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Kinetic Hairpin Oligonucleotide Blockers for Selective Amplification of Rare Mutations

SUBJECT AREAS:
PCR-BASED TECHNIQUES
PROGNOSTIC MARKERS

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Detection of rare mutant alleles in an excess of wild type alleles is increasingly important in cancer diagnosis. Several methods for selective amplification of a mutant allele via the polymerase chain reaction (PCR) have been reported, but each of these methods has its own limitations. A common problem is that Taq DNA polymerase errors early during amplification generate false positive mutations which also accumulate exponentially. In this paper, we described a novel method using hairpin oligonucleotide blockers that can selectively inhibit the amplification of wild type DNA during LATE-PCR amplification. LATE-PCR generates double-stranded DNA exponentially followed by linear amplification of single-stranded DNA. The efficiency of the blocker is optimized by adjusting the LATE-PCR temperature cycling profile. We also demonstrate that it is possible to minimize false positive signals caused by Taq DNA polymerase errors by using a mismatched excess primer plus a modified PCR profile to preferentially enrich for mutant target sequences prior to the start of the exponential phase of LATE-PCR amplification. In combination these procedures permit amplification of specific KRAS mutations in the presence of more than 10,000 fold excess of wild type DNA without false positive signals.

Detection of rare mutations associated with biomarkers is important for early detection, diagnosis, enhanced personalized therapy, and accurate prognosis of cancers. Detection of rare mutations is also important for assessment of residual disease after surgery or radio/chemotherapy and for early identification of treatment resistance^{1–5}. For example, sensitive analysis of KRAS mutations in codons 12 and 13, as well as certain EGFR mutations is critical for choosing which drug to prescribe at each stage of the lung disease^{6–8}. Recently, the American Society of Clinical Oncology (ASCO) and the National Comprehensive Cancer Network have updated their guidelines with a recommendation that therapies including panitumumab or cetuximab be limited to patients with advanced or metastatic colon or rectal cancers carrying wild-type KRAS^{9,10}. In addition, there is increased reliance on detection of rare circulating tumor cells or tumor DNA in blood for identification of very early-stage tumors or for tumors that are difficult or impossible to biopsy^{11,12}.

Real-time polymerase chain reaction (PCR) strategies for detection of minority alleles have been thoroughly reviewed by Milbury et al¹³. These include the use of allele-specific, amplification-refractory mutation system (ARMS) primers to selectively initiate amplification of the mutated genotype¹⁴; COLD-PCR and ICE-COLD-PCR that promote amplification of the mutant amplicon over the wild type amplicon by favoring the preferential denaturation of mutant targets¹⁵; and addition of allele-specific clamps, comprised of PNA^{16–19}, LNA²⁰, or linear oligonucleotides¹⁵, to preferentially inhibit primer extension on wild type targets.

This paper describes a new approach involving kinetic hybridization of a hairpin blocker to selectively amplify mutant targets during Linear-After-The-Exponential (LATE)-PCR. LATE-PCR is an advanced form of non-symmetric PCR in which specially designed primers in limiting and excess concentrations (“limiting primer” and “excess primer”, respectively) are used to amplify double-stranded DNA exponentially followed by linear amplification of single-stranded DNA. To achieve preferential amplification of mutant targets, the loop sequence of the hairpin blocker was designed to be fully complementary to the wild-type allele at the site of the mutation and partly complementary to the 3' end of the upstream limiting primer. By manipulating the time allowed for hybridization of the blocker at certain temperatures during PCR, the blocker preferentially hinders binding of the limiting primer to wild-type targets thereby enabling selective amplification of mutant strands. Such selective inhibition is hereafter referred to as kinetic blocking. The concept of kinetic blocking was derived from the hybridization properties of hairpin-shaped oligonucleotides²¹. Hybridization of such structured oligonucleotides

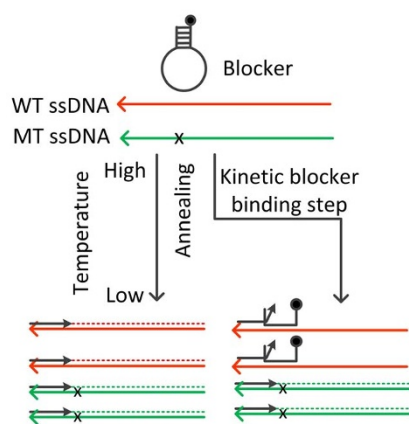


Figure 1 | A schematic description of the Kinetic Blocker selectively amplifying the mutant variant. With the fast annealing profile, the kinetic blocker fails binding on wild type target and therefore both of the targets get amplified. With a kinetic blocker binding step, blocker opens and binds on the wild type target, preventing the primer from binding. Therefore only the mutant type alleles get amplified.

to their targets depends on both opening of the stem of the hairpin and hybridization of the loop to its complementary target sequence. Compared to other methods of selective amplification, kinetic hairpin blockers are inexpensive and flexible to use, i.e., they selectively interfere with amplification of their chosen target sequence depending on how they are used.

The single greatest limitation of all strategies for selective sequence amplification is the occurrence of false positives due to Taq polymerase errors²². One way to decrease these errors is to use a high fidelity polymerase, such as Pfu²³. In the work reported here the problem of Taq DNA polymerase errors was minimized using a mismatched excess primer plus a modified LATE-PCR thermal profile, which together further promote selective amplification of the mutant type. In this system amplification of a rare mutant allele is at least 10,000 times more likely than amplification of a wild type allele in the absence of false positive signals.

Results

The Logic of Hairpin Blockers in LATE-PCR. Hairpin blockers are oligonucleotides with a stem-loop structure. The sequence of the loop is perfectly complementary to the wild type allele. The 3' end of the blocker is capped to prevent extension and several nucleotides at the 5' end of the blocker compete for target binding with the 3' end of the upstream limiting primer. The T_m of the blocker to its fully complementary target sequence is about 10 ~ 12°C higher than the T_m of the blocker to the mutant target, and 15 ~ 17°C higher than the T_m of the upstream limiting primer to both targets. Under these conditions, the blocker preferentially hybridizes onto the wild type template strand and fails to bind the mutant template when the

temperature of the reaction is paused at an optimal blocker binding temperature between the denaturation step and limiting primer annealing temperature, Figure 1. The extent of blocker binding to the wild-type target at this step is governed by the time it takes for the stem of the blocker to open. As a result, blocker efficacy can be optimized by simply adjusting the length of the blocker binding step. When the temperature is then lowered to enable annealing of the limiting and excess primers, the bound blocker prevents binding and extension of the limiting primer on the wild-type target molecules. But, at this lower temperature the blocker is less allele discriminating and can begin to bind to mutant target sequences if the stem is open. To reduce this possibility the temperature is lowered quickly from the blocker binding temperature to the limiting primer binding temperature. Rapid cooling and a lower temperature make the stem more difficult to open resulting in preferential binding and extension of the limiting primer to the mutant strand instead of the blocker. These adjustments in the timing of the thermal cycle result in preferential amplification of the mutant target. In contrast, if no blocker binding step is included in the thermal cycle and the temperature is lowered quickly, the limiting primer hybridizes to, and extends on both the wild type and the mutant templates, Figure 1. Thus, the ability of the blockers to interfere with amplification of their chosen target sequence is determined by how the blockers are used.

Adjusting the Kinetics of Hairpin Blocker by Changing the Length of the Stem. Hairpin oligonucleotides are more allele discriminating than linear probes because closure of the stem provides an alternative thermodynamically stable state of the hairpin molecule²⁴. As a result hairpin oligonucleotides exhibit a large T_m difference, 7–10°C, for hybridization to a perfectly matched versus a mis-matched target²⁵. In the present study we used dual labeled hairpin oligonucleotides (i.e., molecular beacons) to explore the hybridization kinetics of hairpin blockers to their targets. Six blockers B1, B5, B6, B7, B8 and B9 were constructed having the same loop sequence complementary to a region of human KRAS gene at codons 12 and 13 but different stem lengths ranging from 1–9 bases. This target was chosen because mutations in KRAS codons 12/13 are associated with a variety of cancers²⁶.

The T_m s of each blocker hybridizing to synthetic oligonucleotides containing the wild type or a mutant KRAS sequence are summarized in Table 1. The difference between the two T_m s for each blocker, ΔT_m , is its window of allele discrimination. As shown in Table 1, when the length of the stem increased, the effective T_m of the blocker to each target went down an equivalent amount and ΔT_m therefore remained fairly constant. For instance, B1 had an effective T_m of 72°C to the wild type sequence and 61°C to the mutant sequence ($\Delta T_m = 11^\circ\text{C}$), while B5 had an effective T_m of 67°C to the wild type sequence and 57°C to the mutant sequence ($\Delta T_m = 10^\circ\text{C}$). All the ΔT_m s are almost the same, 10–12°C, except for B9 which has a 9 base pair stem and which appears to have a larger ΔT_m . This blocker may have bound to the mutant sequence too slowly to give accurate T_m data.

Table 1 | Equilibrium and kinetic binding of blockers with different stem length on wild type target or mutant type target

Blocker (stem length)	T_m (wild type target) (°C)	T_m (mutant type target) (°C)	ΔT_m	Binding kinetics at 60°C		
				Maximum percentage to WT	Maximum percentage to MT	Time for fully binding (seconds)
B1 (1 bp)	72	61	11	100	80	20
B5 (5 bp)	67	57	10	100	18	120
B6 (6 bp)	64	54	10	80	0	300
B7 (7 bp)	62	52	10	60	0	>300
B8 (8 bp)	61	49	12	55	0	>300
B9 (9 bp)	52	38	14	10	0	>300



Table 1 also shows that the kinetics of blockers binding to the targets slows down with longer stems, from 20 seconds to more than 5 minutes. Blocker B5 was chosen for further use because it bound to 100% of wild type targets but only 18% of mutant targets during the blocking binding step, 60°C. The amount of time required for complete binding of B5 to the wild type target, 2 minutes, was also reasonable in practice.

Effectiveness of a Hairpin Blocker in Rare Mutation Detection in Plasmid DNA. Hairpin blocker B5 was first tested using two plasmid DNA targets containing the wild type KRAS gene or a variant of codon 12, a GGT->GAT mutation. The two plasmids were also engineered to have an additional SNP site downstream from the binding region of the blocker and the limiting primer for convenient identification of the amplification products using a mismatch-tolerant probe against the SNP site. Three LATE-PCR thermal cycle profiles were tested to establish the best conditions for blocking during amplification. In each case the kinetics of double-stranded DNA accumulation in real-time were analyzed using SYBR Green fluorescence at 72°C. The results in Figure 2 show that inclusion of the hairpin blocker, even in the absence of an extended blocker binding step, is sufficient to slow down amplification of 10,000 copies of the wild type sequence to the point that it had a C_q (quantification cycle²⁷) value equivalent to 1,000 copies of the mutant sequence. Inclusion of a 1-minute blocker binding step significantly increased the effect of the blocker on wild type target (seen as an increased C_q value) and also slightly improved the efficiency of mutant target amplification (seen as decreased C_q value). Inclusion of a 2-minute blocker binding step result in distinctly more inhibition of wild type target amplification plus a small additional improvement in the efficiency of mutant sequence and amplification. Quantification of these results shows that the thermal cycling protocol with a 1-minute blocker binding step reduces the amplification efficiency of wild type templates 100 fold relative to mutant target molecules, while a 2-minute blocker binding step favors mutant target amplification 200–1,000 fold over the wild type target.

How Effective are Hairpin Blockers Really? The results presented in Figures 2 raise a fundamental question. Is the selectivity of the kinetic blocker for the wild type sequence only 200–1,000 fold because the blocker fails to bind some wild type target molecules, or, is it only 200–1,000 fold because some wild type target molecules mutate within the blocker binding site due to Taq DNA polymerase errors and thereby escape blocker binding in subsequent rounds of exponential amplification? Taq DNA polymerase is known to make errors at a rate of about $2 \times 10^{-5} \sim 1 \times 10^{-4}$ per nucleotide²⁸.

To answer this question, we first examined the sequence of the downstream SNP by probing it with a mis-match tolerate probe, LTprb. This probe was fully complementary to the wild type sequence with a melting T_m of 55°C. It was partially mismatched to the mutant sequence and therefore had a melting T_m of 43°C. For this test 10 copies of the mutant plasmid were mixed with 10 or 100,000 copies of the wild type. The thermal cycle included a 2-minute/60°C blocker binding step for 55 cycles, as described above. The results show that the hairpin blocker selectively inhibited the wild type target when it was mixed 1 : 1 with the mutant target, but it failed to inhibit synthesis of the wild type target when it was present at a 10,000 fold excess over the mutant target, Figure 3A. This result appears to favor the possibility that the blocker simply fails to bind to all copies of the wild type plasmid DNA when they are present in excess over the mutant plasmid. However, an analysis of the blocker binding sequence itself demonstrates that this conclusion is wrong.

Figure 3B shows the melting temperature profiles of blocker B5 bound to the single stranded products of LATE-PCR amplification in the presence and absence of a 2-minute blocker binding step. In the absence of a blocker binding step during amplification, which we

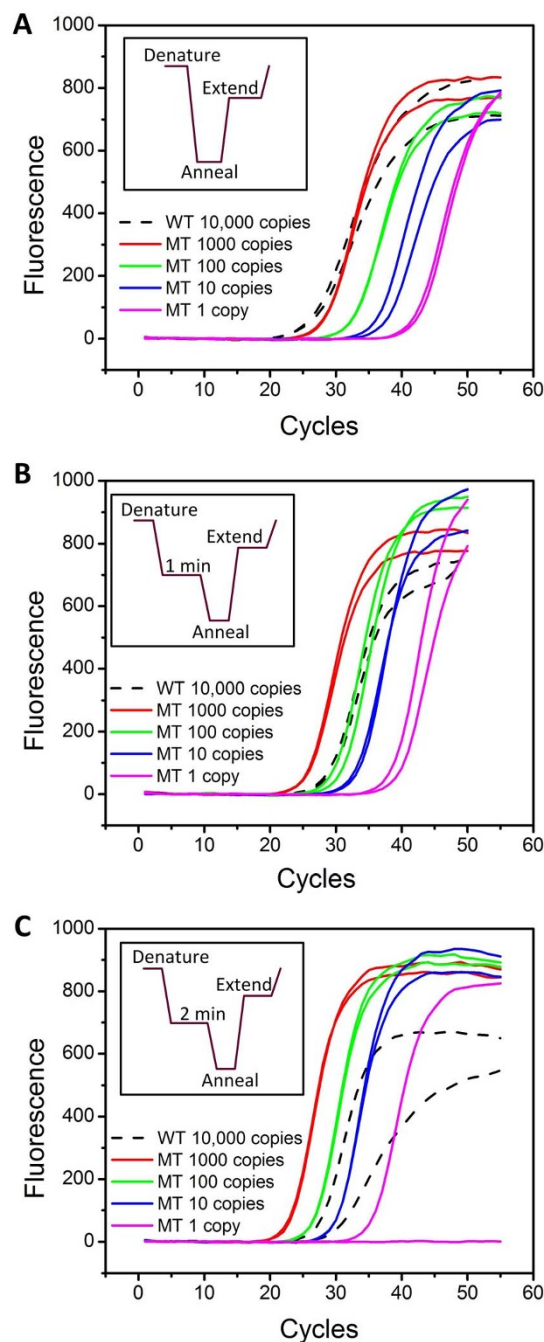


Figure 2 | Real-time SYBR fluorescence reading during LATE-PCR amplifications that include blocker B5 with different length of blocker binding step. (A) No blocker binding step; (B) 1-minute blocker binding step; (C) 2-minute blocker binding step.

have demonstrated having a low blocking efficiency, the blocker binds the wild type product strands at the end of amplification with a T_m of 68°C. In contrast, inclusion of a blocker binding during amplification results in blocker binding to amplified product strands with a T_m less than 60°C, coincident with the peak of blocker binding to the mutant template. In other words, at least some of the wild type sequences appear to have accumulated mutations in the region of blocker binding.

To confirm this conclusion we sequenced single-stranded products of sixteen replicate reactions arising from pure 10,000 copies of wild type target amplified in the presence of the blocker and a 2-minute blocker binding step. As shown in Table 2, in every case the

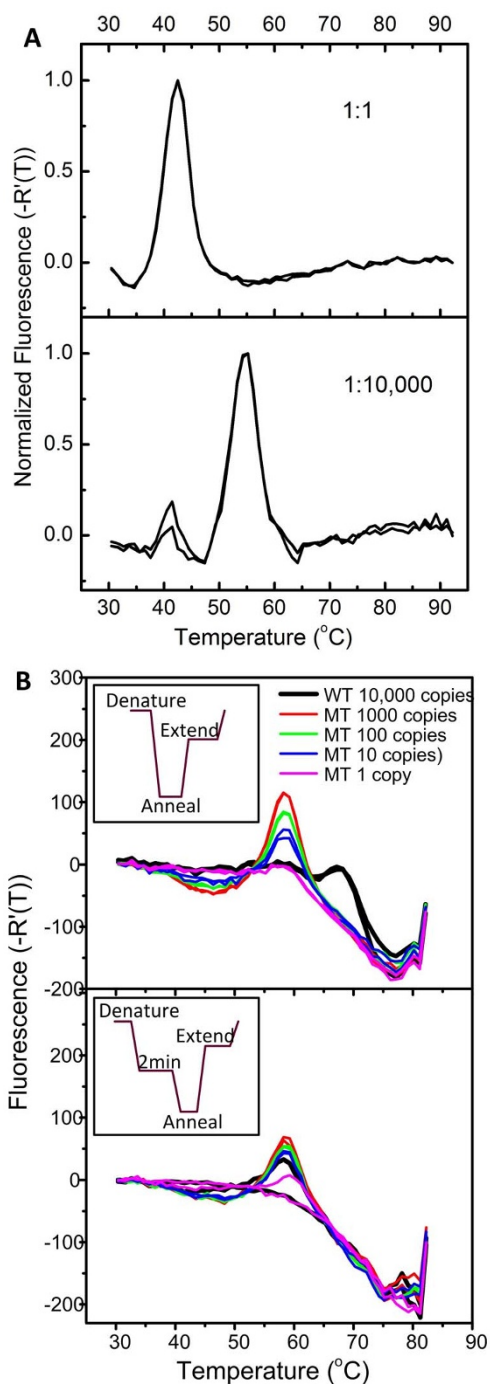


Figure 3 | (A) Melting curves of the downstream probe, LTprb, following LATE-PCR amplification for samples containing mixtures of wild type and mutant type targets. (B) Melting curves of the blocker, B5, as a probe following LATE-PCR amplification for 10,000 wild type targets or 1000, 100, 10 or 1 copy mutant type targets.

sequence of the blocker binding region contained a mutation which significantly lowered the T_m of blocker target binding. Most of the sequence changes were G to A transitions, characteristic of Taq DNA polymerase errors²⁹, and most of the mutations were located toward the center of the blocker binding site where the mutations have the greatest impact on the blocker T_m . Thus, the single-stranded amplicons that appear to be wild type molecules on the basis of their downstream SNP sequence are, in fact, false positives arising from Taq DNA polymerase errors that are selected over the course of amplification.

Strategies for Overcoming the Problem of Taq Polymerase Errors.

The results presented above suggest that kinetic hairpin blockers would actually inhibit wild type target amplification more than 200–1,000 fold, were it not for Taq DNA polymerase errors. Use of a high fidelity enzyme would address this problem but this approach would necessitate re-optimization of all the blocker and primer binding conditions described above to adjust to the buffer reaction conditions for the high fidelity enzyme. We turned instead to another strategy, selective linear pre-amplification. This strategy takes advantage of the fact that (i) preferential blocking of limiting primer hybridization and extension by the kinetic blocker on the original wild-type target strand is not affected by Taq DNA polymerase errors, and (ii) the T_m of the LATE-PCR limiting primer is higher than the T_m of the excess primer. Thus, it should be possible to selectively hybridize and extend the limiting primer on the mutant strand multiple times using a three-temperature cycling profile for denaturing, blocker binding, and limiting primer annealing as long as the temperature never drops to the point where the excess primer can hybridize and generate amplified target strands that could have Taq DNA polymerase errors. To enhance the T_m difference between the limiting and excess primer we replaced the standard excess primer with one that was deliberately mis-matched to its target sequences at its 3' end. The strategy is schematically shown in Figure 4. We also modified the thermal cycle so that the reaction started with several cycles of linear amplification using only the limiting primer.

Figure 5 shows that the modified thermal cycle is divided into 8 segments. In Segment 1 the hotstart antibody is denatured and the Taq polymerase is activated. None of the oligos is bound to a template. In Segment 2 the temperature is lowered to selectively bind the blocker to the wild type template and then is lowered again to permit binding and extension of the limiting primer on the mutant template. The mis-matched excess primer does not bind to either template. Thus, only the mutant limiting primer strand is amplified and, because it does not bind the blocker there is no selection of any additional mutation within the blocker binding target sequence. In theory it is possible to repeat Segment 2 as many times as desired. In practice ten cycles seems a good number as it linearly amplified the mutant type by an order of magnitude within a reasonable time. In Segment 3 all of the oligonucleotides and new template strands are once again melted apart. In Segment 4 the temperature is first lowered to allow the blocker to bind to the wild type template strands and is then lowered again to a temperature sufficient for the mis-matched excess primer to bind to the new mutant limiting primer strands. Segment 4 is repeated four additional times by rapidly oscillating the temperature between 40 and 60 in order to guarantee that all single strands are converted to double strands. Segment 5 is an extension step at 72°C that guarantees that double-strands are full length. Segment 6 is comprised of 50 cycles of amplification at temperatures which block amplification of wild type templates and complete the switch from the exponential phase of LATE-PCR to the linear phase of single-strand production. Linear amplification is followed by analysis of product strand using low temperature Lights-On/Lights-Off probes as described below. Accordingly, Segment 7 is a step at 30°C to permit binding of probe and Segment 8 is an incremental probe/target melt with acquisition of fluorescent signals over the entire temperature range 30°C–95°C.

KRAS Mutation Analysis in a Closed Tube using Lights-On/Lights-Off Probes.

In order to get more detailed information and better discrimination of mutant and wild type signals we used Lights-On/Lights-Off probes³⁰. A Lights-On probe is dual labeled with a fluorophore and a quencher. A Light-Off probe, in contrast, is labeled with only a quencher moiety. When both probes are bound to a target, the Lights-Off probe serves to absorb energy from the fluorophore of its adjacent Lights-On probe. To implement this



Table 2 | T_m s of the blocker binding on targets with different mutated nucleotide. The target sequences were of the samples amplified from 10,000 wild type target with the blocker binding step of 2 minutes

Kinetic Blocker Target Sequences																		T_m (°C)
G	G	A	G	C	T	G	G	T	G	G	C	G	T	A	G	G	C	
-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	68.2
-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	58.2
-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	58.2
-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	58.2
-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	56.7 [#]
-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	56.7 [#]
-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	58.2
-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	58.2
-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	60.8 [#]
-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	59.1 [#]
-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	57.2
-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	56.7 [#]
-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	60.8 [#]
-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	58.2
-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	57.2
-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	56.7 [#]

[#]Calculated T_m .

strategy, we placed a quencher on the blocker to convert it into a Lights-Off probe and designed a Lights-On probe that bound next to it. The T_m of the Lights-On probe was such that if the target is wild type the blocker/Lights-Off probe would bind on the target at a higher temperature than the Lights-On probe, resulting in no net fluorescence increase (i.e., the fluorescence signals from a wild-type target would therefore resemble those from a no template control). On the other hand, if the target is mutant-type, the blocker/Lights-Off probe would bind on the target at a temperature lower than the T_m of the Light-On probe. As the temperature in the reaction went down, the Lights-On probe would generate a signal and then be turned off as the adjacent Lights-Off probe binds at a lower temperature (Figure 6A). In a negative first derivative plot of fluorescence vs. temperature (-dF/dT), the Lights-On probe signal corresponds to a peak whereas the Lights-Off probe corresponds to a valley. In such a plot, a Lights-On probe fluorescent peak would indicate the presence of a mutant target. The lowest temperature of the Lights-Off probe fluorescent valley, which corresponds to the T_m of the Lights-Off blocker bound to a specific mutation, would identify individual mutations. As mentioned above, the wild type target would have the same fluorescent signatures as no template control samples (Figure 6B).

Figure 6C shows proof-of-principle evidence for selective rare mutation detection from the background of wild type using the combination of blocker, mismatched excess primer, modified LATE-PCR thermal profile and Lights-On/Lights-Off probes. As shown, under these conditions no mutant strands of any sort were detected when amplification was initiated using only 10,000 copies of the wild type plasmid, but addition of even a single copy of the mutant plasmid containing the GAT mutant codon resulted in amplification of single strands, a Lights-On signal peak, and a Lights-Off valley with a T_m corresponding to the GAT codon, as confirmed by sequencing, Figure 6D. This finding is consistent with the view that Taq DNA polymerase errors have to occur in the first round of selective exponential amplification in order to measurably alter the composition of the final product. If selective exponential amplification is preceded by just ten cycles of selective linear pre-amplification, the mutant target has a sufficient head start to render invisible any mutants that arise during subsequent cycles due to Taq DNA polymerase errors.

Discussion

Given the clinical importance of rare mutation detection from the background of wild type targets and the convenience of PCR detec-

tion, a number of PCR-based enrichment methods have been reported¹³. COLD-PCR or ICE-COLD-PCR takes advantage of the T_m difference between wild type and mutant type and preferentially melt the mutant type target during PCR to enrich the amplification of mutant type. All the unknown mutations in the mutant target would be revealed after sequencing of mutant amplification products. However, the sensitivity is relatively low (down to only 1%). One of the most widely used approaches with more sensitivity uses allele-specific primers which are perfectly matched to a specific mutant type target and mismatched to all other alleles to enhance a particular mutant type. The detection sensitivity reaches as low as 0.1%. However, once incorporated due to mis-amplification of a wild-type target, those primers turn all amplicons derived from wild type sequences into mutant amplicons. So any wild type target that escapes selection cannot be distinguished from a bona fide mutant. PNA and LNA clamps PCR keep the sequence of the targets but the manufacturing of PNA or LNA are very expensive. Furthermore, all the current PCR-based enrichment methods face the same biological problem which is difficult to overcome: generation of false-positive mutants due to DNA polymerase errors during amplification. For a certain rare mutation detection method to be used as a routine diagnostic tool, it must achieve a balance of high selectivity while maintaining the specificity, accuracy, convenience, and low cost.

Linear oligonucleotide DNA has been an inexpensive material for competing with the primers for binding on the target. We have reported using a linear blocker to sequence all DNA strands generated in multiplex LATE-PCR assays³¹. In that study, a linear blocker was added to prevent all but one of the remaining excess primers in LATE-PCR product serving as the sequencing primer. We observed that when there is a secondary structure in the linear blocker, the blocking efficiency is dramatically decreased in the fast sequencing process. In the present report we took advantage of the slow binding kinetics of the hairpin structured oligonucleotide DNA and used it as a blocker for rare mutation detection.

In order to use the blocker for selective inhibition of the wild type target with no effect on the mutant type amplification it is necessary to introduce a blocker binding step in the thermal cycle. The efficacy of the blocker can then be optimized by adjusting the length and temperature of this special step. If no blocker binding step is included in the thermal cycle and the temperature is lowered quickly, blocker binding fails because the stem of the blocker does not have enough time to open. The limiting primer hybridizes to, and extends on both the wild type and the mutant templates. This feature of kinetic hair-

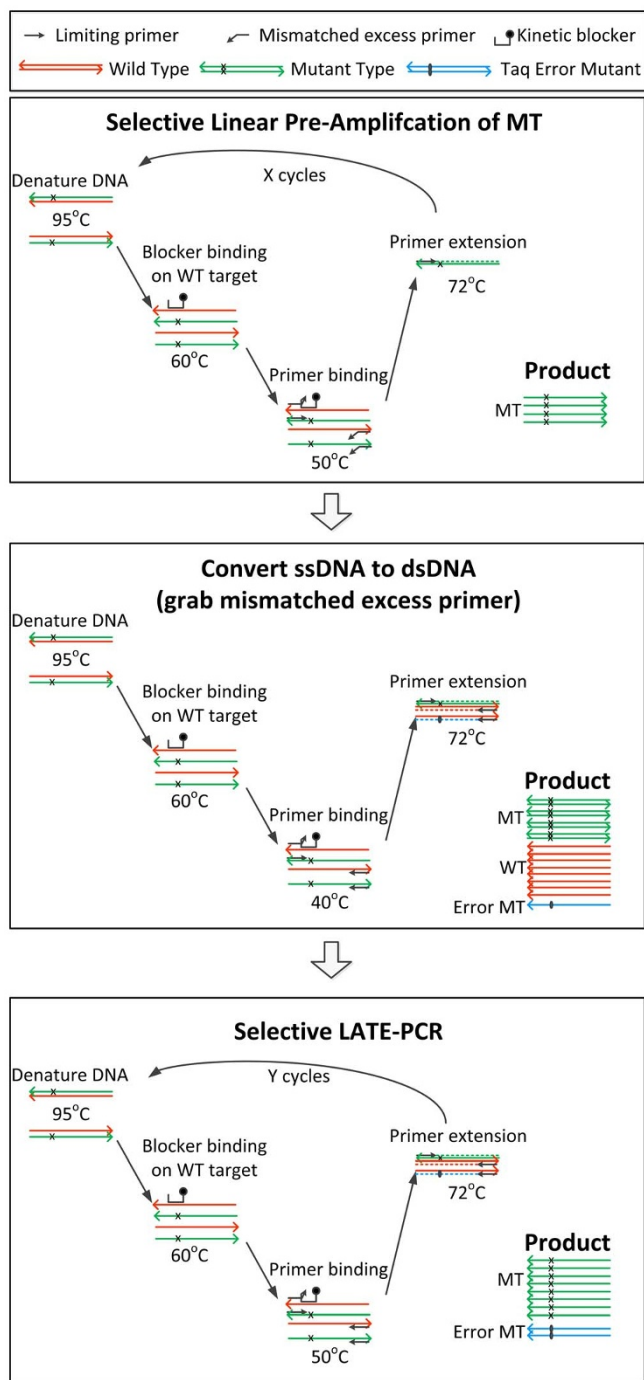


Figure 4 | A schematic description of including a mismatched Excess Primer with the hairpin blocker for a selective linear pre-amplification of the mutant type targets to overcome the false positive signals by Taq polymerase errors.

pin blockers distinguishes them from linear blockers and blockers comprised of LNA or PNA. This feature also makes it possible to establish whether samples that fail to generate a fluorescent signal actually contain only wild type DNA rather than no DNA. All that is required in this case is to skip the blocker binding step and see if a signal is present.

Unlike strategies that depend on the use of mutation-specific ARMS-PCR primers the blocker strategy preserves, rather than erases, the mutation used for selective amplification. This is because the sequence of the limiting primer strand, created by extension of the limiting primer on the mutant target, retains the mutated base

pair, making it possible to distinguish the products of amplification using a fluorescent labeled probe. In the PNA or LNA assays, the mutated nucleotide is also preserved. But these chemically special clamps are expensive and need to be removed in order to sequence or even probe the resulting amplicons.

Amplification of false-positives due to Taq DNA polymerase errors is the single greatest limitation of all strategies that depend on multiple rounds of selection and amplification. These errors actually arise by extension of the reverse primer, or the excess primer in the case of LATE-PCR. In order to circumvent this we deliberately used a mismatched excess primer, thereby reducing its T_m during the first ten rounds of amplification, and we avoided use of this primer by carrying out ten initial cycles at an annealing temperature for limiting primer-only extension. This solution to the problem takes advantage of the fact that the limiting primer T_m is higher than that of the excess primer T_m . The sensitivity of the assay is thus increased about 10 fold.

In addition to various methods of selective sequence amplification, droplet digital PCR has become another method of choice for detection of rare mutation³². Several studies have reported detection of KRAS mutations using this approach in which each droplet contains either a single DNA molecule or no DNA^{33,34}. The detection sensitivity reaches 0.01% ~ 0.001% for certain alleles. The hairpin blocker method can readily be combined with droplet PCR to further improve detection sensitivity. In addition, the combined approach is very attractive because it makes dilution of the sample to single copy of DNA in each droplet unnecessary, thus eliminating the need for having “wasted” droplets that contain no DNA. For instance, if 1000 copies of wild type DNA were present in each droplet, no droplet would generate a signal. In contrast, if 1% of droplets signaled, the frequency of the mutant would be 0.001% (the current detection limit of droplet PCR) and if 0.1% of droplets signaled (i.e., 1 droplet in 1000), the frequency of the mutant would be 0.0001% (1/10⁶). Even lower frequencies of mutant alleles could be detected by rapidly screening several thousands of droplets at a time, as is currently done³³, thereby making detection sensitivity virtually unlimited. Finally, the combined approach would make it easy to construct multiplexed reactions for analysis of several gene targets at the same time in the same droplets.

Methods

Oligonucleotides used in the experiments. Limiting Primer, LP: 5'ACTCTTGCC-TACGC 3' ($T_m=52^\circ\text{C}$ at 50 nM)

Excess Primer, XP: 5'GTGGAGTATTGATAG 3' ($T_m=51^\circ\text{C}$ at 1000 nM)

Mismatched Excess Primer: 5'GTGGAGTATTGATAC 3' ($T_m=45^\circ\text{C}$, when mismatched to the target, and $T_m=51^\circ\text{C}$, when matched to the target at 1000 nM)

Wild-type synthetic target: 5'GTAGTTGGAGCTGGTGGCGTAGGCAAGAGT 3'

Mutant-type synthetic target: 5'GTAGTTGGAGCTGATGGCGTAGGCAAGAGT 3'

Blocker B1: 5' Cal Org 560-GCCTACGCCACCAGCTCC-Dabcy1 3'

Blocker B5: 5' Cal Org 560-CGCCGCTACGCCACCAGCTCCGGCG-Dabcy1 3'

Blocker B6: 5' Cal Org 560-GCGCCGCTACGCCACCAGCTCCGGCG-Dabcy1 3'

Blocker B7: 5' Cal Org 560-CGCGCCGCTACGCCACCAGCTCCGGCG-Dabcy1 3'

Blocker B8: 5' Cal Org 560-CCGCGCCGCTACGCCACCAGCTCCGGCG-Dabcy1 3'

Blocker B9: 5' Cal Org 560-GCCGCGCCGCTACGCCACCAGCTCCGGCG-Dabcy1 3'

Blocker as an Off-probe, Off62: 5' CGCGCCGCTACGCCACCAGCTCCGGCG-BHQ2 3'

On-Probe, On68: 5'Quas670-AAAACCTACCACAAGTTTATATTCAGTCATT-TTCAGTT-BHQ2 3'

Downstream probe, LTrpb: 5'BHQ2-CAAGACATGTACACATAATG-Quasar670 3'

All of the primers, probes and blockers were ordered from Biosearch Technologies (CA, USA). The probes and blockers were purified by double HPLC and the primers were salt-free purified. The binding sites of the blockers and probes to the targets are underlined. The nucleotides that form the stem of a blocker or a probe are bolded. The mutant position in the synthetic targets above is squared.

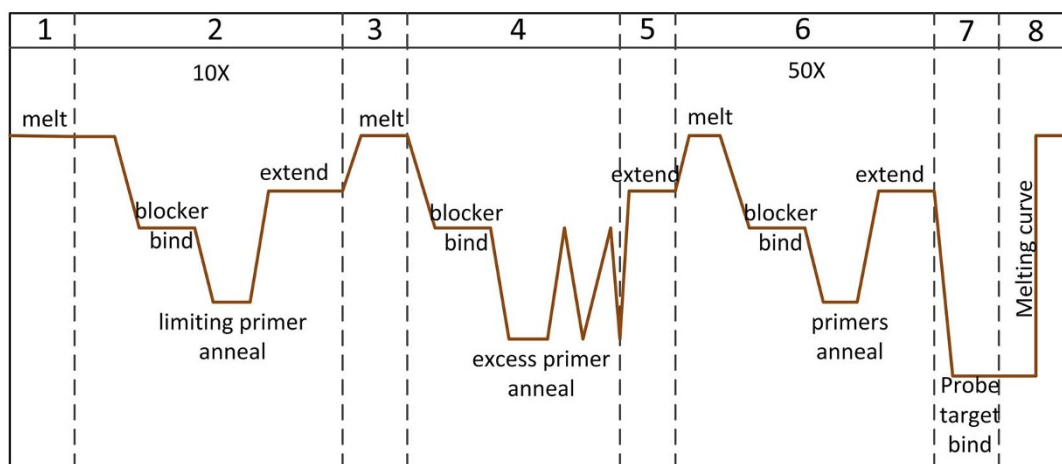


Figure 5 | PCR profiles for employing mismatched excess primer for selectively amplifying mutant type targets.

Target DNA used in the experiments. Plasmid DNAs were used as the targets in this work as a proof-of-principle investigation. The plasmids containing the interesting part of KRAS gene with wild type and mutant type alleles in codon 12 were ordered from Epoch Biolabs Inc. (Sugar Land, TX). An additional artificial single nucleotide polymorphism (SNP) was intentionally included in the mutant target sequence as shown below.

Wild type target excess primer strand is

5'CTGGTGGAGTATTGATAGTGTATTAACCTTATGTGT**G**CATGTTCT-AATATAGTCACATTTTCATTATTTTATTATAAGGCCTGCTGAAAATGAC-TGAATATAAAGCTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGC 3'

Mutant type target excess primer strand is

5CTGGTGGAGTATTGATAGTGTATTAACCTTATGTGT**C**ACATGTTCT-AATATAGTCACATTTTCATTATTTTATTATAAGGCCTGCTGAAAATGAC-TGAATATAAAGCTTGTGGTAGTTGGAGCT**GAT**GGCGTAGGCAAGAGTGC 3'

The blocker binding site is bold underlined and the GGT->GAT mutation in KRAS codon 12 is boxed in. The downstream probe binding site is double underlined and the artificial SNP site within it is shown as a larger bolded letter in the sequence.

Blocker Thermodynamic and Kinetic Investigation. 100 nM each blocker with different stem lengths from 1 bp stem to 9 bp stems (B1, B5, B6, B7, B8, B9) was mixed with 250 nM wild type target or mutant type target to obtain its thermodynamic equilibrium T_m . The solutions were kept at 30°C for 10 minutes to allow complete formation of probe-target hybrids. Then a melt starting at 30°C with 1°C increments at 30 s intervals to 90°C was monitored. The peak position of the negative first derivative of the melting fluorescence gave the T_m of each blocker.

In experiments investigating the kinetics blockers with different stem lengths to wild type or mutant type targets, 100 nM of each blocker was mixed with 250 nM wild type or mutant type target, or no target, established background fluorescence at a certain temperature. The mixtures were then heated to 95°C for 30 seconds to melt

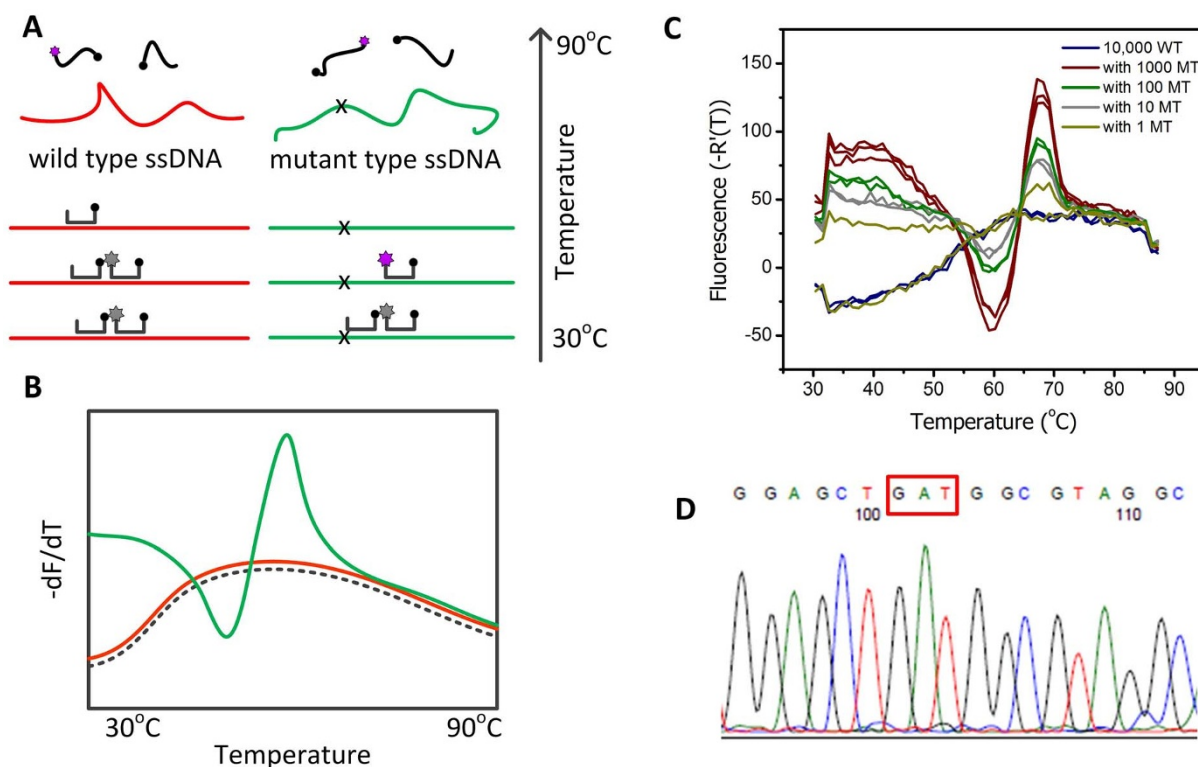


Figure 6 | (A) Schematics of the temperature-dependent hybridization of a set of Lights-On/Lights-Off probes to wild type and mutant type targets, where Lights-Off probe has a higher T_m than the Lights-On probe, and (B) their resulting fluorescent signature (the negative first derivative of fluorescence melting curve); red line, wild-type targets; green line, mutant targets; dotted black line, no template control. (C) Fluorescent signatures of 10,000 copies of pure wild type or a mixture of 10,000 copies of wild type targets with different concentration of mutant type targets. Here On68 and Off62 are consisted in the Lights-On/Lights-Off probe set and Off62 also works as a blocker. (D) Sanger sequencing results for the sample in (C) with single mutant type and 10,000 wild type targets.



the double strands, then were cooled to 60°C and held there for 5 minutes. Fluorescence reading was taken every 5 seconds at 60°C to monitor how much probe was bound to targets with time. All samples were run in duplicate.

All experiments were carried out in a Stratagene MX3005P PCR machine.

PCR Reaction mixtures. LATE-PCR amplifications were performed using 25 µl reaction mixtures containing 1× PCR buffer (Invitrogen, Carlsbad, CA), 3 mM MgCl₂, 200 nM dNTPs, 0.24× SYBR Green (Invitrogen, Carlsbad, CA), 50 nM limiting primer LP, 1000 nM excess primer XP/mmXP, 500 nM blocker B5/Off62, 100 nM molecular beacon probes LTprb/On68, 1 unit of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), and different concentrations of DNA target variants in the range of 1 to 10,000 copies, as determined by serial dilutions. Amplification reactions were run in duplicates.

PCR Profiles. Two excess primers were used in this work, perfectly matched (XP) or mismatched (mmXP). The LATE-PCR profiles used with the perfectly matched excess primer (XP) were as follows: 95°C for 3 min followed by 55 cycles of 95°C/10 sec, 60°C/2 min, 50°C/10 sec, 72°C/20 sec, followed by a 10 min pause at 30°C then a melt starting at 30°C with 1°C increments at 30 sec intervals to 90°C. SYBR signals were monitored during amplification and Quasar 670 signals from the molecular beacon probe were detected during the melting process.

When mmXP was used, the LATE-PCR profiles were as follows: 95°C for 3 min; 10 cycles of 95°C/10 sec, 60°C/2 min, 50°C/10 sec, 72°C/20 sec; 1 cycle of 95°C/10 sec; 1 cycle of 60°C/2 min and 40°C/30 sec with 4 cycles of quick cycling between 60°C and 40°C; 1 cycle of 72°C/2 min; 50 cycles of 95°C/10 sec, 60°C/2 min, 50°C/10 sec, 72°C/30 sec (with SYBR signal reading); followed by a 10 min pause at 30°C, then a melt starting at 30°C with 1°C increments at 30 sec intervals to 90°C. Quasar 670 signals from the Lights-On/Lights-Off probe sets were detected during the melting process.

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Author contributions

The project was designed by L.J.W., Y.W.J. and J.A.S. Experiments were run and analyzed by Y.W.J. The paper was written by Y.W.J., L.J.W. and J.A.S.

Additional information

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