

An internal control applied to RT-PCR detection of HCV and HIV-1 in human pooled plasma and plasma-derived medicinal products

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ABSTRACT: A competitive internal control (IC) adapted to RT-PCR *in-house* assay was developed for HCV RNA detection in human pooled plasma. Also, it was applied in a multiplex RT-PCR for the HIV-1 and HCV RNA screening in human pooled plasma and plasma-derived products. A 258-bp PCR product from the 5' non-coding region of HCV genome was obtained. A competitive IC template was constructed by inserting a 52-bp double strand sequence into the NheI site of the 258-bp amplicon. This sequence was cloned and the obtained plasmid was used to generate a synthetic RNA. The IC/RNA was incorporated in *in-house* HCV and/or HIV PCR technique to monitor the efficiency of extraction, reverse transcription, and PCR amplification steps. IC was also used to detect all major genotypes of HCV and HIV-1 strains with similar sensitivity. The detection limit of the assay for HCV and HIV-1 was 52.7 IU/mL and 164.2 IU/mL, respectively. These techniques have been evaluated in international programs of external quality assurance with highly satisfactory results. This IC is an essential reagent in PCR techniques to detect and identify HCV and HIV-1 in pooled plasma samples involved in the manufacture of plasma-derived products as well as in the field of clinical microbiology with limited resources.

Introduction

Human plasma contains proteins which, following extraction, purification, and formulation into medicinal products constitute a source of great importance in medical practice for the prevention and treatment of a variety of diseases. The potential of human pooled plasma and its plasma derivatives for viral transmission is well recognized, and because of the large number of donations which are pooled, a single contaminated batch of a plasma-derived product can transmit a viral disease to a large number of recipients. The risk of transmission of infectious diseases through blood transfusions has been known over several decades. Different events in the severity and frequency of transmission were associated with blood and its plasma derivatives (Colombo *et al.*,

1985; Dietrich, 1990; James and Mosley, 1995; Lush *et al.*, 1988; Mariani *et al.*, 1987). The hepatitis C virus (HCV), the human immunodeficiency virus type 1 (HIV-1), the hepatitis B virus (HBV), human parvovirus B19 (B19), and the hepatitis A virus (HAV), among others, are mainly associated with viral transmission by human blood and plasma derivatives (Allain *et al.*, 2009; Coste *et al.*, 2005; Dietrich, 1990; Modrow *et al.*, 2011; Roth *et al.*, 2012). Methods to increase the biological safety include donor selection of and testing of individual or pooled blood donations for markers of viral infection. Validation of the viral removal or inactivation should be done in compliance with published guidelines and good laboratory practices (Buchacher and Iberer, 2006; Velati *et al.*, 2008). Current available methodologies for HCV RNA detection include Nucleic Acid Amplification Technology (NAT), which has opened new avenues for microbial detection and characterization. The use of NAT-based techniques, as the polymerase chain reaction (PCR), applied to the detection of blood-borne transmissible viruses, allow a direct and earlier detection of the infectious agent as compared

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with the serological techniques (Cardoso *et al.*, 1999; Coste *et al.*, 2005), thus reducing the risk of transmission (Busch *et al.*, 2000; Gallarda and Dragon, 2000; Kleinman and Busch, 2000; Kleinman, Lelie and Busch, 2009; Stramer *et al.*, 2004).

The European Pharmacopoeia established that those plasma pools destined for fractionation must be tested for HCV RNA by a NAT method able to detect an HCV RNA concentration of 100 IU/mL (EAEMP, 1997; EDQM, 2012). In addition to NAT assays targeting individual viral nucleic acids, multiplex NAT screening assays have been developed which can simultaneously detect DNA or RNA from multiple viruses. However, a major drawback of most published PCR techniques is that they do not contain an internal control (IC) to monitor all procedural steps. A most important aspect of controlling the quality of PCR results is the detection of false-negative results, and the addition of an Internal Standard or IC is used for this purpose (Hoorfar and Cook, 2003; Hoorfar *et al.*, 2003; Hoorfar *et al.*, 2004).

We here report the design and development of an IC/RNA applied to detect and identify HCV RNA in pooled plasma through a competitive *in-house* RT-nested-PCR. In addition, it has been adapted for the detection of HIV-1 RNA in pooled plasma through a non-competitive *in-house* RT-nested-PCR, as well as to the simultaneous detection of both HCV and HIV-1 viruses using a multiplex RT-nested-PCR. Furthermore, these techniques have been optimized for the detection of these viruses in plasma-derived medicinal products, for additional security.

Materials and Methods

Construction of target and IC templates

A 258-bp PCR product from the 5' noncoding region of HCV genome (Accession number: AF009606) obtained from a serum sample spiked with 10000 IU/mL of International Reference Material (IRM) for HCV RNA (2nd International

Standard HCV RNA, code 96/798, WHO/NIBSC, UK, 50000 IU/vial) by RT-PCR, was used as target template. The set of primers used were: HCV-F1 and HCV-R1 (Table 1). This amplification product which contains a NheI restriction site was cloned into the EcoRI site of TOPO TA Cloning[®], pCR[®] 2.1-TOPO[®] (Invitrogen) to generate Topo-258 plasmid.

A competitive IC template was constructed by inserting the following 52-bp double strand sequence 5' CTA GCT GCT GCG CGG CAA TGC CCA AAA GCT TAA TGA CAT CAT GCA CAT GCG G 3' (containing NheI 5' and 3' protruding overhangs) into the NheI restriction site of Topo-258 plasmid to create Topo-310 plasmid. The inserts present in Topo-258 and Topo-310 recombinant plasmids were verified by DNA sequencing using the dideoxy chain termination method (Macrogen Inc., Seoul, Korea).

IC/RNA preparation

For preparation of the synthetic competitor RNA (IC/RNA), the IC template was transcribed *in vitro* from the BamHI linearized Topo-310 plasmid, using 18 U/μL of T7 RNA polymerase, 2.5 mM rNTPs, 40 U/μL ribonuclease inhibitor (RNasin[®]) according to the manufacturer's recommendations (Promega). RNA was purified by silica gel columns (QIAmp[®] Mini Elute[®] Virus Spin Kit, QIAGEN). DNase digestion (DNaseI RNase-free 2000 U/mL, BioLabs, 30 min, 37 °C) was used to remove the plasmid template. Absence of residual DNA was monitored in all PCR assays as a control reaction, excluding the reverse transcriptase used for complementary DNA (cDNA) synthesis, which always produced negative signals. The resulting RNA was quantified by RT-qPCR using a calibration curve performed with different dilutions of linear Topo-310 plasmid (ranging from 5.10²-5.10⁷ of copies/μL). Real-time PCRs were carried out in a ABI 7500 with Sequence Detection Software v1.4 (Applied Biosystems) in a reaction mixture containing 250 nM of each HCV-F2 and HCV-R2 primers for the HCV fragment amplification, 7.5 μL of SYBR[®] Green PCR Master Mix, including

TABLE 1

Primer sequences used for RT-PCR

Sequence (5' - 3')	
HCV-F1	GCC ATG GCG TTA GTA TGA GT
HCV-R1	TGC ACG GTC TAC GAG ACC TC
HCV-F2	GTG CAG CCT CCA GGA CCC CC
HCV-R2	GGG CAC TCG CAA GCA CCC TAT
HIV-1-F-1	CCC TAC AAT CCC CAA AGTC AAG G
HIV-1-R-1	YAC TGC CCC YTC ACC TTT CCA
HIV-1-F-2	TAA GAC AGC AGT ACA AAT GGC AG
HIV-1-R-2	GCT GTC CCT GTA ATA AAC CCG

AmpliTaq Gold® DNA Polymerase, optimized buffer components, dNTPs, SYBR Green I dye, and Passive Reference 1 (ROX dye) (Applied Biosystems), 5 µL of template, and UltraPURE™ DNase/RNase-free Distilled Water (Gibco) to complete 15 µL. The real-time PCR conditions were: an initial cycle at 95 °C for 600 s; 40 cycles of 95 °C for 15 s, 60 °C for 60 s. An additional step from 60 °C to 95 °C (0.2 °C s⁻¹) was added to obtain a denaturation curve. Specificity was verified by melting curve analysis and the presence of a band of the expected size in a 2.3 % (wt/v) agarose gel stained with ethidium bromide. Calibration and melting curves of real-time PCR assays performed with different dilutions containing linear Topo-310 plasmid and aliquots of the cDNA products of IC/RNA stock solution indicated this solution had 4.76 x 10⁷ copies/µL of IC/RNA (data not shown). The IC/RNA stock and the working solutions (~ 4760 copies/µL) were stable for at least three year at -80 °C. The working solution was stable for at least three months at -20 °C (data not shown).

RT-nested-PCR amplification of HCV RNA in plasma specimens and in plasma medicinal-products

Once IC/RNA optimal concentration required to perform a competitive assay was established (4760 copies/µL, see Results), this IC/RNA was added to samples of large or small mixtures (5000 or 500 donations) of plasma specimens containing 100 IU/mL of IRM for HCV RNA. They were extracted, purified by silica gel columns (QIAmp® Mini Elute® Virus Spin Kit, QIAGEN), and then 10 µL of RNA were reverse transcribed into their respective cDNAs at 37°C for 90 min, using 4 µM of HCV-R1 primer, 1 mM of each dNTPs, 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV) with a final volume of 19 µL containing 1x of enzyme buffer (Promega). Samples were then incubated at 95°C for 3 min to inactivate the enzyme.

Five microliters of the cDNA solution were used in the first-round PCR amplification of 258-bp (HCV RNA) and 310-bp (IC/RNA) amplicons. The PCR was conducted in a reaction mixture of 25 µL containing 0.4x PCR-buffer, 1.6 mM MgCl₂, 0.13 mM of each dNTPs, 0.8 U Taq-polymerase (Invitrogen), and 0.8 µM of HCV-F1 primer. Temperature cycling for the PCR run comprised 35 cycles of DNA denaturation for 25 s at 94°C, primer annealing for 25 s at 55°C, and elongation for 25 s at 72°C.

For a conventional RT-nested-PCR assay for HCV RNA, 5 µL of the above PCR reaction products were used in a second round of PCR for amplification of 212-bp (HCV RNA) and 264-bp (IC/RNA) amplicons. The PCR was performed in a reaction mixture of 50 µL containing 0.7x PCR-buffer, 1.8 mM MgCl₂, 0.14 mM of each dNTPs, 1.8 U Taq-polymerase (Invitrogen), and 0.36 µM of each HCV-F2 and HCV-R2 primers (inner primers). Ten microliters of the

PCR mixture were analyzed with 2.3% agarose gel electrophoresis and ethidium bromide staining.

To set up the detection of HCV or HCV and HIV throughout the manufacture of plasma-derived medicinal products the matrix effects were assayed. Protein concentrations were adjusted to 5% for human albumin solutions (HAS), to 12-14% for immunoglobulins used intramuscularly (anti-tetanus toxoid, anti-D and anti-B hepatitis viruses), whereas for intravenously used immunoglobulins, factor VIII, factor IX, anti-thrombin III, and prothrombin complex were carried out without modifications.

RT-nested-PCR amplification of HIV-1 RNA or HCV and HIV-1 RNAs in plasma specimens and in plasma medicinal-products

IC/RNA (4760 copies) was added to each sample of the pooled plasma specimens containing different amounts (ranging from 37 to 36308 IU/mL) of IRM for HIV-1 RNA (2nd International HIV-1 RNA, code 97/650 WHO/NIBSC, UK and HIV-1 RNA Working Reagent for NAT Assays NIBSC) or/and different amounts of IRM for HCV RNA (100-500 IU/mL). To concentrate HIV-1 viral particles, 10 µL of yeast t-RNA (Invitrogen) were added to each sample, and were then centrifuged (18000 rpm, 2°C, 45 min). The RNAs were extracted and purified as already mentioned, and then twenty microliters of RNA were reverse transcribed into their respective cDNAs at 37°C for 90 min, using 1.56 µM of each HCV-R1 and HIV-1-R1 primers, 200 U of M-MLV, 1 mM of each dNTPs and 0.9 x reaction buffer in a final volume of 32 µL. After the enzyme inactivation, and to perform PCR amplification of HIV-1 RNA alone, 5 µL of this cDNA solution were used in the first-round PCR amplification of the 324-bp (HIV-1/RNA) and 310-bp (IC) amplicons. The PCR was conducted in a reaction mixture of 50 µL, containing 1x PCR-buffer, 5 mM MgCl₂, 0.6 mM of each dNTPs, 2.5 U Taq-polymerase (Invitrogen), 0.1 µM of HCV-F1 primer, and 0.08 µM of HIV-1 F-1. Temperature cycling for the PCR run comprised 35 cycles of DNA denaturation for 30 s at 94°C, primer annealing for 30 s at 61°C, and elongation for 30 s at 72°C.

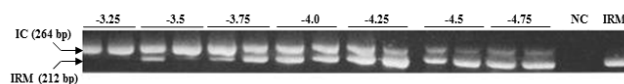


FIGURE 1. Agarose gel electrophoresis of amplification products from human pooled plasma obtained by RT-nested-PCR/HCV containing 100 IU/mL of International Reference Material (IRM) for HCV RNA and different amounts of IC/RNA, processed in duplicate. The numbers over each lane correspond to the logarithm of each IC/RNA stock dilutions (1/1778, 1/3162, 1/5623, 1/10000, 1/17783, 1/31623, 1/56234). NC: a pool of human plasma negative for HCV without IRM/RNA and IC/RNA addition). IRM: 100 IU/mL of IRM/RNA without IC/RNA addition.

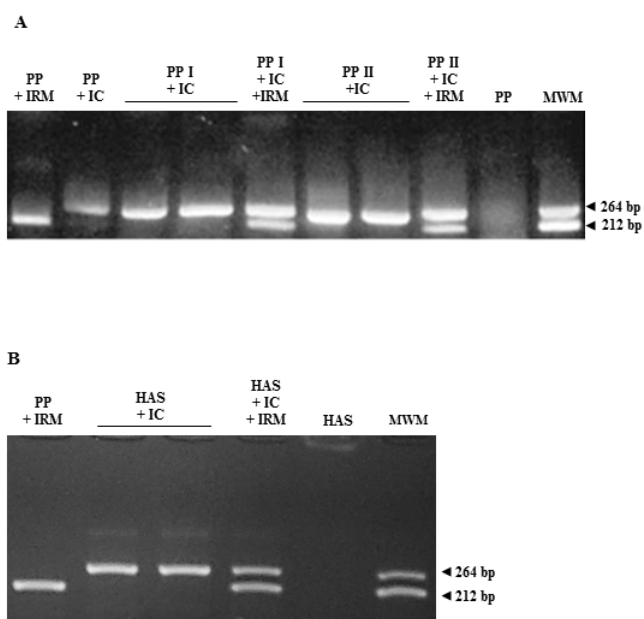


FIGURE 2. A. Agarose gel electrophoresis of amplification products of RT-nested-PCR/HCV from large pools (5000 units) of human plasma specimens (PP) containing: lane 1, 100 IU/mL of International Reference Material (IRM) for HCV RNA without IC/RNA; lane 2, PP without IRM for HCV RNA plus the addition of IC/RNA; lanes 3 and 4, a PPI plus the addition of IC/RNA; lane 5, PPI plus 100 IU/mL of IRM/RNA and IC/RNA; lanes 6 and 7, a PPII plus the addition of IC/RNA; lane 8, a PPII plus 100 IU/mL of IRM for HCV RNA and IC/RNA; lane 9, a PP without IRM for HCV RNA and IC/RNA; lane 10, fragments of DNA of 264 bp and 212 bp. **B.** Agarose gel electrophoresis of amplification products from human albumin solutions (HAS) obtained by RT-nested-PCR/HCV containing: lane 1, 100 IU/mL IRM for HCV RNA without IC/RNA; lanes 2 and 3, a 5% HAS contaminated with IC/RNA; lane 4, a 5% HAS contaminated with 100 IU/mL of IRM/RNA plus IC/RNA; lane 5, a 5% HAS without IRM/RNA and IC/RNA; lane 6, fragments of DNA of 264 bp and 212 bp.

For a conventional RT-nested-PCR assay for HIV-1/RNA, 5 μ L of the above PCR reaction were used in a second round of PCR for amplification of 175-bp (HIV-1/RNA) and 264-bp (IC) amplicons. The PCR was performed in a reaction mixture of 50 μ L containing 1x PCR-buffer, 4 mM MgCl₂, 0.6 mM of each dNTPs, 2.5 U Taq-polymerase (Invitrogen), 0.1 μ M of HCV-F2 and HCV-R2 primers (IC inner primer) and 0.08 μ M of HIV-1-F2 and HIV-1-R2 primers (HIV-1 RNA inner primers). Ten microliters of the PCR mixture were analyzed with 2.3% agarose gel electrophoresis and ethidium bromide staining.

Finally, a multiplex RT-nested-PCR format assay for HCV RNA and HIV-1 RNA was developed. For this purpose five microliters of the above cDNA solution were used in the first-round PCR amplification of 258-pb (HCV RNA), 324-bp (HIV-1/RNA), and 310-bp (IC) amplicons. The PCR was conducted in a reaction mixture of 50 μ L containing 1x PCR-buffer, 5 mM MgCl₂, 0.6 mM of each dNTPs, 2.5

U Taq-polymerase (Invitrogen), 0.37 μ M of HCV-F1 primer (HCV-1/RNA and IC/RNA) and 0.12 μ M of HIV-1-F1 primer (HIV-1/RNA). Temperature cycling for the PCR run comprised 35 cycles of DNA denaturation for 30 s at 94°C, primer annealing for 30 s at 61°C and elongation for 30 s at 72°C.

For a conventional RT-nested-PCR assay for HCV RNA and HIV/RNA, 5 μ L of the above PCR reaction were used in a second round of PCR for amplification of 212-bp (HCV RNA), 175-bp (HIV-1/RNA), and 264-bp (IC/RNA) amplicons. It was performed in a reaction mixture of 50 μ L containing 1x PCR-buffer, 4 mM MgCl₂, 0.6 mM of each dNTPs, 2.5 U Taq-polymerase (Invitrogen), 0.37 μ M of HCV-F2 and HCV-R2 primers (IC plus HCV inner primers) and 0.12 μ M of HIV-1-F-2 and HIV-1-R-2 primers (HIV-1/RNA). Temperature cycling for the PCR assay comprised 35 cycles of DNA denaturation for 30 s at 94°C, primer annealing for 30 s at 65°C and elongation for 30 s at 72°C.

To establish the detection limit in the HCV RNA, HIV-1/RNA, or HCV RNA and HIV-1 RNA assays, semi-logarithmic serial dilutions of their respective IRMs for the RNA/viruses were performed with plasma pools negative for HCV RNA, HIV/RNA, HBV antigen, anti-HIV, and anti-HCV. Then, each dilution was spiked with 4760 copies of IC/RNA. They were extracted, purified, reverse transcribed, and amplified by PCR using the above conditions. Samples without target template were analyzed as blanks by the same experiment procedures.

Results

Assays were performed to verify the absence of template DNA in the stock IC/RNA solution after DNase treatment and before its use in the *in-house* RT-nested-PCR for HCV detection. For such purpose, different aliquots (0.3; 0.5; 1, and 2 μ L) of the stock solution were subjected to PCR with HCV-F2 and HCV-R2 primers, and no residual plasmid DNA was detected in the samples (data not shown).

Optimal concentration of IC/RNA

It was essential to optimize the number of IC molecules added to the sample and the experimental conditions since the IC/RNA sequences compete with the HCV RNA sequence for the primers in the nested-PCR (Hoorfar *et al.*, 2004). The determination of the IC/RNA concentration was based on analytical criteria laid down in the international regulations for the analysis of plasma mixtures proposed for the production of blood products, which correspond to the detection of 100 IU/mL of HCV RNA (EAEMP, 1997; EDQM, 2012). Thus, the amplification efficiency of 100 IU/mL of IRM for HCV RNA in relation to the concentration of IC/

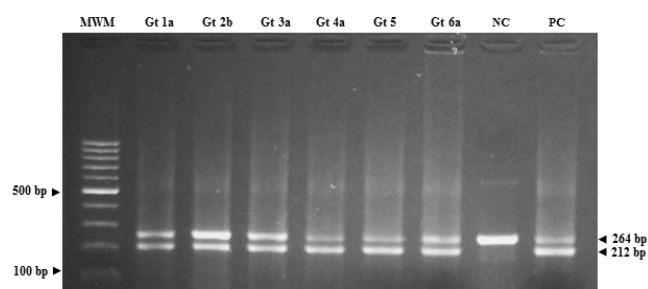


FIGURE 3. Agarose gel electrophoresis of amplification products from a panel of HCV genotypes supplied by National Institute for Biological Standards and Control containing 500-1500 IU/mL obtained by nested-PCR/HCV of: genotype 1a plus the addition of IC/RNA (lane 2); genotype 2b plus the addition of IC/RNA (lane 3); genotype 3a plus the addition of IC/RNA (lane 4); genotype 4a plus the addition of IC/RNA (lane 5); genotype 5 plus the addition of IC/RNA (lane 6); genotype 6a plus the addition of IC/RNA (lane 7); a pool of human plasma negative (NC) for HCV/RNA plus the addition of IC/RNA (lane 8); a pool of human plasma contaminated with 1000 IU/mL of IRM (PC) plus the addition of IC/RNA (lane 9). Molecular weight marker, 100-1000 bp⁻ (lane 1).

RNA added was evaluated. IC/RNA stock solution was serially diluted and then spiked into the 100 IU/mL of IRM. IC/RNA was co-extracted and co-amplified with the IRM in the same reaction tube. Results indicate that the IC/RNA concentration required to perform competitive in-house PCR corresponds to a 10^{-4} dilution of stock solution, matching with equimolar amount of the IC and IRM amplification products. The results of the second round of RT-nested-PCR are shown in Fig. 1.

Detection of HCV RNA in human pooled plasma and plasma-derived medicinal products

The utility of the IC/RNA was shown by testing large (5000 units, Fig. 2A) and small mixtures (500 units, data not shown) of plasma specimens obtained from blood donors. In addition, several plasma derivatives such as HAS (Fig. 2B), immunoglobulins and coagulation factor preparations, among others, were tested (data not shown). Since the final HAS protein concentration is approximately 20% it was necessary to adapt the technique for these matrices. Thus, optimization in protein concentration during nucleic acid extraction was a critical parameter. It was established that HAS concentrates should be diluted to a protein concentration of 5% to achieve an efficient purification of nucleic acids (samples containing a protein concentration higher than 5% hampered the passage of buffer, ethanol, and sample through the silica membranes) (Fig. 2B).

According to the technical specifications, prothrombin complex and factor IX would contain heparin. Also, even if anti-thrombin III does not include heparin in the final formulation, it could contain heparin traces since it is purified

through heparin-sepharose columns. So, the assays should be performed prior to their addition to these preparations, since heparin and other materials such as N-acetyl tryptophan and sodium caprylate (added to stabilize HAS preparations) are well known PCR inhibitors (Wilson, 1997).

Detection of genotypes of HCV of epidemiological importance

One of the requirements for HCV RNA detection by NAT is that the procedures should detect all the major HCV genotypes with a similar sensitivity (Bukh, Purcell and Miller, 1992; Choo *et al.*, 1991). The genotype panel of HCV RNA supplied by National Institute for Biological Standards and Control (NIBSC) has been tested for that purpose. This panel consists of vials containing the six most important genotypes (1a, 2b, 3a, 4a, 5, and 6a) (NIBSC, Health Protection Agency, ENG 3QG, United Kingdom, code 08/264). The results showed that the designed primers can detect the six major hepatitis C virus genotypes (Fig. 3).

Detection of HIV-1 RNA in human pooled plasma and plasma-derived medicinal products

According to the European Pharmacopoeia the detection of HIV/RNA by NAT in mixtures of plasma for plasma derivative production is not a quality requirement (EDQM, 2012). However, the plasma fractionators already opted for its incorporation, since its implementation contributes to optimize the plasma safety, especially in blood donors during serological window period. Even though IC/RNA was designed on the basis of a specific sequence of HCV RNA, it was adapted for the detection of HIV-1 RNA in which the IC/RNA is co-amplified not competitively with HIV-1/RNA. The results showed that both the IC/RNA and HIV-1 RNA were detected in human plasma (Fig. 4A). In addition, several plasma-derived medicinal products such as immunoglobulins and coagulation factors, among others, were successfully tested (data not shown).

Detection of genotypes of HIV-1 of epidemiological significance

The optimized nested-PCR for HIV-1 showed it was capable of detecting all M group subtypes, as well as the N and O groups (WHO International Standard HIV-1 RNA Genotypes, 1st International Reference Panel NIBSC code: 01/466) (Fig. 4B).

Simultaneous HCV and HIV-1 detection by RT-nested-PCR

The simultaneous detection of HCV RNA and HIV-1 RNA may represent an important advantage of a system implemented for screening of a large number of serum or plasma samples (as in blood banks and plasma fractionator industries). The technique has been optimized in such a way that allows the detection and simultaneous identification of HCV RNA and HIV-1 RNA using the IC/RNA. The analytical/clinical meaning of the presence of two viral markers in the

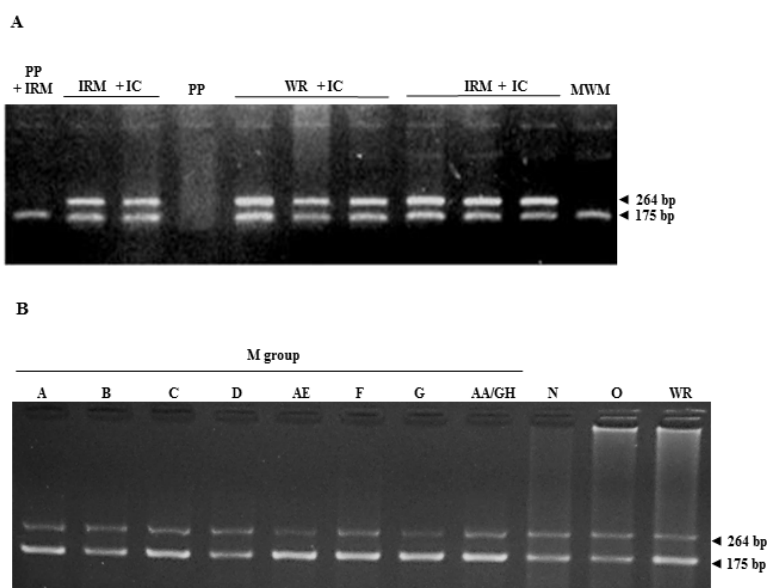


FIGURE 4. A. Agarose gel electrophoresis of amplification products of RT-nested-PCR/HIV-1 obtained from a pool of human plasma (PP) containing: lane 1, 100 IU/mL of International Reference Material (IRM) for HIV-1 RNA without IC/RNA; lane 2, IRM HIV-1 RNA (100 IU/mL) plus the addition of IC/RNA; lanes 3, IRM for HIV-1 RNA (316 IU/mL) plus the addition of IC/RNA; lane 4, PP without IRM for HIV-1 RNA and IC/RNA; lanes 5-7, Working Reagent (WR) for HIV-1 RNA (36308; 363; and 3631 IU/mL, respectively) plus the addition of IC/RNA; lane 8, IRM for HIV-1 RNA (216 IU/mL) plus the addition of IC/RNA; lane 9, IRM for HIV-1 RNA (108 IU/mL) plus the addition of IC/RNA; lane 10, IRM for HIV-1 RNA (37 IU/mL) plus the addition of IC/RNA; lane 11, a fragment of DNA of 175 bp. **B.** Agarose gel electrophoresis of amplification products from a panel of HIV-1 RNA genotypes supplied by National Institute for Biological Standards and Control containing 200-10000 IU/mL obtained by RT-nested-PCR/HIV-1 of M group (lane 1, A; lane 2, B; lane 3, C; lane 4, D; lane 5, AE; lane 6, F; lane 7, G; and lane 8, AA/GH subtypes) plus the addition of IC/RNA; N group plus the addition of IC/RNA (lane 9); and O group plus the addition of IC/RNA (lane 10); WR of HIV-1 RNA(363 IU/mL) plus the addition of IC/RNA (lane 11).

same sample may be related to a co-infection status when the trial is applied in blood banks (in the case of individual donation), while in the plasma derivative industry, the presence of two virus in a same mixture of plasma may be caused by blood of different donors. Fig. 5 shows a representative test in which the IC/RNA was used to detect simultaneously low concentrations of HCV RNA and HIV-1 RNA in a mixture of human plasma.

The detection limit of the assay for HCV and HIV-1 calculated by PROBIT analysis was estimated to be 52.7 IU/mL (38.0–89.6 IU/mL) and 164.2 IU/mL (95.7–413.8 IU/mL), respectively, defined as the 95% detection probability. Assay sensitivity for one virus was not affected by the presence of the other.

External quality assessment programs

Participation in external quality assurance programs is very important to assess the performance of the analysis systems, not only in the analytical phase but also in the entire process involving from the receipt of material to the outcomes

reports (Pisani *et al.*, 2008). Through participation in these programs, it is possible to detect early systematic and precision mistakes, which may frequently affect amplification techniques, and also to permanently update the possible occurrence of new circulating viral species. Together, these programs evaluate the performance of the analytical methods, either commercial or *in-house*, and allow the comparison between different laboratories. The techniques described in this paper were evaluated in international programs detailed below and the results were highly satisfactory.

1. External Quality Assessment Study (EQA): Istituto Superiore di Sanita, Rome, Italy, EQA/3 (2001); EQA/4 (2002) EQA/5 (2003) (Gentili *et al.*, 2003; Pisani *et al.*, 2004; Pisani *et al.*, 2002).
2. Programma ufficiale di Valutazione Esterna di Qualità (VEQ): VEQ / 2010.
3. International Collaborative Study to establish the 2nd WHO International Subtype Reference panel for HIV-1 NAT Assays. Expert Committee on Biological Standardization, Geneva, 15 to 19 October 2011, World Health Organization

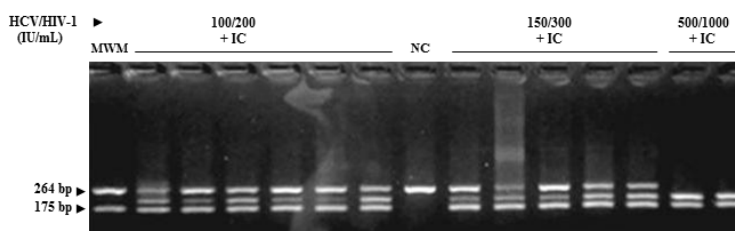


FIGURE 5. Agarose gel electrophoresis of amplification products of HCV RNA and HIV-1 RNA from pooled human plasma obtained by multiplex RT-nested-PCR. Lane 1, fragments of DNA of 264 and 175 bp; lanes 2-7, a pool of human plasma contaminated with International Reference Material (IRM) for HCV RNA (100 IU/mL) and IRM for HIV-1 RNA (200 IU/mL) plus the addition of IC/RNA; lane 8, a pool of human plasma negative (NC) for both virus plus the addition of IC/RNA; lanes 9-13, a pool of human plasma contaminated with IRM for HCV RNA (150 IU/mL) and of IRM for HIV-1 RNA (300 IU/mL) plus the addition of IC/RNA; lanes 14 and 15, a pool of human plasma contaminated with IRM for HCV RNA (500 IU/mL) and IRM for HIV-1 RNA (1000 IU/mL) plus the addition of IC/RNA.

2012 C L Morris, E Wigglesworth and A B Heath Divisions of Retrovirology and Biostatistics, National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Herts EN6 3QG, UK (<http://www.who.int/iris/handle/10665/78050#sthash.7c1psbOb.dpuf>).
4. European Directorate for the Quality of Medicines and HealthCare - Proficiency Testing Scheme (EDQM – PTS): EDQM 2009 / PTS 109 HCV-NAT and EDQM 2012/ PTS 134 HCV-NAT.

Discussion

The European Pharmacopoeia strongly recommends labeling and a traceability system based on documentation throughout the manufacture of plasma derivative products (EDQM, 2012). Thus, the pharmaceutical industry of the plasma derivatives adopted it as part of the quality control of its products. In this regard, safety in the production of plasma-derived medicinal products depends essentially on factors linked to the selection of the quality of plasma. Direct detection of viral nucleic acids using NAT has proven to be a powerful tool applied to the screening of blood donations as well as plasma pools intended for the production of plasma derivatives. However, the application of NAT in laboratories, blood centers, and blood product manufacturers, has been restricted because of a number of difficulties (Romero, 2008). One of them is associated with economic issues since the majority of available commercial methods have proven to be inaccessible for some blood centers of low complexity and resources.

The implementation of the PCR *in-house* techniques has associated a number of technical problems, including those related to false positives, false negatives, lack of automation,

special requirements of infrastructure, and training of analysts, among others. Many of the published *in-house* methods have been generated without considering the use of an IC, which is an indispensable reagent allowing to guarantee the quality of the results through the detection of false negatives and confirmation of the true negatives.

The development of an IC must consider in addition to its design, the optimization and validation of the methods which will be applied (Brightwell, Pearce and Leslie, 1998; Hoorfar *et al.*, 2004; Sachadyn and Kur, 1998). In this sense, the IC of the present work has been designed to act in a competitive way with a HCV RNA sequence and in a non-competitive manner with a HIV-1 RNA sequence. The developed IC/RNA is a chimerical RNA molecule consisting of a sequence pertaining to the 5' non-coding region of the HCV genome and a sequence of unique design inserted into the RNA sequence. Also, the experimental conditions have been optimized so that the IC/RNA is added at the beginning of the trial. This allowed us to detect the presence of false-negative results by tracking the steps of extraction, purification, retro transcription, amplification, detection, and identification of the mentioned viral ribonucleic acids. Additionally, conditions for the IC/RNA storage as well as the special setting for its application in human pooled plasma and plasma derivatives were optimized. The IC concentration has also been optimized to ensure detection of 100 IU/mL HCV RNA, complying with national and international regulations.

The techniques described have been validated, through the use of certified international standards, for the singleplex PCR detection of either HCV or HIV-1, as well as for the simultaneous detection of both HCV and HIV-1 viruses using a multiplex RT-nested-PCR. The participation in studies of international quality control demonstrates that these assays

have specificity, robustness, and a detection limit according to the regulations (52.7 IU/mL and 164.2 IU/mL, for HCV and HIV-1, respectively). The incorporation of the IC/RNA to *in-house* PCR technique for the simultaneous detection of HCV RNA and HIV-1 RNA allows optimizing the technical resource, avoiding individual analysis for each virus.

The decision of each laboratory about the incorporation of NAT, either commercial or *in-house* methods, surely depends on the adopted criteria and policies, legal requirements, the regional epidemiological circumstances, and possibilities to afford it (Busch and Dodd, 2000). The incorporation of ICs in many available techniques of molecular biology is one of the biggest challenges today. Thus, some laboratories have developed their own ICs to implement them in the detection of a wide variety of specimens of microbiological origin (Castelain *et al.*, 2004; Niesters, 2004; Pasloske *et al.*, 1998; Rosenstraus *et al.*, 1998; Villanova *et al.*, 2007).

The possibility to test the presence of these viruses in plasma-derived medicinal products adds another security level to the whole plasma manufacturer industry. It should be noted that the use of the developed IC is currently applied to several plasma derivatives, such as different immunoglobulin products (anti-D immunoglobulin, subcutaneous immunoglobulin), coagulation factors, and in some intermediate steps of the manufacture of plasma derivatives (data not shown).

The incorporation of this IC to either conventional or real-time PCR is of great importance for the industries, particularly in the developing countries, due to the low cost and complexity required for the synthesis and storage of the IC, as well as for its implementation in the techniques above mentioned, as compared to the commercial ones available in the market. The quality of the synthetic molecule, adaptation to PCR techniques for simple and simultaneous detection of HCV and HIV ribonucleic acids, the versatility in the implementation for various biological matrices, the low economic requirements, and the high profits in the analytical field, provide an essential and available reagent to the quality assurance in nucleic acid amplification techniques.

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