Two high-throughput technologies to detect segmental aneuploidies identify new Williams-Beuren Syndrome patients with atypical deletions

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Abstract

Introduction
We developed and compared two new technologies to diagnose a contiguous gene syndrome, the Williams-Beuren syndrome (WBS).

Methods
The first proposed method, named Paralogous Sequence Quantification (PSQ), is based on the use of paralogous sequences located on different chromosomes and quantification of specific mismatches present at these loci using the Pyrosequencing technology. The second exploits quantitative real-time PCR (QPCR) to assess the relative quantity of an analyzed locus.

Results
A correct and unambiguous diagnosis was obtained for 100% of the analyzed samples with either techniques (n=165 and n=155, respectively). These methods allowed the identification of two patients with atypical deletions in our cohort of 182 WBS patients.

Conclusions
We conclude that both PSQ and QPCR are robust, easy to interpret and simple to set-up methods to diagnose segmental aneuploidies, thus representing a competitive alternative for widespread use in diagnostic laboratories. These methods have advantages over Fluorescence *in situ* Hybridization or microsatellites/SNP genotyping for detecting short segmental aneuploidies since the former is costly and labor intensive and the latter dependent on informativeness of the polymorphisms. Both patients with atypical deletions presented mild facial anomalies, mild mental retardation with impaired visuo-spatial cognition, supravalvular aortic stenosis and normal growth parameters. These observations are consistent with the involvement of GTF2IRD1 and/or GTF2I in some of the WBS facial features.

Keywords
Williams-Beuren syndrome, aneuploidy, diagnosis, genomics, real-time PCR, pyrosequencing

Introduction

An increasing number of human diseases are recognized to result from recurrent DNA rearrangements involving unstable genomic regions. Both inter- and intrachromosomal rearrangements are facilitated by the presence of region-specific Low-Copy Repeats (LCRs) and result from nonallelic homologous recombination. These diseases are traditionally diagnosed by FISH (Fluorescence *in situ* Hybridization)[1] and/or microsatellite/SNP genotyping [2]. FISH has the disadvantages of being labor intensive and expensive, whereas microsatellite/SNP genotyping depends on the relative informativeness of the polymorphisms used and on parental DNA to ascertain the deletion.

The completion of the human genome sequence [3-5] has provided an extensive catalog of sequence features that can be exploited for the design of new diagnostic strategies. Here we propose and validate two recently described PCR-based diagnostic approaches to identify contiguous gene syndromes. We compare their efficiency and practicality to detect the 7q11.23 microdeletion in Williams-Beuren Syndrome (WBS) patients [6]. WBS has a prevalence estimated between 1/7500 and 1/20000 [7-9]. Patients are characterized by mental retardation with unique cognitive and personality profile, distinctive facial features, supravalvular aortic stenosis, short stature, connective tissue anomalies, hypertension, infantile hypercalcemia, dental and kidney abnormalities, premature aging of the skin, impaired glucose tolerance and silent diabetes [10-12].

The first method is based on the use of paralogous sequences located on different chromosomes. Because these sequences are under different selective constraint, they accumulate nucleotide substitutions in a locus specific manner. Thus each locus will contain specific internal sequence differences (Paralogous Sequence Mismatches or PSMs), which can be exploited to estimate their relative quantity. Technically, we co-amplify paralogous fragments of identical size that map to different autosomes and quantify the PSMs to detect the presence of chromosome number abnormalities. This technology, named Paralogous Sequences Quantification (PSQ) was recently successfully implemented to detect autosomal trisomies, as well as numerical abnormalities of sex chromosomes [13].

The second methodology, real-time Quantitative Polymerase Chain Reaction (QPCR), allows detection of a PCR product as it accumulates during the reaction [14, 15]. The higher the copy number of a target sequence, the earlier a significant increase in fluorescence will be detected. We can thus estimate the relative quantity of an analyzed locus. By designing multiple assays within and next to a segmental aneuploidy, we should be able to map exactly the extension of an indel. We report the implementation of these technologies to diagnose WBS and to map the breakpoints of atypical rearrangements.

Materials and Methods

Samples

Samples were collected by the C.S.S.-Mendel Institute, Roma (140 Italian WBS patients and 43 Italian controls), the Aghia Sofia Children's Hospital, Athens (31 Greek WBS patients) and the Department of Genetic Medicine and Development, University of Geneva, Geneva (34 French controls from the Departments of Savoie and Haute-Savoie, 11 WBS patients and 15 controls from Western Switzerland) with appropriate informed consent obtained by the physician in charge and approval of the local ethical committee. Genomic DNA was prepared with either the PUREGENE whole blood kit (Gentra Systems) or the QIAamp kit (Qiagen).

Paralogous sequence quantification and pyrosequencing

Paralogous sequence quantification and pyrosequencing were performed as described [13]. The forward and reverse primers and the internal sequencing primers were designed using Oligo 6.55 (Molecular Biology Insights) and the manufacturer's site (http://www.pyrosequencing.com/index.html), respectively. Reverse and sequencing primers were desalted, while the biotinylated forward primers were desalted and purified by HPLC (Sigma-Genosys). PCR reactions with the selected primer pairs (Table S1) were set up in a total volume of 25 µl containing 30 ng of genomic DNA, 400 µM of each primer and 200 µM dNTPs. We used 1.25 units of a standard *Taq* polymerase in the manufacturer provided buffer (Amersham Biosciences), or alternatively the ready made 2xJumpstart PCR mastermix (Sigma-Genosys) with varying levels of $MgCl_2$ and DMSO depending on the assay (Table S1). Three negative controls per plate were added and the PCR products were checked on agarose gel to ensure lack of PCR contamination and correct amplimer size. PCR products were purified, and annealed to an internal sequencing primer close to the PSM site to be quantified (Table S1; Figures S1-S2). The purification and pyrosequencing steps were performed following the instructions of the manufacturer (Biotage). The Pyrosequencing software directly outputs a quantitative value for the proportion of each PSM present in the PCR product (Figure S3). We used the relative abundance of the PSM of the 'query' chromosome as our statistic for all calculations. The methods used to determine the PSM relative abundance that could be confidently diagnosed (99% confidence interval), the sensitivity and the specificity of each assay and to combine assays were described in [13].

Real-time quantitative PCR

Oligos were designed using the PrimerExpress program (Applied Biosystem) with default parameters in every case (Table S2) and ordered from Sigma-Genosys. Amplicon sequences were checked by both BLAST and BLAT against the human genome to ensure specificity. All RT-PCR reactions were performed in two replicates in 9 µl final volume with concentration of 0.96 ng/µl of DNA, 0.42 µM of each primer and 1xSybrGreen mastermix (Applied Biosystems). Reactions were set up in a 384 wells plate format with a Biomek 2000 (Beckman) and run in an ABI Prism7900HT (Applied Biosystems) with the following amplification conditions: 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C 15 sec/60°C 1 min. Raw Ct values were obtained using SDS2.0 (Applied Biosystems). We implemented the geNorm method [16] to measure relative quantities. Four normalization assays mapping to HSA21 (Table S2) and four normalization DNAs were systematically included in each run.

Results

Paralogous sequence quantification

Paralogous sequences were identified by comparing 10 kb-long fragments covering the WBS deletion [17] with the human genome sequence using both BLAT (http://genome.ucsc.edu/) and BLAST (http://www.ensembl.org/Multi/blastview). We identified two pairs of suitable paralogous sequences with one copy to chromosome 7q11.23 and only a single second copy elsewhere in the genome (Figures S1-S2). We aligned all matching sequences, and built a consensus sequence to design three discriminating Pyrosequencing assays (assay BAZ1B and assays CYLN2 and WBSCR3.4M, respectively) for WBS deletion detection. Each assay was tested with a number of PCR conditions on 10 control and 10 WBS samples. From this analysis we selected the conditions based on the following criteria: (i) the Paralogous Sequence Mismatches (PSM) quantification in control individuals should be close to 50%/50%, indicating that both 'loci' amplify with equal efficiency; and (ii) there should be a clear, non-overlapping discrimination between control and WBS samples. For the CYLN2

and WBSCR3.4M assays we found conditions that showed PSM relative abundances close to the theoretical values of 50%/50% for control individuals and 66%/33% for patients (Table S1). In contrast for the BAZ1B assay, and despite testing multiple triads of amplification and sequencing primers (data not shown), we were unable to identify conditions that produced PSM relative abundances close to the theoretical values.

To validate the method on a larger cohort, we tested 128 WBS patient, 16 probably WBS patient (see below) and 37 control samples collected by the C.S.S.-Mendel Institute. Typical results and distribution of PSM relative abundance of normal and affected samples for each assay are shown in Figure S3. We use the relative abundance of the PSM from the "query" chromosome, i.e. the chromosome we want to quantify, here HSA7, as our statistic for all calculations. For both the CYLN2 and the WBSCR3.4M assay, the distribution of the 'query' chromosome PSM relative abundance obtained for both cohorts show normal distributions with medians close to the expected results of 33% for WBS patients (CYLN2 relative abundance: mean=33±2%; WBSCR3.4M:37±2%) and 50% for control individuals (CYLN2:47±2%; WBSCR3.4M:50±2)(see Table 1). The third assay recorded relative abundances of the 'query' chromosome PSM under the expected value for both the controls and the patients samples (mean=41±6, range 39-47% and 27±5%, 12-44%, respectively) and 3% of misdiagnoses (n=160). It was therefore not retained further.

Both retained assays were characterized by no false negative or false positive calls, but some samples fell outside of the 99% confidence interval and could therefore not be unambiguously diagnosed. They were treated either as false positives or as false negatives according to the known FISH result to estimate sensitivity and specificity (Table 1). Combination of both assays resulted in a clearer separation between the control and the patient samples (Figure 1) and a significant improvement of the sensitivity (Table 1).

Table 1 Specificity and Sensitivity of PSQ assays

Assay	CYLN2	WBSCR3.4M	Combined
Mean control	46.7	50.3	50.0
SD control	1.9	2.2	1.6
Mean WBS	32.9	37.3	36.1
SD WBS	2.2	1.7	1.6
Number of samples	163	160	160
Number of uncertain samples	6	4	3
Sensitivity	0.95	0.97	0.98
Specificity	1.00	1.00	1.00

Six subjects of the "probably WBS" cohort, that were clinically diagnosed as WBS but were not analyzed by FISH and /or Southern, showed "query" chromosome relative abundance that classified them in the 99% confidence interval of the control cohort with all three assays (not shown). Consistently, for two of these patients there was only a suspicion of WBS, while a third showed facial features, but no other characteristic phenotypes that can be attributed to this disease (see below).

Quantitative real-time PCR

A quantitative real-time PCR (QPCR) method has been recently successfully implemented to diagnose aneuploid mice [18]. To assess if the technology could be used to diagnose human segmental aneuploidies and to evaluate its qualities/disadvantages vs. the PSQ technology, we applied this second high-throughput technology to diagnose the WBS segmental aneuploidy. Moreover, implementation of this second procedure should allow to clarify ambiguous cases

and atypical deletions and, eventually, exactly map the breakpoints of unusual rearrangements.

We designed 20 QPCR assays spread from HSA7 bp 70012994 to bp 75057642 (Table S2) and preliminarily tested them on DNAs from four controls and three WBS patients. A set of 11 assays were retained and used to analyze 94 WBS patients, 16 probably WBS and 61 controls collected by the C.S.S.-Mendel Institute (55 WBS, 16 probably WBS, 14 controls), the Department of Genetic Medicine, Geneva (8 patients, 47 controls) and the Aghia Sofia Children's Hospital, Athens (31 patients). These assays map either centromerically (2 assays), inside (6) or telomerically (2) to the commonly deleted region. A further assay, named 4_WBSCR16, maps between the two blocks of repeats that flank the telomeric side of the WBS critical region inside the WBSCR16 gene [19]. Mean results and typical examples are presented in Figures 2 and S4, respectively. These assays show mean results close to the expected value of 1 for controls. Consistently, the mean results obtained with the patients samples are close to 1 for assays outside the common deletion and to 0.5 for assays inside the deletion (Table 2). All six diagnostic assays showed no false negative or false positive calls, but a few samples (0.7 to 4.6%) fell outside of the 99% confidence intervals for one (12 samples), three (2 samples) or even four assays (1 sample; Table 2). However even these samples can be unambiguously diagnosed upon combination of the six assays (pairwise t-test: all individual p values <0.0016, n=153, atypical deletions not included, see Table 2). To further validate this method, we performed a blind test on 24 DNAs collected by the Department of Genetic Medicine, Geneva and previously analyzed by FISH. We confirmed the clinical diagnosis of WBS (11 cases) and normals (15 cases) for the 7q11.23 region in all cases (all individual p values <0.0009).

Table 2 Specificity and Sensitivity of QPCR assays

Assay	20_Q8N8C3	19_WBSCR17	17_BAZ1B	15_TBL2	14_WBSCR14	9_ELN	6_RFC2	5_CYLN2	4_WBSCR16	3_HIP1	2_CCL26	6 Dx assays Combined
Mean control	1.01	1.06	0.81	1.06	1.04	0.99	1.04	0.98	0.91	0.86	1.07	0.99
SD control	0.12	0.09	0.10	0.11	0.12	0.10	0.11	0.10	0.09	0.12	0.12	0.13
Mean WBS	1.07	1.02	0.43	0.51	0.53	0.48	0.51	0.48	0.92	0.86	1.09	0.49
SD WBS	0.18	0.13	0.11	0.06	0.07	0.05	0.06	0.06	0.12	0.18	0.13	0.08
Number of samples	153	153	152	153	153	152	152	152	153	153	153	153
Number of uncertain samples			2	2	1	7	4	6				0
Sensitivity			0.98	0.99	0.99	0.96	0.97	0.96				1.00
Specificity			1.00	0.98	1.00	0.96	0.98	0.97				1.00

The six patients of the "probably WBS" group, that exhibit PSM relative abundancies placing them in the control group (see above), showed no evidence of deletion in the 7q11.23 region with the QPCR method either (combination of 6 diagnostic assays, pairwise t-test: all individuals p values <0.006). Thus the results obtained with the two independent methods (PSQ and QPCR) are consistent with the interpretation that these six patients were clinically misdiagnosed. Since no FISH and/or Southern blot results were available for them, we considered that they were clinically misdiagnosed and reclassified them as non-WBS. However, we cannot exclude that these individuals carry an inversion or a subtle deletion, that maps between the assays we used (maximum size 415 kb between WBSCR14 and ELN).

Atypical deletions

Two patients, CO3 and IM3, present an atypical deletion on the telomeric side as measured by both PSQ and QPCR. Their DNAs contain one copy of the BAZ1B (diagnosed with PSQ and QPCR), TBL2 (QPCR), ELN (QPCR) and RFC2 (QPCR) loci, but are unaffected at the WBSCR3.4M (PSQ) and CYLN2 (PSQ and QPCR) assays (Figures 1 and 3). To further narrow down the breakpoints, we designed more QPCR assays between RFC2 and CYLN2 (Table S2). These additional assays allowed mapping the CO3 and IM3 breakpoints to 13011

and 66180 bp long intervals, respectively. Thus these patients present atypical deletions smaller by more than 400 kb, when compared to the extent of the classical WBS deletion (Figure 3)[17]. The breakpoints of IM3 and CO3 map in the region between the RFC2 and CYLN2 genes. All the WBSCR genes centromerically positioned from this point, starting with the RFC2 gene, are deleted, while the two telomeric genes, GTF2IRD1 and GTF2I, are preserved. The situation is more ambiguous for the CYLN2 gene. 12.3 to 25.3 kb separate the mapped CO3 breakpoint from the first known exon of CYLN2. Similarly, the IM3 breakpoint was narrowed down to a region that encompasses the promoter and the three first exons of that gene. Thus both cases display genomic organizations that might impair the normal expression of CYLN2.

Patient CO3, a male, was born by vaginal delivery after an uneventful pregnancy of 32 weeks. Birth weight was 1850 gr (P50-P75), length 43 cm (P50), and head circumference 29 cm (P25). Congenital heart defect was diagnosed at 5 months of age and echocardiography revealed a supravalvular aortic stenosis. Patient IM3, a female, was born by vaginal delivery at term of an uneventful pregnancy. Birth weight was 3100 g, length 49 cm and head circumference 34 cm. Congenital heart defect was diagnosed shortly after birth. Echocardiography showed supravalvular aortic and peripheral pulmonary arteries stenoses that were surgically corrected at 5 years. Both patients showed motor developmental milestones in the normal range, though mild cognitive deficit was evident at 4 years in one patient (CO3). CO3 and IM3 showed difficulties in non-linguistic and visual-motor abilities, overfriendliness and preservation of linguistic abilities. Wechsler Intelligence Scale testing performed at 15 and 10 years of age showed mild mental retardation with IQs of 72 (VIQ 74; PIQ 68) and 71 (VIQ 73; PIQ 66), respectively. Their clinical examination showed mild facial anomalies, including periorbital fullness (mild in CO3) and thick lower lip, normal size and number of teeth and blood glucose levels in the average range.

CO3 is now 30 years old; weight is 77 kg (P90-P97), height 168 cm (P10), head circumference 53 cm (P3). His irises are normal and no signs of premature ageing were noticeable. Ophthalmological and audiological evaluations were normal. Renal ultrasonography showed no malformations. Measurements of blood pressure at rest and during 24-hour ambulatory monitoring were in the normal range. Auxological parameters of IM3 at 11 years of age were normal. Weight was 38,5 kg (P50-P75), height 140 cm (P50), head circumference 50 cm (P3). She presented a hoarse voice, a stellate iris and mild malar hypoplasia. Strabismus was operated at 7 years of age. Audiological evaluation and renal ultrasonography were normal. Measurements of blood pressure at rest and during 24-hour ambulatory monitoring showed mild systolic hypertension.

Discussion

We report the setup and validation of two recently described methods to diagnose the recurrent DNA deletion present in Williams-Beuren syndrome (WBS) patients [6, 17]. The first technique, called Paralogous Sequence Quantification (PSQ), is based on the quantification of two paralogous sequences by pyrosequencing [13, 20]. PSQ allowed us to correctly segregate 161 of the 163 tested samples with no false positives and/or false positive calls. The diagnosis of the remaining two samples was inconclusive as they fell outside of the confidence interval of the combined assay (Table 1). We conclude that the PSQ technology is a reliable, effective and inexpensive tool to diagnose segmental aneuploidies, but that it is not suitable to measure the extent of the deletion in the case of WBS for the lack of exploitable paralogous sequences at the centromeric end of the deletion.

The second method takes advantage of quantitative real time PCR to evaluate relative abundance of a DNA fragment. We designed a set of eleven assays mapping to the 7q11.23 region and tested them on 92 WBS patient and 61 control DNAs. They allowed the correct segregation of the two analyzed cohorts in all of the tested genomes with 100% sensitivity and specificity (Table 2).

The PSQ method is less expensive than the QPCR technology to diagnose segmental aneuploidies. However, PSQ has the weakness to be dependent on the presence of suitable sequences, i.e. sequences paralogous to sequences somewhere else on the genome. This is not a major problem when the goal is to diagnose aneuploidies of entire chromosome or large chromosomal region [13], but it becomes important in partial aneuploidies of small size (<5Mb) like WBS. In contrast, assays for the QPCR technology can be designed on any genomic fragment that is uniquely represented in the human genome. This allows designing a large number of assays along the region to be tested, e.g. a segmental aneuploidy, decreasing the risk of false positives and negatives and increasing the resolution. Moreover, atypical indels can then be characterized further by designing more assays close to the supposed breakpoints, as we did to characterize WBS patients that carry two copies of the CYLN2, GTF2IRD1 and GTF2I genes.

Genetic diagnostic laboratories attached to genetic counseling clinics, routinely use FISH, microsatellite/SNP genotyping and/or Southern to diagnose segmental aneuploidies, including the Williams-Beuren syndrome. FISH and Southern have success rates reaching 99%, but they have the disadvantage of being costly and labor intensive. Microsatellite/SNP genotyping requires the analysis of multiple assays of both the proband and his/her parents to ensure the informativeness of the genotyped polymorphisms. Another drawback inherent to this method is the unwanted prospect of identifying non-paternities. Advantages and disadvantages of these different methods to diagnose segmental aneuploidies are summarized in Table 3.

Table 3: Advantages and Disadvantages of the multiple techniques used to diagnose segmental aneuploidies

	Method				
	QPCR	PSQ	FISH	Microsatellites	Southern
speed [day]	1	1	5 (2)[1]	1	6
cost/assay [€]	30	10	80	15/30/45[2,3]	80
advantages	great adaptability to uncommon rearrangement[4]		possibility to diagnose inversion[5]		possibility to diagnose inversion
disadvantages	impossibility to diagnose inversion	relies on paralogous sequences impossibility to diagnose inversion	labor intensive	relative informativeness of SNPs discovery of non-paternity impossibility to diagnose inversion	requires to work with radioactivity[6] labor intensive

[1]a preliminary results can be given after 2 days
[2]price for QF-PCR
[3]45€/assay includes both parents
[4]see for example the mapping of breakpoints of atypical deletion patient detailed in this report
[5]by 3 color FISH (see for example Osborne et al., 2001)
[6]possibiliy to work with non-radioactive DIG, but with a diminished sensitivity

We further report the detection of two patients, who carry partial deletions that do not extend telomerically further than the RFC2 gene (Figure 3). The status of the relative expression of the neighboring CYLN2 gene remains uncertain, as we can hypothesize that the hemizygosity of the sequences preceding that gene might play an important role in the regulation of its expression. These atypical patients can provide important information for genotype and phenotype correlation. A few WBS individuals with atypical deletion as well as patients with isolated SVAS and partial deletions were previously identified [21-29]. In particular an individual who carried an atypical deletion extending from the centromeric LCR to the CYLN2 or the GTF2IRD1 gene (Figure 3C) and presented an unusual cognitive profile, SVAS, milder facial features, short stature and an IQ of 83, but not the specific visuospatial impairment of WBS patients was reported [23]. Likewise, [27] describes a seven-year-old female with a deletion from the centromeric LCR to RFC2 (status of CYLN2 unclear; Figure 3B) with no dysmorphic features and verbal and performance scores slightly above full deletion WBS. In addition, a Japanese patient with a deletion spanning most of the WBS region with the exception of GTF2IRD1 and GTF2I had abilities to visualize at the spatial and at the global levels that outperformed that of typical WBS despite a VSIQ of 64 [22](Figure 3B). Finally, the affected members of a SVAS kindred without typical Williams facies and visual spatial abilities above that of full deletion WBS were shown to carry smaller deletion from ELN to GTF2IRD1 thus maintaining euploidy of GTF2I [28](Figure 3B). These combined observations allowed to suggest that the GTF2I and/or GTF2IRD1 genes are likely to be linked to the characteristic WBS visual spatial deficits [22, 28]. The milder cognitive deficit evidenced in patients with atypical deletions from the present series is consistent with this hypothesis. However, the presence of some aspects of the characteristic WBS cognitive profile indicates that one should be cautious in drawing firm conclusions on genotype/phenotype correlation from a limited number of subjects all bearing different microdeletions (Figure 3B). This notion is further emphasized by the identification of unusually highly functioning patient that carries the classical deletion [25] and by a family with a balanced translocation t(7;16)(q11.23;q13) within the elastin gene associated with variable expression of the WBS phenotype [30]. Interestingly, both our atypical patients and none of the previously described patients with similar partial deletions [22, 23, 27, 28] showed the facial features characteristic of WBS, suggesting that hemizygosity of genes mapping to the telomeric end of the common deletion might be involved. An additional aspect which can be putatively linked with hemizygosity of telomeric genes is premature aging, since this feature was absent in IM3 at age 30.

Phenotype/genotype correlation is one of the major challenges in clinical human genetic of contiguous gene syndromes. The techniques used in this report, in particular QPCR are suitable to map precisely any aneuploidy, thus giving a reliable means to perform large-scale diagnosis to detect uncommon patients, with a fast reporting time and at a reasonable cost.

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Competing interests
The authors declare no competing interests.

Figure Legends:

Figure 1 Diagnosing WBS by paralogous sequence quantification
Distribution of the relative abundancies of the query chromosome upon combination of the CYLN2 and the WBSCR3.4M assays. Control samples are in burgundy and WBS patients in purple. The positions of samples corresponding to the two patients carrier of an atypical deletion (CO3 and IM3), i.e. euploid for the CYLN2 and WBSCR3.4M assays are indicated by asterisks.

Figure 2 Diagnosing WBS by real-time quantitative PCR (QPCR)
Means and standard deviations of real-time quantitative PCR results obtained for 11 assays from the 7q11.23 region with 61 controls (burgundy square) and 92 WBS patients (purple disks) DNAs. The extension of the classical deletion observed in WBS (double-head black arrow) and the location of the Low-Copy Repeats (LCRs; green arrow, BLOCK C; yellow arrow, BLOCK A; red arrow, BLOCK B; [31]) are indicated.

Figure 3 Atypical deletions
(A) Typical real-time quantitative PCR results obtained for a control (burgundy squares) and a typical WBS patient (purple disks) sample besides the samples of atypical patients CO3 (dark blue disks) and IM3 (red disks).
(B) Schematic partial transcript map of the WBS critical region. Single copy transcripts mapping to the WBS critical region are represented by blue boxes, whereas duplicons within the Low-Copy Repeats are depicted by colored arrows (BLOCK A: yellow; BLOCK B: red; BLOCK C: green; [31]). The name of the hemizygous and normal-copy genes are printed diagonally and vertically, respectively. The extent of the deletion observed in classical WBS (short and long deletions), as well as in the atypical patients discussed in this manuscript are denoted by grey boxes below the transcript map. Unclear deletion statuses are represented by white boxes. Patients symbols and references are indicated on the right.

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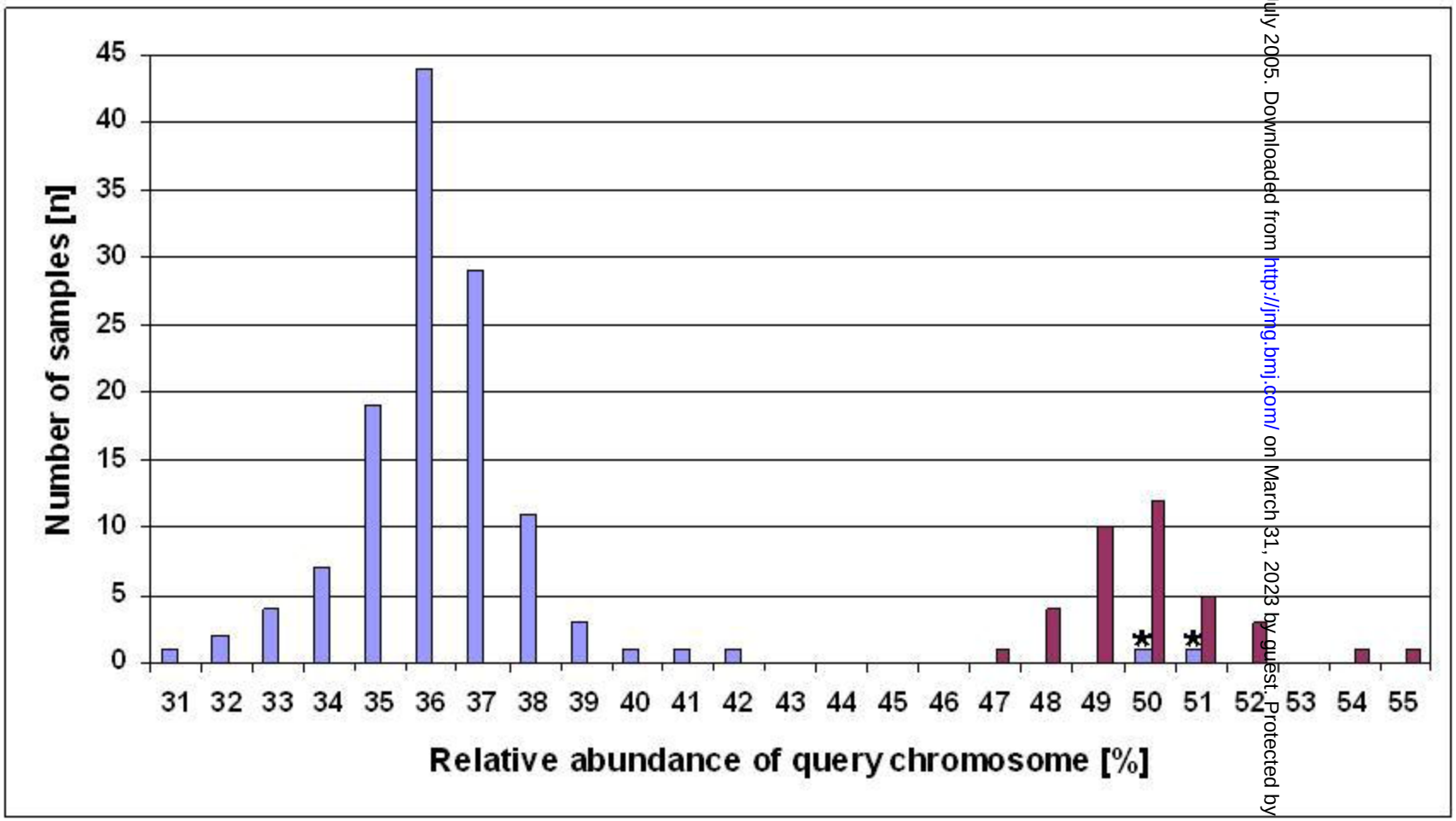

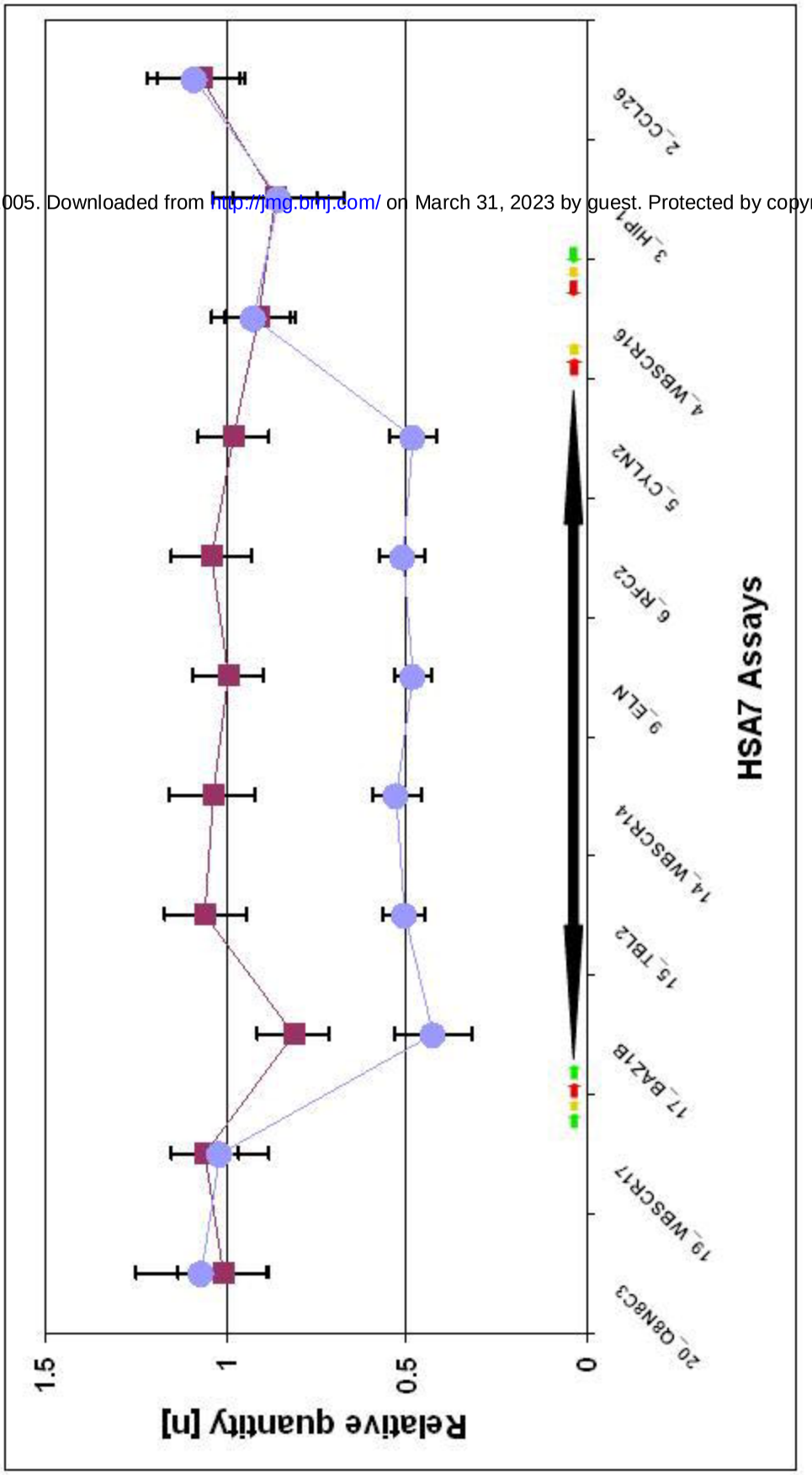

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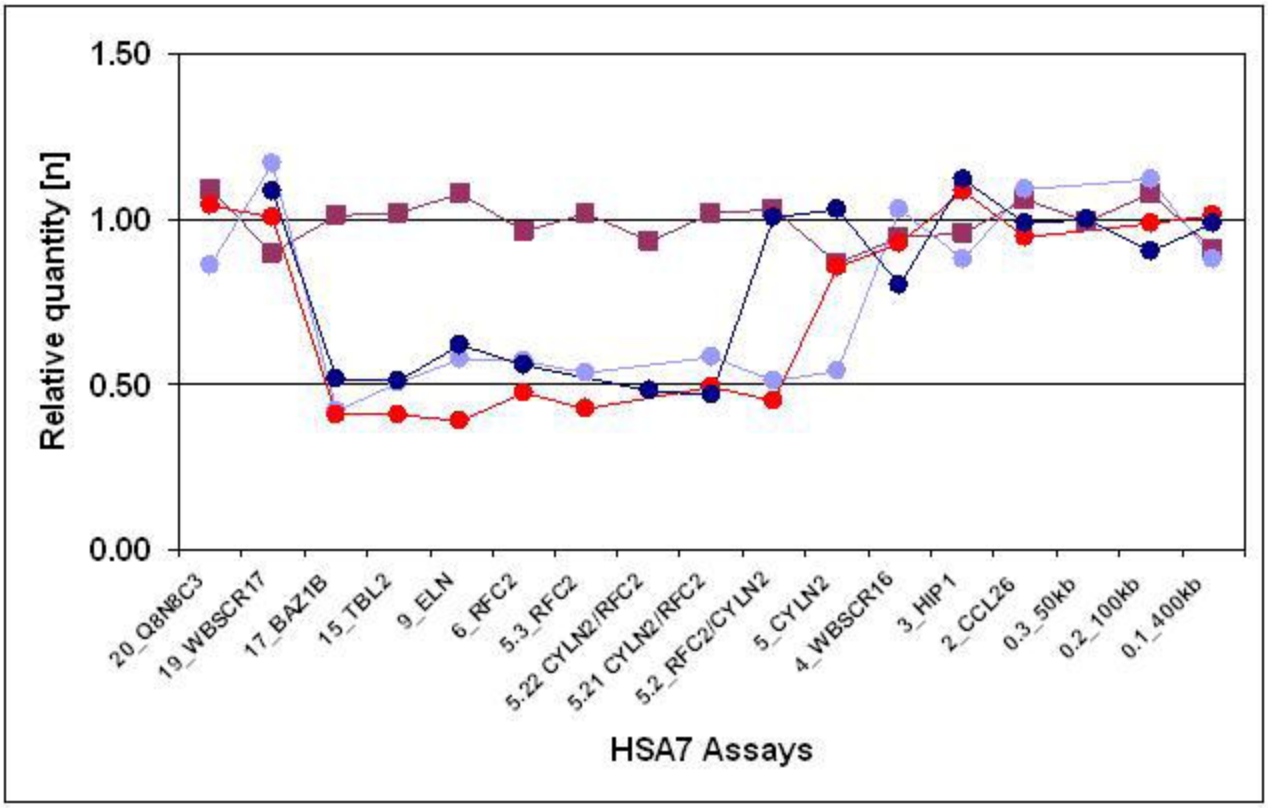
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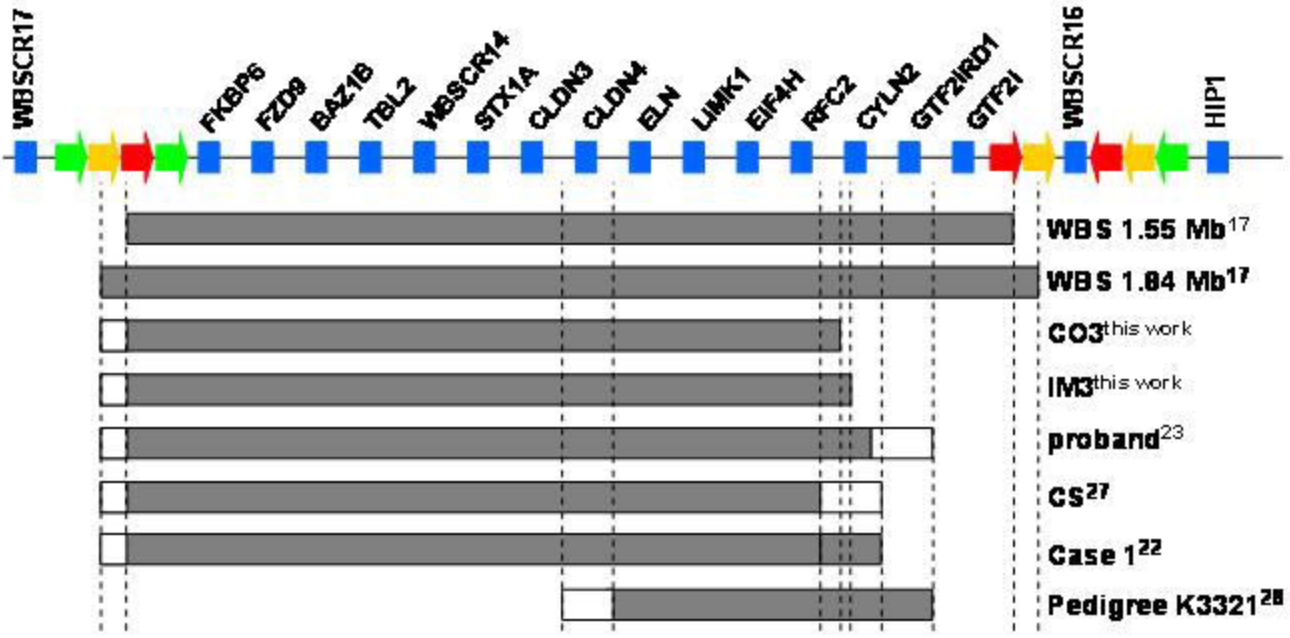
17_BAZ1B
15_TBL2
9_ELN
6_RFC2
5.3_RFC2
5.22 CYLN2/RFC2
5.21 CYLN2/RFC2
5.2_RFC2/CYLN2
5_CYLN2
4_WBSCR16
3_HIP1
2_CCL26
0.3_50kb
0.2_100kb
0.1_400kb
HSA7 Assays

B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FZD9
BAZ1B
TBL2
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STX1A
CLDN3
CLDN4
ELN
LIMK1
EIF4H
RFC2
CYLN2
GTF2IRD1
GTF2I
WBSCR16
HIP1
WBS 1.55 Mb[17]
WBS 1.84 Mb[17]
CO3 this work
IM3 this work
proband[23]
CS[27]
Case 1[22]
Pedigree K3321[28]

Supplementary Online Material
Howald et al.

Supplementary Figure Legends

Supplementary Figure S1

Alignment of genomic sequences from the BAZ1B region with its paralogous sequence from HSA10 (accession: NT_030059.10|Hs10_30314). The sequences corresponding to the PCR amplification primers and the pyrosequencing primer are highlighted in yellow and blue, respectively. The analyzed PSM is marked in black

Supplementary Figure S2

Alignment of genomic sequences from the CYLN2 and WBSCR3.4M region with its paralogous sequence from HSA2 (accession: NT_005403.13|Hs2_5560). The sequences corresponding to the PCR amplification primers are highlighted in red and yellow, while the pyrosequencing primers are highlighted in purple and blue. The analyzed PSMs are marked in black.

Supplementary Figure S3

Diagnosing WBS by paralogous sequence quantification

Typical results (pyrograms and percentage of both Paralogous Sequence Mismatches) of controls (left column) and WBS patients (right column) for all the assays. The name of each assay and the analyzed sequence are indicated on the left. The expected results for euploidy at this marker are indicated by histograms below the pyrograms.

Supplementary Figure S4

Diagnosing WBS by real-time quantitative PCR (QPCR)

Typical results of the real-time quantitative PCR-approach to diagnose the extension of the 7q11.23 deletion in four WBS patients (disks) and four controls (square). The extension of the classical deletion observed in WBS (double-head black arrow) and the location of the Low-Copy Repeats (LCRs; green arrow, BLOCK C; yellow arrow, BLOCK A; red arrow, BLOCK B; (Valero et al., 2000) are indicated.

Figure S1

Figure S2

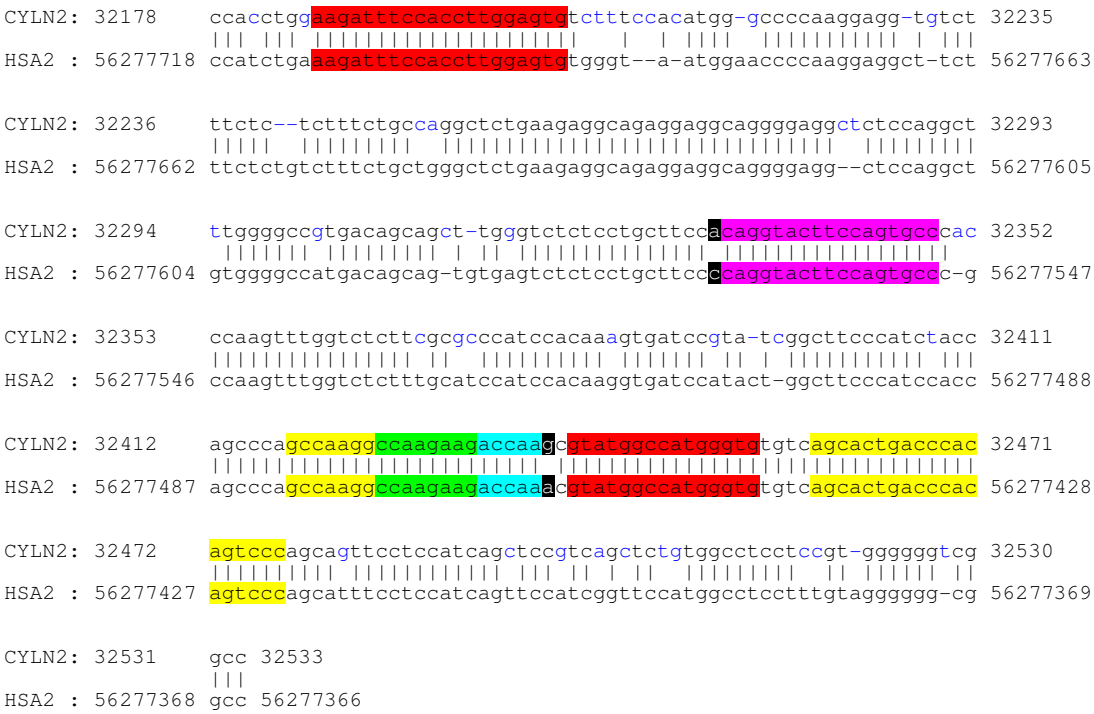
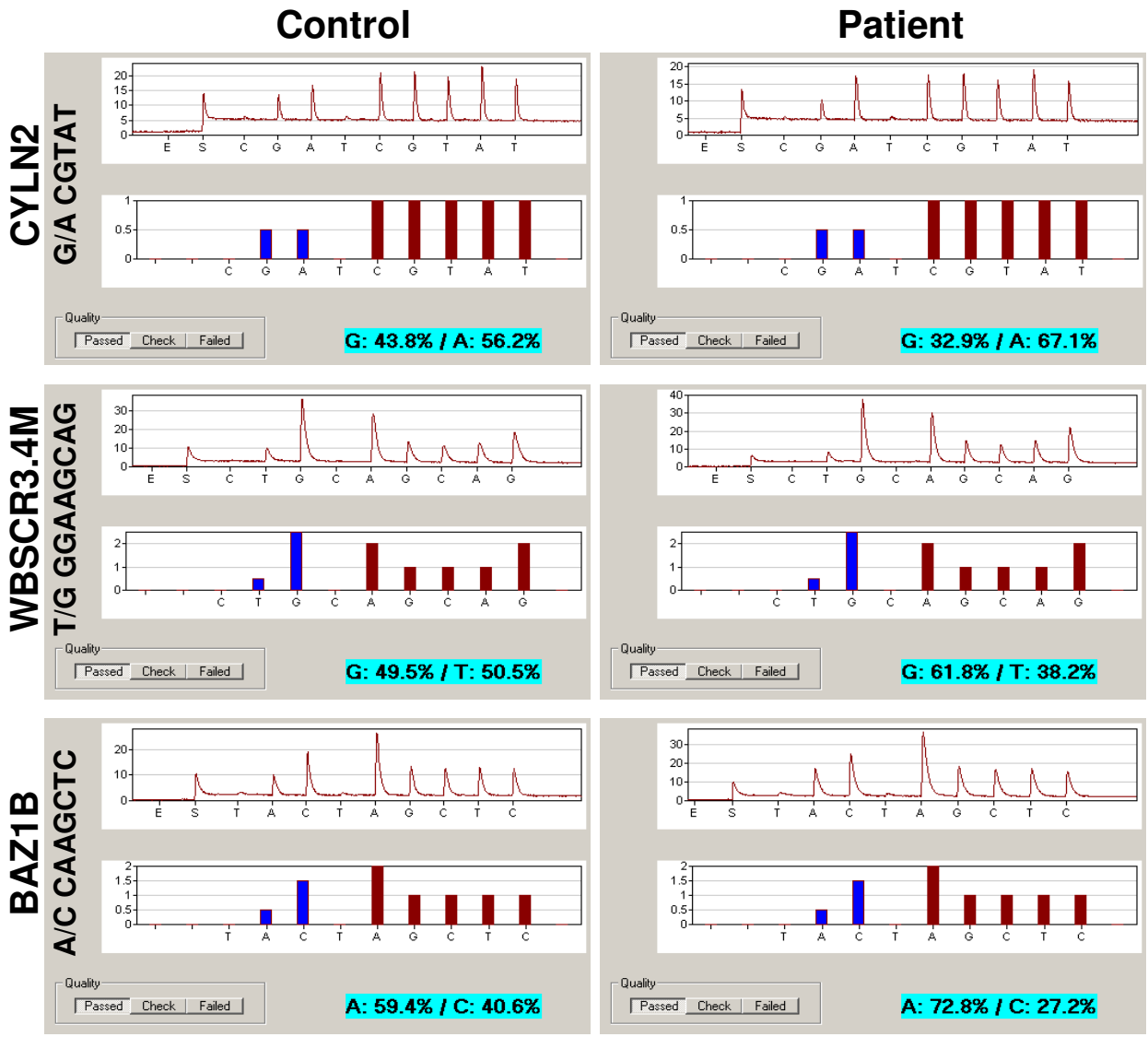


Figure S3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Check
Failed
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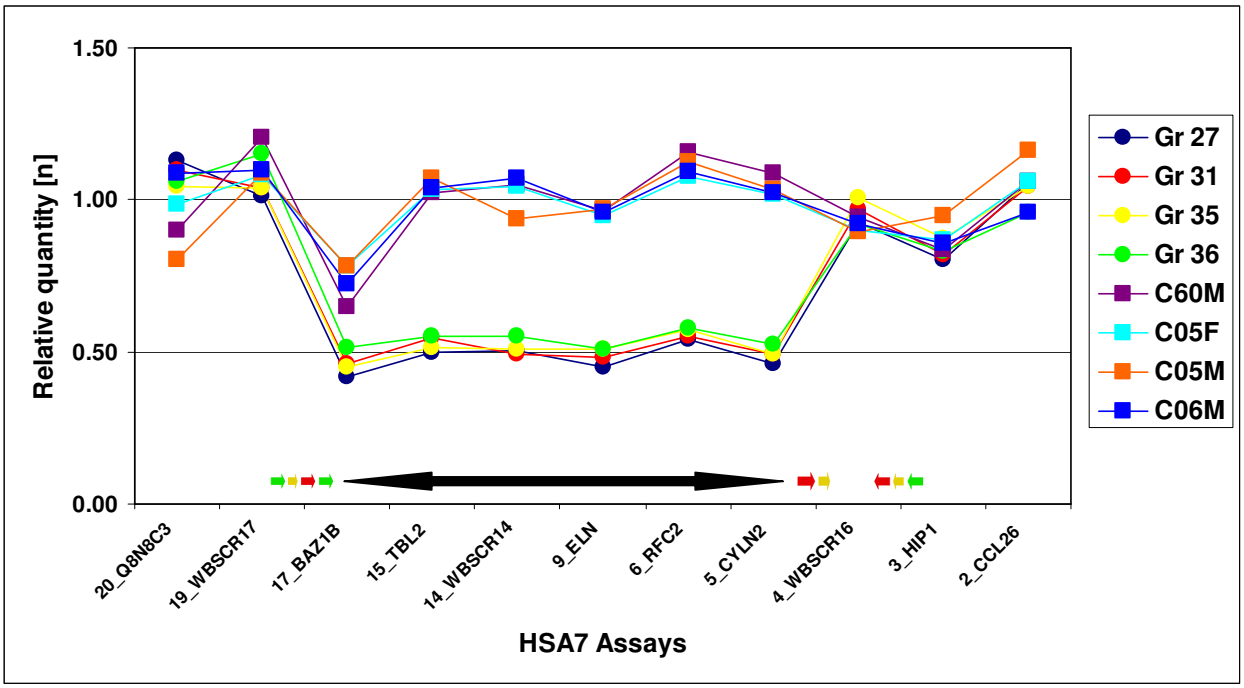
G: 32.9% / A: 67.1%
WBSCR3.4M
T/G GGAAGCAG
G: 49.5% / T: 50.5%
G: 61.8% / T: 38.2%
BAZ1B
A/C CAAGCTC
A: 59.4% / C: 40.6%
A: 72.8% / C: 27.2%

Figure S4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0.50
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19_WBSCR17
17_BAZ1B
15_TBL2
14_WBSCR14
9_ELN
6_RFC2
5_CYLN2
4_WBSCR16
3_HIP1
2_CCL26
HSA7 Assays
Gr 27
Gr 31
Gr 35
Gr 36
C60M
C05F
C05M
C06M

Table S1

TableS1: Primers and conditions used to diagnose WBS by Paralogous Sequence Quantification

	Primers			Conditions				Amplification[1]	
Assay	**ID**	**Sequence**	**Role**	**MgCl2**	**DMSO**	**Buffer**	**Polymerase**	**TD, 10 cycles**	**PCR, 30 cycles**
BAZ1B	14748-BAZ1B(new)Fw py	GGCAAAACTTTGTAGTCTTC	Forward primer	0 mM	5%	1 x *Taq* buffer	*Taq*	95° 30", 63-58.5° 30", 72° 20"	95° 30", 58° 30", 72° 20"
	14750-BAZ1B (new)Rv py	7GATTCTCATGTTCTAGGAGC	Reverse primer						
	14749-BAZ1B(new)SNP py	TTGGAAATTTTGAGCAC	Pyrosequencing primer						
CYLN2	13036Cynl2_WBSF	GCCAAGGCCAAGAAG	Forward primer	1.5 mM	5%	1 x *Taq* buffer	*Taq*	95° 30", 63-58.5° 30", 72° 20"	95° 30", 58° 30", 72° 20"
	13037Cynl2_WBSR	7GGGACTGTGGGTCAGTGCT	Reverse primer						
	13038Cynl2_WBSS	CCAAGAAGACCAA	Pyrosequencing primer						
WBSCR3.4	14754-WBSCR3.4(SNP M)	7AAGATTTCCACCTTGGAGTG	Forward primer			1 x RED-*Taq* buffer	Jumpstart RED-*Taq*	95° 30", 58-53.5° 30", 72° 20"	95° 30", 53° 30", 72° 20"
	14755-WBSCR3.4(SNP M)	TCACCCATGGCCATAC	Reverse primer						
	14756-WBSCR3.4(SNP M)	GGCACTGGAAGTACCTG	Pyrosequencing primer						

[1]The amplification process begins with 5' at 95° and ends with 3' elongation at 72°

Table S2

TableS2: Primers used to diagnose WBS by quantitative PCR

Assay	Forward primer		Reverse primer		mapping to HSA7 bp
	ID	Sequence	ID	Sequence	
2_CCL26	15428_2_CCL26F	TTTGATCATAACACAAGCCAGGA	15429_2_CCL26R	CCCCCAGTTCCCTGCTG	75017006
3_HIP1	15430_3_HIP1F	GTGCCCAGGGTCATCCC	15431_3_HIP1R	AACCTGGAGTCCTGGTTTTGAA	74974601
4_WBSCR16	15432_4_WBSCR16F	CATGCTTTGTGGGAACTGCTT	15433_4_WBSCR16R	AGAGGACAGCAGTGTGAATCCA	73898628
5_CYLN2	15434_5_CYLN2F	CTGCTCTTCCTCCTGTGGCT	15435_5_CYLN2R	AGCTGTTTGCCCAGGTCCT	73169734
6_RFC2	15436_6_RFC2F	TGACAACCCCCTTGCCAC	15437_6_RFC2R	TGCCATATTTGGTAATGCGC	73067913
9_ELN	15442_9_ELNF	GGCATGAGACGCTCCACAT	15443_9_ELNR	TTCTGAGCCAGGGCAACAC	72865074
10_CLDN4	15444_10_CLDN4F	AACACAGGTGGACCATGCTAGA	15445_10_CLDN4R	GGATCTGTTGACCGGCTAATG	72655622
11_WBSCR21	15446_11_WBSCR21F	CAGCTGGGTGGAGCAAGC	15447_11_WBSCR21R	AGGGAGTGCCTTGCTGGG	72561409
14_WBSCR14	15452_14_WBSCR14F	CACGCATTTCTGATTCCCATT	15453_14_WBSCR14R	GCAGGCGGTCTGCGC	72450358
15_TBL2	15454_15_TBL2F	CTGTCTGTCATCTAGCCTCTGCA	15455_15_TBL2R	CCAGGCCCTTCGGCA	72408518
17_BAZ1B	15458_17_BAZ1BF	AGTTACTTGCCGTTTGAATTTGG	15459_17_BAZ1BR	GGCAACTTTCCACACAAAACC	72339015
19_WBSCR17	15462_19_WBSCR17F	AAAATTCTCCTTCTGGCCCC	15463_19_WBSCR17R	TTGCCTAGCACAACCCCC	70206028
20_Q8N8C3	15464_20_Q8N8C3F	CGCAGCTTGCATTCTTAGCTT	15465_20_Q8N8C3R	AGGCTGAGTTTTCCTGACTTGC	70012994
17.1_BAZ1B	15610_17.1_BAZ1B FW	GCTGTTGGCCCAGATAGCAT	15843 17.1_BAZ1B RW	CACCATGGAAGGGCATCAA	72269607
16.1_BCL7B	15611_16.1_BCL7B FW	AGGCGTGCACAGGCTATCA	15844 16.1_BCL7B RW	CTCCCTGGCTCGCATAGGT	72368450
5.3_RFC2	15617_5.3_RFC2 FW	GGATGGTGAAGCCCTGGTT	15850 5.3_RFC2 RW	GGTTGCCATCATCTTGGTTTG	73076964
5.2_RFC2/CYLN2	15618_5.2_RFC2/CYLN2 FW	ACTCATGACACACCGGCTTTC	15851 5.2_RFC2/CYLN2 RW	CAAGCTGCTGTCTCTCCAAGTTT	73103486
4.1_GTF2IRD1	15862 4.1_GTF2IRD1 Fw	GAGGGCTCAGCCCCCATA	15854 4.1_GTF2IRD1 RW	AATGGGAGGCAGCAACCA	73362277
21_100kb outWBScentro	15622_21_100kb outWBScentro FW	CAAACCGCTGTCCATTACAGAA	15855 21_100kb outWBScentro RW	ATGTCAGACTGGGCTCATCAGA	69919994
22_200Kb outWBScentro	15623_22_200Kb outWBScentro FW	GCCTAAAGTGCTTGGCAACAG	15856 22_200Kb outWBScentro RW	TCTCCCCCAACACCATGGT	70254521
23_400Kb outWBScentro	15624_23_400Kb outWBScentro FW	AAGGGATGCTTGAGGCTGTATG	15857 23_400Kb outWBScentro RW	GGAGAAAACTGAGCCGCATT	69619016
0.3_50Kb outWBStelo	15625_0.3_50Kb outWBStelo FW	TGCGCTCCAACGAATCCT	15858 0.3_50Kb outWBStelo RW	GCCAGACAAGCCCAGGTAGA	74799070
0.2_100Kb outWBStelo	15626_0.2_100Kb outWBStelo FW	CCCCAAGCAACACTTCATCA	15859 0.2_100Kb outWBStelo RW	TGGGCCCCAAACCATAGAG	74850630
0.1_400Kb outWBStelo	15627_0.1_400Kb outWBStelo FW	CACCAAAGACTGGCGTTTCC	15860 0.1_400Kb outWBStelo RW	GCGGACCCAGATTTCTTGAC	75207763
5.22_CYLN2/RFC2	15999_5.22_CYLN2/RFC2 F	GGGCATTTGGCCAGAGATG	16000_5.22_CYLN2/RFC2 R	CCTTCCAGGCAAAGCCAAT	73085743
5.21_CYLN2/RFC2	16001_5.21_CYLN2/RFC2 F	GGGCACCCCGAGGTATG	16002_5.21_CYLN2/RFC2 R	CAGATTTGAACGTGAGCTTGCT	73090411
4.01_WBSCR16	16003_4.01_WBSCR16 F	TTGAACCCTCAGGCAAAAGG	16004_4.01_WBSCR16 R	CTTTTAGGCCACAAAAGAACATCTT	73897993
4.02_WBSCR16	16005_4.02_WBSCR16 F	GCTTGATCGATTGAGCCACAT	16006_4.02_WBSCR16 R	CTTGAGCAATCCCACTGATCTG	73894413
15.1_TBL2	17741 15.1_TBL2 F	ACAGGACCCTCTCCGCATACT	17742 15.1_TBL2 R	TTTGCCCCCACCCAATT	72386418
14.3_TBL2	17743 14.3_TBL2 F	CTCTGACTGACCCGCTCCTT	17744 14.3_TBL2 R	GGCCGCAGAAAAACCAGAA	72414194
14.1_WBSCR14	17745 14.1_WBSCR14 F	CAGGAGAGACCACACGCAGTATT	17746 14.1_WBSCR14 R	CCTCTCCCTGCTTGGATGAA	72440902
14.2_WBSCR14	17747 14.2_WBSCR14 F	GACGGGCAGGAAGATTGATG	17748 14.2_WBSCR14 R	GAGAGGAGGGAGTCGAGTACATTTA	72424335
4.3_CYLN2	17749 4.3_CYLN2 F	GACCCCCTATATCCGGGAGTAG	17750 4.3_CYLN2 R	TGGCCAGAGTGATGCTCTTTATC	73225057
5.01_CYLN2	17751 5.01_CYLN2 F	GCCTGGGAGGCATGCTT	17752 5.01_CYLN2 R	CAGGGACATGAACCCGAAGA	73119253
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normalization 21-3	13388hsacgh-21-3A	CCACCTCAAGTATACCGAAGTCCTA	13389hsacgh-21-3B	GGAAGCGCTGCATGTGAA	HSA21 21589275
normalization 21-4	13390hsacgh-21-4A	CAATTCAGGTCAGGTGATAACTCAGTAA	13391hsacgh-21-4B	GCCAGGTTTAGAATGTTTGTCTAAGTC	HSA21 24204262
normalization 21-6	13394hsacgh-21-6A	CGTCACATCACATCTCACTCATTG	13395hsacgh-21-6B	ACCACGTGAAAGGAGGTTTCC	HSA21 30761032