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TECHNICAL ADVANCE

Suppression of Wild-Type Amplification by Selectivity Enhancing Agents in PCR Assays that Utilize SuperSelective Primers for the Detection of Rare Somatic Mutations

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In PCR assays designed to detect rare somatic mutations, SuperSelective primers, by virtue of their short 3′-foot sequences, selectively initiate synthesis on mutant DNA target fragments, while suppressing the synthesis of related wild-type fragments, and the resulting threshold cycle reflects the quantity of mutant targets present. However, when there are ≤10 mutant target fragments in a sample, the threshold cycle that is observed occurs so late that it can be confused with the threshold cycle that arises from samples that contain only abundant related wild-type fragments. We report here that the inclusion of the selectivity enhancing agents tetramethylammonium chloride or *bis*-tetramethylammonium oxalate in SuperSelective PCR assays substantially suppresses the amplification of related wild-type fragments. As a result of this selective suppression, assay sensitivity is increased to such an extent that multiplex PCR assays can be performed in which it is highly unlikely that there will be a false-positive or false-negative result. This advance provides a foundation for the development of rapid, low-cost, multiplex PCR assays for noninvasively assessing the presence of relevant mutations in cancer patients, thereby enabling individually appropriate therapy. *(J Mol Diagn 2018, 20: 415–427; https://doi.org/10.1016/j.jmoldx.2018.03.004)*

Cancer cells are characterized by the occurrence of somatic mutations that change the character of the cells. To effectively treat a patient's cancer, therapeutic agents are needed that affect the cancer cells but do not substantially affect normal cells. Particular mutations in cancer cells alter their encoded protein in a manner that enables effective treatment, whereas other mutations alter their encoded protein in a manner that indicates that particular therapies will be ineffective against those cells. It is thus essential to assess the mutations that are present in a patient's cancer cells to select an individually appropriate therapy. Moreover, because new mutations arise during the course of treatment, it is desirable to frequently monitor the occurrence and abundance of relevant somatic mutations in an individual to assess the effectiveness of

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treatment and to alter that treatment as soon as possible if new relevant mutations arise.

Because frequent testing is desirable, there is great interest in developing techniques that can detect rare somatic mutations by using a relatively noninvasive source, such as a routine blood sample.[1,2] Reproducing cells, such as cancer cells, die frequently by apoptosis and by necrosis, after which time their nucleic acids are digested into small pieces that persist transiently in blood plasma, including fragments of the genomic DNA of the cancer cells that possess the somatic mutations that need to be detected. The problem posed by this approach is that most of the DNA fragments in blood plasma come from normal cells throughout the body, and only a small fraction of the DNA fragments come from the cancer cells. Current techniques for assessing the presence of these rare mutant DNA fragments and for determining the abundance of each mutation include next-generation sequence analysis and droplet digital PCR.[3–5] However, next-generation sequencing, which can simultaneously detect many different mutations, is time consuming, labor intensive, expensive, and lacks sufficient sensitivity to detect rare mutations. Droplet digital PCR, although potentially quite sensitive, is limited to the simultaneous detection of only a few different mutations. It is thus desirable to develop simple, low-cost, extraordinarily sensitive, multiplex, real-time PCR assays that rapidly assess the abundance of relevant mutations, using widely available spectrofluorometric thermal cyclers.

We recently designed PCR primers that selectively initiate synthesis on mutant target sequences, but that are highly unlikely to initiate synthesis on related wild-type sequences, even when the only difference between the mutant and the wild type is a single-nucleotide polymorphism.[6] These SuperSelective primers consist of the following four sequence segments (Figure 1A), described here in order from 5′ to 3′: i) a unique 5′-tag sequence that is not complementary to the sequence of the DNA target fragment and that is chosen so that if the SuperSelective primer initiates synthesis, the resulting amplicons will possess a unique identifying sequence tag; ii) an anchor sequence that is sufficiently long to ensure that under PCR annealing conditions the primer will hybridize strongly to its DNA target fragment; iii) a unique bridge sequence that is chosen to not be complementary to the corresponding intervening sequence in the DNA target fragment; and iv) a short 3′-foot sequence that is perfectly complementary to the corresponding sequence in the mutant DNA target fragments but that mismatches the corresponding sequence in the related wild-type DNA fragments. Because the bridge sequence in the primer is not complementary to the intervening sequence in the DNA target fragment, the hybrid formed by the anchor sequence and the hybrid formed by the short foot sequence are separated from each other by a single-stranded bubble, which effectively separates the primer's polymerization initiation function from the primer's target recognition function.

The main purpose of this design is to cause the initiation of amplicon synthesis to be dependent on the transitory presence of the hybrid formed by the foot sequence. These foot hybrids are so short that, under the annealing conditions of a PCR assay, they are quite weak, and they easily dissociate. Once they dissociate, the relatively long sequences of the bubble tend to keep the feet from re-hybridizing. Therefore, at any given moment during the annealing stages of the PCR assay, only a small fraction of the target sequences are actually hybridized to a foot sequence; and the relatively stronger perfectly complementary hybrids formed by the foot sequences and the mutant target sequences are considerably more abundant than the shorter, mismatched hybrids formed by the foot sequences and the wild-type target sequences. Moreover, these foot hybrids are so weak that they exist for only a fraction of a second before they dissociate[7]; and though the perfectly complementary mutant foot hybrids might persist for a few hundred milliseconds, the much weaker mismatched wild-type foot hybrids persist for only a few milliseconds. Consequently, the likelihood of a foot hybrid encountering a DNA polymerase before it dissociates is substantially higher for mutant foot hybrids than it is for wild-type foot hybrids. Moreover, SuperSelective primers are so specific that they ignore all of the genomic DNA fragments in a sample except the DNA fragments of the intended target gene. However, once amplification is initiated by the incorporation of a SuperSelective primer into an amplicon, subsequent synthesis is highly efficient, because the entire sequence of the SuperSelective primer initiates subsequent rounds of synthesis. Previously, it was found, in real-time PCR assays that use SuperSelective primers, that as few as 10 mutant DNA fragments in a sample that also includes 1,000,000 closely related wild-type DNA fragments give rise to a real-time amplification signal that occurs, on average, a few thermal cycles earlier than a sample that contains only 1,000,000 closely related wild-type DNA fragments.[6]

However, because a signal does arise late in the reaction from the abundant related wild-type DNA fragments present in a sample, there is a chance that the signal arising from a small number of mutant target DNA fragments will also occur late in the reaction and could be confused with the signal arising from the abundant related wild-type DNA fragments (ie, the result will be a false-negative conclusion). Conversely, the signal from a pure wild-type sample could be confused with the signal expected to arise from a sample that contains a small number of mutant target DNA fragments (ie, the result will be a false-positive conclusion). Such misidentifications are more likely to occur when the length of the foot sequence is quite small, because the shorter the foot sequence, and consequently the weaker and shorter-lived the foot hybrids, the lower is the probability that exponential synthesis will begin on a template DNA fragment in a given thermal cycle; as a result, the observed threshold cycle (C_T) will vary. The result of this realization is that the inherent reliable sensitivity of SuperSelective PCR assays is currently limited to approximately 10 to 20 mutant target DNA fragments in the sample being tested.

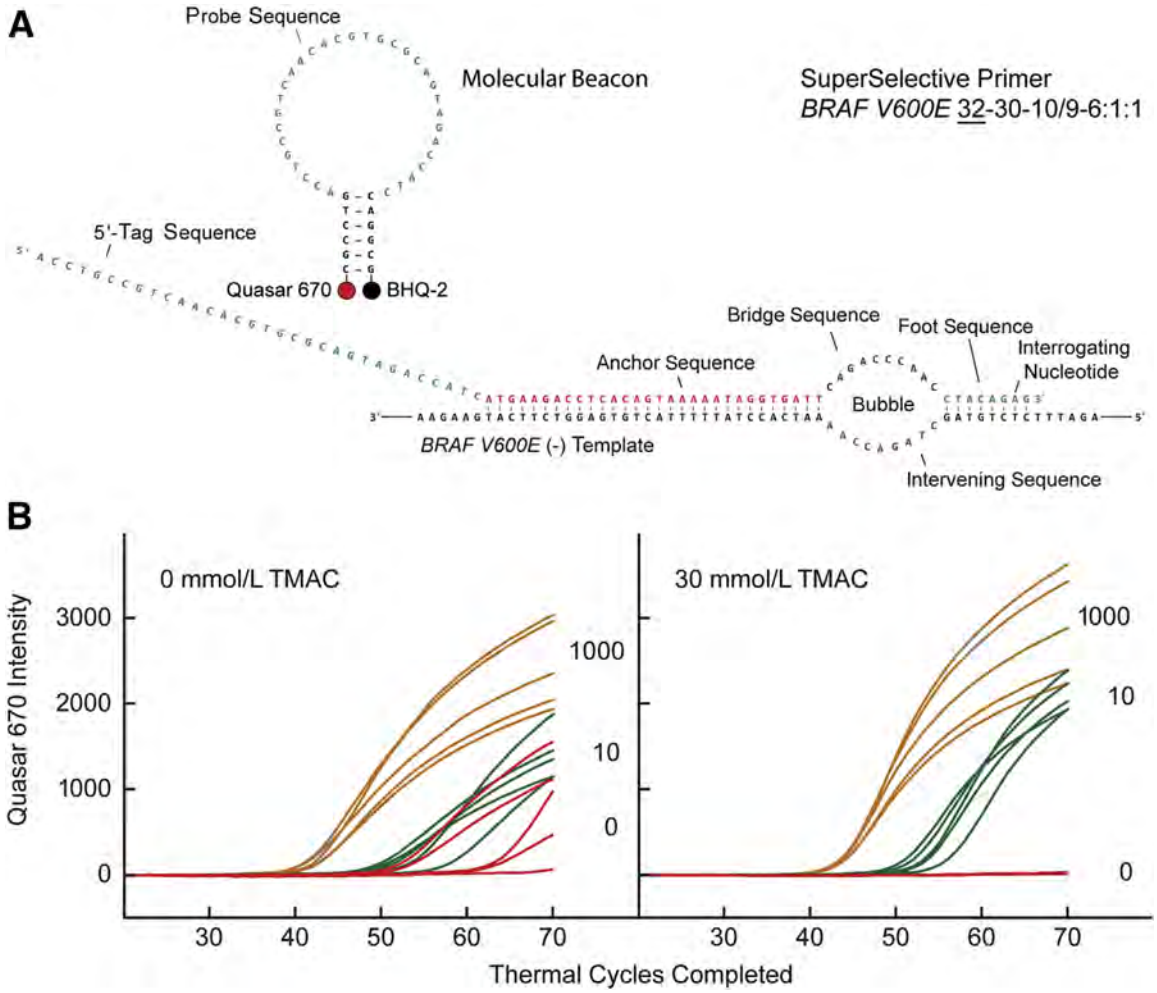

Figure 1 PCR assays that use SuperSelective primers and contain a selectivity enhancing agent, such as tetramethylammonium chloride (TMAC), can detect rare DNA fragments that possess somatic mutations, without interference from extremely abundant DNA fragments from closely related wild types. **A:** Structure of a SuperSelective primer bound to its mutant target fragment, and structure of the molecular beacon that will light up the resulting amplicons. This SuperSelective primer is designated *BRAF V600E* 32-30-10/9-6:1:1, which indicates that it is designed to amplify the *BRAF V600E* mutant sequence; its 5′-tag sequence (**green letters**) is 32 nucleotides long; its anchor sequence (**red letters**) is 30 nucleotides long, its bridge sequence (**blue letters**) is 10 nucleotides long and occurs opposite an intervening sequence (**brown letters**) that is 9 nucleotides long, both of which, along with the 2 bp on either end, form a bubble with a circumference of 23 nucleotides; and its foot sequence is 8 nucleotides long, consisting of 7 nucleotides (**green letters**) that are perfectly complementary to both the mutant sequence and the related wild-type sequence, and an interrogating nucleotide (**purple letter**) that is complementary to the corresponding nucleotide in the mutant sequence, but that mismatches the corresponding nucleotide in the wild-type sequence. **B:** PCR assays that use SuperSelective primer *BRAF V600E* 32-30-10/9-6:1:1 in the presence and absence of TMAC. All of the reactions contained 40,000 *BRAF* wild-type templates and either 1000 *BRAF V600E* mutant templates (**brown lines**), 10 *BRAF V600E* mutant templates (**green lines**), or no *BRAF V600E* mutant templates (**red lines**). Signals from all five replicates of the samples that contained only *BRAF* wild-type templates were completely suppressed by the presence of TMAC.

We therefore explored the inclusion of various Hofmeister salts in the PCR assays in the hope that one of them might enhance the selectivity of the resulting amplification, either by accelerating the signal generated from the rare mutant DNA fragments without substantially accelerating the signal generated from the abundant closely related wild-type DNA fragments or by delaying the signal generated from the abundant closely related wild-type DNA fragments, without substantially delaying the signal generated from the rare mutant target DNA fragments. It was found that the Hofmeister salts tetramethylammonium chloride (TMAC) and *bis*-tetramethylammonium oxalate (TMAO), both of which were previously known to increase hybridization specificity,[8–10] also increase selectivity in SuperSelective primer-based PCR assays, virtually eliminating the signal from abundant closely related wild-type DNA fragments in a sample. As a consequence, the resulting SuperSelective PCR assays are extremely selective and sensitive, and they almost never result in a false-positive or false-negative signal.

The experiments described in this article (most of which explore the effect of TMAC) show that when an appropriate concentration of TMAC is present in SuperSelective PCR assays, the amplification of the wild-type fragments is substantially delayed. Furthermore, the results show that the optimal TMAC concentration depends on the design of the

Table 1 Primers and Molecular Beacons

Primer/molecular beacon	Sequence
Primers for Figure 1	
BRAF V600E 32-30-10/9-6:1:1	5′-ACCTGCCGTCAACACGTGCGCAGTAGACCATCATGAAGACCTCACAGTAAAAATAGGTGATT **CAGACCCAAC**CTACAG*A*G-3′
BRAF Reverse Primer 1	5′-ATCAGTGGAAAAATAGCCTCAATTCTTACCATCC-3′
Primers for Figures 2, 3, and 4	
BRAF V600E 32-24-14/14-6:1:1	5′-ACCTGCCGTCAACACGTGCGCAGTAGACCATCCAGACAACTGTTCAAACTGATGGG **CAAACACATCATCC**GATTTC*T*C-3′
BRAF V600E 32-24-14/14-8:1:1	5′-ACCTGCCGTCAACACGTGCGCAGTAGACCATCTCCAGACAACTGTTCAAACTGATG **TTCAAACACAATCA**GAGATTTC*T*C-3′
BRAF V600E 32-24-18/18-8:1:1	5′-ACCTGCCGTCAACACGTGCGCAGTAGACCATCTGGATCCAGACAACTGTTCAAACT **TTCAAACGCATACAATCA**GAGATTTC*T*C-3′
BRAF V600E 32-24-10/14-8:1:1	5′-ACCTGCCGTCAACACGTGCGCAGTAGACCATCTCCAGACAACTGTTCAAACTGATG **TTCACCATCA**GAGATTTC*T*C-3′
BRAF V600E 32-24-14/14-9:1:0	5′-ACCTGCCGTCAACACGTGCGCAGTAGACCATCATCCAGACAACTGTTCAAACTGAT **TCAAACACAATACC**CGAGATTTC*T*-3′
BRAF Reverse Primer 2	5′-ATAGGTGATTTTGGTCTAGC-3′
BRAF Reverse Primer 3	5′-TTCTTCATGAAGACCTCACA-3′
Primers for Figures 5, 6, 7, and 8	
KRAS G12D 32-28-19/10-8:1:0	5′-ACGTGCCCTCAATACGAGCCCCCTTCACCAACGGCCTGCTGAAAATGACTGAATATAAAC **ACACAGTCTGAGCCCACTC**TGGAGCTG*A*-3′
KRAS G12A 32-28-14/12-8:1:0	5′-ACCTGCCGTCAACACGTGCGCAGTAGACCATCAAGGCCTGCTGAAAATGACTGAATATAA **CAGACGATGCTACC**TGGAGCTG*C*-3′
KRAS wild-type 32-28-14/12-9:0:0	5′-ACCTGCCGTCAACACGTGCGCAGTAGACCATCAAGGCCTGCTGAAAATGACTGAATATAA **CAGACGATGCTACC**TGGAGCTG*G*-3′
KRAS Reverse Primer	5′-GATCATATTCGTCCACAAAATG-3′
Molecular beacons	
Q670 Molecular Beacon	Q670-5′-CGCCTGACCTGCCGTCAACACGTGCGCAGTAGACCATCCAGGCG-3′-BHQ2
FAM Molecular Beacon	FAM-5′-CGCCTGACGTGCCCTCAATACGAGCCCCCTTCACCAACCAGGCG-3′-BHQ1

The template strand for *BRAF V600E* 32-30-10/9-6:1:1 is the complement of the template strand for all other *BRAF* SuperSelective primers. For the SuperSelective primers, 5′-tag sequences are underlined; bridge sequences are shown in bold letters; and the interrogating nucleotide is shown as an underlined italicized letter. For the molecular beacons, the probe sequence is underlined. The molecular beacons are labeled with Quasar 670 (Q670), fluorescein (FAM), Black Hole Quencher 1 (BHQ1), and Black Hole Quencher 2 (BHQ2).

SuperSelective primers that are used. TMAC is shown to be most effective when the interrogating nucleotide of the SuperSelective primer is located at the 3′ end of its foot sequence. Moreover, the optimal TMAC concentration depends on the length of the foot sequence and the size of the bubble that the SuperSelective primer forms with its target DNA fragment. Longer feet and smaller bubbles require more TMAC to achieve an optimal effect. Significantly, in the presence of an appropriate concentration of TMAC, the use of SuperSelective primers possessing somewhat longer feet (9 or 10 nucleotides in length) and a 3′-terminal interrogating nucleotide substantially reduced the variability in the C_T values that were obtained when the number of mutant target fragments in a sample was $\leq$10. Consequently, multiplex, real-time PCR assays that use SuperSelective primers in the presence of TMAC are extraordinarily sensitive, able to detect and quantitate a small number of each mutant target fragment, and are unlikely to produce false-positive or false-negative C_T values. Furthermore, the isolation of target DNA fragments from human plasma does not reduce the sensitivity of SuperSelective PCR assays performed in the presence of TMAC. These results suggest that it is now worthwhile to explore the combined use of SuperSelective primers and selectivity enhancing agents such as TMAC or TMAO in PCR assays that use patient-derived clinical samples.

Materials and Methods

Primers and Molecular Beacons

SuperSelective primer sequences were designed with the aid of the DINAMelt web server[11] and the OligoAnalyzer 3.1 computer program (Integrated DNA Technologies, Coralville, IA) to ensure that under assay conditions they are unlikely to form internal hairpin structures and are unlikely to form self-dimers or heterodimers with the conventional reverse primers. The primers were purchased from Integrated DNA Technologies, and differently colored molecular beacon probes for detecting the amplicons were designed in our laboratory and purchased from LGC Biosearch Technologies (Petaluma, CA). The sequences of the primers and molecular beacons used for the experiments are listed in Table 1.

DNA Templates

Plasmids that contained *BRAF* sequences (either the *V600E* mutant sequence or the wild-type sequence) were prepared by inserting 200-bp gene fragments into pIDTSmart Amp vectors (Integrated DNA Technologies). Plasmids that contained *KRAS* sequences (either the *G12D* mutant sequence, the *G12A* mutant sequence, or the wild-type sequence) were similarly prepared by inserting 390-bp gene fragments into pUCIDT Amp vectors (Integrated DNA Technologies). The sequence of each of these plasmids was confirmed by sequence analysis. Each plasmid was digested by incubation with restriction endonuclease ScaI (New England Biolabs, Ipswich, MA). The 20-μL digestion mixtures contained 4 μg of plasmid DNA, 10 U of ScaI, 100 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, and 50 mmol/L Tris-HCl (pH 7.9). These reactions were incubated for 120 minutes at 37°C, followed by incubation for 20 minutes at 80°C to inactivate the endonuclease.

Digested *KRAS G12A* mutant plasmid DNA was spiked into normal single-donor human plasma (IPLA-N-S; Innovative Research, Novi, MI). The plasma was first centrifuged at 5000 rpm for 5 minutes to remove residual cellular debris, and the digested plasmids were then added, after which total DNA was isolated from the plasma by using a Quick-cfDNA Serum and Plasma Kit (Zymo Research, Irvine, CA), according to the vendor's instructions. The isolated DNA was then placed in a vacuum concentrator to remove residual solvent, and the dried DNA was then resuspended in water.

Human genomic DNA homozygous for the *BRAF* wild-type gene was isolated from cell line H1975 (CRL-5908; American Type Culture Collection, Manassas VA), and human genomic DNA homozygous for the *BRAF V600E* mutation was isolated from cell line A375 (CRL-1619; ATCC). Each of these DNAs was digested by incubation with restriction endonuclease HaeIII (New England Biolabs). The 20-μL digestion mixtures contained 2 μg of mutant or wild-type DNA, 10 U of HaeIII, 50 mmol/L potassium acetate, 10 mmol/L magnesium acetate, 2 μg bovine serum albumin, and 20 mmol/L Tris-acetate (pH 7.9). These reactions were incubated for 120 minutes at 37°C, followed by incubation for 20 minutes at 80°C to inactivate the endonuclease.

PCR Assay Design

In SuperSelective PCR assays, there is a different SuperSelective primer for each mutation that is to be detected, and each of these SuperSelective primers possesses a unique 5′-tag sequence. The amplification of each mutant sequence is followed in real time by the binding of a differently colored molecular beacon probe,[12,13] which only binds to the complement of one of the different 5′-tag sequences that is present in the resulting (−) amplicons. In these reactions, the concentration of each SuperSelective primer is limited, whereas the concentration of each conventional reverse primer is considerably higher.[14,15] Once the SuperSelective primers for a particular mutation are used up, limiting the number of (+) amplicons, the molecular beacon probes do not have substantial competition for binding to the excess (−) amplicons, and they generate a bright, uniquely colored signal for each mutation that is present in the sample. The intensity of each colored signal is measured during the annealing stage of each thermal cycle, and the number of thermal cycles that occur before the intensity of each colored signal reaches a predetermined level (the threshold cycle, C_T) is inversely linearly proportional to the logarithm of the number of mutant DNA fragments originally present in the sample.[16,17]

PCR Assay Components

The PCR assays were performed in 30-μL volumes that contained 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 250 μmol/L dATP, 250 μmol/L dCTP, 250 μmol/L dGTP, 250 μmol/L dTTP, 20 mmol/L Tris-HCl (pH 8.3), 1.5 U of Platinum *Taq* DNA polymerase (Thermo Fisher Scientific, Waltham, MA), 0.5% of the nonionic surfactant Tween 20 (Sigma-Aldrich, St. Louis, MO), and different concentrations of TMAC (Sigma-Aldrich) or TMAO (Sachem, Austin, TX). The amplifications were performed in 200-μL white polypropylene PCR tubes (USA Scientific, Ocala, FL) in an iQ5 spectrofluorometric thermal cycler or in a CFX96 Touch Real-Time PCR Detection System (both manufactured by Bio-Rad Laboratories, Hercules, CA). The reaction mixtures were incubated for 2 minutes at 95°C to activate the DNA polymerase, followed by 60, 65, or 70 thermal cycles that consisted of 95°C denaturation for 15 seconds, 60°C annealing for 20 seconds, and 72°C chain elongation for 20 seconds. Molecular beacon fluorescence intensity was monitored during the annealing stage of each thermal cycle.

Monoplex reactions performed with Platinum *Taq* DNA polymerase contained 60 nmol/L of one of the *BRAF V600E* SuperSelective primers or 60 nmol/L *KRAS G12A* SuperSelective primer, 500 nmol/L of an appropriate conventional reverse primer, and 300 nmol/L of the Q670 molecular beacon. Duplex reactions performed with Platinum *Taq* DNA polymerase contained 60 nmol/L of the *KRAS G12D* SuperSelective primer, 60 nmol/L *KRAS G12A* SuperSelective primer, 1000 nmol/L *KRAS* conventional reverse primer, 300 nmol/L Q670 Molecular Beacon, and 300 nmol/L FAM Molecular Beacon.

DNA Polymerases Other than Platinum *Taq*

SuperSelective PCR assays were also performed with DNA polymerases other than Platinum *Taq* DNA polymerase. These 30-μL reactions contained 60 nmol/L *KRAS G12A* SuperSelective primer, 500 nmol/L *KRAS* conventional reverse primer, 300 nmol/L Q670 Molecular Beacon, and

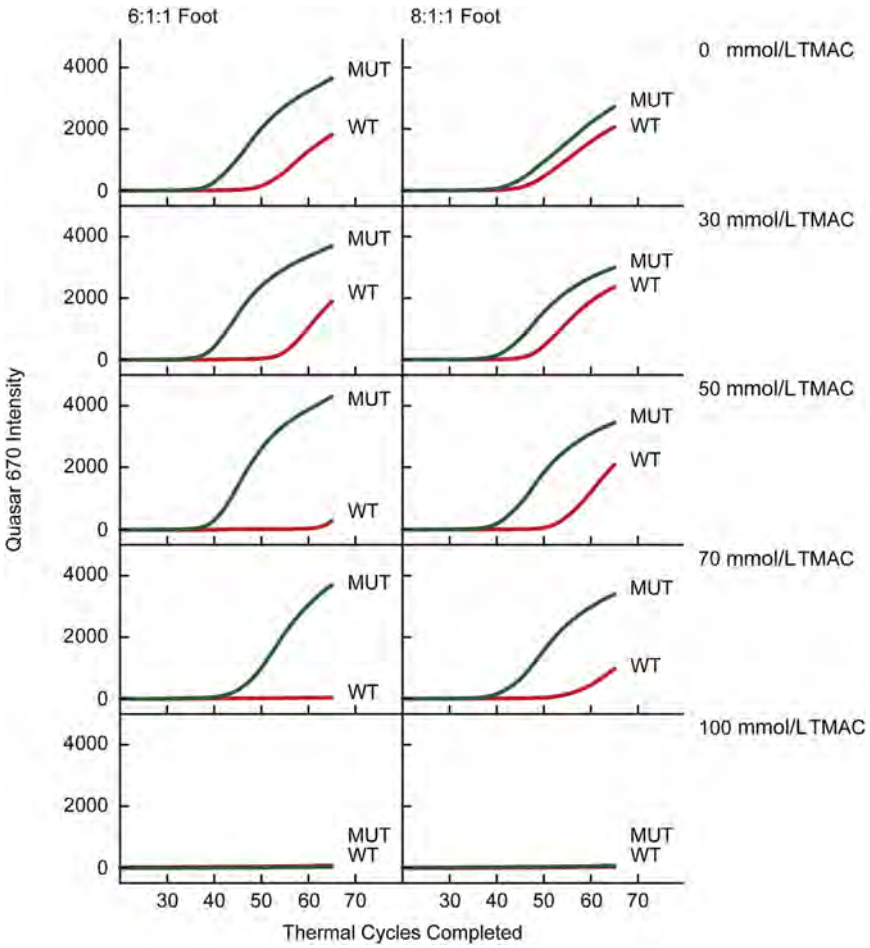

Figure 2 Dependence of selectivity enhancement on the tetramethylammonium chloride (TMAC) concentration and on the length of the foot hybrid formed by the SuperSelective primer. Ten PCR assays were performed with SuperSelective primer *BRAF V600E* 32-24-14/14-6:1:1 (**left column**), and another 10 PCR assays were performed with SuperSelective primer *BRAF V600E* 32-24-14/14-8:1:1 (**right column**). Five different concentrations of TMAC were used: 0 mmol/L, 30 mmol/L, 50 mmol/L, 70 mmol/L, and 100 mmol/L. Each panel shows the real-time results obtained for two different reactions: a reaction that contained 1000 *BRAF V600E* mutant (MUT) templates in the presence of 1,000,000 *BRAF* wild-type (WT) templates (**green lines**) and another reaction that contained only 1,000,000 *BRAF* wild-type templates (**red lines**).

either 100,000 *KRAS G12A* mutant templates or 100,000 *KRAS* wild-type templates. One set of reactions was performed with 1x iQ Supermix (Bio-Rad), which includes hot-start iTaq polymerase and its own proprietary buffer mixture. A second set of reactions was performed with 1× SensiFAST Probe No-ROX Kit (BioLine, Taunton, MA), which includes its own proprietary DNA polymerase and a proprietary buffer. A third set of reactions was performed with 0.75 U of *Taq* DNA polymerase, recombinant (Thermo Fisher Scientific) in the presence of 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 250 μmol/L dATP, 250 μmol/L dCTP, 250 μmol/L dGTP, 250 μmol/L dTTP, and 20 mmol/L Tris-HCl (pH 8.3). All of these reactions were performed in triplicate, either in the absence or in the presence of 0.5% Tween 20 and 60 mmol/L TMAC.

Results

Effect of Adding TMAC to SuperSelective PCR Assays

To explore the effect of TMAC on selectivity, a series of SuperSelective PCR assays were performed on samples that contained different numbers of mutant DNA target fragments in the presence of a large number of closely related wild-type DNA fragments. Figure 1B shows PCR assays that used SuperSelective primer *BRAF V600E* 32-30-10/9-6:1:1 (which possessed a relatively short foot sequence 8-nucleotides long) and *BRAF* Reverse Primer 1. Fifteen PCR assays were performed in the absence of TMAC. Each of these assays contained 40,000 *BRAF* wild-type templates, the amount of which was chosen to be the maximum number of wild-type fragments expected to occur in plasma from a 10-mL blood sample.[18] Five assays also contained 1000 *BRAF V600E* mutant templates, five also contained 10 *BRAF V600E* mutant templates, and five did not contain any *BRAF V600E* mutant templates. The results indicated that the C_T values of the real-time amplification curves of the samples that contained 1000 *BRAF V600E* mutant templates did not vary much. However, the amplification curves for the samples that contained 10 mutant templates and the amplification curves for samples that contained only wild-type templates had variable C_T values, making it difficult to determine whether a sample actually contains mutant templates or whether a sample does not contain any mutant templates. These 15 PCR assays were repeated in a reaction that also contained 30 mmol/L TMAC. Although there was still some variability in the C_T values seen for the reactions

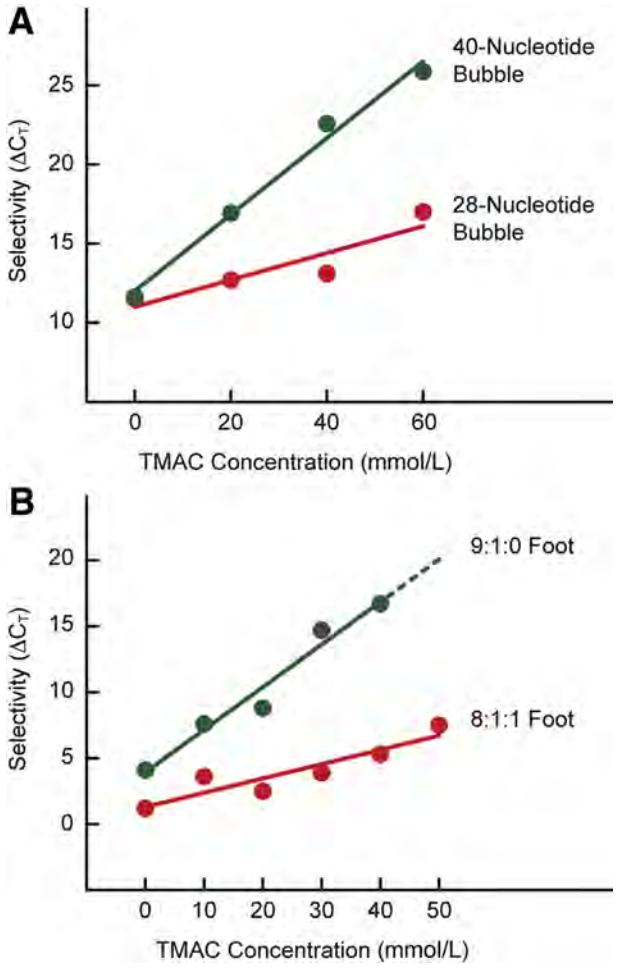

Figure 3 Effect of SuperSelective primer design elements on tetramethylammonium chloride (TMAC) selectivity enhancement. **A:** Dependence of TMAC selectivity enhancement on the circumference of the bubble. The difference in the C_T value obtained in the presence and in the absence of mutant targets (ΔC_T) is plotted as a function of TMAC concentration. Reactions that contained the SuperSelective primer that forms a 40-nucleotide bubble are plotted in green, and reactions that contained the SuperSelective primer that forms a 28-nucleotide bubble are plotted in red. **B:** Dependence of TMAC selectivity enhancement on the location of the interrogating nucleotide. The ΔC_T values for the reactions that contained the SuperSelective primer that possessed a 3′-terminal interrogating nucleotide are plotted in green, and the ΔC_T values for the reactions that contained the SuperSelective primer that possessed a 3′-penultimate interrogating nucleotide are plotted in red.

that contained only 10 mutant templates, none of the five reactions that contained only wild-type templates resulted in the generation of amplicons during the 70 cycles of amplification, suggesting that TMAC serves as an effective selectivity enhancing reagent.

Dependence of TMAC Selectivity Enhancement on the Length of the Foot Sequence

Encouraged by this enhancement in selectivity, the enhancement effect of TMAC was explored in greater detail. Experiments were performed to see the effect of different concentrations of TMAC and to explore whether selectivity enhancement depends on the length of the SuperSelective primer's foot sequence (Figure 2). The results obtained with SuperSelective primer *BRAF V600E* 32-24-14/14-6:1:1 (which has an 8-nucleotide–long foot) were compared to the results obtained with SuperSelective primer *BRAF V600E* 32-24-14/14-8:1:1 (which has a 10-nucleotide–long foot). All reactions contained *BRAF* Reverse Primer 2. In both cases, the interrogating nucleotide was located at the 3′-penultimate position in the foot sequence. In general, increasing the TMAC concentration had little effect on the C_T values of samples that contained 1000 *BRAF V600E* mutant templates in the presence of 1,000,000 *BRAF* wild-type templates. However, as the TMAC concentration increased, the C_T values of the samples that contained only 1,000,000 wild-type templates increased, and when the TMAC concentration was increased to an even higher value, the amplification of the wild type was inhibited to such an extent that no amplicons were detectable above background during the 65 thermal cycles.

A key observation concerning the results of this experiment was that the effect of TMAC depended on the length (and therefore on the strength) of the foot hybrid. A higher TMAC concentration was required when using a SuperSelective primer with a longer foot to obtain the same degree of selectivity enhancement that can be obtained when using a SuperSelective primer with a shorter foot. However, the results also indicated that, depending on the length of the foot, if the concentration of TMAC was too high, the signal from the mutant templates did not occur. Overall, there was an optimal concentration of TMAC to use to obtain the most favorable selectivity, but that optimal concentration depended on the length of the SuperSelective primer foot sequence.

Dependence of TMAC Selectivity Enhancement on the Circumference of the Bubble

Experiments were performed to see whether the selectivity enhancement effect of different concentrations of TMAC depends on the circumference of the bubble formed by the hybridization of the SuperSelective primer to its template fragment. The results obtained with SuperSelective primer *BRAF V600E* 32-24-18/18-8:1:1 (which formed a bubble with a 40-nucleotide circumference) were compared with the results obtained with SuperSelective primer *BRAF V600E* 32-24-10/14-8:1:1 (which formed a bubble with a 28-nucleotide circumference). All reactions contained *BRAF* Reverse Primer 2. Reactions were performed for 60 thermal cycles at different TMAC concentrations. Two reactions were performed for each combination of SuperSelective primer and TMAC concentration: one with 100,000 *BRAF V600E* mutant templates and the other with 100,000 *BRAF* wild-type templates. The C_T values observed for each pair of PCR assays were compared, and the resulting ΔC_T values (difference between the mutant C_T and the wild-type C_T) were plotted in Figure 3A. These results indicate that selectivity is enhanced to a greater degree when the SuperSelective primer forms a bigger bubble.

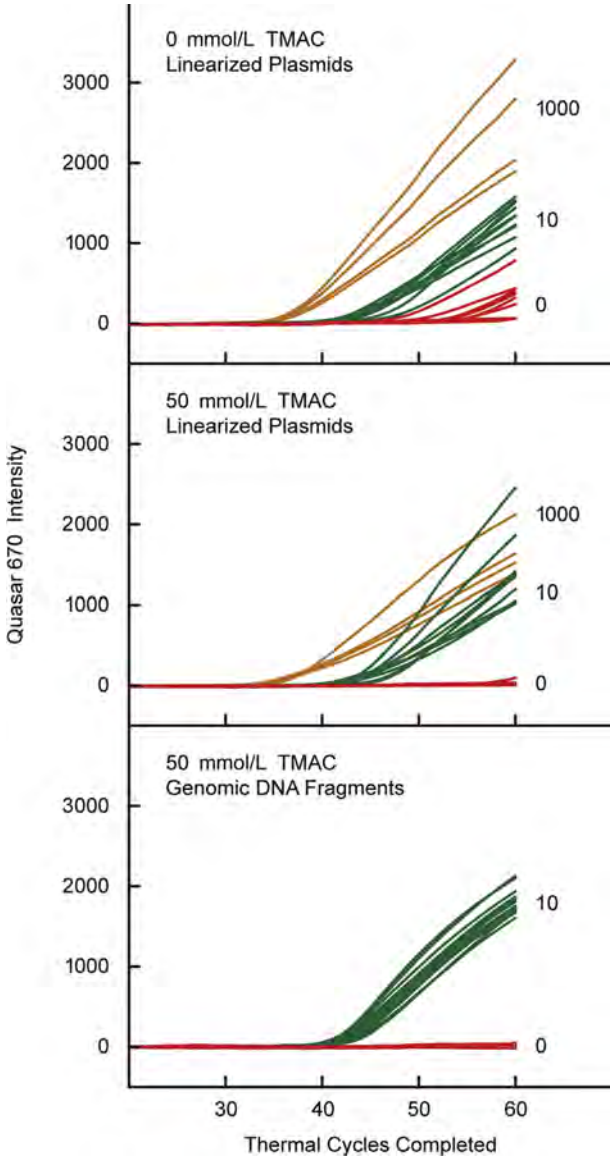

Figure 4 Complete suppression of wild-type signals. Each of the assays shown in the three panels contained SuperSelective primer *BRAF V600E* 32-24-14/14-9:1:0 and conventional *BRAF* Reverse Primer 3. Twenty-four PCR assays, each containing 40,000 *BRAF* wild-type templates, were performed in the absence of tetramethylammonium chloride (TMAC) (**top panel**). Four samples also contained 1000 *BRAF V600E* mutant templates (**brown lines**), 10 samples also contained 10 *BRAF V600E* mutant templates (**green lines**), and 10 samples did not contain any *BRAF V600E* mutant templates (**red lines**). Another set of the same 24 samples were assayed in the presence of 50 mmol/L TMAC (**middle panel**). In addition, 30 samples that contained human genomic DNA were assayed in the presence of 50 mmol/L TMAC (**bottom panel**). Twenty of these samples contained 40,000 copies of human genomic DNA homozygous for the *BRAF* wild-type gene and 10 copies of human genomic DNA homozygous for the *BRAF V600E* gene (**green lines**), and the other 10 samples contained only 40,000 copies of human genomic DNA homozygous for the *BRAF* wild-type gene (**red lines**).

Viewed from a thermodynamic perspective, these results indicate that enhanced selectivity induced by the presence of TMAC is greatest when shorter foot hybrids are formed (lower enthalpy) (Figure 2) and longer bubbles are formed (greater entropy) (Figure 3A). Significantly, these results indicate that the choice of the optimal TMAC concentration to use to suppress the amplification of abundant wild-type templates depends on the design of the SuperSelective primers present in the PCR assay.

Dependence of TMAC Selectivity Enhancement on the Location of the Interrogating Nucleotide

Experiments were performed to see whether the location of the interrogating nucleotide in the foot of a SuperSelective primer alters the selectivity enhancing effect of TMAC (Figure 3B). The results showed that when the interrogating nucleotide is located at the 3′-terminal position of the foot sequence, TMAC has a much greater selectivity enhancing effect. The results obtained with SuperSelective primer *BRAF V600E* 32-24-14/14-8:1:1 (which possessed its interrogating nucleotide at the 3′-penultimate position of its 10-nucleotide-long foot sequence) were compared with the results obtained with SuperSelective primer *BRAF V600E* 32-24-14/14-9:1:0 (which possessed its interrogating nucleotide at the 3′-terminal position of its 10-nucleotide-long foot sequence). All reactions contained *BRAF* Reverse Primer 2. Reactions were performed for 60 thermal cycles at different TMAC concentrations. Two reactions were performed for each combination of SuperSelective primer and TMAC concentration: one with 4000 *BRAF V600E* mutant templates in the presence of 400,000 *BRAF* wild-type templates and the other with only 400,000 *BRAF* wild-type templates.

When mutant templates were present in the samples, increasing concentrations of TMAC had virtually no effect on the C_T values that were observed. Moreover, virtually the same C_T values were obtained no matter whether the SuperSelective primer that was used possessed a 3′-penultimate interrogating nucleotide in its foot sequence or whether the SuperSelective primer that was used possessed a 3′-terminal interrogating nucleotide in its foot sequence. Both SuperSelective primers formed a perfectly complementary 10-nucleotide−long foot hybrid with mutant templates, and the resulting C_T values were not substantially affected by increasing TMAC concentrations. However, when only wild-type templates were present in the samples, increasing concentrations of TMAC caused there to be an increasing delay in the observed C_T values.

The selectivity enhancing effect of increasing TMAC concentrations can readily be seen in Figure 3B, where ΔC_T values (that reflect the difference in the C_T value obtained for a sample that contained only wild-type templates and the C_T value of a corresponding sample that contained mutant templates as well as wild-type templates) was plotted as a function of TMAC concentration. Unlike earlier results obtained in the absence of TMAC,[6] in which the location of the interrogating nucleotide had only a minor effect on selectivity, the presence of TMAC had a much greater selectivity enhancing effect when the interrogating nucleotide was located at the 3′ end of the SuperSelective primer's

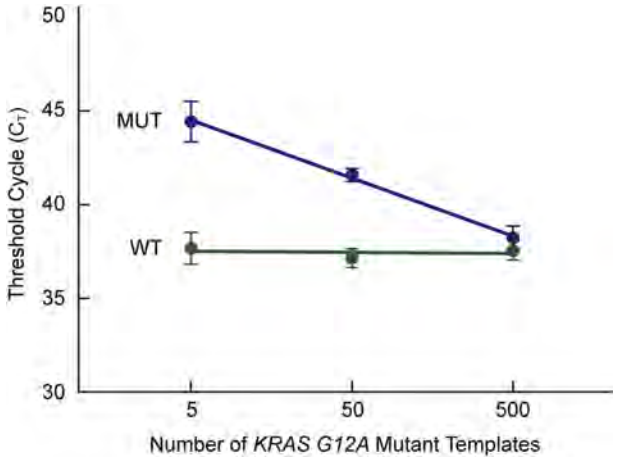

Figure 5 Detection of mutant (MUT) DNA fragments spiked into normal single-donor human plasma before isolating the DNA. Different amounts of *KRAS G12A* mutant DNA were added to human plasma, and the cell-free DNA fragments that were subsequently isolated from the plasma were then used as template for PCR assays in the presence of 50 mmol/L tetramethylammonium chloride (TMAC) that contained either SuperSelective primers for *KRAS G12A* mutant DNA or SuperSelective primers for *KRAS* wild-type (WT) DNA. Each point is the average C_T value obtained from four replicate PCR assays.

foot sequence. Suppression of the signal from the sample that contained only wild-type templates was so strong that when using the SuperSelective primer that possessed a 3′-terminal interrogating nucleotide in the presence of 50 mmol/L TMAC, no signal above background occurred during the 60 thermal cycles. Therefore, PCR assays that combine the use of SuperSelective primers that possess 3′-terminal interrogating nucleotides in their foot sequences with an appropriate concentration of TMAC may completely suppress the generation of signals from samples that possess only wild-type templates, thereby substantially increasing the sensitivity of mutant template detection.

Confirmation that TMAC Suppresses Signals from Closely Related Wild-Type Templates

Multiple real-time PCR assays were performed in the presence and absence of 50 mmol/L TMAC, using a SuperSelective primer that possessed a 3′-terminal interrogating nucleotide in its foot sequence, to confirm that TMAC suppresses signals from wild-type samples (Figure 4). Three groups of PCR assays were performed with SuperSelective primer *BRAF V600E* 32-24-14/14-9:1:0 and with *BRAF* Reverse Primer 3. In both the first and second groups, there were 24 samples that each contained 40,000 *BRAF* wild-type templates. Within each group of 24 samples, four samples also contained 1000 *BRAF V600E* mutant templates, 10 samples also contained 10 *BRAF V600E* mutant templates, and 10 samples did not contain any *BRAF V600E* mutant templates. The first group of 24 samples were assayed in the absence of TMAC, and the second group of 24 samples were assayed in the presence of 50 mmol/L TMAC. In the absence of TMAC, signals from the samples that contained only 40,000 *BRAF* wild-type templates arose and could be confused with the signals that arose from samples that contained 10 *BRAF V600E* mutant templates as well as 40,000 *BRAF* wild-type templates (Figure 4). Significantly, in the group of assays performed in the presence of 50 mmol/L TMAC (Figure 4), signals did not arise from any of the 10 samples that contained only 40,000 *BRAF* wild-type templates, whereas signals arose from each of the 10 samples that contained 10 *BRAF V600E* mutant templates as well as 40,000 *BRAF* wild-type templates. Therefore, the ability of TMAC to suppress signals from closely related wild-type templates enabled extremely sensitive real-time PCR assays to be performed, in which it is highly unlikely that a false-positive result will be obtained.

To confirm that a similar suppression of wild-type signals occurs when the samples contain DNA fragments from the entire human genome (instead of plasmid DNAs), 30 samples that each contained 40,000 copies of restriction enzyme–digested genomic DNA isolated from a human cell line that was homozygous for the *BRAF* wild-type gene were prepared. Twenty of these samples also contained 10 copies of restriction enzyme–digested genomic DNA isolated from a human cell line that was homozygous for the *BRAF V600E* mutant gene. These samples were assayed with SuperSelective primer *BRAF V600E* 32-24-14/14-9:1:0 and *BRAF* Reverse Primer 3 in the presence of 50 mmol/L TMAC (Figure 4). Significantly, no signals arose from the 10 samples that contained only *BRAF* wild-type templates, whereas all 20 samples that also contained 10 *BRAF V600E* mutant templates gave a positive signal.

Confirmation that Rare DNA Fragments Isolated from Human Plasma Can Be Detected

To confirm that the use of SuperSelective primers in PCR assays that contain TMAC are able to detect rare mutant DNA fragments isolated from human plasma samples, either 0, 50, 500, or 5000 copies of digested *KRAS G12A* mutant plasmids were spiked into 1-mL samples of normal single-donor human plasma, and cell-free DNA was then isolated from each sample, as described in *Materials and Methods*. Thirty-two different real-time PCR assays that contained 50 mmol/L TMAC were prepared, each templated with 1/10th of the DNA isolated from one of the four plasma samples (ie, eight reactions contained no *KRAS G12A* mutant target DNAs, eight reactions contained 5 *KRAS G12A* mutant target DNAs, eight reactions contained 50 *KRAS G12A* mutant target DNAs, and eight reactions contained 500 *KRAS G12A* mutant target DNAs). For each set of eight reactions, four reactions were performed in the presence of 60 nmol/L *KRAS G12A* 32-28-14/12-8:1:0 mutant primer, and the other four reactions were performed in the presence of the 60 nmol/L *KRAS* wild-type 32-28-14/12-9:0:0 primer, and all eight reactions contained 500 nmol/L *KRAS* reverse primer.

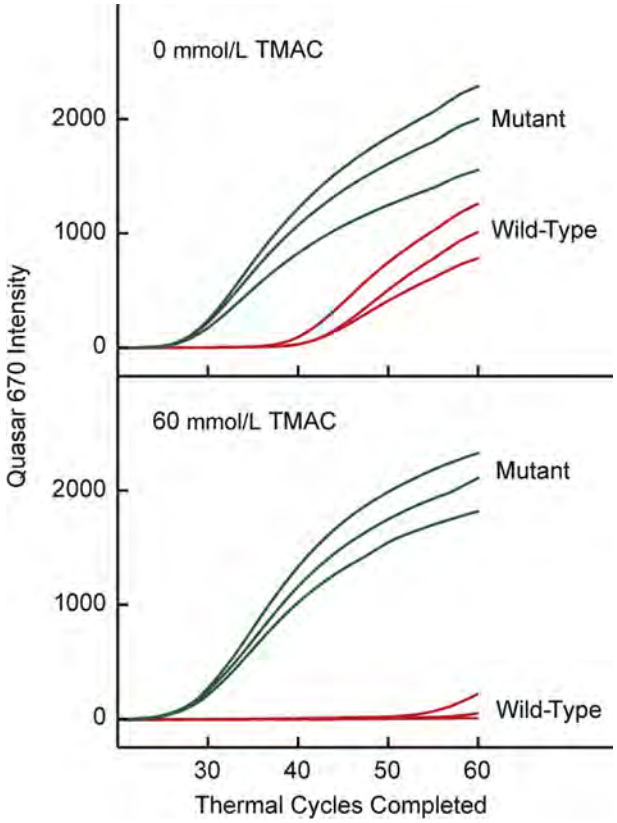

Figure 6 Suppression of *KRAS* wild-type amplification by the addition of 60 mmol/L tetramethylammonium chloride (TMAC) to real-time SuperSelective PCR assays that contained hot-start iTaq DNA polymerase (Bio-Rad). The amplification of 100,000 *KRAS G12A* mutant templates (**green lines**) was not substantially affected by the addition of TMAC. By comparison, the amplification of 100,000 *KRAS* wild-type templates (**red lines**) was virtually completely suppressed by the addition of 60 mmol/L TMAC.

As expected, no substantial signals were obtained during 65 thermal cycles in the four PCR assays that contained the *KRAS G12A* mutant primer in reactions templated with plasma DNA samples to which no *KRAS* mutant DNA fragments were added. However, in the four reactions that contained the *KRAS* wild-type primer templated with plasma DNA to which no *KRAS* mutant DNA fragments were added, signals occurred, with an average C_T value of 38 cycles, confirming the presence of *KRAS* wild-type DNA in the human plasma.

The results of the other 24 reactions that were templated with the DNA isolated from plasma into which *KRAS G12A* mutant DNA fragments were spiked-in before isolation are summarized in Figure 5. When the reactions contained *KRAS* wild-type primers, the resulting C_T values, which reflected the abundance of *KRAS* wild-type DNA fragments in the human plasma sample, were essentially equal. However, when the reactions contained *KRAS G12A* mutant primers, the C_T value of each reaction reflected the number of *KRAS G12A* mutant fragments expected to be present in each sample. Significantly, the observed C_T values for all of the PCR assays that contained SuperSelective primers for the *KRAS G12A* mutant and 50 mmol/L TMAC conformed to the classic inverse linear relationship between the observed C_T value and the logarithm of the number of target molecules in the sample.[16,17]

TMAC Selectivity Enhancement with Other DNA Polymerases

To confirm that TMAC suppresses wild-type signals when DNA polymerases other than Platinum *Taq* DNA polymerase are used, SuperSelective PCR assays were performed with three other commercial DNA polymerases (two of which are sold as a mixture that contains their own proprietary PCR buffer mixtures). These polymerases were hot-start iTaq DNA polymerase (Bio-Rad), SensiFAST DNA polymerase (BioLine), and *Taq* DNA polymerase, recombinant (Thermo Fisher Scientific). Real-time reactions were performed in the presence and absence of 60 mmol/L TMAC that contained either 100,000 *KRAS G12A* mutant templates or 100,000 *KRAS* wild-type templates. The results obtained with hot-start iTaq DNA polymerase (Bio-Rad) are shown in Figure 6. The addition of TMAC had virtually no effect on the C_T values of reactions that contained the mutant templates. However, the addition of TMAC virtually completely suppressed the amplification of reactions that contained the wild-type templates. Similar results were obtained with SensiFAST DNA polymerase (BioLine) and with *Taq* DNA polymerase, recombinant (Thermo Fisher Scientific).

TMAO Is Also an Effective Selectivity Enhancing Agent

TMAO has also been identified as a hybridization specificity enhancing agent for classic PCR assays.[10] Therefore, the selectivity enhancing ability of TMAO was compared with the selectivity enhancing ability of TMAC in SuperSelective PCR assays in which the relevant hybrids were formed by the short foot sequences of the SuperSelective primers. Multiple samples were prepared that contained either 100,000 *KRAS G12A* mutant templates or 100,000 *KRAS* wild-type templates. These samples were tested in real-time PCR assays that contained 60 nmol/L *KRAS G12A* 32-28-14/12-8:1:0 primer and 500 nmol/L *KRAS* reverse primer. One set of reactions contained either 0, 20, 40, 60, or 80 mmol/L TMAC, and another set of reactions contained either 0, 1.0, 1.5, 2.0, 2.5, or 3.0 mmol/L TMAO. The results are summarized in Figure 7. Essentially, both TMAO and TMAC selectively suppressed the amplification of wild-type templates, although the effective concentration of TMAO (up to 2.0 mmol/L) was far lower than the effective concentration of TMAC (up to 60 mmol/L). In the absence of either selectivity enhancing agent, the C_T value of 100,000 wild-type templates occurred approximately 18 cycles later than the C_T value of 100,000 mutant templates. However, in the presence of either 2.0 mmol/L TMAO or 60 mmol/L TMAC, the C_T value of 100,000 wild-type templates occurred approximately 28 cycles later than the C_T

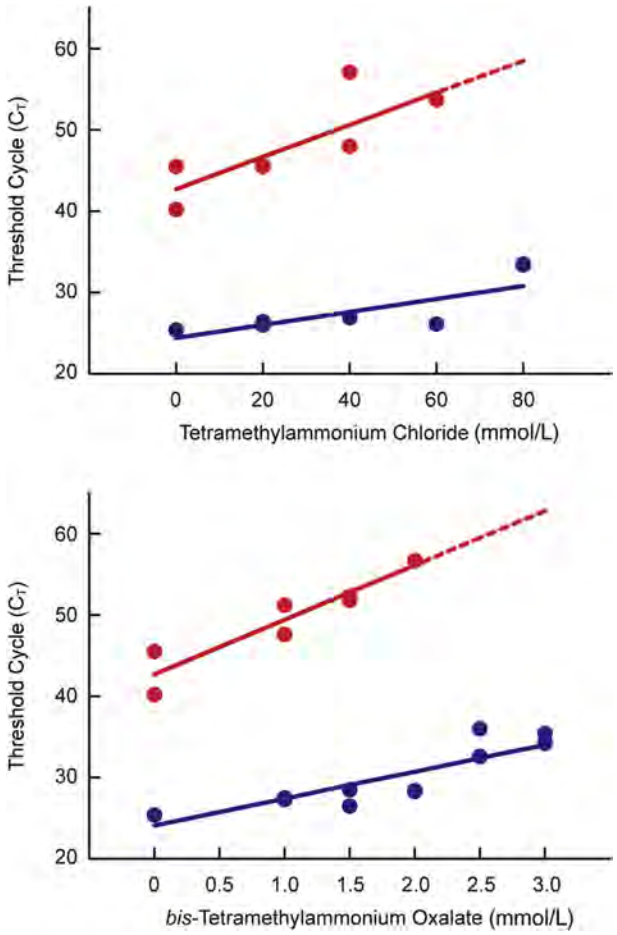

Figure 7 Comparison of the selectivity enhancing effect of *bis*-tetramethylammonium oxalate to the selectivity enhancing effect of tetramethylammonium chloride. Each PCR assay possessed SuperSelective primers for the amplification of *KRAS G12A* mutant DNA fragments. The PCR assays contained either 100,000 *KRAS G12A* DNA templates (blue) or 100,000 *KRAS* wild-type templates (red). The effect of different concentrations of tetramethylammonium chloride and *bis*-tetramethylammonium on the resulting C_T values is shown.

value of 100,000 mutant templates. Moreover, at higher concentrations of either agent, there was no signal from 100,000 wild-type templates through 60 thermal cycles of amplification. Therefore, TMAO is likely to work as well as TMAC in suppressing the signal from abundant wild-type DNA fragments in assays designed to detect extremely rare closely related mutant DNA fragments.

Multiplex PCR Assays for Closely Related Mutant Alleles

To confirm that TMAC suppresses wild-type signals in multiplex assays in the same manner as seen in monoplex assays, four sets of five reactions each were performed with Platinum *Taq* DNA polymerase to distinguish the presence of two different mutations that occur at the same nucleotide position in the *KRAS* gene. Two different SuperSelective primers were present in each assay, 60 nmol/L SuperSelective primer *KRAS G12A* 32-28-14/12-8:1:0 and 60 nmol/L SuperSelective primer *KRAS G12D* 32-28-19/10-8:1:0, as well as 1000 nmol/L *KRAS* Reverse Primer. Each of the two SuperSelective primers possessed a different unique bridge sequence, and the sizes of the bubble that each of these primers formed with their target fragment was adjusted by design (ie, they were fine-tuned) so that the resulting C_T values seen with either SuperSelective primer would be the same when the same number of their target templates are present in a sample.[6] In addition, each of the SuperSelective primers possessed a different unique 5′-tag sequence. To detect and to distinguish the amplicons generated by each of the two different closely related mutant alleles, two differently colored molecular beacons were present in each reaction: 300 nmol/L Quasar 670-labeled Molecular Beacons (which light up amplicons generated from DNA fragments that possessed the *KRAS G12A* mutation) and 300 nmol/L fluorescein-labeled Molecular Beacons (which light up amplicons generated from DNA fragments that possessed the *KRAS G12D* mutation). Every reaction contained 60 mmol/L TMAC.

The results of the four sets of reactions are shown in Figure 8. In the first set of reactions, the five samples contained 1000 *KRAS G12D* templates, 10 *KRAS G12A* templates, and 10,000 *KRAS* wild-type templates; in the second set of reactions, the five samples contained 1000 *KRAS G12D* templates and 10,000 *KRAS* wild-type templates; in the third set of reactions, the five samples contained 10 *KRAS G12A* templates and 10,000 *KRAS* wild-type templates; and in the fourth set of reactions, the five samples only contained 10,000 *KRAS* wild-type templates. The results confirmed that independent amplifications occurred for each of the two different mutations (with their respective amplicons lit up by differently colored molecular beacons), and their resulting C_T values reflected the number of fragments of each mutant initially present in the sample. Significantly, because of the presence of TMAC in those assays in which only one type of mutant was present (lit up by one distinctively colored molecular beacon), there was no signal from the other distinctively colored molecular beacon, indicating that the synthesis of amplicons from the wild-type was completely suppressed. Moreover, in samples that contained only wild-type templates, no colored signals occurred at all. As a control, identical experiments were performed in the absence of TMAC, and signals arose from the wild-type templates that occluded signals from samples that contained 10 *KRAS G12A* templates (data not shown). Taken together, these results confirm that the inclusion of TMAC in SuperSelective PCR assays prevents the occurrence of false-positive results and enhances sensitivity to such an extent that false-negative results are highly unlikely to occur.

Discussion

In the past, the addition of TMAC or TMAO to PCR assays has been found to enhance hybridization specificity and thereby suppress the generation of primer-dimers and other

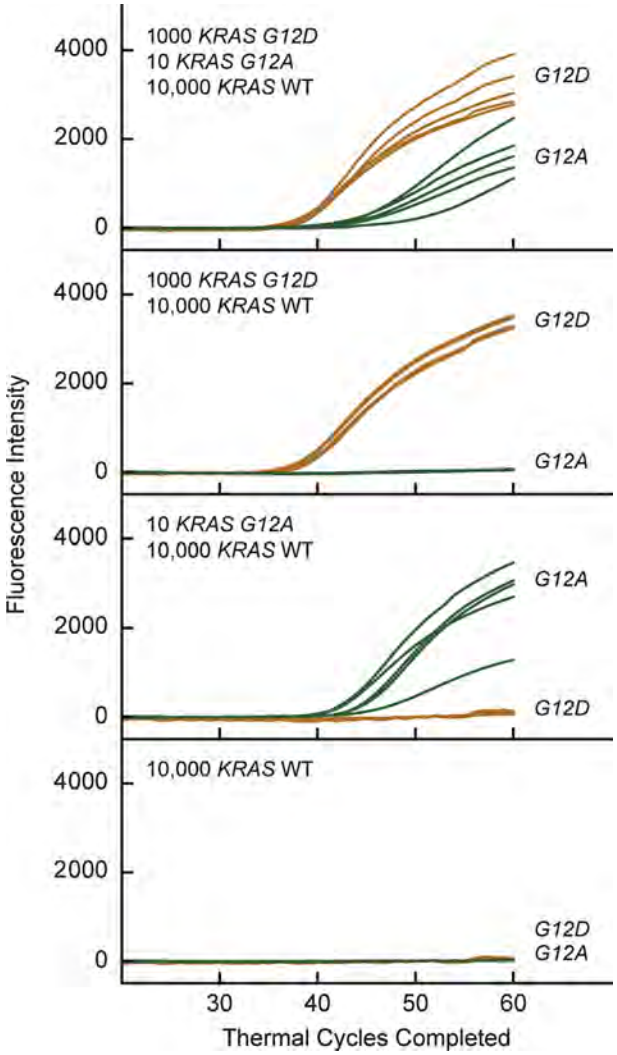

Figure 8 Multiplex PCR assays for samples that contained closely related mutant alleles. Four sets of five reactions were performed, in which the composition of the templates in each sample is indicated by the labels in each panel. Every reaction contained 60 mmol/L tetramethylammonium chloride (TMAC). Amplicons generated from DNA templates that contained the *KRAS G12A* mutation were lit up by Quasar 670-labeled Molecular Beacons (**green lines**), and amplicons generated from DNA templates that contained the *KRAS G12D* mutation were lit up by fluorescein-labeled Molecular Beacons (**brown lines**). Signals from the 10,000 *KRAS* wild-type (WT) templates present in each reaction were completely suppressed by the presence of TMAC.

false amplicons that originate from mismatched hybrids that occasionally occur.[8–10] Our results found that these agents also enhance selectivity between closely related target sequences. Moreover, the results of these experiments show that the selectivity enhancing effect of different concentrations of these agents depends not only on whether the desired mutant foot hybrid is fully complementary and the undesired related wild-type foot hybrid is mismatched, but also that selectivity depends on the length of the desired foot hybrid and on the location of the mismatched base pair or base pairs in the undesired related wild-type hybrid (and in undesired hybrids that form with closely related nontarget sequences). Furthermore, the presence of these selectivity enhancing agents in PCR assays enables somewhat longer foot sequences to be used on the SuperSelective primers, thereby reducing variability in the C_T values obtained when there are a small number of mutant target fragments in the sample.

The mechanism by which these selectivity enhancing agents improve hybridization specificity in PCR assays is not understood.[19] The observation that the location of the mismatch within the foot hybrid has a substantial effect on sensitivity to TMAC or TMAO adds an additional constraint on future mechanistic explanations. Apparently, the mismatched hybrids formed by the foot of the SuperSelective primer and a wild-type fragment are more easily dissociated in the presence of TMAC or TMAO, decreasing their mean persistence time, thereby decreasing the probability that these mismatched hybrids will encounter a DNA polymerase before they dissociate. Furthermore, the location of a mismatch at the 3′ terminus of the foot hybrid appears to make it easier for TMAC or TMAO to dissociate the foot hybrid than a mismatch elsewhere in the hybrid. What is clear is that the addition of TMAC or TMAO to SuperSelective PCR assays suppresses the amplification of closely related wild-type DNA fragments (and closely related nontargeted mutant DNA fragments) to such an extent that the signals that arise during the course of amplification are indicative of the presence of the intended mutant target DNA fragments. Consequently, multiplex PCR assays performed in the presence of an appropriate concentration of TMAC or TMAO, using fine-tuned sets of SuperSelective primers whose foot sequences are preferably 9- or 10-nucleotides long, and whose interrogating nucleotide is at the 3′ end of the foot sequence, are likely to be extremely sensitive and are unlikely to generate false-positive or false-negative results.

Future Directions

It is now possible to develop multiplex PCR assays that combine the use of a fixed concentration of TMAC (say 50 or 60 mmol/L) or a fixed concentration of TMAO (say 1.5 or 2.0 mmol/L) with sets of fine-tuned SuperSelective primers that are each specific for a genetic variation of interest (mutation, deletion, or insertion). Fine-tuning a set of SuperSelective primers is relatively straightforward. The length of the foot sequence of each SuperSelective primer in the set (say 8, 9, or 10 nucleotides) is chosen in preliminary experiments so that the C_T values obtained with the same number of targets in a sample will be approximately the same for each primer. Further small adjustments in the length and rigidity of the bridge sequence in each SuperSelective primer assures that the C_T values obtained for each target sequence are intercomparable.[6]

Because the amplification of related wild-type sequences is suppressed in SuperSelective PCR assays, there are a number of different clinical applications that could be explored. For example, the detection of fetal genes from the father in

maternal blood samples, although quite rare, could be detected without interference from abundant related maternal genes that differ in sequence. Also, in choosing an antibiotic for treatment of a bacterial infection, the early detection of rare antibiotic-resistant bacteria in an otherwise sensitive bacterial population could enable an effective antibiotic to be chosen. Perhaps the most important potential application of SuperSelective PCR assays, although currently quite speculative, is the early detection of somatic mutations associated with cancer in blood samples taken during routine medical examinations, even though symptoms have not yet occurred. For the present, the most promising potential applications involve the personalized care of patients known to have a particular form of cancer, characterized by identifying the presence or absence of particular mutations that indicate the most effective form of treatment.

Multiplex SuperSelective PCR assays performed in the presence of TMAC or TMAO are so sensitive that they may be used for the analysis of noninvasive samples periodically obtained from patients with cancer. These assays would be rapid, low-cost, easy to perform, and can be run on a number of different real-time PCR instruments that are widely available throughout the world. For example, there are quite a few somatic variations, including point mutations and deletions that can occur in the epidermal growth factor receptor (*EGFR*) gene, that are relevant to the treatment of non-small cell lung cancer,[20] and SuperSelective primers are able to detect each of them.

The presence of particular mutations in an initial liquid biopsy from a patient can potentially determine which therapy will be most effective. During treatment, subsequent liquid biopsies can document the effectiveness of that therapy, as the target mutations become less abundant. The beauty of this noninvasive approach is that these multiplex SuperSelective PCR assays should be so sensitive that the occurrence of a resistance mutation can be detected long before cells possessing that mutation cause a recurrence of symptoms. Moreover, the mutational profile of the patient at that time will enable therapy to be altered to take into account that patient's particular somatic-mutational situation. These assays should be sensitive, rapid, and inexpensive, and they should provide an efficient route toward achieving advances in the treatment of different cancers.

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