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MLPA: A Rapid, Reliable, and Sensitive Method for Detection and Analysis of Abnormalities of 22q

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Abstract

In this study, essential test characteristics of the recently described multiplex ligation-dependent probe amplification (MLPA) method are presented, using chromosome 22 as a model. This novel method allows the relative quantification of ~40–45 different target DNA sequences in a single reaction. For the purpose of this study, MLPA was performed in a blinded manner on a training set containing over 50 samples, including typical 22q11.2 deletions, various atypical deletions, duplications (trisomy and tetrasomy), and unbalanced translocations. All samples in the training set have been previously characterized by fluorescence in situ hybridization (FISH) with cosmid or BAC clones and/or cytogenetic studies. MLPA findings were consistent with cytogenetic and FISH studies, no rearrangement went undetected and repeated tests gave consistent results. At a relative change in comparative signal strength of 30% or more, sensitivity and specificity values were 0.95 and 0.99, respectively. Given that MLPA is likely to be used as an initial screening method, a higher sensitivity, at the cost of a lower specificity, was deemed more appropriate. A receiver operator characteristic (ROC) curve analysis was performed to calculate the most optimal threshold range, with associated sensitivity and specificity values of 0.99 and 0.97, respectively. Finally, performance of each individual probe was analyzed, providing further useful information for the interpretation of MLPA results. In conclusion, MLPA has proven to be a highly sensitive and accurate tool for detecting copy number changes in the 22q11.2 region, making it a fast and economic alternative to currently used methods. The current study provides valuable and detailed information on the characteristics of this novel method.

Keywords

MLPA; atypical rearrangements; velocardiofacial syndrome; VCFS; DiGeorge; conotruncal anomaly; 22q11DS

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INTRODUCTION

The presence of chromosome-specific, low-copy repeats (LCRs) on the proximal part of the long arm of chromosome 22 predisposes this region to cytogenetic rearrangements [Shaikh et al., 2001; Edelmann et al., 1999]. To date, the most prevalent abnormality mediated by LCRs in this region is the 22q11.2 deletion, leading to syndromic disorders that include velocardiofacial syndrome (VCFS), DiGeorge syndrome, and conotruncal anomaly face syndrome. This deletion-based disorder is increasingly referred to as “22q11.2 deletion syndrome” (22q11DS). Clinical characteristics of 22q11DS include significant cardiac defects, thymic, parathyroid, craniofacial, developmental, neurological, and behavioral manifestations [McDonald-McGinn et al., 1999; Ryan et al., 1997; Emanuel et al., 2001; Gerdes et al., 1999; Heineman-de Boer et al., 1999; Moss et al., 1999].

In addition, consistent with the expected influence of the LCRs, internal duplications and even a triplication of 22q11.2 have recently been described [Portnoi et al., 2005; Yobb et al., 2005]. The internal duplication is associated with a variable clinical phenotype including some of the features of 22q11DS [Ensenauer et al., 2003]. Further, the breakpoint implicated in generating the Cat Eye Chromosome, a supernumerary inverted duplication of proximal 22q, frequently matches the proximal LCR or one of the more distal LCRs of the 22q11.2 region [Emanuel and Shaikh, 2001; McDermid et al., 1986]. The only recurrent non-Robertsonian translocation in humans, the recurrent t(11;22) (Emanuel syndrome) [Shaikh et al., 1999] has LCR-B as its chromosome 22 breakpoint. Finally, several translocations occur between 22q11.2 and other chromosomes, including a recurrent t(17;22) [Kurahashi et al., 2003], a t(20;22) [de la Chapelle et al., 1981], a t(4;22) [Nimmakayalu et al., 2003], and a t(1;22) [Rhodes et al., 1997], as well as numerous others [Li et al., 1995; Spiteri et al., 2003]. The various rearrangements of 22q11.2 have been extensively reviewed [Emanuel and Shaikh, 2001; Shaikh et al., 2001].

The current estimate of the prevalence at birth of 22q11.2 deletions is approximately 1 in 4,000–6,000 [Tezenas Du Montcel et al., 1996; Botto et al., 2003; Zori et al., 1998; Goodship et al., 1998]. While no information is available on the frequency of duplications and triplications, recent reports suggest a higher prevalence than previously assumed [Yobb et al., 2005]. Taken together, the existing findings imply a high rate of occurrence of different cytogenetic rearrangements involving 22q11.2 that are associated with various ranges of mild and severe clinical manifestations. Thus, proper diagnosis has implications related to clinical outcome, reproductive options, and treatment modalities.

Although standard karyotyping and occasionally multiplex fluorescence in situ hybridization (M-FISH) [Lee et al., 2001] are used to identify translocations involving 22q, the most widely used genetic diagnostic procedure for the detection of 22q11.2 deletions and rearrangements involving changes in copy number is fluorescence in situ hybridization (FISH). Despite the high reliability of such methods, they are laborious and do not have the sensitivity to detect small deletions or duplications (<40 kb) within 22q11.2. Given the high prevalence of 22q rearrangements in the population, a reliable, rapid, sensitive, and less expensive method is necessary to detect these frequent 22q rearrangements.

Recently a novel, commercially available PCR-based technique, multiplex ligation-dependent probe amplification (MLPA), has been favorably described for use in the detection of typical deletions in the 22q11.2 region [Fernandez et al., 2005]. In this study an extensive characterization of this assay has been performed, with specific emphasis placed on determining its utility with regard to the following criteria: 1) sensitivity and reliability; 2) cost effectiveness; 3) ease of use; and 4) suitability for the detection of various types of copy number differences within the 22q11.2 region. MLPA is a previously described method [Schouten et

al., 2002] that concurrently permits the relative quantification of ~40–45 different target DNA sequences. In this work we present the results of MLPA on a training set of previously characterized samples derived from individuals with a variety of rearrangements of the 22q11.2 region. These include typical 22q11.2 deletions, a variety of atypical deletions, 22q11.2 duplications (trisomy and tetrasomy) and unbalanced translocations. First, we determined the concordance of MLPA results with the previously performed FISH results for each sample in the training set. Second, we assessed the reliability of each individual probe in the currently available MLPA DiGeorge Kit (SALSA P023; MRC-Holland; www.mrc-holland.com). Third, the combined data set was used to determine the optimal cutoff values for normalized MLPA signals. Thus, this study was designed to provide further detailed information with regard to essential characteristics of the currently available MLPA kit for the detection of copy number changes in the 22q11.2 region.

MATERIALS AND METHODS

Sample Set

Over the past 20 years the clinical genetics center at The Children's Hospital of Philadelphia (CHOP) has enrolled numerous patients with a variety of deletions and rearrangements of 22q, such as those described in the Introduction, in Institutional Review Board (IRB)-approved research protocols. Characterization of 22q11 rearrangements by FISH analysis with cosmid or BAC clones had been previously performed in our research laboratory. A group of 62 well characterized samples, including typical 22q11.2 deletions, atypical deletions, duplications (trisomy and tetrasomy), and unbalanced translocations, as well as samples without a known rearrangement, were selected to create a training set for the purpose of this study. MLPA was performed in the research laboratory blinded to the molecular genetic status of the samples in the training set. Table 1 shows a detailed description of the cell lines utilized as the training set. The cell lines are designated by in-house identifier numbers assigned by the tissue culture core facility. The training set includes a group of standard 22q11.2DS deletions (34 cases), several recurrent smaller 22q11.2DS deletions (eight cases), several unique 22q11.2DS associated deletions (three cases), a group of unbalanced translocations to 22q (nine cases), several interstitial duplications of 22q11.2 (two cases), several supernumerary der(22)t(11;22)(q23;q11) cell lines (three cases), a Cat Eye syndrome cell line, and several balanced translocations to 22q (two cases).

Laboratory Methods

Genomic DNA (20–500 ng) was denatured (5 min at 98°C) and subsequently hybridized to the MLPA probe set according to the manufacturer's protocols (P023 Kit: DiGeorge Syndrome; MRC-Holland). The P023 kit contains PCR primers for 39 loci, including 11 on the long arm of chromosome 22. They are strategically distributed in and around the region flanked by LCRs A to D. The remaining 28 loci are located on chromosomes 4 (six loci), 7 (one locus), 8 (five loci), 10 (10 loci), 17 (four loci), and 18 (two loci). Each PCR amplifiable locus in this kit consists of two hemiprobcs, each of which contains one-half of the target sequence (the entire target sequence contains ~40–75 nucleotides). While one hemiprobe contains a target-specific sequence of 20–30 nucleotides flanked by a universal primer, the other hemiprobe contains a target-specific sequence at one end (25–43 nucleotides) separated from a universal primer at its other end by a variable length “stuffer” fragment of 19–370 nucleotides. Probe pairs are designed to bind adjacent to one another on the target DNA to be joined by ligase. Upon ligation, a contiguous DNA fragment flanked by universal primer binding sites is produced. The ligated pairs of hemiprobcs become the substrate for amplification through the PCR using the universal primers. As all probe pairs are flanked by identical primer sequences at their 5' and 3' ends, simultaneous amplification using only one primer pair occurs. The relative availability of ligated hemiprobcs at the initiation of the PCR reaction corresponds to the

availability (copy number) of target sequences of the genomic DNA in the sample. The unique variable-length fragment engineered into each of the locus-specific primer pairs permits their electrophoretic separation upon capillary or gel electrophoresis. Signal strength (peak area or height) of PCR products can be measured, allowing copy number of each target sequence to be inferred. Detailed methodology for MLPA is described elsewhere [Schouten et al., 2002].

In the current study, PCR amplification was carried out on an ABI 9700 thermal cycler (Applied Biosystems, Foster City, CA) and electrophoresis was performed using the ABI 3700 DNA analyzer (Applied Biosystems) with Rox 500 size standard and GeneMapper software (GeneMapper Software v3.0; Applied Biosystems).

Statistical Methods

Analysis of results was carried out upon the transfer of GeneMapper results to an Excel (Microsoft; www.microsoft.com) spreadsheet. Failed samples were deleted and primer-dimer/control peaks were removed. Uncorrected MLPA results display a negative correlation between probe size and signal strength. This artifact probably results from preferential injection of smaller fragments through the capillaries of the DNA analyzer. Prior to the normalization of the data, a correction of the artificial downsloping of signal strengths was accomplished using a binomial regression model. Each signal value was divided by its predicted value obtained from the regression model. This method has been described by Mavrogiannis et al. (<http://leedsdna.info/science/dosage/REX-MLPA/REX-MLPA.htm>). Normalization was achieved by division of signal values by the sum of the signal values of all probe sets (loci) for each sample. Finally, normalized signal values were compared to signal values from normal controls by dividing the normalized signal values with the average calculated from the results derived from four to seven control samples; the product of this calculation was termed the dosage quotient. When a target locus in a test sample is disomic (i.e., present in a normal copy number; $n = 2$), the normalized signal value for that locus will be equal to that of the control sample, and thus the dosage quotient will be close to 1.0. A deletion of a locus in a test sample ($n = 1$) should result in a reduction of relative signal strength of $>30\%$, whereas a duplication should lead to an increase in relative signal strength of $>30\%$ (i.e., the delta value is 0.3). Thus, a normal or disomic copy number should generate a normalized signal value of 1, in the case of a deletion or duplication, the normalized signal should be less than 0.7 ($1 - 0.3$) or greater than 1.3 ($1 + 0.3$), respectively. A delta value of 0.3 is a commonly accepted empirically derived threshold value for genetic dosage quotient analysis (e.g., Bunyan et al. [2004]).

Confirmation of each of the results obtained in the MLPA-derived data set, could be accomplished by comparison with existing data derived from previous studies. These studies included extensive FISH mapping of breakpoints, cytogenetic G-band analysis (in cases of translocations), or a combination of both. Under the assumption that the existing data were accurate, each locus signal was then individually labeled as false-negative/positive or true-negative/positive, respectively. For example, a false-positive call would be a MLPA probe signal indicating a deletion or duplication at a locus with a normal copy number as assessed by FISH or cytogenetic analysis.

For each locus signal in a given run: 1) the variation of the signals from that particular locus in the control samples; and 2) the variation of the combined locus signals of that particular sample were calculated and expressed as standard deviations (SDs). These values are referred to as the “probe set (locus) SD” (Probe SD) and “Sample SD.”

Assessment of Probe Set (Locus) Reliability

Differences in reliability of signal outcome are likely to exist between the individual probe pairs, as a result of probe-inherent characteristics such as kinetics of hybridization, ligation,

and amplification. Therefore, the mean Probe SD for each individual probe was calculated from the results of the normal samples. The Levene test for homogeneity of variance was used to assess whether the variability of these values was statistically significant. Subsequently, a correlation between mean Probe SD values and the sum of all spurious results was tested using Pearson regression analysis. If both suppositions can be confirmed, the mean Probe SD for a probe provides useful information with regard to the reliability of that probe.

Assessment of the Optimal Cutoff Values

Receiver operating characteristic (ROC) curve analysis was used to determine the optimal cutoff values. The ROC curve is determined by plotting the sensitivity vs. 1 minus the specificity. Using this approach the Y-axis represents the fraction of correctly recognized deletions and duplications (i.e., sensitivity), while the X-axis represents the fraction of false positives (i.e., the fraction of loci with a normal copy number that are incorrectly labeled as deletions or duplications). The threshold range around the expected value of 1.0, in the case of a normal copy number, can be varied by altering the delta value. The normal threshold range lies between $1.0 \pm \text{the delta value}$. The sensitivity and specificity characteristics were computed for each delta value in a series of delta values. Subsequently, the sensitivity and specificity values associated with each delta value were used to generate the ROC curve. By lowering the delta value, the threshold width decreases and thus the rate of false positives increases and the rate of false negatives decreases. When the delta value is increased the opposite occurs.

Two methods were used to determine the optimal cutoff. The first used an empirical method of selecting a point at which the sensitivity was 0.99. Given the most likely purpose for utilizing the MLPA P023 test, sensitivity was considered more important than specificity. This is because it is likely that positive results would require confirmation by another method such as FISH or quantitative PCR. The second method for determining the optimal cutoff utilized the slope of the tangential line to the ROC curve. The slope represents the weight of a false positive vs. the weight of a false negative. When both outcomes are considered equally important, a delta value corresponding to the point at which the tangential slope to the ROC curve is 45 degrees should be selected. This point is situated in the elbow of the ROC curve. However, since sensitivity was considered to be more important than specificity—i.e., the weight of a false negative is greater—a point to the right of the elbow of the ROC curve, where the tangential slope is less than 45 degrees, should be selected. The associated delta value will generate relatively fewer false negatives (high sensitivity) than false positives (relatively lower specificity). Conversely, the delta value associated with the point at which the tangential slope is greater than 45 degrees is associated with a high specificity whereas the sensitivity is relatively lower.

RESULTS

An overview of results from the MLPA studies on a selection of the samples from the training set (Table 1) is shown in Figure 1. Taking the combined results of all 22q11.2 probes into consideration, MLPA findings were concordant with the prior FISH studies; none of the rearrangements went undetected and in replicates using the same samples consistent results were achieved. In two cases, MLPA findings revealed an atypical proximal breakpoint centromeric of LCR-A, which was unsuspected based upon previous FISH studies. This was subsequently confirmed by additional FISH and molecular studies (see samples CH01-242 and CH02-008 in Fig. 1). Further, the MLPA findings revealed a variant distal breakpoint in one of the same samples (CH02-008) that was also subsequently confirmed by additional FISH studies.

Examination of the individual results for any of the 39 probes in the kit when limited to probes with a Probe SD and Sample SD below 0.20 indicates that the outcomes are very robust; at a

delta value of 0.3, sensitivity is 0.952 with a 95% confidence interval (95%CI) of 0.928–0.969 and specificity is 0.986 with a 95%CI of 0.984–0.988.

Assessment of Probe Reliability

The mean Probe SD for each probe-pair was calculated from the total of 101 control samples that were included in the MLPA experiments described here. A box-plot diagram of signal variability is shown in Figure 2. We utilized the Levene test for homogeneity of variance. Results derived from application of the Levene test indicate that variance in probe signals differed significantly among the probes (Levene test; $P < 0.01$). Further, the Pearson correlation coefficient, computed with the mean Probe SD and the total percentage of false results as independent variables (with delta = 0.22), indicated a strong (positive) correlation (Pearson coefficient: 0.80; two-tailed P-value: 8.6×10^{-10}) (see Figure 3).

For example, the probes 22–1229 and 4–1213 had the highest mean Probe SD (0.096 and 0.105, respectively), while the probes 18–0587 and 22–1521 were identified as having the lowest variability in signals (mean Probe SD: 0.033 and 0.034, respectively). Consistent with the strong positive correlation between the mean Probe SD and the fraction of spurious results, 14–19% of the total of all signals generated by probes 22–1229 and 4–1213 were spurious results, whereas probes 18–0587 and 22–1521 only produced 2 to 3% spurious results.

Assessment of the Optimal Cutoff Values

The empirical assessment of the most optimal cutoff value (expressed as a delta value) consisted of plotting of the percentage of false-positive and false-negative results from all runs as a function of the delta value. The overall ROC curve indicates a rapid rise in sensitivity (data not shown). The area under the ROC curve is 0.9766 (95%CI, 0.967–0.986), indicative of an excellent level of accuracy for the test. In order to identify the elbow of the curve, a magnification of the elbow region is presented in Figure 4. The delta value associated with equal weight of a false positive and a false negative (i.e., specificity and sensitivity are equally important) is 0.16. At this point the tangential slope of the ROC curve is 45 degrees. The delta value considered most optimal for the purposes of this specific test is one for which the percentage of false negatives is 1% or less (i.e., a sensitivity of 0.99). The outcome of the empirical assessment and the ROC curve analysis concur at a delta value of 0.22. The corresponding sensitivity for this delta value is 0.99 (95%CI, 0.977–0.998) and the corresponding specificity is 0.967 (95%CI, 0.963–0.970).

DISCUSSION

The diagnostic interpretation of MLPA results for a given sample is usually performed by examination of the combination of probe signals. Any copy number change mediated by rearrangements involving LCRs A, B, C, or D will more than likely affect more than one probe in the current P023 MLPA kit, and is therefore highly likely to be identified. Indeed, in the present study none of the samples in the training set with known copy-number differences went undetected. In addition, there were no instances (Sample SD < 0.20) in which a deletion or duplication was spuriously suggested by false-positive results for more than two adjoining probes. Moreover, in three instances MLPA proved to be more informative than FISH, revealing an extension of deletions centromeric to LCR-A in two cases and an atypical distal endpoint in one case. From the initial FISH studies, LCR-A was assumed to be the proximal breakpoint in two cases and LCR-D was assumed to be the distal breakpoint in one case. These assumptions were made based on two factors. One was the evidence from numerous other samples that the majority of deletion endpoints resides within one of the blocks of LCR sequence. The other was that when performing a FISH test, just as when assessing a MLPA locus, only a limited region is being queried. However, additional FISH studies confirmed the

findings of MLPA, adding to the characterization of these atypical deletions. These results will allow for additional molecular studies as well as genotype–phenotype assessment to characterize what these atypical deletions add to the 22q11.2DS knowledge base.

However, the sensitivity and specificity of each individual probe must also be considered for their significance. This is primarily the case because short regions of copy number change are likely to affect only one probe instead of two or more. For example, the 22q13.3 deletion in the training set (CH96-160; Table 1) resulted in only one positive (deletion) signal. Also, a sample from the unbalanced t(4;22)(q35.1;q11.2) generated not only five positive (deletion) 22q probes proximal to LCR-B, but also a single positive signal (deletion) of a 4q35 probe, indicative of the deletion of 4q at that locus (CH95-208; Fig. 1). This result clearly distinguishes the difference in the 4q breakpoints for two similar translocations, CH95-208 and CH95-015, providing molecular evidence for the more distal 4q breakpoint in the latter cell line.

The sensitivity and specificity of diagnostic test results for this MLPA kit using a delta value of 0.3 are a reasonable starting point (sensitivity 0.95; specificity 0.99). However, since we anticipate that this test is likely to be used as an initial screening method, we would advocate that using a somewhat higher sensitivity, at the cost of a lower specificity, is more appropriate. In other words, if MLPA is to be used in an initial screening assay, avoiding false negatives or the possibility of missing a real diagnosis is most crucial. Thus, any uncharacteristic positive result after the initial screening test (i.e., a single locus demonstrating a copy-number difference) should be confirmed with a different method (such as FISH). Using this approach, the presence of false-positive results is of lesser consequence. Using the current data set we were able to compute a delta value (0.22) associated with a high sensitivity (0.99) and a very acceptable specificity (0.97). We suggest that this delta value is more appropriate if the test is used for the aforementioned purposes. Given the significant numbers of 22q11.2 deletion tests that are performed in a clinical setting, this would allow for a cost-effective, rapid, diagnostic screen and eliminate many of the more costly cytogenetic and FISH tests performed for detection of deletions. Further, this would allow for the identification of 22q11.2 duplications that often appear as phenocopies of the deletion and are difficult to identify using metaphase FISH alone [Portnoi et al., 2005; Yobb et al., 2005].

Our findings indicate that the current version of the commercially available MLPA diagnostic kit contains several probes that consistently display greater signal variability when compared with the other probes in the set. We have observed that the mean Probe SD, an expression of the extent of variability of individual probes, strongly correlates with the number of spurious results generated by the probe. Thus, the mean Probe SD value for a given locus provides a measure of that probe's reliability. The results provided by our detailed probe analysis can further provide assistance with the interpretation of results. A box-plot diagram of the signal variability of each probe in the currently available P023 kit is provided in Figure 2. In addition, our findings have been communicated to the manufacturer, and several of the less reliable probes in the current kit are planned to be replaced in future versions.

The higher variability in the performance of some probes may be a result of suboptimal probe characteristics, such as the kinetics of hemiprobe hybridization, hemiprobe ligation and fully ligated probe amplification. Conceivably, spurious results implying either a deletion or a duplication may in some cases be the consequence of the presence of a copy number polymorphism (CNP), rather than of a cytogenetic abnormality. Recently, it has been shown that CNPs provide a substantial contribution to the (normal) variation of the human genome [Sebat et al., 2004]. Further, the small size of the MLPA probes (20–30 nucleotides) may cause a failure of hybridization if a small or single base pair polymorphism is present in the target sequence, leading to an unreliable result.

Nonetheless, the advantage of the small size of the probes is that they allow for a more precise assessment of the boundaries of a rearrangement involving an alteration of copy number. For the purpose of the detection of atypical deletions and/or duplications in the 22q11.2 region, this characteristic clearly prevails over the correctable risk of false-positive deletion calls due to polymorphisms in the target sequences. Further, the value of being able to accurately and simultaneously assess multiple short-target sequences provides increased sensitivity over similar diagnostic tests performed with individual BAC or cosmid FISH clones. This is because these larger clones may hybridize even when the target sequence is only partially present, while partial binding and subsequent ligation of MLPA probes is highly unlikely. Further, BAC or cosmid FISH is likely performed with one or two target sites per hybridization. MLPA expands the number of loci queried in a single experiment.

A method somewhat similar to MLPA is multiplex amplification and probe hybridization (MAPH). This protocol does not depend upon ligation of probes prior to amplification or stuffer sequences in the primers, but rather relies on size differences of the targets selected for amplification and stringent posthybridization washes. Thus, the essential difference between MLPA and MAPH is that while in MLPA only paired hemiprobcs bound to target sequences are ligated and subsequently amplified, in MAPH unbound probes can, in theory, be amplified (e.g., if the sample is inadequately washed). Further, the size of MAPH probes is approximately 100–600 bp. Compared to the smaller MLPA probes, the MAPH probes may more easily lead to false-positive results when the probe binds with a partially deleted target sequence. An additional advantage of MLPA over MAPH includes the MLPA requirement for very small amounts of DNA for testing (125 ng is sufficient) [Sellner and Taylor, 2004].

Further, MLPA has significant advantages over other methods such as FISH or array-CGH, based on the fact that it is much less expensive, less labor intensive, and an answer can be achieved with a quicker turnaround time. Finally, after discussion with the manufacturers, additional loci will be added to the MLPA kit. This will allow for a more comprehensive copy-number assessment of the region of 22q11.2 involved in multiple rearrangements.

The results of this study indicate that MLPA is a rapid, relatively inexpensive assay that performs with high sensitivity and specificity. This makes it an appealing alternative to existing methods for the detection of copy-number differences of 22q as well as other regions of the genome.

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FIGURE 1. Overview of results from MLPA and previous FISH studies on a selection of samples from the training set (Table 1). The number to the left indicates the sample class, as designated in Table 1. Shown above the representation of chromosome 22 are the FISH probes (cosmids), designated either by cosmid address or locus name. The FISH probes (orange ovals) and MLPA probes (orange rectangles) are shown in map order based upon data derived from the University of California, Santa Cruz (UCSC) genome browser (<http://genome.ucsc.edu>). All MLPA loci were tested in all samples. FISH studies are indicated by the presence of colored ovals in line with a sample. Copy number for MLPA or FISH probes are indicated by color (grey = normal, two copies; red = deleted, one copy; green = duplicated, three copies; blue = duplicated, four copies).

FIGURE 2.

Box-plot representation of variability of all probes in the P023 MLPA kit. The dosage quotients are plotted on the Y-axis. Since the probe signals in this analysis are exclusively derived from normal samples, the expected dosage quotient for each signal is 1.0. The top horizontal line of the box represents the 75th percentile, the bottom line of the box is the 25th percentile. The horizontal line in the middle of the box is the median. Vertical bars outside the box represent the 10th and 90th percentiles.

FIGURE 3.

Correlation between the mean variability of individual probes (expressed as an SD, calculated from all normal probes) and the percentage of false results (sum of false positives and false negatives). With $\delta = 0.22$, the Pearson coefficient is 0.80, two-tailed P-value: 8.6×10^{-10} .

FIGURE 4.

ROC curve analysis: the Y-axis represents the fraction of correctly recognized deletions and duplications (i.e., sensitivity), the X-axis represents the fraction of false positives (i.e., 1-specificity). Plotted values are calculated for a range of different cutoff thresholds (delta values).

Table 1

Description of the Training Set

Sample identifier	Class ^a	Karyotype	Category of rearrangement	Number of cases
Numerous	1	46,XX or XY,del(22)(q11.2)	Standard 3Mb 22q11.2 deletion (LCR A-LCR B)	34
CH92-247,CH99-199, CH01-246	2	46,XX or XY,del(22)(q11.2)	Recurrent nested 22q11.2 deletion (LCR A – LCRC)	3
CH98-135, CH95-040, CH02-182, CH98-182, CH03-127	3	46,XX or XY,del(22)(q11.2)	Recurrent nested 22q11.2 deletion (LCR A – LCR B)	5
CH04-018	4	46,XX,del(22)(q11.2)	Unique variant 22q11.2 deletion	1
CH01-242	5	46,XY,del(22)(q11.2)	Unique variant 22q11.2 deletion	1
CH02-008	6	46,XY,del(22)(q11.2)	Unique variant 22q11.2 deletion	1
CH96-160	7	46,XY,-22,+der(22)t(1;22)(?;q13)	Translocation to 22qter of acrocentric short arm	1
CH96-010 (GM00980)	8	45,XX,-11,-22,+der(11)(11;22)(11q25;q11.2)	Unbalanced 22q11IDS-associated translocation ^b	1
CH95-215	9	45,XY,-1,-22,+der(1)(1;22)(p36;q11.2)	Unbalanced 22q11IDS-associated translocation	1
CH95-208		45,XX,-4,-22,+der(4)(4;22)(q35.1;q11.2)mat	Unbalanced 22q11IDS-associated translocation ^c	1
CH95-015		45,XY,-4,-22,+der(4)(4;22)(q35.2;q11.2)	Unbalanced 22q11IDS-associated translocation	1
CH94-075		45,XX,-12,-22,+der(12)t(12;22)(p13.3;q11.2)	Unbalanced 22q11IDS-associated translocation	1
CH98-48		45,XX,-17,-22,+der(17)t(17;22)(p13.3;q11.2)	Unbalanced 22q11IDS-associated translocation	1
CH95-103		45,XX,-17,-22,+der(17)t(17;22)(q25.3;q11)	Unbalanced 22q11IDS-associated translocation	1
CH94-073		47,XX,-22,+der(X)t(X;22)(p22.31;q11.2)mat	Unbalanced translocation	1
CH05-52, CH05-50	10	46,XY,dup(22)(q11.2q11.2)	Interstitial 22q11.2 duplication	2
CH95-256,CH97-122, CH97-085	11	47,XX,+der(22)t(11;22)(q23;q11.2)	Supernumerary der(22)t(11;22) syndrome (Emanuel syndrome)	3
CH00-120	12	47,XX,+inv dup(22)(p13->q11.2;q11.2->p13)	Cat-eye syndrome chromosome	1
CH92-193	13	46,XX,t(16;22)(q24;q13.3)	Balanced translocation carrier	1
CH96-13	14	46,XX,t(6;22)(p11.2;q12.2)	De novo balanced translocation carrier	1

^aSee combined MLPA and FISH findings in Figure 1.

^bSpiteri et al. [2003].

^cNimmakayalu et al. [2003].