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Method Article

Optimising a 6-plex tetanus-diphtheria-pertussis fluorescent bead-based immunoassay



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A B S T R A C T

Small volume assays are required for large-scale research studies and in particular paediatric trials, where multiple measures are required from a single sample. Fluorescent bead-based technology (Bioplex/Luminex) allows high through-put and simultaneous quantification of multiple analytes in a single test. This technology uses sets of microspheres, each with a unique spectral address that can be coated with a different antigen of interest. Following the addition of a detector antibody, specific for the isotype of interest and labelled with R-Phycoerythrin, the bioplex reader determines the amounts of antigen-specific antibodies in each test sample relative to a reference standard.

Here we outline the optimisations undertaken to establish a 6-plex fluorescent bead-based immunoassay that can accurately measure human IgG to individual tetanus-diphtheria-acellular pertussis (Tdap) antigens from 2 to 4 ul of human serum/ plasma. This protocol was adapted from previously published methods and aligns with current recommendations for developing pertussis-serological assays. To our knowledge, this is the first Tdap-specific multiplex immunoassay (MIA) established in Australia. All components were optimised and validated in-house including: microsphere preparation conditions, reference serum and QC development, and assay running.

- Determining optimal antigen coating dose and conjugation method.
- Optimising an in-house reference serum with clinically relevant titres.
- Determining assay specificity and reproducibility.

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DOI of original article: [10.1016/j.vaccine.2021.01.041](https://doi.org/10.1016/j.vaccine.2021.01.041)

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<https://doi.org/10.1016/j.mex.2021.101360>

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ARTICLE INFO

Method name: Multiplex fluorescent bead immunoassay

Keywords: Whooping cough, Antibody, IgG, Bioplex, Multiplex, Microsphere, Conjugation, Luminex, MIA, FMIA

Article history: Received 24 January 2021; Accepted 14 April 2021; Available online 22 April 2021

Specifications Table

Subject Area:	Immunology and Microbiology
More specific subject area:	Pertussis vaccinology
Method name:	Multiplex fluorescent bead immunoassay
Name and reference of original method:	[1] Van Gageldonk et al. Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to <i>Bordetella pertussis</i> , diphtheria and tetanus. <i>J Immunol Methods</i> , 2008. 335(1-2): p. 79–89. [2] Van Gageldonk et al. Improved specificity of a multiplex immunoassay for quantitation of anti-diphtheria toxin antibodies with the use of diphtheria toxoid. <i>Clin Vaccine Immunol</i> , 2011. 18(7) 1183–1186.
Resource availability:	<p>Major Equipment and materials</p> <p>1.5 ml copolymer microcentrifuge tubes 1.5 ml PCR clean Eppendorf tubes 12-channel Finn pipettes or equivalent (5–50 ul and 30–300 ul) 5 ml and 25 ml Sterile yellow topped tubes, SARSTEDT 2 ml & 5 mL LoBind microcentrifuge tubes, Eppendorf</p> <p>96 well MV Multiscreen plate, Millipore Bioplex 200 workstation, Bio-Rad Laboratories Disposable reagent reservoirs, 25 ml Finn pipette C1 serological pipettor, Thermo Scientific Light microscope Microcentrifuge Minisart 0.2 um Single Use Syringe Filters, Sterile, non-pyrogenic, hydrophilic, Sartorius, cat no. 16534 K Orbital shaker Schott bottles (100 and 500 mL) Serological Pipettes 5 ml, 10 ml & 25 ml, SARSTEDT Single channel pipettes 2, 10, 20, 100, 200 & 1000 ul Finn pipette or equivalent Syringes – 10 ml, 30 ml, 60 ml Ultrasonic cleaner (sonicator), Ultrasonics Vacuum manifold, Bio-Rad Laboratories Vetri-plast counting slide, Lomb Scientific, cat no. URINSLIDE1 Vortex mixer Weigh boats</p> <p>Reagents</p> <p>Carboxylated microspheres Bio-Rad, 6 unique bead regions are required. The following were assigned but any may be used: PT (1), PRN (3), FHA (5), FIM 2/3 (29), TT (17) and DT (23) Goat Anti-human IgG conjugated with R-phycoerythrin, Jackson ImmunoResearch Sheath Fluid, Bio-Rad Tween-20, Sigma-Aldrich Positive reference sera PT (06/140), TT (TE-3) and DT (10/262), NIBSC Water for injection, Pfizer</p> <p>Proteins (native conformation): PT, PRN and FHA provided by GlaxoSmithKline FIM 2/3 (cat. 186) and DT (cat. 151) sourced from Sapphire Biosciences TT (cat. T3194) sourced from Sigma-Aldrich</p>

(continued on next page)

Buffers

1 x PBS pH 7.4 Gibco

Bead activation buffer (BAB)

1 x PBS containing 5 mg/mL EDC and 5 mg/mL Sulpho-NHS

Bead storage buffer (BSB)

1 x PBS containing 0.1% BSA, 0.05% sodium azide

PBS.T

1 x PBS containing 0.05% Tween 20

10% PBS-BSA

1 x PBS containing 10% (W/V) Bovine serum albumin

Serum diluent

1 x PBS + 0.1% Tween20 + 3% Bovine serum albumin

Definitions and abbreviations

BAB	bead activation buffer
BSA	bovine serum albumin
BSB	bead storage buffer
BSC	biosafety cabinet
CI	confidence interval
CV	coefficient of variance
DT	diphtheria toxoid
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride
EXP	expected
FIM 2/3	fimbriae antigen types 2/3
FHA	filamentous hemagglutinin
IgG	immunoglobulin G
IU	international units
LLOD	limit of detection
LLOQ	lower limit of quantification
MIA	multiplex immunoassay
MFI	median fluorescence intensity
NIBSC	National Institute for Biological Standards and Control
OBS	observed
OOR	out of range
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline - tween
PT	pertussis toxin
PRN	pertactin
QC	quality control
R-PE	R-phycoerythrin
RT	room temperature
Sulpho-NHS	N-hydroxy-sulphosuccinimide
TT	tetanus toxin

Finalised method

The MIA was established and conducted in line with the protocol of Van Gageldonk et al. [1], with some modifications. In brief, Bio-Plex® COOH-microspheres (6.25×10^6) were conjugated with optimised concentrations of antigen in 1 x PBS pH 7.2 (Life Technologies, AUS) as follows: PT, 10 µg/ml; PRN, 75 µg/mL; FHA, 25 µg/ml; FIM 2/3, 50 µg/ml; TT, 50 µg/mL; and DT, 100 µg/ml. Samples were diluted in PBS containing 3% BSA and 0.05% Tween 20 (Sigma-Aldrich). A standard curve was generated using a 10-step 3-fold serial dilution of the in-house reference sera. Blanks and two quality control (QC) samples were included on every plate to calculate % coefficient of variance across all assays. MultiScreen Filter Plates (Merck) were pre-wet with 50 µl PBS containing 0.05% tween 20 (PBS.T), and liquid was removed by vacuum manifold (2–5 mmHg). Diluted samples (25 µl) were mixed with bead-mix (25 µl) (PBS containing 4000 beads/region) in individual wells and incubated on a plate shaker (500 rpm) in the dark for 30 min. Plates were washed twice with 100 µl 0.05% PBS-T before the addition of 100 µl 1:200 RPE-conjugated goat-anti human IgG Fc secondary antibody

(Jackson ImmunoResearch Laboratories Inc.) and incubated as above for a further 30 min. Following washing, the beads were resuspended in 125 μ l 0.05% PBS.T and read using a bioplex-200 machine. Antigen-specific IgG concentrations (mIU/mL) were determined using a 5-PL linear curve generated with bioplex manager software version 5.0.

Microsphere preparation optimisations

Conjugation dose

Optimal bead coating was determined for each antigen by titrating small scale bead conjugations (1.25×10^6 beads). The following coating doses were assessed PT: 10, 25 and 50 μ g/ml; PRN: 10, 25, 50, 75 and 100 μ g/ml; FHA: 10, 25, 50, 75 and 100 μ g/ml; FIM 2/3: 10, 25, 50 and 75 μ g/ml; TT: 50, 75 and 100 μ g/ml; and DT: 50 and 100 μ g/ml.

Antigens were coupled to beads using a two-step carbodiimide reaction. Briefly, beads were activated with bead activation buffer; $1 \times$ PBS pH \sim 7.2 (Life Technologies, AUS) containing 5mg/mL EDC (Thermo Fisher Scientific, USA) and 5 mg/mL Sulpho-NHS (Thermo Fisher Scientific, USA) on an orbital shaker protected from light for 20 min. The beads were then washed twice with PBS (pH 7.2) prior to incubating with antigen for 2 h on an orbital shaker protected from light. After washing twice, beads were blocked with 0.05% PBS.T for 2 h in the dark. Finally, the beads were washed three times with PBS (pH 7.2), resuspended in bead storage buffer; $1 \times$ PBS pH \sim 7.2 containing 0.1% Bovine Serum Albumin (Sigma-Aldrich, USA), 0.05% Sodium azide (Sigma-Aldrich, USA) and stored at 4°C before downstream analysis in MIA.

For each bead-set, a 7-step 3-fold standard curve of serum from an adult donor with recent pertussis infection and a blank control (beads and diluent only) were generated and the MFI at each point compared across the titrations. The extreme ends of the standard curve (low and high) can mask MFI variability, therefore comparisons at the mid-point (S3 to S5) were used to determine when bead saturation occurred. The optimal coating dose (used for the final assay methodology) was defined as the lowest coating dose to achieve bead saturation i.e negligible increases (<1500) in MFI at the standard curve mid-point with increasing antigen coating doses.

Bead saturation was achieved at different coating doses for each antigen and no background issues were identified (Fig. 1). The final conjugation coating doses used for the final methods (6.25×10^6 beads) were as follows: PT 10 μ g/ml, PRN 75 μ g/mL, FHA 25 μ g/ml, FIM 2/3 50 μ g/ml, TT 50 μ g/mL and DT 50 μ g/ml. Adjuvanted (adsorbed) proteins were incompatible with this assay format, likely due to changes in protein conformation from this process (data not shown).

Conjugation incubation time

After determining the optimal coating dose, the efficiency of the conjugation was determined by comparing two commonly used conjugation incubation times (2 h vs. overnight). After conjugation, the MFI for each bead-set was compared using standard curves as described in Section "*Finalised method*". Extending the incubation time had a negligible impact for all antigens, except FHA which had a reduced conjugation efficiency when incubated overnight likely due to adhesion to the plastic. A 2 h incubation time was used for all antigens in the final assay.

Conjugation pH

The effect of pH on conjugation efficiency was investigated by coating beads with the optimal antigen dose as outlined in Section "*Finalised method*" diluted in either PBS at pH 6.2 or pH 7.4. Comparable MFIs were achieved for the two PH conditions (data not shown). Commercially available PBS (pH 7.4) was used in the final assay.

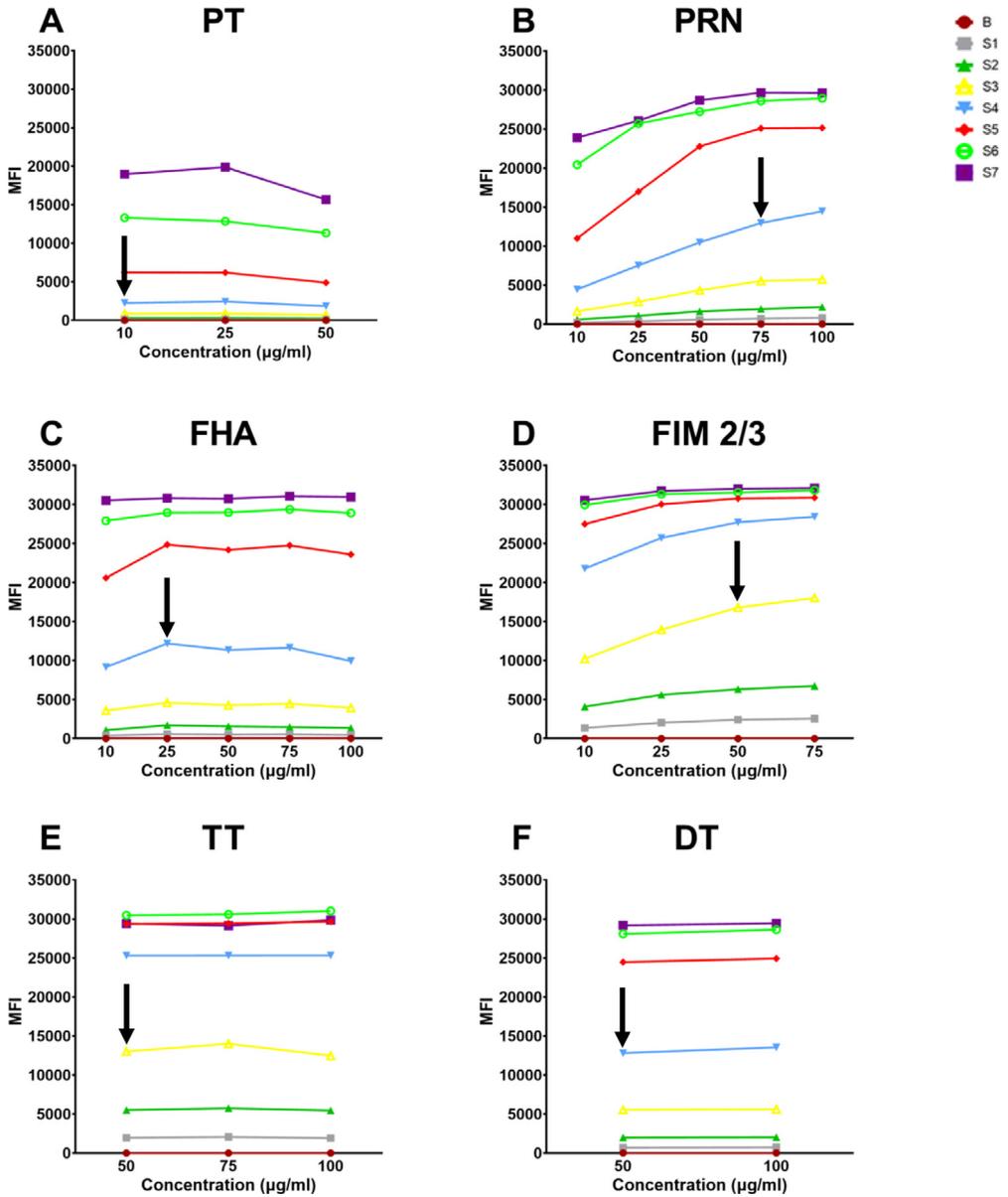


Fig. 1. Optimised bead saturation for each antigen identified by black arrows. A 7-step 3-fold standard curve of serum from an adult donor with recent pertussis infection and a blank control (beads and diluent only) was generated for each bead-set. Each line represents the MFI achieved for the beads at each standard curve dilution and blank, at increasing bead coating doses.

Bead specificity

Interference assay (assessing bead cross-reactivity)

Possible interference between individual antigen-coated beads when assayed together was investigated. Antigen-coated beads were assayed by MIA in duplicate, both together as a plex and individually in a single dilution of donor serum. Serum dilutions from the mid-point of the standard curve for each antigen were used, i.e. PT at 1:180, PRN at 1:540, FHA at 1:540, FIM 2/3 at 1:1620, TT at 1:1620 and DT at 1:540. Single blank wells (beads and diluent only) were compared for plexed or solo beads. MFIs were comparable (< 25% CV) when beads were run solo or in combination, providing evidence they were suitable for multiplexing.

Inhibition assay (assessing bead cross-reactivity)

Aliquots of a high-titre donor serum were depleted of antibodies against individual antigens (PT, PRN, FHA, FIM 2/3, TT and DT) by pre-treating sera with increasing concentrations of antigen (2.5, 5 and 10 µg/mL) for 1 h at RT (18 depleted samples, 3 per antigen). The 18 depleted samples and an untreated positive control serum sample (diluted to be at the middle of the standard curve) were tested with each antigen-coated bead-set. Inhibitions were assessed by calculating the percentage reduction in the MFI of depleted serum compared to the positive un-treated control. This inhibition was then used to indicate homologous (self) and heterologous (non-self) inhibition, i.e. MFI of PT beads in PT-depleted serum (self) or for PT beads in another antigen-depleted serum (non-self). Inhibition of 80% or more to "self" depleted serum and less than 20% to "non-self" antigens were considered acceptable. All beads achieved the acceptable homologous and heterologous inhibition, even when pre-treated with the lowest antigen concentration (Fig. 2). These data provide evidence that all beads were appropriately saturated with their respective antigens and that no cross-reactivity between antigens existed, confirming bead specificity and finalising the conjugation conditions.

Assay running optimisations

Wash buffer

Three commonly used buffers were compared for potential matrix effects: 1) PBS-T (0.05%), 2) PBS-BSA (1%) and 3) PBS-T (0.05%) - BSA (1%) [3]. Two QC sera (donor serum, 1:1000) and a blank control were run in duplicate on 3 separate plates, changing only the wash buffer used between plates. The different buffers produced comparable MFI results for all antigens (data not shown). PBS-T (0.05%) was chosen based on reduced reagent use and faster preparation time.

Incubation times

The optimal time for primary incubations (i.e. incubation of antigen-coated beads with test serum) was assessed using a time series assay. Two QC sera, each at 2 dilutions (donor serum, 1:1000 and 1:2500) and a blank control (beads and diluent only), were run across 6 separate plates, changing only the primary incubation time. Primary incubations were stopped every 15 min, assessing run times from 30 to 120 min. The MFIs for all antigens increased with extended incubation times (data not shown).

Next the effect of extended incubation was investigated by comparing 30 min vs. 2 h primary incubation times including a full standard curve, with the same 2 QC samples and blanks on two separate plates. MFIs increased with extended incubation time at a comparable rate in the reference serum and test samples: therefore, the increased primary incubation time did not affect the calculated IgG concentration (data not shown). Longer incubations were found to limit the dynamic range of the standard curve. As this would result in a higher number of test samples falling out of range, a 30 min primary incubation time was included in the final assay methods.

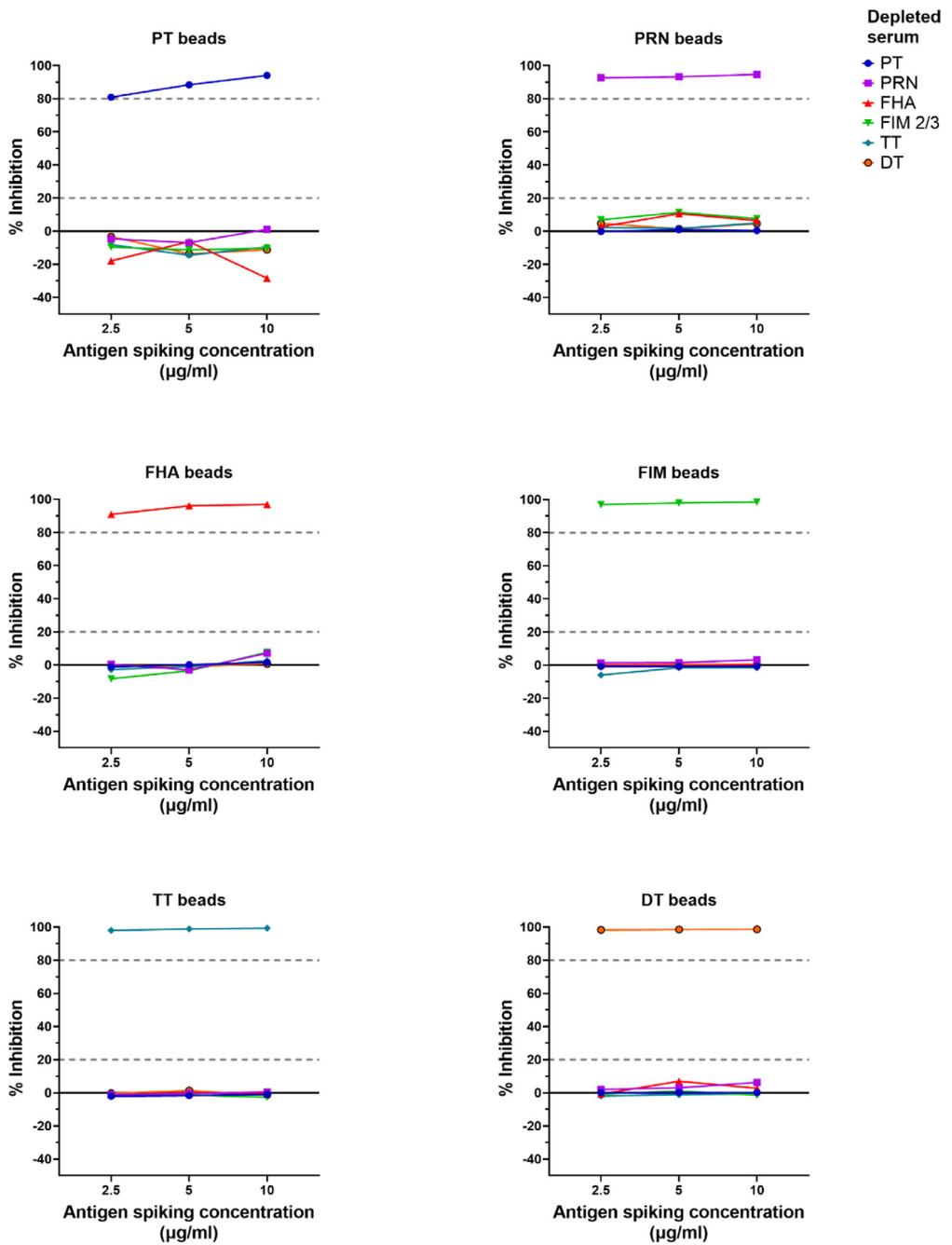


Fig. 2. No cross reactivity observed when beads were incubated with antigens of interest. % median fluorescent intensity (MFI) inhibition of antigen-coated beads in antibody-depleted compared to control serum, across 3 concentrations. Each coloured line represents a different antigen-depleted serum. Serum dilutions were based on the mid-point of the standard curve for adult donor serum.

Table 1

Quantification of the in-house reference serum and two QC sera using NIBSC international reference serum. Results are shown as mean and 95% CI from 3 individual experiments. Pertussis antigens quantified against 06/140, TT against TE-3 and DT against 10/262.

	IgG IU/mL (95% confidence intervals)					
	PT	PRN	FHA	FIM 2/3	TT	DT
NIBSC	335	65	130	46	120	2
Inhouse serum	126.1 (118.5–133.5)	490.5 (436.8–544.1)	416.6 (369.4–463.8)	242.6 (210.8–274.4)	23.3 (21.9–24.8)	1.87 (1.74–1.99)
QC 1	21.0 (20.4–21.5)	113.5 (103.3–123.7)	53.6 (51.3–56.0)	191.7 (165.0–218.4)	1.6 (1.5–1.7)	0.29 (0.27–0.32)
QC 2	58.7 (56.8–60.5)	78.4 (75.7–81.2)	107.9 (104.7–111.1)	553.1 (482.9–623.4)	12.0 (11.2–12.8)	1.86 (1.77–1.94)

Developing an in-house reference serum

Rationale and recruitment

An in-house reference serum was developed. Ethics approval (RGS0000002349) was obtained to recruit volunteers who had a recent pertussis vaccination (Adacel® or Boostrix® within 1 year), or confirmed pertussis infection within 5 years, and were willing to donate a venous blood sample up to 100 mL. Blood was processed as described previously [4].

Candidate screen and quantification

Sera from 12 donors were screened for Tdap-IgG responses by MIA as per Section "Finalised method" (data not shown). Three-fold dilutions starting at 1: 180 were assayed for each donor and the MFI compared to identify high-titre individuals.

A candidate in-house reference serum was created by pooling sera from donors with the highest response ($n = 5$). Two additional donor sera were selected as quality control (QC) samples to track between-assay variability. The in-house reference serum and QCs were quantified against National Institute for Biological Standards and Control (NIBSC) reference sera using the finalised assay methods outlined in Section "Finalised method": 06/140 for pertussis antigens PT, PRN, FHA and FIM 2/3 IgG; TE-3 for tetanus; and 10/262 for diphtheria as previously recommended [5]. Antigen-specific IgG concentrations of the in-house reference serum and QC sera are reported in Table 1.

Standard curve optimisations

The starting dilution and fold-increase was investigated to determine the optimal standard curve using our in-house reference serum, including: (1) 1:100 starting dilution, 4-fold dilutions, (2) 1:150 starting dilution, 3-fold dilutions, and (3) 1:200 starting dilution, 3-fold dilutions. As shown in Fig. 3, a 10-step, 3-fold standard curve with a 1:150 starting dilution showed good linearity across all antigens and was selected for the final assay.

Validation and quality control

Linearity and accuracy

Test samples (serum from adults and children in historical pertussis vaccine trials) were serially diluted (1:150 3-fold, 4 steps) and assayed as for Section "Finalised method" to determine linearity. There was good concordance of calculated PT- IgG values across all dilutions (<25 % CV, data not shown). Similar results were found for plasma samples (data not shown).

The accuracy of the in-house reference serum to quantify Tdap-IgG concentrations of test samples was determined by comparison with values calculated from assays using the NIBSC reference sera.

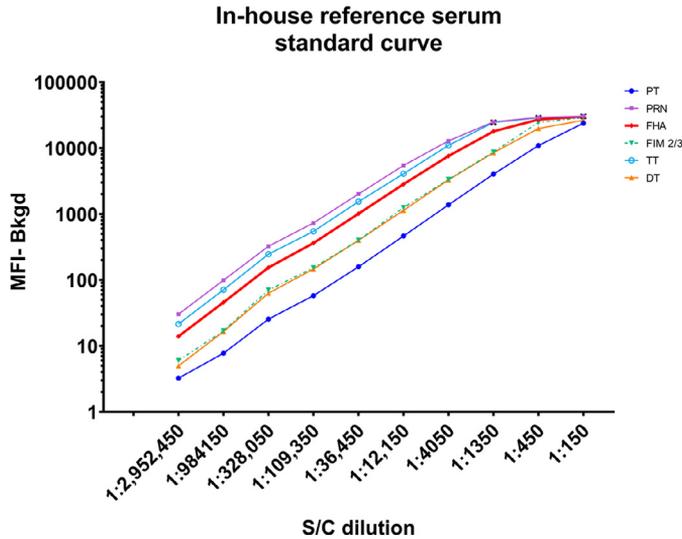


Fig. 3. A 10-step, 3-fold standard curve with a 1:150 starting dilution showed good linearity across all antigens. PT; pertussis toxin, PRN; pertactin, FHA; filamentous haemagglutinin, FIM 2/3; fimbriae types 2/3, TT; tetanus toxin, DT; diphtheria toxoid, MFI-Bkgd; median fluorescent intensity – background, S/C; standard curve.

Table 2.

Assigned tetanus-diphtheria-acellular pertussis IgG concentrations are comparable when calculated using the in-house reference serum and NIBSC international reference serum 06/140 and values calculated were similar across dilutions (<25% CV). PT; pertussis toxin, IU/mL; international units/milliliter, NIBSC; National Institute for Biological Standards and Control, % CV; coefficient of variance.

Dilution factor	Calculated PT - IgG (IU/mL)							
	Sample 1		Sample 2		Sample 3		Sample 4	
	In-house	NIBSC	In-house	NIBSC	In-house	NIBSC	In-house	NIBSC
1:100	6.9	7.5	40.6	45.1	75.7	92.9	-	-
1:1000	4.9	4.8	41.3	44.3	69.0	75.3	157.6	174.6
1:5000	6.0	5.6	39.5	39.4	66.6	67.9	156.2	165.5
% CV	0.8		4.9		5.3		4.0	

A full 10-step standard curve of in-house reference serum and an 11-step standard curve of NIBSC 06/140 was run on the same plate (in duplicate), as well as 8 test sera at 2,3 dilutions (1:500, 1:1000 and 1:5000) in duplicate. All % CVs between replicates and reference serum were all below 6%, which is well below the <25% acceptance criteria. There was good concordance of assigned PT-IgG values between the two reference serum (Table 2). Data presented are representative for all antigens.

Microsphere and plate acceptance criteria

The following criteria were required to validate newly conjugated beads and accept new plate data, as recommended in the Luminex xMap cookbook [6]: background MFI <100, bead aggregation <50%, bead counts >50, % CV between replicates <25%, observed concentrations (standards and QCs) within 25% CV of expected values.

Post-optimisation results

Assay reproducibility

Intra-assay variation

To investigate the deviation within the assay (variation between data points run on the same plate) test samples (serum from an adult Tdap vaccination trial) were run in duplicate as per Section "Finalised method". The % CV between the concentrations for individual replicates was automatically calculated by Bioplex Manager Software 5.0. The average coefficient of variance from 7 plates of data (including 125 samples in duplicate) for each antigen in the MIA was <5%, except FIM 2/3 which was <10%.

Inter-assay variability

Calculated QC concentration data were compiled from 45 separate experiments (run over three years and including 6 batches of beads, 4 batches of RPE and 3 different operators). Less than 10% CV was calculated for all antigens, except FIM 2/3 which was <12%, indicating good assay reproducibility.

MFI data showed more variability, particularly at higher dilutions where the MFI is low. However, this did not impact the assigned concentrations of the standards and QCs, and MFI between runs was within <25% CV for all antigens.

Limit of quantification

Limits of quantification (LoQ) for each antigen/ run were calculated by Bioplex Manager Software 5.0. Assay limits were determined post-optimisation by averaging data compiled from 45 separate experiments over a three-year period (including 6 batches of beads, 4 lots of RPE and 3 different operators). Limits (mIU/mL) were as follows: PT; 0.38, PRN; 0.17, FHA; 0.43, FIM 2/3; 0.24, TT; 0.02 and DT; 0.01.

Summary

When developing multiplexed immunoassays, it is of utmost importance to source good quality proteins in their native conformations, and have a reliable reference standard reporting in IU/mL, as previously recommended [5]. Antigens should be assessed individually prior to plexing, as protein characteristics will influence conjugation efficiency and assay kinetics. Adhesins can be difficult to handle and care should be taken to avoid antigen and bead loss by using Lo-Bind protein tubes, as we report here for FHA. It is critical that specificity tests be carried out at the mid-point of a reference serum with sufficiently high signal for each antigen.

Here we describe the optimisations to establish a reproducible, high through-put serological test that requires minimal sample volumes. This test offers a valuable tool for assessing immunity to pertussis, tetanus and diphtheria in large scale cohort trials, particularly in paediatric populations.

Acknowledgments

We sincerely thank the Light for Riley project of The Immunisation Foundation of Australia for advocating immunisation and generously donating funds for the development of this assay.

Declaration of Competing Interest

All authors confirm no conflicts of interest to declare.

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