Short Technical Report

Improved Sensitivity for Solid-Support Invasive Cleavage Reactions with Flow Cytometry Analysis

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ABSTRACT

A new configuration of the solid-support invasive cleavage reaction provides a small reaction-volume format for high-sensitivity discrimination of nucleic acid targets with single nucleotide differences. With target concentrations as low as 2 amol/assay, the solid-support invasive cleavage reaction clearly distinguishes single base mutations. Two oligonucleotides tethered to the solid support hybridize to the target nucleic acid, forming a tripartite substrate that can be recognized and cleaved by Cleavase®, a structure-specific 5'-nuclease. Each cleavage event yields fluorescence signal on the surface. When microspheres serve as the solid-support surface, analysis by fluorometer imparts real-time information about change in the reaction signal over time. Flow cytometry provides an alternative detection technology that collects endpoint information about the reaction signal on individual microspheres. A reaction volume of 10 µL with as few as 3000 microspheres is sufficient to distinguish single nucleotide differences at target concentrations less than 200 fM. This sensitivity level is within the range required for analysis of SNPs in genomic DNA. In addition, the flow cytometry format has multiplexing potential, making the microsphere-based invasive cleavage assay attractive for high-throughput genomic applications.

INTRODUCTION

Capable of distinguishing nucleic acid sequences that differ at only a single nucleotide position, the invasive cleavage reaction has become a valuable tool for genomic applications, including scoring SNPs (4,5,7,9,11,13), tracking genotypes of viruses and microorganisms (3), and monitoring gene expression (7). The method positions two overlapping oligonucleotides, the probe and upstream oligonucleotide, on the target nucleic acid to create a complex recognized and cleaved by a structure-specific 5' nuclease. Enzyme-substrate recognition provides the assay's exquisite discrimination of closely related nucleic acid sequences (10). Run at a temperature compatible with probe cycling, the assay exhibits signal amplification, with a single target directing cleavage of multiple-probe oligonucleotides.

Previously, we adapted the invasive cleavage reaction to a solid-support format and demonstrated effective probe cleavage whether the probe or upstream oligonucleotide or both were attached to the solid support (8,15). Attachment of the oligonucleotides with long hydrophilic tethers improved reaction rates and enhanced signals (15).

Here we describe adaptations of the solid-support invasive cleavage assay that render it more suitable for diagnostic applications. As illustrated in Figure 1, both probe and upstream oligonucleotide are bound to the solid support in this microsphere-based format. The probe is configured with a 5'-terminal dabcyI and an internal fluorescein; thus, microsphere-bound probe is fluorescence-quenched until target-directed

cleavage releases the dabcyI quencher. After cleavage, fluorescein remains associated with the microsphere. Therefore, increased cleavage is characterized by increased microsphere fluorescence. The oligonucleotide system used in these experiments is based on the apolipoprotein E (ApoE) SNP at position 158, where *cgc* (Arg) and *tgc* (Cys) codons are represented within the population (1).

Analysis of microsphere-based invasive cleavage reactions by fluorometer yields information about the cleavage reaction in real time (15). However, sample size considerations (150–200 µL for our instrumentation) and the technique's sensitivity (approximately 500 fM) limit its potential for analysis of SNPs in genomic DNA. Here we demonstrate that flow cytometer analysis of the microsphere-based invasive cleavage reactions is compatible with a 20-fold smaller reaction volume (10 µL) and target concentrations as low as 200 fM. This high sensitivity of the solid-support invasive cleavage reaction in the flow cytometric format establishes that it is capable of distinguishing nucleic acid targets at concentrations appropriate for SNP analysis in genomic DNA. The potential for multiplexing invasive cleavage assays on tagged microspheres points to the relevance of flow cytometric analyses for future high-throughput genomic applications.

Several solution-phase SNP-genotyping methodologies have demonstrated microsphere-based formats where reaction products are captured and analyzed on coded microspheres (2,6,14). The solid-support invasive cleavage reaction detailed below differs from these methodologies because the micro-

sphere is the location where target-directed probe cleavage occurs, not just the site for capture of previously processed reaction product.

MATERIALS AND METHODS

Materials

Spherotech (Libertyville, IL, USA) donated streptavidin-coated latex microspheres and Rainbow calibration microspheres. Third Wave Technologies (Madison, WI, USA) prepared Cleavase[®] enzyme as described (9). Enzyme dilution buffer, enzyme storage conditions, and oligonucleotide synthesis were specified previously (4). Phosphoramidite reagents for oligonucleotide synthesis were purchased from Glen Research (Sterling, VA, USA), Chill-out[™] 14 liquid wax from MJ Research (Cambridge, MA, USA), and D-biotin from Pierce Chemical (Rockford, IL, USA). Other reagents were obtained from Sigma (St. Louis, MO, USA).

ApoE System

Sequences for Cys probe, Cys target, Arg target, and upstream oligonucleotide are in Reference 15. The Arg probe sequence is 5'-(dabcyl)GC-TT(*FL-dT*)TGCAGGTCATCGG(*Sp*)₁₀(biotin)-3', where *FL-dT* is fluorescein-deoxythymidylic acid and *Sp* is the Glen Research spacer phosphoramidite 18. Each upstream oligonucleotide and probe contain 10 of these hexaethylene glycol spacers, since more effective cleavage (2- to 3-fold faster reaction rates) was observed for microspheres where both oligonucleotides were attached with linkers of the same length than when one of the two oligonucleotides was attached via a longer-length linker (data not shown).

The T_m for upstream oligonucleotide-target hybridization is 83.9°C, while T_m values for probe-target hybridization are 60.0°C and 61.7°C for Cys and Arg probes, respectively. In one experiment, three upstream oligonucleotides with alternative T_m values were tested. Sequences for these oligonucleotides are 5'-(biotin)(*Sp*)₁₀cactgccaggct-3'; 5'-(biotin)(*Sp*)₁₀GTA-CACTGCCAGGCT-3'; and 5'-(biotin)

(*Sp*)₁₀GGTACACTGCCAGGCT-3'. Corresponding T_m values are 55.7°C, 61.8°C, and 65.4°C, respectively.

Preparation of ApoE Cys and Arg Microspheres

Streptavidin-coated microspheres (diameter 3.2 μ m, density 1.05 g/cm³) were coated with upstream oligonu-

cleotide and either Cys or Arg probe essentially as described (15), but before the coating reaction, microspheres were sonicated 30 s with a probe sonicator (4 W; Sonic Dismembrator 60; Fisher Scientific, Pittsburgh, PA, USA). After coating, microspheres were blocked with 10 μ M D-biotin. Unless otherwise stated, the ratio of probe to upstream oligonucleotide was 30:1.

age reaction, cleaved probe readily dissociates from target, while upstream oligonucleotide remains stably associated with target throughout the entire reaction. When the invasive cleavage reaction is run with all components in solution, continuous association of upstream oligonucleotide with target simplifies the assembly of the tripartite cleavable substrate. On a solid support, however, continuous association of an upstream oligonucleotide with target limits the number of probes potentially interacting with that target, since only probes in the immediate vicinity of that upstream oligonucleotide are close enough to form the required structure. Therefore, we hypothesized that allowing cycling of both probe and upstream oligonucleotide might improve cleavage rates for the solid-support reaction. To test

this hypothesis, we designed three alternative upstream oligonucleotides, one with a T_m similar to that of the probe-target T_m and the other two with T_m values approximately 5°C higher or lower than the T_m for probe-target hybridization.

When microspheres coated with the standard Cys probe and one of the upstream oligonucleotides were reacted with Cys or Arg target, microspheres with the standard, high- T_m upstream oligonucleotide performed significantly better than microspheres with any of the alternative upstream oligonucleotides. In fact, microspheres with the upstream oligonucleotide with the lowest T_m demonstrated no target cleavage (data not shown).

Figure 2. Real-time analysis of solid-support invasive cleavage reaction. In a 200- μ L reaction, 76 μ g Cys (A) or Arg (B) microspheres were reacted with 25 pM Cys (black) or Arg (gray) target. Reaction signal was measured every 15 s by fluorometer. Thick and thin lines indicate data from duplicate experiments.

Comparison of Fluorometer and Flow Cytometer Analyses

Fluorometer measurements of solid-support invasive cleavage record real-time signal generated by a tube of suspended microspheres and provide information about the total fluorescence of the sample's entire collection of microspheres. Flow cytometry, on the other hand, records fluorescence on a microsphere-by-microsphere basis.

To compare endpoint measurements for individual microspheres to fluorometer-based endpoint measurements, we

Figure 3. Comparison of reaction analysis by fluorometer versus flow cytometer measurements. (A) 2-fold dilutions of 3.2- μ m Cys microspheres (from 195 to 6 μ g/mL) were reacted for 3 h with 2-fold dilutions of Cys or Arg target (from 100 to 3 pM). Duplicate reactions were prepared for each concentration, and endpoint signals were measured by fluorometer (Cys target, black triangles; Arg target, open triangles). From each reaction, 10 000 microspheres were read by flow cytometer, and median AFU values were determined for each Cys-target reaction (black circles) or Arg-target reaction (open circles). (B) P/N ratios for fluorometry (open squares) and flow cytometry (x) data shown in A.

Figure 4. Reactions with low target concentrations. In a 1.6-mL reaction with 1 μ g/mL Cleavase enzyme, 3 μ g (approximately 170 000) 3.2- μ m Cys microspheres with a 1:1 ratio of probe to upstream oligonucleotide were reacted overnight with 750, 375, or 188 fM target. (A) Fluorescence signals from 10 000 individual microspheres were measured by flow cytometer. Gray and black bars, respectively, indicate median signals for Cys-target and Arg-target reactions. Open bars represent P/N values. (B) Flow cytometry trace for Cys microspheres reacted with 188 fM Cys (white) or Arg (gray) target.

prepared a series of six invasive cleavage reactions containing target and microspheres at a constant ratio of 5600 target strands/microsphere. The most concentrated reactions contained 100 pM target and 195 μ g/mL microspheres in a reaction volume of 200 μ L, while the most dilute reactions contained 3.1 pM target and 6 μ g/mL microspheres in a reaction volume of 6.4 mL. After a 3-h incubation, fluorescence signals for each reaction were measured in the fluorometer, and then microspheres from each reaction were analyzed by flow cytometry.

Increasing the Proportion of Upstream Oligonucleotide Improves P/N Ratios and Assay Sensitivity

Microspheres coated with Cys probe and upstream oligonucleotide at probe:upstream oligonucleotide ratios varying from 1:1 to 30:1 were tested in invasive cleavage reactions with 1 pM target. Microspheres with equal amounts of probe and upstream oligonucleotide on the surface (1:1 ratio) demonstrated a P/N value of 2.4. P/N values for probe:upstream oligonucleotide ratios of 6:1, 12:1, 18:1, 24:1,

and 30:1 were all lower: 2.2, 2.1, 1.7, 1.5, and 1.3, respectively. Higher proportions of probe on the microsphere surface affect P/N ratios adversely, primarily because of the increased fluorescence of reactions with nonspecific target. High concentrations of probe contribute background fluorescence, even when microspheres have not been reacted with target or enzyme. When microspheres coated with a 1:1 ratio of Cys probe and upstream oligonucleotide were tested in invasive cleavage reactions with Cys and Arg target, target concentrations of 750, 375, and 188 fM all demonstrated significant differences in the signals generated by specific (Cys) and nonspecific (Arg) targets (Figure 4A). Even at 188 fM target, the Cys-target and Arg-target flow cytometry peaks were well separated (Figure 4B). These results confirmed that analysis of individual microspheres by flow cytometry was an appropriate method for analyzing solid-support invasive cleavage reactions. In addition, increased separation of positive and negative signals and the resultant higher P/N ratios demonstrated that flow cytometer analysis provided better sensitivity than analysis by fluorometry.

Flow cytometry data showed better differentiation of microsphere signals from Cys-target and Arg-target reactions conducted with low concentrations of target and microspheres (Figure 3A). P/N ratios calculated from flow cytometry data followed the same trend as P/N ratios calculated from fluorometry data, although P/N values for flow cytometry data were consistently slightly higher (Figure 3B).

When microspheres coated with a 1:1 ratio of Cys probe and upstream oligonucleotide were tested in invasive cleavage reactions with Cys and Arg target, target concentrations of 750, 375, and 188 fM all demonstrated significant differences in the signals generated by specific (Cys) and nonspecific (Arg) targets (Figure 4A). Even at 188 fM target, the Cys-target and Arg-target flow cytometry peaks were well separated (Figure 4B).

Solid-Support Assay Configured with Few Microspheres and Low Reaction Volume

The solid-support invasive cleavage experiments described so far were conducted in reaction volumes of at least 200 μ L in microcentrifuge tubes rocked or agitated continuously to prevent microsphere settling. We wondered whether it might be possible to reduce the reaction volume and conduct solid-support invasive cleavage reactions in a thermal cycler without any mixing.

In 10- μ L reaction volumes, from 3000 to 12 000 microspheres coated with a 1:1 ratio of Cys probe and upstream oligonucleotide were reacted overnight with 500 fM target at 54°C in a thermal cycler and then analyzed by flow cytometry. All concentrations of microspheres tested were capable of distinguishing Cys and Arg targets, as demonstrated by the P/N ratios of 1.5, 1.6, 1.8, and 2.1 for reactions with 12 000, 9000, 6000, and 3000 microspheres, respectively. The best P/N ratio was obtained with the lowest number of microspheres.

DISCUSSION

Improving microsphere performance, decreasing microsphere number, reducing reaction volume, and analyzing reac-

tions by flow cytometry have all contributed to significant enhancements of the solid-support invasive cleavage reaction. These enhancements resulted in overall improvement of detection sensitivity from approximately 100 amol in a 200- μ L reaction (500 fM) to 2 amol in a 10- μ L reaction (200 fM). In addition, results from 10- μ L reactions conducted in the thermal cycler demonstrated that microsphere-based invasive cleavage reactions can be conducted in small volumes without microsphere mixing.

The capability of doing small-volume solid-support invasive cleavage reactions means that precious samples can be conserved and that the target nucleic acid can be maintained at reasonably high concentrations. Also, reducing the number of microspheres/reaction has the potential to increase the signal generated by individual microspheres. With this small-volume system for analysis of SNPs represented at femtomolar concentrations, analysis of genomic DNA samples is feasible. With all components in solution phase, the invasive cleavage reaction is capable of SNP analysis directly from genomic DNA without up-front target amplification. The solid-support invasive cleavage assay likewise has potential for scoring SNPs directly from genomic DNA. Thus, microsphere-based reactions offer an effective platform for extending the invasive cleavage reaction from its solution-phase configuration to a solid-support format. In the future, a suspension array (12) of highly multiplexed microsphere reactions or some other array format may be useful for analysis of genomic DNAs by solid-support invasive cleavage reactions.

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