Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Magee-Womens Research Institute and Foundation

2. **Reporting Period (start and end date of grant award period):** 01/01/2010 -12/31/2010.

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Cheryl A. Richards, MBA

4. **Grant Contact Person's Telephone Number:** 412-641-8932

5. **Grant SAP Number:** 4100050901

6. **Project Number and Title of Research Project:** 5 - Epigenetic Analysis of Human Aneuploidy

7. **Start and End Date of Research Project:** 01/01/2010 -12/31/2010.

8. **Name of Principal Investigator for the Research Project:** David Peters, PhD

9. **Research Project Expenses**.

   9(A) Please provide the amount of health research grant funds spent on this project for the entire duration of the grant, including any interest earned that was spent:

   $ 79,250

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Peters	Principal Investigator	10%	$17,539.48
Shaw	Research Assistant	30%	$10,461.85

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Chu	Key collaborator	20%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No_____ X____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___X___ No_____

If yes, please describe your plans:

I anticipate that the data derived from this study will form the basis of a future (June 2011) R21 grant application to the NIH.

12. **Future of Research Project.** What are the future plans for this research project?

My intention is to use the novel method for DNA methylation analysis to further explore differences in the epigenomes of normal versus trisomy 21 tissues.

13. **New Investigator Training and Development**. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes_____ No____X____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian				
Other				
Unknown				
Total				

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No___X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____ _____ No___X_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes___X_____ No_____

If yes, please describe the collaborations:

The work presented at national meetings has helped to engage other researchers across the country and opened a productive dialogue between our University of Pittsburgh group and the NIH-NIAID/NICHD. In addition, the FDA has expressed interest in our projects and collaborator team and this will likely lead to contract-level work.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No ___X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No ___X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.
List the project goals, objectives and specific aims (as contained in the grant application's strategic plan). Summarize the progress made in achieving these goals, objectives and aims <u>for the entire grant award period.</u> Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used.

If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

BACKGROUND AND SPECIFIC AIMS

Despite the intense interest in epigenetics and the importance of its function in normal development there is no literature that characterizes the placental DNA methylome in early gestation. The study proposed therefore presents a unique opportunity to answer critical questions relating to human epigenomics in the context of placental function and aneuploidy. We hypothesize that placental DNA methylation patterns, specifically within the chorionic villus and in cells derived from this tissue, have distinct characteristics that are altered between normal and aneuploid genomes. We further hypothesize that differences in DNA methylation have the potential to be exploited via the development of non-invasive methods based upon selective amplification of fetal DNA from maternal plasma. We will test these hypotheses by carrying out the following specific aims.

1. We will generate high-resolution quantitative maps of DNA methylation in CVS tissue and cultured cells derived from CVS using the Illumina Humanmethylation27 microarray platform. Samples to be used in these experiments have already been collected from normal and aneuploid pregnancies.

2. We will confirm DNA methylation changes identified using microarray based approaches (Aim 1) at the single gene level using Sequenom-based Epityper assays.

RESULTS

DNA was extracted from a total of twenty-four tissue samples/cultured cell samples according to the scheme in Table 1. Briefly, we have thus far analyzed DNA from a total of six karyotypically normal chorionic villus (CV) samples and five trisomy 21 snap frozen CV tissue samples. We further analyzed DNA from a total of four karyotypically normal and four trisomy 21 cultured CV cell samples. Some of the latter were analyzed in duplicate to generate technical replicate data.

DNA was used to interrogate two 12x Humanmethylation27 oligonucleotide microarrays (Illumina). This platform allows the analysis of 27,578 CpG sites per sample at single-nucleotide resolution. The HumanMethylation27 panel targets CpG sites located within the proximal promoter regions of transcription start sites of 14,475 consensus coding sequencing (CCDS) in the NCBI Database (Genome Build 36). In addition, 254 assays cover 110 miRNA promoters

The resulting data were analyzed using statistical methods (see Materials and Methods). Specifically we carried out the following comparisons. First we identified CpG sites that are differentially methylated between karyotypically normal and trisomy 21 samples regardless of whether the data were obtained using fresh CV tissue or cultured CV-derived cells. Two lists of statistically significant genes were identified to represent those CpGs that are more or less methylated in trisomy 21 samples compared to normal samples. The top ten most statistically significant genes in each case are listed in Tables 2A and 2B respectively.

Significantly, we found that hierarchical clustering of all the samples identified a clear distinction between two groups of samples (Figure 1). Rather than delineating differences between normal and trisomy 21 samples, this distinction between clusters was representative of whether the samples were derived from fresh frozen CV tissue or cultured CV-derived cells.

We therefore carried out a second group of analyses in which we separated uncultured and cultured samples into two groups and treated them separately. Table 3A and 3B show the top ten CpG sites found to be more or less highly methylated, respectively, between fresh frozen CV tissue from normal and trisomy 21 pregnancies. Table 3C and 3D show the top ten CpG sites found to be more or less highly methylated, respectively, between cultured CV-derived cells from normal and trisomy 21 pregnancies.

As stated in Aim 2, our secondary goal was to confirm our microarray findings in a gene specific fashion using the Epityper method. While designing these experiments, we were simultaneously undertaking a parallel study in which we were attempting to identify DNA methylation differences between gestational age-matched placental tissues obtained at delivery from normal mothers and mothers with preeclampsia. Preeclampsia is a physiological disorder of pregnancy in which high blood pressure and protein in the urine develop after the 20th week (late 2nd or 3rd trimester) of pregnancy. In this related study, like the current study, we used the Humanmethylation27 microarray platform. Interestingly we discovered that the 48 microarray data sets (derived from 24 normal and 24 diseased placental tissues) could be easily distinguished on the basis of fetal gender. Specifically, when we performed unsupervised hierarchical clustering on these samples we identifed two major clusters of samples. Regardless

of disease state these were distinguished completely by fetal gender. Therefore, we conclude that fetal gender is an extremely strong signal in the context of DNA methylation.

In light of the above discovery, we re-analyzed our trisomy 21 microarray data. In this analysis we compared normal and trisomy 21 CVS samples from only female pregnancies or only male pregnancies. The top ten genes identified in this analysis for each fetal gender is presented in Table 4A (female) and 4B (male). Interestingly there were a significantly larger number of CpG loci identified in the samples from female pregnancies than male pregnancies. In fact we identified 301 loci that reached statistical significance when comparing normal versus trisomy 21 in the female tissues and only 25 in the male tissues.

Regardless of our method of data analysis, one characteristic of our microarray data is that, while differences between cultured and uncultured CVS samples are profound, differences between normal and trisomy 21 samples within either the cultured or uncultured sample groups were more subtle. Despite this apparent lack major of distinction, we did identify DNA methylation differences at CpG loci that were statistically significant between normal and aneuploid tissues and we therefore attempted to confirm these using the Epityper platform marketed by Sequenom. This is a mass spectrometry based system that can detect mass differences between DNA fragments of different sequence. In this assay, purified DNA from a given sample is subjected to treatment with bisulphite. This results in the conversion of cytosine residues to uracil but leaves 5-methylcytosine residues unaffected. Thus, bisulphite treatment introduces specific changes in the DNA sequence that depend on the methylation status of individual cytosine residues, yielding single- nucleotide resolution information about the methylation status of a segment of DNA. It is this information that is detected during the mass spectrometry analysis.

For the Epityper analysis we focused on the CpG loci listed in Table 5. These are genes that were identified as being differentially methylated in normal versus aneuploid uncultured CVS tissues from pregnancies involving female fetuses. We focused on female pregnancies because more differentially methylated sites were identified by microarray analysis. We focused on the uncultured samples because of the marked differences previously identified between cultured and uncultured samples, implying that methylation signatures are altered during cell culture. We felt that the uncultured molecular phenotype was therefore more biologically relevant than its cultured cell counterpart. Furthermore, the differences in DNA methylation between normal and trisomy 21 samples were more profound in the uncultured compared with the cultured samples.

Although the primary microarray data generally identified only one specific CpG locus per gene of interest, the Epityper method is able to assay DNA methylation levels at multiple CpG loci within the surrounding genomic region. This region is specified by the length/location of the PCR fragment that is amplified following bisulphite conversion of DNA prior to mass spectrometry. The Epityper data corresponding to the sentinel CpG loci, which are those previously identified by the microarray analysis, are marked in Table 5 with an asterisk. As shown, the Epityper data generally do not support the findings of the microarray experiments in that CpG loci identified as differentially methylated in the latter are not corroborated by the former. This was true not only with respect to the sentinel CpG site but also the many other adjacent CpG loci that were co-amplified and therefore co-analyzed by the Epityper analysis.

This lack of correlation was observed regardless of whether female samples (Table 5A) or samples of both gender (Table 5B) were analyzed.

The lack of corroboration between the two assay platforms is troubling. It is of course possible that the genes identified by the microarray experiments are not genuinely differentially methylated and are therefore false positive results. In any experimental system that contains relatively few replicates and thousands of data points there are bound to be some false positives and false negative results. However, we have performed large numbers of both Humanmethylation27 microarray and Epityper experiments and we have found that the data generally agree. It should be noted however that the majority of our experience with these platforms has been focused on the analysis of distinct cell or tissue types rather than normal and abnormal samples from the same anatomical tissue source. In such comparisons it is easy to find differences in DNA methylation that are very large, in contrast to the relatively subtle changes identified by the current study.

One question that arises, with respect to the assay platform, is whether the Humanmethylation27 microarrays are of high enough density to collect information at relevant loci. This is because, although many thousands of genes are represented on the microarray, there are very few probes that are present per gene. We know from previous studies in our lab (Chu *et al*, 2009) that DNA methylation levels can change quite dramatically in a spatial context along the chromosome. Therefore it is conceivable that the assay platform is unable to detect significant differences in DNA methylation by not providing enough gene-specific sensitivity. This does not, of course, explain why the microarray and Epityper data sets do not agree.

Because of the rarity and consequent precious nature of our trisomy 21 samples and concerns about the ability of the microarrays to identify real biological differences between normal and trisomy 21 samples, we decided to focus our efforts on the development of a new technology. Ideally, this would be able to assay DNA methylation levels at far more loci than the microarray and at much higher accuracy. Our goal was to combine a previously used method (Chu *et al*, 2009) based on a custom microarray platform developed in our lab with next generation DNA sequencing. In our previous microarray method we generated libraries of methylated and unmethylated DNA fragments using the restriction enzyme isoschizomers HpaII and MspI. These enzymes both cleave at CCGG core recognition sequences but the former will only cut when the DNA is unmethylated and is blocked when it is methylated. In contrast MspI cuts regardless of whether the CCGG site is methylated. By using one or other enzyme to cut each of two aliquots of the same DNA it is possible to compare the restriction fragments to identify differentially methylated sequences. In our previous microarray method we combined this isoschizomer profiling approach with the addition of a second restriction enzyme called TspR1 in conjunction with an Exonuclease III digestion step. This enzyme generates 9bp single stranded DNA overhangs that are resistant to cleavage by Exonuclease III. However, HpaII or MspI generate 2bp over-hangs that are sensitive to Exonuclease III cleavage. Therefore if a TspR1 digested fragment is cleaved internally by HpaII or MspI then it will be lost from the library. Using this approach one is able to generate a methylated CCGG site-enriched fragment library when digestion with HpaII takes place. Libraries digested with MspI and sham-digested libraries serve as positive and negative controls respectively. A schematic diagram highlighting this method is presented in Figure 2.

The novel approach to generating DNA libraries enriched for methylated CCGG sites was carried out in duplicate using a single normal CVS sample. Duplicate libraries digested with HpaII, MspI or undigested sham controls were prepared. The resulting DNA fragment libraries were then used as substrates for the construction of DNA sequencing libraries using Illumina next generation sequencing chemistry. Each of these was then subjected to cycle sequencing on the Illumina GxII platform. Preliminary DNA sequencing data is shown in Table 6. Using this approach we have generated first proof of concept for a novel sequencing based method for comprehensive genome wide DNA methylation analysis, which we believe will prove to be far more sensitive and accurate than the microarray method used previously.

SUMMARY AND CONCLUSIONS

In summary we have used a commercial microarray platform to identify differences in DNA methylation patterns between normal and trisomy 21 genomes from both cultured and uncultured CVS tissues. We find that there is a clear and marked difference between cultured and uncultured specimens but that differences between normal and trisomy 21 samples within these groups are more subtle. We were unable to confirm these aneuploidy-specific differences in the uncultured samples despite controlling for fetal gender, which we have found to have a profound influence on genomic DNA methylation patterns. Because of this lack of agreement between primary microarray data and gene specific follow up analysis by mass spectrometry, we speculated that the microarray platform is not sensitive enough to detect subtle differences in DNA methylation on a genome wide scale. Because of the high value of our affected samples we elected not to perform any more experiments with these until we have further refined our analytical tools. We therefore developed a novel method for genome wide DNA methylation analysis based upon high throughput DNA seuquencing. Preliminary data suggests that this method may have significant potential for the analysis of disease-specific changes in DNA methylation at high resolution on a genome wide scale.

MATERIALS AND METHODS

CV Tissue Handling

All samples used in this study were discarded de-identified tissues. CV samples were obtained between gestational weeks 11 and 13 from the Magee Women's Hospital Cytogenetic Screening Laboratory. Samples were dissected under a microscope and separated from any decidual tissue or flecks of blood. The culture media was removed and the tissue placed in 1.5 -2.0mL microcentrifuge tubes before freezing at -80oC until DNA was extracted.

DNA Extraction from CV Tissue

To extract DNA, one 5mm stainless steel bead and 180µL buffer ATL (from Qiagen's DNeasy Blood and Tissue kit) were added to each CVS sample. The samples were placed in the TissueLyser (Qiagen) Adaptor set 2 x 24, and the TissueLyser was operated for 20 seconds at 30Hz. The DNA was then purified using the DNeasy Blood and Tissue kit (Qiagen) as per the manufacturer's protocol.

DNA Extraction from CV-Derived Cells

DNA then purified using the DNeasy Blood and Tissue kit (Qiagen) as per the manufacturer's protocol.

Infinium Microarray Analysis

The HumanMethylation27 DNA Analysis BeadChip (Illumina) allows interrogation of 27,578 CpG sites based on the NCBI CCDS database (Genome Build 36) and also targets the promoter regions of 110 miRNA genes. Bisulphite conversion of DNA was carried out using the EZ DNA Methylation™ Kit (Zymo Research Corp., CA) to convert unmethylated cytosine nucleotides to uracil. Following denaturation with 0.1N NaOH, converted DNA samples were amplified by incubation at 37ºC for 20 hours in a proprietary amplification reaction mix. Amplified DNA was fragmented using vendor-supplied reagents by incubation for 1 hour at 37ºC. Fragmented DNA sample was precipitated and resuspended in hybridization buffer. Infinium BeadChips were cleaned and activated by washing with ethanol, formamide and vendor supplied pre-hybridization buffers. DNA samples are denatured, applied to the Infinium arrays and hybridized 16-24 hours with rocking at 48ºC. The BeadChip is placed into a flow-through chamber, unhybridized and non-specifically hybridized DNA was washed away and single base extension was performed on bound primers with labeled nucleotides. Hybridized DNA sample was removed by washing using proprietary buffers. Staining steps were performed to attach florescent dyes to the labeled nucleotides and the array surface sealed to protect the dyes from atmospheric degradation. The final array was scanned using an Illumina BeadArray Reader and the data analyzed using Bead Studio 2.0.

Determination of the methylation status of CpG sites using Infinium Array Data

On an Infinium array, each targeted CpG site is interrogated by 2 probes: probe A for unmethylation status, and probe B for methylation status. The A probe signals and B probe signals are normalized separately, using the cyclic loess algorithm. We then compute the log ratio of probe B to probe A: log(B/A), as well as the beta value, which is defined as approximately B/(A+B+100), assuming A,B≥0. Both beta and log(B/A) can be used as a measurement of the methylation level of a CpG site. In particular, a CpG site is hypomethylated if the log(B/A) value of that site is significantly lower than 0. It is hypermethylated if log(B/A) is significantly higher than 0. Student's t tests are used to test if a CpG site is methylated in a group of samples, or if two groups of samples have identical methylation rate at a given CpG site. Empirical Bayesian method is used to estimate the within group variance. P values are adjusted using Benjamini and Hochberg's method to control the false discovery rate (FDR) at 5%. Microarray data were clustered using the agglomerative hierarchical clustering method using the R statistical package. Each sample was treated as a 27,578-dimension vector (there are a total of 27,578 probes in an array). The Euclidean distance between samples was used as the distance metric.

Sequenom Epityper/Quantitative Methylation Analysis

Quantitative Methylation Analysis was performed using the MassARRAY Compact system (Sequenom). 1ug genomic DNA was bisulphite converted using the EZ DNA Methylation (Zymo Research) as per manufacturer's instructions. PCR primers were designed by MethPrimer (www.urogene.org/methprimer) to flank the CpG regions of interest and were purchased from Integrated DNA Technologies (Coralville, IA). Primer sequences are presented

in Table 4. Amplicons for use with the MassARRAY system are typically 200-600 bp in length and are independent of the methylation state of the genomic DNA, meaning they bind to both methylated and non-methylated template (as opposed to methylation-specific primers). Amplification of 1 µL bisulphite treated DNA (20 ng/µL) was performed using HotStar Taq Polymerase (Qiagen) and primers at a final concentration of 200nM each in a 5 µL reaction volume using a 384 well plate. PCR amplification was performed in a PTC225 thermal cycler (MJ Research, Inc.) with the following parameters: 94°C for 15 min hot start, followed by denaturing at 94°C for 20 sec, annealing at 62°C for 30 sec, extension at 72°C for 1 min for 45 cycles, and final incubation at 72°C for 3 min. The PCR products are then treated with shrimp alkaline phosphatase (SAP), *in vitro* transcribed and analyzed according to the manufacturer's instructions (Sequenom).

TspR1/HpaII/MspI Enrichment of DNA Methylated Regions in Genomic DNA

The following oligos 5' – ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TC*T – 3' and 5' - /5Phos/GAT CGG AAG AGC TCG TAT GCC GTC TTC TGC TTG – 3' were resuspended in TE and annealed in 1X T4 DNA Ligase Reaction Buffer (NEB) by heating at 95°C for 5 minutes and then slowly cooled to room temperature for a final concentration of 36µM annealed adaptor. 10ug of DNA was digested with 50U TspRI (NEB). Following reaction cleanup using the MinElute Cleanup kit (Qiagen), all DNA was loaded onto a 1.5% Agarose TAE gel. Fragments between 300bp and 500bp were extracted and purified using MinElute Gel Extraction kit (Qiagen). Fragmented DNA was split into 3 reactions and digested with 30U HpaII (NEB), 30U MspI (NEB), or no enzyme respectively. 25U of ExoIII (NEB) was added to all 3 reactions and reactions were incubated 1 hour at 37oC then immediately cleaned with MinElute Cleanup Kit. Each reaction was end repaired and then terminal A-residues were added using the NEBNext End Repair and the NEBNext dA-tailing modules as per manufacturer's protocols (NEB). Following reaction cleanup using the MinElute Cleanup kit, DNA fragments were then combined with 36uM adaptor mix and 400U T4 DNA ligase (NEB) and incubated for 1 hour at 16°C. Each sample was loaded onto a 1.5% Agarose TAE gel. Fragments between 400bp and 600bp were extracted and purified using MinElute Gel Extraction kit (Qiagen). PCR was performed using the following primers: 5' – CAA GCA GAA GAC GGC ATA CGA GCT CTT CCG ATC*T - 3' and 5' – AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC*T – 3' and Phusion High-Fidelity DNA Polymerase (NEB). PCR conditions were an initial denaturation (98°C 30s), 20 cycles of 98°C for 10s, 65°C for 30s and 72°C for 30s, with a final extension of 72°C for 7min. Following amplification, the PCR reaction was cleaned up using the MinElute PCR Purification Kit (Qiagen).

REFERENCES

Chu T, Burke B, Bunce K, Surti U, Hogge WA, Peters DG. A microarray-based approach for the identification of epigenetic biomarkers for the noninvasive diagnosis of fetal disease. Prenat Diagn. 2009;29(11):1020-30.

Table 1: Samples analyzed by microarray analysis for DNA methylation levels showing sample name, karyotype and maternal age. Samples analyzed in duplicate are indicated in the "Notes" column.

Uncultured				**Cultured**			
Sample Name	**Karyotype**	**Maternal Age**	**Notes**	**Sample Name**	**Karyotype**	**Maternal Age**	**Notes**
09-062	XX	39		CVS 32	XY +21	40	
09-071	XX	44		CVS 51	XX		
09-077	XY	41		CVS 59	XY		
09-106	XY	40		CVS 60-1	XY		
09-129	XY	46		CVS 65-1	XX +21	35	
09-172	XX	34		CVS 66-1	XX		
09-229	XX +21	43		CVS 69-1	XX +21	37	
09-314	XX +21	46		CVS 76-1	XY +21	43	
10-033	XY +21	42		CVS 60-2	XY		DUPLICATE
C-9	XY +21	40		CVS 65-2	XX +21	35	DUPLICATE
C-126	XY +21	41		CVS 66-2	XX		DUPLICATE
				CVS 69-2	XY +21	37	DUPLICATE
				CVS 76-2	XY +21	43	DUPLICATE

Table 2A: Top ten CpG sites more highly methylated in all trisomy 21 samples compared to all normal samples.

The following column headings apply to both Table 2 and Table 3
Norm.pval and t21.pval: p values that the probe is significantly methylated/unmethylated.
Norm.meth and t21.meth: average methylation rate for normal and t21 samples respectively
Diff: difference in average methylation rate between normal and t21 samples.

norm. pval	**T21. pval**	**norm. meth**	**t21. meth**	**Diff**	**Symbol**	**Product**
0.01	0.23	0.26	0.62	0.36	HOXA9	homeobox protein A9 isoform a
0.62	0.17	0.44	0.67	0.23	WT1	Wilms tumor 1 isoform C
0.05	0.17	0.38	0.59	0.21	HOXA9	homeobox protein A9 isoform a
0.49	0.62	0.41	0.57	0.16	VCY	variable charge; Y-linked
0.50	0.49	0.43	0.58	0.16	PRKAR1B	protein kinase; cAMP-dependent; regulatory; type I; beta
0.77	0.02	0.49	0.64	0.15	ANKRD30A	ankyrin repeat domain 30A
0.00	0.57	0.37	0.52	0.15	ARPP-21	cyclic AMP-regulated phosphoprotein; 21 kD isoform 2
0.64	0.02	0.48	0.63	0.15	HCA112	hepatocellular carcinoma-associated antigen 112
0.25	0.31	0.43	0.57	0.15	SRD5A2	3-oxo-5 alpha-steroid 4-dehydrogenase 2
0.03	0.71	0.38	0.52	0.14	SLC9A2	solute carrier family 9 isoform 2

Table 2B: Top ten CpG sites more highly methylated in all normal samples compared to all trisomy 21samples.

norm. pval	t21. pval	norm. meth	t21. meth	Diff	Symbol	Product
0.01	0.78	0.68	0.48	0.20	NAP1L5	nucleosome assembly protein 1-like 5
0.04	0.58	0.63	0.46	0.17	TMC1	transmembrane channel-like 1
0.00	0.22	0.61	0.46	0.15	GRIP1	glutamate receptor interacting protein 1 isoform 2
0.92	0.00	0.50	0.36	0.15	GRID2	glutamate receptor; ionotropic; delta 2
0.79	0.21	0.52	0.41	0.11	CD83	CD83 antigen (activated B lymphocytes; immunoglobulin superfamily)
0.43	0.22	0.54	0.43	0.11	SNRPA	small nuclear ribonucleoprotein polypeptide A
0.22	0.03	0.54	0.43	0.11	ZNF232	zinc finger protein 232
0.26	0.22	0.55	0.44	0.11	ITGA8	integrin; alpha 8
0.05	0.30	0.56	0.47	0.10	SCN9A	sodium channel; voltage-gated; type IX; alpha
0.17	0.91	0.59	0.49	0.09	KCNH5	potassium voltage-gated channel; subfamily H; member 5 isoform 1

Figure 1: Hierarchical clustering of all samples. Samples of normal karyotype are represented by "norm". Trisomy 21 samples are represented by "TR21". Samples designated by the prefix "CVS" are from CV-derived cultured samples. All other samples are from uncultured CV.

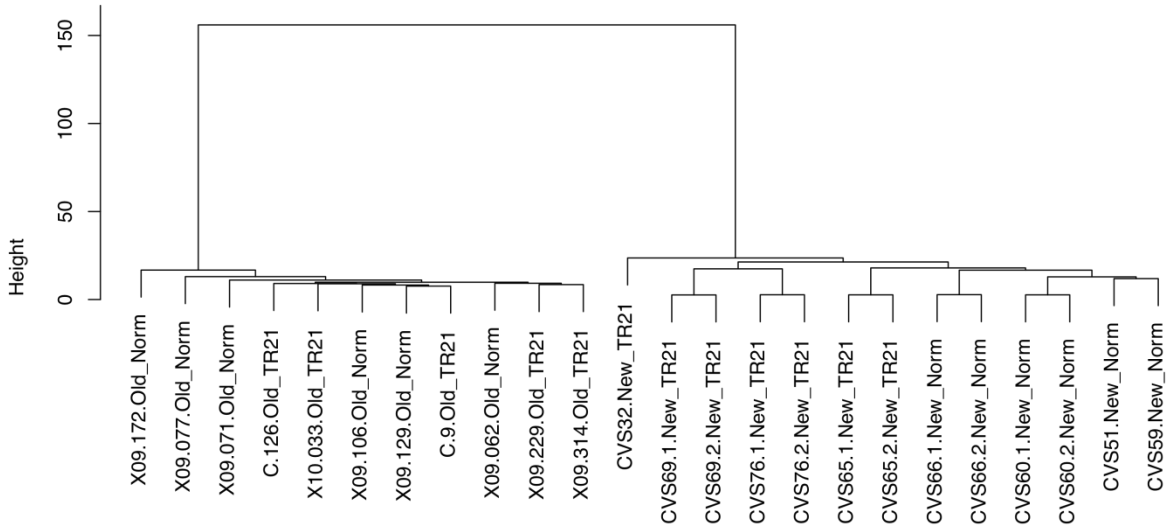

Table 3A: Top ten CpG sites more highly methylated in uncultured trisomy 21 CV samples compared to uncultured normal samples.

Norm. Pval	T21. pval	Norm. meth	T21. Meth	Diff	Symbol	Product
0.87	0.15	0.47	0.74	0.26	VCY	variable charge; Y-linked
0.86	0.00	0.49	0.74	0.25	CALN1	calneuron 1
0.02	0.00	0.40	0.65	0.25	CYP3A4	cytochrome P450; subfamily IIIA; polypeptide 4
0.09	0.05	0.40	0.63	0.24	FAM47B	hypothetical protein LOC170062
0.00	0.76	0.28	0.52	0.23	CRH	corticotropin releasing hormone precursor
0.03	0.54	0.33	0.55	0.22	HOXA9	homeobox protein A9 isoform a
0.01	0.27	0.35	0.56	0.22	ATP1B4	ATPase; (Na+)/K+ transporting; beta 4 polypeptide
0.00	0.02	0.38	0.59	0.21	PRPS1L1	phosphoribosyl pyrophosphate synthetase 1-like 1
0.00	0.73	0.31	0.52	0.21	GPR32	G protein-coupled receptor 32
0.00	0.23	0.35	0.55	0.20	PPM1G	protein phosphatase 1G

Table 3B: Top ten CpG sites more highly methylated in uncultured normal CV samples compared to uncultured trisomy 21 CV samples.

Norm. Pval	T21. pval	Norm. meth	T21. meth	Diff	Symbol	Product
0.05	0.16	0.67	0.37	0.30	NAP1L5	nucleosome assembly protein 1-like 5
0.11	0.32	0.61	0.42	0.19	HTRA4	HtrA serine peptidase 4
0.00	0.01	0.60	0.42	0.18	RPH3A	rabphilin 3A homolog
0.23	0.00	0.55	0.37	0.18	FLJ10916	hypothetical protein LOC55258
0.00	0.53	0.65	0.47	0.17	GRIP1	glutamate receptor interacting protein 1 isoform 2
0.92	0.06	0.51	0.35	0.16	TDG	thymine-DNA glycosylase isoform 2
0.20	0.32	0.58	0.43	0.15	GPR62	G protein-coupled receptor 62
0.22	0.17	0.56	0.42	0.14	SH3BP2	SH3-domain binding protein 2
0.83	0.02	0.51	0.37	0.14	ITGA8	integrin; alpha 8
0.02	0.29	0.59	0.46	0.13	SKIV2L	superkiller viralicidic activity 2-like homolog

Table 3C: Top ten CpG sites more highly methylated in cultured trisomy 21 CV-derived samples compared to cultured normal CV-derived samples.

Norm. Pval	T21. pval	Norm. meth	T21. meth	Diff	Symbol	Product
1.58E-05	0.01	0.24	0.65	0.41	SEC31L2	S. cerevisiae SEC31-like 2 isoform a
0.49	0.04	0.41	0.79	0.38	HOXA9	homeobox protein A9 isoform a
0.09	0.74	0.20	0.58	0.38	CYP2E1	cytochrome P450; family 2; subfamily E; polypeptide 1
0.01	0.31	0.24	0.62	0.37	NOL3	nucleolar protein 3
0.00	0.67	0.18	0.52	0.34	PNMA2	paraneoplastic antigen MA2
0.00	0.21	0.26	0.59	0.33	VIPR2	vasoactive intestinal peptide receptor 2
0.00	0.90	0.19	0.52	0.32	FAM3B	family with sequence similarity 3; member B isoform a
0.30	0.07	0.39	0.71	0.32	NKX6-2	NK6 transcription factor related; locus 2
0.00	0.22	0.27	0.58	0.32	HIST1H3E	H3 histone family; member D
0.00	0.35	0.27	0.58	0.31	HOXA9	homeobox protein A9 isoform a

Table 3D: Top ten CpG sites more highly methylated in cultured normal CV-derived samples compared to cultured trisomy CV-derived samples.

Norm. Pval	T21. pval	Norm. meth	T21. Meth	Diff	Symbol	Product
0.47	0.00	0.53	0.29	0.25	HTR3D	5-hydroxytryptamine receptor 3 subunit D
0.33	0.02	0.56	0.32	0.25	CSPG3	chondroitin sulfate proteoglycan 3 (neurocan)
0.01	0.28	0.66	0.43	0.22	C10orf39	hypothetical protein LOC282973
0.00	0.95	0.72	0.50	0.22	LILRB4	leukocyte immunoglobulin-like receptor; subfamily B (with TM and ITIM domains); member 4
0.23	0.57	0.64	0.42	0.22	TRPC5	transient receptor potential cation channel; subfamily C; member 5
0.96	0.00	0.50	0.29	0.21	BAK1	BCL2-antagonist/killer 1
0.66	0.00	0.52	0.31	0.21	LOC348645	hypothetical protein LOC348645
0.01	0.00	0.59	0.39	0.20	CEACAM3	carcinoembryonic antigen-related cell adhesion molecule 3
0.19	0.35	0.60	0.41	0.19	MAGEA1	melanoma antigen family A; 1
0.16	0.01	0.55	0.37	0.18	XCL2	chemokine (C motif) ligand 2

Table 4A. Humanmethylation27 Infinium microaray data. Top twenty differentially methylated CpG sites identified in comparison between uncultured trisomy 21 CV samples compared to uncultured normal samples (female fetus). “Mean Norm” and “Mean T21” are mean of the proportion of methylated CpGs at the specified locus (probe ID).

Probe ID	adjPvalue	Mean Norm	Mean T21	Gene
Cg00090147	0	0.298	0.615	GATA4
Cg00363813	0	0.193	0.515	HOM-TES-103
Cg01259619	0	0.022	0.337	ITPKB
Cg01942127	0	0.170	0.429	CACNA1D
Cg02919422	0	0.084	0.266	SOX17
Cg06509239	0	0.055	0.349	OSR1
Cg06821993	0	0.070	0.342	CSMD3
Cg08062469	0	0.042	0.105	SPAG5
Cg08145590	0	0.054	0.145	C3orf15
Cg09587549	0	0.085	0.402	PYCARD
Cg09874752	0	0.043	0.167	SFRP5
Cg10976172	0	0.263	0.582	FLJ32130
Cg14015044	0	0.034	0.215	TNFRSF10C
Cg15239123	0	0.060	0.366	SMOC1
Cg15755084	0	0.043	0.275	MSX1
Cg17049328	0	0.518	0.255	GHRH
Cg17665193	0	0.109	0.309	GAD2
Cg19011603	0	0.135	0.315	RP11-311P8.3
Cg19215261	0	0.790	0.938	SLC25A2
Cg20358834	0	0.052	0.403	LRFN4

Table 4B**.** Humanmethylation27 Infinium microaray data. Top twenty differentially methylated CpG sites identified in comparison between uncultured trisomy 21 CV samples compared to uncultured normal samples (male fetus). “Mean Norm” and “Mean T21” are mean of the proportion of methylated CpGs at the specified locus (probe ID).

Probe ID	adjPvalue	Mean Norm	Mean T21	SYMBOL
Cg01980637	0	0.035	0.492	PER1
Cg02566518	0	0.059	0.388	FUT11
cg06819200	0	0.014	0.336	ECE1
cg14617642	0	0.029	0.564	H2AFY2
cg17163751	0	0.034	0.426	H2AFY2
cg22836229	0	0.053	0.423	EFCAB1
cg24473633	0	0.273	0.028	DENND3
cg20322876	0.018	0.460	0.043	FLJ45803
cg16545079	0.020	0.036	0.215	PER1
cg13055278	0.024	0.057	0.226	ID2
cg20166532	0.024	0.193	0.041	EDG1
cg01366419	0.027	0.063	0.299	WBSCR17
cg21561173	0.028	0.069	0.383	C21orf81
cg01204439	0.029	0.287	0.063	TPK1
cg00958560	0.029	0.559	0.229	FLJ46481
cg25760229	0.029	0.061	0.260	CACNA1H
cg17830308	0.029	0.103	0.368	NEUROG1
cg20540428	0.029	0.176	0.491	PPP4R2
cg23367478	0.032	0.159	0.024	PDE3A
cg08731300	0.032	0.046	0.174	FZD8
cg27225570	0.032	0.059	0.225	DUSP15
cg04786857	0.032	0.032	0.165	SPDY1
cg20450764	0.044	0.125	0.349	TSPAN7
cg16509658	0.045	0.089	0.292	GABRB2
cg14221171	0.047	0.282	0.548	TAC1

Table 5A**.** Epityper data for female only uncultured fresh CVS samples. Probe ID numbers and individual CpG sites assayed are shown in the left hand column. Sentinel CpG sites, initially identified as significant in the microarray data are marked with an asterisk (*).

Probe ID and CpG#	Normal mean	T21 mean	pval. wilcoxon	adj.pval. wilcoxon	Gene
cg00090147_CpG_1	0.34	0.46	0.623	0.807	GATA4
cg00090147_CpG_2	0.39	0.38	1.000	1.000	
cg00090147_CpG_3.4	0.37	0.37	1.000	1.000	
cg00090147_CpG_5	0.17	0.19	0.684	0.830	
cg00090147_CpG_6.7	0.16	0.28	0.268	0.521	
cg00090147_CpG_8	0.32	0.44	0.290	0.542	
cg00090147_CpG_9	0.12	0.42	0.489	0.712	
cg00090147_CpG_11	0.15	0.20	0.371	0.627	
cg00090147_CpG_13	0.22	0.24	0.935	0.984	
cg00090147_CpG_15	0.29	0.11	0.667	0.830	
cg00090147_CpG_16	0.36	0.39	1.000	1.000	
cg00090147_CpG_17	0.39	0.37	0.876	0.943	
cg00090147_CpG_18	0.34	0.38	0.530	0.735	
cg00090147_CpG_19	0.34	0.38	0.530	0.735	
cg00090147_CpG_23.24	0.32	0.41	0.028	0.284	
cg00090147_CpG_25*	0.21	0.50	0.006	0.230	
cg00090147_CpG_26	0.38	0.40	0.806	0.903	
cg00090147_CpG_27	0.32	0.44	0.290	0.542	
cg00090147_CpG_28	0.38	0.40	0.806	0.903	
cg00090147_CpG_29.30	0.54	0.55	0.871	0.940	
cg00090147_CpG_31.32	0.37	0.37	1.000	1.000	
cg00090147_CpG_33.34	0.46	0.49	0.807	0.903	
cg00090147_CpG_35	0.31	0.28	0.935	0.984	
cg00090147_CpG_36	0.22	0.24	0.935	0.984	
cg00090147_CpG_37.38.39	0.24	0.44	0.023	0.284	
cg01354473_CpG_1	0.10	0.13	0.512	0.724	HOXA9
cg01354473_CpG_2	0.14	0.16	0.744	0.879	
cg01354473_CpG_3	0.13	0.15	0.369	0.627	
cg01354473_CpG_4	0.06	0.12	0.218	0.479	
cg01354473_CpG_5.6	0.13	0.15	0.369	0.627	
cg01354473_CpG_7	0.20	0.26	0.193	0.432	
cg01354473_CpG_8.9	0.22	0.22	0.566	0.761	
cg01354473_CpG_10	0.20	0.26	0.193	0.432	
cg01354473_CpG_17.18	0.24	0.32	0.432	0.652	
cg01354473_CpG_20	0.23	0.25	0.567	0.761	
cg01354473_CpG_21	0.10	0.16	0.165	0.388	
cg01354473_CpG_22.23.24.25.26	0.51	0.50	1.000	1.000	
cg01354473_CpG_28*	0.13	0.27	0.074	0.307	
cg01354473_CpG_29.30	0.31	0.40	0.343	0.598	
cg01354473_CpG_31	0.16	0.22	0.415	0.652	
cg01354473_CpG_32.33	0.33	0.42	0.515	0.724	

cg01354473_CpG_34.35.36.37	0.45	0.51	0.684	0.830	
cg01354473_CpG_38.39	0.34	0.47	0.268	0.521	
cg01354473_CpG_40	0.13	0.27	0.074	0.307	
cg01354473_CpG_41	0.22	0.37	0.061	0.306	
cg01354473_CpG_42.43.44.45.46	0.38	0.49	0.254	0.506	
cg02919422_CpG_1.2	0.51	0.54	0.755	0.879	SOX17
cg02919422_CpG_4.5	0.09	0.14	0.097	0.327	
cg02919422_CpG_6.7	0.17	0.21	0.807	0.903	
cg02919422_CpG_8	0.06	0.13	0.252	0.506	
cg02919422_CpG_9.10.11	0.15	0.20	0.122	0.347	
cg02919422_CpG_12	0.10	0.13	0.684	0.830	
cg02919422_CpG_13	0.07	0.17	0.122	0.347	
cg02919422_CpG_17.18	0.14	0.18	1.000	1.000	
cg02919422_CpG_19	0.03	0.03	0.868	0.940	
cg02919422_CpG_20	0.09	0.14	0.097	0.327	
cg02919422_CpG_21.22	0.08	0.11	0.119	0.347	
cg02919422_CpG_24.25	0.03	0.03	0.868	0.940	
cg02919422_CpG_30.31	0.22	0.29	0.530	0.735	
cg02919422_CpG_32.33	0.33	0.38	0.432	0.652	
cg02919422_CpG_34	0.08	0.09	0.566	0.761	
cg02919422_CpG_35	0.17	0.19	0.568	0.761	
cg02919422_CpG_36.37	0.09	0.14	0.097	0.327	
cg02919422_CpG_43.44	0.19	0.28	0.166	0.388	
cg02919422_CpG_45	0.10	0.16	0.415	0.652	
cg04032226_CpG_1	0.11	0.23	0.087	0.327	NAGS
cg04032226_CpG_2.3	0.25	0.39	0.073	0.307	
cg04032226_CpG_5.6	0.23	0.39	0.030	0.284	
cg04032226_CpG_7.8	0.14	0.31	0.030	0.284	
cg04032226_CpG_10	0.33	0.52	0.074	0.307	
cg04032226_CpG_11.12.13	0.13	0.28	0.061	0.306	
cg04032226_CpG_16	0.13	0.27	0.034	0.284	
cg04032226_CpG_17.18.19	0.21	0.37	0.051	0.284	
cg04032226_CpG_20	0.33	0.52	0.074	0.307	
cg04032226_CpG_21	0.11	0.31	0.072	0.307	
cg04032226_CpG_22	0.11	0.28	0.042	0.284	
cg04032226_CpG_23.24.25	0.21	0.39	0.073	0.307	
cg04032226_CpG_26	0.16	0.35	0.048	0.284	
cg04032226_CpG_27	0.16	0.38	0.073	0.307	
cg04032226_CpG_28	0.19	0.38	0.048	0.284	
cg04032226_CpG_29	0.11	0.20	0.222	0.479	
cg04032226_CpG_30*	0.33	0.53	0.051	0.284	
cg04032226_CpG_31	0.26	0.38	0.166	0.388	
cg04032226_CpG_32	0.25	0.37	0.167	0.388	
cg04032226_CpG_33	0.23	0.39	0.030	0.284	
cg04032226_CpG_34	0.22	0.39	0.051	0.284	
cg04032226_CpG_35	0.55	0.64	0.122	0.347	

cg04032226_CpG_36	0.12	0.20	0.103	0.327	
cg06303238_CpG_1	0.01	0.00	0.748	0.879	SALL4
cg06303238_CpG_2*	0.05	0.04	0.210	0.466	
cg06303238_CpG_3	0.06	0.10	0.328	0.582	
cg06303238_CpG_4.5.6.7	0.28	0.29	0.871	0.940	
cg06303238_CpG_8.9	0.16	0.20	0.408	0.652	
cg06303238_CpG_10.11	0.07	0.08	0.621	0.807	
cg06303238_CpG_12.13	0.03	0.06	0.460	0.679	
cg06303238_CpG_30	0.04	0.13	0.253	0.506	
cg06303238_CpG_31.32.33	0.13	0.16	0.683	0.830	
cg06303238_CpG_34	0.07	0.11	0.744	0.879	
cg06303238_CpG_35	0.36	0.47	0.106	0.331	
cg07922606r_CpG_1	0.08	0.28	0.009	0.235	HIST1H3E
cg07922606r_CpG_2	0.08	0.28	0.009	0.235	
cg07922606r_CpG_3	0.05	0.19	0.041	0.284	
cg07922606r_CpG_5	0.16	0.29	0.048	0.284	
cg07922606r_CpG_6.7	0.21	0.29	0.149	0.378	
cg07922606r_CpG_8*.9	0.20	0.35	0.061	0.306	
cg07922606r_CpG_10	0.06	0.20	0.085	0.327	
cg07922606r_CpG_11.12	0.23	0.35	0.103	0.327	
cg07922606r_CpG_13	0.23	0.35	0.103	0.327	
cg07922606r_CpG_14.15	0.24	0.35	0.370	0.627	
cg07922606r_CpG_16.17.18	0.27	0.35	0.432	0.652	
cg07922606r_CpG_19	0.19	0.42	0.015	0.284	
cg07922606r_CpG_20	0.10	0.24	0.061	0.306	
cg07922606r_CpG_21.22.23	0.29	0.34	0.755	0.879	
cg07922606r_CpG_24.25	0.18	0.32	0.122	0.347	
cg07922606r_CpG_27	0.08	0.28	0.009	0.235	
cg07922606r_CpG_28	0.09	0.23	0.050	0.284	
cg07922606r_CpG_29.30	0.25	0.37	0.255	0.506	
cg07922606r_CpG_32	0.39	0.44	0.624	0.807	
cg07922606r_CpG_33.34	0.15	0.26	0.221	0.479	
cg07922606r_CpG_35	0.17	0.31	0.073	0.307	
cg07922606r_CpG_36	0.21	0.29	0.149	0.378	
cg08032971_CpG_2	0.72	0.69	0.684	0.830	DMBX1
cg08032971_CpG_3	0.78	0.84	0.343	0.598	
cg08032971_CpG_4	0.39	0.47	0.432	0.652	
cg08032971_CpG_5	0.65	0.77	0.122	0.347	
cg08032971_CpG_6	0.58	0.61	0.639	0.817	
cg08032971_CpG_7	0.65	0.77	0.122	0.347	
cg08032971_CpG_8*	0.36	0.45	0.104	0.327	
cg08032971_CpG_9	0.43	0.47	0.414	0.652	
cg08032971_CpG_10	0.78	0.84	0.343	0.598	
cg08032971_CpG_11	0.83	0.87	0.514	0.724	

cg08062469_CpG_1	0.01	0.04	0.025	0.284	SPAG5
cg08062469_CpG_2	0.06	0.11	0.022	0.284	
cg08062469_CpG_3*	0.06	0.09	0.322	0.582	
cg08062469_CpG_4	0.05	0.10	0.026	0.284	
cg08062469_CpG_5	0.03	0.05	0.155	0.387	
cg08062469_CpG_6	0.05	0.10	0.026	0.284	
cg08062469_CpG_8	0.03	0.05	0.155	0.387	
cg08062469_CpG_9	0.05	0.10	0.026	0.284	
cg08062469_CpG_10	0.09	0.15	0.034	0.284	
cg08062469_CpG_13	0.11	0.23	0.006	0.230	
cg08062469_CpG_14	0.09	0.15	0.034	0.284	
cg08062469_CpG_15.16	0.10	0.16	0.081	0.327	
cg10976172_CpG_1.2	0.57	0.66	0.416	0.652	FLJ32130
cg10976172_CpG_3	0.34	0.44	0.255	0.506	
cg10976172_CpG_4	0.26	0.44	0.088	0.327	
cg10976172_CpG_5	0.45	0.51	0.682	0.830	
cg10976172_CpG_6.7	0.46	0.58	0.149	0.378	
cg10976172_CpG_8	0.21	0.40	0.051	0.284	
cg10976172_CpG_9	0.23	0.37	0.104	0.327	
cg10976172_CpG_10	0.27	0.43	0.074	0.307	
cg10976172_CpG_11	0.34	0.44	0.255	0.506	
cg10976172_CpG_12	0.30	0.39	0.290	0.542	
cg10976172_CpG_13.14.15	0.46	0.58	0.149	0.378	
cg10976172_CpG_16.17.18	0.47	0.61	0.088	0.327	
cg10976172_CpG_19	0.33	0.44	0.167	0.388	
cg10976172_CpG_20	0.30	0.41	0.221	0.479	
cg10976172_CpG_21	0.30	0.39	0.290	0.542	
cg10976172_CpG_22	0.45	0.51	0.682	0.830	
cg10976172_CpG_23.24	0.68	0.72	0.416	0.652	
cg10976172_CpG_25	0.40	0.48	0.463	0.679	
cg10976172_CpG_26	0.53	0.62	0.432	0.652	
cg11830061_CpG_3.4.5	0.94	0.94	0.622	0.807	INSL6
cg11830061_CpG_6	0.68	0.75	0.254	0.506	
cg11830061_CpG_7	0.54	0.67	0.061	0.306	
cg11830061_CpG_8*	0.29	0.38	0.268	0.521	
cg11830061_CpG_9	0.72	0.77	0.744	0.879	
cg11830061_CpG_11	0.68	0.75	0.254	0.506	
cg11830061_CpG_12	0.55	0.64	0.142	0.378	
cg11830061_CpG_13	0.55	0.64	0.142	0.378	
cg11830061_CpG_17.18	0.12	0.15	0.432	0.652	
cg11830061_CpG_19.20	0.26	0.37	0.143	0.378	
cg11830061_CpG_21.22.23	0.16	0.22	0.415	0.652	
cg11830061_CpG_26	0.10	0.18	0.088	0.327	
cg11830061_CpG_27.28.29	0.26	0.36	0.103	0.327	
cg11830061_CpG_30	0.17	0.31	0.037	0.284	
cg11830061_CpG_31	0.18	0.30	0.042	0.284	
cg11830061_CpG_32.33.34.3	0.28	0.37	0.149	0.378	

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cg11830061_CpG_36.37.38.39.40	0.32	0.45	0.049	0.284	
cg11830061_CpG_41	0.18	0.27	0.415	0.652	
cg15239123_CpG_1	0.09	0.19	0.005	0.230	SMOC1
cg15239123_CpG_2	0.02	0.13	0.035	0.284	
cg15239123_CpG_8	0.16	0.12	0.460	0.679	
cg15239123_CpG_9	0.00	0.00	NA	NA	
cg15239123_CpG_10	0.06	0.10	0.191	0.432	
cg15239123_CpG_14	0.16	0.12	0.460	0.679	
cg15239123_CpG_15.16.17.18	0.09	0.19	0.005	0.230	
cg15239123_CpG_19.20.21	0.06	0.12	0.008	0.235	
cg15239123_CpG_22.23	0.12	0.13	0.883	0.946	
cg15239123_CpG_24*	0.08	0.13	0.143	0.378	
cg15239123_CpG_25	0.03	0.05	0.160	0.388	
cg15239123_CpG_26.27	0.07	0.09	0.415	0.652	
cg15239123_CpG_34	0.06	0.06	1.000	1.000	
cg15239123_CpG_36.37.38.39.40	0.30	0.45	0.255	0.506	
cg15239123_CpG_41.42	0.06	0.10	0.191	0.432	
cg15239123_CpG_51	0.09	0.18	0.027	0.284	
cg15755084_CpG_1.2	0.03	0.02	0.765	0.887	MSX1
cg15755084_CpG_3	0.00	0.01	0.561	0.761	
cg15755084_CpG_4.5	0.02	0.02	0.921	0.983	
cg15755084_CpG_6*	0.03	0.11	0.005	0.230	
cg15755084_CpG_7.8.9	0.06	0.08	0.807	0.903	
cg15755084_CpG_11	0.09	0.11	1.000	1.000	
cg15755084_CpG_12.13	0.06	0.13	0.005	0.230	
cg15755084_CpG_14	0.01	0.01	0.246	0.506	
cg15755084_CpG_15.16.17.18	0.25	0.24	0.566	0.761	
cg15755084_CpG_19.20	0.11	0.16	0.051	0.284	
cg15755084_CpG_21.22.23	0.04	0.09	0.142	0.378	
cg15755084_CpG_24.25.26	0.05	0.12	0.163	0.388	
cg15755084_CpG_27.28	0.03	0.04	0.560	0.761	
cg15755084_CpG_29.30	0.13	0.15	0.620	0.807	
cg15755084_CpG_31	0.03	0.04	0.508	0.724	
cg15755084_CpG_32	0.04	0.13	0.021	0.284	
cg15755084_CpG_33	0.07	0.08	0.287	0.542	
cg15755084_CpG_34	0.05	0.06	0.463	0.679	
cg15755084_CpG_35.36	0.17	0.19	0.639	0.817	
cg15755084_CpG_37.38	0.24	0.24	0.289	0.542	
cg15755084_CpG_39.40	0.12	0.17	0.143	0.378	
cg15755084_CpG_41.42.43.44	0.11	0.16	0.051	0.284	
cg15755084_CpG_45	0.09	0.11	1.000	1.000	
cg15755084_CpG_46	0.02	0.07	0.044	0.284	

cg15755084_CpG_47.48	0.10	0.12	0.683	0.830	
cg18230216_CpG_1	0.27	0.34	0.327	0.582	NYX
cg18230216_CpG_2*	0.36	0.37	0.413	0.652	
cg18230216_CpG_3	0.47	0.49	0.755	0.879	
cg18230216_CpG_4	0.33	0.39	0.744	0.879	
cg20723355_CpG_1	0.13	0.20	0.149	0.378	FBX039
cg20723355_CpG_2*	0.11	0.20	0.102	0.327	
cg20723355_CpG_3	0.17	0.17	0.807	0.903	
cg20723355_CpG_4.5	0.31	0.32	0.935	0.984	
cg20723355_CpG_6.7	0.46	0.51	0.755	0.879	
cg20723355_CpG_8	0.26	0.30	0.432	0.652	
cg20723355_CpG_9.10	0.45	0.42	0.871	0.940	
cg20723355_CpG_11.12	0.47	0.41	1.000	1.000	
cg20723355_CpG_13.14.15	0.59	0.54	1.000	1.000	
cg20723355_CpG_16.17	0.41	0.33	0.624	0.807	
cg20723355_CpG_18	0.12	0.15	0.515	0.724	
cg20723355_CpG_19	0.11	0.20	0.102	0.327	
cg20723355_CpG_21	0.26	0.30	0.432	0.652	
cg20723355_CpG_22	0.02	0.09	0.797	0.903	
cg20723355_CpG_23.24	0.51	0.44	0.870	0.940	
cg20723355_CpG_25	0.59	0.47	0.515	0.724	
cg20723355_CpG_26	0.65	0.72	0.684	0.830	
cg20723355_CpG_27.28.29	0.70	0.62	0.370	0.627	
cg20723355_CpG_30	0.37	0.37	0.807	0.903	
cg21296230_CpG_1*.2	0.44	0.46	0.683	0.830	GREM1
cg21296230_CpG_3	0.30	0.34	0.871	0.940	
cg21296230_CpG_4	0.15	0.14	0.744	0.879	
cg21296230_CpG_6	0.02	0.04	0.072	0.307	
cg21296230_CpG_7	0.02	0.05	0.059	0.306	
cg21296230_CpG_8	0.19	0.18	1.000	1.000	
cg21296230_CpG_9	0.26	0.32	0.464	0.679	
cg21296230_CpG_10	0.09	0.13	0.166	0.388	
cg21296230_CpG_11	0.29	0.37	0.192	0.432	
cg21296230_CpG_12.13.14	0.50	0.55	0.415	0.652	
cg21296230_CpG_16.17	0.40	0.45	0.414	0.652	
cg21296230_CpG_18	0.17	0.28	0.051	0.284	
cg21296230_CpG_19	0.29	0.38	0.103	0.327	
cg21296230_CpG_20	0.30	0.34	0.871	0.940	
cg21296230_CpG_21.22	0.19	0.23	0.624	0.807	
cg21296230_CpG_23.24.25	0.17	0.17	1.000	1.000	
cg21296230_CpG_31.32	0.31	0.35	0.806	0.903	
cg21296230_CpG_33	0.30	0.34	0.871	0.940	
cg26186727_CpG_1.2	0.31	0.37	0.328	0.582	NETO1
cg26186727_CpG_3*	0.16	0.21	0.103	0.327	
cg26186727_CpG_4	0.33	0.41	0.515	0.724	

cg26186727_CpG_5	0.31	0.42	0.684	0.830
cg26186727_CpG_6	0.14	0.08	0.327	0.582
cg26186727_CpG_7	0.05	0.11	0.049	0.284
cg26186727_CpG_9	0.18	0.29	0.034	0.284
cg26186727_CpG_11	0.22	0.27	0.325	0.582
cg26186727_CpG_12	0.22	0.27	0.325	0.582
cg26186727_CpG_13	0.12	0.18	0.087	0.327
cg26186727_CpG_20.21.22	0.14	0.28	0.122	0.347
cg26186727_CpG_23	0.12	0.18	0.087	0.327
cg26186727_CpG_24.25	0.12	0.20	0.051	0.284
cg26186727_CpG_26.27.28.29	0.33	0.42	0.103	0.327
cg26186727_CpG_30.31.32.33.34	0.48	0.54	0.328	0.582
cg26186727_CpG_35	0.19	0.22	1.000	1.000
cg26186727_CpG_42.43.44.45.46	0.35	0.48	0.005	0.230
cg26186727_CpG_47.48.49	0.23	0.34	0.018	0.284
cg26186727_CpG_50	0.07	0.11	0.253	0.506
cg26186727_CpG_51	0.11	0.14	0.625	0.807
cg26186727_CpG_52.53	0.12	0.20	0.051	0.284
cg26186727_CpG_54.55.56	0.22	0.29	0.122	0.347
cg26186727_CpG_57	0.05	0.11	0.049	0.284

Table 5B. Epityper data for male and female uncultured fresh CVS samples. Probe ID numbers and individual CpG sites assayed are shown in the left hand column. Sentinel CpG sites, initially identified as significant in the microarray data are marked with an asterisk (*).

Probe ID/CpG#	Normal mean	T21. mean	pval. wilcoxon	adj.pval. wilcoxon	Gene
cg02919422_CpG_1.2	0.49	0.53	0.345	0.502	SOX17
cg02919422_CpG_4.5	0.08	0.14	0.033	0.218	
cg02919422_CpG_6.7	0.14	0.21	0.288	0.460	
cg02919422_CpG_8	0.06	0.13	0.211	0.412	
cg02919422_CpG_9.10.11	0.15	0.21	0.120	0.326	
cg02919422_CpG_12	0.08	0.11	0.255	0.432	
cg02919422_CpG_13	0.07	0.14	0.255	0.432	
cg02919422_CpG_17.18	0.13	0.16	0.622	0.770	
cg02919422_CpG_19	0.02	0.03	0.463	0.611	
cg02919422_CpG_20	0.08	0.14	0.033	0.218	
cg02919422_CpG_21.22	0.08	0.11	0.100	0.301	
cg02919422_CpG_24.25	0.02	0.03	0.463	0.611	
cg02919422_CpG_30.31	0.21	0.30	0.307	0.474	
cg02919422_CpG_32.33	0.29	0.39	0.290	0.460	
cg02919422_CpG_34	0.08	0.12	0.184	0.384	
cg02919422_CpG_35	0.15	0.25	0.075	0.271	
cg02919422_CpG_36.37	0.08	0.14	0.033	0.218	
cg02919422_CpG_43.44	0.19	0.26	0.161	0.367	
cg02919422_CpG_45	0.10	0.16	0.184	0.384	
cg07922606r_CpG_1	0.10	0.22	0.140	0.340	HIST1H3E
cg07922606r_CpG_2	0.10	0.22	0.140	0.340	
cg07922606r_CpG_3	0.07	0.15	0.160	0.367	
cg07922606r_CpG_5	0.16	0.26	0.130	0.327	
cg07922606r_CpG_6.7	0.22	0.27	0.307	0.474	
cg07922606r_CpG_8*.9	0.21	0.36	0.049	0.228	
cg07922606r_CpG_10	0.09	0.17	0.324	0.486	
cg07922606r_CpG_11.12	0.22	0.31	0.185	0.384	
cg07922606r_CpG_13	0.22	0.31	0.185	0.384	
cg07922606r_CpG_14.15	0.24	0.31	0.705	0.844	
cg07922606r_CpG_16.17.18	0.29	0.31	0.910	0.957	
cg07922606r_CpG_19	0.24	0.35	0.130	0.327	
cg07922606r_CpG_20	0.12	0.20	0.241	0.432	
cg07922606r_CpG_21.22.23	0.31	0.32	0.880	0.946	
cg07922606r_CpG_24.25	0.20	0.36	0.069	0.270	
cg07922606r_CpG_27	0.10	0.22	0.140	0.340	
cg07922606r_CpG_28	0.09	0.20	0.196	0.391	
cg07922606r_CpG_29.30	0.26	0.35	0.272	0.440	
cg07922606r_CpG_32	0.39	0.40	0.733	0.857	
cg07922606r_CpG_33.34	0.15	0.22	0.427	0.586	
cg07922606r_CpG_35	0.18	0.26	0.241	0.432	
cg07922606r_CpG_36	0.22	0.27	0.307	0.474	

cg21296230_CpG_1*.2	0.43	0.52	0.185	0.384	GREM1
cg21296230_CpG_3	0.30	0.39	0.198	0.391	
cg21296230_CpG_4	0.15	0.17	1.000	1.000	
cg21296230_CpG_6	0.02	0.04	0.009	0.206	
cg21296230_CpG_7	0.03	0.04	0.108	0.310	
cg21296230_CpG_8	0.17	0.27	0.405	0.564	
cg21296230_CpG_9	0.24	0.37	0.075	0.271	
cg21296230_CpG_10	0.09	0.17	0.021	0.206	
cg21296230_CpG_11	0.28	0.42	0.010	0.206	
cg21296230_CpG_12.13.14	0.48	0.58	0.096	0.299	
cg21296230_CpG_16.17	0.41	0.48	0.272	0.440	
cg21296230_CpG_18	0.18	0.28	0.011	0.206	
cg21296230_CpG_19	0.30	0.43	0.045	0.220	
cg21296230_CpG_20	0.30	0.39	0.198	0.391	
cg21296230_CpG_21.22	0.19	0.30	0.130	0.327	
cg21296230_CpG_23.24.25	0.16	0.23	0.082	0.283	
cg21296230_CpG_31.32	0.29	0.43	0.074	0.271	
cg21296230_CpG_33	0.30	0.39	0.198	0.391	
cg11830061_CpG_3.4.5	0.95	0.94	0.483	0.629	INSL6
cg11830061_CpG_6	0.68	0.76	0.037	0.218	
cg11830061_CpG_7	0.53	0.64	0.037	0.218	
cg11830061_CpG_8*	0.33	0.36	0.487	0.629	
cg11830061_CpG_9	0.70	0.77	0.269	0.440	
cg11830061_CpG_11	0.68	0.76	0.037	0.218	
cg11830061_CpG_12	0.53	0.61	0.045	0.220	
cg11830061_CpG_13	0.53	0.61	0.045	0.220	
cg11830061_CpG_17.18	0.11	0.12	0.705	0.844	
cg11830061_CpG_19.20	0.26	0.34	0.079	0.276	
cg11830061_CpG_21.22.23	0.16	0.21	0.252	0.432	
cg11830061_CpG_26	0.10	0.15	0.109	0.310	
cg11830061_CpG_27.28.29	0.26	0.35	0.033	0.218	
cg11830061_CpG_30	0.17	0.22	0.252	0.432	
cg11830061_CpG_31	0.19	0.30	0.088	0.290	
cg11830061_CpG_32.33.34.35	0.28	0.34	0.152	0.353	
cg11830061_CpG_36.37.38.39.40	0.31	0.38	0.219	0.421	
cg11830061_CpG_41	0.18	0.23	0.437	0.593	
cg08032971_CpG_2	0.72	0.72	0.761	0.873	DMBX1
cg08032971_CpG_3	0.79	0.85	0.095	0.299	
cg08032971_CpG_4	0.41	0.44	0.449	0.605	
cg08032971_CpG_5	0.66	0.79	0.021	0.206	
cg08032971_CpG_6	0.59	0.66	0.212	0.413	
cg08032971_CpG_7	0.66	0.79	0.021	0.206	
cg08032971_CpG_8*	0.37	0.54	0.004	0.182	
cg08032971_CpG_9	0.46	0.53	0.256	0.432	
cg08032971_CpG_10	0.79	0.85	0.095	0.299	

cg08032971_CpG_11	0.84	0.91	0.057	0.259	
cg04032226_CpG_1	0.10	0.19	0.225	0.429	NAGS
cg04032226_CpG_2.3	0.25	0.42	0.041	0.220	
cg04032226_CpG_5.6	0.23	0.32	0.089	0.290	
cg04032226_CpG_7.8	0.16	0.24	0.150	0.353	
cg04032226_CpG_10	0.35	0.55	0.017	0.206	
cg04032226_CpG_11.12.13	0.13	0.31	0.019	0.206	
cg04032226_CpG_16	0.13	0.26	0.103	0.301	
cg04032226_CpG_17.18.19	0.22	0.42	0.019	0.206	
cg04032226_CpG_20	0.35	0.55	0.017	0.206	
cg04032226_CpG_21	0.12	0.34	0.019	0.206	
cg04032226_CpG_22	0.11	0.32	0.012	0.206	
cg04032226_CpG_23.24.25	0.20	0.34	0.055	0.250	
cg04032226_CpG_26	0.16	0.28	0.064	0.265	
cg04032226_CpG_27	0.18	0.30	0.165	0.373	
cg04032226_CpG_28	0.20	0.32	0.104	0.301	
cg04032226_CpG_29	0.12	0.18	0.405	0.564	
cg04032226_CpG_30*	0.33	0.55	0.009	0.206	
cg04032226_CpG_31	0.25	0.34	0.130	0.327	
cg04032226_CpG_32	0.25	0.32	0.173	0.379	
cg04032226_CpG_33	0.23	0.32	0.089	0.290	
cg04032226_CpG_34	0.25	0.37	0.112	0.312	
cg04032226_CpG_35	0.54	0.66	0.041	0.220	
cg04032226_CpG_36	0.14	0.18	0.256	0.432	
cg26186727_CpG_1.2	0.32	0.32	0.820	0.913	NETO1
cg26186727_CpG_3*	0.17	0.17	0.570	0.719	
cg26186727_CpG_4	0.32	0.34	0.677	0.825	
cg26186727_CpG_5	0.30	0.41	0.177	0.384	
cg26186727_CpG_6	0.12	0.07	0.323	0.486	
cg26186727_CpG_7	0.06	0.10	0.037	0.218	
cg26186727_CpG_9	0.20	0.24	0.345	0.502	
cg26186727_CpG_11	0.20	0.24	0.271	0.440	
cg26186727_CpG_12	0.20	0.24	0.271	0.440	
cg26186727_CpG_13	0.12	0.16	0.088	0.290	
cg26186727_CpG_20.21.22	0.14	0.26	0.031	0.218	
cg26186727_CpG_23	0.12	0.16	0.088	0.290	
cg26186727_CpG_24.25	0.11	0.18	0.041	0.220	
cg26186727_CpG_26.27.28.29	0.32	0.39	0.096	0.299	
cg26186727_CpG_30.31.32.33.34	0.46	0.52	0.240	0.432	
cg26186727_CpG_35	0.19	0.21	0.849	0.928	
cg26186727_CpG_42.43.44.45.46	0.35	0.52	0.002	0.182	
cg26186727_CpG_47.48.49	0.22	0.33	0.011	0.206	
cg26186727_CpG_50	0.06	0.10	0.129	0.327	
cg26186727_CpG_51	0.10	0.15	0.240	0.432	
cg26186727_CpG_52.53	0.11	0.18	0.041	0.220	

cg26186727_CpG_54.55.56	0.20	0.29	0.031	0.218	
cg26186727_CpG_57	0.06	0.10	0.037	0.218	
cg08062469_CpG_1	0.01	0.03	0.009	0.206	SPAG5
cg08062469_CpG_2	0.05	0.09	0.012	0.206	
cg08062469_CpG_3*	0.05	0.08	0.137	0.340	
cg08062469_CpG_4	0.05	0.08	0.067	0.265	
cg08062469_CpG_5	0.03	0.05	0.098	0.300	
cg08062469_CpG_6	0.05	0.08	0.067	0.265	
cg08062469_CpG_8	0.03	0.05	0.098	0.300	
cg08062469_CpG_9	0.05	0.08	0.067	0.265	
cg08062469_CpG_10	0.07	0.15	0.004	0.182	
cg08062469_CpG_13	0.11	0.20	0.004	0.182	
cg08062469_CpG_14	0.07	0.15	0.004	0.182	
cg08062469_CpG_15.16	0.10	0.14	0.044	0.220	
cg01354473_CpG_1	0.12	0.14	0.774	0.883	HOXA9
cg01354473_CpG_2	0.14	0.16	0.368	0.531	
cg01354473_CpG_3	0.12	0.14	0.251	0.432	
cg01354473_CpG_4	0.08	0.10	0.386	0.554	
cg01354473_CpG_5.6	0.12	0.14	0.251	0.432	
cg01354473_CpG_7	0.21	0.24	0.806	0.905	
cg01354473_CpG_8.9	0.21	0.22	0.459	0.611	
cg01354473_CpG_10	0.21	0.24	0.806	0.905	
cg01354473_CpG_17.18	0.24	0.31	0.462	0.611	
cg01354473_CpG_20	0.21	0.21	0.967	1.000	
cg01354473_CpG_21	0.09	0.13	0.129	0.327	
cg01354473_CpG_22.23.24.25.26	0.50	0.53	0.487	0.629	
cg01354473_CpG_28*	0.13	0.22	0.066	0.265	
cg01354473_CpG_29.30	0.30	0.43	0.034	0.218	
cg01354473_CpG_31	0.16	0.21	0.307	0.474	
cg01354473_CpG_32.33	0.33	0.44	0.236	0.432	
cg01354473_CpG_34.35.36.37	0.43	0.51	0.288	0.460	
cg01354473_CpG_38.39	0.33	0.43	0.270	0.440	
cg01354473_CpG_40	0.13	0.22	0.066	0.265	
cg01354473_CpG_41	0.22	0.32	0.060	0.265	
cg01354473_CpG_42.43.44.45.46	0.38	0.47	0.218	0.421	
cg18230216_CpG_1	0.33	0.36	0.427	0.586	NYX
cg18230216_CpG_2*	0.36	0.42	0.909	0.957	
cg18230216_CpG_3	0.48	0.45	0.970	1.000	
cg18230216_CpG_4	0.30	0.39	0.161	0.367	
cg00090147_CpG_1	0.36	0.44	0.487	0.629	GATA4
cg00090147_CpG_2	0.39	0.38	1.000	1.000	
cg00090147_CpG_3.4	0.38	0.38	1.000	1.000	
cg00090147_CpG_5	0.21	0.23	0.437	0.593	

cg00090147_CpG_6.7	0.20	0.31	0.141	0.340	
cg00090147_CpG_8	0.33	0.40	0.253	0.432	
cg00090147_CpG_9	0.16	0.25	0.845	0.926	
cg00090147_CpG_11	0.18	0.16	1.000	1.000	
cg00090147_CpG_13	0.24	0.23	0.870	0.943	
cg00090147_CpG_15	0.29	0.11	0.667	0.820	
cg00090147_CpG_16	0.36	0.35	0.713	0.844	
cg00090147_CpG_17	0.42	0.34	0.487	0.629	
cg00090147_CpG_18	0.38	0.37	1.000	1.000	
cg00090147_CpG_19	0.38	0.37	1.000	1.000	
cg00090147_CpG_23.24	0.36	0.39	0.190	0.386	
cg00090147_CpG_25*	0.23	0.44	0.002	0.182	
cg00090147_CpG_26	0.39	0.37	0.712	0.844	
cg00090147_CpG_27	0.33	0.40	0.253	0.432	
cg00090147_CpG_28	0.39	0.37	0.712	0.844	
cg00090147_CpG_29.30	0.54	0.53	0.935	0.980	
cg00090147_CpG_31.32	0.38	0.38	1.000	1.000	
cg00090147_CpG_33.34	0.46	0.44	0.837	0.922	
cg00090147_CpG_35	0.33	0.29	1.000	1.000	
cg00090147_CpG_36	0.24	0.23	0.870	0.943	
cg00090147_CpG_37.38.39	0.28	0.38	0.079	0.276	
cg06303238_CpG_1	0.01	0.02	1.000	1.000	SALL4
cg06303238_CpG_2*	0.05	0.04	0.036	0.218	
cg06303238_CpG_3	0.06	0.08	0.253	0.432	
cg06303238_CpG_4.5.6.7	0.25	0.24	0.909	0.957	
cg06303238_CpG_8.9	0.15	0.19	0.118	0.325	
cg06303238_CpG_10.11	0.06	0.06	0.819	0.913	
cg06303238_CpG_12.13	0.03	0.05	0.357	0.517	
cg06303238_CpG_30	0.04	0.10	0.103	0.301	
cg06303238_CpG_31.32.33	0.12	0.16	0.879	0.946	
cg06303238_CpG_34	0.07	0.10	0.762	0.873	
cg06303238_CpG_35	0.34	0.45	0.034	0.218	
cg10976172_CpG_1.2	0.59	0.67	0.307	0.474	FLJ32130
cg10976172_CpG_3	0.36	0.46	0.272	0.440	
cg10976172_CpG_4	0.29	0.45	0.120	0.326	
cg10976172_CpG_5	0.47	0.53	0.404	0.564	
cg10976172_CpG_6.7	0.49	0.59	0.190	0.386	
cg10976172_CpG_8	0.21	0.37	0.064	0.265	
cg10976172_CpG_9*	0.23	0.36	0.075	0.271	
cg10976172_CpG_10	0.29	0.42	0.172	0.379	
cg10976172_CpG_11	0.36	0.46	0.272	0.440	
cg10976172_CpG_12	0.32	0.41	0.325	0.486	
cg10976172_CpG_13.14.15	0.49	0.59	0.190	0.386	
cg10976172_CpG_16.17.18	0.48	0.61	0.075	0.271	
cg10976172_CpG_19	0.37	0.46	0.344	0.502	
cg10976172_CpG_20	0.33	0.44	0.130	0.327	
cg10976172_CpG_21	0.32	0.41	0.325	0.486	

cg10976172_CpG_22	0.47	0.53	0.404	0.564	
cg10976172_CpG_23.24	0.68	0.71	0.762	0.873	
cg10976172_CpG_25	0.43	0.45	0.806	0.905	
cg10976172_CpG_26	0.57	0.61	0.762	0.873	
cg20723355_CpG_1	0.12	0.22	0.045	0.220	FBXO39
cg20723355_CpG_2*	0.11	0.23	0.018	0.206	
cg20723355_CpG_3	0.15	0.30	0.048	0.228	
cg20723355_CpG_4.5	0.28	0.48	0.028	0.218	
cg20723355_CpG_6.7	0.47	0.61	0.173	0.379	
cg20723355_CpG_8	0.25	0.30	0.241	0.432	
cg20723355_CpG_9.10	0.41	0.57	0.104	0.301	
cg20723355_CpG_11.12	0.45	0.48	0.520	0.668	
cg20723355_CpG_13.14.15	0.57	0.56	0.762	0.873	
cg20723355_CpG_16.17	0.39	0.40	0.733	0.857	
cg20723355_CpG_18	0.11	0.28	0.034	0.218	
cg20723355_CpG_19	0.11	0.23	0.018	0.206	
cg20723355_CpG_21	0.25	0.30	0.241	0.432	
cg20723355_CpG_22	0.02	0.21	0.127	0.327	
cg20723355_CpG_23.24	0.46	0.55	0.185	0.384	
cg20723355_CpG_25	0.53	0.64	0.405	0.564	
cg20723355_CpG_26	0.61	0.81	0.066	0.265	
cg20723355_CpG_27.28.29	0.66	0.73	0.449	0.605	
cg20723355_CpG_30	0.32	0.49	0.045	0.220	
cg15239123_CpG_1	0.08	0.14	0.020	0.206	SMOC1
cg15239123_CpG_2	0.02	0.08	0.110	0.310	
cg15239123_CpG_8	0.16	0.16	0.909	0.957	
cg15239123_CpG_9	0.00	0.00	NA	NA	
cg15239123_CpG_10	0.06	0.08	0.342	0.502	
cg15239123_CpG_14	0.16	0.16	0.909	0.957	
cg15239123_CpG_15.16.17.18	0.08	0.14	0.020	0.206	
cg15239123_CpG_19.20.21	0.06	0.09	0.030	0.218	
cg15239123_CpG_22.23	0.13	0.12	0.624	0.770	
cg15239123_CpG_24*	0.08	0.13	0.016	0.206	
cg15239123_CpG_25	0.02	0.03	0.420	0.582	
cg15239123_CpG_26.27	0.07	0.07	0.676	0.825	
cg15239123_CpG_34	0.05	0.05	0.939	0.981	
cg15239123_CpG_36.37.38.39.40	0.29	0.35	0.570	0.719	
cg15239123_CpG_41.42	0.06	0.08	0.342	0.502	
cg15239123_CpG_51	0.09	0.15	0.033	0.218	
cg15755084_CpG_1.2	0.02	0.04	0.319	0.486	MSX1
cg15755084_CpG_3	0.01	0.01	0.698	0.844	
cg15755084_CpG_4.5	0.02	0.02	0.899	0.957	
cg15755084_CpG_6*	0.03	0.08	0.023	0.215	
cg15755084_CpG_7.8.9	0.06	0.08	0.568	0.719	
cg15755084_CpG_11	0.10	0.11	0.838	0.922	

cg15755084_CpG_12.13	0.09	0.13	0.004	0.182
cg15755084_CpG_14	0.01	0.02	0.598	0.744
cg15755084_CpG_15.16.17.18	0.25	0.25	0.733	0.857
cg15755084_CpG_19.20	0.12	0.15	0.150	0.353
cg15755084_CpG_21.22.23	0.04	0.08	0.170	0.379
cg15755084_CpG_24.25.26	0.07	0.10	0.253	0.432
cg15755084_CpG_27.28	0.06	0.04	0.593	0.744
cg15755084_CpG_29.30	0.13	0.13	0.404	0.564
cg15755084_CpG_31	0.03	0.07	0.321	0.486
cg15755084_CpG_32	0.04	0.11	0.048	0.228
cg15755084_CpG_33	0.07	0.08	0.568	0.719
cg15755084_CpG_34	0.07	0.08	0.704	0.844
cg15755084_CpG_35.36	0.17	0.18	1.000	1.000
cg15755084_CpG_37.38	0.23	0.25	0.075	0.271
cg15755084_CpG_39.40	0.14	0.15	0.594	0.744
cg15755084_CpG_41.42.43.44	0.12	0.15	0.150	0.353
cg15755084_CpG_45	0.10	0.11	0.838	0.922
cg15755084_CpG_46	0.04	0.08	0.018	0.206
cg15755084_CpG_47.48	0.10	0.12	0.790	0.898

Figure 2**.** Schematic diagram of TspR1 method for enrichment and sequencing of methylated DNA fragment libraries. Panel A (left/blue) depicts an unmethylated DNA fragment which is cleaved by both TspR1 (yielding 9 base single stranded overhanging ends [“T”]) and HpaII. Because the CCGG site is not methylated, HpaII can cut. The resulting 2 base overhangs are sensitive to digestion by Exonuclease III and the fragment is lost from the library. In contrast, an identical sequence that is methylated will be resistant to HpaII cleavage. 9 base single stranded TspR1 digested ends will protect the fragment from digestion by Exonuclease III and the fragment will remain in the library and will later be sequenced.

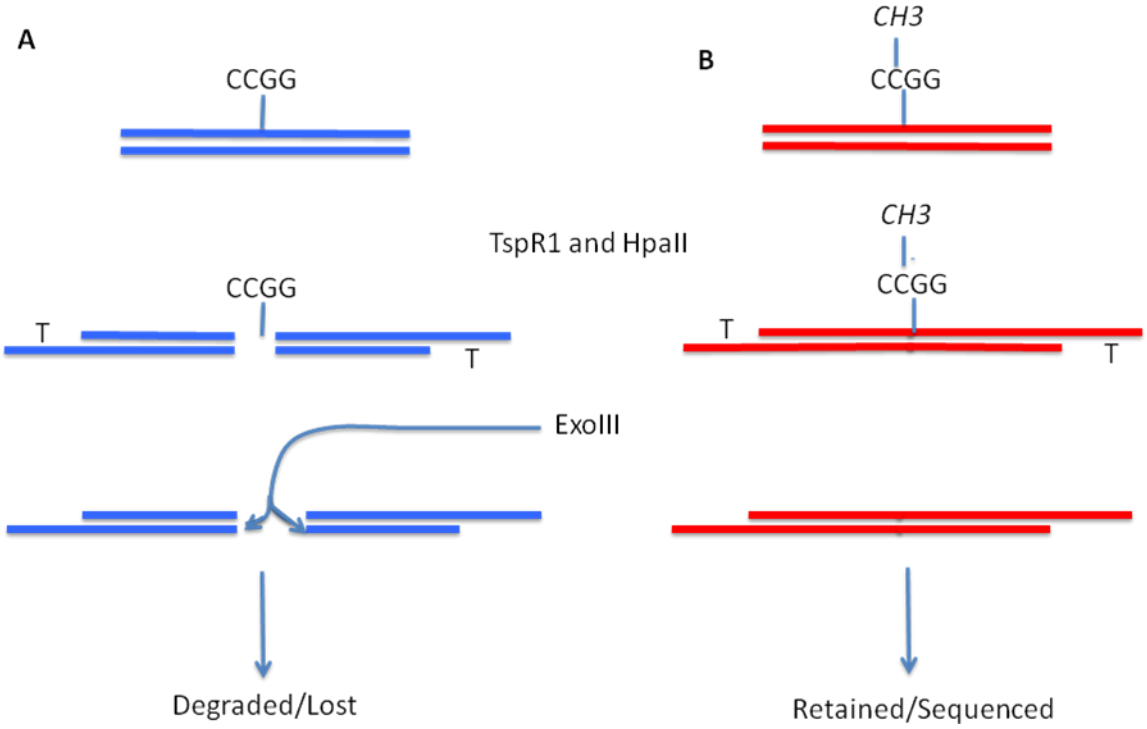

Table 6. Summary data for DNA sequencing of libraries prepared using the new TspR1 method. S1 and S2 are duplicates of HpaII digested DNA. S3 and S4 are duplicates of MspI digested DNA. S5 and S6 are duplicates of sham digested DNA.

Library	Enzyme	Original reads	Matched reads	Distinct matched reads	Matched/ Original	Distinct matched/ Matched
s1	Sham	11386931	6656388	1381483	0.58	0.21
s2	Sham	14111299	10260793	1923606	0.73	0.19
s5	HpaII	23080529	16131370	480194	0.70	0.03
s6	HpaII	24498779	18290725	1137790	0.75	0.06
s7	MspI	9011591	3264193	1026826	0.36	0.31
s8	MspI	9425937	5228484	1302572	0.55	0.25

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American

_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X_ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication, listed in the table, in a PDF version 5.0.5 format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer

Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1.				☐Submitted ☐Accepted ☐Published
2.				☐Submitted ☐Accepted ☐Published
3.				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___X____ No_____

If yes, please describe your plans:

We anticipate that the work completed will lead to a number of future publications that describe the epigenetic consequences of trisomy 21.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

We believe that the experiments performed and the data obtained will provide new insights into the epigenetic consequences of trisomy and will have significant impact on the field of epigenetics by providing a genome-wide experimental approach to the quantification of DNA methylation patterns.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

We expect that the characterization of the epigenetic consequences of trisomy 21 that will arise from the ongoing pursuit of this research study will lead to new diagnostic markers for fetal genetics disease. Specifically, we expect that differences in the early gestational placental DNA methylome that exist between trisomy 21 and normal pregnancies will be used to identify DNA methylation markers that, although originating in the placenta, will be detectable in early gestation in the maternal circulation. This will enable the development of non-invasive tests for fetal genetic disorders.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:

Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No___

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No___

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No___X___

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Peters, David Gerard	Associate Professor
eRA COMMONS USER NAME dpeters	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Liverpool Polytechnic, Liverpool, UK	B.Sc. (Hons)	1990	Biology
University of Liverpool, Liverpool, UK	Ph.D.	1994	Molecular Biology
University of Pittsburgh, Pittsburgh, PA	Post-Doc	1995	Molecular Biology
University of Pittsburgh, Pittsburgh, PA	Post-Doc	1995-98	Human Genetics

A. Personal Statement

The over-arching goal of my research is to develop and validate a new method for the non-invasive diagnosis of fetal aneuploidy using DNA obtained from maternal plasma.

B. Positions and Honors

Professional Experience:

1988-1989 Technician, Department of Biochemistry, Lilly Industries, Liverpool, UK

1990-1994 Graduate Student, Department of Genetics and Microbiology, University of Liverpool, Liverpool, UK

1995-1995 Post-Doctoral Fellow, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA

1995-1996 Post-Doctoral Fellow, Department of Human Genetics, University of Pittsburgh, Pittsburgh, PA

1996-1998 Research Associate, Department of Human Genetics, University of Pittsburgh, Pittsburgh, PA

2001-2002 Visiting Scientist, AstraZeneca Pharmaceuticals, Alderley Park, UK

1999-2004 Scientific Director, University of Pittsburgh Serial Analysis of Gene Expression (SAGE) / Functional Genomics Core Facility

2000-2004 Assistant Professor, Dept. Acute/Tertiary Care, University of Pittsburgh, Pittsburgh, PA

1998-2003 Assistant Professor, Department of Human Genetics, University of Pittsburgh, Pittsburgh, PA

2003-2004 Assistant Professor (Tenure Track), Department of Medicine, Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh, Pittsburgh, PA

2004-2006 Lecturer, University of Liverpool, Department of Pharmacology and Therapeutics, Liverpool, UK.

2006-Present Scientific Director, Centre for Fetal Medicine, Magee-Womens Research Institute, Pittsburgh, PA

2006-Present Associate Professor, University of Pittsburgh, Department of Obstetrics, Gynecology and Reproductive Sciences, Pittsburgh, PA

C. Selected Peer-Reviewed Publications: Recent publications of importance to the field

1. Rajakumar A, Chu T, Handley DE, Bunce KD, Burke B, Hubel CA, Jeyabalan A, Peters DG. Maternal gene expression profiling during pregnancy and preeclampsia in human peripheral blood mononuclear cells. Placenta. 2011;32(1):70-8.
2. Chu T, Bunce K, Hogge WA, Peters DG. A novel approach toward the challenge of accurately quantifying fetal DNA in maternal plasma. Prenat Diagn. 2010;30(12-13):1226-9.
3. Chu T, Bunce K, Hogge WA, Peters DG. Statistical considerations for digital approaches to non-invasive fetal genotyping. Bioinformatics. 2010;26(22):2363-6.
4. Handley D, Peters DG. Noninvasive prenatal chromosomal aneuploidy detection using plasma cell-free nucleic acid. Expert Rev Obstet Gynecol. 2010;5(5):581-9.
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