Presented at the 14th Annual Society for Biomolecular Sciences Conference & Exhibition, April 6-10, 2008, St. Louis, Missouri, USA

Optimizing Nucleic Acid Detection by Altering Detection Chemistry

Heather Harding[1], Bruce Amsden[2], Kerry Oliver[1], and Jo Wegstein[2].

[1]Radix BioSolutions, Ltd., Georgetown, TX

[2]ProZyme, Inc., San Leandro, CA

Abstract

Standard amplified nucleic acid hybridization assays require significant *in silico* and experimental testing to determine the optimal primers and probes for the detection of specific nucleic acid sequences. However, very little analysis or effort is spent optimizing the detection molecules used for a particular hybridization assay. Previously, we have demonstrated that optimization of the specific streptavidin-phycoerythrin conjugate (SA-PE) can increase sensitivity greatly in a nucleic acid hybridization assay using Luminex® xMAP® technology. In the present study, we have examined the effects of changing spacer chemistry between the xMAP microsphere surface and the hybridizing probe, and the primer sequence and the 5'-biotin moiety used to capture the SA-PE conjugate, and varying the SA-PE conjugate structure. The combined effects of these changes demonstrate that varying spacer chemistry and detection conjugate structure significantly enhances the sensitivity of nucleic acid hybridization assays performed on xMAP technology.

Introduction

Reagent selection is a critical parameter during the assay development process to attain appropriate specificity, sensitivity and reproducibility. To optimize the detection of specific nucleic acid sequences, a considerable amount of effort has been expended in developing software programs that optimize probe sequences and assay conditions *in silico* to provide the highest level of specificity, the highest level of sensitivity, and minimize the deleterious effects of secondary and tertiary structure on the hybridization of probe to primer. Following hybridization of the sample nucleic acid to the probe sequence, detection of the hybridization event generally occurs through the binding and subsequent detection of a colorimetric, radioactive, or fluorescent molecule to the complex.

In this study we have utilized a nucleic acid hybridization assay developed for use with Luminex xMAP technology. xMAP technology is a multiplexing technology that utilizes optically encoded microspheres as a substrate to develop and perform multiplexed assays. Capture probes are conjugated to the surface of polystyrene microspheres and hybridization of sample nucleic acids are then detected with a fluorescent detection molecule. To assess the effects of linker chemistry and detection molecule binding, we have developed a nucleic acid hybridization assay using an 18-mer oligonucleotide probe which hybridizes to a 229-base biotinylated amplicon. Detection of hybridization occurs through the subsequent binding of a streptavidin-phycoerythrin (SA-PE) to the biotinylated amplicon.

We have examined the effects of different SA-PE conjugates on assay sensitivity by performing hybridization assays and detecting with multiple commercial SA-PE conjugates available from ProZyme. To address the effects of linker chemistry, capture probes were synthesized with different linker chemistry and covalently conjugated to different microsphere populations. In addition, biotinylated amplicons were produced with different linkers between the end of the primer sequence and the biotin detection molecule.

Our results indicate that optimal selection of SA-PE conjugate and spacer chemistry is capable of enhancing nucleic acid detection by greater than 10-fold.

Methods

Coupling of Oligonucleotides to Microspheres. Oligonucleotide probes were covalently coupled to polystyrene xMAP microspheres as previously detailed (Fulton RJ, McDade RL, Smith PL, Kienker LJ, and JR Kettman, Jr. Advanced multiplexed analysis with the Flow Metrix system, **Clin Chem 43**: 1749-56 (1997)) and stored in TE at 2-8°C in the dark.

DNA Amplification. Human DNA samples were obtained from the UCLA Immunogenetics Center (Los Angeles, CA, Class II Panel C2-053) and amplified via PCR. Specifically, PCR was performed in thin-wall PCR tubes, in 20 µL reactions consisting of 1 µL of template DNA, 1X HotStarTaq Plus Master Mix and 1 µL each of the forward and reverse primers. Two different spacers were used in the primer pairs, referred to as spacer A and spacer B. Samples were amplified and replicate reactions were combined and stored at 2-8°C.

Hybridization. Biotinylated amplicon was hybridized to probe-conjugated microspheres in a 96-well thermowell plate. Specifically, 2,500 microspheres in 25 µL 1.5×TMAC (4.5 M tetramethylammonium chloride, 0.15% SDS; 75 mM Tris-HCl, pH 8.0; and 6 mM EDTA, pH 8.0) were added to 25 µL biotinylated amplicon at varying dilutions (one-half log serial dilutions) diluted in TE (10 mM Tris and 1 mM EDTA, pH 8.0) and mixed. Samples were denatured at 95°C for 5 minutes. The mixture was transferred to a 55°C heat block for 45 minutes. The samples were spun at 2,250 × g, and the supernatant was removed by inverting the plate and tapping the plate on absorbent paper. Samples were resuspended with 100 µL of different SA-PEs (25 µg/mL) for 15 minutes at room temperature. The samples were then spun as before and resuspended in 1X TMAC (3.0 M tetramethylammonium chloride, 0.1% SDS; 50 mM Tris-HCl, pH 8.0; and 4 mM EDTA, pH 8.0). They were then analyzed on a Luminex 100 instrument running Luminex 100 IS v2.3 software.

Results

Figure 1A

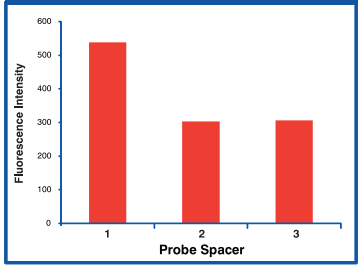


Figure 1B

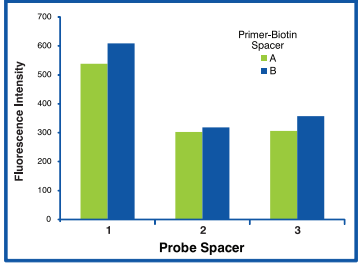


Figure 2

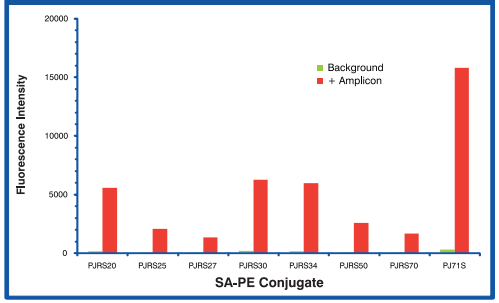
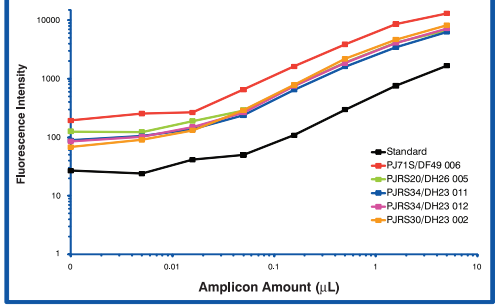


Figure 3



Discussion of Results

Figure 1A demonstrates the effect of three different spacer chemistries between the microsphere surface and the oligonucleotide probe on hybridization efficiency. Amplicon (1 mL) was hybridized to probe-coupled microspheres and detected with a single SA-PE conjugate type. The probe was separated from the microsphere using three different spacer chemistries. This figure demonstrates that optimal spacer chemistries between the surface of the microsphere and the capture probe oligonucleotide can greatly increase hybridization efficiency (signal intensity) of biotinylated PCR amplicon.

Figure 1B demonstrates the effect of two different spacer chemistries between the primer sequence and biotin detection molecule on assay signal intensity. Biotinylated amplicon (1 mL) was hybridized to probe-coupled microspheres and detected with different SA-PE conjugates. The biotin was separated from the amplicon by two different spacer chemistries. Although slight, a significant increase in signal intensity is observed with an optimal spacer between the amplicon and the biotin detection molecule.

Figure 2 demonstrates the effect of varying the SA-PE conjugate on assay signal intensity. Biotinylated amplicon (1 mL) was hybridized to probe-coupled microspheres and detected with different SA-PE conjugates. A single spacer chemistry each for the separation between the probe and the microsphere, and the amplicon and the biotin detection molecule, were used. Optimizing ProZyme SA-PE detection conjugate can increase hybridization signal intensity nearly 10-fold.

Figure 3 demonstrates the effect of different SA-PE conjugates on assay sensitivity. Half-log dilutions of amplicon were hybridized to probe-coupled microspheres and detected with different SA-PE conjugates. A single spacer chemistry each for the separation between the probe and the microsphere, and the amplicon and the biotin detection molecule, were used. Combining the enhancements from Figures 1A, 1B, and 2 produce a greater than 10-fold increase in overall assay sensitivity for nucleic acid detection.

Conclusions

Screening of SA-PE conjugates is a valuable, but often neglected, step in assay development. Optimizing the detection SA-PE conjugate can increase assay signal intensity up to 10-fold. Use of ProZyme's Sampler Kit is a simple first step to cover the range of available conjugates. After initial results, additional candidates can be chosen for further optimization. In addition, optimization of spacer chemistries between the surface of the microsphere and the capture probe oligonucleotide as well as between the amplicon and the biotin detection molecule can provide significant enhancements in assay detection efficiency. The enhancements in signal intensity due to spacer optimization were observed for all SA-PE conjugates tested. In addition to determining the optimal nucleic acid sequence and the optimal hybridization conditions, these results demonstrate that maximizing the detection of specific nucleic acid sequences requires optimizing additional variables in the assay as well.

Radix BioSolutions

PROzyme® GLYKO®