

HIV DRUG RESISTANCE

WHO MANUAL FOR HIV DRUG RESISTANCE TESTING USING DRIED BLOOD SPOT SPECIMENS

THIRD EDITION — OCTOBER 2020



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CONTENTS

Acknowledgements	iv
Summary of changes	v
Introduction	1
Laboratory supplies	4
Preparing DBS from specimens collected by venepuncture	4
Preparing DBS from specimens collected by finger-prick	5
Drying	5
Important considerations for packaging DBS	6
Packaging	6
Handling and storage	6
Transport	7
Transport of DBS specimens stored at ambient temperature	8
Transport of frozen DBS specimens	8
Receipt of DBS at the processing laboratory	8
Using appropriate control reagents for HIV drug resistance testing from DBS	9
Validating HIV drug resistance testing from DBS	9
References	10
Annex 1. RNA extraction and genotyping protocols from the French National Research Institute for Sustainable Development	12
Annex 2. RNA extraction and genotyping protocols from the ILB Laboratory of the United States Centers for Disease Control and Prevention	19
Annex 3. RNA extraction and genotyping (Thermo Fisher Scientific kit) protocols from the ILB Laboratory of the United States Centers for Disease Control and Prevention	45

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SUMMARY OF CHANGES

Emphasis has been added on best practices for DBS preparation, storage and shipping, as well as selection of appropriate positive control. A new procedure for the ThermoFisher genotyping kit has been added (Annex 3).

INTRODUCTION

HIV-1 drug resistance genotyping is an essential component of the WHO global drug resistance prevention and assessment strategy (1,2). Plasma is considered to be the most appropriate specimen type for HIV drug resistance genotyping. However, use of plasma may not be feasible in rural, remote areas in low- and middle-income countries, since preparing and storing it require personnel and laboratory infrastructure that are often lacking. An alternative specimen type for HIV drug resistance genotyping is dried blood spots (DBS). DBS can be made from blood drawn for routine clinical or surveillance purposes without special laboratory processing. DBS are more easily transported to the drug resistance testing laboratory than plasma because they can be shipped at ambient temperature as non-hazardous materials using regular mail or courier services (3).

Despite the potential advantages of DBS as a method of collecting specimens, there are several disadvantages, the foremost being the reduced sensitivity of viral RNA amplification related to input specimen volume constraints. In addition, in specimens with low viral loads, proviral DNA and viral RNA in infected peripheral blood mononuclear cells may contribute a significant proportion of information to genotyping results. Thus, for some people with low viral loads, genotyping results from DBS specimens may not reflect the current status of replicating viruses circulating in the person's plasma as accurately as plasma. Viral RNA in DBS is susceptible to degradation if the DBS are not kept desiccated or left unfrozen for extended periods of time. In this way, DBS must be handled differently if they are to be used for HIV drug resistance genotyping compared with early infant diagnosis, which targets all nucleic acids, including the more stable DNA component.

Several different methods for performing HIV drug resistance genotyping using DBS, including some comparisons of various storage conditions, have been developed and reported in the literature (4–24). Because of its support for HIV drug resistance genotyping as part of a wider global strategy for preventing and assessing HIV drug resistance in low- and middle-income countries, WHO has coordinated efforts to develop, validate and standardize methods for HIV drug resistance genotyping from DBS (25). Table 1 summarizes HIV drug resistance genotyping methods and performance.

DBS have also been extensively studied as a specimen type for viral load testing (26,27). The viral load results obtained from plasma and DBS are comparable, although this depends on the RNA extraction method used (14,28,29). However, the low volume of blood collected on a DBS limits the sensitivity of the viral load determination, and less experienced laboratories may have difficulty in quantitative recovery. In addition, in specimens with very low or undetectable plasma viral load, DBS may contain sufficient levels of proviral DNA or viral RNA from the cellular compartment to lead to a false-positive viral load result from polymerase chain reaction (PCR)-based assays (30). Other WHO guidance documents more extensively discuss special considerations when using DBS for viral load testing (31).

This manual provides current best practice guidance for laboratory HIV drug resistance testing using DBS (Table 1). Because specific reagents, supplies or equipment may not always be available or affordable in all areas where genotyping is being performed, two alternative protocols are provided. Read all sections of the manual before performing any of the laboratory procedures. Pay special attention to the sections on specimen storage and processing, since these procedures might differ from those recommended by other sources for routine analysis of plasma specimens.

Table 1. Overview of published HIV DBS genotyping methods

Study	Genotyping method(s)	Amplicon size	Storage conditions	Sample characteristics
Ziemniak et al. (13)	In-house nested RT-PCR	RT: 663 bp	Ambient, 0–5 months	Treated and untreated patients from the United States, subtype B
Bertagnolio et al. (4)	In house nested RT-PCR	RT: 700 bp	37°C, 85% humidity, three months	Untreated subjects from Mexico, subtype B
Masciotra et al. (8)	ViroSeq™	1.8 kb	–20°C, 18–26 weeks	Mostly treatment experienced, subtype B
McNulty et al. (9)	In-house nested RT-PCR	1 kb	–20°C, 2–3 years	Untreated, subtypes from Cameroon, subtypes A, CRF02
Steegeen et al. (11)	In-house nested RT-PCR	protease: 458 bp RT: 646 bp	–20°C	Treated and untreated patients from Kenya; subtypes A, C, D, CRF16
Buckton et al. (5)	In-house nested RT-PCR	protease: 758 bp RT: 805 bp	–20°C	Clinic patients from the United Kingdom; subtypes A, B, C, CRF02
Hallack et al. (7)	TruGene	1.3 kb	–20°C	Treated and untreated patients from the United States, subtype B
Garrido et al. (6)	In-house nested RT-PCR: RT and gp41 fragments	RT: 726 bp	4°C, no desiccant	Treated patients from Angola; many subtypes
Youngpairoj et al. (12)	ViroSeq™ or in-house nested RT-PCR	1.8 kb or 1 kb	4°C, 1 year	Treatment experienced, subtype B
Monleau et al. (32)	In-house nested RT-PCR	RT: 798 bp	Ambient (+20°C) or +37°C high humidity	HIV-1-infected patients in France
Yang et al. (20)	In-house nested RT-PCR	1062 bp	–20°C or –70°C	Treated and untreated patients from China, Malawi and United Republic of Tanzania
Zhou et al. (19)	In-house nested RT-PCR	1084 bp	Ambient three months (Nigeria) or –70°C (Viet Nam)	Treated and untreated patients from Nigeria and Viet Nam, respectively
Aitken et al. (24)	In-house nested RT-PCR	RT: 591 bp	Not reported	HIV-1-infected people in South Africa
Bronze et al. (23)	In-house nested RT-PCR	RT: 591 bp	–20°C, 13–21 months	HIV-1-infected people in South Africa
Inzaule et al. (22)	In-house nested RT-PCR (United States Centers for Disease Control and Prevention)	1084 bp	–30°C	HIV-1 infected mothers and children in Kenya (Kisumu)
Monleau et al. (21)	In-house nested RT-PCR (ANRS)	protease: 507 bp RT: 798 bp	Ambient 2–4 weeks, –20°C, 16–38 days	Treated patients from Burkina Faso, Cameroon, Senegal, Thailand, Togo and Viet Nam
Salimo et al. (33)	In-house nested RT-PCR (United States Centers for Disease Control and Prevention)	1084 bp	–80°C	HIV-infected children younger than two years
Zhang et al. (34)	ATCC kit ^b	1084 bp	Ambient <2 weeks then –80°C	Treated adults and children in Kenya and United Republic of Tanzania

^a The quality of field-collected DBS is probably substantially inferior to that of laboratory-collected DBS (which are often used in comparison studies) and plasma.

^b Kit now manufactured by ThermoFisher Scientific.

Study	Number of samples tested	Viral load of tested samples (copies/mL)	Amplification success rate ^a	Sequence concordance versus plasma
Ziemniak et al. (13)	9	<50 to 94 600 (median: 17 792)	Overall: 94% Viral load \geq 193 copies/mL: 100%	Not assessed
Bertagnolio et al. (4)	103	Not all tested	90.1% either protease or RT region; 78.2% for both regions	99.9% (in samples with resistance mutations)
Masciotra et al. (8)	60	78 to 676 694 (median: 9 135)	Overall: 83% Viral load >2000 copies/mL: 100% Viral load <2000: 54%	98.8%
McNulty et al. (9)	40	665 to 645 256 (median: 23 715)	Overall: 92% viral load >10 000 copies/mL: 100% viral load <10,000 copies/mL: 73%	98.5%
Steege et al. (11)	29	55 to >100 000	96.6% either protease or RT region; 89.7% for both regions; viral load >100 copies/mL: 100%	Not assessed
Buckton et al. (5)	12	80 to 115 300 (median 10 950)	protease: 83% RT: 100%	Not assessed
Hallack et al. (7)	33	1 178 to 414 212 (median: 11 666)	Overall: 78.8% viral load >6000 copies/mL: 90.5% viral load <6000 copies/mL: 58.3%	99.3%
Garrido et al. (6)	77	1 000 to 850 000	RT: 30% gp41: 43%	Not assessed
Youngpairoj et al. (12)	40	518 to 676 694 (median: 13 680)	ViroSeq™: 57.5% In-house: 95%	94.5% (drug resistance mutations, DBS/in house versus plasma/ViroSeq)
Monleau et al. (32)	12	1 380 to 263 000	100%	Not assessed
Yang et al. (20)	171	Subset of 70 specimens: <400 to 367 875 (median: 8 021)	Overall: 87.1% Subset of 70 specimens: 94%	Not assessed
Zhou et al. (19)	98	Nigeria: 150 to 436 500 (median: 9 332)	Nigeria: 96.1% Viet Nam 95.8%	Nigeria 98.8% Viet Nam 98.9%
Aitken et al. (24)	25	1 020 to 449 000	84%	97%
Bronze et al. (23)	41	270 to 15 519 576	76% for viral load >5000 copies/mL 15% for viral load <5000 copies/mL	Not reported (100% concordance in resistance profiles)
Inzaule et al. (22)	68	>10 000: $n = 44$, 1000–10 000: $n = 13$ <1000: $n = 11$	>10 000 copies/mL: 100% 1000–10 000 copies/mL: 54% <1000: 9%	99.5%
Monleau et al. (21)	124	\geq 1 000 in plasma	77%	<1%, <2% and <3% nucleotide differences seen in 23%, 64% and 83% of the samples, respectively
Salimo et al. (33)	238	Majority >100 000	98.7%	99.5%
Zhang et al. (34)	499	>1 000	92%; ~82% for viral load 1000–5000 copies/mL	Not assessed

SAFETY NOTE:

Working with DBS, whole blood or plasma requires the same biohazard safety precautions. DBS do not introduce new biohazards to laboratory technicians. Universal blood and body fluid precautions should be observed for all dried fluid spot (serum, plasma or whole blood) specimens.

Laboratory supplies

The following supplies and equipment are needed for preparing, storing and shipping DBS. Additional supplies and equipment are required for nucleic acid extraction, RT-PCR and sequencing and are included in the recommended protocols that follow.

- Class II biological safety cabinet
- A solution of 0.5% sodium hypochlorite in a spray bottle (a 1:10 dilution of household bleach)
- Gloves (non-powdered, latex or nitrile) and laboratory coat (cloth or disposable) dedicated for use in each area
- Calibrated micropipettes capable of dispensing volumes in the range of 1–200 μL , with compatible, sterile-filtered tips
- Biohazard waste disposal bin
- Individually packaged 70% isopropyl alcohol wipes or povidone-iodine swabs
- Sterile, disposable, single-use lancet
- Filter paper cards (Whatman 903, Munktell TFN or Ahlstrom grade 226) (35,36) (other sources and types of filter paper cards may also be acceptable but should be thoroughly validated for use in drug resistance genotyping assays first)
- Desiccant sachets
- Humidity indicator (may be combined with desiccant)
- Zip-locked gas-impermeable plastic bags
- Assorted sterile racks

Preparing DBS from specimens collected by venepuncture

1. Anticoagulated (EDTA or ACD) venous blood should be spotted onto filter paper as soon as possible after collection and preferably within 24 hours. Blood without an anticoagulant should be spotted immediately upon collection (<5 minutes after collection), since the blood will begin to clot within minutes. Between the times of collection and spotting, blood should be stored cold, but not frozen, or at room temperature if refrigerated storage is not available.
2. Label the filter paper card with appropriate identification information for the patient. A filter should only be spotted with the blood of a single patient.
3. Handle the filter paper by the edges; do not touch the areas that will be used to collect specimens.
4. For recently collected, fresh whole blood, invert the blood collection tube 2–3 times to mix the whole blood. Carefully open the blood collection tube.
5. Use a micropipettor (with disposable tip) to aspirate 75 μL of whole blood, and without touching the tip to the paper, dispense blood to the centre of one pre-printed circle to fully saturate the circle. Alternatively, use a Pasteur pipette or equivalent clean, disposable means of liquid transfer, to fully saturate the spot to the outline.
6. Repeat this procedure to fill each circle on the card with blood. At least four saturated circles should be obtained for each specimen (although five circles are preferred).

Preparing DBS from specimens collected by finger-prick

1. Put on gloves.
2. Label the filter paper with appropriate identification information (patient's identification and collection date).
3. Handle the filter paper by the edges to avoid cross-contamination. Do not touch the areas that will be used to collect the specimens.
4. Clean the skin area for puncture with antiseptics. Individually packaged 70% isopropyl alcohol wipes may be used to clean the puncture site. Povidone-iodine swabs may also be used.
5. Use a sterile, disposable single-use lancet to puncture the skin to the side of the fingertip. The puncture must be performed with sufficient force and penetration to sustain a flow of at least several drops of blood. For a heel stick, the steps for a finger stick should be followed except that different disposable lancets are recommended. The lancing procedure should yield at least 200 μ l of whole blood.
6. With the finger extended, allow a large, hanging, drop of free-flowing blood to accumulate at the puncture site. To collect the drop, touch the filter paper to the edge of the drop, allowing the blood to be drawn into the card by capillary action. Avoid allowing the finger to touch the card. Then, allow another large drop to form at the puncture site and collect this drop in the next circle. Do not layer successive drops of blood on top of each other. Continue to collect drops in the same manner, filling all the circles completely in the card or until the wound ceases to bleed. If the wound stops flowing before sufficient blood has been collected, the wound may be massaged very gently to encourage blood droplets. Do not squeeze the wound to obtain more blood. If the specimen collection is incomplete and no more blood is being produced from the initial puncture wound, this procedure may be repeated on the adjacent finger.
7. Collect an adequate sample. To do, this you must saturate each circle with blood. For each patient, at least four completely saturated circles must be collected (although five circles are preferable).
8. Supply the participant with a plaster to cover the puncture site, as required.

NOTE:

Do not test any DBS that have any contamination with a foreign substance, contain blood clots or are non-uniformly saturated with blood.

Drying

The time needed to dry a DBS differs according to ambient temperature and humidity conditions. Generally, it is recommended to dry all specimens for at least four hours (though preferably overnight) in a suspended horizontal position on a drying rack in a biohazard safety cabinet with air circulation. If a biosafety cabinet is not available, leave the DBS in an area with no or only limited foot traffic. Do not use an external heat source to dry DBS. When dry, the spots will appear a uniform dark brown. The appearance should be similar to that of a dried bloodstain and no areas of red coloration should be seen (Fig. 1). Proceed to packaging, storage or shipping as soon as possible.

Fig. 1. Dried DBS on a 903 filter paper card



Important considerations for packaging DBS

1. The nucleic acids in DBS are extremely sensitive to degradation in the presence of moisture. This means that specimens must be properly stored in the presence of desiccant packs. Humidity indicator cards and desiccant packs have a colour indicator that changes from blue to pink as humidity increases. All humidity indicator cards and desiccant packs should be immediately replaced if the presence of moisture is indicated.
2. When humidity indicator cards and desiccant packs are changed in specimens that have been stored at 4°C or in the freezer, the bag containing the DBS must be pre-equilibrated to room temperature. Remember: opening DBS packs immediately upon transfer from low-temperature storage will result in condensation on the DBS specimens and storage bags.
3. Before placing desiccant packs into the plastic bag with the DBS, ensure that the desiccant packs have remained dry during storage. Desiccant packs can become moist after use with DBS, but also after storage in a humid environment. Store desiccant packs with humidity indicator cards to assess whether their moisture level has become too high.
4. Desiccant packs can be reused. Moist desiccant packs should be dried in a 65°C oven overnight. Remove from the oven and store in a sealed bag with a humidity indicator.

Note:

Plastic or foil bags used for storage must be gas-impermeable. Bags available from grocery stores or other outlets that do not sell scientific supplies are inadequate since they are not humidity proof.

Packaging

1. Ensure that the DBS are completely dry before packing by providing adequate drying time.
2. Filter paper cards should be individually packaged in a single gas-impermeable, sealable plastic bag containing 2–3 desiccant packs to remove residual moisture along with one humidity indicator card. Ensure that the sample identification and study name are clearly written on both the DBS card and on the plastic bag.
3. Place the humidity indicator card into the bag such that the humidity indicator can be read without opening the bag. Gently apply pressure to the partly sealed bag to expel the air before sealing it completely.
4. Place 5–10 of these small bags into a large plastic bag that also contains a printed manifest with specimen information. The manifest should be written such that there is only one specimen per line and should contain the following information:
 - a. HIV drug resistance survey identification number (refer to the survey concept notes for ID format: for example, ZAF-PDR-2014-0001);
 - b. site identification number;
 - c. date and time blood collected;
 - d. date and time DBS prepared.

If the specimens are being collected as part of a WHO HIV drug resistance survey, refer to the survey protocol for additional information that may be required to be recorded on the specimen manifest.

Handling and storage

- If DBS can be transported to the genotyping lab at ambient temperature within 14 days from the date of collection, they can be kept and/or transported to the genotyping lab at ambient temperature (37). However, the total time at ambient temperature should be minimized and the temperature should be kept as low as possible without freezing. The total time from collection to processing or transfer to long-term storage in a freezer must be less than 14 days.
- Since humidity and ultraviolet light can damage DBS, always keep them in ziplock bags with desiccant, in the dark.
- If the DBS cannot be shipped to the genotyping lab within 14 days, transport the DBS at room temperature to a laboratory with a constant electricity supply and freeze them in a –80°C freezer.

- In settings where -80°C freezers are not available, non-frost-free -20°C freezers can also be used for long-term storage (at least up to two years). Similar to all essential laboratory equipment, the freezer temperatures should be monitored and calibrated at regular intervals.
- If the DBS have been stored refrigerated or frozen, they should only be taken out of cold storage when they are being tested or when desiccant and humidity indicators are being replaced, to prevent condensation from forming in the plastic bags and to avoid unnecessary freeze–thaw cycles.

Table 2 presents a list of publications studying storage conditions for DBS and their main findings.

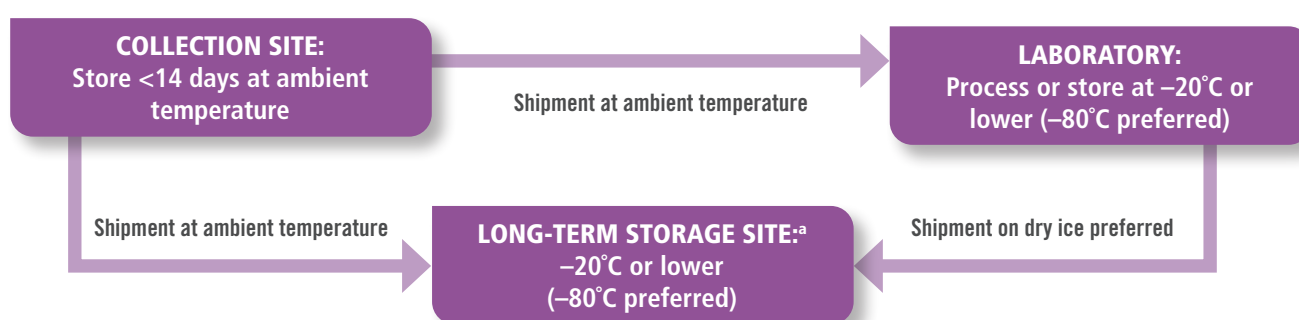
Table 2. Overview of published studies investigating optimal storage conditions for DBS

Study	Storage conditions	Desiccant	Outcomes
Garcia-Lerma et al. (16)	1–16 weeks at $+37^{\circ}\text{C}$ and high humidity, -20°C	Yes	DBS stable at $+37^{\circ}\text{C}$ for only 1–2 weeks. -20°C recommended for long-term storage. -20°C superior for short- and long-term storage
Bertagnolio et al. (4)	3 months at $+37^{\circ}\text{C}$ and 85% humidity	Yes	Good amplification rate (90%)
McNulty et al. (9)	6 years at -30°C 5 years at ambient temperature and -70°C 2–3 years at -20°C	Yes	Complete degradation at ambient temperature; stable at -30°C and -70°C ; -20°C recommended for long-term storage
Nelson et al. (15)	3–6 years at ambient temperature	Yes	Moderately successful amplification rate (69%); 1 log drop in viral load
Wallis et al. (38)	3 months at ambient temperature, $+4^{\circ}\text{C}$ and -20°C	Yes	Some reduction in amplification rate at ambient temperature versus $+4^{\circ}\text{C}$ or -20°C
Parry et al. (37)	2–4 weeks at ambient temperature	Yes	No significant reduction in amplification rate at ambient temperature for 2 weeks (93%) vs. frozen DBS or plasma (97–98%); slight reduction at 4 weeks (89%).
Monleau et al. (32)	1–2 months at ambient temperature and $+37^{\circ}\text{C}$ and high humidity	Yes	11 of 12 DBS amplified after 1 or 2 months at ambient temperature; 10 of 12 or 7 of 12 at $+37^{\circ}\text{C}$
Aitken et al. (39)	4 weeks at -20°C and $+30^{\circ}\text{C}$	Yes	Stable for up to 2 weeks at -20°C or $+30^{\circ}\text{C}$ (viral load $>10\,000$ copies/mL); slight decrease at 2 weeks, $+30^{\circ}\text{C}$ for viral load 1000 copies/mL

Transport

Fig. 2 provides an overview of DBS shipping procedures.

Fig.2. Specimen transport



^a May be the same facility as collection site.

UNDERLYING PRINCIPLES:

1. ≤ 14 days at ambient temperature before processing
2. Long-term storage at -20°C or below, -80°C preferred
3. Keep dry with desiccant at all times
4. Avoid unnecessary freeze–thaw cycles

- Depending on in-country regulations, specimen cards can be shipped to the designated specialized, regional or national drug resistance testing laboratory as non-hazardous materials using regular mail or courier services (3).
- Before shipment, the quality of the collected specimen should be examined and recorded including: integrity of the packaging, condition of the desiccant, humidity indicator reading, overt signs of specimen cross-contamination (two cards in direct contact with one another) and the quantity of DBS.

Transport of DBS specimens stored at ambient temperature

If the DBS have been stored at ambient temperature, either at the collection site or at an intermediate storage facility, and the total time from collection to processing or transfer to long-term storage in a freezer at the destination will be less than 14 days, the recommended procedure for transport to the genotyping laboratory from the collection site to the long-term storage site or the genotyping laboratory is as follows.

- Specimen cards should be maintained in the original gas-impermeable plastic bag with desiccant until the time of transport.
- Change the desiccant before transport, even if the indicator remains blue.
- For DBS that have been stored at room temperature, no special arrangements need to be made to transport DBS as long as they are shipped at least weekly.

Transport of frozen DBS specimens

If the DBS have been stored frozen (-20°C or below), either at the collection site or at an intermediate storage facility, the recommended procedure for transport to the genotyping laboratory depends on the feasibility of shipping on dry ice.

When dry ice is available

- Specimen cards should be maintained in the original gas-impermeable plastic bag with desiccant until the time of transport.
- Change the desiccant before transport if the bags have remained at the collection site longer than 14 days before transport, even if the indicator remains blue.

When dry ice is not available

- Specimen cards should be maintained in the original gas-impermeable plastic bag with desiccant until the time of transport.
- DBS should be removed from the freezer and be allowed to thoroughly equilibrate to room temperature for a minimum of thirty minutes before opening the bag. After equilibrating, the outer bag should be opened and the desiccants contained in each of the small plastic bags replaced with fresh desiccant for shipping. The equilibrated DBS should be placed in a new plastic bag containing a humidity indicator and desiccant and shipped at room temperature.

Receipt of DBS at the processing laboratory

All DBS specimens should be logged in at the laboratory (whether it is logbook or a laboratory information management system). Each specimen record should include notes on specimen quality and packaging.

Before testing, the quality of the collected specimen should be examined and recorded, including the integrity of the packaging, the condition of the desiccant and the humidity indicator reading. Contact the survey coordinator immediately if there are any concerns about the packaging or integrity of the specimens.

Note:

Desiccant packs and humidity indicator cards that have changed to a pink colour should be replaced with fresh ones as soon as practical.

Using appropriate control reagents for HIV drug resistance testing from DBS

Laboratories must ensure that all processes are running optimally before survey specimens are tested. This includes monitoring recent results from low plasma viral load (such as 1000–10 000 copies/mL) positive controls; if DBS have not been tested recently, a panel of positive controls and/or previously tested specimens should be tested. If viral load results are available for survey specimens, those with high plasma viral load (over 10 000 copies/mL) should be tested first, and if amplification rates do not exceed 90%, survey coordinators and WHO laboratory experts should be consulted before proceeding.

To ensure maintenance of appropriate quality control, a positive and negative control specimen must be tested with each batch of DBS tested. Both controls should be tested from the sample extraction stage (from the DBS itself) and until amplification is assessed by agarose gel electrophoresis of the nested PCR amplicons. A valid and acceptable DBS testing run is one in which the negative DBS control is PCR negative and the positive DBS is positive, as determined by agarose gel electrophoresis. However, to validate the sequencing run, if the positive DBS control is not carried forward to sequencing, a positive control DNA template should be sequenced with the specimens. A batch of DBS controls and a sequencing control should be prepared in advance in large quantity, stored along with DBS study specimens and tested in replicate assays before use along with unknown specimens in test batches.

Negative DBS control: DBS made from normal human whole blood (from a known HIV-negative individual)

Positive DBS control: Use one of the following.

- A previously tested DBS specimen, successfully amplified and sequenced at least twice, with known plasma viral load above 1000 copies/mL.
- DBS from a HIV whole-blood specimen with known subtype and a viral load of greater than 10 000 copies/mL. If possible, a low-positive DBS control with viral load between 1000 and 10 000 copies/mL should also be included.
- DBS prepared from a normal whole-blood sample spiked with HIV-1 from a high viral load plasma specimen or grown in cell culture.

Validating HIV drug resistance testing from DBS

Laboratories performing HIV DBS testing in the context of the WHO HIV drug resistance surveys should use a standardized method that has been validated according to HIVResNet guidelines, including participating in an external quality assessment programme. Annex 3 of the *WHO HIVResNet HIV drug resistance laboratory operational framework (40)* outlines the minimum recommended components for validating an in-house genotyping assay. This list of requirements assumes that the laboratory is already designated by WHO for performing HIV drug resistance genotyping from plasma and that the DBS-based assay shares post-RNA extraction procedures that are the same as an existing and validated plasma-based assay.

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ANNEX 1. RNA EXTRACTION AND GENOTYPING PROTOCOLS FROM THE FRENCH NATIONAL RESEARCH INSTITUTE FOR SUSTAINABLE DEVELOPMENT

RNA extraction from plasma, dried blood spots or dried plasma spots (MINIMAG®, bioMérieux)

Source: French Agency for Research on AIDS and Viral Hepatitis (ANRS) (Peeters Laboratory, Montpellier, France)

Contact: Martine Peeters (martine.peeters@ird.fr), Christelle Butel (christelle.butel@ird.fr) or Laetitia Serrano (laetitia.serrano@ird.fr)

Principles and definitions

Abbreviations

RNA: ribonucleic acid

DNA: deoxyribonucleic acid

RNase: ribonuclease

DNase: deoxyribonuclease

The principle for nucleic acid extraction is that of the Boom method, adapted by bioMérieux. This method is a solid-phase extraction technique for nucleic acids that uses silica particles. The use of magnetic silica makes the technique easier and permits increased yields. This method has demonstrated excellent capacity to extract DNA and RNA from several specimen types (whole blood, serum, plasma, stool, sputum, cerebrospinal fluid, etc.). Extraction can be performed using variable volumes of specimen up to 1000 µL. The Boom method is compatible with all anticoagulants (EDTA, citrate and heparin) and enables users to obtain nucleic acids with high purity that are suitable for various applications such as viral load measurement, sequencing and genotyping.

The biological sample is added to a lysis buffer. All nucleic acids are extracted from cellular material or viral particles. RNases and DNases are inactivated. Then magnetic silica particles are added, and nucleic acids are bound under high salt conditions. The silica particles act as a solid phase, with other components being removed through extensive washing using the bioMérieux NUCLISENS® MINIMAG® instrument. Finally, the nucleic acids are eluted.

Refer to bioMérieux's instruction manuals for details on operating the instrument.

Instructions and procedures

1. Safety procedures

All biological materials should be considered to be potentially infectious.

Some buffers contain guanidinium thiocyanate, which is toxic and not compatible with disinfectants containing chlorine bleach. Lab coats, gloves and eye protection are therefore required during handling.

RNA is extremely sensitive to degradation by RNases; RNase-free buffers and materials must be used at all times. Change gloves regularly.

2. Materials and equipment

NUCLISENS® magnetic extraction reagents and buffers (see reference below)

Complete MINIMAG® installation (MINIMAG® instrument, Eppendorf ThermoMixer® and vacuum pump)

DNA, RNA and RNase-free 1.5-mL microtubes with screw cap

DNA, RNA and RNase-free sterile pipette tips with filter

DNA, RNA and RNase-free sterile pipette tips without filter (P200)

Biohazard quality gloves

Disposable lab coats

Micropipettes

+4°C refrigerator

–80°C freezer

Vortex

Centrifuge for 15-mL tubes and microtubes

3. Procedure

3.1 Reagents and buffers

The buffers are ready to use.

Lysis buffer (ref. 280134), 4 times 1 L: stored at room temperature in the dark.

Note: lysis buffer can also be purchased pre-aliquoted in 2-mL tubes (ref. 200292) or 9-mL tubes (ref. 284047). Contains 5 M guanidine thiocyanate. May have a light yellow colour. May form crystals if stored at 4°C, which must be dissolved at 37°C before use.

Magnetic extraction reagents (ref. 200293): stored between 2°C and 8°C. Warm to room temperature before use.

- Silica (SIL): paramagnetic silica particles
- Wash Buffer 1: contains guanidine thiocyanate (resembles lysis buffer)
- Wash Buffer 2: contains an organic solution and a preservative
- Wash Buffer 3: contains an inorganic solution; same composition as the Elution Buffer
- Elution Buffer: contains an inorganic solution.

Magnetic extraction reagents can be purchased separately in 4 times 1-L sizes. However, Wash Buffers 2 and 3 do not contain preservatives.

3.2 Procedure

3.2.1a Plasma specimen lysis step (bioMérieux protocol)

Virus containment (BSL 2+ or 3) laboratory (virus lysis and inactivation)

1. Aliquot 2 mL of lysis buffer into one Falcon 15-mL tube per specimen and label the tube or centrifuge pre-aliquoted bioMérieux tubes for 10 seconds at $1500 \times g$.
2. Centrifuge plasma at $1500\text{--}2000 \times g$ for 3 minutes.
3. Withdraw between 10 and 1000 μL of supernatant, add to the lysis buffer and vortex.

4. Incubate for at least 10 minutes at room temperature.
5. Centrifuge for 1 minute at $1500 \times g$.

3.2.1b Filter paper (DBS, DPS) specimen lysis step

Virus containment (BSL 2+ or 3) laboratory (specimen elution from paper, virus lysis and inactivation)

1. Aliquot 2–4 mL lysis buffer into one Falcon 15-mL tube per specimen and label the tube. Use 2 mL for $2 \times 50 \mu\text{L}$ spots or 4 mL for $4 \times 50 \mu\text{L}$ spots.
2. Cut DBS or DPS spots in half and add to the lysis buffer.
3. Incubate for 30 minutes (maximum 2 hours) at room temperature under agitation.
4. Using a sterile disposable Pasteur pipette, triturate the filter paper in lysis buffer, then transfer the lysis buffer containing the lysed specimen to a new 15-mL tube, leaving behind as much of the paper debris as possible.
5. Centrifuge for 2 minutes at $1500 \times g$ to remove residual paper fibres.
6. Transfer the supernatant containing the lysed specimen to a new 15-mL tube using a sterile disposable Pasteur pipette, being careful not to disturb the paper fibres at the bottom of the tube.

The rest of the protocol is identical no matter what type of specimen was used.

Note: specimens in lysis buffer may be stored for 2 days at room temperature, 4 hours at 37°C or 2 months at -70°C .

3.2.2 Capture of nucleic acids using magnetic silica

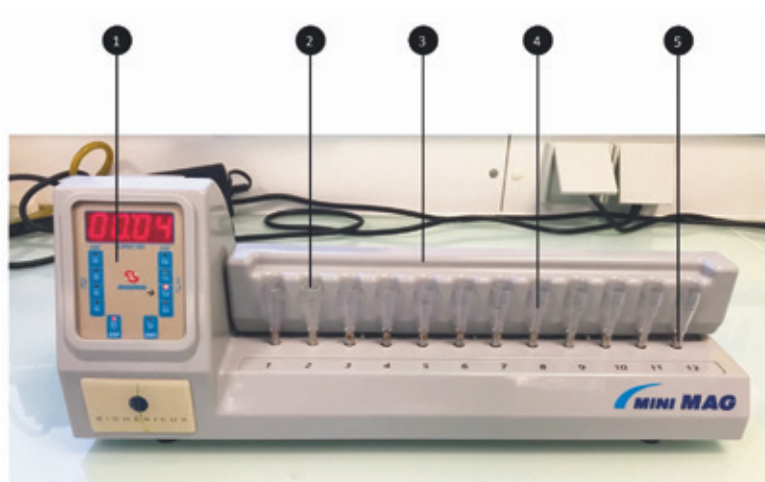
Virus containment (BSL 2+ or 3) laboratory

1. Vortex the magnetic silica.
2. Add $50 \mu\text{L}$ of magnetic silica per tube. Vortex briefly.
3. Incubate for 10 minutes at room temperature.
4. Centrifuge for 2 minutes at $1500 \times g$.

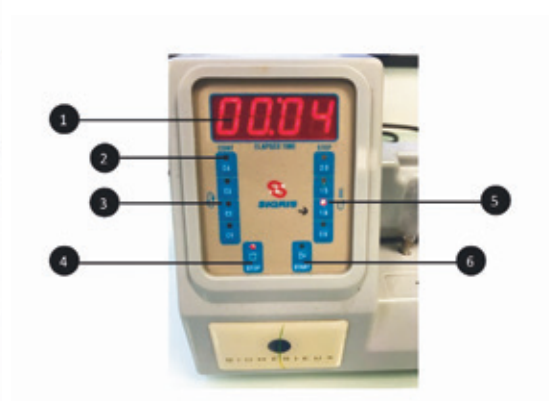
Go to the BSL 2 laboratory (RNA template preparation area; separate from PCR master mix preparation area).

3.2.3 Washing and elution (bioMérieux protocol)

In a BSL 2 laboratory (RNA template preparation area), working on the RNA bench



1. Keypad
2. 1.5 mL microtubes
3. Magnetic rack assembly
4. Racks
5. Mounting pins



1. Timer
2. LED indicator
3. Mixing speed options
4. Stop mixing
5. Staged mixing duration options
6. Start mixing

Preparation

1. Turn on the MINIMAG®, choose the mixing speed option “1 second” (number 5 in the right side of the figure below) and lower the magnetic rack.
2. Turn on the vacuum pump.
3. Turn on the Eppendorf ThermoMixer® and set it to 60°C.
4. Label one 1.5-mL screw-cap tube per specimen and place on the MINIMAG® (without lids). The wash steps using the MINIMAG® are done with the tubes open.
5. Label standard 1.5-mL tubes for elution.

Washing

1. Aspirate the lysis buffer from the 15-mL tube containing the silica base using the vacuum pump and a P200 pipette tip without filter. Use a new tip for each specimen.
2. Add 400 µL of Wash Buffer 1, mix the silica with a pipette, and carefully transfer to the 1.5 mL screw-cap tube on the MINIMAG®.
3. Raise the magnetic rack and wash 30 seconds by pressing the START button and then the STOP button after 30 seconds.
4. Aspirate the supernatant with the vacuum pump.
5. Lower the magnetic rack and add 400 µL of Wash Buffer 1.
6. Wash for 30 seconds as before and aspirate the supernatant.
7. Add 500 µL of Wash Buffer 2.
8. Wash for 30 seconds as before and aspirate the supernatant.
9. Add 500 µL of Wash Buffer 2.
10. Wash for 30 seconds as before and aspirate the supernatant.
11. Add 500 µL of Wash Buffer 3.
12. Wash for a maximum of 15 seconds as before and aspirate the supernatant.

Notes

- After the wash buffers are removed, be careful not to leave drops on the sides of the tubes.
- As the wash steps proceed, reduce the intensity of the vacuum pump to avoid aspirating the silica.

Elution

1. Add 50 µL (25–100 µL) of elution buffer.
2. Remove the tube from the MINIMAG® and cap it.
3. Incubate for 5 minutes at 60°C in the Eppendorf ThermoMixer® at 1400 rpm.
4. Place the tubes on the blue magnetic rack and transfer the eluate to a new labelled 1.5-mL microtube
5. Verify that there is no residual silica in the eluate by replacing the tubes on the magnetic rack. If silica is observed, transfer the eluate into a new labelled 1.5-mL microtube and reverify.

Store the RNA at –80°C or use immediately.

ANRS RT-PCR method for HIV drug resistance genotyping by sequencing using plasma or DBS

Source: French Agency for Research on AIDS and Viral Hepatitis (ANRS) (Peeters Laboratory, Montpellier, France)

Contact: Martine Peeters (martine.peeters@ird.fr) or Marjorie Monleau (marjorie.monleau@ird.fr)

Principles and definitions

Abbreviations

RNA: ribonucleic acid

cDNA: complementary deoxyribonucleic acid

EtBr: ethidium bromide

PCR: polymerase chain reaction

bp: base pair

RT: reverse transcriptase

UV: ultraviolet

See also: ANRS procedures (available at <http://www.hivfrenchresistance.org>) and Plantier JC, Dachraoui R, Lemée V, Gueudin M, Borsa-Lebas F, Caron F et al. HIV-1 resistance genotyping on dried serum spots. *AIDS*. 2005;19:391–7.

Instructions and procedures

1. Safety procedures

All procedures require the use of gloves and lab coats.

Use of DNA amplification by PCR requires a laboratory set-up with separate rooms to avoid molecular contamination:

- a pre-PCR room (RNA extraction);
- a horizontal flow hood or dead-air cabinet for master mix preparation; and
- a post-PCR room, where the thermocycler, post-PCR hood (for nested PCR set-up), amplicon detection system (gel electrophoresis) and material for sequencing are located.

The procedures are carried out in a unidirectional manner to avoid molecular contamination.

Amplicon detection by agarose gel electrophoresis is especially dangerous because of the use of ethidium bromide (EtBr) and ultraviolet (UV) light. EtBr is a carcinogen and mutagen and must be handled with caution. UV light is especially dangerous to the unprotected eye.

2. Materials and equipment

2.1 Reagents and buffers

Invitrogen™ SuperScript™ One-Step RT-PCR System for Long Templates (ThermoFisher Scientific)

HotStarTaq® Master Mix Kit, Qiagen

Primers (see below)

Gloves

Filter pipette tips

Primers for protease fragment

PCR step	Primer name	Primer sequence (5'-3')	Size of fragment
RT-PCR	outer 3prot1AS	GCAAATACTGGAGTATTGTATGGATTTTCAGG	653 bp
	outer 5prot1S	TAATTTTTTAGGGAAGATCTGGCCTTCC	
Nested PCR	inner 3prot2AS	AATGCTTTTATTTTTTCTTCTGTCAATGGC	507 bp
	inner 5prot2S	TCAGAGCAGACCAGAGCCAACAGCCCCA	

Primers for reverse transcriptase (RT) fragment

PCR step	Primer name	Primer sequence (5'-3')	Size of fragment
RT-PCR	outer Mj3S	AGTAGGACCTACACCTGTCA	941 bp
	outer Mj4AS	CTGTTAGTGCTTTGGTTCCTCT	
Nested PCR	inner A35S	TTGGTTGCACTTTAAATTTCCATTAGTCCTATT	798 bp
	inner NE135AS	CCTACTAACTTCTGTATGTCATTGACAGTCCAGCT	

Note: if problems with RT-PCR primers are encountered, alternative primers can be tested (see the ANRS website, <http://www.hivfrenchresistance.org>).

Important notes

It is crucial to test each new lot of the Invitrogen™ SuperScript™ One-Step RT-PCR System for Long Templates (ThermoFisher Scientific) for molecular contamination (Monleau M, Plantier JC, Peeters M. HIV contamination of commercial PCR enzymes raises the importance of quality control of low-cost in-house genotypic HIV drug resistance tests. *Antiviral Ther.* 2010;15:121–6). When a new lot is received, record the lot number and perform the protease fragment PCR procedure using 10 reactions with only water as the template and one positive control. Confirm that the reactions with water are negative.

2.2 Equipment

Thermocycler

Pre-PCR (with air circulation and UV) and post-PCR (UV) hoods

Pipettes

Freezers

Vortex

3. Procedure

This nested RT-PCR protocol is used, starting from extracted RNA, to separately amplify the protease and RT regions of the HIV pol gene, yielding fragments of 507 and 798 bp, respectively.

3.1 Reverse transcription of RNA and first-round PCR: Invitrogen™ SuperScript™ One-Step RT-PCR System for Long Templates

Yields protease and RT fragments of 653 bp and 941 bp, respectively.

Include a negative control for each fragment amplified. In the pre-PCR (no template) room, in the hood, prepare the two master mixes (one for protease and one for RT) using the Invitrogen™ SuperScript™ One-Step RT-PCR System for Long Templates (ThermoFisher Scientific) system.

Master mix for first-round RT-PCR for protease and RT

Per 50 µL RT-PCR reaction:

2 × reaction mix ^a	25 µL
5 mM MgCl ₂ ^b	8 µL
outer primer 3' (20 pmol/µL)	1 µL (20 pmol)
outer primer 5' (20 pmol/µL)	1 µL (20 pmol)
RT/platinum Taq enzyme mix	1 µL
H ₂ O	4 µL
RNA ^c	10 µL

^a Final concentration 1.2 mM MgSO₄ and 0.2 mM of each dNTP.

^b Final concentration 0.8 mM MgCl₂ (total Mg⁺⁺ ion concentration 2 mM).

^c Add RNA in the pre-PCR RNA hood, after aliquoting.

Aliquot 40 µL of master mix to a 0.5-mL PCR tube per specimen or control labelled with the specimen number. Usually two tubes are needed per specimen, one each for the protease and RT.

Place the tubes in the thermocycler; run the following programme:

50°C for 30 minutes (1 cycle)

94°C for 2 minutes (1 cycle)

94°C for 30 seconds, 55°C for 30 seconds and 68°C for 90 seconds (35 cycles)

72°C for 7 minutes (1 cycle)

3.2 Second-round PCR

Note: performed with the HotStarTaq® Master Mix Kit from Qiagen; yields protease and RT fragments of 507 bp and 798 bp, respectively. **Include a second negative control for each fragment.**

Master mix for second-round PCR for protease and RT

Per 50 µL PCR reaction (45 µL of HotStarTaq® Master Mix + 5 µL of first-round PCR product):

HotStarTaq® Master Mix ^a	25 µL
25 mM MgCl ₂ (not supplied with kit) ^b	1 µL
Inner 3' primer (12 or 14 pmol/µL)	1 µL (12 pmol for RT and 14 pmol for protease)
Inner 5' primer (12 or 14 pmol/µL)	1 µL (12 pmol for RT and 14 pmol for protease)
H ₂ O	17 µL
First-round PCR product ^c	5 µL

^a 1 × buffer = 1.5 mM MgCl₂, 200 mM each dNTP, 2.5 U HotStarTaq® DNA polymerase.

^b (+ 0.5 mM MgCl₂); total 2 mM MgCl₂.

^c Add 5 µL of first-round PCR reaction in the post-PCR hood.

Place the tubes in the thermocycler; run the following programme:

95°C for 15 minutes (1 cycle)

94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute (35 cycles)

72°C for 10 minutes (1 cycle)

Then use your current protocols for amplification confirmation, purification of PCR products and sequencing.

Sequencing primers for RT

A(20): 5'-ATTTTCCATTAGTCCTATT-3'

NE1(20): 5'-ATGTCATTGACAGTCCAGCT-3'

The sequencing primers for protease are the same as for the second-round PCR.

Note: if problems with sequencing primers are encountered, alternative primers can be tested (see the ANRS website <http://www.hivfrenchresistance.org>).

ANNEX 2. RNA EXTRACTION AND GENOTYPING PROTOCOLS FROM THE ILB LABORATORY OF THE UNITED STATES CENTERS FOR DISEASE CONTROL AND PREVENTION

NUCLISENS® EASYMAG® using dried blood spots

NCHSTP/GAP International Laboratory

Doc. No.: ILB-300-P27

Rev. No.: 00

Effective date: 22 June 2010

Written by: Mary Garcia and Erin Rottinghaus

Approved by: Bereneice Madison and John Nkengasong

1. TITLE

NUCLISENS® EASYMAG® using dried blood spots

2. PURPOSE

- 2.1 The NUCLISENS® EASYMAG® platform is used for pre-extraction to enable HIV-RNA isolation (purification and concentration) from dried blood spots. The system is based on a generic method of binding nucleic acids from complex biological samples to magnetic silica.

3. REAGENTS

3.1 Components

- 3.1.1 NUCLISENS® 2 mL Lysis Buffer (cap colour: transparent): store at 2–30°C, bioMérieux catalogue no. 200292
- 3.1.1.1 Store at 2–30°C. Pre-warm the reagent for approximately 30 minutes at 37°C before starting the procedure.
- 3.1.1.2 Mix thoroughly at regular intervals by inverting the bottle. Ensure that the crystals have dissolved during reagent preparation.
- 3.1.2 NUCLISENS® EASYMAG® Magnetic Silica (cap colour: white), bioMérieux catalogue no. 280133
- 3.1.2.1 Store at 2–8°C.
- 3.1.2.2 Vortex the tube until a homogeneous suspension is formed before starting the isolation procedure.
- 3.1.2.3 Vortex again at each pipetting step.
- 3.1.2.4 Store and handle the silica well away from magnetic fields.
- 3.1.3 NUCLISENS® EASYMAG® Extraction Buffer 1 (colour code: red): 2–30°C, bioMérieux catalogue no. 280130
- 3.1.4 NUCLISENS® EASYMAG® Extraction Buffer 2 (colour code: white): 2–30°C, bioMérieux catalogue no. 280131
- 3.1.5 NUCLISENS® EASYMAG® Extraction Buffer 3 (colour code: blue): 2–30°C, bioMérieux catalogue no. 280132
- 3.1.6 Nuclease-free water, room temperature (15–30°C)

- 3.2 General storage requirements
 - 3.2.1 Protect against excess heat or light.
 - 3.2.2 Store the extraction reagents in the area of the laboratory dedicated to isolating nucleic acids or in a clean separate room.
 - 3.2.3 Only the required quantities of reagents should be removed from storage. Return unopened reagents to storage immediately.
 - 3.2.4 Opened reagents at room temperature expire within 30 days. Label reagents with the date opened and expiration date.
 - 3.2.5 Storing opened reagents is not recommended.
 - 3.2.6 Do not freeze reagents.
 - 3.2.7 Do not use expired reagents. The software will alert the user when an expired reagent or disposable is detected.

4. EQUIPMENT

- 4.1 NUCLISENS® EASYMAG® instrument, bioMérieux catalogue no. 200111
- 4.2 NUCLISENS® EASYMAG® computer, keyboard, hand-held barcode reader and touch screen, bioMérieux catalogue no. 200111
- 4.3 NUCLISENS® EASYMAG® User Software, bioMérieux catalogue no. 200111
- 4.4 Biohit electronic multichannel pipette, volume range 10 to 5000 µl, bioMérieux e1200 (50–1200µl) catalogue no. 7061217
- 4.5 Centrifuge
- 4.6 Roller mixer
- 4.7 Vortex
- 4.8 Timer

5. SUPPLIES

- 5.1 NUCLISENS® EASYMAG® disposables
 - 5.1.1 Each blister pack contains three sample strips (labelled with a unique barcode) and three aspirator disposables
 - 5.1.1.1 NUCLISENS® EASYMAG® Sample Strips provide 8 reaction vessels. The strips are designed to contain 2 mL of lysis buffer, up to 1 mL of sample, magnetic silica and an internal control.
 - 5.1.1.2 NUCLISENS® EASYMAG® Aspirator Disposables aspirate liquid from the samples strip vessels during the wash cycles.
 - 5.1.2 Store at room temperature, bioMérieux catalogue no. 280135
- 5.2 Additional materials
 - 5.2.1 Whatman 903® specimen collection paper, Whatman catalogue no. 10334885
 - 5.2.2 Dessicants, Multisorb Technologies catalogue no. 02-00040AG37
 - 5.2.3 Bitran specimen storage bags, Fisher catalogue no. 19-240127
 - 5.2.4 6 by 6 weighing paper, Fisher catalogue no. 09-898-12C
 - 5.2.5 Stainless steel surgical scissors, Fisher catalogue no. 50-109-3335
 - 5.2.6 5-mL tubes with caps, VWR catalogue no. 2110047 and 2110039
 - 5.2.7 Sterile disposable aerosol-resistant tips, bioMérieux catalogue no. 783222
 - 5.2.8 8-well strip plates Greiner, bioMérieux catalogue no. 278303
 - 5.2.9 Absorbent tissue or Kimtech® Science™ Kimwipes™, Fisher catalogue no. 06-666-11D
 - 5.2.10 Detergent (such as Extran® MAOI alkaline), Fisher catalogue no. MEX09934
 - 5.2.11 Small, medium and large disposable gloves, Fisher catalogue no. 50820576, 50820577 and 50820578
 - 5.2.12 BD Waste container with cap, Fisher catalogue no. 14-826-127

6. Samples

- 6.1 Preparing dried blood spots
 - 6.1.1 Collect blood in a sterile EDTA anticoagulant tube.
 - 6.1.2 On the same day, spot 100 µL of blood on Whatman 903® Specimen Collection Paper using a calibrated pipette. If blood spots cannot be prepared immediately after drawing blood, store blood tubes overnight at 2–8°C.
 - 6.1.3 Dry the filter paper for at least 3 hours (and a maximum overnight) at room temperature (15–30°C).
- 6.2 Storing dried blood spots
 - 6.2.1 Fold a sheet of weighing paper over each card and store the filter paper containing the dried blood spots with desiccants and humidity indicator in a Bitran specimen storage bag.
 - 6.2.2 Store at room temperature (15–30°C) for a maximum of 2 weeks.
 - 6.2.3 Store at –80°C if greater than 3 weeks.
- 6.3 Specimen transport of dried blood spots
 - 6.3.1 If shipment can be accomplished within 14 days of collection, the specimen may be shipped at room temperature (15–30°C).
 - 6.3.2 If the shipment cannot be accomplished within 14 days after collection, ship on dry ice.

7. Special safety precautions

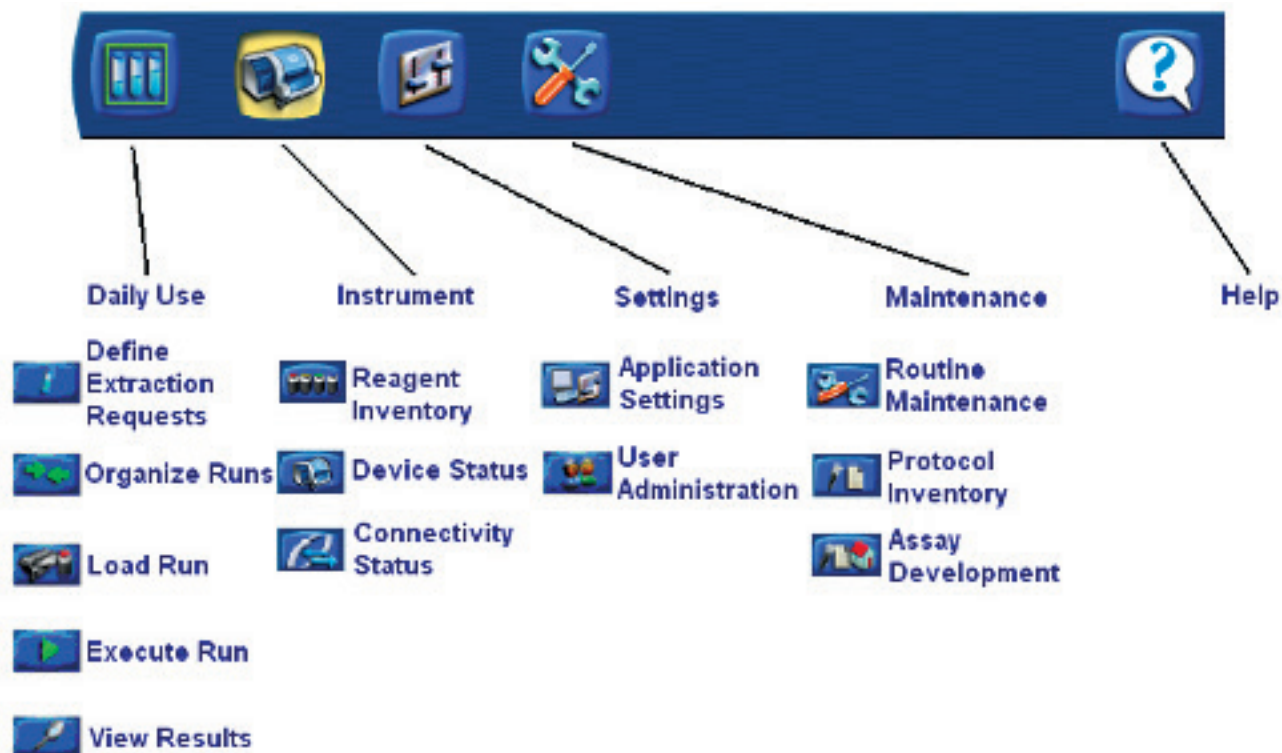
- 7.1 Handle diagnostic equipment, samples, controls and waste as potentially biohazardous materials.
- 7.2 Use personal protective equipment, including gloves, safety goggles and protective laboratory wear.
- 7.3 Ensure that appropriate decontamination is carried out if hazardous materials have spilled onto equipment or surrounding areas.
- 7.4 Hazardous chemicals (such as guanidine thiocyanate) are used in the reagent bay. Avoid contact with skin, eyes and clothing. Do not flush down a drain or mix with bleach. To prevent an unwanted chemical reaction, avoid mixing the liquid waste with other laboratory wastes. Inhaling mists may cause respiratory irritation.
- 7.5 Be familiar with the material safety data sheet for materials used in the procedures.
- 7.6 Do not allow the NUCLISENS® EASYMAG® Lysis Buffer, NUCLISENS® EASYMAG® Extraction Buffer 1 or waste from the instrument to come into contact with acidic materials. Cyanide gas can potentially be released.
- 7.7 Do not remove or raise the protective hood when the instrument is in operation.
- 7.8 Do not touch the heater while the instrument is operating and allow at least 10 minutes after turning off power.
- 7.9 Extreme care should be taken when handling tools and other magnetic material in close proximity to the magnet arrays, since sudden high mechanical forces may be generated.
- 7.10 People wearing a pacemaker should not use this product, since the strong magnetic fields associated with this product may adversely affect or damage the pacemaker.










8. Quality control

- 8.1 NUCLISENS® EASYMAG® Internal Calibrator, store at 2–8°C, bioMérieux catalogue no. 285000
 - 8.1.1 Calibrator containing lyophilized sphere containing synthetic calibrator RNA; this step only needed if RNA is also being used for viral load testing with the NucliSens EasyQ HIV-1 v2.0 Viral Load Kit. If not, substitute with nuclease-free water.
- 8.2 Positive and negative dried blood spots: store at –80°C. Bring to room temperature before testing.

9. Procedure

- 9.1 Refer to the NUCLISENS® EASYMAG® User Manual for a detailed description of the hardware components and software.
- 9.2 Preparing instruments
 - 9.2.1 Start the instrument and log on to the software after the LED on the NUCLISENS® EASYMAG® turns green. The following menu icons will be across the top of the touch screen.








- 9.2.2 Touch the SETTINGS icon  to default into the APPLICATION SETTINGS icon  and choose WORKFLOW . The screen will prompt DEFAULT REQUEST, RUN NAME PREFIX TYPE, RUN NAME PREFIX, SAMPLE ID PREFIX, SAMPLE TYPE (on-board or off-board lysis), WORKFLOW DEFAULTS (on-board lysis incubation)  and ON-BOARD SILICA INCUBATION .
- 9.2.3 Touch the INSTRUMENT icon  to default into the REAGENT INVENTORY icon  and input buffer barcodes.
- 9.2.4 To create a worklist, touch the DAILY USE icon  to default into the DEFINE EXTRACTION REQUEST icon .
 - 9.2.4.1 Samples can be entered manually.
 - 9.2.4.2 The sample matrix can be assigned. The request will default to generic 2.0.1 (other options are available, including specific protocols designed by R&D and user defined protocols).
 - 9.2.4.3 The volume (input) can be assigned.
 - 9.2.4.4 The elution volume must be assigned.
 - 9.2.4.5 If the run includes both lysed and primary samples, the information must be entered.

- 9.2.5 Press the ENTER key on the keyboard or press the NEW EXTRACTION REQUEST icon  after each manual sample addition (this step is unnecessary when using the barcode scanner). The changed settings will remain as the default settings for each new entry.
- 9.2.6 An auto-numbering format can also be used with the AUTO CREATE NEW EXTRACTION REQUESTS icon  to enter a list of samples. Specify the sample ID prefix (use the SETTINGS  screen) and the number of samples (drop-down presets are available or type in numbers from 1 to 24).
- 9.2.7 Save the sample list by touching the SAVE THIS EXTRACTION REQUEST icon .
- 9.2.8 Remove samples by highlighting selected samples and touching the REMOVE THIS EXTRACTION REQUEST icon .
- 9.2.9 Add comments by highlighting one or more samples and touching the ADD REMARK icon . Comments will be printed in the results document. Note: Comments cannot be removed after addition.
- 9.2.10 Touch the ORGANIZE RUNS icon  and then the CREATE RUN icon  to enter the run name. If the DATE function has been previously selected in the RUN NAME PREFIX of the SETTINGS menu , the current date and extraction request number for the day will automatically be filled in. ON-BOARD LYSIS INCUBATION  and ON-BOARD SILICA INCUBATION  can be selected within this window.
- 9.2.11 Assign samples to run with positioning icons   and  . Sample IDs are sorted in numerical and alphabetical order after transferring them to the worklist. The AUTO FILL RUN icon  will assign the first 24 samples in the unassigned sample list to the selected run. If there are fewer than 24 samples, the icon will move all of the available samples to the run.
- 9.2.12 Use the EDIT THIS RUN icon  to change the run name or DELETE THIS RUN icon  to delete the run name. The samples will be moved back into the unassigned sample list and will not be deleted.
- 9.2.13 Touch the LOAD RUN icon . Select the desired run from the dropdown menu. The last run entered in the previous screen will automatically be shown. Comments can be added to the selected run by touching the ADD REMARK icon .
- 9.2.14 Assign a sample reagent vessel to the appropriate run and location within the EASYMAG® (A, B or C) by barcoding the vessel within the BARCODE INPUT area. Other items such as lysis buffer, internal control, silica and internal control diluents can be barcoded within this area as well.
- 9.2.15 To assign kit reagents (lysis buffer, silica, internal control and internal control diluents) to an extraction request, select the ASSIGN KIT REAGENTS tab . From the KIT REAGENT list, select the desired reagent assigned to samples. Select one or more samples from the ASSIGN KIT REAGENT list and press the ADD icon . To remove reagents from an extraction request, highlight the extracts that have one or more reagents to be removed and press the REMOVE icon .
- 9.2.16 Print the worklist with the PRINT WORKLIST icon .


9.3 Preparing samples



9.3.1 Off-board lysis

- 9.3.1.1 Cut out one complete set of blood spots from the filter paper by using a pair of scissors, taking care not to touch the spotted blood. Use a new or clean pair of scissors for each new specimen and decontaminate before reuse.
- 9.3.1.2 Add the blood spot into a NUCLISENS® Lysis Buffer 2-mL tube.
- 9.3.1.3 Incubate the tubes in a horizontal position on a roller mixer for 30 minutes at room temperature.
- 9.3.1.3.1 Position the tubes on the roller mixer to keep the blood spots in constant contact with the Lysis Buffer.
- 9.3.1.3.2 Do not vortex the tubes. Degrading the filter material and releasing fibres can interfere with the extraction process.
- 9.3.1.4 Centrifuge the tubes for 15 seconds at $1500 \times g$ to spin down the fluid.
- 9.3.1.5 Use a fine-tip transfer pipette to transfer the lysate for nucleic acid extraction directly into one of the vessels of a NUCLISENS® EASYMAG® disposable. Leave any possible remains of paper material in the NUCLISENS® Lysis Buffer 2-mL tube.
- 9.3.1.6 Prepare premix:
- 9.3.1.6.1 Vortex magnetic silica. Use programme P1 (550 µl) to add magnetic silica to buffer or nuclease free water.
- 9.3.1.6.2 Vortex silica mixture and use programme P2 (125 µl) to dispense silica mixture into NUCLISENS® EASYMAG® strip wells.
- 9.3.1.7 Premix the preparation when using EasyQ for viral load analysis.
Note: this step only needed if RNA is also being used for viral load testing with the NucliSens EasyQ HIV-1 v2.0 Viral Load Kit. If not, substitute internal calibrator with nuclease-free water.
- 9.3.1.7.1 Use programme P1 to add nuclease-free water to internal calibrator lyophilized sphere.
- 9.3.1.7.2 Vortex magnetic silica and use programme P1 to add magnetic silica to internal calibrator preparation.
- 9.3.1.7.3 Vortex magnetic silica and internal calibrator preparation and use programme P2 to dispense the preparation into EASYMAG® sample strip wells.
- 9.3.1.8 Use programme P3 to add the silica mixture to sample vessels and insert the sample vessel into the instrument.
- 9.3.1.9 Barcode the sample vessel and assign reagent kits.
- 9.3.1.10 Touch the START icon  to begin the run. Note: This is the arrow on the right-side menu.
- 9.3.1.10.1 The instrument will perform five washes, heat and elute.
- 9.3.1.10.2 Eluates will be ready in less than 40 minutes and should be transferred to eight strip tubes for amplification or storage tube within 30 minutes after extraction is completed.
- 9.3.1.11 If the ON-BOARD SILICA INCUBATION icon  has been deselected, the run time will be about 30 minutes.
- 9.3.1.12 To view progress throughout the run, touch the execute run icon . Note: The arrow is on the top of the menu.
- 9.3.1.13 Comments can be added to the selected run by touching the ADD REMARK icon .
- 9.3.1.14 To abort the run, touch the STOP icon .


9.4 Reviewing the results

9.4.1 Once the run is finished, wait for the hourglass to become idle.

9.4.2 The results can be viewed with the VIEW RESULTS icon .

9.4.3 The results can be printed with the PRINT RUN RESULTS icon  or filed as a PDF with the EXPORT THIS RUN icon .

9.4.4 To add a comment to a run, touch the ADD REMARK icon .


9.4.5 To delete the run, touch the DELETE THIS RUN icon .

9.4.6 To rerun samples, highlight samples and touch the INITIATE REPEAT EXTRACTION icon .

Samples will be moved into the unassigned sample list. Note: Comments will not be transferred.


9.5 Shutdown procedure

9.5.1 To turn off the instrument:

9.5.1.1 Close the software, click the LOGOUT/QUIT/CHANGE PASSWORD icon  and allow all the software to power down. Once the light on the EASYMAG® stops flashing green and red and changes to a steady orange, it is safe to then power down the EASYMAG® instrument.

9.5.1.2 Wipe down the instrument with 5% Extran® followed by 70% ethanol and check the drip tray for leakage.



9.5.2 If the instrument shuts down during a run:

9.5.2.1 Clean the guanidinium isothiocyanate from the tubing. Press the MAINTENANCE icon  and follow the prompts for PROTOCOL #1 – CLEAN DISPENSE NEEDLES and #3 – CLEAN ASPIRATE (soap). The protocols are available under the TOOLS icon in the menu (the fourth icon).


9.6 Assay development


9.6.1 To develop specific extraction requests with predefined range of input and elution volumes, default



input and elution volumes and sample types, press the MAINTENANCE icon  and then choose

the ASSAY DEVELOPMENT icon . Press the CREATE NEW ASSAY icon  and input the parameters for ASSAY ID, VERSION NUMBER (needs to be 1.0 to be compatible with NucliSens®), EXTRACTION PROTOCOL to be used, INTERNAL CONTROL REQUIRED, SAMPLE TYPES, DEFAULT SAMPLE TYPE, VOLUME RANGE, DEFAULT VOLUME, ELUATE VOLUMES and DEFAULT ELUATE VOLUME accordingly. The newly created assay is then categorized as a DEVELOPMENT assay. The

initial settings can still be manipulated while in this state by pressing the EDIT ASSAY icon .






9.6.2 Press the PROMOTE ASSAY icon  to promote the selected assay to a TRIAL state. Changes can be applied to the assay.

9.6.3 Press the PROMOTE ASSAY icon  a third time while highlighted on an existing TRIAL assay for a RELEASED version selection. Once the assay is released, changes cannot be made to any of the previous versions.

9.6.4 Press the DEACTIVATE ASSAY icon  or the ACTIVATE ASSAY icon  to respectively remove or add an assay in the available drop-down menu of assays displayed within the DEFINE EXTRACTION

REQUESTS  menu.

9.6.5 Press the DELETE ASSAY icon  to delete any assay within the list.

- 9.7 Create new user
- 9.7.1 Touch the USER ADMINISTRATION icon . Touch the CREATE USER icon  and follow the prompts.
- 9.8 Maintenance procedure
- 9.8.1 Daily
- 9.8.1.1 Shut down the instrument and PC. See subsection 9.5: shutdown procedure.
- 9.8.1.2 To empty the waste container, touch the INSTRUMENT" icon  to default into the REAGENT INVENTORY icon . Then touch the EMPTY WASTE icon  and follow the prompts. There are two silver clasps that secure the bottle to the waste line. Press and hold both down and remove the waste line from the bottle lid. The waste bottle can be decontaminated and entered into the online waste ticket system (click on BIO) for proper disposal. Rinse the empty bottle with water.
- 9.8.1.3 Check the drip tray.
- 9.8.1.4 Check and clean the O-rings for crystals.
- 9.8.1.5 Wipe any lysis spillage with a tissue and clean the area with copious amounts of water.
- 9.8.1.6 Clean the instrument with Extran® MAOI alkaline cleaning solution.
- 9.8.1.7 Spray with 70% ethanol and wipe clean using a fibre-free paper towel.
- 9.8.1.8 Check and initial the ILB-300-F23B NUCLISENS® EASYMAG® maintenance form.
- 9.8.2 Weekly
- 9.8.2.1 Inspect the reagent cap vent filters, carbon filter and waste bottle filter.
- 9.8.2.2 Run maintenance protocol # 1 – clean dispense needles. The protocols can be found under the TOOLS icon in the menu (the fourth icon).
- 9.8.2.3 Initial and date the ILB-300-F23B NUCLISENS® EASYMAG® maintenance form.
- 9.8.3 Before the service engineer visit
- 9.8.3.1 Clean instrument with 5% Extran solution and 70% ethanol.
- 9.8.3.2 Run maintenance protocol # 1 – clean dispense needles.
- 9.8.3.3 Run maintenance protocol # 2 – clean waste (B3).
- 9.8.3.4 Run maintenance protocol # 3 – clean aspirate (soap).
- 9.8.3.5 Run maintenance protocol # 4 – replace liquid (EtOH).
- 9.8.3.6 Run maintenance protocol # 5 – replace liquid (B3).
- 9.8.3.7 Initial and date the ILB-300-F23B NUCLISENS® EASYMAG® maintenance form.

10. Calculations

Not applicable

11. Interpretation and results

- 11.1 After a run, the execution status can be one of the following:
- 11.1.1 Completed – the entire protocol was executed. Typically, all extraction requests were processed without problems. However, individual extraction requests may have warnings or errors. The run as a whole may have warnings but no errors.
- 11.1.2 Failed – the protocol may not have finished execution all the way to the end. An error has occurred for the run itself, an error has occurred for every sample strip or errors have occurred for every extraction request. The system stopped the run.
- 11.1.3 Aborted – the user stopped the run.

12. Reference intervals

Not applicable

13. Method performance specifications

13.1 Limitations

- 13.1.1 Reagents are not guaranteed to be completely free of nucleic acid contamination.
- 13.1.2 Do not mix leftover reagents for another extraction run.
- 13.1.3 The NUCLISENS® EASYMAG® sample strips are designed to extract DNA and RNA. Remove the eluates from the strips as soon as possible and process them in an amplification and detection system.
- 13.1.4 In case of an electrical static discharge, switch off the system and then switch it back on again. Failure to comply may cause the system to malfunction.
- 13.1.5 If the system's power supply is interrupted, data may be lost. Ensure that a reliable power supply is available.

14. References

- 14.1 NUCLISENS® EASYMAG® user manual version 1.1. The Netherlands: bioMérieux; 2005.
- 14.2 NUCLISENS® EASYMAG® 2.0.1 guide. Part # 60-00393-0. Durham: →-bioMérieux-USA.
- 14.3 Pre-extraction protocol for dried blood spots. Protocol # BTL052967. The Netherlands: bioMérieux; 2008.

NUCLISENS® MINIMAG® using dried blood spots

NCHSTP/GAP International Laboratory

Doc. No.: ILB-300-P32

Rev. No.: 00

Effective date: 30 September 2010

Written by: Joshua DeVos and Nick Wagar

Approved by: Bereneice Madison and John Nkengasong

1. TITLE

NUCLISENS® MINIMAG® using dried blood spots

2. PURPOSE

- 2.1 The NUCLISENS® MINIMAG® platform is used for manual isolation (purification and concentration) of total nucleic acids (RNA and DNA) from biological specimens for in vitro diagnostic use. The system is based on a generic method of binding nucleic acids from complex biological samples to magnetic silica.

3. REAGENTS

3.1 Components

- 3.1.1 NUCLISENS® Magnetic Extraction Reagents, bioMérieux catalogue no. 200293
- 3.1.1.1 Store at 2–8°C. Allow the kit to warm to room temperature before use.
 - 3.1.1.2 NUCLISENS® Magnetic Silica (colour code: white)
 - 3.1.1.2.1 Vortex the tube until a homogeneous suspension is formed before starting the isolation procedure
 - 3.1.1.3 NUCLISENS® Wash Buffer 1 (colour code: transparent)
 - 3.1.1.4 NUCLISENS® Wash Buffer 2 (colour code: red)
 - 3.1.1.5 NUCLISENS® Wash Buffer 3 (colour code: yellow)
 - 3.1.1.6 NUCLISENS® Elution Buffer (colour code: yellow)
- 3.1.2 NUCLISENS® Lysis Buffer, bioMérieux catalogue no. 200292
- 3.1.2.1 Store at 2–30°C. Pre-warm the reagent for approximately 30 minutes at 37°C before starting the procedure.
 - 3.1.2.2 Mix thoroughly at regular intervals by inverting the tube. Ensure crystals have dissolved during reagent preparation.
 - 3.1.2.3 Cool down to room temperature.

3.2 General storage requirements

- 3.2.1 Protect reagents against excess heat or light.
- 3.2.2 Store the extraction reagents in the area of the laboratory dedicated to the isolation of nucleic acids or in a clean separate room.
- 3.2.3 Remove only the required quantities of reagents from storage and return unopened reagents to storage immediately.
- 3.2.4 Magnetic silica, Wash Buffer 1 and Wash Buffer 2 can be stored at 2–8°C after opening for 14 days. Label reagents with opened date and expiration date. Wash Buffer 3 and Elution Buffer cannot be reused once opened.
- 3.2.5 Do not freeze reagents.
- 3.2.6 Do not use expired reagents.

4. Equipment

- 4.1 NUCLISENS® MINIMAG® instrument, bioMérieux catalogue no. 200305
- 4.2 Vortex
- 4.3 Centrifuge
- 4.4 Timer
- 4.5 Eppendorf ThermoMixer®

5. Supplies

- 5.1 NUCLISENS® 1.5-mL Micro Tubes with Caps
 - 5.1.1 Each box contains a bag of 500 1.5-mL tubes and a bag of caps
 - 5.1.2 Store at room temperature, bioMérieux catalogue no. 200294
- 5.2 Additional materials
 - 5.2.1 Whatman 903® specimen collection paper, Whatman catalogue no. 10334885
 - 5.2.2 Dessicants, Multisorb Technologies catalogue no. 02-00040AG37
 - 5.2.3 Humidity indicator cards, Multisorb Technologies catalogue no. 02-02291DG03
 - 5.2.4 Bitran specimen storage bags, Fisher catalogue no.19-240127
 - 5.2.5 6x6 weighing paper, Fisher catalogue no. 09-898-12C
 - 5.2.6 Stainless Steel Surgical Scissors, Fisher catalogue no. 50-109-3335
 - 5.2.7 5-mL microcentrifuge tube, USA Scientific Catalogue catalogue no. 3882-7600
 - 5.2.8 Absorbent tissue or Kimtech® Science™ Kimwipes™, Fisher catalogue no. 06-666-11D
 - 5.2.9 Detergent (such as Extran® MAOI alkaline), Fisher catalogue no. MEX09934
 - 5.2.10 Small, medium and large disposable gloves, Fisher catalogue no. 50820576, 50820577 and 50820578
 - 5.2.11 BD Waste container with cap, Fisher catalogue no. 14-826-127
 - 5.2.12 K2EDTA plastic blood collection tubes, BD catalogue no. 367841 (2.0 mL) and catalogue no. 367861 (4.0 mL)

6. Samples

- 6.1 Preparation of dried blood spots
 - 6.1.1 Sterile K2EDTA plastic blood collection tube
 - 6.1.1.1 On the same day, spot 100 µL of blood on Whatman 903® Specimen Collection Paper using a calibrated pipette. If blood spots cannot be prepared immediately after drawing blood, store blood tubes overnight at 2–8°C.
 - 6.1.1.2 Dry the filter paper for at least 4 hours or overnight (preferred) at room temperature (15–30°C).
 - 6.1.2 Finger prick or heel prick
 - 6.1.2.1 Collect each drop of blood in preprinted circular area on Whatman 903® Specimen Collection Paper. Do not layer successive drops of blood in the same spot.
 - 6.1.2.2 Typically, there are five preprinted circles on the Whatman 903® Specimen Collection Paper. Collect a minimum of three spots.
 - 6.1.2.3 Dry the filter paper for at least 4 hours or overnight (preferred) at room temperature (15–30°C).
- 6.2 Storage of dried blood spots
 - 6.2.1 Fold a sheet of weighing paper over each card and store the filter paper containing the dried blood spots, desiccant and humidity indicator in a Bitran specimen storage bag.
 - 6.2.2 Store at room temperature (15–30°C) for a maximum of 2 weeks.
 - 6.2.3 Store at -80°C if greater than 2 weeks.
- 6.3 Specimen transport of dried blood spots
 - 6.3.1 If shipment can be accomplished within 2 weeks of collection, the specimen may be shipped at room temperature (15–30°C).
 - 6.3.2 If the shipment takes longer than 14 days after collection, ship on dry ice.

7. Special safety precautions

- 7.1 Handle diagnostic equipment, samples, controls and waste as potentially biohazardous materials.
- 7.2 Use personal protective equipment, including gloves, safety goggles and protective laboratory wear.
- 7.3 Ensure that appropriate decontamination is carried out if hazardous materials have spilled onto equipment or surrounding areas.
- 7.4 Hazardous chemicals (such as guanidine thiocyanate) are used in the reagent bay. Avoid contact with skin, eyes and clothing. Do not flush down a drain or mix with bleach. To prevent an unwanted chemical reaction, avoid mixing the liquid waste with other laboratory wastes. Inhaling mists may cause respiratory irritation.
- 7.5 Be familiar with the material safety data sheets for the materials used in the procedures.
- 7.6 Do not allow the NUCLISENS® Lysis Buffer, NUCLISENS® Wash Buffer 1 or waste from the procedure to come into contact with acidic materials. Cyanide gas can potentially be released.

- 7.7 Be careful when handling tools and other magnetic material in close proximity to the magnet arrays, since sudden high mechanical forces may be generated.
- 7.8 People wearing a pacemaker should not use this product, since the strong magnetic fields associated with this product may adversely affect or damage the pacemaker.

8. Quality control

- 8.1 NUCLISENS® EASYQ® Internal Calibrator (for viral load): store at 2–8°C, bioMérieux catalogue no. 285007; this step only needed if RNA is also being used for viral load testing with the NucliSens EasyQ HIV-1 v2.0 Viral Load Kit. If not, substitute with nuclease-free water.
 - 8.1.1 Calibrator containing lyophilized sphere with synthetic calibrator RNA
- 8.2 Pooled positive and negative plasma, store at –80°C. Bring to room temperature before testing.

9. Procedure

- 9.1 Refer to the NUCLISENS® MINIMAG® user manual for a detailed description of the system description, warnings and precautions.
 - 9.1.1 The magnetic rack contains high-energy magnets, which generate strong magnetic fields. Keep all loose ferrous material away from the magnetic rack. People with cardiac pacemaker implants should avoid the magnet rack area.
- 9.2 Preparation
 - 9.2.1 Bring the reagents to room temperature.
 - 9.2.2 Examine Lysis Buffer and Wash Buffer 1 for crystals. If present, incubate at 37°C until the crystals have dissolved.
 - 9.2.3 Turn on the Eppendorf ThermoMixer®.
 - 9.2.3.1 Set the temperature at 60°C.
 - 9.2.3.2 Set the mixing speed at 1400 rpm.
- 9.3 Lysis
 - 9.3.1 Centrifuge the Lysis Buffer tube for 10 seconds at $1500 \times g$.
 - 9.3.2 Cut out one complete set of blood spots from the filter paper by using a pair of scissors, taking care not to touch the spotted blood. Use a new or clean pair of scissors for each new specimen and decontaminate before reuse.
 - 9.3.2.1 Add the blood spot into a NUCLISENS® Lysis Buffer 2-mL tube.
 - 9.3.2.2 Incubate the tubes in a horizontal position on a roller mixer for 30 minutes at room temperature.
 - 9.3.2.2.1 Position the tubes on the roller mixer to keep the blood spots in constant contact with the Lysis Buffer.
 - 9.3.2.2.2 Do not vortex the tubes. Degrading the filter material and releasing fibres can interfere with the extraction process.
 - 9.3.2.3 Centrifuge the tubes for 15 seconds at $1500 \times g$ to separate fluid from debris. A pellet will form at the bottom.
 - 9.3.2.4 Use a transfer pipette to transfer the lysate supernatant for nucleic acid extraction directly into a labelled 5-mL microcentrifuge tube. Leave any possible remains of paper material in the NUCLISENS® Lysis Buffer 2-mL tube.
- 9.4 Binding
 - 9.4.1 Internal calibrator for viral load; this step only needed if RNA is also being used for viral load testing with the NucliSens EasyQ HIV-1 v2.0 Viral Load Kit. If not, substitute with nuclease-free water.
 - 9.4.1.1 Reconstitute the Calibrator accsphere from a NUCLISENS® HIV-1 RUO kit with 220 µL of Elution Buffer (yellow cap).
 - 9.4.1.2 Vortex the solution and spin down. Use within 1 hour of preparation.
 - 9.4.1.3 Add 20 µL of Calibrator solution to each labelled 5-mL micro centrifuge tube containing sample.
 - 9.4.2 Vortex the magnetic silica solution thoroughly.
 - 9.4.3 Add 50 µL of silica to the sample and Lysis Buffer; vortex immediately, but gently.
 - 9.4.4 Incubate for 10 minutes at room temperature with no further mixing.

9.5 Washing

- 9.5.1 Centrifuge the sample tubes for 2 minutes at $1500 \times g$.
- 9.5.1.1 Note: Use two different micropipettes (1000 μL , aerosol-resistant disposable tips) in steps 9.5.2 and 9.5.3. Use one exclusively to remove supernatant and the other exclusively for adding wash fluids.
- 9.5.2 Remove the supernatant by aspiration or by careful decantation.
- 9.5.2.1 Caution: Avoid disturbing the pellet.
- 9.5.3 Resuspend the sample in 400 μL of Wash Buffer 1 by pipetting up and down. Avoid introducing bubbles.
- 9.5.4 Transfer the resuspended sample to a labelled 1.5-mL microtube and place in the NUCLISENS® MINIMAG® instrument.
- 9.5.4.1 Note: The magnetic field should be in the OFF position (the magnetic rack should be tilted back).
- 9.5.5 Place the magnetic rack in the up position (magnetic field ON).
- 9.5.6 Wash for 30 seconds at speed STEP 1.
- 9.5.6.1 Allow the silica particles to collect on the wall of the microtube.
- 9.5.7 Remove the supernatant by aspirating at the surface of the liquid.
- 9.5.7.1 Note: Use a fresh tip for each sample to avoid sample carry-over.
- 9.5.8 Tilt back the magnetic rack (magnetic field OFF).
- 9.5.9 Add the wash buffer to the microtube by pipetting directly onto the silica aggregate to improve washing. For the amount, type and volume of wash buffer, see the table.
- 9.5.10 Place the magnetic rack in the up position (magnetic field ON).
- 9.5.11 Repeat until each sample has been washed a total of five times.

Wash 2	400 μL of Wash Buffer 1, wash for 30 seconds at STEP 1
Wash 3	500 μL of Wash Buffer 2, wash for 30 seconds at STEP 1
Wash 4	500 μL of Wash Buffer 2, wash for 30 seconds at STEP 1
Wash 5	500 μL of Wash Buffer 3, wash for 15 seconds at STEP 1

- 9.5.11.1 Complete the wash procedure as quickly as possible.
- 9.5.11.2 Caution: Do not pause while the samples are in Wash Buffer 3. Leaving the samples in Wash Buffer 3 may result in lower yields of nucleic acids.

9.6 Elution

- 9.6.1 Ensure that all fluid has been removed during the last aspiration step.
- 9.6.1.1 Try to remove as much as possible without disturbing the silica pellet.
- 9.6.2 Take the tubes from the instrument and add 25 μL of Elution Buffer onto the silica pellet in the microtubes.
- 9.6.2.1 If only doing extraction and the internal calibrator has not been added, 50 μL of Elution Buffer can be used.
- 9.6.3 Close the microtubes and collect the silica at the bottom of the tube by gently tapping.
- 9.6.3.1 Note: Do not centrifuge to collect the silica since this may compact the silica, which could result in lower extraction yields because resuspending the silica efficiently may become difficult.
- 9.6.4 Place the microtubes in a pre-warmed Eppendorf ThermoMixer® and incubate for 5 minutes at $60 \pm 2^\circ\text{C}$ at 1400 rpm to elute any nucleic acid from the silica.
- 9.6.5 Place the microtubes in the NUCLISENS® MINIMAG® instrument and place the magnetic rack in the up position.
- 9.6.6 Transfer the nucleic acid extracts without silica to a fresh 1.5-mL microtube.
- 9.6.6.1 Take care not to transfer any silica particles.
- 9.6.7 If nucleic acid extracts cannot be used for amplification immediately after preparation, the nucleic acid extracts (without silica) can be stored at $2\text{--}8^\circ\text{C}$ for up to 1 day or at -70°C for up to 1 year. Storage of small quantities of nucleic acid extracts (5 μL) is not recommended.

9.7 Shutdown procedure

- 9.7.1 Unplug the power cord from the instrument:
- 9.7.2 Wipe down the instrument with 5% Extran® followed by 70% ethanol.

10. Method performance specifications

10.1 Limitations

- 10.1.1 Do not mix left-over reagents for another extraction run.
The NUCLISENS® MINIMAG® sample strips are designed to extract DNA and RNA. Remove the eluates from the strips as soon as possible and process them in an amplification and detection system.
- 10.1.2 In case of an electrical static discharge, switch off the system and then switch it back on again.
Failure to comply may cause the system to malfunction.
- 10.1.3 If the system's power supply is interrupted, data may be lost. Ensure that a reliable power supply is available.

11. References

- 11.1 NUCLISENS® MINIMAG® user manual version 1.0, revision 2, bioMérieux, 2008–2009.

HIV-1 drug resistance genotyping testing: in-house assay

1. TITLE

HIV-1 drug resistance genotyping testing: in-house assay

2. Purpose

- 2.1 The purpose of the in-house assay is to identify mutations within the HIV-1 *pol* gene region, which encode amino acid substitutions known to be responsible for resistance to specific antiretroviral drugs.
- 2.2 Identification is accomplished by:
 - 2.2.1 Extracting the viral RNA or total nucleic acid from client plasma, dried plasma spots, dried blood spots or other types of specimens.
 - 2.2.2 Amplifying the HIV *pol* gene by one-step reverse transcription–polymerase chain reaction (RT-PCR) and nested PCR.
 - 2.2.3 Sequencing the resulting amplicons.
 - 2.2.4 Analysing the sequence (from code 6 of protease gene to code 251 of the RT gene).
- 2.3 The assay's sensitivity has been established at 1000 copies/mL for plasma and DBS (Yang et al., 2010).
- 2.4 Clinical indications for ordering the in-house assay:
 - 2.4.1 HIV-1-infected people experiencing drug therapy failure (with increased viral load) before changing therapy.
 - 2.4.2 HIV-1-infected people at initial presentation before initial drug therapy.
- 2.5 The assay is currently for research and surveillance purposes only.

3. Reagents

- 3.1 SuperScript™ III one-step RT-PCR systems with Platinum® *Taq* high fidelity (catalogue no. 12574-036, Invitrogen): store at –20°C.
- 3.2 UltraPure™ DEPC-treated water (catalogue no. 750024, Invitrogen): store at room temperature.
- 3.3 Applied Biosystems™ AmpliTaq Gold LD™ with 10× buffer II (catalogue no. 4338856, Applied Biosystems): store at –15°C to –25°C.
- 3.4 Applied Biosystems™ BigDye XTerminator™ Cycle Sequencing Kit v.3.1 (catalogue no. 4337455, Applied Biosystems): store at –15 to –25°C
- 3.5 POP-6™ Polymer for 3100/3100-Avant™ Genetic Analyzers (catalogue no. 4316357, Applied Biosystems): store at 2–8°C.
- 3.6 POP-7™ Polymer DNA Analyzer (catalogue no. 4363929, Applied Biosystems): store at 2–8°C.
- 3.7 Applied Biosystems™ BigDye XTerminator™ Purification Kit – 50 mL (~2500–20 µL reactions, catalogue no. 4376487, Applied Biosystems): store at 2–8°C.
- 3.8 Separations CENTRI-SEP columns (catalogue no. CS-901, Princeton): store at 15–25°C.
- 3.9 QIAgen® Viral RNA Mini Kit (catalogue no. 52904, Qiagen): store at 15–25°C.
- 3.10 dNTP (catalogue no. 11814362001, Roche Molecular Diagnostics): store at –20°C.
- 3.11 QIAquick™ PCR purification kit (catalogue no. 28106, Qiagen): store at 15–25°C.
- 3.12 ExoSAP-IT for PCR Product Clean-Up (catalogue no. 78201, USB): store at –20°C
- 3.13 UltraPure Agarose (catalogue no. N15510-027, Invitrogen): store at 15–30°C.
- 3.14 5× GelPilot DNA Loading Dye (catalogue no. 239901, Qiagen): store at 2–8°C
- 3.15 Invitrogen™ Low DNA Mass Ladder (catalogue no. 10068-013, Invitrogen): store at –20°C.
- 3.16 GelRed® Nucleic Acid Gel stain (catalogue no. 41003, 10 000× in water, Biotium): stored at 15–25°C. If using ethidium bromide solution (catalogue no. 1510-10ML, 10 mg/mL, Sigma): store in a cool, dry, well-ventilated place.
- 3.17 Ethanol (catalogue no. 459844, 500 mL, Sigma): store in the chemical chamber at 15–20°C.
- 3.18 Primers are synthesized by the United States Centers for Disease Control and Prevention (CDC) Biotechnology Core Facility Branch; store at –20°C. For primer sequences, see Yang et al. *J Clin Microbiol* 48: 3158, 2010.
 - 3.18.1 RT and first-step PCR primers: PrtM-F1 (or PrtM2-F1) and RT-R1.
 - 3.18.2 Nested PCR primers: PRT-F2 and RT-R2.
 - 3.18.3 Sequencing primers: PRT-F2, AV44, 90V1, A35V, A36V and RT-R2.
- 3.19 UltraPure® 10× Tris-Borate-EDTA buffer (catalogue no. 15581-044, Invitrogen): store at 15–30°C.

4. Equipment

- 4.1 Equipment in the specimen preparation area
 - 4.1.1 Biosafety cabinet (BSC, level II)
 - 4.1.2 Benchtop microcentrifuge, such as Eppendorf model 5417C or 5424 up to the speed of $10\,000 \times g$
 - 4.1.3 Benchtop centrifuge, Jouan CR412 or the equivalent
 - 4.1.4 Daigger Twist Shaker, TW3t or the equivalent
 - 4.1.5 37°C water bath
 - 4.1.6 Vortexer
 - 4.1.7 Dedicated adjustable P-10, P-20, P-200 and P-1000 pipettors
 - 4.1.8 Drummond Pipet-Aid® with charger stand or the equivalent
 - 4.1.9 Dedicated benchtop cooler
- 4.2 Equipment in the pre-amplification area
 - 4.2.1 Dead-air PCR work station with UV light
 - 4.2.2 Dedicated adjustable P-10, P-20, P-200 and P-1000 pipettors
 - 4.2.3 UV crosslinker
 - 4.2.4 Vortexer
 - 4.2.5 Benchtop microcentrifuge
 - 4.2.6 –20°C freezer
 - 4.2.7 –80°C freezer
 - 4.2.8 4°C refrigerator
 - 4.2.9 Dedicated ice basket
- 4.3 Equipment in the post-amplification area
 - 4.3.1 Applied Biosystems™ 3130xl Genetic Analyzer or 3730 DNA Analyzer
 - 4.3.2 Dead-air PCR work station or biological safety cabinet with UV light
 - 4.3.3 Benchtop microcentrifuge, such as Eppendorf model 5417C or 5424 up to the speed of $10\,000 \times g$
 - 4.3.4 Drummond Pipet-Aid® with charger stand or the equivalent
 - 4.3.5 Vortexer
 - 4.3.6 Microwave
 - 4.3.7 Benchtop centrifuge with 96-well microwell plate rotor
 - 4.3.8 Dedicated adjustable P-10, P-20, P-200 and P-1000 pipettors
 - 4.3.9 –20°C freezer
 - 4.3.10 4°C refrigerator
 - 4.3.11 Standard top-load balance
 - 4.3.12 Bioimage system or UV transilluminator
 - 4.3.13 Agarose gel separation apparatus and combs
 - 4.3.14 Power supply for gel systems

5. Supplies

- 5.1 Supplies for the specimen preparation area
 - 5.1.1 Aerosol barrier tips
 - 5.1.2 1.5-mL sterile screw-cap conical centrifuge tubes
 - 5.1.3 15- and 50-mL sterile screw-cap polypropylene tubes
 - 5.1.4 Benchtop waste bag and holder
 - 5.1.5 Plastic beaker with 10% bleach
 - 5.1.6 70% ethanol in a spray bottle
 - 5.1.7 Biohazard bag with a holder for tip and tube disposal
 - 5.1.8 Powder-free latex, vinyl or nitrile gloves
 - 5.1.9 Kimtech® Science™ Kimwipes™
 - 5.1.10 Permanent markers
 - 5.1.11 Clean blue laboratory coat
- 5.1.13 Dedicated tube racks
- 5.2 Supplies for the pre-amplification area
 - 5.2.1 Distilled water, spray bottle
 - 5.2.2 Powder-free latex, vinyl or nitrile gloves
 - 5.2.3 Tube racks
 - 5.2.4 1.5-, 2.0- and 7.0-mL sterile RNase- and DNase-free tubes

- 5.2.5 Clean white laboratory coat
- 5.2.6 Kimtech® Science™ Kimwipes™
- 5.2.7 Permanent markers
- 5.2.8 Biohazard bag with a holder for tip and tube disposal
- 5.2.9 Aerosol barrier tips
- 5.2.10 Applied Biosystems™ MicroAmp™ Optical 96-Well Reaction Plate
- 5.3 Supplies for the post-amplification area
 - 5.3.1 Powder-free latex, vinyl or nitrile gloves
 - 5.3.2 Clean white laboratory coat
 - 5.3.3 70% ethanol in a spray bottle
 - 5.3.4 15- and 50-mL sterile screw-cap polypropylene tubes
 - 5.3.5 1.5-mL microcentrifuge tubes
 - 5.3.6 Distilled water
 - 5.3.7 Weigh boats
 - 5.3.8 250- and 500-mL Wheaton screw-cap bottles

6. Samples

- 6.1 Specimen type
 - 6.1.1 The in-house HIV-1 genotyping system is intended for use with dried blood spots (DBS), dried plasma spots (DPS), plasma and serum samples.
- 6.2 Labelling and identification
 - 6.2.1 All specimens arriving in the laboratory must have original identifiers affixed to the specimens.
 - 6.2.2 The laboratory personnel assign and affix the CDC specimen ID (CSID) and CDC unique ID (CUID) to each specimen.
- 6.3 Unacceptable specimens
 - 6.3.1 Unlabelled specimens from the client
 - 6.3.2 Specimens not matching the requisition form
 - 6.3.3 Clotted specimens
 - 6.3.4 Notify the health-care provider referring unacceptable specimens.
- 6.4 Specimen preservation and storage
 - 6.4.1 DBS
 - 6.4.1.1 Prepare within 24–48 hours after the blood is drawn. At least four DBS per client are required.
 - 6.4.1.2 Store at room temperature for up to 2 weeks in a closed container without direct sunlight and at –70°C for greater than 2 weeks.
 - 6.4.1.3 Refer to the instructions for preparing, storing and shipping DBS for HIV drug resistance testing (ILB-300-P19).
 - 6.4.2 Plasma and serum
 - 6.4.2.1 Collect blood in a sterile EDTA tube for plasma and without EDTA for serum. Remove the plasma or serum from the cells within 2–6 hours after collection.
 - 6.4.2.2 Store at –65°C to –80°C for up to 6 months before testing. Do not freeze and thaw plasma more than two times.
- 6.5 Specimen aliquoting
 - 6.5.1 If needed, aliquot plasma and serum samples into tubes affixed with labels. Identify each tube with a CDC aliquoting accession number, original specimen ID and the date of collection.
 - 6.5.2 Perform aliquoting under a biological safety cabinet.
 - 6.5.3 Refer to specimen management procedure (ILB-100-P08) and test directory for details of sample requirements and collection.

7. Special safety precautions

- 7.1 Universal precautions for working with infectious agents must be followed when performing this assay.
- 7.2 Ethidium bromide is a possible carcinogen. Refer to material safety data sheets for handling precautions and instructions.
- 7.3 Equipment with UV lights is required for this procedure. Refer to the procedure for each piece of equipment for specific safety precautions.

8. Quality control

- 8.1 In-house controls
 - 8.1.1 The positive control is made in house with normal human plasma spiked with HIV-1 MN strain viral supernatant from in vitro culture with known viral load measurement, stored at -80°C .
 - 8.1.2 The negative control is (HIV negative) human plasma, stored at -80°C .
 - 8.1.3 The positive control DBS spot is made in-house with 100 μL of spiked blood containing HIV-1 MN strain viral supernatant, stored at -80°C .
 - 8.1.4 The positive and negative controls are prepared and included in each RT-PCR reaction run (exactly as testing specimens), and the results must be within acceptable limits for a run to be valid.
 - 8.1.5 Negative control: no band visible
 - 8.1.6 Positive control: show amplification and visible band during gel electrophoresis
- 8.2 Gel electrophoresis acceptance and rejection criteria
 - 8.2.1 The test sample should be considered void if the major product is not of the appropriate size.
 - 8.2.2 The test sample should be considered void if the major product is of the correct size 1.08 kb but too much smearing exists.
 - 8.2.3 The test sample should be considered void if no PCR product is visible.
 - 8.2.4 The entire run is considered void if the negative control has a product band or smear visible and the positive controls are not the appropriate size or lack PCR products.
 - 8.2.5 If the positive and negative control results are valid and the client sample does not produce a visible band, the test will be repeated with the rescue primer. Increase the initial RNA or total nucleic acid input to 20 μL or increase the RT-PCR products (4 to 8 μL) in the nested PCR reaction. If a negative result is produced after repeated extraction and PCR runs, no further attempt will be made to amplify the specimen.
 - 8.2.6 Data review: a second technician reviews and confirms the electropherograms for each client before submitting to the supervisor. The supervisor reviews the final report and summary sheet before reporting.
- 8.3 Phylogenetic analysis is performed using the sequences generated with successful genotyping results to monitor cross-contamination.

9. Procedure (the entire procedure takes 4–5 days from start to finish)

- 9.1 Workflow precautions
 - 9.1.1 To prevent unamplified samples from contamination with amplicons, different parts of this procedure must be performed in four different physically separated work areas.
 - 9.1.2 Pre-amplification area 1 is used for preparing primers and PCR master mix reactions. A hood with a UV light to eliminate DNA cross-contamination is required.
 - 9.1.3 Pre-amplification area 2 is used for preparing samples. A Biosafety Level 2 hood is used to work with potentially infectious samples.
 - 9.1.4 Amplification area 3 is used to perform RT and first step PCR and to add a template for nested PCR in the in-house assay. A hood with UV light to eliminate DNA cross-contamination is used for adding template for nested PCR.
 - 9.1.5 Post-amplification area 4 used for all steps following PCR amplification.
 - 9.1.6 The workflow of the PCR procedure must be unidirectional, beginning in the pre-amplification area and ending in the post-amplification area, with no return of test plates, equipment or supplies.
- 9.2 Before beginning the assay, fill out the in-house genotyping log (ILB-300-F16B) with sample information.
- 9.3 RNA extraction from plasma or serum follows the same procedures as in ViroSeq™ (ILB-300-P07), TruGene™ (ILB-300-P15), Abbott m2000sp (ILB-300-P24) or NUCLESENS® EASYMAG® (ILB-300-P23) extraction procedures.
- 9.4 Total nucleic acid extraction from DBS using NUCLESENS® EASYMAG® Semi-Automatic System. See RNA EXTRACTION AND GENOTYPING PROTOCOLS.
- 9.5 Preparation of RT-PCR master mixes – reagents are prepared in pre-amplification area 1. Do not bring PCR products to this area.
 - 9.5.1 Turn on the UV light in the PCR chamber for 30 minutes before use.
 - 9.5.2 Thaw the RT-PCR reagents at room temperature except for enzymes. Vortex and microcentrifuge briefly and place in the bench top cooler. Remove the enzyme from the bench top cooler only when needed.

- 9.5.3 Place a sterile 1.5- to 2.0-mL RNase-free tube in the cold block. This tube is used to prepare enough RT-PCR master mix to accommodate the number of specimen and control reactions, reagent control and an extra one to ensure sufficient volume. Prepare the solution based on the following amounts of reagents per reaction.

Reagents	Amount for one RT-PCR reaction
2× Reaction Mix	10 µL
PrtM-F1 (8 µM)	8 µL
RT-R1 (8 µM)	2 µL
DEPC-treated water	3 µL
SS III RT-PCR enzyme	3 µL
Total	40 µL

- 9.5.3.1 Note: If a few samples show no target bands, repeat RT-PCR using rescue primer PrtM2-F1 instead of PrtM-F1.

- 9.5.4 Leave the master tubes on ice.
- 9.5.5 Label one 200-µL thin-walled tube per reaction or label a 96-well plate (according to the testing format) and pre-cool on ice or in a cold block. For each RT-PCR run, positive, negative and RT-PCR reagent controls must be included. Arrange the 3 tubes in the following order: positive, negative and reagent control. Add the tubes to the end of the RT-PCR run. Bring the ready to go master mix tubes into area 2 on ice.
- 9.5.6 While working in the RNA extraction hood, add 10 µL of template (total nucleic acid or RNA) into each labelled tube or plate well. Close the cap and keep the tubes and plate on ice or in a cold block.
- 9.5.7 Denature the templates at 65°C for 10 minutes in a thermocycler.
- 9.5.8 Immediately put the templates on ice (3–5 minutes).
- 9.5.9 Add 40 µL of the master mix into each template tube or respective well and vortex gently. Change the pipette tip between each sample each time. Briefly centrifuge (2 seconds) to collect all the reagents into the bottom of the tube. Bring the tubes or plate to amplification area 3.

9.6 Amplification area 3

- 9.6.1 Pre-warm the thermocycler for 15–20 minutes.
- 9.6.2 Load the tubes or plate into the machine.
- 9.6.3 Select a pre-programmed RT-PCR file or programme the thermocycler as follows (check the accuracy of the programme before pressing the run button):

One RT cycle	
50°C	45 minutes
94°C	2 minutes
40 PCR cycles	
94°C	15 seconds
50°C	20 seconds
72°C	2 minutes
One cycle	
72°C	10 minutes
4°C	Forever

- 9.6.4 The entire RT-PCR reaction takes about 3 hours. Leave the PCR tubes or plate in the thermocycler overnight, but do not leave the PCR product in the thermocycler for more than 18 hours.

Optional stopping point – after the RT-PCR reaction, freeze the RT-PCR product at –20°C for up to 2 weeks or proceed immediately to the nested PCR stage.

9.7 Preparation of nested-PCR master mixes (area 1)

- 9.7.1 Turn on the UV light in the PCR chamber for 30 minutes before use.
- 9.7.2 Thaw the PCR reagents at room temperature except for the enzymes. Vortex and centrifuge briefly and place on ice. Remove the enzyme from the bench top cooler only when needed.

- 9.7.3 Place a sterile 1.5- to 2.0-mL DNase-free tube in a cold block to prepare enough PCR master mix to accommodate the number of planned reactions plus one based on the following amounts of reagents per reaction:

Reagents	Amount for one PCR 100- μ L reaction	Amount for one PCR 50- μ L reaction
10 \times Gold Buffer	10 μ L	5 μ L
MgCl ₂ (25 μ M)	8 μ L	4 μ L
dNTP (10 μ M each)	2 μ L	1 μ L
Prt-F2 (4 μ M)	3 μ L	1.5 μ L
RT-R2 (4 μ M)	3 μ L	1.5 μ L
DEPC-treated water	69 μ L	34.5 μ L
Taq Gold LD	1 μ L	0.5 μ L
Total	96 μL	48 μL

- 9.7.4 Mix the PCR master mix by gentle vortexing then briefly centrifuging (2 seconds) to collect all the reagents into the bottom of the tube.
- 9.7.5 Label one 200- μ L thin-walled tube per reaction or 96-well reaction plate.
- 9.7.6 For the 50- μ L PCR reaction, aliquot 48 μ L of the master mix into each of the properly labelled tubes (For each PCR run, a reagent control must be included. Add the tube to the end of the PCR run).
- 9.7.7 Close the tubes or plate and take them to amplification area 3 and add 2 μ L of RT-PCR products to each of the properly labelled tubes or plate well containing nested PCR master mix in the PCR chamber in amplification area 3.
- 9.7.8 Vortex and centrifuge the tubes briefly and bring them to post-amplification area 4 for PCR.
- 9.8 Post-amplification area 4
- 9.8.1 Pre-warm the thermocycler for 15–20 minutes.
- 9.8.2 Load the tubes into the PCR machine.
- 9.8.3 Select the in-house nested PCR programme or programme the thermocycler as follows (check the accuracy of the programme before pressing the run button):

One cycle	
94°C	4 minutes
40 PCR cycles	
94°C	15 seconds
55°C	20 seconds
72°C	2 minutes
One cycle	
72°C	10 minutes
4°C	Forever

Optional stopping point – after the nested PCR programme, leave the tubes in the thermocycler overnight at 4°C or take the PCR products out of the thermocycler and store them in the 4°C refrigerator and continue to the next step on the following day.

- 9.9 Agarose gel preparation (post-amplification area 4)
- 9.9.1 Agarose gel (1.0%) is used to confirm the PCR amplification results.
- 9.9.2 To prepare 1% (w/v) agarose solution, add 1.0 g agarose and 100 mL of 1 \times Tris borate EDTA to a glass flask or bottle.
- 9.9.3 Carefully microwave the agarose gel solution until boiling (usually 1 minute at full power). Swirl using hot hands or equivalent to prevent injury. Repeat 3 times to insure gel has completely dissolved into solution.
- 9.9.4 Let the dissolved agarose solution cool to 50–60°C using room temperature.
- 9.9.5 Add 10 μ L of GelRed® Nucleic Acid Gel stain (10 000 \times stock, Biotium) to the 100 mL of agarose solution for a 1 \times solution. Mix thoroughly.

- 9.9.5.1 GelRed® is used to replace the highly toxic EtBr solution for staining DNA in agarose gel. If GelRed® is not available, use EtBr solution (add 2 µL of EtBr to the 100-mL agarose solution).
- 9.9.6 Set up the gel tray and comb and carefully pour agarose gel into the gel caster.
- 9.9.7 Remove bubbles or debris and allow the agarose to solidify at room temperature (about 30 minutes).
- 9.10 Preparing samples for agarose gel analysis (post-amplification area 4)
 - 9.10.1 Add 2 µL of 5× GelPilot® DNA Loading Dye into each of the 0.5-mL tubes or on the parafilm.
 - 9.10.2 Add 5 µL of sample to each tube or parafilm and mix with the loading dye.
 - 9.10.3 For a molecular marker standard tube, only add 4 µL of Invitrogen™ Low DNA Mass Ladder.
- 9.11 Agarose gel electrophoresis (post-amplification area 4)
 - 9.11.1 Once the agarose has solidified, gently remove the comb and prepare the gel apparatus to receive running buffer.
 - 9.11.2 Place the gel with sample wells closest to the negative (black) end of the gel box.
 - 9.11.3 Slowly pour 1× Tris borate EDTA buffer to fill line of gel-running apparatus.
 - 9.11.4 Load 5 µL of each sample into the wells (place the molecular marker in the first well and change the pipette tip with each sample to prevent cross-contamination).
 - 9.11.5 Run the gel at 100 V for 30–45 minutes to migrate the gel loading dye front 4–5 cm.
 - 9.11.6 Check to see that the loaded material has run toward the positive (red) end of the gel box sufficiently to visualize the bands and identify specific weights.
 - 9.11.7 Visualize the gel on the imaging system and photograph. Refer to the UVP imaging system procedure (ILB-300-P13). The PCR product should be 1.08 kb in size or fall just below the 1.2 kb marker.
- 9.12 Acceptance and rejection criteria
 - 9.12.1 The test sample should be considered void if:
 - 9.12.1.1 The major product is not the appropriate size.
 - 9.12.1.2 The major product is the correct size 1.08 kb, but too much smearing exists.
 - 9.12.1.3 No PCR product is visible.
 - 9.12.2 The entire run is considered void if the negative control has a product band or smear visible or the positive controls do not have the appropriately sized PCR products or lack PCR products.
 - 9.12.3 If the majority of samples tested show target bands while other samples show no target bands, repeat RT-PCR using rescue primer PrtM2-F1, repeat nested PCR using 4 µL of RT-PCR products with a 50-µL total nested PCR reaction volume or increase the initial RNA or total nucleic acid input to 20 µL.
 - 9.12.4 If a negative result is produced after repeated extraction and PCR runs, no further attempt will be made to amplify the specimen.
 - 9.12.5 If acceptable, continue to DNA purification and preparation for sequencing.
- 9.13 Purification of PCR products using a QIAquick™ PCR Purification Kit
 - 9.13.1 Label one 1.5-mL microfuge tube for each specimen and the positive control.
 - 9.13.2 Add 5 volumes of Buffer PBI to 1 volume of the PCR sample and vortex (for example, 20 µL of PCR product and 100 µL of buffer PBI).
 - 9.13.3 The colour of the mixture should be yellow or similar to the original colour of the PBI solution before the PCR product is added.
 - 9.13.4 If the colour of the mixture is orange or violet, add 10 µL of 3 M sodium acetate (pH 5.0) and mix. The colour of the mixture should turn back to yellow.
 - 9.13.5 Place a QIAquick™ spin column in a provided 2-mL collection tube.
 - 9.13.6 To bind DNA, apply the sample to the QIAquick™ column and centrifuge for 1 minute.
 - 9.13.7 Discard the flow-through. Place the QIAquick™ column back into the same tube.
 - 9.13.8 To wash, add 0.75 mL of Buffer PE to the QIAquick™ column and centrifuge for 1 minute.
 - 9.13.9 Discard flow-through and place the QIAquick™ column back into the same tube. Centrifuge the column for an additional 1 minute.
 - 9.13.10 Place the QIAquick™ column in a clean labelled 1.5-mL centrifuge tube.
 - 9.13.11 To elute DNA, add 50 µL of Buffer EB or water to the centre of the QIAquick™ membrane and centrifuge for 1 minute.
 - 9.13.12 Place the purified DNA on ice.
- 9.14 Purification of PCR products using Applied Biosystems™ ExoSAP-IT™ PCR Product Cleanup Reagent
 - 9.14.1 Label a 0.2-mL PCR tube for each sample. Alternatively, use a 96-well plate for large number of samples.
 - 9.14.2 Add 5 µL of ExoSAP-IT™ to 12.5 µL of the PCR sample and mix after pipetting.

- 9.14.3 Run the plate or the tubes in the thermocycler on the following programme to degrade the remaining primers and nucleotides and inactivate the ExoSAP-IT™, with a reaction volume of 16 µL.

37°C	15 minutes
80°C	15 minutes
4°C	Forever

- 9.14.4 Remove the plate or tubes from the thermocycler immediately after the programme ends and place the purified DNA on ice. If you are not immediately proceeding to cycle sequencing, treated PCR products can be stored in the dark at –20°C until needed.

9.15 Cycle sequencing

- 9.15.1 The purified PCR products are used for the sequencing reaction, which is performed on a thermocycler.
- 9.15.2 Six different primers (three forward and three reverse) are used to sequence the desired 1.08-kb fragment of DNA with Applied Biosystems™ BigDye XTerminators™ and the Applied Biosystems™ 3100 or 3730 DNA Analyzer. This combination of reagents and equipment has a 600-base resolution.
- 9.15.3 The negative control is not sequenced, since it cannot be run on the sequencer detector.
- 9.15.4 The PCR reaction takes about 2.5 hours to run.
- 9.15.5 Thaw each of the sequencing primers (A35V, A36V, AV44, 90V1, PRT-F2 and RT-R2). Place on ice in post-amplification area 4.
- 9.15.6 Set up the sequencing reaction for each sample and the positive control in a sequencing plate or 8-strip PCR tubes.
- 9.15.7 For each reaction:

Water	11 µL
Primer (10 µM)	2 µL (20 pmol)
Applied Biosystems™ BigDye™ Buffer	4 µL
Applied Biosystems™ BigDye XTerminator™	1 µL
DNA template	2 µL
Total	20 µL

- 9.15.8 Each sample is usually assigned to the first 6 wells of a row (A1 to A6) or the last 6 wells of a row (A7 to A12), and each set of the primers to the first 6 wells of a column (1A to 1H) or the last 6 well of a columns (7A to 12H). The wells of H7 to H12 are used for sequencing control. If a plate is not being used, label 0.2-µL PCR tubes with the sample number and primer letter on the cap and side and place the tubes in a rack in the same grid as used for a plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H							P Ctrl	P Ctrl	P Ctrl	P Ctrl	P Ctrl	P Ctrl

- 9.15.9 Cap the tubes or cap the plate with sealing membrane (label each cap or plate). Pulse spin and check that the volumes are consistent.

- 9.15.10 Run the plate or the tubes in the thermocycler in post-amplification area 4 on the following programme with a reaction volume of 20 μL (check the accuracy of the programme before pressing the run button).

94°C	10 seconds	× 25 cycles
50°C	5 seconds	
60°C	4 minutes	
4°C	Forever	

- 9.15.11 Remove the plate or tubes from the thermocycler immediately when the programme is finished. If this is not to be immediately purified, freeze it in the dark at -20°C for up to 3 days.
- 9.16 Sequencing reaction purification
- 9.16.1 Purification of sequencing reactions using the Applied Biosystems™ BigDye XTerminator™ protocol to remove Dye-terminators:
- 9.16.1.1 For 20- μL sequencing reactions, mix 20 μL of Applied Biosystems™ BigDye XTerminator™ and 90 μL of SAM™ solution.
- 9.16.1.2 For an entire 96-well plate, prepare a master mix by adding 2200 μL of Applied Biosystems™ BigDye XTerminator™ and 9900 μL of SAM™ solution to add 110 μL to each well (the mixture provides enough for the entire plate).
- 9.16.1.3 Seal the plate and vortex for 30 minutes on the vertical vortexer.
- 9.16.1.4 Centrifuge the plate at 3000 rpm for 3 minutes before moving to the Applied Biosystems™ 3130xl or 3730 sequencer.
- 9.16.2 The Princeton Separations Centri-Sep™ Spin Columns (catalogue no. CS-901) can also be used to remove excess dye-terminators. Refer to ViroSeq™ (ILB-300-P07) if a Centri-Sep™ column is being used.
- 9.17 Sequencing using an Applied Biosystems™ 3130xl Genetic Analyzer
- 9.17.1 Turn on the computer attached to the 3130xl. Log in as 3100 user (no password). When the computer has booted completely, power on the sequencer. When the sequencer shows a steady green status light, launch the 3130xl Data Collection v3.1 Software. Wait for the software to boot up.
- 9.17.2 Check to ensure that there is no bubble in the gel line. Remove any bubbles if needed. Change the buffer in the anode and needle-soaking containers. Change the water in all containers.
- 9.17.3 A complete run is 16 samples. All 16 wells must have samples, so add buffer to blank wells.
- 9.17.4 After purification by Centri-Sep™ column, dry the sequencing product by the following procedure:
- 9.17.4.1 SpeedVac™ at $30-45^{\circ}\text{C}$ for 1 hour
- 9.17.4.2 Resuspend the samples in 15 μL of Hi-Di™ Formamide.
- 9.17.4.3 Vortex, spin down and load 15 μL in a sequencing plate.
- 9.17.4.4 Cover with an Applied Biosystems™ Septa™, spin down and denature at 94°C for 5 minutes.
- 9.17.4.5 Immediately place the tray on ice for 2 minutes.
- 9.17.5 After purification by the Applied Biosystems™ BigDye XTerminator™ protocol, transfer 50 μL of supernatant into a new sequencing 96-well plate.
- 9.17.6 Place the 96-well plate into the plate base and cover with a white plate retainer. Double check that the holes line up between the Applied Biosystems™ Septa™ and the white plate retainer.
- 9.17.7 Preparing sample sheets
- 9.17.7.1 In the Applied Biosystems™ 3130xl Data Collection Software, select the PLATE MANAGER tab and then click NEW. Create a new 96-well sequencing (SQ) sheet. Fill in the sheet with the sample names, and the run parameters specified in the sequencing sheet set-up protocol for the dye set you are using. Double-check the sheet and then click OK.
- 9.17.8 Repeat the above if running two plates is planned.
- 9.17.9 Press the tray button on the Applied Biosystems™ 3130xl. Wait for the tray to finish moving forward, and then open the instrument doors and place the plate assembly flat on the tray, with the slanted corner forward. Verify that the appropriate plate position indicator in the data collection software has turned yellow.

- 9.17.10 Place the 96-well plate into either plate slot. A1 will be the back right corner. The plate only fits one way. When the plate is in the tray, shut the door. The tray will move back. On the computer in the PLATE VIEW window, plate position indicator A or B will now be yellow depending on the placement of plate (or both if two plates are used).
- 9.17.11 Click the row in the pending plate record table that lists the plate record just created and then click on the plate window to link the sample sheet to samples. The plate position window will change from yellow to green when the sample sheet is linked.
- 9.17.12 The top tool bar will now have a green "run" button. Click on the run button to begin the run. Each run lasts 2.5 hours.
- 9.17.13 At the end of the run, the data are saved to the "extracted runs folder".
- 9.17.14 Close the Applied Biosystems™ 3130xl data collection software.
- 9.17.15 Open the Applied Biosystems™ sequence analysis software to analyse sequences, before using web RECall or ChromasPro software to edit sequences.
- 9.17.16 Push the tray button to bring the auto-sampler for removing the 96-well plate. The plate can be kept and reused if some wells have not been used. Shut the sequencer door and wait for the tray to go back.
- 9.17.17 Except for extended periods of nonuse, do not turn off the sequencer since this can affect its performance.
- 9.17.18 Document maintenance on the Applied Biosystems™ 3130xl Genetic Analyzer maintenance form (ILB-300-F07C).
- 9.18 Sequencing using an Applied Biosystems™ 3730 DNA Analyzer
 - 9.18.1 Before each run:
 - 9.18.1.1 Rinse and change buffers.
 - 9.18.1.2 Check the polymer volume (1 mL).
 - 9.18.1.3 Remove bubbles in the polymer block.
 - 9.18.1.4 Check the plate position on deck.
 - 9.18.1.5 Check that the reservoir Applied Biosystems™ Septa™ is seated and flat.
 - 9.18.1.6 Check the plate assembly.
 - 9.18.1.7 Document the maintenance on the Applied Biosystems™ 3730 DNA Analyzer maintenance form (ILB-300-F16D).
 - 9.18.2 Use DNA-grade sterile water for waste and wash trays.
 - 9.18.3 Open FOUNDATION DATA COLLECTION.
 - 9.18.4 Open all categories listed under GA INSTRUMENTS.
 - 9.18.5 Click on PLATE MANAGER. The window will change to list plate names and status.
 - 9.18.6 Search for plates.
 - 9.18.7 Click NEW if running a new plate.
 - 9.18.8 Click EDIT if editing or adding samples to a processed plate.
 - 9.18.9 After clicking NEW, the NEW PLATE DIALOG window will appear.
 - 9.18.10 Give the plate an ID and NAME.
 - 9.18.11 Set these criteria:
 - 9.18.11.1 Application: sequence analysis
 - 9.18.11.2 Plate type: 96-well
 - 9.18.11.3 Plate sealing: Applied Biosystems™ Septa™
 - 9.18.11.4 Assign an owner and operator name
 - 9.18.11.5 Click OK.
 - 9.18.12 The SEQUENCING ANALYSIS PLATE EDITOR window will appear. Enter the CSID and primer name for each well. Use arrow keys to move down the wells. Example: 20011693333-PrtF2.
 - 9.18.13 Select the RESULTS GROUP 1 where the data should be saved.
 - 9.18.14 To assign all samples at once, highlight the samples and press CTR + D.
 - 9.18.15 Select SEQUENCING-V3 for INSTRUMENT PROTOCOL 1 when using Centri-Sep™ purification.
 - 9.18.16 If Applied Biosystems™ BigDye XTerminator™ purification is being used, select BDX-SEQUENCING-V3 for INSTRUMENT PROTOCOL 1.
 - 9.18.17 Select SEQUENCING-V3 for ANALYSIS PROTOCOL 1.
 - 9.18.18 Click OK. The late should appear in the PLATE MANAGER list as pending.
 - 9.18.19 Open the RUN SCHEDULER window.
 - 9.18.20 In the input stack box, find the plates to be sequenced. Click ADD to add the plates to the input stack list.

- 9.18.21 The plates are added to the list from the bottom up. The number next to the plate tells the order they need to be loaded.
- 9.18.22 When all plates have been added, click DONE.
- 9.18.23 Open the stacker drawer and load the plates. Put the plate that is designated number 1 in first. Add plate number 2 on top, and so on.
- 9.18.24 Each sequencing plate needs to be in a black base (bottom) and covered with a white retainer (top).
- 9.18.25 Close the door when all plates have been loaded.
- 9.18.26 Wait for the green triangle to appear in the top left corner of the FOUNDATION DATA COLLECTION VERSION 3.0 window.
- 9.18.27 Click the green triangle. The plates will start processing. Leave the machine on INSTRUMENT STATUS while running the plates.
- 9.18.28 After all the plates have been processed, remove them plates from stacker drawer.
- 9.18.29 To view sequences, open SEQUENCE ANALYSIS 5.2 from the desktop.
- 9.18.30 Log in using the password SEQUENCING.
- 9.18.31 Open FILE → ADD SAMPLES.
- 9.18.32 Find and select the samples for viewing. Under ADD SELECTED SAMPLES, click OK.
- 9.18.33 Click the green triangle. Samples will base call.
- 9.18.34 To see the chromatogram, click the SHOW box for each sample. A chromatogram will appear below the sample list.
- 9.18.35 When finished, close the window. Burn the data to a CD for further editing.
- 9.18.36 At the end of the run, clean the instrument surface (deionized water and lint-free tissue) and check the database space. Remove the polymer bottle and store at 2–8°C. Document on the Applied Biosystems™ 3730 DNA Analyzer maintenance form (ILB-300-F16). Important: Never use organic solvents to clean the instrument or any of its components.
- 9.18.37 Weekly maintenance
 - 9.18.37.1 Rinse the buffer, water and waste trays with warm water.
 - 9.18.37.2 Document on the 3730 DNA Analyzer maintenance form (ILB-300-F16D).
 - 9.18.38 Periodic maintenance
 - 9.18.38.1 Change the capillary array every 100 runs.
 - 9.18.38.2 Document on the 3730 DNA Analyzer maintenance form (ILB-300-F16D).
- 9.19 Editing sequences
 - 9.19.1 ChromasPro is used to edit sequences and generate consensus sequences.
 - 9.19.2 Refer to genotyping sequence analysis using the ChromasPro software procedure (ILB-300-P20).
- 9.20 Guidelines for identifying mixtures
 - 9.20.1 Two opposite sequence segments containing the possible mixture should each show the same secondary peaks clearly above the local noise (approximately double the noise level) or 20% of the main peak above background noise. In some situations, mixtures not called by the software can meet the above criteria. However, if secondary peaks are present in both directions and are as high as the surrounding background noise peaks (high background sequences), the sequencing needs to be repeated to prevent false mixture calling.
 - 9.20.2 If the segment containing a possible mixture has single sequence coverage only, the segment has to contain a secondary peak that is at least 30% of the primary peak and is three times the local noise level and be able to differentiate between a dye blob and real peaks, which may cause a false mixture.
 - 9.20.3 In rare situations, a mixture pattern does not match the above guidelines. For example, multiple peaks or a shifted peak in a base require repeating the sequencing reaction.

10. Calculations

- 10.1 Sequence editing: refer to ILB-300-P20: HIV-1 genotyping sequence analysis using ChromasPro software procedure
- 10.2 Phylogenetic analysis is performed using BioEdit or MEGA 4 for confirmation of no cross-contamination. See ILB-300-P11: Molecular evolutionary genetics analyses using phylogenetic analysis software procedure.

11. Interpretation and results

- 11.1 After editing all sequences and generating consensus sequences, the resistance data is generated using the Stanford HIV Drug Resistance Database.
- 11.2 For individual client mutation analysis, the Stanford HIVdb Program: Sequence Analysis is used to generate resistance mutation reports.
- 11.3 For population-based surveillance sequences, the Calibrated Population Resistance (CPR) tool is used to generate resistance mutation reports for transmitted resistance.
- 11.4 Simply copy and paste sequences in the analysis box or upload the sequence text file or FASTA file at the following address: http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showMutationForm or <http://cpr.stanford.edu/cpr>.
- 11.5 Results reporting acceptability
 - 11.5.1 The supervisor reviews the controls and results before the results are reported.
 - 11.5.2 The results will not be reported when the controls are unacceptable.
 - 11.5.3 The results are reported as individual client reports with a summary sheet or reported as a summary sheet for population-based surveillance testing. The summary sheet should be signed and be sent back to the CDC to confirm that the results were received.
- 11.6 Turn-around time
 - 11.6.1 The times vary depending on workload and supply availability.
 - 11.6.2 The referring laboratory or requesting clinician can request an approximate turn-around time when submitting the sample.

12. References

- 12.1 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;48:3158–64.
- 12.2 *Current protocols in molecular biology* [journal]. New York: Wiley.
- 12.3 Applied Biosystems™ PRISM 3100 data collection software v1.1.
- 12.4 Applied Biosystems™ DNA sequencing analysis software v3.7.
- 12.5 Applied Biosystems™ 3730/3730xl DNA Analyzer getting started guide.

ANNEX 3. RNA EXTRACTION AND GENOTYPING (THERMO FISHER SCIENTIFIC KIT) PROTOCOLS FROM THE ILB LABORATORY OF THE UNITED STATES CENTERS FOR DISEASE CONTROL AND PREVENTION

1. TITLE

HIV-1 drug resistance genotyping testing: in-house assay using Thermo Fisher Scientific kit

2. PURPOSE

2.1 Principle

- 2.1.1 The purpose of the in-house assay is to identify mutations within the HIV-1 *pol* gene region that encode amino acid substitutions known to be responsible for resistance to specific antiretroviral drugs.
- 2.1.2 Identification of mutations is accomplished by:
 - 2.1.2.1 Extracting the viral RNA or total nucleic acid from client plasma, dried plasma spots (DPS), dried blood spots (DBS) or other types of specimens.
 - 2.1.2.2 Amplifying the HIV *pol* gene by one-step reverse transcription–polymerase chain reaction (RT-PCR) and nested PCR.
 - 2.1.2.3 Sequencing the resulting amplicons.
 - 2.1.2.4 Analysing the sequence (from codon 6 of the protease gene to codon 251 of the RT gene).
- 2.1.3 The assay's sensitivity has been established at 1000 copies/mL for plasma and DBS (Yang et al., 2010).

2.2 Clinical significance

- 2.2.1 HIV-1-infected people experiencing drug therapy failure (with increased viral load) before changing therapy.
- 2.2.2 HIV-1-infected people at initial presentation before initial drug therapy.
- 2.2.3 The assay is currently for research and surveillance purposes only.

3. Scope

- 3.1 This document applies to staff that perform the in-house developed HIV-1 drug resistance genotyping test with the Thermo Fisher Scientific kit in the drug resistance and molecular surveillance laboratory.

4. Responsibilities

4.1 Laboratory personnel

- 4.1.1 Adhere to the approved protocol and notify the laboratory manager and supervisor if there is a deviation from or error in the protocol.
- 4.1.2 Personnel should also notify the manager and supervisor if reagents are expired or have been stored improperly or if equipment is not calibrated or does not function according to established criteria.
- 4.1.3 Properly store and retain specimens, samples and their products.
- 4.1.4 Annually review and sign off on the in-house HIV-1 drug resistance genotyping protocols.

4.2 Laboratory director or team lead

- 4.2.1 Ensure that laboratory personnel document each reagent lot number and expiration date and the use of each instrument and, overall, ensure that documented procedures are followed.
- 4.2.2 Review and approve the in-house HIV-1 drug resistance genotyping protocols.

- 4.3 Quality management team
 - 4.3.1 Advises on regulations and guidelines related to references and working standards.
 - 4.3.2 Ensures that all related documented procedures are available to the end user.
 - 4.3.3 Reviews and approves the in-house HIV-1 drug resistance genotyping protocols.

5. Reagents

- 5.1 Sample extraction. See RNA EXTRACTION AND GENOTYPING PROTOCOLS" above.
- 5.2 RT-PCR and nested PCR master mixes
 - 5.2.1 HIV-1 Genotyping Kit Module 1 (catalogue no. A32317, Thermo Fisher Scientific): store at -20°C
 - 5.2.1.1 Contains pre-mixed master mixes and an aliquot of enzymes
- 5.3 Cycle sequencing master mixes
 - 5.3.1 HIV-1 Genotyping Kit Module 2 (catalogue no. A32318, Thermo Fisher Scientific): store at -20°C
 - 5.3.1.1 Contains pre-mixed master mixes for all sequencing primers
 - 5.3.1.2 Technical note: the sequencing primer names have been modified from those in the publication to match the kit layout and names.

Original name	New name
Prt-F2	SeqF1
SeqF3	SeqF2
SeqF4	SeqF3
RT-R2	SeqR1
SeqR3	SeqR2
SeqR4	SeqR3

- 5.4 Gel electrophoresis
 - 5.4.1 Agarose
 - 5.4.1.1 UltraPure™ Agarose (catalogue no. 16500-500, Thermo Fisher Scientific): store at $15-30^{\circ}\text{C}$
 - 5.4.1.2 TopVision Agarose Tablets (catalogue no. R2801, Thermo Fisher Scientific): store at $15-30^{\circ}\text{C}$
 - 5.4.2 Running buffer
 - 5.4.2.1 UltraPure™ 10× Tris-borate-EDTA buffer (catalogue no.15581-028, Thermo Fisher Scientific): store at $15-30^{\circ}\text{C}$
 - 5.4.3 Nucleic acid stain (pre-cast)
 - 5.4.3.1 GelRed® Nucleic Acid Gel stain, 10 000× in water (catalogue no. 41003, Biotium): store at $15-30^{\circ}\text{C}$ in the dark
 - 5.4.3.2 SmartGlow™ Pre Stain for nucleic acids, 20 000× in water (catalogue no. E4500-PS, Accuris Instruments): store at 4°C in the dark for up to 2 years
 - 5.4.4 DNA ladders
 - 5.4.4.1 Invitrogen™ Low DNA Mass Ladder (catalogue no. 10068-013, Thermo Fisher Scientific): store at -20°C
 - 5.4.4.1.1 Loading dye is not included
 - 5.4.4.2 FastRuler™ Middle Range DNA Ladder (catalogue no. SM1113, Thermo Fisher Scientific): store at -20°C
 - 5.4.4.2.1 FastRuler™ is only used for short separation distances on the gel (large four-comb gels)
 - 5.4.4.2.2 Loading dye is included
 - 5.4.5 Loading dye
 - 5.4.5.1 6× DNA Loading Dye (catalogue no. R0611, Thermo Fisher Scientific): store at 4°C for up to 12 months or -20°C for longer periods
 - 5.4.5.2 6× TriTrack DNA Loading Dye (catalogue no. R1161, Thermo Fisher Scientific): store at 4°C for up to 12 months or -20°C for longer periods
 - 5.4.5.3 5× GelPilot DNA Loading Dye (catalogue no. 239901, Qiagen): store at $2-8^{\circ}\text{C}$
- 5.5 PCR purification
 - 5.5.1 Enzymatic purification (primary method)
 - 5.5.1.1 Illustra ExoProStar enzymatic PCR clean-up (catalogue no.78225, 5000rxn, GE Healthcare): store at -20°C

- 5.5.1.2 Applied Biosystems™ ExoSAP-IT™ for PCR Product Clean-Up (catalogue no.78205, 10 mL, 5000rxn, Thermo Fisher): store at -20°C
- 5.5.2 Spin column purification (backup method)
 - 5.5.2.1 QIAquick™ PCR Purification Kit (catalogue no. 28106, Qiagen): store at $15-25^{\circ}\text{C}$
 - 5.5.2.2 PureLink® PCR Purification (catalogue no. K3100-01, Thermo Fisher Scientific): store at $15-30^{\circ}\text{C}$
- 5.6 Sequencing purification
 - 5.6.1 Applied Biosystems™ BigDye XTerminator™ Purification Kit – 50 mL (~2500 to 20 μL reactions, catalogue no. 4376487, Thermo Fisher Scientific): store at $2-8^{\circ}\text{C}$
 - 5.6.2 Princeton Separations Centri-Sep™ Separation Columns (catalogue no. 401762, Thermo Fisher Scientific): store at $15-30^{\circ}\text{C}$
- 5.7 Sequence detection
 - 5.7.1 POP-7™ Polymer for Applied Biosystems™ 3730-3730xl DNA Analyzer (catalogue no. 4363929, Thermo Fisher Scientific): store at $2-8^{\circ}\text{C}$
 - 5.7.2 Applied Biosystems™ 3730 Running Buffer (10 \times) (catalogue no. 4335613, Thermo Fisher Scientific): store at $15-30^{\circ}\text{C}$
 - 5.7.3 Cathode Buffer Container (CBC) 3500 Series (catalogue no. 4408256, Thermo Fisher Scientific): store at $2-8^{\circ}\text{C}$
 - 5.7.4 Anode Buffer Container (ABC) 3500 Series (catalogue no. 4393927, Thermo Fisher Scientific): store at $2-8^{\circ}\text{C}$
 - 5.7.5 POP-7 Polymer for Applied Biosystems™ 3500/3500xl Genetic Analyzer (catalogue no. 4393714, Thermo Fisher Scientific): store at $2-8^{\circ}\text{C}$
 - 5.7.6 Water, DNA grade (catalogue no. BP24701, 1 L, Thermo Fisher Scientific): store at $15-30^{\circ}\text{C}$
- 5.8 Common reagents (used in multiple areas)
 - 5.8.1 UltraPure™ DEPC-treated water (catalogue no. 750024, Thermo Fisher Scientific): store at $15-30^{\circ}\text{C}$
 - 5.8.2 Nuclease Free Water (catalogue no. AM9932, Thermo Fisher Scientific): store at $15-30^{\circ}\text{C}$
 - 5.8.3 Ethanol (catalogue no. 459844, 500 mL, Sigma): store in the chemical chamber at $15-20^{\circ}\text{C}$

6. Equipment

- 6.1 Equipment in the specimen preparation area
 - 6.1.1 Biosafety cabinet (Biosafety Level 2)
 - 6.1.2 96-well PCR/thermal cycler, such as the Applied Biosystems™ GeneAmp™ PCR System 9700 or equivalent and has a sample ramp rate around $1^{\circ}\text{C}/\text{second}$
 - 6.1.3 Bench top microcentrifuge, such as Eppendorf model 5417C or 5424 up to the speed of $10\ 000 \times g$
 - 6.1.4 Benchtop centrifuge, Jouan CR412 or equivalent
 - 6.1.5 Eppendorf ThermoMixer® or equivalent
 - 6.1.6 Daigger Twist Shaker, TW3t or equivalent
 - 6.1.7 37°C water bath
 - 6.1.8 Vortexer
 - 6.1.9 Dedicated adjustable P-10, P-20, P-200 and P-1000 pipettors
 - 6.1.10 Drummond Pipet-Aid® with charger stand or the equivalent
 - 6.1.11 Dedicated benchtop cooler
- 6.2 Equipment in the pre-amplification area
 - 6.2.1 Dead-air PCR work station with UV light
 - 6.2.2 Dedicated adjustable P-10, P-20, P-200 and P-1000 pipettors
 - 6.2.3 UV crosslinker
 - 6.2.4 Vortexer
 - 6.2.5 Benchtop microcentrifuge
 - 6.2.6 -20°C freezer
 - 6.2.7 4°C refrigerator
 - 6.2.8 Dedicated ice basket
- 6.3 Equipment in the post-amplification area
 - 6.3.1 Applied Biosystems™ 3500xl Genetic or 3730 DNA Analyzer
 - 6.3.2 96-well PCR/thermal cycler, such as the Applied Biosystems™ GeneAmp™ PCR System 9700 or the equivalent and has a sample ramp rate around $1^{\circ}\text{C}/\text{second}$
 - 6.3.3 Dead-air PCR work station or biosafety cabinet with UV light
 - 6.3.4 Bench top microcentrifuge, such as Eppendorf model 5417C or 5424 up to the speed of $10\ 000 \times g$

- 6.3.5 Drummond Pipet-Aid® with charger stand or the equivalent
- 6.3.6 Vortexer
- 6.3.7 Microwave
- 6.3.8 Benchtop centrifuge with 96-well microwell plate rotor
- 6.3.9 Dedicated adjustable P-10, P-20, P-200 and P-1000 pipettors
- 6.3.10 –20°C freezer
- 6.3.11 4°C refrigerator
- 6.3.12 Standard top-load balance
- 6.3.13 Bioimage system or UV transilluminator
- 6.3.14 Agarose gel separation apparatus and combs
- 6.3.15 Power supply for gel systems

7. Supplies

- 7.1 Supplies for the specimen preparation area
 - 7.1.1 Aerosol barrier tips
 - 7.1.2 1.5-mL sterile screw-cap conical centrifuge tubes
 - 7.1.3 15- and 50-mL sterile screw-cap polypropylene tubes
 - 7.1.4 Benchtop waste bag and holder
 - 7.1.5 Plastic beaker with 10% bleach
 - 7.1.6 70% ethanol in a spray bottle
 - 7.1.7 Biohazard bag with a holder for tip and tube disposal
 - 7.1.8 Powder-free latex, vinyl or nitrile gloves
 - 7.1.9 Kimtech® Science™ Kimwipes™
 - 7.1.10 Permanent markers
 - 7.1.11 Clean disposable laboratory coats
 - 7.1.12 Safety glasses
 - 7.1.13 Dedicated tube racks
- 7.2 Supplies for the pre-amplification area
 - 7.2.1 Distilled water, spray bottle
 - 7.2.2 Powder-free latex, vinyl or nitrile gloves
 - 7.2.3 Tube racks
 - 7.2.4 1.5-, 2.0- and 7.0-mL sterile RNase- and DNase-free tubes
 - 7.2.5 Clean disposable laboratory coats
 - 7.2.6 Kimtech® Science™ Kimwipes™
 - 7.2.7 Permanent markers
 - 7.2.8 Biohazard bag with a holder for tip and tube disposal
 - 7.2.9 Aerosol barrier tips
 - 7.2.10 96-well reaction plate
 - 7.2.11 0.2-mL PCR tubes
 - 7.2.12 Safety glasses
- 7.3 Supplies for the post-amplification area
 - 7.3.1 Powder-free latex, vinyl or nitrile gloves
 - 7.3.2 Clean disposable laboratory coats
 - 7.3.3 70% ethanol in spray bottle
 - 7.3.4 15- and 50-mL sterile screw-cap polypropylene tubes
 - 7.3.5 1.5- and 2.0-mL sterile RNase- and DNase-free tubes
 - 7.3.6 96-well reaction plates
 - 7.3.7 0.2-mL PCR tubes
 - 7.3.8 Kimtech® Science™ Kimwipes™
 - 7.3.9 Permanent markers
 - 7.3.10 Biohazard bag with a holder for tip and tube disposal
- 7.3.11 Aerosol barrier tips
- 7.3.12 Safety glasses
- 7.3.13 Distilled water
- 7.3.14 Weigh boats
- 7.3.15 250- and 500-mL Wheaton screw-cap bottles

8. Samples

- 8.1 Plasma or serum collection
 - 8.1.1 Collect 5 mL of whole blood in sterile tubes containing EDTA anticoagulant (lavender-top) and mix adequately according to the manufacturer's instructions. Do not use heparin as an anticoagulant.
 - 8.1.2 Separate plasma and serum from the cells within 2–6 hours of collection.
 - 8.1.3 Centrifuge the tubes at 1000–2000 × *g* at room temperature (15–25°C) for 15 minutes.
 - 8.1.4 Transfer the plasma or serum to a separate sterile 1.5–2.0 mL polypropylene tube (such as Sarstedt 72.694.006).
- 8.2 DBS preparation
 - 8.2.1 Spot anticoagulated EDTA venous blood onto filter paper as soon as possible after collection and preferably within 24 hours of collection. Spot blood without an anticoagulant immediately on collection (<5 minutes).
 - 8.2.2 Dispense 100 µL of whole blood into the filter paper circle.
 - 8.2.3 Obtain at least four saturated circles for each specimen.
- 8.3 DPS preparation
 - 8.3.1 Collect blood in sterile EDTA tube for plasma.
 - 8.3.2 Remove plasma or serum from the cells within 2–6 hours from collection.
 - 8.3.3 Spot 50 µL of plasma onto filter paper.
 - 8.3.4 Obtain at least four saturated circles for each specimen.
- 8.4 Labelling and identification
 - 8.4.1 All specimens arriving in the laboratory must have original identifiers affixed to the specimens.
 - 8.4.2 The laboratory personnel assign and affix the CDC specimen ID (CSID) and CDC unique ID (CUID) to each specimen when they arrive in the designated laboratory.
- 8.5 Specimen preservation and storage
 - 8.5.1 Plasma and serum
 - 8.5.1.1 Store at –65° to –80°C for up to 6 months before testing.
 - 8.5.1.2 Do not freeze and thaw plasma more than two times.
 - 8.5.2 DBS or DPS
 - 8.5.2.1 Package dry filter cards in a single gas-impermeable, sealable ziplock bag containing 2–3 desiccant packs to remove residual moisture along with one humidity indicator card.
 - 8.5.2.2 Ensure that the desiccant packs remain dry during storage.
 - 8.5.2.3 Keep the ziplock bags in the dark since UV light can damage DBS and DPS.
 - 8.5.2.4 If the specimens are processed within 14 days, store at room temperature. If the specimens cannot be processed within 14 days, store at –20°C or colder for up to 2 years.
 - 8.5.2.5 If specimens are processed for longer than 14 days, store at –70°C or colder for up to 5 years.
 - 8.5.2.6 See guidelines on preparation, storage and shipment of DBS for HIV drug resistance testing (ILB-300-P19).
- 8.6 Plasma or serum specimen shipments
 - 8.6.1 Ship the specimens in compliance with the applicable regulations covering the transport of pathogenic agents.
 - 8.6.2 Ship the specimens in frozen conditions with dry ice or in liquid nitrogen.
- 8.7 DBS or DPS shipments
 - 8.7.1 Ship the specimens in compliance with the applicable regulations covering the transport of DBS and DPS.
 - 8.7.2 For shipments that are in transit for less than 14 days, maintain at ambient temperature.
 - 8.7.3 For shipments that are in transit for longer than 14 days, maintain at –20°C or colder by using dry ice.
- 8.8 Unacceptable plasma or serum specimens
 - 8.8.1 Specimens improperly labelled or unlabelled
 - 8.8.2 Specimens with insufficient volume for testing
 - 8.8.3 Specimens without documentation or with discrepant documentation
 - 8.8.4 Specimens in unacceptable preservatives
 - 8.8.5 Specimens that have leaked in transit or otherwise show evidence of contamination
- 8.9 Unacceptable DBS or DPS specimens
 - 8.9.1 Specimens improperly labelled or unlabelled
 - 8.9.2 Specimens without documentation or with discrepant documentation
 - 8.9.3 Specimens packaged without humidity indicators and desiccants
 - 8.9.4 Specimens demonstrating any indication of humidity in the ziplock bags

- 8.9.5 Specimens with insufficient volume for testing
- 8.9.6 Specimens improperly collected
- 8.9.7 Specimens containing blood clots or clumps (DBS specimens)
- 8.9.8 Specimens with a halo around the blood spot indicating contamination (DBS specimens)
- 8.9.9 Specimens that are congruent or show evidence of commingling
- 8.10 Specimen aliquoting
 - 8.10.1 If needed, aliquot the plasma and serum samples into tubes affixed with labels. Identify each tube with a CDC aliquoting accession number, original specimen ID and date of collection.
 - 8.10.2 Perform aliquoting under a biosafety cabinet.
 - 8.10.3 See specimen management procedure (ILB-160-P08) and test directory for details of sample requirements and collection.

9. Special safety precautions

- 9.1 Universal precautions for working with infectious agents must be followed when performing this assay.
- 9.2 Ethidium bromide is a possible carcinogen. See the material safety data sheets for handling precautions and instructions.
- 9.3 Equipment with UV lights is required for this procedure. See the procedure for each piece of equipment for specific safety precautions.

10. Quality control

- 10.1 In-house controls
 - 10.1.1 The positive control is made in-house with normal human plasma spiked with viral supernatant from in vitro culture of a well-characterized HIV-1 isolate containing a known viral load measurement stored at -70°C .
 - 10.1.2 The negative control is (HIV negative) human plasma stored at -70°C .
 - 10.1.3 The positive control for DBS is made in-house with 100 μL of spiked blood containing a viral supernatant of a well-characterized HIV-1 isolate stored at -70°C .
 - 10.1.4 The positive and negative controls are prepared and included in each RT-PCR reaction run (exactly as testing specimens), and the results must be within acceptable limits for a run to be valid.
 - 10.1.4.1 Negative control: no band visible
 - 10.1.4.2 Positive control: show amplification and visible band during gel electrophoresis
 - 10.1.4.3 Repeat the run if the control results are invalid. File an occurrence management form.
 - 10.1.5 To minimize carryover, run specimens and controls in the following order: clinical specimens, positive controls and negative controls.
- 10.2 Gel electrophoresis acceptance and rejection criteria
 - 10.2.1 The test sample should be considered void if the major product is not of the appropriate size.
 - 10.2.2 The test sample should be considered void if the major product is of the correct size, 1.06 kb, but too much smearing exists.
 - 10.2.3 The test sample should be considered void if no PCR product is visible.
 - 10.2.4 The entire run is considered void if the negative control has a product band or smear visible and the positive controls are not the appropriately sized or lack PCR products.
 - 10.2.5 If the positive and negative control results are valid and the client sample does not produce a visible band, the test will be repeated with the rescue primer. Increase the initial RNA and total nucleic acid input to 20 μL or increase the RT-PCR products (2–4 μL) in the nested PCR reaction. If a negative result is produced after repeated extraction and PCR runs, no further attempt will be made to amplify the specimen.
 - 10.2.6 Data review: a second technician reviews and confirms electropherograms for each client before submitting them to the supervisor. The supervisor will review the final report and summary sheet before reporting.
- 10.3 Phylogenetic analysis is performed using the sequences generated to monitor cross-contamination.

11. Workflow chart

- 11.1 Not applicable

12. Procedure (the entire procedure takes 4–5 days from start to finish)

- 12.1 Workflow precautions
- 12.1.1 To prevent unamplified samples from contamination with amplicons, different parts of this procedure must be performed in four different physically separated work areas.
 - 12.1.2 Pre-amplification area 1 is used for preparing PCR master mix reactions. A hood with a UV light to eliminate DNA cross-contamination is required.
 - 12.1.3 Pre-amplification area 2 is used for preparing samples. A Biosafety Level 2 hood is used to work with potentially infectious samples.
 - 12.1.4 Amplification area 3 is used to perform RT and first-step PCR and to also add a template for nested PCR. A hood with UV light to eliminate DNA cross-contamination is used for adding the template for nested PCR.
 - 12.1.5 Post-amplification area 4 is used for all steps following PCR amplification.
 - 12.1.6 The workflow of the PCR procedure must be unidirectional, beginning in the pre-amplification area and ending in the post-amplification area with no return of test plates, equipment or supplies.
 - 12.1.7 To prevent environmental surface or sample cross-contamination, a brief centrifuge step must occur after any vortexing step or before opening sample-containing tubes or plates after removing them from the thermal cycler.
 - 12.1.7.1 The speed and time of spinning down to collect the fluids at the bottom of the well is not critical to the assay.

- 12.2 Before beginning the assay, fill out the in-house genotyping log (ILB-300-F16B) with sample information.
- 12.3 RNA extraction from plasma or serum follows the same procedures as in Abbott m2000sp (ILB-300-P24) or NUCLESENS® EASYMAG® (ILB-300-P23) extraction procedures.
- 12.4 Total nucleic acid extraction from DBS follows the procedures in the NUCLESENS® EASYMAG®, NUCLESENS® MINIMAG® or the bioMérieux EMAG®. See RNA EXTRACTION AND GENOTYPING PROTOCOLS" above.
- 12.5 Preparing the RT-PCR master mixes – reagent preparation takes place in pre-amplification area 1. Do not bring PCR products to this area.

- 12.5.1 Turn on the UV light in the PCR chamber for 30 minutes before use.
- 12.5.2 Thaw the RT-PCR master mix aliquot(s) reagents at room temperature except for enzymes. Vortex all tubes except the enzymes for 3–5 seconds and centrifuge briefly the pre-made master mix and place on ice or in a benchtop cooler. Remove the enzymes from the benchtop cooler only when needed.
- 12.5.3 Place a sterile 1.5- to 2.0-mL RNase-free tube in a cold block. This tube is used to prepare enough RT-PCR master mix to accommodate the number of specimen and control reactions, reagent control and an extra one to insure sufficient volume. Prepare the master mix based on the following amounts of reagents per reaction.

Number of RT-PCR reactions	1	12	24
RT-PCR mix (normal or rescue)	39 µL	468 µL	936 µL
Invitrogen™ SuperScript™ III enzyme	1 µL	12 µL	24 µL
Final volume	40 µL	480 µL	960 µL

- 12.5.3.1 Note: If a few samples show no target band, repeat RT-PCR using rescue RT-PCR master mix (purple cap).
- 12.5.3.2 It is recommended that initial RT-PCR and nested PCR be run in batches of at least 12 samples, including all the controls, to avoid repetitive reagent thawing processes and to reduce the cost of testing each specimen by reducing the ratio of specimens to controls but still follow the standard requirement for appropriate controls in each run.
- 12.5.4 Vortex the master mix after all components have been added and briefly centrifuge to collect the mix at the bottom of the tube. Place the master mix on ice.
- 12.5.5 Label one 200-µL thin-walled tube per reaction or label a 96-well plate (according to testing format) and pre-cool on ice or in a cold block. Each RT-PCR run must include positive, negative and RT-PCR reagent controls. Arrange the three tubes in the following order: positive, negative and reagent control. Add the tubes to the end of the RT-PCR run. Bring the ready-to-go master mix tubes into pre-amplification area 2 on ice.
- 12.5.6 While working in the RNA extraction hood, add 10 µL of template (total nucleic acid or RNA) into each labelled tube or plate well. Close the cap and keep the tubes or plate on ice or in a cold block.
- 12.5.7 Denature the templates at 65°C for 10 minutes in a pre-warmed thermocycler.

- 12.5.8 Immediately put the templates on ice for 3–5 minutes to shock and prevent the RNA secondary structures from reforming.
- 12.5.9 Add 40 µL of the master mix into each template tube or respective well, mix by pipetting 3–5 times and closing the cap. Change the pipette tips between each sample each time. Vortex gently and briefly centrifuge (2 seconds) to collect all the reagents into the bottom of the tube. Take the tubes or plate to amplification area 3
- 12.6 Amplification area 3
- 12.6.1 Pre-warm the thermocycler for 15–20 minutes.
- 12.6.2 Load the tubes or plate into the machine.
- 12.6.3 Select the pre-programmed RT-PCR file or programme the thermocycler as follows with reaction volume of 50 µL and ramp speed as 9600 (check the accuracy of the programme before pressing the run button):

Number of RT-PCR reactions	
50°C	45 minutes
94°C	2 minutes
40 PCR cycles	
94°C	15 seconds
50°C	20 seconds
72°C	2 minutes
One cycle	
72°C	10 minutes
4°C	Forever

- 12.6.4 The entire RT-PCR reaction takes about 3 hours. Leave the PCR tubes or plate in the thermocycler overnight, but do not leave the PCR product in the thermocycler for more than 18 hours.

Optional stopping point – after the RT-PCR reaction, freeze the RT-PCR product at –20°C for up to 2 weeks or proceed immediately to the nested PCR stage.

- 12.7 Preparing nested-PCR master mixes (pre-amplification area 1)
- 12.7.1 Turn on the UV light in the PCR chamber for 30 minutes before use.
- 12.7.2 Thaw the PCR master mix aliquot(s) at room temperature except for the enzyme. Vortex and centrifuge briefly the pre-made master mix aliquots and place on ice or in a benchtop cooler. Remove the enzyme from the benchtop cooler only when needed. Do not vortex the enzyme!
- 12.7.3 Place a sterile 1.5- to 2.0-mL DNase-free tube in a cold block to prepare enough PCR master mix to accommodate the number of planned reactions plus one based on the following amounts of reagents per reaction:

Number of PCR reactions	1	12	24
PCR mix	47.5 µL	570 µL	1140 µL
Applied Biosystems™ AmpliTaq Gold™ LD	0.5 µL	6 µL	12 µL
Final volume	48 µL	576 µL	1152 µL

- 12.7.4 Mix the PCR master mix by gentle vortexing and then briefly centrifuge (2 seconds) to collect all the reagents into the bottom of the tube.
- 12.7.5 Label one 200-µL thin-walled tube per reaction or 96-well reaction plate.
- 12.7.6 For the 50-µL PCR reaction, aliquot 48 µL of the master mix into each of the properly labelled tubes (for each PCR run, a reagent control must be included; add the tube to the end of the PCR run).
- 12.7.7 Close tubes or seal plate and take them to amplification area 3 and add 2 µL of RT-PCR products to each of the properly labelled tubes or plate well containing nested PCR master mix in the PCR chamber in amplification area 3.
- 12.7.8 Vortex and centrifuge the tubes or plate briefly and take them to post-amplification area 4 for PCR.
- 12.8 Post-amplification area 4
- 12.8.1 Pre-warm the thermocycler for 15–20 minutes.
- 12.8.2 Load the tubes into the PCR machine.

- 12.8.3 Select the preprogrammed nested PCR programme or programme the thermocycler as follows with a reaction volume of 50µL and ramp speed of 9600 (check the accuracy of the programme before pressing the run button):

One cycle	
94°C	4 minutes
40 PCR cycles	
94°C	15 seconds
55°C	20 seconds
72°C	2 minutes
One cycle	
72°C	10 minutes
4°C	Forever

Optional stopping point – after the nested PCR programme, leave the tubes in the thermocycler overnight at 4°C or take the PCR products out of the thermocycler and store them in the 4°C refrigerator and continue to the next step on the following day.

- 12.9 Agarose gel preparation (post-amplification area 4)
- 12.9.1 Agarose gel (1.0%) is used to confirm the PCR amplification results.
- 12.9.2 To prepare a 1% (w/v) agarose solution, add 1.0 g of agarose and 100 mL of 1× Tris borate EDTA to a glass flask or bottle.
- 12.9.3 Carefully microwave the agarose gel solution until boiling (usually 1 minute at full power). Swirl using hot hands or equivalent to prevent injury. Repeat three times to ensure that gel has completely dissolved into solution.
- 12.9.3.1 Caution: e-gels containing SYBR™ Safe nucleic acid stain should not be used with this assay since non-specific bands are masked when visualizing the gel with the UV light. The e-gels are marketed for decreased smearing, but the masking of non-specific bands is a negative effect.
- 12.9.4 Let the dissolved agarose solution cool to 50–60°C using room temperature.
- 12.9.5 Add 10 µL of 10 000× GelRed® stock (or 5 µL of 20 000× SmartGlow™ Pre Stain) to the 100 mL agarose solution for a 1× solution. Mix thoroughly.
- 12.9.5.1 GelRed® and SmartGlow™ Pre Stain are used to replace the highly toxic EtBr solution for staining DNA in agarose gel. If GelRed® or SmartGlow™ Pre Stain is not available, use EtBr solution (add 2 µL of EtBr to the 100-mL agarose solution).
- 12.9.6 Set up the gel tray and comb and carefully pour agarose gel into the gel caster.
- 12.9.7 Remove bubbles or debris and allow the agarose to solidify at room temperature (about 30 minutes).
- 12.10 Preparing samples for agarose gel analysis (post-amplification area 4)
- 12.10.1 Add 2 µL of 5–6× DNA loading dye into 0.5-mL tubes, 96-well plate or on the parafilm.
- 12.10.2 Add 5 µL of sample to each tube, well or parafilm and mix with loading dye.
- 12.10.3 For a molecular marker standard tube, only add 4 µL of DNA ladder.
- 12.11 Agarose gel electrophoresis (post-amplification area 4)
- 12.11.1 Once the agarose has solidified, gently remove the comb and prepare gel apparatus to receive running buffer.
- 12.11.2 Place the gel with sample wells closest to the negative (black) end of the gel box.
- 12.11.3 Slowly pour 1× Tris-borate-EDTA buffer to fill the line of the gel-running apparatus.
- 12.11.4 Load 5 µL of each sample to the wells (place the molecular marker in the first well and change the pipette tip with each sample to prevent cross-contamination).
- 12.11.5 Run the gel at 100 V for 30–45 minutes to migrate the gel-loading dye front 4–5 cm.
- 12.11.6 Check to see that loaded material has run toward the positive (red) end of the gel box sufficiently to visualize the bands and identify specific weights.
- 12.11.7 Visualize the gel on the imaging system and photograph. See the UVP imaging system procedure (ILB-300-P13). The PCR product should be 1.06 kb in size or fall just below the 1.2 kb marker.
- 12.12 Acceptance and rejection criteria
- 12.12.1 The test sample should be considered void if:
- 12.12.1.1 The major product is not the appropriate size.

- 12.12.1.2 The major product is the correct size 1.06 kb, but too much smearing exists.
- 12.12.1.3 No PCR product is visible.
- 12.12.2 The entire run is considered void if the negative control has a product band or smear visible or if the positive controls do not have the appropriately sized PCR products or lack PCR products.
- 12.12.3 If the majority of samples tested show target bands while other samples show no target bands, repeat RT-PCR using rescue primer PrtM2-F1, repeat nested PCR using 4 µL of RT-PCR products with 50 µL of total nested PCR reaction volume or increase the initial RNA or total nucleic acid input to 20 µL.
- 12.12.4 If a negative result is produced after repeated extraction and PCR runs, no further attempt will be made to amplify the specimen.
- 12.12.5 If acceptable, continue to DNA purification and preparation for sequencing.
- 12.13 Purification of PCR products using enzymes such as Illustra ExoProStar or ExoSAP-IT for PCR Product Clean-Up (option 1 – best option)
- 12.13.1 Label a 0.2-mL PCR tube for each sample. Alternatively, use a 96-well plate for large number of samples.
- 12.13.2 Add 5 µL of ExoProStar to 12.5 µL of the PCR sample and mix after pipetting.
- 12.13.2.1 It is recommended to use a dilution factor as followed:
- 12.13.2.1.1 2 µL enzyme to 5 µL of PCR product.
- 12.13.3 Run the plate or tubes in the thermocycler on the following programme to degrade the remaining primers and nucleotides and inactivate the enzymes, with a reaction volume of 18 µL.
- 12.13.4
- | | |
|-------|------------|
| 37° C | 15 minutes |
| 80° C | 15 minutes |
| 4° C | Forever |
- 12.13.5 Remove the plate or tubes from the thermocycler immediately after the programme ends and place the purified DNA on ice. If you are not immediately proceeding to cycle sequencing, the treated PCR products can be stored in the dark at –20° C until needed.
- 12.14 Purifying PCR products using such as QIAquick or PureLink PCR Purification Kits (option 2)
- 12.14.1 Note: All centrifugation steps for the purification kit occur at 17 900 × *g* (13 000 rpm).
- 12.14.2 Label one 1.5-mL microfuge tube for each specimen and the positive control.
- 12.14.3 Add 5 volumes of Buffer PBI to 1 volume of the PCR sample and vortex (for example, 20 µL of PCR product and 100 µL of buffer PBI).
- 12.14.4 The colour of the mixture should be yellow or similar to the original colour of the PBI solution before addition of the PCR product.
- 12.14.5 If the colour of the mixture is orange or violet, add 10 µL of 3 M sodium acetate (pH 5.0) and mix. The colour of the mixture should turn back to yellow.
- 12.14.6 Place a QIAquick™ spin column in a provided 2-mL collection tube.
- 12.14.7 To bind DNA, apply the sample to the QIAquick™ column and centrifuge for 1 minute.
- 12.14.8 Discard flow-through. Place the QIAquick™ column back into the same tube.
- 12.14.9 To wash, add 0.75 mL of buffer PE to the QIAquick™ column and centrifuge for 1 minute.
- 12.14.10 Discard flow-through and place the QIAquick™ column back into the same tube. Centrifuge the column for an additional 1 minute.
- 12.14.11 Place QIAquick™ column in a clean labelled 1.5-mL centrifuge tube.
- 12.14.12 To elute DNA, add 50 µL of Buffer EB or water to the centre of the QIAquick™ membrane and centrifuge for 1 minute.
- 12.14.13 Place the purified DNA on ice.
- 12.15 Cycle sequencing
- 12.15.1 The purified PCR products are used for the sequencing reaction, which is performed on a thermocycler.
- 12.15.2 Six primers (three forward and three reverse) are used to sequence the desired 1.06 kb fragment of DNA with Applied Biosystems™ BigDye XTerminators™ and the Applied Biosystems™ 3730 or Applied Biosystems™ 3500xl sequencers. This combination of reagents and equipment has a 600-base resolution.
- 12.15.3 The negative control is not sequenced, since it cannot be run on the sequencer detector.
- 12.15.4 The PCR reaction takes about 2.5 hours to run.
- 12.15.5 Thaw each of the sequencing primer master mixes (SeqF1, SeqF2, SeqF3, SeqR1, SeqR2 and SeqR3). Place on ice in post-amplification area 4.

12.15.6 Set up the sequencing reaction for each sample and the positive control in a sequencing plate or 8-strip PCR tubes.

12.15.7 For each reaction:

Each primer master mix	18 μ L
Purified PCR product	2 μ L
Total volume	20 μL

12.15.8 To run the most samples on one sequencing plate, the best layout is: (example in table layout below)

12.15.8.1 Samples are assigned to the first six wells of a row (A1–A6) or the last six wells of a row (A7–A12).

12.15.8.2 The six primers are assigned to the first six columns (1A–6A) or the last six columns (7A–12A).

12.15.9 Note: If a plate not being used, label 0.2- μ L PCR tubes with the sample number and primer letter on the cap and side and place the tubes in a rack in the same grid as used for a plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1-SeqF1	1-SeqF2	1-SeqF3	1-SeqR1	1-SeqR2	1-SeqR3						
B	2-SeqF1											
C	3-SeqF1											
D	4-SeqF1											
E	5-SeqF1											
F	6-SeqF1											
G	7-SeqF1											
H	8-SeqF1						P Ctrl	P Ctrl	P Ctrl	P Ctrl	P Ctrl	P Ctrl

12.15.10 Cap the tubes or seal the plate with sealing membrane (label each cap or plate). Pulse spin and check that the volumes are consistent.

12.15.11 Run the plate or the tubes in the thermocycler in post-amplification area 4 on the following programme with a reaction volume of 20 μ L and ramp speed of 9600 (check the accuracy of the programme before pressing the run button).

25 cycles	
96°C	10 seconds
50°C	5 seconds
60°C	4 minutes
One hold	
4°C	Forever

12.15.12 Remove the plate or tubes from the thermocycler immediately when the programme is finished. If purifying will not take place immediately, freeze in the dark at -20° C for up to 3 days.

12.16 Sequencing reaction purification

12.16.1 Purification of sequencing reactions using Applied Biosystems™ BigDye XTerminator™ protocol to remove dye-terminators:

12.16.1.1 For 20- μ L sequencing reactions, mix 20 μ L of Applied Biosystems™ BigDye XTerminator™ and 90 μ L of SAM™ solution.

12.16.1.2 For an entire 96-well plate, prepare a master mix by adding 2200 μ L of Applied Biosystems™ BigDye XTerminator™ and 9900 μ L of SAM™ solution to add 110 μ L to each well (the mixture provides enough for the entire plate).

12.16.1.3 Seal the plate and vortex for 30 minutes on the vertical vortexer.

12.16.1.4 Centrifuge the plate at 3000 rpm for 3 minutes before moving to the Applied Biosystems™ 3730 (primary) or 3500xl (backup) sequencer.

12.16.2 The Princeton Separations Centri-Sep™ Spin Columns (catalogue no. CS-901) can also be used to remove excess dye-terminators.

12.17 Sequencing using an Applied Biosystems™ 3730 DNA Analyzer

- 12.17.1 Before each run:
 - 12.17.1.1 Rinse and change the buffers.
 - 12.17.1.2 Check the polymer volume (1 mL).
 - 12.17.1.3 Remove the bubbles in the polymer block.
 - 12.17.1.4 Check the plate position on the deck.
 - 12.17.1.5 Check that the reservoir Applied Biosystems™ Septa™ is seated and flat.
 - 12.17.1.6 Check the plate assembly.
 - 12.17.1.7 Document maintenance on an Applied Biosystems™ 3730 DNA Analyzer maintenance form (ILB-300-F16D).
- 12.17.2 Use the ultrafiltered water from the Elga PURELAB® water system for preparing the 1× EDTA running buffer and the waste and water trays.
 - 12.17.2.1 Backup: DNA-grade sterile water for the water and waste trays.
- 12.17.3 Open the Data Collection Software if not already running.
 - 12.17.3.1 Open it by going to the service console that should already be running and right click on the red circle labelled VIEWER and select START.
- 12.17.4 Open all categories listed under GA INSTRUMENTS.
- 12.17.5 Click on PLATE MANAGER. The window will change to list plate names and status.
- 12.17.6 Search for plates.
- 12.17.7 Click NEW if running a new plate.
- 12.17.8 Click EDIT if editing or adding samples to a processed plate.
- 12.17.9 After clicking NEW, the NEW PLATE DIALOG window will appear.
- 12.17.10 Give the plate an ID and name.
- 12.17.11 Set these criteria:
 - 12.17.11.1 Application: sequence analysis
 - 12.17.11.2 Plate type: 96-well
 - 12.17.11.3 Plate sealing: Applied Biosystems™ Septa™
 - 12.17.11.4 Assign an owner and operator name
 - 12.17.11.5 Click OK.
- 12.17.12 The SEQUENCING ANALYSIS PLATE EDITOR window will appear. Enter the CSID and primer name for each well. Use the arrow keys to move down the wells.
 - 12.17.12.1 The sample name must be: CSID-primer name and contain the full CSID number.
 - 12.17.12.2 Example: 3005050001-PrtF2
- 12.17.13 Select the RESULTS GROUP where the data should be saved.
- 12.17.14 To assign to all samples, highlight the samples and press CTR + D.
- 12.17.15 Select the appropriate INSTRUMENT PROTOCOL based on how cycle sequencing purification was performed:
 - 12.17.15.1 Applied Biosystems™ BigDye XTerminator™ purification:
 - 12.17.15.1.1 BDx-FastSequencing-V3 – 2 hours/plate
 - 12.17.15.1.2 BDx-LongSequencing-V3 – 4 hours/plate
 - 12.17.15.2 Princeton Separations Centri-Sep™ Spin Columns:
 - 12.17.15.2.1 FastSequencing-V3 – 2 hours/plate
 - 12.17.15.2.2 LongSequencing-V3 - 4 hours/plate
- 12.17.16 Select SEQUENCING-V3 for ANALYSIS PROTOCOL.
- 12.17.17 Click OK. The plate should appear in the PLATE MANAGER list as pending.
- 12.17.18 Open the RUN SCHEDULER window.
- 12.17.19 In the input stack box, find the plates to be sequenced. Click ADD to add plates to the input stack list.
- 12.17.20 The plates are added to the list from the bottom up. The number next to the plate tells the order they need to be loaded.
- 12.17.21 When all plates have been added, click DONE.
- 12.17.22 Open the stacker drawer and load the plates. Put the plate that is designated number 1 in first. Add plate number 2 on top, and so on.
- 12.17.23 Each sequencing plate needs to be in a black base (bottom) and covered with a white retainer (top).
- 12.17.24 Close the door when all plates have been loaded.
- 12.17.25 Wait for the green triangle to appear in the top left corner of the DATA COLLECTION SOFTWARE window.

- 12.17.26 Click the green triangle. The plates will start processing. Leave the machine on INSTRUMENT STATUS while running plates.
- 12.17.27 After all the plates have been processed, remove them from the stacker drawer.
- 12.17.28 At the end of the run, clean the instrument surface (deionized water and lint-free tissue). Remove the polymer bottle and store at 2–8°C. Document on the Applied Biosystems™ 3730 DNA Analyzer maintenance form (ILB-300-F16).
 - 12.17.28.1 Important: Never use organic solvents to clean the instrument or any of its components.
- 12.17.29 Weekly maintenance
 - 12.17.29.1 Rinse the buffer, water and waste trays with warm water.
 - 12.17.29.2 Document on the Applied Biosystems™ 3730 DNA Analyzer maintenance form (ILB-300-F16D).
- 12.17.30 Periodic maintenance
 - 12.17.30.1 Change the capillary array every 300 runs, if needed.
 - 12.17.30.1.1 It is recommended to run a spectral every quarter if the number of runs on a capillary is above 300.
 - 12.17.30.2 Document on the Applied Biosystems™ 3730 DNA Analyzer maintenance form (ILB-300-F16D).
- 12.18 Evaluating sequence data before analysis
 - 12.18.1 To view sequences, open SEQUENCE SCANNER from the desktop.
 - 12.18.2 Open FILE and select IMPORT TRACES.
 - 12.18.3 Find and select the samples for viewing.
 - 12.18.4 Once the files have been highlighted, hit the button ADD SELECTED TRACES.
 - 12.18.4.1 The trace files should now appear in the window on the right side of the window.
 - 12.18.4.2 Once the software has loaded the trace files, the main window will refresh.
 - 12.18.5 Open VIEW and select REPORTS.
 - 12.18.5.1 The window will refresh, and the various reports will be displayed on the left side of the screen. The two reports of interest are:
 - 12.18.5.1.1 Quality control report – should open by default
 - 12.18.5.1.1.1 This report allows the user to see various quality control data for each individual trace file like Trace Score, CRL and QV20+.
 - 12.18.5.1.1.2 Clicking on each individual trace file name opens up the respective chromatogram for viewing.
 - 12.18.5.1.2 Plate report
 - 12.18.5.1.2.1 This report enables the user to examine the quality of all the selected trace files as they were programmed in the data collection software for the respective plate.
 - 12.18.5.1.2.2 Clicking on each individual thumbnail opens up the respective chromatogram for viewing.
 - 12.18.6 When finished, close the window.
 - 12.18.7 Copy your data to a USB flash drive so they can be assembled, edited and resistance profile generated.
 - 12.18.7.1 The raw data on the sequencer computers are backed up on the CDC SciCom network, which gets backed up every night.
- 12.19 Editing sequences
 - 12.19.1 ChromasPro or RECall is used to edit sequence and generate consensus sequences.
 - 12.19.2 See RECall HIV-1 sequencing analysis procedure ILB-300-P37 for stand-alone version.
 - 12.19.3 See genotyping sequence analysis using ChromasPro software procedure (ILB-300-P20).
- 12.20 Guidelines for identifying mixtures
 - 12.20.1 Two opposite sequence segments containing the possible mixture should each show the same secondary peaks clearly above the local noise (approximately double the noise level) or 20% of the main peak above background noise. In some situations, mixtures not called by the software can meet the above criteria. However, if secondary peaks are present in both directions and are high as the surrounding background noise peaks (high background sequences), sequencing needs to be repeated to prevent false mixture calling.
 - 12.20.2 If the segment containing a possible mixture has single sequence coverage only, the segment has to contain a secondary peak that is at least 30% of the primary peak and is three times the local noise level. Be able to differentiate between a dye blob from real peaks that may cause a false mixture.

- 12.20.3 In rare situations, a mixture pattern does not match the above guidelines. For example, multiple peaks or a shifted peak in a base requires repeating the sequencing reaction.

13. Method performance specifications

- 13.1 The assay is not to be used as a screening test for HIV or as a diagnostic test to confirm the presence of HIV infection.
- 13.2 A qualified physician with expertise in HIV therapy should interpret and apply the results.
- 13.3 The in-house assay is used for research and surveillance purposes but can also be part of patient management.
- 13.4 Assay sensitivity: the sensitivity of the assay has been established at 1000 copies/mL for plasma and DBS with HIV-1 group M subtypes and circulating recombinant forms.
- 13.5 Assay specificity: the specificity of the assay has been established at 100% with HIV-negative plasma and DBS samples.

14. Calculations

- 14.1 Sequence editing: See ILB-300-P20: HIV-1 genotyping sequence analysis using ChromasPro software procedure
- 14.2 Phylogenetic analysis is performed using BioEdit, MEGA 7 or Geneious for confirming no cross-contamination. See ILB-300-P11: molecular evolutionary genetics analyses using phylogenetic analysis software procedure.

15. Reference intervals

Not applicable

16. Interpretation and results

- 16.1 After all sequences are edited and consensus sequences generated, the resistance data are generated using the Stanford HIV Resistance Database.
- 16.2 For individual client mutation analysis, the Stanford HIVdb Program: Sequence Analysis is used to generate resistance mutation reports.
- 16.3 For population-based surveillance sequences, the Calibrated Population Resistance (CPR) tool is used to generate resistance mutation reports for transmitted resistance.
- 16.4 Simply copy and paste sequences in the analysis box or upload the sequence text file or FASTA file in the following address: <http://sierra2.stanford.edu/sierra/servlet/JSierra?action=sequenceInput> or <http://cpr.stanford.edu/cpr>.
- 16.5 Results reporting acceptability:
 - 16.5.1 The supervisor reviews the controls and results before the results are reported.
 - 16.5.2 The results will not be reported if the controls are unacceptable.
 - 16.5.3 The results are reported as individual client reports with a summary sheet or reported as a summary sheet for population-based surveillance testing. The summary sheet should be signed and be sent back to the CDC to confirm that the results were received.
- 16.6 Turn-around time
 - 16.6.1 The times vary depending on the workload and supply availability.
 - 16.6.2 The referring laboratory or requesting clinician can request an approximate turn-around time when submitting the sample.

17. Reviewing and approving the results

- 17.1 The final reports are generated using the Stanford HIVdb portal, which provides the resistance profile of the submitted sequence. However, the initial laboratory staff member who ran the test will import the raw data from the Applied Biosystems™ sequencer into the RECall sequence editing software.
 - 17.1.1 The laboratory technician will review the sequences of the individual primers that were assembled into one contig and aligned against the HXBII standard.
 - 17.1.2 If the software incorrectly called a mixture or missed a mixture, the staff member corrects the incorrect basecall and continues.
 - 17.1.3 When the primary technician is done reviewing the batch of samples, a second technician will review the sequences to confirm the first technician's edits and confirm that everything is correct.

- 17.2 After both the primary and secondary technicians have reviewed, quality control checks are performed using phylogenetic techniques to verify that no cross-contamination exists and that each sample is unique (<98%) unless paired samples exist in the batch.
- 17.3 After data analysis and quality control checks, the report is generated using the Stanford HIVdb algorithm and saved as a PDF before being printed and given to the supervisor or designee for final review, approval and dissemination.

18. Reporting results and guidelines for notification

- 18.1 After testing is completed and the team lead has reviewed and signs off, the final result for each sample that is part of a particular study is summarized on an approved results reporting template from the document control system.
- 18.2 When all study sample results are recorded, a final quality control check is performed between the summary report and sample reports.
- 18.3 Once the testing is complete, the team lead will return the final results back to the submitter along with a batch or project report (PDF), individual resistance report, if necessary (PDF), individual and grouped sequence files (FASTA) and any other results-related files.

19. Sample retention and storage

- 19.1 The retention and storage of samples and/or derivatives belonging to an approved study will comply with the storage and sample retention requirements of the protocol of the ILB Laboratory.
- 19.2 To maintain specimen quality, nucleic acid extracts will be stored in ultra-low temperature (-70°C) freezers with systems in place to ensure continuity of power supply at all times and 24/7 temperature monitoring.
- 19.3 The original samples removed from the freezer and transferred to the laboratory for testing must be returned immediately to their original assigned storage location after the samples are no longer needed in the extraction procedure.
- 19.4 The samples used in molecular surveillance work for monitoring drug resistance development and transmission will be retained for the following if not specified in a study protocol:
 - 19.4.1 Plasma and DBS sample: 5 years at -70°C , regardless of the test result.
 - 19.4.2 Extracted nucleic acids: 1 year at -70°C
 - 19.4.3 PCR products that contains cDNA from RT-PCR: 5 years at -70°C

20. References

- 20.1 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;48:3158–64.
- 20.2 *Current Protocols in Molecular Biology* [journal]. New York: Wiley.
- 20.3 Thermo Fisher Scientific HIV-1 Drug Resistance Genotyping Kit product insert.
- 20.4 Applied Biosystems™ DNA Sequencing Analysis software v5.2
- 20.5 Applied Biosystems™ 3730/3730xl DNA Analyzer getting started guide.

21. Related documents

- 21.1 ILB-160-P08 Specimen management procedure
- 21.2 ILB-300-P11 Molecular evolutionary genetics analyses using phylogenetic analysis software procedure
- 21.3 ILB-300-P13 UVP imaging system procedure
- 21.4 ILB-300-P16A HIV-1 drug resistance genotyping testing: in-house assay
- 21.5 ILB-300-P19 Preparation, storage and shipment of dried blood spots (DBS) for HIV drug resistant testing
- 21.6 ILB-300-P20 HIV-1 genotyping sequence analysis using ChromasPro software procedure
- 21.7 ILB-300-P23 NUCLISENS® EASYMAG®
- 21.8 ILB-300-P38 bioMérieux EMAG®
- 21.9 ILB-300-P24 HIV-1 quantitation using Abbott m2000
- 21.10 ILB-300-P37 Standalone RECall HIV-1 sequencing analysis procedure
- 21.11 ILB-100-F25A Occurrence management form

22. Appendices

- 22.1 ILB-300-F16A In-house training checklist
- 22.2 ILB-300-F16B In-house genotyping log
- 22.3 ILB-300-F16D 3730 DNA Analyzer maintenance form
- 22.4 ILB-300-F16E 3500xl Genetic Analyzer maintenance form
- 22.5 ILB-300-G16B Qiagen QIAamp® Viral RNA extraction job aid

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