Real-time RT-PCR detection of retroviral contaminations of cells and cell lines

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Abstract

We have developed a fast and sensitive on-line detection method for retroviruses using the PCR technology. The assay utilizes the endogenous reverse transcriptase activity in retroviral particles. In the presence of active reverse transcriptase, bacteriophage MS2 RNA is transcribed into cDNA and is subsequently amplified in a SYBR-Green-type LightCyclerTM reaction. The method allows a qualitative and quantitative monitoring of RT-activity, is several orders of magnitude more sensitive than a standard RT assay and has a time requirement of 2.5 hours from harvest to result. The method is useful for monitoring of cells and cell-derived products, viral vectors and recombinant proteins for the presence of replication-competent retroviruses (RCRs).

Introduction

Several methods have been described for the simple and general detection of retroviruses that make use of the presence of the enzyme reverse transcriptase and its ability to transcribe RNA into cDNA. Among these, radioactive and non-radioactive versions of the 'RT-assay' have found routine use in monitoring for the presence of reverse transcriptase (Goff et al., 1981; RT-ELISA (Roche)). In the last decade, the PCR technique has allowed the introduction of an amplification step and increasing the sensitivity of retroviral nucleic acid (Heinemeyer et al., 1997; De Wit et al., 2000) or reverse transcriptase (RT)-based assays considerably. Early RT based-PCRs, e.g., the 'PERT' assay, combined the DNA amplification step with hybridisation detection (Silver et al., 1993; Pyra et al., 1994). Several improvements have made the RT-PCR based, qualitative detection method more sensitive and reliable. Such improvements encompassed substituting steps for gel detection (Chang et al., 1997) and eliminating false-positive results caused by the endogenous, non-retroviral RT-activity exhibited by certain polymerases (Tönjes et al., 1996; Chang et al., 1997; Maudru and Peden, 1997). Recently, on-line detection

methods have reduced PCR time requirements and enabled the non-radioactive, rapid, quantitative determination of retroviral load in samples. The original assay has been successfully adapted to the Taqman probe format (Perkin Elmer) combined with detection using an ABI 7700 system ('F-PERT') (Lovatt et al., 1999; Arnold et al., 1998). However, for optimum performance training in these methods is necessary. Furthermore, consumables such as buffers and dye-labeled primers are costly and, due to the detection format, the method is sensitive to endogenous DNase activity. More simple formats make use of the incorporation of fluorescent dyes during the elongation step. Based on the incorporation of the fluorescent dye SYBR-Green into DNA, we have developed an alternative retrovirus detection system utilizing the LC-technology. This method has certain advantages compared to current methods.

Materials and methods

Cell lines

NIH3T3 MLV-A and BHK MLV-A are cell lines stably transfected with an amphotropic 4070A provirus vector (Beer et al., submitted). Sp2/0 Ag14 (ACC 146, mouse myeloma), X63Ag8.653 (ACC 43, mouse myeloma), BLV044 (ACC 153, ovine embryonal kidney cells) and AM C6SC8 (ACC152, pig kidney cells) were supplied by DSMZ, Braunschweig (German collection of microorganisms and cell cultures). If not otherwise indicated, cells were propagated as detailed by the supplier. NIH3T3, BHK-A and BHK-B cells were cultivated in DME containing 10% FCS (Bio Whittaker) and 2 mM glutamine. Sp2/0 cells were in Iscove's medium containing 2 mM glutamine and 7.5% FCS. AMC6SC8 were kept in medium 199 (Hank's salts) and 10% FCS. BHK-A and B are hamster BHK21 subclones differing in their glycosylation potential (Karreman et al., 1996). PA317, GPAM12, OmegaE, FlyA13 are packaging cell lines derived from mouse NIH3T3 or human HT1080 cells (FlyA13) (Miller and Buttimore, 1986; Markowitz et al., 1988; Morgenstern and Land, 1990; Cosset et al., 1995). The cells were stably transfected with MLV retroviral vectors and propagated in DME supplemented with 10% FCS and 2 mM glutamine.

Enzymes

Suppliers of enzymes were as follows: Taq Polymerase (AmpliTaQ, Perkin Elmer), AMV reverse transcriptase (Pharmacia), HIV-1 transcriptase (Roche Diagnostics), MLV reverse transcriptase (Superscript, GIBCO BRL).

LightCycler RT-PCR for retroviruses (LC-RT-PCR)

Outline of the real-time PCR assay for retrovirus detection (LC-RT-PCR)

The assay is based on a reverse transcription of RNA, followed by a specific cDNA amplification step (Figure 1a). As template serves the RNA from bacteriophage MS2. Since the bacteriophage is known to exclusively replicate via RNA intermediates, the choice of MS2 as template circumvents the problem of introducing DNA accidentally into the reaction via impure RNA preparations. Provided that viral reverse transcriptase is present in the reaction, RNA is transcribed into cDNA via a MS2 specific primer. Subsequently, MS2-specific primers are used to amplify the cDNA in

the presence of SYBR-Green, a double-stranded DNA fluorescent dye, in a LightCycler™ (Roche). Fluorescence measurement is performed at the end of each cycle.

Sample pretreatment

Supernatants from cell lines were cleared by filtration through 0.45 µm filters (SpinX, Costar). 20 µl of supernatant were combined with 20 µl 1% TritonX100, cooled to -70 °C and treated to two thaw/freezing cycles (37 °C/-70 °C).

The reaction is a two-step procedure consisting of a reverse transcription and a subsequent DNA amplification step in a LightCycler™ (Roche).

Reverse transcription: 4 µl of pretreated samples (or dilutions of reverse transcriptases in H₂O) were used for a transcription reaction in a total volume of 25 µl containing 10 mM Tris/HCl pH 8.3, 2.5, 50 mM CaCl₂, 0.1 mM MnCl₂, 5 mM DTT, 20 U RNasin (Promega), 0.4 mM dNTP (Pharmacia), 100 ng MS2 RNA (Roche), 25 pmol primer MS276 (5'-AGTGCCACTGTTTCGTTTTG-3') at 37 °C for 30 min (RT mix).

PCR amplification: PCR-amplification was performed in a LightCycler™ (Roche) using 4 µl of the RT mix, 2 µl of DNA master SYBR-GreenI reaction mix (Roche), 0.16 µl TaqStart-antibody (Clontech), 0.4 µl primer mix (12.5 pmol MS276, 12.5 pmol MS175 (5'-CCCTCCGTCGCGTTTA-3'), 5 mM MgCl₂ and H₂O to 20 µl. Initial denaturation for 2 min at 95 °C was followed by 50 cycles of DNA amplification (denaturation 94 °C 1 sec, annealing 55 °C 10 sec, elongation 72 °C 10 sec) with fluorescence detection at the end of the elongation step (SYBR Green format). Data evaluation was performed using LightCycler software (Version 3.0) according to the recommendations of the supplier.

Other retrovirus detection methods

MLV specific 'strong stop cDNA PCR test' originates from Towers et al. (1999) and was adapted for use with SYBR-Green and LightCycler™ technology. Comparable results were obtained when either format was used (LightCycler™ SYBR-Green or Taqman). The standard, non-radioactive RT-ELISA (Roche Diagnostics) was performed as described by the supplier.

Titer assay

One day prior to infection NIH3T3 indicator cells were seeded into 24 well plates (3×10^3 cells cm⁻²).

Real time detection of retroviruses RT-PCR (qualitative and quantitative)

(a)

(b)

Figure 1. (a) Schematic drawing of the real-time RT-PCR procedure (LC-RT-PCR). (b) Histogram depicting product accumulation (fluorescence intensity) versus PCR cycle number in reactions starting with different concentrations of mouse leukemia virus reverse transcriptase. 1: 1.5×10^{-1} ; 2: 1.5×10^{-2} ; 3: 1.5×10^{-3} ; 4: 1.5×10^{-4} ; 5: 1.5×10^{-5} units MLV reverse transcriptase/reaction.

Cells were infected in the presence of $8 \mu\text{g ml}^{-1}$ Polybrene with filtered ($0.45 \mu\text{m}$, SpinX, Costar), serially

diluted supernatants from infected and mock-infected cells. Medium was replaced by fresh medium 1 day

Table 1. Sensitivity of LC-RT-PCR. The sensitivity is several orders of magnitude higher than a conventional RT-ELISA

Probe	Detection limit LC-RT-PCR (U/reaction)	Detection limit RT-ELISA (U/reaction)	Viral particles (LC-RT-PCR)
MLV-RT	1.5×10^{-5}	n.d. ^a	15000–75000
HIV-1-RT	1.0×10^{-7}	1×10^{-2}	960–4800
AMV-RT	1.4×10^{-6}	n.d. ^a	1000–5000

^a n.d. = Not determined.

later and changed to selective medium (1 mg ml⁻¹ G418) 2 days after infection. Ten days after infection surviving clones were stained with crystal-violet and counted. Titers are presented in colony-forming units per million cells and day (cfu/Mio cells d).

Results and discussion

Feasibility and sensitivity

The LC-RT-PCR reaction (Figure 1a, outlines in the Material and Methods section) was optimized with respect to magnesium chloride concentration, annealing temperatures and RNA concentration (data not shown).

In order to investigate the feasibility of the assay serial dilutions of MLV-reverse transcriptase were used for real-time RT-PCR detection. Figure 1b depicts results from such an experiment. Fluorescence intensities (product accumulation) are plotted versus the cycle number. The fluorescence signal is proportional to the concentration of newly synthesized DNA. More important, intensities reflect the amount of input reverse transcriptase. The problem of dimer formation by the primers becomes prominent at high cycle numbers and low template amounts. In our assay, the product melting point (84 °C) was clearly different from primer dimer melting peaks. Therefore, the specific product could be verified by 'melting point analysis'.

For evaluation of the sensitivity of the assay, serial dilutions of MLV-RT and several other available reverse transcriptases were subjected to real-time LightCycler RT-PCR. Detection limits are depicted in Table 1. As low as 10^{-7} units/per reaction of reverse transcriptase could be detected in the case of HIV-1 and 10^{-6} units ml⁻¹ in the case of avian leukemia viruses. Given the specific sensitivities of the reverse transcriptases as detailed by the supplier and provided

that 25 to 100 reverse transcriptase molecules reside inside one viral particle, the number of physical particles detected is in the range of 1000–5000 per reaction. It is known that only a small portion of retroviral particles is actually infectious. In the case of MLV from NIH3T3 cells infectious particles represent only 0.5–1% of the total particles released (Anderson, 1983; Beer et al., in preparation). This means that our assay is able to detect at least 2–10 infectious particles. For comparison, the limit of the non-radioactive RT-test performed with serial dilutions of HIV-1 reverse transcriptase (Roche) are included in Table 1. Despite the fact that the reaction time of the RT-ELISA has been increased to 2h, which is recommended by the supplier in the case of low viral titers, the LC-RT-PCR is two orders of magnitude more sensitive than the RT-test and considerably faster (2.5 versus 8 h).

Reproducibility and linearity

To evaluate the accuracy of the assay, dilutions of MLV RT were submitted to LC-RT-PCR. MLV-RT concentrations were plotted versus the 'cycle number', which represents the crossing point of the linear range line of the amplification curve with the base line. The variation coefficients were calculated for triplicate samples from 8 independent experiments (Figure 2). The results demonstrate that the coefficient of variation is in the range of 2–4%. Furthermore, the linear range of the assay encompasses four orders of magnitude. The combination of reproducibility and linearity make the assay useful for quantitative determination of RT activity in virus supernatants.

Specificity

Specificity of RT-PCR was investigated by incorporation of different polymerases or cell extracts into the reaction. It is known for instance that mitochondrial DNA polymerase gamma exhibits low RT activity and may account to false positive results if cell extracts are used for the investigation. No cross-reactions were observed when different polymerases like Taq polymerase, *E. coli* DNA polymerase, Klenow polymerase or T4 DNA polymerase, were used in the RT-PCR. Inclusion of extracts of BHK cells into the reaction did not result in a signal. Artificially occurring or inherent cellular RT activities have been reported to interfere with RT-PCR procedures (Chang et al., 1997). However, they do not represent a severe problem as such RT-like polymerase activities can be suppressed by lowering the pH of the reaction buffer from 8.3 to 5.5

Figure 2. Reproducibility and linearity range of the LC-RT-PCR MLV-RT concentrations are plotted versus the 'cycle number' (crossing point of the extrapolated linear range of the amplification curve with the base line). The coefficient of variation of the assay is indicated in the histogram and ranges at levels between 2–4%. The assay is linear at least in the range of four orders of magnitude.

(Chang et al., 1997) or including nicked DNA as decoy in the RT-PCR (Lugert et al., 1996).

Supernatants of cell lines

To investigate the practicability of the assay, dilutions of supernatants from different cells that contained either replication competent retroviruses or transducing retroviral vectors were submitted to LC-RT-PCR (Table 2). Additionally, the viral infectivity of the supernatants was determined by titer assays. Dilution factors indicate the detection limit achieved with LC-RT-PCR. The detection limits (number of infectious viruses ml^{-1}) were derived from titers and dilution factors.

In addition, supernatants of cell lines derived from cell culture banks with known retroviral status (reverse transcriptase positive/negative) were investigated with LC-RT-PCR for the presence of reverse transcriptase activity. Supernatants of cell lines originating from mouse (myelomas Sp2/0 Ag14; X63Ag8.653), sheep (FLK-BLV044) and pig (AM C6SC8) were investigated. Supernatants of one mouse myeloma (X63-Ag8.653) and the ovine cell line achieved positive signals in LC-RT PCR. At the time of purchase X63Ag8.653 were claimed to be RT-negative by the supplier, but were suspected to contain active retro-

viruses (Deo et al., 1994; Froud et al., 1997) and were also tested positive in a later revision by the supplier. The ovine cell line is reported RT-positive, the pig cell line is suspected to contain endogenous porcine retroviruses (Table 3). Interestingly, supernatants of Sp2/0 cells exhibit negligible RT-activity in the LC-RT-PCR. Interestingly, irrespective of their origin Sp2/0 cells are known to release huge amounts of retroviral particles, albeit with negligible infectivity (Deo et al., 1994; Heinemeyer et al., 1997). Therefore, a strong-stop cDNA PCR specific for MLV type retroviruses (adapted to the LightCycler format) was used to investigate cellular supernatants for the presence of MLV type nucleic acids (Towers et al., 1999). The assay confirmed the presence of MLV strong stop cDNA in both myelomas as well as in the infected controls and, as expected, was negative in the ovine and porcine cell supernatants (data not shown).

Conclusions

A sensitive on-line RT-PCR for the general detection of retroviruses was developed on the basis of the LightCyclerTM technology (LC-RT-PCR). The method is simple and allows access to results within

Table 2. Analysis of supernatants of virus producing cell lines. Virus characteristics: transducing ⁽¹⁾; replication competent ⁽²⁾; amphotropic ⁽³⁾; ecotropic ⁽⁴⁾

Producer virus	Titer (cfu/Mio cells d)	Viruses ml ⁻¹	Dilution factor	Calc. infect. virus ml ⁻¹
PA317 ^(1,3)	1.00×10^4	8.00×10^3	30000	0.3
Omega E ^(1,4)	2.00×10^5	4.38×10^4	1000	43.8
BHK-A ^(2,3)	3.52×10^4	5.00×10^4	800	62.5
BHK-B ^(2,3)	6.52×10^2	1.00×10^3	400	2.5
FlyA13 ^(1,3)	1.26×10^5	2.07×10^4	4000	5.2
GPAm12 ^(1,3)	1.04×10^5	5.21×10^4	20000	2.6

Table 3. Analysis of supernatants from established cell lines for reverse transcriptase activity

Cell line	Type	Status ^a	LC-RT-PCR (U/probe)	cDNA ^b (copies ml ⁻¹)
Sp2/0 Ag14	Mouse myeloma	RT-neg.	4.28×10^{-5}	7.42×10^7
X63Ag8.653	Mouse myeloma	RT-neg.	4.12×10^{-3}	3.96×10^8
FLK-BLV044	Ovine	RT-pos.	3.10×10^{-3}	0
AM C6SC8	Porcine	RT-pos.	4.40×10^{-5}	0
BHK-MLV-A	Hamster	RT-pos.	2.44×10^{-3}	1.83×10^7
NIH-MLV-A	Mouse	RT-pos.	2.04×10^{-3}	6.54×10^6

^a Status reported or tested at the time of purchase.

^b Determined using the strong stop cDNA assay adapted from Towers et al. (1999).

2.5 h. LC-RT-PCR is specific for retroviral reverse transcriptases. LC-RT-PCR exhibits high reproducibility and can be used to quantitate reverse transcriptase activity of virions from cellular supernatants. LC-RT-PCR is useful for determination of viral load in cell supernatants, spiking experiments for validation studies of purification processes and for screening retroviral cell lines for high-level virus-releasing subclones.

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