

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

MARCH, 2010



Table of Contents

Preface	4
Recommendations for use of DBS for Genotyping in the Context of WHO HIV Drug Resistance Surveys	4
How To Use This Manual	5
Required laboratory supplies.....	6
General lab equipment and supplies.....	6
DBS preparation and storage.....	6
RNA extraction.....	7
RT-PCR.....	7
Sequencing.....	7
DBS Collection and Preparation	7
Preparation of DBS from a finger or heel stick	7
Preparation of DBS from specimens collected by venipuncture.	8
Drying DBS	9
Specimen packaging	10
Important considerations for packaging DBS	10
DBS handling and storage	11
Short term storage (≤ 14 days from the time of collection)	11
Long term storage (> 14 days from the time of collection)*	11
Specimen transport	12
Transport of specimens stored at ambient temperature from the collection site to the long-term storage site or the genotyping laboratory.....	12
Transport of frozen specimen from the long-term storage site to the genotyping laboratory.....	14
Receipt of DBS at the processing laboratory	15
Laboratory processing of DBS	15
Extraction of total HIV-1 nucleic acids from DBS prepared on 903 filter paper for HIVDR genotyping	15

Recommended method for HIV pol gene amplification from DBS	16
RT-PCR amplification (Pre-PCR area).....	16
2nd Round (nested) PCR amplification.....	17
PCR product analysis and purification	18
Sequencing reaction protocol for ABI Capillary Sequencers (Post-PCR area).....	19
Appendix.....	21
HIV pol amplification and sequencing primers	21
Use of appropriate control reagents for HIVDR testing from DBS.....	23
Validation of HIV DR testing from DBS.....	23
References	26
ACKNOWLEDGEMENTS.....	29

PREFACE

HIV-1 Drug Resistance (HIVDR) genotyping is an essential component of the WHO Global Drug Resistance Prevention and Assessment Strategy¹. Plasma is considered to be the most appropriate specimen type for HIVDR genotyping. However, use of plasma may not be feasible in rural, remote areas in limited resource settings (RLS), since its preparation and storage requires personnel and laboratory infrastructure that is often lacking. An alternative specimen type for HIVDR genotyping are dried blood spots (DBS). DBS can be made from blood drawn for routine clinical or surveillance purposes without special laboratory processing. The filter paper used is easily obtained and stored, and although procedures for making DBS must be followed precisely, the training required is less intensive than that required for plasma separation. The viral RNA in DBS is stable over longer periods and freezing is not required unless storage over one month is planned. Finally, in RLS, DBS are more easily transported than plasma to the accredited drug resistance testing laboratory because they can be shipped as non-hazardous materials using regular mail or courier services².

Despite the potential advantages of DBS as a method of specimen collection, there are several disadvantages, the foremost being reduced sensitivity of viral RNA amplification. Additionally, in specimens with low viral loads, pro-viral DNA from peripheral blood mononuclear cells (PBMC) may contribute a significant proportion of information to genotyping results. Thus in some patients with low viral loads, genotyping results from DBS specimens may not reflect the current status of replicating viruses circulating in the patient's plasma as accurately.

A number of different methodologies for performing HIVDR genotyping using dried blood (or serum) spots, including some comparisons of various storage conditions, have been developed and reported in the literature³⁻¹⁶. Because of its support for HIVDR genotyping as part of a wider global strategy for prevention and assessment of HIVDR in RLS, WHO has co-ordinated efforts to develop, validate and standardize methods for HIVDR genotyping from DBS.

RECOMMENDATIONS FOR USE OF DBS FOR GENOTYPING IN THE CONTEXT OF WHO HIV DRUG RESISTANCE SURVEYS

The design of WHO HIVDR survey in drug naïve (transmission) and treated (monitoring) surveys is described in detail elsewhere^{17,18}. In situations where plasma cannot be processed and stored under appropriate conditions, DBS may be collected for transmission

surveys and at baseline for HIVDR prevention monitoring surveys, because most patients will have relatively high viral loads. However, because of the limitations mentioned above, DBS should not be used as a specimen type at endpoint in monitoring surveys, when viral loads are expected to be lower, which is below the limit of amplification sensitivity of most DBS-based genotyping assays. In summary:

- Newly diagnosed subjects (i.e. participants in transmission surveys): DBS can be used
- Subjects initiating antiretroviral therapy (i.e. baseline time point in monitoring surveys): DBS can be used
- Patients undergoing antiretroviral therapy (i.e. at endpoint after 12 months in monitoring surveys): DBS are not recommended, plasma should be used instead.

Dried blood spots (DBS) have been extensively studied as a specimen type for *viral load* testing in treatment naïve patients with high viral loads¹⁹. Recent reports indicate that viral load results obtained from plasma and DBS are comparable, although this is dependent on the RNA extraction method used^{13,20,21}. 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 from the cellular compartment to lead to a “false positive” viral load result from PCR-based assays. Because incompletely suppressed, treatment-experienced patients are more likely to have circulating viral loads of <10,000 copies/ml compared to treatment-naïve patients, and because the definition for “failure of DR prevention” at 12 months of antiretroviral therapy is a viral load over 1000 copies/mL in plasma, WHO does not currently recommend use of DBS for viral load measurements in this population; plasma should be used instead.

The methods for handling DBS for both HIVDR genotyping and viral load determination are under investigation, and improvements in sensitivity are likely in the near future. Once the results of these studies are published and have been validated, the recommendations outlined above will be updated accordingly.

HOW TO USE THIS MANUAL

This manual provides current best practice guidance for laboratory HIVDR testing using DBS. Where possible, evidence from relevant peer-reviewed publications has been highlighted. 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. Specific products or procedures which we deem to be most strongly recommended, based on available evidence, have been marked “*” in the text.

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 spots (serum, plasma or whole blood) specimens.

REQUIRED LABORATORY SUPPLIES

The following supplies and equipment are needed for the HIVDR from DBS procedure (note: for several items, suppliers and product names are indicated:

General lab equipment and supplies

- Class II biological safety cabinet
- Solution of 0.5% sodium hypochlorite in spray bottle (a 1:10 dilution of household bleach)
- Gloves (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 1-1000 µl, with compatible, sterile-filtered tips
- Sterile disposable Pasteur pipettes
- Biohazard waste disposal bin
- Sterile 1.5 ml and 0.5 ml micro-tubes
- Sterile 15 ml conical-bottom tubes
- Bench-top and micro-centrifuges, vortex mixer and heat block

DBS preparation and storage

- Sterile, disposable, single-use lancet
- 903 “Protein Saver” filter paper cards* (Whatman)
- Desiccant sachets
- Humidity indicator (may be combined with desiccant)
- Zip-locked gas-impermeable plastic bags

- Assorted sterile racks

RNA extraction

- NucliSENS RNA Isolation kit* (EasyQ or easyMAG; bioMérieux)
- Sterile, nuclease-free water

RT-PCR

- 96-well microtitre and skirted plates
- Adhesive plate sealer
- One-Step RT-PCR kit (Qiagen)
- dNTPs
- Oligonucleotide primers (see below)
- AmpliTaq Gold (Applied Biosystems)
- Ethanol, absolute
- PCR clean-up kit
- Thermal cycler and compatible 0.2ml thin-walled micro-reaction tubes and plates
- Agarose gel electrophoresis equipment: gel tray, gel tank, power pack and combs, TBE buffer, LE Agarose
- UV transilluminator and camera

Sequencing

- Applied Biosystems Genetic analyzer (DNA sequencer)
- Computer with appropriate software installed for DNA sequence analysis
- Big-Dye terminator kit (Applied Biosystems)
- Hi-Di formamide solution (Applied Biosystems)

DBS COLLECTION AND PREPARATION

Preparation of DBS from a finger or heel stick

1. Put on gloves.
2. Use only Whatman 903 filter paper.
3. Label filter paper with appropriate identification information (patient's identification and collection date)
4. Handle the filter paper by the edges in order to avoid cross-contamination. Do not touch the areas that will be used to collect the specimens
5. 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. The puncture must be performed with sufficient force and

penetration to sustain a flow of at least several drops of blood. Use a sterile, disposable single-use lancet to puncture the skin to the side of the finger tip. 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 µl whole blood.

Note: Avoid using capillary tubes to collect blood specimens. Considerable danger of infection exists for laboratory workers from puncture wounds resulting from accidental breakage of the capillary tubes.

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 of 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. It is important that an adequate sample is collected. 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/band-aid to cover the puncture site, as required

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

PREPARATION OF DBS FROM SPECIMENS COLLECTED BY VENIPUNCTURE.

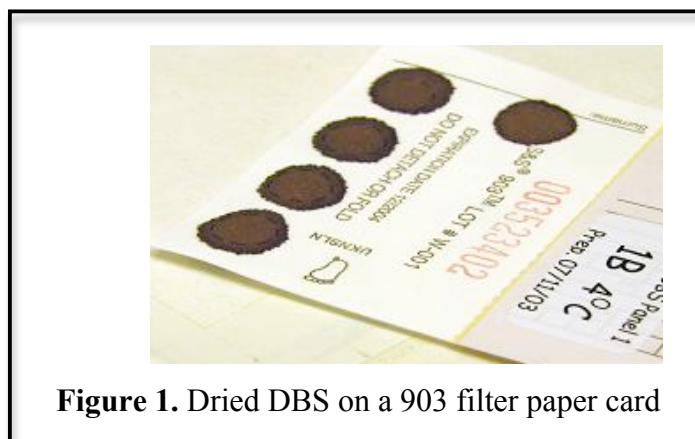
1. Anti-coagulated (EDTA or ACD) venous blood should be spotted onto filter paper as soon as possible after collection, and preferably within 24 hours of collection. Blood without an anti-coagulant should be spotted immediately upon collection (<5 minutes

after collection), as 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 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 micro-pipettor (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 ink outline
6. Repeat this procedure to fill each circle on the card with blood. For each specimen at least four saturated circles should be obtained (although 5 circles are preferred).

DRYING DBS

The time needed to dry a DBS will differ 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 drying rack, if available), or laid flat on a clean paper towel in a biohazard safety cabinet. 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 (See **Figure 1**).



SPECIMEN 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 also on the plastic bag. Use of desiccant packs is recommended as free desiccant material should not come into direct contact with the DBS
3. Place humidity indicator card into the bag in a manner such that the humidity indicator can be read without opening the bag. Gently apply pressure to the partially sealed bag to expel the air before sealing it completely.
4. Place 5-10 of the above 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 which contains the following information:
 - a. HIV Drug Resistance survey identification number
 - b. Site identification number
 - c. Blood collection date/time
 - d. DBS preparation date/time

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

IMPORTANT CONSIDERATIONS FOR PACKAGING DBS

1. In the presence of moisture, the nucleic acids in DBS are extremely sensitive to degradation. This means it is essential to ensure specimens are properly stored in the presence of desiccant packs.* Humidity indicator cards and desiccant packs have a colour indicator which changes from blue to pink as humidity increases. All humidity indicator cards and desiccant sachets should be immediately replaced if the presence of moisture is indicated.* (See recommendation on shipping and storage of specimens²²)
2. When changing humidity indicator cards and desiccant sachets in specimens that have been stored at 4 degrees or in the freezer, it is important to pre-equilibrate the bag containing the DBS 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 or dried plasma spot (DPS), 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 evaluate whether their moisture level has become too high.
4. Desiccant packs can be re-used. 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.

DBS HANDLING AND STORAGE

Short term storage (≤14 days from the time of collection)

- DBS should be transferred to -20°C or lower as soon as possible*; however, when this is not possible they can be kept and/or transported at ambient temperature up to 14 days after collection. After this time, DBS must be either processed for genotyping or frozen at -20°C or below.
- If DBS can be transported to the genotyping lab **within 14 days from the date of collection**, they can be kept and/or transported to the genotyping lab at ambient temperature. However the total time at ambient temperature should be minimized.
- As humidity and UV light can damage DBS, always keep them **in zip-lock bags with desiccant*, in the dark**.
- If direct shipment to the genotyping lab is not feasible within 14 days, transport DBS at room temperature to a laboratory with a constant electricity supply and freeze them in a -70°C freezer or, if not available, in a non-frost-free -20°C (or lower) freezer.

Long term storage (>14 days from the time of collection)*

- DBS cannot be kept and/or transported at ambient temperature for longer than 14 days.
- If genotyping cannot be performed **within 14 days from the date of collection**, DBS should be transported to a central facility where there is a constant electricity supply and a -70°C freezer^{3,5,10}

- In settings where -70°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).
- If 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.

A list of publications studying storage conditions for DBS and their main findings is presented below in **Table 1**.

SPECIMEN TRANSPORT

- Depending on in-country regulations, specimen cards can be shipped to the accredited specialized, regional or national drug resistance testing laboratory as non hazardous materials using regular mail or courier services².
- Prior to 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 (i.e. two cards in direct contact with one another) and the quantity of DBS.

Transport of specimens stored at ambient temperature from the collection site to the long-term storage site or the genotyping laboratory

- Specimen cards should be maintained in the original gas impermeable plastic bag with desiccant until 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.
- 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.

See **Figure 2** for an overview of DBS shipping procedures.

Table 1. Overview of published studies investigating optimal DBS storage conditions

Storage conditions tested				
Study	Time	Temperature/Humidity	Desiccant	Outcomes
Garcia-Lerma ¹⁵	1 to 16 weeks	37°C/high humidity, -20°C	Yes	DBS stable at 37°C only for 1-2 weeks. -20°C recommended for long-term storage. -20°C superior for short <u>and</u> long term.
Buckton ⁴	3 months	-20°C, 4°C, 20°C	Yes	HIV DNA PCR only. No observed degradation in HIV DNA during 3-month study period.
Bertagnolio ³	3 months	37°C/85% humidity	Yes	Good amplification rate (90%)
McNulty ⁸	6 years	-30°C	Yes	Complete degradation at ambient temperature; stable at -30°C and -70°C; -20°C recommended for long-term storage
	5 years	Ambient temperature and -70°C		
	2-3 years	-20°C		
Nelson ¹⁴	3 to 6 years	Ambient temperature	Yes	Moderately successful amplification rate (69%); 1 log drop in viral load.
Wallis ²³	3 months	Ambient temperature, 4°C, -20°C	Yes	Some reduction in amplification rate at ambient temp. vs. 4°C or -20°C

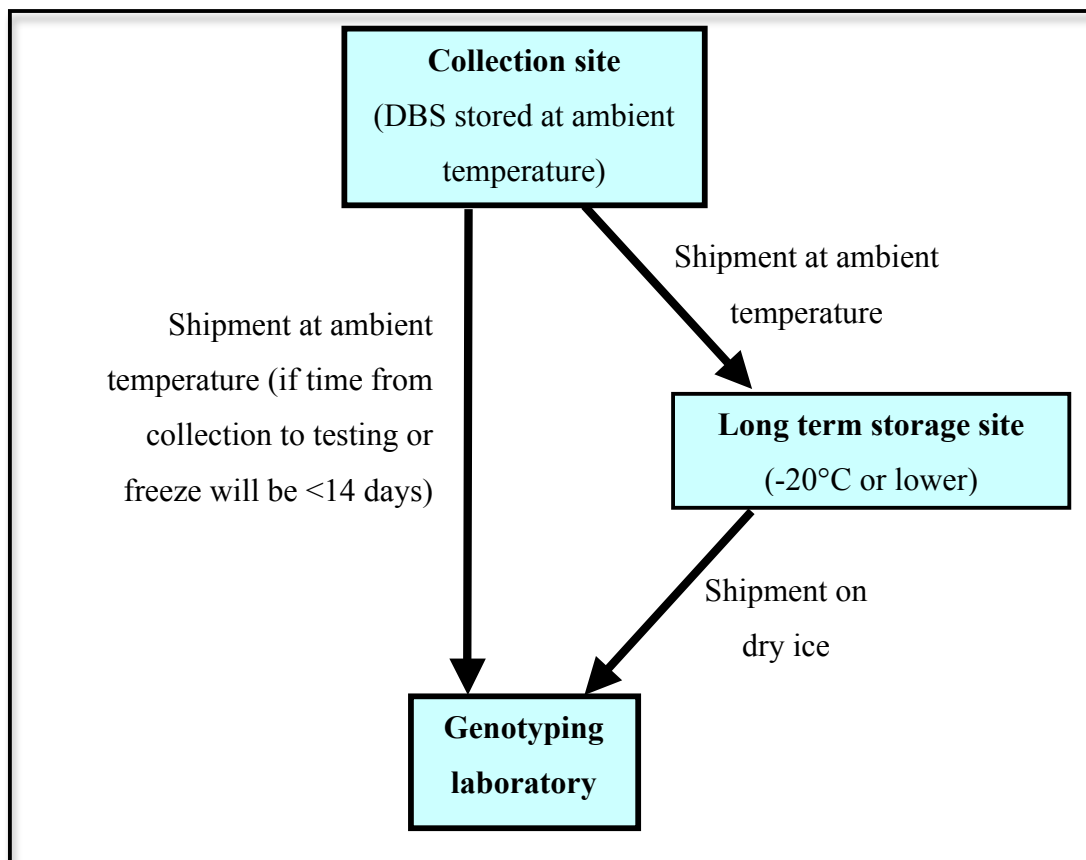


Figure 2: Specimen Transport

Transport of frozen specimen from the long-term storage site to the genotyping laboratory

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 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 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 prior to 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 humidity indicator and desiccant and shipped at room temperature.

RECEIPT OF DBS AT THE PROCESSING LABORATORY

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

Prior to testing, the quality of the collected specimen should be examined and recorded including integrity of the packaging, condition of the desiccant and humidity indicator reading. Contact the survey co-ordinator 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.

LABORATORY PROCESSING OF DBS***Extraction of total HIV-1 nucleic acids from DBS prepared on 903 filter paper for HIVDR genotyping***

- In a dedicated specimen extraction area, punch out spots using a standard office single 6 mm hole-punch device. Two to 3 spots per DBS specimen should be used for each nucleic acid extraction.
- Decontamination of the hole-punch apparatus can be carried out by multiple punching (at least five times) of a clean, unused card^{24,25}.
- While the use of a hole-punch is preferable, if scissors are to be used, single-use scissors are recommended. If scissors or forceps are re-used they must be decontaminated with a 70% solution of ethanol, wiped on clean paper towel and allowed to completely dry between uses. It is suggested to use several sets of scissors

in order to always have a dry, decontaminated set ready to process the next specimen. Cut the spot out with minimal excess filter paper not containing blood. There is no evidence to support further subdivision of the DBS once cut from the filter card.

- Nucleic acid isolation from DBS has been successfully performed using the Boom method²⁶ as employed in Nuclisens (bioMérieux) nucleic acid extraction kits with modifications to the standard protocol for DBS specimens⁷. The procedure is as follows:
 1. Add two DBS spots to 9 ml Nuclisens lysis buffer
 2. Incubate 2 hr at room temperature with gentle rotation
 3. Pellet debris by centrifugation for 5 min at 250xg
 4. Transfer the supernatant to a fresh 15 ml conical tube
- The resuspended DBS supernatant can then be used in place of a plasma sample, following the standard Nuclisens protocol.
- Eluted nucleic acids should be kept on ice, prior to prompt addition to RT-PCR reactions
- Remaining nucleic acid extract maybe stored at -20°C or lower temperatures
- Other extraction methods may be used, but their use should be locally validated following WHO recommendation for DBS assay validation (see Appendix).

RECOMMENDED METHOD FOR HIV POL GENE AMPLIFICATION FROM DBS

WHO is currently co-ordinating the validation of different methods for amplification of HIV pol gene from DBS used by different WHO accredited laboratories. This effort will result in the identification of a preferred methodology. As an interim guidance, the method described below (Source: National HIV & Retrovirology Laboratories in the National Microbiology Laboratories, Public Health Agency of Canada, Ottawa, Canada, a WHO-accredited Specialized Drug Resistance Laboratory) is suggested³.

A list of other published methods for HIVDR genotyping from DBS has been included in the Appendix (see **Table 3**).

RT-PCR amplification (Pre-PCR area)

In the first-round of amplification fragments of the HIV pol gene are amplified in two overlapping pieces using OneStep RT-PCR (Qiagen) reagents and custom primers (see **Figure 3** and **Table 2** in the Appendix). Please also see notes in the Appendix on use of appropriate control reagents when performing HIVDR from DBS.

Standard RT-PCR Master Mix:

Per reaction	Reagent
22 µl	Nuclease-free water
10 µl	5 x Buffer (with kit)
2 µl	dNTP, 10mM (with kit)
2 µl	Forward RT-PCR primer (15pmol/µl stock)
2 µl	Reverse RT-PCR primer (15pmol/µl stock)
2 µl	Enzyme mix
40 µl	Master Mix/Reaction
+ 10 µl	RNA extracted from DBS
50 µl	Total reaction mixture volume

Note. Each specimen requires two separate RT-PCR reactions (i.e. 40 µl of PR master mix and 40 µl of RT master mix need be prepared for each specimen). Each separate PR and RT reaction should be performed in its own labelled, 0.2 ml thin-walled PCR microtube.

Cycling conditions for reverse transcriptase/ 1st round PCR:

Temp	Time	Step/Cycles
50°C	40 min	RT step (1 cycle)
95°C	15 min	RT inactivation (1 cycle)
94°C	30 sec	PCR step (35 cycles)
53°C	30 sec	
72 °C	2.5 min	
72°C	10 min	Final Extension (1 cycle)
4°C	HOLD	

2nd Round (nested) PCR amplification

Preparation of the master mix should be performed in a clean, template free, PCR reagents room. As this 1st round reaction contains amplified product, addition of the template to the master mix should be performed in an area distinct from where the original template was added and must not be carried out in other areas that contain high copy number template such as the DNA sequencing area.

This step is performed using AmpliTaq Gold (Applied Biosystems) polymerase, as follows:

Nested PCR Master Mix:

Per reaction	Reagent
29.5 µl	Nuclease-free water
5 µl	10 x Gold Buffer (with kit)
4 µl	Magnesium chloride solution (25mM, with kit)
2 µl	dNTP solution, 10mM
2 µl	Forward Nested PCR primer (15pmol/µl stock)
2 µl	Reverse Nested PCR primer (15pmol/µl stock)
0.5 µl	AmpliTaq Gold
45 µl	Master mix volume/reaction
+ 5 µl	Of material from 1st round PCR reaction
50 µl	Total reaction mixture volume

Note. Maintain two distinct nested PCR reactions (one for PR and one for RT regions) as in the 1st round PCR each performed in its own labelled 0.2 ml, thin-walled, PCR microtube.

Cycling conditions for 2nd round (nested) PCR:

Temp	Time	Step/Cycles
95°C	10 min	Denaturation (1 cycle)
94°C	20 sec	PCR step (35 cycles)
53°C	30 sec	
72 °C	2.5 min	
72°C	10 min	Final Extension (1 cycle)
4°C	HOLD	

PCR product analysis and purification

In the post-PCR (sequencing) area, take a 5 µl aliquot of each nested PCR reaction, including both control reactions (see Appendix), and analyze by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. **If a specific PCR product appears in the negative control lane, the run must be considered invalid and no further processing of the amplification products should occur. If the negative control lane is free of PCR product, then proceed to the purification step. Failure of the positive control may indicate technical error or degradation of PCR reagents.**

Both the protease and reverse transcriptase nested PCR products should be purified using an appropriate PCR clean-up method employed by your laboratory. Purified PCR products should be resuspended to a final concentration of approximately 15 ng/μl.

Sequencing reaction protocol for ABI Capillary Sequencers (Post-PCR area)

Sequencing reactions are prepared using the ABI BigDye Terminator Cycle Sequencing Kit. Accurate genotypes require good quality bidirectional (both strands) sequencing of overlapping regions. See Appendix for a map of primer locations and overlapping products.

1. Prepare a plate map of samples to be sequenced such that primer sets are in adjacent wells, for example:

	Primer			
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
specimen 1				
specimen 2				
specimen 3				
specimen 4				

2. Set up master mixes on ice (need a master mix per primer used)

Sequencing Master Mix, one required for each primer:

Per reaction	Reagent
2 μl	5x Sequencing buffer
4 μl	Big Dye Terminator solution
1 μl	Sequencing primer (5μM)
12 μl	Nuclease-free water
19 μl	Total

Note: Minimize exposure to light as the dye is light sensitive

3. Add 19 μl of master mix to the wells of 96 well sequencing plate
4. Add 1 μl of purified PCR amplicon (15 ng/μl) to each well.
5. Use 8-well strip caps to cover wells once each column of 8 samples has been added, make sure caps are tight
6. Place chimney top plate into thermocycler to perform sequencing reactions:

Cycling conditions for sequencing reactions:

Temp	Time	Step/Cycles
96°C	1 min	Denaturation (1 cycle)
96°C	10 sec	Polymerization step (25 cycles)
50°C	5 sec	
60°C	4 min	
4°C	HOLD	

7. If not proceeding to the next step immediately, store PCR products at 4°C (short term) or -20°C (long term)
8. Use the plate map of the sequencing samples that has already been prepared. Each cycle sequencing reaction should be purified using a specific DNA sequencing clean-up method, as appropriate to individual laboratories.
9. Dry sequencing reactions using a speed vac at medium heat (or equivalent) for 30-45 minutes or until specimens are dry.
10. Resuspend purified sequencing reaction using 10 µl HI-DI formamide from ABI (stored at 4°C) to each well
11. Quick spin the plate to remove bubbles and to collect all the liquid at the bottom of the wells, and cover with a rubber septum
12. Denature samples for two minutes at 94°C, cool to 4°C
13. Assemble plate into holder and load into ABI DNA analyzer

APPENDIX

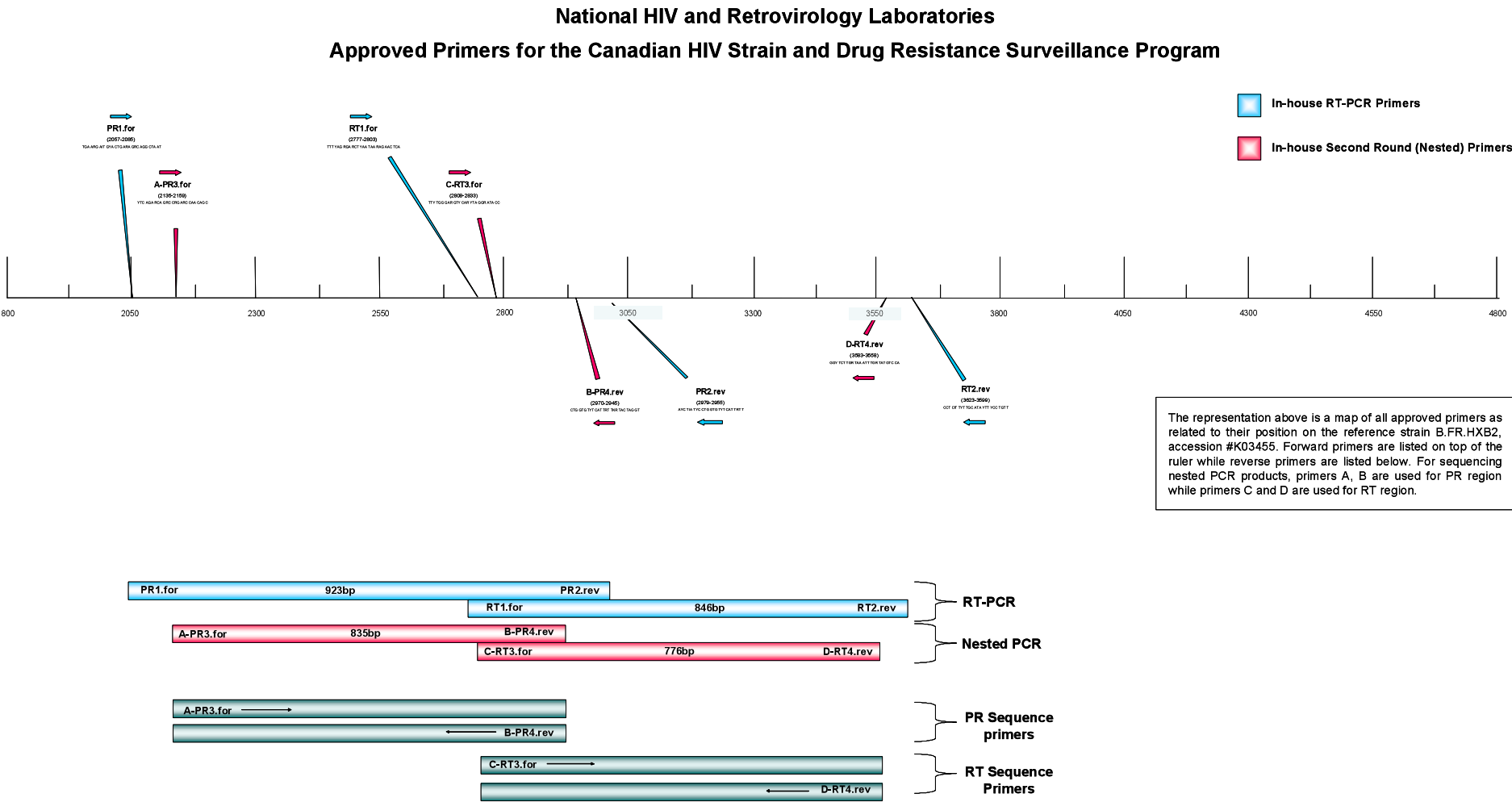
HIV pol amplification and sequencing primers

Table 2. Suggested primers for DBS amplification (from PHAC WHO specialized lab, Ottawa, Canada)

Genomic region	Primer name†	Sequence (5' to 3')
Protease RT-PCR	PR1.for	TGAARGAITGYACTGARAGRCAGGCTAAT
Protease RT-PCR	PR2.rev	AYCTIATYCCTGGTGTATCATTRTT
Reverse Transcriptase RT-PCR	RT1.for	TTYAGRGARCTYAATAARAGAACTCA
Reverse Transcriptase RT-PCR	RT2.rev	CCTCITYTTGCATAYTTYCCTGTT
Protease Nested PCR	PR3.for (A)	YTCAGRCAGRCCRGARCCAACAGC
Protease Nested PCR	PR4.rev (B)	CTGGTGTATCATTRTTKRTACTAGGT
Reverse Transcriptase Nested PCR	RT3.for (C)	TTYTGGGARGTYCARYTAGGRATACC
Reverse Transcriptase Nested PCR	RT4.rev (D)	GGYTCTTGRTAAATTTGRTATGTCCA

† letter designations indicate primers also used for sequencing

Figure 3: Primer location map



Use of appropriate control reagents for HIVDR testing from DBS

To ensure maintenance of appropriate quality control, it is essential to run ONE adequate positive AND negative control specimen with each batch of DBS tested.* Both control reactions should be tested from the sample extraction stage (i.e. from the DBS itself) and until assessment of amplification by agarose gel electrophoresis of the nested PCR amplicons. There is no need to perform DNA sequencing on these controls reactions. 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, in order to validate the sequencing run, if the positive DBS control is not carried forward to sequencing, a positive control of pedigreed 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 on 903 filter paper made from normal human whole blood (i.e. 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
- DBS made from a suspension of in vitro cultured cells (e.g. 8E5 lymphocytes) resuspended in normal human whole blood
- DBS from a HIV whole blood specimen with known subtype and a plasma HIV RNA load of greater than 10,000 copies/ml.

Validation of HIV DR testing from DBS

Laboratories performing HIV DBS testing in the context of the WHO HIVDR surveys should use a standardised methodology that has been validated according to WHO/ResNet guidelines, including participation in an external quality assessment program. The minimum required components of a validation of an in-house genotyping assay are outlined below. This list of requirements is predicated on the assumption that the laboratory is already accredited by WHO for the performance of HIVDR 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. The primary concerns to be addressed during the DBS validation are reproducibility of the sequence produced, amplification sensitivity, representation of mixed

species (especially at viral loads that are close to the amplification sensitivity limit), and contamination.

- Precision: assessment of sequence similarity, including mixtures, by repeated testing of the same sample in the same test run. Recommended design: ≥ 5 replicates of ≥ 3 different samples representing multiple subtypes and resistance patterns. Sequences from each replicate are compared to others from the same specimen and number of discrepancies quantified.
- Reproducibility: assessment of sequence similarity, including mixtures, by repeated testing of the same sample across multiple test runs, and including potential sources of variability such as operator, critical reagent lot number, key pieces of equipment, and time (e.g. over 2 weeks or more). Recommended design: ≥ 5 replicates of ≥ 3 different samples representing multiple subtypes and resistance patterns. May be supplemented by duplicate testing of a larger number of specimens (e.g. 10 to 20). Sequences from each replicate are compared to others from the same specimen and number of discrepancies quantified.
- Amplification Sensitivity: assessment of minimum required copy number (usually reported as equivalent number of RNA copies per mL in plasma) for reproducible amplification and sequencing. Include HIV negative controls interspersed with the positive specimens. Two general design approaches, which are not mutually exclusive, are as follows:
 - Serial dilution of a specimen with high viral load in an appropriate diluent (for DBS, whole blood from an HIV-negative donor) to achieve a range of viral copy number followed by replicate testing of each dilution. Amplification sensitivity may be defined as the viral load at which a majority of amplification reactions are successful.
 - Testing of a large number (>50) of samples over a wide range of copy number, concentrated in the range of the anticipated sensitivity limit; amplification sensitivity may be defined as the percentage of samples that can be amplified within a defined range (e.g. 95% positive for samples with viral load between 1000 and 4000 copies/mL).
- Linearity: assessment of sequence similarity, including mixtures, by testing a known sample over a range of input copy number including the amplification sensitivity limit.

Table 3. Overview of alternative published HIV DBS genotyping methods

Study	Genotyping method(s)	Amplicon size	Storage conditions	Sample characteristics	N samples tested	Viral load of tested samples (copies/mL)	Amplification success rate*	Sequence concordance vs. plasma†
Masciotra 2007 ⁷	Viroseq	1.8 kb	-20°C, 18 to 26 weeks	Mostly treatment experienced, subtype B	60	78 to 676,694 (median: 9135)	Overall: 83% VL>2000: 100% VL <2000: 54%	98.8%
Youngpairoj 2008 ¹¹	Viroseq or in-house nested RT-PCR	1.8 kb or 1 kb	4°C, 1 year	Treatment experienced, subtype B	40	518 to 676,694 (median: 13,680)	Viroseq: 57.5% In-house: 95%	94.5% (drug resistance mutations, DBS/in house vs. plasma/ViroSeq)
McNulty 2007 ⁸	In-house nested RT-PCR	1 kb	-20°C, 2-3 years	Untreated, subtypes from Cameroon, subtypes A, CRF02	40	665 to 645,256 (median: 23,715)	Overall: 92% VL>10,000: 100% VL <10,000: 73%	98.5%
Ziemniak 2006 ¹²	In-house nested RT-PCR	RT: 663 bp	Ambient, 0-5 months	Treated and untreated patients from the US, subtype B	9	<50 to 94,600 (median: 17,792)	Overall: 94% VL≥193: 100%	Not assessed
Bertagnolio ³	In house nested RT-PCR	RT: 700 bp	37°C, 85% humidity, 3 months	Untreated subjects from Mexico, subtype B	103	Not all tested	90.1% either PR or RT region; 78.2% for both regions	99.9% (in samples with resistance mutations)
Hallack ⁶	Trugene	1.3 kb	-20°C	Treated and untreated patients from the US, subtype B	33	1178 to 414,212 (median: 11,666)	Overall: 78.8% VL >6000: 90.5% VL <6000: 58.3%	99.3%
Garrido ⁵	In-house nested RT-PCR: RT and gp41 fragments	RT: 726 bp	4°C, <i>no desiccant</i>	Treated patients from Angola; many subtypes	77	1000 to 850,000	RT: 30% gp41: 43%	Not assessed
Steege ¹⁰	In-house nested RT-PCR	PR: 458 bp RT: 646 bp	-20°C	Treated and untreated patients from Kenya; subtypes A, C, D, CRF16	29	55 to >100,000	96.6% either PR or RT region; 89.7% for both regions; VL > 100: 100%	Not assessed
Buckton ⁴	In-house nested RT-PCR	PR: 758 bp RT: 805 bp	-20°C	Clinic patients from the UK; subtypes A, B, C, CRF02	12	80 to 115,300 (median 10,950)	PR: 83% RT: 100%	Not assessed

*Note: it is likely that the quality of field-collected DBS is substantially inferior to that of lab-collected DBS (which are often used in comparison studies) and especially plasma, with respect to amplification success rates

† mean nucleotide sequence identity, unless otherwise noted

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