Standardized detection of Simian virus 40 by real-time quantitative polymerase chain reaction in pediatric malignancies

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Background and Objectives. Recent studies have detected Simian virus 40 (SV40) DNA in specific human tumors albeit with significant discrepancies in frequency. Possible inefficiency of DNA isolation and different detection methods do not allow comparable and precise quantification of SV40 in human samples. A standardized and common detection method is, therefore, essential for further routine SV40 analysis.

Design and Methods. We established a real-time quantitative (RQ-) polymerase chain reaction (PCR)-based TaqMan assay on the LightCycler system for reproducible detection and quantification of SV40 DNA. We used 500 ng of the COS-1 cell line, containing one single integrated copy of SV40 DNA, as the quantification standard. Amplification of β-globin served as the quality control for DNA integrity. For the extraction of the episomal form of SV40 we compared a column and a precipitation-based DNA extraction method. DNA samples from 149 healthy controls, from 26 fresh frozen childhood cases of acute lymphoblastic leukemia (ALL) (B-cell, B-cell precursor and T-cell ALL) and from 12 paraffin-embedded osteosarcomas were investigated.

Results. The RQ-PCR assay had a linear amplification rate from 10 to 100,000 copies of SV40 in 500 ng of genomic DNA. Very low copy numbers of SV40 DNA were detectable in 2/149 (1.3%) blood samples from healthy German controls. Various amounts of SV40 were detectable in 20/26 (77%) childhood ALL samples of German origin and, in part, high amounts were visible in 11/12 (92%) paraffin-embedded Hungarian osteosarcomas. The column-based DNA isolation method allowed the detection of both the integrated and the episomal forms of SV40.

Interpretation and Conclusions. Our assay provides a standardized and reproducible quantification of SV40 DNA in a wide spectrum of specimens. Exact quantification strongly depends on the source, as well as on the quality, of the DNA used. Quantification of paraffin-embedded DNA generally leads to lower sensitivity of SV40 DNA detection. We strongly recommend this RQ-PCR assay for standardized detection of SV40.

Key words: Simian virus 40 (SV40), large T-antigen (TAG), real-time quantitative PCR, childhood lymphoma, osteosarcoma.

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Simian virus 40 (SV40) is a small DNA polyomavirus of monkey origin, known to possess strong oncogenic potential. It was an unrecognized contaminant of polio vaccines and therefore inadvertently distributed to millions of people between 1955 and 1963.[1] Soon after its discovery it was shown that it can infect and transform human and animal cells *in vitro*.[2,3] When injected into hamsters, SV40 can induce several types of tumors, mainly mesotheliomas, brain-, and bone-tumors and lymphomas.[4,5] The spectrum of tumors associated with SV40 in humans is similar. Its oncogenic potential is thought to be associated with the primary viral gene product, large T-antigen (TAG), responsible for SV40 replication and SV40-mediated cell transformation. TAG is known to promote transformation by binding and inactivating the products of several tumor suppressor genes such as p53 and pRB.[6,7]

There is an increasing number of reports of SV40 DNA being found in specific types of human tumors, and in human lymphoproliferative disorders,[8-15] as well as in lymphoblastoid B-cell lines,[16] although the incidences are somewhat controversial. In part this could be explained by the very low copy number of SV40. DNA extraction methods, possibly not efficient for isolating the small molecular weight episomal form of SV40, as well as differences in DNA quality, differences in virus detection, and different types of specimens investigated, all hamper a controlled analysis. Further difficulties are the use of DNA isolated from paraffin-embedded tissue, possible loss of viral episomes during DNA isolation, loss of detection specificity due to the low copy number of viral DNA, as well as a reduced amplification rate during polymerase chain reaction (PCR). Among all methods used to identify SV40 in

human specimens, the PCR is the preferred and best described.[17] Filter hybridization and sequencing confirmed the authenticity of the SV40 sequences. Although the sensitivity of the various SV40-detection methods and the importance of a suitable extraction method for episomal DNA have often been discussed, so far there is not a standardized method for detecting and quantifying SV40.[18] Since lymphomas and osteosarcomas are two of the four main tumor types experimentally induced by SV40 in rodents, we investigated specimens of these malignancies in order to develop an efficient and reliable real time quantitative (RQ)-PCR-based TaqMan assay on the LightCycler (LC-) System for the detection of SV40 TAG sequences. To achieve the highest accuracy we used (i) control gene amplification for quality control of each sample and (ii) a SV40 based standard curve for quantification.

We quantified DNA samples derived from different types of specimens (fresh or frozen peripheral blood or bone marrow, paraffin-embedded tumors, fresh buffy coat). Additionally we used this RQ-PCR assay to demonstrate that a silica-based DNA extraction method detects both episomal and integrated SV40 and should be preferentially used for routine SV40 research.

Design and Methods

Subjects and specimens

Fresh or frozen samples from bone marrow (BM) or peripheral blood (PBL) from 26 children with acute lymphoblastic leukemia (ALL) (14 had B-ALL, 7 had B-cell precursor (BCP)-ALL, and 5 had T-ALL), from different regions of West Germany, and 12 paraffin-embedded osteosarcoma specimens of Hungarian origin (different regions), were analyzed at the time of diagnosis. The samples contained between 50 and 90% malignant cells. BCP-ALL and T-ALL were provided by different Cooperative ALL (COALL) study group participating clinical centers. One hundred and forty-nine fresh buffy coats from healthy German people derived from the blood transfusion center of the University Hospital in Hamburg were also analyzed. Two cell lines served as SV40-positive controls: SV80 (human skin fibroblast cells)[19,20] carrying a duplication of the 5´portion of the early region, one of which with a point mutation in the coding region, and COS-1, an African green monkey kidney cell line with one integrated copy of the complete early region of SV40 including its transcriptional control region. COS-1 was well described by Gluzmann[21] as having a single SV40 copy and was therefore chosen for the standard. Serial ten-fold dilutions of COS-1 cell line DNA, mixed with SV40-negative DNA from healthy controls, was used to create a standard curve. This mixing guarantees a quantity of 500ng DNA in each dilution step. DNA derived from SV40-negative healthy people as well as DNA derived from normal bone served as negative controls.

DNA extraction

Genomic DNA was extracted from fresh or frozen cells (1×10^7 cells for each sample) by two different isolation methods: (i) using Quiagen silica gel-based spin columns (QiaAmp DNA blood mini/midi kit; Quiagen, Hilden, Germany) according to the *blood and body fluid protocol*, and (ii) a precipitation-based method using the Promega Wizard Genomic DNA Purification kit (Promega, Madison, Wi, USA) according to the supplier's recommendations. DNA from paraffin-embedded tissues (5 pieces of 10 μm for each sample) was isolated using the spin column method. Paraffin pieces were first deparaffinized with xylene and then washed twice with ethanol prior to DNA spin column extraction according to the *QiaAmp tissue protocol*.

Real-time quantitative PCR

Amplification primers were selected for the retinoblastoma (Rb-) pocket binding domain of SV40-TAG: forward primer (F^+) GGGTCTTCTAC-CTTTCTCTTCTTT (nt 4414-4437) and back primer (A) GCAGTGGTGGAATGCCTTT (nt 4524-4542). The resulting 128bp PCR product is SV40-specific, since it lacks a 9bp insert at nt 4517, which is present in BKV and JCV sequences, and it differs therefore in nucleotides from the two human polyoma viruses.[22] The internal TM probe AACCTGTTTTGCTCA-GAAGAAATGCCA (nt 4488-4514) was labeled with the fluorescent reporter dye FAM (6-carboxyfluorescin) at the 5' end and the quencher dye TAMRA (6-carboxytetramethylrhodamine) at the 3' end (TibMolBiol, Berlin, Germany). The Tm of the probe (64.3°C) is at least 5°C higher than the annealing/extension temperature used for PCR, ensuring probe binding to the target template during the extension phase of the reactions. The integrity and quality of the extracted DNA was tested by amplification of the β-globin gene with an internal TM-probe. Only samples with CT values of 20±1 cycles (25±1 cycles for DNA samples extracted from paraffin-wax) were selected for quantification. The RQ-PCR reaction conditions were adapted from those described by zur Stadt *et al.*:[23] 500 ng template DNA, 1U Platinum Taq DNA polymerase (*hot start*, Invitrogen), 2 μL 10× buffer, 5 mM $MgCl_2$, 200 μM of each dNTP, 100nM TaqMan probe, 5 μg bovine serum albumin (BSA), 500nM each primer, and 5% DMSO in a 20 μL reaction. For RQ-PCR of DNA derived from paraffin-embedded tissue 2 units of Taq DNA polymerase were used and the DMSO was omitted. The amplification conditions were as follows: 5 min ini-

tial denaturation at 94°C followed by 50 cycles with 8 s denaturation and 23 s annealing with an annealing temperature of 60°C. The SV40-positive cell line (COS-1) DNA was serially diluted in SV40-negative DNA to produce equal amounts in each dilution sample (500 ng per reaction). The standard curve for paraffin-embedded material was generated from paraffin-embedded SV40-positive cell line DNA. Several RQ-PCR reactions were performed with 6 dilutions (10^{-1} to 10^{-6}), an unrelated SV40-negative DNA control, a negative control and the sample of interest in duplicate or triplicate. SV40-positive ALL samples were additionally analyzed by agarose gel electrophoresis, extracted and purified (*Ultrafree-DA* column; Millipore, Eschborn, Germany) and finally sequenced with the primers used for amplification (ABI Prism BigDye terminator cycle sequencing kit).

Results

Sensitivity and reliability of the RQ-PCR assay

We developed a real time quantitative PCR-based assay for the detection and quantification of SV40 TAG sequences and applied it to evaluate SV40 in a variety of human specimens (fresh, frozen and paraffin-embedded), as well as to different malignancies (lymphoproliferative disorders and osteosarcomas). Our system provides highly precise and reproducible detection and quantification of SV40 over a wide linear range with a minimum detection of 1 copy number per reaction (input of 500 ng sample DNA). The standard curve, with ten-fold serial dilutions of the COS-1 control cell line, shows a reproducible range down to 10 copies of SV40 in 500 ng genomic DNA (Figure 2a and 2b).

Evaluation of the DNA extraction method

The SV40 genome contains 5243 bp[24] either episomal or integrated into the host genome. In the light of the low copy number of SV40 associated with human tumors, as previously reported,[25,26] the method used for extracting the small episomal form of SV40 DNA was considered before PCR set-ups. Therefore we initially prepared 8 B-ALL and 12 T-/BCP-ALL samples, as well as the COS-1 and SV80 cell lines, with two different DNA-isolation protocols. Only the silica gel-based method (QiaAmp DNA mini kit) yielded detectable amounts of all SV40 forms during RQ-PCR analysis (*data not shown*). Several other authors have reported this, but they have not applied it to PCR set-up and analysis.[27,28]

SV40 in childhood samples

Patients' DNA extracted with spin columns had detectable amounts of SV40 (range 10 to 100 SV40 copies per reaction) except 6/26 childhood ALL and 1/12 osteosarcoma (Table 1). We found weak SV40 positivity (10 copies or less) in 2 out of 149 control samples, derived from healthy people and various amounts of SV40 in 20 out of 26 childhood ALL samples. High amounts of SV40 of 10 copies or more were detectable in 11/12 osteosarcoma specimens of Hungarian origin.

Discussion

Our assay represents a standardized method for rapid and reliable detection of SV40 TAG sequences in a variety of human specimens. To our knowledge this is the first report presenting a suitable technique for sensitive and reproducible detection of SV40

Table 1. Detection of SV40 sequences in childhood malignancies and healthy control samples. Quality control was performed on all samples with β-globin specific primer.

Samples	*Type of specimens*	*N.*	*SV40 (pos/all)*	*SV40-quantity (copy no./500 ng DNA)*		
				>100	*10 to 100*	*<10*
Childhood B-ALL	Frozen bone marrow or blood	14	12/14	1	6	5
Childhood BCP-ALL	Frozen bone marrow or blood	7	6/7	1	2	3
Childhood T-ALL	Frozen bone marrow or blood	5	2/5	1	1	
Healthy German population	Fresh buffy coat	149	2/149	–	1	1
Normal bone (German)	Fresh bone biopsies	1	0/1	–	–	–
Osteosarcoma (Hungarian)	Paraffin embedded tissue	12	11/12	7	4	–
COS-1-control-cell line	Fresh cells	1	1/1	1	–	–
SV80-control-cell line	Fresh cells	1	1/1	1	–	–

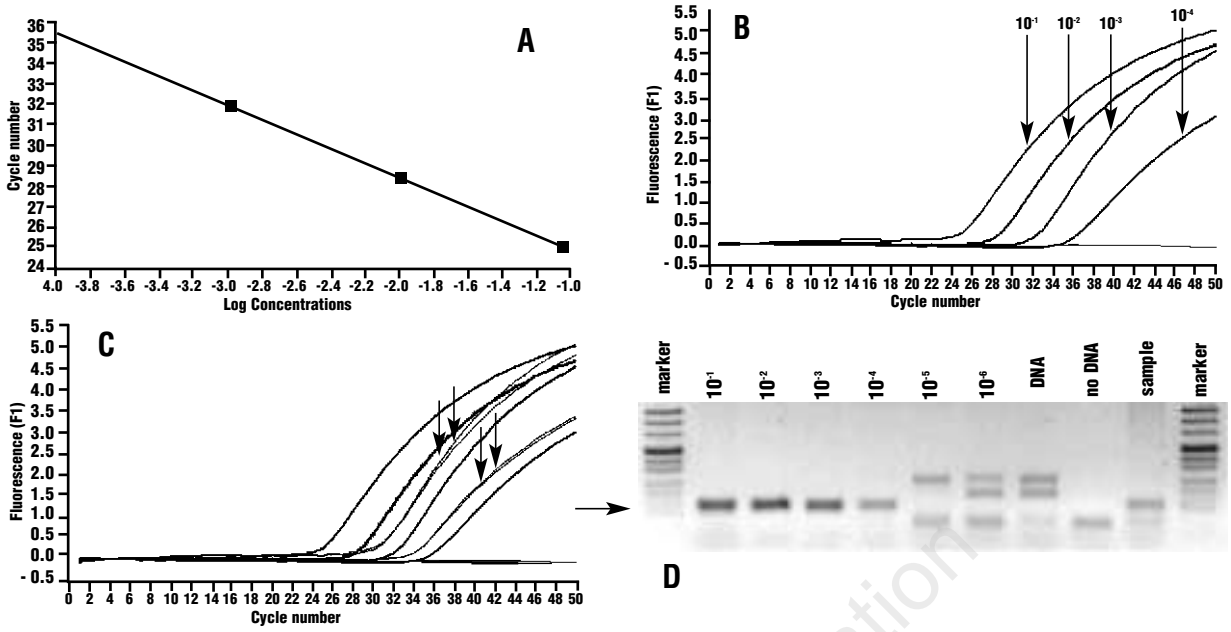
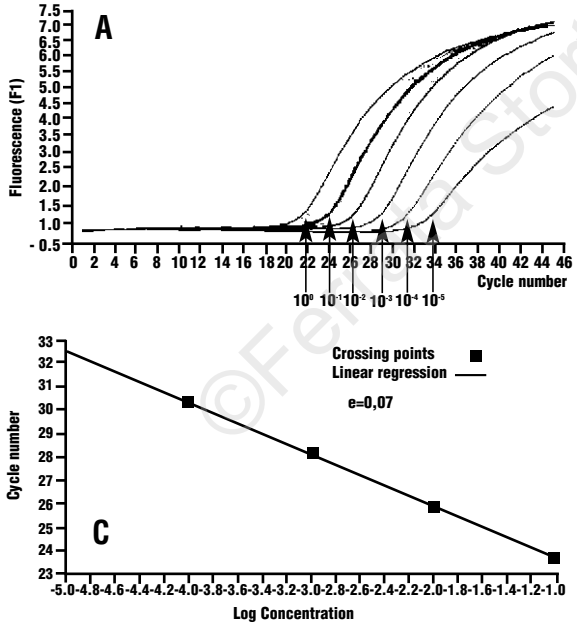


Figure 1. Real-time quantitative LC assay for the detection of SV40 sequences. A. Standard curve for the detection of unknown quantities, e = 0.049; B. Serial 10-fold dilutions of SV40-positive SV80 control DNA with SV40-negative DNA. C. Quantification of two unknown samples measured in duplex reactions (arrows); D. Agarose gel electrophoresis from products amplified by RQ-PCR. Positive samples were cut out of the gel and sequenced. In all cases SV40 was clearly identified.



B

Cycle number	Dilution step	copies/500ng DNA
21	10^{0}	10^{5}
24	10^{-1}	10^{4}
26	10^{-2}	10^{3}
28	10^{-3}	10^{2}
30	10^{-4}	10^{1}
33	10^{-5}	10^{0}

Figure 2. Real-time quantitative LC assay for detection and quantification of SV40 sequences. Calculation of SV40 quantity using serial 10-fold dilutions of COS-1 control cell line. A: Serial 10-fold dilutions of SV40-positive COS-1 control DNA with SV40-negative DNA. Arrows indicate the C_T values of the dilution steps. B: Quantification of SV40: reproducible correlation of cycle numbers of the serial dilution of COS-1 cell line DNA and SV40 copy numbers. C: Standard curve for detection of unknown quantities of the investigated samples, e = 0.07.

sequence in DNA from of paraffin-embedded specimens (Figure 3). Although we developed this assay for the SV40-Tag region, it can easily be extended to other regions of the SV40 genome. The use of 500ng DNA per reaction and the exact quantification with a minimum input of 1 copy per reaction, as well as the routine amplification of a control gene allow sensitive detection of SV40 sequences in a wide range (Figures 1 and 2). When trying to detect low copy numbers it is essential to check the DNA quality not only by an end point detection of a PCR product (i.e. agarose gel electrophoresis) but also by RQ-PCR, measuring products in the log-phase of the amplification. SV40 detection rates down to 1 copy of SV40

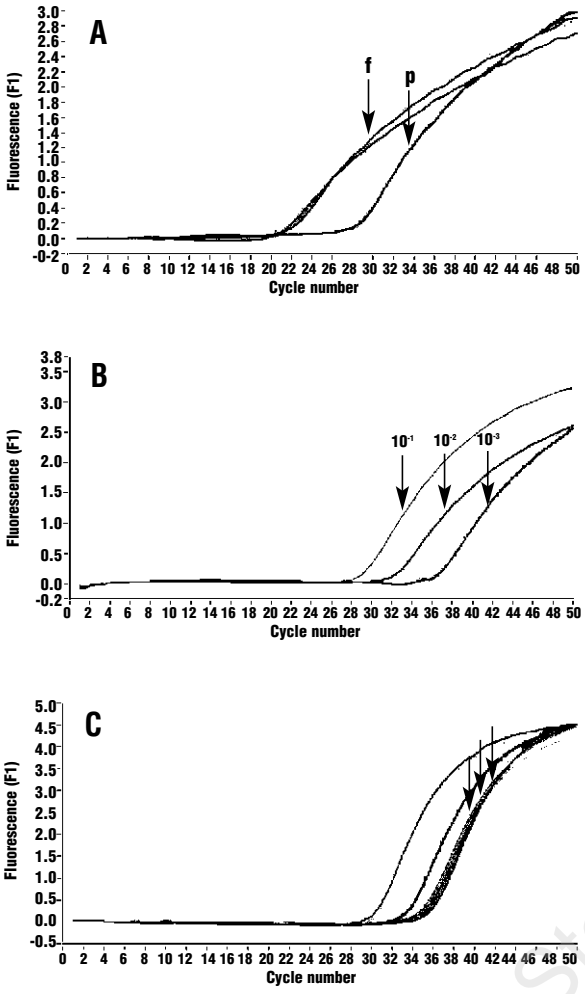


Figure 3. Real-time quantitative LC assay for detection of SV40 sequences from paraffin-derived DNA. A. Quality control of SV80 control DNA derived from frozen (f) and paraffin (p) specimens, and DNA from SV40-negative blood, with β-globin primer; B. Serial ten-fold dilutions ($10^{-1,-2,-3}$) of SV80-paraffin-DNA with SV40-negative DNA; C. Quantification of one unknown paraffin-osteosarcoma-DNA (arrows) in a triple reaction.

are only possible with adequate C_T values during control gene amplification. In our assay C_T values were 20±1 cycle for DNA derived from fresh or frozen cells and 25±1 cycle for DNA derived from paraffin specimens. C_T values of 23 or 26 for quality control amplification would reduce the detection sensitivity 10- or 100-fold, respectively. Less than 10 copies would then falsely be interpreted as negative and, *vice versa*, only intensely positive samples would be observed. The C_T values of positive samples in this investigation vary from 24 to 32. Several additional advantages are offered by the real-time PCR method: first, the potential virus contamination in the laboratory is greatly reduced because amplification, hybridization, and analysis occurs without opening the reaction vial. This will reduce false positive results due to carry-over of PCR products. Secondly, RQ-PCR analysis is a fluorescent-based detection method with simultaneous identification via hybridization with a specific target probe. No further identification with Southern blot and hybridization with radioactive-labeled probes is necessary. This means that laboratory workers do not need to work with radioactive material. Thirdly, RQ-PCR is very time saving and efficient: detection, identification and quantification of SV40 in a triple reaction, including β-globin quality control, takes less than two hours. Temperature cycling in the Light Cycler System is achieved using air, warmed by a heating coil, controlled by thermocouples in a thermal chamber. Given the very rapid temperature-transition rate of up to 20°C/second, temperatures in the chamber can be reached within a few seconds. Therefore ultrarapid cycling is possible and 8 seconds are sufficient for denaturation of the 128 bp DNA in our investigation. Finally, RQ-PCR is more sensitive (down to 1 copy/500 ng sample = 2 copies per µg sample DNA) than conventional PCR. Conventional PCR is only semiquantitative because the amplification products are analyzed by an end-point measurement. In contrast, RQ-PCR assays allow the detection of PCR products in the early phase of amplification, long before the PCR reaches a plateau. This is essential for accurate quantification of the initial input of the target molecules.

In our investigation we found a very high overall prevalence of 77% of SV40 in childhood ALL, compared to the 42-43% incidence reported by Vilchez *et al.* and Shivapurkar *et al.*[8,9] in lymphomas from adults. Childhood lymphomas had not previously been analyzed. These discrepancies may be explained by our highly sensitive assay which detects weakly positive samples (Table 1). Although over 40 different laboratories have reported on the detection of SV40, only a few investigators[29-33] have analyzed SV40 in specimens from a healthy control group. With the exception of one laboratory in Italy[29,33] which analyzed 70 peripheral blood and 38 buffy coat samples, all other investigators analyzed only very small numbers of healthy individuals. To ascertain the prevalence of SV40 in the human population we additionally investigated 149 buffy coats from healthy German people, and found an incidence of SV40 of 1.3%. Despite the high prevalence of SV40 in lymphomas described in this study and the low prevalence found in the healthy control group, a direct association with the tumorigenic potential of SV40 is not unequivocal. Additional studies are required in order to evaluate the importance of the absolute copy number of SV40 in these specimens since we found only a low amount of SV40 in them. The question of whether SV40 is a pathogen or just a passenger in lymphomas

must also be answered by further investigations. We observed quite different individual intensities in samples from Hungarian osteosarcomas. These tumors could likewise be connected with SV40, given the high incidence of the virus and the large amount of SV40 (more than 10 copies per 500 ng DNA) in each specimen. Therefore our quantitative PCR assay gives us a new tool for further investigation of the putative role and incidence of SV40 virus in a wide range of human malignancies.[34,35]

SH,UzS: conception and design of the analysis, interpretation of the data, and writing the paper; SH,SG: RQ-PCR studies; UzS: sequence analysis, SH,UzS,HK: analysis and interpretation of the data; SH,UzS: drafting and final approval of the manuscript; SH,UzS,HK: revising the manuscript.

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