

Rapid Detection of Aneuploidy (Trisomy 21) by Allele Quantification Combined with Melting Curves Analysis of Single-Nucleotide Polymorphism Loci

GENEVIEVE PONT-KINGDON^{1*} and ELAINE LYON^{1,2}

Background: Molecular approaches for the detection of chromosomal abnormalities will allow the development of rapid, cost-effective screening strategies. We present here a molecular alternative for the detection of aneuploidies and, more specifically, trisomy 21.

Methods: We used the quantitative value of melting curve analysis of heterozygous genetic loci to establish a relative allelic count. The two alleles of a given single-nucleotide polymorphism (SNP) were differentiated by thermodynamic stability with a fluorescently labeled hybridization probe and were quantified by relative areas of derivative melting curves detected after fluorescence resonance energy transfer. Heterozygous SNPs provided internal controls for the assay.

Results: We selected six SNPs, heterozygous in at least 30% of a random population, to form a panel of informative loci in the majority of a random population. After normalization to a heterozygous control, samples segregated into three categories; nontrisomic samples had mean allele ratios of 0.96–1.09, whereas trisomic samples had mean ratios of 1.84–2.09 or 0.46–0.61, depending on which allele was duplicated. Within-run mean CVs of ratios were 6.5–27%, and between-assay mean CVs were 13–24%.

Conclusions: The use of melting curve analysis of multiple SNPs is an alternative to the use of small tandem repeats for the detection of trisomies. Because of the high density of SNPs, the approach may be specifically useful for very fine mapping of the regions of chromosome 21 that are critical for Down syndrome; it is also applicable to aneuploidies other than trisomy 21

and to specimens that are not amenable to cytogenetic analysis.

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Down syndrome, one of the most common causes of mental retardation, is observed in 1 of 800 live births (1). Although most cases of Down syndrome are caused by an extra chromosome 21 (trisomy), duplications in a critical region on chromosome 21, the Down syndrome critical region (DSCR),³ have also been associated with the complete or partial clinical syndrome (2, 3). Similar alterations compatible with life include aneuploidies of chromosomes 18, 13, X, and Y.

Prenatal testing for trisomies is available to women of advanced maternal age, women with ultrasound or maternal serum α -fetoprotein findings consistent with Down syndrome, and women with previous fetuses with chromosome abnormalities. The conventional diagnosis of Down syndrome is by chromosome karyotype. A karyotype shows the complete chromosome complement in a cell and detects any chromosomal aneuploidy as well as structural chromosomal rearrangements. The strength of karyotypes is a definitive diagnosis of aneuploidy and the ability to detect any chromosomal abnormality such as rearrangements and large chromosomal deletions and insertions. A drawback to the procedure is the necessity for viable cells to culture for prenatal diagnosis, which may take up to 2 weeks. The requirement for viable cells that can be cultured prevents adequate karyotyping of some products of conception and paraffin-embedded samples. Moreover, karyotypes fail to detect small duplications.

Fluorescent in situ hybridization (FISH) studies of

¹ ARUP Institute for Clinical and Experimental Pathology, 500 Chipeta Way, Salt Lake City, UT 84108.

² Department of Pathology, University of Utah, Salt Lake City, UT 84132.

* Author for correspondence. Fax 801-584-5109, e-mail pontkig@aruplab.com. Received February 27, 2003; accepted April 11, 2003.

³ Nonstandard abbreviations: DSCR, Down syndrome critical region; FISH, fluorescent in situ hybridization; STR, small tandem repeat; SNP, single-nucleotide polymorphism; and HI, heterozygosity index.

either metaphase or interphase cells avoid the need to culture cells and reduce the time required for a diagnosis. However, this technique is time-consuming, requires intact cells and technical expertise, and is unable to detect small duplications.

Molecular methods are being developed to rapidly identify trisomies. The most thoroughly documented molecular technique for detecting trisomies from DNA samples has focused on the use of the polymorphism of short tandem repeats (STRs) (4–6). STRs are composed of a variable number of repeats two to five nucleotides in length. The number of repeats is highly variable among individuals, and STRs have been used extensively for genetic mapping. STRs are amplified by PCR, and products are separated by size on a sequencing gel. The number and intensities of the bands are used to determine the number of copies of each allele, which reflects the number of chromosomes (4). This technique is powerful in its ability to multiplex X, Y, 13, 18, and 21 and is well documented (7, 8), but it has not been widely implemented in clinical laboratories. Recent preliminary data obtained from 11 nontrisomic and 10 trisomic samples with real-time PCR have been published, demonstrating the interest in new molecular techniques for the detection of aneuploidies (9).

The present report extends the application of a quantitative molecular technique that estimates the number of genetic copies by the use of single-nucleotide polymorphisms (SNPs) and fluorescent melting curve analysis to detect aneuploidies. Melting curve analysis allows a combination of mutation detection (10) and quantitative PCR (10, 11). This represents an alternative molecular method for detecting trisomy 21.

Materials and Methods

SAMPLES AND GENOMIC DNA EXTRACTION

We obtained 52 samples that were positive for trisomy 21 and 31 negative samples from the cytogenetics laboratory (University of Utah); all were deidentified according to Institutional Review Board protocol. These samples were either fixed cell pellets (methanol–acetic acid, 3:1 by volume) or amniocytes grown in 25-cm² cell culture flasks. DNA was extracted either with a PUREGENE DNA isolation reagent set for 3–5 × 10⁶ fixed cells (Gentra) or on the MagNa Pure LC instrument (Roche Applied Science) with the MagNa Pure LC DNA Isolation Kit I (Roche Applied Science). For fixed cells, two successive washes were performed with 1× phosphate-buffered saline (137 mmol/L NaCl, 2.7 mmol/L KCl, 8 mmol/L Na₂HPO₄, 2 mmol/L KH₂PO₄; Ambion) before extraction. DNA was eluted in 20–50 µL of elution buffers. An additional 28 nontrisomic deidentified samples were extracted from whole blood with the MagNa Pure LC DNA Isolation Kit I (Roche Applied Science). Because no differences in the performance of the assay were observed among sample types, they are not distinguished in the *Results* section.

SNPs, PRIMERS, AND HYBRIDIZATION PROBES

Sequences of the six selected SNPs are found in two public databases: WIAF 2643 (accession no. G22980) and WIAF 899 (accession no. G42972) at the NIH [Entrez Nucleotide query on the National Center for Biotechnology Information website (<http://www.ncbi.nih.gov>)] and WIAF 1538, WIAF 2215, WIAF 1882, and WIAF 1943 at the Whitehead Institute (<http://www.genome.wi.mit.edu/SNP/human/maps/Chr21.All.html>). The sequences of the PCR primers, available in the SNP databases, are presented in Table 1. PCR primers were constructed by the DNA-Peptide Core facility at the University of Utah. Hybridization probes were designed to allow detection of the SNPs according to previously described guidelines (10) and were constructed by Idaho Technology or by Biosearch Technologies with fluorescein (Biogenix), LCRed640, and LCRed705 (Roche Applied Science) as fluorescent labels. All primers and probes were HPLC-purified, although nonpurified primers can also be used. Sequences are shown in Table 1. Two of the SNPs (WIAF 1882 and 1943) are within the DSCR.

PCR

PCRs were performed on the LightCycler Instrument (Roche Applied Science) under the following conditions. Each PCR was designed to coamplify and detect two SNPs: WIAF 899 and 2643, WIAF 2215 and 1538, or WIAF 1882 and 1943. We added 1 µL of genomic DNA (~50 ng) to a reaction mixture containing 0.5 µM each forward and reverse PCR primer and 0.2 µM each reference and anchor hybridization probe, in 1× LightCycler-DNA Master Hybridization Probes (Roche Applied Science) adjusted to a final MgCl₂ concentration of 2 mM. The reaction mixtures were loaded into glass capillary tubes, and the reactions were performed in the LightCycler with software version 5.32 with automated gain adjustment. The following conditions were used for the reactions: denaturation at 94 °C for 2 s, annealing at 60 °C for 10 s, and extension at 72 °C for 15 s for 35 or 45 cycles. Programmed transition rates were 20 °C/s from denaturation to annealing and from extension to denaturation and 2 °C/s from annealing to extension.

The amplification cycles were followed by three consecutive melting cycles. For each melting cycle, DNA was denatured at 95 °C with no holding time, cooled to 40 °C, and held for 120 s. The temperature was then increased to 80 °C with a transition rate of 0.1 °C/s during the first melting cycle, 0.2 °C/s in the second melting cycle, and 0.3 °C/s in the third melting cycle. Fluorescence was continually monitored during the melts.

ANALYSIS OF MELTING CURVES

Melting curves were converted into negative derivative curves of fluorescence with respect to temperature (–dF/dT) by the LightCycler Data Analysis software (Fig. 1). All analyses were performed with background correction and color compensation. Peak areas under the deriv-

Table 1. PCR primers and probes for allelic quantification of six SNPs on chromosome 21.

SNP name (HI) ^a	Primer sequences and hybridization probes
WIAF-2643 (49%)	Forward primer: AACCCAGTGTGGGAGGAGAA ^b Reverse primer: GTGGTGCTGTGGGGCTAG Rf-F ^c : CAGAATAAATAGAACAGTAGAATG-F An-705: LCRed705-TCACAGATGGGTAATTACACATGTAAATGAGCTC-ph
WIAF-899 (53%)	Forward primer: CAGGCAGGACTTCAGTGTCA Reverse primer: GTCATCTGGGACAGGTCACC Rf-F: TTCCTGTTCCACGAAGAGGAC-F An-640: LCRed640-TTTTGTTCACAATTGGATCACAATGCAGAGGAGTCTGTT-ph
WIAF-1538 (47%)	Forward primer: TGTTTGTGTTCCAGCCACAT Reverse primer: CTCTCAGTTAGCAGCTGGGC Rf-705: LCRed705-CCAATGTTATGTGCGAACTGCATTGTAAAAAG-ph An-F: GCGCACCATTATCATTTAGGCTTGTGGTTTGTGTTTACTCT-F
WIAF-2215 (30%)	Forward primer: GGCTCACAACATCCAC Reverse primer: CATCAAGCACCTGTCTG Rf-F: TGGTCCCCCTGCCGAGGG-F An-640: LCRed640-GTGCGGCTCTGCAAGGTTGGGGGTGGCTTCGTTTGCCTGG-Ph
WIAF-1882 (46%)	Forward primer: TTTTTTGGCTTGTCTGAGA Reverse primer: CAGTGAGCCAGCACTCTTGG Rf-640: LCRed640-GAGGCAGCGCTTACAGGAG-ph An-F: CCCAAGTGCACTAGGCAATGTAAGCTCC-F
WIAF-1943 (52%)	Forward primer: TTTTAAACGAAATCTCACTACTGCA Reverse primer: CTATGCACCATGTACTGTTCTAAGC Rf-F: GCTAATGAATGCACAGATAT-F An-705: LCRed705-GCCTGCAAAATAAATTGAGATTCTATTTTAAAG-ph

^a HIs were experimentally determined on 100 random DNA samples.^b Sequences are given in the conventional 5' to 3' orientation.^c F, fluorescein; ph, phosphate groups used to block the 3' end of oligonucleotides; Rf, reference probe; An, anchor probe.

ative curves were determined with version 3.5 of the LightCycler Data Analysis software based on the sum of two gaussians by nonlinear least-square regression (12). The ratio of the areas under the derivative curves [area of least stable allele (1)/area of most stable allele (2)] was determined after export to an Excel spreadsheet. Nontrisomic heterozygous DNA gave the most equal areas when 25–32 reaction tubes were amplified in a single experiment and the following melting cycles were used: WIAF 899, 1882, and 2215 were analyzed on the F2 channel, and WIAF 2643, 1943, and 1538 on the F3 channel. WIAF 2643 was analyzed with the second melting cycle (0.2 °C/s), in a temperature window from 40 to 66 °C. WIAF 899 was analyzed with the first or the second melting cycle, and the temperature window was 46–73 °C. WIAF 1538 and WIAF 2215 were analyzed with the first melting condition (0.1 °C/s). The temperatures analyzed were 54–72 °C for WIAF 1538 and 47–77 °C for WIAF 2215. WIAF 1882 and 1943 were analyzed with the second melt for WIAF 1882 and the first melt for WIAF 1943; temperatures were 50–75 °C and 45–65 °C, respectively. Known nontrisomic heterozygous DNAs were used as references. In each assay, one of the reference DNA samples heterozygous at the studied SNP was concurrently analyzed, and the area ratio from this sample was used to normalize the area ratios of the experi-

mental sample. All results reported are from samples analyzed in two independent experiments.

Results

SELECTION OF THE SNPs

In theory, a panel of six independent SNPs, each heterozygous in 50% of the population, provides an accuracy >98% if diagnosis is established with only one heterozygous locus. In a clinical test that would require confirmatory data from at least two SNPs, informative data will be obtained for more than 89% of the population. We selected a panel of SNPs predicted to provide at least one heterozygous locus in more than 95% of a random population. Initially, PCR was performed on 21 SNPs available from two public databases [http://www-genome.wi.mit.edu (13) and http://cnp.unige.ch/map/hc21map.html (14)]. PCR was performed with identical cycling conditions and buffers (see *Materials and Methods*) to allow multiplex reactions, and product was obtained for 17 of the SNPs. Fluorescent hybridization probes were designed to distinguish both alleles at 16 of the SNP loci (one SNP was in an AT-rich region not appropriate for probe design). In a first selection step, random DNA samples (<30) were analyzed at the SNP loci to assess both the heterozygosity of the locus and the quality of the melting peaks. Of the 16 loci, results could not be obtained

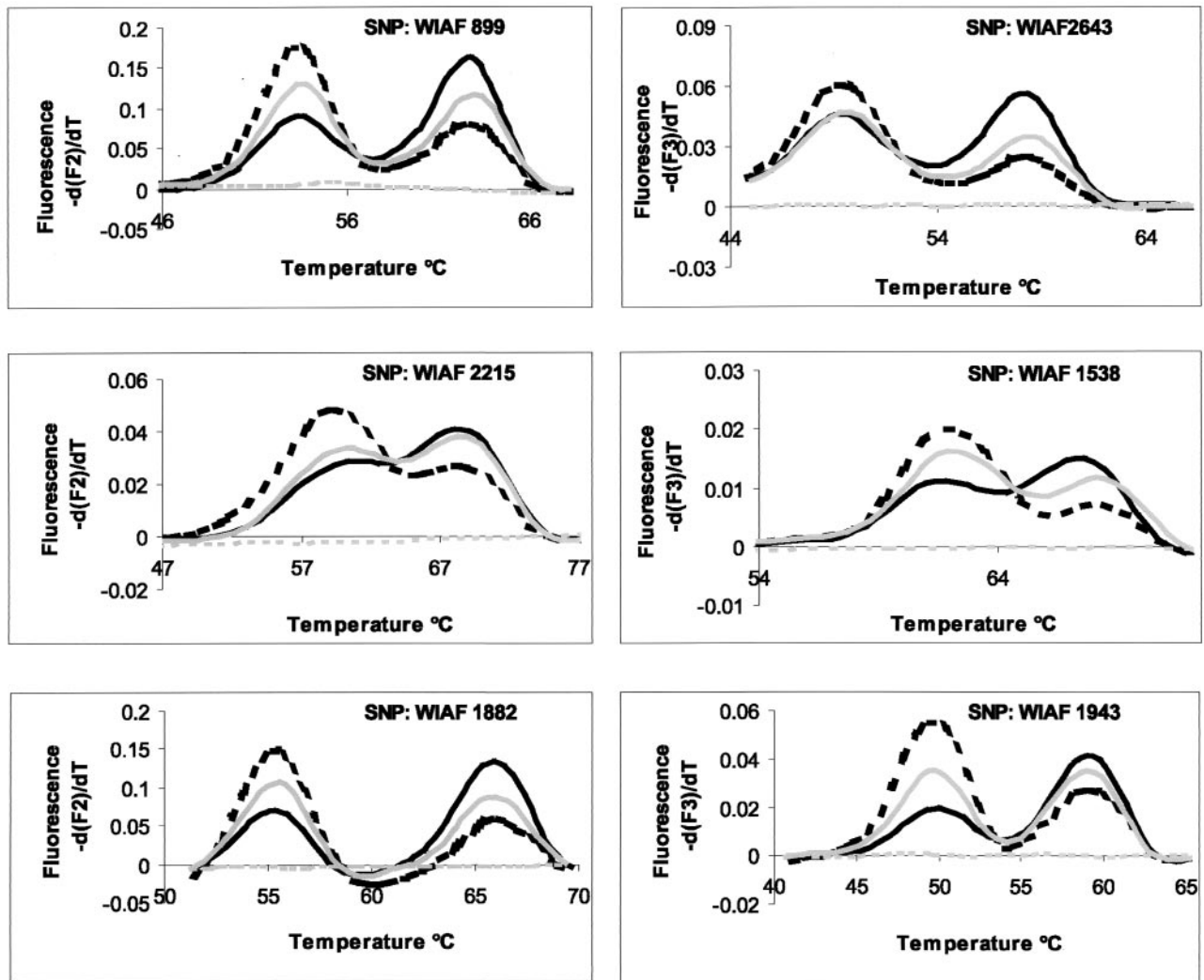


Fig. 1. Derivative melting curves of the six SNPs in trisomy-21-positive and -negative samples.

Derivative melting curves of trisomic samples are shown by the *dashed black* (overrepresentation of the less stable allele) and *solid black* lines (overrepresentation of the most stable allele); *solid gray* line, nontrisomic reference; *dashed gray* line, no-template control.

with two of the fluorescent probes, a published SNP (WIAF 1683) revealed a more complicated polymorphism (A/CAAA), and five SNPs were heterozygous in <30% of the samples. The heterozygosity index (HI) of the eight remaining SNPs was determined on 100 random DNA samples. The mean HI of the SNPs of the Whitehead Institute database is 33% (13). The experimental HI that we found for SNPs that were initially tested from this database varied from 9% to 60%, with a mean of 39.2%. SNPs with a HI >30% were selected (Table 1). Interestingly, recent data incorporated in the CEPH database (<http://www.cephb.fr/cephdb/>) correlate closely with our data; the reported HI of WIAF 899 is 52.5%, that of WIAF 2643 is 57.14%, the HI of WIAF 1538 is 37.5%, and that of WIAF 1943 is 55%. The HIs of three SNPs from the Unige database were <30%, and only one SNP from this

database had a HI of 50%. Unfortunately, melting curves for the analysis of this SNP were not satisfactory, and no SNP from this database was retained in our panel.

A total of 211 DNA samples were tested with all of the SNPs, 100 during the determination of HI, 59 without trisomy 21, and 52 with trisomy 21, for the determination of peak ratios. Only six samples were homozygous at all six selected SNP loci and, therefore, would not provide informative data. This represents less than the 5% expected from this SNP panel. Additionally, 21 samples (10%) were heterozygous at only one of the six loci and would not provide adequate data in a clinical laboratory testing situation in which confirmatory data from at least two SNPs would be required. Taken together, this indicates that this panel can provide informative data for 87% of a random population.

MELTING CURVE ANALYSIS OF HETEROZYGOUS LOCI IN NONTRISOMIC AND TRISOMIC SAMPLES

In this technique, temperature-dependent loss of fluorescence by hybridization probes distinguishes PCR products amplified from the two different alleles of a pair of heterozygous chromosomes. This translates into two "melting peaks" centered on the melting temperature specific for each allele (Fig. 1). In nontrisomic DNA, for each heterozygous SNP, an allele ratio of 1.0 is expected, whereas in trisomic samples, ratios of 0.5 or 2.0 are expected depending on which allele is overrepresented. The representation of each allele was estimated by the area under the curve of the derivative melting curve in a post-PCR melting step.

Allele ratios were calculated on 55 nontrisomic samples and 52 samples with trisomy 21. Originally, 59 nontrisomic samples were tested with the six SNPs, but 4 samples were homozygous at all SNPs and therefore were not used in peak area calculations. All of the trisomy 21 samples and 31 of the nontrisomic samples were obtained from the cytogenetics laboratory in which the trisomy 21 or non-trisomy 21 karyotypes were established. Twenty-eight additional nontrisomic samples were from deidentified DNA extracted from whole blood of nontrisomic patients.

Examples of derivative melting curve data obtained with nontrisomic and trisomic DNA samples analyzed at the six SNP loci are shown in Fig. 1. Examples of trisomic samples with overrepresentation of one or the other allele are shown compared with a nontrisomic reference sample.

To control for experimental differences in area from both alleles in nontrisomic heterozygous samples (10, 11), we normalized the data by use of the allele area ratio determined from nontrisomic reference DNA extracted by phenol-chloroform. This was necessary because of the day-to-day variation observed in the allele ratios of the reference DNAs (Table 2, first column). Some of the variations that we observed might have been produced in part by the use of different reference samples in different experiments, but allele ratio is mainly dependent on optimal PCR and melting conditions. The conditions for optimal allele ratios for each SNP (closest to 1) are reported in the *Materials and Methods*. Melting curve areas of the 55 nontrisomic and 52 trisomy-21-positive experimental samples were analyzed in two independent experiments, and mean (SD) normalized allele ratios were calculated (Table 2). The samples clearly organized into three classes: a class of nontrisomic samples with an allele ratio mean of ~ 1.0 and two classes of trisomic samples (Fig. 2). Samples with overrepresentation of the SNP allele with a single base mismatch to the probe had mean allele ratios varying from 0.46 to 0.61 depending on the SNPs, whereas samples with the overrepresented allele with a perfect match with the probe had allele ratios varying from 1.84 to 2.09 (Table 2). From this set of data, we calculated a range (mean ± 2 SD) that characterized each

Table 2. Allele ratios from nontrisomic and trisomic samples.

Reference DNA (nonnormalized)		Experimental samples (normalized)		
		Nontrisomic	Trisomic-1 ^a	Trisomic-2 ^b
WIAF 899				
Mean	1.09	1.01	0.51	2.03
SD	0.21	0.11	0.08	0.41
Range ^c	NA	0.80–1.22	0.34–0.68	1.20–2.85
n ^d	31	32	11	23
WIAF 2643				
Mean	1.3	1.08	0.61	1.9
SD	0.24	0.12	0.03	0.14
Range	NA ^e	0.83–1.32	0.56–0.67	1.61–2.19
n	28	31	14	19
WIAF 1882				
Mean	1.24	1.09	0.51	2.09
SD	0.21	0.14	0.06	0.34
Range	NA	0.81–1.38	0.39–0.64	1.40–2.78
n	22	23	15	18
WIAF 1943				
Mean	1.18	0.96	0.46	1.84
SD	0.14	0.15	0.13	0.33
Range	NA	0.66–1.26	0.20–0.72	1.17–2.50
n	20	23	6	21
WIAF 2215				
Mean	1.58	0.98	0.6	1.86
SD	0.45	0.13	0.09	0.25
Range	NA	0.72–1.24	0.41–0.78	1.36–2.36
n	29	19	19	7
WIAF 1538				
Mean	1.82	1	0.55	1.86
SD	0.4	0.11	0.08	0.1
Range	NA	0.79–1.22	0.38–0.72	1.66–2.06
n	35	25	22	9
^a Data from SNPs with overrepresentation of the most stable allele.				
^b Data from SNPs with overrepresentation of the least stable allele.				
^c Range, mean \pm 2 SD.				
^d n, number of independent experiments for the reference DNAs and number of samples for each allele.				
^e NA, nonapplicable.				

^a Data from SNPs with overrepresentation of the most stable allele.

^b Data from SNPs with overrepresentation of the least stable allele.

^c Range, mean ± 2 SD.

^d n, number of independent experiments for the reference DNAs and number of samples for each allele.

^e NA, nonapplicable.

SNP (Table 2). Because some overlap existed between the nontrisomic range and the trisomy 21 ranges for three SNPs (WIAF 899, 1882, and 2215), confirmatory data from at least two SNPs were needed for interpretation of the results. All three data points present in the overlapping data area were resolved by additional assays and belonged to samples clearly positive for trisomy 21 at other SNPs.

We tested within- and between-assay variations in allele ratios with the use of two examples per SNP for both nontrisomic and trisomic samples. Because all of the samples were not heterozygous at all six loci, we used three nontrisomic and six trisomic samples. In trisomic samples, an example of each type of allele representation was chosen. Within-assay variation was established by a triplicate set of data for each sample; and for between-assay variation, the samples were analyzed in five inde-

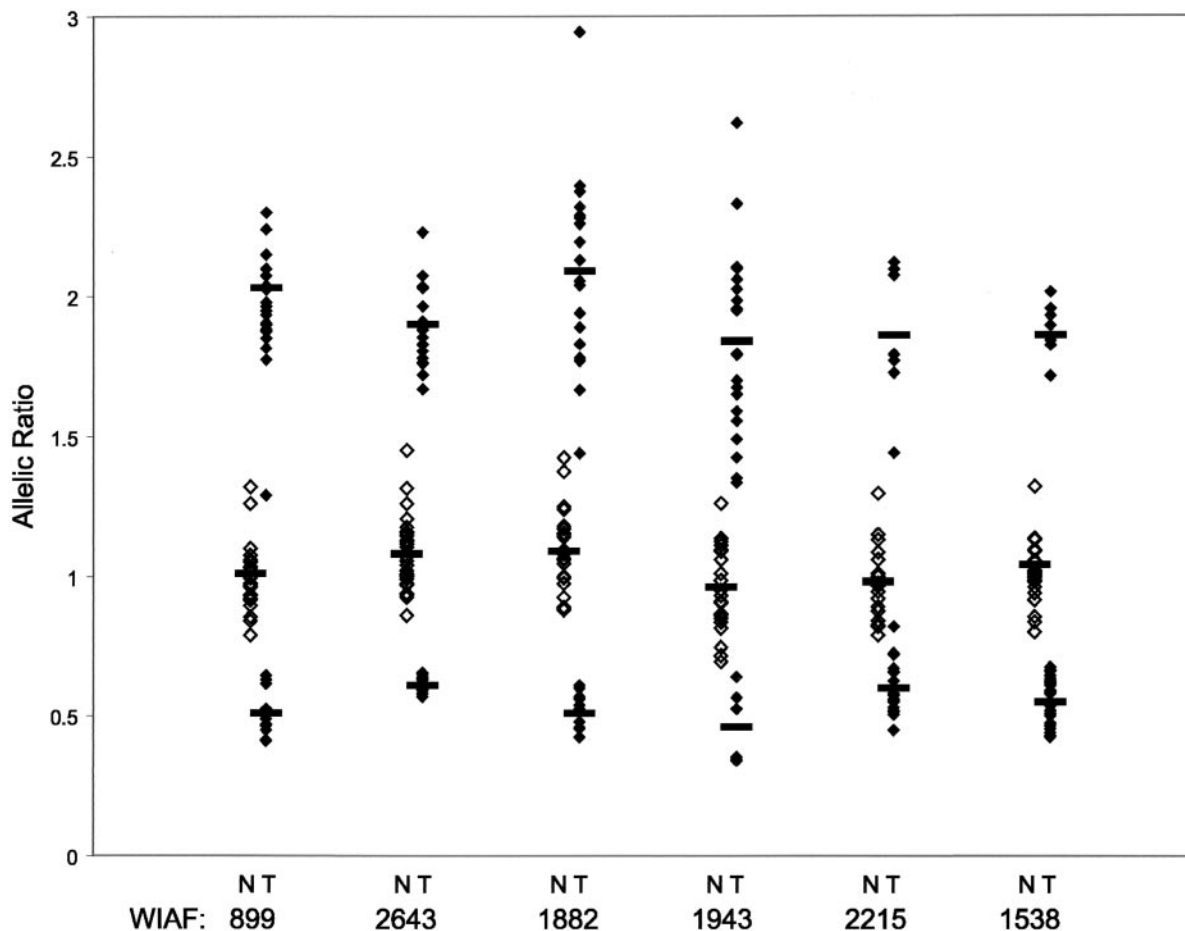


Fig. 2. Allelic ratios of the six SNPs in trisomy-21-positive (T; \blacklozenge) and -negative samples (N; \diamond).

For better visualization, data from both types of sample have been slightly offset. The horizontal bars represent the means for each set of data.

pendent assays (Table 3). CVs were 6.5–27%. Because some of the SNPs (WIAF 2643, 1882, and 1538) displayed higher between-assay imprecision, it was important to use data from more than one heterozygous SNP for accurate interpretation.

Discussion

We have described a novel molecular approach that detects trisomy of chromosome 21 from DNA extracted from amniotic cell cultures and cell pellets fixed for cytogenetics studies. Our results demonstrate the suitability of the quantitative method of end-point analysis of PCR products (10,11) to detect aneuploidies. In this quantitative assay, the use of a panel of heterozygous SNPs bypasses the need for a reference gene.

We described in detail the methodology to analyze six different SNPs with high HIs. This current panel provided an accuracy >89% when we used the strict requirement of at least two confirmatory SNP data per sample. Fifty of the 52 trisomy-21-positive samples and 49 of the 59 nontrisomic samples were clearly identified with this molecular assay. The six SNPs in the panel were selected

from the HI determined on a random population of samples. The heterozygosity of these SNPs may be different in diverse ethnic populations. Other ethnic-specific SNPs could be selected to address this problem.

Like other PCR-based methods, the method presented here has advantages over traditional cytogenetics or FISH-based assays. It should be amenable to additional sample types, such as paraffin-embedded tissues, for archival studies of products of conception or PCR amplification from a single cell for preimplantation genetics (5,15,16). PCR methods are very rapid, and quantification with melting curve analysis can be performed in ~1 h (excluding sample preparation and PCR set-up). In addition, patients suspected of having small partial chromosome duplications not detected by cytogenetics or FISH may be confirmed by molecular analysis of the DSCR. Although the presence of an exclusive critical region is controversial (17,18), authors agree that 2.5 Mb in the 21q22.1–21q22.3 bands (between STR *D21S17* and *ERG*) contain many of the genes that, when present in triplicate, are involved in Down syndrome (3,19). For research purposes, molecular testing can help further

Table 3. Within- and between-run variations

	899			2643			1882			1943			2215			1538		
	Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %
Within-run^a																		
Nontrisomic samples																		
1	0.93	0.34	37	1.16	0.3	26	1.27	0.33	26	0.71	0.01	1.4	1.46	0.36	25	0.73	0.14	19
2	1.16	0.2	17	1.18	0.07	5.5	1.1	0.04	3.6	0.62	0.04	6.5	1.22	0.11	9.0	0.88	0.2	23
Trisomic samples																		
1	1.8	0.66	37	1.97	0.18	9.1	2.21	0.16	7.2	1.54	0.1	6.5	1.94	0.59	30	1.85	0.08	4.3
2	0.48	0.08	17	0.64	0.05	7.8	0.58	0.03	5.2	0.6	0.07	12	0.47	0.06	13	0.57	0.04	7.0
Mean CV, % ^b			27			12			11			6.5			19			13
Between-run^c																		
Nontrisomic samples																		
1	0.91	0.18	20	1.1	0.1	9.1	1	0.2	20	0.8	0.16	20	1.12	0.1	8.9	0.92	0.31	34
2	1.05	0.15	14	1.05	0.14	13	1.01	0.11	11	0.75	0.2	27	1.05	0.12	11	0.89	0.15	17
Trisomic samples																		
1	1.63	0.39	24	1.87	0.18	9.6	0.56	0.06	11	1.58	0.17	11	2.05	0.44	21	1.82	0.28	15
2	0.43	0.03	7.0	0.79	0.31	39	1.98	0.22	11	0.7	0.28	40	0.43	0.07	16	0.51	0.04	7.8
Mean CV, %			16			18			13			24			15			18

^a Data are from two samples per SNP, each measured in triplicate. For trisomic samples, one of each type (overrepresentation of one or the other allele) was chosen.

^b Mean CVs are calculated for each SNP with data from nontrisomic and trisomic samples.

^c Data are from two samples per SNP (same samples as above), each analyzed in five independent experiments.

define the DSCR by fine mapping with SNPs or STRs. Molecular analysis of this area has a great advantage over cytogenetic studies, which may not detect small duplications of the DSCR. Two of the SNPs of the panel (WIAF 1882 and WIAF 1943) are localized in this region. Including SNPs localized in the DSCR in our panel increases the possibility of detecting partial trisomies of chromosome 21. However, results from molecular tests should be considered as screening or preliminary tests and should be confirmed by cytogenetic analysis. Cytogenetic analyses also detect other chromosomal abnormalities that molecular tests would not detect. Molecular methods should be used cautiously and to supplement, not replace, chromosomal analysis.

Quantification by melting curve analysis has advantages over other molecular methods. In comparison with STRs, SNPs are more numerous and can better define the DSCR. Analysis of STRs in the DSCR should also detect partial trisomies (6), but to our knowledge, examples have not been reported, and discovery of partial duplication in chromosome 21 relies essentially on FISH technology (20). SNPs are the most abundant polymorphisms in the human genome with one SNP occurring each kilobase on average (13). A total of 5×10^6 SNPs are estimated to exist in the human genome vs 1×10^5 STRs. Approximately 3000 SNPs are listed on publicly available maps of chromosome 21 in the region that extends from the *CBR1* gene to the *ERG* gene (<http://www.ncbi.nlm.nih.gov>). By contrast, a search for STRs in the Genome Database (<http://gdbwww.gdb.org/gdb/gdbtop.html>) of the same region of the chromosome provides ~20 markers. SNP markers, then, seem the choice for fine molecular mapping of the DSCR. The capital equipment costs for instru-

mentation for melting curve analysis is less expensive than instruments for fluorescent STR analysis. The Light-Cycler allows rapid PCR and analysis in one reaction with no intermediate sample handling. Additionally, the time for rapid PCR and analysis is ~1 h compared with 3–4 h required for the STR analysis. SNPs, on the other hand, have the disadvantage of providing only a biallelic system in contrast to the STR multiallelic system, so that a sample is less likely heterozygous for a SNP than for a given STR. For this study, however, the particular SNP loci were chosen because of their high HIs (30–50%). For comparison, a panel containing six SNP loci, each with a HI of 50%, is needed to provide informative data in more than 98% of the population, whereas the molecular method that uses STRs relies on only two to four markers to provide informative data in more than 97% of the population (4–6).

Real-time PCR with quantification by crossing point is another recently described molecular method (9). PCR and analysis times for this method are comparable to melting analysis. However, quantification by real-time PCR requires comparison with a reference gene amplified from the same sample. Amplification of the target and reference genes should have equal efficiencies. This makes assay optimization more difficult. Lot-to-lot variations in primers may affect PCR efficiencies and may require reoptimization with different lots. Quantification by melting analysis uses an inherent internal control in the SNP. In this assay, the duplicated allele is compared with the nonduplicated allele. The format for the TaqMan assay lends itself to high-throughput testing, whereas the LightCycler is a moderate-throughput instrument. To improve the throughput of our approach, pairs of SNPs

are coamplified and detected by different channels. In principle, up to four SNPs (representing a maximum of eight different alleles) can be multiplexed if both fluorescence and melting temperature are used for allele identification (21, 22). Melting analysis, however, may easily be adapted to a high-throughput format when a 96- or 384-well format thermocycler and the LightTyper (Roche Applied Science), an instrument for end-point melting analysis, are used. With higher throughput capabilities, this assay could easily be expanded to test for common aneuploidies such as trisomy 13 or trisomy 18.

In conclusion, this report describes quantitative PCR combined with melting curve analysis. The accuracy and precision of this method have been shown. The assay could be used as an adjunct for traditional cytogenetics or FISH analysis or when analysis by these methods is not possible. It also can be used as a tool for fine mapping of the DSCR on chromosome 21. The same methods could be easily applied to other aneuploidies or chromosome duplication syndromes.

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