

ABRF  
DNA Sequencing  
Research Group  
February 11, 2006

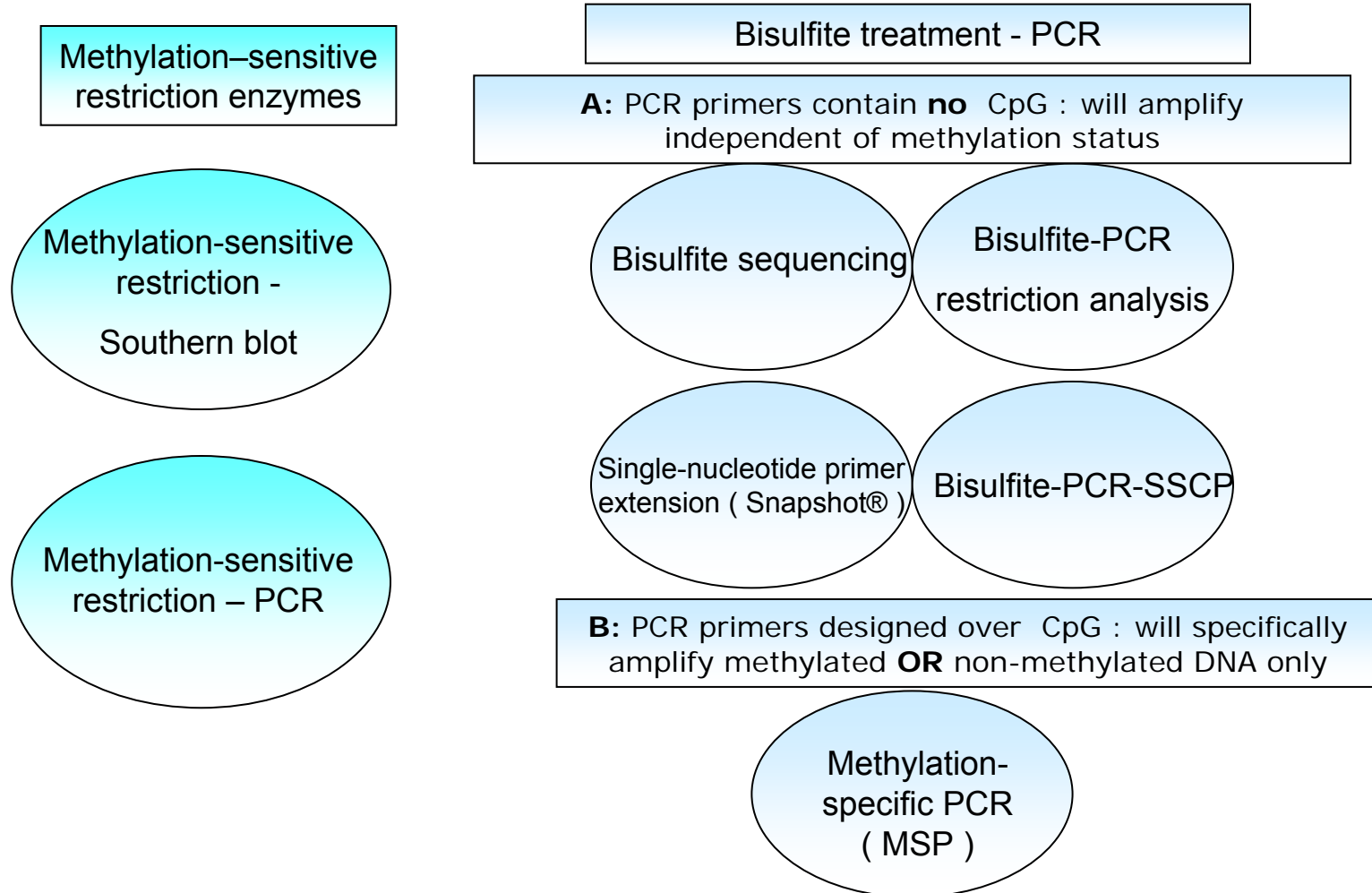
## Improved Protocols for Bisulfite Sequencing and Fragment Analysis of Methylated gDNA

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AB Molecular Biology – Genetic Analysis

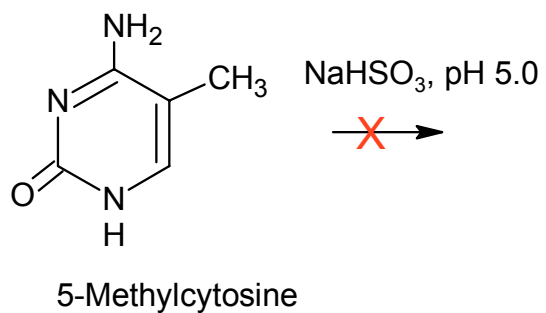
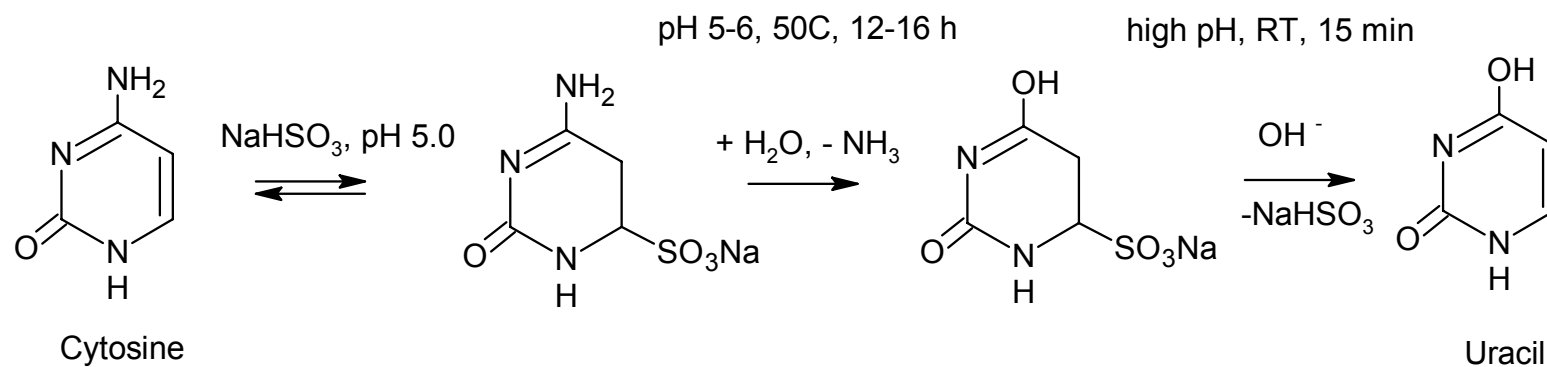
## Epigenetic modification in human genome by DNA-methylation

- 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](http://www.mdanderson.org): methylation in cancer)

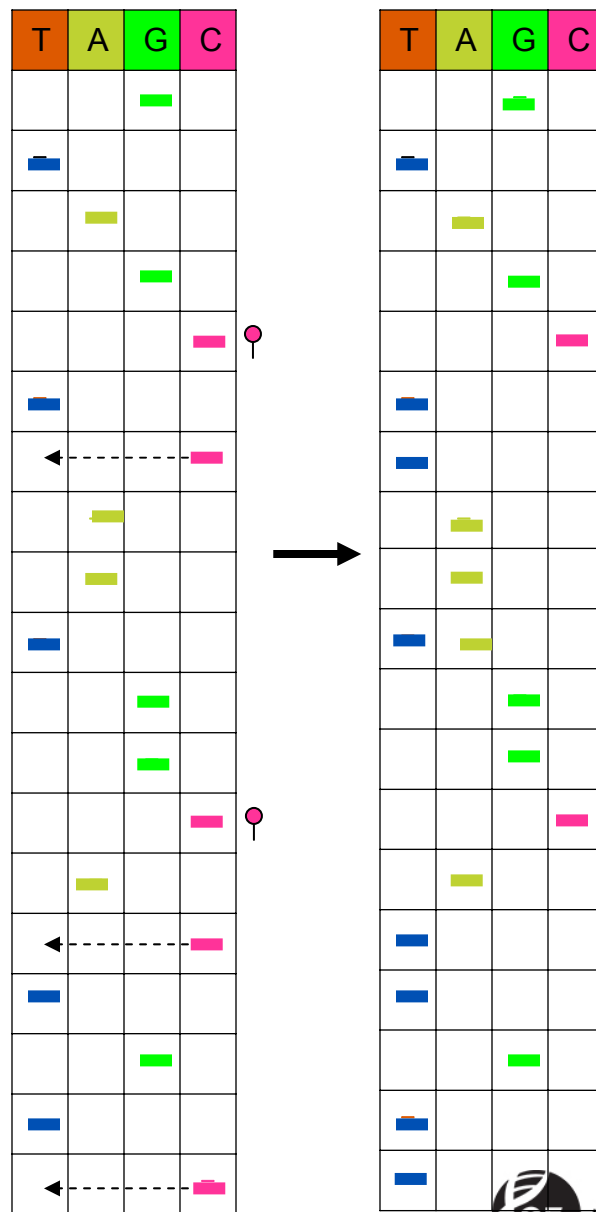
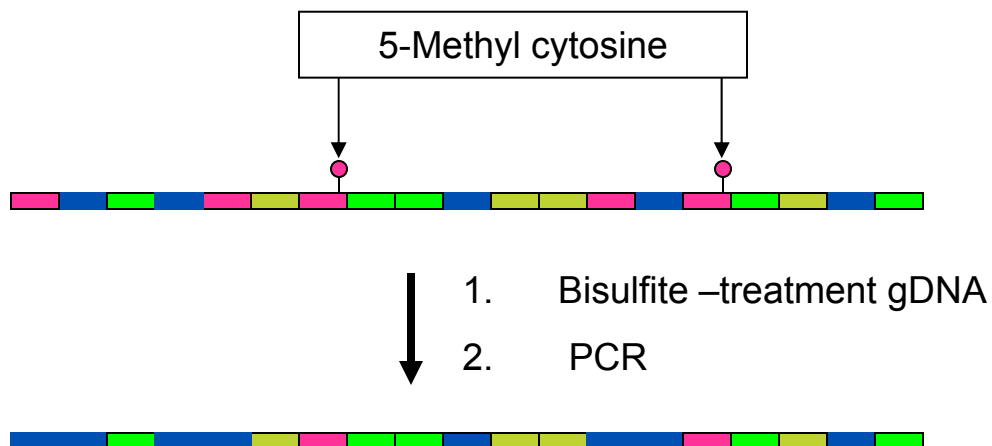
# Methods to analyse methylation status



# Chemistry of bisulfite treatment



# Effect of bisulfite treatment on DNA sequence



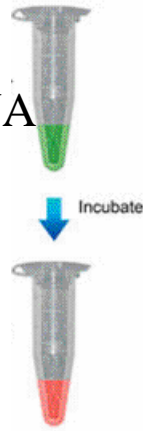
# Bisulfite / PCR / Sequencing workflow

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

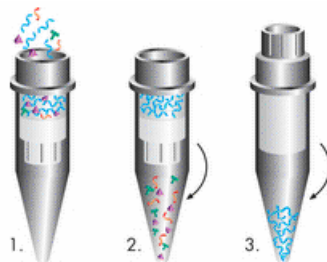
Denature gDNA



Add bisulfite reagent and incubate 15 h @ 50 C

## STEP 2

Purify with Microcon 100



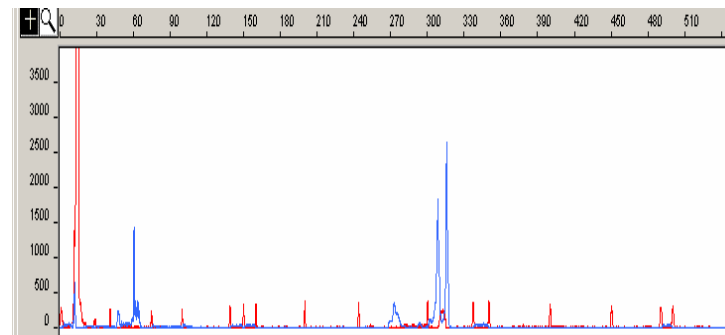
## STEP 3

PCR

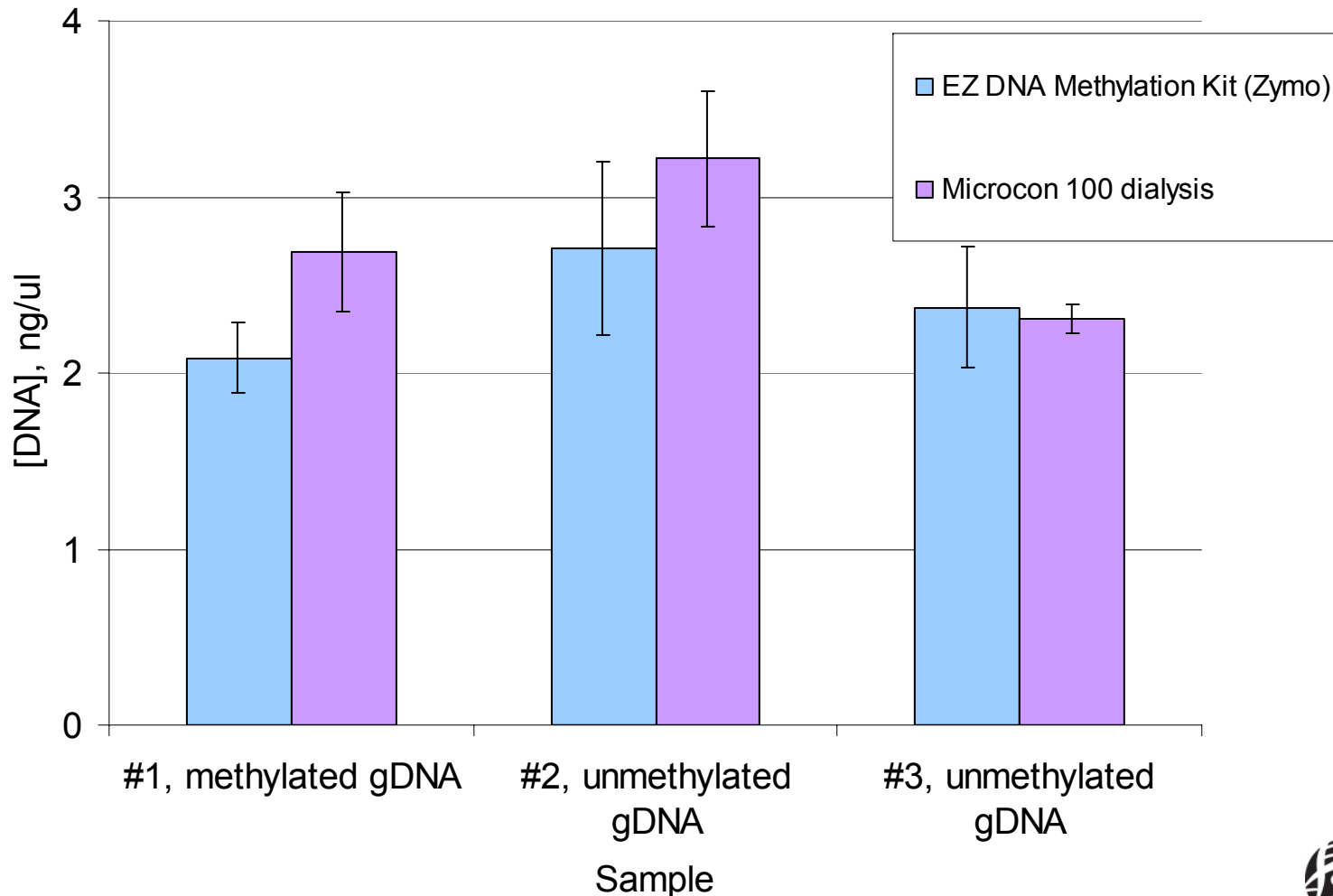


## STEP 4

Fragment Analysis



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



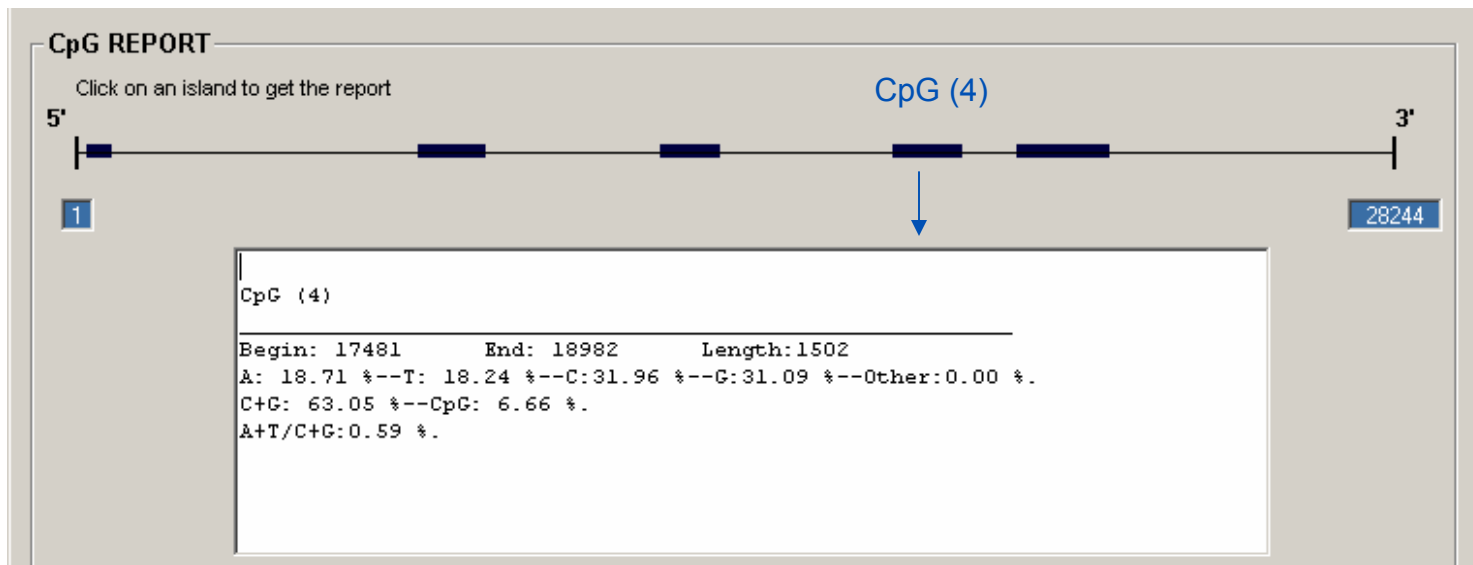
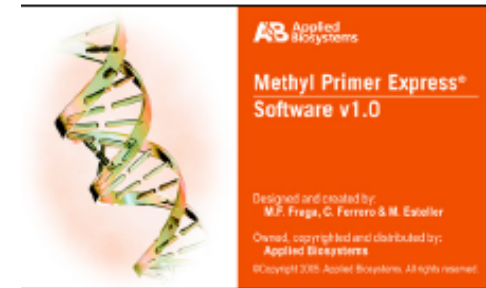
## PCR and cycle sequencing protocols

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

DNA sequencing of bisulfite/PCR product:		Sequencing conditions for universal primers:
Bisulfite/PCR product ( 1-5 ng/ $\mu$ l *)	1 $\mu$ l	96°C /1 min
BigDye® Terminator v1.1 Ready Reaction Mix	8 $\mu$ l	25x ( 96°C /10 s; 50°C / 4:00 min )
Primer (M13 Forward or Reverse, 3.2 $\mu$ M)	1 $\mu$ l	4 °C Hold
Water	10 $\mu$ l	
<b>Total volume</b>	<b>20 <math>\mu</math>l</b>	

\* may require dilution

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

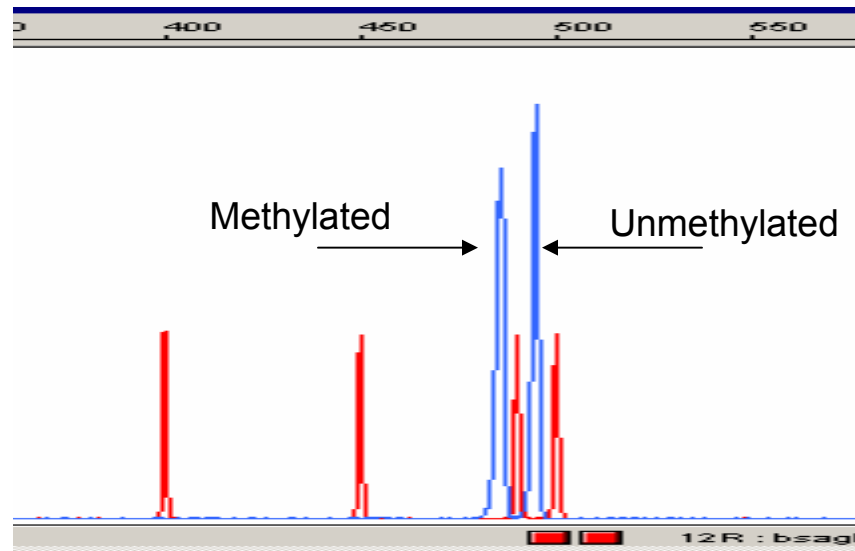


Prediction of CpG islands in 28,244 bp genomic DNA region containing RasSF promoter ( GenBank Acc. # AC002481) using Settings: Min island size 300 bp, GC % > 50, GC Obs/Exp > 0.6





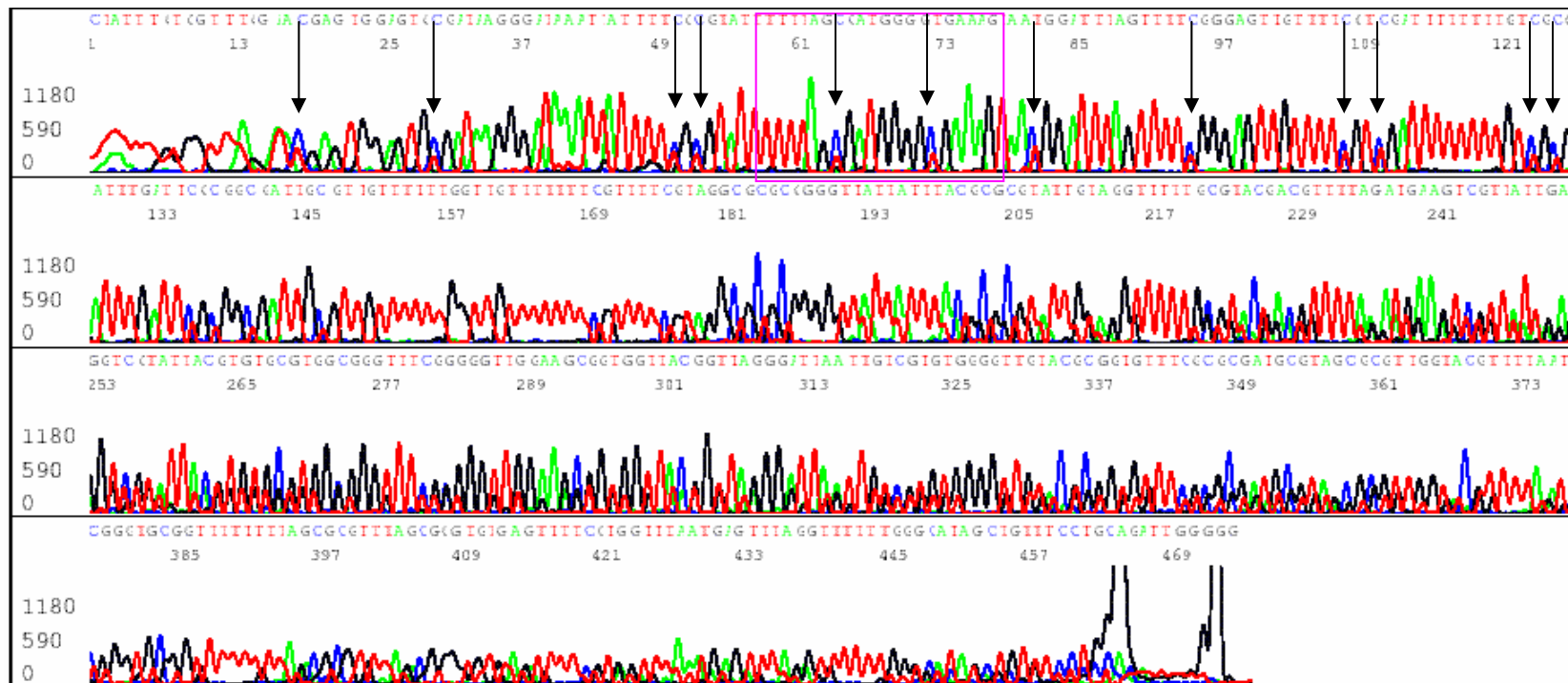
## Fragment analysis of bisulfite PCR product – 1-to-1 test mixture of +/- methylated gDNA's



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

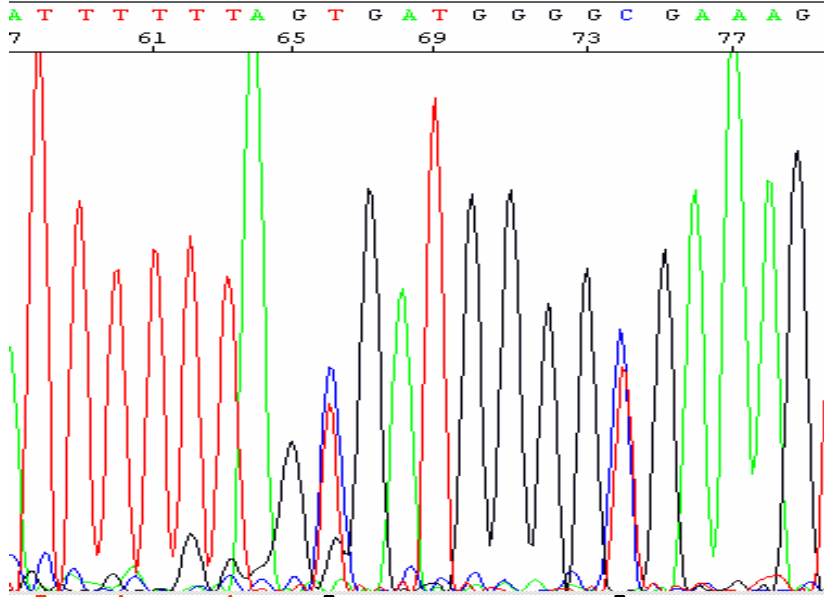


Direct DNA sequencing of the Exo-Sap treated PCR product using BigDye® terminator v1.1 shows:

- 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	



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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For Research Use Only. Not for use in diagnostic procedures.

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