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Characterization of Aberrant Melting Peaks in Unlabeled Probe Assays

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An unlabeled probe assay relies on a double-stranded DNA-binding dye to detect and verify target based on amplicon and probe melting. During the development and application of unlabeled probe assays, aberrant melting peaks are sometimes observed that may interfere with assay interpretation. In this report, we investigated the origin of aberrant melting profiles observed in an unlabeled probe assay for exon 10 of the *RET* gene. It was determined that incomplete 3′ blocking of the unlabeled probe allowed polymerase-mediated probe extension resulting in extension products that generated the aberrant melting profiles. This report further examined the blocking ability of the 3′ modifications C3 spacer, amino-modified C6, phosphate, inverted dT, and single 3′ nucleotide mismatches in unlabeled probe experiments. Although no 3′ blocking modifications in these experiments were 100% effective, the amino-modified C6, inverted dT, and C3 spacer provided the best blocking efficiencies (1% or less unblocked), phosphate was not as effective of a block (up to 2% unblocked), and single nucleotide mismatches should be avoided as a 3′ blocking modification. *(J Mol Diagn 2007, 9:290–296; DOI: 10.2353/jmoldx.2007.060139)*

Unlabeled probe and amplicon melting analysis have been used for detecting single nucleotide polymorphisms and small deletions in the human *RET*, cystic fibrosis, and factor V genes, among others.[1–4] The method uses a 3′ blocked unlabeled probe in combination with the double-stranded (ds)DNA-binding dye LCGreen Plus (Idaho Technology, Salt Lake City, UT) for detection and confirmation of target.[5,6] Unlabeled probes possess no fluorescent moiety; rather, they produce a signature melting profile when used with a dsDNA-binding dye by annealing with single-stranded (ss)DNA produced by asymmetric polymerase chain reaction (PCR). Unlike other detection methods that only interrogate the region of complementarity covered by the probe, an unlabeled probe system can detect polymorphisms outside the probe region by analysis of dsDNA amplicon melting.[7,8]

The high sensitivity and low specificity of a dsDNA-binding dye requires unambiguous melting profiles to accurately identify insertions/deletions or polymorphisms. During unlabeled probe optimization, the majority of aberrant melting peaks are primer dimers or nonspecific amplicons, which can be reduced or eliminated by altering annealing temperatures or by redesigning primer sequences. Once optimized, an unlabeled probe assay produces two melting peaks: the melting of the dsDNA amplicon and the melting of the probe off the ssDNA product derived by asymmetric PCR. During the course of implementing a previously optimized unlabeled probe assay, we have occasionally observed aberrant melting profiles when using older probes or when replacing a proven probe with a newly synthesized probe of identical sequence. Removing the unlabeled probe from these PCR reactions eliminated these aberrant melting profiles, suggesting that the quality of the probe is important for optimal results. It was hypothesized that the aberrant melting profiles might be caused by probe degradation, or probe extension by DNA polymerase because of incomplete 3′ blocking.

In the current study, aberrant melting profiles observed in an unlabeled probe assay specific for exon 10 of the tyrosine kinase receptor gene *RET* were investigated. Mutations in exon 10 lead to multiple endocrine neoplasia type-2 or familial medullary thyroid carcinomas.[9,10] This report determined the identity of aberrant amplification products and melting profiles, and assessed the effectiveness of common 3′ blocking modifications [phosphate, C3 spacer, amino-C6, dideoxynucleotides, and inverted dT (InvT)], used to prevent DNA polymerase extension.[11]

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Table 1. Oligonucleotides Used for Sequencing, Real-Time PCR Amplification, and Detection

Name	Sequence 5′ to 3′	3′ modification
10F	5′-GGGCAGCATTGTTGGGGGAC-3′	None
10R	5′-TGGTGGTCCCGGCCGCCA-3′	None
M13 for −20	5′-GTAAAACGACGGCCAGT-3′	None
Probe-OH	5′-GGAGAAGTACTTCTACGAGCCCGAAGACATC-3′	None
Probe-mis	5′-GGAGAAGTACTTCTACGAGCCCGAAGACATC *G*-3′	G mismatch
Probe-phos	5′-GGAGAAGTACTTCTACGAGCCCGAAGACATC-3′	Phosphate
Probe-C3	5′-GGAGAAGTACTTCTACGAGCCCGAAGACATC-3′	C3 spacer
Probe-C6	5′-GGAGAAGTACTTCTACGAGCCCGAAGACATC-3′	C6 amino
Probe-InvT	5′-GGAGAAGTACTTCTACGAGCCCGAAGACATC-3′	Reverse T

Unmodified oligonucleotides were used as primers and oligonucleotides with 3′ modifications were used as probes. The underlined As in the probe sequences represent mismatches in the probe sequence.

Materials and Methods

Human Genomic DNA Sample

A previously characterized wild-type human genomic DNA sample (hgDNA) was used for these experiments. The clinical sample used was residual and deidentified following the Health Insurance Portability and Accountability Act of 1996 and was used in accordance with University of Utah Institutional Review Board protocol number 7275, which covers research conducted by ARUP Laboratories.

Primers and Probes

All oligonucleotides were manufactured by Integrated DNA Technologies (Coralville, IA). The sequences are summarized in Table 1. ReadyMade primer M13 Forward-20 was used for sequencing of unknown amplification products. Primers used for real-time amplification include 10F, 10R, and probe-OH. Oligonucleotide probes used for unlabeled probe experiments were designed with two internal mutations, represented in Table 1 as underlined As, to differentiate mutant probe extension. The probes possessed the following 3′ blocking modifications: a single nucleotide mismatch (probe-mis), a phosphate (probe-phos), a C3 spacer (probe-C3), an amino-modified C6 (probe-C6), and an inverted dT (probe-InvT). All oligonucleotides were desalted with no postsynthesis purification except for ReadyMade primer, which was polyacrylamide gel electrophoresis purified.

All probes used for blocking efficiency experiments were resuspended on day 0 in RNase/DNase-free water (Quality Biological, Gaithersburg, MD) and adjusted to a 200-μmol/L concentration based on OD_{260} measurements. Each primer was split into two 150-μl aliquots and processed by adding 150 μl of water or 150 μl of 2× TE (20 mmol/L Tris-Cl, pH 8.0, and 2 mmol/L ethylenediaminetetraacetic acid) to obtain a final concentration of 100 μmol/L in water or 1× TE. Oligonucleotides were further split into 20-μl aliquots for subsequent experiments.

PCR Reactions and Melting Analysis

PCR reactions were performed in a LightCycler (Roche Diagnostics, Indianapolis, IN). Each reaction consisted of 1× Roche FastStart DNA hybridization mix (includes dNTPs, dUTP, and 1 mmol/L $MgCl_2$), 0.028 μmol/L forward primer 10F, 0.25 μmol/L reverse primer 10R, 0.5 μmol/L probe, 1× LCGreen Plus, an additional 2 mmol/L $MgCl_2$, 10 U/ml uracil DNA glycosylase (Roche), and ~30 ng (~14 zmol) of hgDNA in a 10-μl reaction volume. Cycling conditions were performed using the following protocol at a transition rate of 20°C per second: (55°C $^{(10:00)}$ + 95°C $^{(10:00)}$ + [95°C $^{(0:01)}$ + 62°C $^{(0:01)}$ + 72°C $^{(0:10)}$] × 55 cycles + 95°C $^{(0:00)}$ + 40°C $^{(0:20)}$ + 50°C → 95°C at 0.5°C per second + 40°C $^{(0:00)}$]), where $^{(0:00)}$ is equal to $^{(\text{minutes:seconds})}$. Melting analysis after amplification was also evaluated using a high-resolution melting analysis platform (HR-1; Idaho Technology, Salt Lake City, UT) with the following melting protocol: 4°C $^{(\geq 10:00)}$ + 60°C → 95°C at 0.3°C per second.

Cloning of PCR Products

PCR products were amplified using the previously described cycling conditions with Roche FastStart DNA polymerase without dUTP. The PCR products were ethanol-precipitated, run on a 4% agarose gel, bands excised, and gel-purified using a QIAQuick spin gel purification kit (Qiagen, Valencia, CA). The gel-purified bands were cloned into pCRII TOPO TA (Invitrogen, Carlsbad, CA) by adding 4 μl of gel-purified amplicon to 1 μl of pCRII TOPO TA plasmid and 1 μl of 6× salt solution (1.2 mol/L NaCl and 0.06 mol/L $MgCl_2$) and allowed to incubate at room temperature for 30 minutes. The cloning solution was transformed into chemically competent TOP10 cells (Invitrogen), following the manufacturer's protocol, plated onto LB-AMP agar plates (50 μg/ml ampicillin), and incubated at 37°C overnight. Single colonies were picked from plates and grown up in 50 μg/ml LB-AMP overnight. Plasmid DNA was isolated using a Qiagen QIAprep spin miniprep kit before sequencing.

Estimation of Blocking Efficiency

The probe blocked with an amino-C6 modification showed limited detectable extension by real-time PCR and was used as a theoretical 100% blocked primer in primer-mixing experiments. Ratios of unblocked probe-OH to probe-C6 were used in real-time PCR reac-