Rapid detection of genomic duplications and deletions using the Agilent 2100 bioanalyzer

Application

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Abstract

Duplications and deletions are known to cause a wide variety of human diseases. Many different methods are used for detecting such rearrangements, most based on either FISH or the quantitative analysis of PCR products. FISH is popular and accurate, but very labour intensive and expensive, while quantitative PCR methods are technically demanding and difficult to design. Recently two related methods (MAPH and MLPA) have been developed and applied to different disorders. Several methods for the analysis of the products were used, using a labeled primer and either gel or capillary electrophoresis. This Application Note describes the Agilent 2100 bioanalyzer in combination with the DNA 500 chip for the detection of copy number changes within the genome using MAPH and MLPA. The speed, ease of data analysis and potential for automation make this an attractive alternative for conventional methods in a diagnostic setting.

Introduction

Alterations in genomic DNA can be broadly divided into 3 main classes: (i) qualitative changes, where the DNA sequence is altered, (ii) changes of order e.g. translocations and inversions, and (iii) quantitative changes, involving the deletion or duplication of a stretch of DNA. Sequencing has long been the gold standard for qualitative changes, and fluorescent in situ hybridization (FISH) and pulsed-field gel electrophoresis (PFGE) are the most widely applied methods for detecting the reordering of genomic segments. Until recently, the 3 methods predominantly used for quantitative analysis were Southern blotting, quantitative or breakpoint-specific PCR, and FISH. Southern blotting is time-consuming and laborious, and it is especially difficult to detect duplications. Quantitative PCR allows the multiplexing of up to 15 products in a single reaction, but has proved technically difficult in practice. The fact that each primer pair has to be optimized when combined in a given PCR

reaction limits the "mix and match" potential. FISH is a visual method, meaning that the number of signals seen corresponds to the copy number of the probe. The disadvantages are the time-consuming preprocessing necessary before the analysis can be performed and the relatively low resolution (approx. 40 kb for a cosmid probe). Although this is more than adequate for larger rearrangements, small (e.g. single exon) deletions and duplications are not usually detected with this method. Two high resolution, high throughput techniques have recently been described for quantitative analysis of DNA, namely multiplex amplifiable probe hybridization (MAPH)[1] and multiplex ligation-dependent probe amplification (MLPA)[2]. Both are based on quantitative amplification using a single pair of primers following probe hybridization to genomic DNA. The difference lies in the probes and the way the genomic DNA is handled. MAPH probes are PCR products that are hybridized to genomic DNA immobilized onto a nylon membrane. After hybridization the unbound probes are removed by stringent washing, with the bound probes being subsequently denatured and amplified in a PCR reaction. As the probes are designed to all have identical sequences at each end, the PCR reaction can be performed with a single primer pair. MLPA is a ligation-based assay, with 2 single stranded DNA sequences being ligated together only if they both hybridize adjacently to their complementary sequences on genomic DNA. As unligated probes can not be PCR amplified it is not necessary to immobilize the genomic DNA (in contrast to MAPH), and the washing steps can also be skipped.

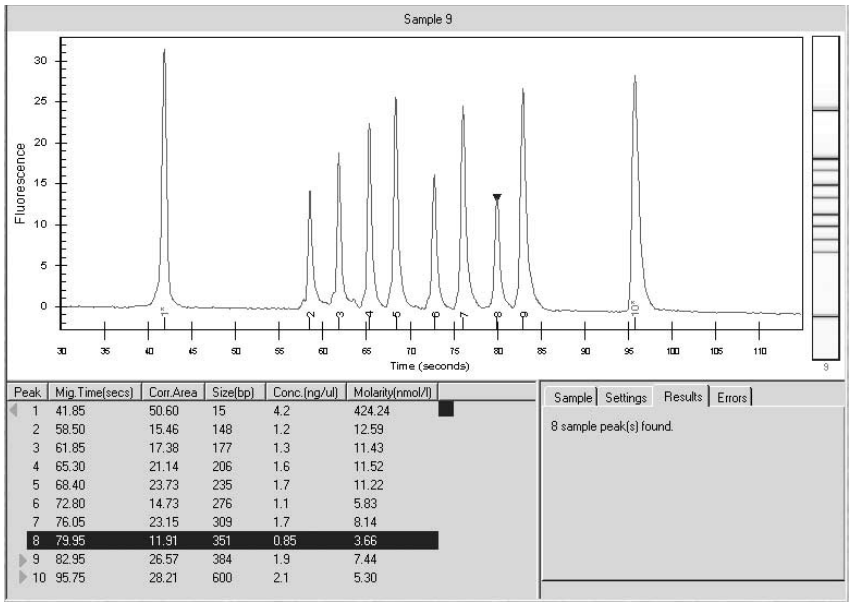

Figure 1
An example of a trace pattern obtained from the 2100 bioanalyzer software. For each peak the length, concentration and area is given.

Different methods for the analysis and the quantitation of the PCR products have been described. The DNA is labeled with either radioactive or fluorescent molecules during the PCR step, and the separation has been performed by electrophoresis, either on a polyacrylamide gel or capillary system. We have tried the possibility of analyzing the MLPA and MAPH products using the Agilent 2100 bioanalyzer in combination with the DNA 500 chip. Parameters under investigation included resolution, reproducibility and the accuracy of quantitation.

Methods

MAPH

A total of 17 female DNA samples were examined, of whom 13 were known to be carriers for mutations in the DMD gene and 4 were non-carriers. The MAPH protocol has been described previously[3], and is also available on http://www.dmd.nl/DMD_MAPH.html For each hybridization several DMD gene exon probes were chosen, both within and outside the suspected breakpoints. At least 2 control autosomal probes were also included in the probe mix.

MLPA

Full details regarding the MLPA protocol are in reference 2, and on the website http://www.mrc-holland.nl/ The MLPA reaction was performed on male and female DNA, as well as DNA from a cell line 49,XXXXY. Bioanalyzer analysis was performed according to the manufacturer's instructions. The peak patterns were visually compared using the Bio Sizing – Data Evaluation program (Agilent Technologies).

Results and discussion

We have previously published work on the analysis of deletions and duplications in the DMD gene

using MAPH[3]. While this concentrated on the analysis of a large group of samples, it is often necessary or sufficient to screen only one or a small number of samples with a few probes. This is particularly the case when a relative of a patient wishes to be screened for carrier status. In this case the mutation to look for is already known, and the question is simply whether a given DNA sample has the same mutation. It is therefore not necessary to examine all 79 exons, but simply compare probes for exons within the potential rearrangement to those outside. To test whether the Agilent system would be suitable for this analysis we first had to determine the practical resolution of separation and the minimum concentration required for accurate analysis. An experiment was performed to separate fragments of a known length. It was found that having a probe separation of > 20 base pairs resulted in peaks that could readily be distinguished (figure 1). We routinely amplify the MAPH products with a 25 cycle PCR reaction. This gave sufficient product to be easily detected. A concentration of approximately 0.5 ng/µl is necessary to give a peak of approximately 5 units in height, the default minimum set in the analysis program. We analyzed a total of 17 samples from females where the carrier status was known[4]. For each sample at least 1 exon was chosen within the deleted/duplicated region, and several exons outside. Changes in peak height correspond to changes in copy number, and in most cases the difference could be easily detected by eye (figure 2). Exon specific peaks were all normalized against unlinked autosomal probes, and the normalized ratios of the exons within the

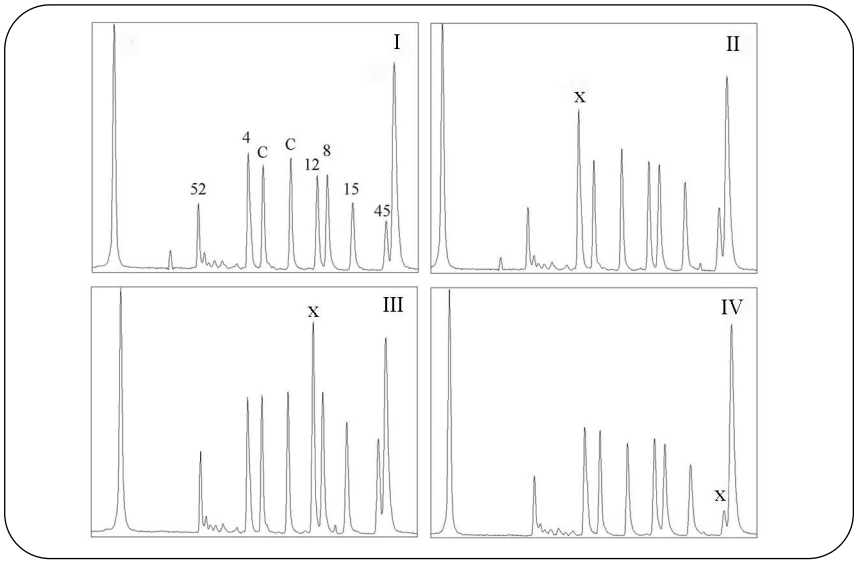

Figure 2
Changes in the peak height and area correspond to changes in copy number of the specific probe. The numbers refer to DMD exons, with autosomal control probes indicated with 'C'. Four different female samples are shown: I – no mutation, II – duplication exon 4, III – duplication exon 12, IV – deletion exon 45. In each case the affected exon is indicated with an X.

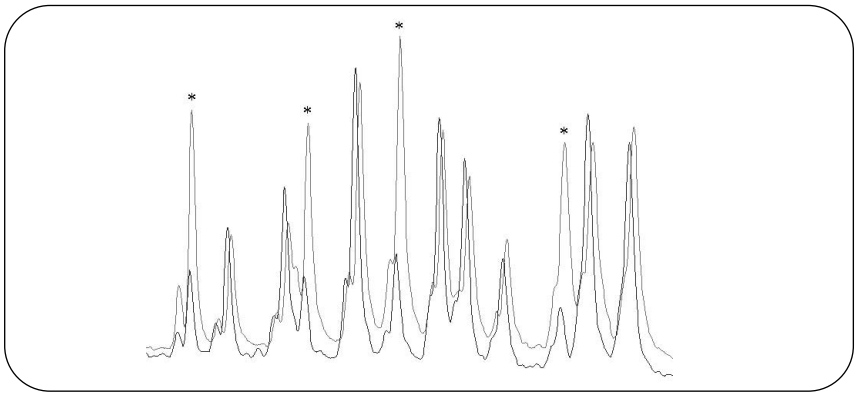

Figure 3
Results from 2 MLPA reactions overlayed using the BioSizing Data Evaluation software. The XXXXY chromosome probes are indicated with an asterisk. The difference between the XY sample (blue) and 4XY (green) are easy to see.

rearrangement were compared with those from exons outside the rearrangement using Student's t-test (table 1). In the 13 cases where the female was a carrier the differences were highly significant ($p < 0.001$), whereas the 4 non-carriers showed no significant difference ($p > 0.1$). To estimate the error rate of the assay the 99 % confidence intervals of the difference were calculated. The ranges are such that the actual error rate is likely to be lower than the predicted rate of 1 %. It should also be possible to detect mosaic cases i.e. when the mutation is not present in all of the cells and is therefore masked by the presence of normal cells. In these cases the p-value may fall into the "retest" range. In such cases alternative methods such as FISH could be employed to confirm this.

#	Mutation in son	Mean ratio within breakpoints (n)	Mean ratio outside breakpoints (n)	99% C.I. of the difference	P value
1	dup 58-63	**1.44**(3)	1.01(11)	-0.58 to -0.28	<0.001
2	del 10-46	*0.47*(10)	0.97(13)	0.30 to 0.70	<0.001
3	dup 44-57	**1.51**(13)	1.07(24)	-0.58 to –0.31	<0.001
4	dup 50-55	**1.39**(6)	0.98(19)	-0.51 to –0.30	<0.001
5	dup 52-55	**1.48**(6)	1.03(13)	-0.61 to –0.29	<0.001
6	dup 51-55	**1.60**(7)	0.99(18)	-0.94 to –0.26	<0.001
7	del 45	*0.39*(3)	1.02(15)	0.46 to 0.80	<0.001
8	del 49-54	*0.51*(10)	1.00(19)	0.39 to 0.59	<0.001
9	del 48-50	*0.53*(5)	1.01(12)	0.41 to 0.55	<0.001
10	dup 2-9	1.01(4)	0.98(11)	-0.16 to 0.11	0.63
11	dup 3-7	**1.43**(6)	0.94(20)	-0.65 to –0.32	<0.001
12	dup 12-13	**1.47**(4)	1.03(17)	-0.64 to –0.23	<0.001
13	dup 2-6	**1.28**(4)	1.01(17)	-0.42 to –0.11	<0.001
14	dup 2-7	1.07(4)	0.94(8)	-0.35 to 0.12	0.13
15	del 52	*0.55*(3)	0.96(12)	0.10 to 0.63	<0.001
16	del 8-43	1.00(4)	0.96(6)	-0.24 to 0.15	0.47
17	dup 12	1.10(3)	1.00(12)	-0.28 to 0.07	0.10

Table 1

The 17 female samples tested. Listed are the ratios derived from probes within and outside the rearrangements. The mean ratio for each sample is given (duplicated in bold, deleted in italics), with the figure in brackets being the number of probes tested. The p-values were determined with Student's t-test, and the associated 99 % confidence intervals (C.I.) of the differences are also shown.

We also looked at testing MLPA reactions on the DNA 500 chip. Initial tests were performed with a test kit provided by MRC Holland (Nederland). The resulting peaks can be analyzed in exactly the same way as those obtained from a MAPH experiment. An example of the results can be seen in figure 3. The difference in the number of X chromosomes can be easily seen as differences in peak height of the appropriate probes. In this regard the software provided (BioSizing Data Evaluation) makes it very easy to overlay different traces and make direct visual comparisons.

Conclusion

These experiments show that the Lab-on-a-chip combined with MAPH /MLPA is a quick and simple way to detect copy number changes in genomic DNA. Particular strengths are the speed and relative automation of analysis. The resolution is sufficient to separate up to 15 probes accurately on the DNA 500 chip, more than sufficient when examining only one particular area of the genome. We consider this technique to be an attractive alternative to FISH in a diagnostic setting, particularly for mutations that cannot be readily detected in such a manner.

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