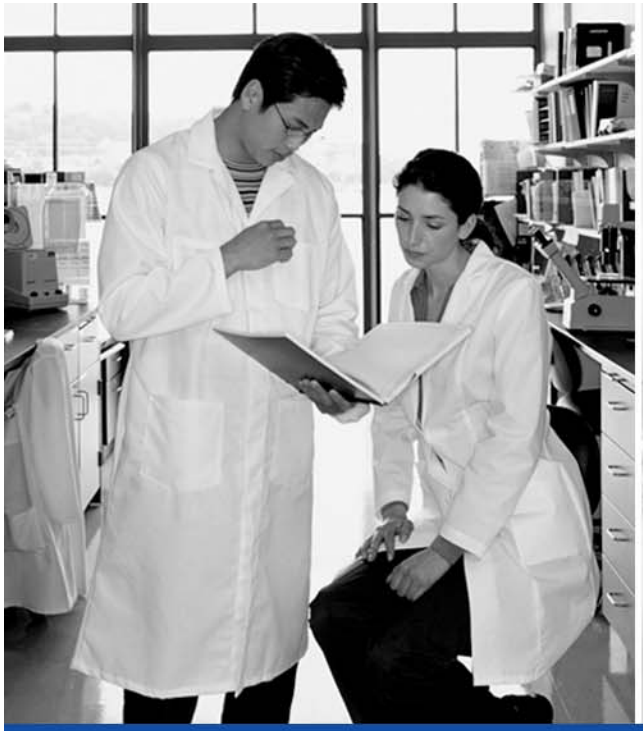

Applied Biosystems

Improved Protocols for Bisulfite Sequencing and Fragment Analysis of Methylated gDNA

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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

Methylation–sensitive restriction enzymes

Methylation-sensitive restriction - Southern blot

Methylation-sensitive restriction – PCR

Bisulfite treatment - PCR

A: PCR primers contain **no** CpG : will amplify independent of methylation status

Bisulfite sequencing

Bisulfite-PCR restriction analysis

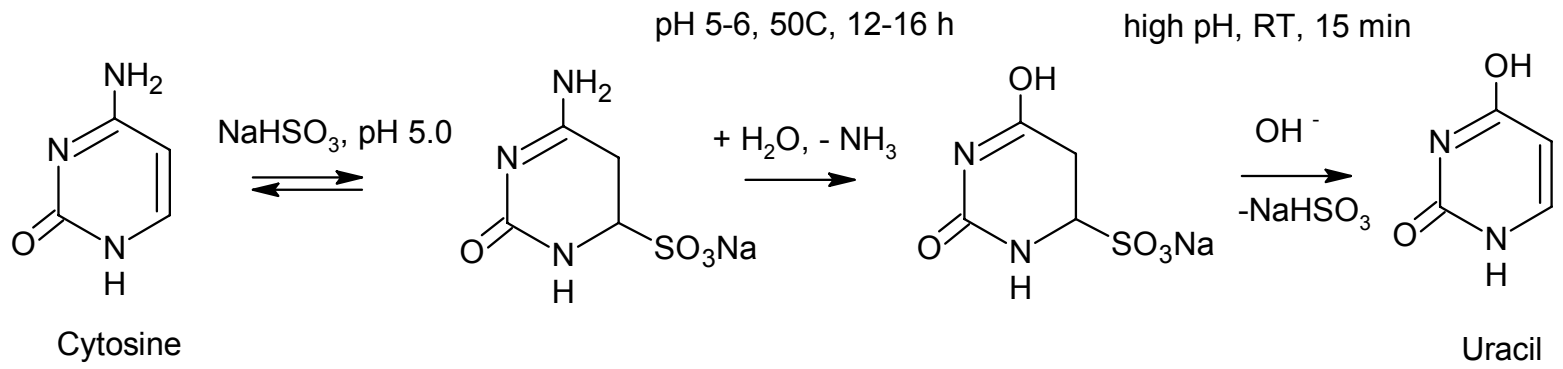
Single-nucleotide primer extension (Snapshot®)

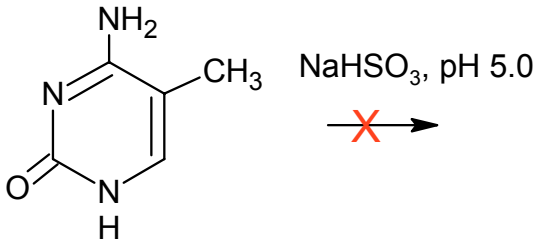
Bisulfite-PCR-SSCP

B: PCR primers designed over CpG : will specifically amplify methylated **OR** non-methylated DNA only

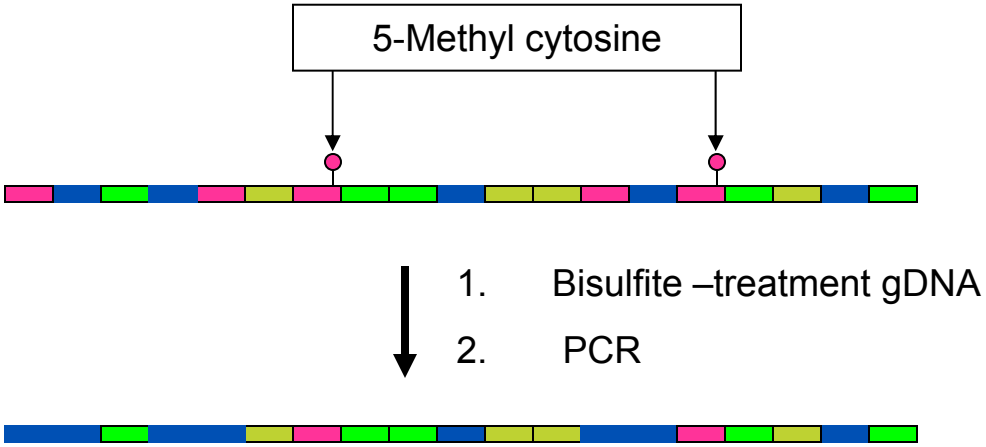
Methylation-specific PCR (MSP)

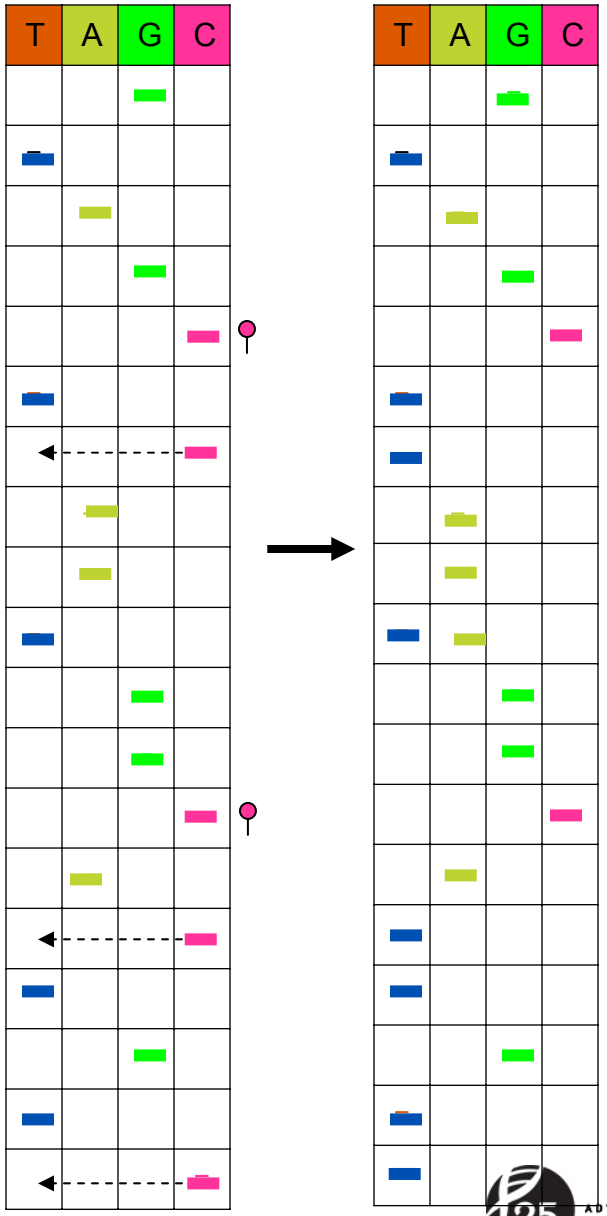
Chemistry of bisulfite treatment





Effect of bisulfite treatment on DNA sequence





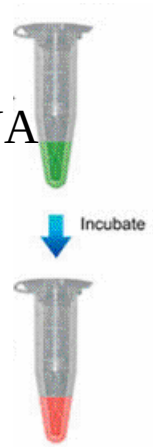
Bisulfite / PCR / Sequencing workflow

Stage	Processing step	Tools
Database target sequence containing CpG	PCR primer design	MethPrimer or Methyl Primer Express® software
gDNA		
↓	1. Bisulfide 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

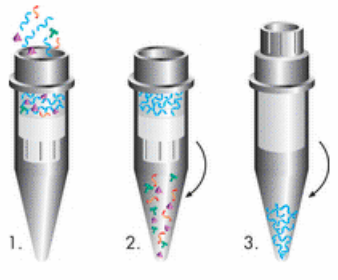
Denature gDNA



Add bisulfite reagent and incubate 15 h @ 50 C

STEP 2

Purify with Microcon 100

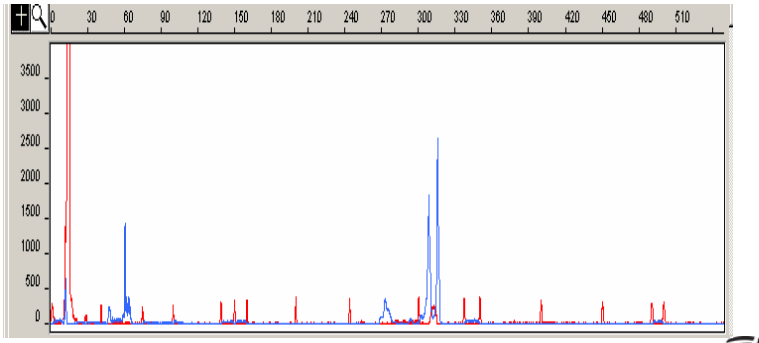


STEP 3

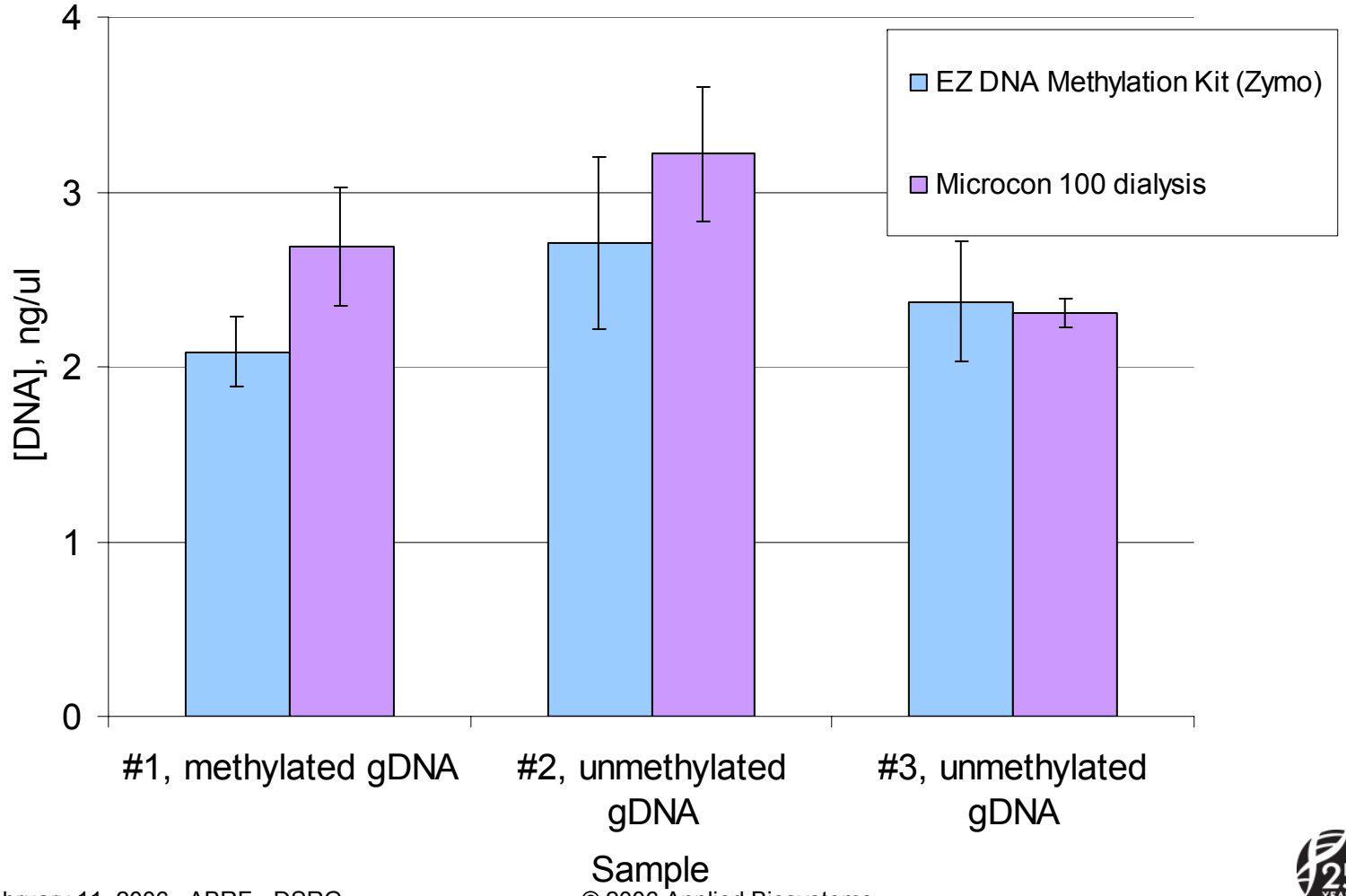
PCR

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:

AmpliTaq Gold® 10X buffer	1.0 µl
dNTP 2.5 mM each	0.8 µl
MgCl2 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

PCR conditions for tailed primers:

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

DNA sequencing of bisulfide/PCR product:

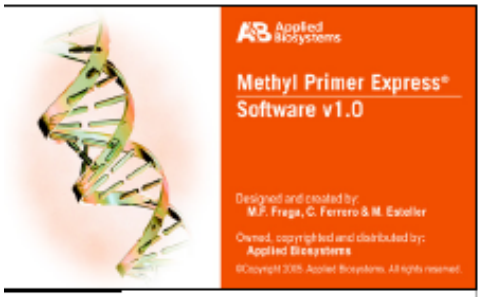
Bisulfite/PCR product (1-5 ng/ul *)	1 µl
BigDye® Terminator v1.1 Ready Reaction Mix	8 µl
Primer (M13 Forward or Reverse, 3.2 uM)	1 µl
Water	10 µl
Total volume	20 µl

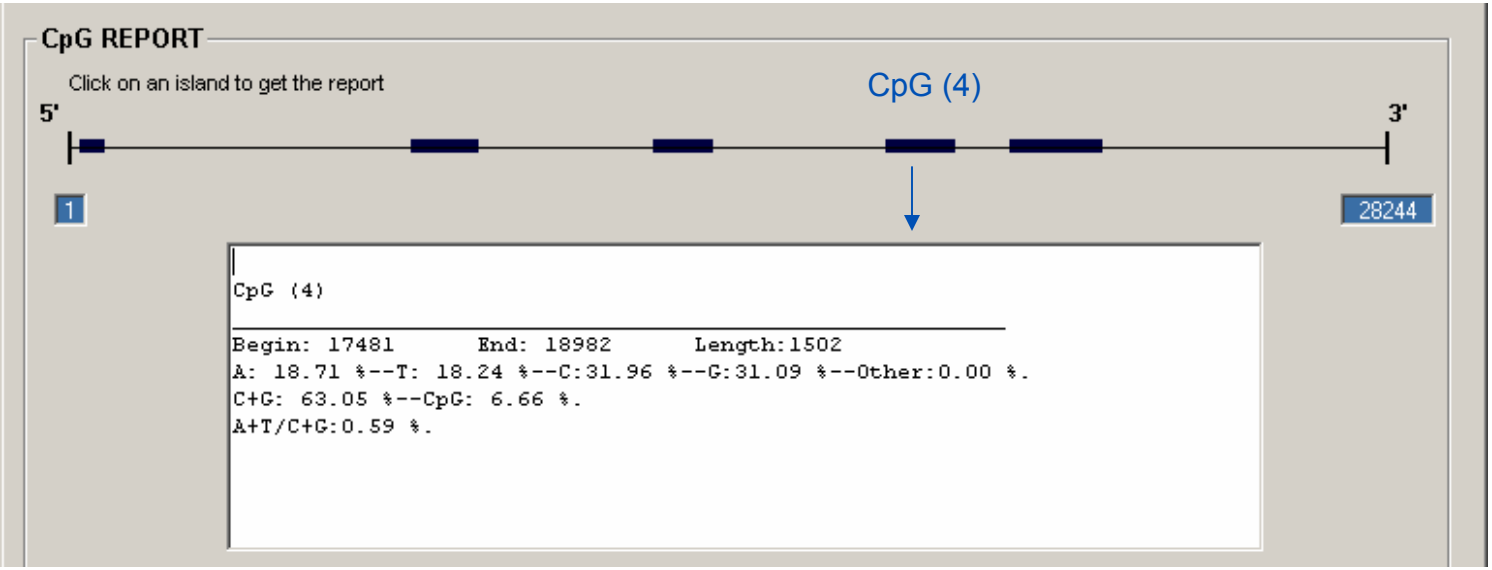
Sequencing conditions for universal primers:

96°C /1 min
25x (96°C /10 s; 50°C / 4:00 min)
4 °C Hold

* may require dilution

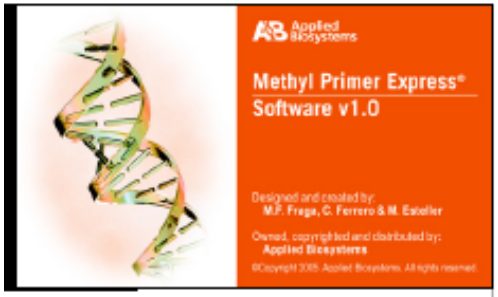
Detection of CpG islands in gDNA (28 kb GenBank file) using Methyl Primer Express® software (AB)



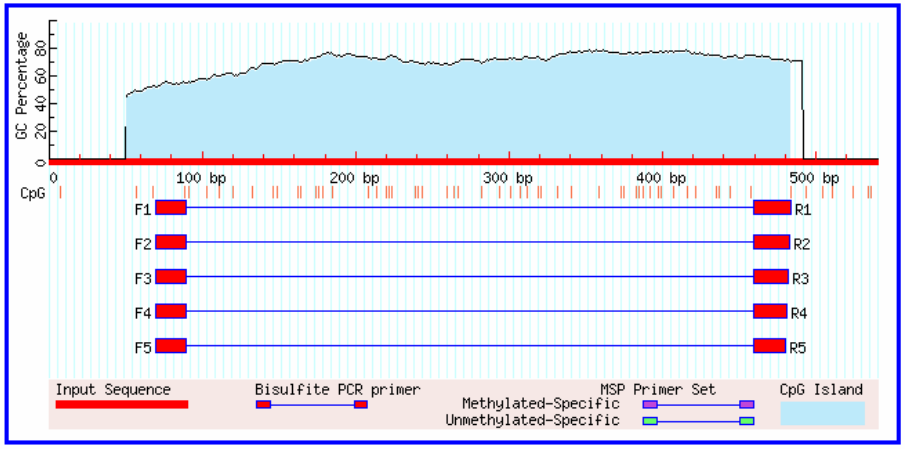


Prediction of CpG islands in 28,244 bp genomic DNA region containg RasSF promotor (GenBank Acc. # AC002481) using Settings: Min island size 300 bp, GC % > 50, GC Obs/Exp > 0.6

Bisulfite sequencing: primer design

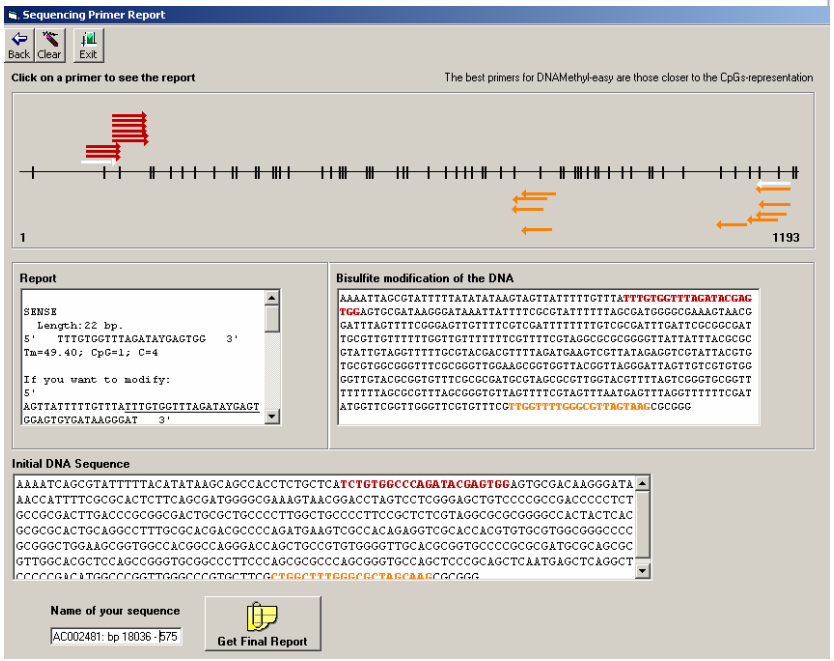


MethPrimer result



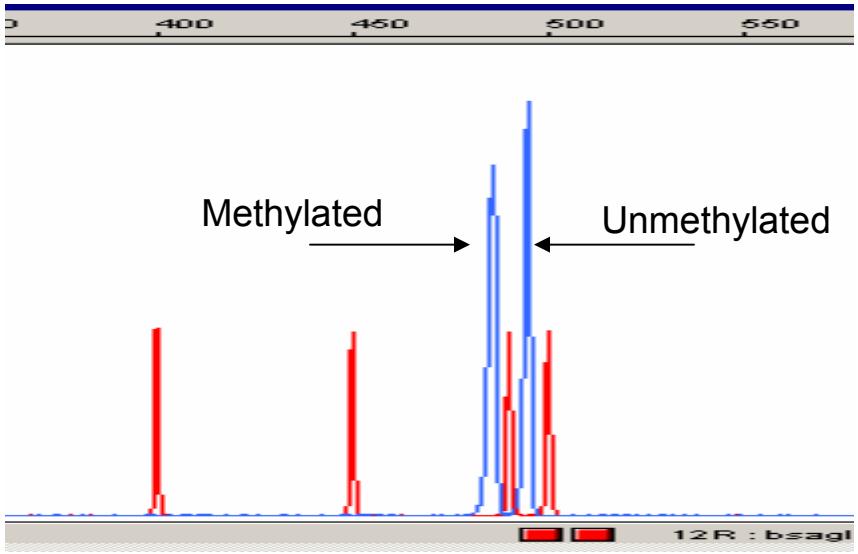

```
Sequence Name: AC002481: bp 18036 - 18575, - strand
Sequence Length: 540

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)
```



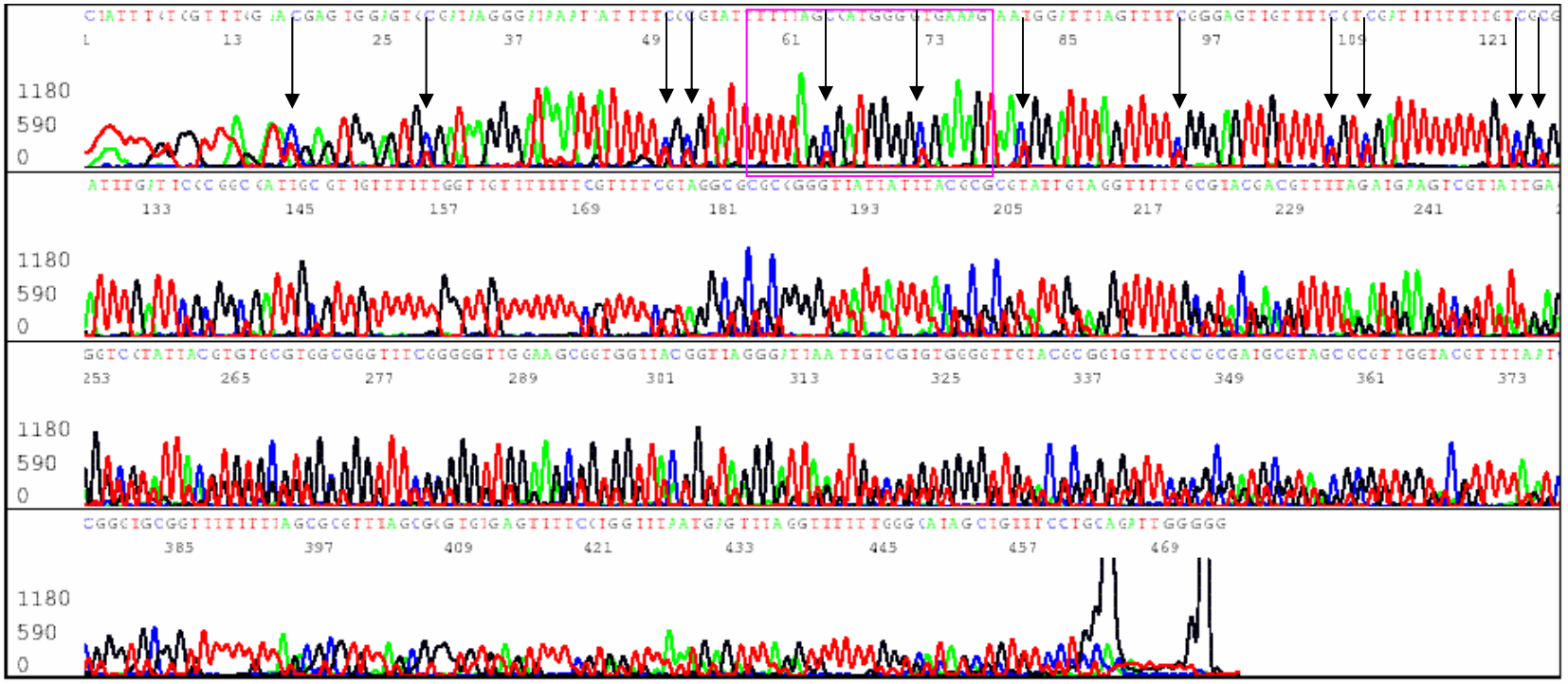
Bisulfide PCR of RasSF promotor CpG island

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 bisulfide-PCR product- 1-to-1 test mixture of +/- methylated gDNA's



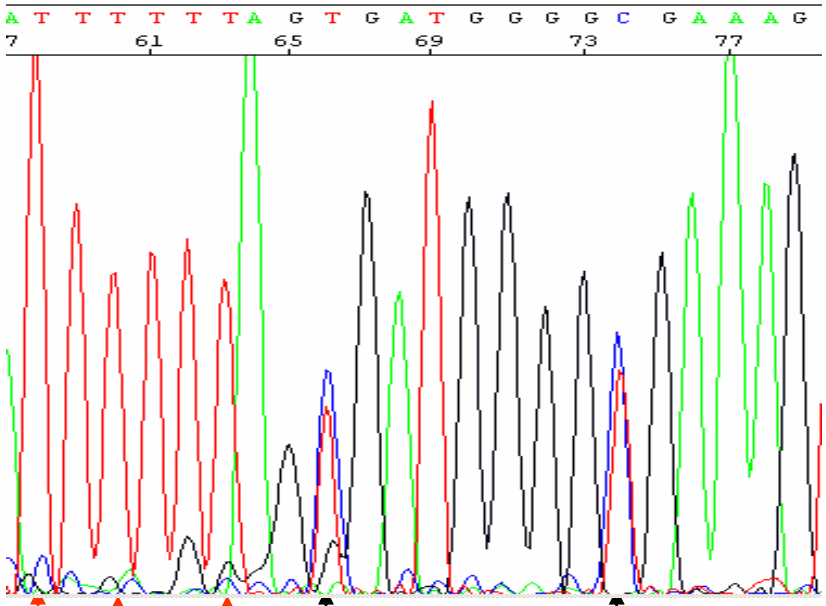
Direct DNA sequencing of the Exo-Sap treated PCR product using BigDye® terminator v1.1 shows:

- 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 bisulfide-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

636 T T T T T T A G C G A T G G G G C G A A A G ← bisulfite converted



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

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