

Research paper

Design and optimization of a multiplex anti-influenza peptide immunoassay

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Abstract

Current flu vaccines are based on killed or attenuated virus vaccines that must be altered each year to include the hemagglutinin and neuraminidase genes from a strain of virus predicted to predominate in the coming year. A vaccine that could protect against multiple strains of influenza A and B would be a major asset in the fight against flu-related mortality and morbidity. To support development of such a vaccine, we have developed a Flu Multiplex Assay based on a Luminex[®] platform to assess serum antibody levels to two conserved peptides derived from influenza A (M2 protein) and influenza B (hemagglutinin protein). The peptides were synthesized with a biotin label and subsequently coupled to two different LumAvidin[™] microspheres. We then tested various sera against both types of peptide in the multiplex assay format. The data show that sera from Rhesus macaques immunized with a single peptide react only with the homologous peptide while Rhesus macaques immunized with both peptides respond well to both peptides. Additionally, we were able to specifically compete reactivity to both peptides. We have tested serial bleeds from 100 pediatric patients at ages ranging from 16 to 56 weeks as well as single bleeds from over 100 healthy adults. No overall trend in titer relative to pediatric age was detected. Both demographics exhibited a minimal response to either the A/M2 or B/HA₀ peptides. However, the average titer for the pediatric serum samples was significantly lower than that found in the adult population. The adult population exhibited a higher prevalence of low reactive samples. Assay reagents and parameters have been optimized and the assay is shown to be repeatable and robust. The assay will be used to support clinical vaccine trials of a bivalent peptide vaccine. © 2008 Elsevier B.V. All rights reserved.

Keywords: Influenza; Multiplex; EIA peptide vaccine

Abbreviations: HA, hemagglutinin; NA, neuraminidase; DMSO, dimethyl sulfoxide; OMPC, outer membrane protein complex; CP, consistency panel; MAbs, monoclonal antibodies; OMPC, *N. meningitidis* outer membrane protein complex; MFI, median fluorescent intensity; WB, wash buffer; RB, run buffer; BC, Buffer Control; LOQ, limit of quantitation; IgG-PE, immunoglobulin G phycoerythrin; CB, coupling buffer; RSD, relative standard deviation.

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1. Introduction

Influenza causes between 20,000 and 40,000 deaths each year in the United States alone (Simonsen et al., 2000; Thompson et al., 2003). Those most at risk are infants, elderly, and immunocompromised individuals (Harper et al., 2005). Currently, immunization with killed whole virus or live vaccines offers the best protection against influenza (Reichert et al., 2001; Greenberg and Piedra, 2004; Hannoun et al., 2004). However, the current whole virus vaccines must be modified each year, in advance of the coming flu season, to include the hemagglutinin (HA) and neuraminidase (NA) genes of a influenza strain predicted to be prevalent. If the prediction is correct then efficacy of up to 90% can be achieved. However, if the prediction is wrong a lesser efficacy is achieved (de Jong et al., 2000; Hsieh et al., 2005). Due to mutations which typically occur in the HA and NA genes it is necessary to revaccinate on a yearly basis with the updated strain. This presents a tremendous cost burden to health care providers, payers, vaccine manufacturers, and the public as well, if a mismatched vaccine strain is chosen and distributed. In addition, logistical issues affecting supply of vaccine can become critical concerns. Therefore, a vaccine that is able to provide protection against multiple strains of influenza without yearly recombination and production process changes would be very valuable.

A vaccine that meets these requirements should direct the immune response to conserved regions of the influenza virus. The immune response should protect from or ameliorate the affects of influenza infection. The vaccine should also be relatively easy to produce in large enough quantities to meet the needs of the population and health care providers. A recombinant peptide vaccine is one way to achieve these goals. We report the development of a multiplex assay to assess serum antibody levels to two conserved peptides derived from influenza A and influenza B. The two peptides, designated as A/M2 and B/HA₀, are derived from conserved regions of the matrix and hemagglutinin proteins, respectively. The A/M2 peptide is derived from the extra cellular domain of the M2 protein and is very conserved across strains of influenza A (Fan et al., 2004; Liu et al., 2005). Vaccination with the A/M2 peptide elicits near complete protection in mouse and ferret models of infection (Fan et al., 2004). The HA component of killed influenza B vaccines contains several neutralizing epitopes that contribute to vaccine efficacy (Ansaldi et al., 2004). The B/HA₀ peptide is derived from a conserved region of the HA molecule of influenza B. Vaccination with this peptide also has elicited complete protec-

tion from lethal influenza challenge in animal models (Bianchi et al., 2005).

The Luminex 100™ assay system has been used successfully to simultaneously measure antibodies to multiple antigens from a single sample (Dias et al., 2005; Ray et al., 2005). Using a LumAvidin™ bead based assay we linked each biotinylated peptide (A/M2, B/HA₀) to a spectrally distinct bead type. We were able to combine the peptide-labeled beads in a true multiplex assay and to show good sensitivity and specificity for anti-peptide antibodies from Rhesus monkeys immunized with different doses of both peptides. The assay is both quantitative and robust. The background reactivity in non-immunized individuals appears to be low.

2. Materials and methods

2.1. Sera

Panel A: Consistency panel (CP) made with low A/M2 and B/HA₀ reactive human sera spiked with 8 increasing amounts of high titered Rhesus monkey sera. Five sets of samples of varying reactivity were created. Each set contained nine samples consisting of a non-spiked low reactive human serum and eight low reactive human samples spiked with increasing concentrations of high-titer rhesus sera. Sets of samples were constructed in this manner to create positive samples of varying reactivity with a human serum background. Panel B: Sera from healthy non-immunized male and female adults ages 18–40 obtained from an outside vendor (Valley Biomedical, Winchester, VA). Panel C: Sera from non-immunized children (100 patients, ages 16–56 weeks, 4–5 samples each) obtained during a previous *Haemophilus influenzae* B (PedvaxHIB®) vaccine trial. Panel D: Sera from Rhesus monkeys immunized with both the A/M2 and B/HA₀ flu peptides. Stock standards and controls consist of high titered pooled sera from Rhesus monkeys immunized with either A/M2 or B/HA₀ monovalent peptides. All animal samples were obtained from studies approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Human samples were obtained from studies that conformed to applicable country or local requirements with regard to ethical committee review, informed consent, and the protection of the rights and welfare of human individuals participating in biomedical research.

2.2. Peptides

Peptides were derived from influenza A matrix protein (A/M2) or influenza B hemagglutinin (B/HA₀). The

peptides were synthesized with a biotin molecule attached (Synpep, Dublin, CA). The A/M2 sequence is SLLTEVETPIRNEWGSRSDSSDK-Biotin and the B/HA₀ is Biotin-EGPAKLLKERGFFGAIAGFLEE (Fan et al., 2004; Bianchi et al., 2005). The original consensus sequence of A/M2 was modified with serine substitutions for cysteines at positions 16 and 18 to inhibit cross linking and improve peptide stability and function. Stock solutions of the A/M2 (350 μ M) and B/HA₀ (1 mM) peptides were solubilized in dH₂O and DMSO, respectively. The peptides were stored frozen, at -70°C , until used. A/M2 or B/HA₀ peptides coupled to *N. meningitidis* outer membrane protein complex (OMPC) and adsorbed to alum beads were used to compete the antibody response to peptide coupled beads in the Flu Multiplex Assay. The OMPC coupled peptides were stored at 4°C in 0.15 M NaCl buffer until use.

2.3. Monoclonal antibodies

Two monoclonal antibodies (MAbs) were used to test specificity of the LumAvidin™ coupled beads. Both the anti-A/M2 monoclonal L18.H7 and anti-BHA₀ monoclonal 08F12 have been shown to bind to the A/M2 and B/HA₀ peptides, respectively, in a specific manner. They have both been shown to provide protection in an influenza infection model (M. Horton, personal communication) (Bianchi et al., 2005).

2.4. Bead coupling

For each biotinylated peptide a distinct LumAvidin™ bead was coupled in the following manner. A bead having a unique spectral identifier was spun down and resuspended in PBS/Tween-20/0.05% Na azide (Coupling Buffer, CB) at 25×10^6 beads per ml. Stock peptide was solubilized in PBS/Tween-20/0.05% Na azide and added for a final concentration of 10 μ M. The beads were rotated in the dark for 30 min at room temperature. Subsequently, the beads spun down and washed $3 \times$ in CB. A 10 μ l sample of beads was diluted 1/1000 and counted using a Z1™ Series Coulter Counter® (Beckman Coulter, Miami, FL). The peptide coupled beads were resuspended at a final concentration of 25×10^6 per ml in CB and stored in the dark at 4°C .

2.5. Multiplex immunoassay

The Flu Multiplex Immunoassay was performed as follows: 96-well filter bottom plates were prewetted with PBS/0.05% Tween-20 (Wash Buffer, WB). The buffer

was removed by placing the filter plate on a vacuum apparatus and aspirating it through the bottom of the filter plate. Serum samples were diluted 1/50 in run buffer (PBS/0.05% Tween-20/10% Normal Goat Serum (Sigma, St. Louis, MO)) (RB). The standard control titration (12 points, 2-fold dilutions), buffer controls (BC), high positive, low positive, and negative controls, and samples were added, 50 μ l per well, to appropriate wells. All standards, controls and samples were assayed in duplicate wells for each dilution used. A/M2 and B/HA₀ peptide coupled LumAvidin™ beads were mixed immediately prior to use at 1×10^5 beads per ml for each bead. The mix of beads was aliquoted, 50 μ l per well, into the 96 well filter bottom plates containing sample or standards. The plates were incubated at 20°C , for 1 h $\pm$ 10 min, while shaking at 700 rpm on a microplate Thermomixer (Eppendorf, Westbury, NY). After the incubation a plate was placed on a vacuum apparatus and the wells were aspirated as previously. Each well was washed with 200 μ l of WB. The wash procedure was repeated twice for a total of three washes. After the wash, anti-human IgG-PE conjugate was diluted in RB, to 5.0 μ g/ml, and added 100 μ l per well. The plates were incubated, for 1 h $\pm$ 10 min, and washed as before. After the last wash, 120 μ l of WB was added to each well and the plates were incubated and shaken as before for 5 min to resuspend the beads. The plates were then placed on a calibrated Bio-Plex™ instrument (Bio-Rad Laboratories, Hercules, CA) and the median fluorescent intensity (MFI) of the sample to each peptide coupled bead was measured. Samples were run in duplicate at 1/100 and 1/1000 final dilutions or as otherwise noted. The MFI response of a sample to a given peptide was compared to a standard reference serum using a weighted four-parameter logistic curve fit. (O'Connell et al., 1992). The standard reference serum is a pool of sera from individual Rhesus monkeys, immunized with either A/M2 or B/HA₀. The standard is assigned arbitrary values expressed in peptide specific units per ml. Sample antibody titers were determined by interpolation of the MFI values against the standard control serum and reported as peptide specific units per ml.

2.6. Competition assay

The Flu Multiplex Immunoassay was performed with an additional competition step as follows: OMPC-linked peptide (A/M2 or B/HA₀) absorbed to alum was used to compete with binding of peptide specific serum antibody to the peptide coupled LumAvidin™ beads. Pediatric (Panel C) and adult (Panel B) sera were prescreened, as described above. Each serum sample

Table 1

Rhesus serum antibody responses to A/M2 or B/HA₀ responses after immunization

Assay	ID#	¹ Dose (μg)	Post dose 1	Post dose 2	Post dose 3
A/M2	1	0.5	3197	10,817	12,541
	² 2	0.5	3469	2722	3019
	3	0.5	950	15,604	22,693
	4	5	1659	2863	42,242
	5	5	2956	3984	2939
	6	5	3382	12,846	3341
	7	50	1067	3644	7806
	8	50	3197	4066	3817
	9	50	7983	15,707	15,555
B/HA ₀	1	0.5	912	529	498
	² 2	0.5	782	967	615
	3	0.5	1393	3310	4537
	4	5	41	219	101
	5	5	1481	10,283	4569
	6	5	917	3081	1530
	7	50	468	985	716
	8	50	1239	3442	2567
	9	50	833	2467	847

¹Dose of each peptide in a bivalent formulation. Post dose 1 immunization at time=0, sample at week 4. Post dose 2 immunization at week 8, sample at week 10. Post dose 3 immunization at week 24, sample at week 26. ²ID# 2 post dose 2 sample from week 12. All pre-vaccination titers were <LOQ.

was diluted 1/50 in RB containing 1 μM concentrations of both OMPC-A/M2 and OMPC-B/HA₀ peptides. We chose our competing peptide concentration based on preliminary experiments with immune Rhesus serum. The amount of peptide that was chosen could consistently and completely compete 200 peptide units of anti-A/M2 reactivity and 10 peptide units of anti-B/HA₀ to

Table 2

MFI responses of mono-immune serum to different peptide coupled or “naked” LumAvidin™ bead preparations mixed at different times before addition of sample

Minutes	A/M2 immune serum response			B/HA ₀ immune serum response		
	A/M2 ²	B/HA ₀ ²	57 ³	A/M2 ²	B/HA ₀ ²	57 ³
0 ¹	7889	31	X	8	9920	X
	8078	33	1174	9	9556	1996
30	7815	114	X	53	8899	X
	8115	105	2363	54	8498	4494
60	7140	169	X	73	7670	X
	7130	148	2596	69	7166	4714
90	5950	186	X	79	6319	X
	5593	164	2260	81	6339	4239

Raw MFI counts from peptide migration experiments. Serum from monovalent peptide immunized Rhesus monkeys. X denotes beads not present in the well. ¹Normal use of beads per SOP (less than 10 min between mixing beads and sample addition). ²Peptide coupled to bead. ³Non-coupled “naked” beads.

below the LOQ (data not shown). The samples were incubated for 1 h with competing peptide and then 50 μl of the peptide sample mix was added to 50 μl of A/M2 and B/HA₀ peptide coupled LumAvidin™ beads. The assay was then performed as described in Materials and methods.

3. Results

3.1. Coupling of beads

LumAvidin™ beads were obtained containing between 1.25×10^6 and 1.8×10^6 avidin per bead (Luminex Corporation, Austin, TX, personal communication). Each avidin was reported to have 4 potential biotin binding sites per molecule. Experiments were conducted to determine the concentration of peptide required to saturate the LumAvidin™ beads. The beads, coupled with differing amounts

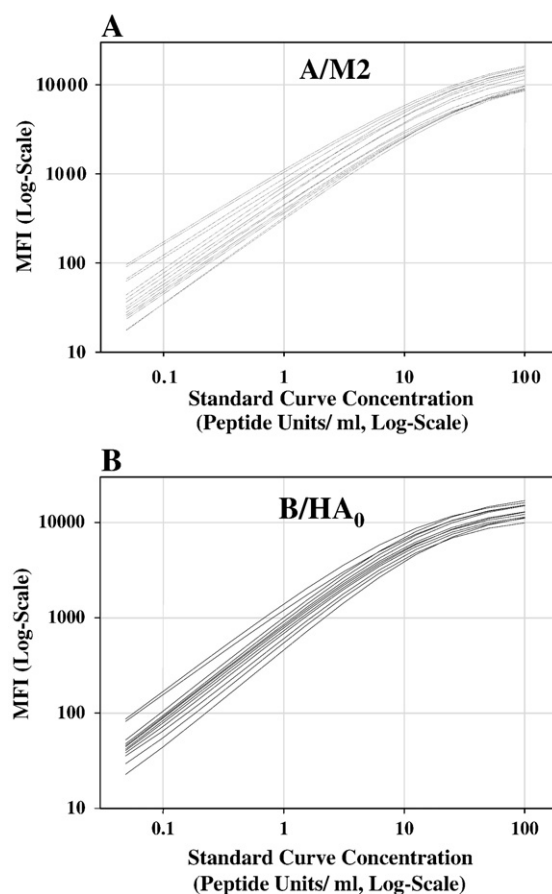


Fig. 1. Average flu standard sera titrations (Log [Standard Curve Concentration] vs. Log [MFI]) against A/M2 (A) and B/HA₀ (B) peptides. Standard sera consisted of normal human sera spiked with sera from Rhesus monkeys vaccinated with 10 μg of either A/M2 or B/HA₀ peptide in alum.

of peptide, were tested against multiple concentrations of a MAb (anti-A/M2, L18.H7 or anti-B/HA₀, 08F12) that react specifically to a given peptide. Anti-Mouse IgG-PE conjugate (Rockland Laboratories, Gilbertsville, PA) was used to detect the MAb. The peptide titrations indicated that saturation of the LumAvidin™ beads occurs above 1 μ M for either peptide (data not shown). A peptide coupling concentration of 10 μ M was conservatively

chosen to ensure full saturation of biotin binding sites for both peptides during the coupling procedure.

3.2. Rhesus monkey immune responses

Post dose 3 sera from Rhesus monkeys immunized with either A/M2 or B/HA₀ peptide monovalent vaccine or with a bivalent peptide vaccine were obtained. The

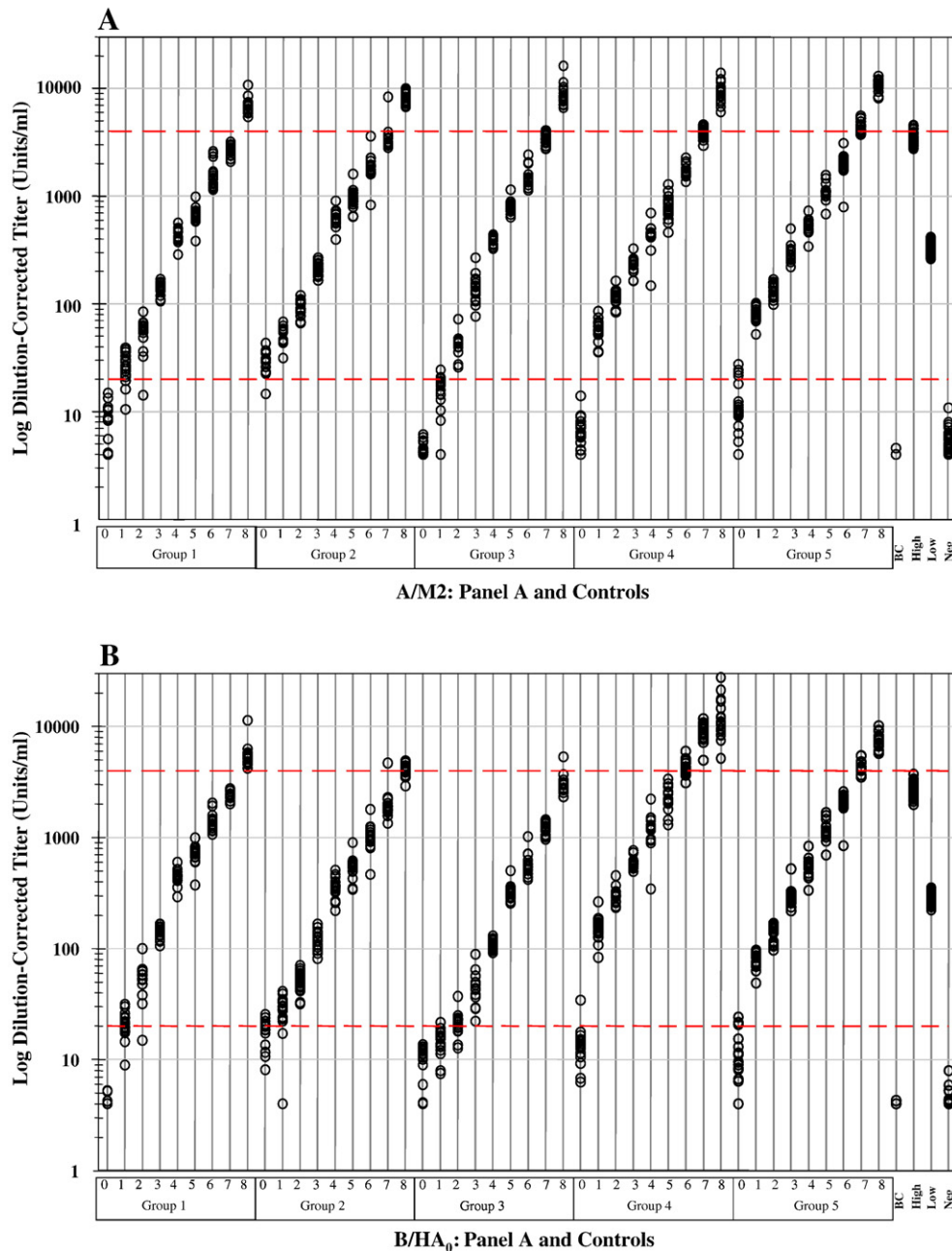


Fig. 2. Dilution corrected anti-peptide titers against A/M2 (A) and B/HA₀ (B) peptides for the CP samples and control samples tested as described in the text. Dashed red lines represent the assay limits of quantitation.

sera were titrated (8-point, 4-fold dilutions, 1/100 start) against single A/M2 or B/HA₀ peptide coupled beads or a mixture of both A/M2 and B/HA₀ coupled beads. Sera from monovalent immunized monkeys reacted only with the beads coupled to the homologous peptide whether in single or duplex format. Sera from bivalent immunized animals reacted against either bead in the single bead format or both beads in the multiplex format. Sera from nine Rhesus monkeys immunized with three doses of A/M2 and B/HA₀ bivalent vaccine were tested in the Flu Multiplex Assay (Table 1). The pre, post dose 1, post dose 2, and post dose 3 titers were calculated. All pre titers were less than 20 peptide units/ml for both peptides. In all post immunization samples at each dose of vaccine a measurable rise in titer of anti-peptide antibody was detected (A/M2, 950–42,242 or B/HA₀, 41–10,283 peptide units/ml).

3.3. Peptide migration

Experiments to determine the stability of the bead coupling chemistry were conducted. A/M2 and B/HA₀ coupled beads were mixed and added to Rhesus serum samples either immediately or the mixed beads were allowed to incubate at room temperature for up to 90 min prior to adding to the assay. In addition we tested “naked” beads that had not been coupled with any peptide, and therefore contained free biotin binding sites, to determine the background bead reactivity of the tested serum. Rhesus serum from A/M2 or B/HA₀ monovalent immunized animals was then titrated against the beads. At time zero the monovalent sera diluted 1/100 demonstrated minimal reactivity (<35 MFI) against the heterologous peptide. There was a small but perceptible rise in the cross reactivity as coupled beads were pre-incubated together prior to use in the assay. The “naked” beads showed an immediate reactivity if incubated with either coupled bead. The reactivity to the “naked” bead more than doubled from the initial read after a 90 min incubation with either peptide coupled bead. The results indicated that a small but detectable amount of peptide was migrating from one coupled bead to another. The migration of peptide from a coupled bead to a naked bead was more pronounced and rapid. The raw MFI data is shown in Table 2.

3.4. Assay performance

After optimizing conditions and setting a standard operating procedure we performed a series of experiments to validate the assay. Briefly, three separate

analysts each coupled both peptides to each of three different lots of LumAvidin™ beads for a total of nine bead batches. Each analyst performed two runs with each bead batch (A/M2 and B/HA₀ coupled beads). Each run contained 3 plates. Panel A was assayed in its entirety on each run. Panels B and C were divided across all 18 runs. One sample from Panel D was titrated (8, 2-fold dilutions, 1/100 start) and assayed on each run. Each plate contained the standard (12, 2-fold dilutions, 100 to 0.05 units/ml), high, low, negative, and buffer controls (BC). The standard, controls, and test samples were diluted with assay buffer containing 10% Normal Goat Serum, and tested in duplicate wells. The response was measured in MFI. The sections below describe our findings.

3.5. Extravariability

Duplicate wells were analyzed to determine extravariability; i.e. constraints on the variability between duplicate MFIs. This serves as a quality control measure for individual sample preparation. The extravariability rule for duplicate MFI readings was established using the range of the 10th root transformed MFI readings on the individual standard, control and test samples from the 18 validation runs. Limits on extravariability of the MFI response in duplicate wells were set as follows: A replicate pair of MFI readings is designated as extra-variable (EXV) if: (1) the range of the untransformed MFIs exceeds 10; (2) the range of the 10th root transformed (Draper and Smith, 1986) MFIs exceeds 0.05; and (3) both replicates fall between the MFIs associated with the quantifiable range of the assay. These same criteria were used for both peptide coupled beads.

Table 3
Percent of samples with titers above the listed cutoff units/ml in the A/M2 or B/HA₀ assays

Titer units/ml	Panel B vs.	Panel C vs.	Panel B vs.	Panel C vs.
	A/M2	A/M2	B/HA ₀	B/HA ₀
	N=137	N=485	N=134	N=484
10	35.0%	7.0%	53.7%	8.9%
20	24.8%	4.3%	35.1%	3.9%
30	19.7%	2.9%	27.6%	3.3%
40	17.5%	2.5%	17.9%	2.5%
50	13.9%	2.1%	13.4%	2.3%
75	5.1%	1.2%	9.7%	1.7%
100	2.9%	1.0%	7.5%	1.4%
250	0.7%	1.0%	1.5%	0.8%
500	0.0%	0.8%	0.0%	0.4%
750	0.0%	0.6%	0.0%	0.2%
1000	0.0%	0.6%	0.0%	0.2%

Table 4
Sensitivity and specificity of Panels B and C samples

Panel	Assay	Sensitivity	Specificity
B	A/M2	44.4	73.6
B	B/HA ₀	58.6	68.6
C	A/M2	2.2	94.2
C	B/HA ₀	16.0	95.3

3.6. Reference standard characteristics

A weighted four-parameter logistic function (O'Connell et al., 1992) was used to model the 12-point, 2-fold serum standard dilution series for each plate (100–0.05 peptide units/ml). During our validation experiments, a standard curve was included on every plate for a total of 54 standard curve titrations. The upper and lower limits of quantitation were calculated following previously described methods (O'Connell et al., 1992). The limits of quantitation (LOQ) of the standard curve were 0.2 to 40 peptide units/ml (non-dilution corrected) for both peptide coupled beads. Representative standard curves are shown (Fig. 1). In the routine operation of the assay, if either: (1) more than two standard concentrations

fail the extravariability specification; or (2) the slope parameter falls outside its 3σ specification limits; or (3) the weighted root mean square error (wRMSE) is greater than its 3σ specification limit, the assay plate is considered invalid.

3.7. Assess ruggedness to analysts and bead lots/bead preparation

The CP (Panel A, described in the Materials and methods) was used to assess the precision and ruggedness of the assay. The CP samples were assayed with 3 different bead lots (A/M2 and B/HA₀ coupled beads) coupled by 3 different analysts for a total of 9 sets. The CP series were run twice per set by each analyst for a total of 18 runs. The analysis was performed using only samples for which titers between 10 units/ml and 4000 units/ml were obtained in at least 2 of the 18 runs. All remaining samples with titers <10 peptide units/ml or >4000 peptide units/ml were assigned a titer of 10 or 4000, respectively. Differences between bead preparations were within 20%, indicating that the assay is rugged to bead batch preparation. Pair wise differences among analysts and bead lots were within 10% for both peptides indicating that the

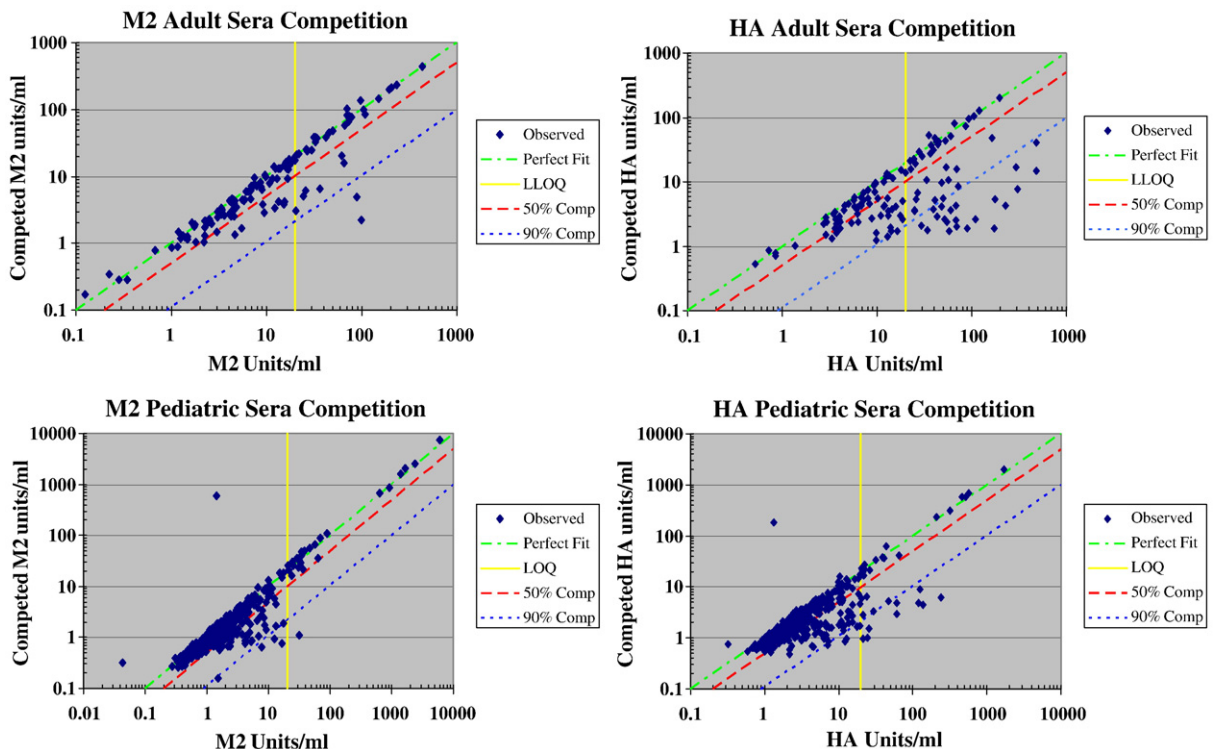


Fig. 3. Non-competed and peptide competed A/M2 and B/HA₀ results (◆) for Panel B (adult) and Panel C (pediatric) populations. The 0%, 50% and 90% competition lines are shown in green (—•—), red (— —), and blue (••••) respectively. The LOQ is represented by the vertical yellow line. All samples were assayed at 1/100 and results are reported as dilution corrected.

assay is rugged to changes in both analyst and bead lot. The overall assay precision was 23% RSD (relative standard deviation) for both peptides, resulting in a statistically meaningful fold-rise in titer of 2.4-fold for both peptides. The variability of calculated titers against A/M2 or B/HA₀ peptides for Panel A and the control samples is shown (Fig. 2).

To assess assay dilutability, one sample from Panel D was tested in a 2-fold dilution series from 1/100 to 1/12,800 in each of the 18 runs. Samples with determinant titers (a titer between the limits of quantitation) at more than one dilution were used to estimate the dilutability of the assay. On average, for each 10-fold increase in dilution, Rhesus sera sample titer decreased by 9.9% and 13.6% for B/HA₀ and A/M2, respectively.

3.8. Population based background

We examined the reactivity of adult and pediatric populations (Panels B and C) in the A/M2 and B/HA₀ assays. The percentage of samples from each panel with reactivity to either peptide is shown in Table 3 for titers ranging from 10 to 1000 peptide units/ml. For both A/M2 and B/HA₀, considerably more adult samples (Panel B) had a titer that was higher than 20 peptide units/ml as compared to the pediatric samples. However, the single most reactive samples were from the pediatric group, some of which achieved a titer of at least 500 units/ml for both A/M2 and B/HA₀. These samples will be addressed in the peptide competition section below.

3.9. Competitions

Results of the competition were compared with those of samples run concurrently without any peptide competition. Samples that exhibited more than a 50% reduction in calculated titer were considered to have specific antibody. A few samples with the highest reactivity to A/M2 and B/HA₀ did not compete in a specific manner indicating that these samples may have non-specific reactivity to the LumAvidin™ beads. This represents a very small proportion of the total population and was not seen in any Rhesus monkey pre-vaccination sera that we examined. Pre-incubation of serum with OMPC adsorbed to alum (no peptide) did not show any competition (data not shown). The data were analyzed at a cutoff corresponding to the LOQ of 20 peptide units/ml. The sensitivity was greatest in Panel B, whereas, the specificity tended to be higher in Panel C (Table 4). Results of the competitions relative to a 50% competition or 90% competition are shown in (Fig. 3). Sample titers are expressed as dilution corrected anti-peptide units/ml.

4. Discussion

Influenza presents the world with an ongoing threat of disease associated with a high morbidity and mortality. Current vaccines can be efficacious, but require constant monitoring of the influenza strains circulating in the population. If the choice of vaccine strain is incorrect than the vaccine may have little efficacy and actually be a drain on health resources. A vaccine that would protect across multiple strains of influenza and not require constant recombination to produce the next year's vaccine would be considerably more economically sustainable. In support of such vaccine development we have developed a multiplexed assay to simultaneously detect antibodies to two different conserved peptide regions from two different strains of influenza virus. The assay was developed to monitor antibody responses to a bivalent peptide vaccine as it progresses through clinical trials. The assay is reproducible and sensitive and should serve the development of influenza vaccines that will not require yearly recombinatorial adjustment.

Our assay utilized LumAvidin™ beads, as purchased from the manufacturer, which have the advantage of being ready to couple to any biotinylated ligand. Minimal experimentation was required to ascertain saturation conditions for either peptide to the chosen bead. Our final concentration (10 μM) ensured saturation of both peptides to the available biotin binding sites.

In any measure of serum antibodies it is necessary to determine the background reactivity of serum to the chosen platform, in this case LumAvidin™ beads. Assaying sera from monovalent peptide immunized Rhesus monkeys on LumAvidin™ beads coupled to homologous or heterologous peptide allowed us to determine the background reactivity of the sample. Our data show that sera from animals immunized with one peptide exhibited strong reactivity on beads with the homologous peptide. The reactivity was minimal on the heterologous peptide beads. Therefore the assay performed in a specific manner.

The LumAvidin™ beads are coated with Neutravidin™ (Pierce Biotechnology, Rockford, IL). The biotin/Neutravidin™ binding is reported as having a disassociation constant $K_a = 10^{15} \text{ M}^{-1}$ (Pierce Biotechnology, Rockford, IL). While this is generally thought to mean that biotin is effectively bound to avidin we conducted experiments to ensure that the peptides would not migrate from one bead to another. We were able to capitalize on the properties of the Bio-Plex system, which can specifically identify the serum reactivity associated with a given bead. We used sera from monovalent peptide immunized Rhesus monkeys and then simultaneously

monitored the sero-reactivity to LumAvidin™ beads with a homologous or heterologous peptide attached. Our data indicate that saturated peptide coupled beads that are mixed immediately prior to use and then added to samples exhibit little or no movement of peptide from one bead to another across the time period of the assay. However, if the beads were mixed and allowed to pre-incubate for 30 to 90 min there was detectable movement of peptide from one coupled bead to another as measured by a slight rise in reactivity of the mono-immune sera to the heterologous bead. We also included “naked” beads in some experiments to determine what the background reactivity was to LumAvidin™ beads alone. The biotin binding sites on the “naked” beads were available. This may explain the rise in reactivity to “naked” beads as soon as the beads were mixed. Therefore, “naked” beads are not suitable for use in background monitoring in the presence of other beads with biotinylated peptides. As a measure to prevent peptide migration we stipulated that peptide coupled beads must be mixed immediately prior to adding to a given assay plate.

After development of the assay we performed validation experiments to characterize sample extravariability, and dilutability, as well as assay ruggedness, sensitivity, and specificity. Our validation experiments consisted of eighteen assays across three different bead lots utilizing three separate analysts. The experiments allowed us to estimate limits of detection, assay variability, assay ruggedness, sample dilutability, sensitivity and specificity. Our sample EXV rules served to detect variability between duplicate wells on a given plate. The rules were developed to apply to MFI values used to quantify sample and control titers. Controls that fail EXV criteria will cause an entire plate to be repeated. Samples exceeding EXV criteria will result in a repeat assay of the sample. In addition we have set 3 sigma failure limits on the standard curve slope and RMSE. If the standard curve parameters are outside the ranges then the plate is considered invalid.

Immunoassay performance is frequently related to the dilution at which samples are assayed. In order to characterize the effect of diluting samples in the flu peptide assay we performed titrations of 18 different sera from Rhesus animals that had been immunized with various doses of bivalent flu peptide vaccine. The effect of diluting the samples on the subsequent calculated titer across a dilution range of 1/100 to 1/12,800 was –13.6% and –9.9% per 10-fold dilution for A/M2 and B/HA₀ respectively. Although a negative dilution effect is unusual, the amount of the effect was considered minimal. Based on titers in peptide immunized Rhesus we do not anticipate having to dilute human samples

more than 1/1000 to achieve a titer within the quantifiable limits of the assay.

We investigated intra (within run) and inter (between run)-assay variability using titers from Panel A samples. These results were used to assess the ruggedness of the assay to changes in analyst, bead lot, and bead batch. The titration series within Panel A were randomized across plates within a run. We did not see any significant difference in the calculated titers between analysts or bead batches. This indicates that the sample results can be replicated regardless of differences between coupled bead lots and analyst.

Prior to development of any vaccine it is essential to understand the population background reactivity of non-immunized human subjects to the vaccine components. We tested sera from normal healthy adult and pediatric populations with our Flu Multiplex Assay. Our results indicate that adults have more total reactivity above the LOQ to both peptides tested than the pediatric samples. However, the pediatric samples exhibited the highest reactivity to date in sera from a non-immunized source. If we apply the LOQ of 20 units/ml as an interim cutoff the adult and pediatric populations show 35.1% and 3.9% of samples above the cutoff for B/HA₀ and 24.8% and 4.3% above the cutoff for A/M2. Non-immunized Rhesus monkeys did not exhibit any reactivity above the LOQ in the assay. We were able to detect significant rises in titers in 9 Rhesus monkeys immunized with different doses (3 animals per dose, 3 immunizations per animal) of bivalent flu vaccine. There was no significant dose response among the 3 dose groups chosen. This was also seen previously in other animal models using this vaccine (Fan et al., 2004; Bianchi et al., 2005). This indicates that we have not achieved the minimum required dose for a significant antibody response with our current bivalent formulation of the vaccine in Rhesus monkeys. Not all of the titers in Rhesus animals after 3 doses exceeded that of all true positives in our screen of naturally occurring anti-peptide antibody in the adult human population. However, significant fold rises should still be detectable even among the highest titered “natural” reactive individuals.

Since we did detect some background reactivity to our peptides in the normal adult and pediatric populations we performed experiments to determine if the serum reactivity was specific for our peptides. Using the adult and pediatric samples described above we performed peptide competitions to determine the number of samples that would show specific competition of the anti-B/HA₀ or anti-A/M2 reactivity. We set our level of competing peptide in preliminary experiments using immune sera from peptide immunized Rhesus

animals. We utilized enough peptide to completely compete 200 units of anti-A/M2 or 10 units of anti-B/HA₀ reactivity. We utilized a level of >50% competition relative to the no peptide competition control as a determining factor for specificity. Using an assay cutoff set at the LOQ, the results showed that sensitivity is quite low in populations expected to have little or no prior infection (i.e. Panel C pediatric samples) with type A or B influenza. However, in the adult population which exhibited a positive reactivity rate of ~30% at the LOQ, a higher sensitivity was seen. The level of naturally occurring antibody to these conserved peptides appears low in the general population. The highest true positive titer (>50% specific competition) in the adult population is <100 units/ml or <500 units/ml for A/M2 or B/HA₀ antibody respectively. In the pediatric population the highest true positive reactivity against A/M2 was <40 units/ml and against B/HA₀ it was <250 units/ml. The high reactive true positive samples represent a small percentage of the normal population. Therefore, our ability to measure antibodies to these peptide antigens represents a new method of following a novel immunogen with protective efficacy against influenza types A and B.

We have developed an assay to simultaneously measure antibodies to two highly conserved peptides derived from influenza A or B. The peptides were chosen due to their ability to elicit protective immune responses in animal models. We have shown that the Flu Multiplex Assay is able to measure antibodies directed against both peptides in a quantitative manner. The assays are sensitive enough to distinguish a 2.6-fold difference in titer which is more than sufficient to measure our expected fold rises in titer over background titers. Our assay will support the continued development of vaccines based on these peptides.

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