Chapter 8 - International Standards and secondary standards

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8.1 GENERAL CONCEPTS

8.1.1 Biologicals and biological assays

The definition of a “biological” in World Health Organization (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.

8.1.2 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 international units (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 IU of activity has been assigned. Such standards are intended for use in the estimation of potency or biological activity 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 Expert Committee on Biological Standards (ECBS). ISs are filled into ampoules/vials with great precision and freeze-dried 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 IU value which does not carry an uncertainty of measurement associated with calibration.

The intended uses of the ISs are for the initial validation of new assays and platforms and the calibration of secondary references for the purpose of assigning them in terms of the IU thus allowing the results of biological measurements to be expressed in the same way worldwide. The preparation, assessment and establishment of ISs follow guidelines published by WHO.[1] Information on how to obtain ISs and other WHO international biological references is available through WHO’s online catalogue. They are stored and dispatched to end-users by WHO custodian laboratories. [2,3]

8.2 SECONDARY AND TERTIARY BIOLOGICAL REFERENCE STANDARDS (WORKING STANDARDS)

Secondary biological standards may include regional, national and local standards. They are developed and established in a different framework to that of the IS. The value assigned to a secondary reference material is usually defined in units directly calibrated and traceable to the higher order IS, i.e. in IU. Unlike the IS, the stated unitage of a secondary standard carries with it a measurement of uncertainty in its calibration (typically stated as 95% Confidence Limits). Secondary biological standards are normally established by e.g. regional and national regulatory laboratories, manufacturers or research laboratories and are used as working calibrators, references and run controls so as not to deplete the IS.

Secondary reference standards may be used to calibrate lower-order, tertiary reference materials in terms of the IU. A tertiary standard may serve as a local or in-house reagent or standard e.g. run control, monitor sample or other assay reference reagent. Being further down the calibration hierarchy, a tertiary reference material will have a greater uncertainty of measurement for its calibration than the secondary standard used as its calibrator.

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 has also published manuals for the preparation and calibration of secondary standards for infectious disease nucleic acid or antigen detection[4] and antibody testing[5]. The WHO manuals have appendices providing examples of potentially useful processes for laboratories developing, storing and distributing secondary biological standards. The examples are not meant to be prescriptive and may be adapted by human papillomavirus (HPV) laboratories as needed.

8.3 ISS FOR HPV DNA

WHO International Standards covering full-length HPV deoxyribonucleic acid (DNA) sequences for HPV types 6, 11, 16, 18, 31, 33, 45, 52 and 58 were established by ECBS following international collaborative studies demonstrating their suitability for use in the standardization and control of HPV DNA assays.[6,7]

Each ampoule of an IS for HPV DNA contains the lyophilized equivalent of 0.5 mL HPV plasmid DNA for the indicated genotype formulated in a background of purified human genomic DNA. The nucleic acid sequences of the cloned full-length HPV genomes are those of the officially established HPV reference clones documented on the International HPV Reference Center (IHRC) website (https://www.hpvcenter.se/human_reference_clones/). Cloning details and summaries of the composition and unitage for the ISs are shown in **Tables 8-1**, **8-2**, and **8-3**. Because the ISs are plasmid-based cell-free preparations of purified DNA, their utility in the standardisation of assays is limited to the amplification and detection steps. Such standards cannot be used to assess sample processing steps such as centrifugation, DNA extraction or purification.

The anticipated users of the WHO International Standards for HPV DNA include public health, manufacturers and research laboratories conducting cervical cancer screening and follow-up testing, epidemiological studies and follow-up studies of vaccines; proficiency study organisers, and diagnostic kit manufacturers. The ISs for HPV DNA and product-specific instructions for their use are available from the National Institute for Biological Standards and Control (NIBSC) online catalogue

https://nibsc.org/products/brm_product_catalogue/sub_category_listing.aspx?category=Diagnostics&subcategory=Human+Papillomavirus. The ISs are available individually by HPV type or as more economical collections of HPV types 16, 18, 6, 11 (NIBSC product code 19/224 https://nibsc.org/products/brm_product_catalogue/detail_page.aspx?catid=19/224) and HPV types 31, 33, 45, 52, 58 (NIBSC product code 19/226 https://nibsc.org/products/brm_product_catalogue/detail_page.aspx?catid=19/226).

Table 8-1 List of HPV plasmids used to prepare the WHO 1st International Standards for HPV DNA.

HPV Type	HPV DNA Plasmid	Reference HPV sequence GenBank ID	Cloned in vector	Restriction enzyme cloning site	GenBank HPV nucleotide cloning site	HPV genome cloning site (HPV gene)	Reference
6	HPV6	X00203	pBR322	*Bam*HI	4722	L2	8
11	HPV11	M14119	pGEM4Z	*Kpn*I	4776	L2	8,9
16	HPV16	K02718	pBR322	*Bam*HI	6150	L1	10
18	HPV18	X05015	pBR322	*Eco*RI	2440	E1	10
31	HPV31	J04353	pT713	*Eco*RI	3361	E4	11,12
33	HPV33	M12732	pBR322	*Bgl*II	2796	E2	13
45	HPV45	X74479	pGEM4	*Hind*III	75	upstream of E6	14
52	HPV52	X74481	pUC19	*Eco*RI	7558	downstream of L1	15
58	HPV58	D90400	pGEM4Z	*Sph*I	1153	E6	9,16

Note: Insertion site (GenBank HPV nucleotide cloning site) is defined as the first nt of the restriction enzyme recognition sequence (not the site of cleavage). Additional information on the HPV reference clones may be found on the IHRC website https://www.hpvcenter.se/human_reference_clones/.

Table 8-2 Composition of the WHO 1st International Standards for HPV DNA genotypes.

IS HPV DNA Type	NIBSC product code	Unitage (IU/ampoule)	Source of purified human DNA background	Concentration after reconstitution as directed in 0.5 mL water (IU/mL)
6	14/256	1 x 10^7	placenta	2 x 10^7
11	14/100	1 x 10^7	placenta	2 x 10^7
16	06/202	5 x 10^6	C33A cells	1 x 10^7
18	06/206	5 x 10^6	C33A cells	1 x 10^7
31	14/258	1.6 x 10^7	placenta	3.2 x 10^7
33	14/260	1.6 x 10^7	placenta	3.2 x 10^7
45	14/104	1 x 10^7	placenta	2 x 10^7
52	14/262	7.9 x 10^6	placenta	1.6 x 10^7
58	14/264	7.9 x 10^6	placenta	1.6 x 10^7

Each ampoule contains the lyophilized equivalent of 0.5 mL HPV plasmid diluted in 10mM Tris buffer pH7.4 containing 1mM EDTA, 5 mg/mL trehalose and purified human DNA (~1 x 10^6 GEq/mL).

Table 8-3 List of the differences in nucleotide sequences of the IS HPV plasmids compared to the respective GenBank HPV reference sequence.

<table>
<tr><th>International Standard HPV plasmid

(plasmid vector_HPV type)</th><th>HPV sequence</th><th>Genome location</th><th>Plasmid vector sequence</th></tr>
<tr><td>pBR322_HPV6</td><td>**A**5720**G**
ins **G**5721
A5724**G**</td><td>L2
L2
L2</td><td>none identified</td></tr>
<tr><td>pGEM4Z_HPV11</td><td>ins **GC**2960-2961
GC4961-4962**CG**</td><td>E2
L2</td><td rowspan="2">**G**8183**C**
G8447**C**
G8884**A**
G9510**C**
del **A**186
ins **G**525
AT9096-9097**TA**
G9342**A**</td></tr>
<tr><td>pT713_HPV31</td><td>del **ACTTGTTCCT**4505-4514</td><td>L2</td></tr>
<tr><td>pBR322_HPV33</td><td>**T**2759**G**</td><td>E2</td><td>none identified</td></tr>
<tr><td>pGEM4_HPV45</td><td>ins **G**4885
del **G**4890</td><td>L2
L2</td><td>**G**8644**A**
ins **TGCA**10299
del **G**10534
A10539**G**</td></tr>
<tr><td>pUC19_HPV52</td><td>del **TTATG**7382-7386</td><td>URR</td><td>none identified</td></tr>
<tr><td>pGEM4Z_HPV58</td><td>none identified</td><td></td><td>**G**8072**C**
G8336**C**
G8773**A**
G9399**C**</td></tr>
</table>

Nucleotide differences were identified by deep sequencing of the HPV plasmids used in the formulation of the ISs for HPV6, 11, 31, 33, 45, 52 and 58. Each nucleotide position has been covered by sequencing at least 50 times; ins: insertion; del: deletion.[7] URR: upstream regulatory region.

8.4 ISS FOR HPV ANTIBODIES

WHO collaborative study reports provide the full details on the formulation, characterization and establishment of the WHO IS for antibodies to HPV 6, 11, 16, 18, 31, 33, 45, 52 and 58 [17-19] Each ampoule is a freeze aliquot of formulation prepared using sera from women naturally infected with the indicated HPV type (**Table 8-4**).

Global utilization of the ISs will enable standardization of L1-based HPV serology assays used in HPV antibody levels of unvaccinated or vaccinated individuals as part of population studies; L1-based HPV vaccine research, development, manufacturing and control; clinical trials or monitoring post-licensure.

The ISs for HPV antibodies are available from the NIBSC online catalogue. Since different ISs may require different volumes of reconstitution, it is important to refer to the product-specific instructions for their use.

Table 8-4 Details of the 1st WHO ISs for HPV antibodies

IS HPV antibodies	NIBSC Product code	Unitage (IU/ampoule)	dH2O volume for reconstitution (mL)	unitage (IU/mL) when reconstituted as directed in instructions for use	Monospecific for reactivity to its HPV type	Reference
Type 6	19/298	7	0.25	28	Yes	17
Type 11	20/174	6	0.25	24	No	17
Type 16	05/134	5	0.5	10	Yes	18
Type 18	10/140	8	0.5	16	Yes	19
Type 31	20/176	3	0.25	12	Yes	17
Type 33	19/290	8	0.25	32	Yes	17
Type 45	20/178	2	0.25	8	Yes	17
Type 52	19/296	14	0.25	56	Yes	17
Type 58	19/300	20	0.25	80	Yes	17

Each ampoule is a freeze-dried preparation using sera from naturally infected individuals.

8.5 PREPARATION OF SECONDARY STANDARDS FOR HPV DNA AND THEIR CALIBRATION IN IU

The WHO manual for the preparation of secondary reference materials for in vitro diagnostic assays designed for infectious disease nucleic acid or antigen detection: calibration to WHO International Standards provides extensive practical guidance on the preparation of secondary and tertiary biological reference materials and on their calibration traceable to WHO International Standards where available.[4] This section of the HPV Laboratory Manual incorporates HPV DNA-specific considerations for applying the guiding principles of the WHO manual.

8.5.1 HPV DNA assays

HPV DNA may be assayed in a range of nucleic acid-based tests, including real-time polymerase chain reaction (PCR). These may be either commercial or in-house assays. Suitable assays for the detection and genotyping of HPV are described in Chapter 6 of this manual. All types of assays 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 HPV6, 11, 16, 18, 31, 33, 45, 52 and 58 DNA should be calibrated in IU against the relevant HPV DNA IS (to produce secondary standards) or against a high-order standard that has itself been calibrated, and traceable to the IS (to produce tertiary standards). 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.

8.5.2 Selection of HPV DNA 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 in cell culture medium may be used as the matrix. This is then spiked with a suitable concentration of HPV plasmid

DNA. Alternatively, a suitable 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.

WHO International Standards for HPV types 6, 11, 16, 18, 31, 33, 45, 52 and 58 DNA are currently full-length genomes cloned into plasmids formulated in a background of purified human genomic DNA (**Tables 8-1 - 8-3**). When calibrating a secondary standard against an HPV DNA IS, serial dilutions of both the IS and secondary standard must be prepared in an appropriate diluent which reflects the biological matrix under test. For example, dilutions could be made in a relevant sample collection medium or assay buffer containing a given concentration of HPV-negative cells or purified human genomic DNA, such as C33A DNA,[6] human placenta DNA,[7] or other HPV-negative human DNA. This will provide a constant cellular DNA background in all diluted samples during assays.

8.5.3 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 (IU/mL) of the secondary standard(s) should also be agreed. This will depend on the intended use of the secondary standard. For example, a high concentration may be required if the secondary standard will be used as a calibrator in a range of assays. A lower concentration may be needed if the standard is intended to serve as a monitor or run control. Identify any need for formulating multiple candidate standards (e.g. candidates with high, medium, low titres). 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.

Preliminary studies on the source material should be undertaken to confirm its identity and determine its concentration and unitage prior to formulation. HPV plasmid DNA may be quantified by A260 and picogreen measurements prior to their use in formulation studies. Whether the source material is, for example, plasmid-based, cell culture-based or derived from clinical samples, the material should 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. Confirmation of the candidate's sequence through e.g. high throughput/deep sequencing may be considered as part of the pre-calibration check. Ideally, the candidate secondary standard should be tested in the different types of assays 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 as reference laboratories 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 and platform of the assay. As described above, standards need to be diluted in a suitable background of human DNA when assessing extraction steps of assays.
- Consideration should be given to cases where the extraction and purification steps are integral to the HPV assay method (e.g. closed sample-in, answer-out platforms). In such cases, the protocol should provide guidance on how to prepare serial dilutions of the study samples for testing (see above examples and reference 7).
- 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. To limit any variability due to differences in diluent formulations prepared by different laboratories, the study organizer should consider preparing bulk diluent for distribution to the study participants. Alternatively, participating labs may be provided with a standard operating procedure for preparing the diluent.

8.5.4 Preparation

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

All vials of the candidate standard should be labelled with:

- the name of the material;
- any assigned production code/batch 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.

8.5.5 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**).

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).

8.5.6 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.[21] Alternatively, the free shareware PLL programme can also be used.

The 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 1 x 107 IU/mL, the relative potency of the test sample (obtained by the statistical analysis) is multiplied by 1 x 107 IU/mL to express the DNA concentration/potency of the test sample in IU/mL.

Quantitative assays:

- Analyse assay outputs, e.g. cycle threshold (Ct) 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.[22] 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 104 detectable units/ml and the IS was estimated to contain 5.2 x 104 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 107 IU/mL, then the unitage of the secondary standard is 4.4 x 106 IU/mL.

8.6 PREPARATION OF SECONDARY STANDARDS FOR HPV ANTIBODIES AND THEIR CALIBRATION IN IU

The WHO manual for the preparation of reference materials for use as secondary standards in antibody testing provides extensive practical guidance on the preparation of secondary and tertiary biological reference materials and on their calibration traceable to WHO International Standards where available.[5] This section of the HPV Laboratory Manual incorporates HPV-specific considerations for applying the guiding principles of the WHO manual.

8.6.1 HPV antibody assays

Antibody 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.

The first generation of licensed L1-based HPV vaccines protect against the 2 major oncogenic HPV types (HPV16 and HPV18). Second generation HPV vaccines include those that also protect against all HPV types that cause >2% of cervical cancers (HPV16/18/45/33/31/52/58). Vaccines targeting even more of the oncogenic HPV types are in development. Some HPV vaccine may also target low risk HPV6 and HPV11 which cause >90% of genital warts. 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 pilot study indicated that the use of an HPV antibody standard improves comparability between laboratories using different assays.[23] Subsequent studies have shown that serology assays for the 9 HPV types represented in current vaccines can be harmonized across laboratories when antibody measurements are determined relative to the IS (ref N, O, P). WHO guidelines state that antibody levels should be reported in International Units (IU) for HPV types for which an International Standard is available.[24]

The ISs have been demonstrated to harmonize immunoassays based on virus like particles (VLPs) and in neutralization tests. For the establishment of ISs, as wide a range of assays as possible is used. The calibration of secondary antibody standards, or tertiary standards, will depend on the assay method(s) routinely performed by the users of the secondary standard. 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 L1-based antigens used.

8.6.2 Selection of HPV antibody 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 blood donations from 1) 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 HPV 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. The exception to this is the IS for HPV11 antibodies, which is also reactive against other types despite efforts to identify monspecific source material. (**Table 8-4**).

As substantial screening and characterization efforts are needed to identify HPV-monospecific serum samples, it is anticipated that most secondary standards will not necessarily need to be HPV-monospecific.

Sera or plasma should 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.

8.6.3 Planning

At the initiation of the project, the intended use, anticipated requirements and the likely usage should be assessed so that the batch of 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.

Preliminary studies on the source sera or plasma should be undertaken to confirm its identity and determine its titre and/or unitage prior to further development and/or aliquoting. These assays should be performed in the different types of assays in which the working standard 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 the performance of validated assays. For serology, VLPs must meet quality control (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.

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. The estimated titre of the samples, as determined in the preliminary assessment of the source material and pooling studies, may be used to gauge which dilutions to test.;
- including replicates of at least one sample in each assay 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.
- use of the diluent for serial dilutions.
- Appendix 6 of the WHO manual for the preparation of reference materials for use as secondary standards in antibody testing provides an example of an SOP for an established bioassay that may be adapted for use by laboratories developing secondary antibody standards.[5]

8.6.4 Preparation

The bulk preparation of candidate working standard should be aliquoted into volumes appropriate for single use. Screw-capped vials with O-rings should be used.

All vials of the candidate standard should be labelled with:

- the name of the material;
- any assigned production code/batch 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.

8.6.5 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**).

8.6.6 Statistical analysis and calibration against the IS for HPV antibodies

Commercially-available statistical software may be used to perform PLL analysis (quantitative assays) or estimating assay end-points (qualitative assays). Statistical programmes are available.[21] Alternatively, the free shareware PLL programme can be used.

The antibody concentration (potency) of the test sample is expressed relative to the calibrating standard (i.e. 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.

8.7 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.

8.8 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).

8.9 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 in the absence of an IS), **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.

8.10 REFERENCES

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