Fluorescent resonance energy transfer (FRET) based detection of a multiplex ligation-dependent probe amplification assay (MLPA) product

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A fluorescent resonance energy transfer (FRET)-based hybridization assay for detecting multiplex ligation-dependent probe amplification (MLPA) products has been developed, extending the diagnostic power of the technique and demonstrating the possibility of combining MLPA with microarrays for the detection of multiple mutations. FRET is one of the most commonly used detection techniques for hybridization assays. To investigate the applicability of FRET based detection of MLPA products, a sandwich assay was designed to detect gene copy number by exploiting an immobilized probe labeled with an acceptor dye, Alexa Fluor 555, which hybridises to specific PCR amplicons, followed by hybridization of a second probe labeled with the donor dye, Alexa Fluor 488. Following excitation of the Alexa Fluor 488, a FRET signal was produced only if a DNA sequence specific to the *BRCA1* exon 13 was present in the test sample. We have verified this assay on a DNA sample of a patient carrying a heterozygous *BRCA1* exon 13 deletion using male genomic DNA as control. Here we demonstrate that the DNA sample containing the heterozygous deletion generated a considerably reduced FRET signal as compared to the control male human DNA. Our results show that the FRET design presented in this study can differentiate between reduced copy numbers any genomic DNA sequence after MLPA analysis, and the reported format is applicable to multiplex detection of MLPA products, using microarrays, or optical biosensor arrays, and future work will focus on the demonstration of this.

Introduction

The multiplex ligation-dependent probe amplification assay (MLPA) is a novel amplification method that can amplify and detect at least 40 different sequences in an easy to perform reaction requiring only 20 ng of human DNA.[1] Since the first publication describing MLPA appeared in the literature in 2002, more than a hundred articles have been published describing a whole range of applications of MLPA for the detection of chromosomal aberrations,[2,3] quantification of DNA methylation[4] and relative expression analysis.[5] In MLPA, it is not the nucleic acid target in the sample that is amplified, but rather probes consisting of two oligonucleotides that hybridise in juxtaposition to the target DNA sequence. Once hybridisation is complete, the two oligo-nucleotides are ligated and subsequently amplified. All MLPA probes have identical end sequences, permitting PCR amplification using a single primer pair and the ligation approach allows the discrimination of single nucleotide differences. Standard MLPA probes are designed such that each amplification product is identified by size and after separation by capillary electrophoresis can be quantified. Changes in relative probe signals between samples reflect changes in copy number of the DNA target sequences.[1] Undoubtedly, MLPA represents a huge advance in multiplex amplification and detection. However, the technique does suffer from drawbacks due to its dependence on length based discrimination, and these size differences can lead to complications in quantitative PCR as smaller fragments are amplified more efficiently[6] and the diagnostic possibilities of MLPA could be improved if a direct quantitative detection without the need for size separation, could be used.

There are several different approaches to detect mutations in high-throughput assays.[7] Sequencing the mutation region directly is the most reliable way to detect mutations after the target area in genome has been amplified, but is time-consuming and hard to perform in a multiplex format. In addition genomic deletions are usually missed by these methods as the second wild type allele is also exponentially amplified. Gel-based systems depend on differences in the electrophoretic mobilities of wildtype and mutant DNA fragments (*e.g.* denaturing gradient gel electrophoresis, single strand conformational polymorphism and heteroduplex analysis) or, alternatively, hybridization-based assays use oligonucleotides immobilized on chips, (*e.g.* microarrays), usually requiring the target to be labelled, or detection in sandwich hybridisation assays (*e.g.* biosensors), where a probe complementary to a target sequence is usually attached to a solid support and another probe labelled for detection, and the target sequence 'sandwiched' between them. The detection systems based on

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direct quantification of hybridized DNA have advantages over gel based or sequencing detection systems when multiple mutation sites should be determined at the same time. Fluorescence (or Förster) resonance energy transfer, FRET, is a powerful technique for characterizing distance-dependent interactions on a molecular scale. FRET is the radiation-free transfer of energy from an excited donor dye to a suitable acceptor dye, a physical process that depends on spectral overlap and correct dipole alignment of the two dyes. When the distance between the two dyes increases the efficiency of energy transfer is markedly decreased.[8] However, FRET has been observed even when two dyes are placed 20 bases apart on an oligonucleotide.[9] FRET has been used in Taqman assays,[10,11] invader assays, beacon type assays[12] and for the detection of point mutations in hybridization assays.[13] A recent report details the first demonstration of the powerful combination of MLPA with microarray detection, based on hybridization with an immobilized probe, but the approach required labeling of the target.[6]

In this article we report on a fluorescent resonance energy transfer (FRET) based detection method for the determination of the presence of specific MLPA products. As shown in Fig. 1, the approach exploits an immobilised probe labelled with an acceptor dye, Alexa Fluor 555, which hybridises with a specific MLPA product, in this demonstrative set of experiments this probe was specific to the exon 13 of the *BRCA1* gene. Consequent hybridisation with a second probe that is labelled with a donor dye, Alexa Fluor 488, brings the two dyes into close proximity, facilitating FRET. FRET between the fluorophores Alexa Fluor 488 and 555 is efficient as they have strongly overlapping donor emission and acceptor excitation fluorescent spectra and the Förster distance, the distance at which half the excitation energy of the donor is transferred to the acceptor, is 70 Å for this FRET pair.[14]

The detection of MLPA products *via* FRET, reported here, illustrates the potential extension of the diagnostic power of MLPA, facilitating multiplexed simultaneous direct product detection using microtitre plates, biosensor platforms and microarrays.

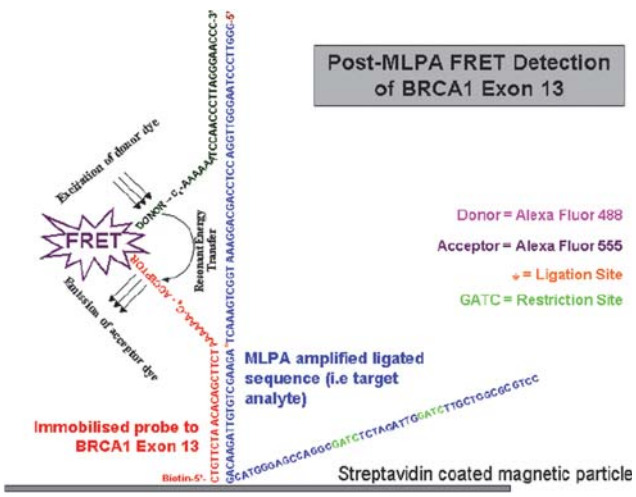

Fig. 1 Schematic of FRET detection of MLPA product.

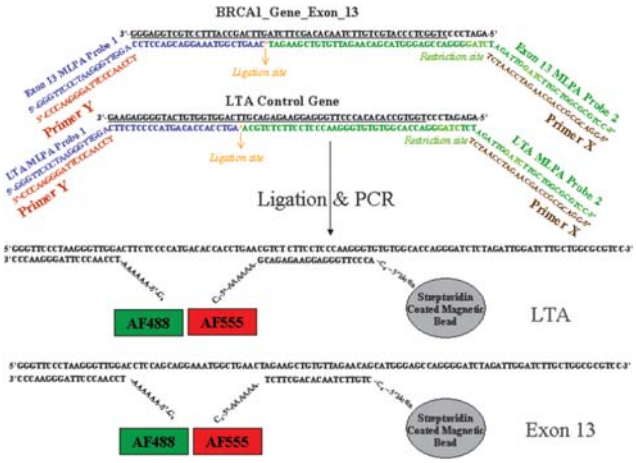

Fig. 2 Schematic of modified MLPA for detection of *BRCA1* exon 13 deletion using synthetic MLPA derived probes and FRET detection as reported here.

Results

To demonstrate the concept we designed completely synthetic MLPA probes for the detection of an exon 13 sequence of the *BRCA1* gene as well as a sequence within the *LTA* gene to act as a control, as outlined schematically in Fig. 2. The MLPA probe designed to hybridize the 5′ of the ligation site included a 19 base complement to the MLPA primer Y sequence and a 24 base target specific part. The 3′ part of the probe was a 5′ phosphorylated synthetic oligonucleotide consisting of a 32 base target specific part, a 4 base recognition site for the restriction endonuclease *MboI*, and a 24 base complement to the MLPA primer X. The restriction site was added in case the 3′-'tail' of the probe amplicon caused steric hindrance. The immobilised probe was a unique oligonucleotide with a 5′ biotinylated terminus and labelled with Alexa Fluor 555 (AF555) at the 3′ end. These unique probes were designed to hybridise to the target specific part at the 3′ side of the MLPA amplicon and were immobilised on streptavidin coated magnetic beads. For FRET detection a universal probe complementary to the 5′ end terminus of the amplicon, the complement to primer Y present in all the MLPA amplicons of the EK1 kit, was labelled with Alexa Fluor 488 (AF488) at the 5′ end.

Approximately 100 ng of DNA from a sample carrying the *BRCA1* exon 13 deletion was used and human male DNA was used as a reference. The MLPA reaction was performed according to Schouten *et al.*[1] and all reagents were obtained from MRC-Holland, Amsterdam (EK1 kit). Following PCR, the MLPA PCR product was incubated with the magnetic beads containing immobilised probe labelled with AF555 and the reporter probe labelled with AF488. Excitation of the AF488 at 492 nm, resulted in a FRET signal of AF555 emission at 565 nm, due to the excitation of the AF555 resulting from the FRET pairs' close proximity (Fig. 3). The system designed here gives a signal when the wildtype sequence is present in the sample. A reduction of signal in a sample indicates that the copy number of the target sequence that MLPA probe hybridizes to is reduced and thus ligation can

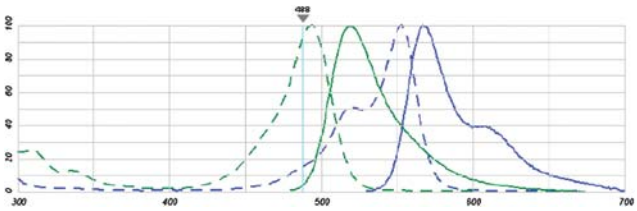

Fig. 3 Fluorescence spectral overlap of Alexa Fluor 488 donor dye (green line, λ_{EX} = 492 nm; λ_{EM} = 519 nm) and Alexa Fluor 555 acceptor dye (blue line, λ_{EX} = 552 nm; λ_{EM} = 565 nm). Dotted lines are the excitation spectrum and joined lines are the emission spectra of the dyes. (Plotted using Fluorescence Spectral Viewer, courtesy of Invitrogen, http://probes.invitrogen.com/resources/spectraviewer/).

not take place. In order to ensure that the reduction of signal is truly due to the decreased copy number, an internal control, the *LTA* gene, was used as reference.

Efficient FRET was observed, and in agreement with previous reports, the fluorescence spectra show three principal features: a maximum at 518 nm, corresponding to the emission from the donor dye, Alexa Fluor 488; a second maximum at 568 nm, from the acceptor dye Alexa Fluor 555 and a common intersection point at 552 nm.

For relative copy number quantification of the *BRCA1* exon 13 the *LTA* gene was used as an internal control. The patient sample containing the heterozygous *BRCA1* exon 13 deletion resulted in a similar FRET signal with the *LTA* probe as the male control sample, but a significantly reduced signal with the exon 13 probe (Fig. 4) indicating a reduced copy number from two to one of that specific sequence. Thus, the amount of ligated MLPA probes will be proportional to the copy number of the DNA sample and reflected in the amount of PCR product. Thus, as observed in Fig. 4, the approach we report here, definitively detects the reduced copy number and could be used quantitatively.

In order to see if improved results were obtained when the Primer X tail was cleaved, a 10 base sequence was hybridised to the restriction site followed by incubation with the *MboI* restriction enzyme and the assay repeated with a sample of male human genomic DNA. No improvement in the produced FRET signal was observed, indicating that this 'tail' does not sterically hinder hybridisation. Hybridisation kinetics were monitored by measurement of FRET signal over time. FRET was observed within 15 min and reached a plateau between 15–30 min. of incubation under mixing conditions at 60 °C. After 30 min the level of signal was constant, comparable to the conditions used in the recent publication of MLPA-array based detection.[6]

Discussion

As previously stated, MLPA represents a huge advance in multiplex amplification and detection and its impact should not be understated. However, the technique does suffer from drawbacks due to its dependence on length based discrimination, and the diagnostic possibilities of MLPA could be improved if a direct quantitative detection without the need for size separation, could be used. The obvious solution is to implement an array detection system based on sequence

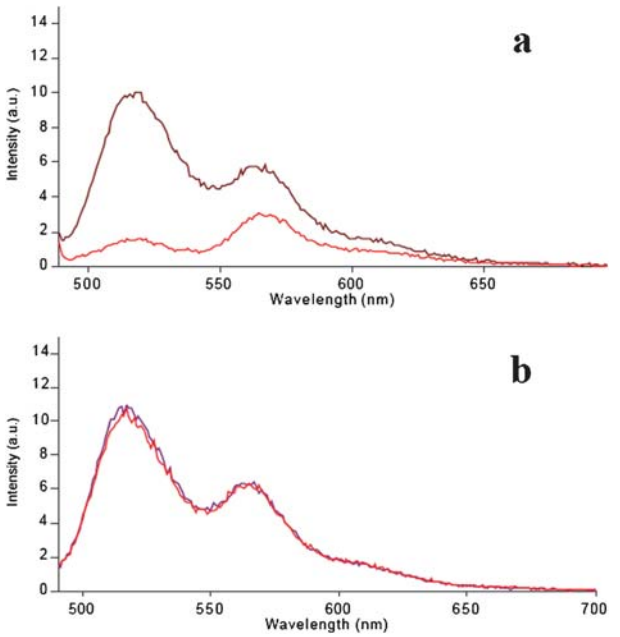

Fig. 4 FRET signals after incubating MLPA products with specific probes immobilized on magnetic beads. The *BRCA1* exon 13 probe produces a FRET signal similar to the *LTA* probe in the male control sample (b), but a significantly reduced signal in the patient sample (a).

recognition rather than size of fragments.[6] The assay format presented here is simply a demonstration of the concept, but could easily be applied to microarrays and biosensor platforms. The MLPA probe oligonucleotides used in this study were about 60 bases and easily synthesized excluding completely the necessity of the use of the *m*13 clones, which could be seen as a further advantage of the approach. Moreover, a recognition system based on specific hybridisation eliminates the requirement for MLPA probes of different lengths, thus theoretically resulting in a better efficiency of MLPA PCR. Here we have demonstrated a heterogeneous assay format for the semi-quantitative detection of MLPA products by using FRET and specific probes immobilised on magnetic beads. The MLPA probes were specifically designed for a sandwich assay to have similar product sizes, both for the investigated *BRCA1* exon 13 sequence and the control gene (and this can be extended to whatever number of exons under study—in MLPA, typically 50 amplicons are analysed) thus minimizing the PCR efficiency differences due to variations in amplicon size.

One of the considerations during the design of the format reported here was the efficiency of FRET signal with 15 base gap between the FRET donor/acceptor dye pair. The interphosphate distances of ssDNA can range between 5.9 Å and 7 Å, depending on the sugar puckering[15,16] and has recently been measured to be an average of 6.3 Å.[17] In the format reported here, taking 6.3 Å as the length of a single base the maximum distance between the donor and acceptor dyes is 24 bases, thus 151.2 Å, but *via* the C_6-AAAAAA spacer incorporated, (taking the length of C–C bond to be 1.54 Å[18]) this distance is expected to be reduced to 69.7 Å. The FRET observed is very efficient and this can be further explained by Livak's observation that for dyes linked to an oligonucleotide in free

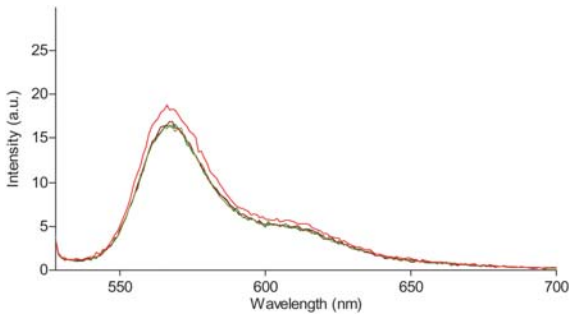

Fig. 5 Emission scan of functionalized beads used in experiments presented in Fig. 1. The beads were excited at 492 nm to check the level of immobilised probe.

solution, the spatial distance between the two dyes is more important than the linear distance separating them on the oligonucleotide.[9] It was not possible to further decrease this distance by increasing the length of the immobilised probe to also hybridise bases beyond the ligation site, as this would lead to false positives *via* binding of non-ligated MLPA probe 1 to the AF555 labelled immobilised probe, which would subsequently hybridise to the AF488 labelled complement to primer Y, resulting in FRET. By choosing an immobilised probe to hybridise to the sequences on the 3′ side of the ligation site, even though non-ligated MLPA probe 2 can bind to the immobilised probe, there will be no complement to primer Y to bind the labelled complement and thus no FRET will be observed. Using this approach only ligated and exponentially amplified MLPA amplicons would be detected *via* FRET, omitting any false positive signals.

Following parameter optimisation, the assay was applied to the detection the *BRCA1* exon 13 deletion in a DNA sample. The assay clearly determined the reduction in the amount of PCR product for the exon 13 with the FRET signal observed being about half that compared to the *LTA* FRET signal.

In conclusion, a FRET based method for direct detection of MLPA products that does not require previous size separation has been developed and demonstrated to differentiate between a sample containing the wild type and a sample containing a heterozygous genomic deletion. The approach reported can be exploited using microtitre plates, biosensor platforms and microarrays, significantly extending the diagnostics possibilities of MLPA. Future work will focus on multiplexed amplification and detection using arrays with specifically designed 'acceptor' dye-labelled immobilised probes for detection of individual exons with a universal 'donor' dye-labelled reporter probe to produce the FRET signal.

Experimental section

MLPA experimental procedure

MLPA was performed using male DNA (Promega) and a DNA sample containing a heterozygous exon 13 deletion of the *BRCA1* gene, which was described previously in Hogervorsd *et al.*[19] and identified among 805 patients tested by MLPA and conventional PCR-based detection method for BRCA1 and BRCA2 regions. The procedure was according to manufacturer's procedures (MRC-Holland). Briefly, 100 ng of genomic template was mixed with MLPA probe mix for *LTA* and Exon_13, incubated overnight at 60 °C and ligated at 54 °C for 15 min. The ligation products of 10 µl were used in the PCR reaction of 35 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 1 min. The products were analyzed on 3% agarose gel electrophoresis and directly used in bead hybridization experiments.

Functionalisation of magnetic beads

Streptavidin-coated magnetic beads (Dynabeads, Myone, C1, Invitrogen inc.) were functionalized with biotinylated hybridization Probe 1 oligos according to manufacturer's

Specific details of MLPA probes, primers, immobilised and reporter probes

Primer X5′-GGACGCGCCAGCAAGATCCAATCT-3′
Primer Y5′-GGGTTCCCTAAGGGTTGGA-3′
BRCA1_Exon_13 Gene Sequence (Genebank number: NM_007294.2)
5′-CCTCCAGCAGGAAATGGCTGAACTA-GAAGCTGTGTTAGAACAGCATGGGAGC-CAG-3′
BRCA1_Exon_13 MLPA Probe-5′
5′-GGGTTCCCTAAGGGTTGGACCTCCAG-CAGGAAATGGCTGAAC-3′
BRCA1_Exon_13 MLPA Probe-3′
5′-TAGAAGCTGTGTTAGAACAGCATGG-GAGCCAGGGGATCTAGATTG-GATCTTGCTGGCGCGTCC-3′
BRCA1_Exon_13 Specific Capture Probe
5′-biotin-CTGTTCTAACACAGCTTCT-AAAAAA-C_6-(Alexa Fluor 555)-3′
LTA Control Gene Sequence (Human lymphotoxin alpha, Genebank number: M16441)
CTTCTCCCCATGACACCACCT-GAACGTCTCTTCCTCCCAAGGGTGTGTGG-CACCA
LTA MLPA Probe-5′
5′-GGGTTCCCTAAGGGTTGGACTTCTCCC-CATGACACCACCTGA-3′
LTA MLPA Probe-3′
5′-ACGTCTCTTCCTCCCAAGGGTGTGTGG-CACCAGGGATCTCTAGATTG-GATCTTGCTGGCGCGTCC-3′
LTA Specific Capture Probe
5′-biotin-ACCCTTGGGAGGAAGAGAC-GAAAAAA-C_6-(Alexa Fluor 555)-3′
Universal Detection Probe
5′-(Alexa Fluor 488)-AAAAAATCCAACCCT-TAGGGAACCC-3′

procedures in hybridization buffer (10 mM Tris (pH = 7.5), 1 M NaCl). The amount of immobilized probe was adjusted for a constant level of probe concentration for each experiment by measuring the emission signal after an excitation at 535 nm (Excitation for Alexa Fluor 555, the acceptor dye of the immobilised probe) (Fig. 5).

FRET detection experiments

All fluorescent measurements were performed in a Cary Fluorimeter at 25 °C constant temperature. The functionalized beads corresponding to 50 nM probe 1 were mixed with MLPA PCR products and 100 nM probe 2. The mixture was denatured at 95 °C for 1 min, cooled to 60 °C and incubated for 15 min. The mixture was cooled to 25 °C and washed with hybridization buffer 2 times *via* magnetic isolation, resolubilized in 100 µl of hybridization buffer and measured for emission at 565 nm after excitation at 492 nm. FRET occurs as the Alexa Fluor 488 is excited at 492 nm and emits at 519 nm, exciting the Alexa Fluor 555 (excited at 535 nm), which emits at 565 nm. Thus if FRET occurs, there should be emission at 565 nm following excitation at 492 nm.

Five independent experiments were performed to determine the variability and specificity of detection by running experiments of LTA and Exon 13 probes separately.

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References

1 J. P. Schouten, C. J. McElgunn, R. Waaijer, D. Zwijnenburg, F. Diepvens and G. Pals, Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependant probe amplification, *Nucleic Acids Res.*, 2002, **30**, e57.
2 B. Janssen, C. Hartmann, V. Scholz, A. Jauch and J. Zschocke, MLPA analysis for the detection of deletions, duplications and complex rearrangements in the dystrophin gene: potential and pitfalls, *Neurogenetics*, 2005, **6**, 21–35.
3 D. J. Bunyan, D. M. Eccles, J. Sillibourne, E. Wilkins, N. S. Thomas, J. Shea-Simonds, P. J. Duncan, C. E. Curtis, D. O. Robinson, J. F. Harvey and N. C. P. Cross, Dosage analysis of cancer predisposition genes by multiplex ligation-dependent probe amplification, *Br. J. Cancer*, 2004, **91**(6), 1155–1159.
4 A. O. H. Nygren, N. Ameziane, H. M. B. Duarte, R. N. C. P. Vijzelaar, Q. Waisfisz, C. J. Hess, J. P. Schouten and A. Errami, Methylation-Specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences, *Nucleic Acids Res.*, 2005, **33**(14), e128.
5 S. Rottenberg, A. O. Nygren, M. Pajic, F. W. van Leeuwen, I. van der Heijden, K. van de Wetering, X. Liu, K. E. de Visser, K. G. Gilhuijs, O. van Tellingen, J. P. Schouten, J. Jonkers and P. Borst, Selective induction of chemotherapy resistance of mammary tumors in a conditional mouse model for hereditary breast cancer, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**(29), 12117–22.
6 F. Zeng, Z. R. Ren, S. Z. Huang, M. Kalf, M. Mommersteeg, M. Smit, S. White, C.-L. Jin, M. Xu, D.-W. Zhou, J.-B. Yan, M.-j. Chen, R. van Beuningen, S.-Z. Huang, J. den Dunnen, Y.-T. Zeng and Y. Wu, Array-MLPA: Comprehensive detection of deletions and duplications and its application to DMD patients, *Human Mutation*, 2008, **29**, 190–197.
7 V. N. Kristensen, D. Kelefiotis, T. Kristensen and A. L. Borresen-Dale, High-throughput methods for detection of genetic variation, *Biotechniques*, 2001, **30**(2), 318–320.
8 X. Chen and P.-Y. Kwok, Template-directed dye-terminator incorporation (TDI) assay: A homogeneous DNA diagnostic method based on fluorescence energy transfer, *Nucleic Acids Res.*, 1997, **25**, 347–353.
9 K. J. Livak, S. J. Flood, J. Marmaro, W. Giusti and K. Deetz, Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization, *PCR Methods Applic.*, 1995, **4**, 357–362.
10 B. J. Bassam, T. Allen, S. Flood, J. Stevens, P. Wyatt and K. J. Livak, Nucleic acid sequence detection systems: Revolutionary automation for monitoring and reporting PCR products, *Aus. Biotechnol.*, 1996, **6**(5), 285–294.
11 I. Tapp, L. Malmberg, E. Rennel, M. Wik and A. C. Syvanen, Homogeneous scoring of single-nucleotide polymorphisms: Comparison of the 5′-nuclease TaqMan (R) assay and molecular beacon probes, *Biotechniques*, 2000, **28**(4), 732.
12 S. Tyagi, D. P. Bratu and F. R. Kramer, Multicolor molecular beacons for allele discrimination, *Nat. Biotechnol.*, 1998, **16**(1), 49–53.
13 N. von Ahsen, E. Schutz, V. W. Armstrong and M. Oellerich, Rapid detection of prothrombotic mutations of prothrombin (G20210A), factor V (G1691A), and methylenetetrahydrofolate reductase (C677T) by real-time fluorescence PCR with the LightCycler, *Clin. Chem.*, 1999, **45**(5), 694–696.
14 R. D. Smiley, T. R. L. Collins, G. G. Hammes and T. S. Hsieh, Single-molecule measurements of the opening and closing of the DNA gate by eukaryotic topoisomerase II, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 4840–4845.
15 W. Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag, New York, NY, 1984.
16 W. K. Olson, Configurational statistics of polynucleotide chains. A single virtual bond treatment, *Macromolecules*, 1975, **8** 272–275.
17 M. C. Murphy, I. Rasnik, W. Cheng, T. Lohman and T. Ha, Probing Single-Stranded DNA Conformational Flexibility Using Fluorescence Spectroscopy, *Biophys. J.*, 2004, **86**, 2530–2537.
18 J. K. Burdett, *Chemical Bonding in Solids*, Oxford University Press, New York, 1995, p. 152.
19 F. B. L. Hogervorst, P. M. Nederlof, J. J. P. Gille, C. J. McElgunn, M. Greppeling, R. Pruntel, R. Regnerus, T. van Welsem, R. Spaendok, F. H. Menko, I. Klujit, C. Dommering, S. Verhoef, J. P. Schouten, L. J. van't Veer and G. Pals, Large genomic deletions and duplications in the BRCA1 gene identified by a new quantitative method, *Cancer Res.*, 2003, **63**, 1449–1453.

