Technical Advance

Locked Nucleic Acids Can Enhance the Analytical Performance of Quantitative Methylation-Specific Polymerase Chain Reaction

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Aberrant DNA methylation of tumor suppressor genes is a frequent epigenetic event that occurs early in tumor progression. Real-time quantitative methylation-specific polymerase chain reaction (QMSP) assays can provide accurate detection and quantitation of methylated alleles that may be potentially useful in diagnosis and risk assessment for cancer. Development of QMSP requires optimization to maximize analytical specificity and sensitivity for the detection of methylated alleles. However, in some cases challenges encountered in primer and probe design can make optimization difficult and limit assay performance. Locked nucleic acids (LNAs) demonstrate increased affinity and specificity for their cognate DNA sequences. In this proof-of-principle study, LNA residues were incorporated into primer and probe design to determine whether LNA-modified oligonucleotides could enhance the analytical performance of QMSP for *IGSF4* promoter methylation in human cancer cell lines using either SYBR Green or fluorogenic probe detection methods. Use of LNA primers in QMSP with SYBR Green improved analytical specificity for methylated alleles and eliminated the formation of nonspecific products because of mispriming from unmethylated alleles. QMSP using LNA probe and primers showed an increased amplification efficiency and maximum fluorescent signal. QMSP with LNA oligonucleotides and either detection method could reliably detect five genome equivalents of methylated DNA in 1000- to 10,000-fold excess unmethylated DNA. Thus, LNA oligonucleotides can be used in QMSP optimization to enhance analytical performance. (*J Mol Diagn* 2008; 10:33–42; DOI: 10.2353/jmoldx.2008.070076)

Aberrant DNA methylation of cytosine residues within CpG dinucleotides clustered within the promoter region

can result in transcriptional inactivation of tumor suppressor genes.¹ These epigenetic changes are frequent events in virtually all human tumors and often occur early in tumor progression.² Studies aimed at identifying tumor-specific methylation profiles that could function as potential diagnostic and/or predictive biomarkers are active areas of investigation in many tumor systems.^{3–7}

Most polymerase chain reaction (PCR)-based methods currently used to detect aberrant promoter methylation use DNA templates that have been chemically modified with sodium bisulfite which selectively converts unmethylated cytosine to uracil and leaves methylated cytosine residues intact.⁸ Methylation-specific PCR (MSP) is a widely used sensitive and specific detection method in which primer design exploits sequence differences between methylated and unmethylated DNA after bisulfite treatment to analyze the methylation status of the CpG sites within a defined region.⁹ In standard MSP, the presence or absence of methylated alleles is determined by evaluating PCR products by gel electrophoresis. Approaches that combine real-time PCR technology with standard MSP have resulted in quantitative methylation-specific PCR (QMSP) methods (ie, MethylLight) that provide advantages such as a closed system to minimize risk of contamination, the ability to quantitate methylated alleles in the sample, high throughput, and a platform suitable for clinical applications.^{3,4,6,10} In most applications, the level of methylated alleles present in a sample can be normalized to a reference gene, such as β -actin (*ACTB*), to control for bisulfite conversion and total input DNA. Quantitation of relative levels of methylation in samples becomes particularly important when low levels of methylation are detected in normal tissues or reactive conditions and allows for the establishment of cutoff values for a positive test based on cost-benefit analysis.

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Similar to other real-time PCR applications, detection methods for QMSP include dual-labeled fluorogenic probes, in which probes are designed to include additional CpG sites, and the nonspecific double-stranded DNA binding dye SYBR Green I.^{10,11}

Maximizing the analytical performance of PCR-based assays is a critical step in assay development before testing clinical samples and can be challenging and time-consuming given the number of variables involved in optimization, including primer design, PCR components, and thermocycler parameters. Important test performance characteristics to consider in the development of real-time quantitative PCR assays include analytical specificity, analytical sensitivity, PCR amplification efficiency, and the linear range throughout which accurate quantitation can be achieved. Additional challenges encountered in QMSP optimization for tumor suppressor genes are attributable to the fact that primers and/or probes must be designed to include CpG sites that provide discrimination between methylated and unmethylated alleles, as well as other cytosine residues that discriminate between converted and unconverted DNA after bisulfite modification.⁹ Maximal discrimination between methylated and unmethylated alleles is achieved when primers are designed to include CpG sites and additional bisulfite-converted cytosines near the 3' end of the primer. The use of a fluorogenic probe as the detection method permits evaluation of the methylation status of additional CpG sites within the promoter and can provide increased specificity for the detection of hypermethylated alleles. However, designing methylation-dependent primers and probes for QMSP can be particularly challenging if the defined region of interest within the CpG island of the tumor suppressor gene has a relatively low density of CpG sites or if the CpG sites are too widely spaced. In such cases, limitations in the number and/or position of available discriminating CpG sites can further hinder optimal primer and probe design and make QMSP optimization for the detection of methylated alleles difficult. Strategies that overcome these challenges could provide increased flexibility for assay design and maximize the analytical performance of QMSP.

Locked nucleic acids (LNAs) are nucleic acid analogs that contain a 2'-O, 4'-C methylene bridge within the ribose ring that imparts a rigid conformational structure that enhances thermal stability and improves bp discrimination.^{12,13} LNAs can be substituted into DNA oligonucleotides at selective sites to enhance hybridization performance and have been used in applications in which mismatch discrimination is critical, such as single nucleotide polymorphism genotyping using allele-specific PCR and fluorogenic probes.^{14,15} Although LNAs have demonstrated superior performance in many molecular applications, their use in primers and probes designed for QMSP and their effect on assay performance have not been previously investigated.

Difficulties encountered in primer and probe design for tumor suppressor genes and in the optimization of analytical specificity and sensitivity of QMSP prompted the investigation of the use of LNA-modified oligonucleotides in QMSP to enhance assay performance. In this proof-of-

concept study, LNA-modified primers and probe were compared to their DNA counterparts in QMSP using either SYBR Green I or fluorogenic probe detection methods to determine the effect of LNAs on the analytical performance characteristics of QMSP for the detection of *IGSF4* promoter methylation in human cancer cell lines. The findings demonstrate that LNA-modified primers and probes can be used to overcome challenges encountered in QMSP optimization and enhance assay performance.

Materials and Methods

Human Cervical Cancer Cell Lines

The human cervical cancer cell lines SiHa and C33a were kindly provided by Dr. Chien-Fu Hung at The Johns Hopkins University, Baltimore, MD, and were originally purchased from American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum and penicillin/streptomycin (50 U/ml) at 37°C in a 5% CO₂ incubator.

Genomic DNA Isolation and Bisulfite Modification

Genomic DNA from SiHa and C33a cells was isolated using the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN) and used as the methylated control (MC) and unmethylated control (UC) genomic DNA templates for *IGSF4* QMSP, respectively. Approximately 1 µg of genomic DNA was bisulfite-treated using the EZ DNA Methylation-GOLD kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. Bisulfite-modified DNA was quantitated by UV spectrophotometer analysis and stored at -20°C until use.

Primers and Fluorogenic Probes

The sequences of the primers and probes for *IGSF4* (GenBank accession number AB017563) used in the QMSP assay are shown in Table 1. All primers were synthesized by Sigma-Proligo (The Woodlands, TX). Dual-labeled fluorogenic probes were purchased from Integrated DNA Technologies (Coralville, IA).

Plasmid DNA Templates

Bisulfite-modified genomic DNA from SiHa and C33a cells was used as the template for the amplification of methylated and unmethylated *IGSF4* promoter sequences, respectively, using the forward primer IGSF4 EXF 5'-GTTTTTYGAGAGTYGGGTTG-3' and reverse primer IGSF4 EXR 5'-CTACCRCCRCACACTAAAAT-3' (190-bp amplicon; position 351 to 540; GenBank accession number AB017563), which were designed to recognize either methylated or unmethylated sequences from

Table 1. Primers and Fluorogenic Probes Used in QMSP Assay

Oligo name	Sequence*	Oligo [†]	Orientation	Position [‡]
IGSF4 FM4	5'-GGCGTTGTGATTGGTTTGTTC-3'	DNA	Forward	-117 to -97
IGSF4 RM4	5'-ACTAACGACGTTAACTACCTCCG-3'	DNA	Reverse	-41 to -19
IGSF4 RM5	5'-CACCTACCTCAAACCTAACGACG-3'	DNA	Reverse	-28 to -7
IGSF4 FM probe	6-FAM-5'-TGTTTCGTTTCGGGTTTCGGAGGTAGTT-3'-BHQ-1	DNA	Forward	-56 to -31
IGSF4 FM4L	5'-GGCGTTGTGATTGGTTTGTTC-3'	LNA	Forward	-117 to -97
IGSF4 RM4L	5'-ACTAACGACGTTAACTACCTCCG-3'	LNA	Reverse	-41 to -19
IGSF4 RM5L	5'-CACCTACCTCAAACCTAACGACG-3'	LNA	Reverse	-28 to -7
IGSF4 FML probe	6-FAM-5'-T+CGTT+CGGGTTT+CGGAGGT-3'-BHQ-1	LNA	Forward	-53 to -35

* LNA residues are underlined and preceded by + signs.

[†] LNA indicates an oligonucleotide that contains at least one LNA residue.

[‡] Position of oligonucleotide relative to the ATG site of *IGSF4*; GenBank accession number AB017563.

the *IGSF4* promoter. These external primers are complementary to sequences within the *IGSF4* promoter that flank the region amplified by the methylation-dependent primers used in QMSP (Figure 1). Plasmid templates were generated by cloning PCR amplicons into the pGEMT Easy Vector (Promega Corp., Madison, WI). Plasmids designated M-IGSF4-pGEM and U-IGSF4-pGEM were sequenced to verify the presence of fully methylated and unmethylated *IGSF4* promoter sequences, respectively, for use as DNA templates in QMSP.

Real-Time QMSP

All experiments were performed using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA), which utilizes a 96-well plate platform. Experiments that used the double-stranded DNA binding dye SYBR Green I as the fluorescent reporter molecule were mainly done using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) unless otherwise specified using Brilliant SYBR Green QPCR Master Mix (Stratagene), SYBR Green PCR Master Mix [Applied Biosystems (ABI), Foster City, CA], or a homebrew PCR buffer,⁴ in which 1× homebrew buffer contained 16.6 mmol/L ammonium sulfate, 67 mmol/L Tris (pH 8.8), 6.7 mmol/L MgCl₂, 10 mmol/L 2-mercaptoethanol, 0.1% dimethyl sulfoxide, 0.2 mmol/L each dNTP, 1 U Platinum Taq (Invitrogen) with added SYBR Green I (Molecular Probes Inc., Eugene, OR). Reactions contained 1× reaction master mix or buffer, 30 nmol/L ROX reference dye (except in reactions with ABI master mix that already included ROX; Stratagene), 300 nmol/L forward and reverse primers, and 50 ng of bisulfite-modified

genomic MC or UC DNA template in a final volume of 25 μl. The real-time PCR conditions were 95°C for 5 minutes, then 45 cycles of 95°C for 15 seconds and 60 to 65°C (depending on the primer set and/or optimization conditions) for 1 minute. Fluorescence data were collected during the annealing/extension step for determination of the cycle threshold (Ct). After amplification, melting curve analysis was performed for PCR product identification that consisted of one cycle of 95°C for 1 minute, 55°C for 30 seconds, and 95°C for 30 seconds with data collection throughout the linear increase in temperature from 55 to 95°C. To ensure the specificity of reactions, each plate contained wells with water only (no template control, NTC), UC DNA, and MC DNA. Serial dilutions of MC DNA template were used to generate standard curves from Ct values to assess the sensitivity, amplification efficiency, and linear range for quantification. Amplification and melting curve data were analyzed using the Stratagene MxPro QPCR software. Representative PCR reactions were analyzed by gel electrophoresis and ethidium bromide staining to confirm the PCR product was of the expected size, and the corresponding melting temperature (T_m) was determined by melting curve analysis. In experiments that used dual-labeled fluorogenic probes for the detection of PCR product, reactions contained 1× iQ Supermix (Bio-Rad), 30 nmol/L ROX reference dye (Stratagene), 300 nmol/L forward and reverse primers, 100 nmol/L probe, and 50 ng of bisulfite-modified genomic DNA template in a final volume of 25 μl. The PCR parameters were the same as those used in experiments that used SYBR Green I except that melting curve data were not collected. All real-time QMSP assays were performed in duplicate or triplicate and at least three independent experiments were performed for each condition tested.

Figure 1. Schematic representation of methylated CpG sites in the *IGSF4* promoter. The positions of the DNA and LNA primers and probes relative to the transcription start site are shown.

Results

Design and LNA Modification of Methylation-Dependent DNA Primers and Probe

The *IGSF4* tumor suppressor gene was used as the test gene in these proof-of-concept studies to investigate the use of LNA-modified oligonucleotides in the optimization of QMSP. The CpG island associated with the *IGSF4* promoter region and exon 1 contains a core region im-

Table 2. QMSP with IGSF4 DNA Primers or LNA Primers Using Buffer Systems with SYBR Green I

Primers	Parameter	SYBR Green I buffer system			
		iQ Supermix (Bio-Rad)	PCR master mix (ABI)	Brilliant QPCR (Stratagene)	Homebrew
FM4/RM4	Ct*	23.20 ± 0.09	24.74 ± 0.10	23.99 ± 0.16	23.20 ± 0.36
	dRn last*	4.51 ± 0.49	4.05 ± 0.20	2.67 ± 0.35	3.90 ± 0.97
	Tm*	79.38 ± 0.06	76.09 ± 0.01	74.33 ± 0.03	81.67 ± 0.25
FM4L/RM4L	Ct*	22.46 ± 0.05	28.00 ± 0.69	25.48 ± 0.49	22.77 ± 0.32
	dRn last*	4.42 ± 0.21	3.64 ± 0.14	2.21 ± 0.21	3.93 ± 1.02
	Tm*	79.89 ± 0.08	76.59 ± 0.01	74.88 ± 0.03	82.25 ± 0.29
	ΔCt†	-0.74	3.26	1.49	-0.43

QMSP experiments were performed with 50 ng of MC DNA template at an annealing temperature of 60°C.

* Tabulated values are the Ct, dRn last, and Tm averages and standard deviations from at least three experiments.

† ΔCt = (average Ct with LNA primers) - (average Ct with DNA primers).

mediately upstream of the ATG site that spans ~180 bp and includes 17 CpG sites; sequencing of bisulfite-modified genomic DNA has demonstrated that methylation of this core region correlates strongly with *IGSF4* inactivation.¹⁶ In the present study, methylation-dependent primers and probes were designed from the core region for use in QMSP using MethPrimer¹⁷ in combination with PrimerQuest software (Integrated DNA Technologies). The sequences of the primers and probes used in the QMSP assay are shown in Table 1. The positions of the primers and probes in the *IGSF4* promoter region relative to the transcription start site are shown in Figure 1. The forward and reverse primer sequences used in these studies together contain four or five CpG sites to discriminate methylated from unmethylated sequences and also include several unmethylated cytosine residues that are converted to uracil after bisulfite treatment to discriminate modified from unmodified DNA. In addition to standard DNA primers, primers for *IGSF4* were designed to contain a single LNA residue (LNA primers). The LNA primer has a sequence identical to its DNA primer counterpart except for the substitution of the LNA residue at the terminal 3' position, which is critical for discrimination of methylated from unmethylated DNA. The dual-labeled fluorogenic DNA probe contains three CpG sites to discriminate methylated from unmethylated sequences as well as unmethylated cytosine residues that are converted to uracil after bisulfite treatment. For the dual-labeled fluorogenic LNA probe, each of the three cytosine residues was substituted with a cytosine LNA residue, and because of the increased melting temperature provided by the LNA residues, the overall length of the probe was reduced by seven bases. In QMSP with fluorogenic probes, the combination of primers and probe contained seven CpG sites. The experimental approach to investigate the use of LNA oligonucleotides in QMSP optimization used bisulfite-modified genomic DNA from two human cervical cancer cell lines in which *IGSF4* is known to be differentially methylated.¹⁶ The *IGSF4* promoter region is hypermethylated in SiHa cells and unmethylated in C33a cells. Therefore, genomic DNA from SiHa and C33a cells was used as MC and UC DNA template, respectively, in QMSP experiments.

QMSP Using SYBR Green I: Analytical Specificity with DNA Primers

The double-stranded DNA binding dye SYBR Green I provides a sensitive, convenient, and cost-effective approach for optimization of quantitative real-time PCR whereby analysis of amplification plots in combination with melting curves can be used to determine the specificity, sensitivity, amplification efficiency, and linear range of quantitation of the assay. Initial experiments to optimize QMSP for the detection of *IGSF4* promoter methylation compared various commercially available master mixes and a homebrew buffer that contained SYBR Green I using IGSF4 FM4 and IGSF4 RM4 DNA primers and 50 ng of bisulfite-modified MC DNA template to identify the buffer system that resulted in efficient amplification of a single, specific product. The reactions were performed using PCR parameters as outlined in the Materials and Methods at an annealing temperature (Ta) of 60°C. Reactions that used iQ SYBR Green Supermix (Bio-Rad) had the lowest cycle threshold (Ct) value and highest maximum fluorescent signal (dRn last value) compared to other buffer systems (Table 2). Melting curve analysis demonstrated that a single product was amplified with each buffer system that was of the expected size (99 bp, data not shown); however, the melting temperatures (Tm) of the products varied, which was most likely attributable to differences in buffer system constituents (Table 2). Given the robust amplification obtained with the iQ SYBR Green Supermix and the convenience and reliability provided by a commercially available buffer system, iQ SYBR Green Supermix was used in subsequent optimization experiments that used SYBR Green I. To determine the analytical specificity of the IGSF4 FM4 and IGSF4 RM4 primers for the detection of methylated alleles, QMSP was performed at an annealing temperature of 60°C using 50 ng of bisulfite-modified UC DNA template or water (NTC). Increased fluorescence was detected in UC DNA and NTC samples at delayed Ct values compared to reactions with MC DNA (Figure 2A). Melting curve analysis demonstrated that the products had lower Tms compared to that of the specific product formed with MC DNA template (Figure 2B). The amplification of nonspecific products detected using SYBR

Figure 2. LNA primers enhance analytical specificity of QMSP with SYBR Green I. **A–C:** QMSP performed with IGSF4 FM4 and IGSF4 RM4 DNA primers and MC DNA template (**black triangles**), UC DNA template (**black squares**), NTC (**black circles**). Amplification plots (**A**) of reactions with DNA primers performed at an annealing temperature (T_a) of 60°C and corresponding melt curves (**B**) are shown. The increased fluorescence detected in reactions with IGSF4 FM4 and IGSF4 RM4 DNA primers and MC DNA template (**black triangles**) represents a single amplified product with a T_m of 79.2°C (melt curve). Nonspecific products with lower T_m s were generated with DNA primers in reactions with UC DNA (**black squares**) and NTC (**black circles**). **C:** Increasing the T_a to 65°C resulted in delayed Ct values of >5 for the nonspecific products; however, they were not completely eliminated. **D–F:** QMSP performed with IGSF4 FM4L and IGSF4 RM4L LNA primers and MC DNA template (**black triangles**), UC DNA template (**black squares**), NTC (**black circles**). Amplification plots (**D**) of reactions with LNA primers performed at T_a of 60°C and corresponding melt curves (**E**) are shown. Use of LNA primers resulted in delayed Ct values of ~7 for the nonspecific products (compare **A** to **D**). Increasing the T_a from 60 to 65°C completely eliminated the formation of nonspecific products in UC DNA and NTC samples (compare **D** to **F**).

Green I as the fluorogenic signal molecule can compromise accurate quantitation and reduce overall efficiency of PCR. To determine whether adjustments in annealing temperature could minimize or eliminate the formation of these products and improve analytical specificity of the reaction, the annealing temperature was increased from 60 to 63°C or 65°C. Although increasing the annealing temperature further delayed the amplification of the nonspecific products, detection of these products was not completely eliminated (data not shown and Figure 2C).

LNA Primers Enhance Analytical Specificity of QMSP with SYBR Green I

LNAs demonstrate increased affinity and specificity for their complementary DNA sequences and have been used in many other molecular applications.¹³ To determine whether LNA-modified primers could enhance the analytical specificity of QMSP for the detection of methylated *IGSF4* alleles, reactions were performed using IGSF4 FM4L and IGSF4 RM4L LNA primers in which a single LNA residue was substituted into each of the primers at the terminal 3' position. When QMSP was performed with LNA primers at an annealing temperature of 60°C, the amplification of nonspecific products with UC DNA template and NTC samples was delayed for approximately seven cycles compared to reactions with DNA primers (Figure 2, compare A and B, to D and E, respec-

tively). Increasing the annealing temperature to 63°C or 65°C further enhanced the specificity of QMSP and completely eliminated the formation of nonspecific products (data not shown and Figure 2F). QMSP performed with LNA primers resulted in Ct values that were slightly less than or similar to those obtained with DNA primers (Figure 2, compare A and C to D and F, respectively). Of interest, comparison of Ct values obtained for reactions using DNA primers and LNA primers in QMSP at an annealing temperature of 60°C differed among buffer systems (Table 2). Similar to reactions performed using iQ Supermix, the Ct values obtained using homebrew buffer with LNA primers were slightly less than those obtained with DNA primers. In contrast, compared to the results obtained with DNA primers, the Ct values obtained using the ABI and Stratagene SYBR Green buffer systems with LNA primers were significantly delayed by 3.26 and 1.49 cycles, respectively (Table 2), which suggests that the performance of LNA primers can vary with different DNA polymerases and buffer systems.

LNA Primers Eliminate the Formation of Nonspecific PCR Products Due to Mispriming from Unmethylated DNA Templates

To determine whether the nonspecific products obtained with DNA primers and UC DNA template were attribut-

Figure 3. QMSP with LNA primers eliminates mispriming from unmethylated DNA templates. **A** and **B**: QMSP performed with IGSF4 EXF and IGSF4 EXR DNA primers and M-IGSF4-pGEM plasmid (**white triangles**) or U-IGSF4-pGEM plasmid (**white squares**) DNA templates. Amplification plots (**A**) and corresponding melt curves (**B**) are shown. A single product was generated from each plasmid template with a different T_m depending on GC content. Based on Ct values, equivalent amounts of M-IGSF4-pGEM and U-IGSF4-pGEM plasmid templates were added to the reactions with IGSF4 DNA and LNA primers. **C** and **D**: QMSP performed with IGSF4 FM4 and IGSF4 RM4 DNA primers and equivalent amounts of M-IGSF4-pGEM plasmid (**white triangles**) or U-IGSF4-pGEM plasmid (**white squares**), 50 ng of MC DNA (**black triangles**) or UC DNA (**black squares**) templates, or NTC (**black circles**). Amplification plots (**C**) and corresponding melt curves (**D**) are shown. Nonspecific products were formed with both the U-IGSF4-pGEM and UC DNA template that had identical T_m s; Ct values were delayed compared to methylated templates. **E** and **F**: QMSP performed with IGSF4 FM4L and RM4L LNA primers and equivalent amounts of M-IGSF4-pGEM plasmid (**white triangles**) or U-IGSF4-pGEM plasmid (**white squares**), 50 ng of MC DNA (**black triangles**) or UC DNA (**black squares**) templates, or NTC (**black circles**). Amplification plots (**E**) and corresponding melt curves (**F**) are shown. Nonspecific products generated with unmethylated templates and NTC were eliminated. Representative experiment; data shown are the average of duplicate samples. All reactions were performed at a T_a of 63°C.

able to mispriming from the bisulfite-modified UC DNA template or the result of amplification of heterogeneously methylated alleles present in C33a cells, plasmids that contained sequences representing the fully unmethylated (U-IGSF4-pGEM) or methylated (M-IGSF4-pGEM) *IGSF4* promoter were used as DNA templates in QMSP. Equivalent amounts of U-IGSF4-pGEM and M-IGSF4-pGEM plasmid DNA were titrated to result in a Ct value equal to that obtained with 50 ng of bisulfite-modified MC DNA (Figure 3A). Melting curve analysis demonstrated a single product was generated from each template and, as expected, because of the increased GC content, the T_m of the product formed using M-IGSF4-pGEM was higher than that of the product obtained with U-IGSF4-pGEM DNA template (Figure 3B). Comparison of QMSP using DNA primers and plasmid DNA or genomic DNA templates revealed that, as expected, specific products were formed with both the M-IGSF4-pGEM and MC DNA templates that had nearly identical Ct values and T_m s (Figure 3, C and D). Importantly, nonspecific products were formed with both the U-IGSF4-pGEM and UC DNA template that had delayed Ct values and identical T_m s suggesting that the nonspecific products were the result of mispriming from the U-IGSF4-pGEM and UC DNA templates (Figure 3, C and D). In contrast, when QMSP was performed using LNA primers, only specific products were generated from M-IGSF4-pGEM and MC DNA templates (Figure 3, E and F). These results demonstrate that the use of LNA primers in QMSP can dramatically

improve the analytical specificity of the assay and eliminate the nonspecific products formed with U-IGSF4-pGEM plasmid DNA, UC DNA, and NTC samples. When the reactions were analyzed by gel electrophoresis and ethidium bromide staining, PCR products of the expected size (99 bp) were detected using either DNA primers or LNA primers with M-IGSF4-pGEM and MC DNA templates (Figure 4; lanes 4, 5, 9, and 10). The nonspecific products formed using DNA primers with both U-IGSF4-pGEM DNA and UC DNA templates were of the same

Figure 4. Agarose gel electrophoresis of QMSP products. Aliquots (10 μ l) of samples from QMSP performed with IGSF4 FM4 and IGSF4 RM4 DNA primers (**lanes 1 to 5**) and IGSF4 FM4L and IGSF4 RM4L LNA primers (**lanes 6 to 10**) were analyzed by gel electrophoresis. DNA templates used in the reactions were as follows: NTC (**lanes 1 and 6**), U-IGSF4-pGEM (**lanes 2 and 7**), UC DNA (**lanes 3 and 8**), M-IGSF4-pGEM (**lanes 4 and 9**), and MC DNA (**lanes 5 and 10**). The nonspecific products formed using DNA primers and unmethylated DNA templates are of the same size as the specific products formed with the methylated templates (99 bp; indicated by the **double asterisk**), whereas the nonspecific product (primer dimer) formed in the NTC reaction is smaller in size (indicated by the **asterisk**). The enhanced specificity of LNA primers eliminated the formation of nonspecific products in reactions with unmethylated DNA templates and NTC (**lanes 6 to 8**). M, 50-bp molecular weight marker.

size as the specific products, whereas the nonspecific product formed with NTC was smaller in size (Figure 4, lanes 1 to 3). As expected, no PCR products were detected in reactions using LNA primers with NTC, U-IGSF4-pGEM, or UC genomic DNA (Figure 4, lanes 6 to 8).

Performance Characteristics of QMSP with SYBR Green I and LNA Primers

To analyze further the performance characteristics of QMSP with SYBR Green I and LNA primers, fivefold serial dilutions of MC DNA (50 ng to 16 pg) were used with IGSF4 FM4L and IGSF4 RM4L LNA primers in QMSP. Repeated measurements demonstrated that the analytical sensitivity of the QMSP assay was 16 pg of MC DNA template (approximately five genome equivalents). Standard curve analysis revealed that the QMSP assay with SYBR Green I was linear throughout the 3125-fold range of template concentrations. The slope of the standard curve was used as a measure of amplification efficiency of the reaction, with a slope of -3.322 indicating a two-fold increase in the PCR amplicon per cycle during the linear phase of the reaction and corresponding to an amplification efficiency of 100%. The standard curve parameters were highly reproducible (mean slope = -3.286 ± 0.129 SD, mean intercept = 28.404 ± 0.2344 SD, mean $R^2 = 0.997 \pm 0.0009$ SD; $n = 5$ experiments).

Development of QMSP for potential use in clinical applications requires that the assay accurately detect methylated alleles in the presence of excess unmethylated alleles because clinical samples frequently consist of heterogeneous mixtures of DNA from tumor and normal tissues. To determine the performance characteristics of QMSP with SYBR Green I and LNA primers under conditions that mimic heterogeneous tissues, samples that contained fivefold serial dilutions of MC DNA (50 ng to 16 pg) in the presence of excess UC DNA were analyzed by QMSP, in which the total amount of DNA template was kept constant at 50 ng. In the representative experiment shown in Figure 5, standard curve analysis revealed a slope of -3.393 which reflected efficient amplification of the PCR product. The linear range of quantitation extended throughout the entire dilution series ($R^2 = 0.997$) with reliable detection of 16 pg of MC DNA in the presence of 3125-fold excess of UC DNA. Under these conditions, the analytical performance characteristics of the QMSP assay with IGSF4 LNA primers and mixtures of MC and UC DNA were similar to those obtained using pure MC DNA. These results confirmed that accurate quantitation of methylated alleles could be achieved using QMSP with SYBR Green I and LNA primers in the presence of 3125-fold excess unmethylated alleles.

QMSP with Dual-Labeled Fluorogenic Probes: LNA Primers and Probe Enhance Analytical Performance of QMSP

The use of dual-labeled fluorogenic DNA probes as the detection method in QMSP can further increase the spec-

Figure 5. Analytical performance of QMSP with SYBR Green I and LNA primers. Standard curve plot showing Ct versus initial quantity of fivefold serially diluted (50 ng to 16 pg) MC DNA with excess UC DNA, in which the total amount of DNA was kept constant at 50 ng, demonstrates that the analytical sensitivity of the assay is 16 pg. The slope of -3.393 reflects efficient PCR amplification, and the correlation coefficient (R^2) of 0.997 indicates that the assay was linear throughout the 3125-fold range of template concentrations. Representative experiment; data shown are the average of duplicate samples.

ificity of the assay for hypermethylated alleles when probes are designed to include additional cytosine residues that discriminate between methylated and unmethylated alleles.¹⁸ In addition, use of fluorogenic probes in QMSP eliminates detection of primer dimers, which can interfere with accurate quantitation. Substitution of LNA residues into fluorogenic DNA probes offer potential advantages over their DNA counterparts by providing enhanced template discrimination and an increase in duplex melting temperature to allow for more flexibility in probe design and assay conditions.¹² For QMSP experiments that used fluorogenic probes for the detection of *IGSF4* methylated alleles, probes were designed to hybridize to sequences within the *IGSF4* promoter and included three CpG dinucleotides to discriminate methylated from unmethylated alleles (Figure 1 and Table 1). For the LNA probe, the three discriminating cytosine residues were substituted with cytosine LNA residues, and, because of the increase in T_m provided by the LNA residues, the LNA probe length was reduced by seven bases. Although the same forward primers (IGSF4 FM4 and IGSF4 FM4L) were used in these experiments, the reverse primers (IGSF4 RM5 and IGSF4 RM5L) were positioned slightly downstream of the IGSF4 RM4 and IGSF4 RM4L primers, to accommodate constraints in probe design and its position within the *IGSF4* promoter, and included two of three original discriminating CpG dinucleotides (see Figure 1). The IGSF4 RM5L LNA primer has a sequence identical to IGSF4 RM5 except for the substitution of the LNA residue at the terminal 3' position (Table 1). The performance of the IGSF4 FM4 and IGSF4 RM5 DNA primers and IGSF4 FM4L and IGSF4 RM5L LNA primers were compared and optimized using SYBR Green I before their use in QMSP with the fluorogenic probes. The results were similar to those shown in Figure 2 and demonstrated that IGSF4 FM4L and IGSF4 RM5L LNA primers enhanced the analytical specificity of QMSP and completely eliminated the forma-

Figure 6. Comparison of the amplification efficiency between QMSP performed with DNA and LNA fluorogenic probes. QMSP was performed at a Ta of 65°C with 50 ng of MC DNA template using IGSF4 FM4L and IGSF4 RM5L LNA primers and either the FM DNA probe (black triangles) or FML LNA probe (black squares). Reactions with the LNA probe show more efficient amplification of methylated alleles with a lower Ct value ($C_t = 25.62$) and higher fluorescence signal (dRn last value = 4.53) compared to reactions with the DNA probe ($C_t = 26.63$, dRn last = 2.29). Representative experiment; data shown are the average of duplicate samples.

tion of nonspecific products that were detected in reactions with DNA primers and UC DNA and NTC samples (data not shown). Based on their enhanced performance in QMSP with SYBR Green I, the IGSF4 FM4L and IGSF4 RM5L LNA primers were used in experiments that tested fluorogenic probes as the method of detection in real-time QMSP. In QMSP that compared the performance of the DNA probe to the LNA probe for the detection of methylated alleles (Figure 6), reactions with the IGSF4 FML LNA probe and MC DNA consistently showed superior results with amplification plots that demonstrated lower C_t values and higher dRn last values compared to reactions with the IGSF4 FM DNA probe (mean $\Delta C_t = -0.97 \pm 0.11$ SD, mean fold change in dRn last = 1.99 ± 0.19 SD; $n = 3$ experiments). QMSP using either the DNA or LNA probes specifically amplified products with the MC DNA template and no fluorescence was detected in UC DNA and NTC samples. When the PCR products were analyzed by gel electrophoresis, a single product of the expected size of 111 bp was detected in reactions with MC DNA confirming the specific amplification of methylated alleles (data not shown).

To analyze further the performance characteristics of QMSP using LNA primers and probe, fivefold serial dilutions of MC DNA (50 ng to 16 pg) were used with IGSF4 FML probe and IGSF4 FM4L and IGSF4 RM5L primers in QMSP. Repeated measurements demonstrated that the analytical sensitivity of the QMSP assay with LNA primers and probe was 16 pg of MC DNA template. The standard curve analysis revealed that the assay was highly reproducible (mean slope = -3.387 ± 0.112 SD, mean intercept = 31.56 ± 0.1668 SD, mean $R^2 = 0.997 \pm 0.0029$ SD; $n = 7$ experiments). Importantly, the excellent performance characteristics of QMSP using the LNA primers and probe were maintained when fivefold serial dilutions of MC DNA (50 ng to 16 pg) were tested in the presence of excess UC DNA, in which the total amount of DNA template was kept constant at 50 ng. As shown in the

Figure 7. Analytical performance of QMSP with LNA fluorogenic probe and primers. Standard curve plot showing C_t versus initial quantity of fivefold serially diluted (50 ng to 16 pg) MC DNA with excess UC DNA, in which the total amount of DNA was kept constant at 50 ng, demonstrates that the analytical sensitivity of the assay is 16 pg. The slope of -3.279 reflects efficient PCR amplification and the correlation coefficient (R^2) of 0.996 shows that the assay is linear throughout the 3125-fold range of template concentrations. Representative experiment; data shown are the average of duplicate samples.

representative experiment in Figure 7, the slope of the standard curve was -3.279 , which reflected efficient PCR amplification. The linear range of the assay was maintained throughout the 3125-fold dilution series ($R^2 = 0.996$) and, importantly, 16 pg of MC DNA was reliably detected in the presence of 3125-fold excess of UC DNA. Additional studies have demonstrated that the linear range of the QMSP assay could be extended up to 100 ng of MC DNA template, and moreover, accurate quantitation of 16 pg of MC DNA could be achieved in the presence of 6250-fold excess (100 ng) of UC DNA (data not shown).

Discussion

Accurate detection of aberrant DNA methylation of tumor suppressor genes in heterogeneous biological samples using sensitive and specific quantitative assays has potential clinical utility for both diagnosis and risk assessment of cancer. Development of QMSP for individual tumor suppressor genes requires design of methylation-dependent probes and/or primers and optimization of PCR conditions to maximize analytical specificity and sensitivity for the detection of methylated alleles and to determine the linear range throughout which accurate quantitation can be achieved before testing clinical samples. The present study investigated the use of LNA-modified methylation-dependent primers and probe compared to their unmodified DNA counterparts in QMSP optimization using either SYBR Green I or fluorogenic probe detection methods to determine whether LNA-modified oligonucleotides could enhance analytical performance of the QMSP assay.

QMSP with SYBR Green I provides a convenient and cost-effective approach for quantitative analysis of promoter methylation of tumor suppressor genes. Similar to standard MSP, methylation-dependent primers are de-

signed to include CpG sites that discriminate between methylated and unmethylated alleles. In the present study, the primers used in QMSP with SYBR Green I included a total of five CpG sites. The results of QMSP with SYBR Green I and DNA primers showed amplification of a single specific product using the MC DNA template. However, nonspecific products with lower Tms were detected at delayed Ct values with UC DNA template and NTC samples. Increasing the annealing temperature from 60 to 65°C delayed the amplification of nonspecific products however they were not completely eliminated. Detection of nonspecific products and primer dimers is a known limitation of quantitative assays that use SYBR Green I and can interfere with accurate quantitation and prevent efficient amplification of target methylated DNA sequences. Previous studies have shown that modification of thermocycler parameters, with an additional PCR step after the extension step, can be used to eliminate primer dimer formation and improve assay performance with SYBR Green I.¹¹ However, strategies aimed at eliminating the formation of nonspecific products in the presence of unmethylated DNA template were not addressed.

LNAs are nucleic acid analogs that demonstrate increased specificity for their complementary DNA sequences and have been used in molecular applications such as single nucleotide polymorphism genotyping to improve mismatch discrimination.^{14,15} The results of QMSP performed with SYBR Green I and LNA primers demonstrated that incorporation of LNA residues at the terminal 3' position of each primer markedly improved the analytical specificity of QMSP with SYBR Green I by completely eliminating the amplification of nonspecific products in reactions with UC DNA or NTC, while maintaining efficient amplification of the specific product generated with MC DNA template. In addition to improved analytical specificity, QMSP with SYBR Green I and LNA primers demonstrated an analytical sensitivity of 16 pg of MC DNA (approximately five genome equivalents). The assay showed a 1000- to 10,000-fold dynamic range even in the presence of excess unmethylated DNA. Recent studies described a novel PCR-based method, MethylQuant, which used real-time PCR and SYBR Green I to quantitate the methylation status at a single CpG site in the genome.¹⁹ In MethylQuant, only one of the primers contains a discriminative base at the 3' end to analyze the methylation status of the cytosine of interest. The authors demonstrated that substitution of an LNA residue at the 3'-discriminative position increased the specificity of the assay to allow for accurate and sensitive quantitation of the methylation status of a specific cytosine that was present at 1% of the overall population.¹⁹ In contrast, in the present study, LNAs were substituted into both primer sequences to determine whether LNAs could be used to enhance the performance of QMSP in which the methylation status of several CpG sites were evaluated.

Advantages of real-time PCR with dual-labeled fluorogenic probes compared to SYBR Green I detection methods include the potential for multiplex reactions with detection of more than one target using probes with different reporter dyes and the lack of primer dimer de-

tection because generation of signal depends on hybridization and hydrolysis of an internal sequence-specific probe. Moreover, in QMSP with dual-labeled fluorogenic probes (ie, MethylLight), the probe can be designed to include additional CpG sites that increases the specificity for detection of CpG island hypermethylation associated with transcriptional silencing.¹⁸ A potential disadvantage of QMSP with fluorogenic probes compared to SYBR Green I includes the added cost of the probe. In the present studies that evaluated QMSP with fluorogenic probes, the combination of primers and probe contained seven CpG sites. For LNA-modified oligonucleotides, LNA residues were incorporated into the terminal 3' position of each primer and at the three discriminating cytosine residues in the probe. Similar to QMSP with SYBR Green I, QMSP with fluorogenic probes also demonstrated enhanced performance with LNA-modified oligonucleotides compared to their DNA counterparts. QMSP with LNA primers and probe showed more robust amplification with a lower Ct and increased maximum fluorescent signal compared to the DNA probe. The analytical sensitivity and dynamic range of the assay were similar to that obtained with SYBR Green I. Importantly, QMSP performed with LNA primers and either SYBR Green I or the dual-labeled fluorogenic LNA probe detected five genomic equivalents of methylated DNA in a background of 1000- to 10,000-fold excess of unmethylated DNA. The results showed that optimization of QMSP with LNA-modified oligonucleotides resulted in assays with excellent analytical performance characteristics.

Interestingly, results of the present studies also showed that the performance of QMSP with SYBR Green I and LNA primers varied among different SYBR Green I buffer systems. Although a single, specific product was obtained with LNA primers and MC DNA template with each buffer, the Ct values were significantly delayed in QMSP performed with the ABI and Stratagene SYBR Green buffer systems. The exact formulations of the various commercially available buffers are proprietary information and not available; however, the range of Tms observed for the specific product suggests that the buffers' components differ significantly. In addition, previous studies have shown that PCR performance with LNA primers may differ depending on the specific Taq DNA polymerase used in the assay.²⁰

Detection of nonspecific products with similar Ct values and identical Tms using unmethylated plasmid and UC genomic DNA templates with DNA primers suggested that the nonspecific products occurred as a result of mispriming from the unmethylated DNA templates. Mispriming with amplification of nonspecific products depends on many factors, including the number and position of CpG sites within the primer sequences, the ratio of unmethylated to methylated DNA template, and thermocycler parameters, such as annealing temperature and number of PCR cycles. In real-time QMSP with SYBR Green I, melting curve analysis can be used to identify nonspecific products that have Tms distinct from the specific product of interest. The melt curves from experiments with DNA primers showed that the nonspecific products amplified with unmethylated DNA templates

had slightly lower Tms and could be distinguished from the specific products amplified with methylated DNA templates. However, when the PCR products were analyzed by gel electrophoresis, these nonspecific products were of the same size as the specific products. These findings have significant implications for the interpretation of standard MSP by gel electrophoresis in which mispriming from unmethylated DNA templates could lead to false-positive results and, further, highlight the potential benefit of using LNA primers in standard MSP as well. In addition, eliminating the formation of nonspecific products could improve PCR amplification efficiency and extend the linear range of the assay for the detection of methylated alleles. In the present studies, the Ct values for the nonspecific products amplified from unmethylated DNA templates ranged from ~33 to 38 depending on the annealing temperature of the reaction. As demonstrated in standard curve analysis performed with LNA primers and mixtures of MC DNA and UC DNA that simulate heterogeneous biological samples, reliable detection of low levels of methylated DNA in a background of unmethylated DNA requires that the dynamic range extends to Ct values in this range. Thus, LNA primers that have increased analytical specificity for methylated alleles can enhance overall assay performance.

In summary, the present studies demonstrate that LNA primers and probes can enhance the analytical performance of QMSP with either SYBR Green I or fluorogenic probe detection methods. These quantitative assays reliably detected five genomic equivalents of methylated DNA in a background of 1000- to 10,000-fold excess unmethylated DNA. LNA-modified primers and/or probes offer additional flexibility for assay design and may be used to overcome some of the challenges encountered in QMSP assay development. Although incorporation of LNA residues into primer and probe design may be most beneficial for certain tumor suppressor genes for which there are limitations in position and/or density of CpG sites within the promoter CpG island, this strategy may have more general applications in primer and probe design for QMSP. To this end, we have used LNA-modified oligonucleotides in the primer and/or probe design and optimization of QMSP for additional tumor suppressor genes, including *DAPK1*, *SPARC*, and *TFPI2*. Achieving maximal analytical performance of QMSP is a critical step in the development of assays that could have potential clinical applications. Studies aimed at evaluating the clinical sensitivity and specificity of the QMSP assay with LNA oligonucleotides for tumor suppressor gene methylation in cervical carcinogenesis are currently under way in our laboratory.

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