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Advanced multiplexed analysis with the FlowMetrix™ system

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The FlowMetrix™ System is a multiplexed data acquisition and analysis platform for flow cytometric analysis of microsphere-based assays that performs simultaneous measurement of up to 64 different analytes. The system consists of 64 distinct sets of fluorescent microspheres and a standard benchtop flow cytometer interfaced with a personal computer containing a digital signal processing board and Windows95®-based software. Individual sets of microspheres can be modified with reactive components such as antigens, antibodies, or oligonucleotides, and then mixed to form a multiplexed assay set. The digital signal-processing hardware and Windows95-based software provide complete control of the flow cytometer and perform real-time data processing, allowing multiple independent reactions to be analyzed simultaneously. The system has been used to perform qualitative and quantitative immunoassays for multiple serum proteins in both capture and competitive inhibition assay formats. The system has also been used to perform DNA sequence analysis by multiplexed competitive hybridization with 16 different sequence-specific oligonucleotide probes.

The first use of flow cytometry for analysis of microsphere-based immunoassays was published in 1977 *[1]*, and recently reviewed by McHugh *[2]*. Because a flow cytometer has the ability to discriminate different particles on the basis of size or color, the possibility of multiplexed analysis with different microsphere populations is suggested. Multiplexed analysis is the ability to perform multiple discrete assays in a single tube with the same sample at the same time. The use of different-sized microspheres for simultaneous analysis of different analytes was originally proposed by Horan et al. *[3]*. Two distinct sizes of microspheres were used for simultaneous detection of two different antibodies by flow cytometry, and subsequently expanded to the use of four different sizes of microspheres to detect four different specificities of anti-HIV antibodies *[4, 5]*. Size discrimination of microspheres allows simultaneous detection of small numbers of analytes, but the inability to distinguish aggregates of smaller microspheres from larger microspheres severely limits the extent of multiplexing that can be achieved. In contrast, differential dyeing of identically sized microspheres with two different dyes, emitting in two different wavelengths, allows aggregates to be distinguished and permits discrimination of at least 64 different sets of microspheres.

The FlowMetrix™ system (Luminex Corp.) performs multiplexed analysis of up to 64 different reactions simultaneously by using a flow cytometer and digital signal processor to perform real-time analysis of multiple microsphere-based assays. The three major components of the system are a benchtop flow cytometer, microspheres, and computer hardware and software. The flow cytometer analyzes individual microspheres by size and fluorescence, distinguishing three fluorescent colors—green (530 nm), orange (585 nm), and red (>650 nm)—simultaneously. Microsphere size, determined by 90-degree light scatter, is used to eliminate microsphere aggregates from the analysis. Orange and red fluorescence are used for microsphere classification, and green fluorescence is used for analyte measurement. The FlowMetrix system is currently configured for the Becton Dickinson FACScan®, a multiparameter flow cytometer that is based on a single 488-nm excitation laser (Becton-Dickinson Immunocytometry Systems).

Microspheres serve as the vehicles for molecular reactions. The microspheres are 5.5-μm polystyrene microspheres that bear carboxylate functional groups on the surface. The microspheres are available in 64 distinct sets that are classified by the flow cytometer by virtue of the

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unique orange/red emission profile of each set as shown in Fig. 1. The microspheres can be covalently coupled to virtually any amine-containing "target" molecule through surface carboxylate groups; alternatively, avidin-coupled microspheres can be used for binding biotinylated molecules. Microspheres of this size provide sufficient surface area for covalent coupling of 1–2 $\times$ 10^6 target molecules per microsphere. In addition, the small size allows the microspheres to remain in suspension for several hours, which is more than sufficient for assay setup and analysis, and also provides near-fluid-phase reaction kinetics.

FlowMetrix hardware and software provide complete control of the flow cytometer and perform real-time classification of the microspheres and analysis of the microsphere-based reactions simultaneously. The hardware is a personal computer interface card that provides communication between the computer and the FACScan. The interface card has an on-board, high-speed digital signal processor that is capable of performing >30 million mathematical functions per second. The interface card connects to the computer interface of the FACScan through a switch, allowing alternate use of the FlowMetrix system or the standard computer system. The software is a Windows95®-based 32-bit application that provides a "multiplexed mode" for automated multiplexed analysis, as well as a "data acquisition mode" for nonautomated gating and data acquisition. Both modes automatically record captured data to flow cytometry standard, FCS 2.0, data files for use with third-party data analysis tools. In addition, statistical analysis generated by the software is recorded to comma-separated-value files that can be read by third-party spreadsheet programs.

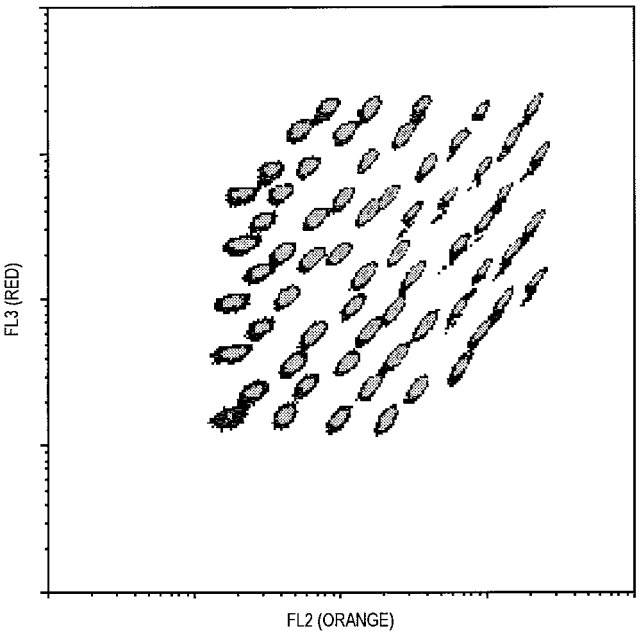

Fig. 1. Fluorescence classification of FlowMetrix microspheres.

Two-dimensional dot plot showing classification of the 64 microsphere sets based on simultaneous analysis of logarithmic orange fluorescence (FL2) and logarithmic red fluorescence (FL3).

Fluorescent antibodies, antigens, or nucleic acid probes provide specific signals for each reaction in a multiplexed assay. Because each fluorescent reactant binds specifically to a target that is present on only one bead set in a multiplexed assay, the soluble reactants do not need to be differentially labeled. All fluorescent molecules are labeled with a green-emitting fluorophore such as Bodipy® (Molecular Probes) or fluorescein isothiocyanate. Any green-emitting fluorochrome can be use as a reporter; however, each fluorochrome has a characteristic emission spectrum, requiring a unique compensation setting for spillover into the orange fluorescence channel.

To prepare a multiplexed assay, individual sets of microspheres are conjugated with the target molecules required for each reaction. Target molecules may be antigens, antibodies, oligonucleotides, receptors, peptides, enzyme substrates, etc. A fluorescent reactant is prepared for each target molecule. Fluorescent reactants may be oligonucleotides, antigens, antibodies, receptors, etc., i.e., any molecule that will bind to the target molecule. After optimizing the parameters of each assay separately in a nonmultiplexed format, the assays can be multiplexed by simply mixing the different sets of microspheres. The fluorescent reactants also are mixed to form a cocktail for the multiplexed reactions. The microspheres are then reacted with a mixture of analytes, such as a serum sample, followed by the cocktail of fluorescent reactants. After a short incubation period, the mixture of microspheres, now containing various amounts of green fluorescence on their surfaces, are analyzed with the flow cytometer. Data acquisition, analysis, and reporting are performed in real time on all microsphere sets included in the multiplex. As each microsphere is analyzed by the flow cytometer, the microsphere is classified into its distinct set on the basis of orange and red fluorescence, and the green fluorescence value is recorded. One hundred individual microspheres of each set are analyzed and the mean value of the green fluorescence is reported. In the present report, an immunoassay for allergen-specific IgE and IgG testing, and DNA hybridization with sequence-specific oligonucleotides for genetic testing are presented. Both assay systems take advantage of the multiplexing capabilities of the system by performing 16 simultaneous analyses in the same reaction.

Materials and Methods

Microspheres. Carboxylate-modified polystyrene microspheres (5.5 μm diameter) were dyed with various amounts of orange-emitting and red-emitting fluorochromes (Luminex Corp.). Eight different concentrations of each of the two fluorochromes were mixed in all 64 possible combinations and the mixtures were used to prepare 64 microsphere sets with unique orange-red emission profiles (Fig. 1). Thus, the flow cytometer measures both the color, or emission wavelength, and the

intensity of each dye. The first 16 microsphere sets produced were used in this study.

Reagents. Grass allergen extracts, canine sera, and affinity-purified goat anti-canine IgE were kindly provided by Bill Mandy, BioMedical Services. Rabbit anti-goat IgG–fluorescein isothiocyanate (FITC), goat anti-canine IgG-FITC, 2-(*N*-morpholino)ethanesulfonic acid (MES), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co.[2] 1-Ethyl-3-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Pierce Chemicals.

Synthetic oligonucleotides. Sixteen oligonucleotides corresponding to allelic sequences within the second exon of the HLA-DQA1 gene and their corresponding complementary sequences were synthesized with standard automated synthesis techniques (Oligos, Etc.). The oligonucleotides were modified at the 5′ end during synthesis with an amino-linker. The amino-linker consists of an amino group attached to a chain of six carbon–carbon linkages. Before the addition of the amino-linker, a chain comprising nine carbon–carbon and aliphatic ether linkages was introduced as a spacer. Double-stranded (ds) oligonucleotides used as competitors in hybridization experiments were prepared by annealing equal amounts of unlabeled complementary oligonucleotides.

Allergen-coated microsphere preparation. Sixteen different grass allergens were conjugated to 16 different microsphere sets through their surface carboxylate groups with a carbodiimide coupling method. Twenty microliters of 4 g/L microspheres (~8×10^6 microspheres) were activated in 100 μL of 50 mmol/L sodium phosphate buffer, pH 7.0, containing 500 μg of EDC and 500 μg of sulfo-NHS. Microspheres were washed twice with 100 μL of PBS, pH 7.4, with centrifugation at 13 400*g* for 30 s to harvest the microspheres. Activated, washed microspheres were suspended in 50 μL of diluted allergen extract (1:100 in PBS, pH 7.4). After 2 h, allergen-coated microspheres were washed twice with 100 μL of 0.2 mL/L Tween 20, 1 g/L BSA in PBS pH 7.4 (PBSTB), suspended in 1 mL of PBSTB, and counted on a hemocytometer. The concentration of microspheres was adjusted with PBSTB to 3×10^9/L and the suspensions stored at 2–8 °C.

Multiplexed assay for grass allergen-specific IgE. Equivalent amounts of each of the 16 grass allergen-loaded microspheres were mixed. Twenty microliters of the mixture (~60 000 microspheres total) were mixed with 60 μL of a 1:3 dilution of canine serum in PBSTB, and the mixture was incubated for 30 min. Microspheres were washed in 200 μL of PBSTB and suspended in 40 μL of a 50 mg/L solution of goat anti-canine IgE. After incubation for 30 min, beads were washed in 200 μL of PBSTB and treated with 40 μL of rabbit anti-goat IgG–FITC at 20 mg/L in PBSTB. After 30 min, the assay was diluted to 300 μL with PBSTB and assayed with the FlowMetrix system. Negative controls included the microspheres with canine serum, without the goat anti-canine IgE and with the rabbit anti-goat IgG–FITC. Allergen-specific canine IgE was determined by subtracting the mean fluorescence intensity (MFI) of the green channel for the negative controls for each grass allergen from the MFI for the tubes including the goat anti-canine IgE.

Multiplexed assay for grass allergen-specific IgG. Twenty microliters (60 000 microspheres) of the microsphere mixture were reacted with 20 μL of a 1:10 dilution of canine serum in PBSTB, and the mixture was incubated for 30 min. Microspheres were washed in 200 μL of PBSTB and suspended in 25 μL of a 50 mg/L solution of goat anti-canine IgG–FITC. After 30 min, the mixture was diluted to 300 μL in PBSTB and assayed with the FlowMetrix system. Negative controls included the microspheres with no canine serum and with the goat anti-canine IgG–FITC. Allergen-specific canine IgG was determined by subtracting the MFI for the negative control for each grass allergen from the MFI for the tubes including canine serum.

Coupling of oligonucleotides to microspheres. HLA-DQA1 allele-specific oligonucleotides corresponding to the noncoding strand [denoted by (−)] were covalently coupled to unique sets of carboxylate-modified polystyrene microspheres by using water-soluble carbodiimide (EDC). Briefly, 100 μL of a 0.001 mol/L solution of oligonucleotide in 0.1 mol/L MES buffer (pH 4.5) was added to 1.0 mL of microspheres (1% solids) in 0.1 mol/L MES buffer (pH 4.5). Fifty microliters of a 10 g/L solution of EDC were added to this mixture, and the solution was mixed vigorously. After incubation at room temperature for 30 min, the same amount of EDC was added again and the solution was mixed. Incubation at room temperature was continued for another 30 min and then the microspheres were pelleted by centrifugation at 11 750*g* for 4 min. Microspheres were washed one time in PBS containing 0.2 mL/L Tween 20, and two times in PBS containing 1 g/L sodium dodecyl sulfate (SDS) by resuspending the microsphere pellet in the solution and then pelleting the microspheres by centrifugation as described. The final microsphere pellet was resuspended in 400 μL of 0.1 mol/L MES buffer (pH 4.5) and stored at 4°C.

Fluorescent labeling of oligonucleotides. HLA-DQA1 allele-specific oligonucleotides corresponding to the coding strand [denoted by (+)] were fluorescently labeled with

[2] Nonstandard abbreviations: FITC, fluorescein isothiocyanate; MES, 2-(*N*-morpholino)ethanesulfonic acid; BSA, bovine serum albumin; EDC, 1-ethyl-3-3(3-dimethylaminopropyl) carbodiimide hydrochloride; ds, double-stranded; NHS, *N*-hydroxysulfosuccinimide; PBSTB, PBS-Tween-BSA buffer; MFI, mean fluorescence intensity; SDS, sodium dodecyl sulfate; and DMSO, dimethyl sulfoxide.

Bodipy FL-X {6-[(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-propionyl)amino]hexanoic acid, succinimidyl ester} (Molecular Probes). Briefly, a 400-μL solution containing 0.000002 mol/L oligonucleotide in 0.1 mol/L sodium bicarbonate and 50 g/L dimethyl sulfoxide (DMSO) (pH 8.2) was mixed with 30 μL of Bodipy FL-X (10 g/L in DMSO) at room temperature for 16–18 h. The mixture was desalted on a PD-10 column (Pharmacia Biotech) equilibrated in TE (0.010 mol/L Tris-HCl + 0.001 mol/L EDTA, pH 8.0) to remove unreacted dye, and stored at 4 °C.

Competitive hybridization assay. Forty femtomoles of each fluorescent oligonucleotide and 24 μg of fragmented salmon sperm carrier DNA were mixed with 500 fmol of ds oligonucleotide in a total volume of 26.7 μL of reaction buffer (0.050 mol/L KCl, 0.020 mol/L Tris-HCl pH 8.4, and 0.003 mol/L $MgCl_2$), and the mixture was heated to 100 °C for 10 min. Twenty-five microliters of 55 °C-equilibrated 2× hybridization buffer (1× hybridization buffer consists of 2.25 mol/L tetramethylammonium chloride, 1.13 g/L SDS, 0.00225 mol/L EDTA, and 0.056 mol/L Tris-HCl pH 8.0) were then mixed with the solution, and the mixture was incubated at 55 °C for 15–30 min. After this incubation, 2.5 μL of the microsphere mixture, containing 8000 microspheres of each of the 16 sets (128 000 microspheres total) in 2× hybridization buffer, were added to the mixture and the solution was vortex-mixed. The hybridization mixtures were incubated for an additional 15 min at 55 °C. Without washing, samples were diluted to 250 μL with 55 °C-equilibrated hybridization buffer. Dilutions were performed immediately before analysis. Simultaneously, competitive hybridizations in the absence of competitor were performed to determine maximal hybridized oligonucleotide values.

Flow cytometric analysis. Assays were performed in a volume of 50 to 100 μL; however, before analysis each reaction was diluted to ~300 μL to provide sufficient sample volume for the flow cytometer. Immediately after dilution, samples were analyzed on a FACScan benchtop flow cytometer (Becton-Dickinson) with FlowMetrix hardware and software for operational control of the flow cytometer and for data acquisition and analysis (Luminex Corp.). At least 100 microspheres were analyzed for each microsphere set. Analysis times averaged ~20 s. The software allows rapid classification of microsphere sets on the basis of the simultaneous gating on orange and red fluorescence. Separate histograms of logarithmic green fluorescence intensity representing each microsphere set were acquired. The MFI of a logarithmic green fluorescence histogram was taken as a measure of the fluorescence associated with the corresponding microsphere set and defined as the value of hybridized oligonucleotide. Percent inhibition of hybridization on a microsphere set was determined with the formula:

$$\% \text{ Inhibition} = \frac{\text{MFI in the absence of competitor} - \text{MFI in the presence of competitor}}{\text{MFI in the absence of competitor}} \times 100$$

Results and Discussion

To establish the utility of the FlowMetrix system for multiplexed analysis, assays have been developed representing both immunoassays for analysis of serum proteins and DNA hybridization-based assays for genetic analysis.

Immunoassays. An area of clinical immunology that is suited particularly well to panel-based analysis is determination of the serum concentrations of allergen-specific IgE antibodies. In many instances, allergic individuals are tested for serum IgE and IgG concentrations specific for a related series of allergens, such as grasses, trees, or bee venoms; in other cases, panels of geographically related allergens are examined. Fig. 2 depicts the results of a multiplexed assay for canine IgE and IgG antibodies specific for 16 different grass allergens. The entire analysis of each serum sample was performed in two tubes, one

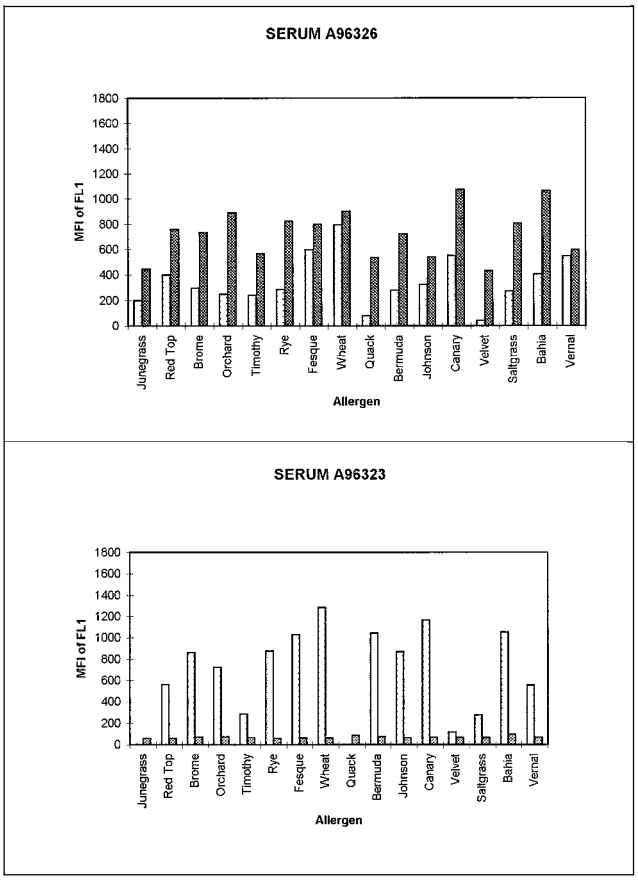

Fig. 2. Allergen-specific IgE and IgG.

Multiplexed analysis of two different canine sera for specific IgE (□) and IgG (■) antibodies to 16 different grass allergens.

for IgE and one for IgG. Each reaction contained 16 different microsphere sets, each set coated with a different allergen. After incubation with serum samples and fluorescent antibodies, the reactions were analyzed with the FlowMetrix system. Removal of excess fluorescent antibodies is not required because of the extremely small sensing volume of the flow cytometer. The analysis time on the flow cytometer is ~15 s for each sample, and the results are reported, instantaneously, in a spreadsheet format. The green fluorescent signal for each microsphere set, reported as MFI, is proportional to the amount of bound antibodies; thus, appropriate calibrators can be used to perform quantitative assays. The data shown in Fig. 2 represent the measurement of allergen-specific antibodies in canine sera; similar principles would apply to measurement of human antibodies. Serum A96326 demonstrated low IgE reactivity to most of the grass allergens as compared with serum A96323. In contrast, serum A96326 contained high concentrations of allergen-specific IgG, compared with allergen-specific IgG in serum A96323 for several of the grass allergens. Perhaps the most important point to be gleaned from these data is that 64 individual measurements were performed, analyzed, and reported from only four reaction tubes.

Genetic analysis. A widely used method for ascertaining the presence of particular sequences in target nucleic acids is hybridization with short, synthetic oligonucleotide probes of known sequence *[6]*. Short oligonucleotides of eight to 20 bp are used because they display a high degree of hybridization specificity *[7–9]*. Molecular hybridization with short oligonucleotides has been applied to numerous problems in research and medicine that require DNA sequence analysis. These include detection of mutations for diagnosis of genetic and malignant diseases *[6*, *10*, *11]* and for classification of HLA alleles for tissue typing *[12]*. The experiments described here involved a series of 16 oligonucleotide probes representing allelic sequences of the human HLA-DQA1 gene (Fig. 3).

Hybridization properties of microsphere-attached oligonucleotides. Parallel hybridization assays necessitate that a single reaction condition is used for hybridization of the oligonucleotide probes to the target nucleic acid. Hybridization solutions containing tetramethylammonium chloride are useful for this purpose since tetramethylammonium chloride minimizes the effects of base composition on duplex association and disassociation rates *[13*, *14]*. The stringency of hybridization is then controlled strictly as a function of the length of contiguous complementarity. All hybridization experiments reported here were performed in the presence of 2.25 mol/L tetramethylammonium chloride.

Before attempting to multiplex the 16 oligonucleotide-substituted microspheres, the basic parameters of microsphere-attached oligonucleotide hybridization were examined with a single microsphere–oligonucleotide. A constant number (8000) of one oligonucleotide-coated microsphere set, 3403−, was hybridized to increasing quantities of complementary (3403+) or unrelated (5501+) fluorescent oligonucleotide at 55 °C for 15 min, and the green fluorescence associated with the microspheres was measured with the FlowMetrix system (Fig. 4A). Microsphere-bound fluorescence increased in a linear manner up to 40 fmol of input complementary fluorescent probe. Identical quantities of input noncomplementary fluorescent oligonucleotide showed little or no hybridization. Each of the 16 DQA1 oligonucleotide pairs showed similar hybridization properties (data not shown), and all future experiments were performed with 8000 of each microsphere and 40 fmol of each fluorescent oligonucleotide.

The kinetics of hybridization of microsphere-bound DQA3403− oligonucleotide to complementary

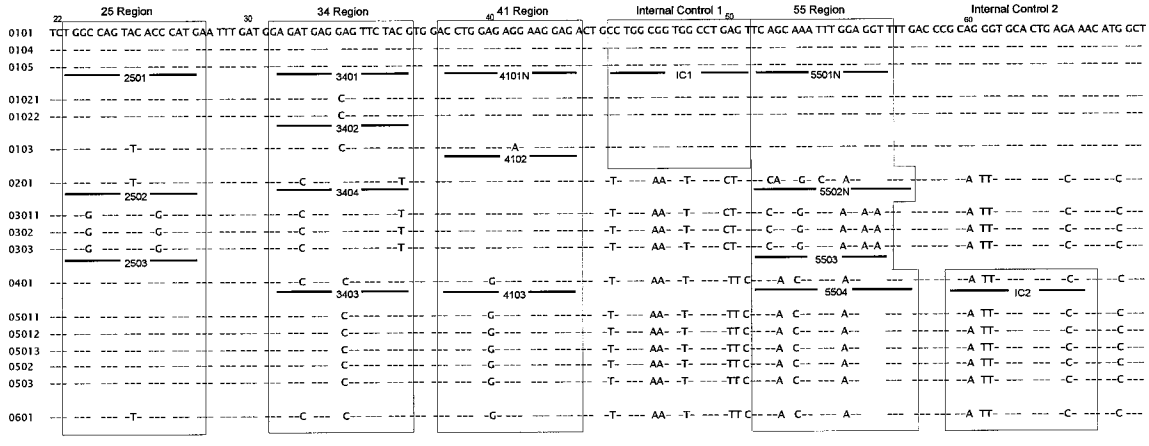

Fig. 3. Nucleotide sequences of HLA-DQA1 alleles and oligonucleotide probes.

HLA-DQA1 nucleotide sequences corresponding to amino acids 22 to 67 are shown with allelic designations on the left. Hyphens (-) indicate identity between sequences, and base changes are noted. The 25, 34, 41, and 55 *boxed regions* are the areas of the HLA-DQA1 gene that have a sequence that contributes to classification of the DQA1 allele. Sequences corresponding to the DQA1 oligonucleotide probes are *underlined* and *numbered*.

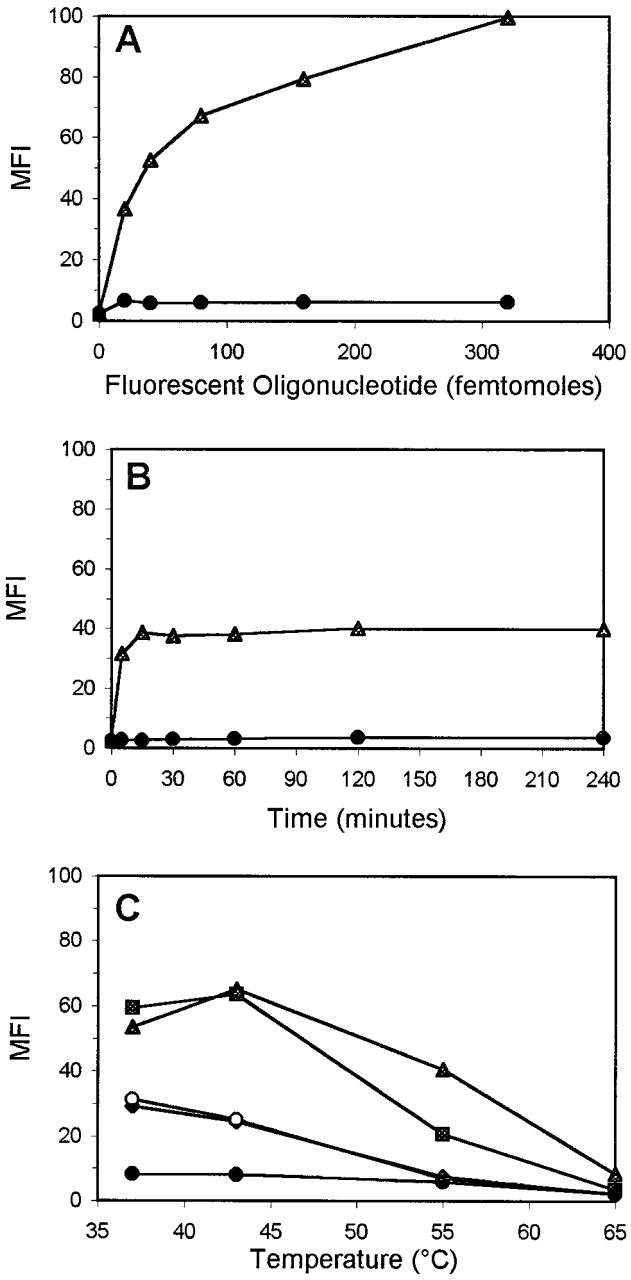

Fig. 4. Hybridization properties of microsphere-bound oligonucleotides.

(*A*) Fluorescent oligonucleotide titration. Microspheres coated with DQA3403− were hybridized to increasing quantitities of fluorescent DQA3403+ (△) or DQA5501+ (●) at 55 °C for 15 min. (*B*) Hybridization kinetics. Microspheres coated with DQA3403− were hybridized to 40 fmol of fluorescent DQA3403+ (△) or DQA5501+ (●) at 55 °C for the indicated times. (*C*) Hybridization temperature. Microspheres coated with DQA3403− were hybridized to 40 fmol of fluorescent DQA3403+ (△), DQA3402+ (■), DQA3404+ (◆), DQA3401+ (○), or DQA5501+ (●) for 15 min at the indicated temperatures.

(DQA3403+) and unrelated (DQA5501+) fluorescent oligonucleotides is shown in Fig. 4B. Hybridization of the microsphere-bound DQA3403− oligonucleotide with its complementary probe is rapid, requiring no more than 15 min to reach completion. During this time period, there is little or no hybridization of the unrelated fluorescent probe. Therefore, a period of 15 min was used routinely for hybridization of the microsphere-attached oligonucleotides to the fluorescent oligonucleotide probes

To confirm that hybridization at 55 °C in 2.25 mol/L tetramethylammonium chloride allowed discrimination between complementary and closely related 18-bp sequences, hybridization reactions were performed at various temperatures with fluorescent probes representing complementary, single, and double mutants, and unrelated sequences. The DQA3403− microsphere set was selected for analysis because the DQA3403 sequence differs from DQA3402 by only a single base and this mismatch is positioned such that the oligonucleotides have 13 contiguous identical bp, the longest stretch of identity between any of the 16 oligonucleotides in the HLA-DQA1 panel. As shown in Fig. 4C, hybridization temperatures <55 °C did not allow discrimination of the point mutant DQA3402 oligonucleotide from the complementary sequence. At 55 °C, the point mutant hybridized with ~50% lower efficiency than the complementary sequence, and the double mutant and unrelated oligonucleotides did not hybridize. No hybridization of any oligonucleotides occurred at 65 °C. Thus, hybridization at 55 °C in 2.25 mol/L tetramethylammonium chloride should allow detection and discrimination of perfectly complementary oligonucleotide duplexes from mismatched ones.

DNA sequence analysis by competitive hybridization. The purpose of these experiments was to establish a general system for multiplexed hybridization analysis of DNA sequences that uses unlabeled, ds target DNA, such as a standard PCR product, as the test material. The assay system contains 16 different microspheres coupled to the 16 different DQA1 oligonucleotide target probes, and the 16 complementary fluorescent DQA1 probes (Fig. 3). In the presence of a complementary competitor sequence, hybridization between the complementary fluorescent probe and its complementary microsphere target will be reduced.

To test the specificity of this system for DNA sequence analysis by competitive hybridization with unlabeled DNA targets, unlabeled oligonucleotides were used as model competitors. Preliminary experiments demonstrated that 500 fmol of ds oligonucleotide resulted in maximal inhibition of fluorescent probe hybridization and that the hybridization was complete within 15 min (data not shown). Each unlabeled ds HLA-DQA1 oligonucleotide was denatured and hybridized to the mixture of the 16 HLA-DQA1 fluorescent oligonucleotides. Then, the mixture was hybridized to the mixture of 16 HLA-DQA1 microspheres. Competition of oligonucleotide duplex formation on microsphere sets with the HLA-DQA1 oligonucleotide competitors was very effective and specific (Table 1). Denatured ds HLA-DQA1 oligonucleotides inhibited hybridization on complementary microsphere sets 62–93%, and inhibited highly homologous or unrelated microsphere sets <21%. Thus, the HLA-DQA1 com-

Table 1. Multiplexed competitive hybridization with oligonucleotide competitors.

Oligonucleotide competitor	Microsphere-bound oligonucleotide															
	2501	2502	2503	3401	3402	3403	3404	4101N	4102	4103	5501N	5502N	5503	5504	IC1	IC2
	Percent inhibition															
—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2501	64	10	5	−1	−6	4	1	4	1	3	5	−9	2	3	1	3
2502	19	77	−7	−2	0	1	−3	−3	−1	−4	−4	−4	−5	4	−2	0
2503	7	1	85	1	2	4	−3	−2	4	−1	1	−1	5	6	−18	−4
3401	−1	−1	−3	76	1	6	1	3	2	2	5	−4	2	5	−12	−2
3402	−1	−8	−12	14	83	11	−5	−5	−3	−10	0	−7	−4	1	−15	−9
3403	−2	−3	−1	0	7	73	−1	2	1	−1	5	−9	−4	2	2	−1
3404	5	5	4	6	2	8	62	10	9	6	10	0	7	9	2	4
4101N	−2	2	−3	6	6	7	8	74	17	21	6	5	7	6	0	6
4102	0	−1	−4	0	−3	5	0	8	79	3	7	−1	5	4	−7	2
4103	−2	11	3	5	6	7	5	4	7	71	0	8	9	6	−16	0
5501N	−3	3	0	2	5	5	1	−1	6	1	79	9	6	4	−17	−1
5502N	3	5	5	5	7	3	−1	1	2	−4	−7	86	4	1	−8	−4
5503	−5	0	−6	1	2	0	−2	−8	0	−7	−9	13	80	−5	−10	−4
5504	3	8	9	5	6	5	2	2	7	6	4	13	4	93	−4	−2
IC1	−4	−1	3	11	7	6	3	11	7	7	9	3	6	6	68	10
IC2	4	3	−4	2	6	1	0	4	3	3	6	7	−3	0	−2	94

petitive hybridization test performed accurately with denatured ds oligonucleotide competitors. This multiplexed hybridization assay has been used to perform HLA-DQA1 tissue typing of PCR-amplified human genomic DNA (P. Smith et al., submitted for publication). The assay types DQA1 alleles in both homozygous and heterozygous samples, and the entire analysis is accomplished in 30 min after PCR amplification.

These studies have demonstrated the ability of the FlowMetrix system to perform highly multiplexed assays for analysis of specific protein–protein interactions, such as immunoassays, and for analysis of specific DNA sequences. The system provides several advantages for analysis of biologically and medically relevant molecules, including speed, economy, and advanced analytical capabilities. The system reduces assay time by performing multiple analyses simultaneously rather than sequentially. The no-wash format of many microsphere-based assays, particularly in the final detection step, is considerably faster than microtiter-based assays that require multiple washing steps to remove excess reagents. In addition, the rapid kinetics of microsphere-based assays allow shorter incubation times than conventional solid-phase assays. The reduced assay time also reduces labor costs for performing multiple analyses. Reagent usage for microsphere-based assays is 10- to 1000-fold less than microtiter-based assays. Multiplexing allows unique analysis of molecular interactions that can only be performed in a multiplexed format. For example, direct comparison of receptor binding avidity for related ligands could be performed under truly competitive conditions where the receptor and all ligand variants are present in the same reaction at the same time.

Most, if not all, assays for biomolecules can be adapted to the FlowMetrix system. Immunoassays have been developed with all assay formats available with microtiter-based technology, including direct binding assays, immunometric or capture/sandwich assays, and competitive inhibition assays. In addition, receptor/ligand analysis and epitope mapping are well suited for the multiplexed format. Multiplexed hybridization of nucleic acids can be used for tissue typing, diagnosis of genetic disease, paternity/forensic testing, tests for oncogenes, tests for foreign pathogens, and tests for mRNA/cDNA expression. Because of the real-time analysis, no-wash format, and solution-phase kinetics of microsphere-based assays, multiplexed enzyme assays also can be performed to analyze both substrate specificity and kinetic data for multiple enzymes or multiple substrates simultaneously. For example, multiple microsphere sets could be prepared, each bearing a different fluorescent peptide sequence. These microsphere sets could then be mixed and used to examine the specificity or relative activity of an endopeptidase for the different substrate sequences. In this case, enzymatic activity would be measured as the loss of fluorescence from the microspheres.

The FlowMetrix system represents a revolutionary new technology that can be applied to virtually any application that requires analysis of molecular interactions, including basic research, clinical diagnostic testing, high-throughput drug screening, environmental testing, and agricultural testing. This system is unique in its ability to provide multiplexed, high-throughput analysis coupled with real-time data analysis. The system offers excellent sensitivity, precision, speed, and economy.

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