INTENDED USE

The **Human Anti-HPVL1 (Gardasil9-Combo) IgG** ELISA Kit is an immunoassay suitable for detecting and quantifying IgG antibody activity specific for **HPV subtypes 6, 11, 16, 18, 31, 33, 45, 52 and 58** L1 proteins in a single sample of serum or plasma. Other biological fluids, including tissue culture medium, may be validated for use. This immunoassay is suitable for:

- Determining **immune status** relative to non-immune controls;
- Assessing efficacy of **vaccines**, including dosage, adjuvantcy, route of immunization and timing;
- Qualifying and standardizing vaccine batches & protocols

The assay is for research use only (RUO) and is not intended nor validated for diagnosing HPV. Reagents contain no virus.

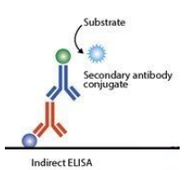
GENERAL INFORMATION

Human papillomavirus (HPV) is a group of more than 200 related viruses, of which more than 40 are spread through direct sexual contact. Several HPV types cause genital warts, and about a dozen HPV types can cause certain cancer types – cervical, anal, oropharyngeal, penile, vulvar and vaginal.

The HPV virus is small and non-enveloped, with a single, circular dsDNA contained within a T=7 icosahedral capsid composed of 72 pentamers. The HPV genome is composed of six early (**E1, E2, E3, E4, E6 and E7)** and two late (**L1 and L2)** proteins**.** The late proteins, translated in the upper layers of the host epithelium, serve as structural proteins that encapsulate the amplified viral genomes.

FDA-approved vaccines that have shown to produce protective antibodies include **Gardasil** (types 6,11,16,18) **& Gardasil 9 (**with add'l types 31,33,45,52 & 58) and **Cervarix** (types 16,18), which are composed of recombinant L1 protein as virus-like particles (VLP).

PRINCIPLE OF THE TEST



The Human Anti-HPV IgG ELISA kit is based on the binding of anti-HPV IgG in samples to HPV antigens immobilized on the microwells, and anti-HPV IgG antibody is detected by anti- IgG-specific antibody-HRP conjugate. After a washing step, substrate (TMB) is added and color (blue) is developed, which is directly proportional to the amount of anti-HPV IgG present in the sample. Stop Solution is added to terminate the reaction (converts blue to yellow), and A450nm is then measured using an ELISA reader. The presence of HPV antibody in samples is determined relative to Calibrators.

PRODUCT SPECIFICATIONS

Specificity
An antigen mixture of purified recombinant HPVL1 virus-like particles (VLP), types 6+11+16+18+31+33+45+52+58, expressed in baculovirus-insect cells, is used to coat plates; thus, this test detects antibodies to any of these 9 HPV serotypes in a single test. HPVL1's from HPV6, 11, 16, and 18 types share ~50% sequence homology. The Anti-human IgG HRP conjugate reacts specifically with human IgG class antibodies; IgA, IgM and IgE antibody would not be measured above background signals.

KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8^{o}C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

To Be Reconstituted: Store as indicated.

Component	Preparation Instructions
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store refrigerated for long term and at lab temperature for short term.
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample Diluent** and store at 2-8^{o}C until the kit lot expires or is used up.
Anti-Human IgG-HRP Conjugate Concentrate (100x) Part: H-HuG.2a11, 0.15ml	Peroxidase conjugated anti-human IgG in buffer with detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8^{o}C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
HPVL1 6, 11, 16, 18, 31, 33, 45, 52, 58 Coated Plate	550-501	8-well strips (12)	Coated with HPVL1 subtype6+11+16+18 +31+33+45+52+58 rec proteins, post-coated with stabilizers.
Anti-HPV Calibrators			
1 U/ml 2.5 U/ml 5 U/ml 10 U/ml	550502B 550502C 550502D 550502E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing anti-HPVL1; in buffer with antimicrobial as stabilizers.
Anti-HPV Positive Control	550-502PC	0.65 ml	Serum with anti-HPVL1 reactivity; [Value range on label]
Low NSB Sample Diluent	TBTm **Not** for HRP dilution	30 ml	Buffer with protein, detergents and anti-microbial. Use as is for sample dilution
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	Dilute sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Human IgG HRP Concentrate.
- Stock bottle to store diluted Wash Solution; 0.2 to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength and ELISA plate washer

ASSAY DESIGN AND SET-UP

Sample Collection and Handling

Serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. For other samples, clarify the sample by centrifugation and/or filtration prior to dilution in Sample Diluent. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.
Caution: Human serum and other bodily fluids may contain infectious material. Always wear gloves when handling human samples, including the standards and controls (which have been tested non-reactive for HbsAg and Anti-HIV), and dispose of these samples and containers as biohazard waste.

Antibody Stability& Dilution

Initial dilution of serum into **Working Sample Diluent** (WSD) is recommended to stabilize antibody activity. This enhances reproducible sampling, and stabilizes the antibody activity for years, stored refrigerated or frozen. Further dilution into **Low NSB Sample Diluent** (LNSD), which provides the lowest assay background, should be at least 10 times the initial dilution and performed the same day as the assay.
Example: Initial (1/5): **10**ul serum + **40**ul WSD [or 0.1ml + 0.4ml]
Further (1/50): **10**ul initial (1/5) + **90**ul LNSD (1/50)

Assay Design
Review Interpretation of Results and Limits of the Assay (p5-7) before proceeding:

- Select the proper sample dilutions accounting for expected potency of positives and minimizing non-specific binding (NSB) and other matrix effects; for example, net signal for non-immune samples should be lower than the **1 U/ml Calibrator**. This is usually **1:200** or greater dilution for human serum/plasma with normal levels of IgG and IgM.
- Run a Sample Diluent **Blank**. This signal is an indicator of proper assay performance, especially of washing efficacy, and is used for net OD calculations, if required. Blank OD should be <0.3.
- Run the Anti-HPV **Positive Control;** the value range is on the label.
- Run a set of **Calibrators**, which validate that the assay was performed to specifications: **10 U/ml** should give a high signal (>1.5 OD); **1 U/ml** should give a low signal which can be used to discriminate at the Positive/Negative threshold (see Interpretation of Results, p. 5).

Plate Set-up
Bring all reagents to room temperature (18-30^{o} C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 8Calibrator wells and 2 wells for each sample control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Add 200-300ul Working Wash Solution to each well and let stand for about 5 minutes. Aspirate or dump the liquid and pat dry on a paper towel before sample addition.

Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. 1st Incubation [100ul – 60 min; 4 washes]

- Add 100ul of calibrators, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 60 minutes.
- Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

2. 2nd Incubation [100ul – 30 min; 5 washes]

- Add 100ul of diluted Anti-Human IgG HRP to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 2.

3. Substrate Incubation [100ul – 15 min]

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.

Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, or read OD at 405-410 nm (results are valid).

4. Stop Step [Stop: 100ul]

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

5. Absorbance Reading

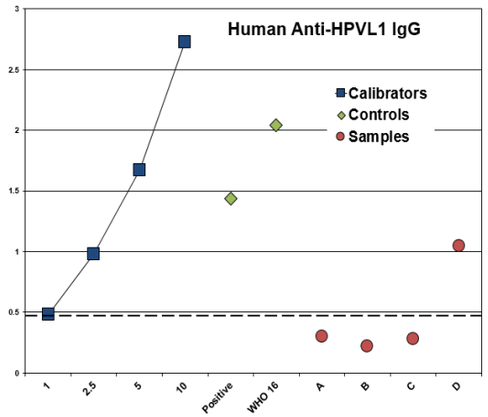
- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

INTERPRETATION OF RESULTS

Method A. Antibody Activity Threshold Index

Compare Samples to **1 U/ml Calibrator** or **Internal Control =Positive/Negative Cut-off**.

Example:



Results

The **sensitivity** of the assay to detect anti-HPV IgG, from either natural infection or vaccination, is controlled so that the **1 U/ml Calibrator** represents a threshold OD for most true positives in human serum diluted to 1:200 or greater. Visual inspection of the data in the above graph shows the following:

Calibrators – dilution curve of an anti-HPV antibody, derived from HPVL1 vaccination, shows the OD range of the assay; high value indicates optimal sensitivity of the assay.
1 U/ml: a 'Cut-off' line has been drawn to indicate a threshold distinguishing between **Positive/Negative**. This is not a clear-cut threshold, rather a low OD area that could represent either low positives or high background negatives.

Positive Control – an antiserum with reactivity to HPVL1 antigens; the value range is on the label. This Control may be used to gauge precision and to normalize between-assay variation.

WHO International Standards for **HPV16** [NIBSC 05/134]:
HPV16 – diluted to **0.1** IU/ml = **2.6** U/ml in the assay.

WHO standards also reacted to a lesser extent with HPV6, 11 and 18 individually, but no units were assigned by WHO.

Samples A,B,C,D – 3 samples (A, B, C) are negative: below the threshold; 1 sample (D) is positive: clearly above the threshold.

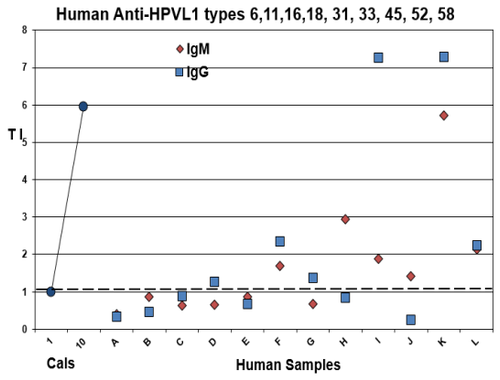
The 1 U/ml Calibrator can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative:

- Divide each Sample net OD by the 1 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

INTERPRETATION OF RESULTS (cont)

Example:

Human Serum IgG & IgM
A panel of sera/plasma from humans of unknown history was tested for anti- HPVL1 IgG & IgM (1:200 dilution in Low NSB Sample Diluent). **Threshold Index** was calculated using the **1** U/ml **Calibrator**.



Results

Anti-HPV IgG: several samples were distinctly positive at 1:200 dilution (above the 1.0 Threshold Index); most samples were negative or borderline.

Anti-HPV IgM: several samples were positive, including 1 (H) that was negative for IgG; many were borderline.

Notes:

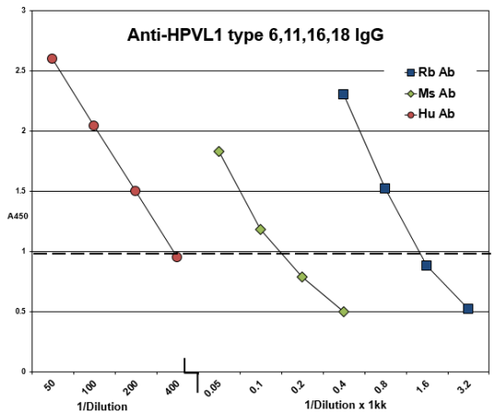
1. **Positives** may be due to prior encounter with HPV, from exposure to an antigen with common epitopes, or from immunization.
2. When the **Positive Index** is **above 5.0,** using a dilution curve to calculate titer is a more accurate quantitation method (see Method C).
3. The **sensitivity** of the assay may be adjusted by changing the sample dilutions: a) increase dilution (e.g., 1:500) to lower the signals of borderline positives to negative; b) decrease dilution (e.g., 1:100) to convert borderline samples to positive. With the latter, the values of negatives may increase, so an alternative threshold should be considered using known negatives to develop a **Positive Index (**see below) or use an **Internal Control** (Page 5).

B. Positive Index
Experimental sample values may be expressed relative to the values of Control or Non-immune samples, by calculation of a **Positive Index**. One typical method is as follows:

1. Calculate the net OD mean + 2 SD of the Control/Non-immune samples = **Positive Index**.
2. Divide each sample net OD by the Positive Index. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

INTERPRETATION OF RESULTS (cont)

C. Antibody Titer
The most accurate method for comparing antibody potencies is by calculation of a titer, using an OD reading midrange in the dilution curves of each antibody as **Index.** In the example below, **IgG** titers were calculated as inverse of the dilution that produced a **1.0 OD** in the assay.



Results
Antisera are from individuals immunized with Gardasil (Merck) HPV 6+11+16+18.

Mouse Anti-HPV: Pool of 5 hyperimmunized mice; Titer = **130k.**

Rabbit Anti-HPV: Hyperimmunized individual; Titer = **1.4 kk.**

Human Anti-HPV: WHO International Standard for HPV16**;** Titer = **375 = 13.3 mIU/ml.**

Limits of the Assay

- The assay detects and quantifies IgG antibodies directed to the L1 protein. Patients may have HPV infection without producing antibodies specific to L1.
- Anti-HPV antibody levels of an infected patient may be below detection threshold related to the time course of the infection, e.g., too early for positive titer development.
- Samples from non-patients may be elevated due to prior exposure to the human papilloma virus.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains dilute sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water. MSDS for TMB, sulfuric acid and BND can be requested or obtained from the ADI website:http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10

Instruction Manual No. M-550-500-PHG

Human Anti-HPVL1 [Gardasil 9 - Combo] IgG ELISA Kit

Cat. No.550-500-PHG, 96 tests

For the Detection and Quantitation of Anti-HPVL1 [Late Protein] IgG for Serotypes **6, 11, 16, 18, 31, 33, 45, 52** and **58**, in Human Serum or Plasma or other Biological Fluids

For research use only, not for diagnostic or therapeutic use.

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ELISA Kit Components	Amount	Part
HPVL1 6,11,16,18, 31, 33, 45, 52, 58 Coated Strip Plate	8-well strips (12)	550-501
Anti-HPV Positive Control	0.65 ml	550-502PC
Anti-HPV Calibrator 1 U/ml	0.65 ml	550-502B
Anti-HPV Calibrator 2.5 U/ml	0.65 ml	550-502C
Anti-HPV Calibrator 5 U/ml	0.65 ml	550-502D
Anti-HPV Calibrator 10 U/ml	0.65 ml	550-502E
Anti-Human IgG HRP Conjugate (100X)	0.15 ml	H-HuG.2a11
Sample Diluent (20x)	10 ml	SD20T
Low NSB Sample Diluent	30 ml	TBTm
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	550-500-PHG