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► **To cite this version:**

J. Ross Hawkins, Malcolm Hawkins, David Edward Barton, Karen Meaney, Miriam Guitart, et al.. Establishment of the first WHO international genetic reference panel for Prader Willi and Angelman syndromes. *European Journal of Human Genetics*, 2011, 10.1038/ejhg.2011.59 . hal-00642414

HAL Id: hal-00642414

<https://hal.science/hal-00642414>

Submitted on 18 Nov 2011

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**Establishment of the first WHO international genetic reference panel for Prader
Willi and Angelman syndromes**

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Running title

Prader Willi and Angelman syndromes genetic reference panel.

Abstract

Prader Willi and Angelman syndromes are clinically distinct genetic disorders both mapping to chromosome region 15q11-q13 which are caused by a loss of function of paternal or maternally inherited genes in the region, respectively. With clinical diagnosis often difficult, particularly in infancy, confirmatory genetic diagnosis is essential to enable clinical intervention. However, the latter is challenged by the complex genetics behind both disorders and the unmet need for characterised reference materials to aid accurate molecular diagnosis. With this in mind, a panel of six genotyping reference materials for Prader Willi and Angelman syndromes was developed, which should be stable for many years and available to all diagnostic laboratories. The panel comprises three Prader Will syndrome materials (two with different paternal deletions, and one with maternal uniparental disomy) and three Angelman syndrome materials (one with a maternal deletion, one with paternal uniparental disomy or an epigenetic imprinting centre defect, and one with a *UBE3A* point mutation). Genomic DNA was bulk extracted from Epstein-Barr virus transformed lymphoblastoid cell lines established from consenting patients, and freeze-dried as aliquots in glass ampoules. Thirty seven laboratories from twenty six countries participated in a collaborative study to assess the suitability of the panel. Participants evaluated the blinded, triplicate materials using their routine diagnostic methods against in-house controls or externally-sourced uncertified reference materials. The panel was established by the Expert Committee on Biological Standardization of the World Health Organization as the 1st International Genetic Reference Panel for Prader Willi & Angelman Syndromes.

Key words

Prader Willi syndrome, Angelman syndrome, certified reference materials, genetic reference panel, WHO international standard.

Introduction

Prader Willi (PWS; OMIM #176270) and Angelman (AS; OMIM #105830) syndromes are clinically distinct genetic disorders, both mapping to chromosome 15q11-q13. Clinical diagnosis of PWS is often difficult due to the relatively non-specific findings, particularly in infancy, and the clinical overlap with other disorders¹⁻³. The incidence of PWS is reported as 1 in 25,000 but since many diagnoses are not at an early age a more realistic estimate is 1 in 10,000 to 1 in 15,000¹. AS clinical diagnosis may be delayed or incorrect since the unique features may not become apparent for several years and there are many other disorders with clinical overlap⁴⁻⁵. The incidence of AS is reported as between 1 in 12,000 to 1 in 20,000, but again may be an underestimate due to the difficulty of clinical diagnosis at an early age⁶.

Genetic testing for PWS and AS has become the standard diagnostic method since clinical criteria, though defined, are not always specific^{7,8}. Confirmed genetic diagnosis allows for clinical intervention and enables determination of recurrence risk in PWS or AS families since this is dependent on the causative genetic mechanism. However, the complexity of genetic testing for PWS and AS is itself compounded by the underlying atypical genetics. Chromosome region 15q11-q13 contains a cluster of imprinted genes under the control of a bipartite imprinting centre (IC: ref. 9); the level of expression being determined by the parental origin of the chromosome. The genes associated with PWS (including *MKRN3*, *MAGEL2*, *NDN* and *SNRPN*) are typically

expressed only on the paternal chromosome, with *NDN* and *SNRPN* known to have differentially methylated CpG islands in the promoter regions which are methylated on the maternal chromosome. Conversely, the gene(s) associated with AS (including *UBE3A*) are usually only expressed on the maternal chromosome whilst the paternally-inherited genes are inactivated. Loss of gene function can be due to a *de novo* deletion within 15q11-q13, uniparental disomy (UPD) of chromosome 15 or an IC defect (epigenetic or microdeletion)¹⁰. There are three common breakpoint (BP) cluster regions within the PWS/AS critical region; a longer class I deletion arising between BP1 and BP3, a shorter class II deletion arising between BP2 and BP3^{11,12}. AS can additionally result from point mutations in *UBE3A*¹³.

A number of testing methods exist for PWS or AS genetic diagnosis, the most common being DNA-based testing for abnormal methylation in 15q11-q13 to detect deletions, UPD and epigenetic IC defects. This approach will detect more than 99% of PWS individuals and approximately 80% of AS individuals. Sequence analysis of *UBE3A* will detect a further approximately 10% of individuals with AS¹⁰. Abnormal methylation is most commonly detected by either methylation-specific PCR (MS-PCR) to determine methylation analysis at the *SNRPN* locus only¹⁴⁻¹⁶ or methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA; MRC-Holland, Amsterdam, The Netherlands; ref. 17-19) to determine the gene copy number and methylation status within 15q11-q13. MS-PCR will confirm the disease diagnosis but not the mechanism, necessitating a follow-up technique such as fluorescence in-situ hybridization (FISH) or microsatellite marker analysis to indicate a deletion or the latter technique to additionally indicate UPD by loss of heterozygosity or an epigenetic IC defect, typically by reference to parental DNA. MS-MLPA will confirm the disease diagnosis and identify class I, class II and IC

deletions. Similarly, to distinguish between UPD and an epigenetic IC defect, microsatellite marker analysis is subsequently required. Other detection methods include Southern blot analysis, methylation-sensitive PCR²⁰, methylation-specific melt curve analysis¹⁸, and denaturing high-performance liquid chromatography, all of which typically determine the methylation status of the *SNRPN* locus only. However, only sequencing can resolve *UBE3A* point mutations.

Genetic testing for PWS and AS is widespread, but due to the genetic complexity of the disorders and the diversity of genetic diagnostic techniques available there is a need for widely available genetic reference materials to ensure a correct and consistent diagnosis. Hence, whilst both disorders are relatively uncommon, the need for confirmatory and accurate genetic diagnostic testing is paramount. EuroGentest, an EU-funded network for genetic testing encouraging the harmonisation of standards and practice, ranks PWS and AS in the top ten disorders for prioritised reference material development based upon test request frequency, number of laboratories offering the test and evidence of need and demand²¹.

There are no internationally certified genetic reference materials available for the genetic diagnosis of PWS or AS. Most laboratories use patient-derived DNA samples which have been characterised in-house as controls. If new to the field, laboratories often rely on small, finite amounts of materials supplied by other diagnostic laboratories which cannot guarantee a continual supply of stable and reliable reference materials. Laboratories may also use residual materials from quality assessment schemes which will again be of limited supply. There are some quality control samples available from the Coriell Institute (Camden, USA), either in the form of cell lines or genomic DNA (gDNA), but these materials are not certified reference

materials and are not approved or intended for *in vitro* diagnostic use. None of these control materials adequately enable worldwide assay performance standardisation.

A panel of six freeze-dried gDNA materials was therefore produced for use as reference materials in the genetic diagnosis of PWS or AS. The panel was established in 2009 as the 1st World Health Organisation International Genetic Reference Panel for Prader Willi & Angelman Syndromes.

Materials and Methods

Patients and Cell Lines

A protocol for approaching patients, obtaining informed consent and anonymising samples was carried out with local research ethics committee approval. Blood samples were collected from six consenting donors as part of the EuroGentest project to produce a PWS and AS genetic reference panel.

The six donors were previously clinically and genetically diagnosed with either PWS or AS: 07/230, female AS maternal deletion (class I); 07/232, female AS *UBE3A* c.1234A>T p.Lys412Stop; 07/234, male AS paternal UPD or epigenetic IC defect; 07/236, male PWS paternal deletion (unbalanced chromosome 15;19 translocation); 07/238, female PWS maternal UPD; 07/240, male PWS paternal deletion (class I). Material 07/234 could not be verified as having either paternal UPD or an epigenetic IC defect as parental DNA was unavailable. However, since UPD and an epigenetic IC defect are indistinguishable in the most commonly used MS-MLPA and MS-PCR methods, the material was considered to enhance the panel.

Lymphoblastoid cell lines were established following EBV transformation of peripheral blood lymphocytes, banked and cell pellets of 10⁸ cells prepared as

previously described²². Each cell line was tested for HIV1, HTLV1, Hepatitis B and Hepatitis C by PCR (TDL Genetics, London, UK).

DNA Extraction and Freeze-Drying

Genomic DNA was extracted from cell pellets using Gentra Puregene® reagents with a Gentra Autopure LS robotic workstation (Qiagen, Crawley, UK). Each gDNA material was prepared at 10µg/ml in 2.0mM Tris, 0.2mM EDTA with 5mg/ml trehalose. Aliquots of 0.5ml were dispensed into glass ampoules, freeze-dried and sealed in nitrogen gas for storage at -20°C. A minimum of 2000 ampoules were filled for each of the 6 materials. The quality of the extracted and freeze-dried DNAs was confirmed by spectrophotometry and agarose gel electrophoresis. Identity testing by DNA profiling was as previously described²².

Stability Monitoring

Accelerated degradation studies on materials stored for two years at +45°C and +56°C were carried out by comparison to materials stored at -20°C and -150°C. DNA concentration was determined by spectrophotometric absorbance analysis (NanoDrop, Wilmington, USA). Q-PCR was performed as previously described²², using Brilliant II SYBR Green Q-PCR Master Mix (Agilent, Stockport, UK) and a Stratagene Mx3005P thermal cycler (Agilent) with cycling conditions of 95°C/10 min followed by 50 cycles of 94°C/30 sec, 58°C/1 min and 72°C/1 min. For agarose gel electrophoresis, Lambda DNA/HindIII markers (Promega, Southampton, UK) and control human genomic DNA (Roche, Burgess Hill, UK) were used.

Collaborative Validation Study

The six materials were sent blinded and in triplicate to each laboratory with instructions for reconstitution and storage. Each laboratory was asked to perform their routine testing method(s) for PWS and AS by testing the 18 coded samples in groups

of 6 on 3 separate days, using different batches of reagents and/or different operators if possible. Laboratories were asked to sequence *UBE3A* exon 9 in the region c.1150 to c.1350 if any AS material appeared to have a normal methylation pattern or no detectable deletions. Participants were asked to report the molecular mechanism and disorder interpretation. Data were to be returned together with full details of techniques used, any in-house or commercial controls and reasons for failure of any of the materials tested. With the exception of one laboratory, all used either MS-MLPA or MS-PCR. For MS-MLPA, laboratories used the then-available version of the MRC-Holland kit (ME028 A1). However, a newer version of the kit was subsequently introduced (ME028 B1) and three laboratories used this to further verify the materials' performance.

Results

Viral Contamination

All cell lines tested negative for HIV1, HTLV1, Hepatitis B and Hepatitis C by PCR; all tested positive for EBV by PCR. However, gDNA extracted using the same purification procedure from other EBV-transformed cell lines did not show EBV infectivity²³. Despite the considered minimal risk, material safety data sheets were provided with each panel.

Characterisation of Freeze-Dried DNA

Three of every one hundred filled ampoules were weighed during production. The coefficient of variation for each fill mass (0.20% to 0.51%; Table 1) indicated a very low level of fill volume variation for each material. DNA homogeneity was determined for each material both within and between ampoules by DNA quantitation. Three ampoules of each material were selected at random and each

reconstituted in 100µl nuclease-free water. Following one hour equilibration at room temperature, the DNA concentration of each ampoule was measured in triplicate. Readings for each ampoule were highly consistent, giving a median coefficient of variation of 2.23% (data not shown). Mean DNA concentrations of each material ranged from 47.75 to 58.21µg/ml, with an overall mean of 53.93µg/ml (%CV 6.93) per ampoule (Table 1). Coefficients of variation between ampoules of a single material ranged from 1.22 to 7.19%.

Residual oxygen levels in the ampoules were comparable to those seen with other freeze-dried DNA reference materials^{22,24}. Residual moisture levels for 07/230 and 07/240 were higher than those obtained with other materials and may be due to limitations on water detection for such low dry masses. The WHO does not set absolute limits on residual moisture or oxygen content, provided adequate long-term stability can be demonstrated²⁵. The mean pH for all reconstituted materials was pH 7.39 (%CV 1.01). The quality of extracted and freeze-dried DNAs was confirmed by agarose gel electrophoresis (data not shown).

No evidence of cross-contamination was apparent in any blood, cell lines, pre-filled DNA or freeze-dried DNA materials as determined by DNA profiling. DNA profiles were fully consistent for each of the materials throughout the production process.

Stability

Materials were stored for two years at +45°C and +56°C to promote accelerated degradation; no significant degradation was apparent at any temperature. Quantitation of reconstituted gDNA determined comparable concentrations for each material across all temperatures, mean 49.36µg/ml (%CV 8.59) and continued acceptable 260/280 absorbance ratios as an indicator of DNA purity, mean 1.78 (%CV 5.85; Figure 1a). Q-PCR determined higher cycle threshold (Ct) values with materials

stored at +56°C compared to -150°C materials, the greatest difference being 0.67 for 07/234, equivalent to a 37% decrease in amplifiable DNA. Agarose gel electrophoresis showed no apparent differences in the electrophoretic profiles of the materials at all temperatures; a high molecular weight band of consistent intensity and the absence of low molecular weight degraded gDNA was apparent for all samples (Figure 1b). Overall it was considered that any limited degradation at +56°C and the absence of degradation at +45°C indicated the materials to be stable for many years.

International Validation of Materials

Seventy seven laboratories were invited to join an international collaborative study to validate the performance of the materials as controls in the genetic diagnosis of PWS and AS; thirty seven laboratories representing twenty six countries participated (Supplemental Table 1). Eleven different methods were used (Supplemental Table 2). Results for each material are detailed in Supplemental Tables 3-8.

MS-MLPA

MS-MLPA determined copy number and CpG island methylation in the 15q11-q13 region. Sixteen laboratories used MS-MLPA; four laboratories supplementing the results with those from a second method. Twelve laboratories reported concordant results for all materials. Four laboratories were unable to report a result for one of the three triplicate samples for up to three of the materials. One of these laboratories additionally reported signals from *NDN* and *SNRPN* probes to have a normal methylation pattern in one of the three triplicate samples for 07/238 resulting in a PWS IC defect interpretation.

Since the collaborative study, a new version of the MS-MLPA kit was issued and three laboratories re-tested the materials. Data presented are mean results for three independently tested ampoules of each material from laboratory 26 (Figures 2 & 3).

Copy number analysis (Figure 2) was by comparison of 32 probes specific for the PWS/AS critical region (probe numbers 17-48) to 16 reference probes ligated to genes outside of the region (probe numbers 1-16; for full probe details see Supplemental Table 9). A deletion in the PWS/AS critical region was determined for three materials; 07/230 (including *NIPAI* to *GABRB3*, class I deletion), 07/236 (including *NIPAI* to at least *APBA2*, deletion beyond BP3) and 07/240 (including *NIPAI* to *GABRB3*, class I deletion), with no copy number changes in 07/232, 07/234 or 07/238. CpG island methylation within the PWS/AS critical region was determined by a second reaction (Figure 3); probes were simultaneously ligated and digested by methylation-sensitive *HhaI* enzyme such that a methylated CpG island should result in a normal ligated product whereas an unmethylated CpG island should be digested and no product formed. Five probes were specific for an imprinted (50% methylated) sequence within the PWS/AS critical region (probe numbers 44-48) and should result in 50% ligated product. Methylation analysis confirmed AS in 07/230 and 07/234 (presence of only unmethylated paternal *NDN* and *SNRPN*) and PWS in 07/236, 07/238 and 07/240 (presence of only methylated maternal *NDN* and *SNRPN*). Material 07/232 had a pattern typical of 'normal' DNA (presence of unmethylated and methylated *NDN* and *SNRPN*). There were no spurious results for any of the probes in the copy number analysis. However, for each material there was at least one spurious probe result in the methylation analysis, although the overall interpretations were not compromised.

MS-PCR

Bisulphite modification of methylated (maternal) *SNRPN* in MS-PCR allowed for differential amplification of the maternal and paternal alleles using allele-specific primers (Figure 4). Twenty one laboratories used MS-PCR; six laboratories

supplementing the results with those from a second method. Seventeen laboratories reported concordant results for all materials. One laboratory could not generate results or did not provide interpretations for many of the samples. The presence of additional faint maternal (in AS) or paternal (in PWS) PCR products for materials 07/230, 07/234, 07/236, 07/238 and 07/240 was reported by four laboratories using all three of the MS-PCR methods¹⁴⁻¹⁶; for one laboratory this did not lead to discordant overall interpretations. These additional weak PCR products in MS-PCR were not consistently present in triplicate samples or across multiple laboratories using the same techniques.

07/232 and UBE3A Sequencing

Five laboratories carried out *UBE3A* sequencing on material 07/232 in addition to either MS-MPLA or MS-PCR; all reported *UBE3A* mutation c.1234A>T p.Lys412Stop. Twenty four laboratories reported that their method (excluding sequencing) did not detect a mutation but did not make a 'Normal' interpretation. One laboratory similarly did not make a 'Normal' interpretation for two of the samples, but was unable to provide a result for one of the samples, and one laboratory also did not make a 'Normal' interpretation for one of the samples, but reported a 'Suggested mosaic AS' interpretation for two samples based upon the presence of a faint paternal PCR product in MS-PCR. One laboratory reported an AS interpretation for one sample but this was based upon discordant methylation data; an interpretation was not made for the other two samples. Five laboratories reported the interpretation as either 'Negative AS' or 'Normal' without suggesting a possible *UBE3A* point mutation. A point mutation in *UBE3A* is present in approximately 10% of AS individuals¹⁰ and thus 07/232 was considered to be an important component of the panel.

Other Techniques

One laboratory did not use either MS-MLPA or MS-PCR, instead reporting concordant results with methylation-specific melting analysis¹⁸. Concordant results were also reported with Southern blot analysis, microsatellite analysis, methylation-sensitive PCR and denaturing high-performance liquid chromatography.

Control Materials

Eighteen laboratories used more than one batch of reagents and testing was carried out by more than one operator in twelve laboratories. The controls used by participants were as follows: 31 laboratories used in-house materials (previously characterised clinical samples from patients and normal controls), 2 laboratories used DNA samples supplied by the Coriell Institute (for 1 laboratory this was in addition to in-house controls), 2 laboratories used CAP survey materials and 2 laboratories did not specify which control material they used. Laboratory 22 stated that no control samples were used.

Discussion

The first International Genetic Reference Panel was approved by the ECBS of the WHO in 2004 and comprised a panel of three materials for the genetic diagnosis of Factor V Leiden²⁴. This certification approach was used for the Prothrombin variant G20210A, Factor VIII intron 22 inversion (EG, unpublished data) and Fragile X²² genetic reference panels. In the current study a similar approach was adopted to produce a genetic reference panel for PWS and AS; cell lines were established from well-characterised patients in order to assure a continued future supply of genetic material, and following large-scale cell culture and DNA extraction, a panel of gDNAs was freeze-dried and sealed in ampoules.

Characterisation of the PWS and AS materials determined the absence of viral contamination and cross-contamination with other materials by PCR and DNA profiling, respectively. Homogeneity studies indicated low levels of variation both within and between ampoules of the same material, though gDNA concentrations varied between the six different materials (47.75 to 58.21µg/ml). The 'Instructions For Use' accompanying each panel prompts the user to measure the DNA concentrations before use. Accelerated degradation studies determined limited gDNA degradation at +56°C by Q-PCR. The absence of degradation at +45°C and the storage temperature of -20°C indicated the materials to be stable for many years. Ongoing monitoring will continue to verify stability at these temperatures.

Thirty seven laboratories participated in an international collaborative study to validate the suitability of the proposed panel of gDNA materials for use in the *in vitro* diagnosis of PWS and AS. The study was designed to determine the performance of the panel in a large number of laboratories using a variety of methods. All materials performed well in the study and most laboratories delivered the expected results. A total of 44 discordant interpretations were made; a frequency of 6.61% for 666 tests (37 laboratories each testing 18 samples). However, this frequency is reduced to 4.35% if the 15 'Normal' or 'Negative AS' interpretations for un-sequenced 07/232 (*UBE3A* point mutation) are excluded. This frequency of discordance is higher than in previous collaborative studies with our other panels (Factor V Leiden, 0.7%²⁴; Prothrombin mutation G20210A, 0.7%; Factor VIII intron 22 inversion, 1.8% [EG, unpublished data]) but similar to Fragile X, 4.9%²², reflecting the challenging nature of PWS and AS the molecular diagnosis and emphasising the need for reference materials. All discordant data were derived from nine laboratories only, and moreover, 14 of the 44 discordant interpretations were from laboratory 22 which

could not provide an interpretation for many of the samples. In no other laboratory did all three samples of the same material fail to work or produce a discordant result. Overall it would appear that discordant results were due to laboratory technique or interpretation rather than an inherent problem with the materials.

Concordant results were reported using a range of techniques: MS-MLPA, MS-PCR, Southern blot analysis, methylation-sensitive PCR, denaturing high-performance liquid chromatography and methylation-specific melt curve analysis. Microsatellite analysis was also performed, although of limited diagnostic value in the absence of parental DNA. Laboratory 22 was not able to provide results from classic comparative genomic hybridisation (CGH), although the materials were successfully validated in-house with array CGH (data not shown).

Normal variation in methylation levels and copy number changes in the PWS/AS critical region have been reported in normal individuals^{10,26,27} and may be detected by MS-MLPA or MS-PCR. Atypical methylation signals for some *SNRPN* and *NDN* probes were reported with MS-MLPA in the collaborative study. For 07/230, the elevated methylation result for *NDN* (probe 44) is likely explained by a known partial methylation which can occur with paternal *NDN* in both patient and normal samples (MRC-Holland; ref. 10). *NDN* is also reported to undergo methylation changes in lymphoblastoid cell lines and thus the result for 07/230 may also be a cell culture artefact²⁸. For materials 07/232, 07/236, 07/238 and 07/240, methylation of the SNORD116 snoRNA cluster (probe 43) was reduced from the expected 100% methylation. Whilst this may be due to normal methylation variations in the human population, with such a frequency (four of the six materials) a cell culture effect on methylation as reported elsewhere for *SNRPN* seems more likely²⁹. The elevated methylation for *SNRPN* (probe 48) in 07/234, and the reduced methylation for *SNRPN*

(probe 46) in 07/240 may be explained as cell culture artefacts or normal methylation variations within patients. However, since the four *SNRPN* probes in MS-MLPA are in close proximity and expected to provide similar results, the recommendation is to take the average methylation, which for both materials provides a conclusive result (0.007 for 07/234, close to 0.000 typical with AS; 0.972 for 07/240, close to 1.000 typical with PWS). And thus these specific probe results do not affect the overall interpretation of any of the materials.

In MS-PCR, the appearance of a very faint second PCR product in samples 07/230, 07/234, 07/236, 07/238 and 07/240 could not conclusively be attributed to the materials as results were variable across the collaborative study. This inconsistent presence of faint reciprocal MS-PCR products and the presence of intermediate signals for particular probes in MS-MLPA for some of the materials may be attributed to normal methylation variations seen in normal individuals and/or a cell culture artefact. However, the 'Instructions For Use' accompanying each panel does highlight the dosage results expected for each material in MS-MLPA and the possibility of additional faint products in MS-PCR to avoid incorrect interpretation. The overall normal methylation pattern for material 07/232 indicates that this DNA may also serve as a 'normal control' in MS-MLPA and MS-PCR analyses.

These materials are well-characterised and prepared in large quantity (2000 ampoules minimum) from a single DNA stock. Since methylation (and copy number) variation is reported in the PWS/AS critical region, the materials are representative of actual patient samples. Some small changes to methylation patterns may not be fully typical of those seen in patients, but are consistent across the ampoule stock and well-characterised. Furthermore, the overall PWS/AS genotype of each material complemented that of the original primary patient material, indicating that any cell

culture/EBV transformation effect on methylation did not alter or obscure the overall disorder-associated methylation status. The production of each material from a large stock of pooled DNA derived from a single batch of cultured cells eliminates the need for regular replacement of the panel and ensures a long-term supply of well-characterised identical materials. However, should the panels ever be exhausted, replacement gDNA materials derived from cell banks would be fully re-characterised in a new collaborative study to ensure any changes to the materials, for example changes in methylation status due to further cell culture passaging, would be captured. The international multi-centre study verified all six materials as suitable for use as genetic reference materials in laboratories carrying out genotyping for PWS and AS; all participating laboratories were in agreement. The need for such materials is demonstrated by the worldwide high frequency of PWS and AS tests performed, and moreover the genetic complexity of the disorders rendering *in vitro* testing particularly challenging. The well-characterised materials can be used to validate *in vitro* diagnostic kits, new in-house methods, or changes to reagents, operator or equipment in existing techniques. Reference materials for use in *in vitro* diagnosis must be CE-marked in the EU, FDA-approved in the USA, or otherwise deemed to be of 'higher order', for example WHO International Standards. The panel was established by the Expert Committee for Biological Standardization of the WHO as the 1st International Genetic Reference Panel for Prader Willi and Angelman Syndromes, Human gDNA (NIBSC code 09/140), in November 2009 and is the only available certified reference material for the *in vitro* diagnosis of PWS and AS.

Acknowledgements

This work was partly funded by the UK Department of Health through the National Genetics Reference Laboratory (Manchester) and an EU FP6 Network of Excellence EuroGentest Contract 512148. All laboratories are thanked for their participation in the collaborative study. Simon Patton (European Molecular Genetics Quality Network, Manchester) is acknowledged for helping source diagnostic laboratories.

Conflict of Interest Statement

A fee is charged for the panel by NIBSC to cover handling and shipping.

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Titles and legends to figures

Table 1. Product summaries for the six Prader Willi and Angelman syndrome genetic reference materials.

Figure 1. Accelerated degradation. Freeze-dried ampoules were stored at temperatures -150°C to +56°C. Two ampoules of each material were retrieved from each temperature after two years. **a. Summary of DNA concentrations and Q-PCR.** Nanodrop-determined DNA concentrations were derived from triple readings from each ampoule of the pair. Mean threshold cycle (Ct) results were derived from one assay from each ampoule of each pair (results derived from a single ampoule only are highlighted in bold italics). **b. Agarose gel.** 0.7% agarose gel of 100ng DNA from each ampoule was resolved. L, size marker Lambda DNA/HindIII; R, 100ng Roche genomic DNA as control.

Figure 2. MS-MLPA copy number analysis. Data are mean results for three ampoules of each material tested by laboratory 26. **a. Summary of all 48 probe results for the ligation reaction.** Results are grouped into unaltered probe peak ratios (no copy number change) and probes showing a reduced peak ratio (deletion). ¹07/230 and 07/240; probes 18-48 including *NIPAI* to *GABRB3*, class I deletion; ²07/234; falsely high peak ratio for one sample with probe 9 due to a pull-up peak resulting in high overall %CV for all probes; ³07/236; probes 17-48 including *NIPAI* to at least *APBA2*, deletion beyond BP3. **b. Scatter plot of all 48 probes for the ligation reaction.** Closed diamonds represent no copy number change (peak ratio approximately 1.0); open diamonds represent copy number change, deletion (peak ratio approximately 0.5). ¹07/234; falsely high peak ratio for one sample with probe 9

due to a pull-up peak resulting in high mean (1.532) and high %CV (63.953) for probe 9. Error bars represent standard deviation of the mean peak ratio of each probe from three tested ampoules.

Figure 3. MS-MLPA methylation analysis. Data are mean results for three ampoules of each material tested by laboratory 26. **a. Summary of results for probes 42-48 only, detecting unmethylated sequence (probe 42), fully methylated sequence (probe 43) and imprinted sequence (probes 44-48) in the wildtype PWS AS region.**¹Percentage methylated in normal individuals, data from MRC-Holland; ²variable methylation in normal individuals; ³imprinted sites are methylated in maternal, unmethylated in paternal. Spurious results are highlighted in bold italics. **b. Scatter plot of all 48 probes for the methylation reaction.** Closed diamonds represent expected methylation results in a patient with a normal methylation pattern in the PWS AS region; open diamonds represent a gain (to peak ratio approximately 1.0) or loss (to peak ratio approximately 0.0) of methylation status. Arrows indicate the actual expected outcome for spurious probe results. ¹07/234; falsely low peak ratio for one sample with probe 9 due to a pull-up peak leading to low mean (0.809) and high %CV (44.983) for probe 9. Error bars represent standard deviation of the mean peak ratio of each probe from three tested ampoules.

Figure 4. MS-PCR analysis. Materials were tested according to the Zeschnigk *et al.*¹⁴ method for differential amplification of the maternal and paternal *SNRPN* exon 1 region following bisulphite modification of methylated (maternal) DNA. Allele-specific primers produce a 313bp maternal PCR product and a 221bp paternal PCR product. PWS, positive control in-house PWS UDP DNA; N, positive control in-

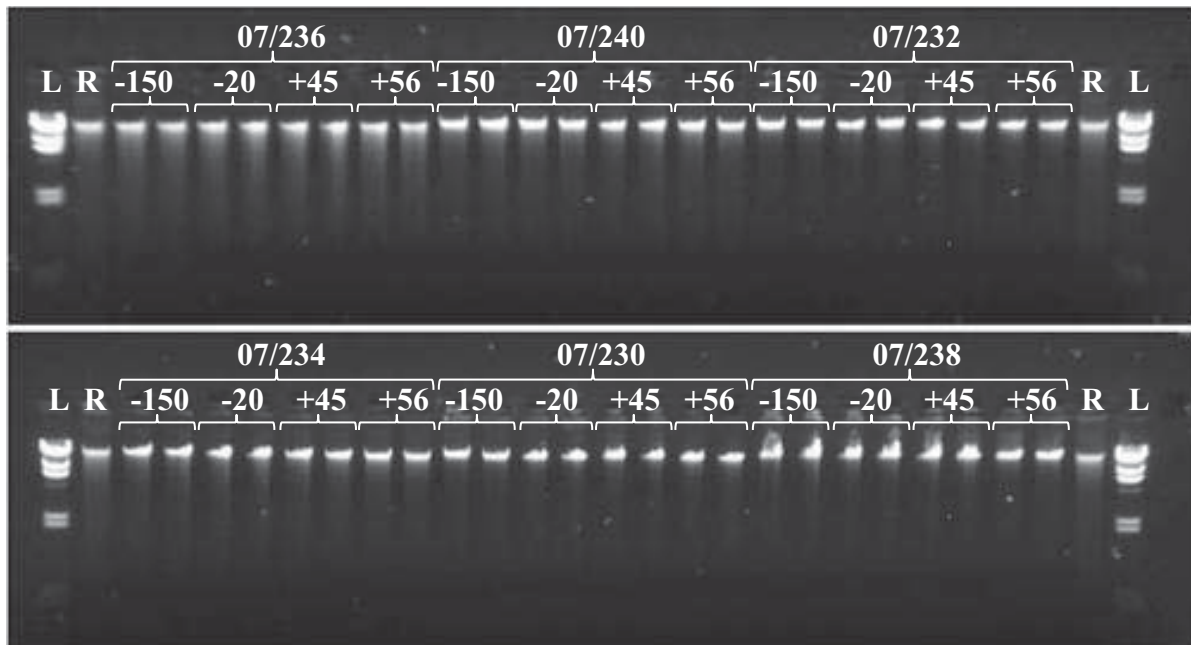
house normal DNA; AS, positive control in-house AS maternal deletion DNA; C,
positive control in-house PWS wildtype methylation DNA; H2O, blank control.

NIBSC material code	07/230	07/232	07/234	07/236	07/238	07/240
Genotype	AS, maternal deletion, class I	AS, <i>UBE3A</i> point mutation	AS, paternal UPD or epigenetic IC defect	PWS, paternal deletion, translocation	PWS, maternal UPD	PWS, paternal deletion, class I
Coefficient of variation of fill mass (%; n=84-165)	0.31	0.51	0.27	0.34	0.20	0.26
Mean % residual moisture after lyophilisation (%; n=6-11)	7.21	2.17	1.75	1.41	0.60	7.11
Mean dry weight (g; n=6)	0.0035	0.0033	0.0037	0.0032	0.0048	0.0024
Mean residual oxygen (%; n=6-11)	0.14	0.18	0.43	0.34	0.48	0.30
Mean OD ratio 260/280 nm (n=27)	1.91	1.92	1.92	1.93	1.89	1.95
Mean DNA conc. post-reconstitution in 100 µl H ₂ O (µg/ml; n=27)	55.03 (%CV 7.19)	47.75 (%CV 5.88)	56.23 (%CV 2.92)	54.88 (%CV 1.22)	58.21 (%CV 3.97)	51.49 (%CV 6.10)
Mean pH (n=9)	7.40	7.35	7.30	7.40	7.42	7.47
Presentation	Sealed, glass DIN ampoules					
Excipient	Trehalose, 5 mg/ml in 2.0 mM Tris, 0.2 mM EDTA buffer					
Storage temperature	-20°C					

a

NIBSC material code	Storage Temperature (°C)															
	-150				-20				+45				+56			
	DNA conc.		Q-PCR		DNA conc.		Q-PCR		DNA conc.		Q-PCR		DNA conc.		Q-PCR	
	Mean conc.	%CV	Mean Ct	%CV	Mean conc.	%CV	Mean Ct	%CV	Mean conc.	%CV	Mean Ct	%CV	Mean conc.	%CV	Mean Ct	%CV
07/230 AS, maternal deletion, class I	49.02	1.39	19.59	0.18	47.45	1.35	19.72	<i>N/A</i>	45.31	8.42	19.67	0.47	46.41	1.02	19.86	0.43
07/232 AS, <i>UBE3A</i> point mutation	43.76	7.52	19.68	0.68	44.17	7.10	19.92	1.60	45.55	7.94	19.72	0.47	44.02	2.91	20.20	0.81
07/234 AS, paternal UPD or epigenetic IC defect	51.10	2.91	19.42	0.00	52.77	2.44	19.27	0.15	51.66	4.80	19.69	0.93	48.60	4.55	20.09	<i>N/A</i>
07/236 PWS, paternal deletion, translocation	49.65	3.49	19.45	0.04	49.46	2.00	19.48	0.94	48.20	4.28	19.72	0.32	48.84	3.51	19.80	0.46
07/238 PWS, maternal UPD	54.13	9.51	19.64	0.04	53.59	6.98	19.51	0.33	57.24	3.14	19.54	0.29	55.36	1.96	19.74	0.64
07/240 PWS, paternal deletion, class I	51.34	2.35	19.50	1.49	51.61	3.48	19.82	1.28	45.42	6.00	19.52	0.14	50.08	2.01	19.60	<i>N/A</i>

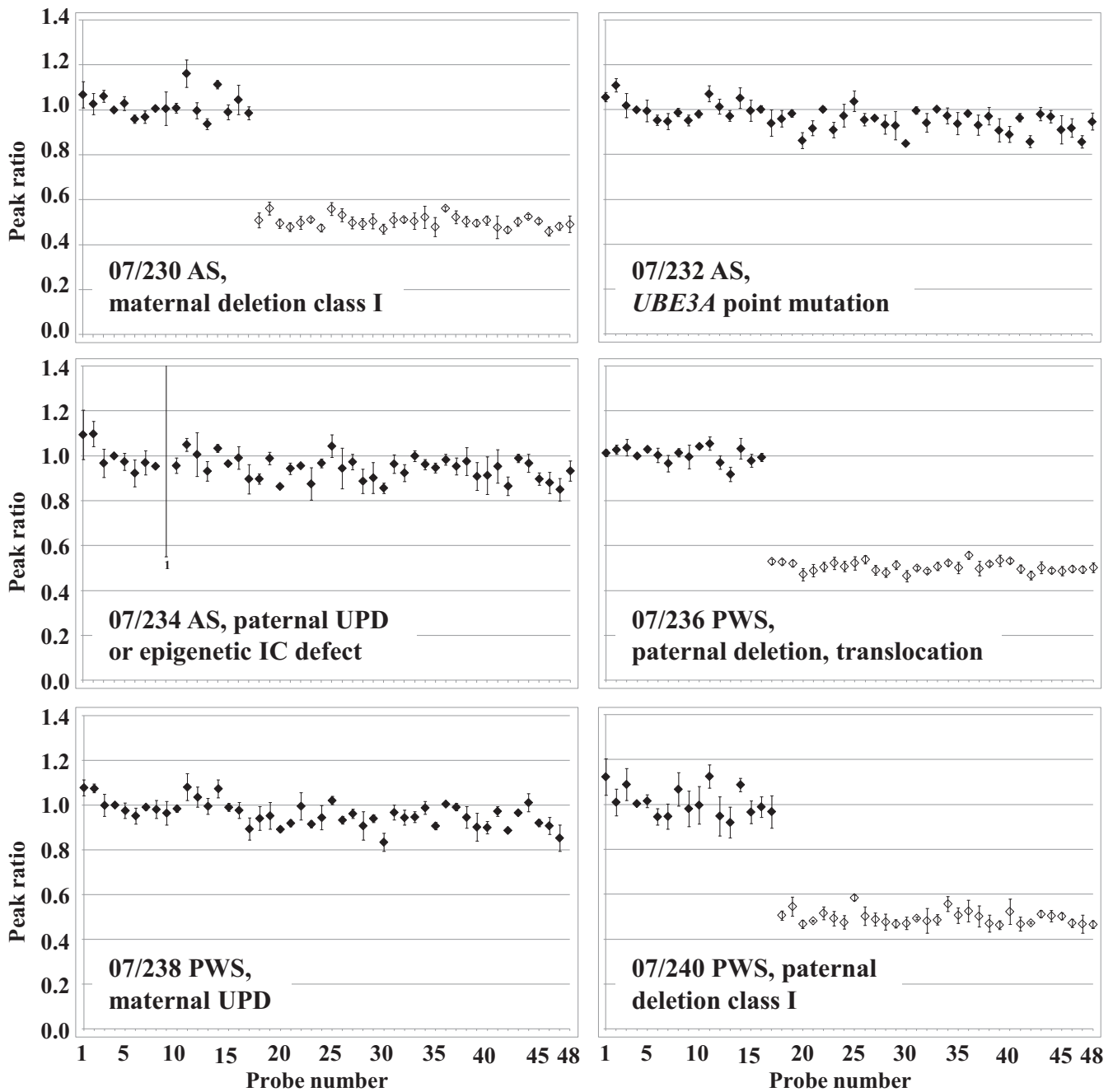
b



a

NIBSC material code	Peak Ratio				Result
	Probes: no copy number change (expected 1.000)		Probes: deletion (expected 0.500)		
	Mean	%CV	Mean	%CV	
07/230 AS, maternal deletion class I	1.022	6.242	0.504 ¹	6.762	Deletion
07/232 AS, <i>UBE3A</i> point mutation	0.964	6.402	/	/	No deletion
07/234 AS, paternal UPD or epigenetic IC defect	0.965	16.401 ²	/	/	No deletion
07/236 PWS, paternal deletion, translocation	1.005	4.149	0.506 ³	5.321	Deletion
07/238 PWS, maternal UPD	0.961	6.559	/	/	No deletion
07/240 PWS, paternal deletion class I	1.011	8.056	0.495 ¹	7.794	Deletion

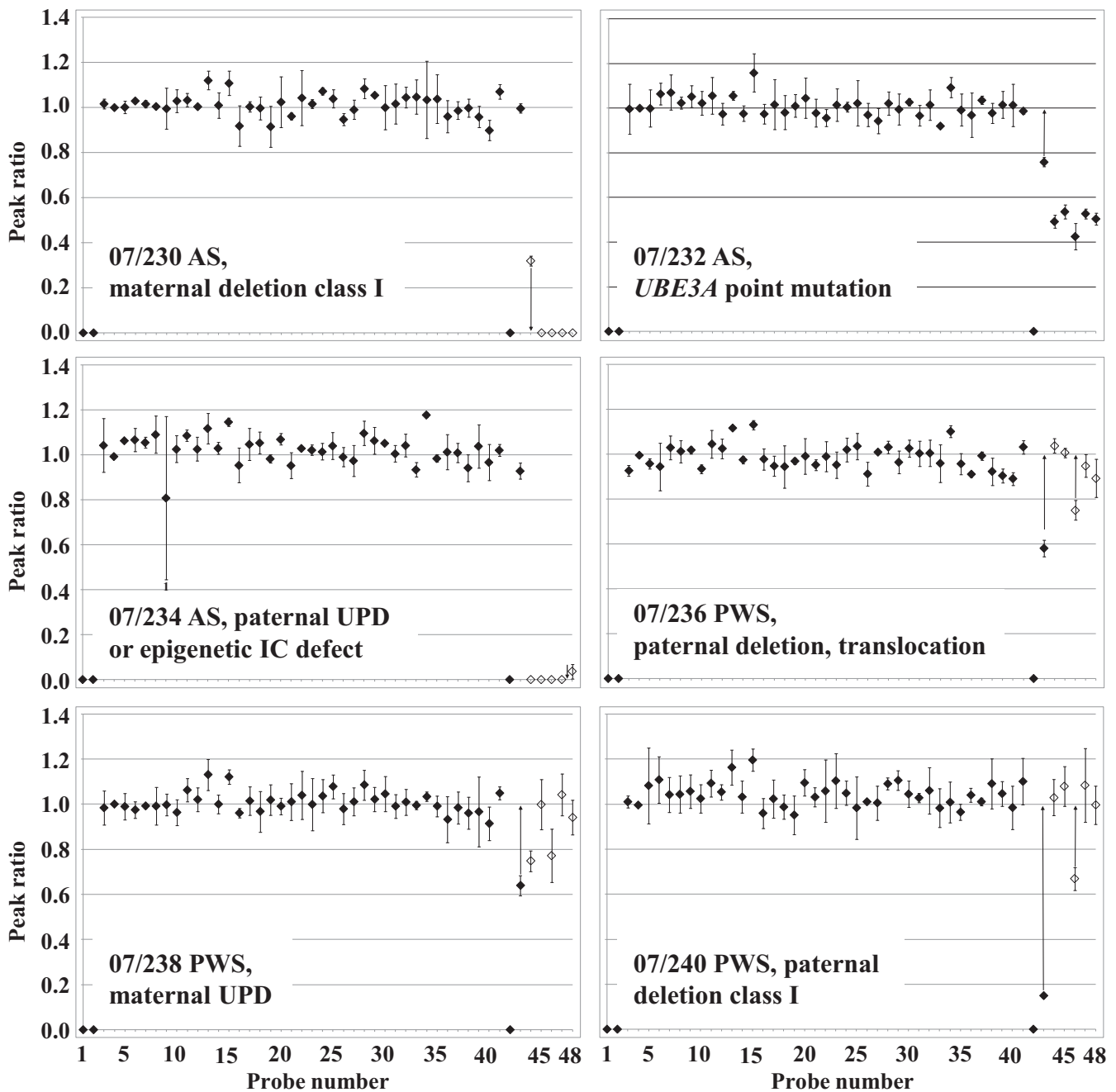
b



a

Probe	Location	% methylated ¹	NIBSC material code												
			07/230		07/232		07/234		07/236		07/238		07/240		
			Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV	
42	<i>UBE3A</i> probe 04641-L04293	<i>UBE3A</i> exon 1	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
43	<i>SNRPN</i> probe 12723-L13798	<i>SNORD116</i> snoRNA cluster	100	0.996	2.096	0.758	2.745	0.929	3.822	0.581	6.229	0.640	6.938	0.149	6.329
44	<i>NDN</i> probe 04027-L13937	<i>NDN</i>	30-50 ²⁻³	0.320	7.036	0.492	5.691	0.000	0.000	1.038	3.098	0.748	6.016	1.030	7.757
45	<i>SNRPN</i> probe 04106-L13905	<i>SNRPN</i> CpG island exon 1	50 ³	0.000	0.000	0.536	5.970	0.000	0.000	1.007	2.045	0.999	11.098	1.080	8.080
46	<i>SNRPN</i> probe 04103-L02951	<i>SNRPN</i> CpG island intron 1	50 ³	0.000	0.000	0.425	13.984	0.000	0.000	0.750	5.849	0.772	15.408	0.669	7.466
47	<i>SNRPN</i> probe 04104-L04294	<i>SNRPN</i> CpG island intron 1	50 ³	0.000	0.000	0.527	4.225	0.000	0.000	0.948	5.328	1.043	8.917	1.084	15.186
48	<i>SNRPN</i> probe 11181-L13997	<i>SNRPN</i> CpG island promoter	50 ³	0.000	0.000	0.504	5.159	0.037	86.988	0.893	9.546	0.942	8.092	0.997	8.479
Overall mean for probes 44-48			0.064	207.45	0.497	10.223	0.007	264.352	0.927	12.260	0.901	16.252	0.972	18.689	
Result			AS		Normal		AS		PWS		PWS		PWS		

b



07/230 07/238 07/234 07/240 07/232 07/236 PWS N AS C H2O

