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

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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 paternally or maternally inherited genes in the region, respectively. With clinical diagnosis often being 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 Willi syndrome materials (two with different paternal deletions, and one with maternal uniparental disomy (UPD)) and three Angelman syndrome materials (one with a maternal deletion, one with paternal UPD 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. In total, 37 laboratories from 26 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 first International Genetic Reference Panel for Prader Willi and Angelman syndromes.

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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 because of the relatively nonspecific findings, particularly in infancy, and the clinical overlap with other disorders.^{1–3} The incidence of PWS is reported as 1 in 25 000; however, as many diagnoses are not made at an early age, a more realistic estimate is 1 in 10 000 to 1 in 15 000.¹ AS clinical diagnosis may be delayed or incorrect as the unique features may not become apparent for several years and there are many other disorders with clinical overlap.^{4,5} The incidence of AS is reported as between 1 in 12 000 and 1 in 20 000, but again this may be an underestimate because of the difficulty of clinical diagnosis at an early age.⁶

Genetic testing for PWS and AS has become the standard diagnostic method as clinical criteria, although 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 as 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, whereas 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 and 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

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approach will detect more than 99% of PWS individuals and ~80% of AS individuals. Sequence analysis of *UBE3A* will detect a further ~10% of individuals with AS.¹⁰ Abnormal methylation is most commonly detected by either methylation-specific PCR (MS-PCR) to determine methylation analysis at the *SNRPN* locus only^{14–16} or methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA; MRC-Holland, Amsterdam, The Netherlands^{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* hybridisation 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 PCR,²⁰ 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 because of the genetic complexity of the disorders and the diversity of genetic diagnostic techniques available, there is a need for widely available genetic reference materials in order to ensure a correct and consistent diagnosis. Hence, although 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 10 disorders for prioritised reference material development based on test request frequency, number of laboratories offering the test and evidence of need and demand.²¹

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, NJ, USA), either in the form of cell lines or in the form of 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 first World Health Organization International Genetic Reference Panel for Prader Willi and Angelman Syndromes.

MATERIALS AND METHODS

Patients and cell lines

A protocol for approaching the patients, obtaining informed consent and anonymising samples was carried out with approval from a local research ethics committee. 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, as 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. Cell lines were banked, and cell pellets of 10⁸ cells prepared as previously described.²² 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 a concentration of 10 µg/ml in 2.0 mM Tris, 0.2 mM EDTA with 5 mg/ml trehalose. Aliquots of 0.5 ml were dispensed into glass ampoules, freeze-dried and sealed in nitrogen gas for storage at –20°C. A minimum of 2000 ampoules was filled for each of the six materials. The quality of the extracted and freeze-dried DNAs was confirmed by spectrophotometry and agarose gel electrophoresis. Identity testing by DNA profiling was carried out as previously described.²²

Stability monitoring

Accelerated degradation studies on materials stored for 2 years at +45 and +56°C were conducted by comparison with materials stored at –20 and –150°C. DNA concentration was determined by spectrophotometric absorbance analysis (NanoDrop, Wilmington, NC, USA). Q-PCR was performed as previously described,²² 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 for 10 min, followed by 50 cycles of 94°C for 30 s, 58°C for 1 min and 72°C for 1 min. For agarose gel electrophoresis, Lambda DNA/*Hind*III 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 six on three separate days, using different batches of reagents and/or different operators, if possible. Laboratories were asked to sequence *UBE3A* exon 9 in the region from 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, the 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 performance of the materials.

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 that was extracted using the same purification procedure from other EBV-transformed cell lines did not show EBV infectivity.²³ Despite the considered minimal risk, material safety data sheets were provided with each panel.

Characterisation of freeze-dried DNA

Out of every 100 filled ampoules, 3 were weighed during production. The coefficient of variation (CV) 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 100- μ l nuclease-free water. Following 1-h equilibration at room temperature, the DNA concentration of each ampoule was measured in triplicate. Readings for each ampoule were highly consistent, giving a median CV of 2.23% (data not shown). Mean DNA concentrations of each material ranged from 47.75 to 58.21 μ g/ml, with an overall mean of 53.93 μ g/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 because of 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 demonstrated.²⁵ The mean pH for all reconstituted materials was 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 2 years at +45 and +56°C to promote accelerated degradation; no significant degradation was apparent at any temperature. Quantification of reconstituted gDNA determined comparable concentrations for each material across all temperatures, mean 49.36 μ g/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 with materials stored at -150°C, 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

were 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

In total, 77 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; 37 laboratories representing 26 countries participated (Supplementary Table 1). A total of 11 different methods were used (Supplementary Table 2). Results for each material are detailed in Supplementary Tables 3–8.

MS-MLPA

MS-MLPA determined the copy number and CpG island methylation in the 15q11-q13 region. Overall, 16 laboratories used MS-MLPA, 4 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, in addition, 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 and 3). Copy number analysis (Figure 2) was carried out by comparison of 32 probes, specific for the PWS/AS critical region (probe numbers 17–48), with 16 reference probes to genes outside the region (probe numbers 1–16; for full probe details see Supplementary Table 9). A deletion in the PWS/AS critical region was determined for three materials: 07/230 (including *NIPA1* to *GABRB3*, class I deletion), 07/236 (including *NIPA1* to at least *APBA2*, deletion beyond BP3) and 07/240 (including *NIPA1* 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

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

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 (%; <i>n</i> =84–165)	0.31	0.51	0.27	0.34	0.20	0.26
Mean % residual moisture after lyophilisation (%; <i>n</i> =6–11)	7.21	2.17	1.75	1.41	0.60	7.11
Mean dry weight (g; <i>n</i> =6)	0.0035	0.0033	0.0037	0.0032	0.0048	0.0024
Mean residual oxygen (%; <i>n</i> =6–11)	0.14	0.18	0.43	0.34	0.48	0.30
Mean OD ratio 260/280 nm (<i>n</i> =27)	1.91	1.92	1.92	1.93	1.89	1.95
Mean DNA conc. post-reconstitution in 100 μ l H ₂ O (μ g/ml; <i>n</i> =27)	55.03	47.75	56.23	54.88	58.21	51.49
	(% CV 7.19)	(% CV 5.88)	(% CV 2.92)	(% CV 1.22)	(% CV 3.97)	(% CV 6.10)
Mean pH (<i>n</i> =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

Figure 1 Accelerated degradation. Freeze-dried ampoules were stored at temperatures ranging from -150 to $+56^{\circ}\text{C}$. Two ampoules of each material were retrieved from each temperature after 2 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 was used to resolve 100-ng DNA from each ampoule. L, size marker Lambda DNA/*Hind*III; R, 100-ng Roche genomic DNA as control.

imprinted (50% methylated) sequence within the PWS/AS critical region (probe numbers 44–48), and thus should result in a 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). A total of 21 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 of the three MS-PCR methods;^{14–16} for three of the laboratories this resulted in a discordant

overall interpretation for one sample only. These additional weak PCR products in MS-PCR were not consistently present in triplicate samples or across multiple laboratories using the same techniques.

Sequencing of 07/232 and UBE3A

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. A total of 24 laboratories reported that their method (excluding sequencing) did not detect a mutation; however, they 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; 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 on the presence of a faint paternal PCR product in MS-PCR. One laboratory reported an AS interpretation for one sample, but this was on the basis of discordant methylation data; 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 $\sim 10\%$ of AS individuals,¹⁰ and thus 07/232 was considered to be an important component of the panel.

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). Notes on probe results for materials ¹07/230 and 07/240: probes 18–48 including *NIPA1* to *GABRB3*, class I deletion; ²07/234: false 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 *NIPA1* 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 ~1.0); open diamonds represent copy number change, deletion (peak ratio ~0.5). ¹07/234: false 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 SD of the mean peak ratio of each probe from three tested ampoules.

Other techniques

One laboratory did not use either MS-MLPA or MS-PCR, instead reported concordant results with methylation-specific melting analysis.¹⁸ Concordant results were also reported with Southern blot analysis, microsatellite analysis, methylation-sensitive PCR and denaturing high-performance liquid chromatography.

Control materials

In total, 18 laboratories used more than one batch of reagents and testing was carried out by more than one operator in 12 laboratories. The controls used by participants were as follows: 31 laboratories used in-house materials (previously characterised clinical samples from patients and normal controls), two laboratories used DNA samples

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 wild-type PWS AS region. ¹Percentage methylation in normal individuals, data from MRC-Holland; ²variable methylation in normal individuals; ³imprinted sites are methylated in maternal and 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 ~1.0) or loss (to peak ratio ~0.0) of methylation status. Arrows indicate the actual expected outcome for spurious probe results. ¹07/234; false 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 SD of the mean peak ratio of each probe from three tested ampoules.

supplied by the Coriell Institute (for one laboratory this was in addition to in-house controls), two laboratories used CAP survey materials and two laboratories did not specify the control material that 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 Leiden.²⁴ 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

Figure 4 MS-PCR analysis. Materials were tested according to the Zeschgnigk *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 313-bp maternal PCR product and a 221-bp 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 wild-type methylation DNA; H₂O, blank control.

studies indicated low levels of variation both within and between ampoules of the same material, although gDNA concentrations varied between the six different materials (47.75–58.21 µg/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 at 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.

A total of 37 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 unsequenced 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 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 because of 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 this is 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 that can occur with paternal *NDN* in both patient and normal samples (MRC-Holland; see Ramsden *et al*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 artefact.²⁸ 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. Although 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 likely.²⁹ 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, as the four *SNRPN* probes in MS-MLPA are in close proximity and are 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). 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 the presence 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. As 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 are 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 multicentre 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 first 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.

CONFLICT OF INTEREST

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

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (<http://www.nature.com/ejhg>)