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A kinetically controlled, isothermal method for the detection of single nucleotide mismatches

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ABSTRACT

We describe an isothermal, enzyme-free method to detect single nucleotide differences between oligonucleotides of close homology. The approach exploits kinetic differences in toe-hold-mediated, nucleic acid strand-displacement reactions to detect single nucleotide polymorphisms (SNPs) with essentially "digital" precision. The theoretical underpinning, experimental analyses, predictability, and accuracy of this new method are reported. We demonstrate detection of biologically relevant SNPs and single nucleotide differences in the let-7 family of microRNAs. The method is adaptable to microarray formats, as demonstrated with on-chip detection of SNP variants involved in susceptibility to the therapeutic agents abacavir, Herceptin, and simvastatin.

The human genome contains a large number of variations (polymorphisms), the most frequent of which are single-nucleotide polymorphisms (SNPs). SNPs reflect phenotypic differences between individuals and thereby signify susceptibility to diseases (disease genetics) and sensitivity to therapeutic agents (pharmacogenomics).[1–5] Thus, one can envision many applications to personalized medicine, therapeutics, and diagnostics. For example, the presence of specific members of a particular microRNA family, differing from other family members by just a single nucleotide, can accurately identify certain cancers.[6,7] Further, SNP variations among genomes of microbial pathogens can be used to track the emergence of highly virulent strains.[8] Thus, improved methods for determining SNPs would have widespread importance in the practice of medicine.

Currently, there are several effective technologies for SNP detection and genotyping, which fall into two broad categories: enzyme-assisted or hybridization-based methods. In enzyme-assisted SNP detection, the power and specificity of various enzymes, such as DNA polymerases, endo- and exo-nucleases, or DNA ligases, are employed to label and readout SNP variants. Despite improvements in this strategy, SNP analysis can be limited by the technical aspects of genotyping, especially pertaining to accuracy, cost-effectiveness, and throughput. In hybridization-based SNP detection, thermostability differences between perfectly matched and mismatched oligonucleotide target/probe pairs are employed. Although this strategy is fairly straightforward, its success is very dependent on many factors, such as the length of the target, sequence of the probe, location of the SNP variant within the probe, and reaction conditions. Because hybridization of SNP-specific probes requires binding with the target oligonucleotide near the melting temperature (T_m) of the resulting DNA duplex,[9–11] energetic differences for single nucleotide mismatches within probe oligonucleotides can be small (i.e., T_m values can be very similar), thereby yielding equivocal results.[12] Herein, we describe a facile strand-displacement hybridization method that is isothermal (room temperature), enzyme-free, and driven by the kinetics (rather than thermodynamics) of DNA target-probe hybridization, which circumvents key limitations of prior SNP-detection methods that are caused by near-equal T_m values and short probe lengths.

The present study builds on the pioneering studies on toe-hold-mediated nucleic acid strand-displacement hybridization.[12] Toe-hold-mediated strand-displacement reactions[13,14,12,15,16] have been elegantly employed in the construction of oligonucleotide-based logic gates,[17–23] dynamic networks,[24–26] and artificial motors;[27–33] in sequence-specific nucleic acid detection;[34–36] as universal translators;[20,21] and in signal amplification protocols.[37–39] This process involves initial binding of an incoming strand to the complementary single-stranded overhang segment of a nucleic acid duplex, termed the toe-hold, to form a three-stranded (branched) complex. Then, in a stepwise fashion, the incoming strand rapidly invades the duplex (branch migration) and displaces the shorter strand from the complex (Fig. 1).[12,14,22,25,38,40] The overall process is thermodynamically favorable, being driven by the higher stability of the final duplex, while the kinetics of the reaction is governed by the

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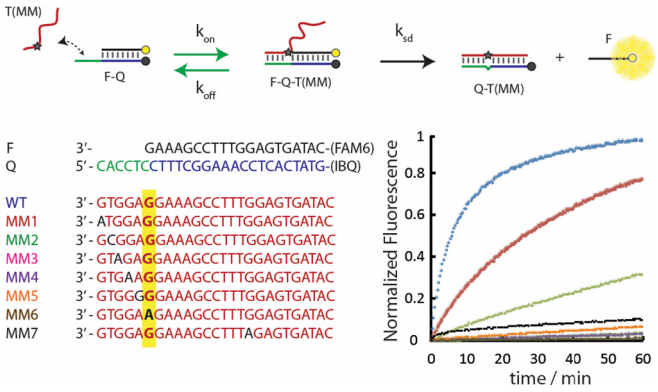

Fig. 1. (a) T(MM)(red) were incubated with F–Q to afford Q-T(MM) with concomitant release of F and an increase in fluorescence. Kinetic constants, k_{on}, k_{off}, and k_{sd} govern the release of F from F–Q. Mismatches in the toe-hold binding domain increase the value of koff, slowing the release of F from F–Q due to the lower accumulation of F–Q-T(MM). (b) The mismatch position in the toe-hold binding domain affects the kinetics of toe-hold-mediated strand displacement. Mismatched nucleotides distal to the branch migration initiation site (branch migration initiation site is shown highlighted) have less pronounced effects on strand displacement, while mismatched nucleotides adjacent to that site impede strand displacement. Sequences used: Targets (red), mutations within each target (black), and toe-hold regions (green) are shown.

rate-limiting step of binding of the incoming strand to the toe-hold. By varying the toe-hold binding energy, the overall rates of strand-displacement reactions can vary by as much as 10^6-fold, as long as the toe-hold binding energy is less than ~9 kcal/mol (i.e., toe-holds of six nucleotides or less in length).[12] We estimated with NuPACK software[41] that a mismatched nucleotide in a short toe-hold segment would decrease the toe-hold binding energy by roughly 4 kcal/mol. Such a sizable energy difference predicts a difference in strand-displacement kinetics of nearly three orders of magnitude. We therefore sought to capitalize on the different kinetic rates of toe-hold-mediated strand displacement to discriminate between SNPs or oligonucleotide sequences of very close homology. While the kinetics of toe-hold-mediated strand displacement have been thoroughly investigated,[12] the utility of toe-hold-mediated strand-displacement reactions in the detection of SNPs has not been fully explored.[42] Others have demonstrated the utility in toe-hold mediated strand displacement reactions using clever DNA origami techniques; however, the dynamic nature of multiple probes in solution led to results that appeared to erode over time, despite the thermodynamic favorability of the matched vs. mismatched duplex.[42]

We first examined the positional effects of mismatched bases on strand-displacement kinetics. We designed a reporter duplex, composed of two strands, one bearing a 6-carboxyfluorescein (FAM6) fluorophore (**F**) and the other bearing an Iowa Black hole quencher (IBQ) moiety (**Q**), where **Q** contains a six-nucleotide toe-hold domain. We then incubated the reporter with a series of oligonucleotides (**T**), based on the cystic fibrosis susceptibility SNP sequence rs-6679677. This series of oligonucleotides (**T**) was designed to have either a mismatched base pair with **Q** at one of the six positions found in the toe-hold domain (**MM1**, **MM2**, **MM3**, **MM4**, **MM5**, **MM6**), a mismatch within the **F–Q** duplex domain (**MM7**), or no mismatch (**WT**) (Fig. 1). Each **T** was added to **F–Q** and the release of **F** was monitored by the increase in fluorescence (Fig. 1). Notably, displacement of **F** was severely hampered when a mismatch occurred at the branch migration site (**MM6**, Fig. 1). However, if a mismatch occurred distal to the point of branch migration initiation, then displacement of **F** would occur slowly but without significant impediment. Previous reports have indicated that mismatch discrimination is possible when **Q–T** bears a mismatch within the **F–Q** duplex domain, but mismatches within the toe- hold domain were not explored.[35] Importantly, we observed that the presence of a mismatch in the toe-hold domain offerssuperior discrimination of single nucleotide differences, as is evident when comparing **MM7** to **MM3**, **MM4**, **MM5**, or **MM6**. The above study demonstrates that kinetic discrimination in isothermal strand displacement reactions can be a simple and powerful means for detecting nucleic acid mismatches.

However, because the product **Q–T** duplex is always thermodynamically more stable than the starting **F–Q** duplex (whether or not there is a mismatch), the initial kinetic differences between matched and mismatch reactions would invariably erode over time. Therefore, this approach will always suffer from a trade-off between analysis time and detection accuracy, making the process less than ideal for routine diagnostic applications. Accordingly, we sought to reconfigure the process into one involving *in situ* competitive strand-displacement reactions, which ought to yield a time-independent readout of the initial kinetics of matched vs. mismatched strand-displacement reactions. This objective can be achieved by competing out the unwanted (signal-degenerating) hybridization of **T(MM)** strands to **F–Q**. To suppress hybridization of **T(MM)** strands with **F–Q**, we introduced another strand, **S**, to serve as a "scavenger strand". We also modified our experimental design to displace and release **Q** rather than **F**, thereby minimizing the background fluorescence (Fig. 2a). In this setup, we have supplied two duplexes, **F–Q**, and **S–Q**, wherein **T(WT)** or **T(MM)** has the potential to hybridize to both duplexes. The importance of **S** is manifested in its ability to suppress hybridization of **T(MM)** to **F**, as **S** is the complementary sequence of **T(MM)**. Due to the stringent sequence and binding requirements of the toe-hold domain for toe-hold-mediated strand displacement, **T(MM)** will hybridize much faster to the **S–Q** complex as opposed to the **F–Q** complex, due to the lack of mismatches in the toe-hold binding domain of **T(MM)-S** (Fig. 2b). In contrast, **T(WT)** will hybridize much faster to **F–Q** as opposed to **S–Q**. Notably, when **T(MM)** is hybridized to **S**, there are no free single-stranded domains, rendering the **T(MM)-S** complex inert and unable to further react with any other single-stranded oligonucleotide species. Thus, **T(MM)** is prevented from binding **F** and causing any increase in fluorescence signal. To differentiate the two sequences **T(MM)** and **T(WT)**, which differ by only a single nucleotide, a three-strand system is thus required, composed of both **F–Q** and **S–Q** species. The addition of **T(MM)** to such a system would not give rise to any output signal irrespective of time, while addition of **T(WT)** would generate a fluorescence signal (Fig. 2c). Previous studies have shown that strand-displacement reactions can be approximated by using second-order reaction kinetics.[16,38] Additionally, the rate of strand-displacement reactions is modulated primarily by the toe-hold binding energy.[12] The apparent rate constant for the bimolecular reaction can be derived by using a quasi-steady-state approximation (Eq. (1)).[43]

$$k' = \frac{k_{sd}}{\frac{k_{sd}}{k_{on}} + exp(\frac{\Delta G_{toe}}{RT})} \quad (1)$$

The apparent rate constant, k', is composed of k_{sd}, k_{on}, and ΔG_{toe}, corresponding to the strand displacement rate constant, toe-hold binding rate constant, and the toe-hold binding energy, respectively. The rate constants, k_{sd} and k_{on}, were systematically investigated by Winfree and co-workers, and calculation of the toe-hold binding energy with programs such as NUPACK[44–46] was found to be highly congruent with experiment.[14] The rate constants, experimentally determined by Winfree and co-workers, allowed us to calculate the toe-hold binding energy by using NUPACK, and to predict the behavior for our three-component system. Assuming that our system behaves like a parallel bimolecular