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Oligonucleotide Primers and Probes: Use of Chemical Modifications to Increase or Decrease the Specificity of qPCR

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Scott D. Rose, Richard Owczarzy, Joseph R. Dobosy
and Mark A. Behlke

Abstract

Although the vast majority of primers and probes employed in qPCR applications today are synthesized using unmodified DNA bases, selective use of chemically modified bases and non-base modifying groups can prevent primer dimer artefacts, improve specificity, and allow for selective amplification of sequences that differ by as little as a single base. A wide variety of chemical modifications have been characterized for use in qPCR. As a general class, the modifications that are in greatest use today increase the binding affinity of the oligonucleotides (i.e. increase the melting temperature, T_m). T_m-enhancing modifications allows both primers and probes to be shorter, improving the differential T_m ($\Delta T_m = T_{m\ match} - T_{m\ mismatch}$) between perfect match and mismatch hybridization. These modifications have widespread application in allele-specific PCR and in the detection of single nucleotide polymorphisms (SNPs). Conversely, a second class of base modifications are in common use that decrease specificity and improve duplex formation in the presence of base mismatches. Although these modifications lower T_m, they have less of an impact on primer stability than do actual mismatched bases. Universal bases permit use of primers and probes in polymorphic loci when it is desirable to detect all sequence variants and minimize mismatch discrimination.

Introduction

The primers and probes used in real-time quantitative PCR (qPCR) are synthetic oligonucleotides and can be manufactured using natural DNA bases or can comprise RNA bases, artificially modified bases, or a variety of pendant chemical groups not found in nature. While most needs are well met using unmodified oligodeoxynucleotides, the use of chemical modifications can improve qPCR performance in a number of areas. This chapter will cover three specific areas of interest, with a primary focus on increasing specificity and allelic discrimination methods:

1 increasing specificity, with a focus on SNP discrimination;
2 preventing primer-dimer or other PCR artefacts;
3 decreasing specificity using universal bases.

Increasing specificity

Specificity relates to the ability of the qPCR reaction to generate a positive signal from a desired nucleic acid sequence while simultaneously not generating a signal from any similar yet distinct sequences also present in the target nucleic acid sample. In some instances this involves discrimination of related sequences within a gene family where multiple base differences are present. In its most extreme form, this involves discrimination of sequences that differ by only a single base, i.e. a SNP. In broadest terms, qPCR approaches to SNP discrimination can be categorized as those methods which employ biased amplification, where one allele is selectively amplified over other alleles, and those methods which do not employ biased amplification, where all related sequences are amplified equally and a detection probe is employed to distinguish base identity.

SNP discrimination using unbiased amplification

PCR-based methods that discriminate between related sequences using unbiased amplification employ primers placed in regions of the sequence that are identical between alleles, positioning the SNP between the two primers. Sequence identity is usually interrogated by differential hybridization with a third probe oligonucleotide. Success is entirely dependent upon having a sufficiently high ΔT_m between the 'match' and 'mismatch' targets so that the 'match probe' hybridizes to the target site during amplification and the 'mismatch probe' does not. A variety of different assay formats exist that employ this strategy, most of which show improved performance using chemically modified oligonucleotides.

Linear dual-labelled probes

One of the more popular formats for quantitative sequence detection is the 5′-nuclease assay, which was first described by Gelfand and colleagues (Cetus Corporation) in 1991 (Holland *et al.*, 1991). This assay employs a fluorescence-quenched dual-labelled linear probe that binds to the target nucleic acid during the annealing phase of each cycle and is degraded during the extension phase of PCR. The probe has a reporter fluorophore on one end and a quencher on the other end, which absorbs fluorescence emission from the reporter dye (the intact probe is 'dark'). The reaction is cycled using a two-step programme with incubation at a single temperature during which time both the primer annealing and polymerase extension steps occur. The T_m of a sequence is the temperature at which 50% of the possible duplexes are formed and 50% remains in the single-stranded state. Generally, the probe is designed to have a T_m 5°C or more above the annealing temperature so that it is almost completely hybridized to the target template strand during DNA synthesis. During elongation, the DNA polymerase encounters the probe which is quickly degraded by the enzyme's 5′–3′ exonuclease activity. Degradation physically separates the reporter fluorophore from the quencher, resulting in the appearance of a detectable signal (the degraded probe is 'bright'). This assay format is often referred to as the TaqMan® assay.

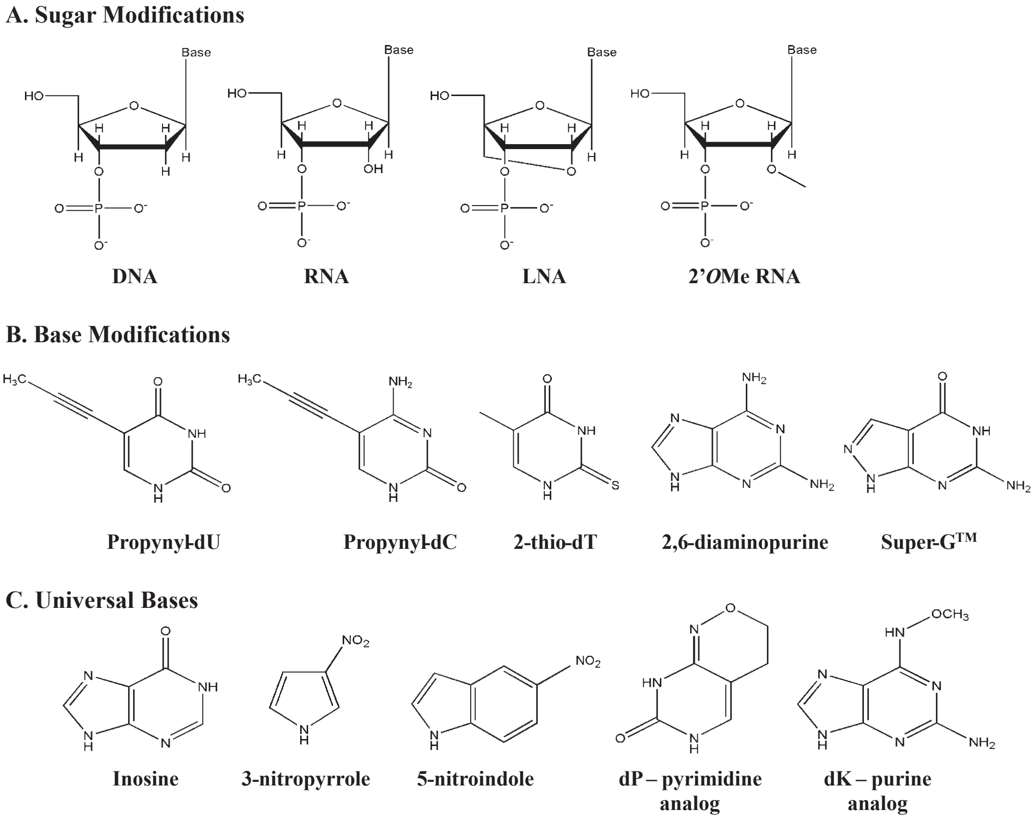
The 5′-nuclease assay was initially developed to measure gene expression levels; it was quickly adapted for use in allelic discrimination. This method allows for a convenient, homogenous closed-tube assay where the same primers are employed to amplify both alleles. Two probes are present in the reaction that are labelled using detectably distinct fluorophores, one specific for allele 'A' and the other specific for allele 'B'. The presence of one or both alleles in a sample can be detected in real time by monitoring rise of signal in two separate fluorescence channels (Livak, 1999). The probe oligonucleotides usually need to be 20 bases or more in length to be completely hybridized to the target nucleic acid at the 60°C annealing/extension temperature (precise length will of course vary with sequence). Unfortunately, single base mismatches in probe sequences this long are often insufficient to produce a large enough ΔT_m between match and mismatch probes to obtain clean separation of signal between alleles. This problem is easily solved by using probes that are made with T_m-enhancing modifications. Modifications that increase T_m permit use of shorter probes at the same reaction temperature. A single base mismatch has a greater impact on duplex stability in short sequences than in long sequences, so the ΔT_m between match and mismatch improves as probe length decreases. Many factors contribute to the T_m and ΔT_m and it is important to be able to accurately predict these values when designing assays.

The ability of a probe oligonucleotide to distinguish between two species solely on the basis of hybridization is a thermodynamic problem that is influenced by the sequence context, the precise match versus mismatch base pair being studied, the length of the probe, and physical parameters of the hybridization reaction, including temperature, probe concentration, and buffer composition. As a general rule of thumb, a good place to start is to design the 'match probe' to have a T_m around 3–5°C above the reaction temperature and the 'mismatch probe' to have a T_m around 5–7°C below the reaction temperature, giving a ΔT_m of 10°C between the match and mismatch hybridization events. For reactions run at a standard temperature of 60°C, this means selecting sequences with T_m values around 64–65°C and 54–55°C for the match and mismatch probes respectively. Obviously having the ΔT_m as large as possible is desirable; however, it is generally not necessary to exceed 10°C and sometimes in actual practice probes with a lower ΔT_m may still perform well. While empiric testing of different probe designs is sometimes necessary, the use of computer assisted design tools that employ the latest thermodynamic parameters can often be used to predict probe sequences that discriminate well without further optimization, or at least greatly accelerates the process of finding good probes.

Typically, reactions are performed at around 60°C with 250 nM probe in a buffer that contains ~50 mM monovalent cation (sodium or potassium), ~3 mM divalent cation (magnesium), and 0.8 mM deoxyribonucleotide triphosphates (dNTPs). It is important to include the total dNTP concentration in thermodynamic calculations since dNTPs bind Mg^{2+} ions and reduce the concentration of free Mg^{2+} present in the reaction. Buffer composition can vary and unfortunately many vendors do not disclose the exact salt content of their PCR reagents, which complicates probe design. Thermodynamic parameters have been determined that allow for the accurate prediction of the T_m of short DNA oligonucleotides (SantaLucia *et al.*, 1996, 1998). In addition, the stabilizing effects of both monovalent and divalent cations are well characterized (Owczarzy *et al.*, 2004, 2008a), and the relative contribution of some fluorescent dyes and quencher have been measured (Moreira *et al.*, 2005). Sufficient detail is known about the thermodynamic effects of DNA base mismatches to model hybridization events of the kind needed to design SNP probes (SantaLucia and Hicks, 2004). A variety of algorithms and sequence analysis tools are available as part of commercial software packages or are freely accessible on the internet and that can help with design of both primers and probes for qPCR applications, including SNP detection (Owczarzy *et al.*, 2008b).

Early approaches to increase T_m and improve SNP discrimination through use of chemical modification focused on alterations of the sugar and base groups. Although 2′-*O*-methyl RNA (2′*O*Me RNA) is often thought of as being a T_m-increasing modification, it actually has increased affinity when binding to an RNA target while the T_m effects with a DNA target are variable. Use of this modification was not found to improve probe performance. In fact, extensive modification with 2′OMe RNA can impair nuclease cleavage of the probe and can be detrimental (Kuimelis *et al.*, 1997). Propynyl pyrimidines are a class of modified base where a propyne group is attached at the C5 position of the pyrimidine ring. This modification was first used to increase binding affinity of antisense oligonucleotides (Wagner *et al.*, 1993), but *in vivo* toxicity limited utility of this application. The propynyl pyrimidines, however, are quite effective in increasing T_m and improve performance of probes used in the 5′-nuclease assay in qPCR (Kuimelis *et al.*, 1997); each base substitution leads to a 0.5–1.5°C increase in T_m. Nearest neighbour parameters have not been reported for these bases; thus T_m predictions cannot be accurately made and empirical testing usually must be done when designing probes of this type. Unfortunately, these modifications are only available for pyrimidines, limiting benefit to pyrimidine-rich probe sequences. To be maximally useful, T_m-enhancing base modifications should be available for all four nucleotides. Structures of the various modifications discussed in this chapter are shown in Fig. 8.1.

Locked-nucleic acids (LNAs) are a T_m-enhancing modification simultaneously developed by Jesper Wengel and his group at the University of Southern Denmark and by Takeshi Imanishi and his group at the University of Osaka. LNAs have a methylene bridge that connects the 2′-O with the 4′-C of the ribose. This bicyclic structure locks the ribose in the 3′-*endo* conformation and increases T_m by 2–6°C per substitution (Kvaerno and Wengel, 1999; Obika *et al.*, 1997; Singh *et al.*, 1998; Singh and Wengel, 1998). LNA nucleotides are commercially available for all four nucleotides (guanine, adenosine, 5-methylcytosine, and thymidine) and have found widespread use in many applications, including antisense, RNA interference, and PCR. Substitution of LNA nucleotides for DNA in probes allows use of much shorter sequences at the standard 60°C reaction temperature and these shorter probes show a significant improvement in the ΔT_m between match and mismatch hybridization compared with parent unmodified (longer) sequences (Letertre *et al.*, 2003; Costa *et al.*, 2004; Johnson *et al.*, 2004; Ugozzoli *et al.*, 2004). The early reports from 2003–2004 which first demonstrated the utility of the LNA modification in dual-labelled hydrolysis probes substituted LNA nucleotides for DNA bases using a variety of different modification patterns with a primary focus on simply improving T_m and reducing probe length. This design strategy was effective; however, no systematic attempt was made to optimize the specific pattern of LNA modifications around the SNP site to maximize the ΔT_m between match and mismatch. In fact, the SNP site was often avoided for modification because it was uncertain if the increased binding affinity of the LNA nucleotide would improve or hurt SNP discrimination if placed directly at the site of mismatch. This question was recently addressed by Owczarzy and colleagues in 2006 (Integrated DNA Technologies), who carefully studied the effect that placement of LNA nucleotides at and around the SNP site had on T_m. Melting temperatures were experimentally measured for a large series of modified and unmodified oligonucleotides hybridized to perfect match or single base mismatch complements. The test set included all possible base variations at the SNP site and the adjacent bases, comparing the effects that LNA substitution had at these sites (You *et al.*, 2006). It was found that simply placing three consecutive LNA nucleotides spanning the SNP site (with the SNP positioned centrally) gave the maximum ΔT_m for all base combinations except for one, where the mismatch is a G:T pair and the guanine base is in the probe. In the single G:T exception, LNA nucleotides actually stabilized the


A. Sugar Modifications

B. Base Modifications

C. Universal Bases

Figure 8.1 Oligonucleotide modifications. (A) Modifications to the ribose. DNA, RNA, LNA (locked nucleic acids), and 2′-*O*-methyl (2′*O*Me) are shown. (B) Base modifications. Propynyl-dU, propynyl-dC, 2-thiothymidine, 2,6-diaminopurine (2,6-DAP, or 2-amino-dA), and Super-G™ are shown. (C) Universal bases. Inosine, 3-nitropyrrole, 5-nitroindole (5-NI), dP, and dK are shown.

mismatch relative to the unmodified sequences, making SNP discrimination worse. LNA modifications can still be used to improve probes having a G:T mismatch by positioning the LNA nucleotides in flanking regions of the probe and reducing probe length. Alternatively, the probe can be designed to the other strand, in which case the mismatch is a T:G pair (with the thymidine base in the probe), and the standard 'LNA triplet' design strategy can be used.

When using the 'LNA triplet' modification pattern described above, it is important to balance the actual T_m of the modified probe so that 'match' hybridization stays around 64–65°C (i.e. 4–5°C above reaction temperature) by trimming bases from each end of the parent unmodified sequence to compensate for the increased binding affinity of the LNA nucleotides. Additional LNA nucleotides can be substituted in the flanking regions of the probe to allow further shortening of the probe sequence, which will continue to improve mismatch discrimination. Ideally, this process would be done using design software that calculates the T_m of different modification patterns and automatically optimizes design. Assistance with estimating the T_m of LNA-modified oligos and design of SNP-discriminating probes can be found on the Exiqon website at: http://lnatools.com/. The interactive tools on this site employ thermodynamic calculations that use experimentally determined parameters and unpublished proprietary rule sets for probe design. Unfortunately, enough inaccuracy exists in these calculations that empiric testing of different probe designs is often required. Experimentally determined nearest neighbour (NN) parameters were published for isolated LNA nucleotides in LNA/DNA hybrids by Celadon Laboratories in 2004 (McTigue *et al.*, 2004). Additional parameters are needed to enable predictions to be made with sufficient accuracy for the T_m and ΔT_m of LNA containing sequences to design probes for use in SNP assays, including parameters for sequential LNA nucleotides and LNA/DNA mismatches. These missing parameters were recently obtained from studies performed at Integrated DNA Technologies (IDT) and were published in 2011 (Owczarzy *et al.*, 2011); the resulting new algorithms enable the accurate calculation of the T_m of complex LNA-modified oligonucleotides of this type and can be accessed at: http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/ or http://biophysics.idtdna.com.

A SNP in the human *SMAD7* gene (NM_005904) was employed here as a model site to demonstrate these principles. This SNP comprises a C/T variation (rs4939827) and the C-SNP is associated with increased risk of colorectal carcinoma (Tenesa *et al.*, 2008). Genomic sequence at this SNP site is shown in Fig. 8.2A. Probes were designed using existing DNA NN parameters and the new LNA match/mismatch parameters described above, keeping the T_m for the perfect match around 65°C. Calculations were made for a reaction mix having a probe concentration of 250 nM, 50 mM KCl, 3.0 mM $MgCl_2$, and 0.8 mM dNTPs (equivalent to a final free Mg^{++} concentration of 2.2 mM). Unmodified probes were designed as a control and modified probes were designed using the LNA 'triplet pattern' at the SNP site. Additional LNA residues were placed in flanking sequence as needed to allow the probes to be shortened from 20–22 bases (for the unmodified probes) to 14 bases; final LNA content ranged from three to four bases. Sequences of the probes designed are shown in Fig. 8.2B. Although not necessary for the sequences studied here, note that probes ending in a 5′-G base should have an additional non-complementary base inserted between the G and the reporter dye, to reduce the impact of G-quenching (which is particularly problematic for fluorescein) (Kaji *et al.*, 2009). Sequences of the SMAD7 probes are shown in Table 8.1. T_m values for the mismatch alleles were calculated using the new thermodynamic parameters and are also shown in Table 8.1. The predicted T_m values for the mismatch targets ranged from 50.9–55.5°C for the LNA-modified probes and resulted in a ΔT_m (match minus mismatch) of 10.0–15.7°C. In contrast, the unmodified probes showed predicted T_m's for the mismatch target ranging from 59.6–64.6°C, with a ΔT_m for match minus mismatch of only 1.5–5.6°C. Accuracy of the new T_m algorithms employed was tested by performing melting curve analysis on the SMAD7 probes. Experimental T_m values were obtained for both match and mismatch targets.

Melting experiments were conducted on a Beckman DU650 spectrophotometer (Beckman-Coulter) with a Micro T_m Analysis accessory, a Beckman High Performance Peltier Controller (to regulate the temperature), and 1 cm path-length cuvettes. UV-absorbance values at 268 nm wavelength were recorded using a PC interfaced to the spectrophotometer and temperature was increased at 0.1 degree increments. Pre-annealed duplexes were melted at 2 μM concentration in 10 mM Tris pH 8.3 (at 25°C), 50 mM KCl, and 2.2 mM $MgCl_2$ (equivalent to a PCR reaction buffer containing 3.0 mM $MgCl_2$ with 0.8 mM dNTPs). The melting profiles were analysed using methods that have been previously described (Owczarzy, 2005). Results are shown in Table 8.1. Experimental melting data were obtained using 2 μM duplex, which will raise T_m by 2–4°C compared

A) *SMAD7* SNP locus, rs4939827

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GGGGCTGTCTCTAATCCACCATGCTCACAGCCTCATCCAAAAGAGGAAA(C/T)AG
GACCCCAGAGCTCCCTCAGACTCCTCAGGAAACACAGACAATGCTGGGGTTTAGAG
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rs4939827 For
GGCTGTCTCTAATCCACCAT

rs4939827 Rev
CCCAGCATTGTCTGTGTTTC

B) Probe Alignments

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5′       FAM-AGGAAACAGGACCC-FQ         3′
5′   FAM-AAAGAGGAAACAGGACCCCA-FQ       3′

5′ CATCCAAAAGAGGAAACAGGACCCCAGAGCTCCCT 3′
3′ GTAGGTTTTCTCCTTTGTCCTGGGGTCTCGAGGGA 5′

3′         MGB-CTTTGTCCTGGGGT-FAM      5′


5′       HEX-AGGAAATAGGACCC-FQ         3′
5′    HEX-AAGAGGAAATAGGACCCCAGAG-FQ    3′

5′ CATCCAAAAGAGGAAATAGGACCCCAGAGCTCCCT 3′
3′ GTAGGTTTTCTCCTTTATCCTGGGGTCTCGAGGGA 5′

3′         MGB-CTTTATCCTGGGGT-VIC      5′
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Figure 8.2 Assays for the C/T SNP rs4939827 in the human *SMAD7* gene. (A) Genomic sequence of the region surrounding the C/T SNP site is shown. For and Rev primer sequences are shown and their binding sites are underlined. (B) Both strands of the genomic sequence of the ‘C’ and ‘T’ alleles are shown aligned with the different probe sequences that were tested. The SNP site is indicated in larger font. DNA bases are uppercase. LNA nucleotides are underlined. Reporter dyes employed (FAM, HEX, VIC) are indicated. FQ indicates the Iowa Black FQ dark quencher. MGB indicates a minor groove binder modification group linked to an Eclipse dark quencher.

Table 8.1 Predicted versus measured T_m values for SMAD7 probes

	Predicted T_m (°C) (0.25μM)			Measured T_m (°C) (2.0μM)		
Probe sequence	Match	Mismatch	ΔT_m	Match	Mismatch	ΔT_m
5′ FAM-AGGAAACAGGACCC-FQ 3′	66.6	50.9	15.7	68.3	54.1	14.2
5′ FAM-AAAGAGGAAACAGGACCCCA-FQ 3′	65.2	59.6	5.6	65.5	56.4	9.1
3′-MGB-CTTTGTCCTGGGGT-FAM 5′	66.0	NA	NA	65.7	56.5	9.2
5′-HEX-AGGAAATAGGACCC-FQ 3′	65.5	55.5	10.0	67.6	59.1	8.5
5′-HEX-AAGAGGAAATAGGACCCCAGAG-FQ 3′	66.1	64.6	1.5	65.1	63.0	2.1
3′-MGB-CTTTATCCTGGGGT-FAM 5′	66.0	NA	NA	67.4	57.7	9.7

Probes specific for the ‘C’ allele were labelled with FAM at the 5′-end. Probes specific for the ‘T’ allele were labelled with HEX or VIC at the 5′-end, as indicated. The SNP site is indicated in larger, bold font. LNA nucleotides are underlined. FQ indicates the Iowa Black FQ dark quencher. MGB indicates a minor groove binder modification group linked to an Eclipse dark quencher. The MGB probes were designed to the opposite strand and are accordingly shown in reverse orientation. Note that predicted T_m values generated using a probe concentration of 0.25μM were used for probe design; measured T_m values are done at 2μM duplex concentration to accommodate needs for spectrometric methods. NA=mismatch predictions not available.

with the 0.25 μM duplex concentration used in qPCR (0.25 μM was used for the original T_m predictions when designing the probes). Measured T_m values were found to all be within a few degrees of the expected values for three of the four probes, well within expected experimental error for these methods. The unmodified 'C'-SNP probe, however, showed a lower than predicted T_m against the mismatched target, resulting in a ΔT_m of over 9°C.

The SMAD7 probes and primers shown in Fig. 8.2 and Table 8.1 were functionally tested in real-time qPCR. Reactions were run on a BioRad CFX384 in 384 well plates using 10 μl reaction volumes. Reactions consisted of either 1X Taqman Genotyping Master Mix (Life Technologies) or a homemade Immolase Master Mix (0.4 U Immolase DNA polymerase (Meridian Bioscience, Cincinnati, Ohio), 1 μl of 10× polymerase buffer (160mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8.3, 0.1% Tween-20), 3 mM $MgCl_2$, 800 μM dNTPs), 250 nM of each probe, 500 nM of the forward and reverse primers, and 2 ng human genomic DNA. Cycling conditions included an initial 10 minutes soak at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Human genomic DNAs that were homozygous C/C or T/T or heterozygous C/T at SNP locus rs4939827 were obtained from the Coriell Institute for Medical Research Cell Repository (http://ccr.coriell.org/, GM07019 (T/T), GM07029 (C/T), GM07034 (C/C)). 'C'-SNP results were collected in the FAM fluorescence channel and 'T'-SNP results were collected in the HEX fluorescence channel. Amplification plots are shown in Fig. 8.3. The LNA-modified probes (Fig. 8.3a) resulted in unambiguous base

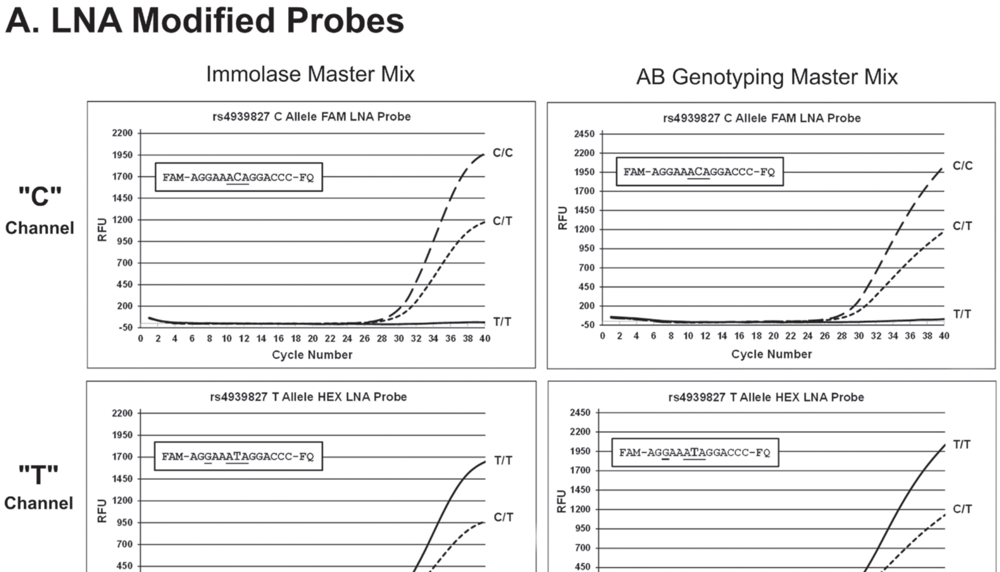
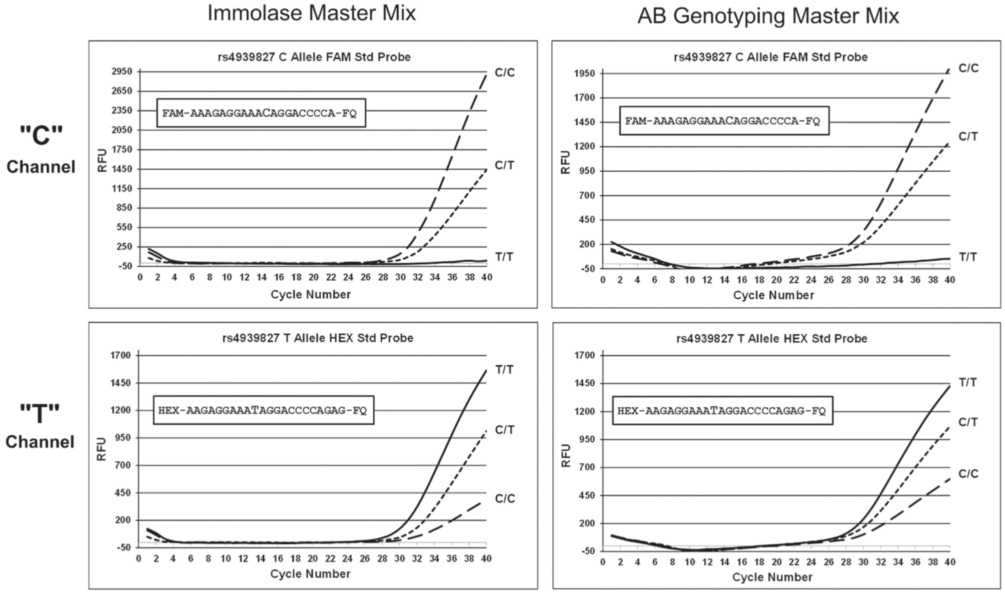
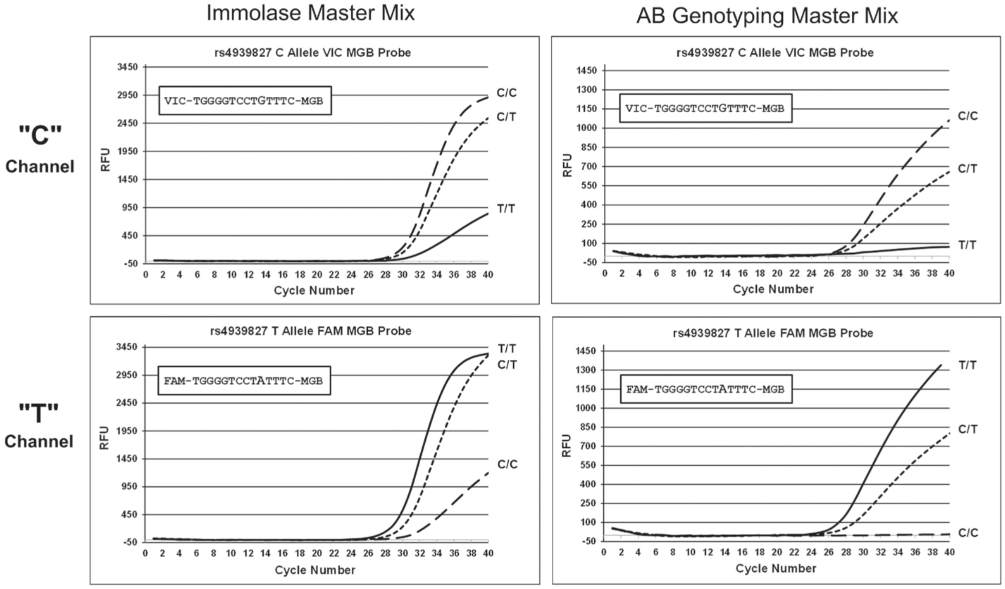


Figure 8.3 5′-Nuclease assay amplification plots for different probe designs at a SNP locus. The 5′-nuclease qPCR assay was performed using 2ng of human genomic DNA homozygous or heterozygous at the SMAD7 rs4939827 locus using probes of different design and two different buffer systems, the Immolase Master Mix or the Applied Biosystems (AB) Genotyping Master Mix. Reactions were run in multiplex format with probes specific for both alleles present in the reaction and were monitored in separate fluorescent channels. Probe

B. Std Unmodified Probes



C. MGB Modified Probes



sequences are inset at the upper left corner of the amplification plots. Reporter dyes FAM, HEX, and VIC are indicated. FQ=Iowa Black FQ dark quencher. MGB=minor groove binder with Eclipse dark quencher. LNA nucleotides are underlined and the position of the SNP base is indicated in larger font. (A) Reactions using LNA-modified dual-labelled probes. (B) Reactions using unmodified DNA dual-labelled probes. (C) Reactions using MGB-modified dual-labelled probes.

discrimination, with the 'C'-probes only detecting amplification with C/C and C/T DNAs and the 'T'-probes only detecting amplification with T/T and C/T DNAs. Similar results were obtained in both of the master mix buffer systems. Importantly, these results were obtained without the need for any empiric optimization and probes were simply designed using the method described above.

The unmodified DNA dual-labelled probes were also tested and results are shown in Fig. 8.3b. The unmodified 'C'-SNP probe gave good discrimination but the unmodified 'T'-SNP probe gave poor discrimination. These results are consistent with the experimentally measured T_m and ΔT_m values obtained for match and mismatch pairs for the unmodified probes (Table 8.1). Interestingly, predictions made using nearest neighbour parameters suggested that both assays would show poor SNP discrimination, whereas in actual practice one worked well and the other failed. Nevertheless, both probes must perform well together, and genotypes could not be reliably called using the unmodified probe assay. Thus, use of ΔT_m-enhancing LNA nucleotides improved the ability of qPCR to distinguish between alleles at this locus, converting a failed assay into a successful assay.

Three additional modified bases were reported by Epoch Biosciences that can be used to improve performance of dual-labelled probes (and primers) in qPCR. The Super A^{TM} and Super T^{TM} bases represent modifications of adenosine and thymidine that increase stability when base paired with unmodified A or T. Substitution of these bases in a probe sequence will increase T_m, particularly for AT-rich sequences (Afonina *et al.*, 2002a; Dempcy *et al.*, 2003). Nearest neighbour parameters have not been reported for these bases, however, so T_m predictions cannot be accurately made to assist with design. The third base, Super G^{TM} (8-aza-7-deazaguanine, or PPG) does not increase T_m but does disrupt the Hoogsteen-bonding potential for the N-7 position of the guanine ring (Kutyavin *et al.*, 2002). Hoogsteen-bonding is required for formation of G-tetrads (or G-quadruplexes), a structure that is naturally found in telomeres and can also cause unwanted aggregation of synthetic oligonucleotides (Simonsson, 2001; Reddy and Hardin, 2003). Substituting Super G^{TM} in G-runs (sequences having four or more sequential guanine residues) can prevent G-tetrad formation and eliminate the various complications that arise from tetrad formation.

Another strategy to increase T_m, shorten probe length, and improve specificity is use of non-nucleoside T_m-enhancing modifying groups. Compounds have been known for many years that will bind DNA duplexes with high affinity, such as intercalators and minor groove binders (Bailly and Henichart, 1991). A novel compound of this class was developed by Epoch Biosciences in 1996 that binds the minor groove of a DNA duplex and significantly stabilizes hybridization of a short DNA oligonucleotide to its complementary sequence. This group is a complex molecule, 1,2-dihydro-(3*H*)-pyrrolo[3,2-*e*]indole-7-carboxylate ($CDPI_3$), which is more conveniently called 'MGB' (Kutyavin *et al.*, 1997). The thermodynamic properties of the MGB vary with sequence, length, and chemistry. When attached to the end of a short A:T 8mer (poly-dT_8:poly-dA_8), the MGB leads to greater than a 40°C increase in T_m. The increase in binding affinity is greater for AT-rich sequences than for GC-rich sequences. T_m changes for phosphorothioate DNA have similar magnitude as unmodified DNA; however, the impact is much less when the MGB is attached to a 2′*O*Me-modified oligonucleotide sequence, possibly because geometry of the minor groove is different in RNA (or RNA:DNA heteroduplexes) than DNA.

The first application proposed for this new chemistry was modification of the 5′-end of PCR primers to enable use of very short oligos (eight to ten bases long) to prime DNA synthesis (Afonina *et al.*, 1997). It was proposed that this would have particular utility in designing primers which were specific for short blocks of highly conserved sequence (for example, in amplifying a broad range of viral serotypes, it is necessary to place primers in very limited areas where sequence is sufficiently conserved between species). Importantly, the MGB group lies in the minor groove of the DNA helix and occupies space corresponding to around six bases, so if this group is positioned at the 5′-end of a primer it will not overlap or interfere with the 3′-terminus of an oligonucleotide that is even as short as eight to ten bases. It was quickly appreciated that the MGB group would also have significant utility if used for dual-labeled probes in the 5′-nuclease assay. In this format, the MGB group is placed at the 3′-end of the probe with a dark quencher positioned between the MGB and the 3′-DNA residue and a reporter fluorophore is placed at the 5′-end. Use of the MGB was demonstrated to permit shortening some probe sequences from 27 bases to as little as 12 bases in length while keeping a constant 65°C T_m, thanks to a 5–20°C contribution from the MGB (Kutyavin *et al.*, 2000, 2003). While probes of this design work well for general gene quantification purposes, the real value becomes apparent when performing allelic discrimination assays. It is thought that the group binds the 3′-end of the probe/target duplex, and, if a SNP site is positioned within the terminal roughly eight bases from the 3′-end (within or near the MGB binding domain), the resulting ΔT_m for match versus mismatch is often in the 10–20°C range, making for very robust SNP assays.

Use of the MGB modification at the 3′-end of a probe for 5′-hydrolysis format qPCR assays was exclusively licensed from Epoch Bioscience by Applied Biosystems and is the basis for the current line of TaqMan® probes. Probes of this design are in widespread use today for all qPCR applications; this format is most often used for low to mid-volume assay throughput needs (De la Vega *et al.*, 2005). The PrimerExpress™ assay design software from Applied Biosystems will provide an estimate of T_m for MGB-modified probes. However, no estimates are given for the ΔT_m when paired with mismatched targets. Applied Biosystems (now Life Technologies) also offers a probe design service via their website and has predesigned or validated probes available for many targets. Predesigned MGB-TaqMan assays are available for most genes and many SNPs from Applied Biosystems and are easily searched/ordered using tools on their website. However, these probes are only provided as fully formulated ‘assays’, where all probes and the primers come pre-mixed in a single tube, making it difficult to study the performance of individual probes. Further, the sequences of the probes are not provided when ordered via this route. Therefore, instead of ordering pre-designed assays, MGB-Taqman probes for the SMAD7 SNP locus were designed using the PrimerExpress™ software and these sequences were obtained from Applied Biosystems as a custom synthesis. Experimental T_m values were determined for the MGB probes using identical methods and buffers as was previously used for the LNA probes. Results are reported in Table 8.1. The ΔT_m comparing match versus mismatch hybridization was 9–10°C, a value grossly similar to that obtained using the LNA modified probes. The SMAD7 MGB-probes were used in allelic discrimination qPCR assays, using the same methods as outlined previously for the unmodified and LNA-modified probes. The results are shown in Fig. 8.3c. Performance of the MGB probes was excellent and complete discrimination of the C/C and T/T genotypes was achieved when using Applied Biosytems genotyping mastermix. However, performance of the MGB probes was very different when reactions were done using a different buffer (the Immolase Master Mix). Anneal/extension temperatures were varied with little benefit (not shown).

It is not clear why the 'C' allele probe showed good functional discrimination while the 'T' allele probe did not, even though both showed similar ΔT_m values (in a buffer having similar ionic composition to the Immolase Master Mix). These results illustrate the important point that buffer composition is a critical variable when performing demanding applications such as SNP discrimination in qPCR. Some commercial reagents contain proprietary components and do not reveal ionic composition, making it difficult to predict performance. Empiric comparison of different reaction mixes may be needed to optimize performance.

The MGB group can also be added to the 5′-end of the probe. In this configuration, 5′-nuclease activity of the polymerase is blocked and the probe instead functions in a hybridization format with fluorescence signal resulting from the reduced quenching that occurs upon hybridization (Afonina *et al.*, 2002a,b). In single stranded form, the end-to-end distance in the random coil conformation is short and the reporter dye is effectively quenched. When hybridized to target, the duplex exhibits more rigid-rod conformation than single strands and the end-to-end length increases, reducing quenching. Fluorescence emission therefore increases from the reporter dye in the absence of probe degradation (Livak *et al.*, 1995).

Other chemical groups can be used to increase T_m and improve probe performance. Behr and colleagues from the University of Strasbourg developed methods to attach cationic spermine groups to oligonucleotides using a novel spermine phosphoramidite. Multiple spermine groups can be joined in serial fashion, creating a polycation. Attachment of this positively charged polymer to one of the ends of an oligonucleotide helps overcome the electrostatic repulsion between two annealing anionic DNA strands, increasing binding affinity (i.e. increasing T_m) (Noir *et al.*, 2008). Methods employing this technology in molecular biology assays have been commercialized by PolyPlus-Transfection, Inc. This modification has been called 'ZNA', for 'zip nucleic acids', when used in synthetic oligonucleotides. Like the effects described previously for the MGB group, addition of a 5′-ZNA modification permits short primers to be used in PCR (Moreau *et al.*, 2009). Similarly, attachment to the 3′-end of a dual-labelled probe is compatible with use in the 5′-nuclease assay and increases probe T_m, enabling use of shorter probes with better performance in allelic discrimination assays than unmodified probes (Paris *et al.*, 2010). Unlike the MGB group, which is usually positioned at the very 3′-end, ZNA probes position the quencher at the 3′-end and typically four spermine groups are placed between the quencher and the 3′-most base of the nucleic acid sequence. This poly-spermine tail flips over and binds the DNA nucleotides near the 3′-end of the probe, which fortuitously also results in the quencher being positioned closer to the reporter fluorophore (at the 5′-end). Forcing a shorter dye-quencher distance increases quenching, lowering background fluorescence and further improves probe performance.

Another variant of a linear hybridization probe for SNP discrimination was described by Yamane (Wakunaga Pharmaceutical Co., Japan) and were called 'MagiProbes' (Yamane, 2002). In this case, a reporter fluorophore and a pyrene quencher were positioned centrally in the probe near the SNP site in adjacent or nearly adjacent positions. When hybridized to a perfect match target, the pyrene group intercalates into the DNA duplex resulting in loss of quencher function and fluorescence emission seen from the reporter. Conversely, when hybridized to a mismatch target, the pyrene group remains external to the duplex and continues to quench the reporter.

Hairpin dual-labelled probes

Hairpin fluorescent dual-labelled probes, called 'Molecular Beacons', were first described by Tyagi and Kramer (Public Health Research Institute, New York, NY, USA) in 1996 (Tyagi and Kramer, 1996). These probes have a reporter fluorophore on one end and a quencher on the other end; a target hybridizing domain occupies the central loop portion of the probe sequence and two short sequences are placed on either end which are self-complementary (and non-complementary to target). In the absence of target, the Molecular Beacon forms a hairpin, which forces the reporter and quencher into close proximity. As a result, quenching efficiency is very high and probes of this design have a very good signal-to-noise ratio. Further, hairpin formation competes with target binding and improves the thermodynamics of match/mismatch discrimination, increasing the relative ΔT_m. With careful adjustment of the hairpin stem length, good SNP discrimination can sometimes be achieved without additional chemical modification (Tyagi *et al.*, 1998; Bonnet *et al.*, 1999; Tsourkas *et al.*, 2003b). As with linear probes, LNA nucleotides can be included within the target binding domain which will allow for use of shorter probe sequences, further improving the ΔT_m. Non-base modifiers (like MGB or ZNA), however, cannot easily be used in Molecular Beacons as these groups would primarily increase stability of the stem and would be detrimental to target binding. Synthesizing probes with 2′-O-Me-RNA in place of DNA increases nuclease stability and permits use of Molecular Beacons in live cells to directly visualize mRNA by fluorescence microscopy (Bratu *et al.*, 2003); note that shorter stem domains must be used with this chemistry as the T_m of the stem is much higher for 2′O-Me–2′O-Me duplexes (Tsourkas *et al.*, 2003a). The use of a 2′-O-Me sugar backbone, however, does not confer any benefit for use in qPCR applications. A version of Molecular Beacons called 'Scorpions' was described by Brown and colleagues (University of Southampton, UK) where the probe is tethered to a PCR primer and configured so that target binding can occur at specific points in the reaction cycle (Thelwell *et al.*, 2000). This design showed good sensitivity and specificity compared with traditional probes and in particular worked well with fast cycling times.

Enzymatic interrogation of SNP identity

The SNP discrimination methods discussed above all rely upon differential hybridization of a probe oligonucleotide with different target nucleic acids to distinguish base identity at the site of interest. It is also possible to perform SNP discrimination using probes with high affinity that bind well to both match and mismatch targets that include modification(s) which permit the probes to interact with an enzyme that is capable of discriminating between paired and unpaired bases at the SNP site. The best example of this class of SNP assay uses RNase H enzymes to selectively cleave probes. RNases H are a family of enzymes that specifically cleave at RNA bases in an RNA:DNA heteroduplex. The enzymes do not cleave single stranded RNA or DNA. Two major classes of RNase H enzymes exist, RNase H1 and RNase H2 (Ohtani *et al.*, 1999). RNase H1 enzymes require a stretch of at least four consecutive RNA bases to show maximal enzyme activity and cleave between RNA residues (Hogrefe *et al.*, 1990). In contrast, RNase H2 enzymes will cleave a substrate having only a single RNA residue in an otherwise DNA sequence and cleavage occurs at the 5′-side of the RNA base at what is actually a DNA linkage (Eder and Walder, 1991; Eder *et al.*, 1993). Cleavage patterns for RNase H1 and RNase H2 enzymes are illustrated in Fig. 8.4. RNase H enzymes have been isolated from a variety of prokaryotic species that are thermostable

(Kanaya, 2001), some of which can be used at elevated temperatures in molecular biology assays or even be included in PCR reactions.

Peter Duck (Meiogenics, MD, USA) and Joseph Walder (University of Iowa and Integrated DNA Technologies) described 20 years ago a method to use chimeric RNA:DNA oligonucleotides as probes to detect complementary sequences with isothermal signal amplification. Probes were made with DNA bases at both ends and contained a stretch of at least four RNA residues in the central region. When hybridized to a DNA target, RNase H1 will cleave within the RNA domain, cutting the probe in half. The shortened probe dissociates from the target, permitting a new probe molecule to hybridize and repeat the cycle (isothermal cycling); cleavage products accumulate and can be detected by a variety of methods (Duck *et al.*, 1990; Walder and Walder, 1995). Harvey and Han (Eximus Biotech, Maryland) adapted this method to a fluorescence format by adding a dye and a quencher to the chimeric probe molecule on opposite sides of the cleavable RNA domain, so that the probe is dark in the intact state and bright after RNase H cleavage (Harvey *et al.*, 2004). Cleavage by RNase H1 is less efficient if a mismatch is present in the RNA cleavage domain between the probe and the DNA target. This method can be linked to PCR by using a thermostable RNase H1 enzyme and the resulting assay can be used to discriminate SNPs (Harvey *et al.*, 2008). In this case, a commercially available RNase H1 enzyme from a *Thermophilus* sp. was employed (Epicenter, Madison, WI).

While this method works, more than one cleavable linkage exists within the 4-RNA central domain and it is not entirely clear where it is best to position the SNP when designing the assay. If an RNase H2 enzyme is employed, the same assay strategy can be used with a probe that has only a single RNA base (i.e. a single cleavable linkage). Hou and colleagues (Shanghai Jiaotong University, China) characterized the ability of the RNase H2 enzyme from *Chlamydia pneumoniae* to discriminate mismatches when a probe containing a single RNA residue was hybridized to targets having mismatches at different positions and

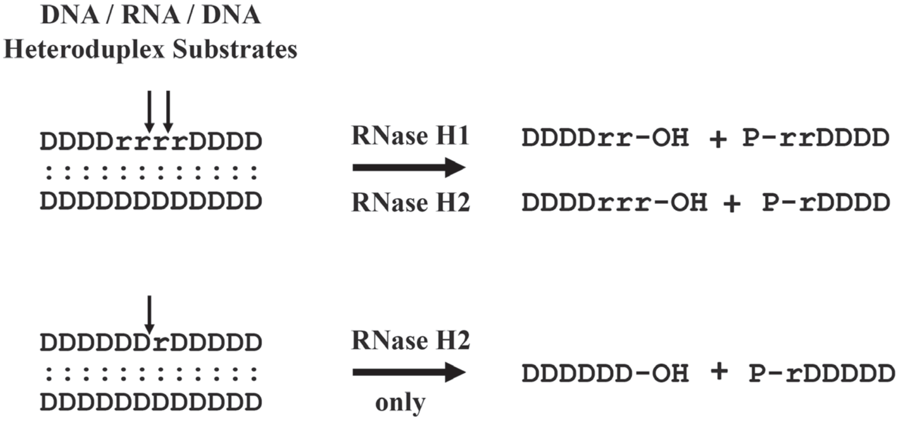


Figure 8.4 RNase H cleavage patterns. Two classes of RNA:DNA heteroduplex substrates are shown. The top row shows a substrate having four consecutive RNA bases and the bottom row shows a substrate having a single RNA base. Arrows indicate expected sites for RNase H cleavage for each substrate. Expected products following cleavage by RNase H1 and/or RNase H2 are shown on the right. D=DNA, r=RNA, OH=3′-hydroxyl, P=5′-phosphate.

found that discrimination was best when the SNP was placed at the RNA base; the bases on either side of the RNA base (at the '+1' and '–1' positions) also showed high selectivity (Hou *et al.*, 2007a). They adapted this assay for use with Molecular Beacons, positioning the RNA base centrally within the target binding loop domain of the probe. Since the *C.p.* RNase H2 enzyme is not thermostable, the Molecular Beacon hybridization/cleavage steps were run post-amplification as an end-point assay (Hou *et al.*, 2007b,c). The same group later improved this method by using a thermostable RNase H2 enzyme from *Thermus thermophilus*, which permitted a homogeneous assay format where the probe and cleaving enzyme were present and functioned in real time during PCR (Liu *et al.*, 2010).

SNP discrimination using biased amplification

PCR-based methods that discriminate between related sequences using biased amplification lead to differential accumulation of product with one allele being preferentially amplified over the other allele. If the different alleles are present at roughly similar levels in the test nucleic acid sample, SNP discrimination methods that use biased or unbiased amplification can perform equally well. However, if one allele is under-represented in the population, unbiased amplification is often only capable of detecting the more prevalent species. Detection of a rare allele in the presence of a high background of wild-type sequence requires use of a method that employs biased amplification, especially if that species is present at levels of 10^{-2} to 10^{-6} or lower. Unlike unbiased amplification, biased amplification normally requires that one of the primers overlaps the SNP site. The system is designed so that the match template supports amplification while the mismatch template does not. Sequence identity is determined by the presence or absence of amplification. Probe-based methods discussed previously usually rely upon differential hybridization between match versus mismatch targets (i.e. thermodynamic discrimination). For biased amplification, primers typically bind to both alleles and discrimination is based upon enzymatic events, e.g. whether or not the oligonucleotide will prime DNA synthesis or not. Most of the methods to perform biased amplification with enzymatic interrogation employ chemically modified oligos, and the chemical modifications enable the process.

Allele-specific PCR

The simplest system to perform allele-specific PCR (ASPCR) employs primers where one primer overlaps the SNP, which is positioned as the 3′-end base of the primer. Differences in the priming efficiency on match versus mismatch templates determine the level of selectivity. In practice, actual discrimination can vary from very low to fairly high and depends largely on the sequence context and precise bases present as the mismatch pair at the SNP site. Also, different polymerases show different degrees of selectivity using the same primers and template (Mendelman *et al.*, 1990). A survey was performed at IDT where we examined the relative discrimination seen in a synthetic amplicon using allele-specific PCR. Four unmodified primers were made having the four possible bases at the 3′-position; four templates were made having the four possible bases at the SNP site. All 16 pairwise combinations were tested in a SYBR® Green format, and reactions were tested individually (not in a multiplex format). The ΔCq (the cycle number at which signal was first detected for the mismatch minus the cycle number at which signal was first detected for the match) ranged from 1.2 to 10.7 cycles, with an average ΔCq of 5.4 for the 12 mismatch pairings (Dobosy *et al.*, 2011). In other words, sometimes this method works well and sometimes it does not. Careful optimization of primer design, reaction temperature, buffer composition, and enzyme choice can improve results. Traditionally, these reactions are performed individually in separate tubes, as there is no simple way to distinguish between the two different allele products when using unmodified primers. Using modified dye labelled primers (such as the hairpin fluorescence-quenched Amplifluor^{TM} system), it is possible to perform assays for both alleles in a single tube. Interestingly, results are often significantly improved in multiplex format and it appears that kinetic competition between alleles increases specificity and reliability (Myakishev *et al.*, 2001).

Use of chemically modified bases can further improve the specificity of ASPCR. In particular, placing an LNA nucleotide at the 3′-end of the allele-specific primer will decrease priming on mismatched templates without compromising efficiency of PCR on matched templates. Usually only a single LNA modification is used on each primer, which is placed at the 3′-end with the LNA nucleotide pairing with the SNP (Latorra *et al.*, 2003; Nakitandwe *et al.*, 2007). This strategy works best when using a DNA polymerase that does not have 3′–5′ exonuclease activity. If a high fidelity DNA polymerase is used that has proofreading function (3′–5 exonuclease activity), then the enzyme can remove the terminal 3′-base from the primer (error correction) and amplification will proceed whether a mismatch is present or not. In this scenario, it is preferred to position the LNA nucleotide at the 'N-2' position, the base adjacent to the 3′-end (Di Giusto and King, 2004). In this design, the LNA modification confers structure to the primer that makes this last linkage relatively nuclease resistant so that the 3′-base is retained in the primer and can contribute to specificity as intended.

The 'amplification refractory mutations system' (ARMS) and 'mismatch amplification mutation assay' (MAMA) are methods that introduce an artificial second mismatch into allele-specific PCR primers as a way to increase selectivity (Newton *et al.*, 1989; Cha *et al.*, 1992). As with standard ASPCR, the SNP under interrogation is positioned at the 3′end of the primer. A second mismatch is introduced in the primer directly adjacent to the 3′-base, resulting in the 'match' primer having a single mismatch with target (the artificial mismatch) and the 'mismatch' primer having two mismatches with the target (the artificial mismatch plus the SNP). At this position, the single internal mismatch is permissive and the 'match' primer does not show any significant decrease in priming efficiency. However, the double mismatch is very inefficient when priming on the mismatch target and ΔCqs of ten or more cycles can often be achieved, even for base mismatches that are unfavourable for standard ASPCR. The primers used in the original versions of these assays are unmodified. In many cases, the specificity of ARMS or MAMA primers can be further enhanced by placing an LNA modification at the SNP site located at 3′ end.

Biased amplification using blocked primers

Another approach to perform biased amplification employs primers that are blocked at the 3′-end which cannot prime DNA synthesis until the block is removed. The blocked 3′-OH group can be removed by cleavage using either an endonuclease (internal cleavage) or a 3′-exonuclease; cleavage activates the primer, allowing PCR to proceed. To function as a SNP assay, the 3′-deblocking step must be selective and only occur when a perfect match is present between the primer and the target at the cleavage site. Specificity of the cleavage process is critical to success.

One successful version of this assay strategy was developed by Liu and Sommer (City of Hope National Medical Center, Duarte, CA, USA) and is called pyrophosphorolysis-activated polymerization, or PAP (Liu and Sommer, 2000, 2002). Like traditional ASPCR primers, PAP primers have the SNP site positioned at the 3′-end of the primer sequence; however, PAP primers are also blocked at the 3′-end. The 3′-end block is removed by the polymerase enzyme via pyrophosphorolysis, which is essentially a reverse polymerization reaction that occurs in the presence of sufficiently high levels of inorganic pyrophosphate (Deutscher and Kornberg, 1969). High levels of inorganic pyrophosphate inhibit DNA polymerases, so careful buffer composition optimization must be done. PAP reactions also employ lower levels of deoxynucleotide triphosphates than are typically employed in PCR (e.g. 50 μM of each dNTP with 300 mM sodium pyrophosphate). Pyrophosphorolysis occurs only when the terminal base of the primer is base-paired with a complementary base on the target; a mismatch base will not function as a substrate for this enzymatic reaction. A variant of the PAP method uses both blocked forward and blocked reverse primers, called Bi-PAP (bidirectional pyrophosphorolysis-activated polymerization). The SNP is positioned at the 3′-end of both primers. As a result, the primers overlap by one base, but this short overlap is not sufficient to allow primer-dimer amplification (the primers are blocked – after deblocking, the primers no longer overlap). For amplification to occur, successful PAP must occur on each primer independently, greatly increasing specificity of the final assay. Bi-PAP has been shown to be capable of rare allele detection at levels as low as 10^{-7}–10^{-8} (Shi *et al.*, 2007). A wide variety of 3′-blocking strategies have been tested and the best results are obtained when the 3′-base is a dideoxynucleotide (ddN). Widespread use of PAP therefore requires the availability of primers with a 3′-terminal dideoxy base for each of the four bases; ddA and ddC synthesis reagents are available as commercial solid supports, so synthesis of primers ending in an A or C base is straightforward. Unfortunately, ddT and ddG supports are not available and primers ending in a T or G base have to be made using expensive reverse synthesis or by enzymatic methods, limiting the general utility of this approach.

Biased amplification using RNase H and blocked-cleavable primers

Another method in this class of assays employs blocked primers that have a single RNA base positioned near the 3′-end of the primer. Following hybridization to a complementary sequence, the terminal blocking group is removed by action of a thermostable RNase H2 enzyme (in contrast with PAP, this time the ‘deblocking enzyme’ acts via internal endonuclease cleavage). RNA-containing blocked-cleavable primers can be used in PCR and provide improved specificity when compared with traditional priming methods, resulting in lower background, reduced mispriming, and elimination of primer-dimer formation (Walder *et al.*, 2009; Dobosy *et al.*, 2011). PCR done using blocked-cleavable primers has been called ‘rhPCR’. Many of the features of RNase H enzymes were reviewed in the previous section. It is important to note that the cleavage reaction requires that the primer must be duplexed with a DNA target. RNase H2 will not degrade single-stranded nucleic acids; it requires an RNA–DNA heteroduplex substrate. When a blocked primer of this design hybridizes to the target, the substrate has a single RNA base embedded in an otherwise DNA sequence. RNase H2 cleaves at the 5′-side of the RNA base at the DNA linkage, leaving a 3′-hydroxyl at this end and a cleavage product which is an activated, functional primer. The second cleavage product contains a 5′-phosphate, the single RNA base, a few DNA bases, and the 3′-blocker; this is an inactive fragment that is short, dissociates from the target, and is 'disposable'. Importantly, the cleavage reaction is sensitive to correct base pairing such that primer deblocking is inhibited by the presence of a base mismatch in the vicinity of the cleavage site. The basic scheme for performing rhPCR with blocked-cleavable primers and RNase H2 is outlined in Fig. 8.5.

Ideally the RNase H2 enzyme employed in rhPCR will be very thermostable. A number of suitable enzymes have been isolated from archaeal sources, including *Pyrococcus furiosus* (Sato *et al.*, 2003), *Methanococcus jannaschii* (Lai *et al.*, 2003), *Thermococcus kodakaraensis* (Haruki *et al.*, 1998; Mukaiyama *et al.*, 2004), and *Pyrococcus abyssi* (Walder *et al.*, 2009; Le Laz *et al.*, 2010; Dobosy *et al.*, 2011). These enzymes require divalent cations for catalysis and can employ either Mg^{++} ions (usually in the 2–5 mM range) or Mn^{++} ions (usually in the 0.3–1.5 mM range). Turnover rate is fast enough that primer annealing and cleavage can be done in real time during PCR using the same primer anneal/extend times that are usually employed (30–60 seconds dwell time) with no loss of priming efficiency. Cycling should be performed as a two-step PCR (e.g. 60°C–95°C steps), using the same temperature for both primer annealing and extension reactions. A three-step PCR (e.g. 60°C–72°C–95°C steps) will be inefficient as primer annealing and deblocking cannot occur during the 72°C extension phase.

For a primer/target heteroduplex to be an efficient substrate for RNase H2 cleavage, a minimum amount of nucleic acid must be present in duplex form both 5′ and 3′ to the RNA base. These requirements have been studied for *Pyrococcus abyssi* RNase H2 and 8–10 DNA bases are needed 5′-to the cleavable cleavage site and four or more DNA bases are needed 3′-to the cleavage site for maximal activity. A variety of methods can be used to block the 3′-end of the primer, including dideoxy nucleotides or non-base modifiers placed attached to the 3′-end of the terminal DNA residue. A C3 spacer group (1,3-propanediol) is inexpensive and works well as a blocking group (Walder *et al.*, 2009; Dobosy *et al.*, 2011). Using these design parameters, a typical rhPCR primer will have a 'DNA primer domain' towards the 5′-end (20–25 bases), a single RNA base, four DNA bases on the 3′-side of the RNA, and a terminal 3′-C3 spacer (or other blocking group). This basic 'first generation' rhPCR design primer will prevent primer-dimer formation and improve specificity. In addition to primer hybridization, mismatches at or near the RNA cleavage site will reduce the rate of cleavage and prevent efficient PCR. It is not necessary that the cleavage event be 'all or none' at the

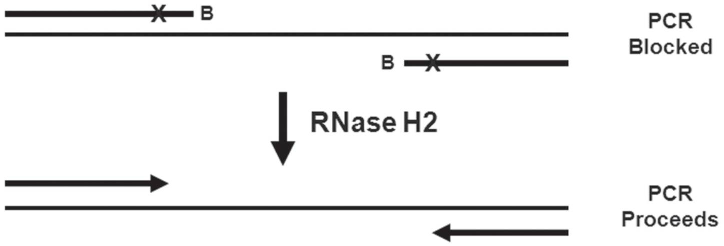


Figure 8.5 rhPCR using blocked-cleavable primers. Blocked primers are aligned against template on the top. X=scissile linkage, which in rhPCR is a single RNA base. B=blocking group, most commonly a C3-spacer (1,3-propanediol). Following cleavage, the RNA base and 3′-nucleic acids are removed and fully active primers remain having an unblocked terminal 3′-hydroxyl group (arrowheads).

RNA base to have a dramatic effect on PCR. If a mismatch reduces primer deblocking from 100% to 50% efficiency, then half of potential PCR priming events never happen and PCR amplification reduces from the standard exponential 2^n (where n = cycle number) to 1.5^n. Over 30 cycles, this represents a 5600-fold reduction in amplification.

The relative ability of a base mismatch to reduce cleavage has been studied for the RNase H2 enzymes from *Thermococcus kodakaraensis* (Liu *et al.*, 2010) and *Pyrococcus abyssi* (Walder *et al.*, 2009; Dobosy *et al.*, 2011). The best sites to place a mismatch (i.e. a SNP) are at positions complementary to the RNA base (position '0') or complementary to the DNA base which is immediately 5′-to the RNA base (position '–1'). RNase H2 cleavage occurs at the linkage between these two bases. From an assay standpoint, however, it is preferable to position the SNP at the RNA base. Since cleavage occurs on the 5′-side of the RNA base, if an unwanted cleavage event does occur in the presence of a mismatch, then the resulting primer will have this base excised and the first base of primer extension by the DNA polymerase will be encoded by the SNP. This ensures that the daughter product will faithfully reproduce the actual sequence present in the target nucleic acid. If the mismatch was positioned at the DNA base (position '–1'), cleavage still occurs immediately 5′-to the RNA base and then the SNP base encoded by the primer remains in the primer and is locked into the extension product, converting the PCR product to the primer sequence.

When the 'first generation' rhPCR primers are used in SNP assays, the observed Δ*Cq*s (mismatch – match) typically range from 5–15 cycles depending on the sequence context and precise base mismatches involved. While it is easy to distinguish the correct genotype present from these results, this level of discrimination is lower than would be expected given the known selectivity of the RNase H2 cleavage event. The basis for this 'lower than expected selectivity' was investigated. Reaction products were cloned from rhPCR done using mismatched templates and sequence was determined. It was unexpectedly found that the amplicons had converted to the primer sequence, which should not have been possible if primer cleavage was only occurring at the 5′-side of the RNA base (the SNP was positioned at this site) (Walder *et al.*, 2009). It appears that a very low frequency (less than 1 in 1000) of cleavage events occur at a non-canonical cleavage position, such that the enzyme cuts on the 3′-side of the RNA base. Cutting at this position results in the primer-encoded SNP base being incorporated in the amplicon, which converts the PCR product to the primer sequence. In subsequent cycles, this product will amplify at 100% efficiency as it is now perfect match to the primer. Thus the frequency of non-canonical cleavage ultimately determines the specificity of rhPCR.

Various patterns of chemical modification were tested in the rhPCR primers to decrease the frequency of non-canonical cleavage on the 3′-side of the RNA base. Substituting non-base groups in the short DNA domain on the 3′-side of the RNA base creates substrates that are less easily cleaved by RNase H2 (such primers require 10- to 100-fold higher enzyme concentrations to achieve reaction efficiency similar to what is seen using a 'first generation' primer); however, the frequency of non-canonical cleavage is reduced and Δ*Cq* values of 10–20+ cycles are easily achieved using this 'second generation' primer design. Currently the favoured 'second generation' primer design incorporates a DNA base, two C3-spacers, and a final DNA base on the 3′-side of the RNA residue. No 3′-blocking group is required with this primer design as the C3-spacers reduce or totally eliminate the ability of the terminal DNA base to prime DNA synthesis. Even if priming did occur, the spacers prevent the primer from functioning as a template for DNA synthesis so second strand synthesis would terminate at the primer, further blocking PCR. This 'second generation' primer design should be employed for allelic discrimination assays; however, the 'first generation' primer design is still preferred for other applications since lower concentrations of enzyme are needed to perform each reaction.

Examples of SNP discrimination using biased amplification

A series of SNP discrimination assays were performed using several of the methods included in the 'biased amplification' section using the same SNP in the *SMAD7* gene previously used in probe studies in this chapter (NM_005904, C/T variation, rs4939827). This site is a relatively challenging SNP assay for these kinds of methods, since one of the mismatch pairings is T:G. The T:G/G:T mismatch pairs are thermodynamically fairly stable and as a result are difficult to distinguish. Primers used included: standard ASPCR, 3′-LNA ASPCR, ARMS, 3′-LNA ARMS, and both the first generation and second generation rhPCR blocked-cleavable primers. Primers are shown in Fig. 8.6 aligned on the target SNP site.

The SNP discrimination assays were run in singleplex format on a Roche LightCycler® 480 in 384 well plates using 10 µl reaction volumes. Reactions consisted of 5 µl of 2′ BIO-RAD iQ™ SYBR® Green Supermix (BIO-RAD, Hercules, CA), 200 nM each of the forward and reverse primers, and 2 ng human genomic DNA. Cycling conditions included an initial 5 minutes incubation at 95°C, followed by 80 cycles of 10 seconds at 95°C and 30 seconds at 60°C (note – reactions were run 80 cycles only to demonstrate the selectivity of the blocked primers; a standard 40–45 cycle run would be used in a typical assay). Human genomic DNAs that were homozygous C/C or T/T at SNP locus rs4939827 were obtained from the Coriell Institute for Medical Research Cell Repository (http://ccr.coriell.org/, GM18562 and GM18537). The quantification cycle number (*Cq*) was determined using the absolute quantification/2nd derivative method. All reactions were run in triplicate and data reported in Fig. 8.6 represents an average of the three data points. The rhPCR reactions included recombinant *Pyrococcus abyssi* RNase H2 in the reaction mix (2.6 mU per 10 µl reaction for the first generation primers and 50 mU per 10 µl reaction for the second generation rC-containing primer and 200 mU for the rU containing primer) (Walder *et al.*, 2009).

At the locus, unmodified ASPCR primers were ineffective and showed < 1 cycle different in *Cq* values seen between match and mismatch targets. Adding a single LNA nucleotide to the 3′-end of this primer design boosted the ΔCq to six to nine cycles. Unmodified ARMS primers performed even better, showing a ΔCq of 9–11 cycles. Adding a 3′-LNA nucleotide to the ARMS primers increased discrimination to 10–13 cycles; however, a three cycle delay in the match *Cq* was observed for the 'T' allele. The first generation blocked-cleavable primers (rhPCR) gave a ΔCq of around 11 cycles and the second generation version of this assay gave the best results in this survey with ΔCq values of 28–30 cycles. It is important to note that these assays were performed in singleplex format. The ASPCR approach often shows improved specificity when run in a multiplex format (which requires modification to add the appropriate dyes/quenchers to the primers to permit multiplex fluorescence detection) where kinetic competition between primers for each allele occurs. Nevertheless it is clear that such biased amplification methods can work well, even at difficult SNP sites like the rs4939827 locus used in the present example.

Assay		Primer Seq	Cq		ΔCq
			match	mismatch	
Control	5′	CAGCCTCATCCAAAAGAGGAAA	26.4	--	--
ASPCR	5′	CAGCCTCATCCAAAAGAGGAAAC	26.7	27.4	0.7
3′-LNA	5′	CAGCCTCATCCAAAAGAGGAAAC	25.9	32.3	6.4
ARMS	5′	CAGCCTCATCCAAAAGAGGAATC	26.5	35.6	9.1
ARMS+LNA	5′	CAGCCTCATCCAAAAGAGGAATC	27.7	41.3	13.6
rhPCR, Gen1	5′	CAGCCTCATCCAAAAGAGGAAAcAGGAx	27.2	38.4	11.2
rhPCR, Gen2	5′	CAGCCTCATCCAAAAGAGGAAAcAxxA	27.2	55.7	28.5
	5′	GCTCACAGCCTCATCCAAAAGAGGAAACAGGACCCC 3′			
	3′	CGAGTGTCGGAGTAGGTTTTCTCCTTTGTCCTGGGG 5′			
Control	5′	CAGCCTCATCCAAAAGAGGAAA	26.4	--	--
ASPCR	5′	CAGCCTCATCCAAAAGAGGAAAT	26.6	27.0	0.4
3′-LNA	5′	CAGCCTCATCCAAAAGAGGAAAT	25.9	35.1	9.2
ARMS	5′	CAGCCTCATCCAAAAGAGGAATT	26.7	37.5	10.8
ARMS+LNA	5′	CAGCCTCATCCAAAAGAGGAATT	30.6	40.6	10.0
rhPCR, Gen1	5′	CAGCCTCATCCAAAAGAGGAAAuAGGAx	27.2	38.1	10.9
rhPCR, Gen2	5′	CAGCCTCATCCAAAAGAGGAAAuAxxA	28.6	59.3	30.7
	5′	GCTCACAGCCTCATCCAAAAGAGGAAATAGGACCCC 3′			
	3′	CGAGTGTCGGAGTAGGTTTTCTCCTTTATCCTGGGG 5′			

Figure 8.6 Biased amplification assays at a C/T SNP in the human *SMAD7* gene. Genomic sequence of the region surrounding the C/T SNP site is shown. SNP discrimination is based upon amplification from the For primer sequences, which are shown aligned with the genomic target. Assays tested include unmodified allele-specific PCR primers (ASPCR), 3′-LNA (variant of ASPCR), ARMS (note second mutation added at the next to last base from the 3′-end, an A–T-change), ARMS with 3′-LNA, first generation rhPCR blocked-cleavable primer with RNase H2 cleavage, and second generation rhPCR blocked-cleavable primer with RNase H2 cleavage. A control primer was also used which did not include the SNP base under interrogation. DNA bases are uppercase. LNA nucleotides are underlined. RNA bases are lowercase. 'X' is a C3 spacer (1,3-propanediol group). Reactions were performed in singleplex format using SYBR® Green detection using 2ng input homozygous C/C or T/T DNA. Cq values obtained for the match and mismatch templates are shown as well as the D*Cq* (mismatch *Cq* minus match *Cq*).

Biased amplification using template-blocking agents

Unlike the methods to perform biased amplification previously discussed, this version uses primers that do not overlap the SNP site and are non-discriminatory. A high affinity oligonucleotide or peptide nucleic acid (PNA) is made that is complementary to the target and overlaps the SNP site, which is positioned between the primers. Like a hybridization probe, the blocking agent must bind to the match target but not bind to the mismatch target under the reaction conditions employed. Unlike fluorescent probes, here the binding must be stable enough to block processivity of the DNA polymerase, interrupting amplification. Both PNA oligomers and LNA oligonucleotides have been shown to work in this application (Shinozaki *et al.*, 2007; Tatsumi *et al.*, 2008). It is essential that either the blocking agent is nuclease resistant or that the DNA polymerase employed lacks a 5′–3′ exonuclease activity. It can be challenging to find oligomers that bind with sufficient affinity to block the polymerase yet do not bind in the presence of a single mismatch. As a result, this method has not found widespread use.

Preventing primer–dimer formation

In spite of efforts to design primers that do not have significant overlap with themselves or each other, sometimes it is necessary for biological reasons to position primers at locations in a sequence which are not ideal and permits primer-dimer formation. 'Hot start' PCR methods have decreased some of the problems seen with primer-dimer formation; however, this does not solve all problems and sometimes it is necessary to use chemically modified primers that have reduced this risk. The blocked-cleavable rhPCR primers discussed above offer one method to prevent primer-dimer formation and will not be discussed further in this section.

Another method to minimize primer-dimer formation was described by Howard Gamper and colleagues from Epoch Pharmaceuticals (Kutyavin *et al.*, 1996; Gamper *et al.*, 2006) and relies upon the base modifications 2,6-diaminopurine (2,6-DAP, or 2-amino-dA) and 2-thiothymine (2-thio-dT). The modified base 2,6-DAP is an adenosine analogue and will pair with thymine bases in the normal fashion. Similarly 2-thio-dT is a thymine analogue which forms a stable base pair with native adenosine. However, 2-thio-dT will not pair with 2,6-diaminopurine. Thus primers made using these modified bases will hybridize to their intended targets and can support PCR with equal efficiency as unmodified primers; however, the modified primers cannot easily hybridize to each other as the 2,6-DAP and 2-thio-dT bases (A and T replacements) will not base pair. These modifications are fairly expensive and this method has not found widespread use, even though it is biochemically quite elegant and works well in practice.

A different approach was described by Gerald Zon, Richard Hogrefe, and colleagues from TriLink BioTechnologies (Lebedev *et al.*, 2008). Instead of using a base modification, the internucleotide linkage was modified at the 3′-most and/or next to last linkage with a 4-oxo-1-pentyl (OXP) phosphotriester (PTE) group. This modification impairs the ability of the oligonucleotides to prime DNA synthesis; however, the modifying group is heat labile and rapidly dissociates from the primers during the initial 95°C heat denaturation step, after which the primers revert to unmodified form and function normally. This prevents primer-dimer formation during the initial warming phase of PCR and is a supplement to standard hot-start methods. In particular, this kind of primer modification should be very useful in one-tube RT-PCR chemistries where the primers are present with an active polymerase and traditional 'hot start' is not employed. This method is inexpensive and primers with this chemistry can be obtained directly from TriLink BioTechnologies (San Diego, CA).

Decreasing specificity using universal bases

The previous sections of this chapter focused on methods to increase specificity. Sometimes it is also necessary to decrease specificity. For example, the SNPs that occur in outbred populations can interfere with assays intended to amplify all variants. This problem can be particularly difficult with infectious disease targets, where different strains or serotypes within the same bacterial species or the same virus may have widely varying sequences. This greatly complicates attempts to make a single assay capable of detecting all important relatives. One approach to deal with this problem is to introduce mixed base sites, where synthetic oligonucleotides are made having a mixture of bases inserted at a specific position so that within the population of oligos some subpopulation will always have a perfect match to the target. This strategy works well if the amount of variability is limited. However, as target variability increases, then the number of different sequence variants of the primers or probes that exist in the mixed population can become very high with the result that the actual molar concentration of perfect match primers is too low to support PCR without having to employ unacceptably high concentrations of oligonucleotide.

A solution to reduce complexity of the oligonucleotides that will bind multiple targets is to incorporate modified bases which can pair with more than one of the natural bases, or at least are minimally destabilizing when paired with different natural bases. An excellent review of this topic was published by Loakes in 2001 (Loakes, 2001). The first 'inert' base used for this purpose was inosine, a naturally occurring hypoxanthine variant. Inosine is commonly used today, but is an inferior 'universal base' because it has very different effects on T_m depending on which base it is mispaired with (Martin *et al.*, 1985). Inosine most stably pairs with cytosine and behaves as a guanine analogue that simply does not have as strong a mismatch thermodynamic cost when paired with other bases as does guanine. The approximate rank order of stability for inosine base paring is C > A > G > T (Kawase *et al.*, 1986). Unlike other universal bases that may show more uniform thermodynamic effects when paired with any native base, inosine has the advantage that it can be inserted in primers and supports template function. When present in a nucleic acid which is used as a template in DNA synthesis, inosine directs incorporation of cytosine, consistent with it being considered a 'G analogue'.

The first artificial base that seemed to have true 'universal base' properties was 3-nitropyrrole; however, this modification was fairly destabilizing when used in primers and was quickly replace with 5-nitroindole (5-NI) (Nichols *et al.*, 1994; Loakes *et al.*, 1995). 5-NI destabilizes short duplexes by 2–5°C per insertion depending on location but shows reasonably little difference between templates having different bases mispaired with it. 5-NI is a good choice to use in hybridization probes as a universal base; however it does not function well in primers. Most polymerases do not recognize 5-NI as a base and chain termination usually occurs when 5-NI is encountered in a template and thus 5-NI containing oligonucleotides do not support PCR.

Additional compounds have been identified that behave as universal purine or pyrimidine bases. The synthetic base analogue 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one (called 'p') is a pyrimidine replacement that will pair with the purine bases A or G. The synthetic base analogue N6-methoxy-2,6-diaminopurine (called 'k') is a purine analogue that will pair with the pyrimidine bases C or T (Bergstrom *et al.*, 1997). These bases are particularly useful as they support template function when used in a primer. The 'p' base directs incorporation of dA (60%) and dG (40%) and the 'k' base directs incorporation of dT (87%) and dC (13%) (Hill *et al.*, 1998). A variety of other base analogues have been synthesized and described in the literature; however, the set described here are commercially available and can be obtained from a variety of sources.

Conclusions

A wide variety of chemical modification can be introduced into synthetic oligonucleotides that alter their properties as PCR primers or probes. Modifications can be made to any part of the nucleic acid molecule, including the nucleobases, the internucleotide linkages, and the ribose ring. The modifications can increase binding affinity (T_m) and are commonly used to shorten primers and probes, improving specificity when this is desired. Conversely, modified bases are available which decrease specificity and are permissive for mismatch pairing. Modifications can also be used to prevent formation of primer-dimers by interfering with the ability of the oligonucleotides to prime DNA synthesis or interact with each other. All of the modifications discussed in this chapter are easily obtained from commercial sources. New modifiers and applications for their use are likely to continue to be developed in the future.

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