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## Method Article

# Multiplex Human Papillomavirus L1L2 virus-like particle antibody binding assay



Kavita Panwar<sup>a</sup>, Anna Godi<sup>a</sup>, Clementina E. Cocuzza<sup>b</sup>, Nick Andrews<sup>c</sup>,  
Jo Southern<sup>d</sup>, Paul Turner<sup>e</sup>, Elizabeth Miller<sup>d</sup>, Simon Beddows<sup>a,f,\*</sup>

<sup>a</sup> Virus Reference Department, UK Health Security Agency, London, UK

<sup>b</sup> Department of Medicine and Surgery, University of Milano-Bicocca, Monza, Italy

<sup>c</sup> Statistics, Modelling and Economics Department, UK Health Security Agency, London, UK

<sup>d</sup> Immunisation and Vaccine-Preventable Diseases Division, UK Health Security Agency, London, UK

<sup>e</sup> Section of Paediatrics, Imperial College London, London, UK

<sup>f</sup> Blood Safety, Hepatitis, Sexually Transmitted Infections and HIV Division, UK Health Security Agency, London, UK

## A B S T R A C T

A variety of *in vitro* techniques are available to estimate the level of antibodies present in human serum samples. Such tests are highly specific and are used to determine prior exposure to a pathogen or to estimate the magnitude, breadth and durability of individual and population level vaccine immunity. Multiplex (or multi-analyte) platforms are increasingly being used to evaluate immune responses against multiple antigens at the same time, usually at reduced per sample cost and a more efficient use of available samples. Consequently, multiplex serology is an essential component of a wide range of public health programmes. Human papillomavirus (HPV) serology is limited to a small number of academic, public health and vaccine manufacturer laboratories globally. Such platforms include indirect binding to the major (L1) capsid protein virus-like particles (VLP), monoclonal antibody competition against L1 VLP and indirect binding to L1 and L2 (minor capsid protein) VLP on multiplex (Luminex®, Meso Scale Discovery®) and standard (ELISA) platforms. The methodology described here utilizes a common multi-analyte platform and L1L2-based VLP expressed *in house*, which allows the simultaneous detection and quantification of antibody responses against nine vaccine-relevant HPV genotypes.

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\* Corresponding author at: Virus Reference Department, UK Health Security Agency, 61 Colindale Avenue, London NW9 5EQ, UK.

E-mail address: [simon.beddows@ukhsa.gov.uk](mailto:simon.beddows@ukhsa.gov.uk) (S. Beddows).

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## ARTICLE INFO

*Method name:* Multiplex Human Papillomavirus L1L2 virus-like particle antibody binding assay

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- A multi-analyte method for the estimation of serological responses against vaccine-relevant genotypes of HPV using virus-like particles (VLP) incorporating both major (L1) and minor (L2) capsid proteins coupled to spectrally distinct microspheres.
- The outlined protocol is a modification of the basic development protocol available from the manufacturer.
- The protocol can be used to estimate HPV type-specific antibody responses and establish serological profiles of natural infection and following vaccination for research, surveillance or public health monitoring purposes.

### Specifications table

Subject Area:	Immunology and Microbiology
More specific subject area	Multiplex assay to evaluate serological responses to distinct, closely related antigens
Method name:	Multiplex Human Papillomavirus L1L2 virus-like particle antibody binding assay
Name and reference of original method:	Bio-Plex 200® suspension array system details and documents (Bio-Rad Laboratories, Hercules, CA and <a href="http://www.bio-rad.com">www.bio-rad.com</a> ). Luminex® xMAP® Cookbook (Luminex Corporation, Austin, TX).
Resource availability:	Reagents and equipment are listed with the name of the suppliers

### Method details

#### Reagents for microsphere coupling

1. Luminex® MicroPlex® Microspheres (bead regions 1, 4, 5, 8, 9, 11, 15, 24, 30, 46) (Luminex Corporation, Austin, TX).
2. HPV L1L2 virus-like particles (L1L2 VLP antigens; see below).
3. N-hydroxysulfosuccinimide (Sulfo-NHS) (Thermo Fisher Scientific, Waltham, MA).
4. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Thermo Fisher Scientific, Waltham, MA).
5. 2[N-Morpholino] ethanesulfonic acid (MES) (Merck, Darmstadt, Germany).
6. Sodium phosphate monobasic anhydrous (Merck, Darmstadt, Germany).
7. 1 x Phosphate buffered saline pH 7.4 (PBS) (Severn Biotech, Kidderminster, UK).
8. ProClin™ 300 (Merck, Darmstadt, Germany).
9. Bovine Serum Albumin (BSA) (Merck, Darmstadt, Germany).
10. Sodium hydroxide (NaOH) 1M (Merck, Darmstadt, Germany).
11. Tween 20 (Merck, Darmstadt, Germany).

#### Reagents for HPV L1L2 VLP serology

1. Luminex® MicroPlex® coupled L1L2 VLP beads.
2. Antibody standards and controls (see below).
3. Goat anti-Human IgG Fc Cross-Adsorbed Secondary Antibody, Biotin (Thermo Fisher Scientific, Waltham, MA).
4. Goat anti-Mouse IgG (H+L) Cross-Adsorbed, Biotin, Polyclonal, Secondary Antibody (Invitrogen, Waltham, MA).
5. Phosphate buffered saline with 1% BSA pH 7.4 (Merck, Darmstadt, Germany).
6. Sheep serum preservative free (Bio-Rad Laboratories, CA, USA).

7. PhycoLink® Streptavidin-R-phycoerythrin (SAPE) (Agilent Technologies, CA, USA).

### Equipment

1. BioTek 50TS Plate Washer (BioTek; Winooski, VT).
2. Bio-plex 200® system + HTF (Bio-Rad Laboratories, Hercules, CA).
3. Filter Plates Millipore; MultiScreen HTS 96 well, 0.45µm (Merck, Darmstadt, Germany).
4. TC20 Automated Cell Counter (Bio-Rad Laboratories, Hercules, CA).
5. PTR-35 Variable Angle Vertical Rotator (Grant Instruments Ltd, Cambridge, UK).
6. XB3 Ultra Sonic Bath (Jencons Scientific Ltd., Leighton Buzzard, UK).
7. IKA™ Vortex 1 Shaker (IKA-Werke, Staufen, Germany).
8. IKA™ MS 3 digital orbital shaker (IKA-Werke, Staufen, Germany).

### Software

1. Bio-Plex Manager v6.1 (Bio-Rad Laboratories, Hercules, CA).

### L1L2 VLP antigens

Non-reporter containing L1L2 VLP were expressed and purified from mammalian cells, according to methods previously published using the Bicistronic vector pXsheLL, where X is the Papillomavirus type from which the codon optimized L1 and L2 genes were derived [1,2]. Purified L1L2 VLP were visualized by SDS-PAGE and the L1 protein concentration determined by comparison with a standard curve derived from known input concentrations of bovine serum albumin (Fig. 1A). Gel analysis was carried out using ImageJ software (U.S. National Institutes of Health; <http://imagej.nih.gov/ij>) to determine the L1 concentration of the gradient fractions. L1L2 VLP formation was confirmed by electron microscopic analysis of negatively stained particles. Typical L1L2 VLP are shown in Fig. 1B. The average  $\pm$  standard deviation (SD) yield of L1L2 VLP from  $n = 30$  preparations (average 3.3 preparations per type) was  $136 \pm 79 \mu\text{g}$  with an average  $\pm$  SD purity of  $90\% \pm 6\%$ . Type-specific antisera generated in BALB/c mice against each L1L2 VLP and a pre-immune pooled sera [1] were used as quality assurance reagents for confirmation of the specificity of individually coupled microspheres using Goat anti-Mouse IgG (H+L) biotinylated secondary antibody (Fig. 1C). Furthermore, routine tracking of run outcomes (see *Performance measures* below) serves to highlight outlier results and therefore acts as an additional layer of quality control for batch release of coupled L1L2 VLP antigens.

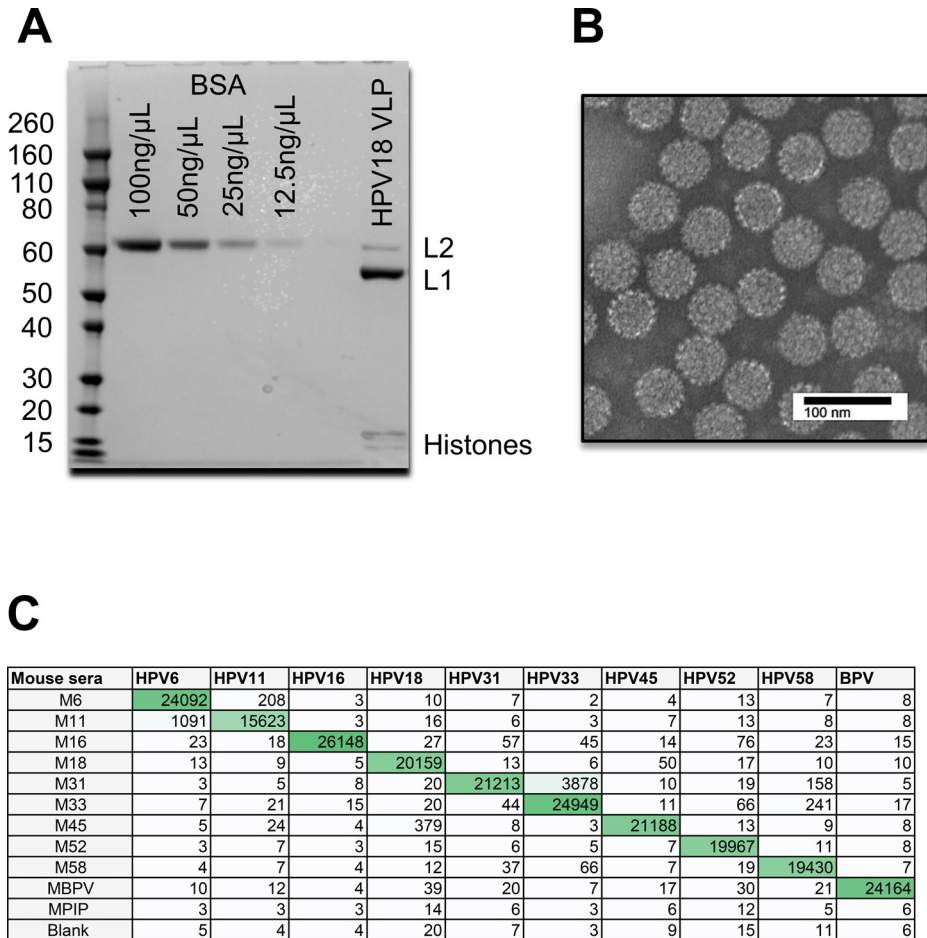
### Antibody standards and controls

An internal standard comprising pooled nonavalent vaccine sera [3] was assigned arbitrary unitage (AU/mL) based upon the magnitude of its binding against each HPV L1L2 VLP and calibrated against the International Standards for HPV16 (IS16; 10 IU/mL; 05/134; National Institute for Biological Standards and Control, UK) and HPV18 (IS18; 16 IU/mL; 10/140) antibodies to allow a readout for these types in IU/mL. The remaining HPV types (HPV6/11/31/33/45/52/58) are reported in AU/mL. A positive internal quality control (IQC-P) was created by admixing this internal standard with normal human serum (Merck; Darmstadt, Germany) and a previously characterized HPV Negative antibody reagent [4] was used as the negative internal quality control (IQC-N).

### Assay procedure

#### (i). Microsphere coupling of L1L2 VLP antigens.

1. Freshly prepare and filter buffers prior to use.
2. Vortex and sonicate Luminex® MicroPlex® vial for 50–60 s in a bath sonicator to obtain an equal distribution of beads. Aliquot 200 µL (approximately  $2.5 \times 10^6$  beads) into standard flip cap 1.5 mL microtube. Couple the following VLP/bead pairs; HPV6/1; HPV11/4; HPV16/5; HPV18/8; HPV31/9; HPV33/15; HPV45/24; HPV52/30; HPV58/46, BPV/11. Other bead regions



**Fig. 1.** Representative HPV L1L2 VLP. A) Coomassie Blue stained SDS-PAGE depicting molecular weight marker, titration of bovine serum albumin as quantitative standards and a typical HPV L1L2 VLP sample including majority L1 capsid protein, minority L2 capsid protein and contaminating histones, likely encapsidated within the L1L2 VLP during maturation. ImageJ software used to estimate L1 concentration from BSA standards (National Institutes of Health, USA); B) negative stained electron microscopic image of HPV L1L2 VLP (Mag. 30,000x; JEM1400 high contrast Transmission Electron Microscope). C) Cross-binding checkerboard of mouse (M) immune sera raised against indicated L1L2 VLP (left column, including pre-immune pool, MPIP) and target L1L2 VLP antigen using Goat anti-Mouse IgG (H+L) biotinylated secondary antibody with the Median fluorescence intensities (MFI) of multiple ( $n = 4$ ) coupling panels reported.

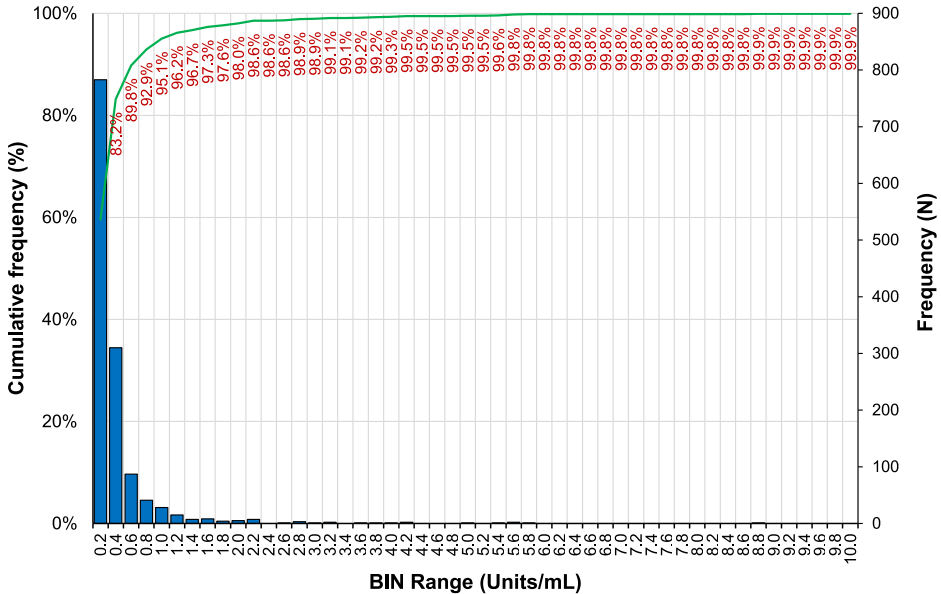
could be selected but these should be spectrally distinct from the other bead regions used to reduce the risk of interference.

3. Centrifuge for 2 min at 9,100 rpm (8000 xg) and re-suspend the bead pellet in 80  $\mu$ L activation buffer, 0.1 M sodium phosphate monobasic anhydrous pH 6.2 (adjust pH with 1M sodium hydroxide, NaOH). Vortex and sonicate for approximately 20 s.
4. Bring 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) to room temperature (17 – 26  $^{\circ}$ C). Add 10  $\mu$ L of freshly prepared 50 mg/mL Sulfo-NHS solution (diluted in distilled de-ionized water) and 10  $\mu$ L 50 mg/mL EDC solution (diluted in distilled de-ionized water). Mix gently by vortex.
5. Incubate beads in the dark for 20 min at room temperature. Gently vortex at 10 min intervals.

6. After activation, centrifuge the beads at 13,000 rpm (15,000 xg) for 2 min. Resuspend in 100  $\mu$ L PBS pH 7.4. Dilute 2  $\mu$ g of L1L2 VLP in PBS pH 7.4 to a volume of 200  $\mu$ L prior to adding to resuspended beads. Vortex gently for 20 s.
  7. Wrap the microtube in foil and mix on a variable angle vertical rotator for 2 h at room temperature.
  8. Add 1 mL of wash buffer containing PBS pH 7.4 with 1% BSA and 0.05% Tween 20 to block any remaining open carboxyl sites. Vortex gently and centrifuge at 13,000 rpm (15,000 xg) for 2 min. Resuspend the beads in 1 mL wash buffer. Vortex and sonicate for 20 s before repeating centrifugation.
  9. After centrifugation, remove supernatant and resuspend L1L2 VLP-coupled beads in 300  $\mu$ L storage buffer (100 mM 2[N-Morpholino] ethanesulfonic acid (MES), pH 6.0 (adjust pH with 1M NaOH), 1% Bovine Serum Albumin (BSA) and 0.2% Proclin™ 300). Vortex and sonicate for 20 s. Transfer L1L2 VLP coupled beads into a low-bind microtube and store in the dark at 2-8 °C.
  10. Enumerate coupled beads using an automated cell counter, as required.
- (ii). *HPV L1L2 VLP Serology assay*
1. Prepare assay buffer (PBS pH 7.4 with 1% BSA and 0.02% Tween 20). Prepare bead buffer by adding sheep serum to assay buffer (10% v/v) as required. Filter and store at 2-8 °C until use. Bring buffers to room temperature prior to use.
  2. Prepare working bead master-mix solution (MM) using 2,500 beads of each target per reaction. Briefly vortex and sonicate required beads for up to 30 s. Prepare bead MM in 5 mL bijoux or low bind microtubes and dilute MM in bead buffer to required sample volume. Vortex and sonicate bead MM briefly to obtain an equal distribution of beads.
  3. Prepare assay run standards, controls and serum sample dilutions in bead buffer in 96 v-welled bottom plates from a starting dilution of 1/50 with a titration series (up to 4 dilutions in a 3-fold, 5-fold or 10-fold series) appropriate to cover the relatively low levels of natural infection antibody as well as the very high levels induced by vaccination as required.
  4. Prepare filter plate by pre-wetting plate in plate washer with 200  $\mu$ L assay buffer for 2 min followed by aspiration of the fluid. Pat plate gently on paper towel to remove microdroplets from the base of the wells.
  5. Add 50  $\mu$ L sample dilution and 50  $\mu$ L bead MM volume to each well of pre-wetted filter plate. Shake plate on orbital plate shaker at 800 rpm for 15 s to distribute the beads. Incubate plate in the dark at room temperature for 60 min.
  6. Place plate on plate washer and wash three times with 200  $\mu$ L assay buffer. Dispense 50  $\mu$ L of assay buffer after final wash and pat plate gently on paper towel.
  7. Prepare secondary antibody solution. Dilute biotinylated goat anti-human IgG Fc highly cross-adsorbed secondary antibody to optimized dilution in assay buffer.
  8. Add 50  $\mu$ L of secondary antibody to sample wells and shake plate on plate shaker. Incubate plate in the dark at room temperature for 30 min.
  9. Wash plate three times with 200  $\mu$ L assay buffer. Dispense 50  $\mu$ L of assay buffer after final wash and pat plate gently on paper towel.
  10. Prepare Streptavidin-R-phycoerythrin (SAPE) solution. Dilute SAPE to optimized dilution in assay buffer. Add 50  $\mu$ L of diluted SAPE to each sample well. Shake plate on plate shaker and incubate in the dark at room temperature for 10 min.
  11. After 10 min wash plate three times with 200  $\mu$ L assay buffer. Dispense 75  $\mu$ L of assay buffer after final wash and pat plate gently on paper towel.
  12. Transfer sample to 96 v-welled bottom plates. Resolve the reactions on the Bio-Plex 200® system.

### *Data analysis*

The internal standard was titrated and subjected to 4PL or 5PL curve fitting (BioPlex Manager™) within a parameter setting of 70-130% recovery range. Individual sera were titrated and dilutions with



**Fig. 2.** Frequency distribution plot of antibody levels including cumulative frequency plot (green line) derived from a panel of 'likely negative' serum samples against antigens in the 9-valent HPV L1L2 VLP Serology Assay. These data represent the pooled data for all sera against all antigens, but individual type-specific profiles are similar. BIN range represents the interval range (0.2 Units/mL) by which the data are categorized.

median fluorescence intensity (MFI) signals between the lower (LLOQ) and upper (ULOQ) limits of quantification were assigned a value by interpolation and adjusted according to the sample dilution. Antibody levels for each sample are reported as the median of these interpolated values. All MFI signals were background normalized by the software (FI-background), where the background signal was the fluorescence intensity of blank wells. The recovery rate for each standard is reported as the observed / expected \* 100. In this case, the mean Obs/Exp was 99.6% (SD 0.4%, range 98.9 – 100.3%;  $n = 15$  representative runs). BPV was included as an irrelevant antigen [5] and samples (23/669; 3.4%) that gave an MFI signal at the 1/100 dilution of  $\geq 50\%$  of the maximum MFI for the plate was excluded from further analysis as a precaution.

## Quality assurance

### Limit of detection determination

The limit of detection (LOD) for each antigen is a crucial metric for the appropriate assignment of seropositivity status for each serum sample and for this we made use of both naïve [2] and pre-vaccine [5] sera as a source of 'likely negative' antibodies. For the present purpose these samples can only be considered 'likely negative' samples as their true HPV infection status can only be assumed. A frequency distribution plot of the antibody levels (Units/mL) for these 'likely negative' samples typically followed a one-tailed normal distribution (Fig. 2). The determination of a serology assay cut-off is typically based upon the Mean + 3SD of the scores obtained [6], but there are a number of other approaches that can be applied to estimate the optimum cut-point to generate the highest specificity. We evaluated four approaches:

- (i). *Generic LOD*: this method applies a fixed LOD applied across all antigens and was set for evaluation purposes at 2, 3, or 4 Units/mL.

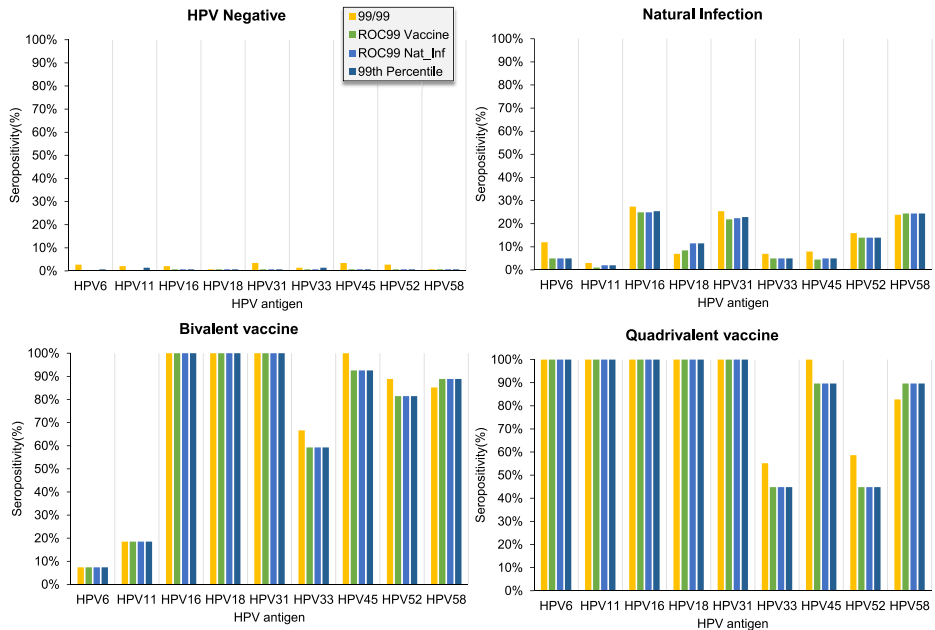
Measure	Observed specificity at LOD derived using indicated algorithm										Impact assessment	
	HPV6	HPV11	HPV16	HPV18	HPV31	HPV33	HPV45	HPV52	HPV58	All	Agreement	Kappa
N	146	146	146	146	146	146	146	146	146	146	1314	
Mean	0.4	0.3	0.2	0.4	0.2	0.5	0.3	0.2	0.2			
sd	0.7	0.8	0.3	1.2	0.3	0.9	0.6	0.3	0.5			
Mean+3sd (U/mL)	2.6	2.7	1.2	3.9	1.1	3.3	2.0	1.0	1.7			
99/99 Tolerance (U/mL)	2.6	2.7	1.2	3.9	1.1	3.3	2.0	1.0	1.7			
ROC99 Vaccine (U/mL)	4.4	5.9	1.7	3.3	1.9	5.1	3.7	1.4	1.5			
ROC99 Nat_Inf (U/mL)	4.2	5.6	1.6	2.1	1.8	5.2	3.6	1.4	1.5			
99th Percentile (U/mL)	4.1	5.6	1.4	1.9	1.7	5.0	3.5	1.3	1.4			
LOD=2 (n)	7	4	0	1	1	7	5	0	1	26		
LOD=3 (n)	3	3	0	1	0	3	3	0	1	14		
LOD=4 (n)	2	3	0	1	0	2	0	0	1	9		
99/99 Tolerance (n)	4	3	3	1	5	2	5	4	1	28		
ROC99 Vaccine (n)	0	0	1	1	1	1	1	1	1	7		
ROC99 Nat_Inf (n)	0	0	1	1	1	1	1	1	1	7		
99th Percentile (n)	1	2	1	1	1	2	1	1	1	11		
LOD=2 (%)	95.2%	97.3%	100.0%	99.3%	99.3%	95.2%	96.6%	100.0%	99.3%	96.0%	95.5%	0.909
LOD=3 (%)	97.9%	97.9%	100.0%	99.3%	100.0%	97.9%	97.9%	100.0%	99.3%	98.9%	96.3%	0.923
LOD=4 (%)	98.6%	97.9%	100.0%	99.3%	100.0%	98.6%	100.0%	100.0%	99.3%	99.3%	97.0%	0.935
99/99 Tolerance (%)	97.3%	97.9%	97.9%	99.3%	96.6%	96.6%	96.6%	97.3%	99.3%	97.9%	95.8%	0.916
ROC99 Vaccine (%)	100.0%	100.0%	99.3%	99.3%	99.3%	99.3%	99.3%	99.3%	99.3%	99.5%	96.4%	0.926
ROC99 Nat_Inf (%)	100.0%	100.0%	99.3%	99.3%	99.3%	99.3%	99.3%	99.3%	99.3%	99.5%	96.4%	0.927
99th Percentile (%)	99.3%	98.6%	99.3%	99.3%	99.3%	98.6%	99.3%	99.3%	99.3%	99.2%	96.4%	0.927

**Fig. 3.** Individual level specificity based upon the ‘false positive’ rate using the indicated algorithm and data from the ‘likely negative’ serum panel ( $n = 146$ ). Impact assessment conducted using natural infection and vaccinee sera ( $n = 108$ ) and associated agreement and Kappa statistics are shown. LOD, limit of detection.

- (ii). *Upper 99/99 tolerance*: this method is based upon a 99/99 upper tolerance limit, being the cut-point estimated to give 99% confidence that at least 99% of the ‘likely negative’ samples test negative. It is considered to be more stringent than the Mean + 3SD. This approach was used by Merck Research Laboratories to set the serostatus cut-off for their Luminex®-based HPV 9-valent L1 VLP binding assay [7]. Of note, for the current dataset, the LODs determined were the same as those generated using the standard Mean+3SD approach.
- (iii). *99th Percentile*: this is based upon the 99th percentile of the negative sera panel antibody levels and is used by the Centers for Disease Control and Prevention in their Meso Scale Discovery (MSD) electrochemiluminescence based HPV L1L2 VLP platform [8].
- (iv). *Receiver Operator Characteristic (ROC)*: this analysis is based upon the use of additional panel(s) of samples representing ‘true positive’ cases to counterbalance the ‘true negative’ antibody levels in order to interpolate a cut-point corresponding to an optimum sensitivity and specificity [6]. We carried out two analyses using: (a) a panel of natural infection samples and (b) three-dose vaccination sera selected at least 12 months post vaccine. ROC *sensitivity* can be considered the optimum cut-point at which a value will correctly record a true positive result as being test positive. However, in this case only the determination of the LOD using the optimum cut-point for 99% *specificity* was relevant.

The LOD determined for each antigen was reapplied to the ‘likely negative’ sample data and individual type specificities were estimated based upon the resulting ‘false positive’ rate. For this purpose, a minimum 98% specificity for each antigen was chosen as a determinant of a successful outcome (Fig. 3). We conducted an impact assessment of the resulting LODs on sample repeatability using a panel of natural infection and vaccine sera ( $n = 108$  samples). Samples were tested twice resulting in high levels of concordance (Agreement >95% and Kappa >0.900) between the assigned seropositivity status (Fig. 3) and a high level of correlation between the magnitude of the antibody levels resulting from the initial and repeat tests (Pearson’s correlation coefficient,  $r = 0.979$ ), regardless of the algorithm used. We next conducted a larger sensitivity analysis by application of the LODs obtained using four algorithms (Tolerance 99/99, ROC99\_Vaccine, ROC99\_Nat\_Inf and 99th Percentile) to a panel of natural infection ( $n = 201$ ), bivalent ( $n = 27$ ) and quadrivalent ( $n = 29$ ) vaccine sera (Fig. 4). Taken together, a fixed LOD and the 99/99 Tolerance method were considered not sufficiently stringent for this purpose while the 99th Percentile and the ROC methods generated appropriate and similar levels of specificity and agreement.

The ROC algorithm using vaccine sera as the antibody positive comparison group was selected as an appropriate algorithm for the determination of antigen-specific LODs and the following values



**Fig. 4.** Sensitivity analysis to estimate the type-specific serostatus of indicated serum panels. The HPV Negative serum panel ( $n = 146$ ) is the panel of 'likely negative' samples used to determine the LODs, while the Natural Infection ( $n = 201$ ), Bivalent Vaccine ( $n = 27$ ) and Quadrivalent Vaccine ( $n = 29$ ) panels are used to compare the seroprevalence estimates resulting from application of the indicated algorithm to determine the LOD.

were derived for this assay: HPV6 4.4 AU/mL; HPV11 5.9 AU/mL; HPV16 1.7 IU/mL; HPV18 3.3 IU/mL; HPV31 1.9 AU/mL; HPV33 5.1 AU/mL; HPV45 3.7 AU/mL; HPV52 1.4 AU/mL; HPV58 1.5 AU/mL.

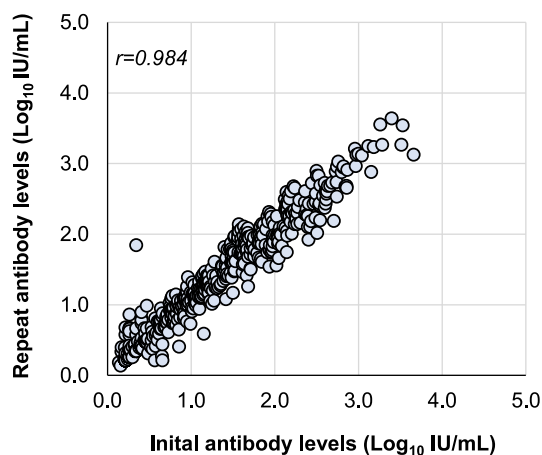
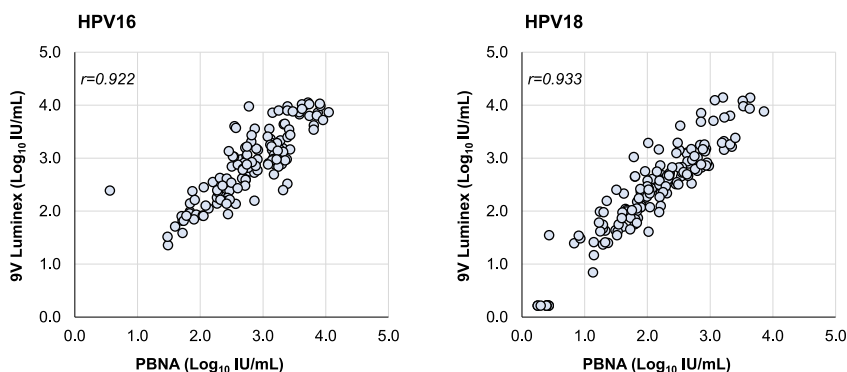
#### Performance measures

The overall CV% for the IQC-P was 21.8% (range 20.8 – 22.9%;  $n = 57$  runs) and demonstrates a measurement uncertainty (2SD intervals) of 1.4-fold from the mean with 95% of tests falling within this range. The IQC-N was negative in all tests. Sample repeatability ( $n = 141$  samples) was excellent with a pairwise serostatus agreement of 97% (Kappa 0.938) and a Pearson's  $r = 0.984$  for the antibody levels (Fig. 5A). For an assessment of external quality assurance, the IS16 and IS18 antibody reagents gave mean (SD;  $n = 4$ ) estimated values of 8.4 (1.4) and 16.5 (0.7) IU/mL, respectively. We compared HPV16 and HPV18 antibody levels (IU/mL) reported from this assay and those reported from the well-established pseudovirion-based neutralization assay [2,5,9], following standardization of the IQC-P to the IS16 and IS18 reagents. There was a clear correlation between the data derived from these two different HPV serology assays for both HPV16 (Pearson's  $r = 0.922$ ;  $n = 152$  samples) and HPV18 ( $r = 0.933$ ) antibody levels (Fig. 5B). These correlation coefficients are similar to those presented in a recent report which also reported good correlations between antibody levels derived from different assays [10] and reinforce the utility of international reference reagents for HPV serology.

#### 4-valent comparability

We have also created a 4-valent version of this assay that exhibits similar quality assurance outcomes. The determination of the LOD was made using the same approach used for the 9-valent assay and the following antigen-specific LODs were derived for this assay: HPV6 2.7 AU/mL; HPV11 3.5



**A****B**

**Fig. 5.** (A) Correlation between antibody levels ( $\text{Log}_{10}$  Units/mL) derived from the initial and repeat tests of vaccine and natural infection sera ( $n = 141$ ) and (B) Compatibility between pseudovirion-based neutralization assay data and 9-valent binding data reported in IU/mL.

AU/mL; HPV16 1.3 IU/mL; HPV18 2.7 IU/mL ( $n=151$ ). The overall CV% for the IQC-P was 19.2% (range 17.9 – 20.7%;  $n = 52$  runs) and demonstrates a measurement uncertainty (2SD intervals) of 1.4-fold from the mean with 95% of tests falling within this range. The negative control (IQC-N) was negative in all tests. Sample repeatability ( $n = 42$  samples) was excellent with a pairwise serostatus agreement of 96% (Kappa 0.922) and a Pearson's  $r = 0.986$  for the antibody levels. For an assessment of external quality assurance, the IS16 and IS18 antibody reagents gave mean (SD;  $n = 4$ ) estimated values of 9.1 (0.7) and 15.1 (1.0) IU/mL, respectively. For an assessment of assay equivalence, natural infection and vaccine sera ( $n = 164$ ) were tested in both the 9-valent and 4-valent assays and the concordance of data for HPV6/11/16/18 antigens evaluated. Seropositivity agreement was excellent (97%; Kappa 0.928) and the data were quantitatively similar (Pearson's  $r = 0.972$ ) (Table 1).

**Table 1**  
Comparability of 4-Valent and 9-Valent assay outcomes.

L1L2 VLP	4-Valent / 9-Valent assay				Agreement	Kappa	Pearson's r
	+/+	-/+	+/-	-/-			
HPV6	29	2	4	129	96%	0.884	0.962
HPV11	26	2	2	134	98%	0.914	0.952
HPV16	60	2	2	100	98%	0.948	0.983
HPV18	43	2	2	117	98%	0.939	0.981
All	158	8	10	480	97%	0.928	0.972

Natural infection and vaccine serum samples ( $n = 164$ ) tested in both the 4-valent and 9-valent assay and evaluation of the pairwise seropositivity (Agreement %, Kappa statistic) and pairwise antibody levels (Pearson's) metrics shown.

### Antigen shelf-life

IQC-P data derived from different batches of coupled beads ( $n = 3-8$  batches per type) and antigen expressions ( $n = 2-4$  batches per type) generated a total of  $n = 349$  observations. Each observation against an antigen was normalized against the mean IQC-P antibody response against that antigen (mean 1.0, SD 0.2). This normalization allowed the data to be pooled and presented against the timeline from each bead coupling. These data support a usable shelf-life of coupled antigen-beads of  $>2$  years.

### Additional observations

Alternative secondary antibodies were evaluated: goat anti-human IgG H&L-Biotin (Thermo Fisher Scientific, Waltham, MA) and directly conjugated goat anti-human PE (Thermo Fisher Scientific, Waltham, MA). The directly conjugated goat anti-human PE demonstrated poor signal-to-background ratios and low antibody levels at a high concentration and was unsuitable for use. The goat anti-human IgG H&L-Biotin could be used as an alternative to goat anti-human Fc-Biotin. The primary antibody step often benefits from an excess of protein, chemical blockers or unrelated antibodies to reduce non-specific charge effects and improve specificity. Sheep serum, polyvinyl alcohol (PVA) (Sigma-Aldrich, St Louis, MO) and polyvinylpyrrolidone (PVP) (Sigma-Aldrich, St Louis, MO) were evaluated for signal-to-background ratios. Addition of 10% sheep serum reduced non-specific signals and stabilized weak signals but no effect was apparent on MFI and background signals with the use of PVA and PVP.

### Method application

This method can be used to provide estimates of seropositivity and antibody levels using human serum collected following natural infection or vaccination for research, surveillance or public health monitoring purposes. We recently used this method to provide data on the magnitude, breadth and durability of the binding antibody response against vaccine (HPV6/11/16/18) and non-vaccine (HPV31/33/45/52/58) type antigens [11]. The readout for HPV16 and HPV18 is in International Units, which allows for comparison between assays [9] and between laboratories [10]. International reference reagents and serum proficiency panels are key tools to improve global harmonization of HPV serology data [12].

### Declaration of Competing Interest

None.

### Data Availability

Data will be made available on request.

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## References

- [1] A. Godi, D. Boampong, B. Elegunde, K. Panwar, M. Fleury, S. Li, Q. Zhao, N. Xia, N.D. Christensen, S. Beddows, Comprehensive assessment of the antigenic impact of human papillomavirus lineage variation on recognition by neutralizing monoclonal antibodies raised against lineage a major capsid proteins of vaccine-related genotypes, *J. Virol.* 94 (2020) e01236–e01220.
- [2] E. Draper, S.L. Bissett, R. Howell-Jones, D. Edwards, G. Munslow, K. Soldan, S. Beddows, Neutralization of non-vaccine human papillomavirus pseudoviruses from the A7 and A9 species groups by bivalent HPV vaccine sera, *Vaccine* 29 (2011) 8585–8590.
- [3] A. Godi, T.J. Kemp, L.A. Pinto, S. Beddows, Sensitivity of human papillomavirus (HPV) lineage and sublineage variant pseudoviruses to neutralization by nonavalent vaccine antibodies, *J. Infect. Dis.* 220 (2019) 1940–1945.
- [4] S.L. Bissett, D. Wilkinson, K.I. Tettmar, N. Jones, E. Stanford, G. Panicker, H. Faust, R. Borrow, K. Soldan, E.R. Unger, J. Dillner, P. Minor, S. Beddows, Human papillomavirus antibody reference reagents for use in postvaccination surveillance serology, *Clin. Vaccine Immunol.* 19 (2012) 449–451.
- [5] E. Draper, S.L. Bissett, R. Howell-Jones, P. Waight, K. Soldan, M. Jit, N. Andrews, E. Miller, S. Beddows, A randomized, observer-blinded immunogenicity trial of Cervarix(R) and Gardasil(R) Human Papillomavirus vaccines in 12-15 year old girls, *PLoS One* 8 (2013) e61825.
- [6] R.H. Jacobson, Validation of serological assays for diagnosis of infectious diseases, *Rev. Sci. Tech.* 17 (2) (1998) 469–526.
- [7] D. Opalka, K. Matys, P. Bojczuk, T. Green, R. Gesser, A. Saah, R. Haupt, F. Dutko, M.T. Esser, Multiplexed serologic assay for nine anogenital human papillomavirus types, *Clin. Vaccine Immunol.* 17 (2010) 818–827.
- [8] G. Panicker, I. Rajbhandari, B.M. Gurbaxani, T.D. Querec, E.R. Unger, Development and evaluation of multiplexed immunoassay for detection of antibodies to HPV vaccine types, *J. Immunol. Methods* 417 (2015) 107–114.
- [9] A. Godi, K. Panwar, M. Haque, C.E. Cocuzza, N. Andrews, J. Southern, P. Turner, E. Miller, S. Beddows, Durability of the neutralizing antibody response to vaccine and non-vaccine HPV types 7 years following immunization with either Cervarix(R) or Gardasil(R) vaccine, *Vaccine* 37 (2019) 2455–2462.
- [10] S.H. Tsang, P. Basu, N. Bender, R. Herrero, T.J. Kemp, A.R. Kreimer, M. Müller, G. Panicker, M. Pawlita, L.A. Pinto, J.N. Sampson, R. Sankaranarayanan, J. Schussler, P. Sehr, M.S. Sierra, E.R. Unger, T. Waterboer, A. Hildesheim, Evaluation of serological assays to monitor antibody responses to single-dose HPV vaccines, *Vaccine* 38 (2020) 5997–6006.
- [11] K. Panwar, A. Godi, C.E. Cocuzza, N. Andrews, J. Southern, P. Turner, E. Miller, S. Beddows, Binding antibody levels to vaccine (HPV6/11/16/18) and non-vaccine (HPV31/33/45/52/58) HPV antigens up to 7 years following immunization with either Cervarix® or Gardasil® vaccine, *Vaccine* 40 (2022) 1198–1202.
- [12] L.A. Pinto, J. Dillner, S. Beddows, E.R. Unger, Immunogenicity of HPV prophylactic vaccines: serology assays and their use in HPV vaccine evaluation and development, *Vaccine* 36 (2018) 4792–4799.