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Human papillomavirus laboratory manual

First edition, 2009

Immunization, Vaccines and Biologicals

World Health Organization

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World Health Organization
Department of Immunization, Vaccines and Biologicals
CH-1211 Geneva 27, Switzerland
• *Fax:* + 41 22 791 4227 • *Email:* vaccines@who.int •

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Contents

Abbreviations and Acronyms

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AIS	adenocarcinoma in situ
bp	base pair
BSL	basic biosafety level
BPV	bovine papillomavirus
C	centigrade
CIN	cervical intraepithelial neoplasia
cm	centimetre
Ct	cycle threshold
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DMEM	Dulbecco´s Modified Eagle Medium
DTT	dithiothreitol
ECBS	Expert Committee on Biological Standardization
ECL®	enhanced chemiluminescent substrate
EDAC	N-Ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDQM	European Directorate for the Quality of Medicines & HealthCare
EDTA	ethylene-diamine-tetra-acetic acid
E1-E7	early proteins of HPV numbered E1–E7 (however no E3)
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
g	gravity
GE	genome equivalents
g	gram
GRL	WHO global reference laboratory
h	hour
HBV	hepatitis B virus
HC2	hybrid capture 2 (Digene®, Qiagen)
HCV	hepatitis C virus
HIV	human immunodeficiency virus

HLA	histocompatibility leukocyte antigen
HPLC	high-performance liquid chromatography
HPV	human papillomavirus
HPV LabNet	WHO HPV Laboratory Network
HR	high risk
HRP	horseradish peroxidase
HS	horse serum
HS-PBS	10% HS in PBS
HSIL	high grade squamous intraepithelial lesion
IARC	International Agency for Research on Cancer
ICH	International Committee on Harmonization
Ig	immunoglobulin
IgG	immunoglobulin G
IS	international standard
IU	international unit
kb	kilobases
kbp	kilobase pairs
kDa	kilodalton
L	litre
L1, L2	late proteins of HPV, major (L1) and minor (L2) proteins of capsid
lab	laboratory
LQA	laboratory quality assurance
LR	low risk
LSIL	low grade squamous intraepithelial lesion
M	molar
Mab	monoclonal antibody
mAmp	milliampere
mg	milligram
min	minute
mL	millilitre
mM	millimetre
µL	microlitre
NAT	nucleic-acid amplification technique
NB	neutralization buffer
NEAA	non-essential amino acids
NIBSC	National Institute for Biological Standards and Control
NIH	National Institutes of Health
nm	nanometer (10^{-9} metre)
NTC	no template control

OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PBS-T	PBS-0.1% Tween 20
PCR	polymerase chain reaction
pg	picogram
PLL	parallel line
PsV	pseudovirion
PTS	Proficiency Testing Scheme (WHO LabNet)
QA	quality assurance
QC	quality control
RBH	reverse blotting hybridization
RLU	relative light units
RNA	ribonucleic acid
RRL	WHO regional reference laboratory
RT	room temperature
RUO	research use only
SDS	sodium dodecylsulphate
SEAP	secreted alkaline phosphatase
s	second(s)
SOP	standard operating procedure
STM	specimen transport media (Qiagen)
SSPE	saline sodium phosphate EDTA
TBE	Tris-Borate-EDTA
TC	tissue culture
Tex	no template extraction control
URR	upstream regulatory region
USA	United States of America
UV	ultraviolet
V	volt
VLP	virus-like particle
WHO	World Health Organization

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The following authors contributed greatly to this document: Dr Morag Ferguson (former Principal Scientist) and Dr Dianna Wilkinson, WHO International Laboratory for Biological Standards, Division of Virology, National Institute for Biological Standards and Control (NIBSC), United Kingdom; Dr Denise Nardelli Haefliger and Dr Roland Sahli, Regional WHO HPV Reference Laboratory (RRL), Switzerland; Ms Carina Eklund and Ms Eva Hedvall, GRL, Sweden; Dr Gitika Panicker, GRL, USA. Additional authors include Professor Anna-Lise Williamson, RRL, South Africa; Dr María Alejandra Picconi, RRL, Argentina; Dr Emna Ennaifer-Jerbi, RRL, Tunisia; Dr Alok Chandra Bharti and Dr Mausumi Bharadwaj, RRL, India; Dr Jarunya Ngamkham and Dr Sukhon Sukvirach, RRL, Thailand; Professor Suzanne Garland, RRL, Australia; Dr Iwao Kukimoto and Dr Kei Kawana, RRL, Japan.

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1. Introduction

1.1 Purpose and target audience of the manual

This manual was developed by the WHO HPV Laboratory Network (WHO HPV LabNet) based on knowledge and experience gained through its international collaborative studies over the past several years. The manual aims to assist in establishing the laboratory support required for implementation and monitoring of human papillomavirus (HPV) vaccination programmes by:

- providing a brief summary of the biology and natural history of HPV and the worldwide burden of HPV-associated diseases;
- discussing the role of laboratories and the WHO HPV LabNet in supporting HPV surveillance and vaccination impact monitoring;
- describing the currently available international standards (IS) and secondary standards for HPV testing and their appropriate use;
- providing guidance on specimen collection and handling for HPV testing;
- providing an overview of HPV serologic and DNA testing and providing example protocols evaluated by the WHO HPV LabNet;
- providing guidance on laboratory quality assurance (QA).

The manual should be useful for all audiences involved in development and implementation of HPV vaccines, particularly those involved in generating or using HPV laboratory data. Additional information on HPV and HPV vaccine can be found in many sources (*1*,*2*).

This document is a living document that will be amended in the light of future advances made in the area, and future global experience of HPV laboratory surveillance and vaccination monitoring.

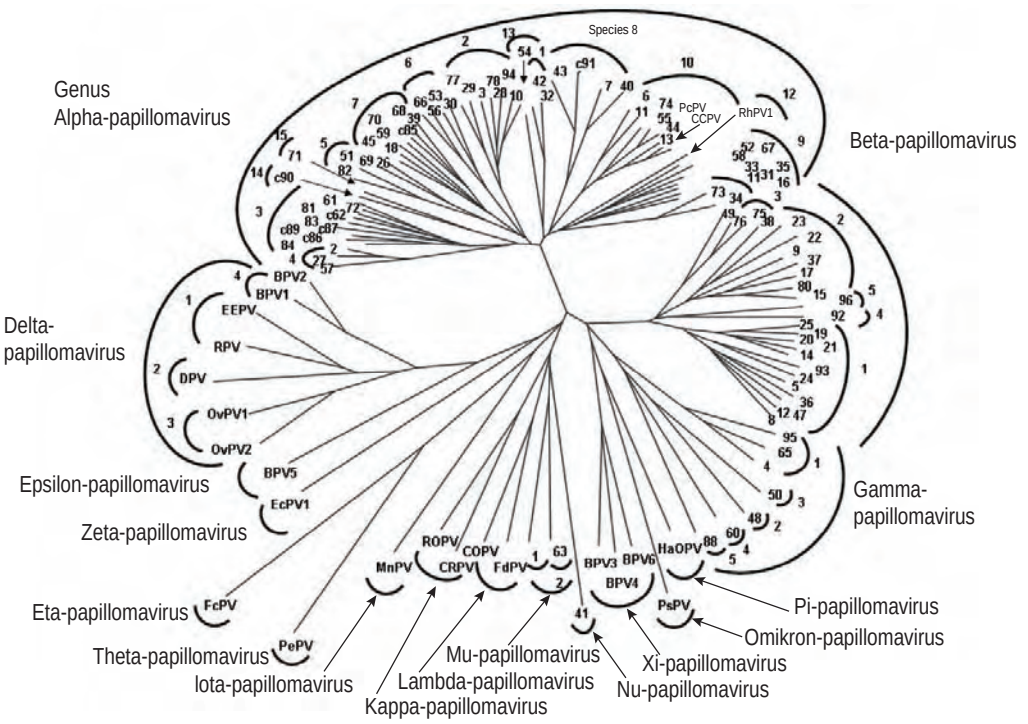
1.2 Biology and natural history of HPV

HPVs are a group of more than 100 closely related non-enveloped double-stranded deoxyribonucleic acid (DNA) viruses in the *Papillomaviridae* family (*3*). HPVs infect epithelial cells of the skin and mucosal surfaces. The circular genome, approximately 8.0 kilobases (kb), is enclosed in a protein shell made from the major (L1) and minor (L2) capsid proteins resulting in virions approximately 55 nm in diameter. All of the coding information is contained in one of the two DNA strands. There are seven open reading frames (ORF) encoding several known viral proteins. The six "early" proteins are E1, E2, E4, E5, E6 and E7. Transcripts encoding the early proteins are detected in

basal and suprabasal epithelial cells in the early portion of the viral replication cycle, and encode proteins that interact with the host cell machinery to allow viral replication and transcription to occur. As a result of their function in the normal viral life-cycle, the E6 and E7 proteins are also the major viral oncogenes, playing a central role in cell immortalization and transformation. The L1 ORF encodes the major capsid protein (55 kDa) that makes up the majority of the virus protein shell and its exposed surface. The L2 ORF encodes the minor capsid protein (77 kDa) that contributes a smaller percentage of the capsid mass, but plays an important role in encapsidating the viral genome. L2 is not significantly exposed to the surface in intact virions. Expression of the "late" structural L1 and L2 genes is restricted to differentiating epithelium where viral assembly occurs. When expressed in cultured cells, the L1 protein will self-assemble into structures resembling HPV, called virus-like particles (VLPs).

Individual HPVs are referred to as "types", distinguished based on their genomic sequence, and numbered in order of discovery. Viruses with sequence divergence of less than 10% from a recognized type are "subtypes" (2%–10% divergence) or "variants" (<2%) of that type. The L1 gene is the region that is most conserved between individual HPV types and is used to form phylogenetic trees used in taxonomy (Figure 1.1) (*3*). The term "genus" is used for the higher order clusters that are named using the Greek alphabet. Within genus, the smaller clusters are referred to as "species" and are named by number.

Figure 1.1 Phylogenetic tree containing the sequences of 118 papillomavirus types



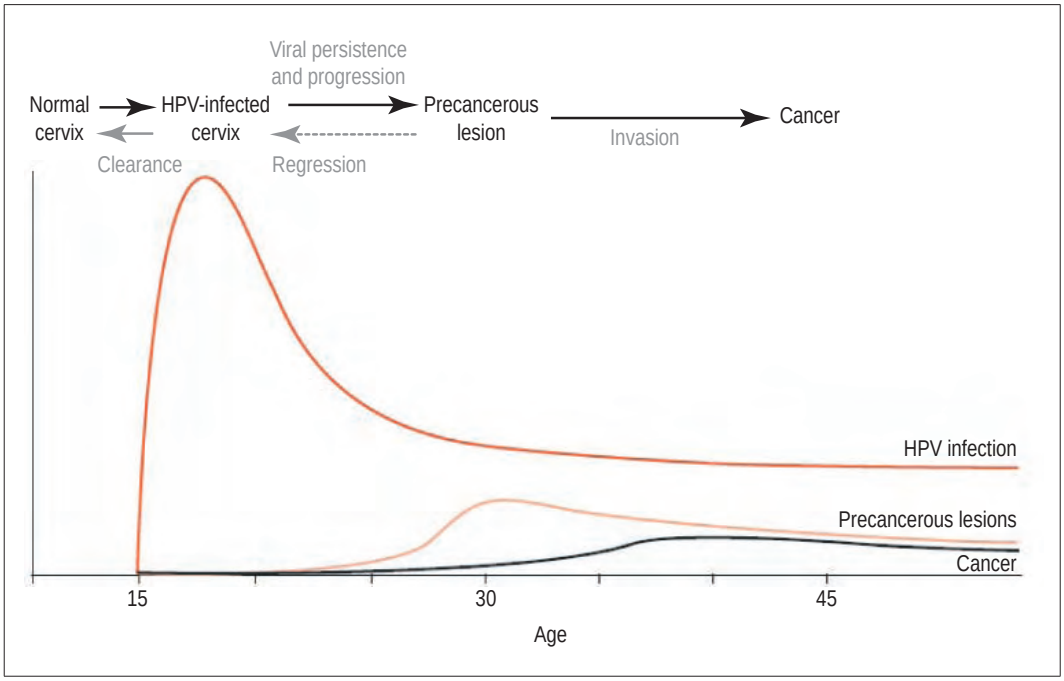
The L1 ORF nucleic acid sequences were used in a modified version of the Phylip version 3.572 and based on a weighted version of the neighbour-joining analysis. The tree was constructed using the Treeview programme of the University of Glasgow. The numbers at the ends of each of the branches identify an HPV type; c-numbers refer to candidate HPV types. All other abbreviations refer to animal papillomavirus types. The outermost semicircular symbols identify papillomavirus genera, e.g. the genus alpha-papillomavirus. The number at the inner semicircular symbol refers to papillomavirus species. To give an example taken from the upper part of the figure, the HPV types 7, 40, 43, and cand91 together form the HPV species 8 in the genus alpha-papillomavirus. [Reproduced, by permission, from de Villiers et al., 2004].

Approximately 35 HPV types are known to infect the human genital mucosa. They can be grouped as "low risk" (LR) or "high risk" (HR) based on their epidemiologic association with cancer (*4*). The LR types such as HPV6 and HPV11 are associated with genital warts or condyloma acuminate, recurrent respiratory papillomatosis, and low-grade cervical intraepithelial lesions (LSIL). HR types such as HPV16 and HPV18 are associated with low- and high-grade cervical intraepithelial lesions (HSIL) and invasive cancer.

Transmission of mucosa-tropic types occurs primarily through sexual contact. HPV is the most common viral sexually transmitted infection, with estimates that up to 75% of sexually active people are infected at some time in their life. The peak age for HPV infection is in the first few years following sexual debut. HPV prevalence in the population decreases in older age as most genital HPV infections resolve without symptoms (typically within 12–18 months). Some infections persist, and persistent infection is more likely to be associated with abnormal cytology and cancer precursors.

Invasive cancer is a rare outcome, many years (generally decades) after infection. Figure 1.2 is a summary of current understanding of the natural history of HPV cervical infection and cancer.

Figure 1.2 Diagram representing the natural history of HPV and age-specific prevalence of HPV-associated disease in women



[Adapted, by permission, from Schiffman & Castle, 2005]

As HPV does not induce cell necrosis and infection is confined to epithelial surfaces, it is relatively protected from the host immune system. Antibodies to type-specific L1 protein conformational epitopes are detectable in less than 70% of HPV-exposed individuals. Nevertheless these low-titre antibodies can serve as a measure of exposure in the population. Clearance of HPV is associated with a cell-mediated response.

Because of its relative insensitivity, HPV serology should not be used for diagnosis on an individual basis, although it can be used to monitor the population as a whole, as an indication of relative cumulative exposure. HPV serology is used mostly for indicating response to vaccination.

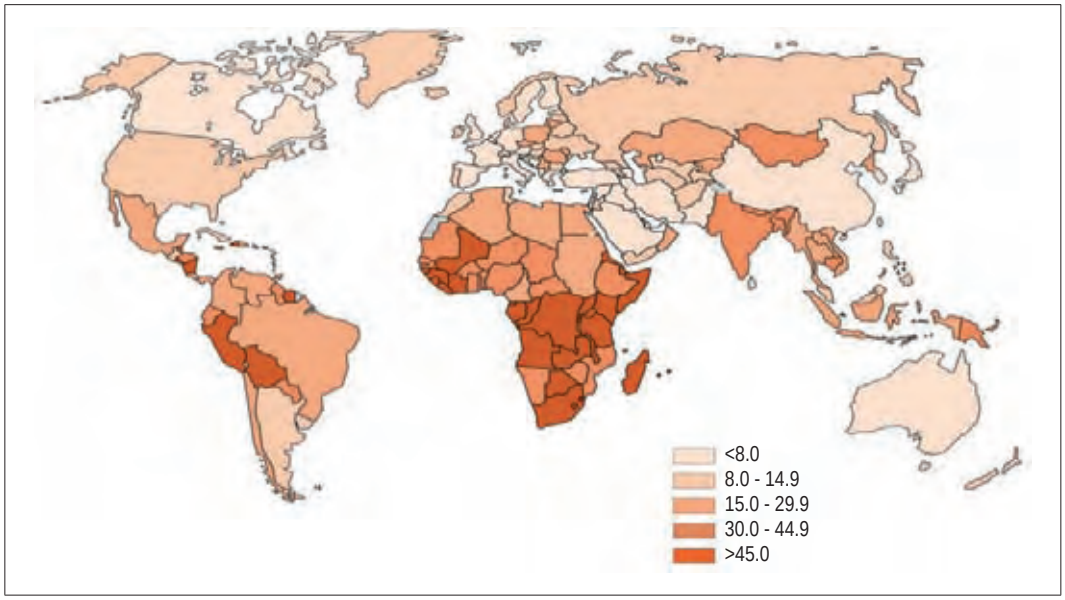
HPV cannot be cultured by conventional methods and is a cell-associated virus; therefore HPV infection is monitored indirectly by detection of HPV DNA in a cellular sample obtained from a particular anatomic site. It should be kept in mind that detection of HPV DNA usually indicates current infection, but surface contamination cannot be excluded. Similarly, failure to detect HPV DNA does not exclude HPV infection, as low-level infections, sampling errors and infections at other anatomic sites cannot be excluded.

1.3 The burden of the disease caused by HPV

HPV has been associated with more than 99% of cervical cancers (*6*). While the type distribution in cervical cancer does vary somewhat worldwide, HPV16 and HPV18 are the most prevalent types, being found in more than 70% of samples from cervical cancer around the world (*7*). The causal relationship between HPV and cervical carcinoma has provided the incentive for the development of vaccines that prevent infection with HPV. By protecting uninfected women against infections with the HPV types that are most associated with cervical cancer, the cervical cancer that would have arisen from those types will be prevented. HPV16 and HPV18 are the two HR types targeted by current vaccine formulations.

Cervical cancer is the leading cause of cancer mortality among women in developing countries. The disease burden contributes to worldwide health inequity, as 80% of cervical cancer deaths occur in under-resourced countries (Figure 1.3) (*8*). Currently, the best way to prevent cervical cancer is through organized gynaecological screening programmes with associated treatment of the detected pre-cancerous lesions. These programmes have not been effectively implemented in some areas of the world where they are most needed, due to barriers of cost, trained personnel and infrastructure.

Figure 1.3 Worldwide incidence of cervical cancer per 100 000 females (all ages), age-standardized to the WHO standard population, 2005



[Adapted, by permission, from WHO, 2008]

In addition to cervical cancer, it is increasingly recognized that HPV is associated with a number of anogenital and oropharyngeal malignancies affecting both males and females. A recent review conducted by the International Agency for Research on Cancer (IARC) concluded that while cervical cancer is caused entirely by HPV, other sexually transmitted cancers are caused by HPV to a varying extent; penis 40%, anus 90%, vulva/vagina 40% and oropharynx 12% (Table 1.1) (*9*). HPV16 and HPV18 (particularly HPV16) account for an even larger proportion of the non-cervical HPV-associated cancers. The estimated worldwide burden of cancer cases caused by

HPV and by HPV16/18 is 5.2% and 3.7% respectively (Table 1.1).

Table 1.1 HPV-attributable cancer in 2002

Site	Attributable to HPV (%)	Of which, HPV16 and/or 18 (%)	Both sexes				
			Total cancers	Attributable to HPV	% all cancer	Attributable to HPV16/18	% all cancer
Cervix	100	70	492 800	492 800	4.54	344 900	3.18
Penis	40	63	26 300	10 500	0.10	6 600	0.06
Vulva, vagina	40	80	40 000	16 000	0.15	12 800	0.12
Anus	90	92	30 400	27 300	0.25	25 100	0.23
Mouth	3	95	274 300	8 200	0.08	7 800	0.07
Oro-pharynx	12	89	52 100	6 200	0.06	5 500	0.05
All sites			10 862 500	561 100	5.17	402 900	3.71

Adapted, by permission, from Parkin DM, 2006

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2. Role of the laboratory in HPV surveillance and vaccine impact monitoring

2.1 WHO position on HPV vaccines

In accordance with its mandate to provide guidance to Member States on health policy matters, WHO publishes a series of regularly updated position papers on vaccines and vaccine combinations against diseases that have an international public-health impact. These papers are concerned primarily with the use of vaccines in the global or regional context and with large-scale immunization of specific high-risk groups; limited vaccination, as executed mostly in the private sector, may be a valuable supplement to national programmes, but is not emphasized in these policy documents. The position papers summarize essential background information on the respective diseases and vaccines, and conclude with the current WHO position concerning their use in the global context (*1*).

In April 2009 WHO published its position paper on HPV vaccines (*2*). The following quotation summarizes its recommendations: "WHO recognizes the importance of cervical cancer and other HPV-related diseases as global public-health problems and recommends that routine HPV vaccination should be included in national immunization programmes, provided that: prevention of cervical cancer or other HPV-related diseases, or both, constitutes a public-health priority; vaccine introduction is programmatically feasible; sustainable financing can be secured, and the cost effectiveness of vaccination strategies in the country or region is considered."

This statement indicates that HPV vaccines should be part of a coordinated strategy to prevent cervical cancer that includes education about HPV and risk factors for acquisition, screening for, and the diagnosis and treatment of, precancerous lesions. Vaccination will not eliminate the need for screening, as not all cancer-associated HPV types are included in current vaccine formulations. The statement also indicates that introduction of the HPV vaccine should not detract from effective screening programmes. However, implementation of a vaccination programme should not be dependent on prior or co-introduction of a screening programme. In addition, following vaccine introduction, the statement recommends that countries monitor vaccine coverage and safety. Countries should consider approaches to monitoring HPV vaccine impact, particularly through establishing or improving reporting to cervical cancer registries. However, HPV vaccine impact monitoring is complex and is not a prerequisite for HPV vaccine introduction. WHO held two meetings in 2009, with experts, WHO Regions and countries worldwide, to consider strategies for HPV surveillance and vaccination impact monitoring. Reports from these meetings are available (*3, 4*).

2.1.1 Unique features of HPV vaccines

While the public-health approach to vaccine introduction and monitoring for the primary prevention of vaccine-preventable diseases in infants has been well established, the HPV vaccine presents several unique features that require additional consideration in the design and monitoring of HPV vaccination programmes.

Vaccination is directed towards prevention of HPV infection, but the public-health outcome of interest is cervical cancer. HPV infection is not treated, so current uses of HPV testing in screening and clinical diagnosis are directed towards detection of HPV-associated precancers that are treated, rather than to diagnose infection per se. As noted in chapter 1, HPV infection is quite common and mostly transient. It is only when the infection becomes persistent that it indicates a risk for neoplasia.

HPV tests in clinical use often do not provide type-specific information and may be sufficiently sensitive for detection of clinical disease but not for detection of HPV infection per se. HPV tests required for documenting type-specific prevalence and monitoring the impact of vaccination are therefore different from clinical tests, in that they should have optimal analytic (rather than clinical) sensitivity and specificity. This limits the usefulness of data from clinical HPV testing in HPV epidemiology and surveillance. Similarly, the high sensitivity HPV tests needed in epidemiology and vaccinology may not be optimal for clinical HPV testing.

As a consequence of the natural history of HPV infection, vaccines targeting infection cannot be expected to have an impact on the most significant public-health outcome, cervical cancer, for 10–20 years from the time of their implementation. In addition, many countries that would benefit most from vaccination do not have well-established cancer registries to provide baseline data on cervical cancer, with which to compare post-vaccination data. Vaccine trials have used cervical cancer precursors [cervical intraepithelial neoplasia grade 2 or 3 (CIN 2/3) and adenocarcinoma in situ (AIS)] as intermediate end-points to demonstrate vaccine efficacy. These end-points could also be used to monitor the impact of vaccination programmes. However, as detection of CIN2/3 and AIS is dependent on if, and how, the population is screened, use of these as markers of vaccine impact will give biased results in populations that do not have a well-organized and population-based cervical cancer screening programme. Use of HPV typing of these lesions to study if the HPV type-distribution changes, could be useful in settings where changes in CIN2/3 and AIS incidences could have occurred because of changes in screening practices.

The mainstays of vaccine impact monitoring for vaccines preventing acute infections, reportable acute disease and diagnostic serology, are not applicable for HPV vaccine impact ascertainment. The chronic and silent nature of HPV infection precludes "outbreak" case-finding to focus monitoring efforts. A short-term biologic impact of vaccination could be seen in the type-specific prevalence of HPV in the target population. This requires HPV detection and typing in a relatively large number of samples selected in a well-defined manner.

The target population for vaccination is adolescents, preferably prior to the time of sexual debut. This presents challenges, and the need for developing new strategies to achieve high coverage, as most current ongoing vaccine strategies are directed to infant immunizations. It will also require different approaches to determining and reporting vaccine coverage.

The prophylactic HPV vaccines are currently the most expensive vaccines that have received a recommendation for routine administration. Because of the cost of the vaccine, many countries are likely to use some form of short-term outcome monitoring in order to ensure that the programme is optimally effective for reducing the risk for HPV-associated diseases.

2.2 The WHO HPV LabNet

2.2.1 Establishment and mission

WHO recognizes the importance of laboratories in achieving the goals of vaccine implementation, and, as a result, has organized worldwide networks of laboratories to provide standardized quality laboratory testing and adequate testing capacity. Poliomyelitis, influenza, measles and rubella, yellow fever and Japanese Encephalitis laboratory networks have been organized around the specific surveillance needs of the respective vaccine preventable disease (*5,6*).

At a WHO meeting held in Geneva 15–17 August 2005, a group of experts recommended the establishment of a global HPV LabNet, to contribute to improving the quality of laboratory services for effective surveillance and HPV vaccination impact monitoring, and to conduct training (*7*). It was envisaged that the HPV LabNet would speed up the introduction of HPV vaccines by facilitating the implementation of validated, standardized laboratory procedures; by developing quality assurance system and proficiency testing, by training personnel, and by providing a network for surveillance.

The HPV LabNet will facilitate the availability of competent laboratory services worldwide for the evaluation of HPV DNA and antibody detection in biological specimens, through capacity-building and strengthening for those who are in need by providing: 1) up-to-date technical information; 2) technical advice and guidance; 3) training on laboratory practice and quality assurance. In addition, WHO reference laboratories may offer, upon request, confirmatory testing of a subset of samples in the context of monitoring vaccine performance in clinical trials and following vaccine introduction.

The WHO HPV LabNet could form an important part of HPV surveillance before and after HPV vaccine implementation. By having internationally comparable assays based on international standards (ISs) available to evaluate vaccine quality and efficacy, and consistency and impact, initiatives in developing countries to formulate new HPV vaccines at reduced cost would be greatly facilitated; inter-laboratory comparability of epidemiological, clinical, and vaccine-related data gathered in various studies throughout the world would also be assured.

Similar to other WHO laboratory networks, it is expected that the structure of the HPV LabNet will be based on three levels of responsibility, each assumed voluntarily by institutions that are subject to independent expert review and site visits led by WHO; namely: global reference laboratories (GRL), regional reference laboratories (RRL), and national/local laboratories. Each member laboratory would have responsibilities in the areas of: 1) scientific and technical advice; 2) quality assurance; 3) training; 4) communication. Specific tasks will be defined for each reference laboratory based on its expertise and capacity (*7*).

Following an open call in 2006 for applications to participate in a WHO global HPV LabNet as GRLs or RRLs, and external expert review of the proposals, the applicants with proven expertise in HPV testing and epidemiology studies have received site visits. To date, the WHO HPV LabNet is formed of two GRLs and eight RRLs distributed in the six WHO Regions worldwide (See Annex). In response to increasing global needs, additional expert laboratories may be assigned as RRLs if necessary. Moreover, expansion of the HPV LabNet to national level reference laboratories will be considered in due course.

The mission of the WHO HPV LabNet is "to contribute to improving quality of laboratory services for effective surveillance and monitoring of HPV vaccination impact, through enhanced, state-of-the-art laboratory support." International collaborative studies indicated that in order to produce HPV data that can be compared and interpreted worldwide, ISs and standardized procedures for HPV test performance are required (*8,9,10*). Therefore, initial work of the HPV LabNet has been focused in two major areas: 1) development and implementation of ISs for HPV DNA and serology; 2) harmonization and standardization of HPV assays for use in surveillance and vaccine evaluation. More information about WHO HPV LabNet activities and projects are available to the public via its WHO website and newsletters (*11*).

2.2.2 International standards and reference reagents

ISs and reference reagents for HPV DNA and serology are being developed by the WHO HPV LabNet in close collaboration with the WHO International Laboratory for Biological Standards, National Institute for Biological Standards and Control (NIBSC) in the United Kingdom. For more details, see chapter 9.

2.2.3 Harmonization and standardization of HPV assays

There are a variety of HPV tests in use worldwide and several of these may be appropriate to use for HPV surveillance and HPV vaccine impact monitoring. However, it is imperative that results are traceable to IS so that data from different parts of the world can be compared. In addition, the ideal assays should have: 1) good sensitivity and specificity as evaluated in international proficiency testing; 2) ease of transfer to laboratories with varying levels of experience and resources; 3) "affordable" cost, to allow use in low-resource settings.

Individual laboratory performance, as well as assay characteristics, can be evaluated based on results of proficiency testing, both for HPV serology and DNA typing assays. In addition, sample-exchange with GRLs to confirm HPV assay results (namely confirmatory testing) is an important part of a QA programme. Proficiency panels are composed of a series of validated samples, traceable to ISs, sent as unknown challenges to HPV LabNet members, and in some instances of global proficiency testing efforts, open to all laboratories worldwide. Results have allowed an evaluation of individual laboratory performances, as well as an evaluation of whether assay platforms used by multiple laboratories are robust in terms of the generation of acceptable results in many laboratories.

Details of assay formats and platforms that have been evaluated in all HPV LabNet laboratories have been included in this manual. As HPV testing is a very dynamic area, it is envisioned that tests with even better performance and lower cost will be developed. However, it is important that there exists a well-defined internationally standardized procedure for evaluation of how new tests compare to old ones. The process of assay evaluation will be ongoing.

2.3 HPV vaccine surveillance

2.3.1 Surveillance goals

HPV vaccines present some unique programmatic challenges for introduction and impact monitoring. The main impetus for HPV vaccination is cervical cancer control. As secondary prevention through cervical cancer screening is also a part of cervical cancer control, in many settings vaccination will be part of a dual approach to cervical cancer control. Vaccines against HPV infections have the potential to be cost-effective and to reduce incidence of cervical cancer, particularly in low-resource settings. In fact, modelling studies suggest that combining HPV vaccination and organized screening programmes may have the highest impact on disease control globally (*12*). HPV vaccination may be particularly attractive in countries without current screening programmes, but may be difficult to introduce and evaluate in those settings. It must also be recognized that screening and vaccination programmes will impact each other, and changes in cervical cancer may be difficult to attribute to one strategy.

Surveillance needs prior to HPV vaccine introduction will vary depending on a country's knowledge of cervical cancer disease burden (age-specific prevalence and HPV type-distribution) and HPV infection in the population (type- and age-specific prevalence). Approaches will vary depending on the infrastructure for cervical cancer screening and treatment, as well as the availability of population-based cancer registries. As there is a worldwide dominance of HPV16/18 in cervical cancer, vaccines that prevent these infections will address the needs of each country. Absence of surveillance data should not delay vaccine introduction. Initiating surveillance will facilitate education for vaccine acceptance and development of the infrastructure (laboratory and personnel) for vaccine monitoring.

As with all vaccination programmes, coverage and safety will need to be monitored in some way. Coverage and safety will largely be ascertained using methods other than laboratory testing, although population-based serosurveys may be of interest as a complementary method for assessing population coverage. Approaches to measuring HPV vaccine impact include monitoring changes in the prevalence of HPV types, the incidence of cervical abnormalities and precancerous lesions, the incidence of and mortality from cervical cancer and the incidence of genital warts. Targets for biologic monitoring of HPV vaccination impact include a variety of end-points, each of which could be population-based or focused to sentinel sites. Careful definition of target population, sample size, biologic specimen collection and testing methods will be required to obtain reliable data. The summary (*4)* and full report of the WHO November 2009 meeting on HPV Vaccine Coverage and Impact Monitoring (*13*) provide a full discussion of options. The WHO HPV LabNet would be involved in endpoints that include HPV testing, however as noted in the WHO meeting reports, these are not required and may not be adopted in some settings.

- **Type- and age-specific HPV prevalence in populations.** For countries using this endpoint, baseline data prior to vaccination will be needed to assess the impact of vaccination. A decrease in prevalence of types targeted by the vaccine should be one of the earliest end-points detected in vaccinated cohorts.
- **Age-specific incidence of cervical cancer and their HPV type-distribution.** For countries using this endpoint, the baseline burden of disease will inform the need for cervical cancer control. Cervical cancer registries will play a central role. Changes in cervical cancer incidence will require decades to be seen.

2.3.2 In-depth studies of HPV vaccines

There are important questions about HPV vaccines that will need to be addressed by in-depth studies. These studies include determining immune correlates of protection, duration of protection and "type-replacement" (possible increase in HPV types not targeted by vaccine formulations). Data from these research studies will inform policy.

2.3.3 Laboratory measures of HPV and immune response to HPV

Examples of laboratory assays will be described in depth in separate sections in this manual. Briefly, options for measures of HPV include serology and DNA detection.

The major types of HPV serology assays are: i) neutralization assays; ii) enzyme-linked immunoassays (ELISAs) that detect type-specific antibodies against conformational epitopes on VLPs. Titres after HPV natural infection are low, with antibodies being detectable in less than 70% of those known to have been infected. As antibodies typically persist, also after clearance of HPV infection, serology can be used in populations as a measure of exposure to HPV. A more common use of particular relevance to vaccinology is in studies of immunogenicity of vaccines , i.e. antibody responses after vaccination. In experimental systems, neutralizing antibodies are established as providing a measure of a protective response to HPV vaccination. However, a minimum level of anti-HPV antibodies required for protection has not been defined for humans.

HPV DNA tests for epidemiologic monitoring and vaccinology are designed for highest analytic sensitivity and specificity and must provide type-specific data. These include a large number of "in-house" laboratory produced assays. Because HPV DNA levels are low in most samples, the assays usually involve target or signal amplification. Commercial assays are also available, and the choices available to laboratories are increasing.

2.4 References

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3. Laboratory quality assurance

3.1 The basis of laboratory quality assurance

Laboratory quality assurance (LQA) is concerned with the organizational processes and the conditions under which laboratory activities are planned, performed, monitored, recorded and reported. Adherence by laboratories to the principles of LQA ensures the proper planning of activities and the provision of adequate means to carry them out. It promotes full and accurate reporting, and provides the means whereby the integrity of activities can be verified.

Setting up a LQA system in a laboratory means defining the organizational structure, responsibilities, procedures, processes and resources necessary to achieve the following objectives:

- prevent risks;
- detect deviations;
- correct errors;
- improve efficiency;
- ensure data quality and integrity.

It is the responsibility of the head of the laboratory to establish, implement and ensure compliance with LQA. However, LQA is the responsibility of all laboratory personnel. There are a number of elements that make up the LQA process, and these are detailed below.

3.1.1 Staff

The HPV laboratory should have all the necessary staff with suitable qualifications and experience to carry out safely and accurately all the functions and responsibilities required of the HPV laboratory. The laboratory should prepare an organizational chart of the HPV laboratory that reflects the hierarchy and lines of authority, including the functions and responsibilities of each person.

Staff should include:

- head of the laboratory;
- head of each section or unit if appropriate, e.g., serology unit, genotyping unit, etc.;
- scientific, technical and auxiliary staff;
- administrative support, maintenance, cleaning and service staff.

Each post should have a job description including functions and responsibilities, academic training required and experience necessary.

3.1.2 Staffing levels

Staffing levels should be adequate to enable all the functions expected of the HPV laboratory to be carried out without compromising safety or the integrity of the processes performed in the laboratory. There should be at least one person with relevant experience to carry out each one of the functions. It is advisable for at least one other person to work together with the experienced person to gain understanding of the activity and build capacity within the laboratory, and allow for backup in the event of staff absence.

To minimize the risk of contamination, all personnel working in areas dedicated to routine molecular diagnostics must be certified before they are allowed to conduct molecular biological work in these premises. Rooms that are particularly sensitive, such as the "clean room" and the "detection room" are only to be entered by certified individuals or cleaning personnel. The following considerations should also be taken:

- to obtain certification to work in areas dedicated to HPV testing, the person should have been educated about the rules that apply for this type of work at the site;
- selected technicians, with permission from the section leaders to conduct education of staff, are responsible for this education;
- other personnel are certified to access areas dedicated to routine molecular biological testing after they have completed adequate education.

3.1.3 Human resources

The fundamental objective of the human-resources policy is to enable reliable staff with scientific and/or technological training to apply appropriate laboratory procedures correctly, and also to be remunerated according to the labour market.

The laboratory must regularly arrange and coordinate training courses to extend and update the skills of both technical and scientific staff according to the needs identified, and as proposed by the heads of department. This training is offered as a means of contributing to the success of the LQA process. A continuing education programme must be developed which includes onsite as well as external training. Documentation should be kept describing the staff training programme. The human resources programme should include the technical evaluation of staff and follow-up of the performance of each staff member, based on the job description.

This system allows for the correction of errors or weaknesses where merited, and can also be used as a tool for promotion. Since, to avoid contamination, molecular biological diagnostics are regulated by certain rules, it is important that all staff working in the assigned areas are certified to do this kind of work.

3.1.4 Space allocation

The HPV laboratory should have adequate space to safely perform all activities, store all necessary equipment, and allow for easy cleaning and maintenance.

There should be enough rooms to enable separation of different activities. To reduce the risk of contamination, different parts of the analysis are performed in specially assigned locations/working spaces, as follows:

- Clean room for preparation of reagents should be separated as much as possible from all other activities and preferably be in a room(s) completely separated from the laboratory where viral or other microbiological activities are being carried out. This is a "DNA free" room. There should be a clear delineation of different working areas. When possible, separate air handling for the clean room is ideal.
- Extraction room.
- Apparatus room.
- Detection room — post polymerase chain reaction (PCR).

The order of the room listing is from a clean environment to a "dirtier". If possible, some of these rooms should be present in more than one location in the laboratory. If a serious contamination occurs, a room may be temporarily shut down and the current analysis continued in an alternative location. All items used in "dirtier" rooms (lab coats, pipettes, tube racks) cannot be transported into the clean room. (See chapter 5 for additional guidance on prevention of PCR contamination).

If possible, there should be a logical arrangement of activities in a laboratory or laboratories to minimize the distance that amplified materials must be carried, and to ensure that these materials are not being transported through clean areas.

Always clean the rooms in the following order: "clean room"; "extraction room"; "apparatus room" and finally "detection room".

- Do not go from the "detection room" to the "extraction room" or "clean room".
- Do not go from the "extraction room" to the "clean room".

This order should also be followed when window-cleaning or other major cleaning is done, but laboratory staff should always be notified about this beforehand.

If space allows, specific areas and preferably specific rooms should be allocated for:

- reagents and consumables storage;
- instruments and equipment;
- washing, preparation and sterilization (clean and dirty);
- specimen receipt and recording;
- specimen processing;
- DNA amplification and typing;
- documentation, archiving and control;
- administrative area;
- disposal of contaminated and medical wastes.

Following are the general characteristics with which the laboratory areas should comply.

- Lighting and ventilation should correspond to the needs of each working area, according to the specific requirements of the activity carried out. The surfaces of the workbenches should be smooth, easy-to-clean and made of material resistant to chemicals.
- Safety systems should cover fire, electrical emergencies, emergency shower and eyewash facilities.
- Hot and cold water, treated water, vacuum, gas, steam and electricity installations should be arranged so that they guarantee adequate use during the work, and also facilitate maintenance and repair operations. Electrical installations should be arranged so that they do not pose any risk to workers, and electrical wires should not cross walkways. A standby generator is desirable for the support of essential equipment such as incubators, biological safety cabinets, freezers etc., especially if power supply is erratic.
- Storage space must be adequate to hold supplies for immediate use and thus prevent clutter on bench tops and in aisles. Additional long-term storage space conveniently located outside the working areas should also be provided.
- Hand wash-basins, with running water if possible, should be provided in each laboratory room, preferably near the door.
- Facilities for storing outer garments and personal items, and for eating and drinking, should be provided outside the working areas.
- Installation of equipment and organization of the laboratories should take biosafety and other safety standards into account.

3.2 Standard operating procedures

Standard operating procedures (SOPs) describe in detail the activities performed in the laboratory so as to provide uniformity, consistency and reliability in each of the activities performed, reduce systematic errors, and provide training and guidance for new staff.

SOPs should be drawn up by specialized technical staff in the laboratory, revised by their immediate supervisor, and approved by the head of the laboratory. SOPs should be prepared for general procedures, for example:

- general: preparation of SOPs, correction of notes and documentation, preparation of protocols and reports;
- test systems: preparation of work areas and maintenance of work areas;
- laboratory operations: receipt, recording and labelling of samples, washing of recyclable apparatus, sterilization of material, storage of samples, labelling of materials and reagents and preparation of media and solutions;
- staff-related matters: training, handling of hazardous materials, laboratory safety and staffing of each laboratory subunit;
- reference materials: identification, characterization, handling, reception, storage and use;
- archives: maintenance, distribution and updating;
- equipment: regular calibration, cleaning and preventive maintenance;
- test methods: methods for processing and testing samples sent to a laboratory.

Each SOP should have the following information on each page:

- logo and name of the organization;
- department or unit issuing the SOP;
- title;
- signature of person who compiled the SOP with date (day, month and year);
- signature of person who reviewed it with date (day, month and year);
- signature of person responsible for the SOP document;
- signature of person who authorized it with date (day, month and year);
- duration of validity;
- number that identifies the revisions version number;
- code;
- page number and total number of pages in the document.

Changes in an SOP should be implemented by specialized technical staff in the laboratory, revised by their immediate supervisor, and approved by the head of the laboratory. Any method that undergoes changes from the standard and official method should be validated before being put into practice. Assay validation is described in chapter 6.

3.3 Documentation

Documentation is the set of quality manuals, SOPs, instructions, forms, reports, analytical protocols and record of data that serve as evidence of the LQA and permit the traceability of data. Responsibility for the preparation and revision of documents should rest with the LQA, or quality assurance department or person appointed, depending on the complexity of the laboratory.

3.4 Equipment and instruments

The laboratory should have the necessary equipment and instruments for the accurate performance of all tests performed. The standard list of equipment for WHO HPV laboratories is listed in Table 3.1. New instruments and equipment should be installed and calibrated, if possible by the distributor or a suitably qualified person. All manuals and operating instructions should be stored in an area accessible to all users. A regular maintenance and calibration schedule should be established, as part of the quality assurance programme. To ensure correct functioning, all users should be completely familiar with the operating, maintenance and validation procedures. Documentation of all malfunctions, maintenance and validation activities should be recorded in a central register.

Table 3.1: Recommended items of equipment in a WHO HPV laboratory

General	Minimum quantity
Autoclave, large (or bench top for small lab)	1
Balance, with power adapter	1
Cabinet, class II safety and replacement filter	2
Centrifuge, low speed, refrigerated	1
Computer with Microsoft Office® and database software like Filemaker® or Access® [or equivalent]	1
Crushed ice machine	1
Pipettes, adjustable 100–1000 µL	2
Pipettes, adjustable 20–200 µL	2
Pipettes, adjustable 2–20 µL	2
Pipettes, adjustable 0.5–10 µL	2
Fax machine	1
Freezer, -20°C, non-frost free	2
Freezer, -80°C	1
Heating block for 1.5 mL and 0.5 mL tubes	1
Incubator, standard	1
Meter, pH, hand held with spare electrodes	1
Mixer, vortex	2
Oven, hot air sterilizing	1
Refrigerator, household, 4°C	2
Stirrer, magnetic with bars	1
Test-tube rack for 16 mm tubes	10
Thermometers, 0°C –100°C	6
Water distiller, double or triple, glass	1
Water deionizer (cartridge)	1
Water-bath	1

	Minimum quantity
For HPV DNA detection	
DNA extraction equipment	1
DNA thermal cycler with ramp function (Applied Biosystem 9600 emulation)	1
Thermomixer (heated shaker)	1
Heater water-bath shaker	1
Angle/speed adjustable shaker	1
Vacuum pump	1
Gel electrophoresis equipment allowing 12-well multichannel pipette loading and power supply	1
UV transilluminator	1
Microwave oven	1
Film developers	1
Digital camera	1
Graduated cylinders	1
Miniblotter (45 lanes, Hoefer or Immunetics)	1
HPV serology (ELISA)	
8 or 12 channel-pipette 50–200 µL or equivalent system	1
Pipet-aid	1
Laboratory micro balance	1
Microplate reader with a 405 or 415 nm filter	1
Microplate washer (optional)	1
HPV serology (neutralization assay)	
Multi-channel pipette 50–300 µL	1
Repeat pipettor (50–200 µL)	1
Laminar-Air-Flow bench	1
Incubator 37°C with carbon dioxide	1
Luminescence reader for 96-well plates	1

The laboratory should have a list of equipment and instruments that include:

- the name;
- brand;
- donor or supplier;
- maintenance company;
- maintenance schedule;
- inventory number;
- serial number;
- model and year;
- location;
- date-of-purchase;
- date of first use;
- copy of manufacturer's handbook.

3.5 Supplies

3.5.1 Reference materials

These comprise material used to calibrate the test procedures and to guarantee uniformity in determining activity, such as proficiency panels for sensitivity testing or validation of typing. A central registry or logbook should be kept containing the following:

- name of the reference material;
- supplier;
- origin;
- lot number;
- date of analysis to determine whether it complies with the stipulated requirements;
- place and conditions of storage;
- expiry date, where applicable;
- storage in an appropriate form (corresponding SOP).

This registry should contain all the information relating to the properties of the reference material. The quality of the reference material should be verified: (a) when the conditions have been altered; (b) routinely, once a year.

3.5.2 Reagents

Reagents can be defined as materials of chemical or biological origin used in laboratory assays. At least a six months' reserve stock of reagents should be held in the laboratory at all times. Given the long delivery times and difficulty of transport to some regions, reagents should be ordered 6 to 12 months ahead of need. Cell-culture media is considered as a reagent.

A registry or logbook should be kept containing the following:

- name of the reference material;
- supplier;
- origin;
- lot number;
- date of analysis to determine whether it complies with the stipulated requirements;
- place and conditions of storage;
- expiry date, where applicable;
- storage in an appropriate form (corresponding SOP).

This registry should contain all the information relating to the properties of the reagents. The quality of the reagents should be verified: (a) when the conditions have been altered; (b) routinely, once a year.

Characteristics of reagents are:

- they should be of appropriate quality;
- they should be obtained from recommended suppliers in their original packaging;
- a record should be kept of purchase, reception and distribution to guarantee continuity, particularly with substances that need to be acquired in advance;
- the reagents should be inspected to ensure that the seals are intact when received in the stockroom or when distributed to the laboratory. These inspections should be recorded with the initials of the person responsible for the inspection, and the date written on the label.
- There should be a specific SOP for the transport, storage and handling of reagents and disposal. Any changes to the composition of reagents or media, or to the lot numbers of biological products (antisera, conjugates etc.) should be fully documented in the registry or logbook.

Water should be considered a reagent and should comply with purity specifications or other technical requirements for use in the laboratory. Reagents prepared in the laboratory should be prepared in conformity with written procedures and, where applicable, according to WHO standard recommendations, validated and labelled appropriately and stating the following:

- identification of the reagent;
- concentration;
- preparation and expiry date;
- storage conditions;
- initials of the technician responsible.

3.6 Laboratory safety

Each laboratory should have available the WHO Laboratory biosafety manual, third edition, 2004 (*1*). This manual describes the essential biosafety, chemical, fire and electrical safety requirements to protect staff, the community and the environment. All staff should be familiar with the contents of this manual and should be required to follow the guidelines. The director is responsible for implementation of, and compliance, with the provisions of the manual.

Safe handling and, ultimately, maximum containment of potential infectious materials in the laboratory is crucial.

- Good microbiological techniques are practised (Table 3.2).

Table 3.2: Good microbiological techniques

Specimens are handled safely
No mouth pipetting is permitted
Pipettes and pipetting aids are used safely
Dispersal of infectious materials is avoided
Contact of infectious materials with skin and eyes is avoided
Ingestion of infectious materials is avoided
Separation of serum is carried out safely
Homogenizers, shakers and sonicators are used safely
Tissue grinders are used safely
Refrigerators are maintained and used safely
Ampoules containing infectious materials are opened safely
Infectious materials are stored safely
Precautions are taken with blood and other bodily fluids
Specimens and infectious materials are shipped safely
Appropriate disinfection and sterilization are carried out
Gloves are worn for procedures that may involve direct contact with blood or infectious material
Hands are washed between procedures and prior to leaving laboratory
Laboratory gowns are worn for work in laboratory
Storage of food or drink in the laboratory or any storage receptacle containing infectious materials is prohibited
Eating, drinking, smoking or applying cosmetics in the laboratory is prohibited

- Facility meets standards for basic biosafety level 2 (BSL-2) laboratory (Table 3.3).
- Access to laboratory is restricted.
- Freezer inventories are current and complete, including nature of material, volume or amount and location in freezer.
- Documentation is current on all stored materials, including geographical source and date of collection.
- All infectious materials are transferred to and from freezers in leak-proof unbreakable containers.
- SOPs are established, and regular training provided on responses to all spills and accidents where infectious material may have been released.
- Laboratory coat (dedicated to a location) and disposable gloves should be worn when doing laboratory work.
- Disposable plastic ware should be used throughout.
- Tips should contain aerosol filters.
- Tubes and tips should be kept in proximity to the working areas.
- Automatic pipettes shall be labelled with the workstation and it is not allowed to move pipettes between workstations.

Table 3.3: Standards for basic BSL-2 laboratory

Ample space is provided for the safe conduct of laboratory work
Walls, ceilings and floors are easily cleanable
Illumination is adequate for all activities
Storage space is adequate to hold supplies for immediate use
Hand wash-basins, with running water if possible, are provided in each laboratory room, preferably near the door
Facilities for storing outer garments and personal items for eating and drinking are provided outside the working areas
A good quality and dependable water supply is available. There are no cross-connections between laboratory and drinking-water supplies
A standby generator is desirable for the support of essential equipment such as incubators, biological safety cabinets, freezers and other essential equipment
Pipetting aids are available to replace mouth pipetting
Biological safety cabinets are available for: - procedures with high potential for producing aerosols, including centrifugation, grinding, blending, mixing, sonic disruption, and opening of infectious materials whose internal pressure may be different from ambient pressure; - handling high concentrations or large volumes of infectious materials
Centrifuges with sealed safety caps are available for centrifuging high concentrations or large volumes of infectious materials in the open laboratory. These caps must be loaded and unloaded in a biological safety cabinet
Screw-capped tubes and bottles are available to hold positive specimens and cultures
An autoclave is available in the same building as the laboratory to sterilize contaminated material

3.7 Audits

The objective of an audit is to carry out a systematic and independent examination to determine whether the quality activities and their results comply with the established documentation, and to confirm whether these activities are appropriate for achieving the desired objectives and whether they have been implemented effectively. Audits may be internal, performed by staff who do not have direct responsibility for the areas audited, or by the LQA department. External audits may be performed in conjunction with the WHO accreditation of HPV laboratories or by national/international authorized bodies.

Audits may apply to:

- the whole quality system;
- some elements of the system (procedures, staff, equipment, working areas);
- processes;
- products;
- services.

Audits should not be confused with quality-control activities. Laboratories should have a regular internal audit programme. Steps in an audit are:

- review of documents;
- drawing up of an audit plan;
- opening meeting between auditor and the area to be audited;
- rapid walk-through of the installation;
- performance of the audit — interviews, checklist, and observation;
- closing meeting;
- audit report.

3.7.1 Audit report

The results of the audit are compiled into a report that indicates the date the audit was performed and contains a description of the observations, deviations or instances of nonconformity, and the recommendations or corrective measures suggested. This report is sent to the director of the area audited, and to the executive director who shall be responsible for ensuring compliance with the resulting recommendations.

3.7.2 Follow-up audit

If nonconformity is encountered, follow-up audits are performed to verify the implementation of corrective actions.

3.8 References

1. *WHO Laboratory biosafety manual. Third edition*. Geneva, World Health Organization, 2004 (http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf, accessed 10 December 2009).

4. Collection and handling of specimens for HPV testing

4.1 Introduction

A consistent method of specimen collection and handling is needed to provide comparable results over time and between studies. This is particularly true for HPV DNA detection and typing assays that require a cellular sample. Only current infection in the anatomic site sampled will be detected. Different methods of sample collection and handling, even from the same anatomic site, can influence the final result, as can method of extraction and volume of extract used in each assay. A variety of sample collection and handling methods will provide satisfactory results. Methods of sampling vary in their cost and ease of implementation. Laboratories should choose one or more methods and apply them consistently. While not discussed in detail in this manual, laboratories need to establish a specimen labelling system so that individual specimens can be tracked through receipt, processing, testing and storage. Identifiers should be unique identification (ID) numbers. Labels for storage are ideally printed on freezer-resistant labels. Incorporating bar code identification of specimens and purchase of bar code readers is an investment that will facilitate speed and accuracy of specimen handling.

4.2 Serum

4.2.1 Principles

Standard methods for collection of peripheral blood and serum are appropriate for HPV serology. Venipuncture should only be performed by qualified personnel trained in the technique.

4.2.2 Equipment and supplies

4.2.2.1 Equipment

- Centrifuge with horizontal rotor and buckets with aerosol barriers to accommodate vacutainer tubes
- Refrigerator, household (4°C, range 2°C –8°C)
- Freezer, frost free (-20°C)

4.2.2.2 Supplies

- Tourniquet
- Sterilizing swabs/wipes
- Band aid and sterile gauze
- Specimen labels
- Screw-cap cryovials for specimen storage (glass cannot be used for freezing serum)
- 5 ml vacutainer tube (no anticoagulant) with 23-gauge needle
- Sterile transfer pipettes
- Gloves, lab gown, protective eyewear
- Biohazard discard bag/box appropriate for sharps
- Racks for vacutainer tubes and serum storage vials

4.2.3 Procedure

- Assemble all materials required to collect blood. Label vacutainer tube with subject identification (ID), date and other information required by study/ surveillance protocol.
- Explain procedure to subject and verify their identity. Seat subject in position that allows comfortable access to antecubital fossa.
- Sterilize antecubital fossa with alcohol wipe. Place and tighten tourniquet. Insert needle in vein, apply vacutainer and release tourniquet as blood enters tube. When full volume is collected, remove vacutainer and needle. Apply pressure over puncture site with gauze and place band aid.
- Discard needle into sharps biohazard bag. Discard used wipes and gauze into biohazard container.
- Place vacutainer tube in rack and allow clot to form for 30 minutes.
- Centrifuge at 1000 x g for 10 minutes to separate the serum.
- Carefully remove the serum with transfer pipette, avoiding extracting red cells, and transfer aseptically to one or more sterile labelled serum storage vials (screw-capped).
- Store serum at 4°C–8°C for a maximum period of seven days, and ship to the laboratory on wet ice. For longer storage, sera must be frozen at -20°°C and transported to the testing laboratory on frozen ice packs. **Repeated freezing and thawing can have detrimental effects on the stability of antibodies, therefore at least two storage aliquots are recommended, and aliquot size should be small enough to minimize number of freeze-thaws.**

Note: DO NOT FREEZE WHOLE BLOOD. Blood can be stored at 2°C-8°C for up to 24 hours before the serum is separated. If no centrifuge is available, blood should be kept refrigerated until there is complete retraction of the clot from the serum, and serum carefully removed as described following centrifugation.

4.2.4 Safety

Blood products are potentially infectious and should be handled under BSL-2 conditions. Gloves, gowns and protective eyewear should be worn during collection and processing (*1*).

4.3 Cervical exfoliated cell samples

4.3.1 Principles

The goal is to provide a cellular sample with preserved DNA from the site of infection. Collection devices and methods used for obtaining samples for routine cervical cytology generally yield an appropriate sample. The cervical transformation zone is the site of origin of most cervical neoplastic lesions, and, as in sampling for cervical cytology, should be targeted for sample collection. This requires a health-care professional trained in performing pelvic examination. Self-collection methods can be used, but the resulting sample includes a larger vaginal contribution. For consistent longitudinal results and across studies, the method of sampling needs to be consistent.

Sample extraction/lysis prior to DNA testing must be matched to the method and collection media used. Commercial/clinical kits for HPV testing may specify collection method and processing. In this situation the manufacturer's recommendation must be followed, unless an alternative method has been verified to provide satisfactory results.

4.3.2 Equipment and supplies

4.3.2.1 Equipment

- Refrigerator
- Pelvic examination table

4.3.2.2 Supplies

- Vaginal speculum (clean and autoclaved or new disposable)
- Gloves
- Cervical brush/broom (see note)
- Dacron swabs
- Collection vials with specimen collection media (see note)
- Specimen labels
- Biohazard discard bag/box

Note: Volume of collection media should be kept constant for each study. Commercial aqueous buffered collection media, such as Digene® specimen transport media (STM) (Qiagen), are convenient and reliable. These generally come in 1.0 mL aliquots in capped tubes that accommodate collection devices. Normal saline may be used (9.0 gm NaCl/L), but must be accurately aliquotted into collection tubes.

Liquid-based cytology media is purchased from the manufacturer, and collection device must follow manufacturer's specification. This media was designed for cytologic preservation, but the methanol-based [PreservCyt® (Hologic Inc., Marlborough, MA)] or ethanol-based [SurePath® (Becton Dickinson)] liquid also preserves DNA for molecular tests. PreservCyt® vials contain 20 mL and SurePath® vials contain 5 mL media.

4.3.3 Procedure

- Assemble all materials required to collect specimen. Label collection vial with subject ID, date and other information required by study/surveillance protocol.
- Explain procedure to subject and verify their identity. Position subject for pelvic examination and place speculum to allow visualization of cervix.
- Remove excess cervical mucous with swab; discard swab into biohazard waste container. Insert cervical brush/cytobroom into endocervix so that outer bristles lie on ectocervix, and rotate device counter-clockwise full circle three times. **Manufacturers of most collection devices provide directions and illustrations demonstrating a recommended technique for specimen collection. This may be substituted for the general guide presented. It is important to use a consistent method.**
- Place collection device in vial with specimen collection media. Collection device is either left in media, or cells are dislodged and device is discarded. This depends on the collection media.
  - For aqueous media like STM: snap or cut the handle of the collection device so that the brush remains in the media and the lid on the vial may be snapped or tightly closed. The bristles should be covered with liquid. The collection device provided by the manufacturer of STM is scored to allow handle to snap easily.
  - For PreservCyt® media: vigorously press the cytobroom against the bottom of the vial at least five times, splaying the bristles and twisting to release all cellular material. Remove cytobroom and discard into biohazard waste. Tighten screw-cap on collection vial, verifying closure lines are in position indicating tight seal. Collection devices used with PreservCyt® media must be made of special material designed to release cells; manufacturer's guidelines must be followed as to appropriate collection device and method of cell release.
  - For SurePath® media: detach the bristle/broom end of the collection device and leave it in the collection vial. Discard the handle into biohazard waste. Tighten the lid on the vial. Collection devices provided by the manufacturer of SurePath® have detachable brooms. Manufacturer's guidelines must be followed as to appropriate collection devices.

- Store samples under conditions appropriate for the collection media, and ship under conditions maintaining storage temperature. **Methanol and ethanol-based media are considered flammable and require special shipping methods.**
  - STM: can be kept at room temperature up to 14 days. Stable at 4°C for three weeks. For longer storage, freeze at -20°C.
  - PreservCyt®: Can be kept at room temperature up to 14 days. Stable at 4°C for at least three months. Freezing is not recommended, although cells may be pelleted and stored at -20°C.
  - SurePath®: Can be kept at room temperature up to 14 days. Stable at 4°C for at least three months. Freezing is not recommended.

Note: If a sample is required for cytologic diagnosis, several options are possible depending on the method of sample collection and Pap test preparation.

For STM media, a separate collection is made for the Pap test, with Pap test sample preceding STM sample.

For PreservCyt® or SurePath® media, the collection device can be smeared on a glass slide to prepare a conventional Pap test, and then placed in the collection vial. Alternatively, the PreservCyt® or SurePath® sample can be used to prepare a liquid-based Pap test as recommended by the manufacturer, and the residual used for HPV testing. The potential for sample contamination is increased, but with care this method is effective. An aliquot for HPV testing may be removed prior to making the slide, but this risks adequate yield for cytology.

4.3.4 Safety

Cervical samples are potentially infectious and should be handled under BSL-2 conditions. The potential for infection is reduced after placement in methanol or ethanol-based collection media. Methanol and ethanol are flammable and require special handling and storage.

4.4 Other samples for HPV testing

4.4.1 General principles

Cellular and tissue samples from sites of suspected HPV infection or HPV-associated diseases may be used for HPV testing. Brief overviews of some options are included, but detailed protocols are not provided. Laboratories must verify the suitability of each sample collection method prior to undertaking studies.

4.4.2 Other cervical samples

Cervical biopsies taken from cervical lesions can be used for HPV DNA detection and typing. This requires coordination with health-care providers to assure that sampling for DNA does not interfere with samples required for optimal histologic diagnosis. The biopsy obtained for HPV testing should be snap frozen in Isopentane bath or placed in normal saline and frozen as soon as possible.

Residual material from diagnostic formalin-fixed paraffin embedded blocks may be used for HPV testing. This has the advantage of not interfering with clinical management and allowing histologic confirmation of the sample being tested. To avoid contamination between blocks, each sample must be cut with a new disposable microtome blade and a negative control section made from an empty paraffin block before the actual sample is sectioned. Obtaining sections for histology immediately before and after unstained sections used for HPV testing, allows histologic verification of the material being tested. However, it should be noted that the cross-linking that occurs with formalin-fixation limits the size of the DNA fragments that can be extracted. "Routine" processing for histopathology is quite variable, and these changes can impact the quality and quantity of the DNA. Some histology laboratories use fixatives other than formalin, many of which, such as Bouin's fixative, make DNA assays impossible.

4.4.3 Self-collected cervical-vaginal specimens

In settings where pelvic examinations are not feasible due to lack of facilities or cultural barriers, participants can be trained to collect a sample from the vaginal pool by inserting a swab into the vagina in a manner similar to inserting a tampon. The self-collected swabs are then handled similar to a clinician collected sample. As the vaginal pool includes cervical cells, HPV in the self-collected sample correlates, but is not a perfect match, to cervical samples. Low-risk (LR) HPV types known to be more prevalent in the lower genital tract and vagina are over-represented (2).

4.4.4 Other anogenital samples

HPV testing of external genitalia, perineum and anus may be of interest, particularly in establishing HPV epidemiology and understanding the natural history of HPV carriage in males as well as females. Sampling approaches vary, but on keratinized surfaces, mild abrasion followed by firm rubbing with a moistened swab is used to increase cellular yield. Sampling the anus requires that a swab or soft brush be inserted to the anal verge, where glandular and squamous epithelium meet. Collection media and sample handling are similar to cervical samples.

Urine has also been used for HPV testing. For females, voided urine, without "clean-catch" precautions, includes cells from the vaginal pool. Urine is less sensitive for HPV DNA detection than are cervical samples, and appropriate controls to check for PCR inhibitors should always be used.

4.4.5 Dry swab or paper smear methods

There is cost associated with purchasing or preparing collection kits and vials. In addition, cold storage and shipment of vials further adds to cost and infrastructure required for sample handling. A filter-paper method for collection and transport of cervical samples has been described (*3*). In this approach the cervical sample is smeared onto filter paper and then dried. The paper can be stored and shipped at ambient temperature. Similarly, dry swabs have been used for self-collected vaginal samples and could be used for clinician collected samples. Extracting DNA from the dry samples requires a step to dissolve or elute DNA from the solid support. Adopting these protocols requires that the laboratory validate the efficiency and reliability of sampling.

4.5 Shipping specimens

Specimens must be shipped under conditions that protect sample integrity for testing and, equally important, protect the safety of all who come in contact with the shipment. There are correct procedures to be followed depending on the material to be transported and the method of transport (air or ground). Shipping biologic specimens requires planning and coordination with the shipping company and the receiving laboratory to assure that specimens arrive and can be handled in a timely manner. Personnel involved in shipping must be trained in the appropriate methods for packing and labelling shipments. Unless specimens have been treated to inactivate all infectious agents, they must be handled as infectious.

Laboratories should follow the most current WHO guidance on regulations for transport of infectious substances. The 2009–2010 document is available on the internet (*4*).

This document includes detailed definitions and instructions for all steps required for safe transport of biologic samples. Most of the samples expected to be received by, or sent from, an HPV laboratory would be considered patient specimens. HPV is a human pathogen, but falls under category B (see text box). Less stringent rules for shipping apply, but triple packaging in appropriate containers is still required (details in WHO guidance) (*4*).

Patient specimens are human materials, collected directly from humans, including, but not limited to, excreta, secretions, blood and its components, tissue, and tissue-fluid swabs being transported for purposes such as research, diagnosis, investigational activities, disease treatment and prevention. These are considered "exempt human specimens" and have less stringent rules for transport. Outer packaging must be labelled "exempt human specimens".

Biological or infectious substances are substances which are known or are reasonably expected to contain pathogens. Pathogens are defined as microorganisms (including viruses) and other agents which can cause disease in humans or animals.

Infectious substances are divided into two categories. HPV is included in category B with less stringent rules for transport.

Materials should be labelled UN 3373.

From: WHO guidance on regulations for transport of infectious substances 2009–2010 (2).

A shipping log detailing the specimen IDs included in the shipment should be included in the package. In addition, a copy of the shipping log should be retained by the laboratory or group originating the shipment. The receiving laboratory should be notified by FAX or email at the time the shipment is made. This notification should include the name and contact information for one or more personnel responsible for initiating the shipment, the transport company and contact information, the shipment tracking number, the expected date of arrival and a copy of the shipping log.

4.6 Receiving specimens

Specimen shipments must be unpacked as soon as possible after they are received by the laboratory. If unpacking must be delayed for some reason, shipments should be stored in a refrigerated area until they can be handled. Shipments should be unpacked in a designated area located away from other laboratory activities. As there is always the possibility of breakage and leakage of specimens, the workbench in the unpacking area must be clean and have a surface covering that is easily disinfected using common laboratory disinfectants (70% ethanol, sodium hypochlorite solution, etc.). It should be equipped with a discard container, alcohol swabs and paper towels. The external surface of the package should be inspected for any physical damage due to water or crushing. As the package is opened, the condition of any packing coolants (cold packs, wet or dry ice) should be noted. Unpacking is ideally handled by two people; one gloved to open the package and check for leakage, breakage or contamination of documents, and to handle the specimens, and the other to record the specimens and note those that were damaged. Serum, samples for DNA extraction, or DNA extracts should be frozen -20°C unless testing is to be performed within a day or two, in which case samples may be refrigerated (4°C).

4.6.1 Recording receipt of specimens

Each specimen must be matched against the shipping log to be sure that all specimens were received and that no additional specimens were included. As soon as the shipment inventory has been completed, personnel responsible for initiating the shipment should be notified that it has been received and if there were any problems noted with it (insufficient coolant, discrepancies between the samples in the shipment and the shipping log, damaged specimens).

The receiving laboratory should create a database for received specimens. This should include:

- study or reason for submission of sample (project or study identification);
- subject identification (in a standard format, usually numeric, often including age and sex);
- specimen identification number (used for all aliquots and recording results);
- date of receipt;
- date of specimen collection;
- specimen type (biopsy, serum, brush etc.);
- volume;
- diagnosis, with date of diagnosis (if available and appropriate);
- residence of subject (as appropriate; town, region, country);
- storage location (unless testing is performed immediately);
- comment (note any problems with sample).

4.7 References

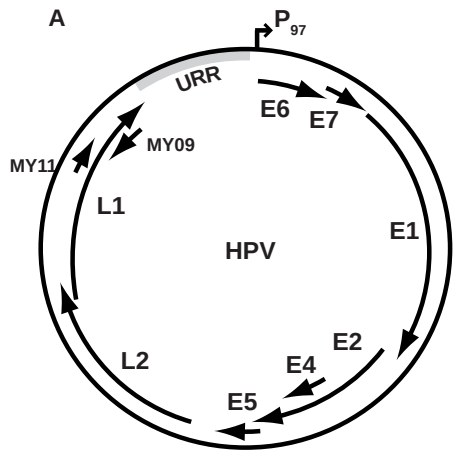
1. *WHO laboratory biosafety manual. Third edition*. Geneva, World Health Organization, 2004 (http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf, accessed 10 December 2009).
2. Gravitt PE et al. Evaluation of self-collected cervicovaginal cell samples for human papillomavirus testing by polymerase chain reaction. *Cancer Epidemiology, Biomarkers & Prevention : a publication of the American Association for Cancer Research, co-sponsored by the American Society of Preventive Oncology*, 2001, 10:95–100.
3. Kailash U et al. A simple 'paper smear' method for dry collection, transport and storage of cervical cytological specimens for rapid screening of HPV infection by PCR. *Journal of Medical Microbiology*, 2002, 51:606–610.
4. WHO Guidance on regulations for the transport of infectious substances 2009–2010. Geneva, World Health Organization, applicable as from 1 January 2009 (http://www.who.int/csr/resources/publications/biosafety/WHO_HSE_EPR_2008_10/en/index.html, accessed 10 December 2009).

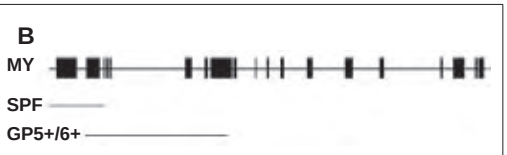
5. DNA extraction and HPV DNA testing

5.1 Principles

HPV cannot be easily propagated by standard in vitro culture systems, and in malignant tissue there are little or no infectious HPV particles. For these reasons, methods to detect and type HPV are based on the detection of HPV nucleic acids, in most assay formats, HPV DNA. All HPV types have a double stranded, circular DNA genome of about 8 kbp (Figure 5.1). Integration of the viral DNA in the host cell chromosomes may occur during tumour development, with concomitant deletions or rearrangements involving the E1/E2 region of the viral genome. Hence, molecular methods aimed at detecting HPV should target the L1, upstream regulatory region (URR) or the E6/E7 genes, which are always retained in infected and neoplastic cells.

Figure 5.1: A. Genetic organization of HPV
B. L1 region targeted by most frequently-used consensus PCR assays





A. Genetic organization of HPV

HPV16 is illustrated, but all HPV genomes have similar organization. The L1 region, upstream regulatory region (URR) and the E6/E7 regions are seldom lost in malignancies, so these regions are most often targeted in assays. The L1 region is most conserved among all HPV types.

B. L1 region targeted by most frequently-used consensus PCR assays

The region of L1 delimited by the MY09 and MY11 primers is illustrated. The vertical bars define 10 base sliding domains sharing 80% homology among the 32 most prevalent types of mucosal HPVs. Targets of the SPF and GP5+/6+ primer sets are illustrated below allowing a comparison of the relative size of the fragments.

5.2 Sample extraction/lysis

HPV is a cell-associated virus, and a cellular sample from the site of infection is required. Methods of specimen collection and processing are described in chapter 4. Lysis with or without extraction is required to release the viral DNA from these samples. Cellular DNA is also released at the same time and can serve as a control for sample adequacy.

A variety of commercial DNA extraction reagents are available. The manufacturer's recommendations for matching specific reagents/kits to specimen type should be adopted. In each case, the manufacturer's recommended method should be used as a starting protocol, but each laboratory must evaluate the success of the protocol on their samples. Adjusting the lysis and digestion steps may be tried to increase yields, but once conditions are selected they should be kept constant. Use of "in-house" extraction reagents is discouraged due to the risk of contamination during reagent preparation, and difficulty maintaining consistent results. DNA extracts are stable for short periods of time at 4°C, but should be stored at -20°C (non frost-free freezer) for long periods of time. Multiple freeze-thaw cycles should be avoided.

Care must be taken to prevent sample-to-sample contamination during extraction and processing of samples. At least one water blank must be extracted with each batch of samples. For high-throughput extractions, these extraction controls should be interspersed, at least one per 20 samples extracted. The extracted water serves as a negative extraction control in HPV testing. It should be stored with sample extracts prior to testing.

DNA should be stored preferably in Sarstedt screw-cap tubes or equivalent (250 µL internal volume). The disadvantage of screw-cap tubes is that cross-contamination of samples can occur with cap exchange. Using extra disposable caps solves this issue for a limited cost. **Do not use snap-cap tubes that may lead to contamination on opening.**

5.3 Overview of molecular methods for detection of HPV DNA

5.3.1 Introduction

Molecular methods for HPV detection can be grouped in two main categories: those that rely on signal amplification to detect the targets, and those that rely on target amplification itself. Most target amplification methods rely on polymerase chain reaction (PCR). Assays for detection of HPV may not always provide type-specific information. Results of HPV testing are greatly impacted by the assay, and comparison between assays is not always possible. This is why all steps of HPV detection and typing used by a global network need careful standardization (e.g. sample collection, extraction and testing).

5.3.2 Signal amplification

The most widely-used signal amplification method for HPV is the Digene® Hybrid Capture® 2 (HC2) HPV Test (Qiagen). The assay uses a simple alkaline lysis to release sample DNA for hybridization to the probe cocktail of RNA probes complementary to 13 HR HPV types [16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68]. Ribonucleic acid (RNA)-DNA hybrids indicate the presence of HPV DNA, as these are never found naturally. A monoclonal antibody specific for RNA-DNA hybrids bound to microtitre plates is used to capture these hybrids. After washing to remove non-specifically bound material, the DNA-RNA hybrids are detected with the same monoclonal antibody conjugated to alkaline phosphatase. Localized enzyme is detected and quantitated with the use of a chemiluminescence detection system. This semi-quantitative assay is designed for clinical use as an indicator of the risk of cervical neoplasia. It is very reproducible with a very good inter-laboratory comparison and very good negative predictive values for high grade lesions. A low-cost version has been designed to be performed in low-resource settings requiring minimal equipment and training (*1*). The assay does not distinguish specific types, and, for this reason, is not envisaged to play a role in epidemiological studies required for HPV type-specific vaccine assessment, and so will not be discussed further in this manual.

5.3.3 Target amplification

Target amplification systems most commonly use a PCR. While type-specific PCR assays have been developed to detect, and in some cases to quantitate, the presence of a single HPV type, epidemiologic studies generally need to address the presence of many of the most important types found in the genital tract. This is achieved by amplifying a portion of the HPV genome that is relatively conserved. When the L1 region is targeted (Figure 5.1B), the assays are referred to as L1 consensus PCR. The first consensus PCR assay was described by Manos and colleagues (*2*). This assay used a set of degenerate primers, MY09/MY11, and resulted in a 450 bp amplicon amenable to typing determination by subsequent molecular techniques like restriction fragment length polymorphism, DNA sequencing and reverse blotting hybridization (RBH) with type-specific oligonucleotide probes. The degenerate MY09/MY11 primers were subsequently replaced by a set of 18 defined primers, PGMY09/11, to improve specificity and sensitivity (*3*). Additional primer sets targeting the same region of L1 are widely used. These include GP5+/6+ producing an amplicon of ~160 bp (*4*), and SPF10, producing an amplicon of 65 bp (*5*). The PGMY09/11 system is commercially available as the Research Use Only (RUO) Linear Array HPV Genotyping Test (Roche), the SPF system is commercially available as INNO-LiPA HPV Genotyping v2 (Innogenetics), and GP5+/6+ systems are commercially available as RUO HPV Genotyping LQ Test (Qiagen) and Multiplex HPV Genotyping Kit (Multimetrix, Heidelberg). The GP5+/6+ and SPF primers may be preferred for paraffin-embedded material to improve sensitivity of the assay with degraded nucleic acids, owing to the small size of the amplicons.

Detection of the amplicon can be done by gel electrophoresis and ethidium bromide or GelRed staining under ultraviolet (UV) light for the PGMY and GP5+/6+ reactions. (Gel detection of PGMY products is illustrated later in the chapter as part of the protocol, see Figure 5.4). DNA hybridization in microplate enzyme-linked immunosorbent assay (ELISA)-type format is preferred for the SPF system owing to the very small size of the amplicon. Detection of the amplicon confirms the presence of HPV, with type- specific hybridization required to determine its type(s). Typing of the consensus PCR products is most commonly accomplished using reverse blotting hybridization (RBH), in which amplicon with affinity label is hybridized to an array of unlabelled type-specific probes, and detected with colorimetric or chemiluminescent methods.

The number and variety of HPV detection and typing assays, using target amplification, being developed and introduced are increasing. Laboratories must validate the sensitivity and specificity of the assay they use, whether they rely on in-house or on commercial assays, to ensure that the assay as performed in their hands allows the laboratory to be proficient in HPV testing. The approach to assay validation is covered in chapter 6. Participation in HPV proficiency testing allows laboratories to verify that they have successfully implemented HPV detection and typing assays.

5.3.3.1 WHO HPV LabNet proficiency studies of HPV DNA typing methods

The WHO HPV LabNet has performed a series of proficiency testing studies since 2007. In 2008, following an announcement on the WHO website, the WHO HPV LabNet proficiency study for evaluating HPV DNA typing methods was launched and made available to the public. The HPV DNA detection and typing proficiency panel was made available upon request to laboratories worldwide (*6*). The study involved many laboratories in the world that are regularly active in research or HPV surveillance, as well as many companies involved in manufacturing HPV tests and HPV vaccines. In total, 81 datasets with HPV typing data were returned to WHO for evaluation. The different assays used are detailed in Table 5.1.

Table 5.1 **Assays used in 2008 WHO HPV LabNet proficiency panel test** Commercial assays have the name of the company written within parenthesis following the name of the assay. “In-house” means that the assay was developed by the laboratory itself, and that no other laboratory used the exact same method. PGMY-RBH is a non-commercial assay used by seven HPV LabNet laboratories with the same SOP following a technical transfer of the assay in early 2008.

HPV assay type	Number of data sets	HPV region targeted (primers)
All assays	81	L1/E1/E6/E7
Linear Array (Roche)	15	L1 (PGMY)
PGMY-RBH	7	L1 (PGMY)
In-house type-specific PCR	7	L1 / E6 / E7
In-house 16 /18 specific PCR	6	E6 / E7
InnoLiPA (Innogenetics)	6	L1 (SPF10)
CLART (Genomica)	6	L1 (PGMY)
DNA chip (Biocore)	4	L1
In-house Lineblot	4	L1 (GP)
In-house PCR Luminex	4	L1 (GP or modified GP)
In-house PCR Luminex	4	E6 / E7
In-house Microarray	3	L1 / E7
PCR-RFLP	3	L1
Microarray (Genetel)	2	L1
DEIA LiPA assays	2	L1 (SPF10)
In-house PCR EIA	2	L1
Microarray (Papillocheck)	1	E1
Type-specific PCR (GenoID)	1	L1
In-house PCR Luminex	1	L1 (PGMY-GP)
PCR Luminex (Multimetrix)	1	L1 (GP)
PCR EIA (GenoID)	1	L1
In-house PCR sequencing	1	L1 (PGMY-GP)

Briefly, in this study, the Roche Linear Array was the most widely-used assay, with results reported by fifteen laboratories. There were a large number of different "in-house" tests used. The "in-house" tests that are based on similar principles are grouped as a single category in Table 5.1 although the exact format used by each laboratory may differ. The non-commercial PGMY-RBH assay was designed and validated by the WHO HPV LabNet regional reference laboratory (RRL) in Switzerland (*manuscript submitted*), and transferred to HPV LabNet laboratories. To evaluate the technical transfer of this assay, the seven HPV LabNet reference laboratories were requested to test the proficiency panel using the PGMY-RBH with the same SOP.

Many datasets showed proficient results. Overall, the performance in this proficiency panel showed as much inter-laboratory variation for laboratories using the same assay as for those using different assays. This indicates that both differences in the assay performance, as well as specific laboratory practices, contribute to proficiency in HPV detection and typing. When the assay is used by only one or few laboratories, it is not possible to make inferences about their general robustness of performance. The PGMY-RBH method is the only assay that has been evaluated through technical transfer to the HPV LabNet laboratories. Successful transfer of this non-commercial assay indicates that it can be used in different laboratories in different regions of the world. The SOP for the PGMY-RBH method is described in section 5.4 to section 5.6.

The WHO HPV LabNet has agreed that a laboratory that performs HPV DNA detection and typing be considered proficient if it is able to detect 50 International Units (IU)/5 µL of HPV16 and HPV18 DNA, and 500 genome equivalents (GE)/5 µL of other HPV types; plus, if it also does not give more than one false-positive result in the panel (*7*). [See chapter 9 on international standards; 1 IU correlated with 1 GE]. It was recommended that genotyping assays should detect, at a minimum, the fourteen most common high-risk (HR) HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and the two LR HPV types targeted by a current HPV vaccine (6 and 11).

5.4 HPV detection with PGMY09/11 PCR

5.4.1 Principle of assay

The PGMY primer PCR system amplifies mucosal HPV types, producing a 450 bp amplicon. Including primers for a cellular target, histocompatibility leukocyte antigen (HLA), producing an amplicon of 230 bp, confirms that DNA has been extracted in sufficient amounts and that inhibitors are not preventing amplification. Amplicons are detected after agarose gel electrophoresis and GelRed staining under UV transillumination, and recorded with a camera. Samples with an HPV band are considered adequate and should be typed by RBH following protocol in section 5.5. Samples without HPV and HLA bands cannot be interpreted and are considered inadequate.

Gel electrophoretic analysis is used to limit the number of samples subjected to RBH to further diminish the cost of typing. If the prevalence of infection is high, then RBH can be used directly after the PCR. In this case, RBH negatives should be verified by gel electrophoresis. A sample with an HPV band that is RBH negative indicates that a type not included in RBH may be present.

5.4.2 Equipment

- Micropipettes (any brand)
- Microcentrifuge (any brand)
- Beakers
- Graduated cylinders
- Thermocycler capable of emulating the Applied Biosystem 9600 instrument (0.8°C per s up and 1.6°C per s down)
- Gel electrophoresis system (for instance Peqlab®, perfect blue horizontal maxi electrophoresis system, 4 x 14-well combs adapted for 12-well multichannel pipette)
- Electrophoresis power pack (any brand capable of delivering up to 300 V, 100 mAmps)
- UV transilluminator and digital camera (any brand)
- Laboratory balance (any brand, capable 10–100 mg precision)
- pH meter (any brand, use a Tris-adapted electrode)
- Microwave oven (any brand)

5.4.3 Consumables

In order to provide a detailed SOP, catalogue numbers of materials found to perform well by the HPV LabNet are provided. A laboratory may use alternative products if they can be validated to yield equivalent results.

- 0.2 mL, 0.5 mL, 1.5 mL microtubes (any brand)
- Racks for microtubes
- 96-well PCR microplates (for instance Thermo fast 96-well from ABgene, AB-1100)
- Adhesive cover (VWR International 732-4819)
- Filter tips (Sarstedt Biosphere: 20 µL, 70.760.213; 200 µL, 70.760.211; 1000 µL, 70.762.211)
- Disposable lab coats
- Disposable gloves
- Disposable absorbent laboratory pads
- Kimwipes

5.4.4 Reagents

In order to provide a detailed SOP, catalogue numbers of materials found to perform well by the HPV LabNet are provided. With the exception of AmpliTaq Gold® polymerase and buffer (evaluated in (*8*)), a laboratory may use alternative products if they can be validated to yield equivalent results.

- Taq polymerase (AmpliTaq Gold®, Applera N8080249)
- PCRII 10X buffer (included with AmpliTaq Gold®)
- 25 mM $MgCl_2$ (included with AmpliTaq Gold®)
- dNTPs (100 mM each, GE Healthcare ref. 28-4065-51 or equivalent)
- Molecular biology grade water (Eppendorf, VWR International 733-0153)
- Tris base (Fluka 93350): to prepare a 1M stock solution pH 7.5, dissolve 12.1 g Tris base in 80mL H_2O. Adjust the pH to 7.5 by adding about 6.5 mL of concentrated HCl (Merck 1.00317). Adjust the volume to 100 mL with distilled H_2O. Sterilize by autoclaving. Use a Tris-adapted electrode for this and pay attention to add HCl drop wise (exothermic reaction) in a fume hood
- Boric acid (Merck 165.1000)
- EDTA (Fluka 03680): prepare a 0.5M solution by dissolving 93.06g EDTA in 400 mL H_2O. Adjust the pH to 8.0 by adding approximately 8 g NaOH (Merck 1.06498). Adjust the volume to 500 mL with distilled H_2O
- Agarose (EuroBio GEPAGA07-65)
- Gel Red (Biotium 41002)
- 70% ethanol (30 mL 100% industrial ethanol (any brand), 70 mL distilled H_2O)
- DNA Zap solution (AMBION, 9891G/9892G)

- 50% glycerol (MP Biochemicals, 193996)
- 0.1% bromophenol blue (Fluka K 463): dissolve 0.25 g bromophenol blue in 50 mL 70% ethanol (made from Merck 1.00983). Mix well until complete dissolution of the powder
- 100 bp electrophoresis ladder (Promega G210A)
- Control cell lines, 293 (ATCC CRL-1573) and CaSki (ATTC CRL-1550)
- Primers listed in Table 5.2 as 100 µM stock from manufacturer of your choice **only if they pass quality control** (see chapter 6). The following companies (by alphabetical order) have provided satisfactory primers: Eurogentec, Life technologies (Invitrogen), Operon/MWG, Microsynth. Other companies like Applera or Sigma have not been evaluated for this purpose by the Swiss RRL. While unlabelled oligos can be simply desalted, biotinylated oligos are purified by high-performance liquid chromatography (HPLC)

Table 5.2: Primers for PGMY amplification

Biotin at the 5' end	
Name	Sequence [5' to 3']
PGMY11-A	GCACAGGGACATAACAATGG
PGMY11-B	GCGCAGGGCCACAATAATGG
PGMY11-C	GCACAGGGACATAATAATGG
PGMY11-D	GCCCAGGGCCACAACAATGG
PGMY11-E	GCTCAGGGTTTAAACAATGG
HLAdQ-F	GTGGTGTAAACTTGTACCA
Unlabelled primers	
Name	Sequence [5' to 3']
PGMY09-F	CGTCCCAAAGGAAACTGATC
PGMY09-G	CGACCTAAAGGAAACTGATC
PGMY09-H	CGTCCAAAAGGAAACTGATC
PGMY09-I	GCCAAGGGGAAACTGATC
PGMY09-J	CGTCCCAAAGGATACTGATC
PGMY09-K	CGTCCAAGGGGATACTGATC
PGMY09-L	CGACCTAAAGGGAATTGATC
PGMY09-M	CGACCTAGTGGAAATTGATC
PGMY09-N	CGACCAAGGGGATATTGATC
PGMY09-P	GCCCAACGGAAACTGATC
PGMY09-Q	CGACCCAAGGGAAACTGGTC
PGMY09-R	CGTCCTAAAGGAAACTGGTC
HMB01	GCGACCCAATGCAAATTGGT
HLAdQ-R	GGTAGCAGCGGTAGAGTT

5.4.4.1 Preparation of reagents

- 10X Tris-Borate-EDTA (TBE): dissolve 162 g Tris base, 50 g boric acid, 9.5 g EDTA in sterile, distilled or nanopure water, to volume slightly less than 1.0 litre. Adjust pH to 8.8 with HCl if needed and add distilled or nanopure water to final volume of 1.0 litre. Store at room temperature (RT) for several months.
- 0.5 X TBE: prepare by diluting 10X TBE in distilled or nanopure water (100 mL of 10 X TBE to final volume of 2 L). 200 mL is used to make an agarose gel, the other to fill the electrophoresis tank. Note that the electrophoresis buffer can be reused several times over a few weeks, provided that the buffer is not left to evaporate and that electrophoretic migration of standards is not altered.
- Gel loading buffer 10X [0.1 M Tris-HCl pH 7.5, 0.1 M EDTA, 50% glycerol, 0.1% bromophenol blue]. For 25 mL solution: to 2.5 mL 1 M Tris-HCl pH 7.5 add 5 mL 0.5 M EDTA, 12.5 mL glycerol and 5 mL 0.5% bromophenol blue.
- Positive control for sensitivity assessment: DNA from tissue-culture cell lines is used as control. Propagation of these lines should follow standard tissue-culture methods using media and conditions recommended by ATCC (*9*). CaSki cells are HPV16 positive and 293 cells are HPV negative human cells. Purify DNA (should not contain RNA) from harvested tissue cultures following the protocol that your laboratory uses for sample extraction. Determine DNA concentration by optical density measurement in a spectrophotometer (for instance the Nanodrop® system) using 50 µg/mL as 1 OD_{260}. The 260/280 ratio should be in the range of 1.7–1.9 and should not exceed 2 as this indicates contamination of the preparation by RNA or low molecular weight nucleic acids that leads to overestimation of DNA concentration.

Note: For copy number evaluation it is assumed that one diploid cell contains 6 pg DNA and that one CaSki cell contains in the order of 600 HPV16 GE; therefore 1 pg CaSki DNA ≈ 100 HPV16 GE. The positive control for sensitivity is not a secondary standard. It serves as a guide for day-to-day assay performance.

- To prepare the 1000 HPV16 GE positive control, dilute CaSki cell DNA to 2 pg per µL in DNA carrier (10 ng 293 cell DNA per µL). This stock ≈ 200 HPV16 GE per µL in background of ≈ 1670 copies of cellular DNA; giving input of ≈ 1000 HPV16 GE and 8400 cell equivalents per 5 µL. The 1000 HPV16 GE control should be stored in 100 µL aliquots at -20°C. **Freezing and thawing cycles must be minimized to avoid degradation of this control.** The 100 and 10 HPV16 GE controls are freshly prepared for each set of PCR reactions by making two consecutive tenfold dilutions of this stock in molecular biology-grade water. **The diluted DNA is lost/degraded even upon short-term storage.**
- PGMY09/HMB01 primer 5 µM working stock: add 50 µL each 12 PGMY09 and HMB01 100 µM primers to 350 µL molecular biology-grade water (1 mL total volume).
- PGMY11 primer 5 µM working stock: add 50 µL each 5 biotinylated PGMY11 100 µM primers to 750 µL molecular biology-grade water (1 mL total volume).
- HLA_DQ primer 5 µM working stock: add 25 µL each 100 µM primer to 450 µL molecular biology-grade water (0.5 mL total volume).

- Distribute each 5 µM working stock in 45–90 µL aliquots and store at -20°C. The number of aliquots and their volumes should be determined by the number of PCR reactions to be performed with a single aliquot, as freezing and thawing of diluted primers should be avoided (for calculations see Figure 5.3 and corresponding Excel® sheet HPV_PCR_reactions_setup_2000.xls available from WHO HPV LabNet laboratories).

5.4.5 Procedure

Strict precautions must be taken to prevent contamination of PCR reagents and specimens with HPV DNA or amplicons. Physical separation of functions in dedicated rooms is mandatory (see section 3.1.4).

These areas include a "DNA-free" or clean room for the preparation of master mixes, and an "assembly or extraction room" where DNA can be extracted from clinical samples and added to PCR master mix. These areas should have signs on the entrance indicating that any person or material potentially contaminated with amplicons should be kept out. Amplification and detection of PCR products must be kept in a separate laboratory (detection room) regarded as a "PCR dirty" area. All equipment, lab coats, reagents and gloves used in the "PCR dirty" area must remain in this area.

- Remove lab coat before entering "DNA-free" clean room or "assembly (extraction)" room. Once inside, wear new lab coat and gloves.
- Never go from "assembly (extraction)" or "PCR dirty (detection)" area where nucleic acids are handled to the "DNA-free" clean area. This applies to the entire working day.
- Reagents and master mixes that do not contain DNA are prepared only in the "DNA-free" clean room. Reagent stocks prepared in-house or from the manufacturer are stored in aliquots in the "DNA-free" clean laboratory.

5.4.5.1 PCR reactions

PCR is performed in duplicate reactions, one with 1.5 mM $MgCl_2$, and the other with 3 mM. The use of two different concentrations of $MgCl_2$ optimizes the analytical sensitivity of detection of the various HPV types and their detection by gel electrophoresis (less background with 1.5 mM and appropriate clinical sensitivity for all types, and somewhat better analytical sensitivity for types 16 and 66; higher background with 3 mM but better analytical sensitivity for types 31, 33, 35, 39, 42 and 56). Three mM $MgCl_2$ may be preferred if only RBH is used after PCR, and appears to increase the robustness of detection and typing (better signal to noise ratio for RBH that allows detection of types despite enhanced chemiluminescent substrate (ECL®) background that can be associated with user's lack of experience).

- Prepare a worksheet indicating positions of samples and controls in a PCR 96-well microplate. Plates are preferred to tubes whenever more than 20 reactions are to be processed. Each set of reactions (1.5 mM versus 3 mM $MgCl_2$) are grouped (Figure 5.2). For each sample set, for example 34 samples, add at least two negative controls (no template or water control (NTC), and one or more extraction blanks (Tex: no template extraction) and more than five positive controls (several CaSki at 1000 HPV16 GE and duplicate tenfold dilutions down to 10 GE). Place the positive controls in well A1 (C1000: 1000 GE), A2–3 (C100: 100 GE), A4–5 (C10: 10 GE), the first extraction control in A6 (Tex1) and the first samples (S1–S5) in the first row. Positive controls at 1000 GE are at incrementing column and row numbers to uniquely identify each row (except for row E).

Figure 5.2 Example of a PCR setup in a 96-well plate with master mixes containing 1.5 and 3.0 mM $MgCl_2$

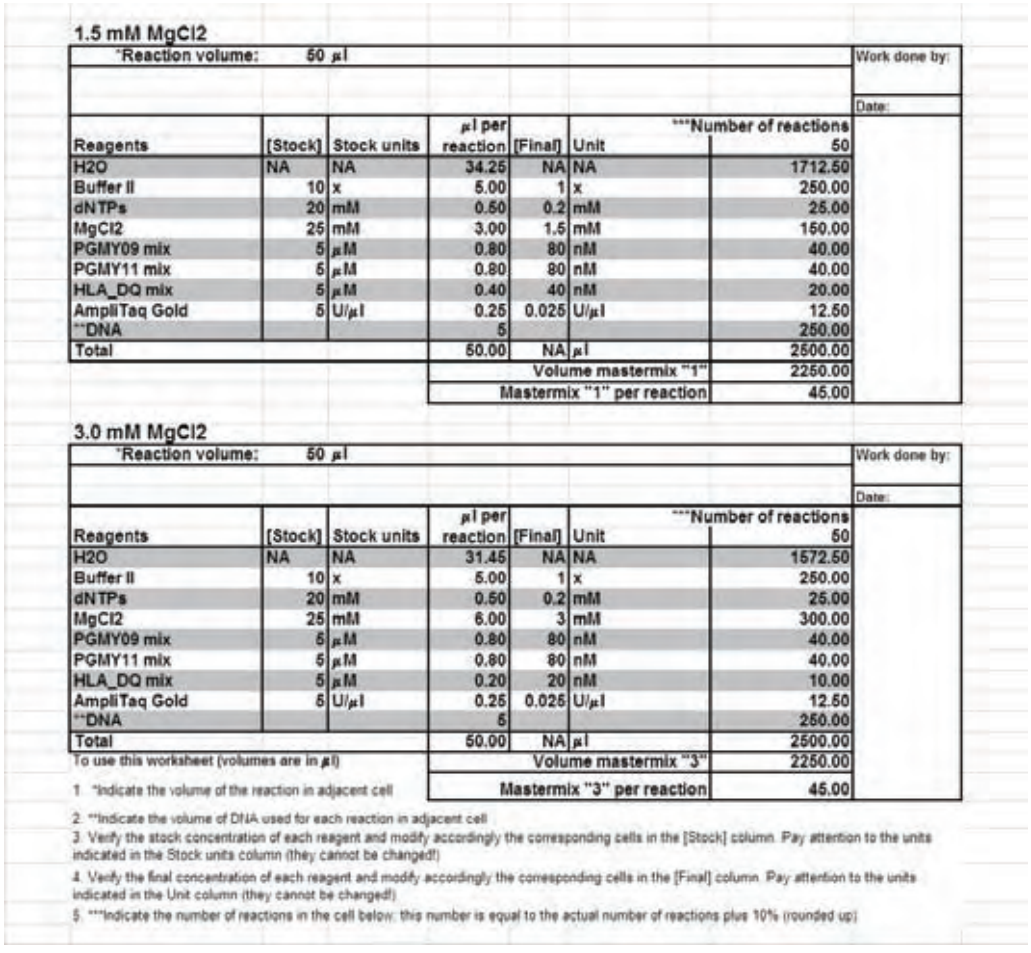
	1	2	3	4	5	6	7	8	9	10	11	12
A	C1000	C100	C100	C10	C10	TEx1	NTC	S1	S2	S3	S4	S5
B	S6	C1000	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16
C	S17	S18	C1000	S19	S20	S21	S22	S23	S24	S25	S26	S27
D	S28	TEx2	S29	C1000	S30	S31	S32	S33	S34			
E	C1000	C100	C100	C10	C10	TEx1	NTC	S1	S2	S3	S4	S5
F	S6	S7	S8	S9	S10	C1000	S11	S12	S13	S14	S15	S16
G	S17	S18	S19	S20	S21	S22	C1000	S23	S24	S25	S26	S27
H	S28	TEx2	S29	S30	S31	S32	S33	C1000	S34			

1.5mM: rows A–D; 3.0mM: rows E–H

The positions of the C1000 positive controls allow identifying uniquely rows containing a majority of samples. This is to identify errors at electrophoresis gel loading.

- PCR is performed with 5 µL DNA in a 50 µL reaction containing 1.25 U Amplitaq Gold, 1X PCR II buffer, 0.2 mM dNTPs, 80 nM each PGMY09 primer and HMB01, 80 nM each biotinylated PGMY11 primer, 20–40 nM each HLAdQ primer and 3.0–1.5 mM $MgCl_2$. Determine the number of samples to be tested and prepare each master mix for that number plus 10% (rounded up). For instance, in the setup depicted in Figure 5.2, the number of reactions is 45 for each $MgCl_2$ concentration so that preparing master mixes for 50 reactions should suffice. Figure 5.3 shows the respective reagent's volumes to use for 1.5 mM and for 3.0 mM $MgCl_2$-containing master mixes ("1" respectively "3"). Note that for the former, the HLA primers are at 40 nM, and that they must be set at 20 nM to avoid interference with HPV amplification in the presence of 3.0 mM $MgCl_2$. The volume of DNA to be added should not exceed 5 µL, and in most studies should remain constant for each sample type. However, for samples with unusually high DNA concentrations, PCR inhibition can occur and a smaller volume, or a dilution, may be required.

Figure 5.3 Excel® sheet for PCR setup



1.5 mM MgCl2

*Reaction volume:	50 µl					Work done by:
						Date:
Reagents	[Stock]	Stock units	µl per reaction	[Final]	Unit	***Number of reactions 50
H2O	NA	NA	34.25	NA	NA	1712.50
Buffer II	10	x	5.00	1	x	250.00
dNTPs	20	mM	0.50	0.2	mM	25.00
MgCl2	25	mM	3.00	1.5	mM	150.00
PGMY09 mix	5	µM	0.80	80	nM	40.00
PGMY11 mix	5	µM	0.80	80	nM	40.00
HLA_DQ mix	5	µM	0.40	40	nM	20.00
AmpliTaq Gold	5	U/µl	0.25	0.025	U/µl	12.50
**DNA			5			250.00
Total			50.00	NA	µl	2500.00
					Volume mastermix "1"	2250.00
					Mastermix "1" per reaction	45.00

3.0 mM MgCl2

*Reaction volume:	50 µl					Work done by:
						Date:
Reagents	[Stock]	Stock units	µl per reaction	[Final]	Unit	***Number of reactions 50
H2O	NA	NA	31.45	NA	NA	1572.50
Buffer II	10	x	5.00	1	x	250.00
dNTPs	20	mM	0.50	0.2	mM	25.00
MgCl2	25	mM	6.00	3	mM	300.00
PGMY09 mix	5	µM	0.80	80	nM	40.00
PGMY11 mix	5	µM	0.80	80	nM	40.00
HLA_DQ mix	5	µM	0.20	20	nM	10.00
AmpliTaq Gold	5	U/µl	0.25	0.025	U/µl	12.50
**DNA			5			250.00
Total			50.00	NA	µl	2500.00
					Volume mastermix "3"	2250.00
					Mastermix "3" per reaction	45.00

To use this worksheet (volumes are in µl)

1. *Indicate the volume of the reaction in adjacent cell
2. **Indicate the volume of DNA used for each reaction in adjacent cell
3. Verify the stock concentration of each reagent and modify accordingly the corresponding cells in the [Stock] column. Pay attention to the units indicated in the Stock units column (they cannot be changed!)
4. Verify the final concentration of each reagent and modify accordingly the corresponding cells in the [Final] column. Pay attention to the units indicated in the Unit column (they cannot be changed!)
5. ***Indicate the number of reactions in the cell below: this number is equal to the actual number of reactions plus 10% (rounded up)

The original file is available from HPV LabNet laboratories.

- Assemble master mixes in "DNA-free" area. Distribute in PCR tubes or 96-well PCR plates or take them to the assembly area in large tubes. Transfer tubes or plates in the assembly area, making sure that they cannot be contaminated (place the plate in a closed box or cover it with a protective foil).
- Distribute 45 µL master mix in each well (tube). Automated device can be used for this, provided they are dedicated to this activity and equipped with barrier tips.
- Remove samples from storage area and bring them to room temperature. Place in a microcentrifuge and spin briefly to remove condensate from caps. Place in rack by increasing reference number.
- Prepare tenfold dilutions of the CaSki 1000 (C1000) positive control in water (100 and 10 GE per 5 µL).

- Work in PCR hood or sheltered area. This area should be regularly cleaned with DNA zap and/or with 70% ethanol. The workspace can be further covered with clean absorbent disposable laboratory pad. Place rack with samples and controls, additional empty rack, pipettes, tips, gloves and bin in workspace. These items should be used only for PCR assembly and should not leave this area. Arrange samples and controls in the order that they are to be added to the PCR microplate.
- Change gloves and bring PCR microplate with master mix to work area.

  Work with one sample at a time, and open it paying special attention not to contaminate the work area by aerosol. The best method is to use screw-cap tubes. If using snap-cap tubes (Eppendorf for instance), use Kimwipe to cover the cap while opening. Transfer 5 µL aliquot and place in correct position of PCR microplate. Close sample tube and move to empty rack. Discard Kimwipe and pipette tip. Proceed to next sample. Gloves should be changed if there is any sample contamination noted. When last sample has been added, seal PCR microplate wells with an adhesive film.
- Move samples to appropriate storage location, discard all tips, gloves and pads in appropriate containers. Clean workspace and racks.
- Transfer material to thermocycler. Amplification programme is: 95°C, 9 min; 45 cycles (95°C, 30s; 55°C, 1 min 30s, 72°C, 2 min); 72°C, 5 min.
- During PCR prepare 200 mL of 2% agarose gel in 0.5 X TBE for 48 to 96-wells depending on the number of combs the electrophoresis tray can accept. Add 4 g agarose to 200 mL 0.5 X TBE and microwave to boil. Cool until it can be handled (leave for 15 min at 60°C in a thermostated water-bath) and add 10 µL GelRed. Pour into gel electrophoresis apparatus. Use four to eight 14-well combs per gel. When the gel is fully cooled and solidified, add electrophoresis buffer (0.5 X TBE) in the electrophoresis chamber to cover the gel by 1 cm. This buffer can be reused and kept in the chamber as long as the electrophoresis profile remains unaffected (several runs). Remove combs slowly, paying attention not to tear the gel underneath the wells.
- Prepare workspace for amplified samples by covering with clean absorbent disposable laboratory pad and arranging multipipette, reagent reservoir, tips, loading buffer, markers, electrophoresis apparatus and discard container. This area should be immediately adjacent to a power supply for electrophoresis.
- Following PCR, the microplate is removed and taken to the workspace. Place a few mL of loading buffer in the reagent reservoir, transfer 10 µL into each sample using a 12-well multipipette and mix very well by up and down pipetting. Ten µL is then deposited into the 12 central wells of the agarose gel. Discard tips and repeat for next row of samples. Apply 3–5 µL markers at the extremities of each row; by doing so, the electrophoresis template does correspond to the PCR template and can be used for results interpretation.
- Place cover on electrophoresis apparatus, apply electrodes and turn on power supply. Electrophorese at 15 watts constant power until bromophenol blue is one cm above the next row of wells.

- Remove gel in tray and place on UV transilluminator. Take a picture of the gel under UV light using the electronic camera, medium aperture (5.6–8), ~600 m s or time adjusted to give a faint but detectable grey background. The digital picture may be improved only in terms of luminescence and contrast (no sharpening). Print image and note position of the positive controls. Save image as .jpg file labelled by date or other appropriate designation.
- Discard electrophoresis buffer in drain or specific container according to your local safety rules, wash gel tray and combs with tap water and rinse with distilled water. Air dry and return to storage. Discard tips, laboratory pad and used gloves in appropriate containers. Return all equipment and reagents. The buffer can be stored in the electrophoresis tank for several weeks and reused as mentioned above.

Note: Use UV-protective eyewear when viewing UV transilluminated gel. Discard gel in dedicated bin according to your local safety rules for Ethidium bromide or Gel Red. The latter is less toxic than Ethidium bromide and less mutagenic. You can refer to the manufacturer's website (10) for the Material Safety Data Sheet. The buffer can be reused and may be stored for several weeks in the electrophoresis tank.

5.4.6 Interpretation of results

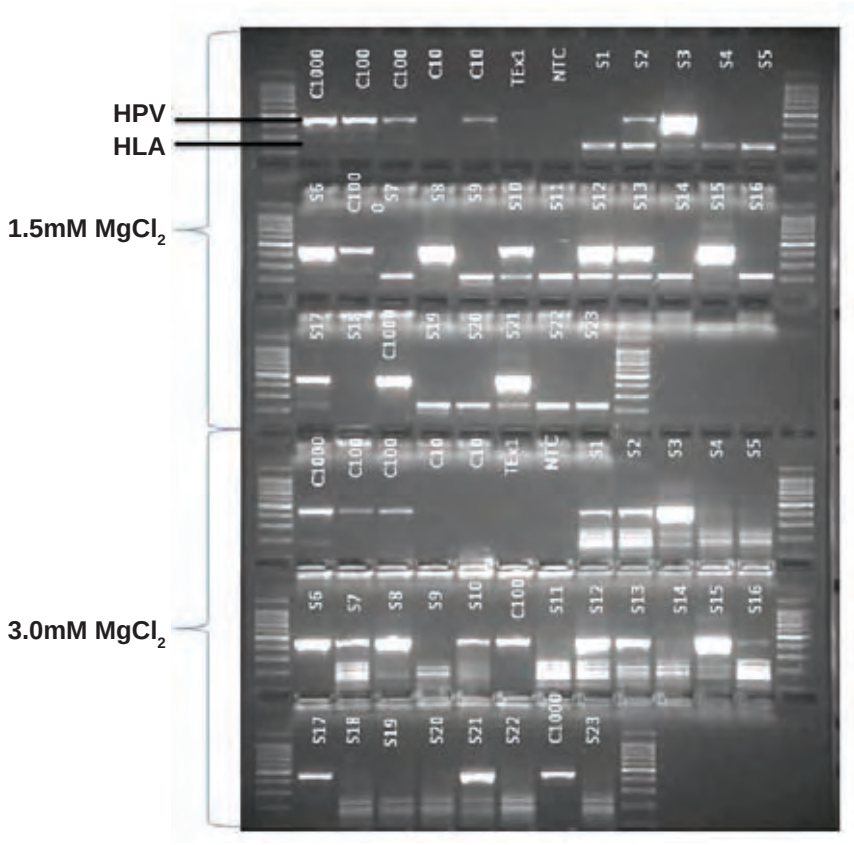
- The HPV band is visible at 450 bp and the HLA band at 230 bp (see Figure 5.4).
- The lanes for all negative controls should not contain HPV or HLA amplicons. A smeary appearance of primer dimers may be evident. **Any HPV or HLA band indicates contamination and the reason must be investigated and assay repeated when the problem is solved. Contamination of extraction control indicates that re-extraction is required to assure results. Contamination of water blank indicates new PCR reagents must be prepared and the assay repeated when contamination has been eliminated. To assess contamination in the laboratory, a set 20–50 negative control reactions are performed. For this reason it is often better and less expensive to simply discard working aliquots of reagents and start again with unopened aliquots.**
- The 450 bp fragment must be visible in the 100 GE positive controls and in one or both C10 samples. **Failure to detect HPV in the 100 GE control indicates that the assay does not have expected sensitivity or the positive control itself is defective. Analysis must be repeated when corrective action is taken. The parameters to verify are: cycling conditions and key reagents (polymerase, nucleotide, primers, positive control). As indicated above, if the problem is not obvious (wrong cycling parameters, wrong enzyme), it is sometimes more economical to simply repeat the entire procedure with new batches of reagents.**

Note: To pass proficiency testing, assays should detect 50 GE of HPV16 and 18 and 500 GE of other types in a 5 µL sample. Secondary standards (see chapter 9) are used to validate the assay and establish sensitivity. The positive controls are used to monitor day-to-day changes that could affect sensitivity.

- If positive and negative controls give expected results, the samples may be interpreted.

- Samples with the 450 bp fragment are presumed to be positive for HPV. These will be verified and typed using reverse blotting hybridization (section 5.5).
- Samples with only the 230 bp fragment are negative for HPV.
- Samples negative for both bands are non-informative. In this case, the reaction should be repeated. A smear in the lane suggests inhibition due to too much DNA, so a smaller volume of extract should be tried. If there is no smear, a larger volume of extract should be tried. If necessary, DNA can be re-extracted after concentrating cells by low-speed centrifugation if cell density seemed low. If these remedies fail, the sample must be considered inadequate for evaluation.

Figure 5.4 Gel electrophoretic analysis of PGMY/HLA amplification products



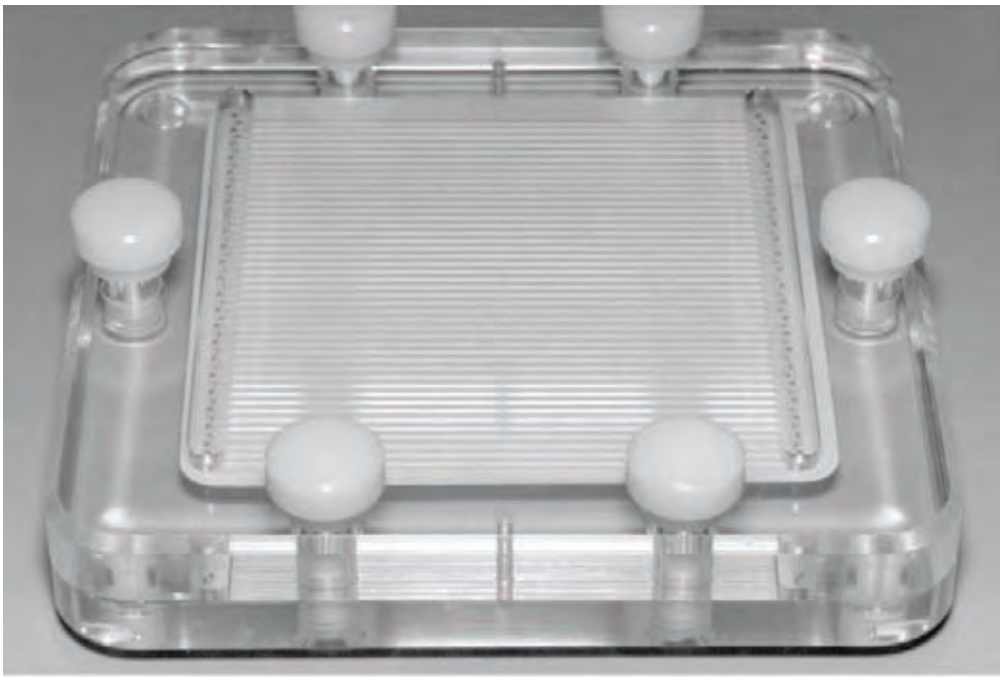
Five µL DNA was subjected to PCR amplification in the presence of 1.5 or 3 mM $MgCl_2$ with the PGMY09/11 and HLA primers as described in 5.4 and 10 µL aliquot of the reaction analysed by gel electrophoresis in 2% agarose gel containing Gel Red. A picture was taken upon UV transillumination. M: molecular weight marker (100 bp ladder); C1000: positive control (1000 GE HPV16 in 10 000 human GE); C100 and C10: positive control (100 respectively 10 GE HPV16); Tex1: extraction blank control; NTC: no template PCR control; S1–S23: clinical samples. The presence of the HLA band indicates that samples are informative (cellular DNA is present and amplifiable). The presence of the HPV fragment indicates HPV positive samples candidate for typing by RBH. Note the more smeary appearance of many negative samples with 3 mM $MgCl_2$ (S4, S5, S11 and others). Gain in sensitivity was observed for sample S1, S7 and S16 with 3 mM $MgCl_2$, and for the C10 positive controls and S10 with 1.5 mM $MgCl_2$. Sample S18 is non- informative with 1.5 mM $MgCl_2$ and HPV negative with 3 mM. The corresponding RBH is shown in Figure 5.7.

5.5 HPV typing with type-specific hybridization and chemiluminescent detection

5.5.1 Principle of as say

After heat denaturation in a low-salt buffer, the biotinylated HPV amplicons are hybridized to a parallel array of HPV type-specific probes covalently bound to a negatively-charged nylon membrane. After washing, the hybrids are revealed by chemiluminescence on an autoradiography film. This method has been adapted from Kaufhold and colleagues (*11*). A miniblotter (Figure 5.5) is used to prepare the probe array and to perform the hybridization.

Figure 5.5: Miniblotter used for the preparation of the probe array



The membrane is placed on a foam pad between the two assembly parts of the apparatus. Upon tightening with the white screws, the negatively- charged nylon membrane defines the bottom of 45 horizontal parallel channels. Probes are oligonucleotides with a 5' aminolink that forms a covalent bond to the membrane that has been activated with EDAC, a chemical crosslinker activating carboxyl groups for amide formation. The membrane is ready for use after inactivation of its surface at alkaline pH. For hybridization, the membrane is turned by 90 degrees relative to the channels, so that the heat-denatured amplicons will cross each probe when loaded into each channel.

5.5.2 Equipment

- 20 µL, 200 µL, 1000 µL micropipettes
- Thermostated water-bath shaker accommodating 20 x 20 cm containers
- Hybridization oven (50°C)
- Block heater for 0.7 mL tubes
- Film exposure cassette equipped with a foam seal. Simple cardboard exposure cassette should be avoided to diminish the risk of membrane drying.
- Dark room equipped for autoradiographic film development
- Shaker
- 20 cm x 20 cm glass container for membrane preparation and washing. Glass containers are preferred to plastic ones for their better heat conductivity.
- Miniblotter, Immunetics MN45 or Hoefer's equivalent (PR645)
- Sealing device
- Aspiration bottles
- Scanner

5.5.3 Consumables

In order to provide a detailed SOP, catalogue numbers of consumables found to perform well by the HPV LabNet are provided. A laboratory may use alternative products if they can be validated to yield equivalent results.

- Filter tips for micropipettes
- Autoradiographic film for ECL® (GeHealthcare RPN 2103K)
- Nonsterile gloves
- Plastic foam pads, PC200, Immunetics USA
- Hybridization membrane Biodyne® C (0.45 µm) 29 cm x 3 m , Pall corporation, 60257 (check for availability or for alternative size with local distributor)
- 0.7 mL microtubes with locking cap
- Whatman 3MM 46 x 57 cm, Whatman, 3030-917
- A5 transparencies for photocopy machines
- Sterile Falcon tubes, 50 mL
- Polyethylene sealing bags
- Aspiration tips (non filter tips for 200 µL pipette)

5.5.4 Reagents

In order to provide a detailed SOP, catalogue numbers of reagents found to perform well by the HPV LabNet are provided. A laboratory may use alternative products if they can be validated to yield equivalent results.

- EDAC (N-Ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride) Sigma #E7750
- 0.5 M EDTA (see 5.4.4)
- 20 X saline sodium phosphate EDTA (SSPE); 3.0 M NaCl, 0.2 M NaH_2PO_4, and 0.02 M EDTA pH 7.4), Invitrogen 15591-035
- Enhanced chemiluminescent (ECL®) detection reagents for nucleic acids, GeHealthcare RPN 3004 for instance. Alternative kits for Western blotting (EuroClone LiteAblot®, EMP010400 for instance) can be used provided they perform adequately with the positive control DNAs. The volume of the ECL® detection reagent for Western blotting should be the same as that for nucleic acids.
- India ink
- Streptavidin peroxidase, GeHealthcare RPN 1231
- 30% H_2O_2 , Merck, 1.08597.1000
- Molecular biology grade water
- Probes as listed in Table 5.3, modified with [5'] Amine_C6 group, synthesized by manufacturer using all measures to prevent cross-contamination. High-performance liquid chromatography **(HPLC) purification should not be used as this increases the risk of cross contamination with shared HPLC device and extra manipulations/concentrations.** Desalted probes from Microsynth have proved satisfactory. They are dissolved in molecular biology grade water under conditions that exclude cross-contamination (similar to those used for PCR setup). They can be kept for several years at -20°C in 100 µM aliquots.
- In addition to these HPV probes, an additional [5′] Amine C6-oligoT_{30}-[3′]-biotin can be used to monitor the quality of steptavidin peroxidase/ECL® detection. Care must be taken to avoid cross-contamination of other probes with this control. This biotin-labelled oligo should be used at a much lower concentration as it simulates detection of a hybridized probe. The amount should be determined empirically, but should be in the order of 20 picomoles per lane.

Table 5.3: Probes for reverse blotting hybridization

Name	Sequence [5'–3']	Comment
HPV06_RHP	TGGAAGATGTAGTTACGGATG	
HPV11_RHP	GCAGATTTAGACACAGATGCA	
HPV16_RHP	GATATGGCAGCACATAATGAC	
HPV18_RHP	CCAGGTACAGGAGACTGTGTA	
HPV26_RHP	TACGCTGACAGGTAGTAGCAG	
HPV31_RHP	AGTATCACTGTTTGCAATTGC	
HPV33_RHP	TGTCACTAGTTACTTGTGTGC	
HPV34_RHP	GCAGTTGTACTTGTGGATTGT	
HPV35_RHP	AGAAGACACAGCAGAACACAC	
HPV39_RHP	GTAGAAGGTATGGAAGACTCT	
HPV40_RHP	ATAGCCTTGTTGGTAAGGAAC	
HPV42_RHP	TGTATCACCAGATGTTGCAGT	
HPV43_RHP	ACAGTAGGGTCAGTAGAGGCA	It may be omitted as the PGMY primers are not adapted to HPV 43 amplification. If kept, it may serve as a non-specific control.
HPV44_RHP	TAGTATATGTAGACGGAGGGG	
HPV45_RHP	GTACTTGGCACAGGATTTTGT	
HPV51_RHP	TTACTTGGAGTAAATGTTGGG	
HPV52_RHP	CTTTCCTTTAGGTGGTGTGTT	
HPV53_RHP	AGACATAGACTGTGTGGTTGC	
HPV54_RHP	TTATTAAAGCTATCCTGCGTG	
HPV55_RHP	GATGGAGACTGAGTTGTAGCA	
HPV56_RHP	TTTCGTGCATCATATTTACTT	
HPV57a_RHP	TACAGTGGCACACAAAGAGAC	Cross-reacts with HPV 42.
HPV57b_RHP	TTCTGTGTTTACAGTGGCACA	Replaces the original HPV57a probe that cross-reacts with HPV 42.
HPV58_RHP	CTTCCTTAGTTACTTCAGTGC	
HPV59a_RHP	AGTAGAGCACACACAGAAAGA	
HPV59b_RHP	AGTAGAAGCACACACAGAAAG	A insertion to increase HPV 59 coverage.
HPV66_RHP	AGTTAATGTGCTTTTAGCTGC	
HPV68_RHP	CTGATTGCAGATAGCGGTATG	Adapted to HPV 68b (ME180 isolate).
HPV69_RHP	GTTTAAAAGTGGCAGATGCAG	
HPV70_RHP	CTATATACAGCAGGTATGGCC	
HPV 82_RHP	TGCAACAGATTGAGTAACAGC	HPVMM4
HPV 83_RHP	AGAGGCTGTGTATTCATTAGC	HPVMM7
HPV 84_RHP	ATTCTGATTCGGTGTTGGTAG	HPVMM8
HPV 73_RHP	GGCATACGTTGTAGTAGAGCT	HPVMM9
HLADQ_RHP	CTCRTCTCCATCAAATTCATG	R: A or G

Note: This list may be updated to match variants found to be important.

5.5.4.1 Preparation of reagents

In order to provide a detailed SOP, catalogue numbers of reagents found to perform well by the HPV LabNet are provided. A laboratory may use alternative products if they can be validated to yield equivalent results.

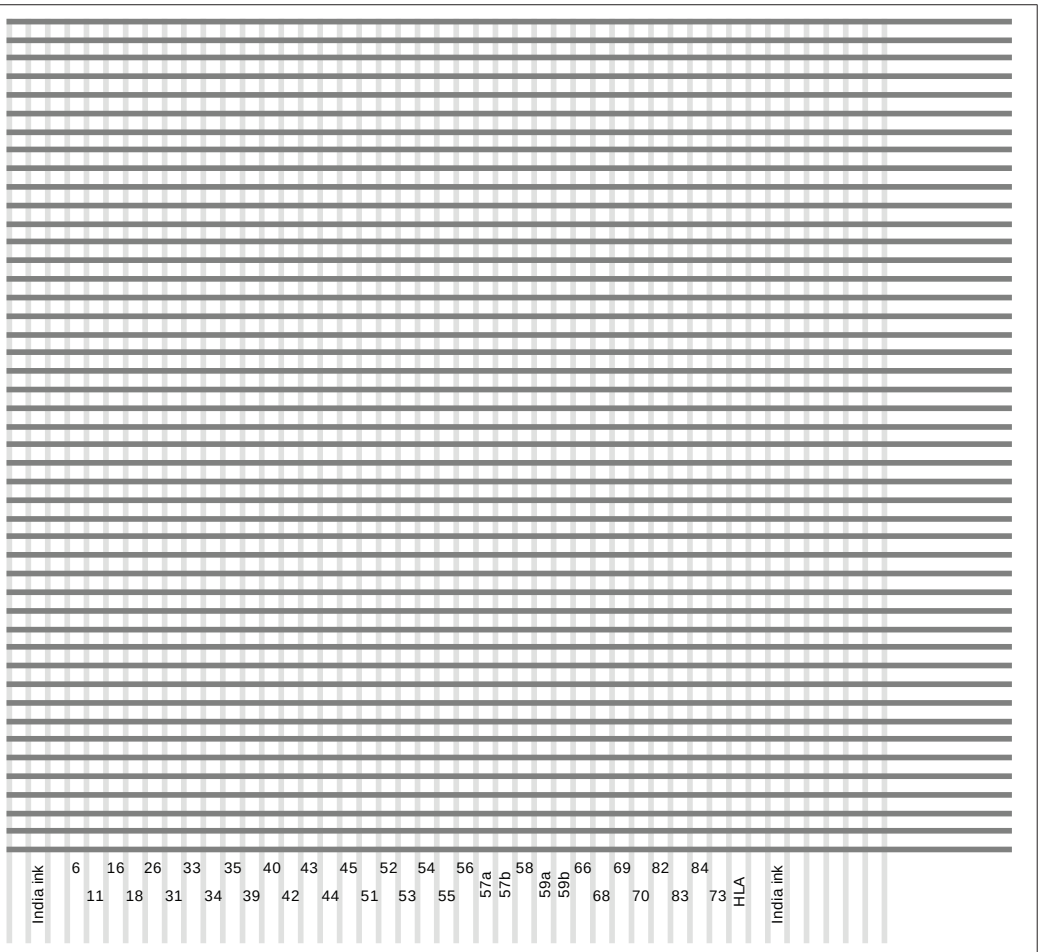
- 5 N NaOH (Fluka): dissolve 100 g NaOH tablets in 500 mL distilled H_2O. **Be careful to first fill the beaker with H_2O and then add the tablets slowly as the reaction generates heat; exothermic. To reduce heating of the solution, the beaker can be placed on ice.** Store at RT in a plastic bottle.
- 20% SDS (Biosolve 19822359): weigh 200 g SDS and dissolve in 1 L H_2O. **Wear a mask as SDS is very volatile and causes irritation. It may be necessary to heat the solution (37°C–45°C) to dissolve SDS completely. If precipitate occurs during storage, reheat and mix well.** Store at RT
- 500 mM $NaHCO_3$ pH 8.4 (Merck 1.06329): dissolve 21 g $NaHCO_3$ in 500 mL H_2O; pH doesn't need to be adjusted.
- 0.1 N NaOH 1% SDS: mix 20 mL 5N NaOH, 930 mL H_2O and 50 mL 20% SDS. Store at RT in a closed plastic container.
- 1% SDS: for 500 mL, mix 25 mL 20% SDS and 475 mL H_2O. Store at RT.
- 16% EDAC: 3.2 g EDAC plus H_2O to 20 mL. Use immediately.
- 2 X SSPE: for 500 mL mix 50 mL 20X SSPE and 450 mL H_2O. Store at RT.
- 8X SSPE, 0.4 % SDS: for 500 mL mix 200 mL 20X SSPE, 290 mL H_2O and 10 mL 20% SDS. Store at RT.
- 2X SSPE, 0.1 % SDS: for 50 mL mix 12.5 mL 8 X SSPE, 0.4% SDS and 37.5 mL H_2O. Store at RT.
- 2X SSPE, 0.5% SDS: for 1000 mL mix 100 mL 20X SSPE, 875 mL H_2O and 25 mL SDS 20%. Store at RT.
- 20 mM EDTA pH 8.0: for 500 mL mix 20 mL 0.5 M EDTA and 480 mL H_2O. Store at RT.
- 3% H_2O_2: for 300 mL mix 30 mL 30% H_2O_2 and 270 mL distilled H_2O. Use immediately.
- Positive control for specificity assessment: PCR reactions performed with control plasmids bearing type-specific sequences or with previously typed clinical sample DNA (less reliable).
- Positive control for run validation: C1000 amplicons from the corresponding PCR run and a mixture of HPV type-specific amplicons derived from control plasmids.

5.5.5 Protocol for preparation of membrane

Wear gloves for all steps.

- Dilute 5 µL of each probe stock (100 µM) in 200 µL of 500 mM $NaHCO_3$ pH 8.4.
- Use a precut 16 cm x 14 cm Biodyne C membrane.
- Activate the membrane in 20 mL of freshly prepared 16% EDAC, in a glass container, for 10 min with gentle agitation. **EDAC is expensive; using half the volume to activate the membrane has proved successful (1.6 gm in 10 mL). In this case, incubate the membrane in this solution in a polyethylene sealed bag.**
- Rinse with distilled water 2 min.
- Place the membrane on the upper part of the miniblotter so that it covers the bottom of the channels. Place a new foam pad on the membrane and assemble the miniblotter. Apply uniform pressure by tightening both parts with opposite screws in a diagonal. Tightening well is important to avoid cross-contamination. Aspirate the liquid that remains in the channels.
- Apply the diluted probes in increasing order (6, 11, 16, etc. with the HLA probe last) according to the array scheme depicted in Figure 5.6. Apply India ink diluted to 10% in H_2O in the channels preceding HPV6 and following HLA to establish asymmetric reference marks. **When applying the probes it is essential to avoid bubbles in the channels to prevent contamination of one channel's contents into another's. This is best accomplished by gently dispensing 120–170 µL to one neck while controlling the liquid to the opposite neck of that channel using a tip connected to a line with a gentle vacuum. The probe solution needs to reach less than 2–3 mm from the top of each neck. Be careful not to empty the channel while applying vacuum!**
- When all probes have been applied, leave for one min at room temperature. Aspirate the probes in the order of application. Disassemble the miniblotter and mark the side of the membrane carrying the probes with permanent ink.
- To inactivate the membrane, place it in a glass container with 250 mL of 10 mM NaOH and incubate under gentle shaking for 10 min at RT.
- Wash the membrane twice in 250 mL 2X SSPE, 0.1% SDS, 5 min at 60°C under gentle agitation.
- Wash the membrane once in 100 mL 20 mM EDTA pH 8.0 at RT for 15 min. Store the membrane in 100 mL 20 mM EDTA pH 8.0 in the refrigerator. The membrane can be stored for weeks or months under these conditions.
- Quality control and validate the membrane (see "Remarks" at the end of this section). Once validated the membrane can be used at least an additional 8–10 times.

Figure 5.6 Array scheme indicating the distribution of probes in adjacent channels (delimited by light grey stripes)



Dark grey stripes indicate the channels oriented perpendicularly after the membrane has been rotated by 90° in the miniblotter, so that the amplicons from PCR reaction can react with each probe. The India ink channels delimit the probe array so that the membrane can be easily oriented in the miniblotter for sample loading. The position of the HPV 73 probe reflects that of its original name (MM9).

5.5.6 Protocol for sample application and hybridization

Wear gloves for all steps. Under no circumstances should the membrane be left to dryness, even partially, or irreversible background will ensue.

- Turn on the hybridization oven and the shaker water-bath to 50°C. Preheat the hybridization buffers in the water-bath.
- Denature 5–15 µL amplicon (from the PCR plate, loading buffer does not interfere with hybridization and provides a blue dye that is extremely helpful to track the samples) in 110 µL molecular biology grade water (0.7 mL microtubes), 5 min at 95°C in an old thermocycler or block heater. Cover the caps of the tubes with a glass beaker to avoid popping up and formation of condensation; if there is some nevertheless, spin down tubes quickly after cooling. Immediately afterwards, place tubes on an aluminium tube holder at 4°C (from the refrigerator or on ice) and add 110 µL 8X SSPE, 0.4% sodium dodecylsulphate (SDS) preheated to 50°C so that SDS does not precipitate. Mix well.

- During denaturation of DNA, assemble the miniblotter as described previously using a new foam pad. **The ink lines must be perpendicular to the channels and 1–2 mm within the binding area defined by their necks.** The probes and filter labels should face up. Aspirate away all liquid in the channels and fill them immediately with 2X SSPE, 0.1% SDS.
- Aspirate the liquid in the second leftmost channel. Apply the first sample, one of the HPV16 controls, in this channel, and then aspirate the liquid in the channel to its right. Do not over aspirate. Apply 2X SSPE, 0.1% SDS next to the HPV16 control as a blank, and repeat the procedure with the samples until all have been applied in consecutive order from left to right **paying attention not to cross-contaminate the channels**. The blue dye helps locating filled channels. Apply 2X SSPE, 0.1% SDS after the last sample of the series and finally two HPV16 controls to set the filter orientation. A mixture of all positive controls that were used for quality control of the filter can be used last as a reference (see quality control of the membrane).
- Place a scotch tape on top of the channels reservoir to limit evaporation. Transfer the miniblotter to the hybridization oven. So as to not contaminate adjacent channels during this manipulation, maintain the miniblotter horizontally throughout the procedure. Protect the miniblotter from airflow (if necessary, depending on the oven), by covering it entirely with a plastic box placed upside down. Leave the miniblotter in the hybridization oven for 1.5 h at 50°C (no more).
- After hybridization, vacuum aspirate the content of each channel in the order of application. Disassemble the miniblotter and immediately place the membrane in 250 mL 2X SSPE, 0.5% SDS preheated at 50°C in a glass container.
- Wash the membrane with gentle agitation at 50°C for 10 min. Repeat this washing step once.
- Cool the shaker water-bath down to 42°C with ice (or use another set at 42°C).

5.5.7 Protocol for hybrid detection

Wear gloves for all steps. Under no circumstances should the membrane be left to dryness, even partially, or irreversible background will ensue.

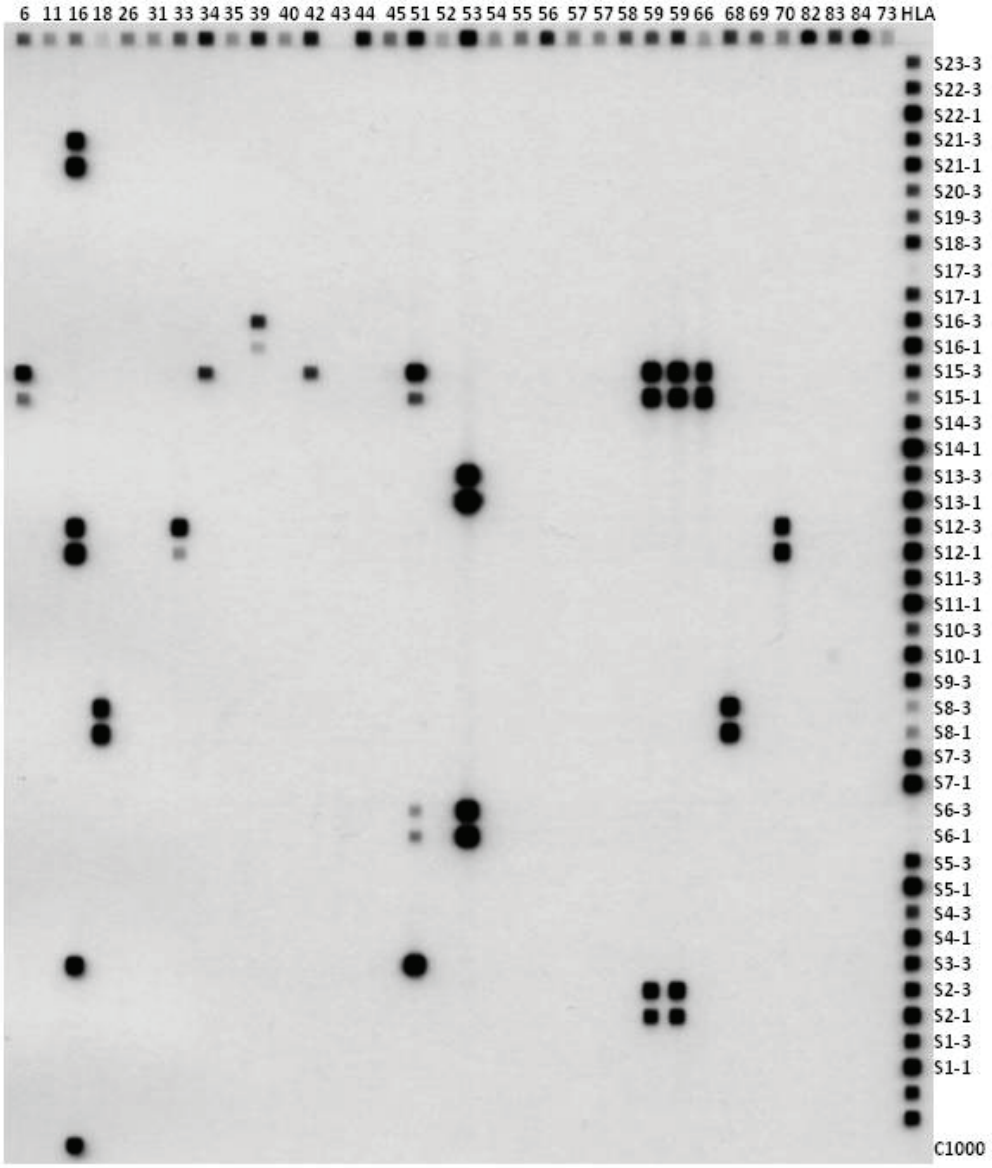
- Immediately after washing, place the membrane in a sealing bag. Add streptavidin peroxidase diluted in 20 mL 2X SSPE, 0.5% SDS (for instance 1/4000, as recommended by the manufacturer, is a good starting point. Optimal signal to noise ratio and sensitivity may require initial optimization of this dilution). Eliminate the bubbles, seal the bag as close to the membrane as possible, and incubate under water in a glass container at 42°C for 45 min with gentle agitation.
- Wash the membrane twice at 42°C in 250 mL of 2X SSPE, 0.5% SDS in a glass container with gentle agitation for 10 min each.
- Rinse the membrane twice for 5 min in 250 mL of 2X SSPE at room temperature in a glass container with gentle agitation.
- Blot the excess liquid briefly until damp between two Whatman 3MM filter paper. **Do not allow membrane to dry.**
- Prepare 4–5 mL of ECL® detection solution according to the manufacturer's instructions and immediately place this solution on to an A5 transparency (this is the recommended volume for the nucleic acid ECL® detection kit and should not be changed if the Western blot ECL® detection kit is used). Transfer the membrane, nucleic acids face down, on to the solution, allowing the liquid to be evenly spread under the membrane, and incubate for 2 min.
- Blot the excess liquid briefly until damp between two Whatman 3MM filter papers. **Do not allow membrane to dry.**
- Place the membrane between two A5 transparencies. In the darkroom under red light add an autoradiography film on top of the assembly in an exposure cassette. Bend one corner of the film to set its orientation relative to the filter.
- Expose the film for 1–2 h (overnight is possible but does not increase sensitivity).
- During exposure, place the miniblotter in 0.1 N NaOH, 1% SDS and leave it in this solution for a few hours, to overnight.
- Wash the channels briefly with a soft toothbrush and rinse the entire miniblotter thoroughly with deionized distilled water.
- Prevent background by treating the miniblotter upper part (carrying the channels), the channels facing up, in 300 mL of freshly-prepared 3% H_2O_2 with gentle agitation for an hour. **This step is crucial to prevent non-specific background in subsequent assays.**
- Rinse for 5–30 min with deionized distilled water, followed briefly by nanopure water. Dry and store the miniblotter unassembled. **Be very careful to avoid scratching the channels.**
- Develop the film and indicate the sample ID next to each histocompatibility leukocyte antigen (HLA) spot. The orientation of the membrane is provided by the black line in relation to the bent corner of the film and the positive controls. Indicate the hybridization date, the membrane ID and usage number, and the technician's name.

- Verify the global signal intensity as indicated in quality assessment for PGMY detection and typing. If the quality control is satisfactory, wash the membrane twice for 40 min in 250 mL 1% SDS at 80°C with gentle agitation. Wash the membrane for 15 min at RT in 250 mL 20 mM EDTA pH 8.0. Store the membrane in 20 mM EDTA pH 8.0 in the refrigerator.
- Assign HPV types to each sample by visual inspection of the developed film on a light box using the array scheme as guide. The non-specific background observed at validation of the membrane with type-specific control amplicons should be used to set the threshold of positivity.
- Record the autoradiogram as a .jpg image, 256 grey levels and 200 dots per inch with a standard scanner. For non-biased evaluation of the signals, this image can be analysed with a density software, National Institutes of Health (NIH) image for instance, to assign grey values, and set the threshold of positivity based on average background value (one or several blank lines can serve for its establishment) plus a multiple of standard deviations.

5.5.8 Interpretation

Due to the very low background of ECL® detection with these membranes, very faint signals can be detected. They should, however, be distinguished from dots or scratches due to static electricity or contaminants. Positives are identified, by their being within their expected matrix position, and their square shape. See Figure 5.7 for examples.

Figure 5.7 ECL detection of PGMY/HLA amplicons from the samples shown in Figure 5.4



The top lane contains a mixture of positive control amplicons to identify the probe position. [Signals vary as amplicons were not adjusted to give the same intensity]. Numbers down the right side indicate sample number and $MgCl_2$ concentration (1=1.5 mM, 3= 3 mM). Sample 6 is interpreted as positive for type 51 and 53 despite the absence of HLA detection. The impact of $MgCl_2$ on amplification is illustrated by samples 10, 12, 15 and 16. Types 6, 31, 33, 34, 39, 42 and 56 amplified better with 3 mM $MgCl_2$, while types 83 and 72 (identified by DNA sequencing) amplified better with 1.5 mM $MgCl_2$.

5.5.9 Remarks

- To exclude handling errors, discard tubes containing samples only after making sure that their position on the developed film corresponds to their position in the rack.
- Discard EDAC in the non-chlorinated chemical waste.
- Prepare and validate a new membrane when membrane has been used 7–8 times.
- Foam pads are expensive. It is possible to reuse them after washing with water and letting them recover their original shape for a few days. **Do not reuse pads that exhibit channel fingerprints.**

5.6 Quality control of PGMY PCR and RBH

The large number of reagents and steps involved in this procedure necessitates a pragmatic approach to quality-control testing and troubleshooting in case of failure. Whenever primers or probes are suspected in a procedure failure, replace the whole set with a new validated aliquot instead of trying to identify the faulty reagent. Reagents and key steps of the procedures that have been associated with failures are:

- cross-contamination of probes at time of manufacture;
- mixing PGMY11 non-biotinylated primers with biotinylated ones;
- faint signals associated with a bad lot of ECL® detection reagent or streptavidin peroxidase (this may occur despite storage as recommended by the manufacturer, in which case the manufacturer should be contacted for troubleshooting and reagent replacement as the ECL® detection kit in particular is expensive);
- drying membrane, even partially;
- not washing the miniblotter with H_2O_2.

5.6.1 Quality-control procedures

5.6.1.1 New primers, PCR reagents and filters

- Perform PGMY PCR up to gel electrophoresis with positive control DNAs (HPV plasmids or previously-verified typed clinical samples) covering the whole spectrum of types on the filter. Use an old, validated lot of primers as reference.
- Hybridize the amplicons to a validated filter (for new PGMY stocks assessment) or to a new filter (for filter/probe assessment with validated PGMY primer stocks).
- Verify the position and hybridization signal on the filter using the HPV probe array scheme.

5.6.1.2 Continuous monitoring with validated reagents

- Verify the positive/negative signal of the positive and negative controls.
- Verify the HLA and overall HPV signal strengths after hybridization.

5.6.2 Interpretation / corrective measures

5.6.2.1 New primers and PCR reagents

- All HPV reactions must be positive after gel electrophoretic analysis. The amplification signal of each sample should be at least as strong as that obtained with the reference primers to validate the new ones. If only the validated lot worked, then the new lot of primers cannot be used. The primer mix must be prepared again. In some instances, this means getting new oligos from the manufacturer. If reactions failed with new lots of PCR reagents, then each component can be investigated individually using a single HPV positive control (C1000), and changed accordingly.
- All HPV reactions that passed the gel electrophoretic test must be positive after hybridization, with corresponding signal strengths. If this is not the case, the biotinylated PGMY11 primers may be the cause, and new primers should be ordered.
- Probe cross-contamination is not acceptable. If suspected, it should be proven by using a set of known, well validated type-specific controls (5.5.4.1). Filters with probe cross-contamination must be discarded. The probes will need to be re-synthesized by the manufacturer if contamination did not occur during construction of the filter.

5.6.2.2 New filters

- Verify that each type represented on the filter is detected with signal that parallels that of the gel image, and that each corresponds to the expected probe on the filter. If signal is too weak and ECL® reagents and streptavidin peroxidase are valid, then the probe concentration must be checked and/or new probes should be synthesized. If the probe distribution is not as expected, the filter must be discarded and a new one prepared after making sure that the probe labels on the tubes are correct.
- Background corresponding to non–specific HPV types should be investigated, taking into account the possibility of probe / positive control DNA cross-contaminations in the laboratory. A new aliquot of known, validated probes, and a set of known, validated type-specific controls, should be used to prepare and validate a new filter.

5.6.2.3 Continuous monitoring

- The PCR control down to 100 HPV DNA GE must always be positive by gel electrophoresis, and the negative controls must always be negative to validate a run. The 10 GE positive control is often weakly positive by gel electrophoresis; sometimes in a stochastic fashion. Using such low signal reactions as control in RBH can be useful to monitor ECL® detection of weak positives.
- The HLA and the overall HPV signal strengths after hybridization must reflect that of gel electrophoresis, and fit with those of previous hybridizations. If not, the ECL® reagents may be the cause, especially the peroxidase, that should never be used beyond its expiration date.

- Weak, non-specific hybridization background parallel to the channel may be tolerated if interpretation of the signal is obvious; otherwise the filter must be discarded.
- Negative controls must, of course, always be negative. Reactions with non-specific background bands near that of authentic HPV amplicons can be observed, in particular with 3 mM $MgCl_2$. These should be evaluated for specificity by RBH and/or DNA sequencing to exclude contamination.

5.7 References

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6. Assay validation

6.1 Introduction

In view of the variety of HPV DNA detection and genotyping assays being used by laboratories worldwide, this manual provides guidance that focuses on the validation of HPV nucleic acid-based assays.

Assays for the detection of HPV DNA must be fit for their intended purpose and be validated for use with the specified samples, i.e. cervical swabs and/or biopsies. The analytical procedure is defined as the complete procedure from start of sample collection through extraction of nucleic acid to completed HPV typing.

Validation is the process of establishing documented evidence to provide a high degree of assurance that a procedure will consistently perform as intended, that is, to demonstrate that a procedure is suitable for its intended purpose. Separate validations are required for different elements of a procedure (e.g. the equipment and reagents used in a test may need validation as well as the test as a whole).

For HPV DNA detection assays all steps of the process must be validated, including sample preparation, DNA isolation, and amplification and detection. Assay validation must be demonstrated for each sample type tested, e.g. cervical swabs, biopsies, urine. The method must also demonstrate lack of interference/ PCR inhibition for all types of samples being tested. The inclusion of an internal control in the material to be extracted will validate assays on individual samples.

Established methods, e.g. tests described in WHO manuals or requirements or pharmacopoeias, can be considered as having been validated. Commercial assay kits which are CE (Conformité Européene) marked, indicating conforming to essential requirements or performance measures of the European Directives on health, safety and environment) and/or meet ISO13485 (quality management standard for medical devices published by the International Organization for Standardization in 2003), have undergone validation. When such tests are used, each laboratory needs to demonstrate satisfactory performance and fitness for use by verification of performance and suitability for its purpose under actual conditions of use in the individual laboratory concerned.

In-house tests need full validation. Appropriate parameters must be assessed as not all are relevant to every test procedure. Once a procedure has been validated, it is expected that it will continue to perform as validated, but this needs to be checked by monitoring test performance. If modifications are made to the method, or problems occur or equipment is changed or relocated, revalidation needs to be performed.

Parameters to be examined will depend on whether the assay is qualitative or quantitative. These may include the following.

- **Sensitivity/limit of detection:** the proportion of samples that contain a specific HPV type that are reported to contain that type or lowest detectable IU (GE) of virus/PCR.
- **Specificity**: the proportion of reported detections of an HPV type where the reported HPV type was truly present in the sample.
- **Accuracy**: the ability to estimate the correct amount of a substance in a sample.
- **Reproducibility**:the ability to obtain the same results on repeat analysis. Reproducibility may be assessed for intra- or inter-lot changes of reagents within the same, or between different techniques or laboratories.
- **Robustness**: a measure of the ability of an assay to tolerate changes in methodology without affecting results, e.g. whether allowed deviations in incubation time will affect results.
- **Interference**: a measure of whether some substances in clinical samples interfere with analysis, e.g. by PCR inhibition. Interference may be assessed by spiking samples with potentially interfering substances, such as crude cell lysate, urine, or transport medium.

Where commercial kits are used for part of, or the complete analytical procedure, some documented validation points already covered by the kit manufacturer can substitute for the validation by the user. Nevertheless, the performance of the kit with respect to its intended use is, in most cases, the result of the kit used plus the performance of the laboratory. The performance of the kit when used in a particular laboratory has to be demonstrated by the user (e.g. precision, accuracy, range, robustness) when the kit is first introduced. Lot-to-lot variation of kit performance is then monitored by routine quality assurance/quality control (QA/QC).

General guidance on assay validation is given in an International Committee on Harmonization (ICH) note for guidance on validation of analytical procedures (*1*). In addition, the European Pharmacopoeia has developed monographs on nucleic acid amplification techniques (*2*) and a specific monograph on the *Validation of nucleic acid amplification techniques (NAT) for the detection of hepatitis C virus (HCV) RNA in plasma pools: guidelines* (*3*) that is also of relevance to validation of HPV assays.

6.2 Quality assurance specific for HPV DNA testing

6.2.1 Documentation

For biological tests, such as nucleic acid testing, specific problems may arise that may influence both the validation and the interpretation of results. All procedures should be documented including methods and reporting forms. These should be assigned a version number and be authorized before use. If any changes are made to the document, a new version must be created and the previous version archived. The SOPs should cover:

- mode of sampling (type of container, etc.);
- preparation of mini-pools (where appropriate);
- conditions of storage before analysis;

- an exact description of the test conditions, including precautions taken to prevent cross- contamination or destruction of the viral DNA, reagents and reference preparations used;
- an exact description of the apparatus used;
- the detailed formulae for calculation of results, including statistical evaluation.

6.2.2 Operator training and qualification

An appropriate operator qualification programme should be implemented for each operator involved in the testing. To confirm successful training, each operator should test at least eight replicate samples of diluents/matrix spiked with HPV DNA to a final concentration of three times the assay detection limit. This test (eight replicate samples) should be repeated twice on two separate days, i.e. a total of 24 tests performed on three different days. The tests should be performed using a properly calibrated and controlled assay. All replicate results should be positive.

6.2.3 Equipment qualification

An appropriate installation and operation qualification programme should be implemented for each critical piece of the equipment used. Confirmation of analytical procedure performance after change of critical equipment (e.g. thermocyclers) should be documented by conducting a parallel test on eight replicate samples of diluents/matrix or negative clinical sample spiked with HPV DNA to a final concentration of three times the assay detection limit. All results should be positive.

6.3 Issues to be considered during performance of an assay

6.3.1 Prevention of contamination

The risk of contamination requires a strict segregation of the work areas for the different steps in the procedure. Points to consider include: movement of personnel; gowning, cleaning, material flow and air supply, and decontamination procedures.

The work area should be sub-divided (see section 3.1.4) into compartments such as:

- master mix area or “clean” (area where exclusively template-free material is handled, e.g. primers, buffers, etc.);
- pre-PCR or “extraction” (area where reagents, samples and controls are handled);
- PCR amplification or “apparatus” (amplified material is handled in a closed system);
- post-PCR or “detection” (the only area where the amplified material is handled in an open system).

6.3.2 Quality control of reagents

All reagents crucial for the methodology used have to be controlled prior to use in routine applications. Their acceptance/withdrawal is based on pre-defined quality criteria.

Primers are a crucial component of the PCR assay and, as such, their design, purity and the validation of their use in a PCR assay require careful attention. Primers may be modified (for example, by conjugation with a fluorophore or antigen) in order to permit a specific method of detection of the amplicon, provided such modifications do not affect accurate and efficient amplification of the target sequence.

6.3.3 Inclusion of run controls

The use of a suitable run control (for example, an appropriate dilution of an HPV working standard/run control or an HPV sample calibrated against the appropriate WHO HPV international standard (IS), NIBSC code 06/202 (HPV16 DNA) or 06/206 (HPV18 DNA) can be considered a satisfactory system for continuous monitoring of performance.

Each run should contain:

- positive controls; these contain a defined amount of the HPV types to be tested. The amount of plasmid to include should correspond to the required detection limit for approval of a test run as a valid test; typically 50 IU of HPV16 DNA, 50 IU of HPV18 DNA and 500 GE, per 5 µL of other HPV types tested for.
  - Inclusion of a second "low positive" control that contains even lower amounts of virus and does not affect classification of a test as valid, can be useful for sensitive monitoring of fluctuations in assay sensitivity.
- Negative control: a sample of a suitable matrix already proven to be free of the target sequences.

6.3.4 Determination of the validity of an individual assay

A valid result is obtained within a test only if the positive control(s) included in the same test run is unambiguously positive, and the negative control(s) is unambiguously negative. The data review should also include a "common sense" evaluation for signs of contamination, such as a weak positive in well adjacent to strong positive, or unusual run of the same result.

A strategy for handling an ambiguous test result should be documented. This may include re-extraction and assay of the sample, re-amplification of the extracted DNA using another assay, DNA sequencing, or submission of the sample to a reference laboratory.

6.3.5 External quality assessment

Participation in external quality-assessment programmes (proficiency panel testing and/or organized referral of part of the analysed samples to a reference laboratory), is an important PCR quality-assurance procedure for each laboratory and each operator.

6.4 Test material

Because of the high sensitivity of PCR, the samples must be protected against external contamination with target sequences. Sampling, storage and transport of the test material are performed under conditions that minimize degradation of the target sequence.

When preparing samples, the target sequence to be amplified needs to be efficiently extracted in a reproducible manner. A variety of physico-chemical extraction procedures and/or enrichment procedures may be employed. The selected extraction procedure should have minimal loss of viral DNA (that is, have high recovery) and result in samples with sufficiently pure DNA to not cause inhibition of PCR reactions. PCR tests for a common housekeeping gene (typically the betaglobin gene), may be used as a control for extraction of amplifiable material without significant inhibitors originating from the test material.

6.5 Validation of an assay

The validation of an assay described here is based on the principles detailed in the ICH guidelines. This will include for both *qualitative* and *quantitative* assays:

- specificity;
- detection limit;
- robustness;
- precision.

In addition, for *quantitative* assay systems the following parameters should also be determined:

- accuracy;
- linearity;
- analytic range.

6.5.1 Validation parameters

If the assay is to be used on different types of samples (e.g. human cells, tissue, blood, plasma, or urine) each type of sample should be validated for the following criteria.

6.5.1.1 Specificity

Specificity is the proportion of reported detections of an HPV type where the reported HPV type was truly present in the sample. Specificity may depend on components that can be present in the sample, e.g. cross-reacting HPV types, bacterial infections and human DNA. The specificity of nucleic-acid amplification analytical procedures is dependent on the choice of primers, the choice of probe for analysis of the final product, and the stringency of the test conditions (for both the amplification and detection steps).

In order to validate the specificity of the analytical procedure, at least 100 samples negative for the HPV type in question should be tested and shown to be non-reactive. The use of plasmid preparations containing potentially cross-reacting HPV types, added to human HPV-negative cell lines and/or to clinical samples known to be HPV-negative, is recommended for assessing specificity. Proficiency panels, such as those prepared by, and available to, the WHO HPV LabNet, may be used to assess specificity. Specificity assessments should also consider that subtypes and variants of HPV may be present in clinical samples.

6.5.1.2 Detection limit

The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample which can be detected (but not necessarily quantified as an exact value).

The detection limit is, for practical purposes, estimated as the minimum amount of HPV DNA per sample which can be detected in 95% of test runs. To determine an assay's detection limit, which may vary in individual analytical test runs, a dilution series of a working reagent, which has been calibrated against the WHO HPV DNA IS, should be tested on at least three different days. At least 24 test results for each dilution is required for statistical analysis of the results. For example, a laboratory could test three dilution series on different days with eight replicates for each dilution, four dilution series on different days with six replicates for each dilution, or six dilution series on different days with four replicates for each dilution. In order to minimize the number of required dilutions, a preliminary test (using, for example, log dilutions of a standard spiked in a diluent/matrix or in a negative clinical sample) could be done in order to obtain a preliminary value for the detection limit (simply the highest dilution giving a positive signal). The range of dilutions can then be chosen around the preliminary detection limit (using, for example, a dilution factor of 0.5 log or less). The concentration of HPV DNA which can be detected in 95% of test runs can then be calculated using standard statistical methods.

The same set of experiments may also be used to demonstrate the intra-assay variation and the day-to-day variation of the analytical procedure.

Note: Analytical sensitivity is synonymous with detection limit. Clinical sensitivity refers to the proportion of samples with disease that are detected by the assay. Clinical sensitivity is dependent on the analytical sensitivity, but may also be affected by the presence of subtypes and variants of the type that is tested for (e.g. mutations in primer target sequences) and by the patient population studied (e.g. patients with CIN having higher viral load). Estimating clinical sensitivity is important for HPV tests used for cervical screening (4), whereas estimation of analytical sensitivity is particularly important for tests used in vaccine trials and in surveillance.

6.5.1.3 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by allowed variations in method parameters, and provides an indication of its reliability during normal usage.

Evaluation of robustness should be considered during the development phase. For nucleic-acid amplification technique (NAT), small variations in the method parameters can be crucial. Examples of critical parameters include the exact concentrations of reagents (e.g. $MgCl_2$, primers or dNTP) and incubation times and temperatures. When data estimating how small variations in such parameters affect detection limit are known, they should be explicitly stated in the SOP. To estimate robustness, the detection limit is estimated (as above) using the maximum and minimum range of allowable variations in method parameters.

6.5.1.4 Absence of cross-contamination

Cross-contamination prevention should be demonstrated by the accurate detection of a panel of at least 20 samples consisting of alternate negative samples and negative samples spiked with high concentrations of HPV (at least 10^2 times the detection limit or at least 10^4 IU/mL).

Known negative samples (e.g. water controls) should always be processed together with test samples, throughout sample treatment and analysis, for all analytic runs.

6.5.1.5 Accuracy

Accuracy expresses the closeness of agreement between the reported amount and the true amount (in an accepted reference standard). Accuracy must be established for all quantitative assays. It is recommended to establish the accuracy across the range of amount of analyte that can be assayed. If test results exceed the maximum for which accuracy has been established, the sample needs to be re-analysed after dilution so that the analyte falls within the concentration range for which accuracy is established.

Accuracy is assessed by assaying dilutions (dilution factor of 0.5 log) of a working standard calibrated in IU against the WHO HPV DNA IS (or the IS itself).

6.5.1.6 Precision

Precision expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample. The precision is defined at three levels.

- Intra-assay precision expresses the precision under the same operating conditions over a short interval of time; it is assessed by using one assay and testing three replicates of appropriate dilutions of an HPV DNA-positive sample suitably calibrated in IU and covering the whole quantitative range of the assay; the coefficient of variation for the individual samples is calculated.
- Inter-assay precision expresses the within-laboratory variations; it is established by assaying replicates (as routinely used for the assay) of appropriate dilutions of an HPV DNA-positive sample suitably calibrated in IU covering the whole quantitative range of the assay under different circumstances (e.g. different days, different personnel, different equipment, different reagents); the coefficient of variation for the individual samples is calculated.

- Inter-laboratory precision (reproducibility) expresses the precision between different laboratories; it is assessed by participation in collaborative studies on HPV DNA assays, e.g. under the WHO HPV LabNet Proficiency Testing Scheme (PTS) or by confirmatory testing in reference laboratories.

6.5.1.7 Linearity

A quantitative assay is considered to be linear when the measured value of an analyte from a series of sample dilutions is linearly proportional to the actual concentration or content of the sample. Assessing the linearity of an assay method should not be confused with that of calibration verification. Instrument calibration uses reference standards to establish a relationship between known concentrations and measured values. The linearity of an assay may be evaluated by using biological samples spiked with analyte. The linearity assessment must be conducted on a calibrated and quality-controlled instrument. Five or more analyte concentrations are necessary, covering the full range of the analyte tested. At least two independent replicate samples should be run in random order. Linearity may be evaluated by plotting the measured values as a function of analyte concentration or content using appropriate statistical methods (*5*).

After an assay has been validated, periodic verification of linearity is good laboratory practice, as non-linear results may indicate reagent or instrument deterioration.

6.5.1.8 Analytic range

The range of an analytical procedure is the span of the upper and lower concentrations of analyte in a sample for which it has been demonstrated that the procedure has a suitable level of precision, accuracy and linearity. The analytic range of an assay is normally derived from linearity studies.

6.6 References

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7. HPV serology — ELISA assay

7.1 Introduction

Antibodies against HPV are a marker of prior exposure to the virus. The detection of antibodies in serum, serology, can be used to study the epidemiology of HPV. Another important use of serology is to test the immunogenicity of vaccines. This section outlines the principles of ELISA assay for HPV-specific antibodies and the requirements in terms of equipment, reagents and procedure.

There are several variations of the ELISA method, such as direct assays that bind HPV VLPs directly on the microplate (Fig.7.1), indirect assays binding HPV VLPs to the microplate via heparin or via anti-VLP antibodies, as well as competitive assay formats and assays that do not use microplates but antigen coated on fluorescent beads (Luminex-based assays). The procedure described below is the simplest and most widely-used version of the HPV VLP-based ELISA and has been evaluated in the WHO HPV LabNet. This description should be regarded as an example of a workable method. Laboratories may choose to perform another serological method or alternative procedure that performs well and is validated according to established quality indicators, if so desired.

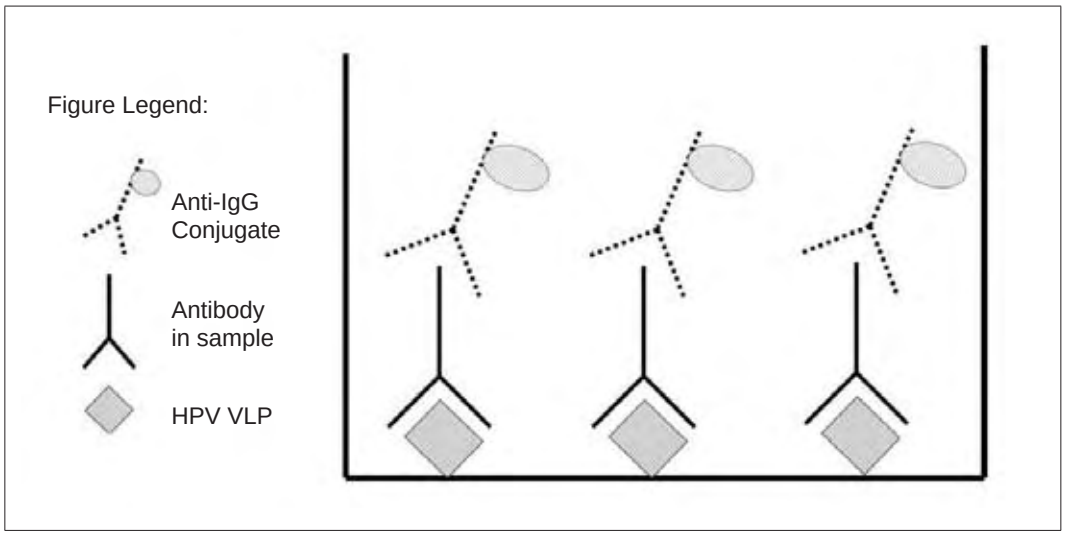
7.2 Principle

VLPs, self-assembled HPV L1 protein capsids that resemble intact virions, are used as antigen in the assay. These VLPs present type-specific conformational epitopes and are the key reagent in determining assay sensitivity and specificity. Human serum samples are allowed to react with the antigen. If HPV type-specific antibodies are present, they will bind to the VLPs adsorbed onto the surface of the well. Bound human immunoglobulin G (IgG) is detected with a secondary antibody conjugated to horseradish peroxidase (HRP). A colorimetric substrate allows wells with HPV antibody-positive samples to be identified by reading the optical density.

Each serum sample can be tested against one or several different HPV types. For each serum sample a negative antigen control is useful, as some sera may be “sticky” and react with uncoated wells. Human antibodies reacting with epitopes of HPV that are not type-specific and exposed on disrupted capsids have been described, although they are not common. Disrupted bovine papillomavirus (BPV) VLPs are useful as a negative control antigen for non-type-specific reactions. If BPV VLP is not available, a plate coated with only PBS should be used as a control plate. Three serial dilutions of each serum sample is assayed (e.g. half-log dilutions; 1/10, 1/31.6 and 1/100), and, along with appropriate serial dilutions of the reference serum sample is tested on each plate.

The amount of antibodies in the sample is estimated relative to the reference serum using the parallel-line method. Samples are also scored as positive or negative using a predetermined "cut-off" level. This system gives good reproducibility (coefficient of variation below 15%) of samples tested on different plates and at different times.

Figure 7.1: Diagram of HPV VLP ELISA



7.3 Equipment, supplies and reagents

7.3.1 Equipment

- Refrigerator at 4 °C to 8 °C
- Freezer at -20 °C
- Microplate reader with a 405 or 415 nm filter
- Microplate washer (optional)
- Adjustable pipettes 10–200 µL
- 8 or 12-channel multi-channel pipette 50–200 µL or equivalent system
- Pipet-aid
- Laboratory micro balance
- Computer with Microsoft Excel® software

7.3.2 Supplies

- Disposable pipette tips
- Sterile transfer pipettes
- Half-area 96-well microplates [e.g. Cat. #3690, Corning, USA]
- 0.45 µm syringe filter
- Syringe
- Funnel
- 3MM Whatman filter paper

- Microfuge or dilution tubes
- Racks for microfuge /dilution tubes
- 50 mL tubes
- Paper towels
- Adhesive plate seals
- Gloves, lab gown, protective eyewear
- Biohazard discard bag/pan
- Ice bucket (optional)

7.3.3 Reagents

In order to provide a detailed SOP, catalogue numbers of materials found to perform well by the HPV LabNet are provided. A laboratory may use alternative products if they can be validated to yield equivalent results.

> **Note:** HPV VLPs are a key reagent for this ELISA assay. This manual does not provide a protocol for their preparation as most HPV laboratories will not be able to prepare VLPs. As there are currently no vendors for HPV VLPs, WHO plans to identify reliable sources of these reagents for HPV LabNet laboratories. The VLP QC method is provided so that laboratories can evaluate quality of VLPs that they prepare themselves, or obtain from sources other than WHO.

- HPV VLPs as test antigen (no commercial source)
- BPV VLP as control antigen (optional, no commercial source)
- Reference reagent [in the case, of HPV16 it should be traceable to the WHO IS Cat. # 05/134, NIBSC, United Kingdom]
- Polyclonal rabbit anti-human IgG/ horseradish peroxidase (HRP) gamma-chain specific [Cat. #P0214, DakoCytomation, Denmark]
- Monoclonal antibody [e.g. H.16 V5, H.18 J4 and H.16 D9, NIBSC, United Kingdom] (needed only for VLP QC)
- Goat anti-mouse IgG-HRP [Cat. #1030-05, Southern Biotech, Atlanta, GA, USA] (needed only for VLP QC)
- Sodium carbonate (Na_2CO_3)
- Sodium bicarbonate ($NaHCO_3$)
- Sodium phosphate dibasic dihydrate ($Na_2HPO_4.2H_2O$)
- Potassium phosphate dibasic (K_2HPO_4)
- Sodium chloride (NaCl)
- Dithiothreitol (DTT) [Sigma D-9163] (needed only for VLP QC)
- Horse serum [Cat. #H1270, Sigma, USA]
- Tween 20 (Polyoxyethylene sorbitan monolaurate)
- ABTS ($C_{18}H_{16}N_4O_6S_4$-$(NH_4)_2$) [Cat. #102946, Roche, USA]
- Citric acid monohydrate

- Sodium citrate dihydrate
- Hydrogen peroxide (H_2O_2)
- Phosphoric acid
- Distilled water
- Ice (optional)

7.3.4 Preparation of reagents

- Carbonate buffer (0.1M, pH 9.6): dissolve 1.65 g Na_2CO_3 and 3.14 g $NaHCO_3$ in 500 ml distilled water. Store at 4 °C for up to six months [provided it is kept sterile].
- 0.2 M Carbonate 10 mM dithiothreitol (DTT) buffer (pH 10.6): dissolve 2.12 g Na_2CO_3 in 100 ml distilled water. Add 0.154 gm DTT. Prepare at time of use (needed only for VLP QC).
- Phosphate buffered saline (PBS), pH 7.2: dissolve 7.65 g NaCl, 1.85 g $Na_2HPO_4.2H_2O$ and 0.06g K_2HPO_4 in 1 L distilled water. Adjust pH to 7.2 using 10% phosphoric acid. Store for three months at 4 °C [provided it is kept sterile].
- Wash buffer (PBS-0.1% Tween 20 (PBS-T)): add 1 mL of Tween20 to 1 L of PBS. Store for three months at 4°C [provided it is kept sterile].
- Horse serum (HS): filter the serum through a funnel with 3mm Whatman filter paper. Aliquot in 50 mL tubes and store at -20 °C for up to one year.
- Blocking and dilution buffer (10 % HS in PBS (HS-PBS)): filter 5 mL HS through 0.45 µm syringe filter. Add to 45 mL PBS at RT for 50 mL of HS-PBS. Store at +4°C up to one month.
- Citrate buffer (0.1M, pH 4.0): dissolve 6.62 g citric acid monohydrate and 5.44 g sodium citrate dihydrate in 1 L distilled water. Store at RT for up to three months [provided it is kept sterile].
- 50X ABTS: dissolve 20 mg of ABTS in 1 mL distilled water. **This should be prepared fresh every day and stored on ice or in a refrigerator until use.**
- HRP substrate: add 240 µL of 50X ABTS and 3.6 µL of 30% H_2O_2 to 12 mL of 0.1 M citrate buffer for two plates. **Prepare the substrate working solution immediately before using it.**

7.3.5 Safety

Blood products are potentially infectious and should be handled under BSL-2 conditions. Gloves, gowns and protective eyewear should be worn during collection and processing.

7.3.6 Good laboratory practice

To avoid problems with the ELISA assay observe the following.

- Do not perform the test in the presence of reactive vapours (i.e. from acids, alkalis or aldehydes), or dust, since this may affect the enzymatic activity of the conjugate.
- Use only thoroughly cleaned glassware, particularly for preparing substrate solutions.
- All pipetting steps should be performed with the utmost care and accuracy.
- To avoid contamination, do not touch the top of the plates or strips with your fingers, and do not touch the walls or bottom of the wells with the pipette tips when adding conjugate or substrate.
- Check for air bubbles after all pipetting steps; if present, remove by gentle tapping.
- Check efficacy of your washing procedure by visually inspecting that there is no residual liquid in the wells, especially after incubation with the conjugate.
- If wells cannot be filled immediately after washing, the plates and strips may be placed upside down on wet absorbent material for no more than 15 minutes.
- Solutions of peroxide should not come into contact with metals or metal ion, since this may give rise to unwanted colour formation.
- Be extremely cautious with the preparation of 10% phosphoric acid. Acid should always be added to water and never the reverse. Wear protective clothing and eyewear.

7.4 Test procedure

The protocol described is for the use of half-area 96-well microplates. Half-area plates can be used to save VLPs, serum and reagents. **If using standard 96-well microplates for the assay, double the volumes of antigen and reagents used in each step of the procedure described below.**

7.4.1 Controls

- An IS for HPV16 antibodies, assigned 5 IU/ampoule has been established by the WHO Experts Committee on Biological Standardization (ECBS), and is available from NIBSC.
- Each laboratory may use its own secondary standard serum to calculate units. The serum should have been tested in parallel with the IS serum to enable traceability to the same IU. The standard serum should be tested in three half-log dilutions, 1/10, 1/31.6 and 1/100 on each plate.
- When other HPV VLPs than HPV16 are tested, each laboratory needs to establish its own reference standard, as an international reference reagent is not yet available. Preparation of a reference serum pool positive for all the types to be tested (e.g. HPV6, HPV11, HPV16, HPV18) can be useful.

Day 1

7.4.2 Planning

- Make a plate layout with the sample ID, and reference serum according to Figure 7.2.

Figure 7.2: ELISA plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Sample1 1/10	Sample1 1/31.6	Sample1 1/100	Sample7 1/10	Sample7 1/31.6	Sample7 1/100	Sample13 1/10	Sample13 1/31.6	Sample13 1/100	Ref. Sample 1/10	
C		Sample2 1/10	Sample2 1/31.6	Sample2 1/100	Sample8 1/10	Sample8 1/31.6	Sample8 1/100	Sample14 1/10	Sample14 1/31.6	Sample14 1/100	Ref. Sample 1/10	
D		Sample3 1/10	Sample3 1/31.6	Sample3 1/100	Sample9 1/10	Sample9 1/31.6	Sample9 1/100	Sample15 1/10	Sample15 1/31.6	Sample15 1/100	Ref. Sample 1/31.6	
E		Sample4 1/10	Sample4 1/31.6	Sample4 1/100	Sample10 1/10	Sample10 1/31.6	Sample10 1/100	Sample16 1/10	Sample16 1/31.6	Sample16 1/100	Ref. Sample 1/31.6	
F		Sample5 1/10	Sample5 1/31.6	Sample5 1/100	Sample11 1/10	Sample11 1/31.6	Sample11 1/100	Sample17 1/10	Sample17 1/31.6	Sample17 1/100	Ref. Sample 1/100	
G		Sample6 1/10	Sample5 1/31.6	Sample6 1/100	Sample12 1/10	Sample12 1/31.6	Sample12 1/100	Sample18 1/10	Sample18 1/31.6	Sample18 1/100	Ref. Sample 1/100	
H												

The outer wells of both the test and control plate are not used. The three dilutions of the reference serum sample are added in wells B11–G11 in duplicate. The three dilutions of the first serum sample are added to wells B2, B3 and B4; the second sample to wells C2, C3 and C4, and so on. Each plate can test 18 unknown samples and one reference sera. An identical plate layout is used for the control plate (uncoated or coated with control antigen).

7.4.3 Coating

- Calculate the volume of cold PBS needed for 50 µL per well of each antigen.
- Calculate the amount of VLP needed. **Note:** The coating concentration of VLP required varies between different VLP preparations. The procedure to establish the coating concentration is described below in section 7.6.
- Disrupt BPV VLP to be used as control antigen by adding the same amount of VLPs as for the test plate from the stock solution to a volume of 500–1000 µL in 0.1 M carbonate buffer (pH 9.6). Incubate at RT (24 ºC± 2) for 4 h.
- Dilute the disrupted BPV VLP to the same concentration as the VLPs on the test plate in 0.1 M carbonate buffer (pH 9.6). Add 50 µL to each well of the control plate. Cover the plate with adhesive plate seal and leave overnight at 4°C. **Note:** As an alternative, if BPV VLPs are not available, omit the VLP steps and add 50 µL of cold PBS to each well of the control plate.
- Dilute the stock of HPV VLPs to be used as test antigen at the required concentration in cold PBS. Add 50µL to each well of the test plate. Cover the plate with adhesive plate seal and leave overnight at 4°C.

Day 2

Perform all steps noted below for both the test and control plate. Warm reagents to RT prior to use. If you have more than one plate, first wash all plates once, and then wash all plates a second time, and so on for the required number of wash steps.

7.4.4 Blocking

- Discard the VLP solution and wash the plate once by adding 150 µl PBS-T to each well using a multi-channel pipette or in a microplate washer. Empty the plate into a biohazard pan and tap it thoroughly against paper towels. Wait one minute and tap once more, to ensure there is no residual liquid in the wells.
- Add 150 µL/well of blocking buffer (HS-PBS) and incubate for 1 h at RT (24 ºC± 2).
- Discard the blocking buffer. Tap the plate thoroughly against paper towels. Wait one minute and tap once more.

> **Note:** It is possible to coat and block all the plates necessary for a certain study and, at this stage, store the plates at -20°C with the wells filled with 200 µL PBS. Thaw before use. Discard PBS and tap against paper towels.

7.4.5 Serum

- Thaw serum samples on ice or in the refrigerator.
- Dilute the serum samples 1/10, 1/31.6 and 1/100 in HS-PBS, e.g. by taking 20 µL serum to 180 µL HS-PBS (1/10); from this dilution take 63 µL to 137 µL HS-PBS (1/31.6), and finally from this dilution take 47 µL to 103 µL HS-PBS (1/100). This volume should be enough to test one sample with two antigens.

- Add 50 µL per well of human serum diluted in a serial dilution at 1/10, 1/31.6 and 1/100 in HS-PBS according to the plate layout. Each serum dilution should be added to both the test and control plate. Incubate for 2 h at RT (24 ºC ± 2). **The reference serum should be tested in duplicate at the same dilutions as the sample.**
- Discard the serum into a biohazard pan, and wash the plates by adding 150 µL of PBS-T per well with a multi-channel pipette or in a microplate washer. Discard wash buffer and tap the plate against paper towels. Repeat wash step for a total of five washes. Tap the plate thoroughly against paper towels. Wait one minute and tap once more.

Note: It is possible to stop at this step. Store the plates with 100 µL PBS-T per well at 4°C and continue the next day.

7.4.6 Anti-human IgG HRP

- Add 50 µL per well of anti-human IgG-HRP diluted 1/1000 in HS-PBS to all plates. Incubate at RT for 1 h. **Avoid air bubbles in the wells. Different commercially- available conjugates differ greatly in performance. The present method is only validated for the conjugate indicated. Use of other conjugates would require titration and assessment for gamma-chain-specificity under these conditions, and separate validation of the assay.**
- Discard the antibody solution and wash the plate by adding 150 µL of PBS-T per well. Discard wash buffer and tap the plate against paper towels. Repeat wash step for a total of five washes. Tap the plate thoroughly against paper towels. Wait one minute and tap once more.

7.4.7 Substrate

- Add 50 µL per well of peroxidase substrate to all plates. Incubate plate at RT for 30 min. **Avoid air bubbles. This is especially important at this step, since bubbles will cause an incorrect absorbance reading.**
- Before reading the plates, shake them carefully as most of the colour will develop at the edge of the wells and the colour should be dispersed over the well to give a correct reading. Wipe the bottom of each plate with a paper tissue cloth to ensure that there is no dirt or moisture that could affect the reading.
- Read plates at 415 nm in a microplate reader. If a 415 nm filter is not available, use a 405 nm filter.
- Two of the dilutions of the reference serum must have an absorbance value above 0.1 to be eligible for use in calculations of the parallel line (PLL) units (see below). If reactivity is too weak after 30 min, the microplate may be read again after 60 min.

7.4.8 Calculation of results

Once the test and control microplates have been read by the microplate reader, the results are exported to Microsoft Excel® for data analysis using the PLL method. A simple programme written in Excel® is used to calculate the PLL values. Such programmes are available in most serological reference laboratories and can be requested from any member laboratory of the WHO HPV LabNet. Programmes of performing PLL calculations are also commercially available, e.g. at www. combistat.com.

7.4.9 Definitions

- Optical density (OD): The absorbance value read by the microplate reader.
- Cut-off value: The PLL value that tells which samples are positive. All values higher than or equal to cut-off are positive.
- PLL value (given in IU, in the case of HPV16): The amount of antibody present in the test serum, relative to the reference standard serum used (the standard serum should have a unit of antibody level assigned to it).
- Correlation: The correlation between the three dilutions. The closer to one, the better the correlation. Correlation is acceptable if it is > 0.9.
- Slope: The downward slope of the three dilutions. The slope is acceptable if the absolute value is >0.4.
- Slope ratio: The ratio of the slope of the standard and the test serum. The slope ratio is acceptable if >0.5.
- Data points: Shows how many data points are outside the linear part of the dilution curve for the serum tested. The serum should not have more than one data point outside the linear range.

7.4.10 How to calculate the results if using PLL shareware programme

- Export absorbance values to a spreadsheet in Microsoft Excel®.
- Calculate final absorbance value for each sample dilution by subtracting the absorbance of the serum on the control plate from that on the test plate. Most sera should have absorbance <0.1 on the control plate, but occasional "sticky" sera may have high values.
- Access the PLL programme and enter the final mean absorbance values of the three dilutions of reference serum in the field indicated in yellow in columns B, C and D of the spreadsheet. Write the sample ID in column A and the final absorbance values of each dilution in corresponding columns B, C and D. As the PLL shareware can only use integers, multiply the mean absorbance values by 1000. The programme will calculate the PLL value and the correlation between data points.

7.4.11 How to interpret your results

- Cut-off values for PLL values/WHO units should be assigned prior to testing. See note below.
- PLL values above the assigned cut-off, if the serum has a correlation >0.9, are considered positive.
- Continue interpretation by evaluating correlation, slope, slope ratio and data points (see definitions above). If a sample does not fulfill the quality criteria it has to be retested.
- If the reference serum has a correlation and/or slope that does not fulfill the criteria, the entire plate has to be retested.
- All samples with a correlation less than 0.9 are retested.
- If the absorbance values of the second or third dilution are higher than the OD value of the first dilution (pro-zone phenomenon), the sample should be diluted further in the retest.
- Samples that have a data point outside cut-off level should also be retested.
- Negative samples with all absorbance values below 0.1 are not retested.
- Pre-assigned cut-off values for HPV serology should be based on ELISA units calculated by the PLL method using the HPV reference reagent as a reference.

Note: International collaborative studies to evaluate the best cut-off to use for HPV16 will be performed in the near future.For other HPV types, where reference reagents are not yet available, we recommend that each laboratory assigns a cut-off using a local standard serum and positive control panels of serum samples (typically subjects with a proven HPV infection of the HPV type under study) and negative control panels of serum samples (typically samples from children in the ages between 2–10, or samples from subjects before sexual debut).

7.5 Quality control of VLPs

Conformationally intact VLPs of good quality are essential for adequate performance of ELISAs. To determine that the VLPs are of sufficient quality to be used for serology, a titration of the VLP against a fixed (excess) concentration of a type-specific monoclonal antibody (Mab) is performed, and type-specific antigenic units are calculated. **The VLPs should have as high a type-specific antigenic activity/milligram as possible. The VLPs should have as low cross-reactive antigenic activity/milligram as possible.** The Mab D9 that broadly reacts with disrupted HPV is used as a control Mab for all HPV types. For HPV16 and HPV18, the monoclonal antibody H16.V5 and H18. J4 respectively, available through the NIBSC in the United Kingdom, should be used.

7.5.1 VLP quality-control test procedure

- Perform the HPV-VLP ELISA described above with the following modifications.
  - Reactivity of intact and disrupted candidate VLP preparations will be compared. For all VLPs, except HPV18, disrupt an aliquot of the candidate VLP stock solution by preparing 0.5–1.0 mL of 1.0 µg/mL VLP in 0.1 M carbonate buffer (pH 9.6) and incubating at RT (24 ºC± 2) for 4 h.
  - Coat candidate VLPs at five different dilutions: 1.0, 0.5, 0.25, 0.125 and 0.062 µg/mL overnight at +4°C. Disrupted candidate VLPs are diluted in 0.1 M carbonate buffer (pH 9.6) and intact candidate VLPs are diluted in cold PBS. If available, a reference batch of the same HPV type VLP previously found to work well is coated in parallel using the same dilutions. For each monoclonal antibody to be tested, there should be two wells coated with only PBS as negative control.

Note: For HPV18 VLPs, disruption is achieved by diluting VLPs to 1 µg/mL in 0.2 M Na_2CO_3 10 mM DTT, pH 10.6, incubating for 30 min at RT, and thereafter diluting to the coating concentrations in the same buffer. Disrupted HPV18 VLPs are coated at 37 °C overnight.

  - Discard VLP solution and add 150 µL of blocking buffer (HS-PBS). Incubate for 1 h at RT.
  - Wash plate once with PBS-T.
  - Discard blocking buffer and add 50 µl of type-specific Mab. Incubate for 2 h at RT.
    - Type-specific Mab for HPV16: V5; dilute to 1/10 000 in HS-PBS.
    - Type-specific Mab for HPV18: J4; dilute to 1/10 000 in HS-PBS.
    - Cross-reactive Mab for All HPV types: D9; dilute to 1/1000 in HS-PBS.
      - Wash plate five times.
      - Add 50 µl anti-mouse IgG-HRP conjugate diluted at 1/2000 in HS-PBS. Incubate for 1 h at RT. **This concentration refers to goat anti-mouse IgG-HRP purchased from Southern Biotech, Atlanta, GA, USA (Cat. #1030-05).**
      - Wash plate five times.
      - Add 50 µL of substrate and incubate for 30 min at RT.
      - Read plate in the microplate reader.
- Perform data analysis as described above. The type-specific antigenic unit is calculated using the PLL method in relation to the reference batch of previously used VLPs.

- The acceptance criteria for the plate using the PLL method are:
  - absorbance value of negative control (PBS) < 0.2;
  - absorbance value of positive control (reference VLP) > 1.0 absorbance at 1 µg/mL;
  - correlation of the reference VLP curve should be >0.95.
- The VLPs are adequate if they have the same or better antigenic units/milligram of VLPs as well as the same or lower cross-reactive antigenic units than the reference batch.

7.6 Determination of coating concentration of VLPs

- For each new batch of VLPs, the coating concentration has to be established. A panel of about 10 serum samples with known reactivity (typically five positive and five negative sera) should be tested with a titration of the new VLP as antigen, together with a reference batch of the same HPV-type VLP previously found to work well.
- Coat VLPs at four different concentrations, typically 3, 2, 1 and 0.5 µg/mL, in cold PBS in parallel with the previously validated concentration of the reference VLP batch. Wells coated with PBS alone are used as negative control or background.
- Perform the HPV-VLP ELISA as described above.
- The reactivity of the old and new batch of VLP with the serum panel should not differ by more than an absorbance value of 0.1, and the individual samples in the panel should remain positive or negative.
- The new coating concentration chosen should give results that are as close as possible to the old VLP batch.

7.7 Control of sensitivity and specificity performance of a new batch of VLP

- A larger panel of serum samples, including samples known to be antibody positive from HPV DNA positive patients, and serum from virginal women or from children 2–10 years of age, are used to control the specificity of the VLP serology.
- If available, use disrupted BPV on separately coated plates. If not, PBS coated plates are used as negative control.
- Perform the HPV VLP-ELISA as described above.
- The reactivity of the sera should correspond to results previously obtained in terms of sensitivity and specificity. Sensitivity (proportion of HPV DNA-positive subjects that are seropositive) should be 50% or better, and specificity should be >98% (proportion of subjects not exposed to HPV that are seronegative). The absorbance of positive samples should be in the same range as obtained with previously-tested VLP batches.

8. HPV neutralization assay

8.1 Introduction

Although there is a relatively good correlation between the presence of anti-VLP conformational antibodies found in the classical VLP-based ELISA, and their neutralizing activity, neutralization assays are the gold standard to assess the protective potential of antibodies induced by papillomavirus vaccines in experimental systems. Papillomavirus neutralization assays rely on neutralization of one of the following: authentic virions; pseudotyped virions that are capsids carrying a reporter gene on their surface, or pseudovirions (PsVs) that have encapsidated reporter genes.

PsVs are used in the neutralization assay described below, as a selected example of a commonly- used neutralization assay that has been found to work in a reliable manner in several HPV LabNet laboratories. The assay was developed by a group led by Dr John Schiller at the National Cancer Institute in the United States of America (*1*). It has similar sensitivity (measured as a proportion of HPV DNA-positive subjects who are assay positive) to standard VLP-ELISAs described in chapter 7. Also, the specificity problems sometimes encountered in VLP-ELISAs with the use of low quality VLPs, are usually less of a problem with a neutralization assay, as only antibodies binding to functional particles are measured. In addition, the neutralization detects capsids-binding antibodies of all immunoglobulin classes, whereas most ELISAs only measure IgG.

8.8.1 Principle

The neutralization assay described here is performed in a high throughput format in 96-well plates with type-specific HPV PsVs that have encapsidated a reporter DNA encoding for secreted alkaline phosphatase (SEAP) under an SV40 promoter. When PsVs bind to 293TT cells that stably express SV40 large tumour antigen, the "infecting" reporter SEAP DNA is expressed and alkaline phosphatase is secreted into the media. Assaying the media for alkaline phosphatase activity measures the PsV infection. Neutralizing antibodies bind to PsV and prevent the PsV from infecting the target cells (note that some antibodies can neutralize even after the virion has bound the cell). PsV neutralization is indicated by a decrease in alkaline phosphatase in the media. The titre of neutralizing antibody is calculated as the reciprocal of the highest dilution of sample to cause 50% reduction in SEAP activity.

8.2 Equipment, supplies and reagents

In order to provide a detailed SOP, catalogue numbers of materials found to perform well by the HPV LabNet are provided. A laboratory may use alternative products if they can be validated to yield equivalent results.

8.2.1 Equipment

- Refrigerator, 4°C to 8°C
- Freezer, -20°C
- Oven 65°C
- Multi-channel pipette
- Centrifuge with rotor for 96-well microplate
- Luminometer (e.g. Dynex MLX Microplate Luminometer or LUMIstar Omega BMG Labtech)
- CO_2 incubator
- Ice -bucket

8.2.2 Supplies

- 96-well round-bottom microplates (e.g. Cat. #3788, Costar®, Corning®, USA)
- 96-well flat-bottom microplates; tissue-culture (TC) treated (e.g. Cat. #3596, Corning®, USA)
- 96-well flat bottom opaque white plate (e.g. Optiplate®, Cat. #6005299, Perkin Elmer, USA)
- Siliconized tips (if not available, use low adherence tips)
- Siliconized tubes (if not available, use polystyrene tubes)
- Heat-resistant adhesive plate seals (e.g. multiscreen plate clear sealing tape, Millipore)

8.2.3 Reagents

- Human embryonic kidney 293TT cells
- HPV16 or HPV18 PsVs with encapsidated pYSEAP
- Neutralizing monoclonal anti-HPV16 or 18 VLP antibody (e.g. H16.V5 and H18.J4)
- Great EscAPe® SEAP chemiluminescence detection kit (Cat. #631701, Clontech, USA)
- Phosphate buffered saline (PBS)
- Dulbecco´s Modified Eagle Medium (DMEM) (e.g. Cat. #11965-092, Invitrogen, USA)
- Fetal calf serum (FCS) (e.g. Cat. #F-0500-A, Atlas Biologicals, USA)
- Non-essential amino acids (NEAA) (e.g. Cat. #11140-050, Invitrogen, USA)
- Glutamax® (e.g. Cat. #35050-061, Invitrogen, USA)
- Antibiotic-antimycotic (e.g. Cat. #15240-062, Invitrogen, USA)
- DMEM without phenol red (e.g. Cat. #21063-029, Invitrogen, USA)
- Trypsin-EDTA (e.g. Cat. #25300-054, Invitrogen, USA)
- Ice

8.2.4 Preparation of reagents

- PBS, pH 7.2: dissolve 7.65 g NaCl, 1.85 g $Na_2HPO_4 \cdot 2H_2O$ and 0.06g K_2HPO_4 in 1 L distilled water. Adjust pH to 7.2 using 10 % phosphoric acid. Store for three months at 4°C [provided it is kept sterile].
- Neutralization buffer (NB): add 1 mL NEAA, 1 mL Glutamax®, 10 mL FCS, 1 mL antibiotic-antimycotic to 37 mL of DMEM without phenol red. Store at 4°C for up to one week [provided it is kept sterile].

8.3 Procedure

Note: PsVs are produced by transfection of 293TT cells with HPV-type-specific plasmids that are available from Dr John Schiller (2). Each purified PsV lot must be tested and titred for SEAP production. Also, for more detailed instructions and explanations on the operating procedure, see the "Papillomavirus neutralization assay" protocol (2).

8.3.1 Overview of assay steps

A schematic diagram of the plate set-up for a HPV16 neutralization assay is shown below as an example (Figure. 8.1).

- Day 1: Plating of 293TT cells in 96-well tissue-culture treated microplate.
- Day 1: Preparation of antibody samples and incubation with PsVs.
- Day 1: Infection of 293TT cells with antibody/PsV mixture.
- Day 4: Detection of SEAP by chemiluminescence.

Figure 8.1: Example of plate layout for HPV16 neutralization assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS						
B	PBS	A 1/10	A 1/20	A 1/40	293TT cells alone	PBS						
C	PBS	B 1/10	B 1/20	B 1/40	293TT cells alone	PBS						
D	PBS	C 1/10,000	C 1/20,000	C 1/40,000	H.16V5 1/20,000	PBS						
E	PBS	D 1/10	D 1/20	D 1/40	H.16V5 1/20,000	PBS						
F	PBS	HPV16 pseudovirion alone	HPV16 pseudovirion alone	HPV16 pseudovirion alone	HPV16 pseudovirion alone	PBS						
G	PBS	PBS	PBS	PBS	PBS	PBS						
H												

Four serum samples A, B, C and D performed in the 96-well tissue culture-treated microplate.

Figure 8.2: Illustration of placement of 293TT cells in microplate [Plate 1]

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS						
B	PBS	293TT cells	293TT cells	293TT cells	293TT cells	PBS						
C	PBS	293TT cells	293TT cells	293TT cells	293TT cells	PBS						
D	PBS	293TT cells	293TT cells	293TT cells	293TT cells	PBS						
E	PBS	293TT cells	293TT cells	293TT cells	293TT cells	PBS						
F	PBS	293TT cells	293TT cells	293TT cells	293TT cells	PBS						
G	PBS	PBS	PBS	PBS	PBS	PBS						
H												

8.3.2 Plating 293TT cells

- Pre-plate 293TT cells in 96-well tissue culture-treated flat bottom plates (TC plate). Plate 30 000 cells/well in 100 µL of NB (Figure 8.2). **Use 293TT cells that are not more than 80% confluent. Freshly trypsinized 293TT cells should be re-suspended in NB.**
- Do not use external wells for the assay, but fill with 200 µL of DMEM (without FCS) or PBS. Cover the plate with adhesive plate seal. **Use of the outer wells of the plate results in evaporation of medium, which creates an artificially high SEAP reading.**
- Incubate plate for at least 2 h (not more than 6 h) at 37°C in incubator with a humidified atmosphere of 5% CO_2 in air.

8.3.3 Preparation of antibodies, samples and incubation with PsVs

Note: Serum and vaginal washes or endocervical secretions can be tested for neutralization. Do not use heparin plasma, because heparin is a potent inhibitor of HPV infection. Perform PsV dilutions in polystyrene tubes or siliconized sterile micro-centrifuge tubes depending on the volume needed. This is very important, since the PsV tends to stick to polypropylene, glass and other materials.

- Thaw test samples, antibodies and PsV stock tubes on ice.
- Use a 96-well round-bottom microplate to make the necessary dilutions. Prepare all dilutions in neutralization buffer. If using serum, start dilutions at 1/10, and for vaginal washes start dilutions at 1/5 (Figure 8.3A, B, C).
- Add 40 µL of the diluted sample (for, e.g. 1/10 for serum or 1/5 for vaginal washes/endocervical secretions) in the first well of the row to be serially diluted (Figure 8.3A). The samples have to undergo two-fold serial dilutions with a final volume of 20µL left in each well (Figure 8.3B).
- Add 20 µL of the appropriate monoclonal antibody dilution in two other wells which would serve as the positive neutralization control (Figure. 8.3A). For example, when conducting a HPV16 neutralization assay, use H16.V5 diluted at 1/20 000 in NB.
- Add 100 µl of neutralization buffer alone in two other wells, which would serve as the negative neutralization control with only 293TT cells (Figure. 8.3A).
- Add 20 µl of neutralization buffer to all other wells (Figure. 8.3A).
- Add 80 µl of HPV16 or HPV18 PsV diluted in neutralization buffer in all wells except those marked 293TT cells alone (Figure. 8.3C). A total of four wells should contain PsVs only, which would provide control relative light units (RLU) for PsVs alone. **Note:** Use siliconized tips or low-adherence tips to dispense PsVs. The dilution of PsVs to be used has to be determined for each preparation as outlined on the Schiller laboratory website noted above. The PsV dilution selected should yield RLU between 50 and 150 if using the Dynex MLX Luminometer. (For Lumistar, RLU between 700 and 2600).
- Cover the plate with adhesive plate seal. Shake plate gently by hand to mix.
- Incubate microplate for 1 h on ice.

Figure 8.3A: Illustration of placement of sample, antibody and NB to wells of microplate [Plate 2]

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		A 1/10 40 µl	A 1/20 20 µl of NB	A 1/40 20 µl of NB	100 µl of NB							
C		B 1/10 40 µl	B 1/20 20 µl of NB	B 1/40 20 µl of NB	100 µl of NB							
D		C 1/10 40 µl	C 1/20 20 µl of NB	C 1/40 20 µl of NB	H.16V5 1/20,000 20 µl of NB							
E		D 1/10 40 µl	D 1/20 20 µl of NB	D 1/40 20 µl of NB	H.16V5 1/20,000 20 µl of NB							
F		20 µl of NB	20 µl of NB	20 µl of NB	20 µl of NB							
G												
H												

Figure 8.3B: Illustration of two-fold serial dilution of serum samples [Plate 2]

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		A 1/10	A 1/20	A 1/40	100 µl of NB							
C		B 1/10	B 1/20	B 1/40	100 µl of NB							
D		C 1/10,000	C 1/20,000	C 1/40,000	H.16V5 1/20,000 20 µl of NB							
E		D 1/10	D 1/20	D 1/40	H.16V5 1/20,000 20 µl of NB							
F		20 µl of NB	20 µl of NB	20 µl of NB	20 µl of NB							
G												
H												

Figure 8.3C: Illustration of the addition of PsV [Plate 2]

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		PSV 80 µL	PSV 80 µL	PSV 80 µL	100 µl NB							
C		PSV 80 µL	PSV 80 µL	PSV 80 µL	100 µl NB							
D		PSV 80 µL	PSV 80 µL	PSV 80 µL	PSV 80 µL							
E		PSV 80 µL	PSV 80 µL	PSV 80 µL	PSV 80 µL							
F		PSV 80 µL	PSV 80 µL	PSV 80 µL	PSV 80 µL							
G												
H												

8.3.4 Pseudo-infection of 293TT cells

- Transfer the content of all the wells (PsV/antibody mixture and controls, 100µL in each well) from the 96-well round-bottom plate (Figure. 8.3C) to the TC plate with 293TT cells (Figure. 8.2). The final volume will be 200µL in each well. **Use siliconized tips or low-adherence tips.**
- Incubate microplate for three days at 37°C incubator with a humidified atmosphere of 5% CO2 in air. **Media does not have to be changed during the 72 h incubation because these cells tend to lift off from the plate. They survive the 72 h without the media change provided they have 200 µL of medium per well.**

8.3.5 Detection of SEAP by chemiluminescence

Note: The protocol below is based on the manufacturer's instruction for use of the Great Escape SEAP chemiluminescent detection kit with a few modifications. The assay reagents mentioned here are all provided as part of the kit. The luminometer settings and RLUs are only applicable to Dynex MLX luminometer and will differ for other models. In the original protocol from the Schiller laboratory, 15 µl of clarified supernatants are used. In this case you would need to use 45 µL of 1X dilution buffer and 1.5 fold higher volumes of all the other components of the SEAP detection kit that are described below.

Also in the original protocol from the Schiller laboratory, a further negative control is performed that consists of adding an irrelevant ascites or purified monoclonal antibody to the 293TT cells.

- Warm all assay reagents to RT before use.
- Gently shake the plate with 293TT infected cells by hand to mix the medium and the cells.
- Transfer 50 µl of medium into a new 96-well round-bottom microplate.
- Spin at 3000 x g for 5 min.
- Meanwhile, add 30 µL of 1X dilution buffer/well in a 96-well flat bottom opaque white microplate in the same positions as in the original 293TT infected cells plate.
- Next, transfer 10 µL of the clarified supernatant from the 96-well round-bottom plate directly into the white microplate. **Care should be taken to not pipette from the bottom of the well.**
- Cover with heat-resistant adhesive plate seal.
- Incubate 30 min at 65°C in the oven.
- Incubate the plate on ice for 5 min.
- Add 40 µL/well of 1X assay buffer.
- Incubate for 5 min at RT.
- Prepare chemiluminescent substrate as directed by diluting the substrate 1:20 in the enhancer solution.
- Add 40 µL of the prepared substrate per well.
- Incubate for 10–20 min at RT.

- Read on luminometer with the glow end-point set at 0.2 sec/well raw data handling average. (For Lumistar, run programme with end-point positioning delay at 0.2, measurement interval time at 0.2, optic at top, gain at 1500 and time to normalize at 0).

8.4 Data analysis and interpretation of results

- Neutralizing monoclonal antibody used as positive control should lead to complete neutralization with an RLU 0–2. (For Lumistar, RLU 10-70).
- 293TT cells alone used as negative control should yield background RLU of 0–2. (For Lumistar RLU 10–70).
- Calculate the mean of RLU for the 293TT cells with pseudovirions alone in four wells.
- Neutralization titre is the reciprocal of the final dilution of serum/vaginal wash that yielded <50% of mean RLU measured with pseudovirions alone.
  - Final dilution = 5 x original dilution as indicated on the example overview plate. **Note:** Final dilution is calculated to take into account the 5-fold dilution of the sample with the pseudovirion i.e. 20 µL of diluted sample +80 µL of pseudovirion.
  - Neutralizing titre = 1 / final dilution.

8.5 References

1. Pastrana DV et al. Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. *Virology*, 2004, 321:205–216.
2. (http://home.ccr.cancer.gov/lco/protocols.asp, accessed 10 December 2009).

9. International standards and secondary standards

9.1 General concepts

9.1.1 Biologicals and biological assays

The definition of a "biological" in WHO documents is "a substance which cannot be fully characterized by physico-chemical means alone, and which therefore requires the use of some form of bioassay" (*1*). Biological assays measure analytes such as antigen, antibody, or nucleic acid content, and do not give a measure of an absolute response of a defined assay procedure.

9.1.2 WHO biological standardization and international standards

WHO establishes international biological reference standards for biological substances that are used in the prevention, treatment or diagnosis of human diseases or conditions. This is to enable their activity to be expressed in the same way throughout the world, in IU or other units as appropriate, and so provide a consistent basis for measurements. WHO biological reference standards are widely used in the development, evaluation, standardization and control of products in industry, by regulatory authorities, and also in biological research in academic and scientific organizations. They play a vital role in facilitating the transfer of laboratory science into clinical practice worldwide, and in the development of safe and effective biological medicines. They facilitate comparisons between laboratories and the determination of the sensitivity of assays (*1*)

An **International Standard** (IS) is a biological preparation to which an **International Unit** (IU) of activity has been assigned. Such standards are intended for use in the estimation of potency of an appropriate test sample by direct comparison with suitable biological test systems. A WHO IS is the highest metrological order biological standard available for a given analyte. They are established by the WHO ECBS. ISs are filled into ampoules/vials with great precision, and meet stringent conditions for residual moisture to ensure long-term stability. Their suitability for use is established in international collaborative studies that are analysed statistically. Based on this analysis the standard is assigned an arbitrary unitage. The preparation, assessment and establishment of ISs follow guidelines published by WHO (*2*).

9.2 Secondary (or working) standards

Secondary biological standards may include regional, national and working standards. They exist in a different framework and the value assigned to the reference material is usually defined in units traceable to the higher order IS, i.e. in IU.

WHO recommendations for the preparation, characterization and establishment of international and other biological reference standards includes a section on the preparation of national standards (*1*). WHO is also developing a manual for the preparation of secondary standards for vaccines that will soon be available to the public. Many of the principles discussed apply to sera and other biologicals.

9.3 ISs for HPV DNA and antibodies

ISs for HPV types 16 and 18 DNA were established by the WHO ECBS in October 2008, each with an assigned potency of 5×10^6 IU per ampoule. They will be used to calibrate in-house or working standards for the amplification and detection of HPV types 16 and 18 DNA (*3*).

A WHO international reference reagent for anti-HPV16 antibodies that was established by WHO ECBS (*4*) was upgraded to an IS in 2009 after data from long-term stability studies had been obtained with an assigned unitage of 5 IU per ampoule. This material will serve as the primary biological standard for antibodies to HPV type 16. This material may be used in immunoassays based on VLPs and in neutralization tests.

These standards are available in the NIBSC catalogue (*5*). Other ISs currently being developed include: i) IS for anti-HPV18 antibodies; ii) ISs for HPV types 31, 33, 45, 52 and 58 DNA: iii) ISs for the DNA of other vaccine-preventable HPV types, namely HPV types 6 and 11.

9.4 Preparation of secondary standards for HPV DNA and their calibration in IU

9.4.1 HPV DNA assays

HPV DNA may be assayed in a range of nucleic acid-based tests, including real-time PCR. These may be either commercial or in-house assays. Suitable assays for the detection and genotyping of HPV are described in chapter 5 of this manual. All types of assay require standardization to facilitate:

- consistent implementation of assays by laboratories;
- inter-laboratory comparisons;
- assay validation;
- ongoing data monitoring of assay performance;
- reproducibility between assay runs;
- reduction in laboratory errors;
- detection of critical loss of sensitivity.

Working standards for HPV16 and HPV18 DNA should be calibrated in IU against the relevant HPV DNA ISs or a high-order standard that has itself been calibrated, and is traceable to the IS. Working standards for other HPV types may be prepared using plasmids diluted to an appropriate level which will facilitate ongoing data monitoring of assay performance.

9.4.2 Selection of source material

Secondary standards should resemble as closely as possible that of the biological samples in the assay systems used to test them. It may, however, be impractical to use HPV DNA-positive clinical samples, such as cervicovaginal swabs or biopsy specimens, as source materials, as this would involve the pooling of a large number of small samples. A suitable material to serve as an HPV DNA standard is likely to be a plasmid formulated in a biological matrix. Such working standards should ideally monitor extraction, amplification and detection steps of an assay. A pool of negative clinical samples or an HPV-negative cell line may be used as the matrix, and be spiked with a suitable concentration of plasmid DNA. Alternatively, buffer containing human genomic DNA could be used as the matrix. In these instances, the HPV DNA is not cell-associated so these standards will not monitor extraction. Consideration should be given to the addition of an internal control in the assay to monitor the efficiency of extraction and assess whether inhibitory factors are present.

The ISs for HPV16 DNA and HPV18 DNA are full-length genomes cloned into plasmid pBR322 formulated in human cellular DNA matrix purified from the C33A cell line. The presence of the C33A DNA mimics the human genomic DNA background that would be present in biological samples. When calibrating a secondary standard against an HPV DNA IS, dilutions of both the IS and secondary standard must be prepared in a diluent containing human genomic DNA, such as C33A DNA or other HPV-negative human DNA. This will provide a constant cellular DNA background in all diluted samples during assays.

9.4.3 Planning

At the initiation of the project, the intended use and anticipated requirements should be assessed, so that the batch of standard produced will last for perhaps 3–5 years. This should take into account the number of vials/ampoules which may be required for calibration and stability studies. Secondary standards are likely to be frozen in multiple aliquots, and each aliquot should be intended for single use to avoid multiple freeze/thaw cycles and the possibility of degradation or contamination. The target titre of the secondary standard should also be agreed. Choosing a target titre of a secondary standard may require an in-house or collaborative study. Such a pilot study would identify inter-assay or inter-laboratory variations in sensitivity and specificity; thus informing the formulation of the titre (*6*).

Preliminary studies on the source material should be undertaken to confirm its identity and determine its concentration and unitage prior to formulation. For example, the source plasmids used in the formulation of the HPV16/18 DNA ISs were quantified by A260 and picogreen methods prior to their use in formulation studies. The same approach can be made for source plasmids for making secondary standards. If the source material is, for example, cell culture-based or clinical samples, then the material can be tested against the IS to determine the unitage.

Any trial formulations/fills should then be tested in HPV DNA assays against the IS to confirm their performance and select the appropriate formulation for the candidate secondary standard. After the definitive formulation and filling, the candidate standards should be assayed prior to their calibration. These assays are necessary to confirm the identity of the candidate secondary standards and to demonstrate that they do not cross-react unexpectedly, and are not contaminated. Ideally, the candidate secondary standard should be tested in the different types of assay in which it will be used. Where possible, validated assay methods should be used. If a validated assay is not available in the laboratory that formulated the secondary standard(s), then one or two outside laboratories that do have validated assays may be nominated to test the candidate secondary standard(s).

The calibration and assignment of unitage to the secondary standard may be undertaken in a single laboratory or in a collaborative study in which multiple laboratories participate. Laboratories invited to participate in the study should be prospective users of the secondary standard and/or possess the expertise to perform the testing. Sufficient assays should be performed to allow statistical analysis. The fewer the participants, the more assays each laboratory may have to perform. The number of assays required to calibrate a secondary standard depends on the precision of the assays and the required precision of the assigned potency.

A study protocol should be prepared and agreed. The study design should consider:

- whether or not to include extraction steps. This depends on the formulation of the material. If the secondary standard is purified plasmid, then it will not be suitable for assessing extraction steps of assays.
- The number of independent assays. Each independent assay should be performed on different days, preferably by different operators using fresh tubes/ampoules of study material and IS.
- Simultaneous testing of all materials within each assay. When feasible, all dilutions of all study samples should be tested simultaneously with dilutions of the IS. If it is unfeasible to test all study samples simultaneously in one assay, dilutions of the IS must be included in each subset of assays. This is to ensure that all study materials can be assigned a unitage that is directly related to the IS.
- Range of dilutions for each sample. The estimated titre of the samples, as determined in the preliminary assessment of the source material and formulation studies, may be used to gauge which dilutions to test.
- Inclusion of replicate sample(s). A replicate of at least one sample should be included in the study to enable estimation of the within-assay variability.
- Repeat testing. Fresh tubes/ampoules of each study material and calibrator should be used in each independent assay.
- Diluent. The diluent used to make serial dilutions of the study materials should contain the same biological matrix as that present in the standard.

9.4.4 Preparation

The candidate working standard should be aliquoted into volumes appropriate for single use. Screw-capped vials should be used.

All vials of the candidate standard should be labelled with:

- the name of the material;
- any assigned code number;
- the storage temperature;
- that this material is "not for use in humans";
- an expiry date, if assigned.

This is particularly important if a standard is to be used in multiple laboratories.

9.4.5 Assays

A series of dilutions of the IS and test samples are prepared and assayed. At least two independent replicate series of dilutions should be prepared, i.e. two series of dilutions of each sample which are each assayed (**not two aliquots from a single series of dilutions**).

For quantitative HPV DNA assays, it is suggested to test three or four dilutions that fall within the linear range of the assay. The material may be tested without dilution, but this is dependent upon the linear range of the assay.

For qualitative HPV DNA assays, the first assay should test tenfold serial dilutions of each sample in order to estimate the HPV DNA end-point (i.e. 10^{-1} serial dilutions, which is equivalent to 1:10 serial dilutions). For the remaining independent assays, a minimum of two half-log serial dilutions (i.e. $10^{-0.5}$ serial dilutions equivalent to 1:3.16 serial dilutions) should be tested on either side of the end-point determined in the first assay. It is therefore not necessary to carry out more than five half-log serial dilutions (centered around the estimated HPV DNA end-point).

9.4.6 Statistical analysis and calibration against the HPV DNA ISs

Commercially available statistical software may be used to perform PLL analysis (quantitative assays) or estimating assay end-points (qualitative assays). Statistical programmes are available (*7*). Alternatively, the free shareware PLL programme described in chapter 7 can also be used.

The DNA concentration of the test sample is expressed relative to the calibrating standard (e.g. IS). For example, if the standard has an assigned unitage of 1×10^7 IU/mL, the relative potency of the test sample (obtained by the statistical analysis) is multiplied by 1×10^7 IU/mL to express the DNA concentration of the test sample in IU/mL.

Quantitative assays:

- Analyse assay outputs, e.g. cycle threshold (C_t) values, as parallel-line assays, to give a "relative potency" of the samples against the IS in IU.
- Alternatively, a standard curve generated by the PCR instrument software using the IS as the standard may be used to determine the unitage of the secondary standard.

Qualitative assays:

- For each laboratory and assay method, pool data from all assays to give a number of positives out of number tested at each dilution step. Calculate the single "end-point" for each dilution series to give an estimate of "detectable units/mL" for each study sample as described in previous studies for nucleic acid standards (for example (*8*)). It should be noted that these estimates are not necessarily directly equivalent to a genuine GE number per mL.
- The ratio of "detectable units/mL" of secondary standard to "detectable units/ml" of IS can then be used to determine the unitage of the secondary standard relative to the IS. For example, if the secondary standard was estimated to contain 2.3 x 10^4 detectable units/ml and the IS was estimated to contain 5.2 x 10^4 detectable units/mL, the ratio of the two samples is 0.44. This ratio multiplied by the unitage of the IS will give the unitage of the secondary standard. Using the example above, if the IS has a unitage of 1 x 10^7 IU/mL, then the unitage of the secondary standard is 4.4 x 10^6 IU/mL.

9.5 Preparation of secondary standards for HPV antibodies and their calibration in IU

9.5.1 HPV antibody assays

Standards calibrated in IU are used in many epidemiological studies and clinical trials, for a range of vaccines such as measles and poliovirus vaccines. Publications report geometric mean titres in IU to ensure results are comparable. Minimum levels of antibodies in IU which are indicative of immunity are available for many viruses and antibody concentrations in IU serve as potency requirements for therapeutic immunoglobulins.

There are currently two licensed HPV vaccines that contain HPV16 and HPV18, with others in development. Standards are needed to allow comparison of the immune responses in clinical trials, for comparable definition of the susceptible (seronegative) populations in vaccine trials, and for epidemiological studies. An international collaborative study indicated that the use of a standard improves comparability between laboratories using different assays (*4*).

For the establishment of ISs, as wide a range of assay as possible is used. The calibration of secondary antibody standards, or working standards, will depend on the assay (e.g. ELISA) in routine use. All assays in which a secondary standard is to be used need to be considered at the outset.

Each serum contains antibodies against a range of proteins and epitopes. Every serum contains these antibodies in different proportions. Every form of assay will detect antibodies in the same sera in different proportions. The results of all immunoassays are highly dependent on the quality and type of antigens used.

9.5.2 Selection of source material

Serum or plasma samples from vaccinees typically have considerably higher titres than serum samples from naturally-infected individuals. It is possible that when serum samples from vaccine recipients are assayed against homologous vaccine VLPs, higher titres will be obtained, because of reactivity with impurities or incorrectly or differently folded VLPs that may be present only in that particular vaccine preparation. Alternatives for secondary standards are therefore either: 1) blood donors (naturally-infected subjects) that are not biased towards any particular vaccine formulation; 2) vaccinees, that will typically contain much higher titres than serum samples from naturally infected subjects.

The IS for HPV16 antibodies contain serum samples from naturally-infected subjects that are seropositive for only a single HPV type, the purpose being to have a clearly defined IU that is not affected by the presence of possibly cross-reactive antibodies against other HPV types. As substantial screening and characterization efforts are needed to identify such HPV-monospecific serum samples, it is anticipated that most secondary standards will not necessarily need to be HPV-monospecific.

Sera or plasma should ideally be tested for markers of human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV) infection, to reduce hazards for use and distribution.

9.5.3 Planning

At the initiation of the project, the intended use, anticipated requirements and the likely usage should be assessed so that the batch of standards produced will last for perhaps 3–5 years. This should take into account the number of vials/ampoules which may be required for calibration and stability studies. Secondary standards are likely to be frozen in multiple aliquots and each aliquot should be intended for single use to avoid multiple freeze/thaw cycles and the possibility of degradation or contamination.

Preliminary studies on the sera or plasma should be undertaken to confirm its identity and determine its titre and/or unitage prior to diluting and/or aliquoting. These assays should be performed in the different types of assay in which it will be used. Validated assay methods should be used, where possible. If assays documented in WHO manuals, or commercial assays are used, these can be considered as validated. Laboratories must demonstrate proficiency in performance of validated assays. For serology, VLPs must meet QC standards described in chapter 7.

The assignment of unitage to the secondary standard may be undertaken in a single laboratory, or in a collaborative study in which multiple laboratories participate. Laboratories invited to participate in the study should be users of the standard and/or possess the expertise to perform the testing. Sufficient assays should be performed to allow statistical analysis. The fewer the participants, the more assays each laboratory may have to perform. The number of assays required to calibrate a secondary standard depends upon the precision of the assays and the required precision of the assigned potency.

Where multiple laboratories will participate, a study protocol should be prepared and agreed. The design of study should consider:

- simultaneous testing of all materials within each assay;
- range of dilutions for each sample;
- include replicates of at least one sample in each assay to enable estimation of the within-assay variability;
- repeat testing using fresh ampoules;
- use of the diluent.

9.5.4 Preparation

The pool of candidate working standard should be aliquoted into volumes appropriate for single use. Screw-capped vials should be used.

All vials of the candidate standard should be labelled with:

- the name of the material;
- any assigned code number;
- the storage temperature;
- that this material is "not for use in humans";
- an expiry date, if assigned.

This is particularly important if a standard is to be used in multiple laboratories.

9.5.5 Assays

A series of dilutions of the IS, and test samples are prepared and assayed. At least two independent replicate series of dilutions should be prepared, i.e. two series of dilutions of each sample which are each assayed (**not two aliquots from a single series of dilutions**).

9.5.6 Statistical analysis and calibration against the IS

Commercially-available statistical software may be used to perform PLL analysis (quantitative assays) or estimating assay end-points (qualitative assays). Statistical programmes are available (*7*). Alternatively, the free shareware PLL programme described in chapter 7 can also be used.

The antibody or DNA concentration (potency) of the test sample is expressed relative to the calibrating standard (e.g. IS). For example, if the standard has an assigned unitage of 10 IU/mL, the relative potency of the test sample (obtained by the statistical analysis) is multiplied by 10 IU/mL to express the concentration/potency of the test sample in IU/mL.

9.6 Storage and stability of secondary standards

Standards should be stored at an appropriate temperature. The temperature should be monitored and recorded on a routine basis. An alternative storage area should be available in case of breakdown. The length of time that the secondary standard will remain suitable for its intended purpose under its defined storage conditions should be considered. The stability of the standard should be assessed periodically by assay against the IS. The frequency of this assessment will depend on the precision of assays and the predicted stability of the standard.

9.7 Dispatch of the secondary standards to end-users

If a working standard is to be used by more than one laboratory, supplies of aliquots should be dispatched under conditions which have been assessed and found appropriate for the stability of the standard. The anticipated time in transit, and ambient temperature, should be considered. The number of vials of standard issued and the recipients should be recorded in case any issues arise. Feedback on the use of the standard which might contribute to ongoing evidence of the stability of the material should also be requested. A copy of the data sheet or "instructions for use" should be included with all shipments. The instructions for use should include:

- the storage conditions;
- the potency of the standard;
- the type of assays in which it may be used;
- relevant safety information;
- the stability of freeze-dried products after reconstitution (if applicable).

9.8 Batch replacement

Replacement of a secondary standard needs to be planned and timely. The process described above should be followed, including calibration against the IS (or high-order calibrating standard), **not** the previous secondary standard. If additional material from the original collaborative study is available, along with appropriate monitored storage conditions, excess candidate bulk material could be held to allow replacement with identical material. The approaches taken to eventual replacement of a standard should be considered when the original proposals for preparation of the intended secondary standard are drawn up.

9.9 References

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10. Data management

10.1 Introduction

An essential part of the work of every laboratory is to record the details of all specimens tested, to record the results of testing, and to report the results. A reference laboratory will also analyse and interpret these results to look for epidemiological patterns or trends, and summarize the results and conclusions in the form of regular reports. The term "data management" covers all of these activities, and is an essential function of any disease surveillance system. Good laboratory data management is crucial for monitoring the impact of HPV vaccination through HPV testing. Poor data management results in wasted time, effort and money, and will make it more difficult to reach the goals of controlling HPV and its associated cancers.

Good data management starts by understanding:

- the meaning of the information generated;
- what information needs to be communicated to those outside the laboratory;
- who needs the information;
- how often they need the information.

Every HPV reference laboratory needs to:

- deliver results to those submitting the specimens in a useable format;
- prepare annual or progress reports of its work for the director or head of its institute and national disease control agencies (as appropriate). For WHO LabNet laboratories, an annual summary report will be submitted to WHO.

Once all of these requirements have been identified, thought can be given to which information must be recorded so that the requirements can most easily be met. This manual describes a set of minimum information (core variables) to be recorded on each specimen from a case of HPV-associated cancer or other HPV-associated disease. As a general rule, the more information that has to be collected and recorded, the greater the chance that the information will be of lower quality, with more omissions and mistakes. It is always easier to collect and accurately record less information than more information, but if less information is collected, it is essential to ensure that it is the **required** information.

The next step in data management is to decide how the information is to be physically recorded and stored. All laboratories maintain specimen registers and books of laboratory results. These are often in the form of paper records, written line-by-line, with information entered into specific columns. Such records are called line-listings, since all the information relating to that specimen or case can be found by reading along the line of information. It is highly desirable that all laboratories establish a computer-record system or an electronic data-management system. A simple spreadsheet system (using software such as Excel®), reflecting the line-listing of paper records, may be sufficient for some laboratories. Although useful for some types of analysis, computer spreadsheets are not very easy to manipulate when using large amounts of information. For large amounts of information it is better to establish a computer database (using software such as EpiInfo®, FoxPro® or Access®). The choice of exactly how to computerize laboratory record-keeping depends on a number of factors, including:

- user preference;
- hardware availability and capacity;
- software availability and cost;
- type of programming required to use the software;
- local expertise to develop and maintain the system.

At minimum, the system chosen should allow rapid and accurate access to chosen or selected records, be able to perform simple calculations, such as frequencies and time intervals, and be able to create tables and graphs. It is often an advantage to establish a "menu system" to help non-advanced users and to make repetitive actions, such as data entry, more efficient. The system must also be well-documented for both users and programmers. The documentation should include clear descriptions of installation procedures, operation, structure, adaptation to specific needs, required maintenance activities, file-management requirements and coding lists, if coded information is used. A good computerized laboratory recording system should contain the following components:

- data entry;
- data cleaning (programmes that detect errors in the information entered);
- routine backup of data;
- routine analysis and reporting (for decision-making, action, monitoring);
- feedback (information to be sent back to the case investigators);
- feedforward (information to be reported to the next level).

In designing any laboratory results recording system, it is essential to involve those who understand the disease-control objectives, strategies, surveillance needs and performance indicators of the activity.

Which information is reported, where it is reported from, and where it is reported to, must be clearly agreed upon by all parties involved in the system. Feedback and feedforward reports will obviously have different formats, and different frequencies. Ideally, all information flow should be hierarchical, going from one level to the next without missing levels. Information can also be "broadcast" (i.e. sent to several sources

at different levels, at the same time). Once a pattern of information flow is established, it is very important that it be followed without exception. It is also important to review the system from time-to-time to make sure that it is doing the job that it was designed to do, and to decide if improvements can be made. If any changes are made to the system, it is essential that all parties involved are informed of the changes and agree to them.

Maintaining laboratory records and keeping them accurate and relevant involve following good management practices and a clear designation of responsibilities. The success or failure of any public health or disease-control initiative depends on establishing and maintaining a good information exchange system, with accurate and timely data being provided for appropriate action. The importance of good laboratory data management cannot be overstated.

10.2 Recording laboratory results

Recording receipt of specimens is detailed in chapter 4 on specimen handling.

Experience has shown that the easiest way to record information on results is to establish a **specimen-based** database, with each line of information relating to one specimen. Thus, for a patient with two specimens collected and processed, there will be two lines of information. Information related to number of patients can then be obtained by relating back to the patient ID or patient code field.

Typical information to be collected and recorded on specimen processing and HPV typing results is shown below. The list would be modified for HPV serology or other HPV testing assays.

- Patient or participant code
- Laboratory specimen number
- Whether the specimen was processed (y/n)
- Date of specimen DNA extraction
- Date of HPV testing
- Method of HPV testing
- HPV testing result (present/absent/not interpretable)
- Date of specimen adequacy testing (beta-globin test or similar, if not included in HPV test)
- Method of specimen adequacy testing
- Specimen adequacy testing result (present/absent/not interpretable)
- Date of HPV typing
- Method of HPV typing
- Typing result: (HPV type number(s). If unknown type, HPVX or GenBank accession number)
- Date results sent to person/institute who submitted sample

10.2.1 HPV typing data

For surveillance and assessment of disease burden purposes, HPV-positive specimens that either; i) originate from patients with HPV-associated diseases, or ii) are part of population-based surveys or other clearly defined surveillance strategies (e.g. sentinel clinics), should be typed in a laboratory that has documented proficiency to carry out HPV typing, as defined in proficiency testing schemes performed under the auspices of WHO.

The WHO HPV LabNet Global Reference Laboratories (GRLs) are committed to performing confirmatory typing of selected samples from regional reference laboratories (RRLs) as well as on "problematic" samples that may be submitted from any laboratory in the world. The minimum recommended information to be sent with the material for confirmatory typing at a GRL includes the following:

- patient or participant code;
- laboratory specimen number;
- date sent to GRL.

10.3 Reporting laboratory activity and results

Laboratory results should be reported in a timely and accurate manner to provide a knowledge base to national infectious disease-control agencies as a basis for continued design of HPV vaccination and HPV surveillance/monitoring strategies, as well as for monitoring laboratory results and performance to identify possible problems and constraints. Details of how and when laboratories report to national agencies and to the head of their institution should be arranged locally.

Apart from describing the general activities of the laboratory and the testing and typing results, it is important that information on errors, e.g. inadequate specimens and inadequate transport of specimens, is mentioned so that improvements can be made.

Typical items expected to be used in evaluation of laboratory activity and results include documentation that:

- acceptable SOPs have been followed;
- laboratory performance has been at an acceptable level. This includes successful results in WHO proficiency panel tests.
- Laboratory activity has been at an acceptable level. The total number of specimens and number of results for each assay type must be documented. The HPV typing results by specimen and specimen type must be documented. Reasons for missing laboratory data should be documented, e.g. reasons for each instance in which a specimen that was received in the laboratory was not processed, reasons for failure to send samples for confirmatory testing, and reasons for missing data.
- The laboratory is complying with WHO biosafety requirements.

Annex

Contacts of WHO HPV LabNet Laboratories (November 2009)

Dr Joakim Dillner

Global WHO HPV Reference Laboratory
Laboratory Medicine Skåne
Malmö University Hospital, Entrance 78
SE-20502 Malmö, **SWEDEN**
E-mail: joakim.dillner@med.lu.se

Dr Elizabeth R Unger

Global WHO HPV Reference Laboratory
Centers for Disease Control and Prevention
1600 Clifton Road, MS G41
Atlanta, GA 30333, **USA**
Email: eru0@cdc.gov

Professor Anna-Lise Williamson

Regional WHO HPV Reference Laboratory, WHO African Region
Institute of Infectious Disease and Molecular Medicine
Faculty of Health Sciences
University of Cape Town
Observatory 7925
Cape Town, **SOUTH AFRICA**
Email: Anna-Lise.Williamson@uct.ac.za

Dr María Alejandra Picconi

PAHO Regional WHO HPV Reference Laboratory, WHO Region of the Americas
Oncogenic Viruses Laboratory, National Reference Laboratory for Papillomavirus
National Institute of Infectious Diseases — ANLIS "Dr. Carlos G Malbrán"
Av. Vélez Sársfield 563
C1282AFF- Buenos Aires, **ARGENTINA**
E-mail: mapicconi@anlis.gov.ar

Dr Emna Ennaifer-Jerbi

Regional WHO HPV Reference Laboratory , WHO Eastern Mediterranean Region
Tunis Pasteur's Institute
13, place Pasteur, BP 74
1002 Tunis le Belvédère, **TUNISIA**
E-mail: emna.jerbi@pasteur.rns.tn

Professor Denise Nardelli-Haefliger

Regional WHO HPV Reference Laboratory, WHO European Region
Institute of Microbiology, CHUV
Bugnon 48, 1011 Lausanne, **SWITZERLAND**
E-mail: dnardell@hospvd.ch

Dr Alok Chandra Bharti/ Dr Mausumi Bharadwaj

Regional WHO HPV Reference Laboratory, WHO South-East Asia Region
Division of Molecular Oncology
Institute of Cytology and Preventive Oncology, (ICMR)
I-7, Sector 39, NOIDA, Ditt. Gautam Buddha Nagar, UP 201 301, **INDIA**
E-mail: bhartiac@icmr.org.in; bharadwajm@icmr.org.in

Dr Jarunya Ngamkham

Regional WHO HPV Reference Laboratory, WHO South-East Asia Region
Research Division, National Cancer Institute
Rama VI Road, Ratchathewee
Bangkok 10400, **THAILAND**
E-mail: jarunyanci@gmail.com

Professor Suzanne Garland

Regional WHO HPV Reference Laboratory, WHO Western Pacific Region
Department of Microbiology & Infectious Diseases
The Royal Women's Hospital
Bio 21 Institute - Level 1, Building 404
30 Flemington Road
Parkville, VIC 3052, **AUSTRALIA**
E-mail: suzanne.garland@thewomens.org.au

Dr Iwao Kukimoto

Regional WHO HPV Reference Laboratory, WHO Western Pacific Region
Pathogen Genomics Center
National Institute of Infectious Diseases
4-7-1 Gakuen, Musashi-Murayama
Tokyo 208-0011, **JAPAN**
E-mail: ikuki@nih.go.jp
Consortium with/

Dr Kei Kawana

Department of Obstetrics and Gynecology
Faculty of Medicine
University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, **JAPAN**
E-mail: kkawana-tky@umin.ac.jp

WHO INTERNATIONAL LABORATORY FOR BIOLOGICAL STANDARDS

Dr Dianna Wilkinson

Division of Virology
National Institute for Biological Standards and Control (NIBSC)
Blanche Lane, South Mimms, Hertfordshire
EN6 3QG, **UNITED KINGDOM**
E-mail: Dianna.Wilkinson@nibsc.hpa.org.uk

The World Health Organization has provided technical support to its Member States in the field of vaccine-preventable diseases since 1975. The office carrying out this function at WHO headquarters is the Department of Immunization, Vaccines and Biologicals (IVB).

IVB's mission is the achievement of a world in which all people at risk are protected against vaccine-preventable diseases. The Department covers a range of activities including research and development, standard-setting, vaccine regulation and quality, vaccine supply and immunization financing, and immunization system strengthening.

These activities are carried out by three technical units: the Initiative for Vaccine Research; the Quality, Safety and Standards team; and the Expanded Programme on Immunization.

The Initiative for Vaccine Research guides, facilitates and provides a vision for worldwide vaccine and immunization technology research and development efforts. It focuses on current and emerging diseases of global public health importance, including pandemic influenza. Its main activities cover: i) research and development of key candidate vaccines; ii) implementation research to promote evidence-based decision-making on the early introduction of new vaccines; and iii) promotion of the development, evaluation and future availability of HIV, tuberculosis and malaria vaccines.

The Quality, Safety and Standards team focuses on supporting the use of vaccines, other biological products and immunization-related equipment that meet current international norms and standards of quality and safety. Activities cover: i) setting norms and standards and establishing reference preparation materials; ii) ensuring the use of quality vaccines and immunization equipment through prequalification activities and strengthening national regulatory authorities; and iii) monitoring, assessing and responding to immunization safety issues of global concern.

The Expanded Programme on Immunization focuses on maximizing access to high quality immunization services, accelerating disease control and linking to other health interventions that can be delivered during immunization contacts. Activities cover: i) immunization systems strengthening, including expansion of immunization services beyond the infant age group; ii) accelerated control of measles and maternal and neonatal tetanus; iii) introduction of new and underutilized vaccines; iv) vaccine supply and immunization financing; and v) disease surveillance and immunization coverage monitoring for tracking global progress.

The Director's Office directs the work of these units through oversight of immunization programme policy, planning, coordination and management. It also mobilizes resources and carries out communication, advocacy and media-related work.

Department of Immunization, Vaccines and Biologicals

Family and Community Health

World Health Organization
20, Avenue Appia
CH-1211 Geneva 27
Switzerland
E-mail: vaccines@who.int
Web site: http://www.who.int/immunization/en/