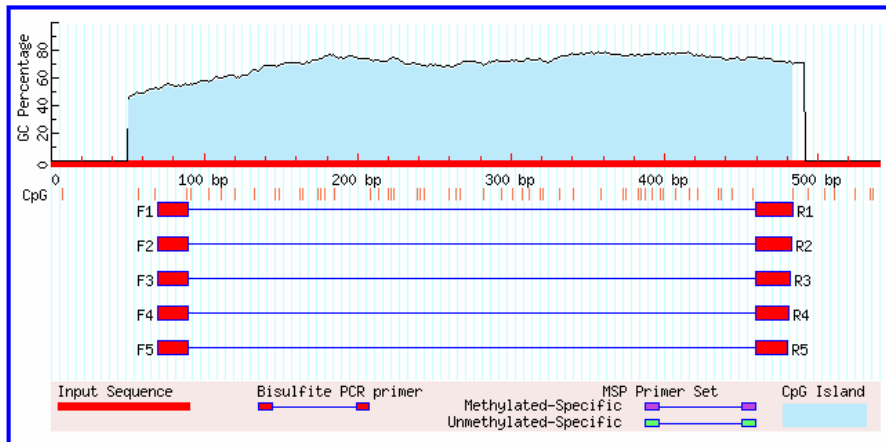


Prediction of CpG islands in 28,244 bp genomic DNA region containing RasSF promoter (GenBank Acc. # AC002481) using Settings: Min island size 300 bp, GC % > 50, GC Obs/Exp > 0.6

Bisulfite sequencing: primer design



MethPrimer result



Sequence Name: AC002481: bp 18036 - 18575, - strand
 Sequence Length: 540

CpG island prediction results
 (Criteria used: Island size > 100, GC Percent > 50.0, Obs/Exp > 0.6)
 1 CpG island(s) were found in your sequence
 Size (Start - End)
 Island 1 433 bp (51 - 483)

Sequencing Primer Report

Back Clear Exit

Click on a primer to see the report The best primers for DNAMethyl-easy are those closer to the CpGs-representation

Report

SEHSE
 Length: 22 bp
 5' TTGTGCTTAGATAYGASTGG 3'
 Tm=49.40; CpG=1; C=4

If you want to modify:
 5' AGTATATTTTGTATTTGTGCTTAGATAYGAST
 3' GGACTCGATAAGGGAT

Bisulfite modification of the DNA

```

AAAAATTAGCCTATTTTATATATAAGTACTATTTTGTGCTTAGATAYGASTGG
TGGAGTGGCATAAGGATAAATTTTCCGCTATTTTATAGCAGTGGGAAAGTAAAGC
GATTTAGTTTTCGGGACTTCTTTTCGCTGATTTTTCGCGCATTTGATTCGGCGGAT
TCCGTTGTTTTTGGTGTCTTTTTCGTTTTTCGAGCCCGCCGGGGTTATTTATCCGC
CTATTCTAGTTTTTCCCTACGCTTTTAGATGAAGCTTATAGAGCTCTATTACGCTG
TCCGTTGGGGTTTTCCCGGTTGGAAGCCGCTGATACGCTTAGGATTAAGTCTCTGTGG
GGTTGTACCGCGTTTTCCCGCATGCGTAGCCGCTTGGTACGTTTACTCGGGTCCGTT
TTTTTAGCCGCTTAGCGGGTTTAACTTTTCGACTTTAATGATTTAGCTTTTTTCGAT
ATGGTTCGGTTGGGTTCTGTTTCGCTGGTTTTGGGCTTAGTAAAGCCGGG
  
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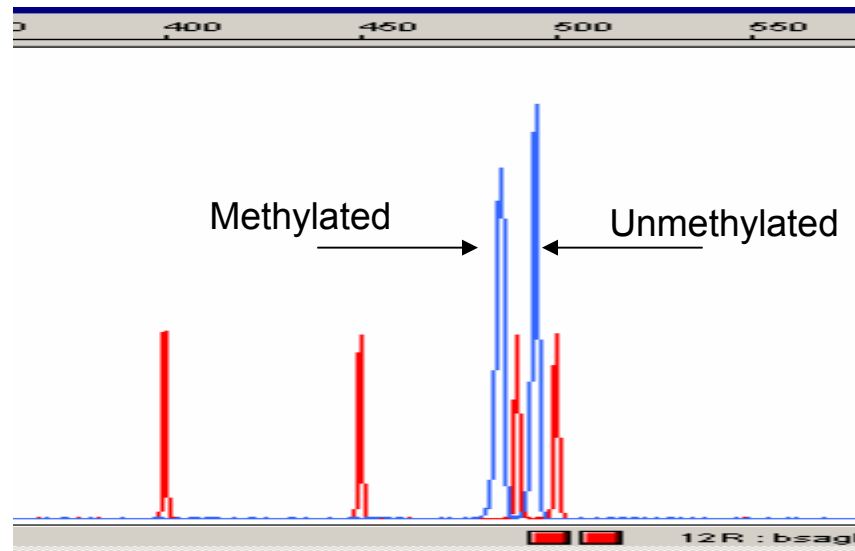
Initial DNA Sequence

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AAAATCAGCGTATTTTACATATAAGCAGCCACCTCTGCTCATCTGTGGCCAGATACGAGTGGAGTGGCACAAGGGATA
AACCATTTTCGGCACTCTTACGCGATGGGGGAAAGTAAAGCAGCCTAGTCTCGGGAGCTGTGCCCGCCAGCCCTCT
GGCGGACTTGAACCCGCGGACTGCGCTGCCCTTGGCTGCCCTTCCGCTCTGATGGCCGGGGCCACTACTAC
GGGGCACTGACGGCTTTGGCAGCAGCCGCAAGATGAAGTCCACAGAGGTCGCCACACGCTGGCGTGGCGGGCC
GGGGCTGGAAGCGGTGGCCACGGCAGGGACAGCTGCCGTGGGGTTGCACGGGTGCCCGCGCATGCGCAGCC
GTTGGCAGCTCCAGCCGGGTGGGGCCCTTCCAGCGCGCCAGCGGGTGCACGCTCCCGCAGCTCAATGAGCTCAGGCT
CCCGGACATGGCCGGTTGGCCCTGCTTGGCTGGCTTGGCGCTAGCAAGCGGGG
  
```

Name of your sequence: AC002481: bp 18036 - E75 **Get Final Report**

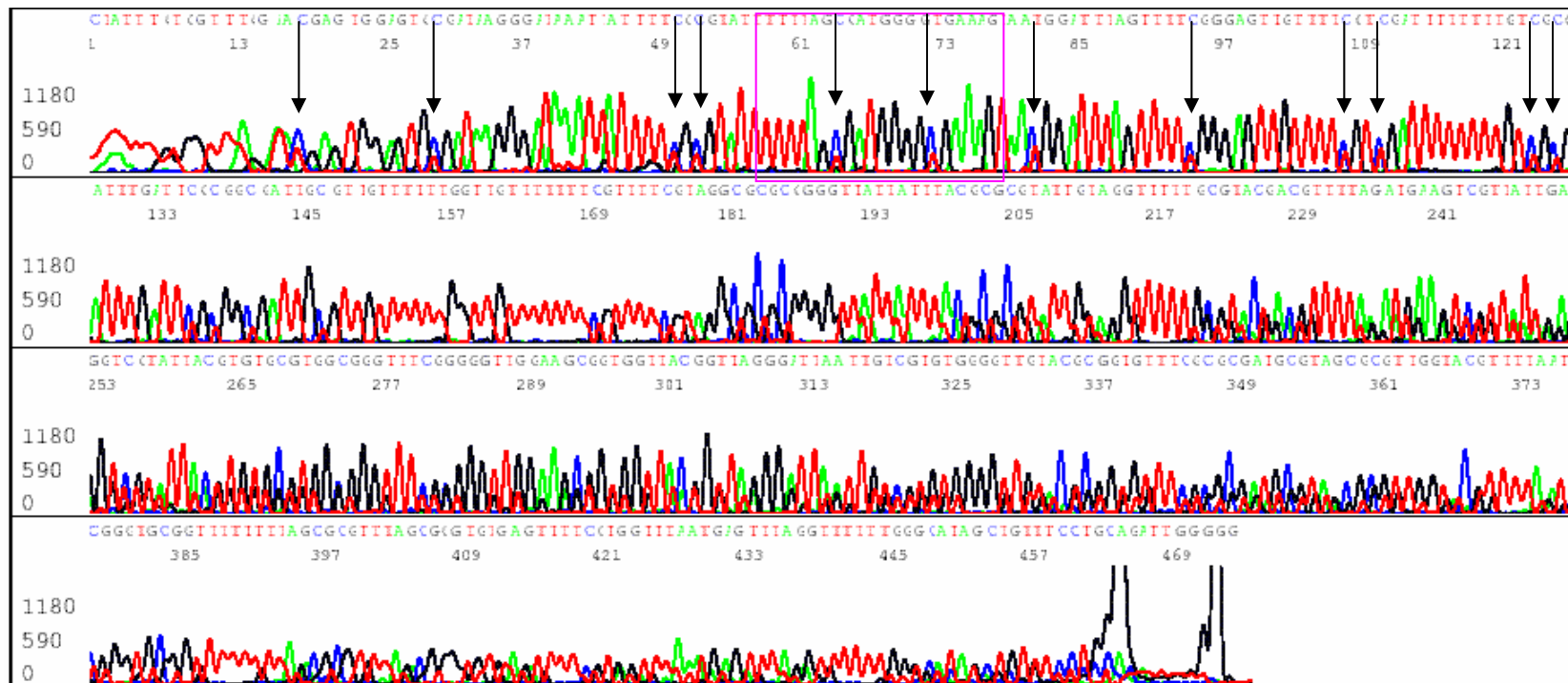
Fragment analysis of bisulfite PCR product – 1-to-1 test mixture of +/- methylated gDNA's



POP-4™ polymer denaturing electrophoresis of the FAM™ -labeled PCR products (+ ROX™ -500 marker) reveals the presence of two products arising from methylated and un-methylated template DNA's.

Note: The 1-to-1 mixture results in almost perfectly equal peak heights. Negative PCR-bias is sometimes seen for the product from methylated template.

Direct sequencing of bisulfite-PCR product- 1-to-1 test mixture of +/- methylated gDNA's



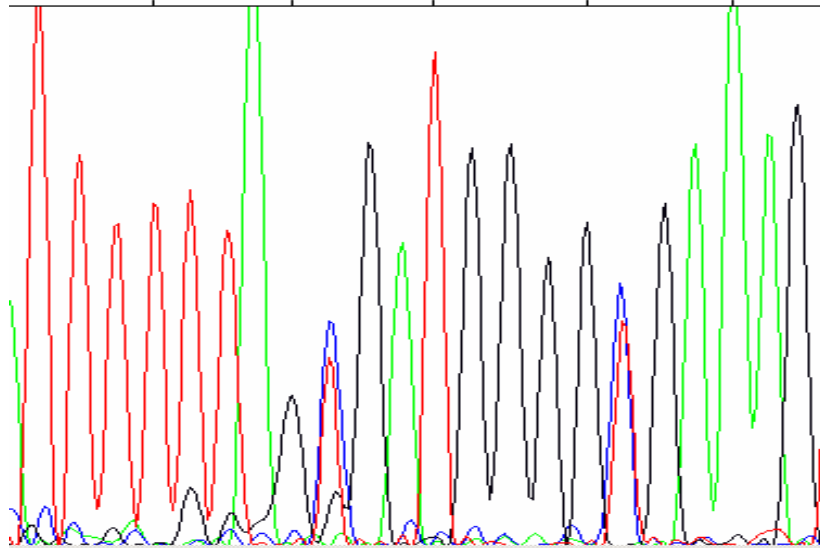
Direct DNA sequencing of the Exo-Sap treated PCR product using BigDye® terminator v1.1 shows:

- Heterozygote T- and C-peaks at every methylated CpG position
- Confirms 100% successful bisulfite conversion of all non-methylated C's in the template

Direct sequencing of bisulfite-PCR product- 1-to-1 test mixture of +/- methylated gDNA's

636	C	T	C	T	T	C	A	G	C	G	A	T	G	G	G	G	C	G	A	A	A	G	← genomic sequence
:		:			:			+	+							+	+					← bisulfite converted	
636	T	T	T	T	T	T	A	G	C	G	A	T	G	G	G	G	C	G	A	A	A	G	

A T T T T T T A G T G A T G G G G C G A A A G
7 61 65 69 73 77



Unmethylated C → T

5-Methyl-C → C
Unmethylated C → T

Direct DNA sequencing of the PCR product shows:

- Heterozygote T- and C-peaks at every methylated CpG position
- Confirms 100% successful bisulfite conversion of all non-methylated C's in the template (internal control)

Recommendations for bisulfite sequencing

- No amplicons with > 9 poly-T
- Improved bisulfite protocol (Anal. Biochem. 2004, 326, 278-280)
- AmpliTaq Gold PCR® Master Mix
- Quantitation of PCR prior to sequencing
- M13-tailed primers
- Full-strength BigDye® Terminator v1.1 Ready Reaction mix
- 2-temperature cycle-sequencing
- SDS/Edge Performa clean-up of sequencing reaction
- Analysis with KB™ basecaller

Acknowledgments

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