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A molecular beacon, bead-based assay for the detection of nucleic acids by flow cytometry

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ABSTRACT

Molecular beacons are dual-labelled probes that are typically used in real-time PCR assays, but have also been conjugated with solid matrices for use in microarrays or biosensors. We have developed a fluid array system using microsphere-conjugated molecular beacons and the flow cytometer for the specific, multiplexed detection of unlabelled nucleic acids in solution. For this array system, molecular beacons were conjugated with microspheres using a biotin-streptavidin linkage. A bridged conjugation method using streptavidin increased the signal-to-noise ratio, allowing for further discrimination of target quantitation. Using beads of different sizes and molecular beacons in two fluorophore colours, synthetic nucleic acid control sequences were specifically detected for three respiratory pathogens, including the SARS coronavirus in proof-of-concept experiments. Considering that routine flow cytometers are able to detect up to four fluorescent channels, this novel assay may allow for the specific multiplex detection of a nucleic acid panel in a single tube.

INTRODUCTION

With the continual emergence of new pathogens, the differential diagnosis or identification of etiological agents is the important first step to control the spread of infection. The SARS coronavirus (SARS-hCoV) tested the ability of the scientific community to develop methods to isolate, identify and characterize an emerging virus (1,2). The most powerful etiological diagnostic was arguably the use of a microarray 'Viro-chip', which was able to quickly reveal that this pathogen was a coronavirus, even as it also found that this particular coronavirus had never been described previously (3). It appears that this type of solid-phase array technology will become more routine as the costs decrease, the procedures become streamlined for practical use and the technology becomes better disseminated. Current tests for nucleic acid detection are based upon real-time PCR assays (4). In these assays, non-specific (SYBR) or probe-specific fluorescence is measured throughout the PCR reaction [reviewed in (5)].

Tyagi and Kramer (6) published work describing single-stranded 'loop-and-stem' molecules carrying both a fluorochrome and a quencher in close proximity. In this configuration, energy emitted by the excited fluorochrome is absorbed by the quencher and dissipated as heat in a process called fluorescence resonance energy transfer (FRET). When the loop binds to a complementary nucleic acid strand, the molecule changes its conformation to distance the fluorochrome from the quencher, allowing unquenched fluorescence. These molecules were called 'molecular beacons' because they emit a fluorescent signal only when the probes are hybridized to their targets (6). It was later shown that it was possible to build multiplexed assays with this method; differently labelled molecular beacons could recognize different targets in the same reaction tube (7). It was also shown that the specificity of the assay was very high, so that probes differing in only 1 nt could be resolved (8). Various assays were then published using the molecular beacons technology, ranging from mRNA *in situ* visualization (9,10) to nucleic acid sequence-based amplification detection (11), and multiplex detection of four pathogenic retroviruses (12). In other applications, molecular beacon probes were designed for use as DNA biosensors by binding molecular beacons to glass beads or cover slips (13), ultra small optical fibre probes (14) and gold surfaces (15), allowing the specific detection of complementary sequences.

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Flow cytometers are common diagnostic tools used in the immunophenotyping of immune system cells or in the characterization of blood malignancies. With a routine configuration, they allow for the contemporary measurement of four different fluorescent wavelengths, in addition to two signals related to cell size and internal complexity. As such, their application is possible only when the fluorescent signal is associated with discrete particles, such as cells in suspension or microparticles. The flow cytometer has recently been used to detect nucleic acids in a multiplexed format using secondary reagents for signal detection (16).

In this report, we describe the construction of molecular beacon-conjugated beads that we have called 'BeadCons', whose specific hybridization with complementary target sequences can be resolved by flow cytometry (see Figure 1). Assay sensitivity is achieved through the concentration of fluorescence signal on discrete particles. We first obtained evidence that the method could work in principle, with the ability to detect single, synthetic target sequences. Then, we set up the system in a multiplex format (array), and applied the method to nucleic acid oligonucleotides mimicking respiratory diagnostic sequences. The results indicated that this method could allow the detection of the corresponding oligonucleotide, even when diluted in a complex mixture of nucleic acids. In fact, the versatility of flow cytometers allows the resolution of very complex analytical mixtures, in which the hybridized beads of a specific size and/or colour can be readily distinguished from the others that are unbound. The short assay time and ease-of-use makes this method a good candidate for a further development of its diagnostic capabilities and use in the routine laboratory.

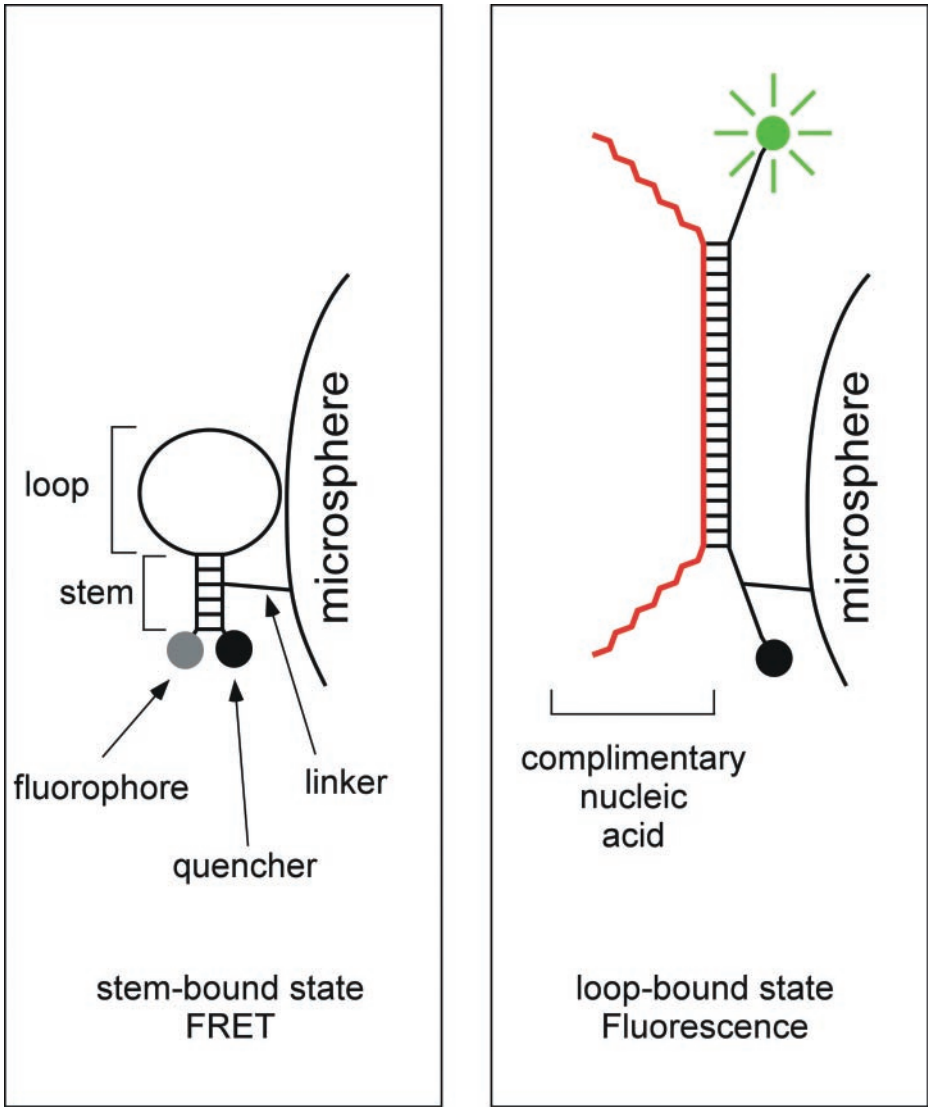

Figure 1. Schematic representation of the interaction of the bead-bound molecular beacon with a complementary nucleic acid. The molecular beacon contains a probing loop sequence embedded within complementary arm sequences. These arms form a hairpin stem that keeps a terminal fluorophore and a terminal quencher molecule in close proximity in the absence of nucleic acid that is complementary to the loop. This state allows for FRET, or a transfer of energy from the fluorophore to the quencher rather than excitation upon absorbance. After target addition, the complementary target forms a duplex with the loop portion of the beacon that pulls the fluorophore and quencher from close proximity, allowing fluorescence. The molecular beacon sequences are linked to the microspheres using a biotinylated thymidine in the stem sequence proximal to the quencher.

MATERIALS AND METHODS

Nucleic acids

Previously published molecular beacon and ssDNA complementary control sequences were synthesized for hepatitis C virus (HCV) (17). Molecular beacons and ssDNA complementary controls were designed for parainfluenza virus type 3 (PIV-3), respiratory syncytial virus (RSV), SARS-hCoV-M and SARS-hCoV-N using Beacon Designer 2.1 software (Premier Biosoft International, Palo Alto, CA). All beacons were designed with a thymidine on the 3′ stem proximal to the quencher molecule, allowing for the addition of biotinylated thymidine in the synthesis process (see Table 1 for beacon sequences). The molecular beacons were ordered with 6-FAM as the reporter dye, BHQ-1 as the dark quencher and the biotinylated thymidine on the 3′ stem (Biolegio, The Netherlands). The molecular beacons for the SARS-hCoV were also ordered with the Cy5/BHQ-2 fluorescence/quencher pair. All molecular beacon and positive control sequences are listed in Table 1.

Bead preparation

Aliquots containing 3.3 μm streptavidin-coated microspheres (G. Kisker GbR, Steinfurt, Germany) were used in a direct conjugation with the biotinylated molecular beacons. Aliquots containing 3.3 and 6.7 μm biotinylated microspheres (G. Kisker GbR) were used in a streptavidin-bridged bead design. Streptavidin (10 μl of 5 mg/ml; Roche) was diluted to a volume of 110 μl in FACSFlow solution (10 mM phosphate-buffered saline, 150 mM NaCl, pH 7.4; Becton-Dickinson), the biotinylated beads (10 μl of 0.5% w/v) were added to the buffer and this mixture was incubated for 5 min at room temperature. The beads were then washed twice with FACSFlow to eliminate unbound streptavidin. The biotinylated molecular beacon (5 μl of 100 μM) was added to the streptavidin-bridged or streptavidin-coated beads (10 μl of 0.5% w/v), diluted to a total of 100 μl in FACSFlow and incubated for 5 min at room temperature. These BeadCons were washed twice in FACSFlow and stored at room temperature until use.

Table 1. Molecular beacon sequences for BeadCons analysis

Target	Sequence[a]
HCV	
MB	5′-6-FAM-GCGAGCCACCGGAATTGCCAGG-ACGACCGCTCGC-BHQ-1-3′
K+	5′-GGTCGTCCTGGCAATTCCGGTG-3′
PIV-3	
MB	5′-6-FAM-ACCATGCGCACCCAGTTG-TGTTGCAGATTCGCATGGT-BHQ-1-3′
K+	5′-AATCTGCAACACAACTGGGTG-3′
RSV	
MB	5′-6-FAM-CGCGATCTTCTCCAGTGTAG-TATTAGGCAATGCGATCGCG-BHQ-1-3′
K+	5′-GCATTGCCTAATACTACACTGGAGAA-3′
SARS-M*	
MB	5′-Cy5-CGCGATAACAGCCTGAAGGAAGCAA-CGAAATCGCG-BHQ-2-3′ (also 6-FAM/BHQ-1)
K+	5′-TTCGTTGCTTCCTTCAGGCTGTT-3′
SARS-N*	
MB	5′-Cy5-CGCGATCCCAAAAGGCTTCTACGCAG-AGGGAAGATCGCG-BHQ-2-3 (also 6-FAM/BHQ-1)
K+	5′-CTTCCCTCTGCGTAGAAGCCTTTTGGG-3′

[a]The loop portion of each molecular beacon (MB) is underlined and the positive controls (K+) used in these studies are listed below each MB. The biotinylated thymidine in each MB 3′stem sequence is listed in bold.

Sample preparation and hybridization

Control samples were analyzed using oligonucleotides complementary to the hybridizing loop sequence (Table 1). The beads were washed with FACSFlow and aliquoted to $\sim 5 \times 10^4$ beads/test in 100 μl. The complementary nucleic acid (10 μl of a 10 μM stock) was then added to the test beads. In the multiplex detection experiment, the test sample contained 0.5 μl of the positive oligo DNA (100 μM stock) diluted in 9.5 μl of a complex mixture of oligonucleotides (equimolar levels of 10 μM each, equalling a 100 μM total concentration; sequences listed in Supplementary Table 1). For the mismatch analysis, 10 μl of a 100 nM stock for each oligonucleotide was used (sequences listed in Supplementary Table 2). The samples were hybridized with gentle agitation for 5 min at room temperature before reading. An aliquot of 200 μl of FACSFlow was added to each tube to run the sample on the flow cytometer.

Flow cytometry and analysis

A minimum of 1×10^4 events were collected for each sample (flow cytometry conditions for the FACSCalibur machine are listed in Supplemental Table 3). The data were analyzed using CellQuest 3.3 (BD Biosciences, San Jose, CA). A positive threshold was set for each BeadCon, based upon the highest fluorescent point seen in the negative control sequence (for all BeadCons, an HIV complementary sequence was used; the sequence is listed in Supplementary Table 2). Mean fluorescence intensities (MFIs) were recorded for the FL1 (6-FAM) and FL3 (Cy5) channels on gated BeadCons populations.

RESULTS

A marked shift in fluorescence intensity is seen using flow cytometry after the addition of loop-specific sequence

BeadCons were prepared as described in Materials and Methods to test whether the system is functional. A molar excess of biotinylated molecular beacon specific for HCV was added to 6.7 μm streptavidin-bound microspheres. It is important to note that the biotinylated thymidine in the molecular beacon is on the stem proximal to the quencher molecule as described previously (13). Target DNA was then added to the washed beads to determine whether there was a shift in the mean fluorescence of the beads. An HCV-specific positive control DNA yielded an MFI of 27.0 ± 1.5 compared with an MFI of 2.06 ± 0.11 for an HIV target sequence (see Figure 2). Therefore, the signal-to-noise ratio (STNR) for the molecular beacons with a direct conjugation to the beads was 13.1, higher than the 2–5 STNR of other molecular beacon systems that use a solid or immobilized phase (18,19), but lower than the standard 25 STNR that is typically seen in solution (6,20,21).

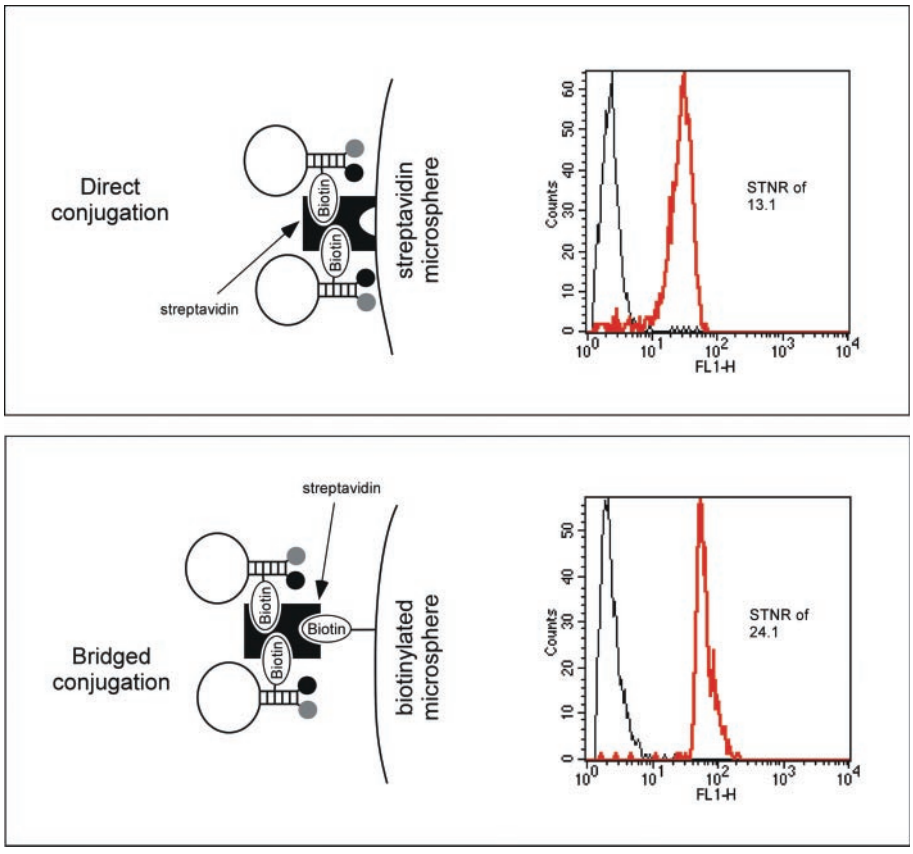

Figure 2. Bridged conjugation of the molecular beacon to the microsphere expands the STNR of the system. Biotinylated molecular beacons were directly conjugated to streptavidin-labelled beads in the original assessment of the assay. An STNR of 13.1 was seen for a 6-FAM/BHQ-1 BeadCon for SARS-hCoV-N gene. The same beacon was conjugated to biotinylated beads through a streptavidin bridge that allowed for an amplification of the fluorescence. The STNR for the bridged beacon, 24.1, is nearly double that of the directly conjugated BeadCon. This result was found in an experimental triplicate, and SDs of the MFI data are listed in the text.

Streptavidin bridging of the molecular beacon increases the STNR

The commercial availability of biotinylated beads led us to test the possibility of using streptavidin to bridge the beacon and bead. Such an approach has previously been used to increase the signal of molecular beacons as functional biosensors (13). Streptavidin was added to 6.7 μm biotinylated beads in excess to limit the possibility of bead cross-linking, with a high enough level of streptavidin so that no two beads would share a single streptavidin molecule. Initial experiments with lower streptavidin levels revealed the production of a doublet population, formed as a streptavidin molecule-bridged two biotinylated beads. When prepared and thoroughly washed beads were hybridized with the HCV-specific positive control DNA, an MFI of 49.8 ± 3.6 was seen compared with an MFI of 2.06 ± 0.15 for the unrelated HIV control. Therefore, an STNR of 24.1 was achieved through the use of a streptavidin bridge, comparable with the typical, optimized results in solution for this fluorophore/quencher set. An asymmetrical PCR product derived from an HCV-infected serum sample yielded an MFI of 14.1 ± 0.9, for an STNR of 6.8 (Supplementary Figure 1). The reduction in STNR is probably due to self-annealing of the amplified asymmetric PCR strand; however, qualitative gating showed that the binding of the single-stranded product was much more effective than that of the symmetric PCR product (negative using the current hybridization conditions).

The assay is sensitive across a four orders of magnitude range

All the test BeadCons were assayed for their dynamic detection range. After an initial 100-fold dilution of a 10 μM stock, 5-fold dilutions of positive oligonucleotide controls were made in FACSFlow buffer and 10 μl were added to the BeadCons that were built using the streptavidin bridging. It must be noted that the PMT voltage setting was slightly increased for the FL1 channel (from 400 to 420) to increase the MFI and, thus, differences between the beads with little or no positivity. A representative detection curve is shown in Figure 3. For this example, the limit of detection (10 SDs above the background MFI of 3.70) for this BeadCon specific for SARS-N was found to be 26 fmol at an MFI of 5.7. The dynamic detection of this assay extended to 100 pmol, revealing a working range of nearly four orders of magnitude. The other BeadCons tested using this method (including those for HCV, PIV-3, RSV and SARS-M) showed an average limit of detection at 37 fmol (range: 26–56 fmol).

Specificity of the BeadCons with mismatched nucleotides

Previous work with molecular beacons demonstrates that single nucleotide polymorphisms can be detected in PCR assays (22). Using a previously published HCV sequence as the

positive control, primers with one, three and five mismatches were studied for their binding characteristics (sequences listed in Supplementary Table 2; for results, see Figure 4). Using this assay under initial conditions, a single nucleotide difference could not be statistically distinguished from the positive control sequence. The same experiment was repeated using a 5 min incubation at 80°C for hybridization, with or without the addition of 25 mM $MgCl_2$. These hybridization conditions allowed a better resolution of nucleotide mismatches, although there was a small loss in the overall STNR in the assay (see Figure 4A). Finally, the analysis was repeated using the SARS-N BeadCon. Using the new hybridization conditions (80°C with 25 mM $MgCl_2$), the single nucleotide mismatch for SARS-N generated a fluorescence that was 24% lower than the complementary positive control. A 3 nt mismatch showed a 91% loss of fluorescence compared with the positive control, at a level very similar to that of the complete mismatch oligo from HIV (see Figure 4B).

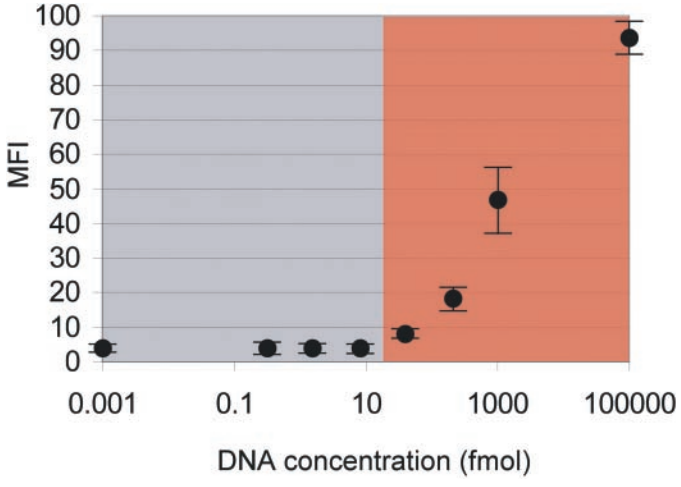

Figure 3. The detection limit of this assay is within the fmol concentration of target nucleic acid. This BeadCon for SARS-CoV-N was sensitive to 26 fmol of complementary DNA using 10^4 beads and the described conditions (triplicate results averaged). Other tested BeadCons had similar limits of detection, ranging from 26 to 56 fmol with an average of 37 fmol.

A multiplex array of respiratory pathogen BeadCons showed specific identification of the corresponding oligonucleotide

To test whether both multiple bead sizes and fluorochromes could be used simultaneously, molecular beacons that incorporated the Cy5/BHQ-2 fluorochrome/quencher set were synthesized for the SARS-hCoV-M and -N open reading frames. The BeadCons for these targets were constructed using the same conditions as for the other probes, with conjugation to streptavidin-bridged 3.3 and 6.7 μm beads. BeadCons against RSV (3.3 μm) and PIV-3 (6.7 μm) were also constructed using the 6-FAM/BHQ-1 fluorochrome/quencher set. An aliquot of 50 pmol of each pathogen oligo sequence was diluted in 950 pmol of a mixture of oligonucleotides with no specificity for the beacon sequence (oligonucleotide sequences are listed in Supplementary Table 2). When these DNAs were added to the BeadCons mixture of four targets, the corresponding BeadCon showed a fluorescent shift above background, confirming presence of that target in the tube (see Figure 5). It should be noted that the 'SARS' tube contained 50 pmol of both SARS-M and SARS-N oligonucleotides, and both BeadCons showed a positive shift in fluorescence. Without specific target addition, the BeadCons failed to fluoresce above background. The MFI, STNR and corresponding SDs are listed for three experimental replicates of the shown, representative dot plots. All fluorescence shifts were significant to the levels shown in the figure by utilizing the unpaired *t*-test using Welch's correction.

DISCUSSION

The development of a highly accessible and easily adaptable multiplex system for the detection of pathogens remains the ultimate goal for the molecular diagnostic laboratory. Real-time PCR has been a very useful tool in the research field as it allows for the rapid, simultaneous detection of pathogens in multiplex. Unfortunately, the multiplex PCR system is complex for assay development, as primer/probe sets must be matched for primer melting temperature (T_m), probe T_m, amplicon length and amplicon T_m. Obviously, each additional target also adds another level of complexity to the assay

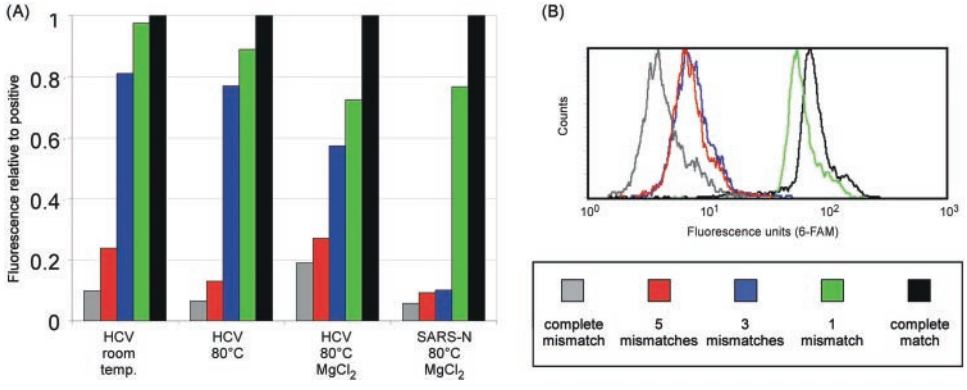

Figure 4. Mismatched nucleotide analysis to test the specificity of the BeadCons. (**A**) A measure of fluorescence relative to that of the positive control for mismatched targets using the indicated conditions. For the previously described HCV molecular beacon, hybridization at 80°C with 25 mM $MgCl_2$ increased the specificity, with a small loss in assay sensitivity. The SARS-N BeadCon showed a higher level of discrimination for multiple mismatches under the same conditions, maintaining an STNR of over 20. (**B**) The histograms of SARS-N BeadCon fluorescence present an illustrative example of the analysis.

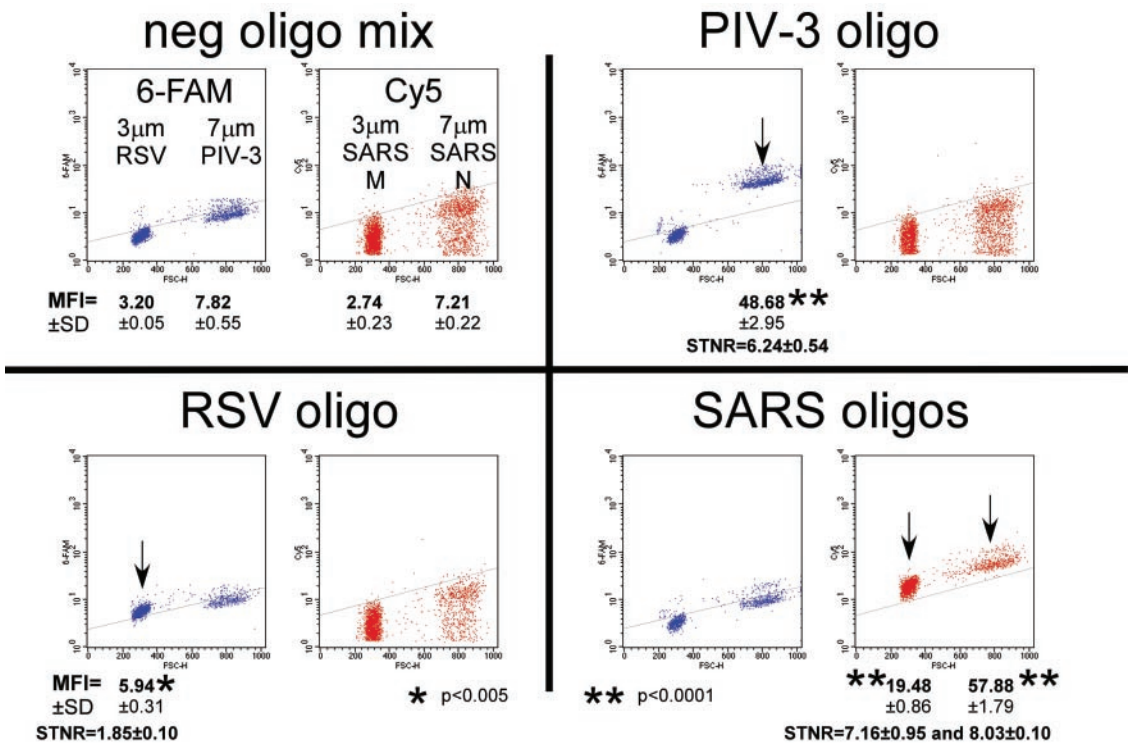

Figure 5. Qualitative detection of three respiratory virus sequences in multiplex. A BeadCons array was generated using two bead sizes and two fluorescence markers as described in the text. The addition of PIV-3, RSV or SARS-h-CoV (genes M and N) DNA control sequences that were diluted in a complex DNA mixture allowed the specific identification of the target compared with negative controls. In effect, each panel shows an internal negative control, as each test was carried out using a complex DNA mixture. The black arrows note the positive shift in fluorescence.

when it comes to primer dimerization and mispriming. Microarrays will probably allow for the rapid screening of thousands of possible pathogens, but the cost, equipment and expertise make current routine use impossible, and this type of solid-phase array technology will not be practical as a clinical diagnostic for some years. There remains a need for the continual development and refinement of assays that can be used to detect nucleic acids in a multiplexed format.

We have developed molecular beacon-labelled microspheres with a read-out on the flow cytometer for the multiplexed detection of nucleic acids in solution. A bridging approach allowed the specific binding of the BeadCon by the proper complementary DNA sequence. The bridging of the BeadCon using a streptavidin molecule increased the STNR 2-fold to levels that are similar to those seen for molecular beacons in solution. The commercial source of the beads that were used in these studies confirmed that the streptavidin beads carried three times as many binding sites for biotin as the biotinylated beads did for streptavidin. Therefore, if each streptavidin of the bridged molecule allows complete accessibility of all three available sites for biotin, the STNR should be roughly about the same. As the STNR was twice as high for the bridged molecule, it is likely that the bridging helps to overcome the surface effects that are often seen using solid state nucleic acid detection systems (19). As the molecular beacons are more accessible for the target, there is a more effective loss of FRET and, thus, an increase in maximal fluorescence.

The individual specificity and sensitivity of each molecular beacon must be developed specifically for this assay. It appears that currently designed methods for use in PCR must be modified if increased specificity is required. For the HCV BeadCon used in our studies at room temperature, a 3 nt mismatch was readily detectable at a level that was virtually indistinguishable from the matched positive control. As this reaction was hybridized and measured at room temperature, mismatched hybridization is expected as it is difficult to distinguish mismatches at temperatures below the 'window of discrimination' for each beacon (20,22,23). In this temperature range, it is possible to discriminate a single nucleotide polymorphism; however, as the temperature decreases to 25°C, the difference in fluorescence between mismatched targets is negligible. The currently designed beacons are used to measure fluorescence at an elevated annealing temperature, which fails to allow mismatched target duplexes and, thus, fluorescence (20). An assay that allows mismatching can be useful in detecting nucleic acids that have point mutations from their homologous sequence. Alternatively, the dynamics of the binding reaction could be altered by changing the Gibb's free energy assignments of the stem and loop regions of the molecular beacon. The SARS-N molecular beacon that we designed showed a better discrimination of mismatched targets, although it appears that the spacing of the mismatches may play a role. The binding properties can be modified by altering the backbone of the beacon (such as for a peptide nucleic acid), substituting high affinity nucleotides for normal ones (such as in a locked nucleic acid), or by changing the

lengths or recognition portions of these sequences (24). These types of molecular beacons will probably be used more in the future, as these complex synthesis technologies are improved and better diffused.

In assay development for each group of targets, it will be necessary to consider whether single nucleotide polymorphism detection will be developed through these means, or if it is better for the system to be tolerant for small nucleotide changes that would allow detection even as the target sequence contains mutations. Unique sequences can be selected for each target, allowing specific detection. In addition, redundancy can be included within the assay by conjugating molecular beacons for multiple sequences of the same pathogen or gene onto a single type of bead. The further development of this assay to detect a single target from a complex mixture may be important in several fields of use. We hope to facilitate differential diagnosis, genetic testing, genotyping and gene expression studies through the use of this technology.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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