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Protocol

A one-step SYBR Green I-based product-enhanced reverse transcriptase assay for the quantitation of retroviruses in cell culture supernatants

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A B S T R A C T

PCR-enhanced reverse transcriptase assays (PERT) are sensitive tools for the detection of retroviruses in biological samples. The adaptation of real-time PCR techniques based on fluorescent probes (F-PERT) has added a reliable quantitative capacity to the assay. In the interest of economy and time, the SYBR Green I-based real-time detection system was used to establish a convenient one-step PERT assay (SG-PERT). This assay can be completed in 2 h, is linear over six orders of magnitude and can be used to quantify retroviruses belonging to divergent species, such as the human immunodeficiency virus type 1 (HIV-1), murine leukemia virus (MLV) and prototypic foamy virus (PFV).

1. Type of research

Assays for the routine quantitation of retrovirus particles are essential tools in research areas addressing the biology of retroviruses and the development and use of viral vectors for gene transfer. To this end, ELISA-based techniques for the quantitation of retroviral Gag proteins are often expensive and require extensive sample dilution, because they are linear only over a limited range of antigen concentration. RT-PCR strategies to detect and quantify virion-associated retrovirus genomes rely on sequence-specific primer design and require RNA isolation procedures which make it expensive and labour intensive. In contrast, biochemical assays which estimate the amount of virion-associated reverse transcriptase (RT-assays) can universally detect and quantify retrovirus particles. The enzymatic activity of RT, present in all retroviruses, is measured using an RNA template which is reverse transcribed *in vitro* by the retroviral enzyme. The resulting DNA product (cDNA) is then quantified as a measure of the amount of virions present. Conventionally, quantitation of the cDNA is achieved by measuring the amount of nucleotides incorporated using radioactively labelled reagents or colorimetric techniques. However, during the last 15 years product-enhanced RT (PERT) assays have been developed using PCR techniques to amplify the cDNA and to allow the detection of even single retrovirus particles (Chang et al., 1997; Fan et al., 2006; Heneine et al., 1995; Pyra et al., 1994; Silver et al., 1993; Yamamoto et al., 1996). Furthermore, the adaptation of real-time PCR techniques based on the fluorogenic 5′-nuclease chemistry (F-PERT) to one- or two-step assays has coupled the high sensitivity given by the PCR to the quantitative capacity given by real-time analysis of product amplification, making it linear over six orders of magnitude (Arnold et al., 1998; Lovatt et al., 1999; Maudru and Peden, 1998; Sears and Khan, 2003). PERT and F-PERT assays can be used to detect low amounts of retroviruses in biological products (Brorson et al., 2001, 2002; Lovatt et al., 1999), HIV in serum samples (Garcia Lerma et al., 1998; Pyra et al., 1994), replication competent recombinant retroviruses in cultures producing vectors for gene transfer (Sastry et al., 2005) and endogenous retroviruses (Bisset et al., 2007; Khan and Sears, 2001).

SYBR Green I-based systems offer a popular alternative to fluorescent probe-based real-time PCR techniques and are based on its ability to produce a 100-fold increase of fluorescence when bound to double-stranded DNA. Because the binding of SYBR Green I to nucleic acid is not sequence-specific, the fluorescent signal produced when in complex with DNA is directly proportional to the length and amount of DNA copies synthesized during the reaction, making this technique very precise and sensitive. Given the popularity of the SYBR Green I-based systems and their cost efficiency, the SYBR Green I chemistry has been adapted to a one-step PERT (SG-PERT). Several attempts were required in order to identify crucial reaction conditions and develop a robust and sensitive

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assay, which is proposed as a fast and economic tool for the routine quantitation of divergent retroviruses.

2. Time required for a 20 sample experiment: 1 h 50 min

(i) Sample preparation: 20 min
(ii) Reaction set-up: 10 min
(iii) RT and PCR reactions: 1 h 10 min
(iv) Data analysis: 10 min

3. Materials

3.1. Cells and viruses

Human endothelial cells HEK 293T, human fibrosarcoma cells HT1080 and Hela-derived TZM-BL indicator cells were grown in DMEM (Invitrogen, Paisley, UK). Human lymphoblastoid Jurkat E6.1 cells were grown in RPMI (Invitrogen, Paisley, UK). Media were supplemented with 10% fetal calf serum (Invitrogen, Paisley, UK) and cell cultures were maintained at 37 °C and 5% CO_2.

HIV-1 was generated using the proviral construct pNL4-3 (Adachi et al., 1986). Recombinant murine leukemia virus (MLV) vector was produced using the gag-pol expression plasmid, pHIT-60 (Soneoka et al., 1995), the VSV-G (G protein from the vesicular stomatitis virus) expression plasmid, pMDG (Naldini et al., 1996) and the retroviral vector, pCNCG-GFP (Neil et al., 2001). Recombinant PFV vector was produced using the vector plasmid, pMH71 (Heinkelein et al., 2003) and pczHFVenv encoding the HFV (human foamy virus) envelope glycoprotein (Pietschmann et al., 1999).

3.2. Special equipment and software

- LightCycler 2.0 (Roche Diagnostics, Burgess Hill, UK).
- LightCycler software 4.0 (Roche Diagnostics, Burgess Hill, UK).
- 7900HT Real-Time PCR system and software (Applied Biosystems, Warrington, UK).

3.3. Chemicals and reagents

- 1 kb plus DNA molecular weight marker, Sybr Green I, deoxynucleotides and Platinum Taq (Invitrogen, Paisley, UK).
- Recombinant HIV-1 reverse transcriptase and AmpliTaq Gold polymerase (Ambion/Applied Biosystems, Warrington, UK).
- Ammonium sulphate, potassium chloride, magnesium chloride, manganese chloride, Tris–HCl, dithiothreitol, Triton X-100, DNase and RNase-free water, glycerol, 1,4-dithioerythritol (DTT) and agarose (Sigma–Aldrich, Gillingham, UK).
- Bovine serum albumin (New England Biolabs, Hitchin, UK).
- Hot-start Taq polymerase (MBI Fermentas, York, UK).
- Brome mosaic virus RNA and RNAsin ribonuclease inhibitor (Promega, Southampton, UK).
- Forward (5′-GGTCTCTTTTAGAGATTTACAGTG-3′) and reverse (5′-CGTGGTTGACACGCAGACCTCTTAC-3′) primer oligonucleotides (Invitrogen, Paisley, UK).
- LightCycler 20 μl capillaries (Roche Diagnostics, Burgess Hill, UK).
- Saquinavir (NIBSC, Potters Bar, UK)
- PFU ultra, PFU turbo and EasyA (Stratagene, Amsterdam Zuidoost, The Netherlands)
- Murex HIV antigen mAB ELISA assay kit (Abbott Murex, Maidenhead, UK).
- X-Gal (5-bromo-4-chloro-3-indolyl-b-galactopyranoside) (Melford, Chelsworth UK).

4. Detailed procedure

4.1. Sample preparation

4.1.1. Production of viruses and recombinant retrovirus vectors

Jurkat E6.1 cells were infected with HIV-1 NL4-3, previously generated by transfection of HEK 293T cells with pNL4-3 and passaged for more than 2 weeks to establish chronic infection.

Recombinant MLV and PFV (prototypic foamy virus) vectors were produced by transfection of HEK 293T cells. Briefly, 1.5 million cells, seeded in T25 flasks 1 day earlier, were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions with pHIT-60, pMDG and pCNCG-GFP (MLV) and pMH71 and pczHFVenv (PFV). Vector-containing supernatants were harvested 48 h after transfection. After clarification by low-speed centrifugation, supernatants were prepared for infectivity assay and SG-PERT analyses by filtering through a 0.45 μm pore filter.

4.1.2. Infectivity assays

TZM-BL or HT1080 target cells were seeded onto 48-well plates (2×10^4 cells/well) 1 day before infection or transduction. Virus and vector supernatants were serially diluted 10-fold in triplicate in culture medium and target cells inoculated with 0.4 ml of each dilution. Virus supernatant was replaced with fresh medium 24 h later and end-point titers evaluated 72 h post-infection (TZM-bl cells) or post-transduction (HT1080 cells). To ensure that HIV-1 infection of TZM-bl resulted in a single cycle of replication, target cells were treated with 1 μM of the protease inhibitor saquinavir. HIV-1 infection of TZM-bl cells was evaluated by staining infected cells with X-Gal as previously described (Pizzato et al., 2001) The infectivities of MLV and PFV GFP vectors were evaluated by counting fluorescent foci by fluorescence microscopy in transduced HT1080 cells. Clusters of stained cells were attributed to a single infection event and titre was expressed as infectious units (i.u.).

4.1.3. Measurements of p24 and HIV-1 RNA in virus supernatants

P24 antigen was measured in cell culture supernatants using the Murex HIV antigen mAB ELISA assay kit following the manufacturer's guidelines. HIV-1 RNA was quantified by Siemens Versant HIV-1 bDNA (branched-chain DNA) assay, after the sample was diluted 10^5-fold in normal human plasma to obtain a concentration falling within the range of the assay.

4.1.4. Preparation and storage of SG-PERT assay buffers

All buffers were prepared using RNAse-free reagents:

2× virus lysis buffer (0.25% Triton X-100, 50 mM KCl, 100 mM TrisHCl pH7.4, 40% glycerol) was stored at −20 °C. RNAse inhibitor (0.4 U/μl) was added to the volume of buffer needed for virus disruption immediately before use.

10× sample dilution buffer (50 mM $(NH_4)_2SO_4$, 200 mM KCl and 200 mM Tris–HCl pH 8.3) was stored in aliquots at −20 °C.

2× reaction mix (10 mM $(NH_4)_2SO_4$, 40 mM KCl and 40 mM Tris–Cl pH 0.3, 10 mM $MgCl_2$, 0.2 mg/ml BSA, 1/10,000 SYBR Green I, 400 μM dNTPs, 1 μM forward primer,1 μM reverse primer, 1.2 μg/ml BMV RNA) was stored in aliquots at −80 °C. Hotstart Taq was added (0.2 U/reaction) immediately before use.

4.1.5. Preparation of virus lysates

Five microliters of undiluted or diluted virus supernatant, or 5 μl of recombinant HIV-1 RT diluted in sample dilution buffer, were mixed with 5 μl of 2× virus lysis buffer in a 1.5 ml microfuge tube. After 10 min incubation at room temperature, 90 μl of sample dilution buffer were added and the tube vortexed to obtain a 10-fold dilution.

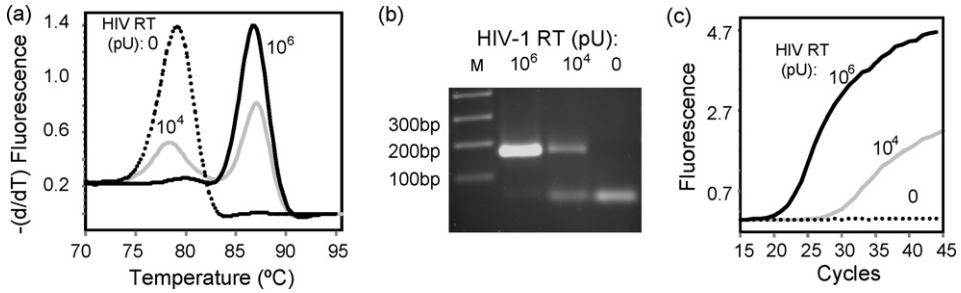

Fig. 1. The SG-PERT assay. (a) Melting curve of PCR products obtained by testing the indicated amount of recombinant HIV-1 RT with SG-PERT. (b) Agarose gel visualizing the DNA products in the SG-PERT reactions analyzed in (a). M: molecular weight marker with indicated molecular sizes. (c) Real-time amplification curves of the reactions visualized in (a) and obtained by acquiring the fluorescence at 83 °C.

4.2. Reaction set-up

Ten microliters of the diluted virus lysate samples were mixed immediately with 10 μl of 2× reaction mix already aliquoted into the reservoir of pre-chilled 20 μl capillaries (lightcycler) or optical tubes (HT7900). The capillaries or tubes were briefly centrifuged to sediment the reaction components and applied to real-time platforms. Thermal cycler conditions used were: 30 min RT reaction at 37 °C, 5 min hot-start Taq activation at 95 °C and 45 cycles of amplification. Each amplification cycle was composed of 5 s denaturation at 95 °C, 5 s annealing at 55 °C, 15 s extension at 72 °C, 7 s acquisition at 83 °C, using the lightcycler; 5 s denaturation at 95 °C, 5 s annealing at 55 °C, 20 s extension at 72 °C, 11 s acquisition at 83 °C, using the 7900HT platform.

Where indicated, the 20 μl PCR products were visualized after electrophoretic separation in 1.8% agarose gel and stained with ethidium bromide.

4.3. Data analysis

Amplification curves and melting temperatures were generated by recording fluorescence at 530 nm and analyzed using the LightCycler software 4. For each amplification curve the software calculates the crossing point, defined as the cycle number at which the fluorescence of a sample rises above the background fluorescence. The fit points method with arithmetic background correction was used to calculate the crossing points and generate the standard curves following the operator's manual. First, the noise band was adjusted using the software automatic option. The maximum number of fit points fitting the linear portion of the curve was then added. The software option "minimize errors" was chosen to automatically adjust the threshold line for the calculation of crossing points. The regression analysis of crossing points versus dilution factors of virus supernatants or recombinant RT was automatically calculated by the software. The slope of the curve, which is referred to as the efficiency of the reaction, indicates the efficiency of the amplification, the perfect value being 2. Melting peaks were calculated automatically by the software.

For data acquired with the 7900HT system, crossing points were calculated automatically by the software.

5. Results

5.1. SG-PERT reaction conditions

A one step real-time based PERT assay was established using the BMV RNA template and primer set which have already been described (Silver et al., 1993) and which amplify a 168 bp fragment at the 3′ end of the BMV genome. After screening several thermophilic DNA polymerases, the hot-start Taq-polymerase from MBI Fermentas was chosen for the PCR amplification because it was found to lack significant RT activity (not shown), as already suggested (Fan et al., 2006; Maudru and Peden, 1997).

Since the binding of SYBR Green I to DNA is sequence-independent, non-specific PCR fragments can contribute to the fluorescent signal recorded by the instrument. A melting curve analysis of the PCR products obtained was, therefore, performed to confirm the presence of the specific DNA fragment which correlates with a distinct melting peak at 86 °C (Fig. 1a). Accordingly, agarose gel electrophoresis analyses of the PCR product obtained in the light cycler confirmed the presence of the predicted 168 bp specific fragment and the absence of non-specific products (Fig. 1b). Primer–dimer formation was often detected in negative or weakly positive samples, characterized by a melting peak at 79 °C (Fig. 1b

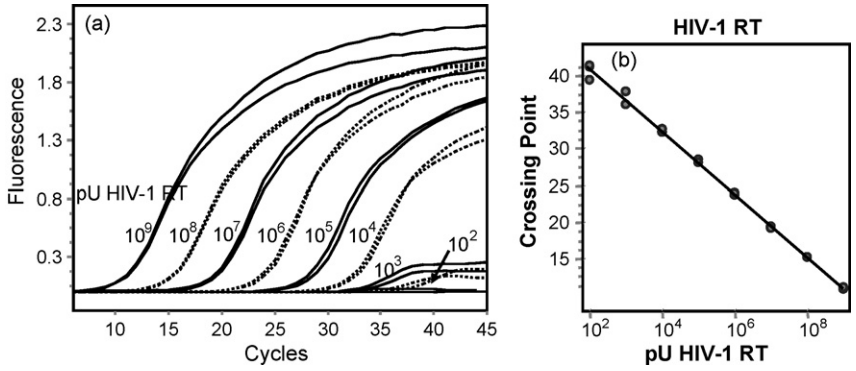

Fig. 2. SG-PERT detection of recombinant HIV-1 RT. (a) Amplification curves obtained with 10-fold serial dilutions of recombinant HIV-1 RT, performed in duplicate. (b) Standard curve showing the input of recombinant HIV-1 RT against the respective crossing points of amplification curves shown in (a) and calculated by the LightCycler software 4. Reaction efficiency is shown.

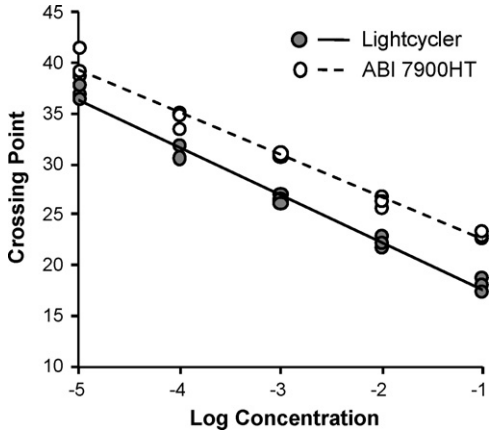

Fig. 3. SG-PERT detection of HIV-1 with two different real-time PCR platforms. Crossing points obtained with the lightcycler and the ABI 7900HT using 10-fold serial dilutions of HIV-$1_{NL4.3}$ retroviral supernatants containing 3×10^5 i.u./ml. Results of triplicate samples and linear regression are shown.

and 1c). To avoid non-specific signal detection of primer dimers, fluorescence was acquired at 83 °C (Fig. 1c). The melting temperature of the PCR product was found to remain constant when recombinant MLV RT or different virus supernatants were tested (not shown), indicating no need to adjust the acquisition temperature.

5.2. Sensitivity of the assay

The sensitivity of the SG-PERT was tested with the lightcycler using 10-fold serial dilutions of recombinant HIV-1 RT, starting from 1 mU. The assay provided a detectable and reproducible signal down to the 10^{-7} dilution equivalent to 100 pU of HIV-1 RT (Fig. 2a), indicating a sensitivity close to that reported by other real-time PCR-based RT-assays for the detection of HIV-1 RT (Arnold et al., 1998; Lovatt et al., 1999; Maudru and Peden, 1997). The diagram generated by plotting the crossing points against the enzyme dilution factor shows linearity over six orders of magnitude (Fig. 2b).

The ability of SG-PERT to detect HIV-1 virus particles in cell culture supernatants was then tested in the lightcycler using cell culture medium derived from Jurkat E6.1 producing HIV-$1_{NL4.3}$. This virus suspension had an infectious titre of 3×10^5 i.u./ml, a p24 content of 355 ng/ml and a virus load of 11.6×10^9 HIV-1 RNA copies/ml. The SG-PERT could detect RT activity down to the 10^{-5} dilution (Fig. 3). Because only 0.5 μl of virus sample is added to the 20 μl reactions, it can be concluded that the assay detects RT activity associated with six HIV RNA copies (three virions) or 1.7×10^{-3} pg of p24. To establish whether SG-PERT can be performed with equal sensitivity using different real-time PCR platforms, the same virus supernatant was tested using the 7900HT apparatus. As shown in Fig. 3, the sensitivity and linear range of the assay remain similar using both real-time PCR platforms.

5.3. Specificity of the assay

The performance of SG-PERT was evaluated for the quantitation and detection of virus particles and recombinant retroviral vectors based on divergent retroviruses. For this purpose, serial dilutions of cell culture supernatants from cells producing HIV and recombinant MLV or PFV GFP transducing vectors were tested in parallel and in triplicate using the lightcycler. End-point infectious titres were first determined to be 4.0×10^6 i.u./ml for the HIV-1 sample, 7.4×10^5 i.u./ml for the PFV sample and 4.2×10^6 i.u./ml for the MLV sample. Results show that for all three types of retrovirus the assay is linear over five to six orders of magnitude and

Table 1
Detection of divergent retrovirus particles and intra-assay variation with SG-PERT.

Virus dilution	Crossing point and coefficient of variation[a]		
	HIV-1	MLV	PFV
Neat	13.47 (0.28%)	17.08 (1.00%)	18.15 (1.26%)
10^{-1}	16.12 (0.31%)	19.18 (0.64%)	21.64 (0.70%)
10^{-2}	19.33 (0.26%)	22.52 (0.76%)	25.09 (0.74%)
10^{-3}	22.92 (0.64%)	26.13 (0.61%)	28.71 (0.98%)
10^{-4}	26.20 (0.19%)	29.70 (1.07%)	32.45 (0.30%)
10^{-5}	29.79 (1.19%)	33.48 (0.66%)	35.64 (2.08%)
10^{-6}	33.10 (0.68%)	36.32 (1.97%)	n.d.
10^{-7}	n.d.	n.d.	n.d.

n.d.: not detected.
[a] Crossing point calculated with the fit points methods. Values are average of triplicate dilutions values. Intraexperimental coefficient of variation is indicated in parenthesis.

detects RT-activity in supernatants containing less than 10 i.u./ml (Fig. 4). As shown in Table 1, for all three types of virus and for all dilutions tested, the coefficient of variation ranges between 0.19% and 2.08%. Reaction efficiencies of 1.995, 1.997 and 1.894 for HIV-1, MLV and PFV, respectively indicate that SG-PERT can be reliably used as a tool for the quantitation of divergent retroviruses.

Retrovirus-free supernatants from uninfected cell cultures were used as negative controls. Rarely and randomly was a weak signal detected with crossing point above 39 PCR cycles. This signal most likely results from the activity of eukaryotic polymerases associated with cellular debris, as reported elsewhere (Voisset et al., 2001). Only samples generating a signal at an earlier PCR cycle than the virus-free control are, therefore, considered positive in these cases.

5.4. Reproducibility of the assay

Reproducibility of the assay was evaluated by testing serial dilutions of the same HIV-1 virus suspension in independent experiments using the lightcycler. Four serial dilutions of an HIV-1 virus supernatant were tested in 18 experiments performed using stocks of lysis buffers and reaction mixtures prepared in three independent occasions. The coefficient of variation ranged from 4.1% to 7.6% (Fig. 5).

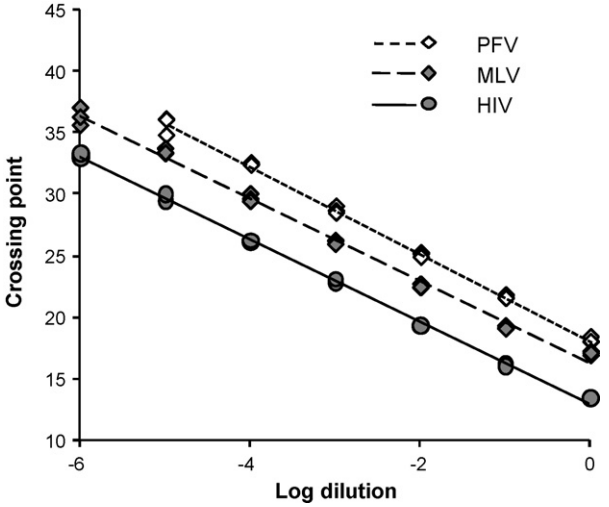

Fig. 4. Detection of divergent retroviruses by SG-PERT. Crossing points and linear regressions obtained with 10-fold serial dilutions of retroviral supernatants performed in triplicate in the lightcycler, using HIV-1_{NL4-3} (infectious titre 4×10^6 i.u./ml), MLV GFP vector (transducing titre of 4.2×10^6 i.u./ml) and PFV GFP vector (transducing titre of 7.4×10^5 i.u./ml).

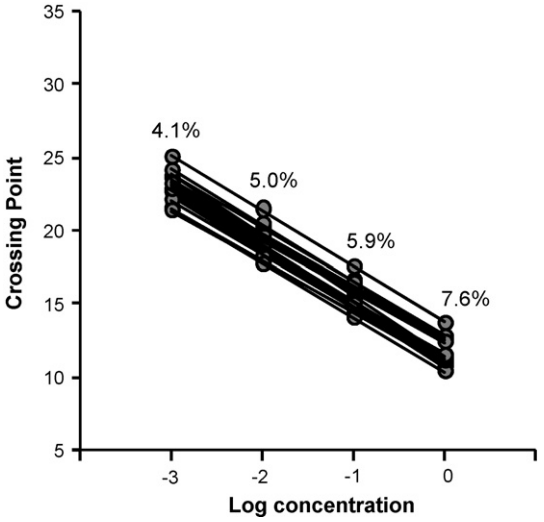

Fig. 5. Intraexperimental variation. Results of 18 SG-PERT independent experiments performed with the lightcycler using the same four HIV-$1_{NL4.3}$ dilutions and three independent lysis and reaction buffer stocks. Coefficient of variation is indicated as percentage of the respective mean crossing point value.

6. Discussion

A SYBR green I-based real-time PERT assay (SG-PERT) for the quantitation and detection of HIV-1 and other divergent retroviruses is described. The assay is linear over six orders of magnitude and requires less than 2 h from sample preparation to data analysis when performed using the lightcycler.

Although PERT was originally established as a two-step assay (Heneine et al., 1995; Lovatt et al., 1999; Maudru and Peden, 1998; Pyra et al., 1994; Yamamoto et al., 1996) the one-step assay, previously proposed using the fluorescent probes techniques (Arnold et al., 1998), adds convenience and minimizes sample handling, which may cause cross-contaminations and decreases quantitative reliability.

Based on the quantitation of viral RNA copies in a cell culture supernatant containing HIV-1 particles, a 1/1500 ratio of infectious units/total HIV-1 particles was established, a value within the range reported in literature (Dimitrov et al., 1993; Kimpton and Emerman, 1992). It was therefore estimated that the SG-PERT assay can detect the presence of three HIV-1 particles in the reaction tube. Because the virus sample represents only 5% of the 20 μl reaction, the assay can reveal RT-activity in HIV-1 supernatants containing 6000 virions or 4 i.u./ml. While this satisfies most needs of a retrovirus research laboratory, the detection capacity of the assay can be increased by the addition of an ultracentrifugation step to concentrate the virus before performing SG-PERT.

The reaction conditions were originally optimised for use with glass capillaries in the lightcycler, which requires the presence of BSA to prevent abstraction of reagents on the glass surface (Teo et al., 2002). However, the same reaction formulation has allowed efficient and quantitative detection of RT activity also with the 7900HT platform, indicating that the protocol described here can be performed with real-time PCR platforms which use plastic tubes instead of glass capillaries. Because the buffers used in this study are entirely home-made, stocks prepared on different occasions might contain small differences which could affect assay efficiency. However, the small inter-assay variation observed between experiments performed using different stocks of lysis and reaction buffers shows that the SG-PERT is reproducible.

While the use of PERT was proposed originally for the detection of retroviruses in clinical specimens (Garcia Lerma et al., 1998; Pyra et al., 1994), undiluted human plasma was found to inhibit potently the one-step SG-PERT (data not shown), suggesting that the assay might not be suitable for analyses of some complex biological samples. However, given its linearity over six orders of magnitude, the simple set-up, the rapid procedure and the low intra- and inter-assay variation, SG-PERT represents an ideal tool for the routine quantitation of retroviruses in cell culture samples. The assay proved also to be a useful tool with which to screen cell lines for retrovirus contamination of tissue cultures (Takeuchi et al., 2008).

This report provides all the information required to perform SG-PERT without the need of acquiring expensive buffers with proprietary formulations. Because the SYBR Green I reagent is relatively inexpensive, the assay described allows significant cost savings compared with the use of most alternative techniques.

6.1. Trouble-shooting

The effects of several chemicals and reagents on the performance of the assay were investigated and are discussed here.

6.1.1. BSA requirement

SYBR Green I is offered currently by different providers in ready-to-use real-time PCR and RT-PCR kits. As the formulation of the reaction mixtures is generally proprietary and, therefore, unavailable, the optimal reaction conditions for the reverse transcription and amplification of BMV RNA in glass capillaries were first investigated using 1 mU of recombinant HIV-1 RT, 5 mM $MgCl_2$ and a 1:20,000 dilution of SYBR Green I, already reported to be compatible with a real-time PCR system (Karsai et al., 2002). After testing numerous buffer formulations, it was established that efficient PCR amplification requires BSA at a final concentration of 0.1 mg/ml (Fig. 6a).

The importance of BSA for the reaction in glass capillaries has been linked to the propensity of the glass surface to sequester some reaction components, such as the taq polymerase (Teo et al., 2002). As shown in Fig. 1, the reaction buffer containing BSA can be successfully used in a real-time PCR platform accepting plastic tubes.

6.1.2. The use of different thermophilic polymerases

Several polymerases from different suppliers were tested. PFU Ultra and PFU Turbo, which are reported to lack RT activity ((Arezi et al., 2003) and products specification from suppliers) could not be adapted to real-time PCR in the light cycler (not shown). Platinum Taq, AmpliTaq Gold, Easy-A performed very efficiently in the light cycler with SYBR Green I, but generated a strong background signal arising early between cycle 20 and 30 in the absence of added retroviral RT activity. Hot-start Taq-polymerase from MBI Fermentas was selected because it consistently failed to reverse transcribe the BMV RNA in 45-cycle PCR reactions.

6.1.3. Concentration and storage of SYBR Green I

Repeated freeze-thaw cycles were avoided by storing multiple aliquots of the SYBR Green I concentrated stock and the 1/100 diluted aliquots used to make the master mix at −80 °C. The reagent was diluted in TE buffer, pH 8.0, as suggested elsewhere (Karsai et al., 2002). More than two freeze-thawing cycles of the 1/100 diluted SYBR Green I aliquots resulted in a significant loss of assay sensitivity (not shown). The assay sensitivity and strength depend on the SYBR Green I concentration. The effects of different dilutions of the dye ranging from 1/60,000 to 1/10,000 (Fig. 6b) were tested. The 1:20,000 dilution was chosen because it combines high sensitivity with a robust signal.

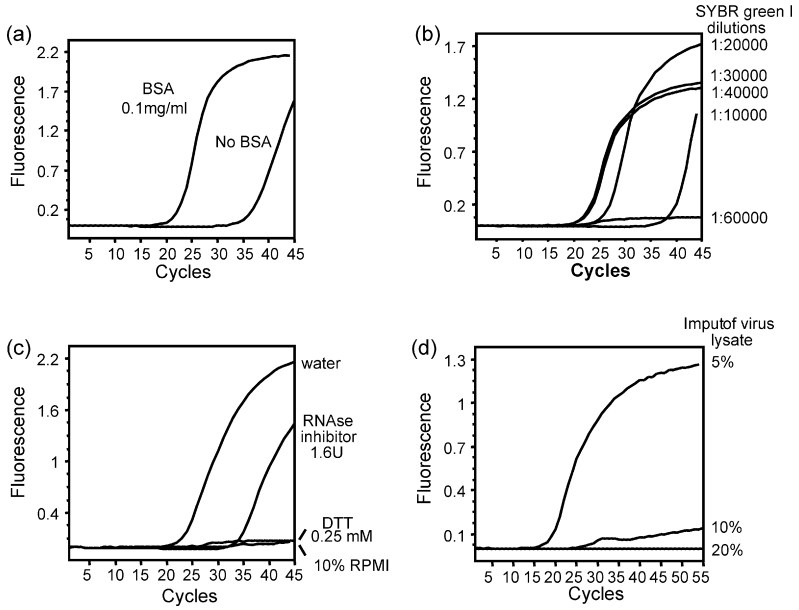

Fig. 6. Crucial effects of different reagents on the assay performance. (a) Positive effect of BSA on the SG-PERT reaction. (b) Performance of the assay with varying SYBR Green I concentrations, as indicated. (c) Inhibitory effects of different components of the virus lysate. (a), (b) and (c) were obtained by testing 1 μU of recombinant HIV-1 RT. (d) Effects of varying amount of virus lysate on the assay performance using HIV-1 NL4-3 containing supernatant. Percentages in (c) and (d) indicate the reagent volume as a proportion of the total reaction volume.

6.1.4. The effect of different components of the virus lysate on the outcome of the assay

After optimising the reaction using recombinant HIV-1 RT, based on previously described protocols (Heneine et al., 1995; Lovatt et al., 1999; Maudru and Peden, 1998; Pyra et al., 1994; Yamamoto et al., 1996), the assay was tested with a suspension of HIV-1 virions lysed in a buffer supplemented with 0.2 U/μl RNAse inhibitor, 1 mM DTT. The assay repeatedly failed to generate any signal, indicating that the virus lysate could contain inhibitory reagents. We tested the effects of single components of the virus lysate on the SG-PERT efficiency using 1 μU of recombinant HIV-1 RT (Fig. 6c). We established that 0.1 mM DTT acts as a powerful inhibitor, in line with previous reports indicating a negative interference with SYBR Green I-based RT-PCR (Lekanne Deprez et al., 2002; Pastorino et al., 2005; Pierce et al., 2002; Varga and James, 2005). The presence of RPMI culture medium in the reaction was also found to affect negatively the assay when it represented 10% of the final reaction volume. Similarly, when 1.6 U of the RNAse inhibitor was present in the 20 μl reaction the assay failed. In contrast, other components of the virus lysates did not significantly affect the performance of SG-PERT. While DTT can be omitted from the RT reaction without loss of enzyme activity (data not shown and Lekanne Deprez et al., 2002), the inhibitory effects of culture medium and RNase inhibitor were avoided by minimizing the amount of virion lysate added to the reaction mix. Given that the presence of RNAse inhibitor is strictly essential, its negative effects were minimized by treating the virion lysate (which represents the main source of RNAses) rather than the total reaction with a concentrated formulation of RNase inhibitor. A robust signal was, therefore, obtained only when the virus lysate was not exceeding 5% of the final reaction volume (Fig. 6d).

6.1.5. Divalent cations

Since efficient reverse transcriptase activity is known to require Mg^{2+}, $MgCl_2$ concentrations ranging from 1.25 to 10 mM were tested and found to have no significant effects on the assay sensitivity (not shown). Although gamma-retroviruses are known to prefer Mn^{2+}, addition of 2 mM $MnCl_2$ to the reaction assay resulted in 100-fold lower efficiency of detection of MLV virus suspensions (not shown).

6.1.6. Absolute quantitation of virus suspensions

For experimental purposes, absolute values of the virus suspensions tested can be retrieved from a standard curve created using known amounts of recombinant RT or serial dilutions of a previously characterized virus stock.

6.1.7. Improved primer design

An alternative set of oligonucleotides (forward: 5′-TAGTTGTTGGGCTTCGCTTT-3′; reverse:5′-TTGTCGGCTTTACCTGCTTT3′) was designed to avoid primer-dimer formation with SG-PERT under the same conditions described in the article. While this modification does not alter the properties of the assay, it further improves the signal generated by low levels of RT activity.

Essential literature references:

- Arnold et al. (1998)
- Chang et al. (1997)
- Lovatt et al. (1999)
- Maudru and Peden (1998)
- Pyra et al. (1994)
- Silver et al. (1993)
- Sears and Khan (2003)

7. Quick procedure

7.1. Sample preparation

- Virus supernatants are harvested, clarified by low-speed centrifugation (400 g) for 10 min and filtered through a 0.45 μm pore filter.
- RNAse inhibitor (0.4 U/μl) is added to 2× virus lysis buffer.
- 5 μl aliquots of 2× lysis buffer are immediately dispensed into 1.5 ml microfuge tubes.

- 5 μl of virus suspensions are added to the tubes and mixed with the 2× lysis buffer.
- Samples are incubated at room temperature for 10 min.

7.2. Reaction set-up (for lightcycler)

- Taq polymerase (0.2 U/reaction) is added to 2× reaction mix.
- Ten microliters aliquots of 2× reaction mix are placed into the reservoir of pre-chilled glass capillaries.
- Virus lysates (10 μl) are diluted with the addition of 90 μl of sample dilution buffer and vortexed.
- Ten microliters of each diluted sample is immediately mixed with the reaction buffer in glass capillaries.
- Capillaries are centrifuged in a microfuge at 3000 rpm for 15 s and inserted into the LightCycler carousel.
- Reaction is performed in the LightCycler using the following parameters:
  - o 30 min RT reaction at 37 °C.
  - o 5 min hot-start Taq activation at 95 °C.
  - o 45 cycles of amplification (5 s denaturation at 95 °C, 5 s annealing at 55 °C, 15 s extension at 72 °C, 7 s acquisition at 83 °C).
  - o Optional melting analysis of PCR products from 60 to 95 °C.

7.3. Data analysis

- Arithmetic background correction and automatic noise band adjustment are performed.
- The maximum number of fit points fitting the linear portion of the curve is added for the calculation of crossing points.
- A standard curve may be obtained using known concentrations of recombinant RT or defined dilutions of a virus suspension to allow absolute quantitation or data comparison between experiments:
  - o A minimum of four standard samples are included in the RT assay.
  - o The threshold line for crossing point calculation is automatically defined by the software in order to minimize errors of the standard curve.

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