WHO PROTOCOL FOR PERFORMANCE LABORATORY EVALUATION OF HBsAg SEROLOGY ASSAYS
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3. **Introduction**

3.1. **Prequalification of in vitro diagnostics**

The World Health Organization (WHO) Prequalification of In Vitro Diagnostics is coordinated through the Prequalification-Diagnostics Assessment team (PQDx). The aim of the WHO Prequalification of In Vitro Diagnostics Assessment is to promote and facilitate access to safe, appropriate and affordable diagnostics of good quality in an equitable manner. Focus is placed on products for high burden diseases and their suitability for use in resource-limited settings.

The WHO prequalification of in vitro diagnostics process includes three main components:

- Review of an pre-submission form and product dossier;
- Performance evaluation of the product;
- Inspection of the manufacturing site(s).

Performance evaluation will be conducted by two different mechanisms as described at: [http://www.who.int/diagnostics_laboratory/evaluations/alternative/en/](http://www.who.int/diagnostics_laboratory/evaluations/alternative/en/). Performance evaluation in List 1 laboratories will be coordinated and paid for by WHO. Performance evaluation in List 2 laboratories will be coordinated and paid for by the manufacturer.

This protocol provides the details of the procedure for performance evaluation of HBsAg serology simple and rapid assays and Enzyme Immunoassays submitted for laboratory evaluation as part of the WHO Prequalification of In-vitro Diagnostic assays on behalf of World Health Organization at the WHO Prequalification Evaluating Laboratory.

*This protocol shall not replace more extensive IVD manufacturer’s protocols required for validation and verification studies of their products.*

3.2. **WHO performance evaluation of in vitro diagnostics for HBsAg detection**

The performance evaluation determines the accuracy of HBsAg assays in comparison with established performance criteria. These characteristics include: analytical and diagnostic sensitivity, diagnostic specificity, negative and positive predictive values, HBV genotypes and HBsAg mutant detection. In addition, a number of operational characteristics are assessed including the suitability for use in laboratories and/or testing settings with limited infrastructure.

Tests for the detection of other HBV markers are not covered in this protocol.

All HBsAg assays submitted for performance evaluation are assessed at a WHO Prequalification Evaluating Laboratory which has been assessed and listed using the WHO Alternative Performance evaluation Mechanism upon the instruction of WHO/PQDx (List 1 laboratories) or a manufacturer (List 2 Laboratories) who has submitted an IVD for WHO assessment.

The HBsAg Specimen Reference Panels consisting of approximately 514 HBsAg positive and HBsAg negative serum/plasma specimens from Europe, Africa, Asia and Latin America plus several well-characterized commercial seroconversion panels and performance panels, a panel of samples containing representative HBV genotypes, HBsAg mutants, a lot-to-lot variation panel, and WHO international biological reference preparations including a genotype panel.
4. Objectives

4.1. Overall objectives

The overall objectives are:

1. To evaluate currently commercially available diagnostics (including EIA and rapid diagnostic tests) for detection of HBsAg against established performance criteria

4.2. Specific objectives

The specific objectives of the evaluation are:

1. To determine the analytical sensitivity, diagnostic sensitivity, diagnostic specificity of currently available assays (EIA’s and rapid diagnostic tests) for the detection of HBsAg as compared to a reference result (two EIA’s including a neutralization method for one EIA)

2. To evaluate the operational characteristics of the HBsAg assays, e.g. ease of performance, specimen type utility, inter-reader variability, reaction endpoint stability, suitability for use in extreme climates (high/low temperatures, high humidity), suitability for use in countries with limited infrastructure (no/limited electricity, no/limited clean water, inadequate means of biosafety disposal)

5. Study Design

5.1. WHO Prequalification Evaluating Laboratory

The WHO Prequalification Evaluating Laboratory shall be one that has undergone assessment using the WHO Alternative Laboratory Evaluation Mechanism which include submission of an Expression of Interest (EoI), Stage 1 audit (Assessment of EoI and specific quality management system (QMS) documentation), Stage 2 audit which include On-site audit of the laboratory to assess compliance with WHO requirements and lastly listed as WHO Prequalification Evaluating Laboratories. [http://www.who.int/diagnostics_laboratory/evaluations/alternative/en/](http://www.who.int/diagnostics_laboratory/evaluations/alternative/en/).

The laboratory shall hold the following certification for quality management within the laboratory: ISO17025 (General requirements for the competence of testing and calibration laboratories), ISO15189 (Medical laboratories: Particular requirements for quality and competence) or equivalent.

The person(s) listed in the EoI letter to WHO will act as the Principal Investigator (PI) for the work performed by the WHO Prequalification Evaluating Laboratory.

5.2. Training, performance evaluation and supervision

The following issues are key to minimizing error and maximizing the value of this evaluation:

- The PI will be responsible for training the laboratory technicians in the evaluation protocol and in the performance of each assay undergoing evaluation;
- Only those personnel who have received specific training for this evaluation will be employed in the evaluation;
- Accurate record keeping is crucial to the success of the evaluation and the PI will be responsible for ensuring that all data required for the evaluation are recorded on the agreed data collection sheets, and are accurate and up to date;
- It is important to plan work in advance and follow standard operating procedures as prepared and controlled by the WHO Prequalification Evaluating Laboratory;
WHO Protocol for Performance Evaluation of HBsAg serology assays

5.3. Safety

HIV, hepatitis B, hepatitis C and other viruses are transmissible by blood and body fluids. Therefore, all types of specimens (including venous and capillary whole blood, serum/plasma, oral fluid, etc.) must be handled as potentially infectious. Appropriate precautions to minimize infectious hazards must be taken at all stages from the collection of specimens to the disposal of used materials from the laboratory. The WHO Guidelines on HIV Safety Precautions, and Guidelines for the Safe Transport of Specimens (WHO/EMC/97.3) and the WHO Prequalification Evaluating Laboratory guidelines on laboratory safety should be carefully followed by the laboratory staff.

5.4. Storage of assays

All reagents must be stored as indicated in the instructions for use. Some assays may not need refrigeration. Calibrated thermometers are placed at each location where reagents and specimens are stored, i.e. ambient, refrigerator and freezer. Temperatures are recorded daily on the WHO Prequalification Evaluating Laboratory forms or by constant monitoring through an electronic system. The lot numbers of the test kits received/used and their expiry dates are recorded on the individual run worksheets.

Two separate production lots (with different lot numbers and different expiry dates) will be requested for evaluation, according to the following definition\(^1\) of a lot: “The amount of material that is uniform in its properties and has been produced in one process or series of processes. The material can be either starting material, intermediate material or finished product.” Furthermore, lots must be sourced from a representative production run and not produced especially for the purpose of this evaluation. WHO will verify this information before the product assessment has been finalized.

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\(^1\)ISO 18113-1:2009 In vitro diagnostic medical devices -Information supplied by the manufacturer (labelling) – Part 1: Terms, definitions and general requirements
6. Specimens

6.1. HBsAg specimen reference panel

6.1.1. Collection of specimens for the HBsAg specimen reference panel
Specimens are collected as serum or plasma (with EDTA as an anti-coagulant or any other relevant anticoagulant). Panel specimens are assigned a unique identification number at the collection site and sent as large volumes to the WHO Prequalification Evaluating Laboratory where they are assigned the specimen identification number. Once the specimens have been processed and labelled they are frozen immediately at -20°C. At the WHO Prequalification Evaluating Laboratory, the specimens are aliquotted into working volumes of 250µl and the remainder into 2ml volumes and stored at -20°C or colder until testing commences. During the period of testing the specimens are stored at 2 - 8 °C and this time period does not exceed one week. After the completion of testing, they are again stored at -20°C. The number of freeze/thaw cycles for each specimen should be within the number specified in the manufacturer’s Instructions for Use, or where not specified then no more than 5 freeze-thaw cycles. Where a false reaction is identified during evaluation then an unused aliquot should be used for retesting.

6.1.2. Characterization of the HBsAg specimen reference panel
The panel consists of minimum of 514 serum/plasma specimens of European, African, Latin American and Asian origin. There are 201 HBsAg positive specimens, and 313 HBsAg negative specimens.

<table>
<thead>
<tr>
<th>HBsAg positive specimens</th>
<th>HBsAg negative specimens</th>
<th>Total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>201</td>
<td>313</td>
<td>514</td>
</tr>
</tbody>
</table>

The HBsAg Specimen Evaluation Panel shall be characterized using a standardized combination of assays i.e. a testing algorithm. These results are used to determine the HBsAg status of each specimen for the purpose of the performance evaluation (Figure 1). Use of any other combination of assays for characterization of the HBsAg specimen evaluation panel shall be communicated, discussed and agreed with WHO beforehand.

Initially, each specimen was tested on the Hepanostika HBsAg Uniform II/ Hepanostika Ultra EIA (bioMérieux) and Monolisa Ag HBs Plus EIA (BioRad Laboratories) in parallel.

Specimens that were non-reactive on both EIAs are not further tested and were assigned as HBsAg negative.

Specimens with discrepant EIA results AND with dually reactive EIA results were further tested on Hepanostika HBsAg Uniform II Confirmatory assay (bioMérieux).

Specimens that were positive by neutralization were assigned as HBsAg positive.

Specimens that were negative by neutralization were assigned as HBsAg negative.

If the characterization results are unclear, the specimen was omitted from the panel.
Figure 1 - Testing Algorithm for characterization of the WHO HBsAg Specimen Reference Panel (Serum/Plasma Specimens)

6.2. Lot-to-lot variation panel
Lot-to-lot variation will be assessed on two separate production lots by testing the same set of dilution series (comprised of 2-fold dilutions of 10 stock HBsAg positive specimens in pooled commercially available normal human plasma in the same testing session. The plasma derived from blood donations will be screened prior to pooling and shown to be negative for markers of HIV, HTLV, HCV and HBV infections, and will also be devoid of anti-HBs and anti-HBc antibodies. The lot-to-lot panel will be tested by the Abbott ARCHITECT® HBsAg (List No. 6C36) to provide IU/ml quantitation for each diluted specimen.

6.3. HBsAg Seroconversion Panel
A seroconversion panel is a series of specimens, sequentially collected over a period of time, from an individual developing serological markers (antigens and antibodies) in response to acute infection. Six commercial seroconversion panels, PHM 903, PHM 907, PHM910, PHM916, PHM928 and PHM934 sourced from SeraCare Life Sciences will be tested using the assay under evaluation in singular on one lot. These panels consist of a total of 45 specimens collected from six individuals during HBsAg seroconversion.

6.4. HBsAg Performance Panels
One HBsAg low titer performance panel containing 15 members, PHA 105 sourced from SeraCare Life Sciences will be tested using the assay under evaluation in singular on one lot.

6.5. WHO international reference preparations
Samples derived from the Second and Third WHO International Standards for HBsAg will be employed.
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The WHO international biological reference preparation panel derived from the 2\textsuperscript{nd} International Standard with the catalogue number 03/262 (Hepatitis B surface antigen, subtype adw2, genotype A, Lyophilized, Dilutional panel [IU/vial: 8.25; 2.06; 0.52; 0.13; 0 (dilution matrix)]) will be tested using the assay under evaluation in singular in one lot.

The 3\textsuperscript{rd} WHO International Standard, catalogue number 12/226 (HBV genotype B4, HBsAg subtypes ayw1/adw2) which has an assigned unitage of 47.3 IU/ampoule will be tested. From this a dilution series of 8, 2, 0.5, 0.125 IU/ml will be prepared in negative human plasma and tested under evaluation in singular on one lot.

7.6 WHO HBV Genotypes’ Panel
The 1\textsuperscript{st} WHO International Reference Panel for HBV Genotypes for HBsAg assays (PEI code number 6100/09) was established by the WHO Expert Committee on Biological Standardization in October 2011. This reference panel consists of 15 different members representing sub-genotypes A1 (Samples 1-2), A2 (Sample 3), B2 (Samples 4-5), C2 (Samples 6-8), D1 (Sample 9), D2 (Sample 10), D3 (Sample 11), E (Sample 12), F2 (Samples 13-14), and H (Sample 15). The panel has been evaluated in an international collaborative study with concurrent testing of the 2nd WHO International Standard for HBsAg (00/588). Each of the processed HBV positive plasma stocks has been diluted in a negative plasma pool to a final HBsAg concentration of approx. 100 IU/ml, based on the chemiluminescent immunoassay Abbott ARCHITECT Quantitative. Each of these 15 samples will be used to prepare dilutions in NHP equivalent to approximately 8.0; 4.0; 2.0; 1.0; 0.5; 0.25IU/ml and tested using the assay under evaluation in singular on one lot.

7.7 WHO HBV Mutants’ Panel
A panel of 12 samples will be included that have been derived from HBV-infected individuals, whose HBsAg has been shown both genotypically (DNA sequencing) and phenotypically (monoclonal antibody reactivities) to have a range of significantly divergent/mutant HBsAg in the diagnostically important ‘a’ determinant. Concentrations have been estimated by testing on the Abbott ARCHITECT® HBsAg (List No. 6C36), and dilutions in NHP have been prepared to provide an indication of whether the device under evaluation is able to detect each of these to approximately 16, 8, 4, 2 and 1 IU/ml.

6.6. External quality control specimen
See section 9 for further details

Table 2 - Overview of specimen panels used in the performance evaluation

<table>
<thead>
<tr>
<th>Panel name</th>
<th>Number of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO HBsAg specimen reference panel</td>
<td>201 HBsAg positive, 313 HBsAg negative</td>
</tr>
<tr>
<td>Lot-to-lot variation panel</td>
<td>22 member dilution series of 9 specimens and one 24 member dilution (222 in total)</td>
</tr>
<tr>
<td>Commercial HBsAg performance panels</td>
<td>1 panel comprising 15 specimens in total</td>
</tr>
<tr>
<td>Commercial HBsAg seroconversion panels</td>
<td>6 panels comprising 45 specimens in total</td>
</tr>
<tr>
<td>HBsAg mutants</td>
<td>12 specimens with five dilutions: 16, 8, 4, 2 and 1 IU/ml (60 in total)</td>
</tr>
<tr>
<td>WHO reference preparation calibrated dilution series for HBV Genotypes for HBsAg</td>
<td>15 members with six dilutions (8, 4, 2, 1, 0.5, 0.25 IU/ml) (90 in total)</td>
</tr>
</tbody>
</table>
assays based on 1st WHO International Reference Panel (PEI code number 6100/09) &nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&n...
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- Commercially acquired HBsAg low titre performance panel;
- HBsAg mutant panel.
- HBV genotype panel

The HBsAg specimen reference panel will be randomized and run in order that approximately one half of the specimen panel will be run with the one lot and the other half of the panel with the other lot. The specimens of the WHO HBsAg specimen reference panel should initially be tested in singular and in a blinded manner.

Lot-to-lot variation will be assessed by testing the same set of dilution series (comprised 2-fold dilutions of 10 stock HBsAg positive specimens) on two separate production lots of the assay under evaluation.

The seroconversion, performance, mutant and genotype panels are then tested in singular on a single lot.

For the purpose of evaluating the rapid diagnostic tests, a ‘test run’ is defined as a consecutive run of simple/rapid diagnostic tests of the same production lot performed during the same ‘session’. A ‘testing session’ might be considered to be a morning or afternoon.

7.2. Recording test results

All test results will be recorded electronically directly from the plate reader and then entered in a Microsoft Excel spreadsheet for further data analysis. Printed records will also be generated from the EIA reader, and these will be used to cross-check a sample of the imported data. For subjectively read assays such as rapid diagnostic tests or line immunoassays, the intensity of band/line/spot is additionally be entered into the data collection sheet. The intensity rating system reads as described in Table 3.

Table 3 - Results legend for data collection sheets for subjectively read assays

<table>
<thead>
<tr>
<th>Scoring index</th>
<th>Intensity reading scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Negative</td>
</tr>
<tr>
<td>1</td>
<td>Very Weak, but Definitely Reactive</td>
</tr>
<tr>
<td>2</td>
<td>Medium to Strong Reactivity</td>
</tr>
<tr>
<td>7</td>
<td>Invalid (including no control line/band/dot/spot visible, or obviously defective test device, no flow, debris present)</td>
</tr>
</tbody>
</table>

Visual interpretation of results of subjectively read assays is made independently by three readers (without the knowledge of the other two sets of results and blinded to the reference result for the specimen) and entered onto the data collection sheets. These results are compared by the operator carrying out the assay so that any potential transcription or reading errors may be identified and rectified immediately. Should recording errors be identified, both the original and corrected result are recorded and initialed by the reader and the operator or supervisor. When the three readers interpret the results differently from each other, the consensus is recorded as that interpretation which occurs two out of three times.
8. Quality control and interpretation of test results

8.1. Test kit controls
Manufacturer-supplied positive and negative test kit controls will be run as indicated in the IFU for all test formats included in each test run for EIAs and at the commencement of each testing session for rapid diagnostic tests and other formats. Where positive and negative test kit controls are not supplied by the manufacturer, as will be the case for many rapid diagnostic tests, the external quality control specimen will act as the control specimen, see later section 9.3.

8.2. Internal control lines for rapid diagnostic tests
Generally, rapid diagnostic tests contain a control band, line or spot that is intended to demonstrate that the test device is operating correctly. Most control bands/lines/spots will become visible with the addition of reagent (i.e. buffer). However, some rapid diagnostic tests will contain a control band/line/spot that becomes visible only with addition of specimen (i.e. presence of IgG). It is imperative that the exact nature of the control band/line/spot is ascertained and recorded in the report. An experiment is performed to check the mode of action of the control reaction, if not explicitly mentioned in the IFU.

8.3. External quality control specimen
The WHO prequalification evaluating laboratory will supply at least one external quality control (QC) specimen which is tested in singular at the beginning of each test session for rapid diagnostic tests and other formats and in triplicate for each test run for EIAs. See definition of testing session in section 5.1. The QC specimen represents a weakly reactive HBsAg positive specimen, and thus may be different for different assays and different assay formats. The QC specimen is made by WHO evaluating Laboratory or acquired commercially, depending on the assay under evaluation.

8.4. Proficiency panels
A proficiency panel must be run successfully for each assay by each operator before the evaluation commences. This must include a 4IU/ml preparation as a minimum detection requirement. This may be the same panel as that used at the time of assay demonstration by the manufacturer or for training purposes.

8.5. Limits of acceptability
All results on test kit controls and QC specimens are entered on the data collection sheets or electronically, if EIA. Should the test kit controls or the QC specimen not give results within the expected ranges, evaluation testing on that assay is suspended until the cause has been identified and a satisfactory solution implemented. Such problems must be communicated immediately to WHO and recorded on the data sheets. The PI is responsible for carefully checking all data entry forms for legibility, accuracy and completeness.

8.6. Interpretation of results
The interpretation of results for each assay under evaluation is made strictly according to the manufacturers’ instructions within the IFU. Invalid test results are recorded on the data collection sheets including where the control line does not appear or in any other way the test result is invalid as defined by the IFU.
9. Analysis of data

9.1. Invalid runs/devices
The number of invalid test runs (if EIA) is recorded as the number of invalid runs as a percentage of the total number of runs performed for clinical specimens only (excluding the commercially acquired panels, lot variation panels, and WHO reference preparation).

The number of invalid devices (if rapid diagnostic test or other format) is recorded as the number of invalid test devices as a percentage of the total number of devices used with clinical specimens (excluding commercially acquired panels, reference preparations, culture supernatant panels, lot to lot variation panels).

Invalid results may mean invalid test results as defined by the IFU where the control line/band/spot does not appear or invalid due to obviously defective test device or defective transfer pipette.

9.2. Inter-reader variability
The inter-reader variability will be calculated when assay readings must be performed without any objective reading instruments i.e. rapid diagnostic tests. Three persons independently interpret each test result. The inter-reader variability is expressed as the percentage of specimens for which initial test results are differently interpreted (i.e. reactive or non-reactive or indeterminate) by the independent readers.

9.3. Performance characteristics from WHO HBsAg specimen reference panel
The following strategies are used to calculate the performance characteristics for each assay under evaluation and is closely linked to the reference testing results gained during specimen panel characterization.

Table 4 - 2 x 2 table for calculation of performance characteristics

<table>
<thead>
<tr>
<th>Results of assay under evaluation</th>
<th>Reference testing results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reactive</td>
</tr>
<tr>
<td>Reactive</td>
<td>a</td>
</tr>
<tr>
<td>(true positives)</td>
<td></td>
</tr>
<tr>
<td>Non-reactive</td>
<td>c</td>
</tr>
<tr>
<td>(false negatives)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>a + c</td>
</tr>
</tbody>
</table>

9.3.1. Sensitivity
Sensitivity is the ability of the assay under evaluation to detect correctly specimens that contain HBsAg (reference results positive). Thus sensitivity is the number of true positive specimens identified by the assay under evaluation as positive (a), divided by the number of specimens identified by the reference assays as positive (a+c), expressed as a percentage.

\[
\text{Sensitivity} = \frac{a}{a + c}
\]
Specificity

Specificity is the ability of the assay under evaluation to detect correctly specimens that do not contain HBsAg (reference results negative). Thus specificity is the number of true negative specimens identified by the assay under evaluation as negative (d), divided by the number of specimens identified by the reference assays as negative (b+d), expressed as a percentage.

\[
\text{Specificity} = \frac{d}{b+d}
\]

Confidence intervals

The 95% confidence intervals will be calculated for both sensitivity and specificity in order to assess the level of uncertainty introduced by sample size, etc. Exact 95% confidence intervals for binomial proportions will be calculated from the F-distribution. [Armitage, 2002; Kirkwood, 2003]

Positive predictive value (PPV)

The probability that when the test is reactive that the specimen does contain HBsAg. PPVs will be calculated using the formula.

\[
\text{PPV} = \frac{(\text{prevalence})(\text{sensitivity})}{(\text{prevalence})(\text{sensitivity})+(1-\text{prevalence})(1-\text{specificity})}
\]

Negative predictive value (NPV)

The probability that when the test is negative that a specimen does not contain HBsAg. NPVs will be calculated using the formula.

\[
\text{NPV} = \frac{(1-\text{prevalence})(\text{specificity})}{(1-\text{prevalence})(\text{specificity})+(\text{prevalence})(1-\text{sensitivity})}
\]

The probability that a test result will accurately determine the true infection status of a person being tested varies with the prevalence of HBsAg infection in the population from which the person comes. In general, the higher the prevalence of HBsAg infection in the population, the greater the probability that a person testing positive is truly infected (i.e., the greater the positive predictive value [PPV]). Thus, with increasing prevalence, the proportion of individuals testing false-positive decreases; conversely, the likelihood that a person whose test result is negative is truly uninfected (i.e., the negative predictive value [NPV]), decreases as prevalence increases. Therefore, as prevalence increases, so does the proportion of individuals testing false-negative.

The PPV and NPV are calculated at prevalence of 0.1%, 1% and 5%.

Discrepant results

Those specimens with results that are consistent with the reference results (i.e. the characterized specimen results) will undergo no further testing. Those specimens with results discrepant from the reference result will be retested in duplicate using the same lot number by the same operator. The results that occur two out of three times will be recorded as the final result. If the result is again discrepant, the specimen will be retested on a second lot number, if available. If the result on the second lot is concordant with the reference result, no further testing will be required. If the result is still discrepant from the reference results, the result will be recorded as is.
Values for initial sensitivity and specificity will be calculated based on the initial results obtained for the assay under evaluation on the first lot. The final sensitivity and specificity will be calculated taking into consideration the results of repeat testing performed on a second lot of the assay under evaluation i.e. if found to be concordant on the second lot will be recorded as such, and if found to be discrepant on the second lot will be recorded as such.

9.5. Interpretation of results from lot-to-lot variation panel
The results of the lot-to-lot panel for the two production lots will be compared and a variation of +/- 1 dilution series is considered acceptable.

9.6. Interpretation of seroconversion sensitivity
The results obtained from seroconversion panels using the assay under evaluation will be compared with those obtained using Monolisa Ag HBs Plus (Bio-Rad Laboratories) EIA; the assay arbitrarily designated the reference for determination of relative sensitivity in these panels. For each seroconversion series (panel) the first specimen in the sequence to become reactive with Monolisa Ag HBs Plus will be assigned the value “0”. Results from the assay under evaluation will be compared with Monolisa Ag HBs Plus by determining the difference between the specimen assigned value “0” and the relative position in the sequence of the first specimen showing a reactive result for the assay under evaluation. For example, if an assay becomes reactive two specimens earlier in a series than Monolisa Ag HBs Plus, the value assigned for that series in that assay would be -2. Similarly, if an assay becomes reactive one specimen later than Monolisa Ag HBs Plus, the value assigned would be +1. The assigned values over the six seroconversion panels (series) will be averaged to determine a mean relative seroconversion sensitivity index for each assay and the 95% confidence interval will be determined.

In addition, each specimen is tested on the following assays: Hepanostika HBsAg Uniform II (bioMérieux) and HBV DNA PCR (Roche Molecular). The HBV DNA data were provided by SeraCare Diagnostics.

9.7. Interpretation of results from performance panels
The number of specimens correctly identified by the assay under evaluation in the HBsAg performance panels will be determined by comparison with the combined reference results from the following assays: Hepanostika HBsAg Uniform II (bioMérieux), Monolisa Ag HBs Plus (Bio-Rad Laboratories) and the Hepanostika HBsAg Uniform II Confirmatory assay (bioMérieux).

9.8. Interpretation of results from WHO international reference preparations
The number of specimens identified by the assay under evaluation for the WHO international reference preparation will be determined by comparison with the combined reference results generated by the WHO Evaluating Laboratory using Hepanostika HBsAg Uniform II (bioMérieux), and Monolisa Ag HBs Plus (Bio-Rad Laboratories).

9.9. Technician's appraisal
The technical aspects of the assay under evaluation will be assessed by the technician who performed the testing. These assessments, along with other selected assay characteristics, contribute to an overall appraisal of each assay’s suitability for use in small laboratories. To enable comparison between assays, a scoring system will be used to rate specified operational characteristics.
9.10. Report preparation
The data analysis and report drafting is carried out by the WHO prequalification evaluating laboratory and sent to WHO for List 1 laboratories. In case of list two laboratories the data and the report will be sent simultaneously to WHO and the Manufacturer in a timely manner. WHO verifies the draft report from List one laboratories and sends to the authorized contact designated by the manufacturer for comment. The company has one month right of reply. The final report is prepared after one month has elapsed. The WHO scientist ensures that the comments of the authorized contact are reviewed and any outstanding issues are resolved before final publication. The final report is prepared and disseminated by WHO. A copy of the final report is sent to the authorized contact designated by the manufacturer and to WHO prequalification evaluating laboratory.

10. Materials and supplies

10.1. Data collection sheets
All data will be reported to WHO/PQT on the following forms:

A. Data collection Microsoft Excel spreadsheet for the rapid diagnostic tests
B. Data collection Microsoft Excel spreadsheet for EIAs (print-outs)
C. Technician’s appraisal worksheet
D. Findings of supplementary testing on discrepant specimens

10.2. Supplies
The manufacturers of products will provide the products and any equipment necessary for the evaluation free of charge.

11. Roles and responsibilities

11.1. Responsibilities of the WHO Prequalification Evaluating Laboratory
1. Act as repository for the WHO HBsAg specimen reference panel, lot-to-lot variation, seroconversion and performance panels;
2. Conduct the laboratory evaluation according to the agreed protocol;
3. Prepare QC specimens and proficiency panels;
4. Prepare draft report on laboratory evaluation;
5. Advise WHO on operational characteristics of assays evaluated.

All source data, data analysis records and all correspondence are retained and archived for a period of at least 10 years.

11.2. Responsibilities of WHO/PQT
1. Technical advice to the PI;
2. Technical and administrative management of the laboratory evaluation;
3. Verify the draft report, seeking of comments from manufacturer;
4. Prepare and disseminate the final report;
5. Formal contacts with authorized contacts of the manufacturers.

Any publication by WHO of the results of these evaluations and the WHO recommendations derived therefrom will, however, be accompanied by the following disclaimer:
The mention of specific companies or of certain manufacturers’ products does not imply that they are endorsed or recommended by the World Health Organization in preference to others of a similar nature that are not mentioned. Errors and omissions exempted, the names of proprietary products are distinguished by initial capital letters.

WHO and the WHO Prequalification Evaluating Laboratory do not warrant or represent that the evaluations conducted with the HBsAg test kits referred to in this document are accurate, complete and/or error-free. WHO and the WHO Prequalification Evaluating Laboratory disclaim all responsibility for any use made of the data contained herein, and shall not be liable for any damages incurred as a result of its use. This document must not be used in conjunction with commercial or promotional purposes.

12. References


World Health Organization Guidelines on HIV Safety Precautions, and Guidelines for the Safe Transport of Specimens (WHO/EMC/97.3)

International Standards
EN 13612:2002 Performance evaluation of in vitro diagnostic medical devices

ISO 17025 (General requirements for the competence of testing and calibration laboratories)

ISO15189 (Medical laboratories — Particular requirements for quality and competence)

13. Other documents required

Master Templates

SOP_PQDx_138 PQT report template for 3rd generation HBsAg EIAs including excel spreadsheets of results

SOP_PQDx_139 PQT report template for 3rd generation HBsAg simple/rapid assays for non-discriminatory detection including excel spreadsheets of results