Analysis of single nucleotide polymorphisms with solid phase invasive cleavage reactions

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ABSTRACT

Using microparticles as the capture surface and fluorescence resonance energy transfer as the detection technology, we have demonstrated the feasibility of performing the invasive cleavage reaction on a solid phase. An effective tool for many genomic applications, the solution phase invasive cleavage assay is a signal amplification method capable of distinguishing nucleic acids that differ by only a single base mutation. The method positions two overlapping oligonucleotides, the probe and upstream oligonucleotides, on the target nucleic acid to create a complex recognized and cleaved by a structure-specific 5′-nuclease. For microarray and other multiplex applications, however, the method must be adapted to a solid phase platform. Effective cleavage of the probe oligonucleotide occurred when either of the two required overlapping oligonucleotides was configured as the particle-bound reagent and also when both oligonucleotides were attached to the solid phase. Positioning probe oligonucleotides away from the particle surface via long tethers improved both the signal and the reaction rates. The particle-based invasive cleavage reaction was capable of distinguishing the ApoE Cys158 and Arg158 alleles at target concentrations as low as 100 amol/assay (0.5 pM).

INTRODUCTION

Currently there is a great deal of interest in solid phase assays for nucleic acids analysis. DNA chips (1), microarrays (2), fiber optic arrays (3) and particle-based assays (4) are all gaining prominence for nucleic acid applications. They offer the multiplexing capabilities required for high throughput analysis since a large number of different assays can be conducted simultaneously on a single sample. Many solid phase techniques are based solely on hybridization, although single base differences are often difficult to uncover with hybridization-only approaches (5–7). Other techniques, such as the oligonucleotide ligation assay, for example, incorporate the specificity of an enzyme to enhance discrimination of sequences that differ at only a single position (8,9). In this paper we describe adapting an alternative enzymatic approach, the invasive cleavage reaction, to a solid phase platform.

The invasive cleavage assay (10) is a probe cycling, signal amplification reaction used for detection of single nucleotide polymorphisms (SNPs) (11) and quantitative determination of gene expression and viral load. The reaction requires two synthetic oligonucleotides, called the 'upstream oligonucleotide' and 'probe', that anneal to the target sequence with an overlap of 1 nt. This creates a bifurcated overlapping complex that resembles a structure generated during strand displacement DNA synthesis, as shown in Figure 1. Structure-specific 5′-nucleases, whose primary cell function is believed to be processing of Okazaki fragments (12,13), cleave the bifurcated substrate at the site of the overlap, releasing the 5′-arm and one base paired nucleotide of the probe (14,15). The cleaved 5′-arm serves as a signal indicating the presence of target in an analyzed sample. By performing the invasive reaction at the

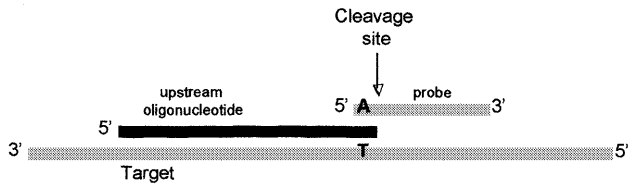

Figure 1. Substrate for invasive cleavage reaction. The probe and upstream oligonucleotides anneal to the target and create a 1 bp overlap. Cleavage of the probe occurs at the 3′-side of the overlapping nucleotide, releasing the probe 5′-flap. The reaction is run near the probe–target T_m with the probe oligonucleotide in excess. Cleavage of a bound probe destabilizes the duplex and results in replacement of the cleaved probe with another uncut probe. Thus a single target directs cleavage of multiple probes.

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Table 1. Oligonucleotides for solid phase invasive cleavage studies

(A) Model system oligonucleotides			
	Upstream		5′-(B)atagagccataaactcaaagtggtaataat-3′
	Probe		5′-(Fl)gagt(Cy3)cctgtgat(B)c-3′
	Target		5′-tgacaaa*atcacaggtact*Cttattaccactttgagtttatggctctat-3′
(B) ApoE oligonucleotides			
	Upstream	No spacer	5′-ccccggcctggtacactgccaggct-3′
		Long spacer	5′-(B)SpSpSpSpSpSpSpSpSpccccggcctggtacactgccaggct-3′
	Cys probe	No spacer	5′-(D)actt(Fl-dT)tgcaggtcatcgg(B)-3′
		Long spacer	5′-(D)actt(Fl-dT)tgcaggtcatcggSpSpSpSpSpSpSpSpSp(B)-3′
	Target	Cys	5′-cgcgatg*ccgatgacctgcagaag*Tgcctggcagtgtaccaggccggggcccgcga-3′
		Arg	5′-cgcgatg*ccgatgacctgcagaag*Cgcctggcagtgtaccaggccggggcccgcga-3′

Underlining in each target sequence indicates the region complementary to the upstream oligonucleotide; italic marks sequence complementary to the probe. The capitalized base is the site of the SNP. D, dabcyl; Cy3, indocarbocyanine-3; Fl-dT, fluorescein-deoxythymidylic acid; B, biotin; Sp, hexaethylene glycol spacer.

probe melting temperature (T_m), multiple cleavage events can be achieved for each target. Typically, an invasive signal amplification reaction generates 30–50 cleaved probes/target/min, resulting in 10^3–10^4-fold signal amplification in a 1–3 h reaction (16). By combining two invasive reactions into a serial assay, the signal amplification can be increased to 10^7-fold, which is sufficient to detect 600 copies of unique sequence in samples of human genomic DNA in 2–4 h using a standard fluorescence plate reader (17).

The unique ability of 5′-nucleases to specifically cleave the overlapping substrate can be utilized for detection of single base mutations. To detect a particular nucleotide in the target sequence, the upstream oligonucleotide and probe are designed to create overlap at this nucleotide, ensuring efficient cleavage of the probe. A substitution at this nucleotide position eliminates the overlap and dramatically reduces the cleavage rate, resulting in mutation discrimination of at least 300:1. Such discriminatory power makes the invasive cleavage assay an excellent tool for identification of SNPs in human genomic DNA (10,17).

The number of different formats that can be applied for signal detection emphasizes the versatility of the invasive cleavage assay. These include electrophoresis (18–20), microplate enzyme-linked immunosorbent assay (ELISA) (10) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry methods (21). More recently, a fluorescence resonance energy transfer (FRET) methodology enabled homogeneous detection of SNPs by the invasive cleavage reaction using zeptomole (10^{-21} mol) amounts of target DNA (17).

Adapting the invasive cleavage assay to a solid phase format presents the possibility of analyzing multiple SNPs in parallel. At the present time, SNP detection using the invasive cleavage reaction is performed in 96-well microplates with nanogram amounts of human genomic DNA per SNP (17). Assuming that the total number of SNPs in the human genome is 3×10^6 (11), a complete genotype analysis of a single individual would require 3×10^4 plates and tens of milligrams of the DNA. A solid phase format could potentially reduce the analysis to a single SNP chip that interrogated a small volume sample of human DNA. We therefore investigated the feasibility of adapting the FRET detection version of the invasive cleavage assay to a solid phase format. So that we could readily monitor the reactions in real time, we selected latex microparticles suspended in reaction solution as the solid phase.

We utilized two different oligonucleotide systems for our FRET studies of solid phase invasive cleavage reactions. First we employed a simple model system where the probe was 5′-end-labeled with a fluorescein molecule quenched by an internal Cy3 molecule (Table 1A). Upon cleavage, the fluorescein was released into solution, providing a measurable signal correlated with the amount of cleavage. Next we analyzed the apolipoprotein E (ApoE) SNP at position 158 (22) in a particle-based assay with a probe whose donor–acceptor pair was in the reverse configuration. In this format, the probe contained a 5′-terminal dabcyl molecule that quenched an internal fluorescein (Table 1B). Upon cleavage, the quenching dabcyl moiety was released into solution, while the resulting signal from the fluorescein remained associated with the solid phase.

Our results demonstrate that the multi-component substrate for the invasive cleavage reaction can indeed be properly assembled, accurately recognized by the structure-specific 5′-nuclease and efficiently cleaved on a solid surface. As in solution phase assays, a single target molecule associates sequentially with multiple probe molecules and facilitates cleavage of these probes, thus yielding linear amplification of the signal.

MATERIALS AND METHODS

Materials

Spherotech (Libertyville, IL) donated streptavidin-coated latex microparticles of density 1.05 g/cm^3. Chill-out 14 liquid wax was purchased from MJ Research (Cambridge, MA) and D-biotin from Pierce Chemical Co. (Rockford, IL). Phosphoramidite reagents for oligonucleotide synthesis were obtained from Glen Research (Sterling, VA). Other chemicals and buffers were from Sigma Chemical Co. (St Louis, MO). The Cleavase

enzyme was prepared and quantified by Third Wave Technologies (Madison, WI) as described (10). Enzyme storage, enzyme dilution buffer and oligonucleotide synthesis were detailed previously (17). Sequences of the oligonucleotides are listed in Table 1.

Unless otherwise stated, invasive cleavage assays were conducted in 10 mM MOPS, pH 7.5, 7.5 mM $MgCl_2$, 0.1% Tween-20, 10 µg/ml yeast tRNA, 5% enzyme dilution buffer, 10 ng/µl Cleavase and, for particle-based assays, 20 µg oligonucleotide-coated particles. To prevent evaporation, 50 µl Chill-out 14 liquid wax was layered on top of each reaction.

Data collection

Real-time FRET measurements of invasive cleavage assays were acquired with a previously described fluorometer constructed on an optical breadboard (23,24). The sample was heated in the temperature controlled tube holder for ~10 min and the reaction was initiated by manual addition of 10 µl enzyme. For each sample, a hundred 10 ms measurements of fluorescence intensity were made at 15 s intervals over a period of at least 30 min. To minimize potential photobleaching, an electronic shutter blocked the excitation beam from the sample except during each read.

Model system

Streptavidin-coated particles (0.86 µm diameter) were coated at 20% surface saturation with model system probe, upstream oligonucleotide or a 50:50 mixture of both. The coating procedure was essentially as described (24), but since both the probe and upstream oligonucleotides were biotinylated, we included an additional biotin-blocking step. After the 48 h coating period, particles were washed once and then resuspended in coating buffer containing 1 µM D-biotin and rotated for 10 min. Subsequent washes were as described, but the final particle resuspension was in 10 mM MOPS, 0.5% Tween-20, 0.5% Nonidet P-40.

In the same manner, streptavidin-coated particles were coated with the model system oligonucleotide at 40, 80 and 100% surface saturation. The particles for all model system studies were the same lot as those of a previous study (24), so the particle capacity at 100% surface saturation was known to be 11 pmol oligonucleotide/cm^2.

Invasive cleavage assays were conducted in 200 µl volume reactions at 45°C, the approximate T_m of the model system probe. Reactions were initiated by manual addition of enzyme in a 10 µl volume. The final concentration of enzyme dilution buffer for the model system assays was 1%.

ApoE system

Streptavidin-coated particles (0.83 µm diameter) were coated with ApoE probe or with a mixture of both probe and upstream oligonucleotide, essentially as described above for the model system, except that the biotin blocking solution contained 10 µM D-biotin. For each batch of particles prepared, a small batch of control particles was prepared under identical conditions but with probe oligonucleotide that did not contain the 5′-terminal dabcyl quencher. During the particle coating procedures, we quantified the amount of fluorescent oligomer in control particle wash solutions and thereby estimated the oligonucleotide surface capacity of these particles to be ~4 pmol/cm^2.

Invasive cleavage reactions were conducted in 200 µl volumes at either 60 or 54°C, as stated. Reactions were initiated by manually adding enzyme to the preheated tube, inverting the tube to mix, layering 50 µl of Chill-out on top and returning the tube to the heating block.

RESULTS

Model system invasive reaction functions in three solid phase configurations

For a functional solid phase invasive cleavage reaction, a three-component substrate consisting of target annealed to upstream oligonucleotide and probe must be assembled on the surface. Additionally, the enzyme must recognize this substrate and form a productive enzyme–substrate complex.

The first indication that this complicated series of associations could indeed occur on a solid phase came when we tested the invasive cleavage reaction in a format where the model system target strand and probe were present in solution while the model system upstream oligonucleotide was bound to microparticles. In this format, reaction kinetics for particle-based cleavage were virtually identical to those of a solution phase reaction containing the same amounts of each oligonucleotide. In control reactions, where target oligonucleotide was omitted from the reaction mixture, no probe cleavage occurred (Fig. 2A).

In a second assay format, the model system probe oligonucleotide was the particle-bound reagent. In a third format, equimolar amounts of both model system oligonucleotides were attached to the particles. The particle-based invasive cleavage reaction was also functional in these assay formats, but after the first 5 min or so of the reaction, cleavage on the solid phase occurred considerably slower than in solution phase reactions with equivalent amounts of each oligonucleotide. Again, in these formats, signal generation occurred only in reactions containing target oligonucleotide and not in control reactions with no target (Fig. 2B and C).

Higher concentrations of solid phase probe oligonucleotide increase reaction rates

Particles coated with model system probe at surface densities of 20, 40, 80 and 100% of the bead maximal oligonucleotide capacity were tested in invasive cleavage reactions. The initial rates of these particle-based reactions increased with surface density of the probe oligonucleotide and were very similar to initial rates of solution phase reactions containing equivalent amounts of each oligonucleotide (Fig. 3A). For reactions with particles saturated with probe oligonucleotide, the shape of the response curve was the same as for solution phase reactions with 75 pM probe (Fig. 3B). For reactions with particles coated with probe at <100% surface density, however, after ~5–10 min the rate on the particles decreased relative to the solution phase rate. The results in Figure 2B, where particles were 20% saturated with probe oligonucleotide, illustrate this trend.

Fluorophore and quencher can be reversed in solid phase reaction

Experiments with the model system demonstrated that the invasive cleavage reaction could indeed be successfully adapted to a solid phase format. Productive tripartite substrate

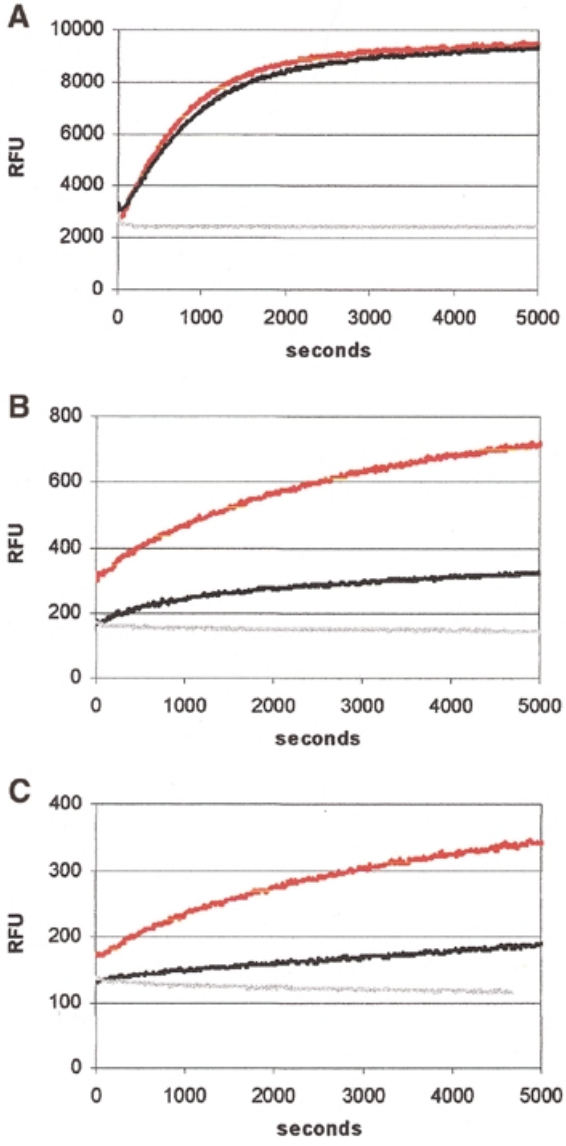

Figure 2. Model system solid phase and solution phase invasive cleavage reactions. Particles were coated with model system oligonucleotides at 20% surface saturation and tested in invasive cleavage reactions. Solution phase reactions (red) contained the same amount of each oligonucleotide as the particle-based reactions (black). Control particle-based reactions (gray) do not contain any target oligonucleotide. Graphs plot signal generation versus time. RFU, relative fluorescence units. (**A**) 15 nM upstream oligonucleotide in solution (red) or particles coated with upstream oligonucleotide (black and gray). Reactions included 500 nM probe in solution and either 1 nM target (black and red) or no target (gray). (**B**) 15 nM probe in solution (red) or particles coated with probe (black and gray). Reactions included 25 nM upstream oligonucleotide in solution and either 100 pM target (black and red) or no target (gray). (**C**) 7.5 nM each oligonucleotide in solution (red) or particles coated with an equal mixture of upstream oligonucleotide and probe (black and gray). Reactions included either 100 pM target (black, red) or no target (gray).

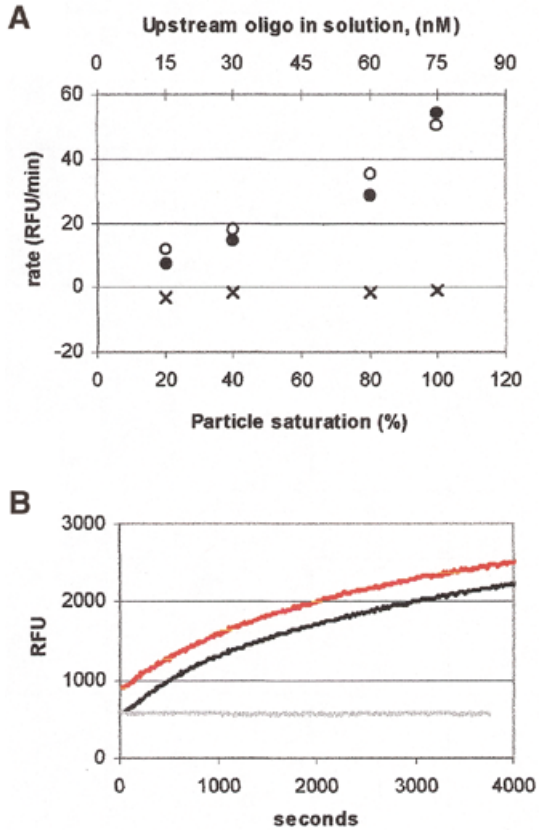

Figure 3. Model system reactions with various concentrations of solution phase and solid phase probe. (**A**) Particles coated at various surface densities with the model system probe were tested in invasive cleavage reactions with 50 nM upstream oligonucleotide and 100 pM target (filled circles) or no target (crosses). Each solution phase reaction (open circles) contained 50 nM upstream oligonucleotide, 100 pM target and an amount of probe equivalent to that in the corresponding particle-based reaction. The lower horizontal axis is for particle-based data; the upper axis is for the solution phase data. Initial reaction rates were calculated from the slopes of data from 30 to 480 s. (**B**) Invasive cleavage assay signal versus time for solution phase assay with 100 pM target (red), particle-based assay with 100 pM target (black) or particle-based assay with no target (gray). The surface of the particles was saturated with probe oligonucleotide.

molecules were assembled on the particle surface and enzyme cleavage resulted in signal amplification. In the model system the fluorescein molecule was released from the particle surface and detected in solution, so that assay configuration would be difficult to adapt to solid phase applications requiring signals at addressable locations. We therefore investigated a second oligonucleotide system based on the ApoE SNP at 158. In that system we reversed the positions of the fluorophore and quenching molecules on the probe. The dabcyl quencher was positioned at the 5′-end of the ApoE probe, while the fluoresceinated nucleotide was at the fifth position (Table 1B). Our objective was to test whether a productive tripartite substrate would assemble when a probe configured with a 5′ quencher and internal fluorescein was attached to the solid phase and whether target-specific cleavage of the probe would be detected by fluorescence increase of the particle-bound fluorescein. In the ApoE system, we utilized a single probe oligonucleotide sequence, which corresponded to Cys158, but we tested two different types of target sequences corresponding to the two human alleles for the SNP at residue 158 (see Table 1B). Cys target molecules, with sequence corresponding to Cys158 ApoE, have a T that is complementary to the A at the 5′-end of the probe. On the other hand, the Arg target, with sequence corresponding to Arg158, is not complementary to

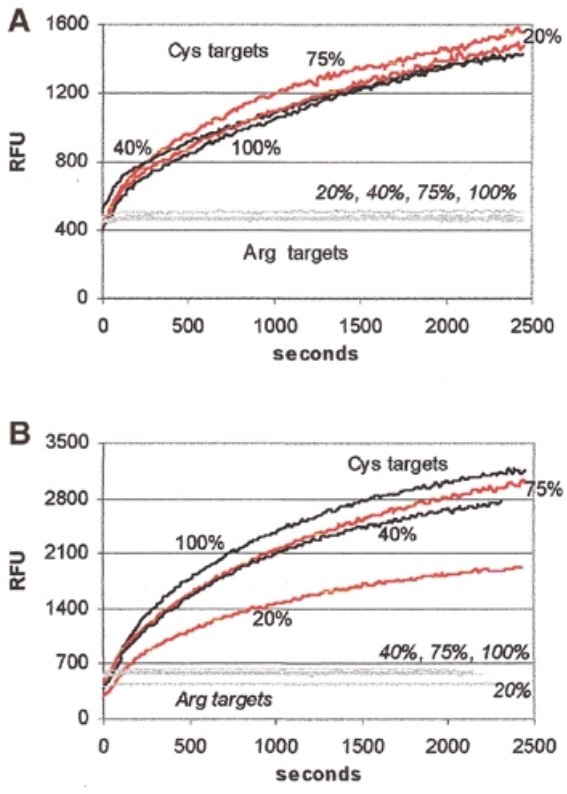

Figure 4. Solid phase ApoE reactions with various surface densities of short spacer or long spacer probe. Particles coated at various surface densities with the no spacer (**A**) or long spacer (**B**) ApoE probe were tested at 60°C in invasive cleavage reactions with 50 nM upstream oligonucleotide and 1 nM Cys target. Black lines represent Cys target data for probe surface densities of 100 and 40%; Cys target data for densities of 75 and 20% are shown as red lines. In the lower portion of each graph, Arg target data is represented by gray lines for all probe surface densities.

the final nucleotide of the probe. In solution phase invasive cleavage reactions, Cys probes are efficiently cleaved when the Cys target is included in the reaction but not when the Arg target is included.

To test whether particle-based ApoE probe could function in the invasive cleavage reaction and discriminate the single nucleotide difference between the Cys and Arg targets, we coated particles with the ApoE probe at four different surface densities and tested each type of particle with the Cys and Arg targets. The Cys target was cleaved well by particles coated with probe at all four surface densities, while the Arg target demonstrated virtually no cleavage (Fig. 4A). Unlike the model system particle-based reactions with surface-attached probe oligonucleotides, surface density of the ApoE probe had little effect on the initial reaction rates or overall signal generation kinetics. We hypothesize that self-quenching of probe fluorophores on the surface of ApoE particles at least partially accounts for this difference between the two systems.

Probes with long tethers exhibit higher signals and improved reaction rates

Attaching oligonucleotides to solid supports via long spacers has been demonstrated to improve solid phase hybridization (5,25). It seemed likely that positioning ApoE probes away from the surface of the particle with a long spacer might also be advantageous for solid phase invasive cleavage reactions. Hoping to minimize potential loss of signal from self-quenching and also improve reaction kinetics, we therefore synthesized a version of the ApoE probe with a long spacer containing 10 hexaethylene glycol units between the end of the probe sequence and the 3′ biotin. In our previous experiments, the 3′ biotin had been directly attached to the probe sequence without any intervening spacer (cf. Table 1).

When we conducted invasive cleavage reactions with particles coated with this long spacer probe at different surface densities, signals were dramatically higher than in reactions with particles coated with the ApoE probe containing no spacer (Fig. 4). As was the case with the model system, where the fluorescein molecule was released into solution, increasing the surface density of the long spacer version of the ApoE probe resulted in increased signal from the reaction. With the long spacer ApoE probe, the solid phase signal observed for the particles with a probe surface density of 40% of the saturating concentration was clearly greater than the signal observed for particles with 20% probe surface density. At the higher probe densities, however, the increase in solid phase signal was less dramatic, perhaps indicating that some fluorophore self-quenching occurred in this system at the increased surface densities, even with the long tethers.

For 1 nM Cys target, initial reaction rates on particles with the long spacer ApoE probe were ~2-fold faster than on particles with the no spacer probe, although the 20% saturated particles demonstrated somewhat less than a 2-fold rate increase (Table 2). With this relatively high concentration of Cys target, the reaction rate was linear for approximately the first 3 min, but became non-linear as the reaction progressed. Initial slopes for the 1 nM Cys target data in Table 2 are calculated from 0–2 min reaction data, a region where all types of particles demonstrated linear slopes with the Cys target. With 1 nM Arg target, however, there was a small increase in signal during the first minute or two of the reaction and then the signal remained essentially flat throughout the remainder of the reaction. The initial burst in signal after enzyme addition appears to be due to temperature and buffer equilibration of the reaction solution, since fluorescence of the fluorescein reporter molecule is extremely sensitive to environmental conditions (26). The Arg target data did not exhibit linear signal increases in the 0–2 min range, so the initial slopes reported in Table 2 for the 1 nM Arg targets are dominated by the equilibration-related signal increase. Slopes calculated from the 1 nM Arg target data in the linear region from 10–30 min are much lower, ranging from 0.2–0.6 relative fluorescence units (RFU)/min for the particles with no spacer probe and from 0.5–0.8 RFU/min for the particles with long spacer probe.

When 100 and 10 pM target concentrations were tested with the particles coated with long spacer ApoE probe oligonucleotide, responses of both the Cys and Arg targets demonstrated an initial burst in fluorescence signal in the first minute or two of the reaction. As described above for the 1 nM Arg target data, this increase appears to be related to equilibration of the solution after enzyme addition. Past the equilibration time, linear kinetics were observed for each Cys target level and each probe surface density (Fig. 5A and B). With 10 pM Cys target, the cleavage rate was ~1 RFU/min, a cleavage rate clearly distinguishable from the rate observed with 10 pM Arg target (Fig. 5C and Table 2).

Table 2. Effect of probe surface density on invasive cleavage reactions

Probe density	Initial slope, 0–2 min (RFU/min)				Ratio of initial slopes long spacer:no spacer	Slope, 10–30 min (RFU/min)			
	Long spacer probe (1 nM target)		No spacer probe (1 nM target)			Long spacer probe (100 pM target)		Long spacer probe (10 pM target)	
	Cys	Arg	Cys	Arg		Cys	Arg	Cys	Arg
100%	102	4.5	214	19	2.1	11	0.14	1.2	–0.041
75%	120	11	229	17	1.9	11	0.12	1.2	0.091
40%	102	13	234	14	2.3	9.1	–0.016	1.2	–0.056
20%	110	16	178	8.9	1.6	7.7	–0.12	0.88	0.019

Particles coated at various surface densities with either long spacer or no spacer ApoE probe were tested in invasive cleavage reactions at 60°C with 50 nM invader and 1 nM, 100 pM or 10 pM ApoE 158 Cys or Arg target. For reactions with 1 nM target (see Fig. 4), initial slopes were calculated from 0–2 min data, as explained in the text. The ratio of initial slopes is based on values for the Cys target. Reactions with 100 and 10 pM target were conducted on particles coated with the long spacer probe and slopes were calculated from 10–30 min data.

Because of better performance we utilized long spacer probes for all further investigations. Also, since the data did not point to any advantage for an oligonucleotide coating level of <100%, we used only surface-saturated particles in subsequent experiments. To determine the optimal reaction temperature for the ApoE system solid phase invasive cleavage reaction, we measured cleavage rates of the particle-bound ApoE probe with 10 pM Cys target. Of temperatures tested in the range 50–60°C, the optimal temperature was 54°C. Thus for further ApoE studies, 54°C became the standard reaction temperature. This temperature is similar to the 54.5°C optimum determined for the ApoE 158 invasive cleavage reaction on a planar surface (data not shown). For the solution phase reaction, the maximal reaction rate was observed at 60°C.

Reactions with both upstream oligonucleotide and probe attached to the surface

For parallel analysis of multiple SNPs, the solid phase invasive cleavage reaction would be greatly simplified if both upstream oligonucleotide and probe were confined on the surface. The results described above for the model system (Fig. 2C) support the feasibility of this approach. To further investigate this format, we synthesized upstream oligonucleotide for the ApoE invasive cleavage reaction with a 5′ biotin separated from the oligonucleotide sequence by 10 hexaethylene glycol spacers (Table 1). Particles were coated with mixtures of the probe and upstream oligonucleotide and tested in invasive cleavage assays with the Cys and Arg targets.

Figure 6A summarizes results from particle-based invasive cleavage reactions conducted with 5 or 10 pM Cys or Arg target. The particles had Cys probe:upstream oligonucleotide ratios of 1:3, 1:1, 3:1, 5:1, 10:1, 20:1 and 30:1. At all ratios tested, the particle-based assay was able to discriminate between the specific Cys target and the non-specific Arg target. In general, particles with higher ratios of probe:upstream oligonucleotide demonstrated greater separation of values for the positive and negative targets.

Titration of target for solid phase invasive cleavage reactions

To test discrimination between Cys and Arg ApoE targets at different target levels, we used the particles coated with probe and upstream oligonucleotide at a ratio of 10:1. Figure 6B shows that the solid phase reaction with these particles demonstrated clear differences in the initial cleavage rates for the Cys and Arg targets down to 0.5 pM (100 amol/assay). Although the slope for the reaction with 0.5 pM Cys target was essentially 0, it was significantly different from the slope for the reaction with Arg target at the same concentration.

DISCUSSION

Possibility of utilizing invasive cleavage reactions for microarray applications

Currently the invasive cleavage reaction is a powerful tool for solution phase analysis of nucleic acid mutation. The reaction must be configured on a solid phase, however, if this tool is to be used for microarray applications.

An assay format with a non-cycling upstream oligonucleotide attached to the surface would be suitable for solid phase processing of one assay at a time. If a number of probes with distinguishable fluorophores were developed, this format could be scaled up so that a small number of assays could be conducted simultaneously in a single well. However, it would not be a simple matter to apply this assay format to microarray applications, where thousands of assays are run concurrently.

On the other hand, an assay format with surface-attached probe could be adapted for microarrays, which require that positive signals be read at addressable locations. With probes designed to have the quencher on the cleaved 5′-flap, this assay format would result in fluorescing probes on the solid phase for positive samples but fluorescence quenched probes for negative samples. A disadvantage of this assay format for microarray applications, however, is that upstream oligonucleotides for each array location would need to be added to the reaction with the sample and enzyme.

The most convenient assay format for microarray applications would be tethering of both required oligonucleotides to the solid phase so that only sample and enzyme need be added for array processing. With paired probe and upstream oligonucleotides at each array location, fluorescence would indicate a sample positive for the mutation specified at that array location, while absence of fluorescence would indicate absence of the specified mutation.

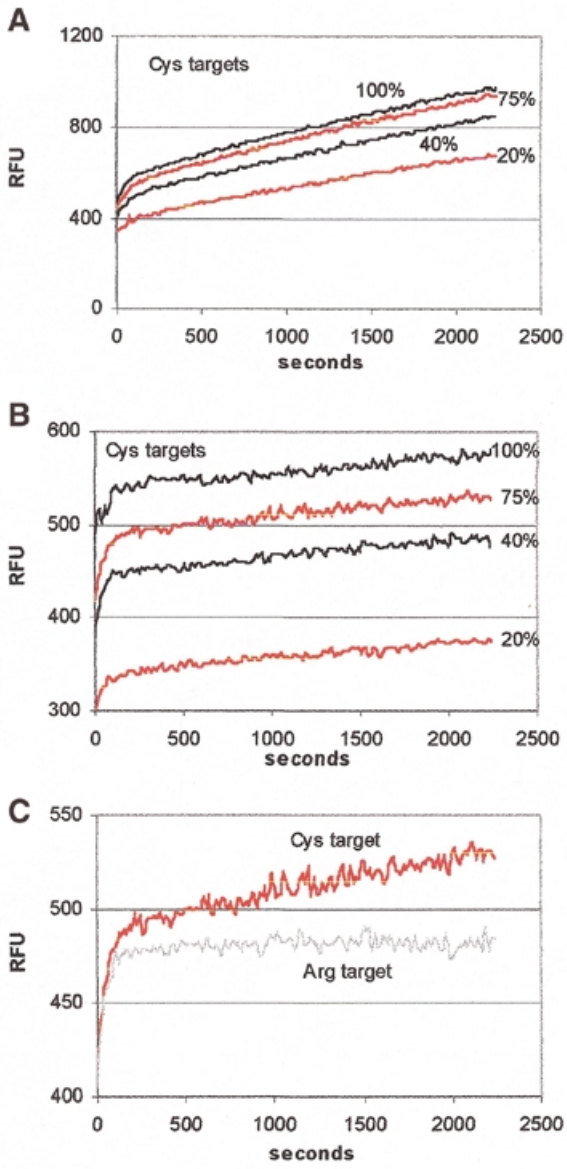

Figure 5. Solid phase ApoE reactions with 100 or 10 pM target concentrations. Particles coated at various surface densities with long ApoE probe were tested at 60°C in invasive cleavage reactions with 50 nM upstream oligonucleotide and (**A**) 100 or (**B**) 10 pM Cys target. Black lines represent Cys target data for oligonucleotide surface densities of 100 and 40%; Cys target data for densities of 75 and 20% are shown as red lines. Data for the Arg targets are not included in (A) or (B). (**C**) Invasive cleavage reactions with particles of 75% surface density were conducted as in (B) but with 10 pM Cys (red) or Arg (gray) target.

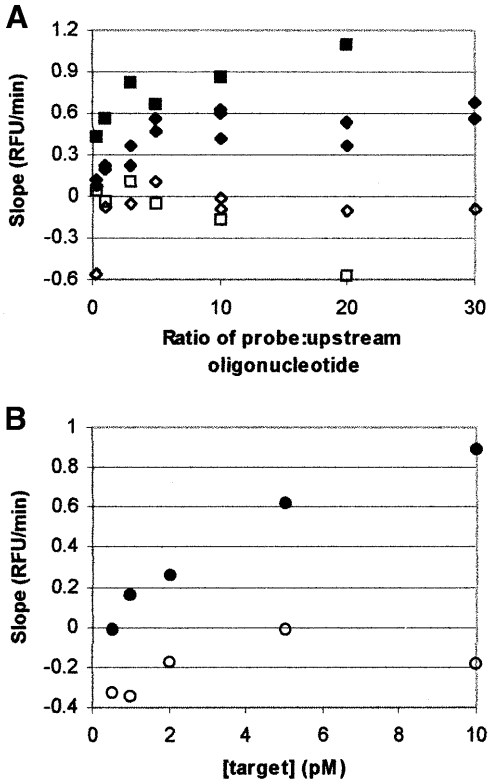

Figure 6. Solid phase ApoE reactions on particles coated with mixtures of long spacer probe and upstream oligonucleotide. Particles were tested in invasive cleavage reactions at 54°C and slopes were calculated from 10–30 min data. (**A**) Particles coated with various ratios of ApoE probe and upstream oligonucleotide were tested with the following targets: 5 pM Cys (filled diamond); 5 pM Arg (open diamond); 10 pM Cys (filled square); 10 pM Arg (open square). (**B**) Particles coated with ApoE probe and upstream oligonucleotide at a 10:1 ratio were tested with various concentrations of Cys target (filled circle) or Arg target (open circle).

Feasibility of solid phase invasive cleavage reactions

All solid phase assay formats we tested enabled target-specific detection, regardless of which oligonucleotide(s) was attached to the surface or how the fluorophore and quencher were oriented on the cycling probe. Thus, in all formats and orientations tested, the properly assembled three-component substrate (target hybridized to upstream oligonucleotide and probe) was specifically recognized and cleaved by the enzyme.

Positioning the solid phase oligonucleotides away from the surface via an extended spacer arm improved both the signal and the rate of reaction. Invasive cleavage reactions on particles coated with mixtures of ApoE probe and upstream oligonucleotide at various ratios from 1:3 to 30:1 all discriminated the specific Cys target from the non-specific Arg target at target concentrations as low as 5 pM, i.e. 1 fmol target/assay. With particles coated with a 10:1 ratio of ApoE probe:upstream oligonucleotide, the invasive assay yielded distinguishable results for the two targets at even lower target concentrations.

These preliminary studies provide the basis for future studies aimed at constructing a microarray capable of parallel analysis of multiple SNPs with a solid phase invasive cleavage reaction. Investigating alternative solid phase materials, examining covalent attachment of oligonucleotides, exploring other detection methodologies and optimizing reaction conditions should all yield improvements in the solid phase invasive cleavage assay. A SNP microarray capable of processing subattomole amounts of target is clearly within the realm of possibility.

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