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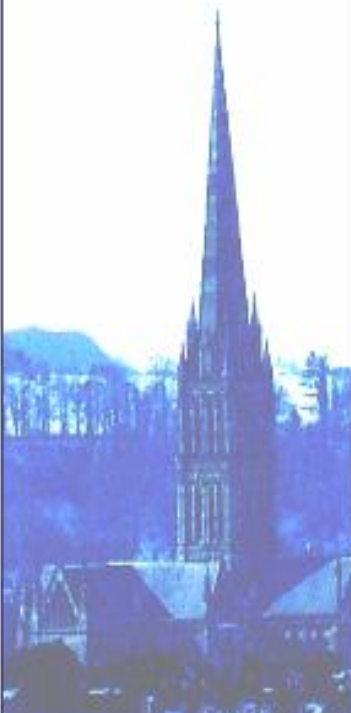
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**NHS**

**Technology  
Assessment**


**Evaluation of Pyrosequencing® for  
quantitative analysis of CpG  
methylation at imprinted gene loci:**

**analysis of SNRPN gene methylation as a  
model system**



**NGRL WESSEX**

**December 2005**

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### Contributors

Name	Role	Institution
Helen White	Clinical Research Scientist	NGRL (Wessex)
Vicky Durston	MTO	NGRL (Wessex)

### Peer Review and Approval

This document has been reviewed by two experts and their comments have been incorporated in the final version. Biotage AB have also been given the opportunity to comment on the content of the report.

### Conflicting Interest Statement

The authors declare that they have no conflicting financial interests

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or by contacting

National Genetics Reference Laboratory (Wessex)  
Salisbury District Hospital  
Odstock Road  
Salisbury  
SP2 8BJ  
UK

E mail: [ngrl@soton.ac.uk](mailto:ngrl@soton.ac.uk)  
Tel: 01722 429016  
Fax: 01722 338095

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## ABSTRACT

**Background:** Several studies have reported the use of Pyrosequencing® for quantitative methylation analysis of multiple CpG sites at imprinted gene loci. To evaluate the use of Pyrosequencing as a tool to analyse DNA methylation in diagnostic practice we have developed a new diagnostic test for PWS/AS which uses Pyrosequencing to analyse and quantify twelve CpG sites within the 5' end of the SNRPN gene. Angelman syndrome (AS) and Prader-Willi syndrome (PWS) are two distinct neurodevelopmental disorders which are caused primarily by deficiency of specific parental contributions at an imprinted domain within the chromosomal region 15q11.2-13. In the majority of cases, lack of paternal contribution results in PWS either by paternal deletion (~70%) or maternal uniparental disomy (UPD) (~30%). Most cases of AS result from the lack of a maternal contribution from this same region, by maternal deletion (~70%) or paternal UPD (~5%). Analysis of allelic methylation differences at the SNRPN locus discriminates the maternally and paternally inherited chromosome 15 and can be used as a diagnostic test for AS and PWS.

**Methods:** Sodium bisulphite-treated genomic DNA was PCR amplified from the SNRPN gene. Using Pyrosequencing, the resulting artificial C/T polymorphisms at CpG sites were individually quantified. Anonymised DNA samples from PWS (n=40), AS (n=31) and control samples (n=81) were analysed in a blinded fashion using two PCR and three Pyrosequencing reactions. Results from the Pyrosequencing assays were compared to those obtained using a methylation specific PCR (MS-PCR) protocol that is used commonly in diagnostic practice.

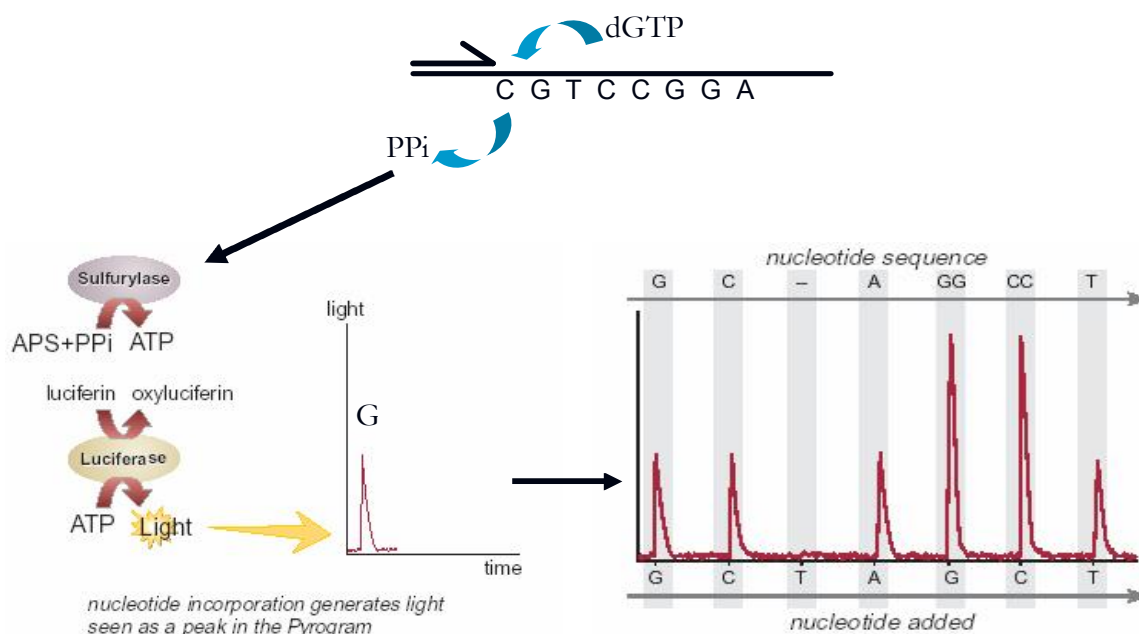
**Results:** The Pyrosequencing assays had a sensitivity and specificity of 100% and provided quantification of methylation at 12 CpG sites within the SNRPN locus. The resulting diagnoses were 100% concordant with those obtained from the MS-PCR protocol.

**Conclusions:** We conclude that Pyrosequencing is a rapid and robust method for quantitative methylation analysis of the SNRPN locus and can be used as a diagnostic test for PWS and AS.

# 1. INTRODUCTION

Several studies have reported the use of Pyrosequencing™ technology for quantitative methylation analysis of multiple CpG sites at imprinted gene loci (Uhlmann *et al.*, 2002; Colella *et al.*, 2003; Tost *et al.*, 2003a; Dupont *et al.*, 2004). To evaluate the use of Pyrosequencing as a tool to analyse DNA methylation in diagnostic practice we have developed a new diagnostic test for PWS/AS which uses Pyrosequencing to analyse and quantify twelve CpG sites within the 5' end of the SNRPN gene. We have used three Pyrosequencing reactions to analyse SNRPN methylation in a group of patients referred to the Wessex Regional Genetics Laboratory for PWS/AS testing (n=71) and a cohort of normal controls (n=81) in whom the SNRPN methylation status had previously been determined using MS-PCR (Zeschnigk *et al.*, 1997a).

Pyrosequencing™ technology is a real time sequencing method for the analysis of short to medium length DNA sequences (Ronaghi *et al.*, 1998). Four enzymes and specific substrates are used to produce light whenever a nucleotide forms a base pair with the complementary base in a DNA template strand. Incorporation of a nucleotide into the strand results in the release of pyrophosphate which is quantified using a luciferase reaction (Figure 1). The signal produced is proportional to the amount of pyrophosphate released and hence methylation at a CpG site can be detected and quantified by analysing the chemically induced C/T sequence differences using the Pyrosequencing Allele Quantification (AQ) software (Biotage AB). We have used three Pyrosequencing reactions to analyse SNRPN methylation in a group of patients referred to the Wessex Regional Genetics Laboratory for PWS/AS testing (n=71) and a cohort of normal controls (n=81) in whom the SNRPN methylation status had previously been determined using MS-PCR (Zeschnigk *et al.*, 1997a).



**Figure 1:** Principle of Pyrosequencing (adapted from <http://www.pyrosequencing.com>).

Angelman syndrome (AS) and Prader-Willi syndrome (PWS) are two clinically distinct neurodevelopmental disorders that are caused by alteration of parentally imprinted genes localised in a 2Mb domain in the chromosomal region 15q11-13. Loss of imprinted genes from the paternally inherited chromosome 15q11-13 region results in PWS. In approximately 70% of cases this is due to an interstitial deletion on the paternally derived chromosome 15 and in approximately 30% of cases PWS results from uniparental disomy (UPD) where both chromosomes 15 are maternal in origin (Nicholls *et al.*, 1989a,b; Robinson *et al.*, 1991; Mascari *et al.*, 1992). In AS, the lack of maternal epigenetic contribution from 15q11-13 can occur as a result of maternal deletions or paternal UPD (~70% and 5% of cases respectively). In approximately 25% of AS cases there is biparental inheritance of chromosome 15 and a normal pattern of allelic methylation in the 15q11-q13 region. In this group,

mutations in the UBE3A gene have been shown to be a cause of AS (Kishino *et al.*, 1997; Matsuura *et al.*, 1997). In a small number of cases (1% in PWS and 2-4% in AS) aberrant imprinting and gene silencing are thought to be responsible for disease and 27% of these cases are found to be mosaic (Buiting *et al.*, 2003).

At a molecular level, the paternal and maternal copies of this imprinted region can be distinguished by examining DNA methylation. Several differentially methylated sites have been identified in 15q11-q13 and the two regions most commonly investigated for the diagnosis of PWS/AS are D15S63 (PW71) and the promoter region of the small nuclear ribonucleoprotein associated polypeptide N (SNRPN) gene. Methylation analysis of these regions has been recognised as a clinically and scientifically valid diagnostic test for PWS/AS by the ASHG/ACMG Test and Transfer Committee (ASHG/ACMG, 1996). The most studied site is the CpG island at the 5' end of the SNRPN gene which appears to be completely methylated on the maternal chromosome while the paternal chromosome remains unmethylated (Zeschnigk *et al.*, 1997b). Southern blot analysis of DNA cleaved with methylation sensitive restriction enzymes has been routinely used as a diagnostic test for PWS/AS but has intrinsic disadvantages such as problems with partial cleavage which in the case of *NotI* digestion of SNRPN can lead to false positive PWS and false negative AS results. Currently, the majority of European diagnostic laboratories test PWS and AS referrals using methylation specific PCR (MS-PCR). MS-PCR is performed using DNA that has been treated with sodium bisulphite. This converts cytosine, but not 5-methyl-cytosine, to uracil and hence allelic methylation differences are detectable as sequence differences. Amplification using primers which are specific for methylated and unmethylated DNA allows the paternal and maternal alleles to be discriminated. Two different MS-PCR protocols are most widely used in Europe (Kubota *et al.*, 1997; Zeschnigk *et al.*, 1997a) but analysis of data from the German external quality assessment schemes from 2001/2002 has shown that the method published by Zeschnigk *et al.* is more reliable at detecting mosaic DNA methylation in Angelman syndrome since the test is semi-quantitative (Horsthemke *et al.*, 2003).

## **2. MATERIALS AND METHODS**

### **2.1 Bisulphite Treatment of DNA Samples**

Total genomic DNA samples (n=152) were collected comprising of normal controls (n=81), PWS patients (maternal UPD, n=14; paternal deletion, n=25; abnormal methylation but not maternal UPD or paternal deletion, n=1) and AS patients (paternal UPD, n=2; maternal deletion, n=24; abnormal methylation but not paternal UPD or maternal deletion, n=5). 2µg genomic DNA from each sample was bisulphite treated on the same day according to the manufacturer's instructions using the EZ DNA Methylation kit (Zymo Research). Bisulphite treated DNA was eluted from the column using 10µl of elution buffer and was diluted 1:10 prior to use. DNA samples were analysed for methylation differences at the SRNPN locus using both our current diagnostic methylation specific PCR (modified from Zeschnigk *et al.*, 1997a) and the three Pyrosequencing assays. Sequences of MS-PCR, Pyrosequencing PCR and sequencing primers (Biomers) are listed in Table 1.

### **2.2 Methylation specific PCR and GeneScan Analysis**

Amplicons were generated in a 25µl reaction volume with 0.8pmol of common primer, 0.8pmol maternal primer, 0.24pmol of paternal primer, 0.18mM dNTPs (Promega), 1X Hotstar Buffer with MgCl<sub>2</sub> (Qiagen), 1U Hotstar Taq (Qiagen) using 1µl of bisulphite treated DNA. PCR conditions were 94°C for 15 min; 29 cycles with denaturation at 94°C for 20s, annealing at 60°C for 35s and elongation at 72°C for 35s; 1 cycle at 72°C for 7 min; and a final hold at 4°C. Thermocycling was performed using a PTC-0225 DNA Engine Tetrad (MJ Research). All samples were analysed in triplicate.

Fluorescently labeled amplicons were analysed using an ABI 3100 Genetic Analyser and the percentage methylation was calculated by dividing the peak area of the maternal specific peak by the combined peak areas of the maternal and paternal specific peaks (figure 2).

**Table 1:** Oligonucleotide sequences and Pyrosequencing assay details

a) PCR primers for methylation specific PCR (MS-PCR) and Pyrosequencing PCR reactions

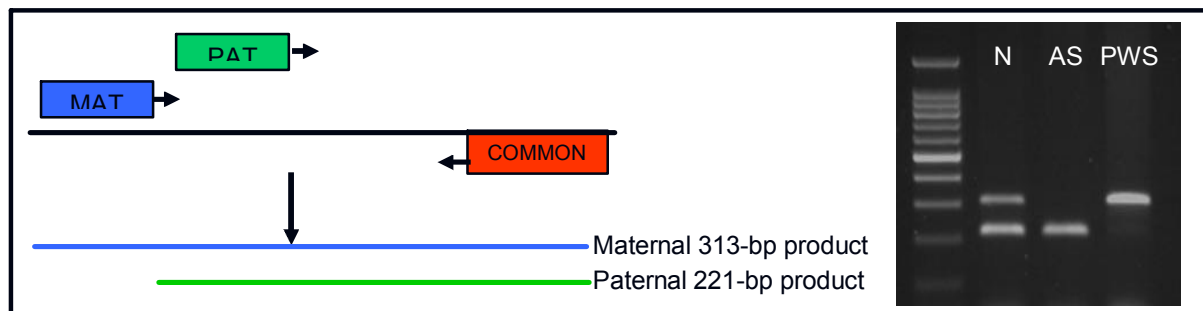
Oligo Name	Assay	CpG in primer binding site <sup>1</sup>	Sequence 5' to 3'
Common	MS-PCR	None	FAM - CTCCAAAACAAAAAAGTTTAAAACCCAAATTC
Maternal	MS-PCR	B - F	TATTGCGGTAAATAAGTACGTTTGC
Paternal	MS-PCR	L - P	GTGAGTTGGTGTAGAGTGGAGTGGTTGTTG
A1031F	Pyrosequencing (Forward assay)	None	AGGGAGTTGGGATTTTTGTATT
A1032RB	Pyrosequencing (Forward assay)	None	Biotin - CCCCAAATCTCTTAAAAA
A1035FB*	Pyrosequencing (Reverse assay)	None	Biotin - AGGGAGTTGGGATTTTTGTATT
A1036R*	Pyrosequencing (Reverse assay)	None	CCCAAATCTCTTAAAAA

b) Primers for Pyrosequencing assays. The sequence to analyse is immediately 3' to the sequencing primer binding site on the biotinylated strand. The positions of the CpG sites analysed are shown in brackets (**bold font**). The dispensation order of the nucleotides includes several dispensations, where no signal should be observed, these are shown in *italics*. The dispensation orders used were those determined by the software in the Simplex SNP entry function (Biotage AB). Bisulphite control positions are marked in brackets. <sup>1</sup>As assigned by Zeschnigk *et al.* (1997b).

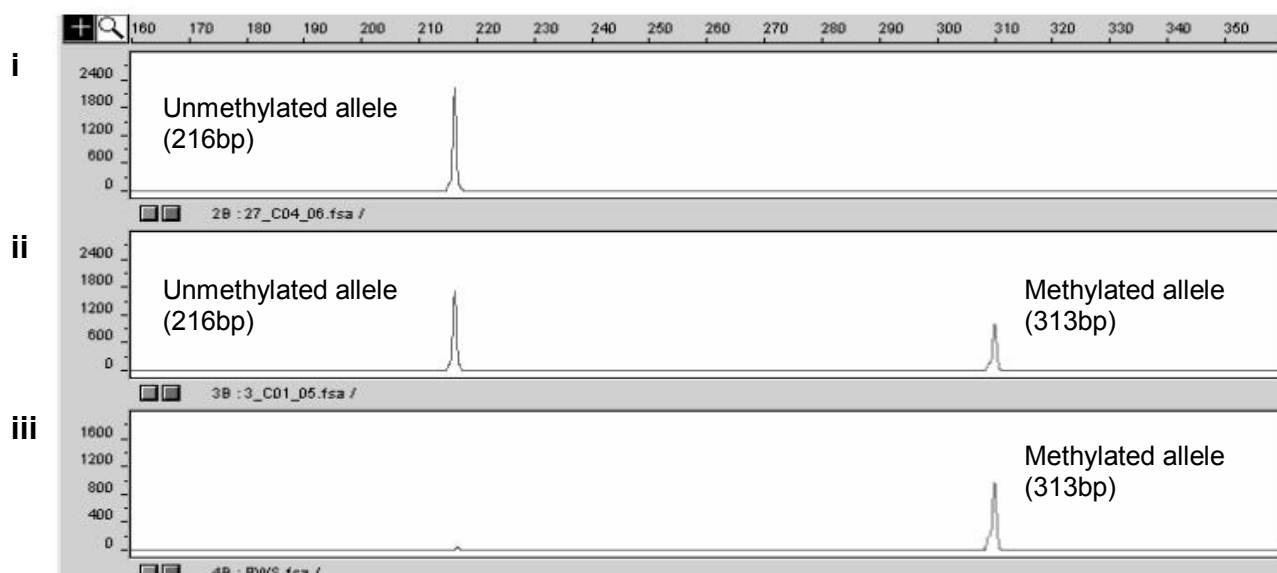
\* Assays available from Biotage AB (PyroMark™ PWS/AS).

Oligo Name	CpG sites Analysed <sup>1</sup>	Sequence 5' to 3'	Sequence to analyse	Dispensation order
A1033FSeq	G - J	GTAGAGGTAGGTTGG	<b>YG</b> ( <b>Y</b> ) ATGTTTAGG <b>Y</b> GGGGATGTGT <b>Y</b> GAAG	<u><i>ATC</i></u> <b>CG</b> <u><i>TC</i></u> <b>TG</b> ( <b>TC</b> ) <u><i>GATGT</i></u> <b>TCAG</b> <u><i>TC</i></u> <b>TGGATGTGCTG</b> <u><i>TC</i></u> <b>TG</b>
A1037RSeq*	R - U	ACACAACCTAACCTTACCC	<b>R</b> CTCCAT <b>CRCR</b> CTCACT ( <b>R</b> ) ACC <b>R</b> CTCC	<u><i>TGA</i></u> <b>GCTCAG</b> <u><i>TCGA</i></u> <b>ACGA</b> <u><i>GTCACT</i></u> ( <b>GA</b> ) <u><i>ACGA</i></u> <b>GC</b>
A1038RSeq	C - F	CCAACCTACCTCTAC	<b>R</b> ACC <b>R</b> CRCA ( <b>R</b> ) AC <b>R</b> TACTT	<u><i>CGA</i></u> <b>GCGA</b> <u><i>ACGA</i></u> <b>GCA</b> ( <b>GA</b> ) <u><i>ACGA</i></u> <b>GTAC</b>

a)



b)



**Figure 2:**

**a)** Schematic diagram of MS-PCR assay adapted from Zeschnigk *et al.*, 1997a. The maternal primer binds specifically to the methylated strand and incorporates CpG sites B to E and the paternal primer binds specifically to the unmethylated strand and incorporates CpG sites L to P.

**b)** Representative GeneScan traces from the MS-PCR assay for:

i) an AS patient. The presence of the paternal (unmethylated allele) PCR product at 216 bp and absence of the maternal (methylated allele) PCR product at 313 bp indicates complete loss of maternal methylation which is consistent with a diagnosis of AS.

ii) a normal control. The presence of the paternal PCR product at 216 bp and maternal (methylated allele) PCR product at 313 bp indicates % methylation which is consistent with a normal methylation profile.

iii) a PWS patient. The presence of the maternal (methylated allele) PCR product at 313 bp and absence of the paternal (unmethylated allele) PCR product at 216 bp indicates complete loss of the paternal allele which is consistent with a diagnosis of PWS

### 2.3 PCR amplification for Pyrosequencing reactions

Amplicons were generated in a 50µl reaction volume with 10pmol of forward and reverse PCR primers, 0.2mM dNTPs (Promega), 2.5mM MgCl<sub>2</sub>, 1X Buffer II (Applied Biosystems), 1U AmpliTaq Gold (Applied Biosystems) using 1µl of a 1:10 dilution of bisulphite treated DNA. PCR conditions for all reactions were 94°C for 7 min; 45 cycles with denaturation at 94°C for 30s, annealing at 58°C for 30s and elongation at 72°C for 30s; 1 cycle at 72°C for 7 min; and a final hold at 15°C. Thermocycling was performed using a PTC-0225 DNA Engine Tetrad (MJ Research). Two PCR amplifications were performed for each sample to allow analysis of 12 CpG sites (Zeschnigk *et al.*, 1997a). Primers A1031F and A1032RB were used for the forward pyrosequencing assay 1033 (analysis of CpG sites G to J) and primers A1035FB and A1036R were used for reverse pyrosequencing assays 1037 (analysis of CpG sites R to U; figure 1b) and 1038 (analysis of CpG sites C to F). All samples were analysed in triplicate.



Single-stranded biotinylated PCR products were prepared for sequencing using the Pyrosequencing™ Vacuum Prep Tool. 3µl Streptavidin Sepharose™ HP (Amersham) was added to 37µl Binding buffer (10 mM Tris-HCl pH 7.6, 2M NaCl, 1 mM EDTA, 0.1% Tween 20) and mixed with 20µl PCR product and 20µl high purity water for 10 min at room temperature using a Variomag Monoshaker (Camlab). The beads containing the immobilised templates were captured onto the filter probes after applying the vacuum and then washed with 70% ethanol for 5 sec, denaturation solution (0.2M NaOH) for 5 sec and washing buffer (10 mM Tris-Acetate pH 7.6) for 5 sec. The vacuum was then released and the beads released into a PSQ 96 Plate Low containing 45µl annealing buffer (20 mM Tris-Acetate, 2 mM MgAc<sub>2</sub> pH 7.6), 0.3µM sequencing primer. For assays 1033 and 1038 0.3µl single stranded binding protein (SSB, Promega, 2.2µg/µl) was added to eliminate secondary structure in the template DNA. The samples were heated to 80°C for 2 min and then allowed to cool to room temperature.

Since this evaluation was performed the composition of the Pyrosequencing kits has been altered. The new PyroGold kits contain single stranded binding protein and therefore SSB no longer needs to be added to assays 1033 and 1038. Assay 1037 is now commercially available from Biotage AB PyroMark PWS/AS)

## **2.4 Pyrosequencing reactions and data analysis**

Pyrosequencing reactions were performed according to the manufacturer's instructions using the PSQ 96 SNP Reagent Kit (Biotage AB) which contained the enzyme and substrate mixture and nucleotides. Assays were performed using the nucleotide dispensation orders shown in Appendix 1. The degree of methylation at each CpG site was determined by analysing the artificial C/T SNP using the Allele Frequency Quantification function in the SNP Software (Biotage AB). The CpG sites analysed by forward assay 1033 and reverse assays 1037 and 1038 were G-J, R-U and C-F respectively (according to Zeschnigk *et al.*, 1997b, figure 3)

## **2.5 Ascertainment of PCR amplification bias for Pyrosequencing reactions**

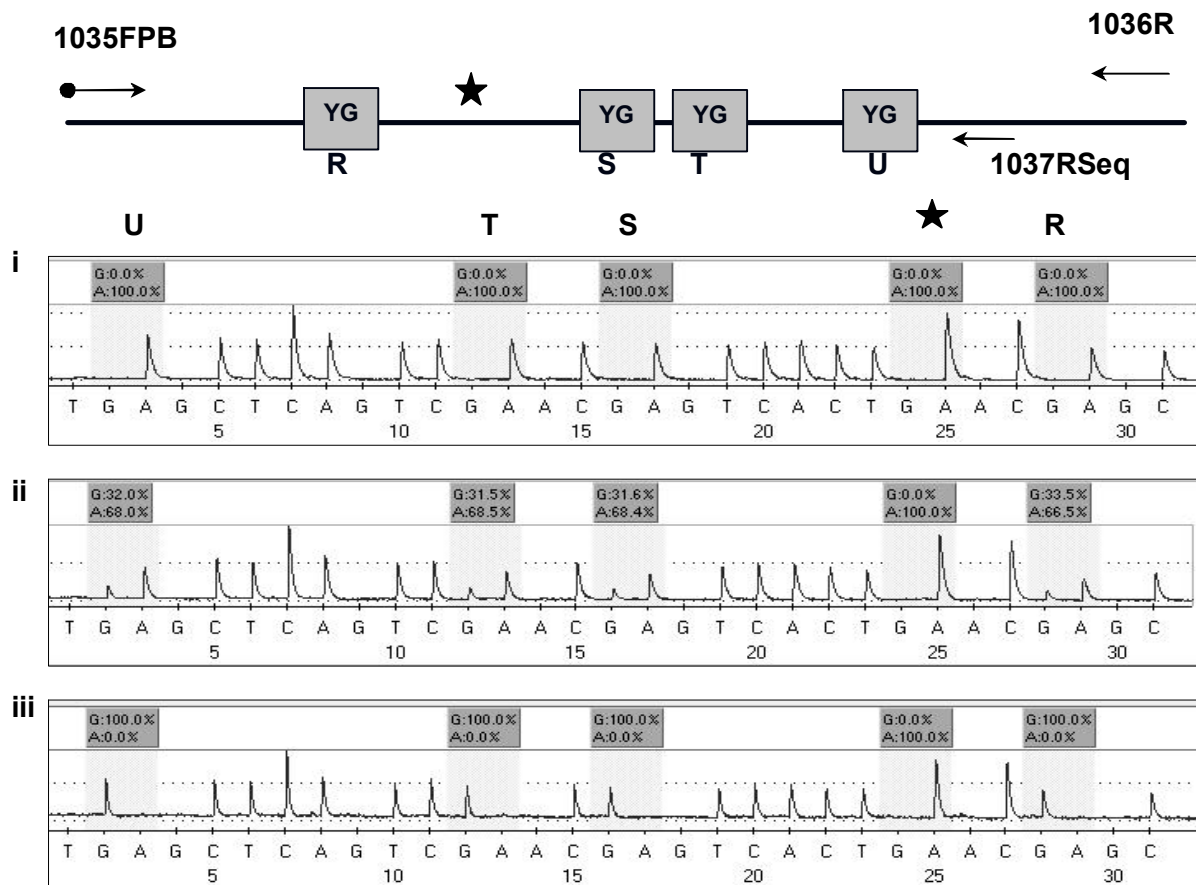
The difference in sequence content between the methylated and unmethylated alleles after bisulphite treatment can influence the melting and annealing properties of the PCR amplification. Calibration curves were constructed for the forward and reverse Pyrosequencing PCR reactions to determine the extent of PCR amplification bias (Tost *et al.*, 2003a). DNA from a PWS and AS patient was quantified and 2µg of each sample was bisulphite treated. The bisulphite treated DNA samples were then mixed prior to PCR to generate samples with 0% - 100% methylation (in 10% increments). The samples were PCR amplified and subjected to Pyrosequencing. To ensure that the Pyrosequencing assays were providing unbiased quantification, PCR products generated from a PWS patient and an AS patient were mixed prior to Pyrosequencing to generate samples with 0% - 100% methylation (in 20% increments).

## **2.6 Effect of bisulphite treatment**

To determine whether variation in quantification at CpG sites occurred between batches of bisulphite treatment normal DNA samples (n=16) were treated on different days using different batches of the EZ DNA Methylation kit (Zymo Research)

## **2.7 Effect of DNA concentration**

To determine whether the amount of template DNA added to the PCR affected the reproducibility of quantification of methylation 2µg DNA from normal control samples (n=4) was bisulphite treated. Assuming 100% recovery, PCR was performed in triplicate using 50, 20, 10, 5 and 2.5ng of template DNA. Pyrosequencing was carried out using assay 1037 and the data were analysed using AQ software (Biotage AB).



**Figure 3:** Schematic diagram of Pyrosequencing assay 1037 showing the region amplified using primers 1035FPB (biotinylated) and 1036R. Using sequencing primer 1037 the CpG sites R, S, T and U can be analysed using AQ software. Representative programs are shown for:

i) an AS patient. AQ values (in boxes) for the methylated (G) allele at all CpG sites are 0% which indicates the absence of the maternal (methylated) allele. This is consistent with a diagnosis of AS

ii) a normal control. AQ values (in boxes) for the methylated (G) allele range from 31.5 – 33.5% which, after correction for PCR amplification bias, equates to 50% methylation. This is consistent with a normal methylation profile where both the methylated (maternal) and unmethylated (paternal) alleles are present.

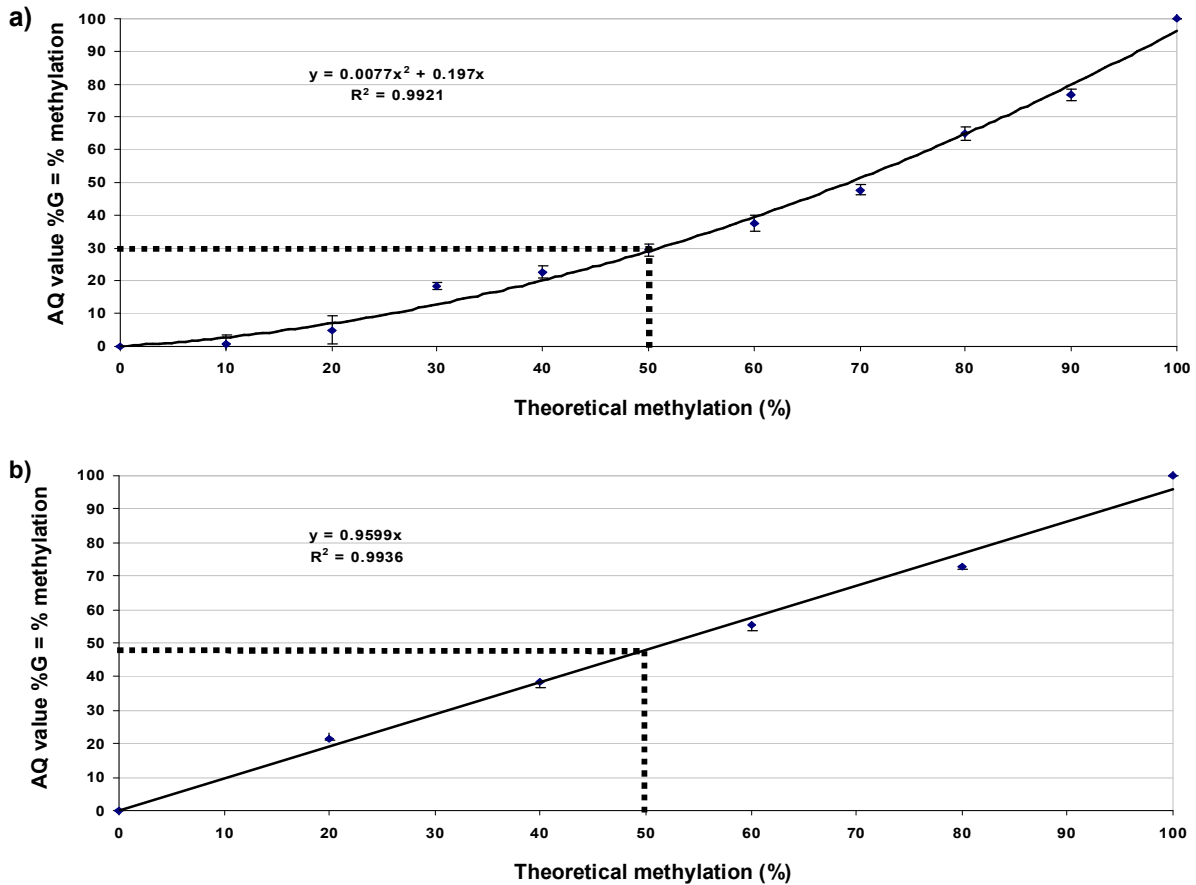
iii) a PWS patient. AQ values (in boxes) for the methylated (G) allele at all CpG sites are 100% which indicates the absence of the paternal (unmethylated) allele. This is consistent with a diagnosis of PWS

In all case the bisulphite control (star) shows complete conversion of the unmethylated cytosine residue since the AQ value is 100% for the unmethylated (A) allele.

### 3. RESULTS

#### 3.1 Pyrosequencing PCR amplification bias

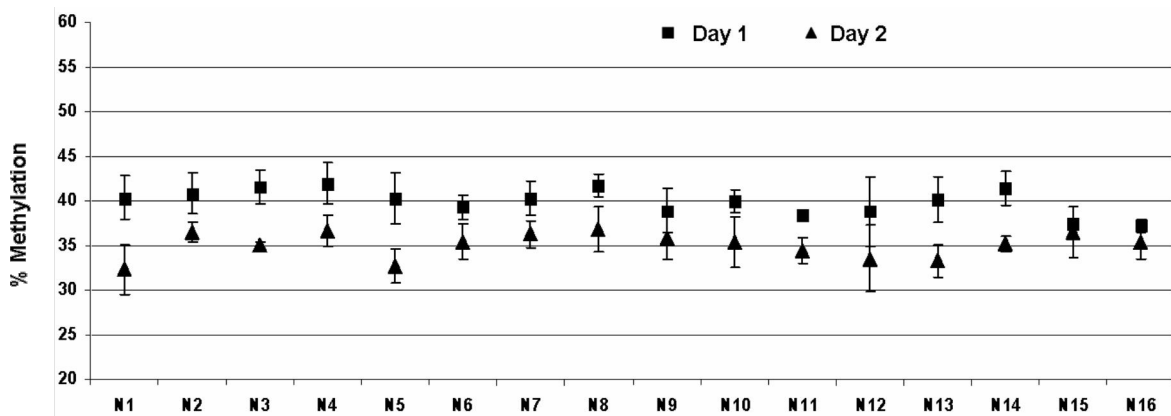
Figure 4 shows the calibration curves generated for the Pyrosequencing reverse assay 1037 where DNA from a PWS and AS patient were mixed prior to PCR (figure 4a) and when PCR products amplified from an PWS and AS sample were mixed after PCR (figure 4b). The curve generated from the mixed template DNA samples (figure 4a) shows a polynomial fit of the second order with a correlation coefficient of 0.9921, rather than a linear trend, demonstrating that there has been preferential PCR amplification of the unmethylated allele. This must be taken into account when absolute quantitation of methylation for a PWS/AS sample is required. However, the highly linear relationship ( $R^2=0.9936$ ) observed for the Pyrosequencing assay run using mixed PCR products obtained from a PWS and an AS patient show that the Pyrosequencing reaction is absolutely quantitative (figure 4b). The calibration curves for the forward Pyrosequencing assay 1033 produced similar results (data not shown).



**Figure 4:** Calibration curves for Pyrosequencing assay 1037 constructed by a) mixing equal concentration of PWS and AS patient DNA samples prior to PCR to generate samples with theoretical methylation from 0 – 100% in 10% increments and b) mixing PCR products obtained from a PWS and an AS patient after amplification to generate samples with theoretical methylation from 0 - 100% in 20% increments

### 3.2 Effect of bisulphite treatment on absolute quantitation of methylation

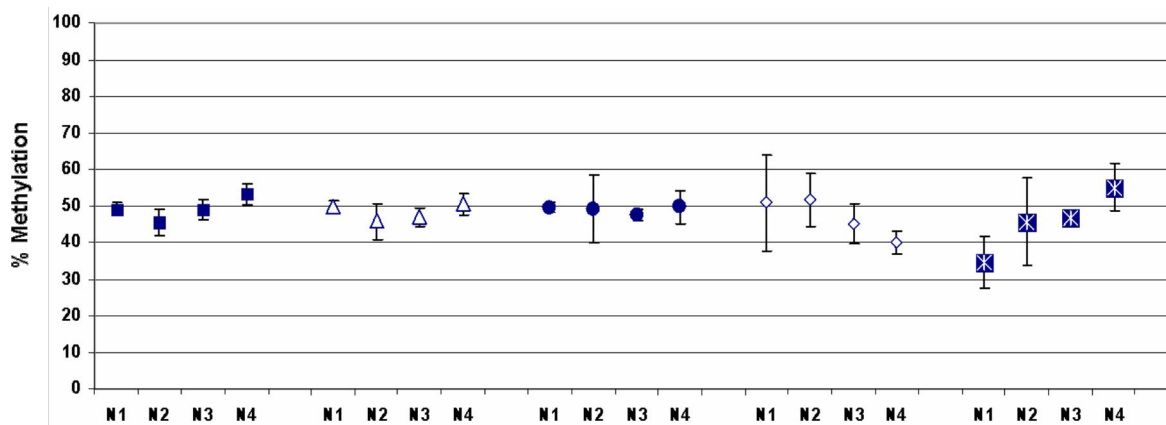
Figure 5 shows that bisulphite treatment of DNA samples on different days can produce subtle differences in quantitative results when using the 1037 forward Pyrosequencing assay. The mean difference in quantitation of methylation at CpG site U was 4.5% with a range of 0.9 – 8%. However this variation was less pronounced with the Pyrosequencing assays 1033 and 1038 (data not shown).



**Figure 5:** Graph showing the effect of bisulphite treatment on absolute quantitation of CpG methylation at CpG site U using Pyrosequencing assay 1037. Analysis of sixteen normal control DNA samples (N1 – N16) bisulphite treated on day 1 (black squares) and day 2 (white squares). The % methylation values are the AQ values for % G and have not been corrected for methylation bias. The error bars indicate the standard deviation for triplicate analyses.

### 3.3 Effect of DNA concentration on reproducibility

The amount of DNA added to the Pyrosequencing PCR can affect the reproducible quantitation of methylation. Low amounts of starting DNA template may introduce bias into the PCR reaction since preferential amplification of either allele during the first few rounds of amplification can occur (Dupont *et al.*, 2004). Figure 6 shows the effect of DNA concentration when using assay 1037, similar results were obtained using assays 1033 and 1038 (data not shown). The average standard deviations at position U for 50ng, 20ng, 10ng, 5ng and 2.5ng of template DNA were 2.8, 3.0, 4.1, 7.2, and 6.7 respectively. These data show that a minimum of 10ng of template DNA should be added to the PCR if reproducible results are to be obtained.

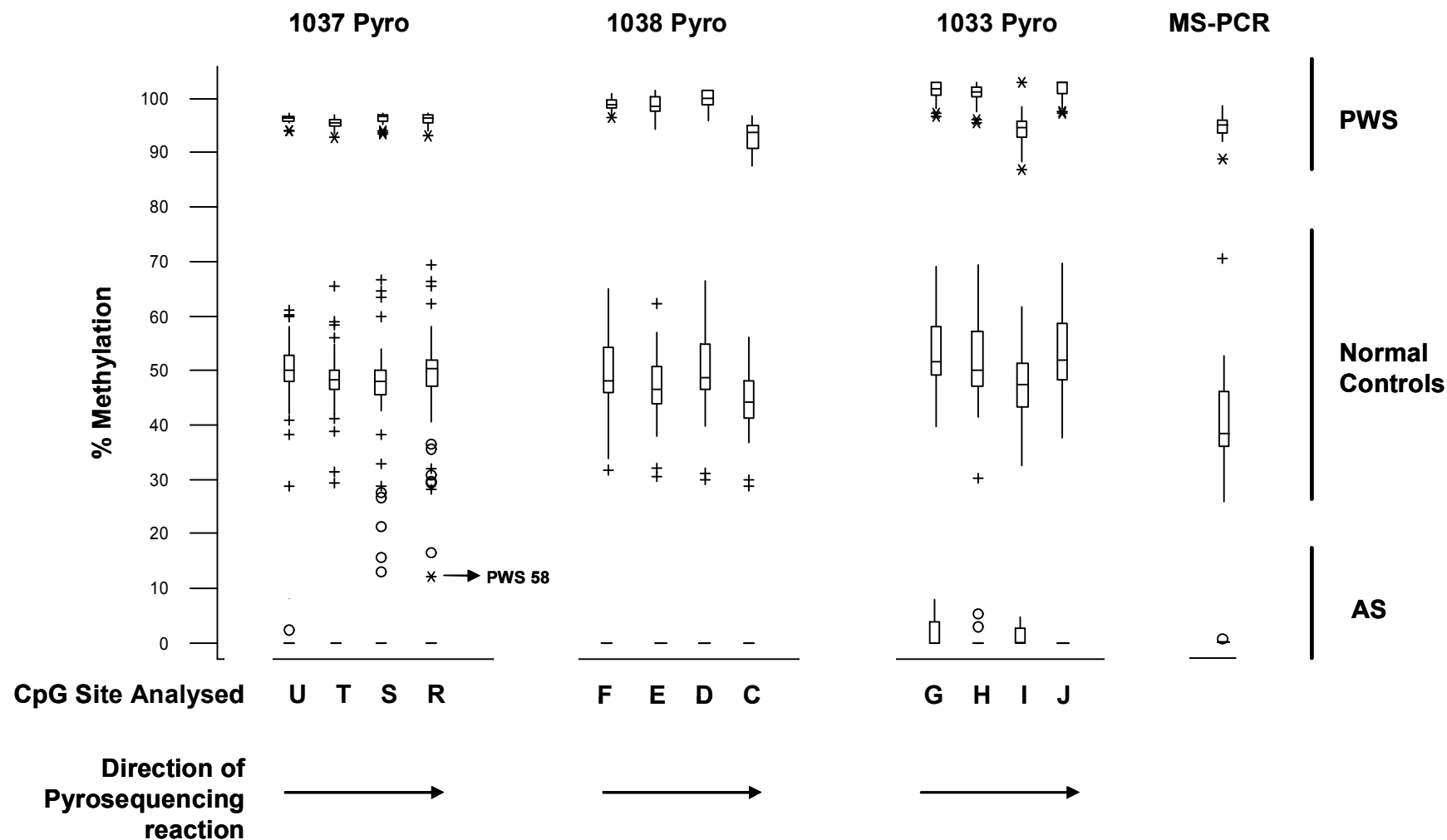


**Figure 6:** Graph showing the effect of DNA concentration on absolute quantitation of CpG methylation at CpG site U using Pyrosequencing assay 1037. Analysis of 4 normal control samples (N1 – N4) where 50ng (blue squares), 20ng (white triangles), 10ng (blue circles), 5ng (white diamonds) and 2.5ng (white stars on blue background) template DNA were added to the PCR. The % methylation values have been corrected for methylation bias. The error bars indicate the standard deviations for triplicate analyses.

### 3.4 Pyrosequencing assays

Forward and reverse Pyrosequencing assays were performed on triplicate PCRs from all DNA samples in a blinded fashion. Quantitation of methylation at twelve CpG sites was achieved using three Pyrosequencing assays performed using either the forward (1033) or reverse (1037, 1038) PCR assays. Data were analysed using AQ software (Biotage AB) which calculated the percentage of methylated (maternal) and unmethylated (paternal) alleles as chemically induced C/T polymorphisms (or G/A) respectively at each CpG site. The percentage methylation (maternal contribution) was recorded as the %C for forward assays and %G for the reverse assays and each position was given a quality score by the software; passed, checked (require manual check to determine quality of data) or failed. Replicates that had failed or that had checked results for 3 or more CpG sites were excluded from the analysis. Data were corrected for PCR amplification bias using the calibration curves for each PCR assay. The samples were scored as being indicative of AS, PWS or unaffected and the samples were then unblinded and results were compared with the original diagnosis. All 152 samples were correctly identified and the results, divided into diagnostic categories, are shown in figure 7. The box plots provide a graphical representation of the distribution of the % methylation values for PWS, AS and normal controls with the lowest value, highest value, median value, and the size of the first and third quartile.

Analysis of CpG sites U, T, S and R using assay 1037 revealed some unexpected methylation patterns for 5 AS patients and 1 PWS patient. In 5 AS cases with maternal deletions, methylation was detected at CpG sites S and R at levels ranging from 13 – 36.5 %. These results were confirmed by Pyrosequencing the CpG sites in the opposite orientation (data not shown). We also identified a PWS patient (PWS58, maternal UPD) who showed unexpected loss of methylation at CpG site R. Further examination of the Pyrogram and subsequent DNA sequencing revealed that this patient had a homozygous C to T mutation at CpG site R. These six samples showed expected methylation profiles for assay 1033, 1038 and MS-PCR. Outlier samples were observed in the normal control population (indicated by + in figure 7). In particular, two samples showed consistently lower levels of detectable methylation than other normal control samples in all assays (including MS-PCR). Triplicate analyses of these samples were reproducible with standard deviations of < 1.5 for each assay. It is possible that these samples reflect true population variation.



**Figure 7:** Box plots showing distribution of AQ data corrected for PCR amplification bias for each diagnostic category for Pyrosequencing assays 1037, 1038 and 1033 and the distribution of % methylation obtained using the fluorescent MS-PCR protocol. The data are shown for each CpG site analysed in each assay. The boxes indicate the distribution of data between the 1st and 3rd quartiles (Q1 and Q3). The line in the box indicates the median value and the whiskers extend to 1.5 times the interquartile range of Q1 and Q3. Outlier samples are indicated as open circles (AS), plus symbols (normal controls) and asterisk (PWS). The five AS samples with apparent methylation at CpG sites S and R (assay 1037) can be seen as open circles and PWS sample 58 with a homozygous C to T mutation at CpG site R is also identified. Outlier samples observed at individual CpG sites (except CpG sites R and S) for the AS and PWS population were due exclusively to the presence of a checked result at a single CpG position which caused the mean result of the triplicate analysis to deviate from the expected 100% methylation for PWS patients and 0% methylation for the AS patients. None of these samples were classified as outliers for more than a single CpG site within an individual assay. Outliers in the normal control population may be an artefact of bisulphite treatment or variable DNA quality, which is often found with diagnostic samples, or may reflect normal population variation.

With the exception of CpG sites S and R in assay 1037, the three Pyrosequencing assays were capable of providing unambiguous classification of PWS, AS or normal control samples. The mean and standard deviation of % methylation for each CpG site for each group of patients are shown in table 2. The three diagnostic categories are clearly distinct and no overlapping % methylation values are observed when the range of % methylation for each category is defined as being three standard deviations from the mean. Classification of the samples as PWS, AS or unaffected were 100% concordant with data obtained from MS-PCR.

		AS (n=31) (% Methylation)			Normal Controls (n=81) (% Methylation)			PWS (n=40) (% Methylation)		
	CpG Site <sup>1</sup>	Mean	SD	Mean +/- 3xSD	Mean	SD	Mean +/- 3xSD	Mean	SD	Mean +/- 3xSD
1038	C	0	0	0	44.5	5.3	28.6 – 60.4	92.9	2.4	85.7 - 100
	D	0	0	0	49.8	5.9	32.1 – 67.5	99.9	1.4	95.7 - 100
	E	0	0	0	47.2	5.2	31.6 – 62.8	98.7	1.8	93.3 - 100
	F	0	0	0	49.7	5.9	32.0 – 67.4	98.9	1.1	95.6 - 100
1033	G	1.6	2.2	0 – 8.2	53.2	6.0	35.2 – 71.2	100	1.6	95.2 - 100
	H	0	0	0	51.8	6.8	31.4 – 72.2	100	1.9	94.3 - 100
	I	0.9	1.4	0 – 5.1	47.3	6.2	28.7 – 65.9	94.2	3.2	84.6 - 100
	J	0	0	0	53.2	5.5	36.7 – 69.7	100	1.8	94.6 - 100
1037	R*	0	0	0	50.1	5.7	33 - 67.1	96.2	0.9	93.7 – 98.9
	S*	0	0	0	48.1	5.3	32.1 - 64	96.4	1.0	93.4 – 99.4
	T	0	0	0	48.3	4.8	33.9 – 59.4	95.5	0.9	92.8 – 98.2
	U	0.1	0.5	0 - 1.6	50.2	4.6	36.4 - 64	96.3	0.8	93.9 – 98.7
MS-PCR	B-F L-P	0	0	0	40.2	7.3	18.3 – 62.1	94.8	1.9	89.1 - 100

**Table 2:** Mean and standard deviations of the % methylation detected at each CpG site for Prader-Willi, Angelman and normal control samples. Each category can be clearly discriminated since the ranges of %methylation for each group (presented as the mean plus or minus three standard deviations) show no overlap. \* data for the 5 unusual AS syndrome cases and the PWS patient (PWS58) with a homozygous C to T mutation at CpG R have been excluded from this analysis, <sup>1</sup> As assigned by Zeschnigk *et al.* (1997b).

### 3.5 MS-PCR

Triplicate analysis of all DNA samples by MS-PCR (Zeschnigk *et al.*, 1997a) was performed in a blinded fashion. The percentage methylation was calculated by dividing the peak area of the maternal specific peak by the combined peak areas of the maternal and paternal specific peaks. Figure 7 shows the box plots for the MS-PCR data and table 2 shows the mean and standard deviation of % methylation for each diagnostic category. As for the Pyrosequencing assays, the three diagnostic groups remain clearly defined after MS-PCR when the diagnostic range of %methylation for each category is defined as being three standard deviations from the mean. An outlier sample was observed in the normal control population that demonstrated higher than normal levels of methylation. Pyrosequencing of this sample showed % methylation within the normal range using assays 1033, 1037 and 1038.

### 3.6 Cost-effectiveness and Speed of Analysis

Costings for the Pyrosequencing assays and fluorescent MS-PCR using 2005 list prices were comparable with the cost per sample being £1.20 (GBP) and £1.17 respectively, excluding system costs and machine maintenance contracts. The analysis times for Pyrosequencing and MS-PCR were similar with results being obtained 1 hour after PCR if less than 16 samples were analysed. If >16 samples are analysed Pyrosequencing becomes the more efficient technology to use since 96 samples can be reported 1 hour after PCR whereas using a 3100 Genetic Analyser (Applied Biosystems) the reporting time would be extended to 6 hours after PCR.

## 4. DISCUSSION

PWS and AS both occur with an incidence of approximately 1:15000 – 1:20000 (Robinson *et al.*, 1991; Cassidy *et al.*, 1984; Butler, 1990; Clayton-Smith and Pembrey, 1992) and result from the loss of the paternal (unmethylated) copy of 15q11-13 (PWS) or loss of the maternal (methylated) copy at the same region (AS). Individuals with PWS have phenotypic features that include infantile hypotonia, mild to moderate mental retardation, hypogonadism, hyperphagia with obesity, short stature and obsessive-compulsive behaviour (Holm *et al.*, 1993). Those with AS exhibit severe mental retardation, inappropriate outburst of laughter, protruding tongue, microcephaly, ataxia and abnormal electroencephalogram patterns (Williams *et al.*, 1995).

In newborns and young children PWS and AS are often difficult to diagnose on the basis of clinical examination alone and molecular and/or cytogenetic analysis is required for definitive diagnosis. Rapid, simple and accurate molecular tests that are capable of diagnosis of PWS and AS by detecting allelic methylation differences are therefore of clinical importance. A recent cost comparison has shown that DNA methylation studies followed by confirmation with fluorescent *in situ* hybridization (FISH) analysis (to detect 15q11-13 deletions) is the most cost effect diagnostic strategy when compared with simultaneous FISH and methylation analysis or with FISH followed by DNA methylation analysis for deletion negative FISH results (Monaghan *et al.*, 2002). In cases where DNA methylation is abnormal, but FISH for 15q11-13 deletions is negative, the presence of UPD can be confirmed using microsatellite analysis. Abnormal methylation in the absence of 15q11-13 deletions and UPD may be indicative of an imprinting centre mutation.

Several molecular strategies have been employed to study allelic methylation differences in PWS/AS; Southern blotting (*e.g.* Ditttrich *et al.*, 1992; Kubota *et al.*, 1996), MS-PCR (*e.g.* Zeschnigk *et al.*, 1997a; Kubota *et al.*, 1997; Muralidhar and Butler, 1998; Buller *et al.*, 2000; Kosaki *et al.*, 1997), PCR following restriction digestion of bisulphite treated DNA (Velinov *et al.*, 2000) and methylation specific multiplex ligation dependent probe amplification (MS-MLPA) (Nygren *et al.*, 2005). In general these methodologies are qualitative with the exception of MS-PCR and MLPA which can be semi-quantitative. Bisulphite treatment of DNA converts unmethylated cytosines to uracil, leaving methylated cytosines unchanged. The resulting sequence changes can be analysed as C/T SNPs since thymidine incorporates at uracil positions during PCR. These chemically induced polymorphisms can be more accurately quantified using a variety of techniques including dHPLC (Baumer *et al.*, 2001), MALDI mass spectrometry (Tost *et al.*, 2003b), adaptations of SnaP-shot™ (Uhlmann *et al.*, 2002) and Pyrosequencing® (Uhlmann *et al.*, 2002; Colella *et al.*, 2003; Tost *et al.*, 2003a; Dupont *et al.*, 2004). In this study we have described the use of Pyrosequencing as a novel method to detect and quantify allelic methylation differences at the SNRPN gene locus which can be used for the rapid and accurate diagnosis of PWS/AS.

Using Pyrosequencing we have analysed bisulphite treated DNA samples from 81 normal controls, 40 patients with PWS and 31 patients with AS and compared the results to those obtained from a commonly used MS-PCR protocol (Zeschnigk *et al.*, 1997a). We have demonstrated that the assays can be used for the accurate diagnosis of PWS/AS and can also be used to simultaneously quantitate the degree of methylation at multiple CpG sites in a single assay. Data from the three assays showed complete segregation of the three diagnostic categories (table 2) and can therefore be used to diagnose 100% of PWS cases and ~80% of AS cases including mosaics. Pyrosequencing assays were designed such that PCR and Pyrosequencing primers were located in regions without CpG sites reducing the possibility of extreme PCR amplification bias and eliminating bias during the Pyrosequencing reactions themselves. This is different to the PCR design strategy used in MS-PCR where primers are designed to anneal specifically to either the methylated or unmethylated strand. In this case bias is minimized by the introduction of higher concentrations of the primer used to amplify the methylated allele. Construction of calibration curves for the Pyrosequencing assays (figure 4)

show PCR amplification bias that is indicative of preferential amplification of the unmethylated allele. This bias means that the lowest detectable level of methylation using these assays is approximately 5%. It is important to be aware that if results from the AQ software are to be used in a truly quantitative manner the AQ data require correction using the appropriate equation from the curve generated by mixing DNA from a PWS and AS patients prior to PCR. This is particularly important if mosaicism is suspected. Therefore appropriate calibration controls should be included if absolute quantification of data is required.

Factors which may affect the reproducibility of absolute quantification of individual CpG sites include bisulphite treatment and DNA concentration/quality. We found that the DNA samples bisulphite treated on different days show subtle variation in % methylation values obtained, ranging from 0.8 – 9% for CpG site U in assay 1037 (figure 5). Variability can be controlled for by ensuring that calibration curves are constructed from DNA bisulphite treated at the same time as the test samples. Low concentrations (<10ng) of template DNA (and presumably poor DNA quality) can also affect absolute quantification since PCR amplification bias can be introduced in the first few cycles of PCR. We suggest that all samples should be amplified in triplicate so that variations in amplification bias can be easily detected. Samples with variable replicates or with more than two checked or any failed positions after analysis with the AQ software should be treated with caution and re-tested using higher concentrations of template DNA.

Methylation analysis using Pyrosequencing has several theoretical advantages over MS-PCR since it allows quantification of methylation at multiple CpG sites and the assays have several inbuilt quality controls. Pyrosequencing assays are designed to include a bisulphite treatment control, where the analysis of a cytosine which is not present at a CpG site is analysed as a C/T polymorphism. Since this cytosine residue always remains unmethylated it should undergo full conversion to uracil following bisulphite treatment. Figure 3 shows the position of the bisulphite control for assay 1037 (indicated by the star) and demonstrates that full bisulphite conversion has occurred since the AQ software has calculated 100% T at this position. AQ data generated using the PSQ MA system is scored for confidence as either passed, checked or failed which alerts users to the quality of the assay. The parameters taken into account are the agreement between the observed and expected sequence, the signal to noise ratios and also the peak width. The CpG sites are presented in sequence context and therefore sequence variants will be identified. Several 'reference peaks' are also incorporated into the analysis that add confidence to the data collection. Additional information can be gained about subtle methylation changes at individual CpG sites as in the case of the 5 AS patients exhibiting methylation at CpG site R and S, and also detection of mutations is possible as in the case of the PWS patient who harboured a homozygous C to T point mutation at CpG site R. Such information could be useful both clinically and in a research context to further understand the epigenetics of PWS/AS.

Using appropriate calibration controls it is possible that the rare mosaic cases of PWS/AS could be accurately diagnosed since rigorous application of the Pyrosequencing assay provides absolutely quantitative analysis of methylation at multiple CpG sites when all AQ data is scored as passed. When attempting to diagnose mosaic cases it is important that the normal range of methylation has been determined. Our normal control population showed variation in detectable methylation and several outliers were observed when data were analysed using the box plots. This variation may be an artifact of bisulphite treatment or variable DNA quality, which is often found with diagnostic samples, or may reflect normal population variation. The two normal control samples which had consistently low levels of methylation detected in all assays could not be investigated further as our study population was anonymised and no clinical information was available. AQ data from these samples passed (for all replicates) at all CpG sites and the triplicate analyses had low variation (SD <1.5).

Outliers in the normal controls were observed most commonly for the 1037 assay. The interquartile ranges at the CpG sites for this assay are smaller than those observed for the other assays making outlier samples more obvious. The outlying samples did not produce AQ values that were checked and generally the standard deviations of the samples were <5 and the samples did not exceed the diagnostic cut off set as being three standard deviation from the mean. Therefore it is possible that these samples represent normal population variability. Outlier samples observed at individual CpG sites (except CpG sites R and S) for the AS and PWS population were due exclusively to the presence of a checked result at a single CpG position which caused the mean result of the triplicate analysis to deviate from the expected 100% methylation for PWS patients and 0% methylation for the AS patients (figure 7). None of these samples were classified as outliers for more than a single CpG site within an individual assay.



Pyrosequencing methylation assays were easy to design and optimize and provided detection and quantification of DNA methylation at the SNRPN gene locus allowing the unambiguous diagnosis of PWS/AS in a diagnostic setting. The cost and time of analysis of MS-PCR and Pyrosequencing are similar but Pyrosequencing has advantages in terms of quality control and the additional information gained from the assay data. Results from this study suggest that Pyrosequencing could be developed as a diagnostic tool for other methylation disorders, such as Beckwith–Wiedemann syndrome, where mosaicism is more commonly observed and absolute quantitative analysis of methylation at individual CpG sites would be essential.

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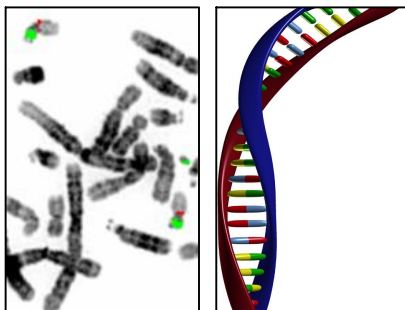
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National Genetics Reference Laboratory (Wessex)  
Salisbury District Hospital  
Salisbury SP2 8BJ, UK

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