Confirmation of Single Exon Deletions in *MLH1* and *MSH2* Using Quantitative Polymerase Chain Reaction

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Deletions of one or more exons in the mismatch repair genes *MLH1* and *MSH2* have been implicated in a significant fraction of hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome). Multiplex ligation-dependent probe amplification (MLPA) detection of deletions of multiple sequential exons is widely accepted; however, there is concern over the reliability of MLPA results showing single exon deletions. Given the clinical implications of a diagnosis of Lynch syndrome, it is important to use an alternative technique to confirm single exon deletions. To verify single exon deletions, we developed a SYBR Green-based quantitative polymerase chain reaction (PCR) assay. Clinical DNA samples containing deletions in 33 of the 35 exons in *MLH1* and *MSH2*, previously screened by MLPA, were evaluated by quantitative PCR. Gene dosage ratios were determined by both the relative standard curve method and by the $2^{-\Delta\Delta C_T}$ method. Deleted exons had gene dosage ratios of 0.4 to 0.6, while nondeleted exons exhibited ratios of 0.8–1.3. We found 100% concordance between the quantitative PCR and MLPA results, including confirmation of all single exon deletions. The $2^{-\Delta\Delta C_T}$ method was as accurate as using standard curves for the calculation of ratios. Single exon deletions in *MLH1* and *MSH2* can be verified using quantitative PCR. Assays using this method are simple to design and easy to perform, making them ideal for confirmatory testing. (J Mol Diagn 2008, 10:355–360; DOI: 10.2353/jmoldx.2008.080021)

The detection of relatively large intragenic deletions has become increasingly important in patient screening for many disorders, including cystic fibrosis,^{1–3} Rett syndrome,⁴ and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome).^{5,6} In Lynch syndrome, 10 to 27% of pathogenic mutations have been attributed to large deletions in the *MLH1* and *MSH2* genes.^{5,7,8} Genomic rearrangements are particularly prevalent in the

MSH2 gene, where they comprise up to 45% of mutations.⁹ Since large deletions cannot be detected by standard exonic sequencing, other methods such as Southern blotting,^{5,10} long-range polymerase chain reaction (PCR),¹¹ quantitative multiplex PCR,⁸ and multiplex ligation-dependent probe amplification (MLPA)^{12–15} have been used to detect deletions spanning one or more exons.

The use of MLPA for detection of large deletions has recently become widespread because it provides a robust and relatively simple method to simultaneously look at gene dosage of up to 40 targets.¹² However, while MLPA detection of deletions of multiple sequential exons is widely accepted, there is concern over the reliability of MLPA results showing single exon deletions.^{10,13} Given the clinical implications of diagnoses of diseases such as Lynch syndrome, it is important that any detection method have built-in redundancy to guard against false-positive results. For samples with multiple contiguous deleted exons, dosage ratios of adjacent deleted exons provide internal confirmation of results. However, for MLPA results showing a single exon deletion, it is important to use an alternative technique for confirmation.

To this end, we examined the feasibility of using SYBR Green-based quantitative PCR to detect deletions as a rapid confirmation of single exon deletions in the *MLH1* and *MSH2* genes. Samples shown by MLPA to contain deletions were tested to verify whether quantitative PCR could distinguish samples containing heterozygous deletions from samples with nondeleted exons. We also compared two alternative gene dosage calculation methods.

Materials and Methods

DNA Samples and Multiplex Ligation-Dependent Probe Amplification

Ten DNA samples previously determined by MLPA to contain large deletions in either the *MLH1* or *MSH2* gene were generously provided by the Mayo Clinic. Collectively, the samples represented heterozygous

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Table 1. Deletions Determined by MLPA

Sample	Deletion
1	<i>MLH1</i> exons 1–13
2	<i>MLH1</i> exon 13
3	<i>MLH1</i> exon 14
4	<i>MLH1</i> exon 16
5	<i>MLH1</i> exons 16–19
6	<i>MSH2</i> exons 1–6
7	<i>MSH2</i> exons 2–14
8	<i>MSH2</i> exons 3–8
9	<i>MSH2</i> exon 8
10	<i>MSH2</i> exons 8–15

deletions in 18 of the 19 *MLH1* exons and 15 of the 16 *MSH2* exons. The deletions ranged in size between 1 and 13 exons (Table 1). All single exon deletions were further confirmed by the Mayo Clinic using Southern blot analysis.

MLPA analysis was also performed at our institution to verify the deletions. This analysis was performed using 300 ng of each DNA sample with the SALSA MLPA Kit P003 for *MLH1* and *MSH2* (MRC-Holland, Amsterdam, The Netherlands). All steps were performed according to the manufacturer's recommendations. Products were separated by capillary electrophoresis, and analysis of

the results was performed using the GeneMarker software, version 1.6 (SoftGenetics, State College, PA). Exons with peak height ratios of less than 0.7 relative to the corresponding peak of the control samples were scored as deletions.

Quantitative Polymerase Chain Reaction

Primer Design

Primers for each exon in the *MLH1* and *MSH2* genes and for a fragment of the β_2 -microglobulin gene were designed using Primer Designer 5 (Scientific and Educational Software, Cary, NC). The primers were designed to have a T_m of $\sim 60^\circ\text{C}$ and generate amplicons approximately 110 bp in length. Initially, one to three primer sets were chosen for each exon and standard curves were generated, as described below. Following amplification, melting curve analysis was performed to verify the absence of nonspecific products. Primers for analysis of patient samples were chosen based on the following two criteria: absence of nonspecific products and a PCR efficiency of ~ 2 . Sequences of the chosen primers are listed in Table 2.

Table 2. Primer Sequences for Quantitative PCR

Gene	Exon	Forward primer sequence	Reverse primer sequence
β_2 -microglobulin	2	5'-GGTGCCTGATATAGCTTGAC-3'	5'-GCTGGATGACGTGAGTAAAC-3'
	1	5'-CTGAAGGCACCTCCGTTGAG-3'	5'-TGGCCGCTGGGATAACTTC-3'
	2	5'-GTTAAAGAGGGAGGCGCTGAAG-3'	5'-GAAGCGCACAAACATCCTG-3'
	3	5'-GCAGTCCTTTGAGGATT-3'	5'-GACAATGTTCATCACAGGAGGATA-3'
	4	5'-CAGCAGTGAGTTTTCCTTCAGTC-3'	5'-GCACATATACCTGTATGCACACTT-3'
	5	5'-GAGCAAGTTACTCAGATGGA-3'	5'-ACTCTCCCATGTACCATTCCTTA-3'
	6	5'-GTTTCAGGTGGAGGACCTTTT-3'	5'-AGACCCACTCCCAGATTTTG-3'
	7	5'-GTGTGTGTTTTTGGCAACTC-3'	5'-CAAAACCATCCCCAGTAAACC-3'
	8	5'-AGAGACAGTAGCTGATGTTAGG-3'	5'-GACATACCGACTAACAGCATTTC-3'
	9	5'-GAGGATAAAACCTAGCCTTCA-3'	5'-TGGATTTCCTCATGTGGTTCT-3'
	10	5'-CAGTGATGCAGCCTATTG-3'	5'-AAAGAGGAGAGCCTGATAGAAC-3'
	11	5'-TGCAGCAGCACATCGAGAG-3'	5'-CAAAAGCCCCAGAGAGTAGC-3'
	12	5'-CAAGTCTGACCTCGTCTTCTAC-3'	5'-AGAGGCTCGAGAAATGCATC-3'
	13	5'-CCCGGAGAAGGATCATTAACC-3'	5'-TTGAGGCCCTATGCATCCC-3'
	14	5'-TGGCACAGCATCAAACCAAG-3'	5'-CCCTGGACCATTTGTTGTAGTAG-3'
	15	5'-CTCCCATTTTGTCCCAACTG-3'	5'-TATCTGGTAGAAGCATCTTCAC-3'
	16	5'-CTCCGTAAAGCTTGCTCCTTC-3'	5'-GTCCTTCTTTGGGACCATCTTC-3'
	17	5'-TGCAAGCAGGAAGGGAACC-3'	5'-GACCTCAGTGGCTAGTCGAAG-3'
	18	5'-GCGCTATGTTCTATTCATCCG-3'	5'-TATGAGGTCTGTCTTAGTC-3'
	19	5'-CAAAACAGGAGGCTTATGAC-3'	5'-GAGCGCAAGGCTTTATAGAC-3'
<i>MSH2</i>	1	5'-GGCGGAAACAGCTTAGTG-3'	5'-TCTCCAAGTGCAGCGTCTC-3'
	2	5'-TGCAGAGTGTTGTGCTTAG-3'	5'-CTCCTTGGATGCCTTATTC-3'
	3	5'-GAGGCTCTCCTCATCCAGATTG-3'	5'-GGCCTGGAATCTCCTTATCAC-3'
	4	5'-AGAGGAGGAATCTGATCAG-3'	5'-TCATCTGCTCTCCCTTTTG-3'
	5	5'-CACTGTCTGCGTAATCAAG-3'	5'-AGGTAAAGGGCTCTGACTG-3'
	6	5'-AGATACCACTGGCTCTCAGTC-3'	5'-CCATGAGAGGCTGCTTAATCC-3'
	7	5'-TGCAGAATTGAGGCAGAC-3'	5'-ACCTGATAGAGTCCGTAAC-3'
	8	5'-TTGTTGGCAGTTTGTGCTGAC-3'	5'-TGCATACCTGATCCATATCT-3'
	9	5'-CTGTTTGCAGGTGGAACCAAC-3'	5'-GATCTCTGGCTGCACCTTA-3'
	10	5'-CCAGTGACAGTTTGGAT-3'	5'-CGACTTGCAAACTGTTG-3'
	11	5'-ATGAAGAAGCCAGGATGC-3'	5'-GCCAGGTGACATTCAGAAC-3'
	12	5'-CAGGCATGCTTGTGTGA-3'	5'-CCCCACAAAGCCCAAAA-3'
	13	5'-TTGTGCCATGTGAGTCAG-3'	5'-AACGTGGAGACTCCTTTC-3'
	14	5'-GGGTTAGCATGGGCTATATC-3'	5'-GGCCAAGGCAGTAAGTTC-3'
	15	5'-CTCACGCTTCCCAAAATTC-3'	5'-GGGCTTCTGTGTAGCACAC-3'
	16	5'-GCCCTTTACTGAAATGTCAGAAG-3'	5'-CCATTACTGGGATTTTCACGTAG-3'

Standard Curves

DNA from a wild-type sample was serially diluted and amplified for each target exon at concentrations of 100, 50, 25, and 12.5 ng per reaction. Each concentration was run in duplicate. Crossing threshold (C_T) values were determined by the LightCycler software, version 3.5 using the second derivative maximum. The efficiency of each PCR reaction was determined by the following formula: $E = 10^{-1/\text{slope}}$.¹⁶

Amplification

Fifty nanograms of each DNA sample were amplified in a 20- μ l reaction containing 0.5 μ mol/L each primer, 3 mmol/L MgCl_2 , and 1X LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science, Indianapolis, IN). Amplification was performed on the Roche LightCycler and entailed a preincubation of 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 1 second, annealing at 62°C for 5 seconds, and extension at 72°C for 10 seconds. Samples were amplified in duplicate for both the target exon and the reference gene (β_2 -microglobulin).

Quantitative PCR for each of the *MLH1* exons was performed on samples 1 to 5 and for each of the *MSH2* exons on samples 6 to 10. Given the location of deleted and nondeleted exons (as shown in Table 1), a total of 54 deleted and 121 nondeleted exons were evaluated by quantitative PCR.

Calculation of Ratios

Gene dosage ratios for each exon were calculated using two methods: relative quantification using standard curves and the $2^{-\Delta\Delta C_T}$ method. C_T values were determined by LightCycler software and the average C_T of the duplicate reactions was used in the gene dosage ratio calculations. Both the standard curve and the $2^{-\Delta\Delta C_T}$ calculations are derived from the same equation, as previously described^{17–19}; however, the first method accounts for differences in PCR efficiencies, whereas the $2^{-\Delta\Delta C_T}$ method assumes all efficiencies to be 2.

For relative quantification using standard curves, the concentration of the target exon and β_2 -microglobulin are determined by comparison of the resulting C_T values to their respective standard curves. The ratio of target exon to β_2 -microglobulin for the patient sample is then divided by the ratio achieved for a control sample.

In the $2^{-\Delta\Delta C_T}$ method the PCR efficiencies for all reactions are assumed to be 2, and the gene dosage ratio is calculated using the following equation: $2^{-[\Delta C_T(\text{target}) - \Delta C_T(\text{ref})]}$, where $\Delta C_T(\text{target})$ equals the difference between the C_T values for the patient sample and the control sample for the target exon and $\Delta C_T(\text{ref})$ equals the difference between the C_T values for the patient sample and the control sample for the reference gene. Calculated gene dosage ratios were compared using a paired *t*-test.

Figure 1. MLPA results showing a multiexon deletion of *MSH2* exons 8–15 (sample 10) (A) and a single exon deletion of *MSH2* exon 8 (sample 9) (B). Nondeleted exons have ratios of ~1 (green squares), whereas deleted exons have ratios of ~0.5 (red squares).

Results

Multiplex Ligation-Dependent Probe Amplification

All previously identified mismatch repair gene deletions were confirmed by MLPA at our institution (Figure 1 for representative examples of multiple and single exon deletions).

Quantitative PCR

Standard curves were used to calculate PCR efficiencies (see Materials and Methods). The PCR efficiency of the reaction for β_2 -microglobulin was 2.04. PCR efficiencies of the reactions for *MLH1* and *MSH2* exons ranged from 1.80 to 2.27. Representative real-time amplification curves are shown in Figure 2.

The combined 10 patient samples had a total of 54 deleted exons (as determined by MLPA). Quantitative PCR results of these exons showed gene dosage ratios of 0.38 to 0.56 as calculated by relative quantification using standard curves. There were a combined 121 nondeleted exons, and quantitative PCR results of these exons gave gene dosage ratios of 0.79 to 1.29. By the $2^{-\Delta\Delta C_T}$ methodology, deleted exons showed gene dosage ratios of 0.42 to 0.55, and nondeleted exons showed ratios of 0.78 to 1.28 (Table 3).

Figure 2. Real-time amplification curves for samples 1 to 5 for $\beta 2$ -microglobulin (A) and *MLH1* exon 13 (B). Each sample was run in duplicate. The starting concentration of all samples was 50 ng per reaction; thus, similar C_T values for the reference gene, $\beta 2$ -microglobulin, are observed. In the *MLH1* exon 13 amplification, samples without a deletion in this exon (samples 3 to 5) have C_T values of ~ 22.5 . Samples with a deletion in this exon (samples 1 and 2) have C_T values that are approximately one cycle later at ~ 23.5 .

Comparison of Calculation Methods

A comparison of the ranges, means, and standard deviations of the ratios achieved using the standard curve and the $2^{-\Delta\Delta C_T}$ methodologies is shown in Table 3. The gene dosage ratios calculated by the two methods differed by <0.08 for individual data points in all cases, and a paired t -test showed no statistical difference between the two calculation methods.

Discussion

This report demonstrates the utility of using SYBR Green-based quantitative PCR for the verification of single exon

deletions. Calculated gene dosage ratios provided an unambiguous distinction between deleted and nondeleted exons, with 100% concordance between the results of quantitative PCR and MLPA. Deletions were successfully detected in all of the exons in *MLH1* and *MSH2* for which samples with deletions were available (33 of the 35 exons). Gene dosage ratios of all nondeleted exons were clearly differentiated from the ratios of deleted exons.

Multiplex ligation-dependent probe amplification is a very powerful tool for simultaneously evaluating coding regions of entire genes for large deletions.^{6,10} Due to the complex nature of the MLPA assay, however, this technique can be sensitive to DNA quality, concentration, and extraction method.¹⁰ Given the implications of an incorrect disease diagnosis for individuals and potentially affected family members, some form of confirmation of a deletion is desirable. In the case of multiexon deletions, this confirmation is provided by the results from neighboring exons, since the MLPA probe sets for each exon bind independently of each other. For example, in the multiexon deletion depicted in Figure 1A, the finding that exon 10 is deleted is supported by the finding that exons 9 and 11 are deleted; in this way deletion of contiguous exons can be seen to confirm the deletion found for each individual exon. However, the internal validation present in the detection of contiguous multiexon deletions is lacking for single exon deletions, as demonstrated by the MLPA analysis in Figure 1B. Sequencing the exon in question can be performed to verify the absence of variants that may hinder the binding of the MLPA probes and cause a false-positive result; however, this method does not provide confirmation of a true-positive result. Thus, for single exon deletions, it is important to employ an alternative technique for confirmation of results.

Assays using the SYBR Green quantitative PCR method presented here are well suited to confirming single exon deletions because they can be rapidly designed and performed. Suitable primers are easily designed since amplification products need only span a small (~ 110 bp) region of each exon. Additional specificity can be achieved by using hybridization probes; however, we were able to attain sufficient specificity by designing primers with high T_M s and using an annealing temperature of 62°C . Real-time PCR on the LightCycler can be performed in under an hour and requires few control samples, resulting in a simple and rapid confirmatory test.

Alternative detection methods, which can also be used in the initial detection of deletions, arguably impose un-

Table 3. Gene Dosage Ratios Determined by Quantitative PCR

Calculation method	Range	Average	Standard deviation
Deleted exons ($n = 54^*$)			
Standard curve	0.38–0.56	0.47	0.04
$2^{-\Delta\Delta C_T}$	0.42–0.55	0.47	0.03
Nondeleted exons ($n = 121^\dagger$)			
Standard curve	0.79–1.29	0.99	0.08
$2^{-\Delta\Delta C_T}$	0.78–1.28	0.99	0.08

*All exons within samples 1 to 10 that were previously shown to be deleted by MLPA.

† All exons within samples 1 to 10 that were previously shown to be nondeleted by MLPA.

necessary difficulties for a confirmatory test. Southern blots are both time-consuming and expensive to perform. Methods using multiplex quantitative PCR require extensive optimization to achieve similar exponential phases of the PCR reactions of all targets in a single tube.²⁰ Methods using long-range PCR or RT-PCR, which are often used in research studies, may not be amenable to use in a clinical setting. Long-range PCR across a possibly deleted region may be technically difficult or impossible, and RT-PCR requires a sample of patient RNA. Additionally, RT-PCR may not be possible in cases involving a deletion of the extreme 5' or 3' exon, and RNA has been shown to be subject to nonsense-mediated decay in the presence of knockout mutations.²¹ Finally, MLPA assays using either multiple probe sets per exon or secondary MLPA assays using alternative probe sets directed at the same exons are more expensive and time-consuming to perform than the quantitative PCR described in this study.

Quantitative PCR is further simplified if, as in this study, the $2^{-\Delta\Delta C_T}$ method can be used for calculating ratios of patient samples. While the standard curve methodology, which corrects for differences in PCR efficiency between the target exon and the reference gene, is the gold standard for quantification calculations, both methods produced near identical results in our data set. For assays quantifying gene dosage ratios in germline defects, the assumption of efficiencies of 2 used in the $2^{-\Delta\Delta C_T}$ method may have been appropriate for multiple reasons. First, small amplicons with similar PCR efficiencies can be designed. Second, there is a relative lack of complexity present in the quantitative detection of germline deletions in contrast to other quantitative PCR assays, such as those used for the detection of RNA transcript levels or infectious diseases. In particular, assays detecting germline deletions do not need to cover large dynamic ranges; rather, they need only determine twofold differences. Therefore the differences in PCR efficiency may be less consequential in this application of quantitative PCR. The effect of PCR efficiency differences on the calculation is further minimized by standardizing the input DNA concentration of the clinical and control samples. In fact, Pfaffl¹⁸ states that when input DNA is standardized, $\Delta C_T(\text{ref})$ can be assumed to be 0, further simplifying the equation. However, the inclusion of a reference gene amplification provides for correction of DNA quality or any inhibitors that may be present. While not a problem in this study, changes in copy number and primer site polymorphism in the reference gene could affect the results of quantitative PCR. This could be addressed by using a nondeleted exon in the gene of interest as the reference.

In summary, the detection of large deletions is becoming increasingly important as a component of full gene analysis in the detection of pathogenic mutations in a myriad of diseases. Many methods are well suited to the detection of large deletions, and MLPA in particular has recently come into widespread use. For samples with MLPA results showing a single exon deletion, quantitative PCR can be used to verify results. We have presented here the primers and PCR conditions necessary to detect deletions in *MLH1* and *MSH2*; however, the same design principles can be applied to other targets such as *CFTR*,

MECP2 (Rett syndrome), and *ENG* and *ALK1* (hereditary hemorrhagic telangiectasia), which also may require confirmation of single exon deletions. The simple design, accuracy, and rapid manner in which SYBR Green-based quantitative PCR can be performed makes it particularly ideal for confirmatory testing in a clinical setting.

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References

1. Bombieri C, Bonizzato A, Castellani C, Assael BM, Pignatti PF: Frequency of large CFTR gene rearrangements in Italian CF patients. *Eur J Hum Genet* 2005, 13:687–689
2. Niel F, Martin J, Dastot-Le Moal F, Costes B, Boissier B, Delattre V, Goossens M, Girodon E: Rapid detection of CFTR gene rearrangements impacts on genetic counselling in cystic fibrosis. *J Med Genet* 2004, 41:e118
3. Hantash FM, Redman JB, Starn K, Anderson B, Buller A, McGinniss MJ, Quan F, Peng M, Sun W, Strom CM: Novel and recurrent rearrangements in the CFTR gene: clinical and laboratory implications for cystic fibrosis screening. *Hum Genet* 2006, 119:126–136
4. Schollen E, Smeets E, Deflem E, Frys JP, Matthijs G: Gross rearrangements in the MECP2 gene in three patients with Rett syndrome: implications for routine diagnosis of Rett syndrome. *Hum Mutat* 2003, 22:116–120
5. Wijnen J, van der Klift H, Vasen H, Khan PM, Menko F, Tops C, Meijers Heijboer H, Lindhout D, Moller P, Fodde R: MSH2 genomic deletions are a frequent cause of HNPCC. *Nat Genet* 1998, 20:326–328
6. Armour JA, Barton DE, Cockburn DJ, Taylor GR: The detection of large deletions or duplications in genomic DNA. *Hum Mutat* 2002, 20:325–337
7. Wagner A, Barrows A, Wijnen JT, van der Klift H, Franken PF, Verkuiljen P, Nakagawa H, Geugien M, Jaghmohan-Changur S, Breukel C, Meijers-Heijboer H, Morreau H, van Puijenbroek M, Burn J, Coronel S, Kinarski Y, Okimoto R, Watson P, Lynch JF, de la Chapelle A, Lynch HT, Fodde R: Molecular analysis of hereditary nonpolyposis colorectal cancer in the United States: high mutation detection rate among clinically selected families and characterization of an American founder genomic deletion of the MSH2 gene. *Am J Hum Genet* 2003, 72:1088–1100
8. Charbonnier F, Raux G, Wang Q, Drouot N, Cordier F, Limacher JM, Saurin JC, Puisieux A, Olschwang S, Frebourg T: Detection of exon deletions and duplications of the mismatch repair genes in hereditary nonpolyposis colorectal cancer families using multiplex polymerase chain reaction of short fluorescent fragments. *Cancer Res* 2000, 60:2760–2763
9. Baudhuin LM, Ferber MJ, Winters JL, Steenblock KJ, Swanson RL, French AJ, Butz ML, Thibodeau SN: Characterization of hMLH1 and hMSH2 gene dosage alterations in Lynch syndrome patients. *Gastroenterology* 2005, 129:846–854
10. Baudhuin LM, Mai M, French AJ, Kruckeberg KE, Swanson RL, Winters JL, Courteau LK, Thibodeau SN: Analysis of hMLH1 and hMSH2 gene dosage alterations in hereditary nonpolyposis colorectal cancer patients by novel methods. *J Mol Diagn* 2005, 7:226–235
11. Nakagawa H, Hampel H, de la Chapelle A: Identification and characterization of genomic rearrangements of MSH2 and MLH1 in Lynch syndrome (HNPCC) by novel techniques. *Hum Mutat* 2003, 22:258
12. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G: Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res* 2002, 30:e57
13. Wehner M, Mangold E, Sengteller M, Friedrichs N, Aretz S, Friedl W,

- Propping P, Pagenstecher C: Hereditary nonpolyposis colorectal cancer: pitfalls in deletion screening in MSH2 and MLH1 genes. *Eur J Hum Genet* 2005, 13:983–986
14. Sellner LN, Taylor GR: MLPA and MAPH: new techniques for detection of gene deletions. *Hum Mutat* 2004, 23:413–419
15. Gille JJ, Hogervorst FB, Pals G, Wijnen JT, van Schooten RJ, Dommering CJ, Meijer GA, Craanen ME, Nederlof PM, de Jong D, McElgunn CJ, Schouten JP, Menko FH: Genomic deletions of MSH2 and MLH1 in colorectal cancer families detected by a novel mutation detection approach. *Br J Cancer* 2002, 87:892–897
16. Rassmussen R: Quantification on the LightCycler. *Rapid Cycle Real-Time PCR, Methods and Applications*. Edited by Meuer S, Wittwer CT, Nakagawara K. New York, Springer Press, 2001, pp 21–34
17. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 2001, 25:402–408
18. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001, 29:e45
19. Schneider M, Joncourt F, Sanz J, von Kanel T, Gallati S: Detection of exon deletions within an entire gene (CFTR) by relative quantification on the LightCycler. *Clin Chem* 2006, 52:2005–2012
20. Diebold R, Bartelt-Kirbach B, Evans DG, Kaufmann D, Hanemann CO: Sensitive detection of deletions of one or more exons in the neurofibromatosis type 2 (NF2) gene by multiplexed gene dosage polymerase chain reaction. *J Mol Diagn* 2005, 7:97–104
21. Kuzmiak HA, Maquat LE: Applying nonsense-mediated mRNA decay research to the clinic: progress and challenges. *Trends Mol Med* 2006, 12:306–316