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Multiplex SNP Discrimination

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ABSTRACT Multiplex hybridization reactions of perfectly matched duplexes and duplexes containing a single basepair mismatch (SNPs) were investigated on DNA microarrays. Effects of duplex length, G-C percentage, and relative position of the SNP on duplex hybridization and SNP resolution were determined. Our theoretical model of multiplex hybridization accurately predicts observed results and implicates target concentration as a critical variable in multiplex SNP detection.

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Personalized medicine, medical diagnosis, and prognosis based upon the unique genotype of individuals require the diagnostic capability of detecting and discerning multiple genetic markers (targets) within the genome of each individual. Multiplex assays, involving the simultaneous hybridization and detection of multiple genetic markers, promise to be a cornerstone of modern genetic testing and personalized genotyping. However, before the potential of multiplex assays and the prospects for personalized medicine can be fully realized, a more complete understanding of the underlying physics and chemistry of multiplex hybridization must be established. Such an analytical understanding provides superior insight into design and interpretation of multiplex hybridization reactions (1). In this communication, our analytical approach has been applied to the design of a multiplex assay for the discrimination of single nucleotide polymorphisms.

Single nucleotide polymorphisms (SNPs) that occur in the sequences of the human genome constitute the most ubiquitous and subtle genetic variations between individuals and different populations of individuals. With rapid accumulation of personal genomic sequence information, the influence of SNPs on many important phenotypes, and the role of SNPs in disease susceptibility and the response to therapeutic treatment, are coming to light. This emergence of information makes it ever more important to develop high-throughput, and sensitive, reliable, and rapid SNP genotyping assays that can clearly discriminate and resolve SNPs from their perfectly matched duplex counterparts.

Nucleic acid diagnostic assays based on multiplex hybridization offer unprecedented capabilities for systematic high-throughput screening, discrimination, and analysis of large numbers of DNA (and RNA) sequences. However, multiplex hybridization reactions are much more complex than those composed of only two complementary single strands. Increased complexity in a multiplex hybridization environment arises from the vast number of mispairing interactions that can occur, leading to crosshybridization and mismatched duplex formation (1). Complexity of the multiplex environment and the resulting competition between perfect match and mismatch strands strongly influence both kinetic and equilibrium behaviors and severely alter amounts of overall reaction products. Several authors have attempted to describe effects of such competition and associated hybridization errors on multiplex reaction results (1–9).

Initial theoretical and numerical studies of multiplex hybridization kinetics have revealed various distinct temporal behaviors with unexpected consequences due to competition in reactions between perfect match and mismatch duplexes (1). These results suggested that the effects of competitive hybridization might be utilized for optimizing SNP detection. Experiments described below were performed to investigate conditions for observation of optimum discriminatory behavior of individual probe/target subsystems in a complex (multiplex) hybridization environment.

Experimental setup consisted of six subsystems and two controls spotted to a glass microarray slide with the general design and sequences shown in Fig. 1. Each subsystem was composed of different probe pairs. The targets for each subsystem were designed to form either a perfect match duplex, or a duplex containing a single basepair mismatch with each of the probes in the subsystem. Two control probes having the same G-C composition were also spotted at various positions on the microarray. Target strands labeled with Cy-3 were hybridized in multiplex fashion (i.e., all in the same mix) to microarrays containing 14 probes (six probe pairs and two controls) located in at least four different places on the microarray; in total, each array contained 234 individual probe spots. Experiments were conducted at 25°, 40°, and 55°C.

At incubation times of 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min, a microarray was removed, washed, fixed, and read. The average fractional intensities were determined and are displayed in Fig. 2. Solid lines drawn through the data points are scaled predictions of our theoretical (numerical) model. For each subsystem, the predicted curve for the

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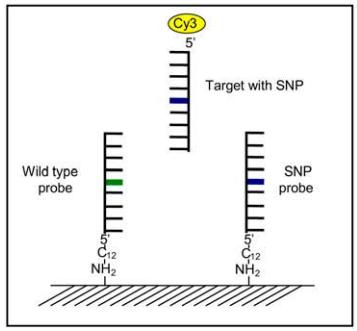

	Probe Sequence 5′⟶3′	Function	Target Sequence 5′⟶3′	Function
(a)	AATCACATTGCT[G]ACTGAACAAGT	SNP probe	ACTTGTTCAGT[C]AGCAATGTGATT	SNP target
	AATCACATTGCT[A]ACTGAACAAGT	PM probe		
(b)	GTACACCGCCCT[G]GCTTGCACACA	SNP probe	TGTGTGCAAGC[C]AGGGCGGTGTAC	SNP target
	GTACACCGCCCT[A]GCTTGCACACA	PM probe		
(c)	AGTTGACTA[G]CCGAAGAT	SNP probe	ATCTTCGG[C]TAGTCAACT	SNP target
	AGTTGACTA[A]CCGAAGAT	PM probe		
(d)	GTGATGTCT[G]CACGTGAG	SNP probe	CTCACGTG[C]AGACATCAC	SNP target
	GTGATGTCT[A]CACGTGAG	PM probe		
(e)	GTGACACATAAGTACAT[G]	SNP probe	[C]ATGTACTTATGTGTCAC	SNP target
	GTGACACATAAGTACAT[A]	PM probe		
(f)	ATAAGCACATTGACACAAAGGAT[G]	SNP probe	[C]ATCCTTTGTGTCAATGTGCTTAT	SNP target
	ATAAGCACATTGACACAAAGGAT[A]	PM probe		
(g)	TTTGCGGTCAAAATTGGATCAACTGCTACA	control probe	TGTAGCAGTTGATCCAATTTTGACCGCAAA	control target
	GTCACACTAGGCGCCACTGTGCGTGACATC	control probe	GATGTCACGCACAGTGGCGCCTAGTGTGAC	control target

FIGURE 1 Design of probes (*left*) and sequences (*right*). Target strands were synthesized on the 100 nM scale with Cy-3 on the 5′ end. DNA probe and target strands were synthesized, with standard purification, and used directly as obtained from the commercial supplier (IDT, Coralville, IA). Probes were spotted using a 50 μM DNA solution (the actual concentration on the spot is not precisely known, but is assumed to be the same at each spot). Sequences contained 18 or 24 bases and varied in G-C% from 30% to 60%. SNP positions (indicated by brackets) were either in the middle or on the 3′ end.

perfect match duplex was first normalized, and then uniformly scaled to the equilibrium value, averaged over all temperatures, of the perfect match intensity of that subsystem. Scaling factors varied from 0% to 40%. Temperature in the fitting was allowed to vary from 25° to 55°C, with best results achieved at an average of 35 $\pm$ 10°C. As seen in the figure, a reasonably good fit of the data is obtained for both perfect match and SNP duplexes.

Clear resolution between perfect match and SNP duplexes, where the perfect match duplex intensity was significantly higher than that of the SNP duplex, was seen in nearly every case. In those cases where resolution was less clear, the SNP (mismatch) occurred on the ultimate 3′ end of the probe molecule. Collectively, results in Fig. 2 indicate that better discrimination occurs for SNPs that lie in the middle of shorter duplexes. Recall, these reactions took place in a multiplex hybridization environment where all targets were present simultaneously in the reaction mixture. Such consistent resolution further indicates that for the probe/target subsystems that were designed and utilized, crosshybridization

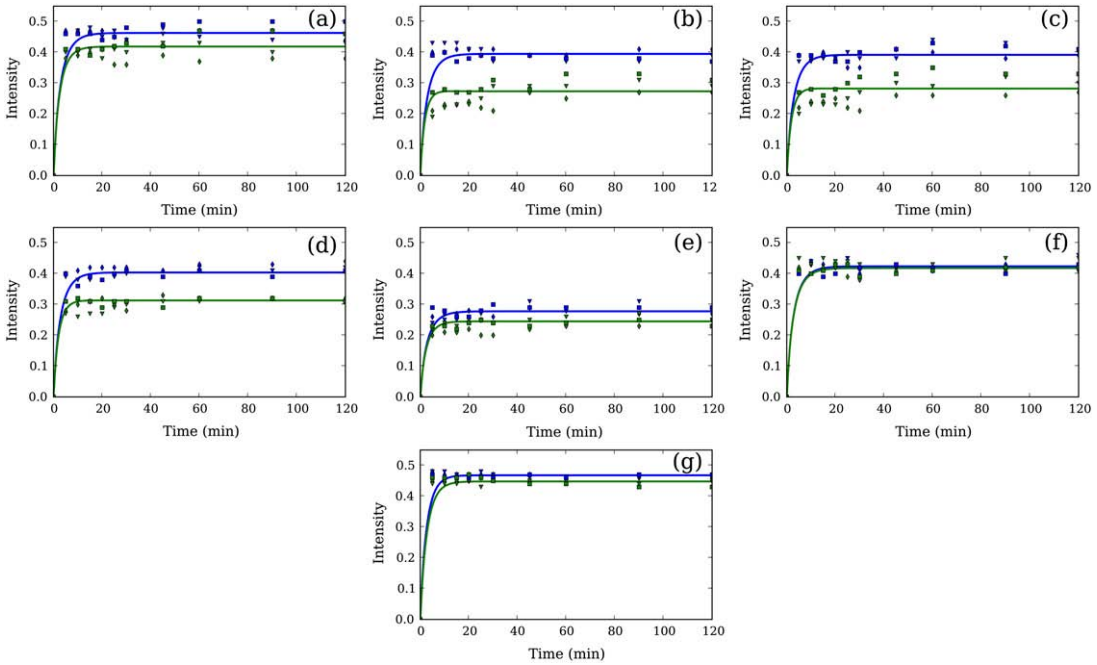

FIGURE 2 Plots of intensity versus hybridization time for each of the targets shown in Fig. 2 with the probe pairs (*a–f*) and two controls (*g*). The six target strands and two controls were hybridized by adding a 10 μM solution of target to microarrays incubated at 25°, 40°, and 55°C (*diamonds*, *triangles*, and *squares*, respectively) and allowed to react for varying times from 5 min to 2 h before washing, fixing, and reading. Solid curves depict scaled best fits of simulated kinetics from the theoretical model; theoretically predicted curves were fit to experimental data for each subsystem (see text). Blue colors represent perfect match duplex intensities, green represents SNP intensities. Average standard deviation is approximately the size of the symbols.

between subsystem components was not a significant factor. Under these conditions, the effects of temperature from 25° to 55°C are minimal.

Although SNP discrimination was evident, optimum discriminatory behavior was not observed in these initial experiments. With the goal of achieving enhanced SNP discrimination, kinetic simulations of the multiplex system were performed and calibrated with experimental data. Results for a representative target-probe set are shown as solid curves in Fig. 3. The upper blue and green curves were calculated assuming a relatively high theoretical target-probe concentration ratio. These curves fit the data in Fig. 2, and are shown as they appear in the model calculation (before scaling). The lower pair of solid curves in Fig. 3 shows results of the simulated experiment at significantly lower target concentration. The model predicts enhanced discriminatory behavior should be observed if the target-probe concentration ratio used in the calculations is reduced by a factor of 10^4. This enhanced resolution suggests that a lower abundance of available target leads to a competitive dynamic between perfect match and SNP probes, which in turn leads to displacement of less favorable SNP reactions.

To test this prediction, an identical series of hybridization experiments was performed with the same microarray design and same target strand mixture added, except that the concentration of added targets was reduced to 400 pM, a 2.5×10^4 lower relative concentration of target. Representative results are shown as square symbols in Fig. 3. A complete analysis and report of these, and additional relevant microarray experiments that have been performed, will be described in a subsequent publication (D. J. Fish, M. T. Horne, G. P. Brewood, and A. S. Benight, unpublished).

In summary, results that are presented reveal several interesting features of multiplex SNP hybridization and

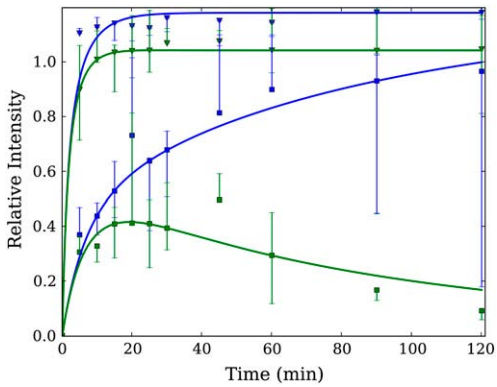

FIGURE 3 Theoretical and experimental results of SNP discrimination experiments. Solid curves display predicted behavior at a high concentration ratio (*top set of curves*) and a lower ratio (*bottom set*). Data points show best fit (within one standard deviation) of experimental results to numerical prediction. Triangle markers indicate high concentration ratio, square markers indicate lower ratio. Blue color refers to perfect match duplex intensity, green to mismatch.

detection: 1), Hybridization is essentially complete, and SNP resolution is obtained in 1–2 h. 2), Better resolution is obtained when the SNP is located near the middle of the strand versus the ultimate end. 3), Better SNP resolution is obtained when shorter sequence fragments are employed. 4), Better resolution is obtained in higher GC% sequences. 5), Target concentration is a critical experimental parameter in achieving optimal discrimination of SNPs.

This study clearly demonstrates how our rigorous theoretical model provides guidance in experimental design as well as interpretation. Other experimental observations of enhanced discrimination of SNP duplexes due to competitive dynamics were also recently reported (10). These, along with results reported here, validate the efficacy of our theoretical approach to experimental design. Such analysis should prove invaluable in the future design of specific genotype assays, and find a central role in enhancing microarray performance and advancing personalized medicine.

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