Serological Assays for Early Detection of HPV-associated Oropharyngeal Cancer

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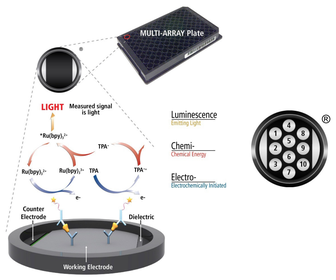
1 Abstract

Research-use-only (RUO) sample-sparing MULTI-ARRAY serology immunoassays have been developed for human antibodies to several HPV-16 early antigens. These assays are intended to support research of oropharyngeal cancer (OPC) linked to the human papillomavirus (HPV). The HPV virus can infect the mouth and throat and cause cancers of the oropharynx, i.e. the back of the throat, including the base of the tongue and tonsils. HPV-related cancer incidence is increasing dramatically among men and is unlikely to decrease in the foreseeable future, despite vaccination efforts. As a result, this may become one of the most common cancers in middle-aged men in the United States by 2045. The HPV-16 genome consists of six early genes (E1, E2, E4, E5, E6, and E7) and two late genes (L1 and L2) that constitute the viral capsid. Antibodies against the late HPV-16 antigens are common in individuals with HPV infections and are observed in approximately 15% of the general population. Antibodies to early HPV-16 antigens are less common (about 1% of general population) but are commonly present in those with OPC. These antibodies are present many years before cancer develops; thus, they are ideal biomarkers for early detection of OPC. Immunoassays for antibodies against HPV-16 early antigens E1, E2, E6, and E7 were developed in a high-throughput multiplexed format using electrochemiluminescence (ECL) detection, and MULTI-ARRAY 10- spot 96-well plates. Each well has an array presenting recombinant antigens manufactured by MSD. The assay uses 25 uL of 2,500x diluted serum or plasma. The assay format is simple: diluted sample is incubated in the well with the antigen array followed by a wash and detection of bound anti-HPV antibodies using an anti-human IgG antibody labeled with the SULFO-TAG ECL label. Assay performance was evaluated with approximately 200 commercially sourced samples from apparently healthy individuals and 14 samples from individuals known to be positive for at least one HPV-16 early antigen using an established reference method (Programmable Protein (RAPID) ELISA, Anderson 2015). There was excellent agreement between the two methods. This high-throughput MULTI-ARRAY assay may be useful in research applications requiring screening of a large number of samples for HPV-16 antibodies.

2 Methods

MSD® electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



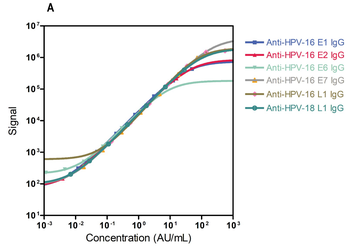
MULTI-ARRAY Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

3 HPV-16 E-Protein Serology Assay

A multiplexed serology panel was developed to detect antibodies against HPV-16 E-proteins associated with oropharyngeal cancer. Plates are provided with antigens on spots in the wells of a 96-well plate. Antibodies in the sample bind to the antigens on the spots and anti-human IgG antibody conjugated with MSD SULFO-TAG is used for detection. Selected samples of the screened serum were used to build serology calibrator 1. The calibrator allows for quantification of serum antibody concentrations. Commercially-sourced plasma or serum samples were tested at 2,500-fold dilution on the serology panel

A



B

		LLOD and TOC concentration in MSD arbitrary units (AU/mL)	
		IgG	
Antigens	Fold Sample Dilution	LLOD	TOC
HPV-16 E1	2,500	0.004	49.6
HPV-16 E2		0.005	16.0
HPV-16 E6		0.005	4.7
HPV-16 E7		0.005	72.1
HPV-16 L1		0.006	557
HPV-18 L1		0.005	28.4

Assay Protocol

1. Add diluent (25 µL/per well) to the plates coated with antigens.
2. Add sample (25 µL/per well of 2,500-fold diluted serum or plasma).
3. Incubate 2 hours at room temperature (RT).
4. Wash and add detection antibody solution (25 µL per well). Incubate 1 hour at RT.
5. Wash and add MSD Gold Read Buffer B (150 µL per well). Analyze with MSD instrument.

Figure 1. (A) Typical calibration curves for the assays in the serology panel. These representative graphs show the wide dynamic range of the serology assays.
(B) Table shows the assignments for Top of Curve (TOC) concentrations for IgG antibodies to antigens in the calibrator and the estimated Lower Limits of Detection (LLOD). Values in this table are not corrected for sample dilution.

4 Individual Serum and Plasma Sample Testing

More than 300 commercially-sourced serum and plasma samples from apparently healthy individuals and from individuals with lung, breast, gastric or ovarian cancer, and 14 samples from individuals known to be positive for serological response to at least one HPV-16 early antigen were tested for HPV-16 E1, E2, E6, E7, HPV-16 L1 and HPV-18 L1 immunoglobulin G (IgG) antibodies.
For oropharyngeal cancer-associated E proteins, preliminary cutoff values for positive serology were defined as the 95th percentile of signals from approximately 200 serum and plasma samples from apparently healthy individuals. Larger studies will be required to set a more clinically appropriate cutoff (e.g., 98-99th percentiles).
Age, gender, and HPV vaccine status are not known for the samples tested in this study. We did not show a cutoff for L1 serology. A reasonable estimate for expected seropositivity for HPV-16 L1 in the general population is ~15%.

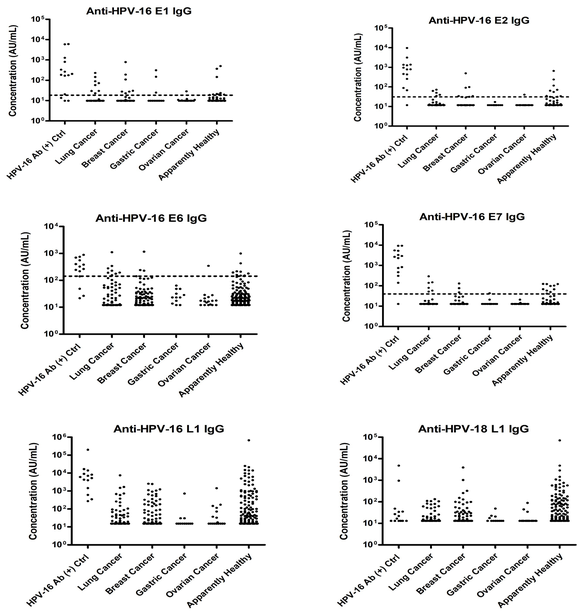


Figure 2. Concentrations with assigned AU/mL for assays detecting antibodies against HPV-16 E proteins, HPV-16 L1, and HPV-18 L1 in commercially-sourced samples. Concentrations are corrected for sample dilution. Samples with concentrations below LLOD are assigned those respective values.
For E-proteins, dashed black lines show the 95th percentile of signals from approximately 200 serum and plasma samples from apparently healthy individuals.

	Anti-HPV-16 E1 IgG	Anti-HPV-16 E2 IgG	Anti-HPV-16 E6 IgG	Anti-HPV 16 E7 IgG
Positivity Rate in HPV-16 (+) Control Samples (N=14)	79%	93%	71%	93%
Positivity Rate in Breast Cancer Samples (N=80)	9%	8%	4%	4%
Positivity Rate in Gastric Cancer Samples (N=12)	25%	0%	0%	8%
Positivity Rate in Ovarian Cancer Samples (N=17)	6%	6%	6%	0%
Positivity Rate in Lung Cancer Samples (N=62)	13%	8%	13%	10%
Positivity Rate in Apparently Healthy (n=183)	5%	5%	5%	5%

Table 1. Sensitivity of seropositivity for cancer-associated HPV-16 E-Proteins at a 95% specificity.

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5 Samples With Positive Reactivity Against HPV-16 Early Antigen

Sample ID	Anti-HPV- 16 E1 IgG	Anti-HPV- 16 E2 IgG	Anti-HPV- 16 E6 IgG	Anti-HPV- 16 E7 IgG	Number of Pos Reactivities
HPV-16 Ab (+) Control 01	Pos	Pos	Neg	Pos	3
HPV-16 Ab (+) Control 02	Pos	Pos	Pos	Pos	4
HPV-16 Ab (+) Control 03	Pos	Pos	Neg	Pos	3
HPV-16 Ab (+) Control 04	Neg	Pos	Pos	Pos	3
HPV-16 Ab (+) Control 05	Pos	Pos	Neg	Pos	3
HPV-16 Ab (+) Control 06	Pos	Pos	Neg	Neg	2
HPV-16 Ab (+) Control 07	Pos	Pos	Pos	Pos	4
HPV-16 Ab (+) Control 08	Pos	Pos	Pos	Pos	4
HPV-16 Ab (+) Control 09	Neg	Neg	Pos	Pos	2
HPV-16 Ab (+) Control 10	Pos	Pos	Pos	Pos	4
HPV-16 Ab (+) Control 11	Pos	Pos	Pos	Pos	4
HPV-16 Ab (+) Control 12	Pos	Pos	Pos	Pos	4
HPV-16 Ab (+) Control 13	Neg	Pos	Pos	Pos	3
HPV-16 Ab (+) Control 14	Pos	Pos	Pos	Pos	4
Breast Cancer 02	Neg	Neg	Neg	Pos	1
Breast Cancer 03	Pos	Neg	Neg	Neg	1
Breast Cancer 10	Pos	Pos	Neg	Neg	2
Breast Cancer 12	Pos	Neg	Neg	Neg	1
Breast Cancer 15	Pos	Pos	Pos	Neg	3
Breast Cancer 16	Pos	Pos	Neg	Pos	3
Breast Cancer 23	Neg	Neg	Pos	Neg	1
Breast Cancer 25	Neg	Pos	Neg	Neg	1
Breast Cancer 31	Pos	Pos	Neg	Neg	2
Breast Cancer 33	Neg	Pos	Neg	Neg	1
Breast Cancer 34	Pos	Neg	Neg	Neg	1
Breast Cancer 37	Neg	Neg	Neg	Pos	1
Breast Cancer 39	Neg	Neg	Pos	Neg	1
Gastric Cancer 04	Pos	Neg	Neg	Neg	1
Gastric Cancer 06	Neg	Neg	Neg	Pos	1
Gastric Cancer 07	Pos	Neg	Neg	Neg	1
Gastric Cancer 12	Pos	Neg	Neg	Neg	1
Lung Cancer 11	Pos	Neg	Pos	Neg	2
Lung Cancer 16	Pos	Neg	Neg	Neg	1
Lung Cancer 17	Neg	Neg	Pos	Neg	1
Lung Cancer 19	Pos	Pos	Pos	Pos	4
Lung Cancer 20	Neg	Neg	Pos	Neg	1
Lung Cancer 13	Neg	Neg	Pos	Neg	1
Lung Cancer 14	Neg	Neg	Neg	Pos	1
Lung Cancer 17	Pos	Pos	Pos	Neg	3
Lung Cancer 18	Pos	Pos	Neg	Neg	2
Lung Cancer 21	Pos	Pos	Pos	Pos	4
Lung Cancer 22	Neg	Neg	Neg	Pos	1
Lung Cancer 23	Neg	Pos	Neg	Neg	1
Lung Cancer 24	Pos	Neg	Neg	Neg	1
Lung Cancer 29	Pos	Neg	Neg	Neg	1
Lung Cancer 36	Neg	Neg	Neg	Pos	1
Ovarian Cancer 02	Pos	Neg	Neg	Neg	1
Ovarian Cancer 09	Neg	Pos	Neg	Neg	1
Ovarian Cancer 12	Neg	Neg	Pos	Neg	1
Apparently Healthy 07	Neg	Neg	Neg	Pos	1
Apparently Healthy 10	Neg	Pos	Neg	Neg	1
Apparently Healthy 12	Pos	Neg	Neg	Neg	1
Apparently Healthy 38	Neg	Neg	Pos	Neg	1
Apparently Healthy 42	Neg	Neg	Neg	Pos	1
Apparently Healthy 45	Neg	Pos	Neg	Neg	1
Apparently Healthy 48	Neg	Pos	Neg	Neg	1
Apparently Healthy 53	Neg	Neg	Neg	Pos	1
Apparently Healthy 54	Neg	Neg	Neg	Pos	1
Apparently Healthy 56	Neg	Neg	Neg	Pos	1
Apparently Healthy 57	Neg	Neg	Neg	Pos	1
Apparently Healthy 61	Neg	Neg	Neg	Pos	1
Apparently Healthy 69	Pos	Neg	Neg	Neg	1
Apparently Healthy 77	Pos	Pos	Neg	Neg	2
Apparently Healthy 82	Pos	Neg	Neg	Neg	1
Apparently Healthy 86	Neg	Pos	Neg	Neg	1
Apparently Healthy 91	Neg	Neg	Pos	Neg	1
Apparently Healthy 92	Pos	Pos	Neg	Pos	3
Apparently Healthy 97	Neg	Pos	Neg	Neg	1
Apparently Healthy 102	Pos	Neg	Neg	Neg	1
Apparently Healthy 106	Pos	Neg	Neg	Neg	1
Apparently Healthy 108	Pos	Neg	Neg	Neg	1
Apparently Healthy 125	Neg	Neg	Neg	Pos	1
Apparently Healthy 127	Neg	Neg	Pos	Neg	1
Apparently Healthy 131	Pos	Neg	Neg	Neg	1
Apparently Healthy 134	Neg	Neg	Pos	Neg	1
Apparently Healthy 136	Neg	Neg	Pos	Neg	1
Apparently Healthy 141	Neg	Neg	Pos	Neg	1
Apparently Healthy 143	Neg	Pos	Neg	Neg	1
Apparently Healthy 145	Neg	Neg	Pos	Neg	1
Apparently Healthy 158	Neg	Neg	Pos	Neg	1
Apparently Healthy 169	Neg	Pos	Neg	Neg	1
Apparently Healthy 170	Neg	Neg	Neg	Pos	1
Apparently Healthy 171	Pos	Pos	Pos	Pos	4

Table 2. At the preliminarily selected cutoff for antigen seropositivity of 95% of an apparently healthy population, presumably true positive samples are reactive for several E proteins. In contrast, many of the remaining samples are positive only for one antigen. Larger studies are required to establish criteria to accurately classify true positives, such as requiring positivity for more than one E protein.

6 Conclusions

- Sensitive serology assays for oropharyngeal cancer associated HPV-16 E-proteins were developed and there was excellent agreement between the two methods.
- The assay format is simple, appropriate for high-throughput screening, and uses only 25 µL of 2,500-fold diluted serum or plasma per determination.

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7 Acknowledgement

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