AD_____

Award Number: DAMD17-03-1-0642

TITLE: Epigenetic Inheritance of Breast Cancer

PRINCIPAL INVESTIGATOR: Susan J. Clark, Ph.D.

CONTRACTING ORGANIZATION: Royal Prince Alfred Hospital
Camperdown, NSW 2050, Australia

REPORT DATE: September 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2004	Final (1 Sep 2003 - 31 Aug 2004)

4. TITLE AND SUBTITLE
Epigenetic Inheritance of Breast Cancer

5. FUNDING NUMBERS
DAMD17-03-1-0642

6. AUTHOR(S)
Susan J. Clark, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Royal Prince Alfred Hospital
Camperdown, NSW 2050 Australia

E-Mail: S.Clark@Garvan.Org.Au

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Hypermethylation of tumour suppressor genes is only thought to occur in the somatic cell in sporadic tumours. However, we propose that methylation of the promoter of tumour suppressor genes, such as *BRCA1*, may also occur in the germ-line. Germ-line inheritance of this epigenetic silent state would therefore contribute to familial breast cancer. In this study we aimed to address if methylation of the tumour suppressor gene *BRCA1* is inherited through the germ-line and is associated with the hereditary breast cancer. We developed a high-throughput sensitive real-time methylation assay that allowed us to screen for *BRCA1* methylated DNA. We used this assay to screen for *BRCA1* methylation from DNA isolated from archival blood and biopsy samples of women that have a family history of breast cancer but have no defined *BRCA1* or *BRCA2* mutations.

14. SUBJECT TERMS
DNA Methylation, tumour suppressor genes, BRCA1 epigenetic

15. NUMBER OF PAGES
20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

It is well established that germ-line mutations in tumour suppressor genes such as *BRCA1* predispose women to breast cancer (1). In fact, 20-30% of families with familial breast cancer have identified mutations in the *BRAC1* and *BRCA2* genes. However *BRCA1* and *BRCA2* mutations do not account for the whole spectrum of hereditary breast cancer. It is well established that methylation of the CpG rich promoter regions of tumour suppressor genes, including *BRCA1*, is associated with gene silencing and tumour progression (2, 3). Indeed the impact and significance of the epigenetic silencing of *BRCA1* is functionally equivalent to carrying a germ-line *BRCA1* mutation. Hypermethylation of tumour suppressor genes is only thought to occur in the somatic cell in sporadic tumours. However, we propose that methylation of the promoter of tumour suppressor genes, such as *BRCA1*, can be also be sporadically hypermethylated in the germ-line. Germ-line inheritance of this epigenetic silent state would occur by a similar mechanism as the inheritance of an imprinted gene and thus contribute to familial breast cancer.

Hypothesis: That epigenetic inheritance of methylated and silent tumour suppressor genes predisposes families to hereditary cancer.

Objective/Overall aim: To study if methylation of the tumour suppressor gene *BRCA1* is inherited through the germ-line and is associated with the hereditary breast cancer.

Specific Aims:

1. To develop a high-through put sensitive real-time assay to assess the methylation state of BRCA1.
2. To determine the methylation state of the *BRCA1* gene in the DNA from archival blood, and biopsy samples from at least 100 women with familial breast cancer that have no defined mutations in the *BRCA1or BRCA2* gene.

BODY

Task1: Develop real-time bisulphite methylation assay for detection of BRCA1 methylation.

1. Design primers and probe for high throughput quantitative bisulphite methylation assay using real-time PCR for the detection of methylated *BRCA1* CpG island promoter.
    a. We have designed a novel real-time PCR assay, a technique called Head-Loop PCR methylation assay,that can specifically be used for the detection of methylated *BRCA1* CpG island promoter. The principal of the technique is outlined in Figure 1 and the primers and probe are listed in Table 1.
    b. This technique was shown to be highly specific. Figure 2 shows that only methylated *BRCA1* DNA molecules are amplified in a mixture of methylated and unmethylated DNA template.
    c. The efficiency of the amplification was shown to be excellent in detecting methylated DNA down to 0.2 % (R= 0.99665) (Fig 3).
    d. The assay is also highly sensitive. Figure 4 shows that down to 10 molecules of methylated DNA can be detected when spiked into 1ml of blood.
    e. The headloop *BRCA1* assay also was demonstrated to detect breast cancer from matched normal breast in biopsy samples (Fig 5).

2. Design primers and optimise conditions for genomic bisulphite sequencing of the *BRCA1* CpG island promoter
    a. We have designed primers for bisulphite sequencing of the *BRCA1* promoter (Table 1). These primers have successfully been used to determine the methylation profile of the CpG island promoter. Figure 6 shows examples of the tract from a methylated Headloop amplification (Fig 6A) and from an unmethylated direct PCR sequence (Fig 6B).

Task2: Determine if hypermethylation of the BRCA1 tumour suppressor gene is inherited in familial breast cancer

1. Retrieve archival blood samples and biopsy samples from females with *BRCA1* related familial breast cancer that have no defined germline mutations in *BRAC1*.

a. We have analysed the methylation state from 28 breast biopsy cancer and matched normal samples and from blood from the same patients. These patients had *BRCA1* related familial breast cancer but no defined mutations in *BRAC1 or BRCA2*. Figure 7 summarises the results. 7/28 breast cancer biopsy samples contained methylated *BRCA1* DNA. Of these only 1/28 showed methylation in the matched normal sample. Interestingly this same patient showed methylation of *BRCA1* in the blood. 2 other patients were found to have methylated *BRCA1* sequences in the blood but were not methylated in the breast tissue.

b. We extended the analysis to include 96 archival breast cancer samples and found methylation of *BRCA1* in 72/96 (75%) (Fig 8). Of these only 28 had matched normal samples and only one was shown to be methylated. We compared these results to published data for BRCA1 methylation and found a great disparity in the literature depending on the method used for methylation analysis (Fig 9). Using our method we detected a significantly higher proportion of methylation in breast tumours. The choice of method is critical in interpreting methylation data as the sites tested can vary as well as the sensitivity of each assay.

KEY RESEARCH ACHIEVEMENTS

Developed a sensitive and novel real-time PCR based assay for detection of *BRCA1* methylation.

Hypermethylation of the *BRCA1* tumour suppressor gene was found to be principally due to somatic mutations in familial breast cancer

One example however was found which indicated that hypermethylation of the *BRCA1* tumour suppressor gene may have been inherited through the germline.

REPORTBLE OUTCOMES

Manuscript in preparation reporting the novel methylation assay

The data was presented at the kConFab annual meeting in Couran Cove, Qld Australia in September, 2004

Funding will be applied for 2005 to the NSW Cancer Institute to extend this study on more extensive families

Wenjia Qu was employed on this grant and will continue to work on this topic through support from the Garvan Cancer program.

CONCLUSION:

We have developed a high-through put sensitive assay for the detection of methylated *BRCA1* from biopsy samples and blood. Using this assay we have screened women with familial breast cancer to determine if any carried a methylated *BRCA1* copy through the germ line. We found a high proportion (78%) of biopsy cancer samples were methylated. Of these only 1/28 (0.03%) showed methylation in both matched normal and blood. It is therefore possible that this patient may have inherited a germline methylation mutation of the *BRCA1* gene. To test for this we need to assay for *BRCA1* methylation in other family members. Unfortunately these samples are not available in the archival collection. A new study is now being organised assay more samples from other family members. Intriguingly methylation was found in blood of 2 patients with no methylation in the breast cancer or normal tissue suggesting that a low level of methylation of BRCA1 may occur in normal blood.

Results from this study support the possibility of epigenetic inheritance and that inheritance of a methylated *BRCA1* allele may predispose the families to hereditary cancer. However such inheritance must be very low as only 0.03% of samples showed any indication of *BRCA1* methylation in blood and normal and cancer samples. Somatic changes in methylation of *BRCA1* appear to be the primary source of epigenetic change in the cancers studied.

REFERENCES

1. Venkitaraman AR. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell. 2002 108:171-82.
2. Esteller M, Fraga MF, Guo M, Garcia-Foncillas J, Hedenfalk I, Godwin AK, Trojan J, Vaurs-Barriere C, Bignon YJ, Ramus S, Benitez J, Caldes T, Akiyama Y, Yuasa Y, Launonen V, Canal MJ, Rodriguez R, Capella G, Peinado MA, Borg A, Aaltonen LA, Ponder BA, Baylin SB, Herman JG. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet. 2001 10:3001-7.
3. Dobrovic A, Simpfendorfer D.Methylation of the BRCA1 gene in sporadic breast cancer.Cancer Res. 1997 57:3347-50.
4. Rand K, Qu W, Ho T, Clark SJ, Molloy P. Conversion-specific detection of DNA methylation using real-time polymerase chain reaction (ConLight-MSP) to avoid false positives. Methods. 2002 27:114-20.
5. Clark SJ, Harrison J, Paul CL, Frommer M.High sensitivity mapping of methylated cytosines. Nucleic Acids Res. 1994 22:2990-7.

Table 1: Primers and Probes for BRCA1

Table 1

BRCA1 topstrand HeadLoop primers and probes	
BTF2	tgagaggttgttgtttagiggtagtttt
BTR1	cccccitccaaaaaatctcaa
BTHL2	acacttttccattaccacaaaatgagaggttgttgtttagiggtagtttt
BRCA1TMC1	Vic-cgcgcaatcgcaat-MGB
BRCA1 bottom strand bis primers and probes	
BBF1	ATTTTATAGTTTGTTTTTiGTTTAGGAAGTTTT
BBR1	TCTAAAAAACTACTACTTAACiATAACCCCTTAA
BBFHL1	AACTACACAACATAAACTCACTAAAATTTTATAGTTTGTTTTTiGTTTAGGAAGTTTT
BBRHL1	TGTGTTTTTTTGTTGTTATGGAAATTTCTAAAAAACTACTACTTAACiATAACCCCTTAA
BRCA1BMC1	VIC-ATTTTCGCGTTTTTTCGTTGT-MGB
Primers for direct sequencing	
BBR1	TCTAAAAAACTACTACTTAACiATAACCCCTTAA
BTR1	cccccitccaaaaaatctcaa

Fig 1. Methylation Detection: HeadLoop PCR

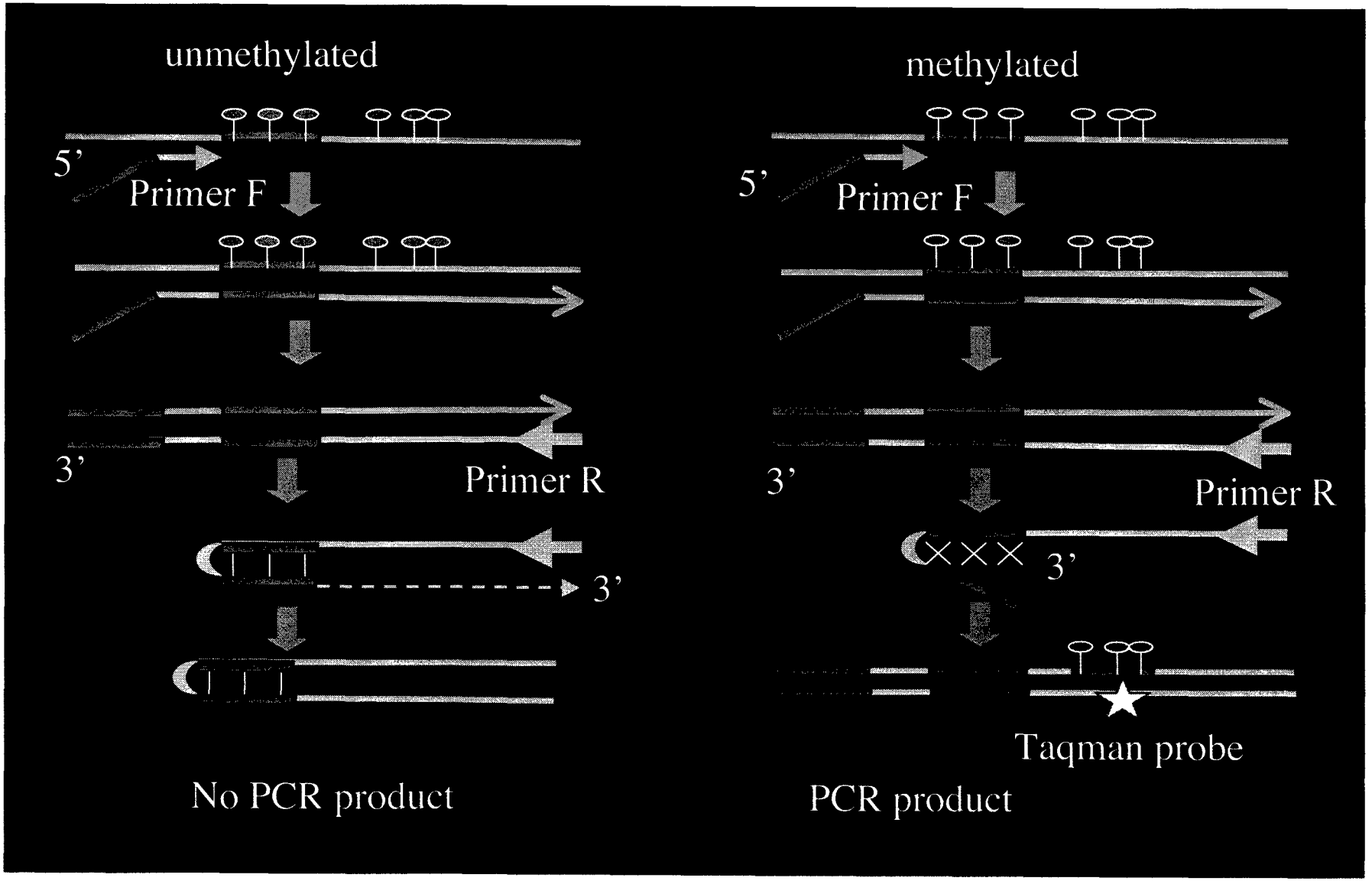


Fig 2. Headloop PCR Specificity

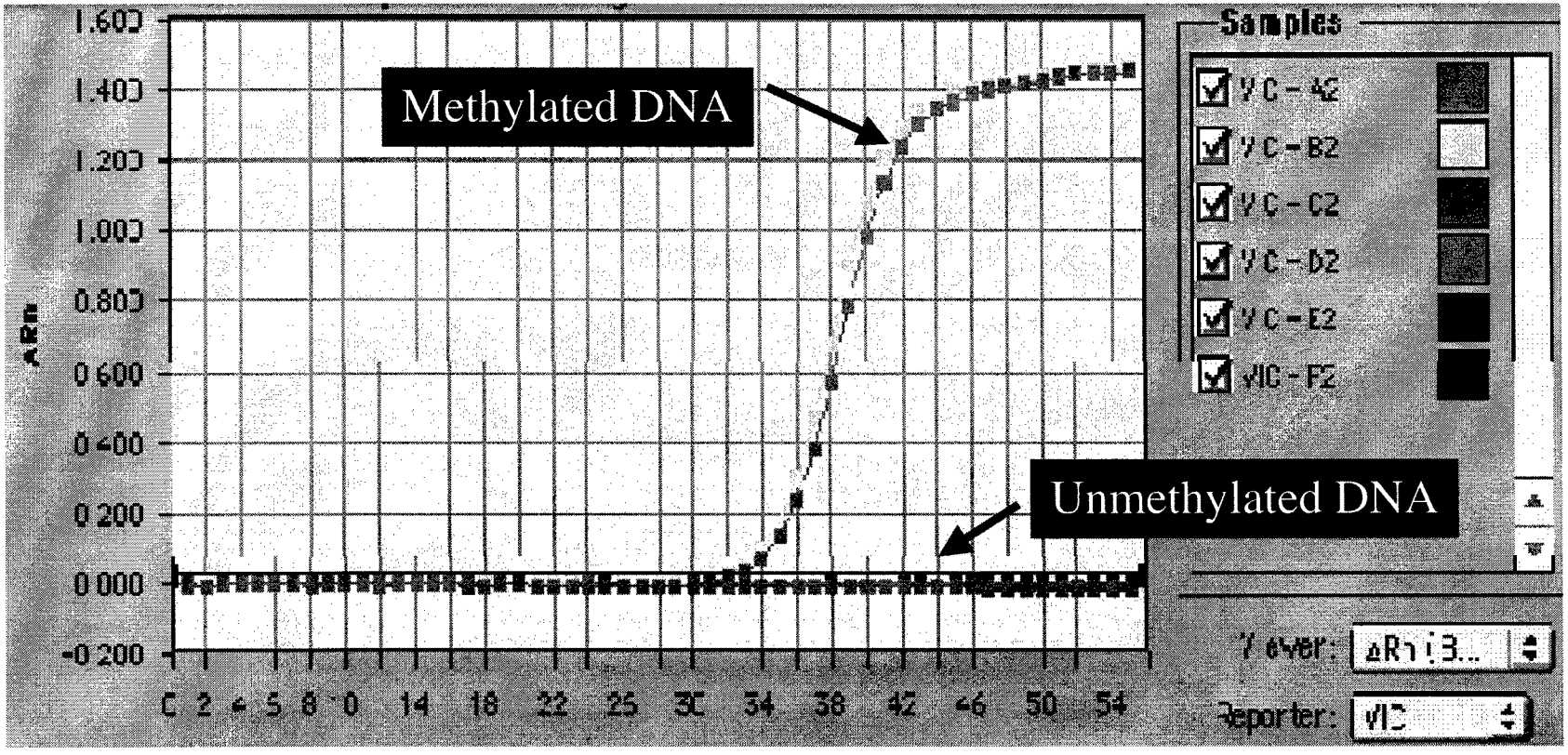
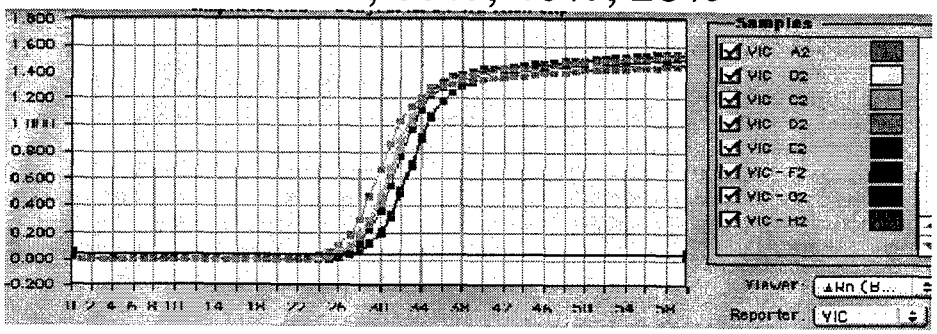
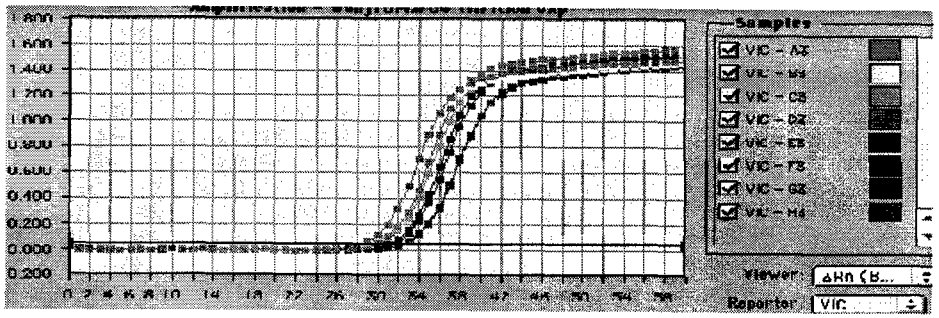


Fig 3. Efficiency of Headloop PCR

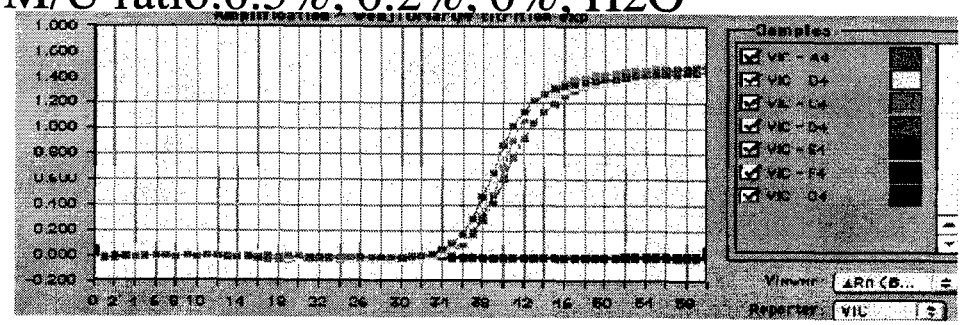
M/U ratio:100%, 60%, 40%, 20%



M/U ratio:10%, 5%, 2%, 1%



M/U ratio:0.5%, 0.2%, 0%, H2O



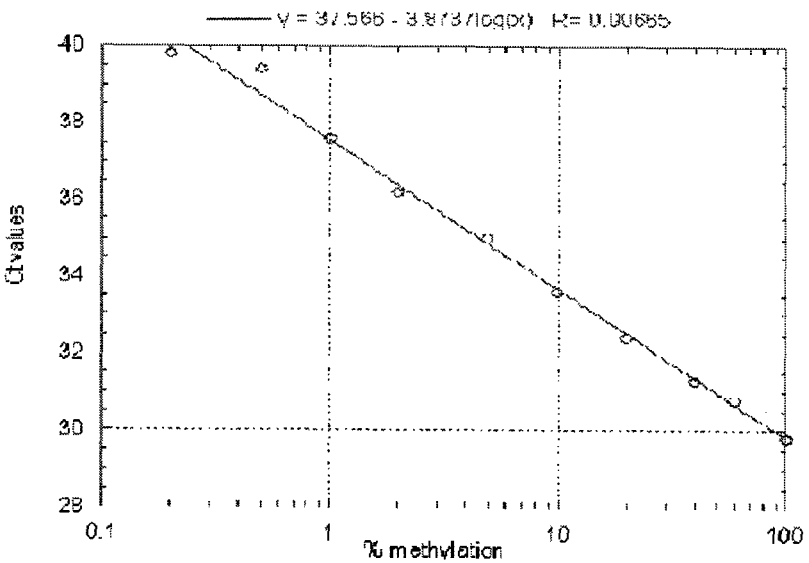


Fig 4. Headloop PCR Sensitivity

10^5 , 10^4 , 10^3, 10^2, 20, 10 LNCaP cells/ml blood

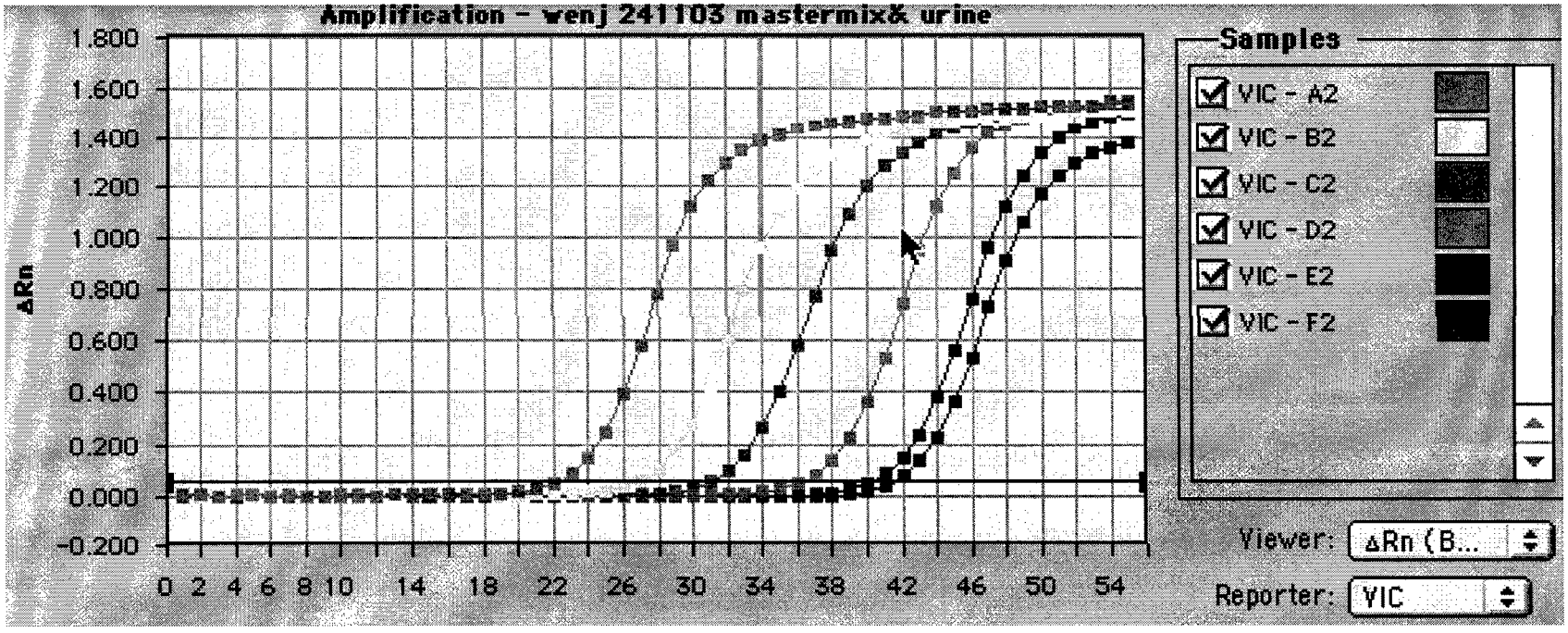
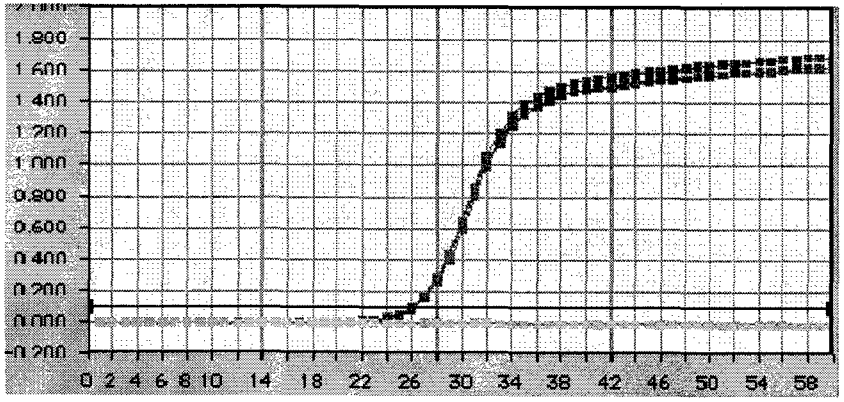
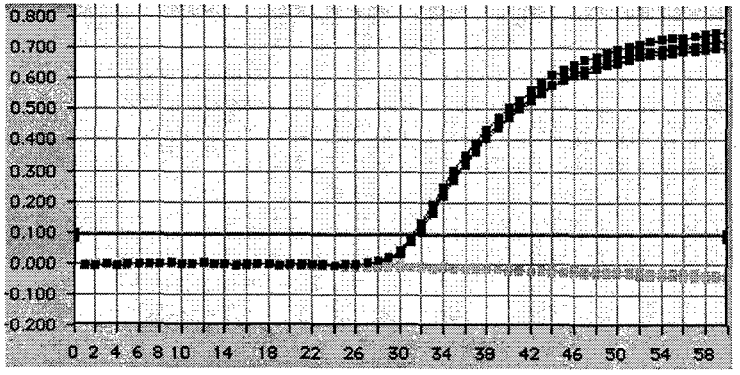


Fig 5. Headloop PCR distinguishes breast tumours

Cancer & Normal: 1



Cancer & Normal: 2



Cancer & Normal: 3

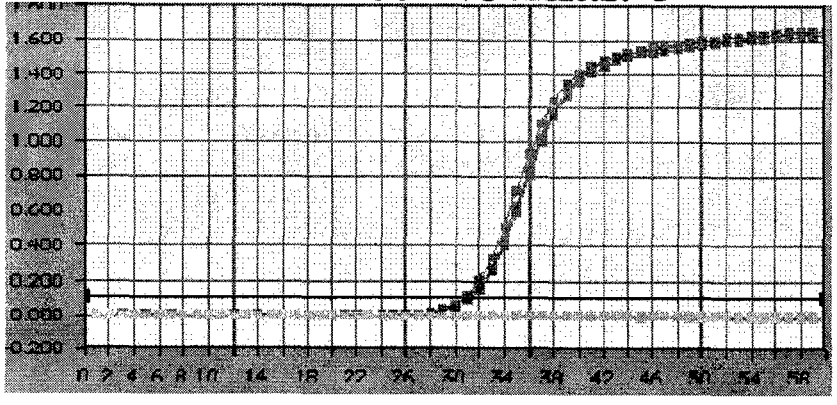
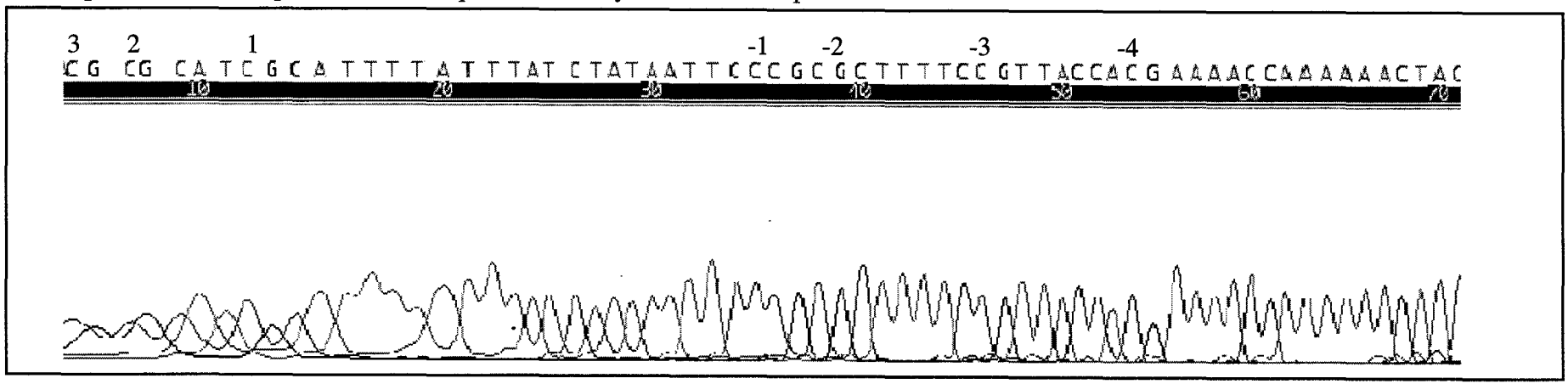


Fig. 6 Bisulphite Direct Sequencing Profile

A. Top strand HeadLoop PCR: Direct sequence of methylated BRCA1 CpG island



B. Direct sequence of unmethylated BRCA1 CpG island

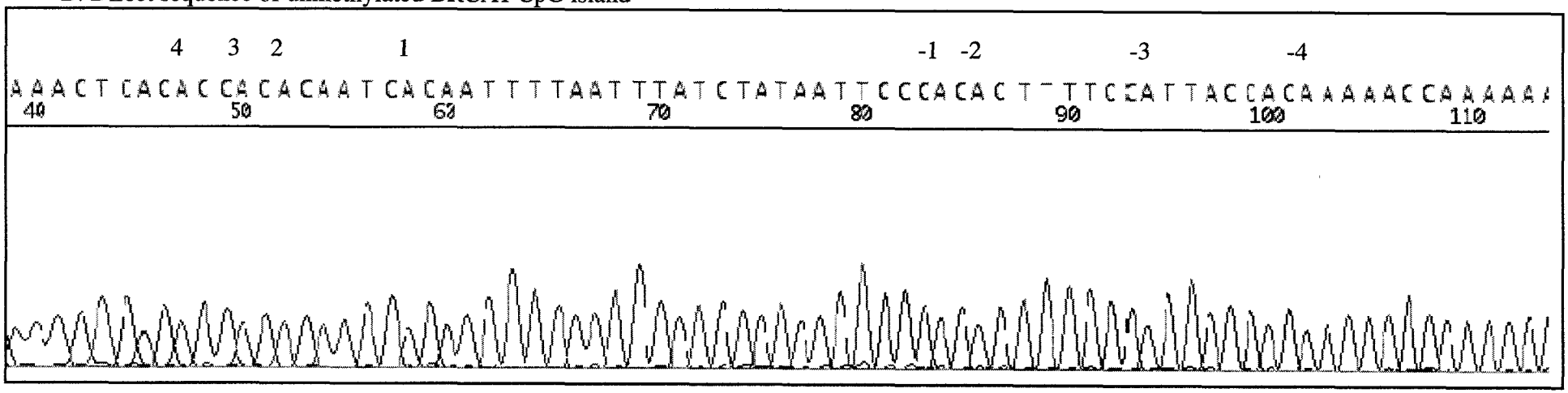


Fig. 7 BRCA1 Methylation Assay

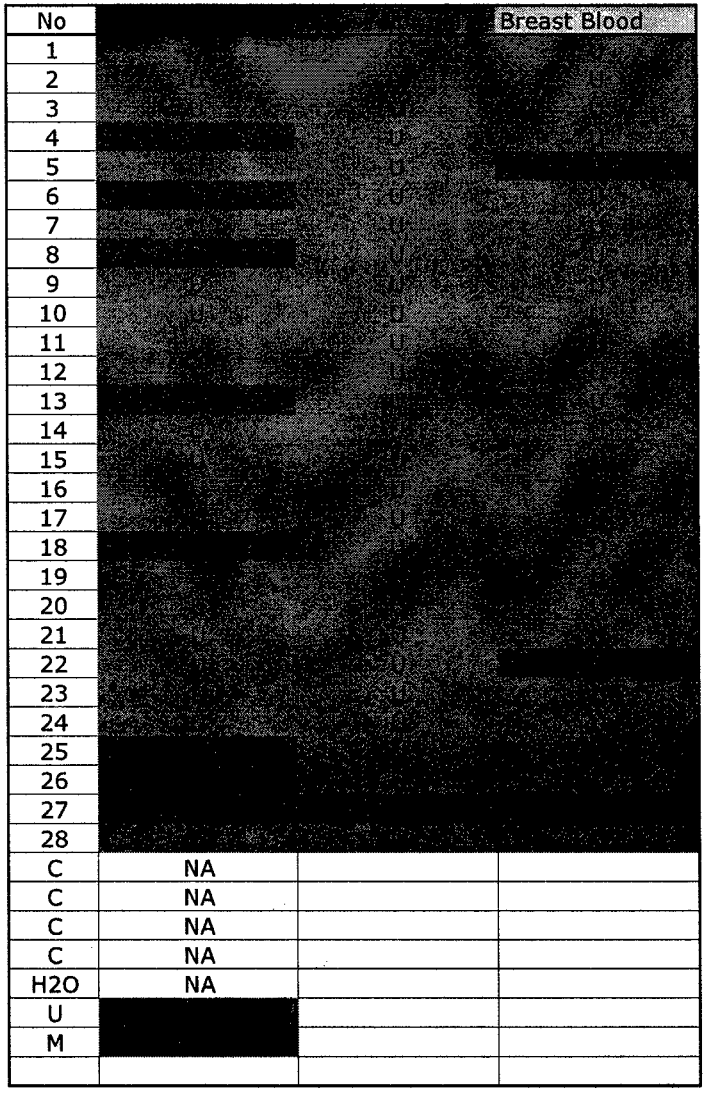


Fig 8. Headloop BRCA1 PCR: Degree of methylation in breast tumours

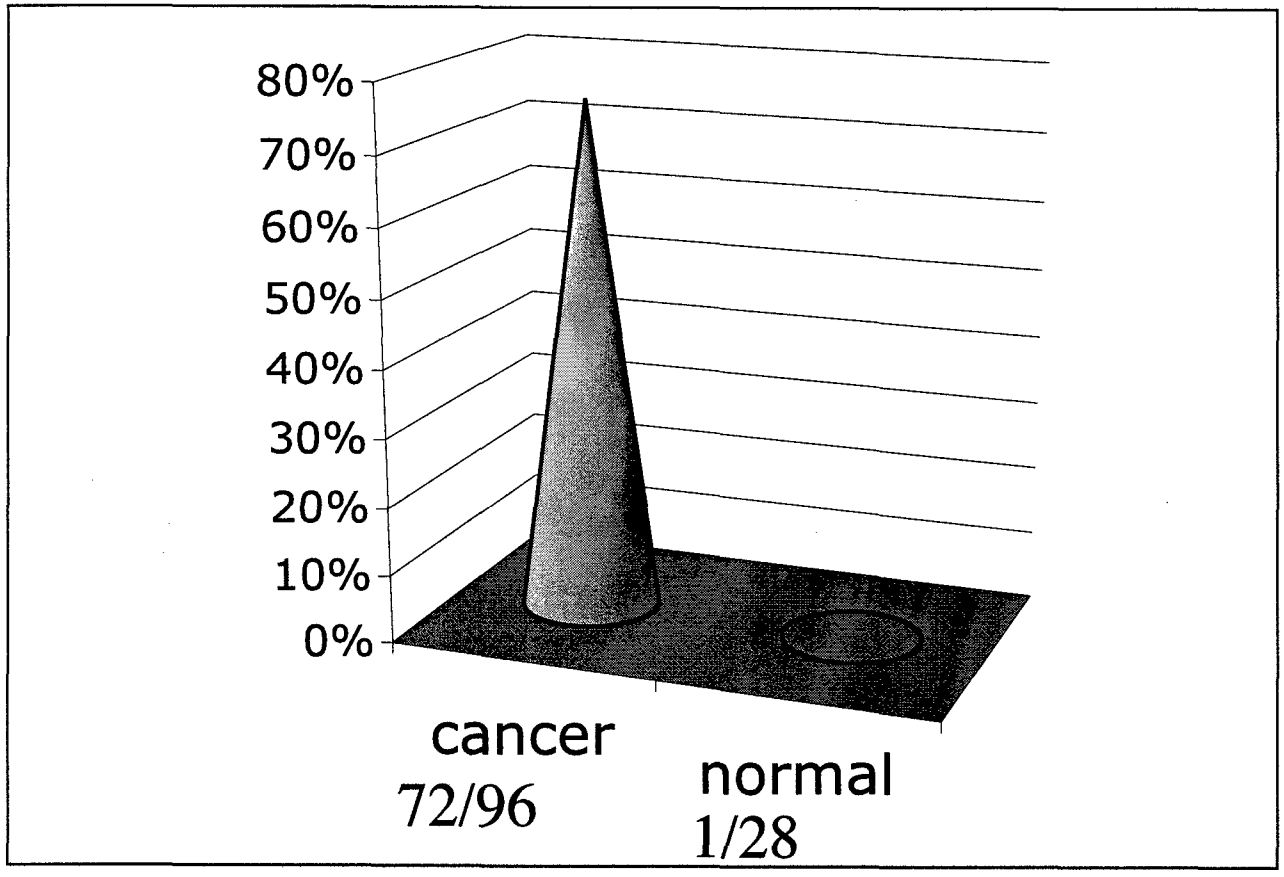


Fig 9 Methylation of BRCA1 by different assays

Method of Detection	***CpG sites***	***BRCA1 Methylation in sporadic tumours***	***%BRCA1 Methylation***	***Reference***
Southern	5HpaII sites -336 to +672		28%	Dobrovic *et al* 1997
Southern	3HpaII sites -166 to +484	0/27	0%	Magdiner *et al* 1998
Bisulphite sequencing	-260 to +61	2/6	33%	Mancini *et al* 1998
Bisulphite sequencing	-567 to +44	3/21	14%	Rice *et al* 2000
MSP	-45 to +44	11/84	13%	Esteller *et al* 2000
Headloop PCR	Critical sites	72/96	75%	Clark *et al* 2004 (unpublished)