HIV DRUG RESISTANCE

WHO HIVResNet HIV DRUG RESISTANCE

LABORATORY OPERATIONAL FRAMEWORK

SECOND EDITION — SEPTEMBER 2020

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WHO HIVResNet HIV drug resistance laboratory operational framework, second edition

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EXECUTIVE SUMMARY

The WHO HIV Drug Resistance Network (HIVResNet) HIV drug resistance laboratory operational framework describes how WHO HIVResNet laboratories function to support national, regional and global HIV drug resistance surveillance by providing accurate genotyping results in a standardized format according to WHO specifications. The aim of the operational framework is to ensure:

- accurate collection, handling, shipment and storage of specimens collected in countries implementing HIV drug resistance surveillance; and
- The availability of quality-assured HIV genotyping laboratory services producing comparable and reliable results at the national, regional and global levels.

WHO requires all countries implementing HIV drug resistance surveys to send specimens for HIV drug resistance genotyping to laboratories designated by WHO for this purpose. This publication describes:

- the structure of the WHO HIVResNet Laboratory Network;
- the roles and responsibilities of the different types of Network laboratories;
- the requirements for application;
- the application review and evaluation process; and
- the requirements for submitting HIV drug resistance data to WHO.

The WHO HIVResNet HIV drug resistance laboratory operational framework consists of the following four elements.

1. National strategy for laboratory support for HIV drug resistance surveillance.
2. The WHO HIVResNet Laboratory Network is responsible for ensuring the delivery of quality-assured HIV genotyping data at the national, regional and global levels. The Laboratory Network includes different categories of membership, with different tasks and responsibilities:
    - national HIV drug resistance laboratories (usually one per country);
    - regional HIV drug resistance laboratories (ideally, at least one per WHO region); and
    - specialized HIV drug resistance laboratories.
3. Standards for specimen collection, handling, shipment and storage. The Laboratory Network provides guidance documents and laboratory procedures to support the standardization of all components of the operational framework.
4. Laboratory technical support for capacity building.

Summary of updates

This publication updates the WHO HIVResNet HIV drug resistance laboratory operational framework published in 2017 and reflects technical and strategic developments over the past three years. Although the core principles and structure of the Network remain unchanged, adjustments have been made to the following areas:

- tasks and responsibilities of Network laboratories (subsection 1.3.1, items 2, 6 and 9 and subsection 1.3.2, item 5);
- changes to the minimum required criteria for a laboratory to obtain designation as a member of the Laboratory Network (subsection 1.3.1, items 6b and 9);
- clarification of the potential reasons for suspending a laboratory (subsection 2.4);

- consideration of next-generation sequencing methods (subsection 4.5.4);
- updates to the standard operating procedures for post-testing quality assurance of HIV sequence data related to integrase and next-generation sequencing (Annex 2); and
- assay validation recommendations included as a new annex (Annex 3).

1. PRINCIPLES OF THE HIVResNet LABORATORY NETWORK

1.1 Background

The WHO strategy and Global Action Plan on HIV drug resistance describe how to evaluate drug resistance among people living with HIV before starting treatment, among those receiving antiretroviral therapy and among infants younger than 18 months. Surveillance methods are designed to provide national prevalence estimates of HIV drug resistance in these populations and to support optimal regimen selection and programmatic actions. The HIV drug resistance laboratories are evaluated in the context of this strategy. More details about the WHO HIV drug resistance strategy are available at: http://www.who.int/hiv/topics/drugresistance/en.

WHO requires that all genotyping for HIV drug resistance surveys be performed at laboratories designated by WHO for this purpose. Genotyping data generated in laboratories that are not designated by WHO may not be considered for inclusion in global reporting of the prevalence and patterns of HIV drug resistance. The genotyping laboratories designated by WHO are members of the HIVResNet Laboratory Network. This publication describes the structure of the Laboratory Network, the roles and responsibilities of the different types of Network laboratories, the requirements for application, the application review and evaluation process and the requirements for submitting HIV drug resistance data to WHO.

1.2 Principles of the WHO HIVResNet HIV drug resistance laboratory operational framework

The Laboratory Network supports the implementation of the global HIV drug resistance strategy and aims to ensure high-quality data by:

- developing and updating laboratory guidance to describe the complete process of specimen collection, handling, shipment, storage, genotyping, quality assurance and data management;
- participating in an integrated and harmonized external quality assurance scheme (Annex 1);
- assisting in capacity building and training in laboratories that are seeking to improve their infrastructure and ability to achieve Laboratory Network membership;
- engaging in research to develop simple and affordable methods for HIV drug resistance testing; and
- assisting WHO in assessing the laboratories seeking Laboratory Network membership.

Although membership in the Laboratory Network may be important for continued government funding of the laboratories and international recognition of their work, Network laboratories are not formally accredited by WHO as collaborating centres and therefore have no official WHO status. Importantly, Laboratory Network membership governs the performance of HIV drug resistance genotyping only for public health surveillance purposes and does not imply that a laboratory has met procedural or regulatory requirements to certify the validity of sequencing results for the clinical management of individual people such as treatment decisions by the care provider. Regulatory standards associated with providing results for clinical purposes are generally much more stringent and governed by national regulatory bodies. The members' national governments, nongovernmental organizations, foundations and other contributions support the Network.

The WHO HIVResNet HIV drug resistance laboratory operational framework consists of the following four elements.

1. **National plan for laboratory support for HIV drug resistance surveillance.** The national HIV drug resistance working group should choose a WHO-designated laboratory to perform testing for HIV drug resistance surveys. This laboratory is preferably located within the country where the survey was implemented. In countries without a WHO-designated laboratory for HIV drug resistance, a regional or specialized laboratory in another location can provide genotyping services.
2. **WHO HIVResNet Laboratory Network.** The Laboratory Network is responsible for delivering quality-assured HIV genotyping data. Standardizing laboratory procedures in all laboratories designated by WHO for HIV drug resistance surveillance activities helps to ensure that the results from different surveys are comparable to each other. The designation process qualifies laboratories for the purpose of providing quality-assured results to a country implementing HIV drug resistance surveillance.

WHO-designated genotyping laboratories are members of the Laboratory Network. Membership within the Network falls into one of the following three categories, with different tasks and responsibilities:

- national HIV drug resistance laboratories, usually one per country;
- regional HIV drug resistance laboratories, usually one for each WHO region; and
- specialized HIV drug resistance laboratories.

WHO coordinates the Laboratory Network in consultation with the HIVResNet Working Group, which includes representatives of the specialized and regional laboratories and external experts in HIV laboratory science.

3. **Standards for specimen collection, handling, shipment and storage.** Standardizing the collection, handling, shipment and storage of specimens for HIV drug resistance testing is a critical step for producing accurate, comparable results. Accurate genotypic testing depends on appropriate methods of specimen collection and handling, suitable transport from the collection site to the central laboratory and storage under appropriate conditions.

   One of the responsibilities of the national HIV drug resistance working group is to ensure that the national plan for specimen collection, handling, shipment and storage follows WHO HIVResNet guidance before HIV drug resistance surveys begin. Preparing and implementing the plan requires close cooperation between virologists, epidemiologists and clinicians at the national level as well as between the WHO-designated genotyping laboratories and local laboratory personnel.

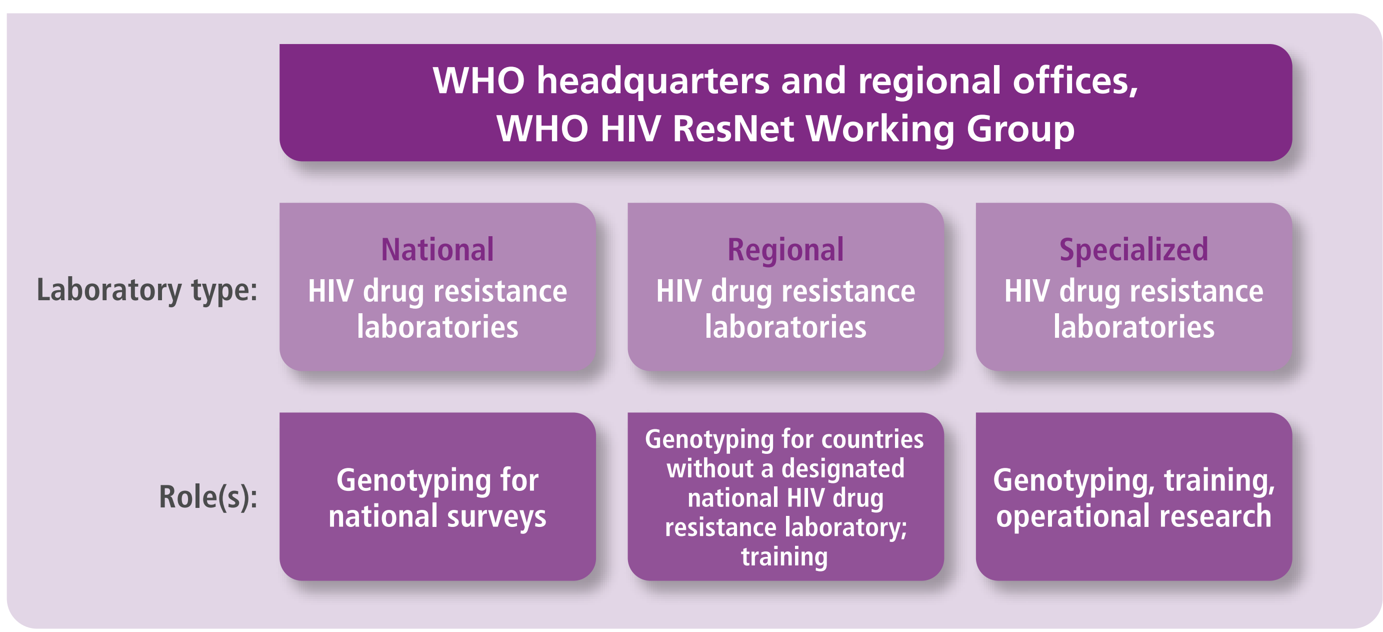
4. **Laboratory technical support for capacity building.** One goal of the Laboratory Network is to maximize the transfer of knowledge and expertise from member laboratories to those that have not yet been designated by WHO. For this purpose, WHO facilitates the link (twinning) between the member laboratory and applicant (trainee) laboratories.

1.3 Structure and function of the Laboratory Network

WHO grants membership in the Laboratory Network to a laboratory after it demonstrates that it meets the criteria described in this publication. A laboratory is assessed for membership through critical review of procedures and documentation, genotyping results produced by the laboratory, laboratory assessment visits conducted by one or more members of the WHO HIVResNet Working Group and successful participation in a quality assurance system recognized by WHO. A laboratory that is awarded membership must demonstrate, at regular intervals, that it continues to meet the criteria. WHO may require reassessment at any time, based on the laboratory's performance in the Laboratory Network.

The Laboratory Network has three levels of institutions: national, regional and specialized (Fig. 1).

Fig.1. Structure of the HIVResNet Laboratory Network



1.3.1 National HIV drug resistance laboratories

WHO designates a laboratory as a national HIV drug resistance laboratory to perform HIV drug resistance testing for surveillance activities in the country in which it is located. Before designation by WHO, the national health ministry must first nominate the laboratory for the purpose of supporting a country's surveillance activities. Although a national HIV drug resistance laboratory is preferably a public health laboratory with an active role in HIV surveillance, the health ministry may also designate other types of laboratories as candidate laboratories for HIV drug resistance genotyping.

The process and detailed requirements for designation as a national HIV drug resistance laboratory are outlined below.

Tasks and responsibilities

1. The national HIV drug resistance laboratory performs genotyping of specimens collected during HIV drug resistance surveys conducted in the country in which it is located and provides accurate HIV sequences to the national HIV drug resistance working group in a timely manner.
2. The cost of the genotyping assay is not expected to exceed US$ 150. This is the all-inclusive cost per test for protease, reverse transcriptase and integrase that would be requested by the testing laboratory and included in the survey implementation budget. All laboratories are encouraged to keep these costs as low as possible, for example by developing (and validating) less expensive assays.
3. Preferably, the national HIV drug resistance laboratory performs viral load testing for acquired drug resistance surveys, since specimen quantities are often insufficient to enable shipment to a separate laboratory for such testing. If specimens are collected as dried blood spots (DBS), the laboratory should consult with WHO regarding the most appropriate type of viral load assay to use.
4. The national HIV drug resistance laboratory participates in a WHO-recognized external quality assurance programme for genotyping and is able to support the cost of the testing and shipment of the annual proficiency panel, if required.
5. The national HIV drug resistance laboratory applies appropriate quality assurance and quality control procedures, including genetic distance analysis that detects molecular contamination, according to WHO recommendations (see Annex 2).
6. The national HIV drug resistance laboratory sends HIV genotyping results in FASTA file format to the national HIV drug resistance working group and to WHO headquarters for ongoing quality assurance. Sequence data should be transmitted to WHO via the HIV drug resistance surveillance database (https://www.who.int/hiv/topics/drugresistance/hiv-drug-resistance-database/en). WHO will provide database access and training to the national HIV drug resistance laboratory supporting national surveys.
7. The national HIV drug resistance laboratory supports the country in performing HIV drug resistance analysis using the Stanford University HIVdb algorithm (https://hivdb.stanford.edu/hivdb/by-sequences). The interpretation of sequence data according to WHO requirements is automated as part of the WHO HIV drug resistance surveillance database.
8. Representatives from the national HIV drug resistance laboratory participate actively in WHO HIVResNet Laboratory Network calls and meetings.
9. If DBS are being used as the specimen type for national surveillance, the national HIV drug resistance laboratory must complete a DBS assay validation according to the recommendations outlined in Annex 3. Similarly, if integrase inhibitors are part of national treatment guidelines, the national HIV drug resistance laboratory must complete an integrase assay validation that follows the same recommendations.

Requirements

Before designation by WHO, the national health ministry must first nominate the laboratory for the purpose of supporting a country's surveillance activities. Upon designation by WHO, a national HIV drug resistance laboratory becomes a member of the WHO HIVResNet Laboratory Network. Although a national HIV drug resistance laboratory is preferably a public health laboratory with an active role in HIV surveillance, the health ministry may also designate other types of laboratories as candidate laboratories for HIV drug resistance genotyping.

The following five criteria must be met to apply for membership.

1. Existence of a national plan for implementing surveillance of HIV drug resistance. The country in which the laboratory is located must have a national strategy in place for the implementation of WHO-recommended HIV drug resistance surveys. This strategy must be consistent with WHO recommendations.
2. Following the establishment of the national HIV drug resistance surveillance plan, the need for laboratory support must be considered. One national WHO drug resistance laboratory per country is usually appropriate, if the laboratory is likely to meet the

required standards (criterion 5). Justification for more than one national HIV drug resistance laboratory per country must be provided by the country and approved by WHO headquarters.

3. The health ministry is responsible for nominating candidate national and regional HIV drug resistance laboratories. The health ministry must nominate the laboratory as the candidate national laboratory for the purpose of genotyping specimens collected during HIV drug resistance surveillance in that country or region. The health ministry must provide a letter of nomination that clearly states the ministry's commitment to use this laboratory for WHO HIV drug resistance surveys and to support the costs of the laboratory testing.

4. At least one year of experience in genotyping HIV and more than 100 specimens tested annually with satisfactory results.

5. Minimum infrastructure for HIV drug resistance genotyping in place

   a. Separation of work areas, with map and workflow plan consistent with molecular diagnostic work: relevant anti-contamination laboratory spaces for polymerase chain reaction (PCR).

   b. Reliable access to the minimum required equipment (sequencer, PCR machine, refrigerators and –20°C and –80°C freezers). If a sequencer is not present and an external facility is used for sequencing, documentation that this facility follows Good Laboratory Practice and participates in an appropriate external quality assurance programme with satisfactory results must be provided.

   c. Standard operating procedures in place covering all aspects (pre-analytical, analytical and post-analytical) of genotyping procedures: specimen receipt, assessment, rejection criteria and storage; genotyping procedures, including quality control and quality assurance management; biohazardous waste disposal and biosafety; data quality control measures and management; and reporting procedures. Standard operating procedures must be developed internally following Clinical Laboratory Standards Institute standards (or adapted from another laboratory in the network), specific to the context in which the laboratory is located (not copied from package inserts) and controlled according to quality assurance standards (version control, senior-level management review and approval, system in place for training and following updates, etc.).

   d. An automated or semiautomated data management system that precludes reliance on manual transcription or re-entry of data such as specimen identifiers and resistance interpretations.

Criteria 6–9 must then be met for the laboratory to be designated.

6. Demonstrated proficiency with sequence data quality assurance, data management and reporting (to be evaluated using the "dry panel", after other mandatory criteria are met).

   a. Adoption of WHO post-testing quality assurance procedures or demonstrated equivalent process. Post-testing steps include base calling using RECall, quality assurance and quality control checks using the British Colombia Centre for Excellence in HIV/AIDS quality control tool, Stanford University HIVdb website and resistance interpretation and reporting (Annex 2).

   b. Chromatogram and sequence data for a large number of specimens (50–100) will be included in a "dry panel" for evaluation of data analysis and management steps.

7. Site assessment score ≥85%.

8. Successful participation in the WHO HIV drug resistance proficiency testing programme. The Viral Quality Assurance Laboratory analysis and criteria will be used, with some modifications (including testing for PCR cross-contamination).

9. Demonstrated capacity to sequence integrase (exception: the national HIV drug resistance laboratory in countries not using DTG or other integrase inhibitors in public health antiretroviral therapy programmes).

1.3.2 Regional HIV drug resistance laboratories

A regional HIV drug resistance laboratory is an institution designated by its national health ministry to support the region's HIV drug resistance surveillance activities. Upon designation by WHO, the regional HIV drug resistance laboratory becomes a member of the WHO HIVResNet Laboratory Network. Ideally, there should be at least one designated regional HIV drug resistance laboratory in each WHO region. Rarely, more than one regional HIV drug resistance laboratory for each region may be warranted. Experience as public health laboratories, although not compulsory, is an asset. The regional HIV drug resistance laboratory may serve as the national HIV drug resistance laboratory in its own country.

Tasks and responsibilities

The following tasks and responsibilities are in addition to those listed above for the national HIV drug resistance laboratory.

1. The regional HIV drug resistance laboratory functions as a genotyping facility for countries that do not have a designated national HIV drug resistance laboratory. It must provide support and backup as needed to national HIV drug resistance laboratories in countries that are implementing WHO-recommended HIV drug resistance surveys.
2. The regional HIV drug resistance laboratory, in coordination with WHO and/or a designated specialized HIV drug resistance laboratory, facilitates the training, education and capacity building of laboratory personnel from national HIV drug resistance laboratories within the region. The regional HIV drug resistance laboratory hosts laboratory technicians from candidate laboratories and trains them to become competent in HIV genotyping.
3. Representatives from the regional HIV drug resistance laboratory are available to visit the national HIV drug resistance laboratories for technical assistance when necessary.
4. Representatives from the regional HIV drug resistance laboratory are available to participate in assessing candidate laboratories within the specified region, including on-site inspection visits.
5. The regional HIV drug resistance laboratory must complete a DBS assay validation and an integrase assay validation according to the recommendations outlined in Annex 3.

Requirements

WHO will consider new applications for regional HIV drug resistance laboratories only if the laboratory can provide an unmet function in the Network.

The health ministry must provide a letter of agreement indicating that the candidate laboratory has been identified to test specimens collected from other countries during WHO-recommended HIV drug resistance surveys and to provide training and capacity building to other laboratories in the region.

The mandatory criteria listed above in the section on national HIV drug resistance laboratories also apply to new applications for regional HIV drug resistance laboratories. In addition, the following additional criteria must be met in order for the laboratory to be designated:

- the capacity for sequencing the integrase region is mandatory;
- demonstrated capacity to perform genotyping using DBS; and
- a cost per test for protease, reverse transcriptase and integrase of US$ 150 or less.

Capacity for genotyping from DBS and of integrase is demonstrated by:

- experience: at least six months of experience with at least 100 specimens successfully amplified and sequenced;
- a procedure is in place and validated according to WHO recommendations; and
- successful participation in the WHO HIV drug resistance proficiency testing programme.

1.3.3 Specialized HIV drug resistance laboratories

Only laboratories that provide an unmet function in the network may submit an application to become a specialized HIV drug resistance laboratory.

Applicant laboratories must be well established in the international scientific community, with existing national and international responsibilities and experience in reference virology, training, surveillance and relevant scientific publications. Ideally, a specialized HIV drug resistance laboratory should already host WHO reference activities outside of HIV drug resistance and have sufficient medical, scientific and technical resources to respond to demands for training, laboratory testing and advice on short notice.

All the mandatory criteria described in the section on regional HIV drug resistance laboratories apply to specialized HIV drug resistance laboratories, except the requirements for a national plan for implementing HIV drug resistance surveillance and the health ministry designation.

Tasks and responsibilities

In addition to the tasks and responsibilities listed above for the regional HIV drug resistance laboratory, the specialized HIV drug resistance laboratory must be willing to:

1. participate in the WHO HIVResNet Laboratory Network Working Group and contribute actively to developing the WHO HIVResNet Laboratory Network;
2. provide support, technical assistance and back-up to national or regional HIV drug resistance laboratories, where needed;
3. serve as a regional HIV drug resistance laboratory (and therefore fulfil the roles and responsibilities described above) to countries within a WHO region that do not have a designated regional HIV drug resistance laboratory;
4. serve as a national HIV drug resistance laboratory (and therefore fulfil the roles and responsibilities described

above) to specified countries that do not have a national designated genotyping laboratory, and:

a. the regional laboratory is not able to assist; and/or

b. a relationship between the specialized HIV drug resistance laboratory and the specified country is already in place.

Specialized HIV drug resistance laboratories should also actively participate in one or more of the core activities listed below. These activities may not be equally distributed between the laboratories, with some laboratories being the sole provider of certain functions, according to availability, commitment and expertise. Nevertheless, each specialized HIV drug resistance laboratory must be willing to take responsibility for at least one core activity.

Core activities of specialized HIV drug resistance laboratories

1. Capacity building and training
    - Coordinate the development of training materials and educational programmes for laboratories within the HIVResNet Laboratory Network.
    - Organize technical workshops at the regional level, as necessary.
2. Operational research
    - Participate in collaborative studies to develop and validate methods aimed at improving the feasibility of genotype testing under field conditions.
    - Participate in research aimed at improving the sensitivity, specificity, applicability, turnaround time, reporting and affordability of HIV drug resistance testing in HIV drug resistance surveys (for example: developing methods for genotyping integrase and sharing and supporting the implementation of these methods in other Network laboratories).
3. External quality assurance

Note: To date, external quality assurance testing has been provided through the Virology Quality Assurance programme, under contract from the United States National Institutes of Health. However, should this cease to be the case, the specialized HIV drug resistance laboratories will be expected to perform this function as outlined below:

- coordinating the participation of designated laboratories in a WHO-recognized external quality assurance programme for genotyping, including proficiency panels;
- coordinating the performance evaluation of laboratories participating in any WHO-recognized proficiency testing programme;
- assisting in developing and supplying WHO-recognized proficiency panels;
- harmonizing WHO-recognized quality assurance systems and identifying methods for attaining comparative results; and
- coordinating the development and distribution of standardized reagents and validation panels for all laboratories in the Network, as needed.

Requirements

WHO identifies and designates a small number of laboratories as specialized HIV drug resistance laboratory based on:

- the excellence of their performance;
- their recognized expertise on selected key topics relevant to the development of the HIVResNet Laboratory Network; and
- their capacity, resources, commitment and motivation.

Experience as public health laboratories, although not compulsory, is an asset.

1.3.4 WHO HIVResNet Laboratory Working Group

The WHO HIVResNet Laboratory Working Group includes members of the designated specialized HIV drug resistance laboratories, regional HIV drug resistance laboratories and national HIV drug resistance laboratories and a select number of experts in HIV drug resistance surveillance. WHO, in consultation with the Working Group, coordinates the Laboratory Network.

Tasks and responsibilities

1. Harmonize and support the Laboratory Network by providing technical advice, assistance with advocacy and resource mobilization
2. Assist in assessing and strengthening laboratories that have the capacity to function as national, regional or specialized HIV drug resistance laboratories
3. Meet as needed to review the technical performance of the Network and provide assistance in formulating policy
4. Provide updates to the laboratory operational framework publication as needed

1.3.5 Role of WHO

WHO has two specific areas of responsibility: coordination and financial support.

1. Coordination

    - Facilitate linkage, communication and the flow of data between national HIV drug resistance laboratories, the regional HIV drug resistance laboratory and specialized HIV drug resistance laboratories when necessary
    - Organize laboratory assessments and grant membership in the Laboratory Network
    - Organize meetings of the WHO HIVResNet Laboratory Working Group when needed
    - Communicate information relevant to the Laboratory Network as needed, but at least annually
    - Assure the overall quality of the entire survey results by reviewing sequences and epidemiological data submitted for global reporting purposes by network laboratories and country working group and by supporting country and regional analysis

2. Financial support

    Although the specialized HIV drug resistance laboratory, regional HIV drug resistance laboratory and host governments bear much of the financial responsibility for assisting countries in conjunction with this project, WHO will assist in identifying sources of funding for the following, as needed:

    - costs related to the initial laboratory assessment visit, including the cost of travel and per diem of a laboratory expert to visit the candidate national HIV drug resistance laboratory or regional HIV drug resistance laboratory, and subsequent visits for providing technical assistance may also be supported; and
    - shipping proficiency panels in the context of the external quality assurance programme.

    WHO may provide letters of endorsement to the national HIV drug resistance laboratories, regional HIV drug resistance laboratories and specialized HIV drug resistance laboratories for grant applications seeking financial support from other sources for activities related to HIV drug resistance surveillance.

1.4 HIV drug resistance genotyping procedures and quality assurance

Genotyping (sequencing) for HIV drug resistance can be performed either by using commercial kits that include reagents, controls and software to generate results or by using in-house developed ("home-brew") assays. For in-house assays, laboratories select their own primers for amplification and sequencing and use generic reagents and software for sequence analysis. Laboratories use a large variety of in-house sequencing assays, and several methods have been published *(1–4)*. Because of their lower cost, in-house assays are often preferred. However, they are associated with additional requirements for quality control and quality assurance. Although many laboratories in resource-limited settings have experience in genotyping, the lack of standardized procedures and quality assurance steps limits the production of comparable and reliable results.

After the sequencing procedures are completed, the results must be carefully reviewed to identify unexpected or anomalous sequences that might be related to procedural errors or laboratory artefacts. Laboratories with less experience in this area should collaborate with regional or specialized laboratories and WHO HIVResNet virologists. The recommended procedures for post-testing sequence quality assurance (Annex 2) and the HIV drug resistance laboratory training package, developed jointly by WHO and the United States Centers for Disease Control and Prevention in 2010 (https://www.who.int/hiv/pub/drugresistance/lab_training/en/), provide more information on HIV drug resistance genotyping and quality assurance.

2. LABORATORY STATUS DEFINITIONS

2.1 Candidate laboratory

A candidate laboratory is an institution at the national, regional or global level that has expressed interest in joining the WHO HIVResNet Laboratory Network and has fulfilled all the mandatory application criteria. An expression of interest can be provided to WHO via its country, regional or headquarters offices.

2.2 Network-designated laboratory

A laboratory is designated as being a member of the Laboratory Network once it has met all the mandatory criteria (see Section 1).

2.3 Laboratories failing to achieve WHO designation

A laboratory that does not meet the mandatory application criteria or that meets the mandatory application criteria but fails to achieve the passing score based on the evaluation and/or does not pass the proficiency panel test is considered not designated. If specimens are collected and awaiting genotyping, arrangements must be made for an existing designated regional HIV drug resistance laboratory or specialized HIV drug resistance laboratory to perform tests on all WHO survey specimens. A laboratory that does not achieve designation may work with the regional laboratory coordinator, WHO headquarters staff or another designated laboratory to:

- identify areas where improvement is needed;
- develop and implement a work plan;
- monitor laboratory progress; and
- continue steps to achieve designation in the future.

A laboratory with the status of "not designated" is eligible to reapply at a later date, once it has addressed the deficiencies noted following the original assessment. Depending on the delay between the original and second application, a new site assessment may be required.

2.4 Suspended laboratory

A designated laboratory may be suspended for one or more reasons. A laboratory at risk of being suspended will be notified and provided with the necessary support to address the problems noted. Suspended laboratories are required to cease genotyping activities for WHO surveys until designation has been restored. After suspension, a laboratory may reapply for designation at the discretion of WHO once it has addressed the deficiencies that led to the suspension.

The reasons for suspension include, but are not limited to:

- failure to maintain good standing with respect to the mandatory designation criteria (see above);
- failure to meet target performance standards on two consecutive proficiency panels;
- other serious lapses in quality that are not addressed in a timely manner, such as:
  - detection of molecular contamination in a survey data set or proficiency panel;
  - abnormally high amplification failure rate without appropriate investigation and corrective action;
  - changes in laboratory infrastructure or personnel that negatively affect factors considered in the assessment visit score; and
- lack of responsiveness and demonstration of commitment to the WHO HIV drug resistance mission.

3. LABORATORY NETWORK MEMBERSHIP REQUIREMENTS AND REVIEW PROCEDURE

Membership in the Laboratory Network demonstrates that the laboratory has the capacity to provide quality-assured HIV protease, reverse transcriptase and integrase sequences for HIV drug resistance surveillance activities in a timely manner. The membership application review process also provides a learning opportunity, a mechanism for identifying resource and training needs and a measure of progress.

Evaluation for Laboratory Network membership takes place through on-site laboratory assessment, review of procedures and documentation, review of genotyping assay validation and external quality assurance testing results and ongoing successful participation in a WHO-recognized external quality assurance system. WHO-designated laboratories are members of the global WHO HIVResNet Laboratory Network. A member laboratory must demonstrate that it continues to meet the criteria at regular intervals.

To apply for Laboratory Network membership, laboratories must meet the national, regional or specialized mandatory application criteria, which will be verified during the assessment visit. To obtain membership, each laboratory must achieve a satisfactory score based on the assessment and pass the WHO-recognized proficiency panel (Fig. 2).

The procedure for obtaining designation is described below.

3.1 Application phase

Before assessment, a set of application criteria helps to determine whether all crucial elements for evaluation during the assessment are present. WHO will also assess the need for adding a laboratory at the requested level (national HIV drug resistance laboratory, regional HIV drug resistance laboratory or specialized HIV drug resistance laboratory) in the applicant's country or region. For example, new applications will not be considered for a regional laboratory in a region that is already served by a sufficient number of regional HIV drug resistance laboratories, nor for a national HIV drug resistance laboratory in a country that is not actively planning to initiate HIV drug resistance surveys according to WHO recommendations.

The checklist and questionnaire (Annex 4) forms the basis for application, along with supporting documentation as detailed in the questionnaire. The questionnaire is used to collect information about the laboratory, including

Fig.2. Overview of the application, evaluation and designation process

Candidate HIV drug resistance genotyping laboratory

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Application submitted to WHO

↓

Minimal criteria demonstrated and verified by WHO

↓

Dry panel testing
External quality assurance programme enrolment and prequalification

↓

External quality assurance annual proficiency panel(s)[a]

↓

Site visit[b]

↓

Dossier review by WHO headquarters and HIVResNet laboratory advisory group

↓

Designation as Network laboratory

[a] Annual proficiency panel(s) refers to the panel that is active at the time the laboratory reaches this stage, including a plasma-based panel for all laboratories as well as a DBS-based panel for regional HIV drug resistance laboratories and specialized HIV drug resistance laboratories.

[b] In some cases, the site visit may take place before the annual proficiency panel testing is complete.

standard operating procedures, specific training of personnel and evidence confirming the laboratory's adherence to expected performance standards. This questionnaire should be returned to WHO, along with all the requested materials, including a map of the physical layout of the laboratory and standard operating procedures as requested in the questionnaire. Materials should be submitted electronically to the Department of Global HIV, Hepatitis and Sexually Transmitted Infections Programmes using the following email address: hiv-aids@who.int.

3.2 Assessment phase

After the questionnaire and requested documentation are reviewed and found to be satisfactory, WHO will:

- make the dry panel materials and instructions available for download;
- send a WHO-recognized proficiency panel to the candidate laboratory, with the proficiency panel shipment, testing and evaluation taking several months to one year; and
- arrange an on-site assessment visit for auditing the questionnaire by WHO personnel and/or a representative from the WHO HIVResNet Laboratory Network Working Group that usually takes several weeks to coordinate.

During the on-site assessment, the following aspects are evaluated.

1. Complete laboratory infrastructure and equipment for genotyping
    - The office and laboratory space is clean, well kept and adequate for current workload.
    - Appropriate equipment is in place, functional, in good condition and maintained regularly; this includes sequencer(s), PCR machine(s), refrigerators, –20°C and –80°C freezers, electric power back-up, etc.
    - Space configuration, workflow and contamination control is consistent with good laboratory practices.
    - Genotyping capacity is adequate, including sequence editing and review of results by an experienced supervisor.
    - Computational capability, including hardware, software and Internet access.
    - Regular and consistent temperature monitoring records for incubators, refrigerators, PCR machines and freezers.
    - Sufficient inventories and timely replenishment of supplies.
    - Minimum biosafety level of two in the areas where the specimens are handled (standard procedure for handling biohazardous materials).
2. Adequate expertise of laboratory personnel
    - Laboratory supervisor with specific training in molecular virology.
    - Laboratory technicians with training and experience in HIV drug resistance genotyping, including reviewing and editing sequences.
    - Presence of a dedicated safety officer.
    - Adequate number of trained staff to handle the anticipated workload.
    - The standard operating procedures clearly state that the supervisor or designee critically reviews test results and confirms the results report by signing it.
3. The laboratory
    - Clear and accountable laboratory management structure.
    - The HIV drug resistance activities in the laboratory are financially sustainable.
    - Genotyping assay costs are kept as low as possible.
4. Laboratory experience in genotyping
    - >1 year of experience in HIV genotyping.
    - ≥100 specimens genotyped by sequencing annually.
5. Demonstrated use of standard operating procedures covering all procedures, including:
    - specimen receipt, assessment, rejection criteria and storage
    - internal quality control
    - all steps of genotyping tests, including sequencing and workflow
    - handling and manipulation of infectious human material, including handling infectious waste
    - data management
    - post-testing sequence quality assurance, including detection, containment and control of molecular contamination

6. Proficiency panel testing

    - Successful participation in HIV drug resistance proficiency testing programmes from providers other than WHO in the past year. Copies of the reports are requested.

In addition, the laboratory must pass a WHO-recognized proficiency panel and the dry panel before being granted Laboratory Network membership (see Annex 1).

The candidate laboratory will become a Laboratory Network member if it meets all the mandatory criteria (see subsection 1.3.1) and achieves a passing score of ≥85 of 100 based on the additional criteria evaluated during the on-site visit.

For regional HIV drug resistance laboratories, the following additional requirements apply.

- Regionally recognized experience and leadership in HIV laboratory science: the laboratory must identify a minimum of three public health HIV laboratories within the region as references (please specify the contact people, contact details and type of collaboration for each). WHO will contact the laboratories to confirm the suitability of the applicant laboratory to function as a regional laboratory.
- Adequate experience in providing training and establishing collaborations in the laboratory sciences in the past three years.
- Ability to provide reference virology services to other laboratories, including enhanced capacity for specimen receipt from other countries, specimen storage and management.
- Capacity for genotyping of integrase and using DBS.

For specialized HIV drug resistance laboratories, the following additional requirements apply.

- The candidate laboratory must have internationally recognized experience and leadership in HIV drug resistance genotyping. The laboratory must provide a list of five public health laboratories as references. WHO will contact the laboratories to confirm the suitability of the candidate laboratory to function as a specialized laboratory.
- The candidate laboratory must prove that it conducts original research in laboratory aspects of HIV drug resistance, as demonstrated by:
    - the laboratory's peer-reviewed publications from the past five years in which laboratory staff are included as authors; and
    - a list of funding awards for ongoing collaborative HIV drug resistance research in the past five years.
- The laboratory must demonstrate willingness to share information and work cooperatively with WHO and with other Laboratory Network laboratories.
    - The laboratory should provide a description of previous collaborative work with WHO in the field of HIV and specifically on HIV drug resistance.
    - If applicable, the laboratory must provide a description of any potential problem that may occur by sharing data and information with WHO and other laboratories in the Laboratory Network.
    - The laboratory must attach a detailed description on its potential role and function within HIVResNet.

Experience in providing laboratory support to large-scale HIV drug resistance surveillance programmes is not a mandatory criterion but will be considered positively during the assessment.

Laboratories that conduct research or coordinate activities on the following topics will receive special consideration, as these topics are pertinent to the aims of the Laboratory Network:

- designing and validating novel resistance assays and relevant surveillance technologies such as point mutation assays, cheap methods for genotyping, genotyping using dried fluid spots, next-generation sequencing etc.;
- developing, distributing and evaluating quality assurance systems;
- producing and organizing training materials and modules and educational programmes for HIV drug resistance;
- reference laboratory activities for HIV drug resistance surveillance (list of number of specimens tested for the HIV drug resistance surveillance and number of countries assisted); and
- DBS research, including DBS method development and testing (please attach a description of the planned studies, if applicable).

3.3 Designation phase

WHO and, if needed, the HIVResNet Laboratory Network Working Group will evaluate the laboratory assessment documents and results of the assessment site visit and decide whether the candidate laboratory has met the criteria for designation. Candidate laboratories will be granted a status of "designated", "not designated" or "pending" (if one or more required elements are missing). Designated laboratories become members of the Laboratory Network. Designation is specific for assay and specimen type (ViroSeq™ or other kit and/or in-house method, plasma and/or DBS).

WHO reviews the status of all designated laboratories on an ongoing basis. This review takes into account the laboratory's activities and performance, the results of the annual WHO-approved proficiency panel testing and may include a site assessment visit when needed (for example, if there are significant changes to laboratory facilities or staff).

4. GUIDANCE FOR HIV DRUG RESISTANCE GENOTYPING

4.1 Introduction

Nucleotide sequence analysis (genotyping) of relevant portions of the HIV genome, such as the complete protease region and most of the reverse transcriptase and integrase, can identify mutations associated with HIV drug resistance. In addition, the genetic sequence can also be used to derive HIV-1 subtype, although complete characterization of some recombinant forms may not be possible.

Genotyping by sequencing of bulk PCR products identifies the predominant virus populations in the viral quasi-species. Depending on the method used, an individual variant sequence must be present at levels above approximately 20% for reproducible detection. This means that if a particular variant in the virus population of a specimen is present at a level below the threshold of detection for the assay method, it will not be detected reliably. More sensitive technologies such as deep sequencing, real-time allele-specific PCR or hybridization are available. However, allele-specific PCR is able to detect mutations at only a limited number of positions in the sequence and does not result in a complete genotypic profile. Deep sequencing is becoming more accessible and is discussed further below.

HIV drug resistance genotyping identifies mutations associated with reduced susceptibility to one or more antiretroviral drugs. Studies demonstrate that access to genotyping results can be useful in the clinical management of HIV infection by providing information to guide the selection of appropriate subsequent therapies. Since the number of mutations known to be associated with HIV drug resistance is high and various interactions between mutations have been identified, the interpretation of a genotypic resistance profile for clinical purposes can be complex. Several different genotyping interpretation algorithms have been developed for clinical applications. The algorithms were developed using mainly information on HIV-1 subtype B strains, although the natural polymorphisms in non-subtype B strains can influence the final results of drug resistance interpretation algorithms. WHO surveys of pretreatment HIV drug resistance among people initiating antiretroviral therapy, people for whom antiretroviral therapy has failed (acquired HIV drug resistance) and among infants younger than 18 months use the Stanford University HIVdb interpretation algorithm. HIVResNet published a list of mutations suitable for surveillance of transmitted drug-resistant HIV *(5)*.

4.2 Specimen types for HIV drug resistance genotype testing

The current specimen types collected for HIV drug resistance genotyping are plasma or DBS. The national HIV drug resistance working group should consider using DBS for HIV drug resistance surveys in which plasma cannot be separated and frozen in an appropriate manner, maintaining frozen specimens at a uniform temperature is problematic or shipment of frozen specimens to the genotyping laboratory is not feasible. Although HIV genotyping based on plasma provides the HIV drug resistance profile of the population of replicating viruses, the results generated from DBS may also reflect a contribution from archival HIV DNA found in latent cellular reservoirs. Although theoretically this may lead to discordance in results generated from DBS and plasma *(6)*, studies show that genotyping results from plasma and DBS are comparable *(7–10)*, although subtle differences have been observed *(11)*.

The decision to use DBS for surveys of HIV drug resistance is a country decision and is taken in consultation with WHO and laboratories with DBS experience, with full understanding of the limitations implicit in the amplification of HIV from DBS. For additional information, including recommended procedures for collecting, storing, shipping and processing DBS for HIV drug resistance genotyping, see the *WHO manual for HIV drug resistance testing using dried blood spot specimens (12)*.

Dried plasma spot and dried serum spot specimens are not appropriate specimen types for HIV drug resistance monitoring surveys.

Using DBS for viral load testing, a component of surveys for acquired HIV drug resistance, also involves important considerations. Because of the presence of viral DNA in cells in whole blood that are not present in plasma, the potential exists for false positives or overestimation of viral load from DBS *(13)*. The preference is for viral load assays that are selective or specific for RNA over DNA. For additional information, see the WHO HIV website (https://www.who.int/hiv/en).

4.2.1 Plasma specimen processing

Specimens should be processed and stored as soon as possible to ensure the quality of the specimens for genotyping. Centrifugation, pipetting and aliquoting must follow standard laboratory biosafety precautions at

a laboratory equipped to manipulate infectious clinical samples, with adequate sample storage and inventory facilities.

During the time between collection and separation, whole-blood specimens should remain as cold as possible without freezing (such as at 4°C or on ice), but may also be kept at room temperature (15–30°C) for limited periods of time (such as less than six hours). If the room temperature exceeds 30°C, an isotherm box should be used to store specimens at 15–30°C.

After separation, plasma specimens should be constantly at refrigeration temperature (4°C) until the aliquots are frozen. Plasma for genotyping must be processed or frozen at –20°C to –80°C within 48 hours after the blood is drawn. Freezing at –80°C is preferable but –20°C is acceptable; not having a –80°C freezer is not an impediment to storing specimens for HIV drug resistance surveys. Approximately 1 ml of plasma can be obtained from 2–3 ml of whole blood. Plasma specimens should be considered potentially infectious and transported according to international regulations. The WHO and the United States Centers for Disease Control and Prevention provide information on shipment of hazardous material at: https://www.who.int/influenza/gisrs_laboratory/logistic_activities/en and http://www.cdc.gov/od/eaipp/shipping. In all cases, national working groups should verify proper and safe shipping conditions with the courier service they plan to use and be in contact with the genotyping laboratory before shipping specimens.

Transport methods depend on a country's infrastructure. Field personnel are often responsible for transporting clinical specimens from an antiretroviral therapy site to a central laboratory for CD4 cell counts or other tests. Timely transport for specimens may require additional resources if processing and storage conditions at the antiretroviral therapy site are not suitable.

Plasma specimens should be frozen at the survey site only when the frozen state is guaranteed to be maintained during the period of transport to a national storage laboratory or to the HIV drug resistance testing laboratory. Cooler boxes and ice packs are never sufficient to maintain the frozen state; dry ice or liquid nitrogen are necessary for transport within the country once specimens are frozen. If local logistics do not permit proper plasma collection, processing and storage, DBS should be strongly considered as the specimen type for the survey, especially among untreated people.

After freezing, if appropriate freezer facilities are available for storage centrally, survey plasma specimens may remain frozen (–20°C) for many years before being sent to the genotyping laboratory. Specimens must be transported to the genotyping laboratory on dry ice or liquid nitrogen, without thawing of plasma specimens. The adequate preparation for shipping should therefore include a courier service experienced in transporting clinical specimens on dry ice, including replenishing dry ice during transport and during customs processing or via transport in liquid nitrogen containers.

All customs import and export permits must be obtained before shipping. An acknowledgement or notification system should be set up involving the survey coordinator, the transport system and the receiving genotyping laboratory to ensure the prompt delivery of continuously frozen specimens. Notifications may be by email or fax, using the shipping manifests for this purpose.

4.2.2 Nucleic acid extraction from liquid plasma and DBS

High-quality purified nucleic acid is essential for successful genotyping. Most extraction methods are evaluated and applied using plasma and are generally designed to isolate RNA or DNA or both from specific types of specimens. The type of clinical specimen and the type of nucleic acid to isolate should be considered in selecting the extraction method or technology. Standard nucleic acid extraction procedures can be used to isolate HIV RNA from plasma. Many reagents and methods based on well-established procedures for nucleic acid extraction are commercially available. Several of these methods can also extract RNA from whole blood or DBS. WHO recommends using an established, commercially available extraction method that has been validated for the type of specimen being used and the type of nucleic acid being collected. The minimum input volume should be 100 µl of plasma or two DBS spots. Preferably, a larger volume, up to 1 ml of plasma, is used to increase the amplification sensitivity of the genotypic assay. See the *WHO manual for HIV drug resistance testing using dried blood spot specimens (12)* for details of the recommended extraction procedures for DBS.

4.3 Amplification and sequencing

Once purified, the nucleic acid requires amplification by PCR and subsequent sequencing. Two commercial genotyping kits are available: ViroSeq™ HIV-1 (Abbott Molecular) and the Applied Biosystems™ HIV-1 Genotyping Kit (Thermo Fisher Scientific). Many experienced genotyping laboratories have their own in-house ("home-brew") amplification and sequencing procedures and reagents. HIV drug resistance testing through genotyping is a complicated procedure that requires a high level of technical experience and a properly designed molecular laboratory. Given the high number of laboratory manipulations involved, the procedure is prone to variation and requires a high level of standardization, both in the people performing the laboratory testing and in analysing and interpreting the genotyping result. Further, the various HIV-1 subtypes have extensive genetic differences. This means that the primers used for

amplifying and sequencing specimens need to be validated for adequate performance on various subtypes.

Published studies have evaluated the performance of both the commercially available kits on various HIV-1 subtypes *(14–24)*. For in-house assays, this information may not always be easily accessible. Given the heterogeneous distribution of the various subtypes throughout the world, both home-brew protocols and commercial assays must perform adequately for a range of subtypes. Both commercial genotyping kits come with dedicated software to support the analysis and editing of the electropherograms. This software may not be applicable to analysing sequence data obtained from in-house assays.

Laboratories considering implementing HIV drug resistance genotyping for the first time should consider starting with a commercial kit. Before implementing commercial assays, laboratories should also obtain information on how these assays perform on the local circulating variants. After establishing a commercial kit assay, laboratories should evaluate any home-brew assays by comparing the results to those obtained from the commercial kit before implementing for routine use.

The following are recommended for amplification and sequencing.

- Home-brew or in-house assays should be implemented only after adequate validation, including evaluation of performance with various HIV-1 subtypes.
- A high level of standardization is necessary for laboratories performing genotyping. This includes standardizing personnel training, peer-reviewed standard operating procedures, workflow and other aspects of operation.
- The minimal regions for which collecting sequence information is essential are:
  - protease: codons 10–93;
  - reverse transcriptase: codons 41–238; and
  - integrase: codons 51–263.

4.4 Data management and traceability of results

All specimen handling and manipulation should be traceable for each step of the procedure. This means that the administrative process should be well defined and described. This includes:

- registering a unique specimen identification code in accordance with the recommendations of the WHO survey protocol;
- registering any subsequent specimen codes while processing the specimen in the laboratory;
- registering the final result in relation to the original specimen identification;
- storing all laboratory results at the laboratory, including the raw sequencing data (electropherogram files), with regular backup and traceability at any moment; and
- storing raw laboratory data for a minimum of five years.

4.5 Preferred genotyping methods

WHO recommends one of the genotypic testing procedures shown below; several home-brew protocols are available with different primer sets for amplification and/or sequencing. Laboratories performing genotyping should be aware that successfully implementing these procedures is not a trivial process.

4.5.1 ViroSeq™ HIV Genotyping Kit

Vendor: Abbott Molecular (https://www.abbottmolecular.com/us/products/viroseq-hiv-1-genotyping-system.html)

The kit consists of protocols and reagents for extracting the sample and amplifying and sequencing the entire protease coding region and most of the reverse transcriptase region (amino acids 1–320). A separate kit for integrase is also available.

The requirements include:

- PCR-grade laboratory design;
- ultracentrifuge for concentrating virus:
- sequence detection hardware: capillary electrophoresis equipment is recommended, since it is simple to use and generally well suited for diagnostic use;
- training to ensure that the equipment is properly used;
- gel-based sequencers are an alternative to capillary systems, but require extensive training and experience and are less suitable for diagnostic use; and
- sequence analysis software (provided by Abbott).

4.5.2 Applied Biosystems™ HIV-1 Genotyping Kit

Vendor: Thermo Fisher (https://www.thermofisher.com/ng/en/home/life-science/sequencing/sanger-sequencing/applications/genotyping-hiv-detect-drug-resistance.html)

The Applied Biosystems™ HIV-1 Genotyping Kit is used for detecting HIV genomic mutations that confer resistance to specific types of antiretroviral drugs, to aid in monitoring the development and transmission of HIV-1 drug resistance. It is based on methods developed by the United States Centers for Disease Control and Prevention *(25,26)*. It can detect viral resistance in plasma or DBS obtained from individuals infected with multiple HIV-1 subtypes and circulating recombinant forms with a viral load equal to or greater than 1000 copies/ml.

Two modules are provided for genotyping, allowing for both detection and sequence analysis:

- module 1: reverse transcriptase-PCR and nested PCR; and
- module 2: cycle sequencing.

The requirements include:

- PCR-grade laboratory design;
- sequence detection hardware: capillary electrophoresis equipment is recommended, since it is simple to use and generally well suited for diagnostic use;
- training to ensure that the equipment is properly used;
- RNA purification must be performed using reagents or kits available from other vendors; and
- sequence analysis software, which is not provided; a base-calling software package developed by the British Columbia Centre for Excellence in HIV/AIDS called RECall is available without cost to WHO genotyping laboratories (see Annex 2).

4.5.3 Home-brew or in-house sequencing methods

Several laboratories specializing in HIV-1 drug resistance have developed in-house or home-brew methods using reagents that are not marketed in the form of a genotyping kit. These methods may require purchasing commercial reagents and expensive sequencing hardware. Reagents and procedures are less standardized than with commercial kits, both within and between laboratories. A considerable advantage is that reagent price per sample is significantly less than for commercial kits. Home-brew methods are more flexible than kit-based methods, especially since changes (such as alternative primers) can be more easily implemented when required.

The requirements include:

- PCR-grade laboratory design;
- extensive inter-laboratory validation of all aspects of the sequencing procedure, including extraction and sequencing hardware;
- performance on various subtypes may vary per laboratory and depends on the primers included in the specific home-brew protocol; and
- sequence analysis software, which is not provided; a base-calling software package developed by the British Columbia Centre for Excellence in HIV/AIDS called RECall is available without cost to WHO genotyping laboratories (see Annex 2).

4.5.4 Next-generation sequencing

Next-generation sequencing is an emerging technology that has several potential advantages compared with standard Sanger sequencing, including increased sensitivity for low-abundance variants, improved ability to quantify different variants and reduced cost if throughput is high enough. Several Laboratory Network laboratories are considering, or have completed, a transition from standard Sanger-based sequencing methods to a procedure based on next-generation sequencing for routine genotyping of HIV and other pathogens. As with any new method, existing quality assurance and quality control procedures must be adapted to make them compatible with using next-generation sequencing for HIV drug resistance genotyping. Several publicly available data processing pipelines developed specifically for HIV drug resistance have been developed *(27)*.

To simplify the transition to next-generation sequencing, and in the absence of definitive data regarding a clinically relevant threshold for low-abundance drug-resistant HIV variants, the HIVResNet Laboratory Working Group has recommended a phased approach that begins with configuring next-generation sequencing assays to mimic the sensitivity of Sanger-based methods. In practice, this means that the threshold for low-abundance variants should be set at 20% *(28)*. In this light, next-generation sequencing can be thought of as an alternative, in-house (home-brew) assay that is required to generate results with the same performance characteristics as existing Sanger-based methods. This includes generating a nucleotide sequence in FASTA file format with the same minimum coverage: codons 10–93 for protease, 41–238 for reverse transcriptase and 51–263 for integrase. In this first phase, it is acknowledged that the potentially useful advantages of next-generation sequencing will be ignored, such as the increased sensitivity for drug-resistant variants present at frequencies below 20%, resolution of complex ambiguous codons containing more than one mixed base and quantification of variant proportions; these aspects will be investigated more completely in a later phase.

4.5.5 Choice of resistance testing methods

For surveillance purposes, any of the genotyping assays listed above are acceptable. Each laboratory should choose which method to use based on considering laboratory personnel experience, kit and reagent availability, cost and infrastructure. If laboratory capacity is not available in the country and planners want to develop such capacity, a WHO-designated laboratory can assist in developing the protocol. All Laboratory Network laboratories should have detailed and approved laboratory protocols for procedures to enable comparable genotyping information to be collected.

4.6 Interpreting HIV drug resistance

For surveys of pretreatment HIV drug resistance, acquired HIV drug resistance and HIV drug resistance among infants younger than 18 months, the impact of mutations is assessed by using the Stanford University HIVdb algorithm (https://hivdb.stanford.edu/hivdb/by-sequences). Classifications of "potential low-level resistance" are considered as "susceptible" for the purposes of WHO surveys. Classifications of "low-level", "intermediate" and "high-level" should be combined into one "resistant" category.

To avoid confusion, laboratories should not provide alternative interpretations of sequence data to national working groups.

4.7 Laboratory biosafety

Laboratories should observe universal precautions for all blood and body fluid specimens. The United States Centers for Disease Control and Prevention described them *(29)* and further expanded and updated them *(30,31)*. In addition, all laboratory personnel should be familiar with the WHO *Laboratory biosafety manual (32)* and other WHO recommendations (https://www.who.int/in-vitro-diagnostic/biosafety-guidelines/en). Every laboratory should have a copy of these guidelines and observe the recommendations.

5. GUIDANCE FOR SUBMITTING THE RESULTS TO NATIONAL HIV DRUG RESISTANCE WORKING GROUPS AND TO WHO

After HIV drug resistance testing is completed, including the procedures described in the WHO laboratory standard operating procedures for post-testing quality assurance of HIV drug resistance genotyping (Annex 2), the designated laboratory should submit the results to the national HIV drug resistance working group or designated national focal person for the survey. If the designated laboratory does not know the focal point of the national HIV drug resistance working group or survey, it should contact WHO headquarters for clarification. Sequences should also be submitted via the WHO HIV drug resistance database (https://www.who.int/hiv/topics/drugresistance/hiv-drug-resistance-database/en).

Drug susceptibility interpretations must be based on the drug resistance mutations found in each sequence using the Stanford University algorithm (https://hivdb.stanford.edu/hivdb/by-sequences) for surveys of acquired or pretreatment HIV drug resistance or surveys of HIV drug resistance among infants younger than 18 months. Analyses using other methods or other standardized algorithms should not be provided to the country HIV drug resistance working groups or to WHO.

All WHO-designated laboratories are strongly recommended to submit sequences to WHO headquarters for additional review before submitting them to a national authority. Genotyping results are returned in FASTA format to facilitate standardized quality assurance and HIV drug resistance analysis, which can be facilitated by using the WHO HIV drug resistance database. Specific procedures for submitting sequence data and associated information are being developed. The essential components of the data package to be submitted to WHO are:

- the quality-assured sequences in FASTA format;
- a summary report describing sequence anomalies detected during analysis, if any, and how they were resolved;
- the output of the Stanford quality assurance and resistance analysis;
- the British Columbia Centre for Excellence in HIV/AIDS quality assurance tool output (see Annex 2).

The quality of the sequence data is one component of the annual performance evaluation for continued Laboratory Network membership.

5.1 Data ownership

The results of national HIV drug resistance surveillance activities belong to the country from which the specimens were obtained, and national, regional and specialized laboratories must remember that sequencing results may never be published under any circumstances without the express consent of that country. In addition, specimens may not be used for research projects for which they were not initially intended, and any remnant specimens must be returned or destroyed after the results have been finalized and quality assured by the national programme and WHO headquarters.

5.2 Data flow from designated laboratories

The genotyping laboratory should submit HIV drug resistance testing (and viral load, if also performed by the genotyping laboratory) results simultaneously to the HIV drug resistance national working group and WHO, using the WHO HIV drug resistance database. There may be queries and cleaning of data, if necessary, and discussions between WHO, the laboratory and country counterparts to arrive at the final agreed quality-assured dataset. In some instances, this will involve the national working group epidemiologist, since demographic data from individual people may be required to finalize quality assurance queries. In many cases, the epidemiological data are not available to the laboratory, and WHO can act as a mediator to bring the two data sources together to complete the quality assurance process.

Once the quality-assured dataset is final, the designated drug resistance testing laboratory sends it to the country HIV drug resistance working group. Country analyses and reporting should use only the final quality-assured sequence and epidemiological dataset.

Some regional HIV drug resistance laboratories and specialized HIV drug resistance laboratories have considerable expertise in quality assurance and may be able to provide national HIV drug resistance laboratories with support and supervision when performing quality assurance following the WHO-suggested standard operating procedures for quality assurance. In such cases, the national HIV drug resistance laboratory should liaise with WHO to identify a specialized laboratory with quality assurance expertise and twin with this laboratory to optimize its quality assurance assessment.

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ANNEX 1. WHO HIV DRUG RESISTANCE EXTERNAL QUALITY ASSURANCE AND INTERNAL QUALITY CONTROL

1. External quality assurance

To obtain and maintain membership in the HIVResNet HIV Drug Resistance Laboratory Network, a laboratory must participate in a WHO-recognized genotyping external quality assurance programme that includes proficiency panel testing. WHO and its partners and/or selected specialized HIV drug resistance laboratories are responsible for developing and distributing proficiency panels to member laboratories and those applying for membership. Initially, however, laboratories should enrol in one or more existing nationally or internationally recognized external quality assurance programmes. Laboratories should successfully pass a minimum of one proficiency panel per year. External quality assurance programmes should take into account the distribution of HIV genetic variants and subtypes in different areas of the world as well as other viral characteristics. Since 2007, proficiency panels were provided by the Virology Quality Assurance (VQA), under a contract from the United States National Institutes of Health/National Institute of Allergy and Infectious Diseases. A summary of proficiency panel results was published in 2012 (https://academic.oup.com/cid/article/54/suppl_4/S266/309451).

The recommended characteristics for the HIV drug resistance genotyping proficiency panel are:

- The proficiency panel should include only HIV types derived from diluted clinical samples (preferred) or cell culture propagation.
- The proficiency panel should contain samples with several drug resistance-associated mutations in the reverse transcriptase, protease and integrase regions.
- The proficiency panel should include a minimum of five different samples.
- Various subtypes should be represented in the panel: at least one subtype B, one subtype C and one non-B, non-C virus.
- Samples should have a minimum viral load of 2000 copies/ml.
- The viruses chosen should be compatible with all commercial assays. All samples from the panel should be well characterized using validated in-house assays and/or commonly used commercial kits before distribution.
- A minimum of 10 laboratories should test each panel to yield a meaningful consensus sequence.

1.1 Data analysis and scoring

The sequencing data submitted by laboratories testing the proficiency panel are analysed by comparison with the group consensus sequence according to one or both of two alternative procedures. The VQA analyses most routine proficiency panel testing. The VQA method is described here: https://www.hanc.info/labs/labresources/vqaResources/ptProgram/VQA%20Document%20Library/VQA%20GENO%20Scoring%20Document_v1_3.pdf. The alternative method developed at WHO is described below.

A consensus sequence is prepared by first aligning the sequences submitted by all participants in the programme. At each position in the alignment, the nucleotide (or nucleotide mixture) observed in >80% of the submitted sequences is included in the consensus. If no nucleotide or mixture is observed in >80% of the sequences, then that position is not included as part of the consensus sequence during the analysis.

Nucleotide sequence concordance of each laboratory’s results with the consensus sequences (over the region spanning amino acids 10–93 of protease, 41–238 of reverse transcriptase and 51–263 of integrase) is reported as the number of concordant nucleotides of the total number of unambiguous bases in the consensus.

Concordance at the major and minor drug-resistance mutation sites is also determined. The Stanford University HIV drug resistance algorithm defines drug-resistance mutation sites, and the sites are periodically updated (http://hivdb.stanford.edu). Drug-resistance mutation site scores are calculated and expressed as the number of concordant drug-resistance mutation codons of the total number of drug-resistance mutation codons not containing an ambiguity in the consensus.

Laboratories will receive a summary of overall sequence concordance and drug-resistance mutation site scores for each specimen in the panel as well as average scores for each dataset and sequence alignments for each specimen protease and reverse transcriptase, with discrepancies highlighted and scores calculated.

The definition of "concordance" depends on the context and involvement of mixtures.

- When mixtures are not present in either the consensus or the test sequence, the same base must be reported to be considered concordant, whether or not the change results in an amino acid mutation and whether or not it is considered wild-type (same as the consensus subtype B reference) or mutant (any other amino acid).
- If a mixed base is present in either the consensus or the test sequence, it is treated according to the impact on the encoded amino acid(s), as outlined in the table below. In addition, for comparisons involving mixtures to be counted as concordant, the represented bases in the mixture must be compatible with the unmixed base or corresponding mixture (for example, R versus A or C versus Y but not G versus Y or R versus M).

If frameshift mutations are encountered in any test sequence, they will be handled as follows.

- Deletion (missing nucleotide): a dash is put into the test sequence, and the rest of the sequence is aligned against the consensus sequence as normal. When the alignment score is calculated, each dash is given a gap penalty of 10 (the alignment score reduced by 10) and flagged in a separate column on each report.
- Insertion (extra nucleotide): dashes are put in the sequence to which the participant sequence is being aligned (the consensus sequence), and a gap-opening penalty of 10 is given. This process is done separately and is not represented in sequence form on any of the worksheets, and only the scores are shown. This is also flagged in a separate column on each worksheet.

Scoring matrix for positions involving mixtures (1 = concordant, 0 = discordant)

		Consensus					
		Wild-type unmixed	Mutant unmixed	Mixed[a] (A, wild-type)	Mixed (A, mutant)	Mixed (B, wild-type + mutant)	Mixed (C, >1 mutant)
Test sequence	Wild-type unmixed	1	0	1	0	0	0
	Mutant unmixed	0	1	0	1	1	0
	Mixed* (A, wild-type)	1	0	1	0	0	0
	Mixed (A, mutant)	0	1	0	1	1	0
	Mixed (B, wild-type+ mutant)	0	1	0	1	1	0
	Mixed (C, >1 mutant)	0	0	0	0	0	1

[a] Mixture types:
A: the mixture results in codons that encode only one amino acid; discrepancies at the mixed base position are not counted.
Example: consensus = GTR (WT = GTA), test sequence = GTG; or consensus = ACG, test sequence = ACR; count both as concordant. However, if consensus = GAR, test sequence = GAM, count as discrepant.
B: the mixture results in the presence of two or more amino acids, one of which is the wild-type.
Example: consensus = AYT (WT = ATT), test sequence = ACT: count as concordant; however, if the test sequence = ATT, counted as discrepant.
C: the mixture results in two or more amino acids, none of which is the wild-type.
Example: consensus = TWC (WT = ACC), test sequence = WCC or TRC: count as concordant.

- Neither insertions nor deletions will affect the drug-resistance mutation site score (unless they occur in a drug-resistance mutation codon), but they would affect the alignment scores and the overall decision on the success or failure of the participant.

The acceptance criteria require >99% nucleotide concordance scores over the entire sequence and at the drug-resistance mutation sites. If polymerase chain reaction (PCR) contamination or specimen mix-up or mislabelling is detected (for example by sequence homology analysis between samples and consensus sequences), the submission will be considered a failure, and incident investigation and corrective action reporting will be required.

2. Internal quality control

Internal quality control measures include the use of positive and negative control reagents during sample extraction, amplification and sequencing. Although internal quality control is commonly intended for these procedures, some measures of good laboratory practice (regarding collection, handling, shipment and storage of samples) need to be stressed during the training of technical personnel and must be verified during the designation process and at subsequent site visits. Internal quality control also comprises stringent control of changes in assay procedures, including equivalency testing and potentially revalidation of an assay when procedures, equipment, reagents or facilities are changed.

2.1 Recommendations

- Internal quality control, consisting of positive and negative control materials, is necessary throughout the entire laboratory procedure, from the start of specimen extraction to the final sequencing.
  - A negative control, ideally in the same specimen matrix as the test specimens (that is, plasma or dried blood spots (DBS)), must be included in every batch. Additional negative controls such as blank reverse transcriptase or polymerase chain reaction (PCR) reactions can also be very helpful in identifying procedural steps in which molecular contaminants may be introduced. If a PCR product is generated from any negative control, all test samples in that batch must be considered invalid. An incident investigation should be conducted and appropriate corrective action taken to prevent recurrence of the contamination. Patient specimens should not be processed until the investigation and implementation of corrective actions has been completed.
  - One or more positive controls (such as low, medium and high viral load plasma or DBS) should be tested regularly to monitor assay performance over time. For PCR amplification, if a positive control (especially medium or high viral load) is negative, the possible causes should be investigated. PCR products from patient specimens in the same batch can be carried forward. Specimens for which PCR products were not obtained should be reprocessed after the assay performance has been confirmed to be optimal. If the sequence of a positive control does not match the expected sequence (such as more than 0.5% different), then a specimen labelling error is suspected and all test samples in that batch must be considered invalid.
  - Examples of positive control specimens:
    - plasma or DBS from HIV-positive patients, previously genotyped at least three times, with viral load in the range of 1000–5000 (low), 5000–50 000 (medium) and >50 000 (high) copies/ml;
    - HIV-1 virus from cell culture, diluted to a final viral load in the ranges above, in HIV-negative plasma or whole blood (for DBS);
  - *Note:* do not use undiluted cell culture supernatant virus as a positive control. The viral load of such supernatants is usually very high and is not as informative regarding assay performance as controls with lower viral load. Further, storing or handling such virus stocks represents a contamination risk in the laboratory.
- Laboratory results for specimens can be accepted only when the results of the internal controls meet the predetermined acceptance criteria.
- The quality of each sequence must be considered when accepting or rejecting a laboratory result. Annex 2 describes the sequence characteristics that should be evaluated and recommended acceptance criteria. Sequences not meeting

these criteria should receive additional review by the supervisor, since in rare circumstances these findings may be correct (such as end-stage infection with many quasi-species, rare HIV drug resistance mutations and coinfection with multiple strains).

- All patient sequences derived from a single survey should be checked against each other and common laboratory strains for potential contamination. WHO and the British Columbia Centres for Excellence developed an online program for HIV drug resistance sequencing quality control. This tool accepts sequences in FASTA format (for example, as generated by RECall) and performs a series of quality control checks, including genetic distance (https://recall.bccfe.ca/who_qc/) through pairwise sequence comparison to ensure that each patient sequence is sufficiently different from others in the survey and from reference viruses (such as positive control viruses) that may be in the laboratory. See Annex 2 for additional details.

ANNEX 2. WHO LABORATORY STANDARD OPERATING PROCEDURES FOR POST- TESTING QUALITY ASSURANCE OF HIV DRUG RESISTANCE GENOTYPING

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Introduction

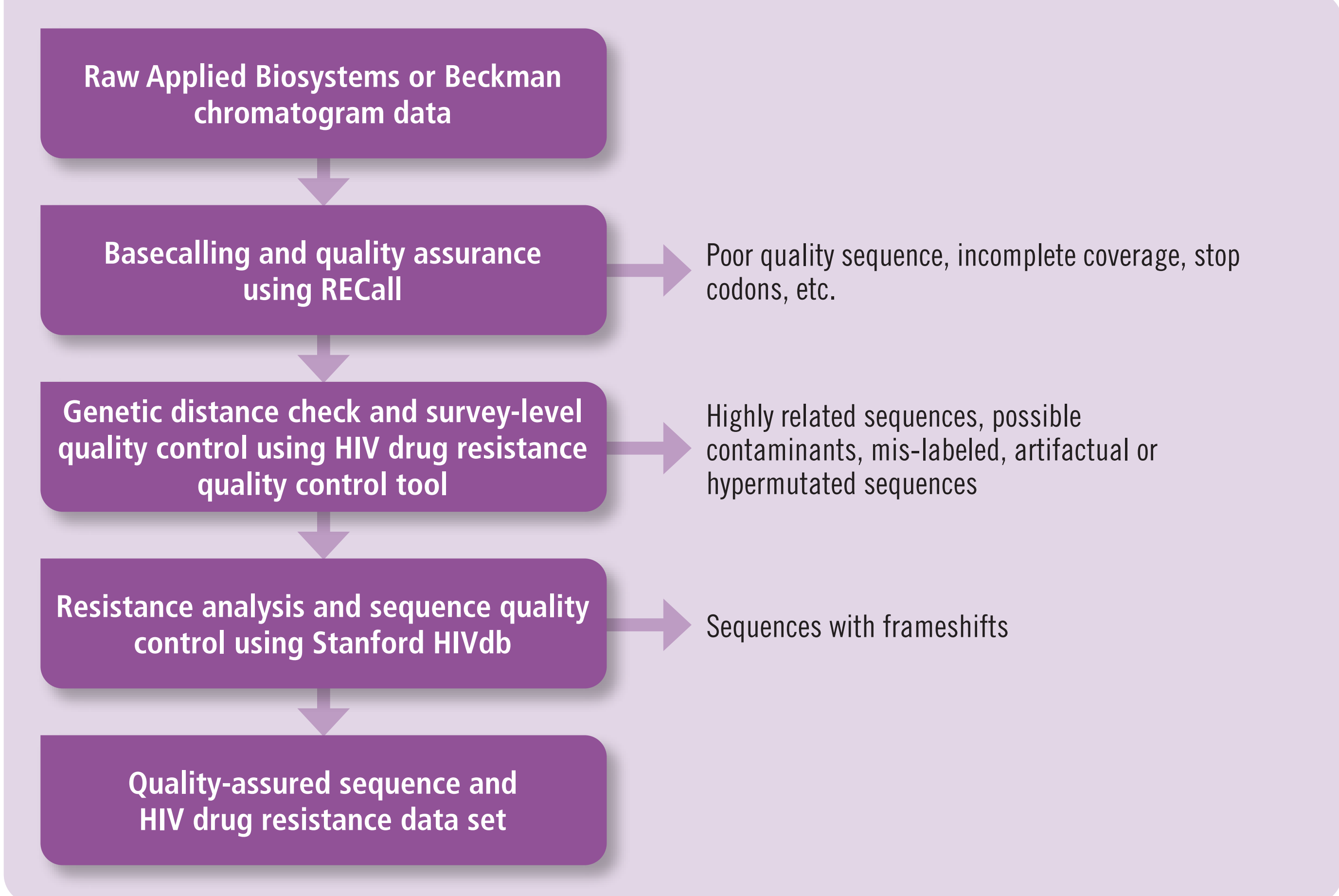
Quality assurance of HIV-1 sequence data generated by RNA extraction, reverse transcription–polymerase chain reaction (RT-PCR), sequencing and data analysis is an essential step towards producing high-quality, accurate and standardized surveillance information on drug resistance. This document is an update to the post-testing quality assurance standard operating procedures originally developed by WHO in 2014. These standard operating procedures aim to facilitate the incorporation of rigorous quality assurance procedures in all WHO-designated HIV drug resistance genotyping laboratories that are part of the WHO HIVResNet HIV Drug Resistance Laboratory Network.

Standardization of procedures is useful to ensure maximal comparability of results generated in laboratories all over the world. Although adoption of the procedures described here is not an absolute requirement for laboratories to become or remain members of the Laboratory Network, laboratories that do not or are unable to adopt them must demonstrate that alternative procedures will generate comparable results. Such laboratories should consult with WHO to determine the best method to use to demonstrate comparability.

The intended audience for this document is all Laboratory Network member laboratories, other public health laboratories considering applying for Laboratory Network membership and national HIV drug resistance working group members concerned with laboratory operations and data quality. The document contains three main sections: (A) HIV-1 genotyping sequence analysis using Web RECall, an online automated base calling program; (B) sequence quality assurance using the WHO/British Columbia Centre for Excellence in HIV/AIDS HIV drug resistance quality control tool; and (C) drug resistance analysis using the Stanford Calibrated Population Resistance (CPR) tool and HIVdb web program.

This summary provides an overview of required procedures for post-testing quality control and quality assurance of HIV drug resistance genotyping results for WHO-designated laboratories. These procedures rely on freely available tools accessible on the Internet. There is some overlap in the functionality of these tools, and WHO is in the process of evaluating the most appropriate way to synthesize the results from the different tools to generate final survey results. Fig. 1 shows the flow of recommended steps.

Fig.1. **Overview of the recommended sequence quality assurance process**



Quality control for HIV drug resistance genotyping includes checking of or the presence of stop codons, highly unusual ("atypical") mutations, out-of-frame insertions or deletions, missing sequences, excessive ambiguity, mixed bases and APOBEC mutations. Quality control checks are carried out using an online quality control tool at both the batch level while analysing sequences in RECall and the survey level after all sequences for a specific survey have been generated. Batch-level checks are important because they enable raw data to be inspected and corrected in real time when needed; earlier repeat amplification or sequencing reactions are also possible. Required survey-level checks help to identify expected and unexpected genetic relatedness between pairs of sequences that may not have been analysed in the same batch.

Sequence naming convention

Individuals enrolled in surveys must be assigned a survey identification (survey ID) number, or unique survey ID, by the country implementing the survey. The laboratory performing genotyping on these specimens should follow this naming convention. This number will identify the sequence generated by the genotyping assay and contains the following elements delimited by a dash character (–):

- country abbreviation: the International Organization for Standardization (ISO) standard three-letter abbreviation (https://www.iso.org/obp/ui/#search/code);
- survey type: TDR for a transmitted HIV drug resistance survey,[1] PDR for a pretreatment drug resistance survey, ADR12 or ADR48 for an acquired drug resistance survey (12-month or ≥48-month time point) and INF for surveys of infants younger than 18 months; other survey abbreviations may be needed in special cases; consult with WHO for the abbreviation to use;
- four-digit year survey started;

[1] WHO no longer recommends implementing surveys of transmitted HIV drug resistance; however, some countries are in the process of completing these surveys at the time this document is being published.

- site abbreviation (a three-letter abbreviation for the site, unique within the country; by default, the first three letters of the site name unless this is not unique or sufficiently descriptive); and
- four-digit unique numbers: a consecutive unique number assigned to a participant at that site.

Importantly, do not use the underscore character (_) in the survey ID.

Examples

- If the "University HIV Clinic" was a site that participated in a national acquired drug resistance survey in South Africa in 2014 (12-month time point), a participant's ID would look like this: ZAF-ADR12-2014-UNI-0001.
- If the "University HIV Clinic" was a site that participated in a national acquired drug resistance survey in South Africa in 2014 (≥48-month time point), a participant's ID would look like this: ZAF-ADR48-2014-UNI-0001.
- If the "Quality HIV Clinic" was a site that participated in a national pretreatment drug resistance survey in South Africa in 2014, a participant's ID would look like this: ZAF-PDR-2014-QUA-0001.

All sites providing specimens for the survey should use the identical survey ID format. Identical survey IDs to those used at the country level are essential when completing data reporting templates, which will be provided separately by WHO.

In some laboratories, established validated procedures may require assigning an accession number to track the specimen through the genotyping procedures. This may lead to the automatic naming of sequences using this accession number. In this case, the sequence files must be renamed according to the convention above using the automated quality control tool at the survey-level quality control step before reporting to the country or WHO.

HIV-1 genotyping sequence analysis using Web RECall

RECall is an automated base calling program developed at the British Columbia Centre for Excellence in HIV/AIDS.[1] RECall is available as a stand-alone program for Windows PCs and as an online web service. WHO recommends using the web version because it does not require the user to install and configure the software; however, it does require a reliable high-speed Internet connection. In addition to base calling, RECall includes several quality control checks, including checks for:

- raw chromatogram quality;
- single-stranded coverage;
- sequence length;
- stop codon;
- excessive mixtures;
- bad insertions;
- ambiguous nucleotide/amino acids (Xs);
- APOBEC mutations;
- atypical mutations; and
- genetic distance.

Laboratories in the Laboratory Network should use Web RECall if they are not already using the stand-alone version of RECall or another automated base-calling software that is known to generate comparable results. Some of the quality control checks performed by RECall at the level of each batch are repeated in subsequent steps using the nucleotide sequence data from the whole survey. If the laboratory cannot use the web version for any reason, it should use the latest version of the stand-alone software customized for WHO (as of March 2020, this is version 2.28); please contact WHO to ensure use of the most recent stand-alone version.

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[1] Woods CK, Brumme CJ, Liu TF, Chui CK, Chu AL, Wynhoven B et al. Automating HIV drug resistance genotyping with RECall, a freely accessible sequence analysis tool. J Clin Microbiol. 2012;50:1936–42.

Sequence quality assurance using the WHO/British Columbia Centre for Excellence in HIV/AIDS HIV drug resistance quality control tool

It is important to analyse nucleotide sequence data generated from all specimens from each survey together, along with as many contemporaneously and previously tested specimens as possible, for genetic relatedness. This analysis has several purposes:

- to detect laboratory contamination: inadvertent amplification of a laboratory strain of HIV or cross-contamination between specimens handled in the same batch or processed on the same equipment; and
- to identify potentially anomalous sequence relationships between pairs or groups of sequences from the same survey.

This analysis is carried out using an online quality control tool developed by WHO and the British Columbia Centre for Excellence in HIV/AIDS. The aspects of sequence data that are examined by the WHO/British Columbia Centre for Excellence in HIV/AIDS HIV drug resistance quality control tool include the following:

- concatenation of separately generated protease, reverse transcriptase and integrase sequences;
- survey ID format and sequence renaming, if necessary;
- genetic distance;
- atypical mutations;
- APOBEC mutations;
- stop codons; and
- laboratory strain similarity.

Drug resistance analysis using Stanford HIVdb

The final step in post-testing quality assurance for genotyping is interpretation and assessment for drug resistance using the automated tools available on the Stanford University HIVdb website. HIVdb should be used for all types of surveys, including cross-sectional surveys of HIV drug resistance among people initiating antiretroviral therapy (pretreatment drug resistance), cross-sectional surveys of acquired HIV drug resistance and surveys of HIV drug resistance among infants younger than 18 months.

HIVdb includes many of the quality control checks described above as well as subtyping (based on the closest genetic distance match to a set of reference sequences), a list of major resistance-associated mutations and a complete list of mutations versus consensus B reference, resistance scores and assessments for each drug, and a tabulation of the presence/absence of all resistance-associated mutations.

Transferring sequence data to WHO

Performing genotyping assays according to standardized methods and with uniform quality assurance and quality control criteria applied in the post-testing phase is the basis of accurate and reliable survey results on which public health and antiretroviral therapy programme actions may be based. In the spirit of standardizing quality assurance, submitting to WHO headquarters the data from surveys performed following WHO methods is recommended regardless of the source of funding for the surveys. Specifically, viral load and HIV drug resistance testing results should be submitted by the laboratory to both the national HIV drug resistance working group and WHO headquarters for quality assurance at the same time. This process will facilitate:

- confirmation that anomalous or suspicious sequences have been handled appropriately;
- the resolution of complex issues raised by anomalous data;
- the communication between designated laboratories, countries and the WHO region and headquarters to solve potential problems and build capacity at the country level; and
- the generation of a single final data set for survey analysis and subsequent reporting and publication.

Sequences submitted to WHO will not be included in any WHO publication without the permission of the country from whose survey they originated.

Specific procedures for transmitting sequence data and associated information are being developed. The essential components of the data package to be submitted to WHO are the quality-assured sequences in FASTA format; a summary report describing the sequence anomalies detected during analysis, if any, and how they were resolved; and the outputs from the Stanford resistance analysis.

A. HIV-1 genotyping sequence analysis using Web RECall

Background

Web RECall[1] is a Web-based system developed for analysing chromatogram data generated by Applied Biosystems™ Genetic Analyzers and other sequencers that produce a standard chromatogram file (*.scf). For background, see: https://webrecall.fandom.com/wiki/BCCFE_Web_RECall_Wiki.

Web RECall provides a means to review and edit HIV sequence data to determine drug resistance. It uses an algorithm designed to interpret chromatogram data and generate consensus sequences that may include nucleotide mixtures at various positions. Web RECall can provide sequence analysis of the HIV protease and reverse transcriptase region as well as other regions of HIV including integrase and envelope (gp41 and gp120).

Requirements

Computer (Windows or Macintosh operating system)

A Windows personal computer with a Pentium processor or the equivalent; or a Macintosh personal computer with an Intel processor or the equivalent.

Mozilla Firefox, version 3.5 or later; or Google Chrome, version 49 or later

Web RECall may not be compatible with other web browsers. Firefox can be obtained from: http://www.mozilla.org/en-US/firefox/new. Chrome can be obtained from: https://www.google.com/chrome.

High-speed Internet connection

If a reliable connection of sufficient speed is not available, please contact WHO for information regarding a stand-alone version of the software.

Web RECall account

Users in WHO laboratories must obtain an account from the WHO RECall administrator with a unique user name and password to use the software. The administrator is a virologist designated by WHO headquarters who determines the settings used for sequence evaluation and quality acceptance criteria that are applied to all users.

Procedure

Procedure limitations and requirements

- The accuracy of base calling highly depends on the quality of the raw chromatogram data. If the quality is poor, the sequence may be "failed" by the software. If this occurs, the data most likely are not of sufficient quality to ensure the accuracy of the result, and the sequencing and/or RT-PCR amplification may need to be repeated. Frequent failures resulting from poor-quality data likely indicate the need for improving the genotyping procedure; the extensive expertise of the laboratory network can be an important resource should this situation arise.
- A laboratory should include a positive control sample of known sequence with each batch of raw sequence data that are analysed. For example, an appropriate positive control would comprise raw chromatogram files for a clinical specimen that has been previously analysed multiple times so that a consensus sequence can be generated and used to compare to the sequence from each new test of the control.
- Raw chromatogram data files generated by automated sequencers with the file extension .abi or .scf need to be used.

[1] Woods CK, Brumme CJ, Liu TF, Chui CK, Chu AL, Wynhoven B et al. Automating HIV drug resistance genotyping with RECall, a freely accessible sequence analysis tool. J Clin Microbiol. 2012;50:1936–42.

Important: chromatogram files must be named according to WHO convention: survey ID_primerID_other (where "other" can be run date, operator name, etc.). The survey ID is the survey ID described in the overview (for example, ZAF-PDR-2014-CEN-0001); a chromatogram file generated using primer "A" on 10 March 2020 might be named "ZAF-PDR-2014-CEN-0001_A_20200310.ab1". If problems arise when processing data, check the naming convention. Note that the survey ID uses a dash as a delimiter, whereas the chromatogram file name for the web version of RECall uses the underscore character. Contact WHO for recommendations regarding programs that help automate file renaming (for example, replacing underscores with dashes or vice versa on a batch of .ab1 files).

1. User settings

   1.1. Open Firefox or Chrome and go to https://recall.bccfe.ca/.

   1.2. Log in using the user name and password provided by the WHO RECall administrator.

   1.3. Click on SETTINGS at the top right of the page. Reset password or change email address if desired. Select FASTA for both DOWNLOAD SEQUENCES AS and EMAIL SEQUENCES AS. Leave SEND TO unchecked. The results will be downloaded rather than emailed after the review is complete.

   1.4. Click on BACK at the top or bottom of the page to return to the main page.

2. Sequence submission

   2.1. On your computer, select the complete set of .ab1 or .scf sequence files for the samples that you want to analyse.

   2.2. Right click on the selection of files and select SEND TO from the menu, then select COMPRESSED (zipped) FOLDER to package the sequence chromatogram for submission. (In Macintosh OS, this is called COMPRESS N ITEMS, where N is the number of files selected).

      2.2.1. RECall also accepts .tar and .tar.gz compressed files.

   2.3. Open Firefox or Chrome and go to https://recall.bccfe.ca/.

   2.4. Log in with your user name and password.

   2.5. Click on the BROWSE button in the UPLOAD SAMPLE DATA section. Navigate to the folder containing the compressed archive with chromatogram data to analyse, select it and then click on OPEN.

Upload sample data

1) Please select your sample files:

Upload: Browse... No file selected. [Max: 30Mb]

We accept ABI and SCF files in a .zip, .tar or .tar.gz file

2.6. Choose the reference sequence to use for analysis in the CHOOSE A REFERENCE SEQUENCE section. The default reference sequence for United States Centers for Disease Control and Prevention (CDC) and WHO laboratories when analysing HIV protease and reverse transcriptase is CDC-WHO_HIVDR. This reference sequence spans protease amino acid 6 through reverse transcriptase amino acid 251. A separate reference sequence is available for integrase (IN_50-265 or IN_1-288).

2) Choose a reference sequence:

Sequence name: CDC-WHO_HIVDR ?

2.7. Click the PROCESS DATA button to send the files to the data server for processing.

2.7.1. The amount of time to complete submission and processing will vary depending on how many samples are in the package and the speed of the Internet connection. In locations with slow Internet speed, the data processing may continue for several hours. Do not leave the page or attempt to perform other operations on the RECall site while a batch is processing.

2.7.2. Once the processing is complete, the list of samples will appear on the right side of the window under the section titled PAST SAMPLES in a folder named by the submission date, reference sequence name and the number of samples in parentheses.

2.7.3. Rename the folder. To do so, select it and click the RENAME button below and name it as needed. A name including something informative about the survey is recommended (such as "ZAF-PDR-2014-CEN batch 1").

3. Evaluation of processed specimens

3.1. Click once on the batch folder to expand it and view all the submitted samples. Each sample is color-coded by RECall.

3.1.1. A green background indicates that RECall's sequence analysis was successful and passed quality control parameters and only minimal review by staff is necessary. Very little, if any, manual editing is required for these samples.

3.1.2. An orange background indicates that the sequence requires careful manual review at the key resistance mutations or other listed positions with possible errors because of lower confidence of the automated base calls. Some manual editing is likely to be required. Pay special attention to the list of errors found by manually inspecting each codon where an error is found.

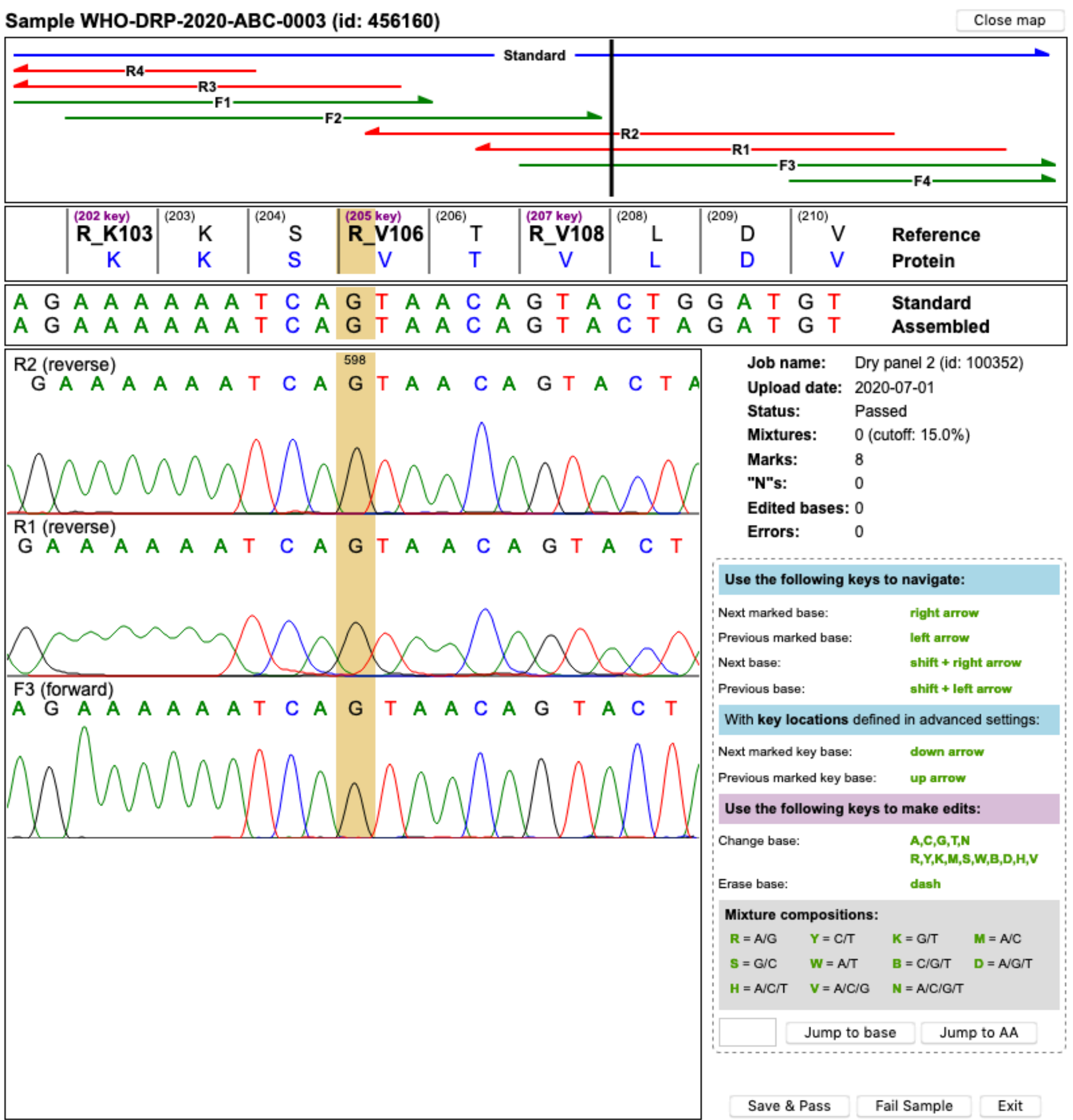
3.1.3. A red background indicates that the sequence analysis was unsuccessful because of one or more problems but might be rescued by manual review and/or repeat testing. Repeating sequencing and/or amplification reactions for these samples are often likely, possibly with alternative primers.

3.1.4. A black background indicates that sequencing failed. These sequences cannot be rescued and retesting is required, possibly with alternative primers.

Key resistance mutation sites in all sequences with orange or red backgrounds, and all positions called out in the list of errors, should be checked manually. The laboratory director or senior technical supervisor must approve any edits or changes made to the key resistance mutations.

The RECall administrator predetermines the quality control parameters relating to mixture detection thresholds, the allowable number of bases of single coverage and the list of key resistance-associated mutations.

3.2. To view a specific sample, double-click on the sample name or select it and click the “view” button. A new window will open with the edited, aligned consensus sequence, the standard reference sequence and the amino acid translation of the consensus sequence above the chromatograms (see example below). Codons are identified by the reference amino acid and position (protease 6–99 first, preceded by “P_”, then reverse transcriptase 1–251, preceded by “R_”) in black (and **bold** for key resistance positions), and the test sequence amino acid underneath in blue if it matches the reference or in red if it differs.



3.3. Click on OPEN MAP to view a map of the primers and how they were aligned and assembled to the reference (standard) sequence. While scrolling through the assembled sequence, the black bar will move through the map, indicating where the cursor (brown shading) is on the sequence.

3.4. For all samples marked in green, review the base calls at all key mutation (resistance) sites marked by RECall as requiring review by pressing the DOWN or UP arrow key. If no key mutation sites have been marked, pressing the DOWN key will move the screen to the last position (RT251).

3.5. For all samples marked in orange or red, review the base calling at all sites marked by RECall as requiring review by pressing the RIGHT or LEFT arrow key. In addition, review all positions listed under ERRORS (such as suspicious atypical amino acids, insertions and deletions, etc.).

3.5.1. For errors at a specific location, clicking on the text of the error will jump the chromatogram viewer to that position. Be sure to inspect all identified errors.

3.6. To scroll through the assembled sequence by a half window at a time to verify all the base calling, press the PAGE UP or PAGE DOWN key.

3.7. To navigate through the sequence one base at a time, hold down the SHIFT key and press the left or right arrow key.

3.8. To navigate to a particular nucleotide or amino acid position, enter a number in the box at the bottom right and click on JUMP TO BASE.

3.9. To change a base, select the base you want to change and input the correct base. To erase a base, input a dash (hyphen or minus sign). The changed base will then have a red line underneath it, indicating it was edited.

3.10. If the assembled sequence meets acceptance criteria, click SAVE & PASS to return to the sample selection menu. This will mark the sample as having passed quality assurance and sequence checks and that it is ready for export as a FASTA file. If the sample does not pass acceptance criteria, click FAIL SAMPLE. Failed samples will not be included in the set of exported sequences.

4. Export consensus sequence and job summary files

4.1. When all samples in the batch are edited, select the desired folder from the list of PAST SAMPLES and click the DOWNLOAD button. When prompted, choose SAVE and choose a location for the file. This will download a compressed (zipped) file containing:

- A FASTA file ("job_*jobnumber*.fas") containing the nucleotide sequences for all passed samples in one file. This file is used for the next step in the quality control process.
- An Excel file ("job_*jobnumber*_summary.xls") containing information about various quality indicators for all submitted samples:
  - job summary: pass/fail status, number of marks, mixtures, ambiguous bases (Ns), edits and a description of errors detected for each sample in the batch
  - quality: for each sample and primer, the average Phred quality score
  - similarity check: list of pairs of sequences with less than 0.5% difference
  - mutation list: amino acid substitutions and corresponding codon mutations for each sequence
  - mixture relative peak heights for each position where a mixture was detected above the threshold percentage.
- A csv file ("resistance_summary.csv") containing a summary of the drug susceptibility interpretations for all passed samples based on the Stanford HIVdb algorithm.
- A pdf file ("jobnumber_tree.pdf") with an image of a neighbour-joining tree. The tree includes all test sequences from the batch and any previously tested sample from previous batches that has less than 1.5% nucleotide differences compared with any sequence in the current batch. Batches are identified by job number, which is appended to the sample name (survey ID_job-12345).
- pdf file (survey ID.pdf) of Stanford HIVdb drug resistance interpretations for each passed sample.

4.2. If desired, rename the RECall output files with a name more informative about the project.

B. Sequence quality assurance using the WHO/British Columbia Centre for Excellence in HIV/AIDS HIV drug resistance quality control tool

Background

Molecular diagnostic assays that rely on PCR to amplify small amounts of target nucleic acids in clinical specimens are sensitive to possible molecular contamination between specimens or by laboratory contaminants. Quality assurance for such assays involves pre-testing steps (such as operator training and standard operating procedures), in-process controls (such as unidirectional workflow) and post-testing evaluation. For assays whose results involve generating a nucleic acid sequence, such as HIV drug resistance genotyping, carefully examining all sequences tested for relatedness in the same batch or over a recent period of time in the same laboratory, is a powerful approach to detect potentially inaccurate or otherwise anomalous results. Anomalous results may also be caused by errors in specimen collection or labelling. Sequences identified as anomalous using genetic distance or phylogenetic analysis must be flagged for investigation. Lack of a satisfactory explanation for anomalous results will result in excluding the sequences in question from the final study analysis.

WHO and the British Columbia Centre for Excellence in HIV/AIDS jointly developed an online program for quality control of HIV drug resistance sequencing. This tool accepts sequences in FASTA format (a format generated by RECall as described above) and performs a series of quality control checks.

- An alternative, although less customized tool, is the free open-source software program called MEGA[1] (Molecular Evolutionary Genetic Analysis). MEGA can be used for basic as well as more advanced phylogenetic analysis. Additional details on use of MEGA are available at http://www.megasoftware.net/manual.php. WHO will provide a procedure for using MEGA for assessing pairwise genetic distance upon request.

Ideally, each newly generated sequence is compared with other sequences generated in the laboratory; however, given the very large number of sequences, this is often impractical. WHO recommends a tiered system as follows:

1. At an absolute minimum, all sequences from patients included in the survey must be compared with each other.
2. Preferably, include sequences from unrelated specimens tested contemporaneously (in the same batches as survey specimens or in other batches tested on the same days).
3. Also include sequences from as many previously tested specimens as practically feasible.

Comparisons 2 and 3 above will require including sequences that are not part of the WHO survey being analysed.

Requirements

Computer (Windows or Macintosh operating system)

A Windows personal computer with a Pentium processor or the equivalent; or a Macintosh personal computer with an Intel processor or the equivalent.

Mozilla Firefox version 3.5 or later; or Google Chrome version 49 or later

The quality control tool has not been extensively tested with other Internet browsers. Firefox can be obtained from: http://www.mozilla.org/en-US/firefox/new. Chrome can be obtained from: https://www.google.com/chrome.

Internet connection

A high-speed Internet connection is recommended.

[1] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731-2739.

Test sequences in FASTA format

Nucleic acid sequences are often stored in FASTA format. FASTA format is simply a text file with the name of the sequence preceded by a ">" character (the "header"), followed by the sequence on a new line or lines. All characters before the next ">" character are considered part of the preceding sequence. For example:

```
>ZAF-PDR-2014-CEN-0001
ACGTACGTACGT…
>ZAF-PDR-2014-CEN-0002
ACATACCTACGT…
```

A hard return must follow the sequence name and the last nucleotide of the sequence. The text file must have the file extension .fas or .fasta. This can be added when saving the file in the SAVE AS… dialog box or by manually renaming the file, for example by changing a .txt file extension to .fas. File extensions must be explicitly visible to facilitate manual editing of file extensions. For more information about file extensions, see https://support.microsoft.com/en-us/help/4479981/windows-10-common-file-name-extensions or https://support.apple.com/guide/mac-help/show-or-hide-filename-extensions-on-mac-mchlp2304/mac.

Many sequence analysis software tools have options to export nucleotide sequences in FASTA format (including RECall). In most cases, little to no further manipulation is required, especially if a correct file-naming convention has been followed.

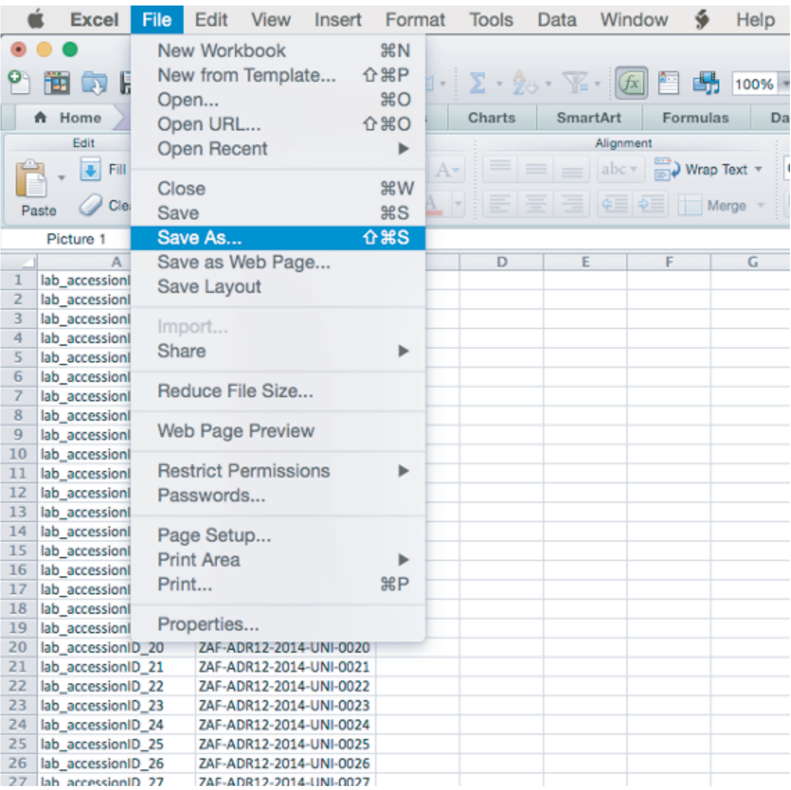
Sequences from protease, reverse transcriptase and/or integrase can be uploaded separately if desired. The quality control tool will concatenate and align sequences with the same FASTA sequence name. Currently, the FASTA sequence name must be identical in the separate sequences for this to take place.

Procedure

1. If the required sequence-naming convention has been followed, skip step 1 and proceed to step 2. If the required sequence-naming convention has not been followed for any reason, the sequence names will be changed automatically by providing a list of the existing and new names in tabular format and saved as a comma-separated values (.csv) file.

    1.1. Using Microsoft Excel, prepare a table that lists the existing sequence IDs in the first column and the corresponding new ID in the second column. Column headers are not required.

    1.2. Click on the File menu and choose SAVE AS…
    This will appear different in various versions of Excel. The example below is for Excel 2011 on Mac OS.



   1.3. In the dialog box that appears, navigate to the same directory (folder) as the one in which the sequence files are saved, choose comma-separated values (csv) as the file type (format), name the file, and click SAVE.

2. The standard list of laboratory strains is displayed at the bottom of the page, and full sequences are available in Appendix 1. If other cloned HIV strains are frequently used in the laboratory where genotyping is performed, add the sequence(s) corresponding to protease amino acid 6 to reverse transcriptase amino acid 251 and/or integrase amino acid 50 to 265 to the sequences being analysed.

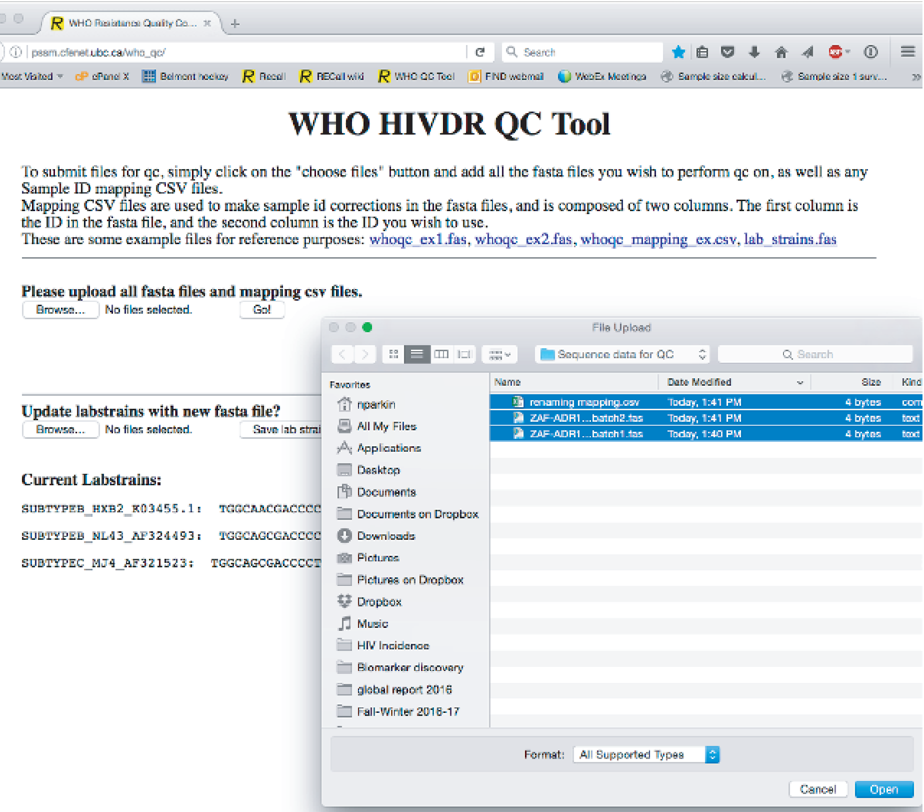
3. Upload the sequences to be analysed for potential quality control problems

   3.1. Open Firefox or Chrome and go to https://recall.bccfe.ca/who_qc/

   3.2. Under the PLEASE UPLOAD ALL FASTA FILES AND MAPPING CSV FILES header, click on BROWSE… and navigate to the folder containing the sequences in FASTA format.

   3.3. Select the file(s) to analyse. To analyse multiple files together (such as one for each batch analysed in RECall but that all correspond to the same survey and to other samples processed during the same time period), hold down the control (CNTRL in Windows) or command key (CMD in Mac OS) while clicking on multiple files.

      3.3.1. If the sequences have not been named according to the recommended format, at this point also select the name-mapping file generated in csv format in step 1.



   3.4. Click OPEN. The name of a single selected file, or the number of multiple files selected, will appear next to the BROWSE button.

   3.5. Optional: if you are analysing sequences that are not part of a WHO survey and have not been named according to the WHO sample ID format, click the SKIP SAMPLE ID CHECK checkbox.

   3.6. Click GO! to launch the analysis. A web page will appear containing a hyperlink to the results, with text similar to: "Your job is currently being processed; save this link to check the status: https://recall.bccfe.ca:80/who_qc/upload_results/1483561347"

   3.7. Click on the results hyperlink or refresh the page to view the results.

The amount of time required to complete the analysis depends on the number of sequences submitted. Several hundred sequences may take several minutes.

4. Review the output in your Web browser and download the results as a csv file by clicking on DOWNLOAD RESULTS. Review each section in the report and use the checklist (Appendix 2) to record the completeness of the review.
    - 4.1. Processing errors: list of errors encountered by the program.
    - 4.2. Warnings and notes: information about possible problems with alignment, missing sequence regions and sequences with excessive numbers of APOBEC or atypical mutations.
    - 4.3. Sequence IDs: a list of sequence IDs that do not conform to the required WHO format. Change the sequence IDs in the FASTA file and record the details of each change in a table listing the old and new IDs.
    - 4.4. Stop codons: a list of sequences containing stop codons as mixtures (such as W212*/W) or unmixed (W212*). Sequences with stop codons, especially unmixed, should be flagged for investigation. Sequences with unmixed stop codons that are confirmed not to be base calling errors should be repeated from RT-PCR if possible or excluded from the survey if not.
    - 4.5. APOBEC mutations: a list of sequences containing one or more APOBEC mutations, indicating whether these mutations are at a drug resistance-associated site ("DR site") or not ("non-DR site"). Each APOBEC mutation is listed on its own row; counting the number of rows with the same sequence ID yields the total number per sequence. A sequence with one or more APOBEC mutations at a drug resistance site and a total of four or more APOBEC mutations should be flagged for investigation. A list of APOBEC mutations at drug-resistance mutation sites is available at: https://hivdb.stanford.edu/page/release-notes/#data.files.
    - 4.6. Atypical mutations: a list of sequences containing one or more highly unusual, or atypical, mutations. Each atypical mutation is listed on its own row. As with APOBEC mutations, counting the number of rows with the same sequence ID yields the total number per sequence. A sequence with three or more atypical mutations in protease ("Amino location" begins with "P_"), or five or more in reverse transcriptase ("Amino location" begins with "R_"), should be flagged for investigation.
    - 4.7. Samples <0.5% genetic distance (from different patients): a list of pairs of sequences not expected to be highly related to each other based on their sequence IDs but with <0.5% differences. All such pairs of sequences should be flagged for investigation.
    - 4.8. Samples <0.5% genetic distance (from laboratory strains): a list of sequences that are suspiciously highly related (<0.5% differences) compared with one or more laboratory strains. All such sequences should be flagged for investigation.
5. Follow-up actions. WHO recommends the following steps for all potential anomalies identified in step 4.
    - 5.1. Review the raw chromatogram data (such as by using RECall) at the positions listed as having stop codons, APOBEC mutations or atypical mutations to confirm that the anomaly is not a result of a base calling error or poor-quality data.
        - 5.1.1. If edits to the base calling are made, re-export the FASTA file and repeat steps 3 and 4 above.
    - 5.2. If specimen quantities are sufficient, repeat the genotype test starting from RNA extraction. All members of anomalous pairs or clusters should be repeated if possible.
        - 5.2.1. If specimen quantity does not permit a repeat genotype to be performed, contact the HIV drug resistance working group from the country where the survey was conducted to determine whether additional specimens are stored elsewhere and can be sent for amplification and sequencing.
    - 5.3. Confer with the HIV drug resistance working group from the country where the survey was conducted to obtain as much epidemiological information as possible (such as clinic site, specimen collection date or relationships between the survey participants in question such as known transmission pair, couple, etc.).
    - 5.4. Consult HIVResNet virologists to explore possible explanations.
    - 5.5. If no reasonable explanation for the anomalous sequence results arises from this investigation, consult with HIVResNet virologists designated by WHO headquarters to determine whether to remove the suspicious sequences.

C. Sequence drug resistance analysis using Stanford HIVdb

Background

The translation of nucleotide sequence data into useable information related to drug resistance is a multi-step process. It involves converting the data into a predicted amino acid sequence, comparing it to a standard reference (drug-sensitive) sequence, evaluating the combination of known resistance-associated mutations detected using one of several different interpretation systems and generating a report indicating the predicted susceptibility to each antiretroviral drug. One such interpretation system is implemented and maintained at Stanford University as a freely accessible website (http://hivdb.stanford.edu). This website allows users to analyse sequence data for several purposes related to drug resistance. WHO selected this resource as a standardized way to interpret sequence data for public health surveillance of HIV drug resistance.

The HIVdb resistance analysis program provides drug resistance and susceptibility assessment using a mutation scoring system and five levels of predicted susceptibility. The HIVdb program is used for cross-sectional surveys of drug resistance before (pretreatment surveys) and after treatment (acquired drug resistance surveys) and in surveys of HIV drug resistance among children younger than 18 months. The program includes an assessment of sequence quality and shares many features with RECall and the British Columbia Centre for Excellence in HIV/AIDS HIV drug resistance quality control tool.

Requirements

Computer (Windows or Macintosh operating system) with Internet connection

Nucleotide sequence file

All sequences must be in one text file in FASTA format. The file should not contain more than 500 sequences.

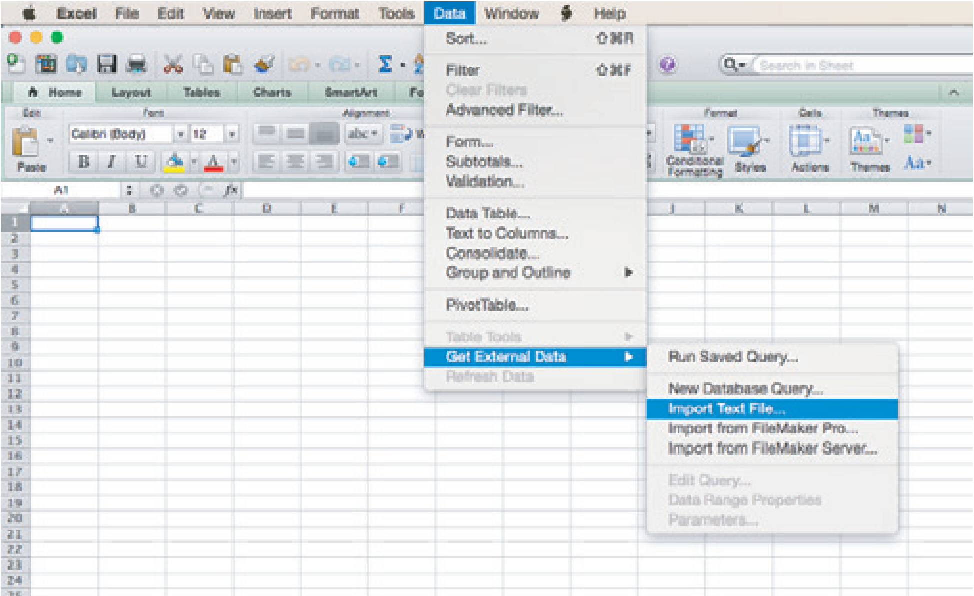
Microsoft Excel

Office 2007 version or later preferred (.xlsx). Older versions of Excel may not be able to open the tables of results from large sequence sets.

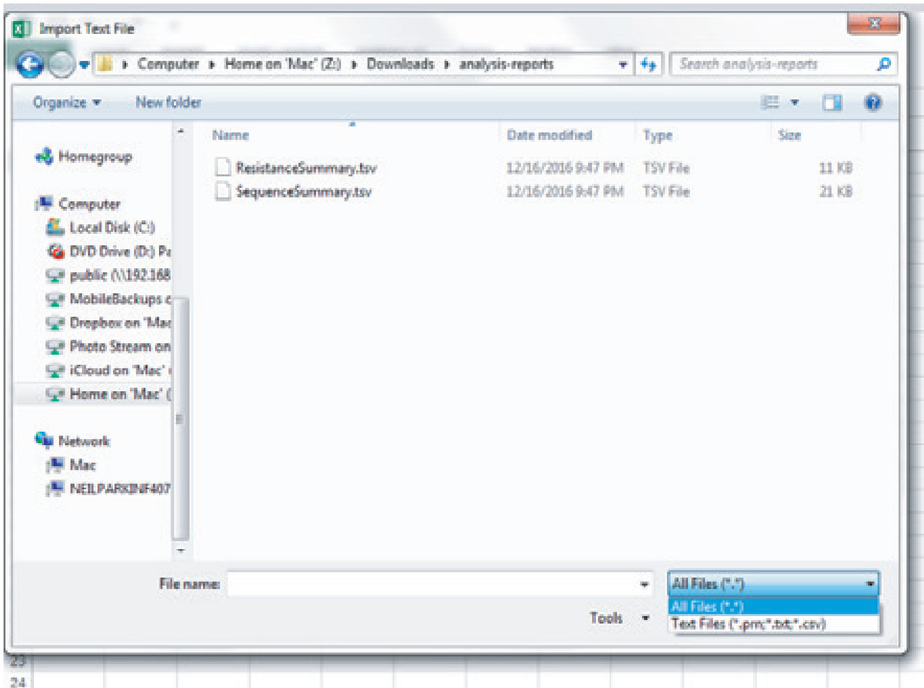
Procedure

1. Open an Internet browser and go to: https://hivdb.stanford.edu/hivdb/by-sequences.
2. Click the CHOOSE FILE button under INPUT SEQUENCES and navigate to the file containing the sequences to be analysed.
3. Select the SPREADSHEETS (TSV) option under OUTPUT OPTIONS. Output files are tab-separated values (.tsv) format.
4. Next to SELECT OUTPUTS, select SEQUENCE QUALITY SUMMARIES and DRUG-RESISTANCE SUMMARIES (other outputs may be used as well but are optional).
5. Click on ANALYZE.
6. Once the analysis is complete, the results will download as a .zip file in the location set for downloads in the browser's preferences. It may be necessary to disable pop-up windows to complete the download. The zip file will have a name similar to "analysis-reports.1477959654164.zip". Open (unzip or extract) the "analysis-reports" zip file (the exact steps may differ between operating systems and software configurations).
7. Import the results into a Microsoft Excel file. Multiple methods can accomplish this. The method outlined here is expected to be the least subject to differences between platforms and individual computer installations.
    7.1. Open a new Excel file.

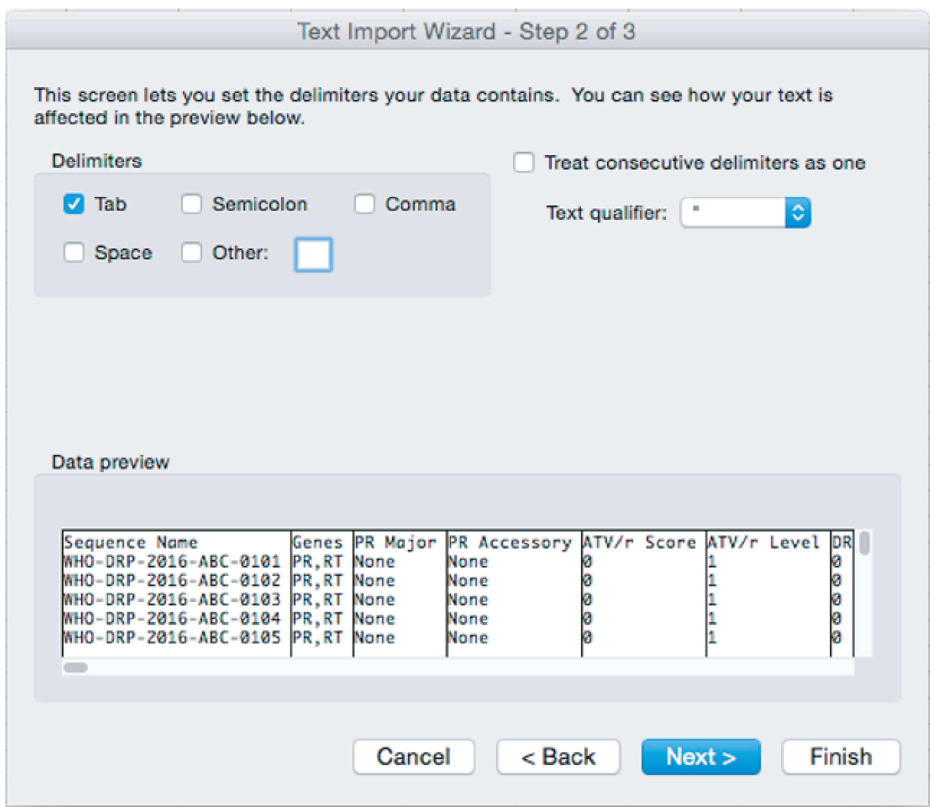
7.2. For Macintosh: from the DATA menu, choose GET EXTERNAL DATA and IMPORT TEXT FILE…. From the dialogue box that opens, choose ALL FILES from the ENABLE drop-down menu and choose the SequenceSummary.tsv file.



For PC: From the DATA ribbon, choose FROM TEXT. In the dialogue box that opens, choose ALL FILES from the drop-down menu at the bottom left and then select the SequenceSummary.tsv file.



7.3. In the Text Import Wizard window, choose DELIMITED, then choose TAB as the delimiter. Rename the tab SEQUENCESUMMARY instead of SHEET1.



7.4. Create a new sheet in the Excel file and repeat steps 7.7.2 and 7.7.3 for the ResistanceSummary.tsv file. Rename the tab RESISTANCESUMMARY instead of SHEET2.

7.5. Save the Excel file using a name that includes the survey code (such as ZAF-acquired drug resistance-2014 CEN).

8. Examine the SequenceSummary output carefully. Any sequence with a result that meets one or more of the criteria in the table below should be flagged for review or repeat testing. Column headers in the spreadsheet that are not in the table below can be ignored.

Column header	Criterion for flagging
PR Start	>10
PR End	<93
RT Start	>41
RT End	<238
IN Start	>51
IN End	<263
Num Frame Shifts	>0
Num Insertions	>0
Num Deletions	>0
Num Stop Codons	>0 (if unmixed)
Num APOBEC Mutations	> 0 at a drug resistance site and ≥4 or more total in protease, reverse transcriptase or integrase[a]
Num Unusual Mutations	≥ 3 in protease, ≥ 5 in reverse transcriptase, ≥ 4 in integrase

[a] The total number of APOBEC mutations listed in the Stanford output may not match the number found in the British Columbia Centre for Excellence in HIV/AIDS HIV drug resistance quality control tool if there are mutations at drug resistance sites that could also be APOBEC mutations. In this case, use the number reported by the British Columbia Centre for Excellence in HIV/AIDS HIV drug resistance quality control tool.

Appendix 1. Reference sequences

The reference sequences below correspond to commonly used laboratory strains HXB2 and NL4-3 (for subtype B) and MJ-4 (for subtype C). These should be used when checking for PCR contamination with the British Columbia Centre for Excellence in HIV/AIDS HIV drug resistance quality control tool. Additional reference sequences for other subtypes and CRFs are available in the LANL repository (http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html#ref and http://www.hiv.lanl.gov/content/sequence/HIV/REVIEWS/RefSeqs2005/RefSeqs05.html).

All reference sequences should be trimmed to span protease amino acids 6–99, reverse transcriptase 1–251 and integrase 1–288.

Protease (6–99) and reverse transcriptase (1–251):

```
>SUBTYPE_B_HXB2_PRRT_K03455.1
TGGCAACGACCCCTCGTCACAATAAAGATAGGGGGGCAACTAAAGGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGA
AGAAATGAGTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAAGACAGTATGATCAGATAC
TCATAGAAATCTGTGGACATAAAGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGGAAGAAATCTGTTGACT
CAGATTGGTTGCACTTTAAATTTTCCCATTAGCCCTATTGAGACTGTACCAGTAAAATTAAAGCCAGGAATGGATGGCCCAAAAGT
TAAACAATGGCCATTGACAGAAGAAAAAATAAAAGCATTAGTAGAAATTTGTACAGAGATGGAAAAGGAAGGGAAAATTTCAAAAA
TTGGGCCTGAAAATCCATACAATACTCCAGTATTTGCCATAAAGAAAAAAGACAGTACTAAATGGAGAAAATTAGTAGATTTCAGA
GAACTTAATAAGAGAACTCAAGACTTCTGGGAAGTTCAATTAGGAATACCACATCCCGCAGGGTTAAAAAAGAAAAAATCAGTAAC
AGTACTGGATGTGGGTGATGCATATTTTTCAGTTCCCTTAGATGAAGACTTCAGGAAGTATACTGCATTTACCATACCTAGTATAA
ACAATGAGACACCAGGGATTAGATATCAGTACAATGTGCTTCCACAGGGATGGAAAGGATCACCAGCAATATTCCAAAGTAGCATG
ACAAAAATCTTAGAGCCTTTTAGAAAACAAAATCCAGACATAGTTATCTATCAATACATGGATGATTTGTATGTAGGATCTGACTT
AGAAATAGGGCAGCATAGAACAAAAATAGAGGAGCTGAGACAACATCTGTTGAGGTGGGGACTTACCACACCAGACAAAAAACATC
AGAAAGAACCTCCATTCCTTTGGATGGGTTATGAACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGCTGCCAGAAAAAGAC
AGC
>SUBTYPE_B_NL43_PRRT_AF324493
TGGCAGCGACCCCTCGTCACAATAAAGATAGGGGGGCAATTAAAGGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGA
AGAAATGAATTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAAGACAGTATGATCAGATAC
TCATAGAAATCTGCGGACATAAAGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGGAAGAAATCTGTTGACT
CAGATTGGCTGCACTTTAAATTTTCCCATTAGTCCTATTGAGACTGTACCAGTAAAATTAAAGCCAGGAATGGATGGCCCAAAAGT
TAAACAATGGCCATTGACAGAAGAAAAAATAAAAGCATTAGTAGAAATTTGTACAGAAATGGAAAAGGAAGGAAAAATTTCAAAAA
TTGGGCCTGAAAATCCATACAATACTCCAGTATTTGCCATAAAGAAAAAAGACAGTACTAAATGGAGAAAATTAGTAGATTTCAGA
GAACTTAATAAGAGAACTCAAGATTTCTGGGAAGTTCAATTAGGAATACCACATCCTGCAGGGTTAAAACAGAAAAAATCAGTAAC
AGTACTGGATGTGGGCGATGCATATTTTTCAGTTCCCTTAGATAAAGACTTCAGGAAGTATACTGCATTTACCATACCTAGTATAA
ACAATGAGACACCAGGGATTAGATATCAGTACAATGTGCTTCCACAGGGATGGAAAGGATCACCAGCAATATTCCAGTGTAGCATG
ACAAAAATCTTAGAGCCTTTTAGAAAACAAAATCCAGACATAGTCATCTATCAATACATGGATGATTTGTATGTAGGATCTGACTT
AGAAATAGGGCAGCATAGAACAAAAATAGAGGAACTGAGACAACATCTGTTGAGGTGGGGATTTACCACACCAGACAAAAAACATC
AGAAAGAACCTCCATTCCTTTGGATGGGTTATGAACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGCTGCCAGAAAAGGAC
AGC
>SUBTYPE_C_MJ4_PRRT_AF321523
TGGCAGCGACCCCTTGTCTCAATAAAAGTAGGGGGCCAAATAAAGGAGGCTCTTTTAGACACAGGAGCAGATGATACAGTATTAGA
AGAAATGAGTTTGTCAGGAAAATGGAAACCAAAAATGATAGGAGGAATTGGAGGTTTTATTAAAGTAAGACAGTATGATCAAATAC
CTATAGAAATTTGTGGAAAAAAGGCTATAGGTACAGTGTTAATAGGACCTACTCCTGTCAACATAATTGGAAGAAATATGTTGACT
CAGCTTGGCTGCACTCTAAATTTTCCAATCAGTCCTATTGAAACTGTACCAGTAAAATTAAAGCCAGGCATGGATGGCCCTAAGGT
TAAACAATGGCCATTGACAGAAGAAAAAATAAAAGCATTAACAGAAATTTGTGCAGAAATGGAAAAGGAAGGAAAAATTACAAAAA
TTGGGCCTGAAAATCCATATAATACTCCAGTATTTGCAATAAAAAAGAAAGACAGTACTAAGTGGAGAAAATTAGTAGACTTCAGG
GAACTTAATAAAAGAACTCAAGACTTTTGGGAGGTTCAATTAGGAATACCGCACCCAGCAGGGTTAAAAAAGAAAAAATCAGTAAC
AGTATTAGATGTGGGGGATGCATATTTTTCAGTTCCTTTAGATGAAGGCTTCAGGAAATACACTGCATTCACCATACCTAGTATAA
ACAATGAAACACCAGGGATTACATATCAATATAATGTGCTTCCACAGGGATGGAAAGGATCACCAGCAATATTCCAGAGTAGCATG
ACAAGAATCTTAGAGCCCTTTAGGACACAAAATCCAGAAATAGTCATCTATCAATATATGGATGATTTGTATGTAGGATCTGATTT
AGAAATAGGGCAACATAGAGCAAAAATAGAGGAATTAAGAAACCATCTATTAAAGTGGGGATTTACCACACCAGACAAGAAACATC
AGAAAGAACCCCCATTTCTTTGGATGGGGTATGAACTCCATCCTGATAAATGGACAGTACAGCCTATACAGCTGCCAACAAAGGAT
AGC
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```
>SUBTYPE_B_HIVMN_PRRT_M17449
TGGCAACGACCCATTGTCACAATAAAGATAGGGGGGCAACTAAAGGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGG
AGAAATGAATTTGCCAAGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAAGACAGTATGATCAGATAA
CCATAGGAATCTGTGGACATAAAGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGGAAGAAATCTGTTGACT
CAGCTTGGGTGCACTTTAAATTTTCCCATTAGTCCTATTGAAACTGTACCAGTAAAATTAAAGCCAGGAATGGATGGCCCAAAAGT
TAAACAATGGCCATTGACAGAAGAAAAAATAAAAGCATTAATAGAAATTTGTACAGAAATGGAAAAGGAAGGAAAAATTTCAAAAA
TTGGGCCTGAAAATCCATACAATACTCCAGTATTTGCCATAAAGAAAAAAGACAGTACTAAATGGAGAAAATTAGTAGATTTCAGA
GAACTTAATAAGAAAACTCAAGACTTCTGGGAAGTTCAATTAGGAATACCACATCCTGCAGGGTTAAAAAAGAAAAAATCAGTAAC
AGTACTGGATGTGGGTGATGCATATTTTTCAGTTCCCTTAGATAAAGACTTCAGGAAGTATACTGCATTTACCATACCTAGTATAA
ACAATGAAACACCAGGGATTAGATATCAGTACAATGTGCTTCCACAGGGATGGAAAGGATCACCAGCAATATTCCAAAGTAGCATG
ACAAAAATCTTAGAGCCTTTTAGAAAACAAAATCCAGACATAGTTATCTATCAATACATGGATGATTTGTATGTAGGATCTGACTT
AGAAATAGGGCAGCATAGAGCAAAAATAGAGGAACTGAGACGACATCTGTTGAGGTGGGGATTTACCACACCAGACAAAAAACATC
AGAAAGAACCTCCATTCCTTTGGATGGGTTATGAACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGCTACCAGAAAAAGAC
AGC
>SUBTYPE_B_BRU-LAV-1_PRRT_K02013.1
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GGAT
```

Appendix 2. Quality control checklist

Use this checklist to confirm that each category of potential quality control problems has been checked for each batch of sequences (corresponding to all sequences from a survey).

Batch information and identification (note the batch ID, survey ID, date, operator, etc. here.)

- ☐ **Sequence IDs:** a list of sequence IDs that do not conform to the required WHO format. Change the sequence IDs in the FASTA file and record the details of each change in a table listing the old and new IDs.
- ☐ **Stop codons:** a list of sequences containing stop codons as mixtures (such as W212*/W) or unmixed (W212*). Sequences with stop codons, especially unmixed, should be flagged for investigation. Sequences with unmixed stop codons that are confirmed not to be base calling errors should be repeated from RT-PCR if possible or excluded from the survey if not.
- ☐ **APOBEC mutations:** a list of sequences containing one or more APOBEC mutations. indicating whether these mutations are at a drug resistance–associated site (DR site) or not (non-DR site). Each APOBEC mutation is listed on its own row; counting the number of rows with the same sequence ID yields the total number per sequence. A sequence with one or more APOBEC mutations at a drug resistance site and a total of four or more APOBEC mutations should be flagged for investigation.
- ☐ **Atypical mutations:** a list of sequences containing one or more highly unusual, or atypical, mutations. Each atypical mutation is listed on its own row. As with APOBEC mutations, counting the number of rows with the same sequence ID yields the total number per sequence. A sequence with three or more atypical mutations in protease ("Amino location" begins with "PR_"), five or more in reverse transcriptase ("Amino location" begins with "RT_"), or four or more in integrase ("Amino location" begins with "IN_"), should be flagged for investigation.
- ☐ **Samples <0.5% genetic distance (from different patients):** a list of pairs of sequences not expected to be highly related to each other based on their sequence IDs but with <0.5% difference. All such pairs of sequences should be flagged for investigation.
- ☐ **Samples <0.5% genetic distance (from lab strains):** A list of sequences that are suspiciously highly related (<0.5% differences) compared to one or more lab strains. All such sequences should be flagged for investigation.

ANNEX 3. RECOMMENDED METHODS FOR VALIDATING AN IN-HOUSE GENOTYPING ASSAY FOR SURVEILLANCE OF HIV DRUG RESISTANCE

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1. Introduction

The development of in-house genotyping assays in a given laboratory is often carried out independently, requiring investing considerable effort and resources to optimize the procedures. Performance characteristics are variably determined by regulatory agencies, market competitive forces, limitations inherent in the technology chosen and the demands of the intended clinical applications. However, when studies and surveys are performed on a national, regional or even global scale using in-house assays, it is important to have confidence that the results from different participating laboratories are all of high quality and are comparable to each other. One solution to this problem would be to require all laboratories to use the same method, but this is not practical given local differences in reagent and equipment supply, HIV subtype variability, personnel training and the pre-existence of already established and validated procedures controlled by local regulatory authorities. An alternative approach is to establish a validation framework and common performance characteristic targets that will provide maximum confidence in the comparability of results generated while allowing for variation in the methods used.

WHO Laboratory Network laboratories that use in-house assays have been designated based on assays in use for at least one year with plasma as the specimen type. The various laboratories have generated varying quantities of assay validation data, depending on the accepted standards of the investigator, institution or relevant regulatory body. Many laboratories are now in the process of adapting their assays to support testing using dried blood spots (DBS) in addition to plasma, expanding the region sequenced to include integrase and moving to next-generation sequencing platforms. These variations of the existing method should be extensively characterized in a consistent manner before being used to generate data for HIV drug resistance surveillance purposes.

Laboratories seeking designation as a laboratory in the HIVResNet Laboratory Network for HIV drug resistance genotyping with DBS, or including integrase, or using a new method such as next-generation sequencing, must validate the procedure in their laboratory before testing any specimens for WHO surveys. The requirement for assay validation is separate from (but complementary to) the annual proficiency panel testing as part of an external quality assurance programme and an internal quality assurance and quality control programme that includes evaluation of new equipment and new lots of critical reagents before being released for use in testing real specimens, to ensure consistency of assay performance.

The purpose of this publication is to outline how assay validation should be structured and to describe the parameters to be used when validating an in house-genotyping assay. Importantly, these recommendations have been developed with national-scale, population surveillance applications in mind and may not be applicable when the intended use is to guide individual patient treatment decisions. In general, the scale and stringency of assay validation is considerably higher for the individual patient management application.

2. Assay validation, verification and equivalency testing

The scale of experiments (breadth of variables tested and number of specimens included) required to characterize assay performance depends on several factors. In general, a new laboratory-developed assay requires very extensive characterization (full-scale validation) to help to define its performance, while the performance of a kit purchased from a manufacturer that uses a rigorous quality management system for its production (for example, ISO 13485:2016 or equivalent) requires a smaller-scale method verification intended to confirm that the manufacturer's specifications for test performance can be reproduced when the procedure provided by the manufacturer is followed precisely. Once the assay is validated, if the procedure or reagents used for a laboratory-developed assay are changed, confirmation that the performance has not been negatively affected must be demonstrated through either equivalency testing, if the change is relatively minor, or by revalidation for major changes (Table 1).

Table 1. Validation, verification and equivalency testing

New method or change to existing method?	Manufactured kit[a] or laboratory-developed method?	Type of validation
New	Laboratory-developed	**Full-scale validation**
New	Manufactured kit	**Method verification**
Change (minor)	Laboratory-developed	**Equivalency testing**
Change (major)	Laboratory-developed	**Full-scale validation**

[a] Manufactured for RUO, or FDA/CE-approved; changes to a kit procedure makes it a laboratory-developed test.

Examples

- Use of the ABI (ThermoFisher/CDC) kit is considered a laboratory-developed procedure, since it does not include the RNA extraction step.
- The ViroSeq™ kit for integrase (RUO) is considered a manufactured kit, since it includes all reagents and methods for the entire process and has been manufactured under rigorous controls.
- Adapting the ViroSeq™ kit for protease and reverse transcriptase for use with DBS would be considered a laboratory-developed procedure since DBS is a new specimen type.
- Adapting a laboratory-developed test from one laboratory to another requires full-scale validation, even if such validation was carried out in the first laboratory.

For each aspect being tested (see below), experiments must be carefully planned and acceptance criteria must be predefined. Acceptance criteria may be set at a level considered the minimum acceptable for the intended application; if the results exceed the criteria, these parameters can be used in a descriptive fashion to characterize the assay.

A written validation protocol and standard operating procedures with detailed step-by-step instructions should be finalized before experiments are started and certainly before the results are analysed. A complete validation plan comprises the following elements:

- description of need, intended use and context;
- outline of the genotyping procedure, referring to standard operating procedures for the details; and
- for each section of the validation:
  - a description of the specimens to be used;
  - a detailed procedure for carrying out the tests – for example, number of replicates, organization of the testing with respect to batch, different days of testing, etc.;
  - analysis plan; and
  - acceptance criteria.

No changes to the validation protocol or experimental procedures are allowed while the validation experiments are in progress. The validation should be performed under the same conditions (facility, equipment, reagents, standard operating procedures and personnel) that will be used for real specimens. The policies for repeat testing of specimens should be the same as those applied for unknown samples, such as what to do following failure to amplify or detection of contamination or sequence errors using routine quality assurance procedures.

Failure to pass any single criterion means the overall validation also fails. The reasons for failure should be investigated and appropriate corrective action (such as changing the protocol) taken must be documented. The validation (or the relevant portion of it) can then be repeated. Simply repeating the experiments with the same assay and no other changes until the validation criteria are met is not acceptable.

3. Requirements after a validated procedure is changed

Changing a validated procedure requires equivalency testing (if the change is minor) or assay revalidation (if the change is major), to be sure that the change does not adversely affect assay performance. Equivalency testing involves parallel testing of at least 10 (but preferably 20) specimens using the old and new methods or retesting 10–20 previously tested specimens (preferably less than six months previously). For the original assay validation, an analysis plan and acceptance criteria should be predefined. Acceptance criteria should be chosen such that it can be reasonably claimed that the change did not adversely affect assay performance. Examples of a minor change include: a new supplier of a well-defined chemical with similar specifications (such as sodium chloride or Tris buffer), or use of a new instrument identical to one already in use (such as a second PCR machine or sequencer of the same model number and vendor). Examples of a major change include: change in enzyme used for reverse transcription or PCR, a change in a critical reagent concentration, new primers for reverse transcription, PCR amplification or sequencing, introduction of automation such as liquid handlers or nucleic acid extraction instruments or introducing a new type of instrument such as a PCR machine or sequencer. It may be possible to test the new reagent or instrument in a limited number of steps rather than through the entire assay; for example, a new sequencer or sequencing primers can be tested in parallel with the existing validated one(s) using residual PCR products from extraction and amplification reactions, if the quantities are sufficient. However, for changes affecting an upstream step (such as extraction or amplification), all downstream steps should be completed to check for unforeseen effects on quality.

When a change is considered because it leads to a significant improvement in some aspect of performance, additional data are often required to support the validity of the improvement. For example, if a new reverse transcription-PCR (RT-PCR) protocol is adopted as a means of improving the amplification sensitivity, the accuracy of the sequences generated by the new procedure in samples that were previously RT-PCR-negative should be assessed to ensure that they represent replication-competent viruses and not less relevant forms such as archived, possibly defective proviral DNA. If the new procedure is intended to improve the sensitivity for detecting drug-resistant variants present at low abundance (such as <10%), the fact that these variants are truly present and not being falsely detected should be established through independent means.

4. Validating a full-scale genotyping assay

Once a laboratory has decided to validate a genotyping assay, the following steps should be followed:

- needs assessment including a target product profile or target performance characteristics consistent with the intended use;
- assay development (optimize method and prepare standard operating procedures);
- pre-validation (Is the assay ready for testing? Are appropriate specimens available to determine if targets can be met?);
- preparing a validation plan, including selecting specimens, a plan for the analysis and acceptance criteria;
- perform validation experiments under routine conditions;
- data analysis and reporting; and
- improving the assay and revalidation (if necessary).

At a minimum, for genotyping assays intended for use in WHO HIV drug resistance surveillance, the validation must include testing of the following four aspects:

- accuracy (such as agreement with the results from a gold-standard assay):
- amplification sensitivity (minimum required viral load for reproducible amplification and sequencing);
- precision (intra-assay variability); and
- reproducibility (inter-assay variability).

In addition, it is highly recommended to test:

- linearity (reproducibility over a range of input copy numbers); and
- sensitivity for detection of mixtures.

4.1 Accuracy

Accuracy is the agreement between a result and an expected reference value (for genotyping assays, the nucleotide sequence). The reference or comparator sequence should be generated using a gold-standard method that has been thoroughly validated and accepted as a reliable assay in the field. Reference sequences could be generated via parallel testing of the same specimens at approximately the same time as the new method validation, or previously, as long as the specimen has been well stored and not altered in any way. Reference sequences could have been generated in a laboratory different from the one in which the new method is to be performed. For HIV drug resistance genotyping, acceptable gold standards include:

- the sequence derived from kit-based assays that have been approved by the United States Food and Drug Administration or equivalent regulatory agencies, such as the ViroSeq™ or TruGene™ kit for protease or reverse transcriptase (although the TruGene™ kit is no longer available, it can be used as a standard for comparison if the sequence was generated previously using this kit according to the manufacturer's instructions);
- the sequence derived from an in-house assay that has already been thoroughly validated; and
- a consensus sequence derived from replicate testing in multiple laboratories with validated in-house assays: for example, if specimens are obtained from the VQA proficiency testing panels, the VQA consensus sequence.

Accuracy testing should be performed using specimens from at least 20 different people for each specimen type being used (plasma or DBS). Some test results from other sections of the validation (see below) can be used for assessing accuracy. The specimens chosen for accuracy testing should ideally comprise multiple laboratory-associated mutations to include the accuracy of detection of all mutations that affect the clinical interpretation of the sequence, such as all mutations with non-zero penalty scores in the Stanford HIVdb interpretation algorithm. However, this is usually not practical because of the large number of such mutations. Instead, as many as possible of the mutations identified as occurring most frequently among people for whom commonly used antiretroviral therapy regimens have failed should be included. Lists of these mutations are available at https://hivdb.stanford.edu/pages/poc.html.

The data should be analysed and reported as the percentage nucleotide sequence identity between the sequences generated by the new test method and the reference standard and the percentage of the laboratory mutations known to be present (or absent) that were detected (or correctly not detected). The treatment of mixtures should be predetermined and described in the validation protocol, since establishing separate acceptance criteria for precision at positions where mixtures are versus are not observed may be necessary. For example, in a strict approach, an R in one sequence versus an A or G at the same position in a comparator sequence would be counted as a mismatch; whereas in a more lenient approach, it would be allowed. In a hybrid approach, a mixed versus unmixed (but compatible) base is allowed only if it occurs in a position of a codon that does not change the encoded amino acid. The acceptance criteria thresholds may differ depending on the approach chosen. In particular, if specimens with abnormally high numbers of mixed bases (such as >2%) are used in the validation, the acceptance criteria may need to be made less stringent. The sequences from each replicate are compared with others from the same specimen and the number of discrepancies is quantified.

When the accuracy of detection of drug-resistance mutations is calculated, the presence and absence of drug resistance mutations should be considered – in other words, the detection of a drug resistance mutation not present in the gold standard should be counted as an error, unless this is the result of improved sensitivity of the new method. In this case, additional verification data should be provided to demonstrate that the newly detected mutation is indeed present and is not artefactual. The denominator for laboratory mutation counts should be the total number of mutations that are considered in the interpretation step (all mutations with non-zero penalty scores in the Stanford HIVdb interpretation algorithm).

Resistance-associated positions in the Stanford HIVdb algorithm (version 8.9):

Protease: 10, 11, 20, 23, 24, 30, 32, 33, 43, 46, 47, 48, 50, 53, 54, 58, 73, 74, 76, 82, 83, 84, 88, 89, 90

Reverse transcriptase: 41, 62, 65, 67, 68, 69, 70, 74, 75, 77, 98, 100, 101, 103, 106, 108, 115, 116, 138, 151, 179, 181, 184, 188, 190, 210, 215, 219, 221, 225, 227, 230, 238

Integrase: 51, 66, 74, 92, 95, 97, 118, 121, 138, 140, 143, 145, 146, 147, 148, 151, 153, 155, 157, 163, 230, 263

- Proposed acceptance criteria: ≥90% of comparisons (base calls and drug-resistance mutation calls) for each specimen (between the test result and the reference or standard result) must be at least 98% identical, with non-matching mixtures counted as a difference or at least 99% identical with non-matching mixtures not counted as a difference.

4.2 Amplification sensitivity

Amplification sensitivity is defined as the percentage of successful (accurate and reproducible) genotyping tests among specimens with a specific viral load range. This distinguishes it from the limit of detection, which is the minimum viral load required for amplification and sequencing. For WHO HIV drug resistance surveillance, genotyping of specimens with viral load below 1000 copies/ml is not required.

If possible, specimens representing all subtypes that are expected to be commonly encountered during testing of the people included in WHO drug resistance surveys should be included in this section of the validation and analysed separately (according to subtype). Keep in mind that regional and specialized drug resistance laboratories may need to test specimens from countries other than the one in which the laboratory is located, necessitating broad subtype coverage.

Two design approaches, which are not mutually exclusive, are described below. Amplification sensitivity can be assessed using either approach or a combination; the important point is to have a sufficient number of independent tests (at least 10 and preferably 20 or more) using specimens with viral loads in the low range (such as 1000–5000 copies/ml) so that a confident claim can be made with regard to the assay performance target. Each laboratory's access to specimens with the desired viral load will largely determine the balance between the approaches.

1. Specimens with high viral load are serially diluted in an appropriate diluent (usually HIV-negative plasma, or for DBS, whole blood from an HIV-negative donor) to achieve a range of known viral copy numbers, followed by replicate (preferably at least four each in the range that approaches the sensitivity limit) testing of each dilution. At least two of the sets of diluted replicates should have viral load in the range 1000–5000 copies/ml. To exclude the possibility of unusual results derived from a single specimen with non-representative characteristics, the dilution series should be prepared from at least two and preferably more different viruses – at least in the viral load range close to the sensitivity limit. Viral loads should be confirmed after dilution and not assumed to be the calculated value based solely on intended dilution factor.

2. A large number (such as more than 40) of specimens are tested with known viral loads that are spread over a wide range of copy numbers but concentrated in the range of the anticipated sensitivity limit (such as at least 10 and preferably 20 or more in the range 1000–5000 copies/ml).

Although not all replicates tested for the purpose of defining amplification sensitivity need to be carried through the rest of the assay (purification, sequencing and analysis), comparing the sequences, and especially the representation of mixed bases, between replicates at high and low viral load can be informative. These data could contribute to the assessment of assay linearity. In addition, several replicates from the lowest viral loads should be sequenced to confirm that the amplified PCR product is actually derived from the intended specimen and to guard against the possibility of PCR contamination. When amplification success is scored based on agarose gel band intensity, the criterion should be based on sufficient quantity of DNA produced to support the downstream sequencing steps.

The proposed acceptance criteria for protease, reverse transcriptase and/or integrase are:

- plasma: more than 90% of the specimens or replicates are positive for viral load between 1000 and 5000 copies/ml and >95% for viral load exceeding 5000 copies/ml; and
- DBS: more than 50% of the specimens or replicates are positive for viral load between 1000 and 5000 copies/ml and >90% for viral load exceeding 5000.

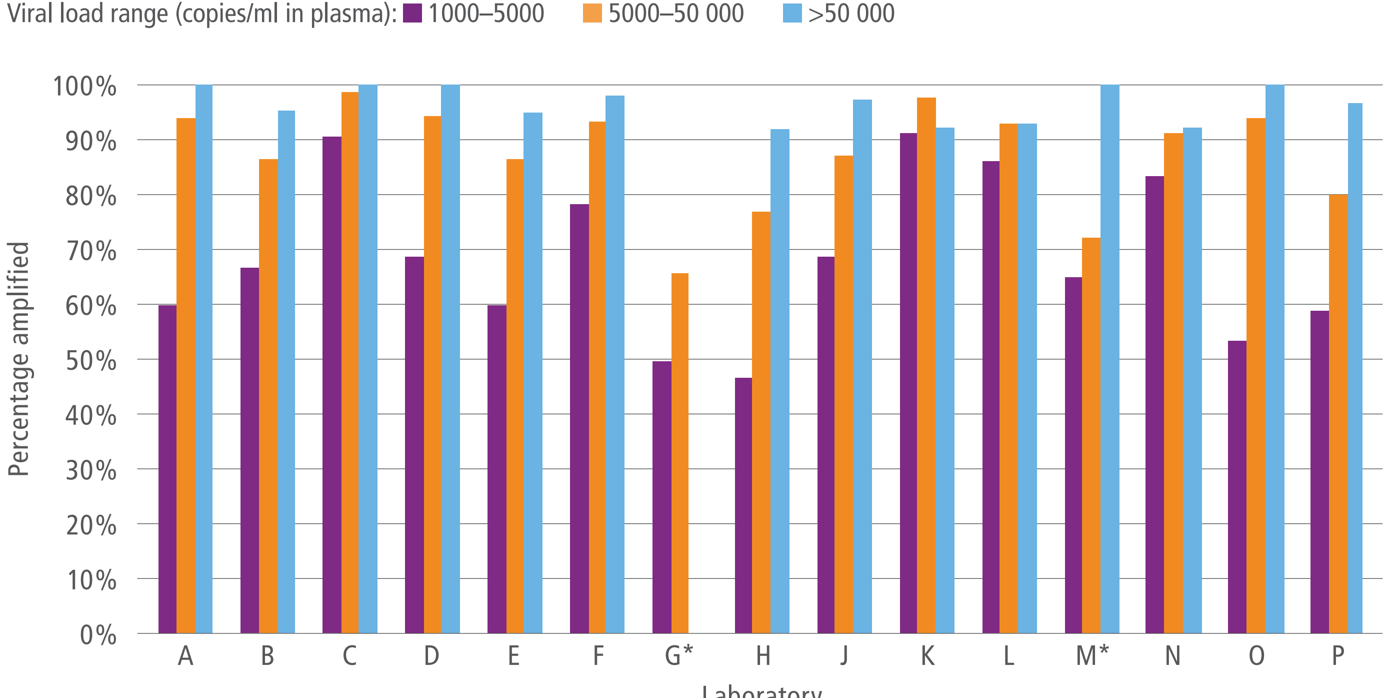
Negative amplification results with specimens having viral loads exceeding 1000 copies/ml for plasma or 2000 copies/ml for DBS should be investigated, for example as a possible signal of reduced sensitivity for certain subtypes.

The proposed target for amplification sensitivity with DBS is based on the results observed to date in experienced laboratories in the Laboratory Network and on the WHO definition of treatment failure (viral load >1000 copies/ml). The amplification success rate is expected to be lower for DBS than for plasma because of limitations on the specimen input volume (about 75 µl of plasma per 150-µl blood spot). For this reason, it is preferable to extract RNA from two spots per test. Fig. 1 summarizes the representative amplification sensitivity results from laboratories in the Laboratory Network.

4.3 Precision and reproducibility

The intra-assay precision of a genotyping test can be assessed by repeated testing of the same specimen in the same test

Fig.1. Amplification sensitivity using DBS



* DBS assay not successfully validated in these laboratories.

run. Five or more replicates of at least three different clinical specimens, preferably representing multiple subtypes and resistance patterns, are tested, with all replicates of a particular sample being processed in parallel in the same batch, by the same operator and with the same lots of critical reagents.

The inter-assay precision (also referred to as reproducibility) of a genotyping test can be assessed by repeated testing of the same specimen across multiple test runs, preferably including real potential sources of variability such as operator, critical reagent lots, key pieces of equipment, and time (such as over at least two weeks). Five or more replicates of at least three different clinical specimens, preferably representing multiple subtypes and resistance patterns, are tested, with replicates of a particular sample being processed in different batches. One set of replicates from intra-assay precision testing can be used for testing inter-assay precision. The results from this type of replicate testing may be supplemented by duplicate testing of a larger number of specimens (such as at least 20).

For both precision and reproducibility testing, the variability in the result is determined based on nucleotide sequence similarity between pairs of replicates (all possible pairwise comparisons) or by comparison with a consensus sequence derived from the replicate results. Five results produce 10 possible pairwise comparisons per sample.

- Proposed acceptance criteria: ≥90% (9 of 10) of pairwise comparisons for each specimen must be at least 98% identical (with non-matching mixtures counted as a difference).

4.4 Linearity

Linearity refers to the reproducibility of the result (sequence) over a wide range of input copy numbers, including near the amplification sensitivity limit. Linearity testing may be combined with sensitivity testing. Particular attention should be paid to the lowest viral load at which mixtures are reproducibly detected. Theoretically, at low viral load, although a product is amplified, the PCR product is likely clonal: derived from a single or only a few individual templates and thus not representative of the quasi-species of the person. At this viral load or below, the mixtures are expected to be fewer or absent, and thus the sequence of a specimen containing significant levels of multiple species will not be reproducible. To avoid this situation as much as possible, the input copy number should be maximized at each step. For example, the largest possible volume of plasma should be used for extraction, and the largest possible volume of extracted RNA should be used for RT-PCR.

- The acceptance criteria need not be predefined; these experiments may be considered exploratory. Knowledge about the limitations may be useful for interpreting survey results.

4.5 Sensitivity for detecting mixtures

A different type of sensitivity of genotyping assays is related to the determination of the lowest proportion of an individual sequence variant that can reliably be detected in a mixture of viruses. Using dye-terminator sequencing methods, this is typically not lower than 20%, although this depends on the sequence and context; in some cases, it may be considerably higher or lower, since contextual effects can make some bases easier to detect as secondary peaks than others. Sensitivity for detecting mixtures can be assessed by preparing mixtures of well-characterized viruses at predefined proportions and testing through all steps of the assay. Alternatively, plasmid DNA of molecular clones can be used and taken through the sequencing steps only (although this does not test variability introduced by RNA extraction, reverse transcription or PCR amplification). Regardless of what substrate type is used, viruses should have several distinct resistance-associated mutations that are different between them (for example one virus with only protease mutations and a second one with only reverse transcriptase mutations) (Table 2).

- The acceptance criteria need not be predefined; these experiments may be considered exploratory.

Table 2. Number of tests required for validating an assay

	Number of specimens	Replicates per specimen	Total tests	Evaluation	Comments
Accuracy	20	1	20	% nucleotide identity versus standard	Include five with low viral load
Sensitivity (amplification): high viral load[a]	10	2	20	Successful amplification	For viral load >10 000 copies/ml; can also be 10 samples tested once each
Sensitivity (amplification): low viral load[a]	5	4	20	Successful amplification	For viral load 1000–5000 copies/ml; can also be 20 samples tested once each
Intra-assay precision	3	5	15	Pairwise nucleotide sequence comparison	All replicates tested on the same batch
Inter-assay precision	3	5	15	Pairwise nucleotide sequence comparison	Each replicate tested on a different batch
Raw total number of tests			**90**		
Adjustment for overlap between:					
Accuracy and sensitivity	10	1	10		Five each with low or high viral load
Intra- and inter-assay precision	3	1	3		One replicate of intra-assay precision used for inter-assay precision
Sensitivity and precision	3	2	6		Three samples with high viral load used for both
Adjusted total number of tests			**71**		

[a] Complete testing (including sequencing) not required for all replicates.

5. Verifying a method

This approach is similar in concept but smaller in scale compared to the full-scale validation described above. The acceptance criteria are the same as for validation, although separate analysis with reference to the performance claims provided by a kit manufacturer should also be performed. The changes compared with the validation described above are as follows.

5.1 Accuracy

Test 10 specimens instead of 20; focus on the priority drug-resistance mutations expected to be found most commonly in specimens from WHO surveys. At least 10 results need to be generated for the acceptance criteria (>90% of comparisons) to translate to only one outlier result being allowed (9 of 10 results with acceptable results is okay, but 8 of 9 is not).

5.2 Amplification sensitivity

For the dilution series, duplicates instead of four replicates of each dilution of two different specimens should be tested; with undiluted specimens, 20 instead of 40 are needed, as long as they are concentrated in the lower range of viral loads (1000–10 000 copies/ml).

5.3 Precision and reproducibility

Intra-assay precision: test four replicates each for two different specimens.

Inter-assay precision: test 10 specimens in two different batches on different days, if possible using different operators and lots of critical reagents.

6. Equivalency testing

Equivalency testing is usually performed when a relatively minor change is made to a laboratory-developed assay (see above for examples) or as a lot-release procedure for new production lots of reagents already in use.

6.1 Accuracy

This is the same as verifying a method but with a minimum of 10 specimens; the standard sequences for comparison are usually derived from specimens recently tested with the validated method before the change.

6.2 Amplification sensitivity

Perform the extraction, reverse transcriptase and PCR steps using five specimens with low viral loads (1000–10 000 copies/ml).

This approach does not apply if a change is made to the procedure using a manufactured kit, since such a change would by definition represent a deviation from the kit procedure and thus make it a laboratory-developed test instead.

6.3 Reproducibility

Inter-assay precision: test three specimens in two different batches on different days, if possible using different operators and lots of critical reagents (Tables 3 and 4).

Table 3. Numbers of tests required for verifying a method

	Number of specimens	Replicates per specimen	Total tests	Evaluation	Comments
Sensitivity (amplification): high viral load[a]	5	2	10	Successful amplification	For viral load >10 000 copies/ml; can also be 10 samples tested once each
Sensitivity (amplification): low viral load[a]	5	2	10	Successful amplification	For viral load 1000–5000 copies/ml; can also be 20 samples tested once each
Intra-assay precision	2	4	8	Pairwise nucleotide sequence comparison	All replicates tested on the same batch
Inter-assay precision	10	2	20	Pairwise nucleotide sequence comparison	Each replicate tested on a different batch
Raw total number of tests			**90**		
Adjustment for overlap between:					
Accuracy and sensitivity	10	1	10		Five each with low or high viral load
Intra- and inter-assay precision	2	1	2		One replicate of intra-assay precision used for inter-assay precision
Sensitivity and precision	2	2	4		Two samples with high viral load used for both
Adjusted total number of tests			**42**		

[a] Complete testing (including sequencing) not required for all replicates.

Table 4. Numbers of tests required for equivalency testing

	Number of specimens	Replicates per specimen	Total tests	Evaluation	Comments
Accuracy	10	1	10	% nucleotide identity versus standard	Include five with low viral load
Sensitivity (amplification): high viral load[a]	0	0	0		
Sensitivity (amplification): low viral load[a]	5	4	20	Successful amplification	For viral load 1000–5000 copies/ml; can also be 20 samples tested once each
Intra-assay precision	0	0	0		
Inter-assay precision	3	5	15	Pairwise nucleotide sequence comparison	Each replicate tested on a different batch
Raw total number of tests			**45**		
Adjustment for overlap between:					
Accuracy and sensitivity	5	1	5		Five each with low or high viral load
Intra- and inter-assay precision	0	0	0		One replicate of intra-assay precision used for inter-assay precision
Sensitivity and precision	0	0	0		Two samples with high viral load used for both
Adjusted total number of tests			**40**		

[a] Complete testing (including sequencing) not required for all replicates.

7. Potential sources of specimens for validating an assay

The selection of specimens for use in validation experiments such as those described here is critical for the success and relevance of the validation itself. Factors to consider carefully include cost, availability of large quantities, viral load, subtype, matrix (such as type of anticoagulant), resistance mutation pattern, degree of heterogeneity (mixtures) and representation of the types of specimens expected to be encountered during subsequent testing in the laboratory. The specimens should be well characterized through replicate testing in pre-validation experiments to guide the establishment of acceptance criteria and to avoid unforeseen problems later. In the accuracy and sensitivity experiments in particular, several examples of HIV-1 subtypes regularly received in the laboratory should be included. For regional and specialized laboratories, subtypes from countries outside of the location of the laboratory itself should be included, since these laboratories are expected to test specimens from many geographical areas.

Specimens may be obtained from several sources including the following:

- AIDS Research & Reference Reagent Program, United States National Institutes of Health (https://www.aidsreagent.org);
- Centre for AIDS Reagents, National Institute for Biological Standards and Control, United Kingdom Medicines and Healthcare Products Regulatory Agency (http://www.nibsc.org/science_and_research/virology/centre_for_aids_reagents/reagents_catalogue.aspx);
- External Quality Assurance Program Oversight Laboratory (EQAPOL), Duke University: (https://eqapol.dhvi.duke.edu);

- Rush Medical College (https://www.rushu.rush.edu/research/departmental-research/microbial-pathogens-and-immunity-research);
- commercial sources:
  - BocaBiolistics (http://www.bocabio.com);
  - SeraCare Life Sciences (http://www.seracare.com);
  - HemaCare (http://www.hemacare.com);
  - Discovery Life Sciences (https://www.discoverylifesciences.com);
- local clinics and hospitals (be sure to follow appropriate informed consent regulations);
- academic collaborators; and
- other WHO network laboratories (http://www.who.int/hiv/topics/drugresistance/laboratory/en/index1.html).

8. Special considerations for validating assays based on next-generation sequencing

Several laboratories in the Laboratory Network are considering or have completed a transition from standard Sanger-based sequencing methods to next-generation sequencing procedures for routine genotyping of HIV and other pathogens. As with any new method, existing quality assurance and quality control procedures must be adapted to make them compatible with the use of next-generation sequencing for HIV drug resistance genotyping. Among the quality assurance and quality control aspects of the WHO HIVResNet HIV drug resistance laboratory operating framework that are potentially affected, assay validation parameters are one of the most important.

To simplify the transition to next-generation sequencing, and in the absence of definitive data regarding a clinically relevant threshold for low-abundance drug resistance variants, the WHO HIVResNet Laboratory Working Group has recommended a phased approach that begins with configurating next-generation sequencing assays to mimic the sensitivity of Sanger-based methods. In practice, this means that the threshold for low-abundance variants should be set at 20%[1]. In this light, next-generation sequencing can be thought of as an alternative, in-house (home-brew) assay that is required to generate results with the same performance characteristics as existing Sanger-based methods. This includes the generation of a nucleotide sequence in FASTA file format with the same minimum coverage: codons 10–93 for protease, 41–238 for reverse transcriptase and 51–263 for integrase (if integrase is included, separate guidance will be provided regarding the need for integrase sequence data). In this first phase, it is acknowledged that potentially useful advantages of next-generation sequencing such as increased sensitivity for drug resistance variants present at frequencies below 10–20%, resolution of complex ambiguous codons containing more than one mixed base and quantification of variant proportions will be ignored; these aspects will be investigated more completely in a later phase. The phased approach will also simplify, though not eliminate, concerns related to constraints on input copy number inherent in lower thresholds for low abundance variants.

In addition to the validation components outlined above, genotyping assay validations based on next-generation sequencing should also pay particular attention to the following attributes:

- including multiple subtypes to test the alignment step;
- detecting and correctly reporting insertions and deletions, especially those affecting the prediction of resistance (insertions near reverse transcriptase amino acid 69) and of K65R, K103N and other drug resistance mutations located in homopolymeric regions;
- variant detection sensitivity (must be at least 15%);
- input copy number required to support variant detection sensitivity.

For example, if sufficient individual reads are generated (and a minimum of five required) to support a theoretical detection sensitivity of 1%, the total input copy number must be well above 500 to ensure that this variant is detected reproducibly. An input copy number of 500 may translate to an original viral load of 10 000 copies/ml or higher, depending

[1] Parkin NT, Avila-Rios S, Bibby DF, Brumme CJ, Eshleman SH, Harrigan PR et al. Multi-laboratory comparison of next-generation to Sanger-based sequencing for HIV-1 drug resistance genotyping. Viruses. 2020;12:E694.

on the volume of plasma processed (usually between 0.1 and 0.5 mL) and other bottlenecks such as the proportion of extracted nucleic acids used for amplification (often only 20%) or the efficiency of extraction or reverse transcription steps. Conversely, to support a theoretical detection sensitivity of 15%, the total input copy number must be well above 33 to ensure that this variant is detected reproducibly. An input copy number of 33 may translate to an original viral load of 660 copies/ml or higher.

ANNEX 4. QUESTIONNAIRE FOR COLLECTING INFORMATION ON THE CAPACITY AND EQUIPMENT OF HIV SEQUENCING LABORATORIES

Laboratory name:

Street address:

City, state:

Country:

Director of department or institution:

Email:

Laboratory director:

Email:

Contact person for assessment visit:

Position of contact person:

Email:

Phone:

Date questionnaire completed:

Checklist

Mandatory criteria (laboratory self-assessment)	✔
National plan for implementing surveillance of HIV drug resistance (national drug resistance laboratories and regional drug resistance laboratories only)	
Designation letter from the health ministry (national drug resistance laboratories and regional drug resistance laboratories only)	
At least one year of experience in genotyping HIV, and ≥100 specimens tested annually with satisfactory results (≥3 years and ≥200 specimens for regional drug resistance laboratories and specialized drug resistance laboratories)	
Minimum infrastructure for HIV drug resistance genotyping in place	
Capacity for dried blood spot (DBS) genotyping (regional drug resistance laboratories and specialized drug resistance laboratories only)	
Capacity for genotyping integrase	

Mandatory criteria (to be assessed by WHO)	✔
Need for laboratory support established	
Demonstrated proficiency with quality assurance, management and reporting of sequencing data	
Successful participation in the WHO HIV drug resistance proficiency testing programme	
Additional criteria for regional drug resistance laboratories and specialized drug resistance laboratories only	
Regionally recognized experience and leadership in HIV laboratory science	
Adequate experience in providing training and establishing collaborations in laboratory sciences in the past five years	
Good general knowledge of sequencing, including techniques other than commercially available kits	

Documentation to be submitted to WHO*	✔
Letter of support from the health ministry indicating that the laboratory has been identified to test specimens collected during WHO-recommended HIV drug resistance surveys (national drug resistance laboratories); for regional drug resistance laboratories, the letter should indicate that that the candidate laboratory has been identified to test specimens collected from other countries during WHO-recommended HIV drug resistance surveys and will provide training and capacity building to other laboratories in the Region	
Maintenance records and service contract for major equipment.	
Map of the genotyping facility	
CVs of genotyping laboratory personnel (including supervisor) documenting their qualifications and experience in molecular biology	
Description of the management structure of the genotyping laboratory personnel	
Information on the financial sustainability of HIV drug resistance genotyping activities in the past five years	
Records and documentation of the sequencing tests performed in the past two years, including both in-house methods and commercial kits	
Proficiency panel testing reports from providers other than WHO in the past year	
Standard operating procedures. including: (1) receipt, assessment and storage of specimens; (2) internal quality control; (3) all steps of genotyping tests, including sequencing; (4) handling and manipulating infectious human material, including handling infectious waste; (5) data management; and (6) post-testing sequence quality assurance.	
Validation reports on DBS and integrase assays (regional drug resistance laboratories and specialized drug resistance laboratories only)	

* The laboratory should submit all documentation in electronic format (Adobe PDF preferred).

Questionnaire

As part of the efforts to organize the WHO HIVResNet Laboratory Network, the existing capacity of your laboratory for HIV drug resistance sequencing will be evaluated. Please answer the following questions by checking the appropriate boxes or filling in the appropriate number or text.

A. General information

1. Is the laboratory performing drug resistance sequencing for HIV?

☐ Yes ☐ No

2. For what purpose is HIV drug resistance sequencing performed? (Check all that apply)

☐ Clinical care

☐ Research

☐ Public health or epidemiological purposes

3. How many years of experience does the laboratory have in performing HIV drug resistance sequencing?

_________ Years

4. How many HIV-1 drug resistance sequencing tests did the laboratory perform in each of the past two years?

Number performed last year: _________ Year: _________

Number performed year before last: _________ Year: _________

5. Is the laboratory integrated into the health ministry?

☐ Yes ☐ No

6. If not, is it a private laboratory?

☐ Yes ☐ No

Specify type:

. . . .

. . . .

. . . .

B. Personnel

7. Indicate the personnel (scientific and technical personnel) available to perform HIV drug resistance sequencing and specify the ability to perform in-house home-brew assays. Please include the qualifications and training undertaken (add additional rows as needed). Indicate the time dedicated to sequencing per month by each individual. Attach the CVs of the personnel working in the genotyping laboratory.

Name	Qualification and training[a]	Time dedicated	Years experience with HIVDR genotyping

[a] For example, general technician, technician molecular diagnostics, biomedical scientist or physician.

8. Is a safety officer in place?

☐ Yes ☐ No

If yes, name of safety officer:

. . . .

9. Please provide an organizational chart showing the management structure of the laboratory or institution (attach a separate file). The chart should include all personnel listed in question 7.

C. Quality management system

10. Does the laboratory have standard operating procedures in place for HIV drug resistance sequencing?

☐ Yes ☐ No

If yes, please provide the title and number of standard operating procedures for all steps of the HIV drug resistance sequencing procedure. (Add additional rows as needed.) Please submit copies of the standard operating procedures for HIV drug resistance for review along with this questionnaire.

Standard operating procedure number	Standard operating procedure title

11. Are standard operating procedures managed using a quality assurance or document control system or under regulatory oversight (such as ISO or CAP/CLIA)?

☐ Yes ☐ No

If yes, provide details:

. . . .

. . . .

12. Does the laboratory participate in an external quality assurance programme for HIV drug resistance sequencing?

☐ Yes ☐ No

If yes, please specify:

Name of the programme: . . . .

Name of the provider: . . . .

Date of participation: . . . .

Please submit a summary of the proficiency panel results from the past two years along with this questionnaire.

13. Indicate the frequency of power failure in number of times per year: ________ per year

Is backup available in case of power failure?

☐ Yes ☐ No

D. Biosafety

14. Does the laboratory have well-documented procedures for handling and manipulating infectious human material, including handling infectious waste?

☐ Yes ☐ No

If yes, please provide the name and number of the standard operating procedure or laboratory protocol and submit a copy for review:

Name and number: . . . .

15. Are laboratory disinfection procedures in place?

☐ Yes ☐ No

16. If yes, please list the workspace or equipment disinfected, procedures and disinfecting agents used and frequency of disinfection in the following table (add additional rows as needed).

Workspace or equipment	Procedure and disinfecting agent	Frequency

E. Dedicated molecular diagnostic workspace and workflow

17. Are there separate laboratories, rooms or work areas for molecular diagnostic activities?

☐ Yes ☐ No

If yes, please answer questions 17a and 17b.

17a. Is there a separate, dedicated room for specimen extraction and master mix preparation that remains free of contaminating DNA (pre-amplification)?

☐ Yes ☐ No

17b. Is there a separate, dedicated room for polymerase chain reaction (PCR) amplification and for handling amplification products and high-copy number DNA (post-amplification)?

☐ Yes ☐ No

18. Do all laboratory personnel respect a strict unidirectional workflow?

☐ Yes ☐ No

Please submit copies of standard operating procedures or laboratory procedures for workflow and provide a map of the genotyping facilities.

19. Are procedures in place for cleaning and molecular decontamination of the laboratory?

☐ Yes ☐ No

If yes, please submit copies of standard operating procedures or laboratory procedures for cleaning and molecular decontamination. If a written document is not available, please use the table below to report the type and frequency of cleaning and molecular decontamination procedures or strategy the laboratory uses. (Add additional rows as needed.)

Workspace or equipment	Procedure and decontaminating agent	Frequency

F. Equipment

20. Indicate the type, year of purchase and the frequency of maintenance and calibration of the equipment present in the pre-amplification area.

Pre-amplification area equipment	Type	Year of purchase	Maintenance type and frequency
Bench with sink and tap water			
Biohazard flow, class IIb			
Dead air cabinet (preparation mixes)			
Dead air cabinet (nucleic acid extraction)			
Freezer –20°C			
Microcentrifuge 12 500–15 000 × *g*			
Vortex			
Dedicated set of micropipettes			
Ultracentrifuge[a] 21 000–25 000 × *g*			

[a] In case the extraction procedure requires pelleting of virus.

21. Are the centrifuges anti-aerosol?

☐ Yes ☐ No

22. Indicate the type, year of purchase and frequency of maintenance and calibration of the equipment present in the post-amplification area.

Post-amplification equipment	Type	Year of purchase	Maintenance type and frequency
Bench with sink and tap water			
Dead air cabinet (nested reaction)			
Thermal cyclers			
Agarose gel apparatus			
Photo documentation of agarose gel			
DNA sequencer			
Computer			
Computer program (editing)	Version:		
Microcentrifuge 450–550 × *g*			
Vortex			
Dedicated set of micropipettes			
Freezer –20°C			
Refrigerator 4°C			

23. Indicate the type, year of purchase and frequency of maintenance and calibration of additional equipment.

Equipment	Type	Year of purchase	Maintenance
Freezer –20°C			
Freezer –80°C			
Autoclave			

24. Describe how the freezer temperature control is monitored:

. . . .

. . . .

25. Indicate the presence of the following materials for biosafety in the separate workspaces.

Workspace	Gloves	Paper lab coats
Pre-amplification and extraction		
Pre-amplification and mix preparation		
Post-amplification		

26. If paper lab coats are used, how frequently are they changed? ________ Times per month

26a. If paper lab coats are not used, are cloth lab coats used?

☐ Yes ☐ No

26b. If Yes, how frequently are the cloth lab coats cleaned? ________ Times per month

27. Supplies

27a. Are current inventories maintained?

☐ Yes ☐ No

27b. Is a system in place for replenishing supplies?

☐ Yes ☐ No

28. Computational capability

28a. Is a computer available in the laboratory?

☐ Yes ☐ No

28b. Is Internet access available?

☐ Yes ☐ No

G. Specimens

29. What types of specimens does the laboratory use for HIV drug resistance sequencing? (Check all that apply.)

- ☐ Ethylenediaminetetraacetic acid (EDTA) plasma
- ☐ Citrate plasma
- ☐ Serum
- ☐ Dried blood spot (DBS)
- ☐ Dried plasma spot (DPS)
- ☐ Dried serum spot (DSS)

30. If the laboratory uses dried blood, plasma or serum spots for HIV drug resistance sequencing, please indicate the type of membrane and manufacturer:

- ☐ Membrane 903 filter, manufacturer: ....
- ☐ Membrane FTA filter, manufacturer: ....
- ☐ Other membrane, manufacturer: ....

31. If the laboratory uses dried blood, plasma or serum spots for HIV drug resistance sequencing, please indicate the storage conditions and detailed information on the processing of the specimen, including extraction, amplification and sequencing (quantities used, conditions, etc.). If a standard operating procedure or written laboratory protocol is available, you may submit a copy and indicate "see enclosed document" in the space below.

....

....

....

32. If the laboratory uses dried blood, plasma or serum spots for HIV drug resistance sequencing, please indicate the number of specimens tested annually in the last 2 years and years of experience in genotyping using DBS, DPS and DSS.

Number of specimens tested annually in past two years: ....

Years of experience genotyping using DBS, DPS and DSS: ....

H. Specimen registration

33. Indicate the information present on the stored specimens used for HIV drug resistance sequencing.

- ☐ Unique specimen identification code
- ☐ Patient identification code
- ☐ Identification code for the specimen collection centre
- ☐ Specimen collection date
- ☐ Specimen collection time
- ☐ Other: ....

34. Indicate the number of aliquots stored for each patient: ________ Volume: ________

35. Indicate the system used for registering specimens. (Check all that apply.)

- ☐ Paper registry
- ☐ Computer registry
- ☐ Other (specify): . . . .

36. Indicate the information collected in the registry:

- ☐ Type of specimen
- ☐ Unique specimen identification code
- ☐ Patient identification code
- ☐ Patients' date of birth
- ☐ Patients' age group
- ☐ Patients' antiretroviral therapy history
- ☐ Number of pregnancies (for women)

Other patient data: . . . .

- ☐ Identification code for the specimen collection facility
- ☐ Specimen collection date
- ☐ Specimen collection time
- ☐ Date specimen was sent to the sequencing lab
- ☐ Date specimen was received in the sequencing lab
- ☐ Specimen viral load
- ☐ Condition of the specimen
- ☐ Specimen volume and number of dried fluid spots
- ☐ Specimen volume and number of dried fluid spots
- ☐ Specimen storage location

Other: . . . .

I. Sequencing methods

37. Please list the HIV-1 nucleic acid extraction method, manufacturer (if applicable), specimen type and starting volume for all specimen types used for HIV drug resistance sequencing.

Extraction method	Manufacturer	Specimen type	Starting volume

38. Method for your laboratory sequencing of HIV-1. (List all methods used.)

☐ Kit-based assay:

Name/version: . . . .

Name/version: . . . .

☐ Home-brew (developed in-house) assay

39. If the laboratory uses a kit-based HIV genotyping assay, describe the deviations from the standard procedure, if any:

. . . .

. . . .

40. If the laboratory uses a home-brew (developed in-house) assay, provide information on the primers and method:

a. RT and PCR primers: ☐ From a published reference ☐ Designed by laboratory

b. Sequencing primers: ☐ From a published reference ☐ Designed by laboratory

c. RT assay conditions: ☐ From a published reference ☐ Designed by laboratory

d. First PCR assay conditions: ☐ From a published reference ☐ Designed by laboratory

e. Second PCR assay conditions: ☐ From a published reference ☐ Designed by laboratory

If applicable, the laboratory must provide documentation of the references used

41. Has the method been validated in the laboratory?

☐ Yes ☐ No

If yes, please attach a summary of how the method was validated, including information on the specimen types validated, the number of specimens tested and the method of evaluation or reference assay used for comparison.

42. Minimal region sequenced on both strands (both directions):

For protease: codons ________ to ________

For reverse transcriptase: codons ________ to ________

For integrase: codons ________ to ________

43. Is the viral load of specimens submitted for genotyping known?

☐ Yes ☐ No

44. Assay sensitivity: what is the minimal viral load required for sequencing?

Plasma: ________ copies/ml DBS: ________ copies/ml

Proportion of samples with viral load between 1000 and 5000 copies/ml that can be amplified

Plasma: ________ DBS: ________

45. Is the preservation time for reagents controlled?

☐ Yes ☐ No

46. How are specimens or derivatives kept cold during sequencing?

. . . .

. . . .

47. What is the mean turn-around time and throughput for sequencing?

Turn-around time: ________ days Comments: ________________________________

Throughput: ________ sample/week Comments: ________________________________

48. Is a positive run control included in every run?

☐ Yes ☐ No

If yes, indicate the step(s) in which positive controls are included, the specimen type of the control and the viral load, if known:

Step: ________________ Type of specimen: ________________ Viral load: ________________

Step: ________________ Type of specimen: ________________ Viral load: ________________

49. If a positive run control is included in every run, please complete the following:

One positive control is used per ________ specimens.

What measures are in place in case of a negative result in the positive control?

. . . .

. . . .

50. Is a negative run control included in every run?

☐ Yes ☐ No

If the answer is yes, please complete the following:

One negative control is used per ________ specimens.

What measures are in place in case of a positive result in the negative control?

. . . .

. . . .

51. Are filter tips used for reaction set-up?

☐ Yes ☐ No

If yes, indicate the steps in which they are used:

. . . .

. . . .

J. Sequence editing

52. Indicate the software used for sequence editing: ______________________________

53. Which of the following are taken into account when evaluating the raw sequence data? Check all that apply.

- ☐ Signal intensity; limit: ______________
- ☐ Signal-to-noise ratio
- ☐ Reading forward and reverse strand
- ☐ Amount of editing needed; limit: ______________
- ☐ Other; specify: . . . .

54. How is sequence editing performed? Check all that apply.

- ☐ Manual reading
- ☐ Software-associated editing; specify software: ______________
- ☐ Other; specify: . . . .

55. Are the edited sequence results confirmed by a second or independent person?

☐ Yes ☐ No

If yes, please provide documentation for this procedure.

56. Does a supervisor critically review results?

☐ Yes ☐ No

K. Data management

57. Indicate the information registered during the processing and sequencing of the specimen.

- ☐ Dates of the various steps in specimen processing (extraction, amplification and sequencing)
- ☐ Detailed information on the specimen processing (quantities used and conditions)
- ☐ The results of each step
- ☐ Other attempts in case of failure
- ☐ The personnel performing each step of specimen processing
- ☐ Storage of interim material (extracted nucleic acids and PCR product)
- ☐ Lot numbers of kits and materials

58. What type of registry are these data records kept in?

- ☐ Paper registry
- ☐ Computer or electronic registry

59. Are the data and results archived?

☐ Yes ☐ No

If yes, how long are the data and results kept?

☐ Specimen registries	Length of time: ______________
☐ Laboratory processing registry	Length of time: ______________
☐ Raw sequence data	Length of time: ______________
☐ Final sequence result	Length of time: ______________
	Format: ______________

60. Are backup procedures for sequences data in place?

☐ Yes ☐ No

Describe the backup method and the frequency of backup:

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L. Financial sustainability

61. Describe the mechanism(s) for funding operations in the laboratory, including the annual budget.

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62. What is the cost of genotyping? Provide the cost per test, assuming a volume of about 500 specimens per year.

Cost for general testing: ______________

Cost expected to be reimbursed by an external funder for WHO surveillance work: ______________

Comments about costs (such as including or excluding labour costs, volume dependent, etc.):

. . . .

. . . .

Additional remarks, if any:

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