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Automatic analysis of multiplex ligation-dependent probe amplification products (exemplified by a commercial kit for prenatal aneuploidy detection)

For use in routine prenatal diagnostics, we developed software and methods for automatic aneuploidy detection based on a commercial multiplex ligation-dependent probe amplification (MLPA) kit. Software and methods ensure a reliable, objective, and fast workflow, and may be applied to other types of MLPA kits. Following CE of MLPA amplification products, the software automatically identified the peak area for each probe, normalized it in relation to the neighboring peak areas of the test sample, computed the ratio relative to a reference created from normal samples, and compensated the ratio for a side effect of the normalization procedure that scaled all chromosomally normal DNA peak areas slightly up or down depending on the kind of aneuploidy present. For the chromosomes 13, 18, 21, X, and Y, probe reliability weighted mean ratio values and corresponding SDs were calculated, and the significance for being outside a reference interval around ratio 1.0 was tested. $p \leq 1\%$ suggested aneuploidy and $1 < p \leq 5\%$ suggested potential aneuploidy. Individual peaks, where the normalized area was situated more than 4 SD from the corresponding reference, suggested possible partial deletion or gain. Sample quality was automatically assessed. Control probes were not required. Having used the software and methods for two years, we conclude that a reliable, objective, and fast workflow is obtained.

Keywords: Multiplex ligation-dependent probe amplification / Prenatal aneuploidy detection
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1 Introduction

Multiplex ligation-dependent probe amplification (MLPA) is a novel technique allowing relative quantitation of about 40 different DNA sequences in a single reaction [1]. Many different commercial MLPA kits are available [2–6].

In our routine cytogenetics laboratory we perform more than 2000 MLPA reactions anually. All prenatal samples are processed in parallel by MLPA aneuploidy detection and conventional cytogenetics. We aim to complete and send MLPA aneuploidy results within two days. As there is no commercially available software for automatic analysis of MLPA results, we developed computer-assisted analysis of the electrophoresis data, improving and facilitating the interpretation of MLPA analysis. Several groups have developed semiautomated methods [2–6]. In this paper we describe our fully automated method, which despite the suppliers' recommendations is based on interassay instead of intra-assay evaluation of MLPA probe amplification products.

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Abbreviations: MLPA, multiplex ligation-dependent probe amplification; **RFU**, relative fluorescent units

2 Materials and methods

2.1 MLPA analysis

Approximately 40 oligonucleotide probes are hybridized to sample DNA, ligated and amplified by PCR in one reaction. The relative copy number of target sequences is reflected by the amount of probe amplification products. Each probe has a unique length, and electrophoresis is used for identification and quantitation of each amplified probe product.

The most recent aneuploidy detection kit, SALSA P095 (MRC-Holland, Amsterdam, The Netherlands) serves as an example in the following method description. The probe mix is a set of 40 probes consisting of 8 specific probes for each of the human chromosomes 13, 18, 21, and X, 3 probes specific for chromosome Y, and 5 additional fragments for quality control. Each probe is sepa-

rated from its neighbors by at least 6–9 bp. Four of the quality control fragments are amplified even if ligation fails, and the peaks representing these products are smaller than the other peaks when ligation works and the required minimum of 20 ng test DNA is present. The fifth quality control fragment represents a synthetic 2q14 fragment that is ligated and amplified like the 35 chromosome specific probes. In our hands the first control fragment sometimes became mixed up by a primer dimer peak, therefore we only used the other three control fragments for quality control of ligation and the amount of sample DNA.

2.2 Samples

DNA from 3 to 4 mL amniotic fluid or 5 mg chorionic villi was isolated using a QIAamp kit (Qiagen, Hilden, Germany), and 20–100 ng DNA was used in the MLPA aneuploidy protocol [3]. Here, 558 karyotypically normal cases and 4 cases with chromosome abnormalities are presented. All the normal cases fulfilled the quality requirements mentioned later, but to illustrate the effect of analyzing low amounts of DNA the normal cases were divided into two groups. In the first group the level of ligated probe peaks relative to the three nonligated control fragments was lower than 150%, whereas in the second it was higher. The low DNA group included 59 cases, while the other group constituted the normal reference including 499 cases.

2.3 Instrumentation

PCR reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Electrophoresis was performed on an ABI 3100 system, which analyzes up to 16 samples at a time. ABI Prism GeneScan Analysis software produced quantitated measurements for each detected peak (fragment length was measured in bp, the peak height and corresponding peak area in relative fluorescent units (RFU) ranging from 1 to 8100).

2.4 Software for automatic MLPA aneuploidy analysis

The fragment length, area, and height of each detected peak for each sample of a single run were exported automatically to individual text files having names identical to the sample names used on the ABI 3100 system. The sample exportation software was programmed by use of the ABI Sample File Toolkit (included in the system). Subsequently, software programmed in Microsoft Access (Microsoft, Redmond, WA, USA) automatically picked up case files one by one, analyzed the peak data, and printed one-sheet reports. The software is freely available at www.chromosomelab.dk.

2.4.1 Peak normalization

As the ligated probe peak areas produced by the ABI 3100 system often decreased with increasing fragment length, each peak area was normalized in relation to a group of neighboring peaks. The peaks were divided into four normalization groups according to fragment length. The synthetic 2q14 fragment and the first ten chromosome specific probes defined the first group, followed by three groups with nine, eight, and eight probes, respectively. Each group contained two peaks from each of the chromosomes 13, 18, 21, and X. There were only three Y chromosome specific peaks, two in the first group and one in the second. Each peak area was normalized by dividing it with the mean peak area of the group.

2.4.2 Normal reference data

Based on the 499 normal samples (239 female and 260 male), the mean fragment length for each of the 40 peaks was computed. The mean normalized peak area and the corresponding SD for each peak were computed separately for female and male samples, because the X and Y peaks affect every peak-computation due to normalization.

2.4.3 Automatic peak recognition

For each peak of a test sample the fragment length was compared to all mean lengths of the reference data set by a distance measure that divided the difference by the square root of the actual peak area. By using this distance measure most small nonspecific amplification products were rejected, since they got a relatively high distance value, even if they were close to one of the mean fragment lengths in the reference data set. Other nonspecific amplification products were avoided by requiring all probe peaks to be $\geq$ 90 RFU (except the peaks representing the Y chromosome). Peaks were classified in two rounds with the purpose of avoiding nonspecific amplification products to be classified as Y peaks when the sample was female. During the first round, peaks closer than 2 bp to the nearest reference peak mean were classified by use of the distance measured. This first round was used to catch locations where the GeneScan Analysis software had interpreted the Genescan-500 Rox standards wrongly and made local or global offsets to the

fragment lengths. The fragment lengths of the reference data were subtracted from the actual fragment lengths. Before the second round, the resulting list of differences were smoothed by a (1,1,1,1,1)-linear filter and added to the reference means. All peaks were classified again, but this time the corrected reference means were used and the maximum distance was decreased to 0.90 bp to avoid Y peaks for female samples. Samples with identified Y peaks were assumed to be male samples unless the mean of the Y-peak areas was less than 9% of the mean of the four peaks appearing prior to each classified Y peak.

2.4.4 Computation of probe signals relative to the reference data

The ratio between each normalized peak area and the corresponding mean normalized peak area of the appropriate female or male reference data set theoretically reflects the DNA copy number of the corresponding sample DNA sequence. However, it was necessary to compensate for a side effect of the normalization procedure that scaled all normal DNA peak areas slightly down when trisomy peaks were present (and up when monosomy was present); thus, each peak ratio was finally divided by the median ratio value of all peaks. In this way disomy, trisomy, and monosomy peaks showed ratios in the range of 1.0, 1.5, and 0.5, respectively.

2.4.5 Computation of representative statistics for chromosomes 13, 18, 21, X, and Y

Peaks with relatively large area variations were given a lower weight (mean/SD of the corresponding reference data peak area) when computing the mean ratio and SD of the chromosome specific peaks. Prior to application, the weights were scaled, in order to let the sum of chromosome specific weights equal the number of peaks for the chromosome.

2.4.6 Ploidy evaluation of chromosomes 13, 18, 21, X, and Y

It was tested how significantly (one-tailed p-level) the mean ratios for chromosomes 13, 18, 21, X, and Y differed from 1.0. For chromosomes 13, 18, 21, and female X an interval of 1.0 ± 0.1 was used, for male X 1.0 ± 0.13 was used, and for chromosome Y 1.0 ± 0.24 was used. These intervals were in the range of ratio $1.0 \pm$ (3.2–4.6) SD of the corresponding chromosome ratios in the reference data set (see Table 1). $p \leq 1\%$ suggested aneuploidy, $1 < p \leq 5\%$ suggested possible aneuploidy. Samples where p was $>10\%$ were deemed normal with respect to ploidy. Samples showing $5 < p \leq 10\%$ were regarded possible mosaic, or having partial gain or deletion of a chromosome.

2.4.7 Evaluation of partial deletion or gain of chromosome 13, 18, 21, X, and Y

Individual peaks having the normalized area placed more than 4 SDs from the corresponding reference suggested possible partial deletion or gain.

2.4.8 Quality assessments

The sample was rated "poor technical quality" if the results did not meet the following requirements: (i) The total area of the ligated probe peaks should be at least 50% of the total area of the primer dimer peaks (these were peaks detected at fragment lengths between approximately 35 bp (lower limit of ABI 3100 output) and 64 bp (length of the smallest control fragment). (ii) The mean area of the ligated probe peaks should be at least 65% of the mean area of the three nonligated control fragment peaks. (iii) The mean height of the first 20 ligated probe peaks should be higher than 450 RFU, and the mean of the last 16 should be higher than 280 RFU (Applied Biosystems recommends to keep intensities between 200 and 4000 RFU), and the ratio of these mean

Table 1. Statistics of the chromosome mean ratios of the 499 normal reference samples

Chromosome	Samples	Mean	SD	Minimum	Maximum	Reference interval
13	499	1.001	0.0227	0.906	1.093	1.0 ± 0.10 (4.4 SD)
18	499	1.001	0.0216	0.905	1.099	1.0 ± 0.10 (4.6 SD)
21	499	1.001	0.0222	0.929	1.087	1.0 ± 0.10 (4.5 SD)
X-female	239	1.001	0.0255	0.925	1.065	1.0 ± 0.10 (3.9 SD)
X-male	260	1.001	0.0305	0.885	1.087	1.0 ± 0.13 (4.3 SD)
Y-male	260	1.001	0.0746	0.767	1.173	1.0 ± 0.24 (3.2 SD)

heights should be below 3.0. (iv) The maximum ratio CV (*i.e.*, SD/mean) for a chromosome should be below 0.35, and the mean of these CVs should be below 0.20. All the above stated requirements were based on experience. Furthermore, the system counted the number of results being close to the "poor quality" limits, and it marked peaks found more than 0.5 bp from the expected fragment length, peaks being unexpectedly wide (*i.e.*, not being close to predicted values based on linear regression of all peak widths of the sample), and the ones higher than 7000 RFU. The system also warned if an expected peak was missing or if only a few of them were higher than 1000 RFU.

2.4.9 Report

Finally, a report was printed for each prenatal sample showing the chromosome position of the actual probes, peak measurements and ratio for each peak, summary statistics including the mean ratio values and *p*-levels (when $p < 25\%$) for the chromosomes 13, 18, 21, X, and Y, putative diagnosis, quality measurements, and quality warnings. Individual peaks having the normalized area placed more than 4 SD from the corresponding reference were marked. For $p > 10\%$ the system suggested: "Normal 13, 18, 21, XX" or "Normal 13, 18, 21, XY." For $p \leq 5\%$, the system suggested the following diagnoses, "Trisomy 13" (or 18, 21, X), "Monosomy 13" (or 18, 21, X), "Male with extra X" (or Y), "Male without an X (or Y)," "69,XYY," and "69,XXY or contamination by maternal DNA." The least significant ratio of X and Y needed only a $p \leq 10\%$ and $p \leq 15\%$, respectively, to produce the last two diagnoses. For abnormal samples, the expected ratio of the reported diagnosis was printed to help detection of mosaicism, *etc.* (*e.g.*, the expected Y ratio for a "Male with extra Y" is 2.0).

For samples having all three Y peaks, but at an average normalized area $< 25\%$ of the reference data set, two reports were printed: one in relation to female reference data and one in relation to male reference data.

The automatic diagnosis and quality evaluation produced by the software is only intended to assist in making the MLPA aneuploidy diagnosis. The final diagnosis is made by an M.D.

3 Results

Here we report methodically aspects of automatic MLPA analysis exemplified by a new aneuploidy detection kit (P095 kit, MRC-Holland, www.mrc-holland.com).

Compared to the MLPA kit supplier's standard normalization method that normalizes a peak area by dividing it with the sum of all areas, the four-group normalization technique described here decreased the variation of each individual probe ratio. For the 239 normal female reference samples, the mean of the ratio SDs of the 33 chromosome probes was reduced from 0.098 to 0.066 (33% reduction). For the 260 male reference samples, the mean ratio SD of the 36 probes was reduced from 0.102 to 0.074 (27% reduction).

By computing the sample mean ratio of a chromosome on the basis of probe "reliability" weights, the noise that may arise from "unreliable" probes is reduced. For the 499 normal reference samples, the SDs of the sample mean ratios for chromosomes 13, 18, 21, female X, male X, and Y were reduced by 4.6, 1.8, 2.6, 6.6, 2.6, and 5.5%, respectively, when weighting was applied. Further summary statistics of the chromosome mean ratios of the 499 normal reference samples are shown in Table 1 along with the applied reference interval for normal cases. It is obvious that all ratios are inside the reference interval for this data set. Table 2 shows that the SDs increase for each chromosome from 20 to 81% when the 59 karyotypically normal cases with small amounts of DNA were analyzed and compared to the 499 normal reference samples. Another effect of small amounts of DNA is that the mean ratio of male Y chromosomes decreases.

Table 2. Statistics of the chromosome mean ratios of the 59 normal low DNA samples

Chromosome	Samples	Mean	SD	Increase of SD compared to Table 1
13	59	1.001	0.0324	43%
18	59	1.008	0.0291	35%
21	59	1.010	0.0402	81%
X-female	20	1.009	0.0407	60%
X-male	39	0.989	0.0519	70%
Y-male	39	0.930	0.0893	20%

Figures 1–4 show essential clippings from the final report-sheet.

Figure 1 shows details of a trisomy 13 chorionic villus sample. The weighted mean ratio 1.35 was significantly higher than ratio 1.1 ($p = 0.000\%$), but it did not reach the theoretical 1.5 level for trisomies. All peaks were marked by an * illustrating that their areas were more than 4 SD from the reference areas.

Figure 2 shows details of a partial 18q gain of a chorionic villus sample (46,XX,der(4)t(4;18)(p16;q21)). The weighted mean ratio 1.22 was not significantly higher than ratio 1.1 ($p = 9.68\%$). Four peaks were marked by an * illustrating that their areas were more than 4 SD from their reference areas.

			Normalized Peak Area						
Peak Label	Peak no.	Peak Area	Peak Area	Ref. Mean	Ref. SD	Ref. Weight	Ratio	Dist. in SD	Ratio P
13q32.1	10	38768	1.911	1.418	0.079	1.17	1.35	6.3 *	
13q13.3	15	28659	1.413	1.084	0.064	1.10	1.30	5.1 *	
13q14.2	20	32592	1.647	1.254	0.060	1.36	1.31	6.5 *	
13q21.33	25	24861	1.256	0.865	0.066	0.85	1.45	5.9 *	
13q34	29	22130	1.604	1.235	0.076	1.05	1.30	4.8 *	
13q13.1	33	21364	1.548	1.175	0.087	0.88	1.32	4.3 *	
13q14.2	37	24346	1.840	1.307	0.115	0.74	1.41	4.6 *	
13q34	41	19980	1.510	1.043	0.080	0.84	1.45	5.8 *	
Mean		26588	1.591	1.173	0.078	1.00	**1.35**		
Coefficient of variance							**0.05**		**P= 0.000%**
High significance P= 0.000%									**Trisomy 13**
Male Ref. Ratio 1.35 is found. Thereotically 'Trisomy 13' has ratio 1.5									

Figure 1. Details of a trisomy 13 chorionic villus sample.

			Normalized Peak Area						
Peak Label	Peak no.	Peak Area	Peak Area	Ref. Mean	Ref. SD	Ref. Weight	Ratio	Dist. in SD	Ratio P
18q21.1	12	52411	1.671	1.229	0.052	1.40	1.36	8.5 *	
18q21.32	16	40352	1.286	0.892	0.061	0.87	1.44	6.5 *	
18q11.2	20	29076	0.943	0.968	0.052	1.10	0.97	-0.5	
18q23	24	54952	1.781	1.203	0.071	1.01	1.48	8.1 *	
18p11.32	28	20005	1.005	1.073	0.063	1.01	0.94	-1.1	
18q21.33	32	18881	0.948	0.662	0.052	0.76	1.43	5.5 *	
18q11.2	36	28264	1.365	1.248	0.069	1.07	1.09	1.7	
18p11.21	40	21270	1.027	0.999	0.075	0.79	1.03	0.4	
Mean		33151	1.253	1.034	0.062	1.00	**1.22**		
Coefficient of variance							**0.19**		**P= 9.68%**
P= 9.68% is NOT significant!									**Increased ratio of chromosome 18**
Female Ref. Ratio=1.22 (but a normal ratio 1.0 was expected)									

Figure 2. Details of a partial 18q gain of a chorionic villus sample (46,XX,der(4)t(4;18)(p16;q21)).

			Normalized Peak Area						
Peak Label	Peak no.	Peak Area	Peak Area	Ref. Mean	Ref. SD	Ref. Weight	Ratio	Dist. in SD	Ratio P
21q22.13	15	27126	2.104	1.477	0.081	1.13	1.42	7.7 *	
21q21.1	20	25771	1.999	1.239	0.096	0.79	1.61	7.9 *	
21q21.1	25	23525	1.693	1.262	0.063	1.24	1.34	6.8 *	
21q11.2	30	18755	1.349	1.062	0.051	1.28	1.27	5.6 *	
21q22.11	34	16213	1.909	1.155	0.087	0.82	1.65	8.7 *	
21q21.3	38	14531	1.711	1.014	0.071	0.88	1.69	9.8 *	
21q22.3	43	14481	1.758	1.019	0.073	0.86	1.72	10.1 *	
21q22.11	47	15678	1.904	1.003	0.062	1.00	1.90	14.5 *	
Mean		19510	1.804	1.154	0.073	1.00	**1.55**		
Coefficient of variance							**0.14**		**P= 0.04%**
High significance P= 0.036%									**Poor Quality! Trisomy 21**
Male Ref. Ratio 1.55 is found. Thereotically 'Trisomy 21' has ratio 1.5									

Figure 3. Details of a trisomy 21 amniotic sample having a low amount of sample DNA.

			Normalized Peak Area						
Peak Label	Peak no.	Peak Area	Peak Area	Ref. Mean	Ref. SD	Ref. Weight	Ratio	Dist. in SD	Ratio P
Xq12	15	24932	0.796	0.645	0.051	1.08	1.23	3.0	
Xq23	21	17677	0.564	0.501	0.056	0.76	1.13	1.1	
Xp21.3	26	20402	0.809	0.642	0.047	1.16	1.26	3.6	
Xp11.4	31	20647	0.819	0.646	0.046	1.18	1.27	3.7	
Xq28	35	11870	0.830	0.620	0.061	0.86	1.34	3.5	
Xp22.12	39	11970	0.837	0.785	0.058	1.14	1.07	0.9	
Xq25	43	8390	0.797	0.606	0.053	0.97	1.32	3.6	
Xp21.1	47	5938	0.564	0.457	0.046	0.84	1.23	2.3	
Mean		15228	0.752	0.613	0.052	1.00	**1.23**		
Coefficient of variance							**0.07**		**P= 0.90%**
Yp11.31	17	12215	0.390	0.563	0.053	1.11	0.69	-3.2	
Yp11.31	22	6917	0.221	0.420	0.060	0.74	0.53	-3.3	
Yq11.21	27	12290	0.488	0.668	0.061	1.16	0.73	-3.0	
Mean		10474	0.366	0.551	0.058	1.00	**0.67**		
Coefficient of variance							**0.15**		**P= 12.24%**
High significance P= 0.90%									**Contamination by mat. DNA or 69,XXY?**
Male Ref. Theoretically X and Y ratios of 69,XXY are 1.33 and 0.66									

Figure 4. Details of a 69,XXY amniotic sample.

Figure 3 shows details of a trisomy 21 amniotic sample having a low amount of sample DNA. The weighted mean ratio 1.55 was significantly higher than ratio 1.1 (p = 0.036%). All peaks were marked by an * illustrating that the area was more than 4 SD from the reference area. The results are marked poor quality because the mean area of the ligated probe peaks divided by the mean area of the three nonligated control fragment peaks was 0.65 (*i.e.*, not $>$0.65 as required). The weighted ratio CV 0.14 was almost three times higher than the corresponding CV of the trisomy 13 sample (Fig. 1), stressing the poor quality of the sample.

Figure 4 shows a triploid amniotic sample having two X chromosomes and one Y chromosome. Technically, the method cannot distinguish between this condition and a normal male sample contaminated by maternal blood, yet, the weighted mean ratios of chromosome X and Y, respectively, correspond to the theoretical ratios of 1.33 and 0.66. For chromosome X the weighted mean ratio 1.23 was significantly higher than ratio 1.13 (p = 0.90%), whereas the chromosome Y ratio 0.67 was not significantly lower than 0.76 (p = 12.24%), but there is enough significance ($\leq$ 15%) to turn on the diagnosis "Contamination by mat. DNA or 69,XXY."

4 Discussion

MLPA is a rapid technique for aneuploidy detection [2], and the methods described above ensure an easy workflow with objective results well suited for high output rou-

tine settings. We have previously shown that the combination of MLPA and a previous version of our software also ensures reliable results [3].

Peak recognition could have been performed by adding Genotyper software to the ABI 3100 installation, but we found it easier to control the peak recognition and at the same time perform all the other necessary computations in a single program.

Some groups base MLPA ratio computations on peak heights instead of peak areas [4, 5]. However, theoretically area measurements should be used. Another reason for using peak areas is that peak width normally increases with increasing fragment length; hereby the slope of decreasing peak areas becomes smaller than the slope of decreasing peak heights. Thus, the peak area variation within a normalization group is smaller than the peak height variation.

The manufacturer of the P095 kit suggests, for this special kit, to do normalization by dividing each peak area by the sum of two or three nearby probes. We wait to perform normalization method experiments until we get enough abnormal cases, as it is essential that a normalization method works for both normal and abnormal samples. Until now most users of other probe sets have reported to use the supplier's general standard normalization method that divides a peak area by the sum of all areas, but other methods have been reported, *e.g.*: (i) normalization by four nearby probes [5], and (ii) division of each peak area by the sum of control probes included in the kit [6], however, the P095 kit does not include control probes.

Instead of just computing the ratio of each normalized peak area in relation to a mean (or median [5]) of the corresponding normalized area of normal samples dose quotients may be used. Dose quotients compare ratios of test peaks to control peaks of a sample to the same ratios obtained for normal samples [4]. But again, the P095 kit does not include control probes.

We have successfully used the above methods (not published) with slight modifications for the following MLPA kits that also do not include control probes: SALSA P019, P020, and P036 (Human Telomer kits), and SALSA P064 MR1 (Mental Retardation kit). Yet, with regard to the telomere kits only detection of individual aberrant peaks is feasible, similar to the case shown in Fig. 2.

The weighting of each probe ratio according to its reliability did only improve the chromosome ratios slightly. For the 499 normal reference samples the SDs of the sample mean ratios for the individual chromosomes was reduced by 1.8 to 6.6%. However, weighting by probe reliability is essential for other kits like the P001 kit (not published).

The supplier of the MLPA kits proposes to do intraassay evaluation of samples, *i.e.*, include normal reference cases in each run, but our computational method based on interassay evaluation has several advantages: (i) local normalization of probe areas; (ii) compensation for a side effect of the normalization procedure that scales all normal DNA peak areas slightly up or down when aneuploidy is present; (iii) weighting each probe ratio according to its reliability when computing the chromosome mean ratios and SDs; (iv) use of statistics that compares the (weighted) mean ratio and SD of a chromosome to reference limits based on normal samples; and (v) quality assessment that excludes samples of poor quality as well as samples that deviate from the samples of the reference data set.

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