WHO PREQUALIFICATION TEAM:
DIAGNOSTICS

World Health Organization

Technical Specifications Series
for submission to WHO Prequalification – Diagnostic Assessment

TSS-9 Immunoassays to detect HIV antibody and/or antigen

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Abbreviations

ANA	anti-nuclear antibodies
CRF	circulating recombinant form
CV	coefficient of variation
HAMA	human anti-mouse antibody
ICD	immune complex dissociation
IFU	instructions for use
IU	international units
IVD	in vitro diagnostic
NAT	nucleic acid amplification technology
RNA	ribonucleic acid
ROC	receiver operator characteristic
SLE	systemic lupus erythematosus
TSS	Technical Specification Series
URF	unique recombinant form
VLP	viral like particle
WHO	World Health Organization

A Introduction

The purpose of this document is to provide technical guidance to in vitro diagnostic (IVD) medical device manufacturers that intend to seek WHO prequalification of:

- third generation immunoassays (e.g. enzyme, chemiluminescent, fluorescent) for the detection of antibodies to human immunodeficiency virus (HIV);
- fourth generation immunoassays intended to detect antibodies and antigens of HIV, and
- immunoassays intended to detect HIV p24 antigen.

It does not cover the requirements for rapid diagnostic tests for HIV which are described in a separate technical specifications series (TSS) document. The standard international definitions of generations of HIV assays are used in this document: 1st generation using native virus in any assay format; 2nd generation using synthetic antigens (recombinant or peptide) in any assay format; 3rd generation using synthetic antigens, specifically in an antigen sandwich format (technically and preferably according to the original patents using antigens prepared in a different host at each side of the sandwich to improve assay specificity); 4th generation using synthetic antigens in an antigen sandwich format to detect antibodies along with a component to detect HIV antigen.[1]

[1] https://apps.who.int/iris/bitstream/handle/10665/179870/9789241508926_eng.pdf;jsessionid=B076BDB67306ADAC2FD7E36C62C6F024?sequence=1

Where possible, WHO analytical and clinical performance study requirements are aligned with published guidance, standards and/or regulatory documents. Although references to source documents are provided, in some cases WHO prequalification has additional requirements.

For the purpose of this document, the verbal forms used follow the usage described below:

- "shall" indicates that the manufacturer is required to comply with the technical specifications.
- "should" indicates that the manufacturer is recommended to comply with the technical specifications but it is not a requirement.
- "may" indicates that the technical specifications are a suggested method to undertake the testing but it is not a requirement.

A documented justification and rationale shall be provided by the manufacturer when the WHO prequalification submission does not comply with the required technical specifications outlined in this document.

For WHO prequalification purposes, manufacturers shall provide evidence in support of the clinical performance of an IVD to demonstrate that reasonable steps have been taken to ensure that a properly manufactured IVD, being correctly operated in the hands of the intended user, will detect the target analyte consistently and fulfil its indications for use.

WHO prequalification requirements summarized in this document do not extend to the demonstration of clinical utility, i.e. the effectiveness and/or benefits of an IVD, relative to and/or in combination with other measures, as a tool to inform clinical intervention in a given population or healthcare setting.[2] WHO prequalification focus on analytical and clinical performance of a specific IVD. To demonstrate clinical utility, a separate set of studies is required. Clinical utility studies usually inform programmatic strategy and are thus the responsibility of programme managers, ministries of health and other related bodies in individual WHO Member States. Such studies are applicable to all assays with the same intended use and do therefore not fall under the scope of WHO prequalification.

B How to apply these specifications

For the purposes of WHO prequalification, 3rd and 4th generation immunoassays shall comply with the specifications in Part 1 and Part 2 of this document. For immunoassays which detect HIV antigen, submissions shall comply with the specifications in Part 1 and Part 3.

C Other guidance documents

This document should be read in conjunction with other relevant WHO guidance documentation, including:

WHO prequalification documents[3]

- Technical Guidance Series for WHO Prequalification - Diagnostic Assessment

[2] See GHTF document GHTF/SG5/N6:2012 Clinical Evidence for IVD medical devices – Key Definitions and Concepts for more information: http://www.imdrf.org/docs/ghtf/final/sg5/technical-docs/ghtf-sg5-n6-2012-clinical-evidence-ivd-medical-devices-121102.pdf

[3] Available at http://www.who.int/diagnostics_laboratory/evaluations/en/

- Sample Product Dossiers for WHO Prequalification - Diagnostic Assessment
- Instructions for Compilation of a Product Dossier, WHO document PQDx_018

WHO Consolidated Guidelines on HIV testing services [4]

D Performance principles for WHO prequalification

D.1 Intended use

An IVD intended for WHO prequalification shall be accompanied by a sufficiently detailed intended use statement. This should allow an understanding of at least the following:

- The assay type and what is detected (e.g. to detect antibodies to HIV-1, HIV-2 and/or HIV-1 p24, HIV-2 p26 antigen, etc.);
- The clinical indication and function of the IVD (e.g. for screening blood donations, as an aid to diagnosis of HIV infection), and whether it is qualitative, semiquantitative or quantitative;
- The testing population for which the functions are intended (e.g. adult blood donors, neonates, attendees at maternity hospitals, sexually transmitted infection clinic or service attendees);
- The intended operational setting (e.g. blood banks, hospital laboratories, clinical laboratories);
- The intended user;
- The intended specimen type; and
- Any limitations to the intended use.

If the assay is being claimed for use as a confirmatory assay, then this should be validated at a regional level in the intended testing population. More extensive clinical studies are required which are outside the scope of the document. The document does not address IVDs that discriminate between the detection of HIV-1 and HIV-2 infection.

D.2 Diversity of specimen types, users and testing environments and impact on required studies

For WHO prequalification submission, clinical performance studies shall be conducted using each specimen type (e.g. serum, plasma) claimed in the instructions for use (IFU).

Prequalified immunoassays are likely to be used by laboratory professionals in low- and middle-income countries. Depending on the intended use of an immunoassay, analytical and clinical performance studies shall be designed to consider not only the diversity of knowledge and skills across the population of such individuals, but also the likely operational settings in which testing will occur.

Laboratory demonstration of equivalence between specimen types without evidence of clinical validation is insufficient. For example, studies that comprise the testing of left-over/repository specimens by research and development staff at a manufacturer's facility shall not, on their own, be considered sufficient to meet many of the clinical performance study requirements summarized in this document.

[4] Available at http://apps.who.int/iris/bitstream/handle/10665/179870/9789241508926_eng.pdf?sequence=1

D.3 Applicability of supporting evidence to IVD under review

Performance studies shall be undertaken using the specific, final (locked-down) version of the immunoassay intended to be submitted for WHO prequalification. For WHO prequalification, design lock-down is the date that final documentation, including quality control and quality assurance specifications, is signed off and the finalized method is stated in the IFU. Where this is not possible, a justification shall be provided, and additional supporting evidence may also be required. This may occur in the case of minor variations to design where no impact on performance has been demonstrated (see WHO document PQDx_121 Reportable Changes to a WHO Prequalified In Vitro Diagnostic Medical Device).[5] If the protocol section of the IFU has been changed in any way, both the protocol provided to laboratory for studies as outlined in Part 2 and Part 3 and that in the final version intended for users shall be provided with the submission to WHO prequalification.

The version of the IFU used for performance evaluations submitted to WHO prequalification shall be stated. If the test procedure in the IFU is changed in any way after completing performance verification and validation studies the change shall be reported to WHO, including a rationale for the change, and an explanation of why the study results support the claimed performance.

Specific information is provided in this document for the minimum numbers of lots required for each study. Where more than one lot are required, each lot shall comprise different production (or manufacturing, purification, etc.) runs of critical reagents, representative of routine manufacture. It is a manufacturer's responsibility to ensure, via risk analysis of its IVD that the minimum numbers of lots chosen for estimating performance characteristics considers the variability in performance likely to arise from the interlot diversity of critical components and their formulation or from changes that could occur during the assigned shelf life of the IVD. Differences found between lots during the analytical and clinical performance studies shall be reported. Where the manufacturer supplies the instrumentation required to conduct the assay, (e.g. a closed system), safety and performance data shall be provided in the dossier using this instrumentation.

Performance shall be established in comparison to a well-established device (e.g. WHO prequalified, FDA-approved, CE-marked or otherwise approved by a stringent conformity assessment body). Discrepant specimens should be resolved as far as reasonable. Comparison with a similar device is insufficient for resolution of discrepant specimens. Reference methods must not contain antigens or antibodies from the same source as the comparator or IVD under evaluation. Estimation (and reporting) of IVD performance shall include the rate of invalid tests and the 95% confidence interval around the estimated values for key performance metrics, as appropriate.

For certain analytical studies it may be acceptable to use contrived specimens (e.g. where normal human specimens have been spiked with those containing HIV antibodies or antigen). Clinical studies should be based on testing in natural specimens in Part 2 and Part 3.

[5] http://apps.who.int/iris/bitstream/handle/10665/251915/WHO-EMP-RHT-PQT-2016.01-eng.pdf;jsessionid=30D5BF0B09FFDA3B38A1698E65C8B496?sequence=1

For IVDs that include a claim for detection of multiple analytes, evidence of performance shall be provided for each claimed analyte. It should be noted that, depending on the design of an IVD, evidence generated in a similar, related product will usually not be considered sufficient by WHO to support performance claims in an IVD submitted for WHO prequalification.

For example, the antibody detection performance of a 4th generation enzyme immunoassay cannot be inferred from a 3rd generation enzyme immunoassay of identical components and that of an enzyme immunoassay intended to detect HIV antigen cannot be inferred from a 4th generation enzyme immunoassay even if the antigen detection components in the two are identical.

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Part 1: Establishing non-clinical evidence (analytical performance characteristics) for 3rd, 4th generation and HIV antigen immunoassays.

The requirements in the tables below are not applicable to HIV rapid diagnostic tests.

Aspect	Testing requirements	Notes on testing requirements	Source documents
1.1 Stability of sample(s)			
1.1.1 Specimen collection, storage and transport	1. Real time studies shall be determined for each specimen type taking into account: • storage conditions (duration at different temperatures, temperature limits, freeze/thaw cycles) • conditions and time duration before arriving in the laboratory (e.g. transport) • specimen collection and/or transfer devices intended to be used with the IVD • a minimum of 10 specimens tested (see note 4) • specimen concentrations shall cover the measuring range (low, medium and high reactivity positive) and include the different analytes (anti-HIV and/or P24 antigen) to be tested	1. Evidence shall be provided which verifies the maximum and minimum allowable time between specimen collection processing of the specimen and its addition to the IVD 2. In case the use of archived specimens is considered for Part 2 and 3 of this document, evidence of stability shall be demonstrated 3. Data generated by the manufacturer on other similar proprietary IVDs for the detection of the same analyte may be submitted to support the specimen stability claims 4. Spiked (natural positive spiked into natural negative specimens of the claimed specimen type) specimens may be used	
1.2 Validation of specimens			
1.2.1 Demonstration of equivalence of specimen types , contrived specimens and clinical specimens	1. At least 25 positive and 25 negative specimens shall be tested for each claimed specimen type (see note 1) • The equivalence of specimen types shall be determined for all claimed analyte types independently (e.g. anti-HIV antibodies, p24 antigen) 2. If equivalence is claimed between different anticoagulants, testing shall be conducted in at least: • 25 positive (anti-HIV antibody or p24 antigen) specimens of each claimed anticoagulant • 25 negative specimens of each claimed anticoagulant	1. If specimens other than plasma and serum are claimed, then agreement of results shall be demonstrated as part of the clinical study using paired specimens 2. The relationship between IVD performance in claimed specimen types and reference materials used for analytical studies shall be established. The design of subsequent studies shall take that relationship into account 3. If weak, true positive specimens are not available, spiking all the negative individual donors with the same small (less than 5% v/v) amount of a known positive specimen and analysing the results for within (i.e. between specimen types) and between donor variability is acceptable • the corresponding unspiked or negative specimens should be analysed to evaluate potential outliers leading to potential false reactivity in larger tested populations 4. A third of the positive specimens chosen should be near (x 2 to 3) the	Technical Guidance Series for WHO Prequalification – Diagnostic Assessment TGS-3 (1) European Commission decision on CTS (2) Health Products and Food Branch, Health Canada (3)

Aspect	Testing requirements	Notes on testing requirements	Source documents
		immunoassay cut-off and the rest across the dynamic range	
1.3. Metrological traceability of calibrator and control material values			
1.3.1 Metrological traceability of control material values for antigen detection immunoassays	Only applicable for antigen detection immunoassays: 1. When the intended use of the IVD includes a quantitative measurement, the metrological traceability of the provided calibration material(s) to a conventional international calibrator(s) shall be determined (e.g. to WHO International reference panel HIV (antibody), WHO International Standard HIV-1 P24 Antigen)	1. In some jurisdictions there is a requirement for use of a 'National Testing Panel' for lot release and IVD validation. Such a national requirement does not remove the need for evidence of traceability to a validated reference material as described here	ISO 17511 (4) ISO 15198 (5) TRS 1004 Annex 6 (6)
1.4. Precision (repeatability, reproducibility)			
1.4.1 Repeatability, reproducibility	Precision (repeatability and reproducibility) shall be estimated for each analyte type (i.e. anti-HIV antibodies, HIV antigen) using panels of at least the following specimens: 1. 1 negative specimen 2. 1 low reactivity positive spiked specimen (2-3 x assay cut-off) 3. 1 medium reactivity positive spiked specimen. Each panel member shall be tested: 1. in 5 replicates per test 2. over 5 days (not necessarily consecutive) with one run per day (alternating morning/afternoon) 3. repeated in total with 3 different lots (at least 2 lots should be tested at each of the sites) 4. tested at each of 3 different sites. For all precision studies, the effect of operator-to-operator variation on IVD performance should be included as part of the precision studies except for fully automated IVDs where the effect of operator is negligible (see notes 5 and 6). Testing shall be conducted: 1. by trained laboratory staff representative of intended users in addition to members of manufacturer's staff unassisted	1. E.g. within- or between-run, -lot, -day, -site, etc. 2. The testing panel should be the same for all operators, lots and sites 3. Each lot shall comprise different production (or manufacturing, purification, etc.) runs of critical reagents 4. Studies shall be statistically designed and analysed to identify and isolate the sources and extent of any variance 5. The effect of operator-to-operator variation on IVD performance may also be considered as a human factor when designing robustness studies (see 1.10 Usability/human factors) and should be addressed as part of clinical studies in representative populations (see 0) 6. Operators' profiles shall be detailed in the submission for example affiliation and skill level 7. Results provided should be reported as mean, standard deviation, and coefficient of variation (CV) for each specimen	CLSI EP05-A3 (7) EN 13612 (8) CLSI EP12-A2 (6) Health Products and Food Branch, Health Canada (3)

Aspect	Testing requirements	Notes on testing requirements	Source documents
	2. using only those materials provided with the IVD (e.g. IFU, labels and other instructional materials).		
1.5. Analytical sensitivity			
1.5.1 Limit of detection for HIV antigen, (where appropriate)	1. Analytical sensitivity estimated as the concentration of HIV-1 p24 or HIV-2 p26 antigen detectable at the assay cut-off 2. The determination shall comprise a minimum of 24 replicate tests (3 replicates per dilution) of an 8-member doubling dilution panel of a suitable biological reference material (e.g. WHO International Standard HIV-1 p24 Antigen, NIBSC code 90/636 or a secondary standard calibrated against it) 3. Testing shall be conducted using a minimum of 2 different lots. 4. The assay limit of detection shall be evaluated in all claimed matrices (e.g., serum, plasma). One type of plasma may be used and then the rest validated through matrix equivalency	1. Sensitivity for HIV antigen shall be given with a 95% confidence interval that consider lot to lot variation 2. For the international standard(s) the result shall be expressed in international units (IU) as an analytical end-point sensitivity with a 95% confidence interval 3. If the listed international standards are no longer available, then the version of the international standard used shall be stated 4. Each lot shall comprise different production (or manufacturing, purification, etc.) runs of critical reagents	European Commission decision on CTS (2) Health Products and Food Branch, Health Canada (3) CLSI EP12-A2 (9)
1.6. Analytical specificity			
1.6.1 Potentially interfering substances	The potential for false results (false nonreactive and false reactive results) arising from interference by the substances/conditions listed below shall be determined (see note 1) using: 1. A minimum of 100 specimens 2. substances/conditions represented, by at least 5–10 specimens from different individuals 3. Testing shall be undertaken in both HIV-negative, and anti-HIV and/or HIV p24 antigen detectable specimens as appropriate, unspiked or spiked with each potentially interfering substance at physiologically relevant dosages/levels	1. The risk assessment conducted for an IVD shall identify substances where the potential for interference can reasonably be expected for the analyte to be detected (i.e. HIV antibodies and/or HIV antigens) in the areas of intended use and not simply rely on published lists of such compounds and conditions which might be of limited relevance in resource limited settings 2. By conducting appropriate risk assessment, testing can be conducted on specimens spiked with the substances/conditions identified as likely to be significant and testing of potentially irrelevant substances/conditions avoided 3. Under some circumstances stringent risk evaluation may eliminate the requirement to test some of the items in the lists but any such decision shall be documented in any submissions to WHO and considered in the risk-benefit statements • any observed interference or cross-reactivity shall be investigated and performance limitations of the ivd reported in the ifu and taken into consideration in the required risk - benefit statements • any effect must be evaluated against the probability of that effect	Health Products and Food Branch, Health Canada (3) European Commission decision on CTS (2) CLSI EP07-A3 (10) CLSI EP37 (11) ISO 14971 (12)
1.6.1.1 Endogenous	Testing shall include: 1. Human antibodies to the expression system (for recombinants), e.g. anti-Escherichia coli (anti-*E.coli* positive) 2. Human anti-mouse antibody (HAMA) 3. Effects of multiple blood transfusions 4. Pregnancy (including multiparous women) 5. Haemoglobin, lipids, bilirubin and protein		

Aspect	Testing requirements	Notes on testing requirements	Source documents
	6. Elevated immunoglobulin concentrations 7. Rheumatoid factor 8. Sickle-cell disease 9. Biotin (see note 6) 10. Other autoimmune conditions including systemic lupus erythematosus (SLE) and anti-nuclear antibodies (ANA)	occurring and causing clinically significant issues in the intended population 4. Interference studies should be performed with HIV-positive specimens with an analyte response (antigen or anti-HIV) near the cut-off 5. Evaluation of endogenous interfering substances may be addressed as part of the clinical studies (as applicable) 6. If biotin is commonly used as a supplement and the technology of the test employs streptavidin, then biotin levels of up to 1200 ng/ml should be tested as part of this study[6]	
1.6.1.2 Exogenous	1. Medicines, relevant to the populations intended to be tested including: antiretroviral, anti-parasitic, antimalarial and anti-tuberculosis medicines 2. Common over-the-counter analgesia medications (aspirin, paracetamol)		
1.6.2 Cross-reactivity	The potential for false-positive results arising from cross-reactivity (see note 1) shall be determined using at least 5–10 of each: 1. Non-HIV viral infections, including: hepatitis B, C infection, acute hepatitis A infection, cytomegalovirus, acute Epstein–Barr virus, varicella zoster virus, measles, influenza A and B, tick-borne encephalitis 2. Other human retroviruses, including: human T-lymphotrophic cell virus-1 and -2 3. Bacteria/parasites, including: malaria, visceral leishmaniasis, tuberculosis and human African trypanosomiasis 4. Recent vaccinations including against: influenza, hepatitis B, yellow fever 5. Vaccine-induced HIV seropositivity (see note 4)	1. The types of conditions/disease tested for shall be risk-based, taking into consideration the operational setting as well as the intended population for the analyte being detected in the areas of intended use and not simply rely on published lists of such cross-reactivity which might be of limited relevance in resource limited settings • see 1.6.1, notes 2. Any observed cross-reactivity shall be investigated, and performance limitations of the immunoassay reported in the IFU and taken into consideration in the required risk-benefit statements 3. For evaluation of cross-reactivity, should be performed using specimens with high content of the interfering substance. 4. HIV vaccine containing antigen will lead to antibody positive results.	
1.7. High dose hook effect			
1.7.1 Prozone/High dose hook effect for antibody	For each claimed analyte, the potential for a prozone/high dose hook effect shall be determined: 1. Using at least 3 different lots 2. using multiple, highly-reactive specimens (minimum of 20)	1. Specimens shall be chosen that have a high analyte concentration, as determined using a sensitive IVD method other than the IVD under evaluation and from a different manufacturer. This second method shall be of a design not subject to prozoning	Health Products and Food Branch, Health Canada (3)

[6] Clinical Biochemistry 65 (2019) 61-63 Gifford J.L et al . Strategies for mitigating risk posed by biotin interference on clinical immunoassays & Clinical Biochemistry 65 (2019) 64-65 Trambas, C.M.Further assessment of the prevalence of biotin supplementation and its impact on risk

Aspect	Testing requirements	Notes on testing requirements	Source documents
detection immunoassays	3. Using at least two different concentrations of each specimen (diluted by at least a factor of 10) 4. If a prozone is detected it shall be noted in the IFU	• an increase in signal upon dilution of a specimen implies a prozone effect	Butch, AW (13) Technical Guidance Series for WHO Prequalification – Diagnostic Assessment. TGS-6 (14)
1.7.2 Prozone/High dose hook effect for antigen detection immunoassays	For each claimed analyte, the potential for a prozone/high dose hook effect shall be determined: 1. Using at least 3 different lots 2. Using multiple, highly-reactive specimens (minimum of 20) 3. Using at least two different concentrations of each specimen (diluted by at least a factor of 10) 4. If a prozone is detected it shall be noted in the IFU.	1. Specimens shall be chosen that have a high viral load (low Ct level) as determined by nucleic acid amplification technology (NAT) in a format with a design not subject to prozoning • an increase in signal upon dilution of a specimen implies a prozone effect 2. If there is evidence of a fall in signal, this information shall be added to the IFU and mandatory mitigation actions shall be described	
1.8. Validation of the assay cut-off			
1.8.1 Validation of the assay cut-off	1. The way in which any cut-off used in the IVD was established must be demonstrated including: • the statistical methods (e.g., Receiver Operator Characteristic [ROC]) to generate results; and • the testing performed to define a grey-zone/equivocal zone if applicable	1. "Cut-off" may refer to the value used to assign reactive or negative status to a result	
1.9. Validation of the assay procedure			
1.9.1 Validation of the assay procedure	1. For each claimed analyte, evidence shall be provided on how the required reagent volumes and concentrations were determined and validated.	1. Some of these aspects may be evaluated within section 1.10 Usability/human factors studies	IMDRF IVD Marketing Authorization Table of contents (15)
1.9.2 Validation of reading times	For IVDs where a reading interval is specified (i.e. time when result can first be read; time beyond which result should not be read), 1. Validation of critical time points shall be provided for each analyte type (i.e. anti-HIV antibodies, HIV antigen) using spiked specimen panels of at least: • 1 negative specimen • 1 low reactivity positive specimen (near assay cut-off) • 1 medium reactivity positive.	1. The ranges of humidity tested for shall be risk-based, taking into consideration likely operational settings. 2. The intended operating temperature, upon which reading time has been validated, shall be clearly stated in the IFU. • Some of these aspects may be evaluated within 1.10 Usability/Human factors	WHO Prequalification – Diagnostic Assessment PQDx_018 (16)

Aspect	Testing requirements	Notes on testing requirements	Source documents
	2. Using a minimum of 2 different lots 3. If applicable (e.g. for manual methods without temperature controls), performance studies shall be conducted at the midpoint and the extremes of the claimed operating range • the effect of humidity as claimed in the IFU shall also be investigated.		
1.9.3 Procedural controls	The IVD shall have a procedural control(s) for each of the intended analytes (i.e. HIV¬1, ¬2, HIV antigen). 1. The validity of procedural control values shall be demonstrated 2. If the control material has a defined minimum (or maximum) value, it shall be demonstrated that critical specimens (e.g. seroconversion specimens, antigen at the claimed detection limit) will be detected as intended when the IVD functions at that value	1. State-of-the-art semi-automated immunoassay should have independent means of monitoring addition of specimen and other reagents 2. In resource limited settings coloured reagents are an important process aid for manually loaded assays	ISO 15198 (5)
1.9.4 Sample carryover	Where the manufacturer claims use of a specific instrumentation then carryover must be evaluated on that type of instrumentation.		
1.10. Usability/human factors			
1.10.1 Flex studies	The influence of potential user errors in method parameters and effects from the environments of intended use on IVD performance shall be evaluated: • when reasonable excursions from IFU parameters occur (note 1) • when environmental factors vary within foreseeable ranges (note 2) The influence of the following factors on expected results (both reactive and nonreactive) shall be considered. This list is not exhaustive: **Robustness**: any numerical factor in the IFU provided and/or identified by risk assessment such as: 1. Specimen and/or reagent volume; 2. Operating temperature, pressure and humidity. 3. Time between opening packaging or preparing reagents and	1. Refer to WHO document PQDx_018 "Instructions for compilation of a product dossier" for other flex studies that may be relevant, taking into consideration the range of operational and environmental conditions consistent with intended use in resource limited settings 2. The factors listed opposite should be investigated in ways that not only reflect, but also exceed, likely operating conditions in lower- and middle-income countries so that the limitations of the device can be understood. • for example, in addition to investigating deviations of temperature within those claimed in the IFU, temperature ranges should be investigated that exceed those of claimed operating conditions and which could cause test failure (incorrect/invalid results). 3. The impact of lighting can be multiple, e.g. for enzyme immunoassays, the substrate is light sensitive and needs to be freshly prepared. Hence, lighting impacts on the preparation time and the incubation environment.	IEC 62366 (17) USP chapter 12 (18) VIM (19) FDA (20)

Aspect	Testing requirements	Notes on testing requirements	Source documents
	starting the assay 4. Any incubation times, mixing speeds (e.g. For shaking incubators; rockers), temperatures 5. Incubation temperatures 6. Reading time: the time after stopping the final incubation for which the result is stable **Ruggedness** 7. IVD sturdiness including robustness of packaging and labelling. IVD in final packaging shall be subjected to drop-shock testing 8. Permanence of component labels: print legibility, adhesiveness 9. Effects of lighting and humidity (see note 3) 10. Residual volumes and characteristics of liquids (potential evaporation, pH changes, microbial growth, antimicrobial efficacy) **Where the manufacturer provides instrumentation:** 11. Ruggedness 12. Impact of dust and mould on componentry (e.g. optics)	4. Robustness testing generally takes the form of statistically designed experiments to evaluate the effect of simultaneous "small but deliberate changes" in method parameters	
1.11. Stability of the IVD			
1.11.1 Stability general requirements	Replicate testing shall be undertaken using a panel consisting of at least the following: 1. 1 low and 1 medium reactivity positive specimen for each analyte (e.g. anti-HIV-1, anti-HIV-2 and HIV antigen) as claimed 2. 1 specimen near the detection limit of antigen, where applicable (e.g. for HIV Antigen and 4th Generation HIV assays), 3. 3 negative specimens 4. Quality control performance specimens developed by the manufacturer for monitoring the reactivity of critical active ingredients. Those specimens are in most cases not natural samples (see note 2) 5. The manufacturer's batch release testing shall include at least 100 specimens negative for the relevant analyte (see note 3) 6. Where the claimed specimen type is either serum or plasma, the	1. The stability testing panel should include functional user controls and natural (i.e. undiluted) specimens. Where this is not feasible, stock specimens may be diluted and used 2. The IVD shall pass the quality control performance test at the end of the claimed stability 3. The IVD shall pass the Quality Control Testing for batch release at the end of its shelf life 4. Each lot shall comprise different production (or manufacturing, purification, etc.) runs of critical reagents (If different reagent-container sizes are used in packs with different volumes of reagent (e.g. different volumes for single use and multiple use), stability evidence (real time, in-use) should be obtained on all variants, even if the contents of the containers are identical) 5. Claims for stability shall be based on the last successful data point	ISO 23640 (21) CLSI EP25 (22) ASTM D4169 (23) Technical guidance series – Diagnostic Assessment TGS-2 (24)

Aspect	Testing requirements	Notes on testing requirements	Source documents
	evaluation may be conducted in either of these specimen types.	from the least stable lot. the maximum stability claim shall have a confidence margin included 6. The numbers of invalid tests with each lot shall be reported	
1.11.2 Shelf life (including transport stability)	1. Testing of a minimum of 3 lots of product manufactured to: • the final locked-down design, • in the final primary packaging, • to validated manufacturing scale and • released to the final quality assurance specifications 2. The lots shall be transport stressed (simulated) before real time studies are undertaken (note 1) • with cyclic temperature variation, • IVD in final packaging subjected to drop-shock testing. 3. Stability of labelling shall be determined (note 2)	1. Determination of transport stability shall be performed using simulated extreme stress conditions, ensuring that the application of those conditions is consistent and controlled • transport stress shall be applied before assigning lots to shelf-life studies to mimic the real-life situation 2. Stability of labelling is to be determined under the conditions of intended and expected use • in addition to routine transport stress (shock testing of defined types) the resilience of labels (e.g. strength of attachment, print stability, legibility over time, damp tolerance) shall be evaluated as part of shelf-life verification 3. Accelerated studies do not replace the need for real time data for shelf-life and in-use stability	
1.11.3 In-use stability (open pack or open vial stability)	1. Testing of a minimum of 1 lot manufactured to: • the final locked-down design, • in the final primary packaging, • to validated manufacturing scale and • released to the final quality assurance specifications 2. There shall be evidence that once the IVD is removed from its primary packaging, it is stable at the expected temperature and humidity ranges for a defined period at the beginning and end of its assigned shelf life. 3. Liquid components, once opened, shall have a validated life and number of stated uses under environmental (including microbial) conditions expected.	1. The lots tested should include • 1 lot shortly after manufacture • 1 lot towards the end of the assigned shelf-life (this may be the same lot) 2. In-use stability studies cover • short-term effects (e.g. after taking a microplate out of its packaging), • medium term effects (e.g. reading times, between adding specimens and reagents to microwells before beginning the next stage of the method) and • long-term effects (e.g. open pack stability on-board instrumentation, open bottle with repeated removal of aliquots over time by hand over time). 3. Statistically designed experiments should be used to allow evaluation of any interactions between environmental conditions 4. Most aspects of in-use stability may be considered as part of section 1.10 Usability/human factors	

Part 2 Establishing clinical evidence (clinical performance characteristics) for 3rd and 4th generation immunoassays

Aspect	Testing requirements	Notes on testing requirements	Source documents
2.1. Performance panels			
2.1.1 Subtype panels	Testing of WHO International Reference Preparations and/or commercial HIV subtype panels shall include: 1. All HIV-1 subtypes (e.g. A, B, C, D, G, etc.) HIV-2, HIV-1 group O, and common circulating recombinant forms (CRFs) 2. At least 10 of each of the most common subtypes (Subtype C, Subtype A, Subtype B, CRF02_AG, CRF01_AE, CRF07_BC and Subtype G, including HIV-1 (O)) 3. At least 3 of each of the less common subtypes (and several other CRFs and unique recombinant forms (URFs)). 4. For IVDs including a claim for detection of HIV antigen, appropriate specimens for the same range of subtypes as for antibodies shall also be included in the testing panel (see note 4) 5. To substantiate a claim for detection of HIV-1 (O) at least 5 typed HIV-1 (O) specimens shall be evaluated(see note 5)	1. Testing should be performed using a minimum of 2 different lots 2. All confirmed type- and subtype-positive specimens shall be detected by the IVD. 3. All reasonable attempts shall be made to test rare subtypes. 4. Use of panels of viral-like-particles (VLPs) or viral cultures may be considered acceptable as may evaluation of the antigen detection aspect of the IVD in the absence of part of the antibody detection aspect so that natural samples including both antibody and antigen can be evaluated. 5. At least 70% of HIV-1 (O) specimens are detected by anti-HIV-1 immunoassay with only group M antigenic components. Hence, finding a small number of HIV-1 (O) specimens reactive does not validate a claim for universal HIV-1 (O) detection.	Health Products and Food Branch, Health Canada (3)

2.1.2 Seroconversion panels	1. A minimum of 25 commercial or well characterized anti-HIV-1 seroconversion panels shall be tested which: • individually start with a negative bleed(s) and have narrow bleeding date intervals • overall contain least 40 early seroconversion specimens (see note 2) 2. Testing shall be conducted using a minimum of 3 different lots. 3. Seroconversion sensitivity shall be reported to the user in the IFU.	1. Panels should have been collected at short intervals to cover the seroconversion period and should also cover the whole window period. 2. Early seroconversion: either of • p24 antigen and/or HIV RNA-positive (Fiebig stage II) • Fiebig stage III, (negative for HIV antibody by confirmatory assays, western blot or line immunoassay, positive by most sandwich (3rd generation antibody assays))fal. 3. Full-seroconversion: both of: • p24 antigen and/or HIV RNA-positive • Fiebig stage, IV or V (indeterminate or positive by confirmatory assays). 4. With some 3rd generation IVD and many 4th generation IVD on some seroconversion series there is an initial seroconversion with a rising signal followed by a fall in signal as the immune response matures and then a rise to final signal strengths. • This should not occur at all in state-of-the-art 3rd generation IVD and shall not approach negative in 4th generation IVD 5. Each lot shall comprise different production (or manufacturing, purification, etc.) runs of critical reagents.	European Commission decision on CTS (2) Health Products and Food Branch, Health Canada (3) Fiebig E.W.,Wright D.J., Rawal B.D. et al. (25)
2.1.3 Mixed titre panels	1. Testing of panel of specimens with a range of analyte concentrations (e.g. antibody 'mixed titre' panel).		
2.2. Diagnostic sensitivity and specificity			
2.2.1 Diagnostic sensitivity and specificity study general requirements	1. Diagnostic sensitivity and specificity shall be determined for each claimed specimen type. 2. Testing shall be conducted: • in different geographical settings (minimum of 2 regions, see note 1), • by laboratories representative of different intended use settings (e.g. primary, district, regional, and national laboratory settings[7])	1. Prequalified HIV immunoassay are generally used by trained laboratory staff in resource limited settings. This should be considered when preparing evaluation protocols 2. Lots shall comprise different batches of critical components 3. Appropriately stored, well characterized sera that have not undergone more than one freeze-thaw cycle may also be used for clinical evaluation testing if necessary assuming that freezing specimens has been validated during analytical studies (see section 1.1.1). These shall be a random selection of consecutively chosen	

[7] Available at http://apps.who.int/iris/bitstream/handle/10665/179870/9789241508926_eng.pdf?sequence=1

Aspect	Testing requirements	Notes on testing requirements	Source documents
	• using more than 1 different lot in each laboratory (see note 2) 3. Discrepant or unexpected results shall be fully evaluated	specimens • at collection of fresh specimens, an aliquot of an appropriate specimen type should be frozen at -70°C for use should HIV RNA testing be required • if necessary, the sensitivity for antigen detection may be verified using specimens archived at the chosen centres assuming that such specimens have been validated during analytical studies see section 1.1.) 4. The protocol should specify the criteria for unbiased patient selection with associated risk analysis but in general there should be no exclusions except for ethical reasons: • the patients should be classified, and results analysed accordingly (e.g. first time or repeat blood donors, concomitant infections, age, gender, concomitant medicines including antiretrovirals, recent vaccinations). 5. Samples for both comparator and test methods shall be taken from the same specimen container. 6. Assessment shall be made of the potential for false reactive results in common between immunoassays: • confirmatory methods must not contain antigens or antibodies from the same source as the reference nor test methods 7. Problematic specimens including those with unexpected results, but which otherwise meet selection criteria for a study, shall not be systematically excluded from analysis. 8. Indeterminate results shall not be systematically excluded from the denominator data for analysis 9. All invalid test results shall be recorded. 10. Estimates of diagnostic/clinical sensitivity and specificity shall be reported with 95% confidence intervals.	
2.2.2 Diagnostic sensitivity	Testing of at least 500 subject specimens confirmed HIV antibody positive consisting of: 1. 400 HIV-1	1. At least 50% of the results from which the diagnostic sensitivity is calculated shall be from fresh specimens 2. Some or all of the antigen positive specimens may be from those	European commission decision on CTS

Aspect	Testing requirements	Notes on testing requirements	Source documents
	2. 100 HIV-2 3. 100 HIV antigen positive	found positive for HIV antibodies during the evaluation 3. The comparator method should be a state-of-the art immunoassay (ideally, a 4th generation immunoassay) • the immunoassay shall not be from the same manufacturer as the IVD under evaluation 4. All initially reactive specimens on reference or test IVD shall be subjected to full characterization of the HIV status: • the algorithm shall include a reference 4th generation immunoassay distinct from the comparator immunoassay, a method of evaluation of the occurrence of HIV-2 and an immunoassay for antigen detection if any of the methods are 4th generation • reactive specimens on comparator or test IVD, negative or positive, false or true, shall be tested in duplicate on all available lots of the test IVD and the reproducibility noted. 5. Performance characteristics shall be reported using initial results, only. The results of further testing of specimens with discrepant results shall be reported separately as additional information about IVD performance	(2) Health Products and Food Branch, Health Canada (3)
2.2.3 Diagnostic specificity	1. Testing of at least 1000 confirmed nonreactive specimens collected in a routine hospital setting with each of two lots analysed independently and with different specimens tested on each 2. If blood screening is claimed, at least 5000 confirmed nonreactive donor plasma specimens shall be tested	1. At least 80% of the results from which the diagnostic specificity is calculated shall be from fresh specimens. 2. If the intended use includes both serum and plasma the specimens for evaluation of specificity shall comprise equal numbers of serum and at least one plasma type 3. Results shall be expressed separately for each specimen type and for each specimen type per intended use (no aggregation of results).	

Part 3 Establishing clinical evidence (clinical performance characteristics) for HIV antigen detection immunoassays

Does not apply to 3rd and 4th generation immunoassay

Aspect	Testing requirements	Notes on testing requirements	Source documents
3.1. Performance panels			
3.1.1 Seroconversion panels	1. At least 10 seroconversion series covering Fiebig stages II, III and IV shall be investigated (see note **Error! Reference source not found.**) 2. Reactive specimens shall be verified as antigen positive by the associated HIV antigen confirmatory reagents (see section 3.3) 3. When a protocol for immune complex dissociation is used results for each specimen shall be presented with and without dissociation 4. Criterion: results to be comparable with those of a state-of-the-art HIV antigen immunoassay or peer reviewed literature	1. 10 well characterized seroconversion series which together provide at least 15 Fiebig stage II specimens and at least 25 stage III + IV	
3.1.2 Subtype panels	1. Testing of the following reference panel: 1st WHO International Reference Panel for HIV-1 p24 antigen and HIV-2 p26 antigen	1. All specimens shall be correctly identified	
3.2. Diagnostic sensitivity and specificity			
3.2.1 Diagnostic sensitivity and specificity study general requirements	1. Diagnostic sensitivity and specificity shall be determined. 2. Testing shall be conducted: • in different geographical settings (minimum of 2 regions – see note 3) • by laboratories representative of different intended use settings (e.g. primary, secondary and tertiary laboratory settings) • using a minimum of 2 different lot in each laboratory (see note 2) • reactive, discrepant and unexpected results shall be resolved	1. Prequalified HIV immunoassay are generally used by trained laboratory staff but in resource limited settings. This should be considered when preparing evaluation protocols 2. Lots (finalized design, to validated scale and final quality control protocols) shall comprise different batches of critical components 3. The sensitivity for antigen detection may be verified using appropriately stored, well characterized sera that have not undergone more than one freeze-thaw cycle archived at the chosen centres if such specimens have been validated during analytical studies (see Section 1.1.1) 4. The protocol should specify the criteria for unbiased patient selection with associated risk analysis but in general there should be no exclusions except for ethical reasons	

Aspect	Testing requirements	Notes on testing requirements	Source documents
		5. The patients should be classified and results analysed accordingly (e.g. first time or repeat blood donors, concomitant infections, age, gender, concomitant medicines including antiretrovirals, recent vaccinations) 6. Samples for both comparator and test methods shall be taken from the same specimen container 7. The comparator method should be a state-of-the art HIV antigen test with associated immune complex dissociation and confirmatory method. • comparator method shall not be from the same manufacturer or contain antibodies from the same source as the IVD under evaluation 8. Performance characteristics shall be reported using initial results only. The results of further testing of specimens with discrepant results shall be reported separately as additional information about IVD performance 9. Assessment shall be made of the potential for false reactive results in common between immunoassays: 10. Problematic specimens including those with unexpected results but which otherwise meet selection criteria for a study, shall not be systematically excluded from analysis 11. All indeterminate results shall be included in the denominator data for analysis 12. All invalid tests shall be recorded 13. Estimates of diagnostic/clinical sensitivity and specificity shall be reported with 95% confidence intervals	
3.2.2 Diagnostic sensitivity	Testing of 50 confirmed positive specimens • at least 50 % should require immune complex dissociation	1. At least 50% of the results from which the diagnostic sensitivity is calculated shall be from fresh specimens 2. All initially reactive specimens on comparator or test IVD shall be subjected to full characterization of the HIV status. 3. The algorithm shall include • a reference HIV antigen immunoassay and accessories distinct from the comparator immunoassay,	

Aspect	Testing requirements	Notes on testing requirements	Source documents
		• a 4th generation HIV immunoassay, • if antigen is detected, a method to differentiate between HIV-1 and HIV-2 • all reactive specimens on comparator or test IVD, (false or true) shall be tested in duplicate on as many lots as possible	
3.3.Immune complex dissociation and confirmatory reagents			
3.3.1 Immune complex dissociation	1. If an immune complex dissociation procedure is available, • the function of the ICD should be verified in comparison with the ICD for a reference HIV antigen immunoassay • it should be demonstrated that the use of the ICD does not affect the result from a non complexed specimen, allowing for small dilution effects • and the ICD should comprise at least reagents for dissociating and for control of the dissociation	1. An immune complex dissociation accessory (ICD) should be available to accompany the HIV antigen immunoassay	
3.3.2 Confirmatory reagents	1. Confirmatory reagents for serological confirmation of reactive specimens should be available 2. All true and false-positive specimens as detected by the respective HIV antigen assay shall be evaluated in comparison with the true status of the specimen determined by a reference HIV antigen immunoassay and its confirmatory reagent • criterion: The confirmatory reagent shall confirm 100% of all true positive specimens 3. The confirmatory reagent shall function accurately with non-specific reactions found on the HIV antigen immunoassay (note 5) 4. At least 10 specimens falsely reactive on the HIV antigen immunoassay shall be evaluated (see note 5) • criterion: 100% of false reactions shall correctly identified	1. The confirmatory process shall comprise at least reagents for neutralization and for control 2. Confirmation of reactive antigen results is usually performed by quenching the reaction with anti-HIV antibodies • other serological methods verified and described are acceptable • although many antigen reactive specimens will be further evaluated by NAT, this is not always possible in resource limited settings and a serological method is required 3. The confirmatory reagent shall confirm all types and subtypes of HIV-antigen claimed for the antigen immunoassay • this may be with fully characterised natural antigens in clinical specimens or with matrix spiked with recombinant proteins 4. All antigen reactive specimens found during analytical and clinical evaluations should be accurately classified by the confirmatory accessory in comparison with the true status of the specimen as determined by a reference HIV antigen immunoassay and its confirmatory test	

Aspect	Testing requirements	Notes on testing requirements	Source documents
		• the confirmatory procedure requires a dilution of the specimen. this means that low positive sample may get diluted to a level below the analytical sensitivity 5. Specimens may be found during development work of the immunoassay or during performance evaluation. In case of specific interferences these samples may be spiked with the interfering component.	

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