

HIV DRUG RESISTANCE

# WHO/HIVRESNET HIV DRUG RESISTANCE LABORATORY OPERATIONAL FRAMEWORK

DECEMBER 2017





**WHO/HIVRESNET  
HIV DRUG  
RESISTANCE  
LABORATORY OPERATIONAL  
FRAMEWORK**

**DECEMBER 2017**

WHO/HIVResNet HIV drug resistance laboratory operational framework

ISBN 978-92-4-151287-9

© World Health Organization 2017

Some rights reserved. This work is available under the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 IGO licence (CC BY-NC-SA 3.0 IGO; <https://creativecommons.org/licenses/by-nc-sa/3.0/igo>).

Under the terms of this licence, you may copy, redistribute and adapt the work for non-commercial purposes, provided the work is appropriately cited, as indicated below. In any use of this work, there should be no suggestion that WHO endorses any specific organization, products or services. The use of the WHO logo is not permitted. If you adapt the work, then you must license your work under the same or equivalent Creative Commons licence. If you create a translation of this work, you should add the following disclaimer along with the suggested citation: "This translation was not created by the World Health Organization (WHO). WHO is not responsible for the content or accuracy of this translation. The original English edition shall be the binding and authentic edition".

Any mediation relating to disputes arising under the licence shall be conducted in accordance with the mediation rules of the World Intellectual Property Organization.

**Suggested citation.** WHO/HIVResNet HIV drug resistance laboratory operational framework. Geneva: World Health Organization; 2017. Licence: CC BY-NC-SA 3.0 IGO.

**Cataloguing-in-Publication (CIP) data.** CIP data are available at <http://apps.who.int/iris>.

**Sales, rights and licensing.** To purchase WHO publications, see <http://apps.who.int/bookorders>. To submit requests for commercial use and queries on rights and licensing, see <http://www.who.int/about/licensing>.

**Third-party materials.** If you wish to reuse material from this work that is attributed to a third party, such as tables, figures or images, it is your responsibility to determine whether permission is needed for that reuse and to obtain permission from the copyright holder. The risk of claims resulting from infringement of any third-party-owned component in the work rests solely with the user.

**General disclaimers.** The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of WHO concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted and dashed lines on maps represent approximate border lines for which there may not yet be full agreement.

The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by WHO in preference to others of a similar nature that are not mentioned. Errors and omissions excepted, the names of proprietary products are distinguished by initial capital letters.

All reasonable precautions have been taken by WHO to verify the information contained in this publication. However, the published material is being distributed without warranty of any kind, either expressed or implied. The responsibility for the interpretation and use of the material lies with the reader. In no event shall WHO be liable for damages arising from its use.

# TABLE OF CONTENTS

<b>Acronyms and Abbreviations</b> .....	<b>3</b>
<b>Acknowledgements</b> .....	<b>3</b>
<b>Executive Summary</b> .....	<b>4</b>
<b>1. Principles of the HIVResNet Laboratory Network</b> .....	<b>5</b>
<b>1.1 Background</b> .....	<b>5</b>
<b>1.2 Principles of the WHO/HIVResNet Laboratory Operational Framework</b> .....	<b>5</b>
<b>1.3 Structure and function of the WHO/HIVResNet Laboratory Network</b> .....	<b>6</b>
1.3.1 National HIVDR laboratories .....	7
1.3.2 Regional HIVDR laboratories .....	7
1.3.3 Specialized HIVDR laboratories .....	8
1.3.4 WHO HIVResNet Laboratory Advisory Group .....	9
1.3.5 Role of WHO .....	9
<b>1.4 Summary of HIVDR genotyping procedures and quality assurance</b> .....	<b>10</b>
<b>2. WHO/HIVResNet Laboratory Network membership</b> .....	<b>11</b>
<b>2.1 Laboratory status definitions</b> .....	<b>11</b>
2.1.1 Candidate laboratory .....	11
2.1.2 Laboratory Network member laboratory .....	11
2.1.3 Provisionally designated laboratory .....	11
2.1.4 Laboratories failing to achieve WHO designation .....	11
2.1.5 Suspended laboratory .....	11
<b>2.2 Network membership requirements and review procedure</b> .....	<b>12</b>
2.2.1 Application phase .....	12
2.2.2 Assessment phase .....	12
2.2.3 Designation phase .....	13
2.2.4 Continued membership .....	13
<b>3. Guidelines for the submission of results to national HIVDR working groups and to WHO</b> .....	<b>14</b>
<b>3.1 Data ownership</b> .....	<b>14</b>
<b>3.2 Data flow from designated laboratories</b> .....	<b>14</b>
<b>ANNEX 1: Guidelines for HIV drug resistance genotyping</b> .....	<b>15</b>
<b>1. Introduction</b> .....	<b>15</b>
<b>2. Specimen types for HIVDR genotype testing</b> .....	<b>16</b>
<b>3. Amplification and sequencing</b> .....	<b>18</b>
<b>4. Data management and traceability of results</b> .....	<b>19</b>
<b>5. Preferred genotyping methodologies</b> .....	<b>19</b>
<b>6. HIVDR interpretation</b> .....	<b>21</b>

<b>ANNEX 2: Laboratory Safety</b> .....	<b>22</b>
<b>ANNEX 3: WHO HIVDR external quality assurance and internal quality control</b> .....	<b>24</b>
<b>1. External Quality Assurance</b> .....	<b>24</b>
<b>2. Internal Quality Control</b> .....	<b>26</b>
<b>ANNEX 4: Requirements for application to WHO for HIV Drug Resistance Laboratory Network membership</b> .....	<b>27</b>
<b>1. National HIVDR Laboratories</b> .....	<b>27</b>
<b>2. Regional HIVDR Laboratories</b> .....	<b>30</b>
<b>3. Specialized HIVDR Laboratories</b> .....	<b>30</b>
<b>ANNEX 5: Questionnaire for the collection of basic information on HIV sequencing laboratory capacities and equipment</b> .....	<b>32</b>
<b>ANNEX 6 : World Health Organization laboratory standard operating procedures (SOP) for post-testing quality assurance of HIV drug resistance genotyping: 2016 update</b> .....	<b>49</b>
<b>Introduction</b> .....	<b>49</b>
<b>Sequence file naming convention</b> .....	<b>50</b>
<b>A. HIV-1 genotyping sequence analysis using Web RECall</b> .....	<b>51</b>
<b>B. Sequence quality assurance using the WHO BCCfE HIVDR QC tool</b> .....	<b>51</b>
<b>C. Drug resistance analysis using Stanford CPR and HIVdb</b> .....	<b>52</b>
<b>Transfer of sequence data to WHO</b> .....	<b>52</b>
<b>A. HIV-1 genotyping sequence analysis using Web RECall</b> .....	<b>53</b>
Background .....	<b>53</b>
Requirements .....	<b>53</b>
Procedure .....	<b>53</b>
<b>B. Sequence quality assurance using the WHO BCCfE QC tool</b> .....	<b>58</b>
Background .....	<b>58</b>
Requirements .....	<b>58</b>
Procedure .....	<b>59</b>
<b>C. Sequence drug resistance analysis using Stanford CPR and HIVdb</b> .....	<b>62</b>
Background .....	<b>62</b>
Requirements .....	<b>62</b>
Procedure .....	<b>63</b>
<b>Appendix 1: Reference sequences</b> .....	<b>66</b>
<b>Appendix 2: QC checklist</b> .....	<b>67</b>

# ACRONYMS AND ABBREVIATIONS

<b>ART</b>	Antiretroviral therapy	<b>NDRL</b>	National HIV Drug Resistance Laboratory
<b>ARV</b>	Antiretroviral drugs	<b>PCR</b>	Polymerase chain reaction
<b>BCCfE</b>	British Columbia Centre for Excellence in HIV/AIDS	<b>PP</b>	Proficiency panel
<b>CDC</b>	Centers of Disease Control and Prevention (United States)	<b>PR</b>	Protease
<b>CPR</b>	Calibrated Population Resistance	<b>QA</b>	Quality assurance
<b>CRF</b>	Circulating recombinant form	<b>QC</b>	Quality control
<b>DBS</b>	Dried blood spot	<b>RDRL</b>	Regional HIV Drug Resistance Laboratory
<b>DNA</b>	Deoxyribonucleic acid	<b>RNA</b>	Ribonucleic acid
<b>DPS</b>	Dried plasma spot	<b>RT</b>	Reverse transcriptase
<b>DSS</b>	Dried serum spot	<b>SDRL</b>	Specialized HIV Drug Resistance Laboratory
<b>EQA</b>	External quality assurance	<b>SOP</b>	Standard operating procedures
<b>HIV</b>	Human immunodeficiency virus	<b>WHO</b>	World Health Organization
<b>HIVDR</b>	HIV drug resistance		
<b>HIVResNet</b>	HIV Drug Resistance Network		
<b>MOH</b>	Ministry of Health		

## ACKNOWLEDGEMENTS

This document was written by Neil Parkin (Data First Consulting, Inc., Consultant, HIV/AIDS Department), Michael R. Jordan (Tufts University School of Medicine, Consultant, HIV/AIDS Department) and Silvia Bertagnolio (WHO, HIV/AIDS Department).

This document was developed with critical input from many WHO-designated laboratories and the WHO HIVDR Laboratory Working Group. In particular, contributions of the following individuals were indispensable:

- Richard Harrigan, James Brooks and Conan Woods, British Columbia Centres of Excellence, Vancouver, BC, Canada;
- Rami Kantor, Brown University, Providence, RI, USA;
- Martine Peeters, UMI 233, TransVIHMI, IRD and UM1, Montpellier, France;
- Paul Sandstrom, Public Health Agency of Canada;
- Rob Schuurman, Department of Virology, University Medical Center Utrecht;
- Robert Shafer and Soo-Yon Rhee, Stanford University, Stanford, CA, USA;
- Anne-Mieke Vandamme, Katholieke Universiteit Leuven, Leuven, Belgium;
- Chunfu Yang and Joshua DeVos, US Centers of Disease Control and Prevention, Atlanta, GA, USA;
- Current and previous members of the WHO HIVDR Laboratory Network and advisory Group.

# EXECUTIVE SUMMARY

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

- Accurate collection, handling, shipment and storage of specimens collected in countries implementing HIVDR surveillance;
- Availability of quality-assured HIV genotyping laboratory services producing comparable and reliable results at the national, regional and global level.

WHO recommends that all countries implementing HIVDR surveys send specimens for HIVDR genotyping to laboratories designated by WHO for this purpose. This document 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;
- The requirements for the submission of HIVDR data to WHO.

The WHO/HIVResNet Laboratory Operational Framework consists of the following four elements:

1. National strategy for HIVDR surveillance laboratory support;
2. WHO/HIVResNet Laboratory Network. The HIVResNet Laboratory Network is responsible for ensuring the delivery of quality-assured HIV genotyping data at the national, regional and global level. The Network includes different categories of membership, with different tasks and responsibilities:
  - National HIVDR Laboratories (NDRL; usually one per country);
  - Regional HIVDR Laboratories (RDRL; usually one per WHO region);
  - Specialized HIVDR Laboratories (SDRL).
3. Standards for specimen collection, handling, shipment, and storage. The Laboratory Network provides guidance documents and laboratory procedures to support standardization of all components of the Laboratory Operational Framework;
4. Laboratory technical support for capacity building.

## Summary of updates

This document is an update of the WHO HIV Drug Resistance Laboratory Strategy published in 2010 and reflects the accumulated knowledge and experience of the Laboratory Network over the last six years. While the core principles and structure of the Network remain unchanged, adjustments were made to the following areas:

1. Standard operating procedures (SOPs) for post-testing quality assurance of HIV sequence data ([Annex 6](#));
2. Minimum required criteria for a laboratory to apply for designation as a member of the Laboratory Network ([Annex 4](#));
3. Addition of a "dry" proficiency panel consisting of raw chromatogram data to support laboratories' capacity to detect quality anomalies and flag them for investigation or repeat testing;



# 1. PRINCIPLES OF THE HIVRESNET LABORATORY NETWORK

## 1.1 Background

The WHO HIV drug resistance (HIVDR) strategy evaluates drug resistance in populations of individuals infected with HIV prior to treatment initiation, among those receiving antiretroviral therapy, and in infants less than 18 months old. Surveillance methods are designed to provide national prevalence estimates of HIVDR in these populations and support optimal regimen selection and programmatic actions. More details about the WHO HIVDR strategy can be found at: <http://www.who.int/hiv/topics/drugresistance/en/>

WHO recommends that all countries implementing HIVDR surveys perform HIVDR genotyping at laboratories designated by WHO for this purpose. The genotyping laboratories designated by WHO are members of the HIVResNet Laboratory Network. This document 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 the submission of HIVDR data to WHO.

## 1.2 Principles of the WHO/HIVResNet Laboratory Operational Framework

The WHO/HIVResNet Laboratory Operational Framework provides a framework within which the WHO HIVResNet laboratories function to support national, regional, and global HIVDR surveillance by the timely provision of accurate genotyping results which meet WHO specifications and are in a standardized format. The aim of the WHO HIVDR Laboratory Operational Framework is to ensure:

- Accurate collection, handling, shipment and storage of specimens collected in countries implementing HIVDR surveillance;
- Availability of quality-assured HIV genotyping laboratory services producing comparable and reliable results at the national, regional and global level.

The WHO/HIVResNet Laboratory Operational Framework consists of the following four elements:

1. **National strategy for HIVDR surveillance laboratory support.** The national HIVDR working group should choose a WHO-designated laboratory to perform testing for HIVDR surveys. This laboratory can be located within the country or at the regional or global level. In countries without a WHO-designated laboratory for HIVDR, or in countries developing capacity and proceeding towards WHO designation, a designated regional or specialized laboratory can provide genotyping services.
2. **WHO/HIVResNet Laboratory Network.** The HIVResNet Laboratory Network is responsible for the delivery of quality-assured HIV genotyping data at the national, regional and global levels. Standardization of laboratory procedures in all laboratories designated by WHO for HIVDR surveillance activities helps achieve these goals. The designation process qualifies laboratories for the purpose of providing quality-assured results to a country implementing HIVDR surveillance.

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

- National HIVDR Laboratories (NDRL; usually one per country);
- Regional HIVDR Laboratories (RDRL; usually one per WHO region);
- Specialized HIVDR Laboratories (SDRL).

The Network is coordinated by WHO, in consultation with the HIVResNet Advisory Group that is composed of representatives of the specialized and regional laboratories and external experts in the field of HIV laboratory science.

### 3. Standards for specimen collection, handling, shipment and storage.

Standardization of collection, handling, shipment and storage of specimens for HIVDR testing is a critical step for production of accurate, comparable results. Accurate genotypic testing depends on appropriate methods of specimen collection and handling, suitable transportation from the collection site to the central laboratory and storage under appropriate conditions.

One of the responsibilities of the national HIVDR working group is to ensure that the national plan for specimen collection, handling, shipment and storage follows WHO HIVResNet guidelines, before HIVDR surveys begin. The preparation and implementation of 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 staff.

The Laboratory Network provides guidance documents describing specimen collection/handling/shipment/storage and laboratory procedures to support standardization of all components of the Laboratory Operational Framework.

### 4. Laboratory technical support for capacity building.

One goal of the WHO/HIVResNet Laboratory Network is to maximize 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.

- Participating in an integrated and harmonized external quality assurance (EQA) scheme that operates in all Network member laboratories (Annex 3);
- Assisting in capacity building and training in laboratories that are seeking to improve their infrastructure and ability to achieve Network membership;
- Engaging in research to develop simple and affordable methods for HIVDR testing; and
- Assisting WHO in the assessment of laboratories seeking Network membership.

Although membership in the WHO Laboratory Network may be important for continued governmental 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, WHO Laboratory Network membership governs the performance of HIVDR 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 individual patient clinical management such as treatment decisions by the patient's care provider. Regulatory standards associated with provision of 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 Laboratory Network.

**WHO grants full membership in the Network** to a laboratory after it demonstrates that it meets the criteria listed in this document. A laboratory that is awarded full membership must demonstrate, at regular intervals, that it continues to meet the criteria. Assessment for full membership takes place 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 Advisory Group, and successful participation in a quality assurance system recognized by WHO. WHO may require re-assessment at any time, based on the laboratory's performance in the Network.

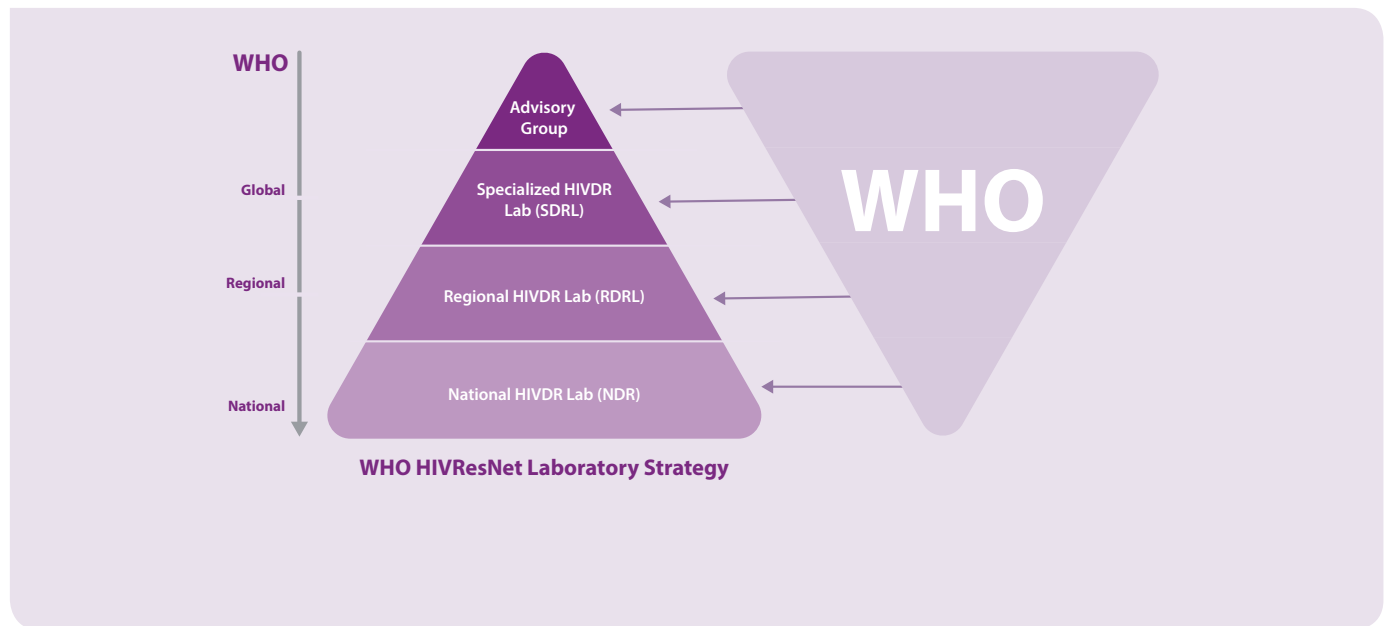
The WHO/HIVResNet Laboratory Network consists of three levels of institutions: National, Regional and Specialized. The overall structure of the Laboratory Network is shown in Fig1.

## 1.3 Structure and function of the WHO/HIVResNet Laboratory Network

The WHO/HIVResNet Laboratory Network supports the implementation of the global HIVDR 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 (Annex 1-2);

**Fig 1. Structure of the HIVResNet Laboratory Network.**



### 1.3.1 National HIVDR laboratories

#### *Requirements*

WHO designates a laboratory as a NDRL to perform HIVDR testing for surveillance activities in the country in which it is located. Before designation by WHO, the national Ministry of Health (MOqH) must first nominate the laboratory for the purpose of supporting a country's surveillance activities.

*Annex 4* outlines the process and detailed requirements for designation as a NDRL. Upon recognition by WHO, a NDRL becomes a member of the WHO/HIVResNet Laboratory Network. Although a NDRL is preferably a public health laboratory with an active role in HIV surveillance, the MOH may also designate other types of laboratories as candidate laboratories for HIVDR genotyping.

#### *Tasks and Responsibilities*

1. The NDRL performs genotyping of specimens collected during HIVDR surveys and provides accurate HIV sequences to the National HIVDR working group in a timely manner;
2. Preferably, the NDRL performs viral load testing for surveys of acquired HIVDR since specimen quantities are often insufficient to allow for 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;
3. The NDRL participates in a WHO-recognized EQA programme for genotyping and is able to support the cost of the testing and shipment of the annual proficiency panel, if required;
4. The NDRL applies appropriate quality assurance/quality control (QA/QC) procedures, including genetic distance or phylogenetic analysis that detects molecular contamination, according to WHO recommendations;
5. The NDRL sends HIV genotyping results in FASTA file format to the national HIVDR working group and to the WHO headquarters for on-going QA;
6. The NDRL supports the country in performing HIVDR analysis using the WHO list of mutations for surveillance of TDR (embedded in the Stanford Calibrated Population Resistance [CPR] tool: <http://cpr.stanford.edu/cpr.cgi>) or the Stanford University HIVdb algorithm (<https://hivdb.stanford.edu/hivdb/by-sequences/>) for all other types of HIVDR surveys;
7. Representatives from the NDRL participate in HIVResNet Laboratory Network regional meetings. These meetings may occur as frequently as once a year and offer an opportunity for discussion of programme development and problem-solving.

### 1.3.2 Regional HIVDR laboratories

#### *Requirements*

A RDRL is an institution designated by its national MOH to support the region's HIVDR surveillance activities. Upon recognition by WHO, the RDRL becomes a member of the WHO/HIVResNet Laboratory Network. Ideally, there should be at least one designated RDRL in each WHO region. In rare cases, more than one RDRL for each region may be

warranted. The RDRL should preferably be in the same region as the surveyed countries. Experience as public health laboratories, although not compulsory, is an asset. The RDRL may serve as the NDRL in its own country.

### *Tasks and Responsibilities*

The following tasks and responsibilities are **in addition** to the ones listed above for NDRL:

1. The RDRL functions as a genotyping facility for countries within the region that do not have a designated NDRL. It must provide support and backup as needed to NDRLs in countries that are implementing WHO-recommended HIVDR surveys;
2. The RDRL, in coordination with WHO and/or a designated Specialized HIVDR Laboratory, facilitates the training, education and capacity building of laboratory personnel from NDRLs within the region. The RDRL hosts laboratory technicians from candidate laboratories and trains them to become competent in HIV genotyping;
3. Representatives from the RDRL are available to visit the NDRLs for technical assistance when necessary;
4. Representatives from the RDRL are available to participate in assessment of candidate laboratories within the specified region, including on-site inspection visits.

### **1.3.3 Specialized HIVDR laboratories**

#### *Requirements*

A small number of laboratories are identified and designated as SDRL by WHO based on:

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

Experience as public health laboratories, although not compulsory, is an asset. As with the national and regional laboratories, WHO carefully assesses candidate laboratories.

### *Tasks and Responsibilities*

In addition to the tasks and responsibilities listed above for NDRL and RDRL, the SDRL must be willing to:

1. Participate in the WHO HIVResNet Laboratory Network Advisory Group and contribute actively to the development of the WHO HIVResNet Laboratory Network;
2. Provide support, technical assistance and backup to National or Regional HIVDR Laboratories, where needed;
3. Serve as a RDRL (and therefore fulfil the roles and responsibilities described above) to countries within a region that do not have a designated RDRL;
4. Serve as a NDRL (and therefore fulfil the roles and responsibilities described above) to specified countries where there is no national designated genotyping laboratory, and:
  - a. The regional laboratory is not able to assist; and/or
  - b. A special relationship between the SDRL and the specified country is already in place.

SDRLs also should actively participate in one or more of the four 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 SDRL must be willing to take responsibility for at least one of the four core activities.

#### *SDRL Core Activities*

##### **1. Capacity building/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 methodologies aimed at improving the feasibility of genotype testing under field conditions;
- Participate in research aimed at improving the sensitivity, specificity, applicability, turn-around-time and reporting of HIVDR testing in HIVDR surveys.

### 3. External quality assurance system

**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 (US NIH). However, should this cease to be the case, the SDRLs will be expected to perform this function as outlined below:

- Coordinate the participation of designated laboratories in a WHO-recognized EQA programme for genotyping, including proficiency panels (PP);
- Coordinate the performance evaluation of laboratories participating in any WHO-recognized proficiency testing programme;
- Assist in the development and supply of WHO-recognized PP;
- Harmonize WHO-recognized QA systems and identify methods for attaining comparative results;
- Coordinate the development and distribution of standardized reagents and validation panels for all laboratories in the Network, as needed.

### 4. Dried blood spot activities

Function as a genotyping reference laboratory for DBS specimens by:

- Coordinating the development of a validated protocol for DBS specimens and sharing the protocol among the laboratories in the Network in order to reach consensus;
- Performing HIVDR testing for countries without a designated laboratory for DBS testing;
- As needed, supporting the development of a DBS-based PP.

#### 1.3.4 WHO HIVResNet Laboratory Advisory Group

The Advisory Group includes a select number of experts in the field of HIVDR surveillance and members of the designated SDRLs, RDRLs and NDRLs. WHO, in consultation with the Advisory Group, coordinates the HIVResNet Laboratory Network.

### Tasks and Responsibilities

1. Harmonize and support the HIVResNet Laboratory Network by providing technical advice, assistance with advocacy and resource mobilization;
2. Assist in the assessment and strengthening of laboratories that have the capacity to function as National, Regional or Specialized HIVDR Laboratories;
3. Meet as needed to review the technical performance of the Network and provide assistance in policy formulation;
4. Provide updates to the Laboratory Operational Framework as needed..

#### 1.3.5 Role of WHO

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

##### 1. Coordination

- Facilitate the linkages, communication and flow of data between NDRL, RDRL and SDRL when necessary;
- Organize laboratory assessments and grant membership in the Laboratory Network;
- Organize meetings of the HIVResNet Laboratory Advisory Group when needed;
- Communicate information relevant to the Network as needed, but at least annually;
- Assure the overall quality of 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 analyses.

##### 2. Financial support

Although the SDRL, RDRL and often the host governments bear much of the financial responsibility for assistance to 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 identified within the Network to visit the candidate NDRL or RDRL. Subsequent visits for the provision of technical assistance may also be supported;



- Shipment of PP in the context of the EQA programme;
- Operational research studies related to core Network priorities;
- WHO may provide letters of endorsement for grant applications to the NDRLs, RDRLs and SDRLs regarding financial support from other sources for HIVDR surveillance-related activities.

## 1.4 Summary of HIVDR genotyping procedures and quality assurance

Genotyping (sequencing) for HIVDR 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<sup>1-4</sup>. Because of their lower cost, in-house assays are often preferred. However, they are associated with additional requirements for quality control (QC) and quality assurance (QA). Although many laboratories in resource-limited settings have experience in genotyping, the lack of standardized procedures and QA steps limits the production of comparable and reliable results. Following completion of the sequencing procedures, careful review of results is necessary 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 QA (Annex 6) as well as the HIVDR Genotyping Training Package, developed jointly by WHO and the United States Centers for Disease Control and Prevention (US CDC) in 2010 (<http://www.who.int/hiv/pub/drugresistance/labtraining/en/index.html>), provide more information on HIVDR Genotyping and Quality Assurance.

---

1. Buckton AJ, Bissett SL, Myers RE, Beddows S, Edwards S, Cane PA, et al. Development and optimization of an internally controlled dried blood spot assay for surveillance of human immunodeficiency virus type-1 drug resistance. *The Journal of antimicrobial chemotherapy*. 2008;62(6):1191-8.

2. Steegen K, Demecheleer E, De Cabooter N, Nges D, Temmerman M, Ndumbe P, et al. A sensitive in-house RT-PCR genotyping system for combined detection of plasma HIV-1 and assessment of drug resistance. *Journal of virological methods*. 2006;133(2):137-45.

3. Zhou Z, Wagar N, DeVos JR, Rottinghaus E, Diallo K, Nguyen DB, et al. Optimization of a low cost and broadly sensitive genotyping assay for HIV-1 drug resistance surveillance and monitoring in resource-limited settings. *PLoS One*. 2011;6(11):e28184.

4. Ziemiak C, Mengistu Y, Ruff A, Chen YH, Khaki L, Bedri A, et al. Use of dried-blood-spot samples and in-house assays to identify antiretroviral drug resistance in HIV-infected children in resource-constrained settings. *J Clin Microbiol*. 2011;49(12):4077-82.

## 2. WHO/HIVRESNET LABORATORY NETWORK MEMBERSHIP

Membership in the Laboratory Network demonstrates that the laboratory has the capacity to provide quality-assured HIV protease (PR) and reverse transcriptase (RT) sequences to the National HIVDR database 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 full membership takes place through on-site laboratory assessment, review of procedures and documentation, review of genotyping assay validation and EQA testing results, and on-going successful participation in a WHO-recognized EQA 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.

### 2.1 Laboratory status definitions

#### 2.1.1 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, has fulfilled all of the mandatory application criteria and has been notified by WHO that it is a candidate laboratory.

#### 2.1.2 Laboratory Network member laboratory

All WHO-designated HIVDR genotyping laboratories are members of the Laboratory Network. Under special circumstances and with the approval of the HIVResNet advisory group, non-designated laboratories that serve a special function may also be Network members.

#### 2.1.3 Provisionally designated laboratory

A laboratory may be provisionally designated in certain circumstances, following a previous successful designation as a Network Laboratory, including during a time period when investigations into quality standards deficiencies are ongoing, or when responses to required improvements are being prepared for evaluation by WHO. Provisionally designated laboratories are required to suspend genotyping activities for WHO surveys until full designation has been restored.

#### 2.1.4 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 laboratory (e.g. the nearest RDRL) 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;
- Continue steps to achieve designation in the future.

A laboratory with the status of "not designated" is eligible to re-apply for the assessment between one and two years after the previous assessment, if the laboratory considers that all criteria are then met. If the laboratory does not reapply within two calendar years of the previous assessment, it will no longer be eligible to re-apply.

#### 2.1.5 Suspended laboratory

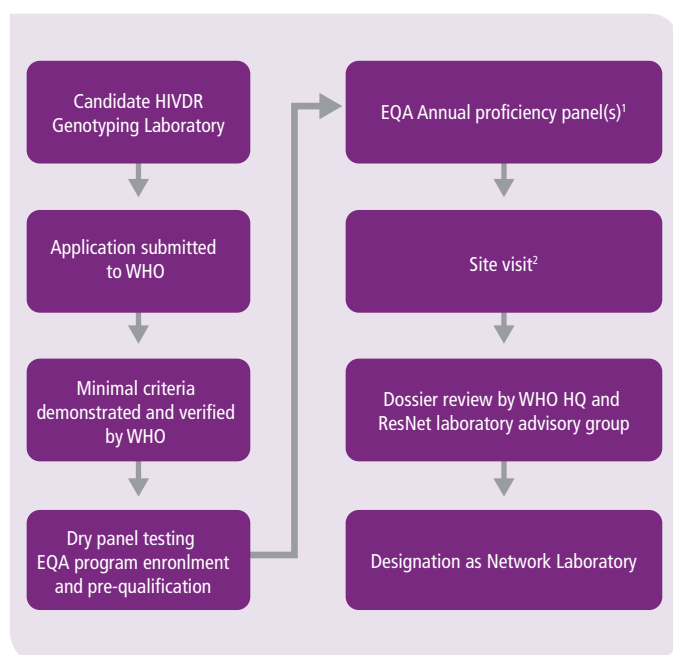
A designated laboratory may be suspended if it fails to demonstrate on-going proficiency through the annual proficiency panel testing programme or if other serious lapses in quality occur. A laboratory at risk of being suspended will be notified and provided with the necessary support to address the problems noted. Suspended laboratories are required to cease genotyping activities for WHO surveys until full designation has been restored. After suspension, a laboratory may re-apply for designation only once and only at the discretion of WHO.

## 2.2 Network membership requirements and review procedure

The evaluation of HIVDR laboratories occurs in the context of the WHO global strategy for HIVDR surveillance (<http://www.who.int/hiv/pub/drugresistance/hiv-drug-resistance-2015-update/en/>) which is a component of the Consolidated Strategic Information Guidelines For HIV in the Health Sector (<http://www.who.int/hiv/pub/guidelines/strategic-information-guidelines/en/>).

To apply for WHO 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 (see Fig. 2). Evidence that the laboratory meets the criteria is essential. Annex 4 shows the minimum requirements for application and criteria for designation as a NDRL, RDRL, or SDRL.

**Fig. 2. : Overview of the Application, Evaluation and Designation Process.**



The procedure for obtaining designation is described below:

### 2.2.1 Application phase

An application process is in place to maximize the efficiency of the assessment. Prior to performing an assessment, a set of application criteria helps determine if all crucial elements for

evaluation during the assessment are present. WHO will also assess the need for adding a laboratory at the requested level (NDRL, RDRL or SDRL) 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 RDRL, nor for a national DRL in a country that is not actively planning to initiate HIVDR surveys according to WHO concept notes.

The **Application Checklist** (Annex 4) helps candidate laboratories determine their readiness for assessment. This checklist is intended to provide guidance to the laboratory on the requirements for proceeding to the laboratory assessment site visit. The completed application checklist, including supporting documentation specified on the checklist, should be sent to WHO. This can be done either directly to the HIV Global HIVDR Programme (WHO headquarters) or through the WHO Country/Regional Office. Materials should be submitted electronically. All application criteria should be fulfilled, and requested documentation provided, in order for the laboratory to be considered for further assessment. If the initial application is deficient in any required documentation, the laboratory can apply again when the missing elements have been provided. WHO headquarters will notify the applicant as to whether all criteria have been met and if so, will begin the assessment procedure.

### 2.2.2 Assessment phase

If the application is considered satisfactory, the candidate laboratory will:

- Complete the pre-assessment questionnaire (see Annex 5). This questionnaire is used to collect information about the laboratory including 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 standard operating procedures (SOPs) or laboratory protocols as requested in the questionnaire. Materials should be submitted electronically to the WHO HIV Department using the following email address: [hiv-aids@who.int](mailto:hiv-aids@who.int);
- After the questionnaire and requested documentation are reviewed and found to be satisfactory, WHO will send a WHO-recognized proficiency panel to the candidate laboratory and arrange an on-site assessment visit by WHO personnel and/or a representative from

1. 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 RDRL and SDRL.

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



the HIVResNet Laboratory Network Advisory Group. During this visit the questionnaire will be audited. The assessment visit usually takes several weeks to co-ordinate, and the proficiency panel shipment, testing and evaluation can take several months to a year.

### 2.2.3 Designation phase

WHO and, if needed, the HIVResNet Laboratory Network Advisory Group will evaluate the laboratory assessment documents and decide whether the candidate laboratory has met the criteria for designation. Candidate laboratories will be granted a status of “designated”, or “not designated”. Designated laboratories become members of the HIVResNet Laboratory Network. Designation is specific for assay and specimen type (i.e. TruGene, ViroSeq or in-house, and for plasma and/or DBS).

### 2.2.4 Continued membership

WHO reviews the status of all designated laboratories annually. This review takes into account the laboratory’s activities and performance during the preceding year(s), the results of the annual WHO-approved PP testing, and may include a field assessment visit when needed (for example, if there are significant changes to laboratory facilities or staff).

WHO-designated laboratories are strongly recommended to submit all final sequences from HIVDR surveys to WHO headquarters for analysis prior to or at the same time as finalization of country HIVDR surveillance reports and FASTA files for return to national working groups. All data are reviewed for overall quality. The quality of the sequence data is one component of the annual performance evaluation.

A list of currently designated HIVDR genotyping laboratories can be found on the WHO website: <http://www.who.int/hiv/topics/drugresistance/en/>

# 3. GUIDELINES FOR THE SUBMISSION OF RESULTS TO NATIONAL HIVDR WORKING GROUPS AND TO WHO

After completion of HIVDR testing, including the procedures described in the World Health Organization Laboratory Standard Operating Procedures (SOP) for Post-Testing Quality Assurance of HIV Drug Resistance Genotyping ([Annex 6](#)), the designated laboratory submits the results to the national HIVDR working group or designated national focal person for the survey. If the designated laboratory does not know the focal point of the national HIVDR working group or survey, it should contact WHO headquarters for clarification. Laboratories must provide an interpretation based on drug resistance mutations found in each sequence using either:

1. The Stanford University algorithm (<https://hivdb.stanford.edu/hivdb/by-sequences/>) for surveys of acquired or pre-treatment HIVDR or surveys of HIVDR in infants <18 months old, or
2. The most recent version of the WHO surveillance mutations list (<http://cpr.stanford.edu/cpr.cgi>) for surveys of transmitted drug resistant HIV.<sup>1</sup>

Analyses using other methods or other standardized algorithms should never be provided to the country HIVDR working groups or to WHO.

All WHO-designated laboratories are strongly recommended to submit sequences to WHO headquarters for additional review preferably prior to, or at the same time as, submission to a national authority. Genotyping results are returned in FASTA format to facilitate standardized QA and HIVDR analysis. Specific procedures for submission of sequence data and associated information are under development. 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 Stanford QA and resistance analysis output, and
- The British Columbia Centre for Excellence in HIV/AIDS QA tool output (see [Annex 6](#)).

## 3.1 Data ownership

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

## 3.2 Data flow from designated laboratories

The genotyping laboratory should submit HIVDR testing (and viral load, if also performed by the genotyping laboratory) results simultaneously to the HIVDR national working group and WHO. 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-upon quality-assured dataset. In some instances, this will involve the national epidemiologist, as individual patient demographic data 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 QA process.

Once the quality-assured dataset is final, it is sent to the country HIVDR Working Group by the designated drug resistance testing laboratory. Country analyses and reporting should use only the final quality-assured sequence and epidemiological dataset.

Some SDRL are expert in QA and may be able to provide national laboratories with needed support and supervision when performing QA following the WHO suggested QA SOP. In such cases NDRL should liaise with WHO to identify a specialized laboratory with QA expertise and twin with this laboratory to optimize its QA assessment.

1. WHO no longer recommends implementation of surveys of transmitted HIVDR (TDR); however, some countries are in the process of completing these surveys at the time of publication of this document.

# ANNEX 1: GUIDELINES FOR HIV DRUG RESISTANCE GENOTYPING

## 1. INTRODUCTION

Nucleotide sequence analysis (genotyping) of relevant portions of the HIV genome, typically the complete protease (PR) region and most of the reverse transcriptase (RT), can identify mutations associated with HIV drug resistance (HIVDR). In addition to using this profile for HIVDR determinations, the genetic sequence can also yield HIV-1 subtype, although complete characterization of some recombinant forms may not be possible.

Genotyping by population sequencing identifies the predominant virus populations in the viral quasispecies. 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 polymerase chain reaction (PCR) or hybridization, are now 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 currently not well standardized and requires significant capital investment.

Genotyping identifies mutations associated with reduced susceptibility to one or more antiviral 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 HIVDR is already more than 150 and various interactions between mutations have been identified, the interpretation of a genotypic resistance profile for clinical purposes can be very complex. Several different genotyping interpretation algorithms have been developed for clinical application. 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. The World Health Organization (WHO) HIV Drug Resistance Network (HIVResNet) published a list of mutations suitable for surveillance of transmitted DR-HIV<sup>1</sup>. WHO surveys of pre-treatment HIVDR in patients initiating antiretroviral therapy (ART), in patients failing ART (acquired HIVDR), and in infants less than 18 months of age use the Stanford University HIVdb interpretation algorithm.

---

1. Bennett DE, Camacho RJ, Otelea D, Kuritzkes DR, Fleury H, Kiuchi M, et al. Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. *PLoS One*. 2009;4(3):e4724.

## 2. SPECIMEN TYPES FOR HIVDR GENOTYPE TESTING

Presently, the specimen types collected for HIVDR genotyping are plasma or dried blood spots (DBS). The National HIVDR working group should consider the use of DBS for HIVDR surveys in which either the volume of residual specimens is limited, plasma cannot be separated and frozen in an appropriate manner, maintaining frozen specimens at a uniform temperature is problematic, or where shipment of frozen specimens to the genotyping laboratory is not feasible. While HIV genotyping based on plasma provides the HIVDR profile of the population of replicating viruses, results generated from whole blood specimens (for example, DBS specimens) may also reflect a contribution from archival HIV deoxyribonucleic acid (DNA) found in latent cellular reservoirs. Although theoretically this may lead to discordance in results generated from DBS and plasma<sup>1</sup>, studies show that genotyping results from plasma and DBS are comparable<sup>2-5</sup> although subtle differences have been observed.<sup>6</sup>

The choice of DBS for HIVDR surveys of acquired HIVDR is a country decision and is taken in consultation with the 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 collection, storage, shipping and processing of DBS for HIVDR genotyping, refer to the WHO manual for HIVDR testing using DBS specimens: <http://www.who.int/hiv/topics/drugresistance/laboratory/en/index.html>.

Dried plasma spot and dried serum spot specimens are not appropriate specimen types for HIVDR monitoring surveys. For more detailed guidance regarding specimen types, please refer to the survey-specific concept notes prepared by WHO.

Using DBS for viral load testing 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 positive or overestimation of viral load from DBS<sup>7</sup>. Preference is for viral load assays that are selective or specific for ribonucleic acid (RNA) over DNA.

For additional information see <http://www.who.int/hiv/pub/arv/viral-load-testing-technical-update/en/>.

### 2.1 Plasma specimen processing

Processing and storage of specimens as soon as possible is important 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 (e.g., at 4°C or on ice), but may also be kept at room temperature (15-30°C) for limited periods of time (e.g., less than six hours). If room temperature is >30°C, an isotherm box should be used to store specimens between 15°C and 30°C.

After separation, plasma specimens should be constantly at refrigeration temperature (4°C) until aliquots are frozen. Plasma for genotyping must be processed and frozen at -20°C to -80°C within 48 hours after the blood draw. Freezing at -80°C is preferable but -20°C is acceptable and not having a -80°C freezer is not an impediment to storing specimens for HIVDR 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 US Centers of Disease Control and Prevention (CDC) provide information on shipment of hazardous material on the following two links: [www.who.int/ihr/publications/who\\_hse\\_ihr\\_2012.12/en/](http://www.who.int/ihr/publications/who_hse_ihr_2012.12/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 prior to shipment of specimens.

1. Chew CB, Potter SJ, Wang B, Wang YM, Shaw CO, Dwyer DE, et al. Assessment of drug resistance mutations in plasma and peripheral blood mononuclear cells at different plasma viral loads in patients receiving HAART. *J Clin Virol.* 2005;33(3):206-16.
2. Ziemiak C, Mengistu Y, Ruff A, Chen YH, Khaki L, Bedri A, et al. Use of dried-blood-spot samples and in-house assays to identify antiretroviral drug resistance in HIV-infected children in resource-constrained settings. *J Clin Microbiol.* 2011;49(12):4077-82.
3. Wei X, Youngpairaj AS, Garrido C, Zahonero N, Corral A, de Mendoza C, et al. Minority HIV mutation detection in dried blood spots indicates high specimen integrity and reveals hidden archived drug resistance. *J Clin Virol.* 2011;50(2):148-52.
4. Bertagnolio S, Parkin NT, Jordan M, Brooks J, Garcia-Lerma JG. Dried blood spots for HIV-1 drug resistance and viral load testing: A review of current knowledge and WHO efforts for global HIV drug resistance surveillance. *AIDS Rev.* 2010;12(4):195-208.
5. Hallack R, Doherty LE, Wethers JA, Parker MM. Evaluation of dried blood spot specimens for HIV-1 drug-resistance testing using the Trugene HIV-1 genotyping assay. *J Clin Virol.* 2008;41(4):283-7.
6. Duc NB, Hien BT, Wagar N, Tram TH, Giang le T, Yang C, et al. Surveillance of transmitted HIV drug resistance using matched plasma and dried blood spot specimens from voluntary counseling and testing sites in Ho Chi Minh City, Vietnam, 2007-2008. *Clin Infect Dis.* 2012;54 Suppl 4:S343-7.
7. Parkin NT. Measurement of HIV-1 viral load for drug resistance surveillance using dried blood spots: literature review and modeling of contribution of DNA and RNA. *AIDS Reviews* 2014;16(3):160-171.

Transport methods depend on a country's infrastructure. Frequently, field staff is responsible for transporting clinical specimens from an ART 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 ART site are not suitable.

Plasma specimens should be frozen at the survey site only when maintenance of the frozen state is guaranteed during the period of transport to a national storage laboratory or to the HIVDR 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 at baseline.

After freezing, if appropriate freezer facilities are available for storage centrally, survey plasma specimens may remain frozen (-20°C°) for up to three years before being sent to the genotyping laboratory. Transport to the genotyping laboratory must take place on dry ice or liquid nitrogen, without thawing of plasma specimens. Therefore, adequate preparation for shipping should be via a courier service experienced in the transport of clinical specimens on dry ice including replenishing dry ice in route and during customs processing, or via transport in liquid nitrogen containers.

Procurement of all customs import and export permits is necessary prior to shipping. An acknowledgement/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.

## 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, DNA, or both, from specific types of specimens. In the selection of the extraction method/technology, the type of clinical specimen and the type of nucleic acid to isolate should be considered. 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 use of 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 100µl of plasma or two DBS spots. Preference is for larger volumes (up to 1ml plasma) in order to increase the amplification sensitivity of the genotypic assay.



### 3. AMPLIFICATION AND SEQUENCING

Once purified, the nucleic acid requires amplification by PCR and subsequent sequencing. Presently two commercial genotyping kits are available: ViroSeq HIV-1 (Abbott Molecular) and GeneThink HIV-1 (Research Think Tank). In addition, the US CDC HIV-1 Pan Group-M Drug Resistance Genotyping Assay will be available for purchase from Thermo Fisher. Many experienced genotyping laboratories have their own in-house ("home-brew") amplification and sequencing procedures and reagents. HIVDR 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 needs a high level of standardization, both in terms of the persons performing the laboratory test as well as in the analysis and interpretation of the genotyping result. Furthermore, extensive genetic differences of HIV-1 exist between the various HIV-1 subtypes. This means the primers used for amplification and sequencing of specimens require validation for adequate performance on various subtypes.

Published studies have evaluated the performance of both the commercially available kits on various HIV-1 subtypes<sup>1-11</sup>. 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 the analysis of sequence data obtained from in-house assays.

Laboratories considering implementation of HIVDR genotyping for the first time should start by using a commercial kit. Before implementing commercial assays, laboratories should also obtain information on the

performance of these assays on the local circulating variants. After establishment of a commercial kit assay, laboratories should evaluate any home-brew assays by comparison of results to those obtained from the commercial kit prior to implementation for routine use.

#### *Amplification and sequencing recommendations:*

- Kit-based sequencing procedures are preferable to home brew assays for laboratories that are newly initiating genotyping;
- "Home-brew" or in-house assays should be implemented only after adequate validation, including evaluation with regard to performance with various HIV-1 subtypes;
- Establishing a high level of standardization is necessary for laboratories performing genotyping. This includes standardization of staff training, peer reviewed SOPs, workflow and other aspects of operation;
- The minimal regions for which collection of sequence information is essential are:
  - PR: codons 10 to 93
  - RT: codons 41 to 238
  - IN: codons 51 to 263

- 
1. Eshleman SH, Hackett J, Jr., Swanson P, Cunningham SP, Drews B, Brennan C, et al. Performance of the Celeris Diagnostics ViroSeq HIV-1 Genotyping System for sequence-based analysis of diverse human immunodeficiency virus type 1 strains. *J Clin Microbiol.* 2004;42(6):2711-7.
  2. Maes B, Schrooten Y, Snoeck J, Derdelinckx I, Van Ranst M, Vandamme AM, et al. Performance of ViroSeq HIV-1 Genotyping System in routine practice at a Belgian clinical laboratory. *Journal of virological methods.* 2004;119(1):45-9.
  3. Ribas SG, Heyndrickx L, Ondoa P, Franssen K. Performance evaluation of the two protease sequencing primers of the Trugene HIV-1 genotyping kit. *Journal of virological methods.* 2006;135(2):137-42.
  4. Tong CY, Mullen J, Kulasegaram R, De Ruiter A, O'Shea S, Chrystie IL. Genotyping of B and non-B subtypes of human immunodeficiency virus type 1. *J Clin Microbiol.* 2005;43(9):4623-7.
  5. Fontaine E, Riva C, Peeters M, Schmit JC, Delaporte E, Van Laethem K, et al. Evaluation of two commercial kits for the detection of genotypic drug resistance on a panel of HIV type 1 subtypes A through J. *Journal of acquired immune deficiency syndromes (1999).* 2001;28(3):254-8.
  6. Grant RM, Kuritzkes DR, Johnson VA, Mellors JW, Sullivan JL, Swanstrom R, et al. Accuracy of the TRUGENE HIV-1 genotyping kit. *J Clin Microbiol.* 2003;41(4):1586-93.
  7. Hallack R, Doherty LE, Wethers JA, Parker MM. Evaluation of dried blood spot specimens for HIV-1 drug-resistance testing using the Trugene HIV-1 genotyping assay. *J Clin Virol.* 2008;41(4):283-7.
  8. Jagodzinski LL, Cooley JD, Weber M, Michael NL. Performance characteristics of human immunodeficiency virus type 1 (HIV-1) genotyping systems in sequence-based analysis of subtypes other than HIV-1 subtype B. *J Clin Microbiol.* 2003;41(3):998-1003.
  9. Kuritzkes DR, Grant RM, Feorino P, Griswold M, Hoover M, Young R, et al. Performance characteristics of the TRUGENE HIV-1 Genotyping Kit and the Opengene DNA Sequencing System. *J Clin Microbiol.* 2003;41(4):1594-9.
  10. Beddows S, Galpin S, Kazmi SH, Ashraf A, Johargy A, Frater AJ, et al. Performance of two commercially available sequence-based HIV-1 genotyping systems for the detection of drug resistance against HIV type 1 group M subtypes. *J Med Virol.* 2003;70(3):337-42.
  11. Mracna M, Becker-Pergola G, Dileanis J, Guay LA, Cunningham S, Jackson JB, et al. Performance of Applied Biosystems ViroSeq HIV-1 Genotyping System for sequence-based analysis of non-subtype B human immunodeficiency virus type 1 from Uganda. *J Clin Microbiol.* 2001;39(12):4323-7.

## 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:

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

## 5. PREFERRED GENOTYPING METHODOLOGIES

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

### 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 sample extraction, amplification and sequencing of the entire PR coding region and most of the RT region (amino acids 1-320). A separate kit for integrase is also available.

#### *Requirements*

- PCR grade laboratory design;
- Ultracentrifuge for concentrating virus;
- Sequence detection hardware: capillary electrophoresis equipment is recommended, as it is simple to use and generally well suited for diagnostic use. Proper use of the equipment requires training;
- Gel-based sequencers are an alternative to capillary systems, but require extensive training and experience and are less suitable for diagnostic use;
- Sequence analysis software (provided by Abbott).

### 5.2 GeneThink™ HIV-1 Genotyping Kit

Vendor: Research Think Tank, <http://www.researchthinktank.com/>

The integrated genotyping system consists of a kit, protocols, controls, reagents, sequencing instrument, product support and a fully integrated reporting software for the amplification and sequencing of the protease coding region (amino acids 10-99) and the clinically relevant portion of the RT region (amino acids 41-237). A separate kit for integrase is also available.

#### *Requirements*

- PCR grade laboratory design;
- Sequence detection hardware (RTT MolecularDx USA, Inc.). Moderate experience and training are required, though the system is well suited for diagnostic use;
- Sequence analysis software provided by the company, an automated rules-based clinical resistance report and/or research report.

## 5.3 US CDC HIV-1 Pan Group-M Drug Resistance Genotyping Assay

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

The HIV-1 Drug Resistance Genotyping Kit is for use in detecting HIV genomic mutations that confer resistance to specific types of antiretroviral drugs, as an aid in monitoring HIV-1 drug resistance development and transmission. It is based on methods developed by the US CDC.<sup>1,2</sup> 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 & sequence analyses:

- Module 1: RT-PCR & Nested PCR;
- Module 2: Cycle Sequencing.

### Requirements

- PCR grade laboratory design;
- Sequence detection hardware: capillary electrophoresis equipment is recommended, as it is simple to use and generally well suited for diagnostic use. Proper use of the equipment requires training;
- RNA purification must be performed using reagents or kits available from other vendors;
- Sequence analysis software (not provided). A base-calling software package developed by the BCCfE in HIV/AIDS called RECall is available without cost to WHO genotyping laboratories (see Annex 6).

## 5.4 “Home-brew” or “in-house” Sequencing Methods

Several laboratories specializing in HIV-1 drug resistance have developed “in-house” methods (often referred to as “home-brew” methods) that use 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, particularly in that changes (such as alternative primers) can be more easily implemented when required.

### Requirements

- PCR grade laboratory design;
- Extensive inter-laboratory validation of all aspects of the sequencing procedure, including extraction and sequence hardware;
- Performance on various subtypes may vary per laboratory and is dependent on the primers included in the specific “home-brew” protocol;
- Sequence analysis software. A base-calling software package developed by the BCCfE in HIV/AIDS called RECall is available without cost to WHO genotyping laboratories (see Annex 6).

## 5.5 Choice of resistance testing methodology

For surveillance purposes, any of the genotyping assays listed above are acceptable. The choice of which method to use should be made by each laboratory based on consideration of laboratory personnel experience, kit and reagent availability, cost, and infrastructure. If laboratory capacity is not available in country and planners wish to develop such capacity, a WHO-designated laboratory can assist with protocol development. All HIVResNet Laboratory Network laboratories should have detailed and approved laboratory protocols for procedures to allow the collection of comparable genotyping information.

1. Yang C, McNulty A, Diallo K, Zhang J, Titanji B, Kassim S, et al. Development and application of a broadly sensitive dried-blood-spot-based genotyping assay for global surveillance of HIV-1 drug resistance. *J Clin Microbiol.* 2010;48(9):3158-64.

2. Inzaule S, Yang C, Kasembeli A, Nafisa L, Okonji J, Oyaró B, et al. Field evaluation of a broadly sensitive HIV-1 in-house genotyping assay for use with both plasma and dried blood spot specimens in a resource-limited country. *J Clin Microbiol.* 2013;51(2):529-39.



## 6. HIVDR INTERPRETATION

For surveys of pre-treatment HIVDR, acquired HIVDR, and HIVDR in infants less than 18 months old, assessment of the impact of mutations is through use of 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.

WHO and the HIVResNet published a list of HIVDR mutations to be used for surveillance of transmitted HIVDR<sup>1</sup>. All WHO surveys assessing transmitted drug-resistant HIV should use this HIVDR surveillance mutations list as well as the CPR tool on the Stanford University HIVdb website (<http://cpr.stanford.edu/cpr.cgi>).

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

---

1. Bennett DE, Camacho RJ, Otelea D, Kuritzkes DR, Fleury H, Kiuchi M, et al. Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. *PLoS One*. 2009;4(3):e4724.

# ANNEX 2: LABORATORY SAFETY

## GUIDELINES TO PREVENT TRANSMISSION OF HIV IN THE LABORATORY

Laboratories should observe universal precautions for ALL blood and body fluid specimens. These precautions are described in the supplement to the US Centers of Disease Control and Prevention (CDC) Morbidity and Mortality Weekly Report<sup>1</sup>. They are further expanded and updated in subsequent publications<sup>2,3</sup>. Every laboratory should have a copy of these guidelines and observe the recommendations. Additional guidance for safe laboratory practices follows here.

1. Employ appropriate barrier precautions to prevent skin and mucous membrane exposure when contact with blood or other body fluids of any person is anticipated;
  - Wear gloves when performing venipuncture and other vascular access procedures;
  - Use gloves for performing finger stick tests on children and adults and heel stick tests on infants;
  - Change gloves and wash hands after contact with each patient;
  - Place all specimens of blood and body fluids in containers that will prevent leakage during transport. Avoid contaminating the outside of the container and the laboratory form, which accompanies the specimen. (Note: Whole blood dried on filter paper has not been shown to present a hazard when mailed in paper envelopes. See the specimen collection and storage section for more information);
  - Wear gloves when processing blood and body fluid specimens. Remove gloves and wash hands with soap and water upon completion of specimen processing.
2. If hands or other skin surfaces become contaminated with blood or other body fluids, wash them immediately and thoroughly with soap and water;
3. Employ a biological safety cabinet for procedures that have a high potential for generating droplets (blending, sonicating, vigorous mixing);
4. Use mechanical pipetting devices to manipulate all liquids in the laboratory. **DO NOT PIPETTE BY MOUTH**;
5. Take precautions to prevent injuries caused by needles, scalpels, and other sharp instruments;
  - Do not recap needles, bend or break needles by hand, or remove needles from disposable syringes;
  - Discard all sharp instruments in puncture resistant containers located close to the work area;
  - Limit use of needles and syringes to situations in which there is no alternative.
6. Decontaminate laboratory work surfaces at least daily with a freshly prepared chemical germicide such as a 1:10 dilution of household bleach (this dilution has a final concentration of 0.5% sodium hypochlorite). If bleach is used, dilutions should be mixed daily, as bleach loses its effectiveness within 24 hours when diluted. Other commercially available disinfectants can also be used (dilute as indicated by manufacturer);

1. CDC. Recommendations for prevention of HIV transmission in health-care settings. Centers for Disease Control; 1987 Aug 21. Report No.: 0149-2195

2. CDC. Update: universal precautions for prevention of transmission of human immunodeficiency virus, hepatitis B virus, and other bloodborne pathogens in health-care settings. Centers for Disease Control; 1988 Jun 24. Report No.: 0149-2195

3. CDC. Recommendations for preventing transmission of human immunodeficiency virus and hepatitis B virus to patients during exposure-prone invasive procedures. Centers for Disease Control; 1991 Jul 12. Report No.: 1057-5987

7. To decontaminate equipment that may come in contact with blood or body fluids:
  - Disinfect refrigerators by cleaning thoroughly and then wiping with 1:10 dilution of household bleach;
  - Disinfect centrifuge components by swabbing head, bowl and carriers with 70% ethanol;
  - Autoclave or soak specimen racks in a 1:10 dilution of household bleach for 5 minutes and then rinse thoroughly with water;
  - Discard as hazardous waste any disposable components of instrument systems that come in contact with patient specimens. Clean non-disposable components with 70% ethanol;
  - Allow disinfectant to remain in contact with surfaces for at least five minutes at an ambient temperature for optimal effectiveness against blood or plasma;
  - If equipment needs maintenance, clean and decontaminate it in the laboratory before transporting it to the manufacturer for repair.
8. Use special precautions in handling microbiological laboratory waste, pathology waste and blood specimens or blood products. To decontaminate spills of blood and body fluids:
  - Wear disposable gloves;
  - Cover visible blood or body fluids with paper towels and soak with a 1:10 dilution of household bleach. Allow to stand for at least five minutes;
  - Discard contaminated towels in infectious waste containers;
  - Wipe down the area with clean towels soaked in a 1:10 dilution of household bleach;
  - Incinerate or autoclave all waste before disposal in a sanitary landfill. Solutions containing bleach may corrode the autoclave; therefore these solutions may be poured down a drain connected to a sanitary sewer;
  - After decontaminating, carefully pour bulk blood, suctioned fluids, excretions, and secretions down a drain connected to a sanitary sewer.
9. Wash hands thoroughly after completing laboratory activities. Remove protective clothing before leaving the laboratory.

# ANNEX 3: WHO HIVDR EXTERNAL QUALITY ASSURANCE AND INTERNAL QUALITY CONTROL

## 1. EXTERNAL QUALITY ASSURANCE

To obtain and maintain membership in the World Health Organization (WHO) HIV drug resistance (HIVDR) Laboratory Network, a laboratory must participate in a WHO-recognized genotyping external quality assurance programme that includes proficiency panel (PP) testing. WHO and its partners and/or selected Specialized HIVDR Laboratories (SDRL) are responsible for developing and distributing PP 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 (EQA) programmes. Laboratories should successfully pass a minimum of one PP per year. EQA programmes should take into account the distribution of HIV genetic variants and subtypes in different areas of the world, as well as other virological characteristics.

The recommended characteristics for the HIVDR genotyping PP are:

- PP should include only HIV viruses derived from diluted clinical samples or cell culture propagation;
- PP should contain samples with several drug resistance-associated mutations in both the reverse transcriptase (RT) and protease (PR) regions;
- PP 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 should be included;
- Samples should have a minimum viral load of 2000 copies/ml;
- Viruses chosen should be compatible with all commercial assays. All samples from the panel should be validated using commonly used commercial kits before distribution;
- PP may include a maximum of one sample containing an equal mixture of two defined virus variants;
- A minimum of 10 laboratories should test each panel in order to yield a meaningful consensus sequence.

### 1.1 Data analysis and scoring

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 PR, 41-238 of RT, and 51-263 of IN, is reported as the number of concordant nucleotides out of the total number of unambiguous bases in the consensus.

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

Laboratories will receive a summary of overall sequence concordance and DRM site scores for each specimen in the panel, as well as average scores for each dataset and sequence alignments for each specimen PR and RT, with discrepancies highlighted and scores calculated.

The definition of "concordance" is dependent 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 (e.g., R vs. A or C vs. Y, but not G vs. Y or R vs. M).

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

		Consensus					
		Wild-type unmixed	Mutant unmixed	Mixed* (A, wt)	Mixed (A, mut)	Mixed (B, wt+mut)	Mixed (C, >1 mut)
Test Sequence	Wild-type unmixed	1	0	1	0	0	0
	Mutant unmixed	0	1	0	1	1	0
	Mixed* (A, wt)	1	0	1	0	0	0
	Mixed (A, mut)	0	1	0	1	1	0
	Mixed (B, wt+mut)	0	1	0	1	1	0
	Mixed (C, >1 mut)	0	0	0	0	0	1

### \*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*

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 aligned against the consensus sequence as normal. When calculating the alignment score, each dash is given a gap penalty of 10 (i.e., 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 (i.e., 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;

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

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

## 2. INTERNAL QUALITY CONTROL

Internal quality control (IQC) measures include the use of positive and negative control reagents during sample extraction, amplification and sequencing. Although IQC 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 have to be verified during the designation process and at subsequent site visits. IQC also comprises stringent control of changes in assay procedures including equivalency testing and potentially re-validation of an assay when procedures, equipment, reagents or facilities are changed.

### 2.1 Recommendations

- IQC, consisting of positive and negative control materials, is necessary throughout the entire laboratory procedure, from the start of specimen extraction to the final sequencing. For example, a limited number of samples should be processed in each extraction, amplification and sequencing round (a maximum of twelve samples including a positive and a negative control). This recommendation pertains in particular to laboratories using manual processes and may not be practical for laboratories that use automated instrumentation for these activities;
- Laboratory results for specimens can be accepted only when the results of the internal controls meet the predetermined acceptance criteria;
- The quality of the sequence is a consideration when accepting or rejecting a laboratory result. Characteristics of acceptable sequences are:
  - Less than 5% of the raw sequence file requires editing;
  - Less than 2% of nucleotides are mixed.

Sequences not meeting these criteria should receive additional review by the supervisor, as in rare circumstances these findings may be correct (e.g., end-stage infection with many quasispecies, many HIVDR mutations, co-infection 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 HIVDR sequencing quality control (QC). This tool accepts sequences in FASTA format (e.g., as generated by RECall) and performs a series of QC checks including genetic distance ([http://pssm.cfenet.ubc.ca/who\\_qc](http://pssm.cfenet.ubc.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 (e.g., positive control viruses) that may be in the laboratory. See Annex 6 for additional details.

# ANNEX 4: REQUIREMENTS FOR APPLICATION TO WHO FOR HIV DRUG RESISTANCE LABORATORY NETWORK MEMBERSHIP

## 1. NATIONAL HIVDR LABORATORIES

### 1.1 Mandatory criteria to apply for membership

In order to apply for membership, the following mandatory criteria must be met:

1. Existence of a national plan for implementation of HIV drug resistance (HIVDR) surveillance. The country where the laboratory is located must have a national strategy in place for the implementation of World Health Organization (WHO) recommended HIVDR surveys. This strategy must be consistent with WHO recommendations;
2. Following the establishment of the National HIVDR surveillance plan, the need for laboratory support must be considered. One National WHO DR laboratory per country is usually appropriate, if the laboratory is likely to meet the required standards (criterion #5). Justification for more than 1 National Drug Resistance Laboratory (NDRL) per country must be provided and approved by WHO headquarters;
3. The Ministry of Health is responsible for nominating candidate National and Regional HIVDR laboratories. The Ministry of Health (MOH) must nominate the laboratory as the candidate National laboratory for the purpose of genotyping specimens collected during HIVDR surveillance in that country or region. The MOH must provide a letter of nomination that clearly states the commitment by the Ministry to use this laboratory for WHO HIVDR surveys and to support the costs of the laboratory testing;
4. At least one year experience in genotyping HIV AND more than 100 specimens tested with satisfactory results;
5. Minimum infrastructure for HIVDR 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 minimum required equipment (sequencer, PCR machine, refrigerators, -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 (GLP) 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. SOPs must be developed internally following Clinical Laboratory Standards Institute standards (or adapted from another lab in the network), specific to the context in which the laboratory is located (i.e., not copied from package inserts), and controlled according to quality assurance (QA) standards (version control, senior level management review and approval, system in place for training and following updates, etc.);
  - d. An automated or semi-automated data management system that precludes reliance on manual transcription or re-entry of data such as specimen identifiers, resistance interpretations, etc..



Criteria 6-7 must then be met in order 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 QA Procedures or demonstrated equivalent process. Post-testing steps include base calling using RECall, QA/quality control (QC) checks using the British Columbia Centre for Excellence in HIV/AIDS QC tool, Stanford HIVdb website, and resistance interpretation and reporting;
  - 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. See dry panel description and acceptance criteria for more details (under development).
7. Successful participation in the WHO HIVDR Proficiency Testing Programme. The VQA analysis and criteria, with some modifications (including testing for PCR cross-contamination), will be used.

## 1.2 Additional criteria for evaluation during the assessment

1. Complete laboratory infrastructure and equipment for genotyping:
  - 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, and electric power back up
  - Space configuration, workflow and contamination control is consistent with good laboratory practices
  - Adequate genotyping capacity, including sequence editing and confirmation
  - Computational capability, including hardware, software and Internet access
  - Regular and consistent temperature monitoring records for incubators, refrigerators, PCR machine and freezers
  - Sufficient inventories and timely replenishment of supplies
2. Adequate expertise of laboratory personnel:
  - Minimum biosafety level of two in the areas where the specimens are handled (standard procedure for handling bio-hazardous materials);
  - Laboratory supervisor with specific training in the area of molecular virology;
  - Laboratory technicians with training and experience in HIVDR genotyping, including review and editing of sequences;
  - Presence of a dedicated safety officer;
  - Adequate number of trained staff to handle anticipated workload;
  - Standard operating procedures (SOP) clearly state that supervisor/designee critically reviews test results and confirms the result report by signing it.
3. The laboratory:
  - Clear and accountable laboratory management structure;
  - Financial sustainability of the HIVDR activities in the laboratory;
  - The 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 SOPs 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 the handling of infectious waste;
  - Data management;
  - Post-testing Sequence Quality Assurance, including detection, containment and control of molecular contamination.



## 6. Proficiency panel testing:

- Successful participation in HIVDR 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 Network membership (see [Annex 3](#)).

**The candidate laboratory will become a Laboratory Network Member if it meets all the mandatory criteria (1.1 above) AND achieves a passing score of  $\geq 85/100$  based on the additional criteria (1.2 above) AND successfully passes the WHO HIVDR proficiency panel programme.**

## 1.3 Checklist: Mandatory criteria

- National plan for HIVDR surveillance implementation;
- Need for laboratory support established;
- Ministry of Health designation letter;
- At least 1 year of experience in genotyping HIV, AND  $\geq 100$  specimens tested with satisfactory results;
- Minimum infrastructure for HIVDR genotyping in place;
- Demonstrated proficiency with sequence data quality assurance, management and reporting;
- Successful participation in the WHO HIVDR Proficiency Testing Programme.

## 1.4 Documentation to be submitted to WHO

The laboratory should submit the following documentation in electronic format (Adobe pdf preferred):

- Letter of support from the MOH indicating the laboratory has been identified to test specimens collected during WHO-recommended HIVDR surveys;
- Maintenance records and service contract for major equipment;
- Map of the genotyping facility;

- CVs of genotyping laboratory personnel (including supervisor) documenting qualifications and experience in molecular biology;
- Description of the management structure of the genotyping laboratory personnel;
- Information on the financial sustainability of HIVDR genotyping activities in the past five years;
- Record/documentation of the sequencing tests performed in the last two years. Both in-house methods and commercial kits will be considered;
- Copies of the reports of proficiency panel testing from providers other than WHO in the past year;
- Copies of Standard Operating Procedure (SOPs) including: (1) specimen receipt, assessment and storage; (2) internal quality control; (3) all steps of genotyping tests, including sequencing; (4) handling and manipulation of infectious human material, including the handling of infectious waste; (5) data management; and (6) post-testing Sequence Quality Assurance.

Together with the application checklist, the laboratory must complete a pre-assessment questionnaire of lab equipment and infrastructure (see [Annex 5](#)). The laboratory must send an electronic copy of the questionnaire and application checklist, as well as the documentation specified on the checklist, to:

WHO HIV Department  
[hiv-aids@who.int](mailto:hiv-aids@who.int)

If the initial application is deficient in any required documentation, the laboratory can apply again when the missing criteria are provided. WHO HQ will notify the applicant whether all criteria are met and will begin the assessment procedure.

Upon completion of the checklist and fulfilment of all the mandatory application criteria, WHO will schedule an assessment visit. If the assessment visit finds that the majority of the evaluation criteria are met, WHO will coordinate shipment of a WHO-recognized proficiency panel.

WHO and the Laboratory Advisory Group of the HIVDR Laboratory Network will evaluate the performances of the assessed laboratory and provide recommendations regarding Network membership.

## 2. REGIONAL HIVDR LABORATORIES

WHO will consider new applications for Regional DR Laboratories (RDRL) only if the laboratory can provide an unmet function in the network (refer to RDRL Terms of Reference).

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

The seven mandatory criteria listed above in the section on National HIVDR Laboratories also apply to new applications for Regional HIVDR Laboratories. In addition, an eighth criterion applies to Regional HIVDR Laboratory applications:

1. Capacity for dried blood spot (DBS) genotyping is demonstrated by:
  - a. Experience with DBS (at least six months of experience in DBS-based genotyping with at least 100 DBS specimens successfully amplified and sequenced);
  - b. A procedure for genotyping from DBS is in place and validated according to WHO recommendations;
  - c. Successful participation in the WHO HIVDR DBS Proficiency Testing Programme.

During the assessment, the following additional requirements apply to Regional HIVDR Laboratories:

- 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 persons, contact details and type of collaboration for each lab). WHO will contact the laboratories to confirm the suitability of the applicant laboratory to function as a regional laboratory;
- Adequate experience in provision of training and establishment of collaborations in laboratory sciences in the last 3 years;
- Ability to provide reference virology services to other laboratories.

## 3. SPECIALIZED HIVDR LABORATORIES

Only laboratories that provide an unmet function in the network may submit an application to become a Specialized HIVDR Laboratory (SDRL; refer to SDRL Terms of Reference).

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 SDRL should already host WHO reference activities outside of HIVDR and have sufficient medical, scientific and technical resources to respond to demands for training, laboratory testing and advice on short notice.

All of the mandatory criteria described in the section on Regional HIVDR Laboratories apply for SDRL, except the requirements for a national plan for implementation of HIVDR surveillance and the Ministry of Health designation.

During the assessment, the following additional requirements apply to Specialized HIVDR Laboratories:

- The candidate laboratory must have internationally recognized experience and leadership in HIVDR 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 lab to function as a specialized laboratory;
- The candidate laboratory must prove that it conducts original research in laboratory aspects of HIVDR, as demonstrated by:
  - The laboratory's peer-reviewed publications from the last 5 years in which laboratory staff are included as authors;

- A list of funding awards for on-going collaborative HIVDR research in the last 5 years.
- The laboratory must demonstrate willingness to share information and work cooperatively with WHO and with other HIVDR Network laboratories.
- The laboratory should provide a description of previous collaborative work with WHO in the field of HIV, and specifically on HIVDR;
- If applicable, the laboratory must provide a description of any potential problem that may occur by sharing data/information with WHO and other laboratories in the network;
- The laboratory must attach a detailed description on its potential role and function within the HIV Drug Resistance Network.

Experience in providing laboratory support to large-scale HIVDR 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 aim of the HIV Drug

#### Resistance Laboratory Network:

- Design and validation of novel DR assays and relevant surveillance technologies (e.g., point mutation assays, cheap methodologies for genotyping, genotyping using dried fluid spots, deep sequencing, etc.);
- Development, distribution and evaluation of quality assurance systems;
- Production and organization of training materials/modules and educational programmes for HIVDR;
- Reference laboratory activities for HIVDR surveillance (list of number of specimens tested for the HIVDR surveillance and number of countries assisted);
- DBS research including DBS method development and testing (please attach description of planned studies, if applicable).

# ANNEX 5: QUESTIONNAIRE FOR THE COLLECTION OF BASIC INFORMATION ON HIV SEQUENCING LABORATORY CAPACITIES AND EQUIPMENT

Laboratory Name	_____
	_____
Street Address	_____
	_____
City	_____
Country	_____
Director of Department or Institution	_____
Email	_____
Laboratory Director	_____
Email	_____
Contact person for HIVDR assessment visit	_____
	_____
Position of contact person	_____
Phone	_____
Fax	_____
Email	_____
Date of questionnaire completion	_____

As part of efforts to organize the World Health Organization HIV Drug Resistance Global Laboratory Network, the existing capacities for HIV drug resistance sequencing of your laboratory will be evaluated. Please answer the following questions by checking the appropriate boxes or filling in the appropriate number or text.

---

If desired, this questionnaire can be filled out using Microsoft Word; please contact WHO to obtain a copy of the questionnaire in this format.

## A. GENERAL INFORMATION

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

- Yes
- No

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

- Clinical care
- Research
- Public health/Epidemiological purposes

3. How many years of experience does the laboratory have performing HIVDR sequencing?

\_\_\_\_\_ years

4. How many HIV-1 DR 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 Ministry of Health?

- Yes
- No

6. If not, is it a private laboratory?

- Yes
- No

Specify type: \_\_\_\_\_

## B. PERSONNEL

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

Name	Qualification/training*	Time dedicated

\*e.g., General Technician, Technician Molecular Diagnostics, Biomedical Scientist, Physician.

8. Is a safety officer in place?

- Yes  
 No

9. Please provide a management structure of the laboratory/institution. (Attach a separate file).

10. Please provide indications for the financial sustainability of the laboratory.

---



---



---



---



---



**12.** Does the laboratory participate in an External Quality Assurance programme for HIVDR sequencing?

- Yes
- No

If **Yes**, please specify:

Name of Programme: \_\_\_\_\_

Name of Provider: \_\_\_\_\_

Date of participation: \_\_\_\_\_

*Please submit a summary of Proficiency Panel results from the last 2 years along with this questionnaire.*

**13.** Indicate the frequency of power failure:

\_\_\_\_\_ per year

Is backup available in case of power failure?

- Yes
- No

## D. BIOSAFETY

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

- Yes
- No

If **Yes**, please provide name/number of the SOP or Laboratory Protocol and submit a copy for review:

Name/number: \_\_\_\_\_

**15.** Are laboratory disinfection procedures in place?

- Yes
- No



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

Workspace/equipment	Procedure/disinfecting agent	Frequency

## E. DEDICATED MOLECULAR DIAGNOSTIC WORKSPACE AND WORKFLOW

**17.** Are there separate laboratories/rooms/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/high-copy number DNA (post-amplification)?

- Yes
- No

**18.** Is a strict “unidirectional workflow” respected by all laboratory personnel?

- Yes  
 No

If **Yes**, please submit copies of SOPs/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 SOPs/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/strategy that the laboratory uses. (Add additional rows as needed.)

Workspace/equipment	Procedure/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/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 <sup>2</sup> 21,000-25,000 g			

<sup>2</sup> in case 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/tap water			
Dead Air Cabinet (nested reaction)			
Thermal cyclers			
Agarose gel apparatus			
Photo documentation of agarose gel			

Post-amplification Equipment	Type	Year of purchase	Maintenance type and frequency
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 organized:

---



---



---

**25.** Indicate the presence of the following materials for Bio-Safety in the separate workspaces by a "+".

Workspace	Gloves	Paper lab coats
Pre-amplification/extraction		
Pre-amplification/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. SPECIMEN

**29.** What types of specimens does the laboratory use for HIVDR sequencing? (Check all that apply.)

- Ethylenediamine tetra-acetic 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/Serum Spots for HIVDR 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/Serum Spots for HIVDR sequencing, please indicate storage conditions and detailed information on the processing of the specimen, including extraction, amplification and sequencing (quantities used, conditions, etc.). If an SOP 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/Serum Spots for HIVDR sequencing, please indicate the number of specimens tested annually in the last 2 years and years of experience in genotyping using DBS/DPS/DSS.

Number of specimens tested annually in last 2 years: \_\_\_\_\_  
Years of experience genotyping using DBS/DPS/DSS: \_\_\_\_\_

## H. SPECIMEN REGISTRATION

**33.** Indicate the information present on the stored specimens used for HIVDR 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 specimen registration. (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
- Patient date of birth
- Patient age group
- Patient antiretroviral treatment 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/number of dried fluid spots
- HIV confirmation date
- Specimen storage location

Other: \_\_\_\_\_

# I. SEQUENCING METHODS

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

Extraction method	Manufacturer	Specimen type	Starting volume

**38.** Method for HIV-1 DR sequencing. (List all methods used.)

- Kit-based assay: Version: \_\_\_\_\_  
Version: \_\_\_\_\_
- Home-brew (in-house developed) assay

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

---



---



---

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

a. Reverse transcriptase (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. 1st PCR assay conditions:

- from a published reference  
 designed by laboratory

e. 2nd PCR assay conditions:

- vd reference  
 designed by laboratory

*If applicable, lab must provide documentation of references used.*



**41.** Has the method been validated in the laboratory?

- Yes
- No

If **Yes**, please attach a summary of the method validation 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:

For protease: Codons \_\_\_\_\_ to \_\_\_\_\_

For reverse transcriptase: Codons \_\_\_\_\_ to \_\_\_\_\_

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

- Yes
- No

**44.** What is the minimal viral load required for sequencing?

\_\_\_\_\_ copies/ml

How was it determined?

---

---

---

---

**45.** Is the preservation time for reagents controlled?

- Yes
- No

**46.** How are specimens or derivatives kept cooled during sequencing?

---

---

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

\_\_\_\_\_ days

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: \_\_\_\_\_ VL: \_\_\_\_\_

Step: \_\_\_\_\_ Type of specimen: \_\_\_\_\_ VL: \_\_\_\_\_

49. If a Positive Run Control is included in every run, please complete the following:

One positive control per \_\_\_\_\_ specimens is used.

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 per \_\_\_\_\_ specimens is used.

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/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/independent person?

- Yes
- No

If **Yes**, please provide documentation of this procedure.

56. Does a supervisor critically review results?

- Yes
- No

## K. DATA MANAGEMENT

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

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

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

- Paper registry
- Computer/electronic registry

**59.** Are the data and results archived?

- Yes
- No

If **Yes**, for what length of time are the data and results kept?

- Specimen registries
- Laboratory processing registry
- Raw sequence data
- Final sequence result

Length of time: \_\_\_\_\_

Length of time: \_\_\_\_\_

Length of time: \_\_\_\_\_

Length of time: \_\_\_\_\_

Format: \_\_\_\_\_

**60.** Are backup procedures for sequences data in place?

- Yes
- No

Describe the backup method and the frequency of backup:

---



---

**ADDITIONAL REMARKS, IF ANY:**

---



---



---



---



---

Assessment done by: \_\_\_\_\_

Date of assessment visit: \_\_\_\_\_

Time spent on assessment: \_\_\_\_\_

# ANNEX 6: WORLD HEALTH ORGANIZATION LABORATORY STANDARD OPERATING PROCEDURES (SOP) FOR POST-TESTING QUALITY ASSURANCE OF HIV DRUG RESISTANCE GENOTYPING

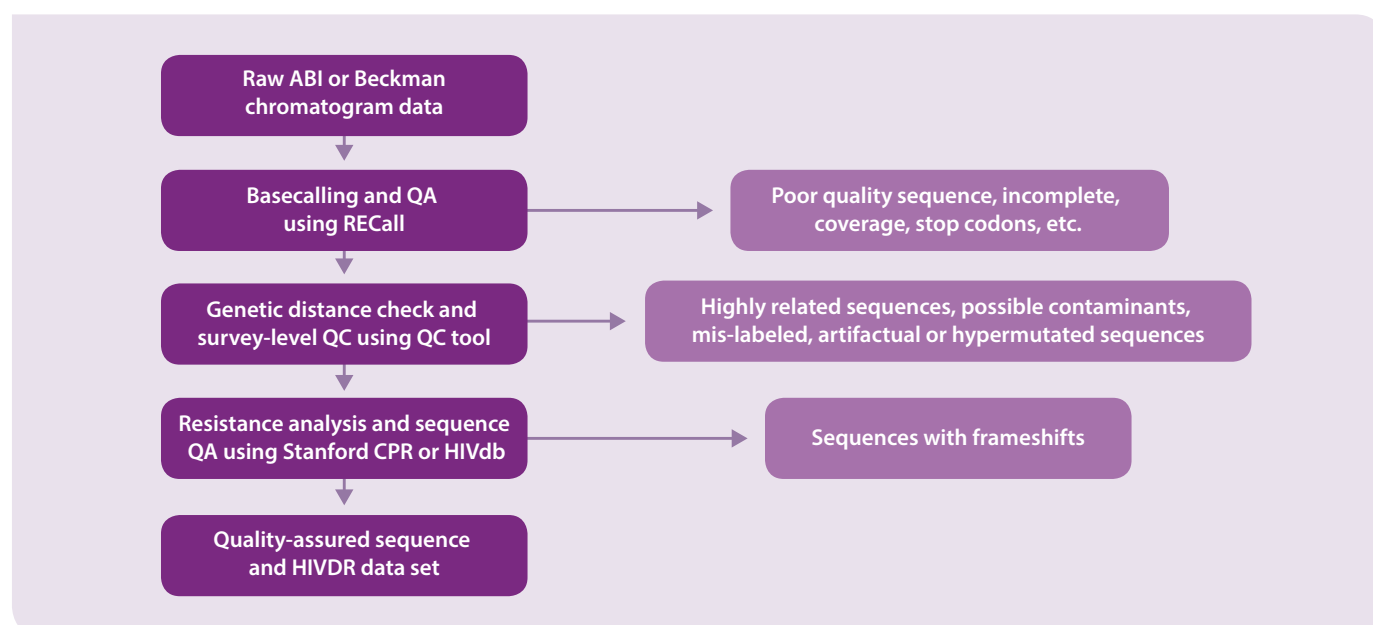
## INTRODUCTION

Quality assurance (QA) of HIV-1 sequence data generated by ribonucleic acid (RNA) extraction, reverse transcription-polymerase chain reaction (RT-PCR), sequencing and data analysis is an essential step towards production of high quality, accurate and standardized drug resistance surveillance information. This document is an update to the post-testing QA Standard Operating Procedures (SOPs) originally developed by World Health Organization (WHO) in 2014. These SOPs aim to facilitate the incorporation of rigorous QA procedures in all WHO-designated HIV drug resistance (HIVDR) genotyping laboratories that are part of the WHO HIVDR Laboratory Network.

Standardization of procedures is useful to ensure maximal comparability of results generated in laboratories all over the world. While adoption of the procedures described here is not an absolute requirement for laboratories to become or remain members in the WHO HIVDR 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 in order to demonstrate comparability. The intended audience for this document is all WHO HIVDR Laboratory Network member laboratories, other public health laboratories considering applying for Network membership, and National HIVDR 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 (BCCfE) in HIV/AIDS HIVDR quality control (QC) 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 QC and QA of HIVDR 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. The flow of recommended steps is below.



QC for HIVDR genotyping includes checking of or the presence of stop codons, highly unusual (“atypical”) mutations, out-of-frame insertions or deletions, missing sequence, excessive ambiguity, mixed bases, and APOBEC mutations. QC checks are carried out using an online QC tool at both the batch level while analysing sequences in RECall, as well as at the survey level after all sequences for a particular survey have been generated.

## Sequence file naming convention

Individuals enrolled in surveys must be assigned survey identification (SID) 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 of the following elements delimited by a dash character (“-”):

- Country abbreviation: the International Organization for Standardization (ISO) standard 3-letter abbreviation (<http://www.worldatlas.com/aatlas/ctycodes.htm>);
- Survey type: TDR for transmitted HIVDR survey<sup>1</sup>, PDR for pre-treatment DR survey, ADR12 or ADR48 for acquired DR survey (12 month or 48+ month time point) INF for surveys of infants <18 months of age, and PMS for prospective monitoring survey<sup>2</sup>. Other survey type abbreviations may be needed in special cases; consult with WHO for the abbreviation to use;
- Four-digit year survey started;
- 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);
- Four-digit unique patient number, i.e., a consecutive unique patient number assigned to a participant at that site;
- For PMS of acquired HIVDR **only**, the letter B should be appended to the patient number for baseline specimens, and the letter E for endpoint;

Batch-level checks are important because they allow inspection and correction of raw data in real time where needed; earlier repeat amplification or sequencing reactions are also possible. Required survey-level checks help identify expected and unexpected genetic relatedness between pairs of sequences that may not have been analysed in the same batch.

*Importantly, do not use the underscore character (“\_”) in the SID.*

*Examples:*

- If the “Central HIV Clinic” was a site that participated in a national TDR survey in South Africa in 2014, a participant’s TDR-SID would look like this: ZAF-TDR-2014-CEN-0001;
- If the “University HIV Clinic” was a site that participated in a national ADR survey in South Africa in 2014 (12 month time point), a participant’s ADR12-SID would look like this: ZAF-ADR12-2014-UNI-0001;
- If the “University HIV Clinic” was a site that participated in a national ADR survey in South Africa in 2014 ( $\geq 48$  month time point), a participant’s ADR48-SID would look like this: ZAF-ADR48-2014-UNI-0001;
- If the “Quality HIV Clinic” was a site that participated in a national PDR survey in South Africa in 2014, a participant’s PDR-SID would look like this: ZAF-PDR-2014-QUA-0001;
- If the “Quality HIV Clinic” was a site that participated in a prospective monitoring survey in South Africa in 2014, a participant’s PDR-SID for the baseline specimen would look like this: ZAF-PMS-2014-QUA-0001-B.

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

1. WHO no longer recommends implementation of surveys of transmitted HIVDR (TDR); however, some countries are in the process of completing these surveys at the time of publication of this document.

2. WHO no longer recommends implementation of prospective monitoring surveys of acquired HIVDR (PMS); however, some countries are in the process of completing these s.surveys at the time of publication of this document.

In some laboratories, established validated procedures may require assignment of 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 QC tool at the survey-level QC step prior to reporting to the country or WHO.**

## A. HIV-1 genotyping sequence analysis using Web RECall

RECall is an automated base calling program developed at the BCCfE<sup>1</sup>. 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 installation and configuration of the software by the user; however it does require a reliable high-speed Internet connection. In addition to base calling, RECall includes several QC checks, including checks for:

- Raw chromatogram quality;
- Single stranded coverage;
- Sequence length;
- Stop codon;
- Excessive mixtures;
- Bad insertions;

- Ambiguous nt/amino acids (xs);
- Apobec mutations;
- Atypical mutations;
- Genetic distance.

WHO Laboratories 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 QC 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 software customized for WHO (as of June 2016, this is version 2.27); please contact WHO to ensure use of the most recent stand-alone version.

## B. Sequence quality assurance using the WHO BCCfE HIVDR QC 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. The purpose of this analysis is several-fold:

- To detect laboratory contamination (i.e., inadvertent amplification of a laboratory strain of HIV, or cross-contamination between specimens handled in the same batch or processed on the same equipment);
- To identify potentially anomalous sequence relationships between pairs or groups of sequences from the same survey;
- To confirm expected genetic relationships between sequences from the same patient collected at different times (PMS surveys only).

This analysis is carried out using an online QC tool developed by WHO and the BCCfE. The aspects of sequence data that are examined by the WHO/BCCfE QC tool include the following:

- Concatenation of separately generated protease (PR) and reverse transcriptase (RT) sequences;
- Survey ID format and sequence renaming, if necessary;
- Genetic distance;
- Atypical mutations;
- APOBEC mutations;
- Stop codons;
- Lab strain similarity.

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(6):1936-42.

## C. Drug resistance analysis using Stanford CPR and HIVdb

The final step in post-testing QA for genotyping is interpretation and assessment for drug resistance by using the automated tools available on the Stanford University HIVdb website. Two tools are useful for this analysis: the CPR tool and the HIVdb resistance analysis program. CPR should be used **only** for surveys of **transmitted** HIVDR in recently infected individuals. HIVdb should be used for **all other types** of surveys including both baseline and endpoint of prospective monitoring surveys (PMS), cross-sectional surveys of HIVDR in patients initiating therapy (PDR), cross-sectional surveys of acquired HIVDR (ADR), and surveys of HIVDR in paediatric populations (infants less than 18 months of age).

**CPR** (version 6) includes many of the QC checks described above in addition to generating a list of Surveillance Drug Resistance Mutations (SDRM) and a complete list of mutations compared to consensus B reference.

**HIVdb** also includes many of the QC checks described above as well as subtyping (based on closest genetic distance match to a set of reference sequences); a list of major resistance-associated mutations and 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. The subtyping analysis performed by HIVdb has known limitations, especially with respect to detection of unique and circulating recombinant forms. An alternative, more rigorous tool is available on the REGAdb website at: <http://www.bioafrica.net/regagenotype/html/subtypinghiv.html>. Another alternative is the SCUEAL package at [http://www.datamonkey.org/dataupload\\_scueal.php](http://www.datamonkey.org/dataupload_scueal.php).

## Transfer of sequence data to WHO

The performance of genotyping assays according to standardized methods and with uniform QA/QC 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 standardization of QA, the submission to WHO headquarters of data from surveys performed following WHO methods is recommended regardless of the source of funding for the surveys. Specifically, viral load and HIVDR testing results should be submitted by the laboratory to both the HIVDR national working group and WHO headquarters for QA at the same time. This process will facilitate:

1. Confirmation that anomalous or suspicious sequences have been handled appropriately;
2. Resolution of complex issues raised by anomalous data;
3. Communication between designated laboratories, countries and the WHO region and headquarters to solve potential problems and build capacity at the country level;
4. 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 transmittal of sequence data and associated information are under development. 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 that were 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<sup>1</sup> is a Web-based system developed for the analysis of chromatogram data generated by Applied Biosystem Genetic Analysers and other sequencers that produce a Standard Chromatogram File (\*.scf). For background, see: [http://webrecall.wikia.com/wiki/BCCFE\\_Web\\_RECall\\_Wiki](http://webrecall.wikia.com/wiki/BCCFE_Web_RECall_Wiki).

Web RECall provides a means to review and edit HIV sequence data for drug resistance determination. 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 PR and RT region as well as other regions of HIV including integrase (IN) and envelope (gp41 and gp120).

## Requirements

### Computer (Windows or Macintosh operating system)

Windows personal computer with Pentium processor or equivalent; or Macintosh personal computer with Intel processor or equivalent.

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

**Web RECall is not compatible with any other Internet browser.** 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 username and password in order to use the software. The administrator is a virologist designated by WHO headquarters in Geneva. Settings used for sequence evaluation and quality acceptance criteria are set by the administrator and are applied to all users.

## Procedure

### Procedure Limitations and Requirements

- The accuracy of base calling is highly dependent 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 accuracy of the result and the sequencing and/or RT-PCR amplification may need to be repeated. Frequent failures due to poor quality data likely indicate the need for improvements to 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 consist of 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;
- Use of raw chromatogram data files generated by automated sequencers with the file extension .ab1 or .scf is necessary.

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(6):1936-42.

**IMPORTANT:** Chromatogram files **must** be named according to WHO convention: SID\_primerID\_other (where "other" can be run date, operator name, etc.). The SID is the survey ID described in the overview (for example, ZAF-TDR-2014-CHC-0001); a chromatogram file generated using primer "A" on a particular 10 March 2015 might be named "ZAF-TDR-2014-CHC-0001\_A\_20150310.ab1". If problems arise when processing data,

**check the naming convention.** Note that the SID uses a dash as a delimiter, while the chromatogram filename 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 abi files).

## 1. User settings

- 1.1 Open Firefox or Chrome and go to <http://pssm.cfenet.ubc.ca/>;
- 1.2 Log in using the username 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. Results will be downloaded rather than emailed after 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 file.
- 2.3 Open Firefox or Chrome and go to <http://pssm.cfenet.ubc.ca/>;
- 2.4 Log in with your username 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:**   [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 Centers of Disease Control and Prevention (CDC) and WHO laboratories when analysing HIV protease and reverse transcriptase is "CDC-WHO\_HIVDR". This reference sequence spans PR amino acid 6 through RT amino acid 251; A separate reference sequence is available for IN ("IN\_50-265" or "IN\_1-288").

2) Choose a **reference sequence**:

**Sequence name:**

- 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, it may be necessary to allow the data processing to 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 (e.g., "ZAF-TDR-2014-CHC 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, 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, due to 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 due to one or more problems, but might be rescued by manual review and/or repeat testing. Repeating sequencing and/or amplification reactions for these samples is 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.

**Note:** 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.

**Note:** The RECall administrator pre-determines 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;

**Sample WHO\_DRP\_2016\_ABC\_0044 (id: 167067)** Open map

(285)	(286)	(287)	(288)	(289)	(290)	(291)	(292)	(293)	<b>Reference Protein</b>
R_S191	R_D192	R_L193	<b>R_E194</b>	R_I195	R_G196	R_Q197	R_H198	R_R199	
S	D	L	<b>P</b>	I	E	Q	H	R	

G	A	T	C	T	G	A	C	T	T	A	G	A	A	T	A	G	G	G	C	A	G	C	A	T	A	G	<b>Standard Assembled</b>
C	A	T	C	T	G	A	C	T	T	A	<b>C</b>	<b>C</b>	<b>A</b>	A	T	A	G	A	A	C	A	G	C	A	T	A	G

**F (reverse)**

C A T C T G A C T T A **C C A** A T A G A A C A

**A (forward)**

A T C T G A C T T A **C C A** A T A G A A C A

**B (forward)**

C A T C T G A C T T A **C C A** A T A G A A C A C

**Job name:** Mar-10-CDC-WHO\_HIVDR (id: 41173)

**Upload date:** 2016-03-10

**Status:** Passed

**Mixtures:** 13 (cutoff: 15.0%)

**Marks:** 7

**"N"s:** 0

**Edited bases:** 0

**Errors:** 1

\* Suspicious atypical mutation P at amino 288 found.

**Use the following keys to navigate:**

Next marked base: right arrow

Previous marked base: left arrow

Next base: shift + right arrow

Previous base: shift + left arrow

**With key locations defined in advanced settings:**

Next marked key base: down arrow

Previous marked key base: up arrow

**Use the following keys to make edits:**

Change base: A,C,G,T,N  
R,Y,K,M,S,W,B,D,H,V

Erase base: dash

**Mixture compositions:**

R = A/G    Y = C/T    K = G/T    M = A/C

S = G/C    W = A/T    B = C/G/T    D = A/G/T

H = A/C/T    V = A/C/G    N = A/C/G/T

288 Jump to base Jump to AA

Save & Pass
Fail Sample
Exit

**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 QA 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 editing of all samples in the batch is complete, 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:
- 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 QC process;
  - 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% differences;
    - 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;
  - csv file ("*resistance\_summary.csv*") containing a summary of the drug susceptibility interpretations for all passed samples, based on the Stanford HIVdb algorithm;
  - pdf file ("*jobnumber\_tree.pdf*") with an image of a neighbor-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 to any sequence in the current batch. Batches are identified by "job number", which is appended to the sample name (*i.e.*, *SID\_job-12345*);
  - pdf file ("*SID.pdf*") of Stanford HIVdb Drug Resistance interpretations for each passed sample.
- 4.2** If desired, rename RECall output files with a name more informative of the project.

# B. SEQUENCE QUALITY ASSURANCE USING THE WHO BCCFE QC 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. QA for such assays involves pre-testing steps (e.g., operator training, SOPs, etc.), in-process controls (e.g., unidirectional workflow), and post-testing evaluation. For assays whose results involve generation of a nucleic acid sequence, such as HIV Drug Resistance Genotyping, careful examination of 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 analyses must be flagged for investigation. Lack of a satisfactory explanation for anomalous results will result in exclusion of the sequences in question from the final study analysis.

WHO and the BCCfE jointly developed an online program for HIVDR sequencing QC. This tool accepts sequences in FASTA format (a format generated by RECall as described above) and performs a series of QC checks.

- An alternative, though less customized tool, is the free open-source software program called MEGA<sup>1</sup> (Molecular

Evolutionary Genetic Analysis). MEGA can be used for basic as well as more advanced phylogenetic analysis. Additional details on use of MEGA can be found at <http://www.megasoftware.net/manual.php>. WHO will provide a procedure for use of MEGA for assessment of pairwise genetic distance upon request.

Ideally, each newly generated sequence is compared to 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 to each other; this includes baseline and endpoint specimens from the same patient in prospective monitoring surveys;
2. Preferably, include sequences from unrelated specimens **tested contemporaneously** (i.e., 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 inclusion of sequences that are not part of the WHO survey being analysed.

## Requirements

### Computer (Windows or Macintosh operating system)

Windows personal computer with Pentium processor or equivalent; or Macintosh personal computer with Intel processor or equivalent.

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

**The QC 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.

### Test sequences in FASTA format

Nucleic acid (and protein) 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-TDR-2014-CHC-0001
ACGTACGTACGT...
> ZAF-TDR-2014-CHC-0002
ACATACCTACGT...
```

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.



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 about file extensions, see <http://support.microsoft.com/kb/865219>

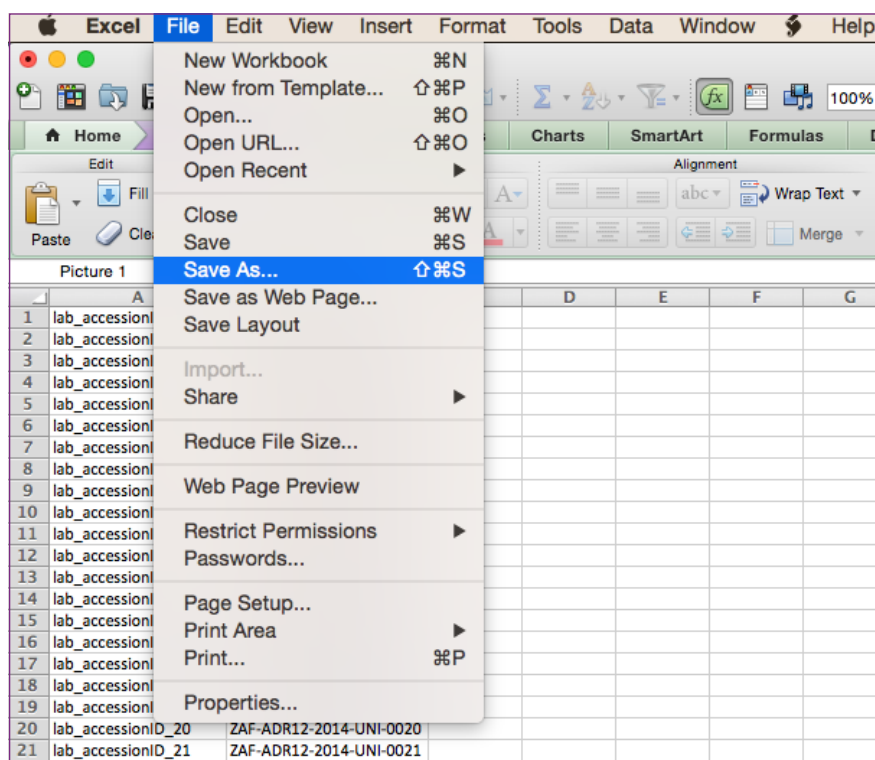
or [http://support.apple.com/kb/PH10845?viewlocale=en\\_US&locale=en\\_US](http://support.apple.com/kb/PH10845?viewlocale=en_US&locale=en_US).

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.

## 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...** ;

**Note:** 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. Check that the list of lab strains displayed at the bottom of the page includes at least HXB2 and NL4-3 (for subtype B) and MJ-4 (for subtype C). If other cloned HIV strains are frequently used in the laboratory where genotyping is performed, add the sequence(s) corresponding to PR amino acid 6 to RT amino acid 251 to the sequences of the three lab strains above and upload these additional sequences. The sequences for HXB2, NL4-3, and MJ-4 can be found in [Appendix 1](#). To upload a new set of lab strain sequences:

**2.1** Prepare the file containing the lab strain sequences in FASTA format;

**2.2** Open Firefox or Chrome and go to [http://pssm.cfenet.ubc.ca/who\\_qc/](http://pssm.cfenet.ubc.ca/who_qc/);

**2.3** Under the **Update lab strains with new fasta file?** header, click on **Browse...** and select the file containing the lab strain sequences (in FASTA format);

**2.4** Click on **Save lab strains fasta.**

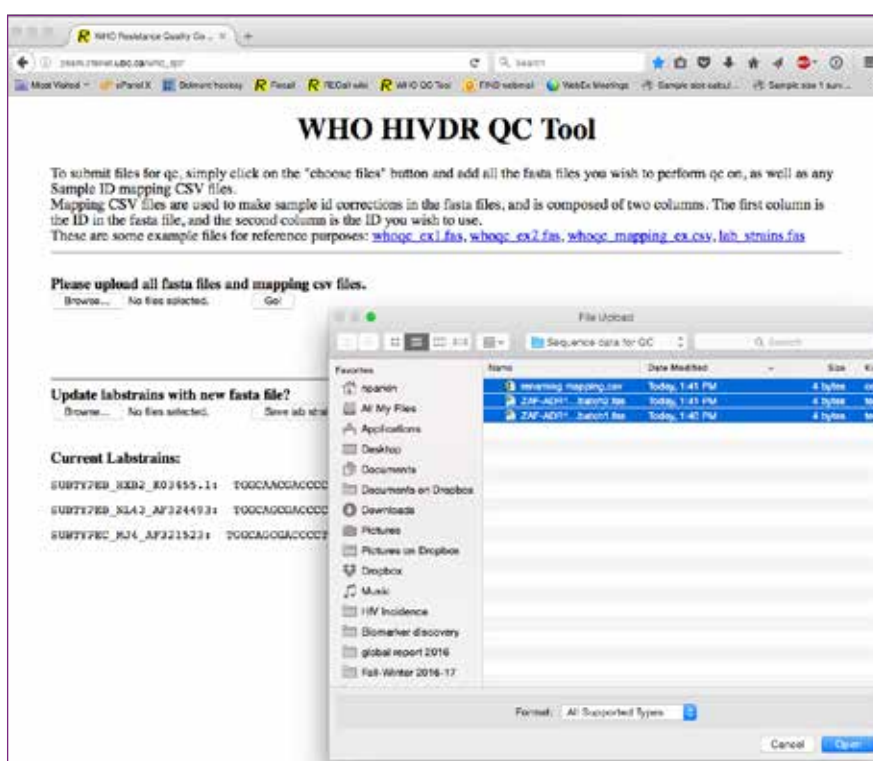
**3.** Upload the sequences to be analysed for potential QC problems;

**3.1** Open Firefox or Chrome and go to [http://pssm.cfenet.ubc.ca/who\\_qc/](http://pssm.cfenet.ubc.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 (e.g., 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 (**ctrl** 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 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** 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: [http://pssm.cfenet.ubc.ca:80/who\\_qc/upload\\_results/1483561347](http://pssm.cfenet.ubc.ca:80/who_qc/upload_results/1483561347)"*

**3.6.** Click on the results hyperlink, or refresh the page to view the results.

**Note:** The amount of time required to complete the analysis is dependent on the number of sequences submitted. For several hundred sequences, it may take several minutes.



4. Review the output in your Web browser, or download the results as a csv file by clicking on the **Download results** button. Review each section in the report and use the checklist ([Appendix 2](#)) to record completeness of the review;

**4.1 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;

**4.2 Stop Codons:** A list of sequences containing stop codons as mixtures (e.g., 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;

**4.3 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 DR site AND a total of 4 or more APOBEC mutations should be flagged for investigation. A list of APOBEC mutations at DRM sites can be found here: <https://hivdb.stanford.edu/page/release-notes/#data.files>;

**4.4 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;

**4.5 Samples > 2.5% genetic distance (from the same patient):** A list of pairs of sequences expected to be highly related to each other based on their sequence IDs (for example, ZAF-PMS-2014-CHC-0002-B and ZAF-PMS-2014-CHC-0002-E), but with >2.5% differences. All such pairs of sequences should be flagged for investigation;

**4.6 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;

**4.7 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;

5. **Follow-up actions.** WHO recommends the following steps for all potential anomalies identified in step 4:

**5.1** Review the raw chromatogram data (e.g., 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 HIVDR Working Group from the country where the survey was conducted to determine if additional specimen is stored elsewhere and can be sent for amplification and sequencing;

**5.3** Confer with the HIVDR Working Group from the country where the survey was conducted to obtain as much epidemiological information as possible (e.g., clinic site, specimen collection date, or relationships between survey participants in question such as known transmission pair, couple, etc.);

**5.4** Consult with HIV Drug Resistance Network (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 CPR AND HIVDB

## Background

The translation of nucleotide sequence data into useable information related to drug resistance (DR) is a multi-step process. It involves conversion of the data into a predicted amino acid sequence, comparison to a standard reference (drug sensitive) sequence, evaluation of the combination of known resistance-associated mutations detected using one of several different interpretation systems, and generation of a report

indicating predicted susceptibility to each antiretroviral (ARV) 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 DR. WHO selected this resource as a standardized way to interpret sequence data for public health surveillance of HIVDR.

Two separate tools on the website are applicable to WHO survey data analysis:

1. **CPR tool.** This analysis specifically evaluates sequences from treatment-naïve subjects for assessment of the prevalence of transmitted drug resistant HIV. It uses a list of mutations (the SDRM list) to categorize viruses as having or lacking evidence of ARV drug selection pressure. The SDRM list includes major DR-associated mutations and accessory mutations that are not considered polymorphic (i.e., that are often found in sequences from viruses never exposed to drug selective pressure and thus could lead to over-estimation of rates of DR HIV transmission). This method of analysis is **always** used in WHO surveys of transmitted drug resistant HIV (TDR surveys)<sup>1</sup>. The CPR tool also includes an assessment of sequence quality, many features of which are shared by RECall and HIVdb. This method of analysis is not used when determining major DR outcomes of surveys of PDR, ADR, or infant <18 months of age;
2. **HIVdb resistance analysis program.** This program provides a drug resistance/susceptibility assessment using a mutation scoring system and five levels of predicted susceptibility. The HIVdb program is used in prospective WHO surveys of acquired DR (both at baseline and endpoint), in cross-sectional surveys of DR before (pre-treatment surveys) and after treatment (acquired DR surveys), and in surveys of HIVDR in children <18 months old. The program includes an assessment of sequence quality and shares many features with RECall and CPR.

## 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 tables of results from large sequence sets.

## Procedure

### 1. CPR Tool analysis (for surveys of transmitted drug resistance<sup>1</sup>);

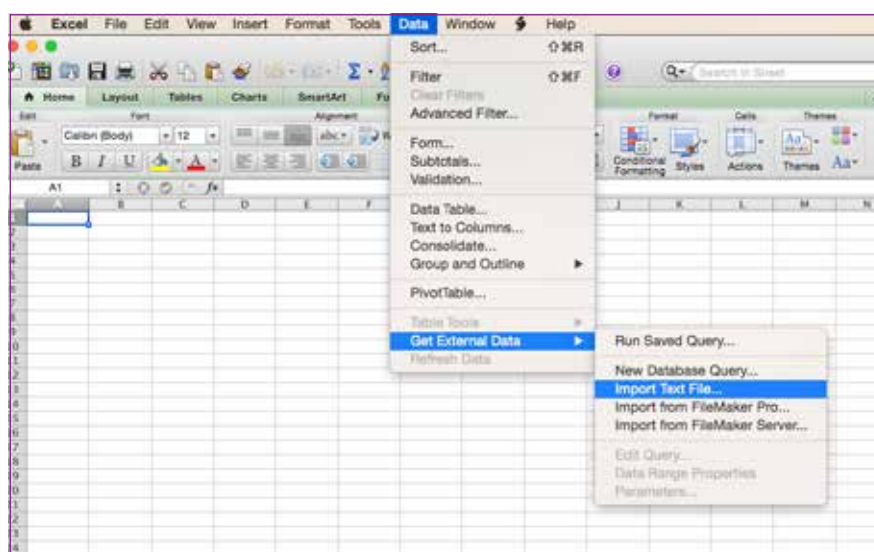
- 1.1 Open an Internet browser and go to: <http://cpr.stanford.edu/cpr.cgi>;
- 1.2 Click the **Choose File** button under **Text File Upload** and navigate to the file containing the sequences to be analysed;
- 1.3 Make sure to select the latest version of the Surveillance Drug Resistance Mutations list: as of December 2016, this is **SDRM2009**;
- 1.4 Click on **ANALYZE**;
- 1.5 When the analysis is complete, results will display in the browser window. Click on the **In Excel** link at the top to download the results;
- 1.6 Rename the file using a descriptive title reflecting the survey name and the suffix “\_cpr.results”.

### 2. HIVdb Resistance Analysis (for surveys of pre-treatment HIVDR, acquired HIVDR and surveys of HIVDR in infants <18 months).

- 2.1 Open an Internet browser and go to: <https://hivdb.stanford.edu/hivdb/by-sequences/>;
- 2.2 Click the **Choose File** button under **Input Sequences** and navigate to the file containing the sequences to be analysed;
- 2.3 Select the **Spreadsheets (TSV)** option under **Output Options**. Output files are tab-separated values (.tsv) format;
- 2.4 Next to **Select Outputs**, select **Sequence Quality Summaries** and **Drug-resistance Summaries** (other outputs may be used as well, but are optional);
- 2.5 Click on **Analyse**;
- 2.6 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 (exact steps may differ between operating systems and software configurations);
- 2.7 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.

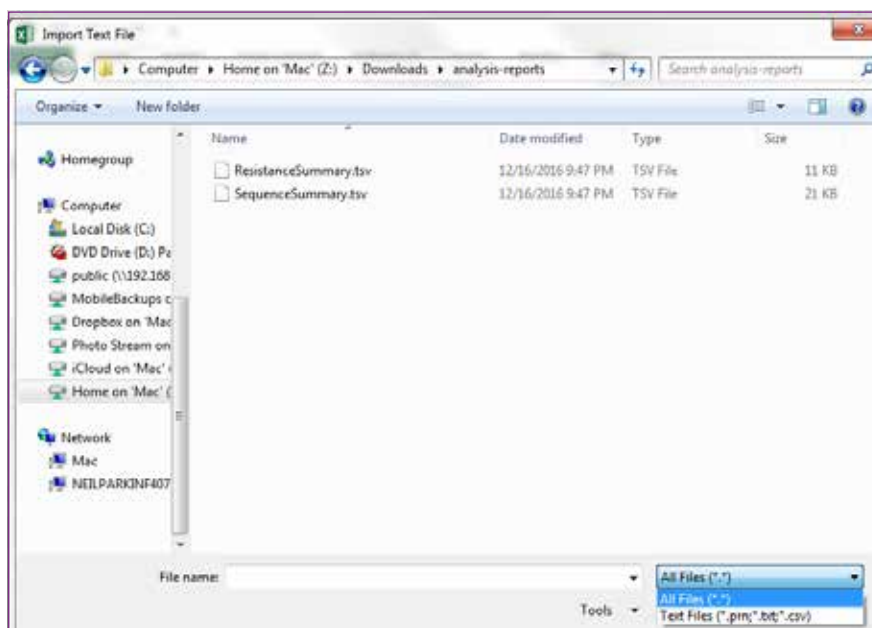
2.7.1 Open a new Excel file;

2.7.2 For Macintosh: From the **Data** menu choose **Get External Data** and **Import Text File...** From the dialog box that opens, choose All Files from the **Enable** drop-down menu and choose the SequenceSummary.tsv file;

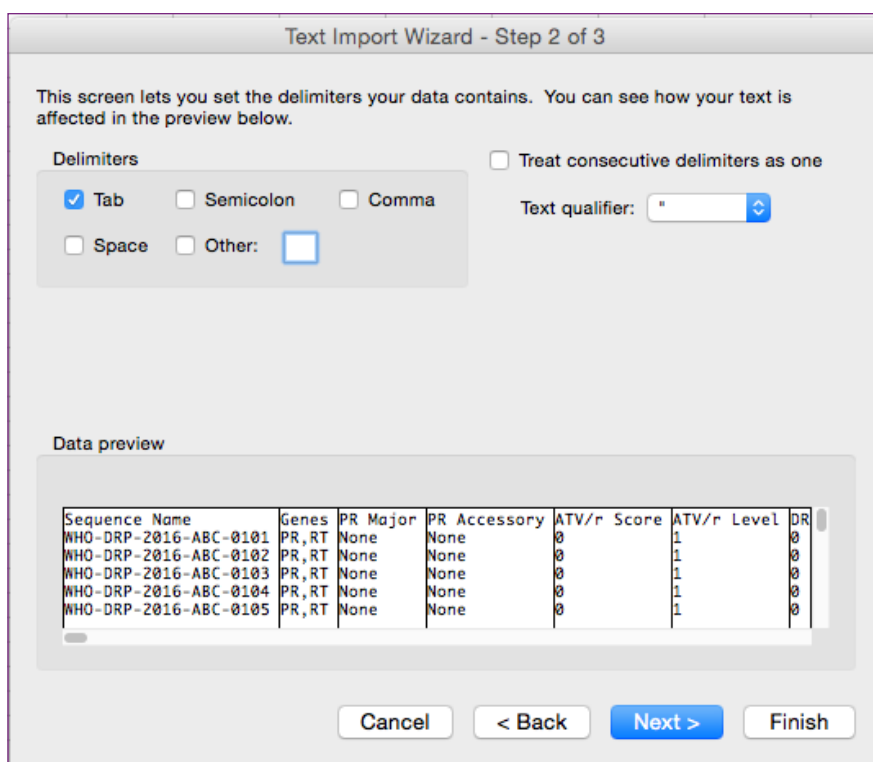


1. WHO no longer recommends implementation of surveys of transmitted HIVDR (TDR); however, some countries are in the process of completing these surveys at the time of publication of this document.

For PC: From the **Data** ribbon choose **From Text**. In the dialog box that opens, choose **All Files** from the drop-down menu at the bottom left, then select the SequenceSummary.tsv file;



**2.7.3** In the Text Import Wizard window, choose **Delimited**, then choose Tab as the delimiter. Rename the tab **SequenceSummary** instead of **Sheet1**;



**27.4** 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**;

**2.7.5** Save the Excel file using a name that includes the survey code (such as ZAF-ADR-2014-CHC).

**2.8** 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 DR site AND > 4 or more total*
Num Unusual Mutations	> 3 in PR or > 5 in RT

\*Note that the total number of APOBEC mutations listed in the Stanford output may not match the number found in the BCCfE QC tool if there are mutations at DR sites that could also be APOBEC mutations. In this case, use the number reported by the BCCfE QC tool.

# APPENDIX 1: REFERENCE SEQUENCES

The reference sequences below correspond to commonly used lab 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 BCCfE QC tool. Additional reference sequences for other subtypes and CRFs can be found 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 PR amino acids 6-99 and RT 1-251.

## PR (6-99) and RT (1-251):

>SUBTYPEB\_HXB2\_PRRT\_K03455.1

TGGCAACGACCCCTCGTCAACAATAAAGATAGGGGGGCAACTAAAGGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGAGTTTGGCCAGGAAGATGGAACCAAAAAATGATAGGGGGAATT  
GAGGTTTTATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGTGGACATAAAGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGGAAGAAATCTGTTGACTCAGATTGGTGCACITTTAA-  
ATTTCCATTAGCCCTATTGAGACTGTACAGTAAAATTAAGCCAGGAATGGATGGCCAAAAGTTAAACAATGGCCATTGACAGAAGAAAAATAAAGCATTAGTAGAAATTTGTACAGAGATGGAAAAGGAAGG-  
GAAAATTTCAAAAATGGGCTGAAAATCCATACAATACTCCAGTATTTGCCATAAAGAAAAAGACAGTACTAAATGGAGAAAATTAGTAGATTTAGAGAACTTAATAAGAGAAGTCAAGACTTCGGGAAGTTCAAT-  
TAGGAATACCCATCCCGCAGGGTTAAAAAGAAAAAATCAGTAACAGTACTGGATGTGGGTGATGCATATTTTCAGTTCCCTTAGATGAAGACTTCAGGAAGTATACTGCATTACCATACCTAGTATAACAATGAGA-  
CACCAGGGATTAGATATCAGTACAATGTCTCCACAGGGATGGAAGGATCACCAGCAATATTCCAAGTAGCATGACAAAAATCTTAGAGCCTTTAGAAAAACAAAATCCAGACATAGTTATCTATCAATACATGGAT-  
GATTTGTATGAGGATCTGACTTAGAAATAGGGCAGCATAGAACAATAAGAGGAGCTGAGACAACATCTGTTGAGGTGGGGACTTACCACACCAGACAAAAAATCAGAAAAGAACCTCCATTCCTTTGGATGGGTTA-  
TGAACCTCATCTGATAAATGGACAGTACAGCCTATAGTGTCCAGAAAAAGACAGC

>SUBTYPEB\_NL43\_PRRT\_AF324493

TGGCAGCGACCCCTCGTCAACAATAAAGATAGGGGGGCAATTAAGGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGAAATTTGCCAGGAAGATGGAACCAAAAAATGATAGGGGGAATTGGA-  
GGTTTTATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGCGGACATAAAGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGGAAGAAATCTGTTGACTCAGATTGGCTGCACITTTAAATTT-  
TCCATTAGTCTATTGAGACTGTACAGTAAAATTAAGCCAGGAATGGATGGCCAAAAGTTAAACAATGGCCATTGACAGAAGAAAAATAAAGCATTAGTAGAAATTTGTACAGAAATGGAAAAGGAAGGAAAA-  
ATTTCAAAAATGGGCTGAAAATCCATACAATACTCCAGTATTTGCCATAAAGAAAAAGACAGTACTAAATGGAGAAAATTAGTAGATTTAGAGAACTTAATAAGAGAAGTCAAGACTTCGGGAAGTTCAATTAGGA-  
ATACCACATCTCGCAGGGTTAAAAAGAAAAAATCAGTAACAGTACTGGATGTGGGCGATGCATATTTTCAGTTCCCTTAGATAAAGACTTCAGGAAGTATACTGCATTACCATACCTAGTATAACAATGAGACACCAG-  
GGATTAGATATCAGTACAATGTCTCCACAGGGATGGAAGGATCACCAGCAATATTCCAGTAGCATGACAAAAATCTTAGAGCCTTTAGAAAACAAAATCCAGACATAGTATCATCAATACATGGATGATTTGTA-  
TGTAGGATCTGACTTAGAAATAGGGCAGCATAGAACAATAAGAGGAAGTACTGAGACAACATCTGTTGAGGTGGGGATTACCACACCAGACAAAAAATCAGAAAAGAACCTCCATTCCTTTGGATGGGTTATGAACTC-  
CATCTGATAAATGGACAGTACAGCCTATAGTGTCCAGAAAAAGACAGC

>SUBTYPEC\_MJ4\_PRRT\_AF321523

TGGCAGCGACCCCTTGTCTCAATAAAGTAGGGGGCAATAAAGGAGGCTCTTTAGACACAGGAGCAGATGATACAGTATTAGAAGAAATGAGTTTGTCCAGGAAGATGGAACCAAAAAATGATAGGAGGAATTGGA-  
GGTTTTATAAAGTAAGACAGTATGATCAATACCTATAGAAATTTGTGAAAAAGGCTATAGGTACAGTGTAAATAGGACCTACTCTGTCAACATAATTGGAAGAAATATGTTGACTCAGCTGGCTGCACITTTAAATTT-  
TCCAATCAGTCTTATTGAACTGTACAGTAAAATTAAGCCAGGCATGGATGGCCCTAAGGTTAAACAATGGCCATTGACAGAAGAAAAATAAAGCATTAAACAGAAATTTGTGAGAAAATGGAAAAGGAAGGAAAA-  
ATTACAAAATTTGGGCTGAAAATCCATATAACTCCAGTATTTGCAATAAAGAAAAAGACAGTACTAAGTGGAGAAAATTAGTAGACTTCAGGGAACCTAATAAAGAACTCAAGACTTTTGGGAGGTTCAATTAGGA-  
ATACCGCACCCAGCAGGGTTAAAAAGAAAAAATCAGTAACAGTATTAGATGTGGGGGATGCATATTTTCAGTTCCCTTAGATGAAGGCTTCAGGAATACACTGCATTACCATACCTAGTATAACAATGAAACACCA-  
GGGATTACATATCAATATAATGTCTCCACAGGGATGGAAGGATCACCAGCAATATTCCAGTAGCATGACAGAATCTTAGAGCCTTTAGGACACAAAATCCAGAAAATAGTATCATCAATATATGGATGATTTGTA-  
TGTAGGATCTGATTAGAAATAGGGCAACATAGAGCAAAAATAGAGGAATTAAGAAAATCTTAAAGTGGGGATTACCACACCAGACAAAAGAACATCAGAAAAGAACCCCATTCCTTTGGATGGGTTATGAACTC-  
CATCTGATAAATGGACAGTACAGCCTATAGTGTCCAGAAAAAGACAGC

## IN (1-288):

>SUBTYPEB\_HXB2\_IN\_K03455.1

TTTTAGATGGAATAGATAAGGCCCAAGATGAACATGAGAAATACACAGTAATTGGAGAGCAATGGCTAGTATTAACTGCCACCTGTAGTAGCAAAGAAATAGTAGCCAGCTGTGATAAATGTCAGCTAAAAG-  
GAGAAGCCATGCATGGACAAGTAGACTGTAGTCCAGGAATATGGCACTAGATTGTACACATTTAGAAGGAAAAGTTATCTGTAGCAGTTCATGTAGCCAGTGGATATAGAAAGCAGAAATTTCCAGCAGAAAA-  
GGGCAAGAAACAGCATAATTTCTTTAAATTAGCAGGAAGATGGCCAGTAAAAACAATACATACTGACAATGGCAGCAATTTACCGTGTCTAGGTTAGGCGCCGCTGTTGGTGGGCGGGATCAAGCAGGAATTTG-  
GAATTCCTACAATCCCCAAGTCAAGGAGTAGTAGAATCTATGAATAAAGAATTAAGAAAAATATAGAGCAGGTAAGAGATCAGGCTGAACATCTTAAGACAGCAGTACAATGGCAGTATTATCCACAATTTAAAA-  
GAAAAGGGGGGATTGGGGGTACAGTGCAGGGGAAAGAATAGTAGACATAATAGCAACAGACATACAACTAAAGAATTACAAAAACAATTAACAAAATTTCAAAAATTTTCGGGTTTATTACAGGGACAGCAGAAATC-  
CACTTTGAAAAGGACAGCAAGCTCTCTGAAAAGGTGAAGGGGCGAGTAGTAATACAAGATAATAGTGACATAAAGTAGTGCCAAGAAGAAAAGCAAGATCATTAGGGATTATGAAAAACAGATGGCAGGTGAT-  
GATTGTGTGGCAAGTAGACAGGATGAGGAT

>SUBTYPEB\_NL43\_IN\_AF324493

TTTTAGATGGAATAGATAAGGCCCAAGAAGAACATGAGAAATACACAGTAATTGGAGAGCAATGGCTAGTATTAACTGCCACCTAGTAGCAAAGAAATAGTAGCCAGCTGTGATAAATGTCAGCTAAAAGG-  
GGAAGCCATGCATGGACAAGTAGACTGTAGCCAGGAATATGGCAGCTAGATTGTACACATTTAGAAGGAAAAGTTATCTGTAGCAGTTCATGTAGCCAGTGGATATAGAAAGCAGAAATTTCCAGCAGAGACAG-  
GGCAAGAAACAGCATACTCTCTTAAATTAGCAGGAAGATGGCCAGTAAAAACAGTACATACAGCAATGGCAGCAATTTACCAGTACTACAGTTAAGGCCGCTGTTGGTGGGCGGGATCAAGCAGGAATTTG-  
GCATTCCTACAATCCCCAAGTCAAGGAGTAAATAGTAATCTATGAATAAAGAATTAAGAAAAATATAGAGCAGGTAAGAGATCAGGCTGAACATCTTAAGACAGCAGTACAATGGCAGTATTATCCACAATTTAAAA-  
GAAAAGGGGGGATTGGGGGTACAGTGCAGGGGAAAGAATAGTAGACATAATAGCAACAGACATACAACTAAAGAATTACAAAAACAATTAACAAAATTTCAAAAATTTTCGGGTTTATTACAGGGACAGCAGAGATC-  
CAGTTTGGAAAAGGACAGCAAGCTCTCTGAAAAGGTGAAGGGGCGAGTAGTAATACAAGATAATAGTGACATAAAGTAGTGCCAAGAAGAAAAGCAAGATCATTAGGGATTATGAAAAACAGATGGCAGGTGAT-  
GATTGTGTGGCAAGTAGACAGGATGAGGAT

>SUBTYPEC\_MJ4\_IN\_AF321523

TTTCTAGATGGAATAGATAAGGCTCAAGAAGAGCATGAAAGGTATCATAGCAATGGAGAGCAATGGCTAGTATTAACTGCCACCTAGTAGCAAAGAAATAGTAGCTAGCTGTGATAAAGTGTAGTCAAAG-  
GGGAAGCCATGCATGGACAAGTAGACTGTAGCCAGGAATATGGCAATGATTGTACACATTTAGAAGGAAAAGTTATCTGTAGCAGTCCATGTAGCCAGTGGCTATAGAAAGCAGAGGTTATCCAGCAGAAAA-  
CAGGACAAGACAGCAGCATACTATACTAAAATAGCAGGAAGATGGCCAGTCAAAGTAAATACATACAGCAATGGCAGTAATTTACCAGTGTGCAGTCAAGGCAGCTGTTGGTGGGCGGGATCAAGCAGGAATTTG-  
TGGGATTCCTACAATCCCCAAGTCAAGGAGTAGTAGAATCCATGAATAAAGAATTAAGAAAAATATAGAGCAGGTAAGAGATCAGGCTGAACATCTTAAGACAGCAGTACAATGGCAGTATTATCCACAATTT-  
TAAAAGAAAAGGGGATTGGGGGTACAGTGCAGGGGAAAGAATAATAGATATAATAGCAACAGATATCAAACTAAAGAATTACAAAAACAATTAACAAAATTTCAAAAATTTTCGGGTTTATTACAGGGACAGCAGAGATC-  
GACCTTATTGAAAAGGACAGCAAACTACTCTGAAAAGGTGAAGGGGCGAGTAGTAATACAAGATAATAGTGACATAAAGTAGTGCCAAGAAGAAAAGCAAGATCATTAGGGATTATGAAAAACAGATGGCAGGTGAT-  
GCTGATTGTGTGGCAAGTAGACAGGATGAAGAT

# APPENDIX 2: QC CHECKLIST

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

Batch information and identification (Note 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 (e.g., 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 DR 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 "P\_"), or five or more in reverse transcriptase ("Amino location" begins with "R\_"), should be flagged for investigation.
- Samples > 2.5% genetic distance (from the same patient): A list of pairs of sequences expected to be highly related to each other based on their sequence IDs (for example, ZAF-PMS-2014-CHC-0002-B and ZAF-PMS-2014-CHC-0002-E), but with >2.5% differences. All such pairs of sequences 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% differences. 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.





**For more information, contact:**

World Health Organization  
Department of HIV/AIDS  
20, avenue Appia  
1211 Geneva 27  
Switzerland

E-mail: [hiv-aids@who.int](mailto:hiv-aids@who.int)

[www.who.int/hiv](http://www.who.int/hiv)

ISBN 978-92-4-151287-9



9 789241 512879