EXPERT REVIEWS

In-house HIV-1 RNA real-time RT-PCR assays: principle, available tests and usefulness in developing countries

Expert Rev. Mol. Dign. 8(5), 635–650 (2008)

François Rouet†, Hervé Ménan, Johannes Viljoen, Nicole Ngo-Giang-Huong, Kishor Mandaliya, Diane Valéa, Truong Xuan Lien, Sivapragashini Danaviah, Dominique Rousset, Amandine Ganon and Eric Nerrienet

†*Author for correspondence*
Laboratoire de Virologie, Centre Muraz, BP390
Bobo-Dioulasso 01, Burkina Faso
Tel.: +226 2097 0102
Fax: +226 2097 0457
franrouet@yahoo.fr

The principle of currently available licensed HIV-1 RNA assays is based on real-time technologies that continuously monitor the fluorescence emitted by the amplification products. Besides these assays, in-house quantitative (q) real-time reverse transcription (RT)-PCR (RT-qPCR) tests have been developed and evaluated particularly in developing countries, for two main reasons. First, affordable and generalized access to HIV-1 RNA viral load is urgently needed in the context of expected universal access to prevention and antiretroviral treatment programs in these settings. Second, since many non-B subtypes, circulating recombinant forms and unique recombinant forms circulate in these areas, in-house HIV-1 RNA RT-qPCR assays are ideal academic tools to thoroughly evaluate the impact of HIV-1 genetic diversity on the accuracy of HIV-1 RNA quantification, as compared with licensed techniques. To date, at least 15 distinct in-house assays have been designed. They differ by their chemistry and the HIV-1 target sequence (located in *gag, Pol-IN* or LTR gene). Analytical performances of the tests that have been extensively evaluated appear at least as good as (or even better than) those of approved assays, with regard to HIV-1 strain diversity. Their clinical usefulness has been clearly demonstrated for early diagnosis of pediatric HIV-1 infection and monitoring of highly active antiretroviral therapy efficacy. The LTR-based HIV-1 RNA RT-qPCR assay has been evaluated by several groups under the auspices of the Agence Nationale de Recherches sur le SIDA et les hépatites virales B et C. It exists now as a complete standardized commercial test.

Keywords: developing country • genetic diversity • HIV-1 • PCR • quantitative • real-time • reverse transcription • RNA • viral load

Viral load (VL) is defined as the quantification of one specific viral nucleic acid sequence in one specific organism compartment. Plasma HIV-1 RNA VL is directly correlated with the number of viral particles circulating in plasma, thus reflecting the active HIV-1 replication in HIV-1-infected subjects [1]. Because of the variability of plasma HIV-1 RNA VL measurements and their wide dynamic range, results are expressed in HIV-1 RNA copies/ml of plasma with a $\log_{10}$ transformation. Since 1995, many nucleic acid-based assays have been developed for the quantification of HIV-1 RNA in plasma. They use three major technologies: reverse transcriptase (RT)-PCR, nucleic acid sequence-based amplification (NASBA) and a signal amplification methodology termed branched-chain DNA (bDNA). Initial HIV-1 RNA assays were based on end point determinations, with detection/quantification carried out after the amplification step by hybridization of amplified products with specific labeled probes and subsequent detection by enzyme immunoassay or chemiluminescence [2]. At present, all currently available PCR-based HIV-1 RNA assays are focused on real-time technologies continuously monitoring the fluorescence emitted by the amplification products at each PCR cycle [3]. The HIV-1 RNA quantification is performed during the exponential phase of PCR, thus providing a more reliable measurement than conventional end point techniques. With these newer assays, the lower detection limit has been improved (~50 copies/ml).

In industrialized countries, numerous studies have shown that plasma HIV-1 RNA levels are independent predictors of disease progression in

 10.1586/14737159.8.5.635 ISSN 1473-7159

adults and children [4,5]. The determination of plasma HIV-1 RNA VL is performed routinely in various clinical situations:

- Early pediatric diagnosis [6];
- Diagnosis and evaluation of primary infection in adults [7];
- Follow-up of asymptomatic untreated HIV-1-infected subjects;
- Virological monitoring before initiation (or switch) of highly active antiretroviral (ARV) therapy (HAART);
- Virological follow-up at 1–2 months after initiation (or switch) of HAART and then every 3–4 months [8].

The essential goal of HAART is the complete suppression of viral replication (<50 copies/ml).

In developing countries, where the vast majority (95%) of HIV-1-infected subjects lives, it is expected that by 2010, 7.1 million people will receive ARV treatment [9]. Although access to ARV treatment has significantly increased in the last few years, the availability of HIV-1 RNA VL measurements remains quite limited [10]. There are many barriers to implementation of HIV-1 RNA VL testing in resource-limited countries. These include lack of basic essential equipment, important human resources shortages, a limited number of training programs and inadequate logistical support [11,12]. Another critical point is represented by the cost of commercially available HIV-1 RNA assays (reagent costs >US$20) that are approved by the European Commission and the US FDA [13,14]. In addition, it is difficult to assess the usefulness of alternative tools (such as the ultrasensitive p24 Ag assay and the Cavidi ExaVir™ Load RT test) in these settings. Follow-up of patients treated with HAART using US p24 Ag levels showed different kinetics than those obtained with HIV-1 RNA concentrations [15]. The Cavidi assay is a time-consuming method that does not allow the scaling-up of HIV-1 RNA measurements necessary in developing countries [16]. Results expressed in $\log_{10}$ fg HIV-1 p24/ml plasma and in fg RT/ml plasma, respectively, are less familiar to clinicians although recent software changes are able to convert these figures to the more conventional copies/ml.

However, there are ongoing initiatives to address the lack of virological HIV-1 RNA monitoring in resource-constrained settings. The latest WHO guidelines for HIV treatment in resource-limited settings specifically recognize the urgent need for practical, reliable and affordable tools for measuring plasma HIV-1 RNA VL. In combination with adherence support, HIV-1 RNA VL testing strengthens treatment success. It is the only currently available tool that enables the early detection of therapeutic failure which also delays the emergence and spread of HIV-1 drug-resistant strains that jeopardize the success of ARV treatment programs. It may also prevent unnecessary switches to expensive second-line therapies. This benefit of low-cost HIV-1 RNA VL assays is thus potentially huge. In this context, an increasing number of research groups have recently developed and successfully evaluated distinct in-house low-cost real-time PCR assays allowing accurate HIV-1 RNA quantification in plasma.

This review focuses on these in-house assays. The principle of quantitative (q) real-time PCR (qPCR) technologies is described, including the numerous chemistries and open real-time PCR machines. We then describe in more detail the main currently available in-house plasma HIV-1 RNA real-time PCR tests, with a special focus on the Agence Nationale de Recherches sur le SIDA et les hépatites virales B et C (ANRS) test developed and evaluated by our groups. An important concern discussed in this review is related to HIV-1 genetic diversity. HIV-1 has been subdivided into three groups (M, N and O) [17]. Within the group M responsible for the global AIDS pandemic, nine subtypes (A, B, C, D, F, G, H, J and K) have been identified. Recombination between subtypes has generated many circulating recombinant forms (CRFs; at least 37 different CRFs have been characterized so far) [18] and more than 200 unique recombinant forms (URFs). The extremely high HIV-1 genetic drift, particularly in sub-Saharan Africa [19], impacts on the sensitivity of currently available nucleic acid-based assays and emphasizes the need for reliable assays which accurately quantify non-B HIV-1 subtypes/CRFs/URFs.

Real-time reverse transcription PCR

Principles

Real-time RT-qPCR has become the method of choice for RNA quantification. In the mid-1990s, two teams pioneered the analysis of PCR kinetics and demonstrated the possibility of simultaneous amplification and detection/quantification of PCR products, eliminating the need to run gels [20–22]. Kinetic RT-qPCR techniques continuously collect the fluorescence signal emitted by the amplification products at each PCR cycle (in real time; reviewed in depth in references [23–31]), as opposed to end point detection. They combine thermal cycling, fluorescence detection and application-specific software to measure the cycle-by-cycle accumulation of PCR products in a single-tube homogeneous reaction. The fluorescent signal is proportional to the amount of amplified products. They offer significant improvements to the quantification of the target because the fluorescence data are acquired during the exponential phase of the amplification where PCR conditions are optimal and reproducible, before possible saturation and/or inhibition of PCR when the reaction reaches the plateau phase. RT-qPCR assays show increased sensitivity over a wide dynamic range, as compared with traditional quantification techniques. Their homogeneous formats are also attractive alternatives to conventional PCR tests because they do not require any post-PCR processing, leading to a significant decrease of hands-on time and risks of contamination. They are therefore ideally suitable for high-throughput screening applications as amplicons remain sealed in a tube throughout the reaction, obviating the need for several separate work areas, as required by end-point PCR techniques. Furthermore, the use of probes labeled with distinct reporter dyes allows the quantification of multiple target genes in a single multiplex reaction [32].

RT-qPCR data are typically displayed as sigmoidal-shaped amplification plots, in which the fluorescence on the y-axis is plotted against the PCR cycle number on the x-axis (Figure 1). The

amplification plots can be subdivided into four major phases, the linear ground (or baseline) phase, early exponential phase, log-linear (also known as exponential) phase and plateau phase [28]. The linear ground phase is the background level in early cycles (usually measured between cycles three to 15) where there is no detectable increase in fluorescence due to amplification products. The average fluorescence value detected at baseline is calculated at this time and subtracted from the fluorescence value obtained from amplification products. At the early exponential phase, the amount of fluorescence has reached a threshold where it is significantly higher (usually ten-times the standard deviation of the baseline) than background levels. The cycle at which this occurs is known as a cycle threshold (C_t) or crossing point. The C_t serves as a tool for calculation of the starting template amount in each clinical sample; the more target in the starting material, the lower the C_t. During the log-linear phase, PCR reaches its optimal amplification period with the PCR approximately doubling after every cycle in ideal reaction conditions. Finally, the plateau phase is reached when one or more reaction components become limiting and the fluorescence intensity is no longer useful for data calculation.

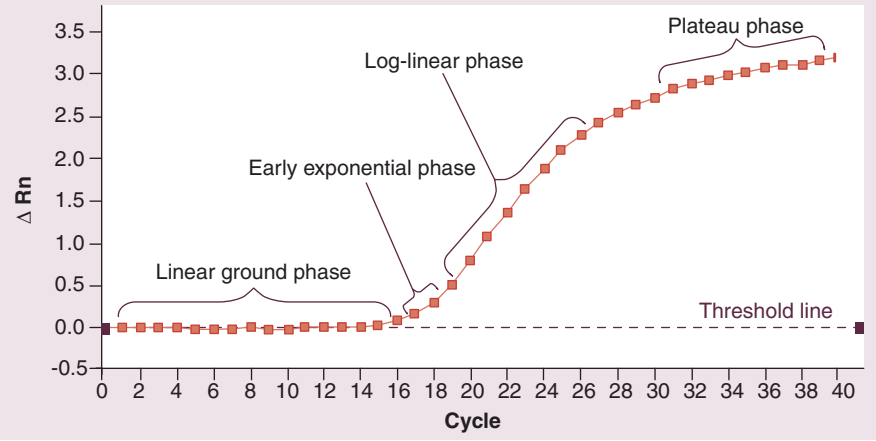

Figure 1. The four phases of the PCR amplification curve. The curve can be divided into four different phases: the linear ground, early exponential, log-linear and plateau phases. Rn is the intensity of fluorescent emission of the reporter dye divided by the intensity of fluorescent emission of the passive reference dye (ROX). ROX serves as an internal reference for normalization of the fluorescence. It allows correction of well-to-well variation due to pipetting inaccuracies and fluorescence fluctuations. ΔRn is calculated as the difference in Rn values of a sample and either no template control or background. ΔRn is an indicator of the magnitude of the signal generated by the PCR.

Real-time qPCR realizes the conversion of the continuous fluorescent signals detected into numerical value for each sample. Absolute quantification determining the absolute amount of target (expressed as copy number with $\log_{10}$ transformation) is the method used for viral quantification where C_t values are translated into quantitative results by constructing a standard curve. Relative quantification (determining the ratio between the amount of target and an endogenous reference molecule), used for gene expression analysis [33] as well as comparative quantification using mathematical models [34,35], are not discussed in this review.

Practically, absolute quantification uses a dilution series of at least five different concentrations of an external standard to generate a standard curve. C_t values of the different standard dilutions are plotted against the logarithm of input amount of standard material, allowing the calculation of the concentration of unknown clinical samples based on their C_t values (Figure 2). The fluorescent signal is directly proportional to the amount of accumulated PCR product, enabling sensitive and accurate quantification of target sequences. This method assumes an external standard and samples have approximately equal amplification efficiencies.

When generating a standard curve by using an external standard, the hallmarks of an optimized qPCR run are based on two capital parameters.

The slope obtained for the standard curve provides an indication of the efficiency of the real-time PCR [36,37]. Traditionally, the PCR efficiency is calculated with the following formula: efficiency = $[10^{(-1/\text{slope})}] - 1$. An optimal PCR efficiency (~ doubling of the amount of PCR products during each cycle) is indicated by a slope equal to -3.322. The absolute value of the slope is the same as the ideal spacing of the fluorescent signals obtained with a tenfold serial dilution of the standard. Slope values of standard curves must fall between -3.1 to -3.8. Standards which give a slope differing greatly from these values should be discarded. A slope of less than -3.322 (e.g., -3.8) is indicative of a reaction efficiency less than 1. Generally, most amplification reactions do not reach 100% efficiency due to experimental limitations (suboptimal concentrations of probe/primers, secondary structures in the probe, primers or amplicon, etc.). A slope of greater than -3.322 (e.g., -3.1) indicates a PCR efficiency that appears to be higher than 100%. Typically, it indicates miscalibrated pipettors or coamplification of nonspecific PCR products (such as primer dimers). The presence of inhibitors in the PCR reaction can also result in an apparent increase in efficiency. This is because samples with the highest concentration of template also have the highest level of inhibitors, which cause a delayed C_t.

The Pearson's correlation coefficient (r) or the coefficient of determination (R^2) provides information on the linearity of the standard curve. Its value must be greater than 0.990 (for r) or greater than 0.980 (for R^2). Nonlinear standard curves can indicate pipeting errors leading to imprecise dilutions.

For absolute quantification, another possibility is the use of an internal standard, added to each clinical specimen at a known copy number and carried through the specimen preparation and PCR amplification (Figure 3) [38]. This competitive duplex PCR compensates for effects of inhibition and controls the extraction, reverse transcription and amplification processes. However, as compared with an external standard, the use of an internal standard is more expensive because it must be added to each clinical

specimen. Furthermore, two distinct probes (one specific to the target and one specific to the internal standard) labeled with different fluorescent reporter dyes are required.

During each real-time PCR run, several types of controls must be included. A negative control (no template control [NTC]) checks the specificity of the technique and enables detection of contamination. A simple method to confirm the absence of PCR inhibitors is to dilute a sample in which inhibitors are suspected. Another method to detect PCR inhibitors is to co-amplify, in addition to the target gene, housekeeping genes (β-actin, glyceraldehyde-3-phosphate dehydrogenase, 18S ribosomal RNA, etc.) used as endogenous references [37]. PCR inhibitors are represented by reagents used during the extraction step (such as ethanol) and components present in biological samples (such as hemoglobin, heparin, IgG, urea, lipids and lactoferrin) [39–42].

Finally, critical factors for successful RT-qPCR are represented by:

- Template quantity, quality and optimal assay design;
- The reverse transcription step whose efficiency can be variable;
- Guidelines for data analysis (criterion of validity of the standard curve, necessity of controls, etc.) [30]. All these technical aspects are crucial to maximize reproducibility by quality-assessing every component of the RT-qPCR assay [31]. The quality of RNA templates, assay designs and protocols, data normalization and analysis constitute essential technical steps required to generate accurate and reproducible results.

Real-time chemistry

Numerous homogeneous fluorescent reporting chemistries have recently been developed and new innovative ones are still in development. They can be divided into two categories: DNA intercalating dyes (nonspecific chemistry) and dye-labeled sequence-specific probes or primers [28].

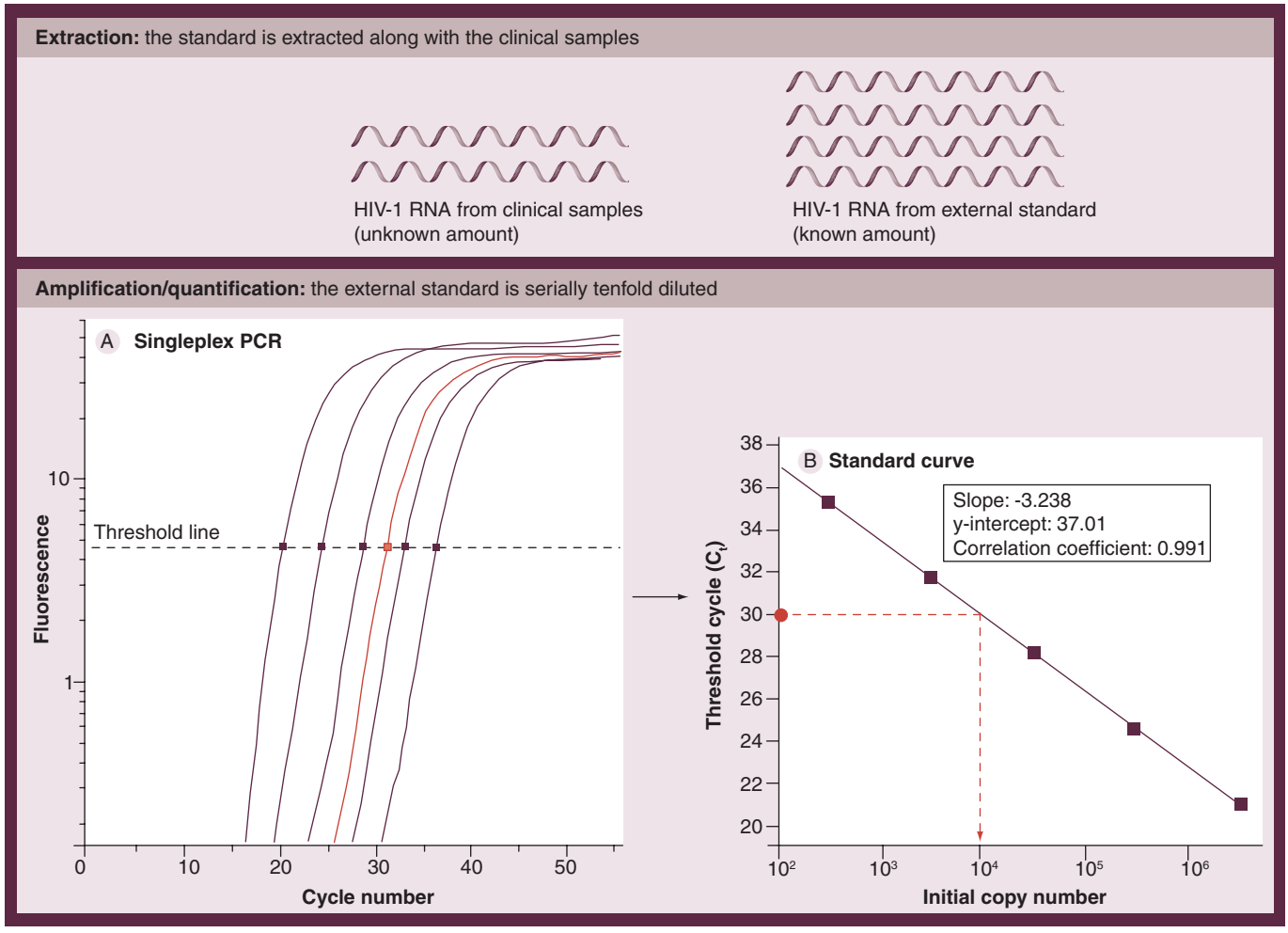

Figure 2. Quantitative real-time PCR using an external standard. (A) Amplification plots. The crossing points with the threshold line are the C_t values. In black, serial dilutions of five different HIV-1 RNA concentrations of an external standard. In red, sample with unknown HIV-1 RNA concentration. **(B)** Standard curve. The C_t values are plotted versus the $\log_{10}$ of the initial number of template copies in the standard samples. In red, the C_t value equal to 30 leads to a concentration of 4.0 $\log_{10}$/ml.
C_t: Cycle threshold.

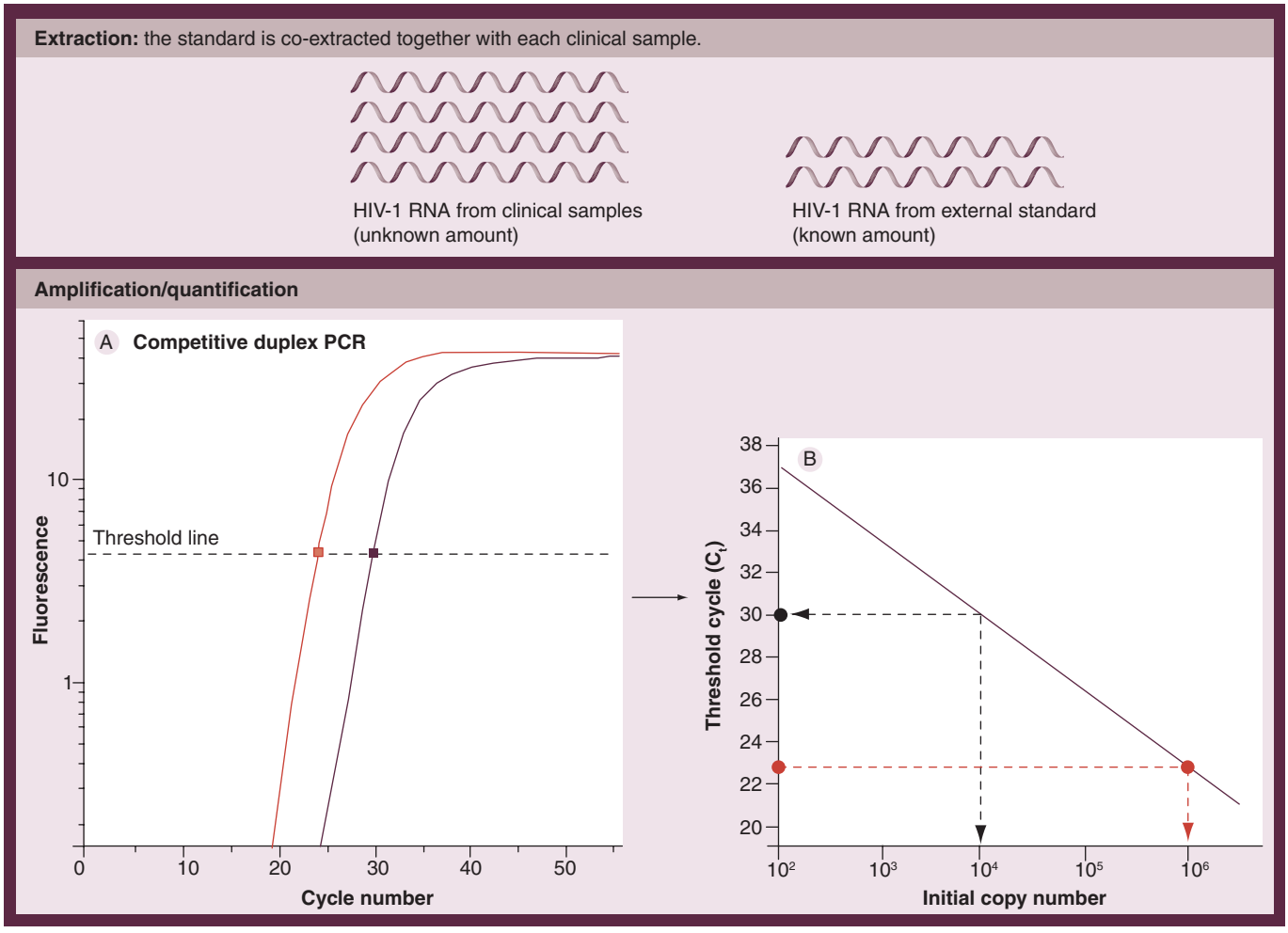

Figure 3. Quantitative real-time PCR using an internal standard. (A) Amplification plots. The crossing points with the threshold line are the C_t values. In black, plot amplification for the internal standard. In red, plot amplification for the clinical sample with unknown HIV-1 RNA concentration. **(B)** Calculation of the HIV-1 RNA concentration in the clinical sample. The C_t values are plotted versus the $\log_{10}$ of the initial number of template copies in the standard samples. In black, a C_t value equal to 30 corresponds to the 4.0 $\log_{10}$/ml concentration of the internal standard. In red, the C_t value equal to 23 leads to a concentration of 6.0 $\log_{10}$/ml in the clinical sample. C_t: Cycle threshold.

DNA intercalating dyes

DNA intercalating dyes such as asymmetric cyanines (SYBR® Green, BEBO, BOXTO, YOYO-1, etc.) emit enhanced fluorescence when they bind to any double-stranded (ds) DNA generated during the PCR reaction [43]. The fluorescence of the bound dye increases up to a 1000-fold as compared with the free dye and, therefore, is well suited for monitoring the product accumulation during PCR.

Their indiscriminate binding to any dsDNA molecules (including undesired primer dimers) can affect the accuracy of quantification. Accurate results will therefore be obtained only by using a really specific PCR (ability of the test to generate the only expected PCR product), which is determined entirely by its primers. RT-PCR product verification can be achieved by plotting fluorescence as a function of temperature to generate a DNA melt curve. If there are two or more peaks, it means that the amplification was not specific for a single DNA target. Furthermore, in HIV-1 infection where an extremely wide dynamic range of plasma HIV-1 RNA concentrations exists (from <50 HIV-1 RNA copies/ml of plasma among treated patients to several millions of copies/ml in untreated children), SYBR Green-based assays do not appear sensitive and reproducible enough. Indeed, sensitivity and reproducibility are influenced by statistic parameters such as Poisson's law and C_t values are less reproducible when working with very low copy numbers.

Fluorophore-labeled sequence-specific probes or primers

To date, there are two types of fluorescently labeled sequence-specific probes: linear versus structured probes (Figure 4). Linear probes are easier to design than structured probes. All probes use fluorescence resonance energy transfer (FRET) that corresponds to energy transfer from an excited molecule (donor) to a neighboring molecule (acceptor/quencher). FRET occurs when the donor and acceptor molecules are in close proximity (10–100 Å). All probes operate according to the same principle: a fluorescent signal is obtained when the probe hybridizes to its target sequence. These probes are labeled by fluorescent dyes showing distinct

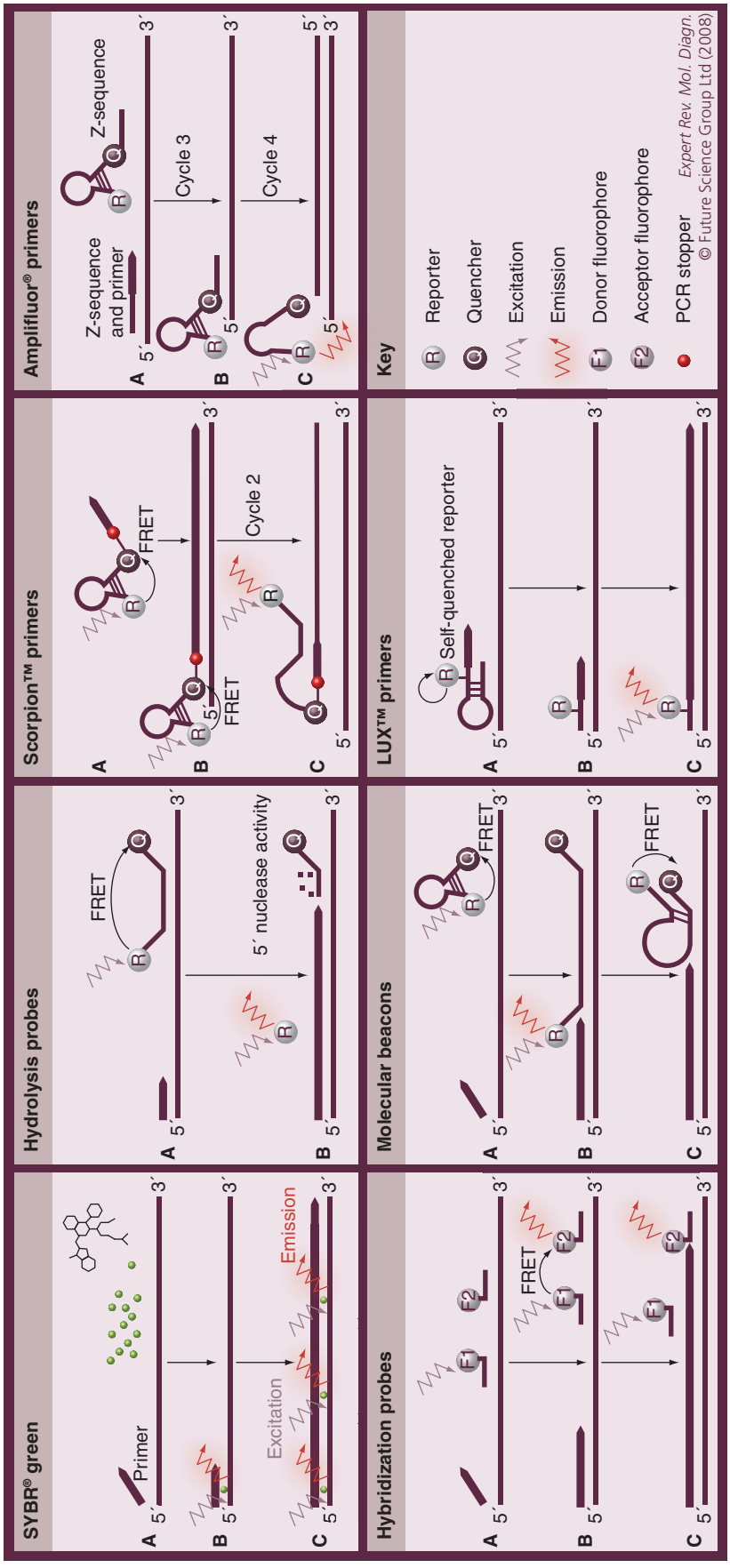

Figure 4. Quantitative real-time PCR detection chemistries.
FRET: Fluorescent resonance energy transfer.

excitation and emission spectra. The wide variety of fluorescent dyes available makes real-time multiplex PCR possible (Cy3, Cy3.5, Cy5, Cy5.5, Dabcyl, FAM, HEX, JOE, NED, Rhodamine 6G, Rhodamine Green, Rhodamine Red, ROX, TAMRA, TET, Texas Red, VIC, etc.).

Linear probes

Hybridization probes

Hybridization assays employ four or three oligonucleotides. The 4-oligo method uses two sequence-specific unlabeled primers and two sequence-specific fluorescently labeled probes, the upstream probe carrying at its 3′-end a donor fluorophore and the downstream probe an acceptor fluorophore at its 5′-end [44]. After denaturation, these two probes hybridize to their target sequence in a head-to-tail fashion. This brings the two dyes in close proximity (1–5 nucleotides), allowing an increase in FRET from the donor to the acceptor fluorophore. Therefore, fluorescence is detected during the annealing phase of PCR and is proportional to the amount of PCR product synthesized during the PCR reaction. The 3-oligo method is similar but the 5′ primer is labeled with a donor fluorophore at its 3′-end, taking the place of one probe.

Hydrolysis probes

Hydrolysis probe assays (also called TaqMan® assays) use a TaqMan probe, in addition to two sequence-specific primers. A TaqMan probe contains a reporter dye at its 5′-end and a second fluorescent quencher dye located at its 3′-end. When the probe is intact, it allows the quencher to reduce the reporter fluorescence intensity by FRET. During the combined annealing/extension phase of PCR, the TaqMan assay utilizes the dsDNA specific 5′ 3′ exonuclease activity of the Taq DNA polymerase to hydrolyze the probe bound to its target sequence [45,46]. This separates the reporter and quencher dyes and releases quenching of reporter fluorescence emission that is proportional to the amount of accumulated PCR product. Most hydrolysis probes have a melting temperature (Tm) of around 68–70°C, 8–10°C higher than Tm of the primers pair. This ensures that the probe is bound to its target before the primer extension step.

Typically, the length of hydrolysis probes is around 25–35 nucleotides. However, minor groove binders (MGBs; such as 1,2-dihydro-(3H)-pyrrolo[3,2e]indole-7-carboxylate [$CDPI_3$] can be added to the 3′-end of these probes [47]. Consequently, MGBs lead to increased probe stability and allow the design of shorter probes (12–20 nucleotides) with the same Tm. They are appropriate for the PCR amplification of sequences that possess a high degree of variability (such as HIV-1).

Structured probes

Molecular beacons

In addition to two sequence-specific primers, molecular beacon assays use a DNA hybridization probe containing a fluorophore reporter attached to its 5′-end and a quencher dye located at its 3′-end [48]. These probes form a hairpin structure (stem-and-loop structures) in solution causing a reduction in fluorescence emission via FRET. The loop part of the molecule is designed to anneal specifically to a 15–30-bp region of the target sequence. The stem (5–8 bp in length) formed by two inverted repeats serves to bring reporter and quencher molecules in close proximity. When the molecular beacon anneals to its complementary target sequence, it forces the stem apart and results in the formation of a probe/target hybrid more stable than the stem. Consequently, the reporter and quencher are separated, generating a fluorescent signal in the annealing step that is proportional to PCR product. Finally, molecular beacons are displaced from the target during the elongation step and return to the hairpin structure.

Scorpion™ primers

Scorpion™ primer assays use two primers. One of these primers is a bifunctional molecule consisting of a fluorophore reporter molecule on the 5′-end, followed by a stem-and-loop structure (complementary to target sequence), quencher dye, PCR stopper (nonamplifiable monomer to prevent amplification of stem–loop sequence via PCR) and a PCR primer on the 3′-end [49]. In the absence of the target, scorpions constitute a nonfluorescent complex (the quencher absorbs the fluorescence emitted by the fluorophore). During annealing, the hairpin primer binds to the template and is then extended. During subsequent denaturation, it opens the hairpin loop, which binds to its internal target sequence, whereas the reporter separates from the quencher and the fluorophore leading to increased fluorescence. The resulting fluorescence signal is proportional to the amount of amplified product in the sample.

LUX™ primers

Light upon extension (LUX™) primer assays use two primers, one of which is a self-quenched single-fluorophore-labeled primer (20–30 bp in length) [50]. The fluorogenic primer is designed to form a hairpin structure which quenches the fluorophore completely (this chemistry does not require a quencher dye). The other primer is unlabeled. When the primer is incorporated during the annealing phase of the PCR reaction, the fluorophore is no longer quenched, allowing an increase in fluorescent signal.

Amplifluor® primers

This chemistry (also known as Sunrise and UniPrimer™) uses three primers, including two target-specific primers and one universal primer (UniPrimer), designed to form hairpin structures [51]. The first target-specific primer contains a 5′-extension sequence called the Z-sequence that is also found at the 3′-end of the UniPrimer. When unbound, the hairpin is intact, causing reporter quenching via FRET. When bound, the quencher and reporter are apart allowing reporter emission.

Instrumentation

In parallel with the development of numerous chemistries, real-time PCR instrumentation technology has also advanced very rapidly in the last 5 years. Real-time PCR machines consist of specialized thermal cyclers equipped with fluorescence detection modules that monitor the fluorescence as amplification occurs. More precisely, they include:

- A thermal cycler (heating block or heated air or a combination of the two);
- An excitation light source to excite the fluorophores (laser or halogen lamps or light-emitting diode);
- A photodetector (charge-coupled device cameras, photomultiplier tubes, or other types of photodetectors) to register photon emissions that are proportional to the concentration of the amplification product;
- A data-collection and data-analysis software.

At present, there are many integrated, versatile real-time PCR platforms, which makes choosing a suitable instrument somewhat difficult. The main differences between them are the excitation and emission wavelengths that are available, speed and the number of reactions that can be run in parallel. Reaction containers differ as well. The most popular are 96-well microtiter plates which are widely available. There are also 384-well plate instruments available on the market. Some instruments use other containers such as glass capillaries and plastic tubes.

Current trends in real-time PCR devices include the following (for further detail, see [201] or the respective manufacturer's website for the most up-to-date model information):

- Ultracompact footprint (miniaturization) fits any laboratory setting;
- The use of long-life light-emitting diode-based optical systems require very minimal maintenance, which is of primary importance in resource-limited countries;
- New machines are designed for high throughput and use smaller volumes of reagent and sample;
- PCR reactions can be performed much faster (<30 min by using the fastest machines);
- More user-friendly accompanying software for data acquisition and analysis has significantly improved for most instruments;

- The cost of instruments varies quite substantially, depending mainly on throughput and the number of colors they can handle; unless a sophisticated machine is required, accurate real-time PCR reactions can be performed by using the cheapest instruments (starting price: ~ US$20,000). For indication, the price of the Nuclisens EasyQ® Analyzer as a point of reference for the corresponding commercial bioMérieux assay is approximately US$80,000 in Burkina Faso.

Finally, one of the main advantages of these open machines is represented by their high flexibility allowing their use for quantification of many viruses, most of which are prevalent in developing countries: plasma HIV-1 RNA from strains belonging to group M or group O, HIV-1 proviral DNA from PBMC [52], HIV-2 [52,53], HBV [54], HCV [55], HSV-2 [56], HTLV-1 [57], CMV [58], EBV [59], HPV [60] and so forth.

In-house plasma HIV-1 RNA RT-qPCR assays: currently available tests

To our knowledge, there are presently at least 15 distinct in-house HIV-1 RNA RT-qPCR assays designed, developed and described in the literature [61–78]. As summarized in Table 1, they differ in chemistry as well as in choice of HIV-1 target sequence. They also utilize distinct extraction methods and different standards for quantification. At present, these assays are not approved by the European Commission or US FDA and are utilized for research only.

Of these, four tests appear particularly attractive in terms of test design and have recently been large-scale evaluated by testing many genetically diverse (notably non-B) HIV-1 strains.

In South Africa, Reckwiashwili and coworkers have developed and evaluated a LUX primer-based assay, with or without a noncompetitive internal control (IC) [68,69]. Viral RNA was extracted from 200 µl of plasma, by using automated (MagNA Pure LC instrument, Roche) or manual (QIAamp® Viral RNA Mini Kit, Qiagen) methods. The external standard used for quantification constituted an *in vitro* transcribed RNA molecule, which incorporated 128 bp of a conserved region of the HIV-1 *gag* gene. Runs were performed on the LightCycler® instrument version 2 (Roche).

The sensitivity threshold of this technique was 400 HIV-1 RNA copies/ml. There was no PCR inhibition when using the IC. Good specificity of the LUX assay was demonstrated on 50 seronegative patients (no false-positive results were obtained). By testing 142 HIV-1 positive patients predominantly infected with HIV-1 subtype C, good agreement was obtained between the LUX assay and the COBAS Amplicor HIV-1 Monitor™ test. The mean difference between the HIV-1 RNA VL values of the LUX assay minus the COBAS Amplicor values was +0.03 $\log_{10}$/ml. A total of 18 samples (out of 102) showed discrepant results with a difference of more than 1.0 $\log_{10}$/ml (five were lower by the LUX assay and 13 were lower by the Roche technique). In a cohort of 55 patients treated with HAART, the LUX assay showed similar declines in HIV-1 RNA VL as compared with the COBAS Amplicor test. Given that subtype C largely predominates in

Table 1. In-house quantitative plasma HIV-1 RNA real-time reverse transcription PCR assays*.

Chemistry	Country	Amplified gene	Standard‡	Sensitivity threshold	Ref.
SYBR® Green	Italy	*gag*	bDNA standard	50 cp/ml	[61]
	Italy	*gag*	bDNA standard	500 cp/ml	[62]
Hydrolysis probes	USA	*gag*	pQP1 plasmid	1 cp/ml	[63]
	USA	*gag*	pWISP98–85 plasmid	80 cp/reaction	[64]
	Thailand	*gag*	Culture supernatant	125 cp/ml	[65]
	China	*gag*	Armored RNA	173 IU/ml	[66]
LUX™ primers	Thailand	*gag*	Synthetic HIV-1 RNA	10 cp/ml	[67]
	South Africa	*gag*	Synthetic HIV-1 RNA	400 cp/ml	[68,69]
Scorpions™	USA	*Pol*	SG3 plasmid	1 cp/ml	[70]
Hydrolysis probes	Germany	*Pol-IN*	Accurun® standard	281 IU/ml	[71]
Molecular beacons	Netherlands	LTR	Not mentioned	500 cp/ml	[72]
Hydrolysis probes	UK	LTR	WHO international standard (97/656)	680 IU/ml	[73]
	Germany	LTR	pCR 2.1 plasmid	31.9 IU/ml	[74]
	Ivory Coast	LTR	Culture supernatant	300 cp/ml	[75]
	France	LTR	Culture supernatant	300 cp/ml	[76]
	Argentina	LTR	Culture supernatant	300 cp/ml	[77]
	Kenya	LTR	Optiquant™ standard	300 cp/ml	[78]

*Classification according to the amplified target gene and the chemistries used (list established in September 2008).
‡All are external standards except dual-specific armored RNA described by Huang *et al.* [66] and used as internal control.
bDNA: Branched-chain DNA; cp: Copies; LUX™: Light upon extension.

South Africa, this test should be further evaluated on other HIV-1 clades. According to preliminary cost estimation, the LUX assay was approximately two- to three-times less expensive (US$22.5 per test) than the approved commercial assays currently used for the public sector of South Africa (US$45–75).

Muller and colleagues in Germany have described an RT-qPCR assay targeting a conserved region (160 bp) within the *Pol*-IN gene [71]. This test was based on TaqMan chemistry and enabled the simultaneous quantification of HIV-1 group M, O and N. RNA was isolated from 140 µl of plasma using the QIAamp Viral RNA Mini Kit. As an IC reaction, endogenous glyceraldehyde-3-phosphate dehydrogenase transcripts were detected in a duplex configuration. The test was performed utilizing the ABI Prism® 7700 Sequence Detection System (Applied Biosystems, CA, USA). The external standard curve was generated using the FDA-approved Accurun® 315 HIV-1 RNA-positive controls series 500 (BBI). A calibration against the WHO International HIV-1 RNA standard (NIBSC N° 97/656) was also performed [79]. Data from quantitative assays showed that 1 IU was equivalent to 0.62 HIV-1 RNA copies/ml [80].

The detection limit was calculated as 281 IU/ml of HIV-1 RNA. The rate of PCR inhibition was found to be as low as 0.37%. Based on the testing of 1206 confirmed HIV-1 RNA-negative blood donor samples, assay specificity was found to be 100%. By testing 46 HIV-1 subtype reference samples from five distinct panels (representing 36 group M isolates, nine group O isolates and one group N strain), quantitative results obtained with the in-house assay and the Abbott RealTime HIV-1 technique were highly correlated ($R = 0.861$; $p < 0.001$). The agreement of results was lower within group O when compared with group M. In addition, 28 clinical specimens were also found to be positive for HIV-1 RNA by both the Abbott and the in-house assays. HIV-1 RNA results were highly correlated ($R = 0.940$; $p < 0.001$) with a mean difference of +0.02 $\log_{10}$/ml.

In Germany, Drosten *et al.* have also reported an ultrasensitive RT-qPCR technique targeting the LTR gene and using hydrolysis probes [74]. Two probes were used in order to accurately quantify HIV-1 strains belonging to groups M and O. An ultrasensitive extraction of HIV-1 RNA was performed using 1.2 ml of plasma (centrifugation for 1 h at 21,000 g). Virus pellets were then used for HIV-1 RNA extraction by the QIAamp Viral RNA Mini Kit. A competitive IC (called srLTRic) was used for quantification, employing an 80-copy reaction as a working concentration. The ABI 7000 or ABI 7700 real-time PCR machines were used for this analysis. Additionally, a calibration against the WHO International HIV-1 RNA standard was performed.

The detection limit was 31.9 IU of HIV-1 RNA/ml of plasma. The IC showed inhibition in 3.7% of samples (n = 454; 40 different runs). By testing 33 HIV-1 subtype reference samples (30 group M, three group O and one group N), the mean difference of HIV-1 RNA VL values obtained with the LTR assay and the Roche Amplicor® test was +0.31 $\log_{10}$/ml. Comparative testing between the LTR assay and commercially approved techniques was performed on patient samples from Brazil (n = 1186), South Africa (n = 130), India (n = 44) and Germany (n = 127). HIV-1 RNA values obtained with the in-house real-time PCR assay were highly correlated with values obtained with Amplicor, Nuclisens or Versant® techniques ($R = 0.9$; $p < 0.001$ for all). Drosten and coworkers estimated the price of their test as being less than US$10 for reagents per sample. Large-scale feasibility studies of this technique are ongoing in Brazil and South Africa [81].

Finally, our groups have developed and evaluated, under the auspices of the ANRS, a RT-qPCR assay targeting a conserved region within the LTR gene. Viral RNA extraction was performed from 200 µl of plasma, by using a manual method (QIAamp Viral RNA Mini Kit, Qiagen). Runs were performed by using the ABI 7000 real-time PCR instrumentation.

The first generation (G1) of this LTR-based assay was transferred to Abidjan (Ivory Coast) and assessed for 806 plasma samples collected within four ANRS research programs [75]. The external standard was a culture supernatant of an HIV-1 subtype B strain quantified by the Versant® bDNA version 3.0 (kindly provided by the Virology Laboratory at Necker Hospital, Paris, France). By using 200 µl of plasma, the threshold of the assay was 300 copies/ml (2.48 $\log_{10}$/ml). The HIV-1 RNA values obtained with this real-time PCR test were highly correlated with those obtained by the Bayer Versant kit ($n = 320$; $R = 0.901$; $p < 0.001$) and the Roche Monitor test ($n = 97$; $R = 0.856$; $p < 0.001$) and homogeneously distributed according to HIV-1 genotypes (mostly CRF02_AG). For early diagnosis of pediatric HIV-1 infection, sensitivity (n = 57) and specificity (n = 210) of this LTR-based assay were both 100%, when compared with the Versant results. Following initiation of HAART, the kinetics of HIV-1 RNA levels were highly comparable, with a similar proportion of adults (n = 46) and children (n = 36) below the detection limit during follow-up with our LTR assay and the Versant technique. The test was performed at US$12.5 per sample (including reagents and consumables), instead of more than US$100 per test for the bDNA technique. The G1 assay was subsequently used in many ANRS programs conducted in Republic of Côte d'Ivoire [82–89] and Burkina Faso [90].

However, some HIV-1 strains from Cameroon, Thailand and France were not detected by the G1 assay. Upon analysis of the LTR sequences, our teams decided to use a reduced-length MGB probe and to shorten the forward primer sequences. Thus, a second generation assay (G2) was developed at the Virology Laboratory at Necker Hospital and evaluated as follows on 898 specimens positive for HIV-1 RNA by commercial assays (Amplicor or Versant test) [76]:

- 518 samples containing HIV-1 of known subtypes, including 88 from two subtype panels and 430 harboring B (n = 266) and non-B (n = 164) group M HIV-1 subtypes from clinical specimens;
- 380 samples from ten different countries (Argentina, Cambodia, Cameroon, Central African Republic, France, Republic of Côte d'Ivoire, Madagascar, Morocco, Thailand and Zimbabwe).

Overall, of 430 specimens quantified by the Amplicor test, 427 were also quantifiable by the G2 real-time PCR assay (sensitivity: 99.3%). The differences between the values obtained

using the G2 real-time PCR assay and the Amplicor test were +0.10 and +0.23 $\log_{10}$/ml, for B and non-B subtypes, respectively. HIV-1 RNA values obtained by G2 real-time PCR were highly correlated with those obtained by the Amplicor test for B and non-B subtypes (R = 0.892 and 0.892, respectively) and for samples from diverse countries (R = 0.867 and 0.893 for G2 test vs Amplicor technique and G2 test vs Versant assay, respectively). However, for 35% of specimens harboring non-B subtypes, values produced by the real-time PCR assay and the Amplicor test differed by at least 0.51 $\log_{10}$/ml (30% were lower by the Amplicor test whereas 5% were lower by the G2 test). Discrepant results were also obtained among samples harboring B subtypes (14% had lower values by the Roche test vs 7% by the G2 test).

This G2 ANRS test has now been commercialized (Generic HIV Viral Load®) and is currently distributed by the manufacturer Biocentric (Bandol, France). The standard curve is established by using the commercial Optiquant® quantification panel HIV-1 RNA N°6 (Acrometrix Inc., CA, USA) which is calibrated against the WHO International HIV-1 RNA standard. After ultracentrifugation of 0.5 ml plasma, the lower detection limit of the G2 test is approximately 50 copies/ml [Unpublished data]. The first evaluation of the commercial assay was performed in Mombasa, Kenya [78], by comparing it with the reverse transcriptase activity assay (ExaVir™ Load v2.0, Cavidi Tech, Uppsala, Sweden) and the ultrasensitive HIV-1 Amplicor Monitor assay (Roche). In this study, 170 samples were analyzed (20 from HIV-1 seronegative and 150 from HIV-1 seropositive subjects of whom 50 received HAART). The Generic HIV Viral Load test demonstrated a sensitivity of 100% and a specificity of 90%. Linear regression analyses revealed good correlation between the Roche assay and the Generic HIV Viral Load test (R = 0.935; $p < 0.001$). Although a high genotypic HIV-1 diversity was observed in this East African area (presence of subtypes A, C, D, G, CRF16_AD and URF), no impact of HIV-1 subtype on the performance of the Generic HIV Viral Load could be detected in this study, in comparison with the Cavidi assay, which is supposed to be independent of the HIV-1 subtypes/CRFs/URFs. The cost per Generic HIV Viral Load test was approximately US$20 but could be reduced to US$10 by reducing the recommended reagent volume. It is currently used in sub-Saharan Africa (Burkina Faso, Ivory Coast, Cameroon, Kenya, South Africa) and Asia (Cambodia [91,92] and Vietnam).

Expert commentary

Overall, the analytical and clinical performances of the four in-house plasma HIV-1 RNA RT-qPCR assays described herein appeared at least as good as those observed with commercial approved assays. The sensitivity thresholds ranged from 31.9 IU/ml (US protocol carried out by Drosten *et al.*) to 400 copies/ml. The rates of PCR inhibition were extremely low (from 0 to 3.7%). In-house RT-qPCR assays and commercially approved tests gave highly correlated and concordant HIV-1 RNA quantitative results. The mean differences between the assays were largely under 0.5 $\log_{10}$/ml, which is clinically acceptable. In-house assays appeared as suitable as approved assays for early diagnosis of pediatric HIV-1 infection and monitoring HAART efficiency. Contrary to approved HIV-1 RNA assays utilizing expensive closed platforms, these tests are performed by using open real-time PCR machines with affordable starting prices. In-house HIV-1 RNA RT-qPCR tests appeared much cheaper than comparable approved assays (~US$10 for reagents per sample and <US$20 including the service license payable to Roche). Indeed, Roche Diagnostics Corporation required that PCR royalties must be paid to them if testing is performed for commercial purposes [93].

As with any molecular virology technique (including licensed HIV-1 RNA assays), the disadvantages of in-house HIV-1 RNA RT-qPCR assays are that they require skilled technicians, solid infrastructure (air conditioned, need for refrigeration, electricity supply, etc.) and relatively expensive instrumentation (even if the prices of open real-time machines have significantly reduced, as previously indicated). Laboratories must participate in external quality assurance (EQA) programs such as ANRS (ten samples per year), Quality Control for Molecular Diagnostics (QCMD; ten samples per year), Virology Quality Assurance (20 samples every 8 months) or UK National External Quality Assessment Service (two samples per year) programs.

Given the high HIV-1 genetic diversity, it is crucial to conduct large-scale and continuous evaluations of specimens harboring distinct B and non-B subtypes and of different panels from diverse geographic origins. In this respect, work performed by Drosten and colleagues and by our groups showed that these in-house RT-qPCR assays can be considered as accurate methods for quantifying most clinically relevant HIV-1 strains (B and non-B) circulating worldwide. It must be pointed out that the different in-house RT-qPCR assays described in this review targeted different conserved regions within distinct genes (*gag* for the LUX assay designed by Reckwiashwili *et al.*, *Pol*-IN in the test developed by Muller and colleagues and LTR for the assay developed by Drosten and coworkers and by our teams). There are two identical LTR sequences within the HIV-1 genome, each comprising a U3, R and U5 region. They are essential for viral transcription, integration and gene expression [94]. These functional genes are poorly recombinant and contain highly conserved regions suitable for an accurate HIV-1 RNA quantification [95,96].

Other important issues are the feasibility and standardization of in-house plasma HIV-1 RNA RT-qPCR assays in resource-constrained settings. As mentioned earlier, the G2 ANRS assay is now currently available as a ready-made kit for distribution. The advantages of this commercial format are numerous.

The test is now fully standardized. The same extraction procedure (QIAamp Viral RNA Mini Kit, Qiagen, Courtaboeuf, France), the same enzyme, PCR buffer and RT mix (SuperScript™ III Platinum®, One-Step qRT-PCR kit, Invitrogen Corp., Carlsbad, CA, USA), the same primers and MGB probe (Applied Biosystems), the same chemistry (hydrolysis probe), the same thermocycling profile and the same quantification standard (OptiQuant® quantification panel HIV-1

RNA N°6, Acrometrix, Benicia, CA, USA) are included in the kit. Consequently, it maximizes reproducibility by using quality-assessed reagents. The manufacturer's instructions included in the kit define standard operating procedures and common guidelines for data analysis. A reproducibly low positive (target value: 10,000 IU/ml) control has also been included in the kit and its value, assessed during each run, must be between 3.7 and 4.3 $\log_{10}$/ml. Finally, the interlaboratory comparability of quantitative HIV-1 RNA results obtained through the ANRS EQA program by different laboratories using this kit has significantly increased (Unpublished data).

Each batch of this kit is evaluated in the virology laboratory at Necker Hospital (Paris, France). If results of such analytical validation are found to be satisfactory, the batch is released for distribution; if not, it is discarded.

Instead of purchasing separate reagents from distinct manufacturers, the availability of the Generic HIV Viral Load kit eased the purchase process from developing countries. This test is easy to use because all reagents are ready made.

Finally, we are of the opinion that this assay is not an in-house HIV-1 RNA RT-qPCR assay anymore, but a fully-fledged HIV-1 RNA VL assay. A preapproval of the Generic HIV Viral Load assay is being examined by WHO.

Five-year view

In developing countries where treatment and prevention of mother-to-child transmission programs are moving on towards universal access by 2010, expanded access to plasma HIV-1 RNA VL measurements is definitively required for monitoring efficacy of lifelong ARV treatment and providing early infant HIV-1 diagnosis. In this scaling-up, it should be understood by the scientific community and governmental healthcare providers that such technology, whether in-house or licensed, requires a certain level of infrastructure and human resources. New innovative chemistries and versatile real-time PCR machines (using microarrays and nanotechnologies) may become available in future in African and Asian reference laboratories [97].

As previously described by our teams, it is important to understand for what purpose HIV-1 RNA VL measurements are needed [13]. It could be for qualitative measurement for pediatric diagnosis performed on plasma or dried blood spots (DBS) or quantitative measurement in a variety of settings with different requirements. For example, a semiquantitative model would be more suitable for large-scale government ARV rollout programs where automation, throughput and cost would be more important factors to consider than very accurate low-limit detection. According to a public health approach, a low-limit detection of 10,000 copies/ml has been suggested to be acceptable in such a setting. However, this 10,000 copies/ml threshold remains questionable at an individual level where a lower limit of detection of less than 50 copies/ml in plasma is quite desirable.

Although manual extraction methods are commonly utilized in research laboratories, many automated extraction platforms exist that can be used for in-house tests to improve turnaround time and decrease labor, where large numbers of samples have to be processed. Examples of automated extractors include X-tractor Gene™ (Corbett Life Science) and QIAcube (Qiagen).

Analysis of DBS collected on filter paper has become increasingly popular during recent years as an attractive alternative to plasma and is likely to remain so for a number of reasons, especially in developing countries. In infants, it is much easier to obtain blood by heel, finger or toe prick than by venipuncture. In some countries, such as South Africa, venous blood may only be collected by registered nursing practitioners or phlebotomists, whereas DBS may be taken by trained volunteers. DBS collection is much easier in remote rural areas and does not require centrifugation or freezing and is also easier to store and to transport to reference laboratories located in main cities. DBS is also useful in adults for monitoring HIV-1 resistance genotyping [98] and response to ARV treatment [99], which would facilitate sample collection in models of home-based care and treatment delivery currently under evaluation. Thus, DBS is an important tool to allow expanded access to HIV-1 RNA VL measurements. In the Africa Center Virology Laboratory (Durban, South Africa), we have obtained very promising preliminary results when comparing 69 HIV-1 RNA results obtained on DBS versus plasma specimens by using the HIV Generic Charge Virale kit (Unpublished data). The DBS sensitivity was under 90% (as compared with plasma samples). Ou and coworkers in Cameroon and Uganda have successfully used this approach for early infant HIV-1 diagnosis by detecting total nucleic acid by an in-house real-time PCR [100].

Another sample type where an open system can be used is breast milk. Recently, the new WHO Consensus Statement on HIV and Infant Feeding promotes exclusive breast feeding for the first 6 months of life [101]. Therefore, much research is needed and ongoing to determine HIV-1 RNA VL in breast milk in order to identify factors associated with postnatal mother-to-child transmission.

One key issue remains the impact of HIV-1 genetic diversity on the accuracy of HIV-1 RNA VL results. Recent studies reported an underquantification (>0.5 $\log_{10}$/ml) of some non-B (and also B) HIV-1 strains by some approved assays [102,103]. Because of the unpredictability and the extent of ongoing HIV-1 genetic drift that occurs across the entire retroviral genome within a same subtype or CRF, it is crucial to continuously evaluate the accuracy of HIV-1 RNA quantification obtained with approved assays, in comparison with in-house tests. The HIV-1 virological imbroglio highlights that there is no perfect technique for HIV-1 RNA quantification. Any test may underquantify or fail to detect one given subtype and/or complex recombinant form. Facing this challenge, in-house HIV-1 RNA RT-qPCR assays can be considered and used as academic reference tools in order to evaluate (and improve if necessary) the analytical performance of approved HIV-1 RNA assays. They also allow an independent thought from established manufacturers' assays in which mismatches cannot be identified since the primer and probe sequences are not known by customers. By contrast, in the case of HIV-1 strains underquantified or missed by any particular in-house assay, the target region sequences (in *gag* or *pol*-IN

or LTR) can be analyzed by the researcher-users in order to identify mismatches and then modify the probe and/or primers sequences accordingly.

Finally, the goal of having an open versatile real-time PCR research tool is to be flexible and adaptable to analyze a variety of viruses (not only HIV-1 but also HIV-2, HTLV-1, HBV, HCV, CMV, EBV, etc.) on a variety on samples (not only plasma but also DBS, breast milk, cerebrospinal fluid, cervicovaginal secretions, sperm, etc.) using any desired extraction method (not only manual but also automated) customized to the type of sample and any type of chemistry.

Financial & competing interests disclosure

François Rouet is supported by the French Ministry of Foreign Affairs. The authors have neither financial interests in nor affiliation with the Biocentric manufacturer.

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this review manuscript.

Key issues

- Kinetic reverse transcriptse (RT)-quantitative (q)PCR techniques continuously monitor the fluorescence emitted by the amplification products at each PCR cycle, as opposed to end point detection. Their homogeneous format is attractive because it eliminates the need for post-PCR processing.
- These assays can use different attractive chemistries (SYBR® Green and specific chemistries such as hybridization probes, hydrolysis probes, molecular beacons, Scorpions™, Sunrise™ primers and LUX™ primers). They are performed on compact versatile real-time PCR platforms with affordable starting prices and user-friendly software. These open machines allow the quantification of many viruses which can be prevalent in developing countries, such as HIV-1.
- To date, at least 15 distinct in-house RT-qPCR assays designed for performing plasma HIV-1 RNA quantification have been developed. They differ in their chemistry as well as the choice of the HIV-1 target sequence (*gag* or *Pol-IN* or LTR).
- Our teams have developed and evaluated, under the auspices of the Agence Nationale de Recherches sur le SIDA et les hépatites virales B et C (ANRS), one of these assays. It targets a conserved region within the *LTR* gene.
- The first generation (G1) of this test has been successfully evaluated in Abidjan (Ivory Coast) for performing the early diagnosis of pediatric HIV-1 infection and monitoring HAART efficacy among treated patients (adults and children).
- Given that few HIV-1 strains were underquantified with the G1 test, a second generation (G2) test was developed by using a shortened minor groove binder (MGB) probe and by modifying the primer 5′-sequence. The G2 test was successfully evaluated on 898 specimens harboring distinct B and non-B subtypes and from panels and samples from ten distinct countries (Argentina, Cambodia, Cameroon, Central African Republic, France, Ivory Coast, Madagascar, Morocco, Thailand and Zimbabwe).
- The G2 ANRS test has been commercialized (Generic HIV Viral Load®, Biocentric, Bandol, France). Its first evaluation has been successfully carried out in Mombasa (Kenya). This marketing step has promoted the assay standardization and facilitated its purchase from developing countries.
- Overall, the analytical and clinical performances of in-house plasma HIV-1 RNA RT-qPCR assays appear at least as good as those observed with commercially approved assays. Their feasibility is also good. They can be performed by using dried blood spots.
- Due to the continuously increasing genetic diversity of HIV-1 viruses, there is no perfect technique for plasma HIV-1 RNA quantification. In-house HIV-1 RNA RT-qPCR assays can be considered as academic reference tools in order to evaluate (and improve if necessary) the analytical performance of commercial HIV-1 RNA assays approved by the European Commission or US FDA. It is of crucial importance in sub-Saharan Africa where complex recombinant HIV-1 strains emerge continuously and spread rapidly.

References

Papers of special note have been highlighted as:
• of interest

1 Ho DD, Neumann AU, Perelson AS *et al.* Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* 373, 123–126 (1995).

2 Peter JB, Sevall-Sanders J. Molecular-based methods for quantifying HIV viral load. *AIDS Patient Care STDS* 18, 75–79 (2004).

3 Wittek M, Stürmer M, Doerr HW, Berger A. Molecular assays for monitoring HIV infection and antiretroviral therapy. *Expert Rev. Mol. Diagn.* 7, 237–246 (2007).

▶4 Mellors JW, Margolick JB, Phair JP *et al.* Prognostic value of HIV-1 RNA, CD4 cell count, and CD4 cell count slope for progression to AIDS and death in untreated HIV-1 infection. *JAMA* 297(21), 2349–2350 (2007).

▶5 Shearer WT, Quinn TC, LaRussa P *et al.* Viral load and disease progression in infants infected with human immunodeficiency virus type 1. *N. Engl. J. Med.* 336, 1337–1342 (1997).

6 Delamare C, Burgard M, Mayaux MJ *et al.* HIV-1 RNA detection in plasma for the diagnosis of infection in neonates. *J. Acquir. Immune Defic. Syndr.* 15, 121–125 (1997).

7 Blattner WA, Oursler KA, Cleghorn F *et al.* Rapid clearance of virus after acute HIV-1 infection: correlates of risk of AIDS. *J. Infect. Dis.* 189(10), 1793–1801 (2004).

▶8 Polis MA, Sidorov IA, Yoder C *et al.* Correlation between reduction in plasma HIV-1 RNA concentration 1 week after start of antiretroviral treatment and longer-term efficacy. *Lancet* 358(9295), 1760–1765 (2001).

▶9 Katabira ET, Oelrichs RB. Scaling up antiretroviral treatment in resource-limited settings: successes and challenges. *AIDS* 21(Suppl. 4), S5–S10 (2007).

▶10 Calmy A, Ford N, Hirschel B *et al.* HIV viral load monitoring in resource-limited regions: optional or necessary? *Clin. Infect. Dis.* 44(1), 128–134 (2007).

• **First review clearly demonstrating the clinical need for viral load testing in developing countries.**

11 Fiscus SA, Cheng B, Crowe SM *et al.* HIV-1 viral load assays for resource-limited settings. *PLoS Med.* 3(10), e417 (2006).

12 Rouet F, Rouzioux C. The measurement of HIV-1 viral load in resource-limited settings: how and where? *Clin. Lab.* 53(3–4), 135–148 (2007).

13 Rouet F, Rouzioux C. HIV-1 viral load testing cost in developing countries: what's new? *Expert Rev. Mol. Diagn.* 7, 703–707 (2007).

- **Proposes different strategies for an expanded access to HIV-1 viral load measurements in resource-limited settings according to three expected requirement levels.**

14 Elbeik T, Dalessandro R, Loftus RA, Beringer S. HIV-1 and HCV viral load cost models for bDNA: 440 Molecular System versus real-time PCR AmpliPrep (R)/TaqMan (R) test. *Expert Rev. Mol. Diagn.* 7(6), 723–753 (2007).

- **Significant paper that describes models to assess cost per reportable result for HIV-1 RNA viral load.**

15 Bonard D, Rouet F, Toni TA *et al.* Field evaluation of an improved assay using a heat-dissociated p24 antigen for adults mainly infected with HIV-1CRF02_AG strains in Cote d'Ivoire, West Africa. *J. Acquir. Immune Defic. Syndr.* 34, 267–273 (2003).

16 Malmsten A, Shao XW, Sjodahl S *et al.* Improved HIV-1 viral load determination based on reverse transcriptase activity recovered from human plasma. *J. Med. Virol.* 76, 291–296 (2005).

17 Robertson DL, Anderson JP, Bradac JA *et al.* HIV-1 nomenclature proposal. *Science* 288, 55–56 (2000).

18 Powell RL, Zhao J, Konings FA *et al.* Circulating recombinant form (CRF) 37_cpx: an old strain in Cameroon composed of diverse genetically distant lineages of subtypes A and G. *AIDS Res. Hum. Retrovirus* 23, 923–933 (2007).

19 Peeters M, Toure-Kane C, Nkengasong J. Genetic diversity of HIV in Africa: impact on diagnosis, vaccine development and trials. *AIDS* 17, 2547–2560 (2003).

20 Higuchi R, Dollinger G, Walsh PS, Griffith R. Simultaneous amplification and detection of specific DNA sequences. *Biotechnology (NY)*, 10, 413–417 (1992).

21 Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR: real time monitoring of DNA amplification reactions. *Biotechnology (NY)* 11, 1026–1030 (1993).

22 Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl Acad. Sci. USA* 88, 7276–7280 (1991).

23 Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* 25(2), 169–193 (2000).

24 Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* 29, 23–39 (2002).

25 Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J. Biomol. Tech.* 15, 155–166 (2004).

26 Bustin SA, Benes V, Nolan T, Pfaffl MW. Quantitative real-time RT-PCR – a perspective. *J. Mol. Endocrinol.* 34, 597–601 (2005).

27 Bustin SA, Mueller R. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. *Clin. Sci. (Lond.)* 109, 365–379 (2005).

28 Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. *Biotechniques* 39, 75–85 (2005).

- **In-depth review of real-time PCR technologies.**

29 Niesters HG. Quantitation of viral load using real-time amplification techniques. *Methods* 25(4), 419–429 (2001).

30 Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. *Nat. Protoc.* 1, 1559–1582 (2006).

31 Kubista M, Andrade JM, Bengtsson M *et al.* The real-time polymerase chain reaction. *Mol. Aspects Med.* 27, 95–125 (2006).

- **In-depth review of real-time PCR technologies.**

32 Vet JAM, Majithia AR, Marras SAE *et al.* Multiplex detection of four pathogenic retroviruses using molecular beacons. *Proc. Natl Acad. Sci. USA* 96, 6394–6399 (1999).

33 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCr and the $2^{-DD\,C_T}$ method. *Methods* 25, 402–408 (2001).

34 Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45 (2001).

35 Liu W, Saint DA. A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. *Anal. Biochem.* 302, 52–59 (2002).

36 Lalam N. Estimation of the reaction efficiency in polymerase chain reaction. *J. Theor. Biol.* 242, 947–953 (2006).

37 Kontanis EJ, Reed FA. Evaluation of real-time PCR amplification. Efficiencies to detect PCR inhibitors. *J. Forensic Sci.* 51, 795–804 (2006).

38 Pasloske BL, Walkerpeach CR, Obermoeller RD, Winkler M, Dubois DB. Armored RNA technology for production of ribonuclease-resistant viral RNA controls and standards. *J. Clin. Microbiol.* 36, 3590–3594. (1998).

39 Akane A, Matsubara H, Nakamura S, Takahashi S, Kimura K. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstrains, a major inhibitor of polymerase chain reaction (PCR) amplification. *J. Forensic Sci.* 39, 362–372 (1994).

40 Al-Soud WA, Jonsson LJ, Radström P. Identification and characterization of immunoglobulin G in blood as major inhibitor of diagnostic PCR. *J. Clin. Microbiol.* 38, 345–350 (2000).

41 Al-Soud WA, Radström P. Purification and characterization of PCR-inhibitory components in blood cells. *J. Clin. Microbiol.* 39, 485–493 (2001).

42 Izraeli S, Pfleiderer C, Lion T. Detection of gene expression by PCR amplification of RNA derived from frozen heparinized whole blood. *Nucleic Acids Res.* 19, 6051 (1991).

43 Lekanne-Deprez RH, Fijnvandraat AC, Ruijter JM, Moorman AF. Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depend on cDNA systhesis conditions. *Anal. Biochem.* 307, 63–69 (2002).

44 Bernard PS, Wittwer CT. Homogeneous amplification and variant detection by fluorescent hybridization probes. *Clin. Chem.* 46, 147–148 (2000).

45 Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. *Genome Res.* 6, 995–1001 (1996).

46 Heid CA, Stevens J, Livak KJ, Williams PM. Real-time quantitative PCR. *Genome Res.* 6, 986–994 (1996).

47 Afonina I, Zivarts M, Kutyavin I *et al.* Efficient priming of PCR with short nucleotides conjugated to a minor groove binder. *Nucleic Acids Res.* 25, 2657–2660 (1997).

48 Weusten JJ, Carpay WM, Oosterlaken TA, van Zuijlen MC, van de Wiel PA. Principles of quantitation of viral loads using nucleic acid sequence-based amplification in combination with homogeneous detection using molecular beacons. *Nucleic Acids Res.* 30, e26 (2002).

49 Solinas A, Brown LJ, McKeen C *et al.* Duplex Scorpion primers in SNP analysis and FRET applications. *Nucleic Acids Res.* 29, E96 (2001).

50 Nazarenko I, Lowe B, Darfler M *et al.* Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. *Nucleic Acids Res.* 30, e37 (2002).

51 Nazarenko IA, Bhatnagar SK, Hohman RJ. A closed tube format for amplification and detection of DNA based on energy transfer. *Nucleic Acids Res.* 25, 2516–2521 (1997).

52 Gueudin M, Damond F, Braun J *et al.* Differences in proviral DNA load between HIV-1- and HIV-2-infected patients. *AIDS* 22(2), 211–215 (2008).

53 Damond F, Collin G, Descamps D *et al.* Improved sensitivity of human immunodeficiency virus type 2 subtype B plasma viral load assay. *J. Clin. Microbiol.* 43, 4234–4236 (2005).

54 Liu WC, Phiet PH, Chiang TY *et al.* Five subgenotypes of hepatitis B virus genotype B with distinct geographic and virological characteristics. *Virus Res.* 129(2), 212–223 (2007).

55 Halfon P, Bourliere M, Penaranda G, Khiri H, Ouzan D. Real-time PCR assays for hepatitis C virus (HCV) RNA quantitation are adequate for clinical management of patients with chronic HCV infection. *J. Clin. Microbiol.* 44(7), 2507–2511 (2006).

56 Nagot N, Foulongne V, Becquart P *et al.* Longitudinal assessment of HIV-1 and HSV-2 shedding in the genital tract of West African women. *J. Acquir. Immune Defic. Syndr.* 39, 632–634 (2005).

57 Dehee A, Cesaire R, Desire N *et al.* Quantitation of HTLV-I proviral load by a TaqMan real-time PCR assay. *J. Virol. Methods* 102(1–2), 37–51 (2002).

58 Leruez-Ville M, Ouachee M, Delarue R *et al.* Monitoring cytomegalovirus infection in adult and pediatric bone marrow transplant recipients by a real-time PCR assay performed with blood plasma. *J. Clin. Microbiol.* 41, 2040–2046 (2003).

59 Brengel-Pesce K, Morand P, Schmuck A *et al.* Routine use of real-time quantitative PCR for laboratory diagnosis of Epstein–Barr virus infections. *J. Med. Virol.* 66(3), 360–369 (2002).

60 Lefevre J, Hankins C, Pourreaux K, Voyer H, Coutlee F. Real-time PCR assays using internal controls for quantitation of HPV-16 and beta-globin DNA in cervicovaginal lavages. *J. Virol. Methods* 114(2), 135–144 (2003).

61 Gibellini D, Vitone F, Gori E, La Place M, Re MC. Quantitative detection of human immunodeficiency virus type 1 (HIV-1) viral load by SYBR green real-time RT-PCR technique in HIV-1 seropositive patients. *J. Virol. Methods* 115(2), 183–189 (2004).

62 Gibellini D, Gardini F, Vitone F *et al.* Simultaneous detection of HCV and HIV-1 by SYBR Green real time multiplex RT-PCR technique in plasma samples. *Mol. Cell Probes* 20, 223–229 (2006).

63 Palmer S, Wiegand AP, Maldarelli F *et al.* New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. *J. Clin. Microbiol.* 41(10), 4531–4536 (2003).

64 Shapshak P, Duncan R, McCoy CB, Page JB. Quantification of HIV *gag* RNA using real time reverse transcriptase PCR. *Front. Biosci.* 10, 135–142 (2005).

65 Watanaveeradej V, Sirirattanaphoomee S, Chantratita W *et al.* Quantification of HIV-1 RNA load by one-tube-one-step and real time PCR assay with TaqMan probe. *J. Med. Assoc. Thai.* 88, S206–S213 (2005).

66 Huang J, Yang CM, Wang LN *et al.* A novel real-time multiplex reverse transcriptase-polymerase chain reaction for the detection of HIV-1 RNA by using dual-specific armored RNA as internal control. *Intervirology* 51(1), 42–49 (2008).

67 Promso S, Srichunrusami C, Utid K *et al.* Quantitative detection of human immunodeficiency virus type 1 (HIV-1) viral load by real-time RT-PCR assay using self-quenched fluorogenic primers. *Southeast Asian J. Trop. Med. Public Health* 37, 477–487 (2006).

68 Rekhviashvili N, Stevens G, Scott L, Stevens W. Fluorogenic LUX primer for quantitation of HIV-1 by real-time RT-PCR. *Mol. Biotechnol.* 32, 101–110 (2006).

69 Rekhviashvili N, Stevens W, Marinda E *et al.* Clinical performance of an in-house real-time RT-PCR assay using a fluorogenic LUX™ primer for quantitation of human immunodeficiency virus type-1 (HIV-1). *J. Virol. Methods* 146(1–2), 14–21 (2007).

70 Saha BK, Tian BH, Bucy RP. Quantitation of HIV-1 by real-time PCR with a unique fluorogenic probe. *J. Virol. Methods* 93(1–2), 33–42 (2001).

71 Muller J, Eis Hubinger AM, Daumer M *et al.* A novel internally controlled real-time reverse transcription-PCR assay for HIV-1 RNA targeting the pol integrase genomic region. *J. Virol. Methods* 142(1–2), 127–135 (2007).

72 de Baar MP, van Dooren MW, De Rooij E *et al.* Single rapid real-time monitored isothermal RNA amplification assay for quantification of human immunodeficiency virus type 1 isolates from groups M, N, and O. *J. Clin. Microbiol.* 39, 1378–1384 (2001).

73 Candotti D, Temple J, Owusu Ofori S, Allain JP. Multiplex real-time quantitative RT-PCR assay for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus type 1. *J. Virol. Methods* 118(1), 39–47 (2004).

74 Drosten C, Panning M, Drexler JF *et al.* Ultrasensitive monitoring of HIV-I viral load by a low-cost real-time reverse transcription-PCR assay with internal control for the 5′ long terminal repeat domain. *Clin. Chem.* 52(7), 1258–1266 (2006).

- **Significant paper studying the impact of HIV-1 genetic diversity on HIV-1 RNA quantification by using an in-house real-time PCR assay, in comparison with licensed HIV-1 RNA assays.**

75 Rouet F, Ekouevi DK, Chaix ML *et al.* Transfer and evaluation of an automated, low-cost real-time reverse transcription-PCR test for diagnosis and monitoring of human immunodeficiency virus type 1 infection in a West African resource-limited setting. *J. Clin. Microbiol.* 43, 2709–2717 (2005).

- **First demonstration of real-time PCR technology for HIV-1 monitoring and diagnosis in a resource-limited country.**

76 Rouet F, Chaix ML, Nerrienet E *et al.* Impact of HIV-1 genetic diversity on plasma HIV-1 RNA quantification: usefulness of the Agence Nationale de Recherches sur le SIDA second-generation long terminal repeat-based real-time reverse transcriptase polymerase chain reaction. *J. Acquir. Immune Defic. Syndr.* 45, 380–388 (2007).

77 Schvachsa N, Turk G, Burgard M *et al.* Examination of real-time PCR for HIV-1 RNA and DNA quantitation in patients infected with HIV-1BF intersubtype recombinant variants. *J. Virol. Methods* 140(1–2), 222–227 (2007).

78 Steegen K, Luchters S, De Cabooter N *et al.* Evaluation of two commercially available alternatives for HIV-1 viral load testing in resource-limited settings. *J. Virol. Methods* 146, 178–187 (2007).

79 Holmes H, Davis G, Heath A, Hewlett I, Lelie N. An international collaborative study to establish the 1st international standard for HIV-1 RNA for use in nucleic acid-based techniques. *J. Virol. Methods* 92(2), 141–150 (2001).

80 Davis C, Heath A, Best S *et al.* Calibration of HIV-1 working reagents for nucleic acid amplification techniques against the 1st international standard for HIV-1 RNA. *J. Virol. Methods* 107, 37–44 (2003).

81 Preiser W, Drexler JF, Drosten C. HIV-1 viral load assays for resource-limited settings: clades matter. *PLoS Med.* 3, e538 (2006).

82 Dabis F, Bequet L, Ekouevi DK *et al.* Field efficacy of zidovudine, lamivudine and single-dose nevirapine to prevent peripartum HIV transmission. *AIDS* 19, 309–318 (2005).

83 Rouet F, Fassinou P, Inwoley A *et al.* Long-term survival and immuno-virological response of African HIV-1-infected children to highly active antiretroviral therapy regimens. *AIDS* 20(18), 2315–2319 (2006).

84 Chaix M-L, Ekouevi Koumavi D, Rouet F *et al.* Low risk of nevirapine resistance mutations in the prevention of mother-to-child transmission of HIV-1: Agence Nationale de Recherches sur le SIDA DITRAME Plus, Abidjan, Côte d'Ivoire. *J. Infect. Dis.* 193, 482–487 (2006).

85 Chaix ML, Ekouevi DK, Peytavin G *et al.* Impact of nevirapine (NVP) plasma concentration on selection of resistant virus in mothers who received single-dose NVP to prevent perinatal human immunodeficiency virus type 1 transmission and persistence of resistant virus in their infected children. *Antimicrob. Agents Chemother.* 51(3), 896–901 (2007).

86 Danel C, Moh R, Anzian A *et al.* Tolerance and acceptability of an efavirenz-based regimen in 740 adults (predominantly women) in West Africa. *J. Acquir. Immune Defic. Syndr.* 42(1), 29–35 (2006).

87 Danel C, Moh R, Minga A *et al.* CD4-guided structured antiretroviral treatment interruption strategy in HIV-infected adults in west Africa (Trivacan ANRS 1269 trial): a randomised trial. *Lancet* 367(9527), 1981–1989 (2006).

88 Tonwe-Gold B, Ekouevi DK, Viho I *et al.* Antiretroviral treatment and prevention of peripartum and postnatal HIV transmission in West Africa: evaluation of a two-tiered approach. *PLoS Med.* 4, e257 (2007).

89 Seyler C, Adje Toure C, Messou E *et al.* Impact of genotypic drug resistance mutations on clinical and immunological outcomes in HIV-infected adults on HAART in West Africa. *AIDS* 21(9), 1157–1164 (2007).

90 Nagot N, Ouedraogo A, Foulongne V *et al.* Reduction of HIV-1 RNA levels with therapy to suppress herpes simplex virus. *N. Engl. J. Med.* 356(8), 790–799 (2007).

91 Janssens B, Raleigh B, Soeung S *et al.* Effectiveness of highly active antiretroviral therapy in HIV-positive children: evaluation at 12 months in a routine program in Cambodia. *Pediatrics* 120(5), E1134–E1140 (2007).

92 Ferradini L, Laureillard D, Prak N *et al.* Positive outcomes of HAART at 24 months in HIV-infected patients in Cambodia. *AIDS* 21, 2293–2301 (2007).

93 Espy MJ, Uhl JR, Sloan LM *et al.* Real-time PCR in clinical microbiology: applications for a routine laboratory testing. *Clin. Microbiol. Rev.* 19(1), 165–256 (2006).

94 de Arellano ER, Martin C, Soriano V, Alcami J, Holguin A. Genetic analysis of the long terminal repeat (LTR) promoter region in HIV-1-infected individuals with different rates of disease progression. *Virus Genes* 34(2), 111–116 (2007).

95 de Baar MP, Abebe A, Kliphuis A *et al.* HIV type 1 C and C' subclusters based on long terminal repeat sequences in the Ethiopian HIV type 1 subtype C epidemic. *AIDS Res. Hum. Retrovirus* 19, 917–922 (2003).

96 van Opijnen T, Jeeninga RE, Boerlijst MC *et al.* Human immunodeficiency virus type 1 subtypes have a distinct long terminal repeat that determines the replication rate in a host-cell-specific manner. *J. Virol.* 78, 3675–3683 (2004).

97 Khodakov DA, Zakharova NV, Gryadunov DA *et al.* An oligonucleotide microarray for multiplex real-time PCR identification of HIV-1, HBV, and HCV. *Biotechniques* 44, 241–248 (2008).

98 Plantier JC, Dachraoui R, Lemee V *et al.* HIV-1 resistance genotyping on dried serum spots. *AIDS* 19(4), 391–397 (2005).

99 Waters L, Kambugu A, Tibenderana H *et al.* Evaluation of filter paper transfer of whole-blood and plasma samples for quantifying HIV RNA in subjects on antiretroviral therapy in Uganda. *J. Acq. Immune Defic. Syndr.* 46, 590–593 (2007).

100 Ou C-Y, Yang H, Balinandi S *et al.* Identification of HIV-1 infected infants and young children using real-time RT PCR and dried blood spots from Uganda and Cameroon. *J. Virol. Methods* 144, 109–114 (2007).

101 Bland RM, Little KE, Coovadia HM *et al.* Intervention to promote exclusive breast-feeding for the first 6 months of life in a high HIV prevalence area. *AIDS* 22, 883–891 (2008).

102 Damond F, Roquebert B, Benard A *et al.* HIV-1 plasma viral load discrepancies between the Roche Cobas Amplicor HIV-1 monitor v1.5 and Roche Cobas Ampliprep/Cobas TaqMan HIV-1 assays. *J. Clin. Microbiol.* 45, 3436–3438 (2007).

103 Gottesman BS, Grossman Z, Lorber M *et al.* Comparative performance of the amplicor HIV-1 monitor assay versus NucliSens EasyQ in HIV subtype C-infected patients. *J. Med. Virol.* 78(7), 883–887 (2006).

Website

201 Gene-Quantification.com & Gene-Quantification.info – The reference in qPCR – Academic & Industrial Information Platform www.gene-quantification.info

Affiliations

- François Rouet, PharmD, PhD
Laboratoire de Virologie, Centre Muraz, BP390 Bobo-Dioulasso 01, Burkina Faso
Tel.: +226 2097 0102
Fax: +226 2097 0457
franrouet@yahoo.fr
- Hervé Ménan, PharmD, PhD
Centre de Diagnostic et de Recherches sur le SIDA (CeDReS), CHU de Treichville, 22 BP 1175, Abidjan 22, Côte d'Ivoire
Tel.: +225 2125 8459
Fax: +225 2124 9206
rvmenan@yahoo.fr

- Johannes Viljoen, MD
Africa Centre Virology Laboratory (ACVL), Doris Duke Medical Research Institute, University of KwaZulu-Natal, Durban, South Africa
Tel.: +27 31 260 4013
Fax: +27 31 260 4015
jviljoen@africacentre.ac.za
- Nicole Ngo-Giang-Huong, PharmD, PhD
Institut de Recherche pour le Développement (IRD) URI 174/AMS Laboratory, Faculty of Associated, Medical Sciences, Department of Clinical Microbiology, 110 Inthawaroros Road, Muang Chiang Mai 50200, Thailand
Tel.: +66 53 894 431
Fax: +66 53 894 220
nicole@phpt.org
- Kishor Mandaliya, MD, FRCPath
Coast Provincial General Hospital, BP 90086 Mombasa 80100, Kenya
Tel.: +254 414 73 978
Fax: +254 412 31 6347
kmandaliya@gmail.com
- Diane Valéa, MSc
Laboratoire de Virologie, Centre Muraz, BP390 Bobo-Dioulasso 01, Burkina Faso
Tel.: +226 2097 0102
Fax: +226 2097 0457
diane.valea@voila.fr
- Truong Xuan Lien, PhD
Department of Biological Analysis, Pasteur Institute Ho Chi Minh City, 167 Pasteur, Ho Chi Minh City, Vietnam
Tel.: +84 822 2883
Fax: +84 882 4335
truongxuanlien@gmail.com
- Sivapragashini Danaviah, MSc
Africa Centre Virology Laboratory (ACVL), Doris Duke Medical Research Institute, University of KwaZulu-Natal, Durban, South Africa
Tel.: +27 31 260 4013
Fax: +27 31 260 4015
sdanaviah@africacentre.ac.za
- Dominique Rousset, MD, PhD
Unité de Virologie, Centre Pasteur du Cameroun – BP1274, Yaoundé, Cameroun
Tel.: +237 22 231 015
Fax: +237 99 046 282
rousset@pasteur-yaounde.org
- Amandine Ganon, MD
Laboratoire de Virologie, CHU Necker-Enfants Malades, 149, rue de Sèvres, 75015 Paris Cedex
Tel.: +33 144 494 961
Fax: +33 144 494 960
amandine.ganon@nck.aphp.fr
- Eric Nerrienet, PhD
Laboratoire VIH/Hépatites, Institut Pasteur Cambodia, Phnom Penh, Cambodia
Tel.: +855 1233 3105
Fax: +855 2372 5606
enerrienet@pasteur-kh.org