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14. ABSTRACT The promoter regions of some genes, in particular tumor suppressor genes, are frequently hypermethylated in cancer, but not normal cells. We are conducting a nested case-control study (within the NYUWHS cohort) to assess the potential of serum DNA hypermethylation markers as a tool for early detection of breast cancer. Case-control selection criteria have been designed, the first 200 subject (of 452 subjects in total) selected and cases and their 3 controls matched for age and date of blood donation. DNA has been extracted for these first 200 subjects (50 case-control sets) and stored in aliquots at -20°C until further analysis. DNA methylation analysis requires two basic steps. DNA is chemically modified using sodium bisulfite, creating methylation specific sequence variation that is detectable using quantitative methylation-specific real-time PCR (QMSP). QMSP reactions have been optimized and sensitivity to one genome copy has been attained. Work on the methodological issues surrounding the sodium bisulfite protocol continues. Early detection is an important determinant of breast cancer prognosis and survival. This study is the first to examine aberrant promoter methylation patterns in pre-diagnostic serum samples, taking a step closer to the development of a panel of markers to be incorporated into screening strategies.					
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Annual Progress Report – DNA Hypermethylation Patterns in Serum as a Tool for Early Breast Cancer Diagnosis

Introduction:

The promoter regions of some genes, in particular tumor suppressor genes, are frequently hypermethylated in cancer, but not normal cells. This methylation is thought to be an early event in carcinogenesis. Through necrosis and apoptosis, tumors release genomic DNA into the systemic circulation. Analysis of this DNA found in the serum/plasma of breast cancer cases, allows for the detection of promoter hypermethylation, with results showing good concordance with paired tumor tissue samples. We proposed to assess the potential of serum DNA hypermethylation markers as a tool for early detection of breast cancer. To date, no study has been conducted using serum collected prior to breast cancer diagnosis. Such a study can only be conducted using the resources of a large cohort with access to blood samples collected prospectively in healthy women, such as the NYU Women's Health Study (NYUWHS).

The NYUWHS enrolled 14, 274 women aged 35-65 between the years 1985 and 1991. Serum was collected from each participant and stored for future biochemical analyses. At the time of the last complete round of follow-up, 1,006 cases of breast cancer had been diagnosed. This project is a nested case-control study within this cohort. Women for whom we have a blood sample collected within the 6 months preceding breast cancer diagnosis (n=113) will form the case group. For each case, controls will be selected and matched for age at, and date of, blood donation. The analysis of the promoter methylation status of a panel of six cancer-related genes (*RASSF1A*, *GSTP1*, *RAR β 2*, *ER β* , *DAPK* and *CDKN2A*) was proposed.

Body:

Training Plan: The first two tasks listed in the statement of work (coursework and rotations and written preliminary/qualifying exam) were completed prior to the submission of this grant. The third task was completed on October 6th, 2006 when I successfully defended my dissertation proposal to my PhD advisory committee (Drs Zeleniuch-Jacquotte, Shore, Wirgin, Klein, Liu and Cairns). The discussion generated during the defense lead to a number of modifications to the proposal outline.

- 1) A second control group consisting of women with a history of benign breast disease (BBD), as indicated by a history of breast biopsy, was added to the proposal. It was agreed that the inclusion of two control groups is necessary to assess the ability of the methylation panel to detect breast cancer. The inclusion of these two control groups will allow the determination of how well the methylation markers are able to distinguish between normal and malignant tissue (cases versus healthy controls comparison) and malignant and BBD (cases versus controls with a history of benign breast disease comparison).

- 2) Because this is a fast changing field of research, it was suggested that the gene panel remain flexible pending the publication of new results that may favor the use of alternate genes.
- 3) It was also decided that due to the precious nature of this study's samples that the complete analysis (for all 6 genes) should be conducted on 50 sets (1 case and its 3 controls) to allow for a preliminary assessment of how the study is progressing, before analyzing all 113 sets.

During the year, while working out the details of the methylation analysis I also had the opportunity to go to the American Association for Cancer Research (AACR) Annual Meeting. This allowed me to attend and contribute to discussions of the most up-to-date information on epigenetics. Shortly after attending this meeting I also traveled to the Fox Chase Cancer Center in Philadelphia, where I spent the day in the laboratory of my external PhD committee member and project consultant, Dr. Paul Cairns. Dr. Cairns is an expert in the field and an important asset to this project.

Work Plan:

Task 1: Case-control Selection

A total of 1,006 invasive breast cancer cases were diagnosed prior to 7/1/03, the start date of our latest complete follow-up. A total of 3,074 women with a history of benign breast disease have also been identified. Cases are women for whom we have a blood sample collected within the 6 months prior to breast cancer diagnosis (n=113). For each case, two sets of controls were selected. In the first set, two healthy controls were selected at random from women who were alive and free of any cancer and who had no history of BBD. In the second set, one control subject was randomly selected among healthy cancer-free women with a history of BBD. Controls were matched to cases for age and date of blood donation $\pm$ 6 months.

A series of selection criteria and priorities were created to facilitate the selection of appropriately matched controls. In an ideal match the control's age is within $\pm$ 6 months of the case's age and the date of blood donation in the control is within $\pm$ 6 months of the date of blood donation of the case. To be included in the "healthy" control group, subjects must have been free of benign breast disease at baseline and ANY cancer for the duration of the study to date. Those women in the "Benign Breast Disease" control group needed to be free of ANY cancer for the duration of the study.

The primary objective of this study is to determine whether the promoter methylation status of a panel of genes can be used for the early detection of breast cancer. This makes the cancer free status of the controls the most important selection criterion. To conduct the appropriate comparison between case and control methylation status, needed to meet the study objective, it is important to know that the control did not become a case later on in the study and therefore may have had undiagnosed, early stage breast cancer at the time of blood donation. This is especially true in the case of promoter hypermethylation

given that it is believed that these changes occur early on in the development of the tumor.

When an ideal match was not possible, a series of relaxation criteria were established. The first relaxation was to extend the matching for date of blood donation to ± 9 months while keeping all other criteria the same. If control selection was still not possible then the variation in date of blood donation was increased in 3 month increments up to ± 18 months. At this point, if a control was still not available, the variation in age was increased in 6 month increments, up to ± 2 years.

In the first 50 case-control sets, of the 150 controls selected only 7 required the relaxation of selection criteria. For 4 controls the difference in dates of blood donation was extended to ± 9 months, and for 3 controls to ± 12 months.

Task 2: DNA Isolation

DNA was isolated from the first 50 sets (200 samples) from 1 ml aliquots of serum using the QIAamp DNA Blood Midi Kits (Qiagen, Valencia CA) as described by the manufacturer with a few minor modifications due to the expectation of small amounts of DNA being isolated. These changes have been extensively validated in Dr. Wirgin's laboratory where analysis of NYUWHS DNA has been conducted for the past four years. Samples from each case-control set were isolated in the same batch, on the same day and stored for the same length of time before DNA conversion by sodium bisulfite. Isolated DNA is stored in six 45 μ l aliquots, the volume of DNA needed for sodium bisulfite conversion, to eliminate any unnecessary freeze-thaw. Each aliquot is sufficient for the methylation analysis of two genes of interest and the reference gene.

Task 3: Method optimization and DNA methylation analysis

DNA methylation analysis requires two basic steps. First the DNA must be chemically modified using sodium bisulfite, converting unmethylated cytosines to uracil while leaving methylated cytosines unchanged. This treatment leads to the generation of detectable methylation specific sequence variation. Once treated, DNA is amplified using fluorescence based, quantitative real-time PCR (QMSP) using the AB7300 (Applied Biosystems, Foster City CA).

3.1 Gene Selection:

Epigenetics is a fast changing field of study. We are rapidly learning more about how and when DNA methylation is occurring and the consequences of such modifications. This study is designed to test the ability of the methylation status of a panel of genes to detect breast cancer before the disease has been clinically diagnosed. Selection of the appropriate gene panel for this analysis is integral to the success of the study. It is for this reason that the panel has remained flexible. Over the course of the last year an extensive literature review was conducted to ensure that the best genes are selected for the methylation panel. After much consideration and examination of the most up-to-date

literature, as well as further discussion with Dr. Cairns, some small changes to the original panel have been made. The new panel is as follows: *RASSF1A*, *HIN-1*, *GSTP1*, *APC*, *p16* and *RAR β 2*. The last 2 genes in this panel (*p16* and *RAR β 2*) remain subject to change, as new information is made available. Primer and probe sequences were obtained from previous publications (Appendix 1).

3.2 Sodium Bisulfite Treatment:

Originally, sodium bisulfite conversion was going to be carried out using the method by Herman et al (1). However, since the time of the original grant submission a number of kits have become available for the sodium bisulfite treatment of DNA. After consultation with those in Dr. Klein's laboratory, in which two different kits had been used, the Qiagen Epitect Bisulfite conversion kit (Qiagen, Valencia CA) was selected. Using the QIAGEN kit will significantly increase the speed with which the samples may be analyzed, the number of samples that can be treated at one time and will reduced the amount of sample lost during the treatment procedure. Kits were tested using standards of fully methylated and fully unmethylated DNA (Millipore, Billerica MA).

The long-term stability of sodium bisulfite treated serum DNA is not well known. It is recommended that treated DNA be used as soon after treatment as possible (stored for no more than 1 week). It is therefore necessary to treat the DNA as the methylation analysis is conducted. Samples from each case-control set are treated in the same batch, on the same day and stored for the same length of time upon conversion. Further, standards for each plate are treated in the same batch as the samples for that plate. Usually, samples are analyzed on the same day of sodium bisulfite conversion to eliminate the effects of storage completely.

3.3 QMSP:

The initial focus was the familiarization of the procedure and the determination of the appropriate assay conditions. This included optimization of primer and probe concentrations and master mix composition (amount of dNTPs and MgCl₂). Through initial PCR runs, it was confirmed that the primer and probe concentrations (600nM and 200nM respectively) used by Eads et al (2) were appropriate for the current system.

During a visit to Dr. Cairns' lab at Fox Chase Cancer Center in June, the details of the procedure and modifications to improve QMSP results were discussed and preliminary runs were examined. After this meeting and consultation with those working in Dr. Wirgin's laboratory here at NYU, a switch to the TaqMan® Universal PCR Master Mix No AmpErase® (Applied Biosystems, Foster City, CA) was made. This marginally increases the cost of each reaction, but significantly improves reaction efficiency and consistency.

The final composition of the master mix consists of 1X TaqMan® Universal PCR Master Mix No AmpErase®, 600nM of each primer (forward and reverse) and 200nM MGB probe, with a final reaction volume of 25µl. Amplification conditions were as follows: 10 minutes at 95°C and then 95°C for 15 seconds followed by 60°C for 1 minute, for 50 cycles. It was determined that the same master mix composition and amplification conditions may be used for each gene under investigation, greatly reducing the optimization time for each gene, and allowing the analysis of more than one gene at a time.

3.3.1 Standard Curves

Before running study subject samples, the generation of a consistent standard curve was needed. The relative quantification of promoter methylation requires running standard curves of dilutions of known amounts of methylated DNA and probing for the gene of interest along-side *ACTB*. *ACTB* is used to standardize for the amount of DNA template in each reaction, when using unknown subject samples. Dilutions of methylated DNA are run from 10,000 copies (6600 pg DNA/µl) down to 1 genome copy (0.66 pg DNA/µl). Running a standard curve down to 1 gene copy allows the investigator to be confident that if no methylation is seen, that the result is not a false negative due to lack of assay sensitivity (3). Standard curves for *RASSF1A* (Figures 1A and B), *HIN-1* (Figures 2A and B), *GSTP1* (Figures 3A and B), *APC* (Figures 4A and B) and *ACTB* (Figures 5A and B) have been run and show good consistency and accuracy. Standard curves with high r-squared values and slopes close to -3.33 are the most accurate. Optimization of the remaining two genes (*p16* and *RARβ2*) will follow shortly.

Once the first 50 sets have been analyzed for these first four genes, a preliminary analysis will be conducted to ensure that the study goals are being met and that all technical and analytical problems are addressed, before continuing with the remaining samples.

3.3.2 Analytical Design

Standard curves for the genes of interest and for the control gene, *ACTB*, are included in each plate. This allows for the quantification of promoter methylation relative to a fully methylated control and the control of sample DNA input. Further, standard curves are generated from the same stock solution (3.3ng/µl) and can therefore act as a control for plate-to-plate variability.

To optimize assay efficiency with respect to the limited amount of sample DNA, two target genes are run for each sample on one plate (using one aliquot of isolated DNA). This is run along side the *ACTB* control and allows the same control to be used for both genes, thereby decreasing the amount of sample DNA needed for *ACTB* control reactions overall.

Cases and their 3 controls (2 healthy, 1 BBD) are run on the same plate. This ensures that any differences in methylation seen between the 3 groups is not due to plate-to-plate variability or differences in DNA storage time.

An initial run of 12 subjects (3 case-control pairs) was conducted and analyzed for *RASSF1A* and *HIN-1* promoter methylation. None of the samples were found to be methylated in the target genes, but all samples amplified with *ACTB* (Figure 6). This indicates that there is sufficient DNA in the sample for detection. Nevertheless, raising the sample volume from 5µl to 10µl is being considered. Controls are expected to have low levels of circulating DNA, increasing the sample volume will help ensure that there are no false negatives due to low concentrations of sample DNA. This analysis is working at the detection limit of the technology. By including a larger sample volume and ensuring that the assay can detect down to one genome copy (as indicated by the standard curves run on each plate), the occurrence of false negatives can be minimized (3).

3.3.3 Troubleshooting:

Unmethylated DNA is included as a negative quality control on each plate to reduce the probability of false positive sample results. Inclusion of this control monitors the specificity of the primers and probes for methylated sodium bisulfite treated sequences as well as the efficiency of the bisulfite treatment reaction itself. When the sodium bisulfite conversion is incomplete, there can be the generation of false positive results, where unmethylated DNA (i.e. the negative control) is amplified using methylation specific primers. The negative control should only be amplified by *ACTB*, whose primers and probe are not methylation specific, this indiscriminant amplification is what allows it be used to quantify the amount of DNA template in each sample.

The unmethylated negative control (CpGenome Universal Unmethylated DNA, Millipore, Billerica MA Cat#S7822) consists of DNA from two sources, human genomic DNA and DNA isolated from a human fetal cell line. For this analysis, negative controls consist of a mixture of these two DNA sources. A large volume of negative control DNA was prepared and aliquoted so that the negative control for each set of reactions was coming from the same source.

During assay optimization it was found that the negative control was amplifying for *RASSF1A*, *HIN-1* and *APC*, but not for *GSTP1* (Figure 7). This may be due to any of three things: 1) the sodium bisulfite conversion is incomplete, 2) the negative control is not actually fully unmethylated and/or 3) the primers and probes have not been designed to be methylation specific. If one gene was being amplified in the negative control, then this problem may be over-looked and a new control found for that one gene. However, because 3 of the genes being optimized at this time were found to be methylated in the negative control, this suggests that there may not just be a problem with the control, but one with the bisulfite conversion procedure as well. In order to determine which component was leading to this problem, a series of experiments were conducted.

Experiment 1: Sodium Bisulfite Treatment Using the Herman Method

Negative control DNA (100 copies/5µl or 66pg/µl), positive controls (run in a standard curve) as well as HMEC DNA (human mammary epithelial cells, 200pg/µl) were sodium bisulfite treated using the method of Herman et al (1) and analyzed using real-time PCR as described. HMEC cells are cultured, normal, non-transformed breast cancer cells and were included as an alternate negative control. Dr. Klein's laboratory has been working extensively with these cells, examining promoter methylation using methylation specific PCR (MSP), including the methylation of genes examined in the current study (*HIN-1*, *GSTP1*, *p16*, *RARβ2*).

Briefly, in the Herman method of sodium bisulfite treatment, DNA is denatured with 0.2M NaOH for 10 min at 37°C. Samples are then mixed with 10mM hydroquinone and 3M sodium bisulfite (pH = 5) and incubated under mineral oil at 50°C for 16-20 hours. DNA is then washed and purified using Wizard DNA purification resin (Promega, Madison WI), washed with 80% isopropanol and eluted with TE (Tris-EDTA buffer) at 75°C for 1 min. Finally, samples are treated with 0.3M NaOH for 5 min at room temperature and precipitated by ethanol (1).

Neither the negative control nor the HMEC DNA was amplified by any of *RASSF1A*, *HIN-1* or *APC* (data not shown). These results seemed to point to a problem with the sodium bisulfite kit. However, using this method involved the ethanol precipitation of sample DNA after DNA clean-up. During this process a large amount of DNA is lost. This meant that the sensitivity of the real-time PCR was not reaching that which was achieved using the QIAGEN bisulfite treatment kit. Using the Herman method, it was not possible to detect down to one genome copy, and in fact, where as using the 100 copy dilution for the negative control using the QIAGEN method places the negative control in the middle of the standard curve, using the Herman method, this was actually at the lower detection limit of the assay. There was therefore some concern that these results were actually false negatives due to loss of DNA during processing.

Experiment 2: Possible Modifications to Improve the QIAGEN kit

There are two potential points of weakness in the QIAGEN kit. These are the possibility of incomplete conversion by sodium bisulfite and/or incomplete desulfonation of DNA during the clean-up process. With this in mind a number of modifications were made to the QIAGEN kit protocol.

The QIAGEN protocol (Appendix 2) calls for a 5 hour heat cycling incubation with sodium bisulfite that ends with a hold at 20°C. The first modification was to increase the bisulfite incubation time from 5 hours to overnight (the Herman protocol calls for a 16 hour incubation) held at a temperature of 50°C rather than 20°C. Increasing the temperature allows the conversion to continue once the heat cycling is complete. This modification should address the issue of incomplete sodium bisulfite conversion. The second modification was to take half the samples and complete the remaining portion of the QIAGEN protocol and to take the other half and treat it with NaOH during the

desulfonation step. The QIAGEN protocol calls for an incubation of 15 minutes with 500µl of their desulfonation buffer. In the modified protocol samples were first incubated with 100µl 0.3M NaOH for 5 minutes (used as the desulfonation step in the Herman protocol) followed by the addition of 400µl of the QIAGEN buffer for the remaining 10 minute incubation.

Modifications in the QIAGEN protocol did not alter the analysis of the positive controls (i.e. the sensitivity and Ct values were the same, data not shown). Negative control DNA run using both modifications was found to be amplified for *RASSF1A* and *APC*, but not for *HIN-1*. The modifications to the QIAGEN protocol did not alter the PCR results, except that *HIN-1* was no longer amplified in the negative control.

Experiment 3: HMEC DNA Treated Using the QIAGEN Method

Until this point HMEC DNA had not been run after treatment using the QIAGEN method. HMEC DNA (6.6ng/µl) run using the QIAGEN kit to compare to the results found using the Herman method in Experiment 1.

Using the QIAGEN method the HMEC DNA amplified for *RASSF1A* and *HIN-1* (Figure 8). For *GSTP1* the samples had a very high Ct value (Ct = 47). It is likely that this sample is actually a negative. No amplification was seen for *APC*.

Experiment 4: Higher Concentration of Sample DNA Using the Herman Method

In this experiment the same HMEC DNA used in Experiment 1 (sodium bisulfite treated using the Herman method) was run. In this experiment however, a much higher concentration was used (6.6ng/µl, the highest standard concentration). This experiment was done because the concentrations used in Experiment 1 proved to be on the lower range of assay sensitivity when using the Herman method.

HMEC DNA (6.6ng/µl) was found to amplify for *RASSF1A* but not for *HIN-1*, *APC* or *GSTP1* (Figure 9).

Experiment 5: Testing Primer and Probe Methylation Specific Design

Primers and probes are designed to be specific for methylated DNA that has been sodium bisulfite treated. Sequences were obtained from previously published reports using real-time PCR methods similar to the one being used in this study (Appendix 1).

To test whether the probes and primer sets being used were specific to sodium bisulfite treated DNA, untreated DNA from MDA-MB-486 breast cancer cell lines were run. These cells had been analyzed in Dr. Klein's laboratory using traditional methylation specific PCR (MSP). Regardless of the source of the DNA, if the sample has not been sodium bisulfite treated, it should not be amplified when using primers and probes designed to specifically amplify methylated DNA that has been sodium bisulfite treated.

Untreated DNA from MDA-MB-486 cells was not amplified by *RASSF1A*, *APC* or *GSTP1*. However, amplification of *HIN-1* was seen (Figure 10). This indicates that there may be a problem with the design of the primer-probe set for *HIN-1*.

Summary:

It remains unclear whether the false positive results seen in the negative control are due to, incomplete sodium bisulfite conversion, the use of an inappropriate negative control or poor primer and probe design. This study is using a very limited amount of sample (1ml of serum per subject) that must be used for the methylation analysis of up to six genes. The advantage to using the QIAGEN kit is that much less sample is lost during DNA clean-up after bisulfite treatment. It is with the QIAGEN kit that the standard curves reveal the high sensitivity of the assay, detecting down to 1 genome copy.

Further steps are planned to determine the component of the analysis behind the unwanted amplification. First, whole genome amplification will be used to create unmethylated DNA in the laboratory. This will ensure that the issue does not lie with the negative control being methylated. This DNA will then be sodium bisulfite treated using both the QIAGEN and the Herman methods, followed by methylation specific real-time PCR, and the results compared. High concentrations of sample DNA (both of the negative control and HMEC DNA) will be used to ensure that the template is well within the detection range of the Herman method. New *HIN-1* probe and primers will also be designed.

The Herman method of sodium bisulfite treatment is labor intensive, time consuming and requires large amounts of sample DNA (1-2 μ g). If it is determined that the QIAGEN method can not be used, modifications may be made to the Herman method. By purchasing a vacuum manifold, the number of samples that can be treated at one time can be increased and the time it takes to treat them reduced. This will also allow ~20 samples to be treated at one time (comparable to that of the QIAGEN method), rather than treating each sample individually, which is how the protocol is currently carried out. Further, to address the issue of loss of DNA during the ethanol precipitation step, this step may be replaced with a column based purification method.

Once these problems have been resolved the analysis will be continued as planned and as outlined earlier in this report. The primary concern of assay sensitivity, due to the small amount of DNA available for each subject, has been addressed. Standard curves show the ability to detect down to one genome copy and that DNA is detectable in the NYUWHS samples using this method.

Key Research Accomplishments:

- Familiarization of analytical procedures (DNA isolation, sodium bisulfite conversion and QMSP)
- Case-control selection
- Optimization of QMSP reactions

- Troubleshooting the QMSP Method
- Full funding for laboratory supplies and personnel (see below)
- Presentation of proposal at an international meeting (see below)

Reportable Outcomes:

Presentations:

Annual meeting of the International Collaborative Group on Hormones and Cancer in Gavi, Italy, May 2007.

This presentation to the international group of investigators in Gavi, Italy was both a significant learning opportunity and a chance to discuss the future directions of this project. As a scientist in training the experience of witnessing the intricacies of international collaborations and the necessity of such collaborations in molecular epidemiology was invaluable. With respect to the current project, an outline was presented to the research group from Umea Sweden and a subsequent study, based on the results of this study, and including samples from their extensive national repository was proposed. They expressed enthusiasm at the possibility of collaboration and are looking forward to the results of the current investigation.

Grants Received as a result of this Award:

NYU Cancer Institute Translational Research Pilot Study Grant (Title: Serum Epigenetic Markers for the Early Diagnosis of Breast Cancer, P.I: Dr. Anne Zeleniuch-Jacquotte)
Funding Period: 1 year (01/07-12/07)
Amount: \$30,000

Susan G. Komen For The Cure, Basic, Clinical and Translational Research Grant (Title: Serum Epigenetic Markers and the Early Detection of Breast Cancer, P.I: Dr. Anne Zeleniuch-Jacquotte).
Funding Period: 2 years (07/01/07 – 06/30/09)
Amount: \$186,724

The Komen grant funds support laboratory supplies and efforts for the mentor of this project and the study data manager. The NYU Pilot Study grant allows for the addition of the BBD control group.

Conclusion:

At the conclusion of the first year of funding my thesis research is underway and funded for the next two years. Along with securing funding, I have had the opportunity to travel to conferences, one in Italy, where my proposal was presented to an international group of researches and to AACR, where I had access to the most up to date information in my field. Work will continue to resolve the issue surrounding the negative control and methylation analysis of study samples will begin before the end of October. Though this

study is in its early stages, progress is on target to meet the work plan outlined in the proposal. Once methodological issues have been resolved, progress will be fast.

Promoter methylation of tumor suppressor and other cancer related genes is a frequent and early event in breast cancer carcinogenesis. Studies in women diagnosed with breast cancer have found that promoter methylation status is measurable in both tissue and blood samples with the results showing good concordance. These studies lay the foundation for the use of methylation markers as targets for the early detection and diagnosis of breast cancer.

This study seeks to determine whether these methylation markers, detected in serum, may be used for the early detection of breast cancer. It will be the first study to examine aberrant promoter methylation patterns in pre-diagnostic serum samples providing insight into the time course of promoter hypermethylation events during breast cancer development.

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Appendices:

Appendix 1: Primer-Probe Sequences

Gene	Reference	Primer/Probe Sequence
RASSF1A	Widschwendter et al (4) Müller et al (5)	Forward: 5'-ATTGAGTTGCGGGAGTTGGT-3' Reverse: 5'-ACACGCTCCAACCGAATACG-3' Probe: 6FAM5'-CCCTTCCCAACGCGCCCA-3'TAMRA
HIN-1	Jeronimo et al (6)	Forward: 5'-GTACGGTCGTGAGCGGAGC-3' Reverse: 5'-GAAACTTCTTATACCCGATCCTC-3' Probe: 6FAM5'-GCCGACCTCGCCCGCGCTCCTAAA-3'
GSTP1	Widschwendter et al (4) Müller et al (5) Eads et al (7)	Forward: 5'-GTCGGCGTCGTGATTTAGTATTG-3' Reverse: 5'-AAACTACGACGACGAAACTCCAA-3' Probe: 6FAM5'-AAACCTCGCGACCTCCGAACCTTATAAAA-3'TAMRA
APC	Eads et al (8)	Forward: 5'-GAACCAAAACGCTCCCCAT-3' Reverse: 5'-TTATATGTTCGGTTACGTGCGTTTATAT-3' Probe: 6FAM5'-CCCGTCGAAAACCCGCCGATTA-3'TAMRA (antisense)
CDKN2A	Widschwendter et al (4) Müller et al (5) Eads et al (8)	Forward: 5'-TGGAGTTTTCGGTTGATTGGTT-3' Reverse: 5'-AACCAACGCCCCGCACCTCCT-3' Probe: 6FAM5'-ACCCGACCCCGAACCGCG-3'TAMRA
RAR β 2	Schmiemann et al (9)	Forward: 5'-CGAGAACGCGAGCGATTTC-3' Reverse: 5'-GACCAATCCAACCGAAACGA-3' Probe: 6FAM5'-CCTTCCGAATACGTTCCGAATCCTAC-3'TAMRA
ACTB	Widschwendter et al (4) Müller et al (5) Eads et al (8)	Forward: 5'-TGGTGATGGAGGAGGTTTAGTAAGT-3' Reverse: 5'-AACCAATAAAACCTACTCCTCCCTTAA-3' Probe: 6FAM5'-ACCACCACCAACACACAATAACAAACACA-3'TAMRA

Appendix 2: QIAGEN Sodium Bisulfite Treatment Procedure:

Bring all samples to room temperature

- 1) Dissolve the required number of Bisulfite Mix aliquots by adding **800µl RNase-free water** to each aliquot

Vortex until completely dissolved (may take 5 minutes)

If necessary heat to 60°C and vortex again

Do not place Bisulfite Mix on ice

- 2) Prepare bisulfite reactions in 200µl PCR tubes according to the table below

Add each component in the order listed

Component	Volume per reaction (µl)
DNA solution (1ng-500ng)	Variable (maximum 40µl)*
RNase-free water	Variable*
Bisulfite Mix (dissolved in step 1)	85
DNA Protect Buffer	15
Total Volume	140

*Combined volume of DNA solution and RNase-free water must total 40µl

- 3) Close PCR tubes and mix the bisulfite reaction thoroughly

Store tubes at room temperature

NOTE: DNA Protect Buffer should turn from green to blue after addition to DNA-Bisulfite Mix (indicates sufficient mixing and correct pH)

- 4) Perform the bisulfite DNA conversion using a thermal cycler, complete cycle should take approximately 5 hours.

Denaturation – 5 minutes at 99°C

Incubation – 25 minutes at 60°C

Denaturation – 5 minutes at 99°C

Incubation – 85 minutes at 60°C

Denaturation – 5 minutes at 99°C

Incubation – 175 minutes at 60°C

Hold at 20°C

Converted DNA can be left in the thermal cycler overnight without any loss of performance

DNA CLEAN-UP:

- 1) **Centrifuge** the PCR tubes briefly
Transfer to clean 1.5ml microcentrifuge tubes
Precipitates are OK
- 2) Add **560µl** freshly prepared **Buffer BL** containing **10µg/µl carrier RNA**
Mix by **vortexing**
Centrifuge briefly
- 3) Place an EpiTect spin column and collection tube in a suitable rack
Transfer the whole mixture into EpiTect spin column
- 4) **Centrifuge** column at maximum speed for 1min.
Discard flow through
Place spin column back into the collection tube
- 5) Add **500µl Buffer BW** (wash buffer) to spin column
Centrifuge at max speed for 1min.
Discard flow through
Place spin column back into the collection tube
- 6) Add **500µl Buffer BD** (desulfonation buffer) to the spin column
Incubate for 15min. at room temperature (with the lid closed)
Avoid transfer of precipitate into spin column
- 7) **Centrifuge** column at max speed for 1min.
Discard flow through
Place spin column into collection tube
- 8) Add **500µl Buffer BW** and centrifuge at maximum speed for 1min.
Discard flow through
Place spin column into collection tube
- 9) **Repeat** step 8 once
- 10) Place the spin column into a new 2ml collection tube
Centrifuge the spin column at max speed for 5min.
Remove any residual liquid
- 11) Place spin column into a new, clean 1.5ml micro-centrifuge tube
Add **20µl Buffer EB** to the center of the membrane
Elute purified DNA by **centrifugation** for 1min at approx. **15,000 × g** (12,000 rpm)
- 12) **Transfer** spin column to a new 1.5ml micro-centrifuge tube

Add **20µl Buffer EB** to the center of the membrane
Centrifuge for 1min. at max. speed
Combine both eluates

- 13) To store for up to 24 hours – store at 2-8°C
For storage longer than 24 hours (up to 12 weeks) - -20°C

Supporting Data:

Figure 1A: RASSF1A Real-Time PCR Amplification

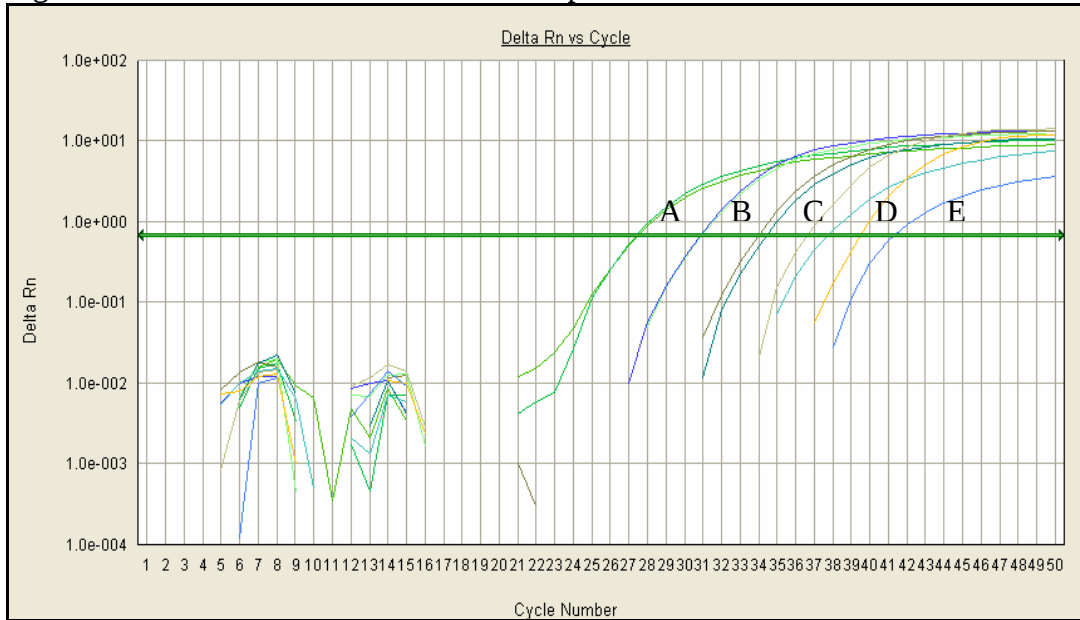


Figure 1B: Sample RASSF1A Standard Curve

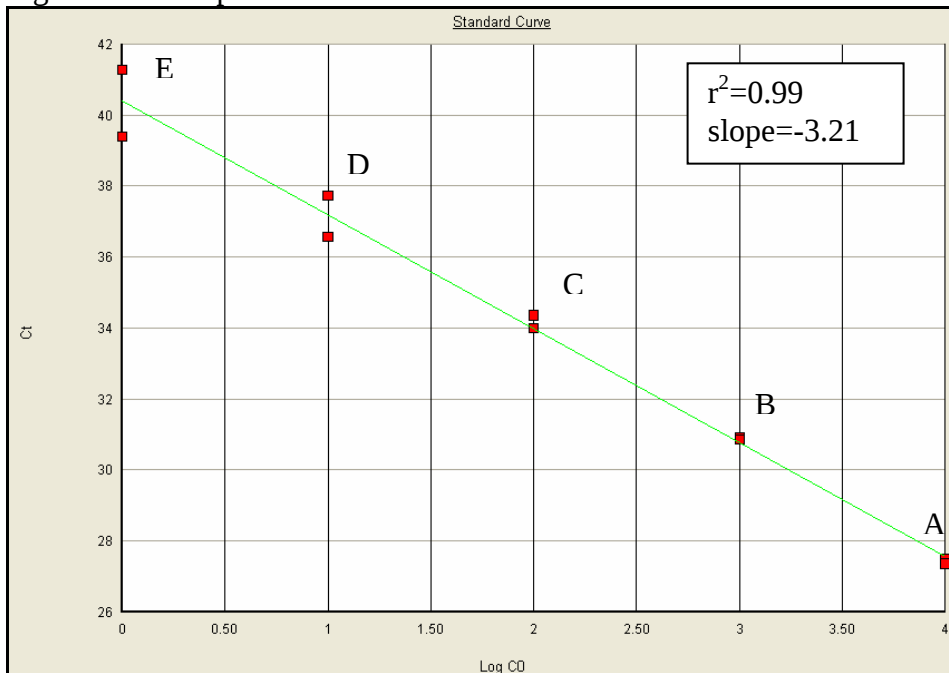


Figure 2A: HIN-1 Real-Time PCR Amplification

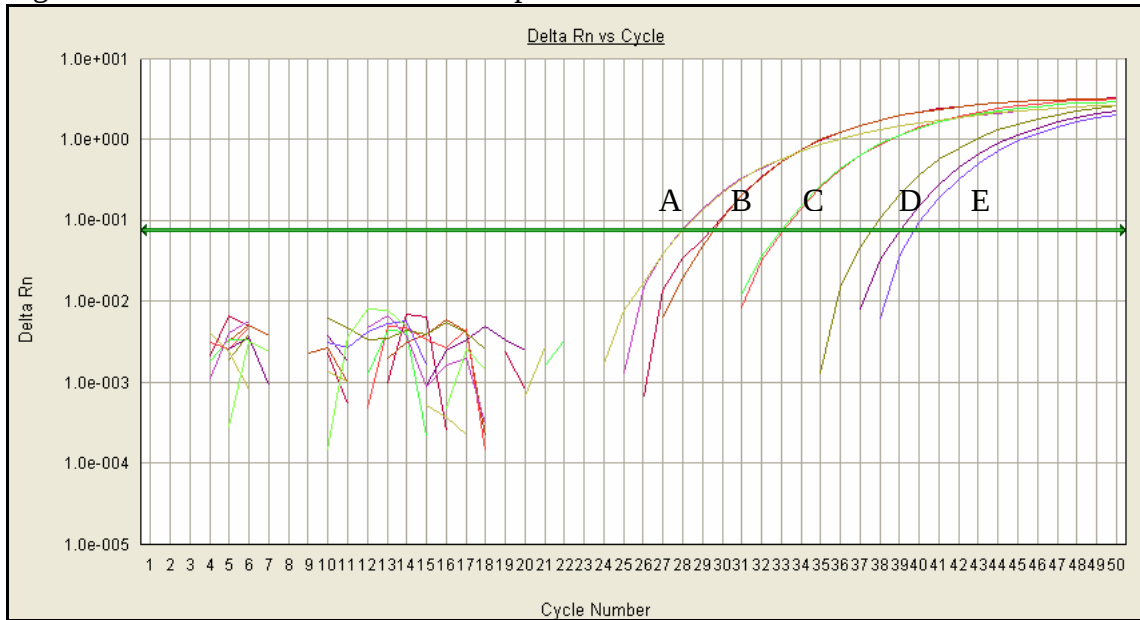
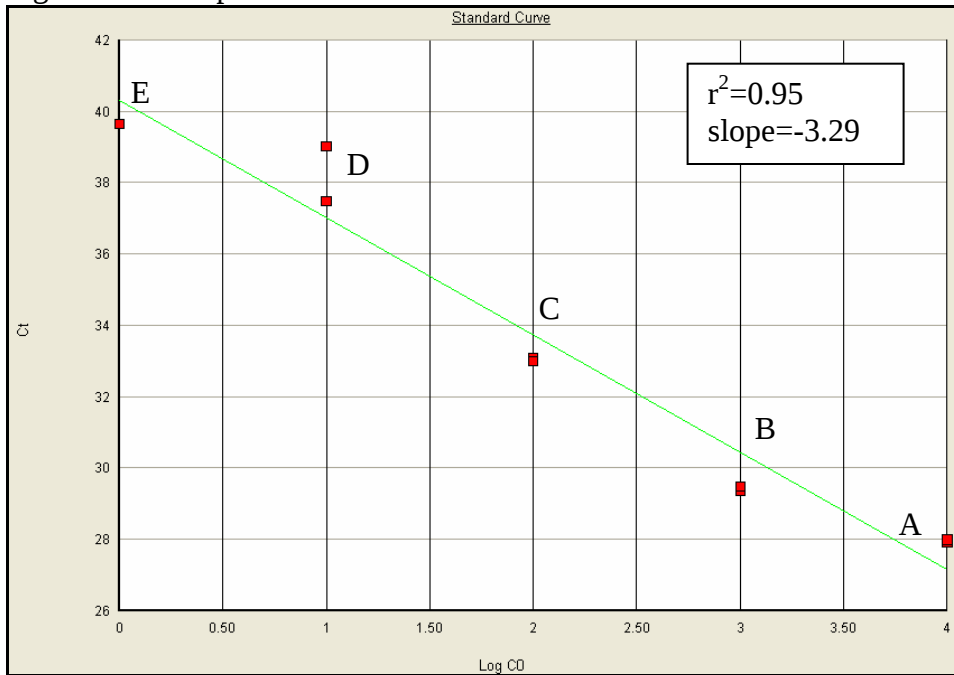
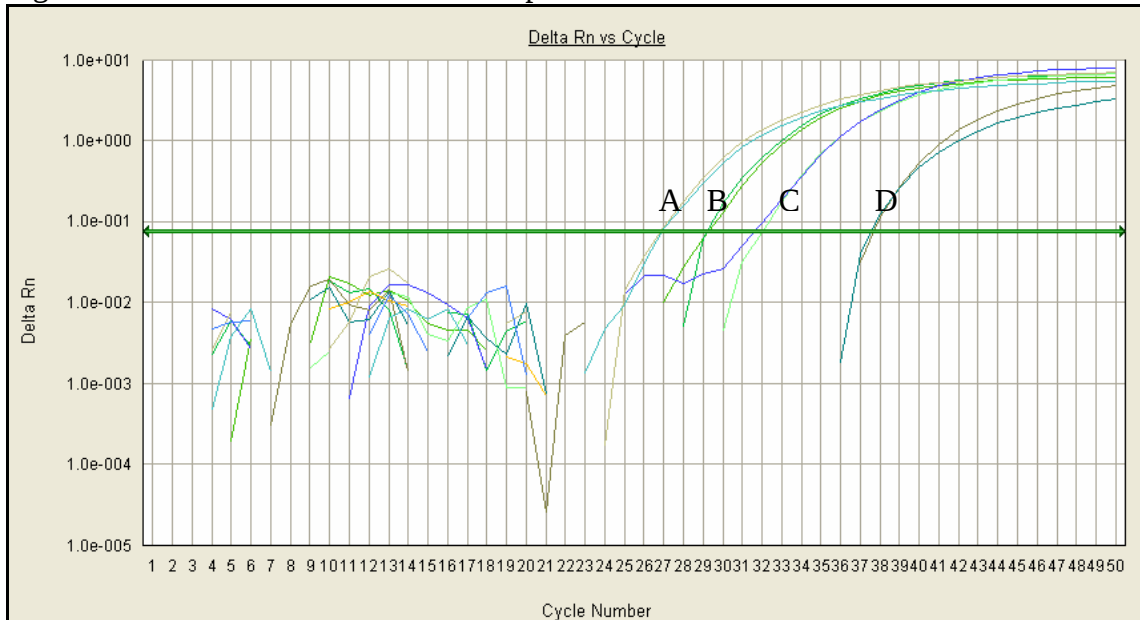


Figure 2B: Sample HIN-1 Standard Curve



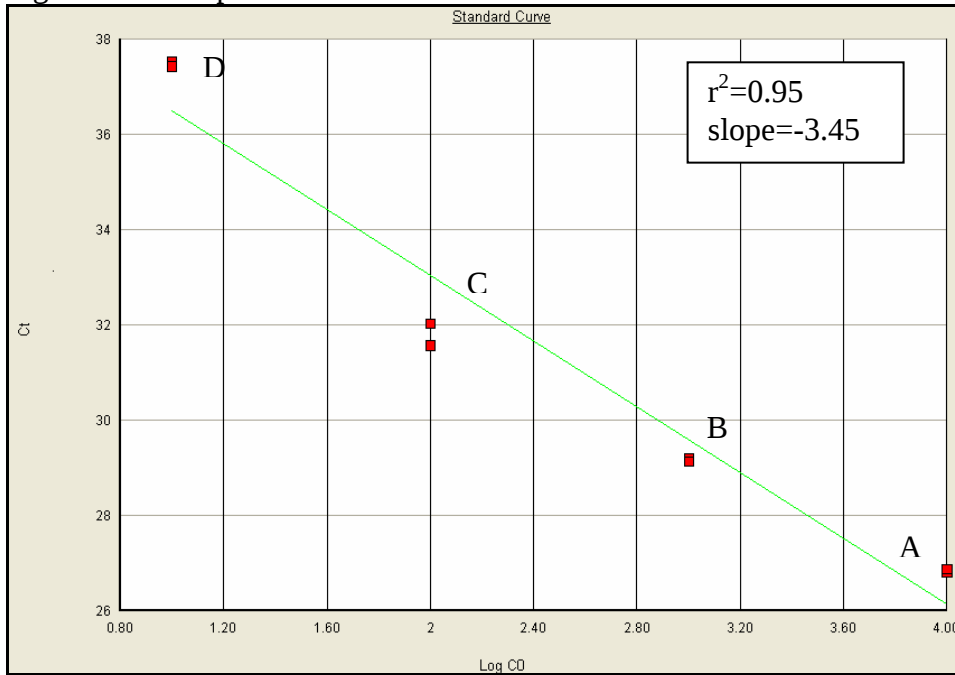
A: 10,000 genome copies, B: 1000, C: 100, D: 10, E:1

Figure 3A: GSTP1 Real-Time PCR Amplification



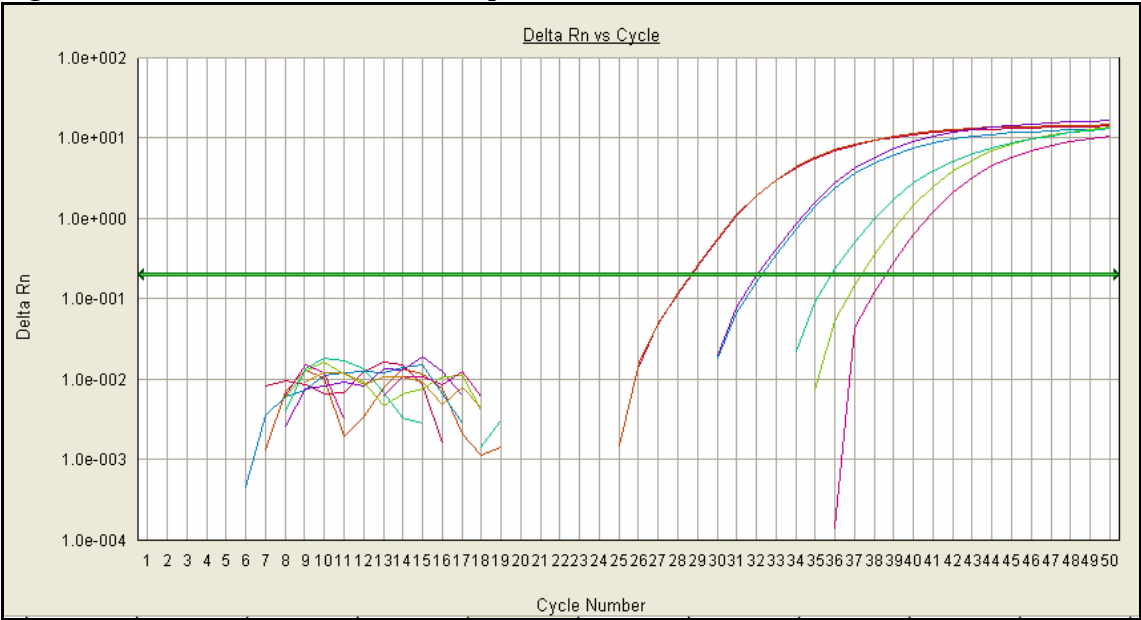
A: 10,000 genome copies, B: 1000, C: 100, D: 10

Figure 3B: Sample GSTP1 Standard Curve



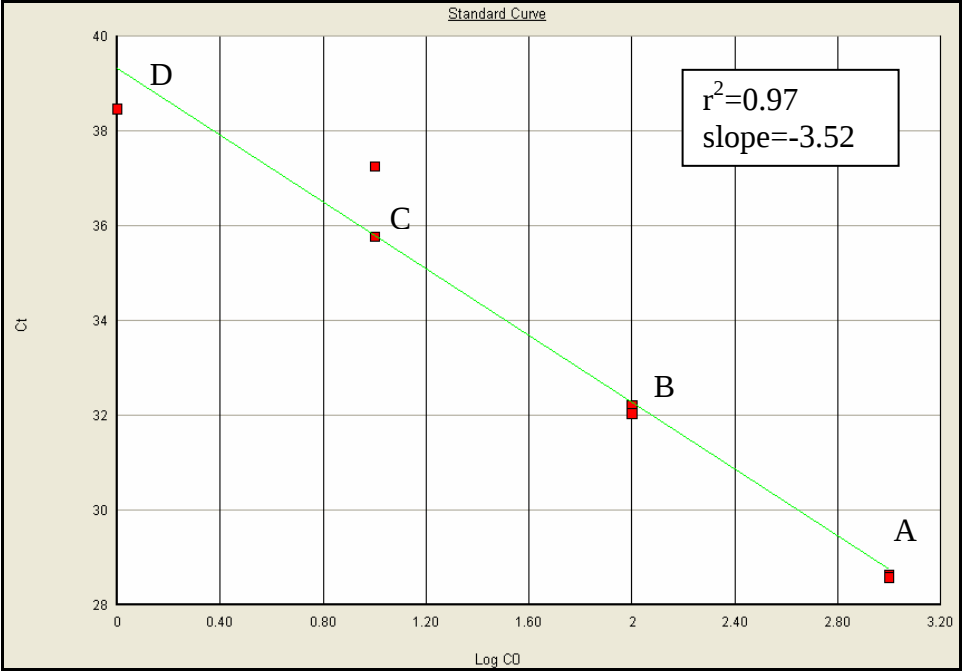
A: 10,000 genome copies, B: 1000, C: 100, D: 10

Figure 4A: APC Real-Time PCR Amplification



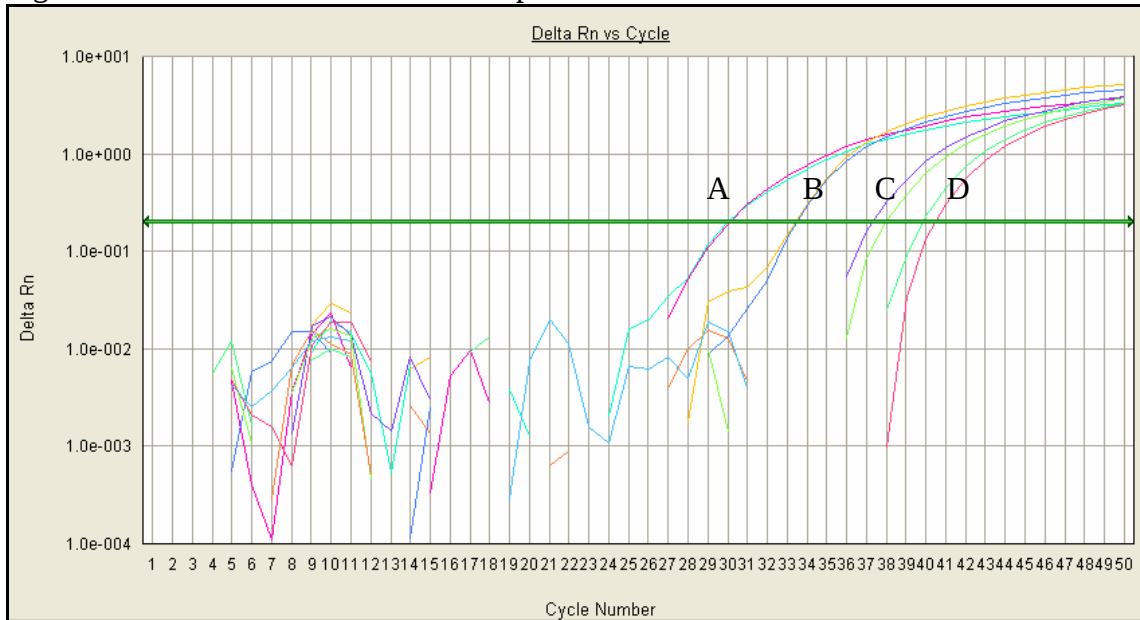
A: 10,000 genome copies, B: 1000, C: 100, D: 10

Figure 4B: Sample APC Standard Curve



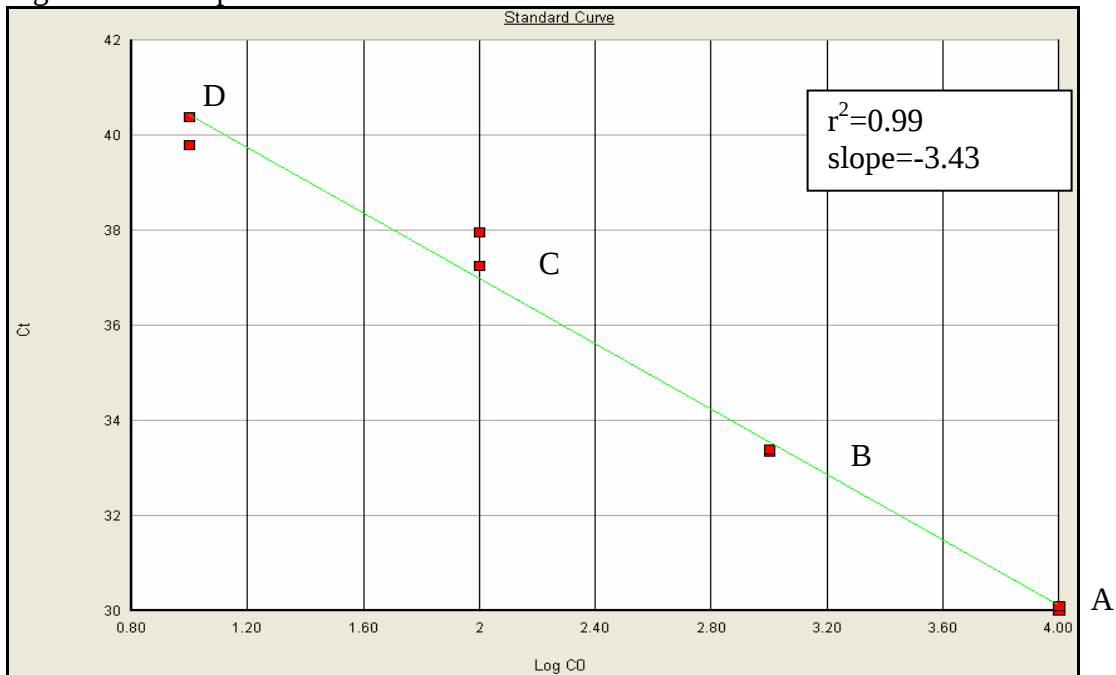
A: 10,000 genome copies, B: 1000, C: 100, D: 10

Figure 5A: ACTB Real-Time PCR Amplification



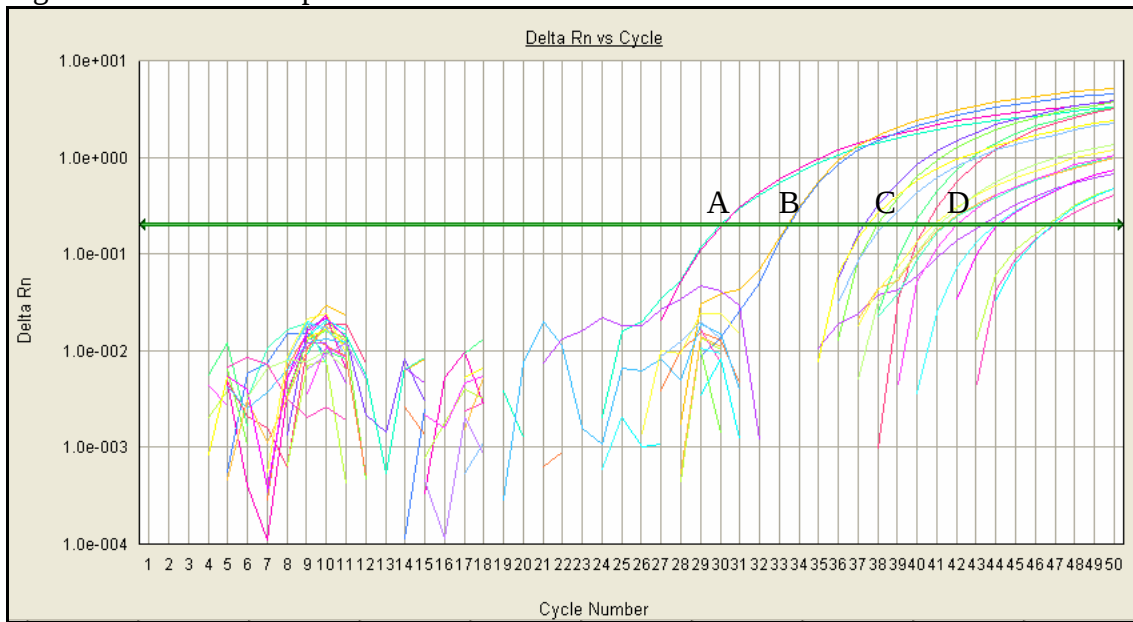
A: 10,000 genome copies, B: 1000, C: 100, D: 10

Figure 5B: Sample ACTB Standard Curve



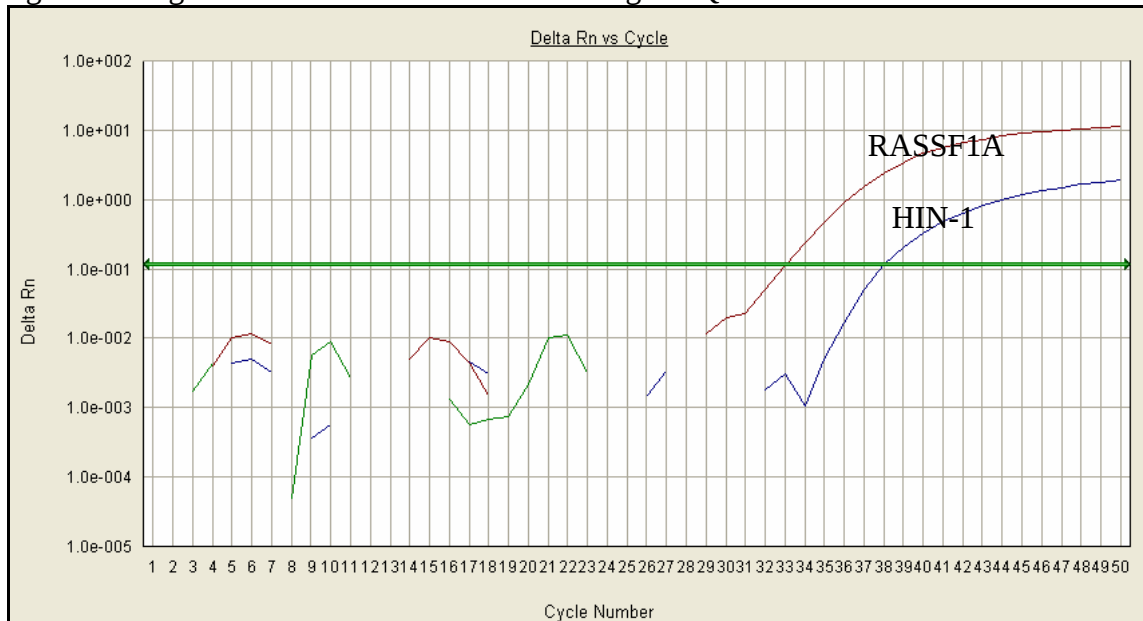
A: 10,000 genome copies, B: 1000, C: 100, D: 10

Figure 6: First 12 Samples and ACTB Standards



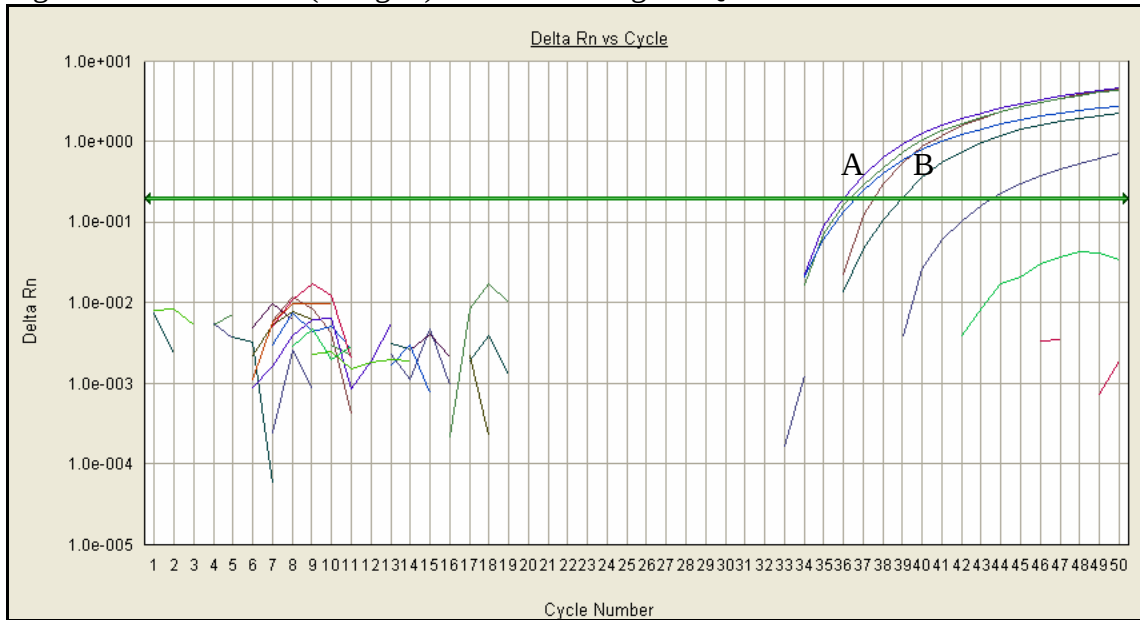
A: 10,000 genome copies, B: 1000, C: 100, D: 10 of positive control DNA

Figure 7: Negative Control DNA Modified Using the QIAGEN Method



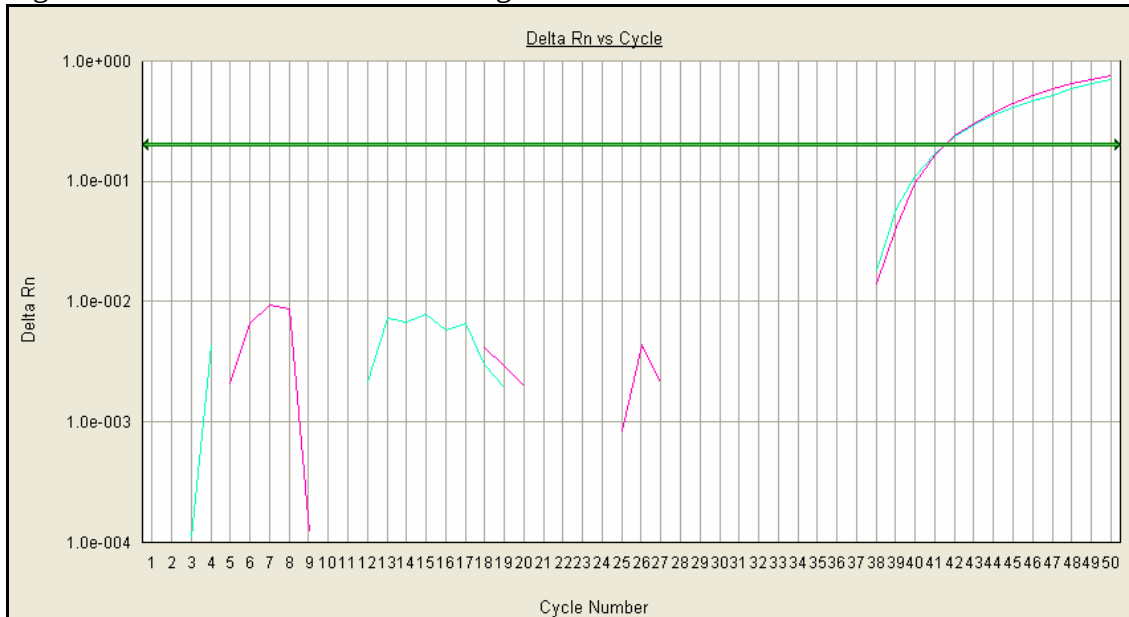
Amplification using primers and probes for RASSF1A and HIN-1

Figure 8: HMEC DNA (6.6ng/ul) Modified Using the QIAGEN Method



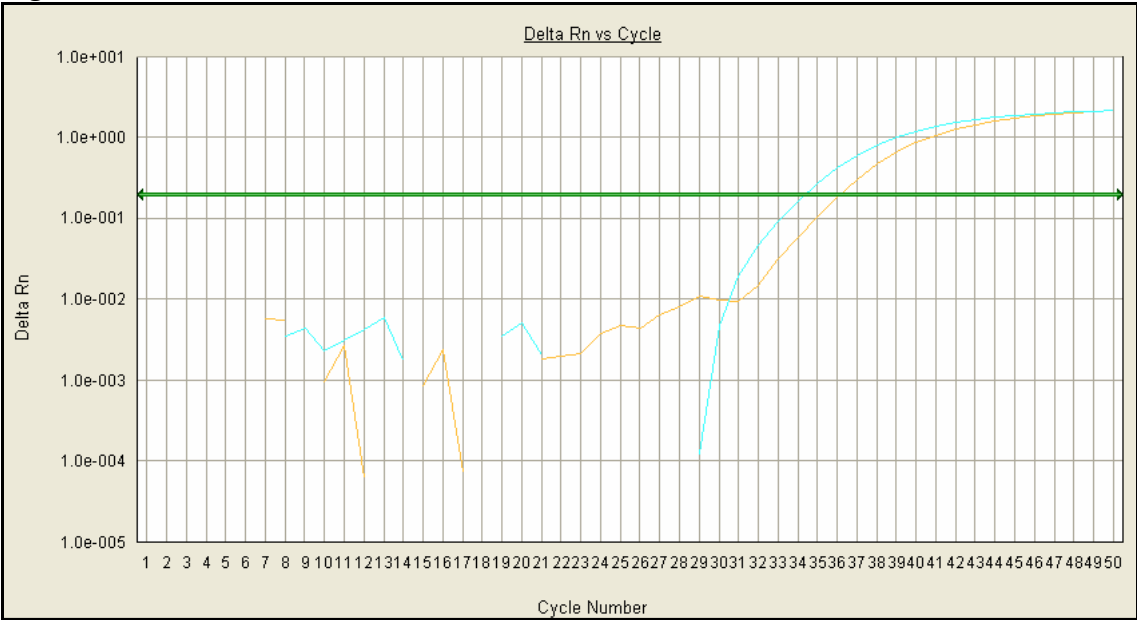
Amplification using primers and probes for A: RASSF1A and B: HIN-1

Figure 9: HMEC DNA Modified Using the HERMAN Method



Amplification using primers and probe for RASSF1A only

Figure 10: Untreated MDA-MB-486 DNA



Amplification using primers and probe for HIN-1 only

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