

Chapter 2

Guidelines for the Qualitative Detection of Viral Genomes in Dried Blood Spots

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Abstract

Dried blood spots (DBSs) are a useful alternative to blood sampling especially in children or for screening high-risk populations in developing countries. DBS blood collection can be employed in the diagnosis of viral infections by PCR or RT-PCR and also in viral genome sequencing. In addition, the advent of multiplex PCR approaches has led to further diagnostic and methodological improvements allowing simultaneous detection of two or more different viral genomes in the same sample and amplification reaction. This chapter describes general guidelines for the qualitative viral genome amplification and detection in DBS providing an example application of a qualitative real-time SYBR Green-based multiplex RT-PCR assay targeting two major viral pathogens, HIV-1 and HCV.

Key words: Real-time RT-PCR, Dried blood spots, SYBR Green, HIV, HCV

1. Introduction

PCR direct detection of viral genomes in clinical samples has dramatically enhanced the performance of clinical virology laboratories leading to major improvements in patient management and the correct choice of therapy (1–3). PCR has evolved into a more useful procedure represented by real-time PCR that has further increased PCR applications in viral infection diagnosis offering several advantages over classic PCR. Real-time PCR shows shorter analysis time, lower contamination risk, and the possibility to perform reliable and easy quantitative detection of multiple targets using different fluorophores. Software has been developed to manage assay results and many real-time PCR dedicated instruments are available (4–6). Classic and real-time PCR can detect either one target (“monoplex” PCR) or multiple targets (“multiplex” PCR) in a single tube for the concomitant detection of two or more

viruses in clinical samples. The multiplex PCR assays save time and money by identifying mixed infections (5, 7–10). Although some commercial kits have appeared in recent years, specific multiplex PCR settings are complex because they require a careful optimization of specific PCR amplification reagents. Both monoplex and multiplex platforms can be applied on different samples and then even on DBS sampling where the blood sample is directly placed on filter paper and dried facilitating blood collection and storage even for molecular analysis (11, 12). This sampling alternative is very useful in specific patient groups such as children from whom it is relatively difficult to obtain large blood volumes. DBS is also suited to developing countries where health system infrastructure may be wanting (13, 14). This chapter describes general guidelines for DBS preparation and amplification of viral targets providing an illustrative application of a SYBR Green-based real-time multiplex assay for HIV-1/HCV detection where this procedure has been successfully applied in practice (15).

1.1. General Guidelines for DBS Preparation and Viral Nucleic Acid Amplification

1.1.1. Clinical Sample Preparation

Blood Collection

DBS assessment and subsequent qualitative determination of viral genomes can be summarized in three major points: preparation of DBS, nucleic acid extraction/purification, and specific viral target amplification and detection (Table 1).

Blood is collected from patients by venipuncture in an EDTA-containing vacutainer tube (at least 1 mL of blood is recommended) or with a sterile lancet either from the heel or finger tip. Finger tip and heel blood collections are especially indicated for pediatric patients from whom it is often difficult to obtain optimal volumes of blood. The operator must collect and handle the blood sample taking into account that all human blood samples could potentially be infectious for blood-borne pathogens and hence standard “universal” precautions should be taken before, during, and after the blood collection (http://www.cdc.gov/ncidod/dhqp/bp_universal_precautions.html) and also when processing blood derived samples such as DBS.

Dried Blood Spot Assessment

The blood is placed on a solid support, represented by filter paper, just after the blood collection at room temperature or, at least, within 6 h to achieve reliable results. Specific filter papers (i.e., S&S n°903, Whatman BFC 180) can be used to retain the blood on their surface with similar performances without any interference with the following steps of nucleic acid extraction and amplification.

The blood must be spotted carefully onto a paper support without excessive sample spreading: the optimal blood volume is 50 µL and several blood spots (i.e., six or more) are recommended for each patient in order to obtain sample replicates useful for eventual re-testing or for recording. When the blood is collected with a sterile lancet either from the heel or from finger tip, it is not

Table 1
Flowchart of qualitative real-time PCR/RT-PCR in DBS

DBS assessment	A. Sample collection	1. Collect blood sample by venopuncture or with a sterile lancet from heel or finger tip
	B. Drying procedure	2. Place blood sample (50 µL) on a paper support minimizing blood spreading. It is recommended to prepare at least six blood spots
	C. Storage	3. Dry blood spots at room temperature for 3 h in a safety cabinet or, alternatively, at 37 °C for 1 h in a bio-containment box placed in a drying oven
Nucleic acid extraction and purification	D. Excision	4. Refrigerate DBS at +4 °C or alternatively freeze (at –20 °C or less) in an individual envelope if the extraction and amplification assay is not immediately performed.
	E. Extraction and purification kit	5. Excise three punches from DBS
Amplification and detection of molecular target(s)	F. Real-time PCR/RT-PCR technique	6. Clean puncher between the samples to avoid carry over contamination
	G. Controls	7. Select nucleic acid extraction method. The choice of procedure is related to the kind of nucleic acid target involved (DNA/RNA). We strongly recommend the use of DBS validated commercial kits
		8. Assess real time PCR/RT-PCR technique. The choice of technique is related both to nucleic acid target and amplification format. All critical parameters must be titrated to obtain better sensitivity and specificity performance (see also Subheading Monoplex and Multiplex Procedure)
		9. Analyze qualitative data using the software dedicated to real time apparatus only when all controls are fully satisfied
		10. Run each sample, positive and negative controls in duplicate. Check the extraction performance analyzing the presence of specific cellular mRNA or single copy gene DNA. Amplification run is validated when all controls are properly identified

possible to establish the exact blood volume spotted but, in this case too, the operator should try to minimize blood spreading. Following this stage, blood spots can be dried at room temperature for 3 h in a safety cabinet or, alternatively, at 37 °C for 1 h in a biocontainment box placed in a drying oven with the paper in a horizontal position. Drying time, at room temperature, may be longer if the humidity is high and then is essential to check visually that the sample is fully dried to avoid the failure of assay.

Storage of Dried Blood Spots

A major advantage of blood sampling like DBS is that the samples can be handled and/or shipped easily because they can be stored for a long time before processing. The storage of DBS is a crucial point

in DBS management and can be done with different procedures. Both DNA and RNA are well conserved for long periods when DBS samples are refrigerated (+4 °C) in an individual envelope such as a hybridization bag or in a zip-closure bag with desiccant packs or frozen (−20 °C or less) showing a reliable recovery of RNA or DNA for amplification after at least 1 year of storage (12, 16, 17).

DBS exposure to higher temperatures such as room temperature or 37 °C seems to be variably associated with a progressive degradation of RNA, whereas DNA remains more stable in these conditions. Several studies have demonstrated that RNA stability progressively declines at 37 °C and after two months RNA loss is very consistent (18). Room temperature storage in a dedicated envelope with desiccant packs does not consistently affect RNA stability for at least 3 months (19), even though other studies show a good RNA stability for at least 1 year (16, 20). These conditions allow the possibility of DBS shipping to reference laboratories from rural areas where the cold chain of transport is not attainable.

1.1.2. Extraction and Purification of Nucleic Acids from DBS

Preliminary Steps

To extract the nucleic acids from a paper support, the sample must be excised from the support in paper disks using a paper puncher. To avoid carryover contaminations, between the different DBS samples, the puncher must be thoroughly cleaned with 70 % ethanol followed by PBS wash and by repeated punching of clean filter paper between consecutive samples. This approach is sufficient to rule out carry over contamination even though other alternative procedures have recently been described (21). The carryover contamination may, however, be monitored by placing reference negative samples between DBS achieved from patients.

Nucleic Acid Extraction

The choice of nucleic acid extraction and purification procedure is obviously related to the viral nucleic acid target and several methods are able to extract DNA, RNA or both. Although it is possible to use in-house extraction techniques (22–24), it is strongly recommended to employ commercial DNA and/or RNA extraction kits (i.e., Qiagen, Siemens, Roche, Invitrogen, etc.) that generally assure a higher recovery and purity of nucleic acids without the presence of amplification inhibitors. Importantly, these manufacturers have assessed effective nucleic acid extraction protocols specifically applied to DBS. These commercial kits generally allow a reliable recovery of nucleic acids greater than 75–80 %. We encourage the use of these commercial kits because in-house extraction procedures may exhibit some problems of reliability and nucleic acid recovery effectiveness.

1.1.3. Amplification and Detection of Nucleic Acids Purified from DBS

Correct DBS preparation and optimal nucleic acid extraction and purification procedures are essential for the subsequent amplification and detection steps that can be performed by classical or real-time PCR/RT-PCR. The real-time approach is more functional for the

detection of viral genomes due to its flexibility and technical characteristics. Real-time PCR or RT-PCR will disclose one (monoplex) or more (multiplex) specific viral genomes in the same run using different chemistries (i.e., SYBR Green, Taqman, etc.).

Monoplex and Multiplex Procedure

As shown in several reports, a single viral target can be detected in DBS using or slightly adapting qualitative or quantitative current classical or real-time protocols already validated on nucleic acids purified from blood or other body fluids without significant changes in the mixture composition or amplification cycles. Although real-time qualitative amplification monoplex assays are widely used in pathogen diagnostics to qualitatively discriminate specific nucleic acid targets, it is essential to determine assay performance such as the low-end sensitivity and dynamic range of amplification. To estimate the sensitivity limits, the protocol needs to assay blood samples with a known scalar number of viral genomes. The lowest viral concentration detected in over 95 % of replicates indicates the sensitivity limit. In parallel, as previously indicated (25), it is important to detect the dynamic range of amplification analyzing the calibration curve determined by amplification of reference sample dilutions. The range must cover at least three orders of magnitude (25). The assessment of RT-PCR or PCR multiplex procedures (especially real-time multiplex) is more complex because the multiplex protocols suitable for adaption to DBS using the same format are not yet as widely available as for monoplex PCR. For example, a well-known multiplex format is the Taqman approach where oligo probes (specific for each pathogen and labeled with distinct fluorescent dyes with separate excitation and emission wavelengths) discriminate the different pathogens after appropriate optimization of the real-time instrument, fluorescent calibration and scanning. On the other hand, even fluorochrome dyes binding non sequence-specific dsDNA such as SYBR Green may be used in the multiplex platforms. This approach is less complex and expensive than Taqman because it needs a lower number of oligos for the amplification reaction but distinct viral amplicon melting temperature values must be obtained for accurate identification of the specific target (4, 26–28). Nevertheless, a reasonable starting point for the assessment of multiplex real-time RT-PCR or PCR procedures may be to attempt to combine two or more effective target-specific monoplex techniques with the same format. This approach can be useful but unfortunately the addition of extra primers and probes may result in a loss of performance with reduced sensitivity and/or formation of unspecific amplification products. To overcome this drawback, an in-depth analysis of the target region is required to determine primers/probes compatible with the multiplex assay. Several oligo primer design software programs are available to disclose the more promising oligos for specific multiplex platform (e.g., Oligo, Primerplex). The amplification effectiveness

of selected primers/probes must be challenged in an amplification mixture where a careful optimization of the concentrations of the oligos and key PCR reagents, such as the magnesium chloride (tested generally between 1.5 and 8 mM), DNA polymerase (1–3 U/reaction), dNTPs and reverse transcriptase (only for multiplex RT-PCR), is essential to achieve reliable and sensitive results. These parameters can be evaluated by a chessboard procedure where scalar concentrations of these molecules are challenged among them on known reference samples positive for the target viruses to identify the optimal amplification mixture. In addition, it is noteworthy that some companies have assessed pre-formed master mix solutions that can be directly used in several multiplex contexts or appropriately modified (7). The sensitivity limit and dynamic range of amplification can be determined using a reference sample positive for the viruses of interest with a procedure similar to those indicated in the monoplex approach.

Since multiplex formats detect mixed viral infections, it is conceivable that the two (or more) viral targets, present in the DBS at very different concentrations, can suffer a relevant competition where the less represented target may be undetectable. To overcome this drawback, a chessboard procedure challenging scalar opposite dilution of two or more viral genomes in reference samples can reveal the conditions affecting the amplification of less represented viral target. This analysis is pivotal because it is possible that, in a mixed infection, the burden of a specific virus may be generally higher than in another. This problem can be solved or at least minimized by decreasing the primer concentration of preferentially amplified target. When the monoplex or multiplex qualitative real-time assays are set on reference samples, it is important to verify the assay specificity on a large group of positive and negative samples. Moreover, qualitative real-time assays must include positive and negative controls in each run. Positive controls with high and low viral concentrations are recommended. In particular, the low positive control should be added at a concentration near the lower limit of detection to monitor the sensitivity performance of amplification. Positive controls can be prepared for example, by pooling negative blood sample with reference positive plasma. On the other hand, negative controls are represented by a negative blood sample. To check nucleic acid extraction in each sample, cellular genes are amplified in the same or parallel amplification tube: GAPDH, β -actin can be chosen for RT-PCR, whereas for PCR it is important to select single copy genes such as HLA or β -globin. Viral detection analysis must be performed with the dedicated software of real-time instrument and the manufacturer's instructions must be followed to optimize analysis performance.

The next section provides an illustrative example of qualitative real-time multiplex amplification procedures represented by simultaneous amplification of HIV-1/HCV genomes in DBS using a SYBR Green-based qualitative real-time RT-PCR.

2. Materials

All reagents should be stored at room temperature (unless otherwise indicated) and all solutions must be nuclease-free. All waste disposal regulations should be followed when disposing of waste materials.

2.1. Dried Blood Spot Preparation

1. EDTA-containing vacutainer blood collection tubes.
2. Whatman No. 3 filter paper (Whatman, Maidstone, UK) (see Note 1 and Subheading 1.1.1).
3. Micropipettes and pipette tips with aerosol barrier.
4. Drying oven instrument.

2.2. Nucleic Acid Extraction

1. Single-hole paper punch (3 mm diameter).
2. Water baths or heating blocks (56, 70, and 85 °C).
3. Microcentrifuge (Eppendorf 5415D microcentrifuge, Eppendorf, Hamburg, Germany).
4. Micropipettes and pipette tips with aerosol barrier.
5. Vortexer.
6. Microcentrifuge tubes (1.5 and 2 mL).
7. QIAmp Mini spin column (Qiagen, Hilden, Germany) (see Notes 2 and 3).
8. ATL, AL, AW1, and AW2 buffers (Qiagen) (see Note 2).
9. Proteinase K solution (600 mAU/mL; Qiagen). Store at 2–8 °C (see Note 2).
10. Ethanol (100 and 70 %).
11. Sterile PBS (phosphate buffered saline) buffer.
12. Deionized, double-distilled, nuclease (RNAase and DNAase)-free water.

2.3. SYBR Green Multiplex Real-Time RT-PCR Amplification

1. Light Cycler instrument (Roche, Mannheim, Germany).
2. Capillaries (20 µL; Roche).
3. 2× Quantitect SYBR Green PCR master mix (Qiagen). Store at –20 °C (see Note 4).
4. Reverse transcriptase (RT) mix (Qiagen). Store at –20 °C (see Note 4).
5. Taq polymerase (Invitrogen, Carlsbad, USA). Store at –20 °C.
6. dNTPs mix (10 mM). Store at –20 °C.
7. MgCl₂ (25 mM).
8. Oligonucleotide primers. Store at –20 °C. For sequences see Table 2.
9. Deionized double-distilled water.

Table 2
Oligonucleotide sequences

Oligos	Sequences	Amplicon length
HIV-1 gag (29) K03455	5'-TGCTATGTCAGTTCCCCTTGGTTCTCT-3' (SK431; 1473–1499) 5'-AGTTGGAGGACATCAAGCAGCCATGCAAAT-3' (SK462; 1358–1387)	142 bp
HCV 5'NCR (30) AF009606	5'-GTCTAGCCATGGCGTTAGTATGAG-3' (HCVP1; 77–100) 5'-ACCCTATCAGGCAGTACCACAAG-3' (HCVP2; 280–302)	226 bp
β-Actin NM_001101.3	5'-GACAGGATGCAGAAGGAGATCACT-3' (ACT1; 1015–1038) 5'-TGATCCACATCTGCTGGAAGGT-3' (ACT2; 1135–1156)	142 bp

3. Methods

Blood specimens must be considered to be infectious and manipulated under strict biosafety precautions. Essential precautions include the availability of adequate hand washing facilities, work practice controls, proper use of protective equipment such as gloves, laboratory coats, the routine decontamination of work areas, and the proper disposal of waste material (see also Subheading 1.1). The following protocol is based on specific steps allowing the amplification of specific targets of HCV and HIV-1 genomes from dried blood spots.

**3.1. Preparation
of Dried Blood Spots**

1. Collect at least 1 mL of venous blood by venipuncture in EDTA-containing vacutainer tube (see Subheading 1.1.1).
2. Transport the sample tube to the virology laboratory at room temperature (see Subheading 1.1.1).
3. Carefully apply 50 µL aliquots of whole blood to Whatman No. 3 filter paper using a micropipettor and sterile tips with aerosol barrier (see Subheading 1.1.1).
4. Let the blood spotted filter papers dry at 37 °C for 1 h in a biocontainment box placed in a drying oven (see Subheading 1.1.1).

**3.2. Nuclear Acid
Extraction
and Purification from
Dried Blood Spots**

1. Use a single-hole paper punch to excise three filter punches where DBS is visible (each punch about 3 mm in diameter; see Subheading 1.1.2).
2. Place the three punched-out circles into a microcentrifuge tube (1.5 mL) and add 180 µL of ATL buffer (see Note 2).
3. Incubate at 85 °C for 10 min in a water bath. Briefly centrifuge to remove the drops from inside the lid.

4. Add 20 μL of proteinase K solution to the sample and mix by vortexing.
5. Incubate at 56 °C for 1 h in a water bath. Briefly centrifuge to remove the drops from inside the lid.
6. Add 200 μL of AL buffer to the sample. Mix thoroughly by vortexing.
7. Incubate at 70 °C for 10 min in a water bath. Briefly centrifuge to remove the drops from inside the lid.
8. Add 200 μL of 100 % ethanol to the sample, and mix thoroughly by vortexing. Briefly centrifuge to remove the drops from inside the lid.
9. Carefully apply the mixture to the QIAamp Mini spin column (placed in a 2 mL sterile collection tube) without wetting the rim (see Note 3).
10. Centrifuge at $6,000 \times g$ for 1 min. Place the QIAamp Mini spin column in a clean sterile 2 mL collection tube and discard the tube containing the filtrate.
11. Add 500 μL of AW1 buffer in the QIAamp Mini spin column without wetting the rim.
12. Centrifuge at $6,000 \times g$ for 1 min and place the QIAamp Mini spin column in a clean sterile 2 mL collection tube and discard the collection tube containing the filtrate.
13. Add 500 μL of AW2 buffer to the QIAamp Mini spin column without wetting the rim. Close the cap and centrifuge at $16,000 \times g$ for 3 min.
14. Place the QIAamp Mini spin column in a new clean and sterile 2 mL collection tube and discard the collection tube containing the filtrate.
15. Centrifuge at $16,000 \times g$ for 1 min.
16. Place the QIAamp Mini spin column in a clean and sterile 1.5 mL microcentrifuge tube and discard the collection tube containing the filtrate.
17. Carefully add 30 μL of deionized, double-distilled, RNAase and DNAase-free water to the center of the filter and incubate at room temperature for 2 min, and then centrifuge at $6,000 \times g$ for 1 min.
18. The eluate can be processed immediately or stored at $-70\text{ }^{\circ}\text{C}$ until use.

3.3. SYBR Green-Based Real-Time RT-PCR Amplification and Detection

All materials and equipment must be rigorously clean and sterile. A separate set of micropipettes and microcentrifuge tubes must be used exclusively for this procedure.

Table 3
Amplification reaction mixture

Reagents for each sample	Volume
2× Quantitect SYBR Green PCR Master Mix	10 µL
10 mM dNTPs	0.4 µL
Taq polymerase (5 U/µL)	0.4 µL
25 mM MgCl ₂	0.2 µL
HIV-1 oligonucleotide mix (25 µM each) (see Note 6)	0.32 µL
HCV oligonucleotide mix (25 µM each) (see Note 6)	0.16 µL
RT Mix	0.25 µL
Deionized, double-distilled, nuclease-free water	0.27 µL
Total	12 µL

Table 4
SYBR Green real-time multiplex RT-PCR cycling conditions

Steps	Cycles	Temperature and times
Retrotranscription	1	50 °C for 20 min
Taq activation	1	95 °C for 15 min
Amplification	45	94 °C for 5 s 60 °C for 15 s 72 °C for 30 s 78 °C for 3 s (see Note 8)
Melting	1	from 60 to 95 °C with an increase of 0.4 °C/s
Cooling	1	25 °C for 2 min

1. Add the following reagents (Table 3) for each sample in a nuclease-free microcentrifuge tube (see Notes 4 and 5):
2. Add 8 µL of purified sample and transfer the whole PCR amplification mixture to the Light Cycler capillary and perform the amplification reaction in the Light Cycler instrument (see Note 7) following the procedure shown in Table 4.
3. Data analysis is performed by LightCycler 5.3.2 software (see Note 7). The melting peaks are detectable at 81.6 °C and 86.5 °C for HIV-1 and HCV respectively. The controls are summarized in Table 5.

Table 5
SYBR Green real-time multiplex RT-PCR controls

<i>Negative controls</i>	Two HIV-1/HCV negative blood samples in duplicate (see Note 9 and Subheading 1.1.3)
<i>Positive controls</i>	HIV-1/HCV high positive blood sample in duplicate (see Note 9 and Subheading 1.1.3) HIV-1/HCV low positive blood sample in duplicate (see Note 9 and Subheading 1.1.3)
<i>Nucleic acid extraction control</i>	Amplification of β -actin mRNA in each tested sample in a parallel separate run (see Note 10 and Subheading 1.1.3)

4. Notes

1. We place the dried blood spots on Whatman No. 3 filter paper but other filter papers such as S&S n°903 (Schelchter and Schuell, Dassel, Germany) or Whatman BFC 180 (Whatman) are useful alternative.
2. The ATL, AL, AW1, AW2 buffers, QIAamp mini columns, and proteinase K are supplied in the QIAamp DNA mini kit (Qiagen, cat n°51304). Before using for the first time AW1 and AW2 buffers (supplied in a concentrated solution) must be diluted with 100 % ethanol (44 mL of AW1 plus 25 mL of 100 % ethanol and 43 mL of AW1 plus 30 mL of 100 % ethanol) and are stable for 1 year at room temperature. Proteinase K solution is stable for up to 2 months when stored at 2–8 °C. Storage at –20 °C prolongs the stability of protease up to 6 months but repeated freezing and thawing of protease K must be avoided. For this reason, we recommend the storage of suitable aliquots of proteinase K at –20 °C. AL and AW1 buffers contain guanidine hydrochloride and it is thus essential to not add bleach or acidic solutions directly to the sample-preparation waste because it can form highly reactive compounds. For more information, please consult the material safety data sheets of AL, ATL, AW1, AW2, and proteinase K solutions online (<http://www.qiagen.com/support/msds.aspx>).
3. We use as nucleic acid extraction and purification procedure (fully described in Subheading 3.2, steps 2–18) the same protocol with minor adjustments indicated for dried blood spots in the QIAamp DNA mini kit. The QIAamp mini spin columns effectively bind both RNA and DNA nucleic acids (31), potentially allowing the extraction of any viral genome by DBS. Hence, these columns can represent a good tool to purify any

virus to develop future real-time multiplex PCR or RT-PCR for the diagnosis of mixed viral infections. In our model, HIV-1 is advantageously detected because the presence of proviral DNA and viral RNA genome can be determined in the same amplification reaction.

4. Both Quantitect SYBR Green PCR Master Mix and RT mix are available in the Quantitect SYBR Green RT-PCR kit (Qiagen cat n°204243). These solutions are stable at -20°C for at least 1 year. The material safety data sheets are available online (<http://www.qiagen.com/support/msds.aspx>).
5. The RT-PCR mixture of this multiplex assay was optimized with a chessboard procedure by which the concentrations of magnesium chloride, Taq, dNTPs, and HIV-1 and HCV oligos were adjusted.
6. The oligonucleotide sequences recognize the major HIV-1 subtypes and HCV genotypes as checked by BLAST program (<http://blast.ncbi.nlm.nih.gov/>).
7. For the use and maintenance of LightCycler and dedicated software, it is essential to follow the instructions in the instrument manual. The technique may be applied to other commercial real-time PCR dedicated instruments: in this case, it is sufficient to scale-up the contents of all reagents appropriately when other real-time instruments with higher volumes of amplification reaction mixture are employed.
8. The single fluorescence detection is performed at 78°C to rule out any possible interference of rare unspecific products such as the formation of primer-dimers.
9. Both negative and positive controls are treated as the samples obtained from patients and are analyzed in duplicate. Negative control samples are represented by HIV-1/HCV negative blood samples from healthy donors. Positive control samples can be obtained mixing HIV-1/HCV positive plasma with negative healthy donor blood sample and spotted onto the filter. The positive reference plasma can be supplied by specific sources (e.g., NIBSC, London, UK, Siemens, Munich, Germany). Alternatively, HIV-1/HCV positive blood samples can be used when the quantitation of viral genomes has been previously performed by gold standard techniques such as (b-DNA, quantitative real-time RT-PCR, etc.). The positive controls are represented by high and low positive controls (4). The high positive control is represented by DBS with HIV-1/HCV at concentration of 1×10^5 HIV-1 copies/mL and 5×10^5 HCV copies/mL, whereas a low positive control is indicated by an HIV-1/HCV positive sample with 1×10^3 HIV-1 copies/mL and 5×10^3 HCV copies/mL. The analysis of scalar HIV-1/HCV reference blood samples showed

that, in our hands, this SYBR-green based real-time assay consistently detects 4×10^2 copies/mL and 2.5×10^3 copies/mL of blood for HIV-1 and HCV respectively (15).

10. β -Actin mRNA was selected as the target to determine the effectiveness of extraction and purification of nucleic acids (32). The oligonucleotide primers for β -actin are described in Table 2.2. The amplification mixture solution and the amplification conditions are the same as those shown in Tables 2.3 and 2.4 except for the volumes of oligos and water used in the mixture solution described in Table 2.3: 0.25 μ L of β -actin primer mix (25 μ M each) and 0.5 μ L of water.

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