An internally controlled, triplex quantitative PCR assay

for human polyomaviruses JC and BK

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Abstract
We have developed a triplex TaqMan-based quantitative PCR assay for the human polyomaviruses JC (JCPyV) and BK (BKPyV). The assay simultaneously detects and quantifies both JCPyV and BKPyV in human clinical samples, and it includes an internal amplification control (IAC) consisting of murine polyomavirus (MuPyV) plasmid DNA. We developed the assay for the Roche LightCycler 480 platform with the reporter dyes VIC, 6-FAM, and Cy5 for the MuPyV, BKPyV, and JCPyV, respectively. The assay had a high specificity for BKPyV and JCPyV when either viral genome was present alone, or in mixed samples over a range of $10^1$ to $10^7$ copy numbers per reaction. The analytical sensitivity was 50 copies for BKPyV and 10 copies for JCPyV. The use of the MuPyV internal control ensured monitoring of the quality of the extraction and of PCR inhibition, even in samples such as CSF and plasma in which controls based on host genes cannot be effectively used. In addition, we developed a similar assay using a different dye configuration (6-FAM, VIC, and NED) that could be used on an ABI 7500 Fast platform. This assay had similar sensitivities as the LightCycler 480 configuration for BKPyV and JCPyV when either viral genome was present alone, but the sensitivity of detection of BKPyV was greatly decreased when an excess of JCPyV (more than 100-fold) was present in the sample. This internally controlled combined assay offers greater convenience and cost effectiveness compared with separate assays for each virus and can also detect unexpected PyV activations by testing for both viruses in all samples.
Introduction

The human polyomaviruses BK (BKPyV) and JC (JCPyV) are widespread in human populations, with initial infection occurring in childhood via the respiratory or gastrointestinal route (6, 20) followed by latency in urinary tract tissues, circulating B-lymphocytes (BKPyV and JCPyV) and the central nervous system (JCPyV) (20, 35). Initial infections with both viruses are usually mildly symptomatic or asymptomatic, as is the persistent state in immunocompetent hosts (20).

In certain cases associated with immunosuppression, these PyV can reactivate and cause severe pathology. For example, reactivation of BKPyV is associated with hemorrhagic cystitis in bone marrow transplant recipients (22, 24, 25) and can also cause transplant organ failure in renal transplantation patients receiving immunosuppressive therapy (7, 17, 23, 26, 30, 33). The reactivation of JCPyV is associated with the development of progressive multifocal leukoencephalopathy, a fatal condition associated with AIDS-related immunosuppression (13, 20, 35). Both JCPyV and BKPyV can induce tumors in laboratory animals (19), but their role in human cancers is uncertain (20).

Although BKPyV is most commonly associated with urogenital tract disorders and JCPyV with central nervous system infections, the distinction between the pathologies caused by these viruses is not absolute. For example, JCPyV is readily detectable in the urinary tract of both healthy and immunocompromized individuals (12) and can be associated with renal disease (8, 21). Furthermore, BKPyV can cause fatal meningoencephalitis in AIDS patients (1) and has been found in the CSF of patients with central nervous system disease (12). In addition, the simultaneous reactivation of JCPyV and BKPyV in patients with renal disease has been reported (17).
Due to the overlapping pathogenicities of these two human PyV, it is important to have an assay that will detect either or both viruses in clinical samples. Moreover, because both JCPyV and BKPyV infect the majority of the population and are shed at varying levels by both healthy hosts and by patients affected by disease (12), it is important to quantify the viral load in samples so that trends can be monitored over time. Our objective in this study was to develop and evaluate a method to detect both JCPyV and BKPyV simultaneously in human clinical samples using quantitative PCR.

Materials and Methods

DNA extraction. DNA was extracted from clinical samples that were sent to the National Microbiology Laboratory for detection of polyomavirus DNA. Plasma/serum samples (up to 1 ml), or CSF samples (280 µl) were extracted using an EasyMag automated processing unit (BioMerieux, St. Laurent, QC, Canada) and eluted in 60 µl of EasyMag elution buffer. At the lysis step, 20 µg of yeast tRNA (Sigma, St. Louis, MO) was added. Urine samples (10 ml) were preprocessed by centrifugation at 2500 x g for 5 minutes. The cell pellet was resuspended in 570 µl of phosphate buffered saline (200 mM phosphate, 150 mM NaCl pH 7.4), and 280 µl of the suspension was added to EasyMag lysis buffer along with 20 µg of yeast tRNA as above. Alternatively, urine cell suspensions and CSF samples were extracted manually using a Viral RNA mini kit (Qiagen, Mississauga, ON, Canada), and plasma/serum samples were extracted using a Qiagen Blood Midi/Maxi kit. Extracts were eluted in 60 µl of elution buffer.

PCR primers and TaqMan probes. PCR primers specific for BKPyV and murine polyomavirus (MuPyV) (37) along with corresponding TaqMan probes were designed
using Primer Express 2.0 (ABI, Foster City, CA) (Table 1). The PCR primer/TaqMan probe set that was used to detect JCPyV is described by Ryschkewitsch et al. (34). PCR primers were obtained from Invitrogen (Carlsbad, CA) and TaqMan probes were obtained from ABI (BKPyV, MuPyV and JCPyV VIC-MGB) or Sigma-Genosys (Oakville, ON, Canada) (JCPyV Cy5-BHQ2). The assays for JCPyV and BKPyV targeted the large T antigen, while the MuPyV assay amplified a region of the MuPyV genome that is outside of the large T antigen (Table 1).

**Triplex quantitative PCR.** Quantitative PCRs used the LightCycler 480 (Roche Diagnostics, Laval, QC) or the ABI 7500 Fast system. Amplification used LightCycler 480 mastermix (Roche Diagnostics) or TaqMan mastermix (ABI). For both formats, the final concentration of each PCR primer was 0.3 µM and the concentration of each probe was 0.2 µM. Internal standard (plasmid DNA containing the MuPyV genome cloned in vector pBSKS+) was added to qPCRs at 100 copies/reaction prior to amplification or prior to sample extraction. For amplification on the LightCycler 480, the reaction volume was 20 µl and the cycling conditions were 95°C, 5 minutes (1x), followed by 45 cycles of 95°C, 10 seconds; 60°C, 30 seconds, and 72°C, 30 seconds (data collection step). For the ABI 7500 Fast system, the final reaction volume was 25 µl and the cycling conditions were 50°C, 2 minutes; 95°C, 10 minutes (1x), followed by 40 cycles of 95°C, 15 seconds and 60°C, 1 minute (data collection step). Results were quantified by interpolation on a standard curve consisting of serial dilutions of plasmid DNA containing the complete genome of BKPyV (in pBR322) or JCPyV (in pUC19). For triplex analysis of clinical samples, the standard curve consisted of equivalent mixtures of both JCPyV and BKPyV plasmid DNA.
**High resolution melt (HRM) analysis of BKPyV and JCPyV amplicons.** Amplicons were generated from various mixtures of JCPyV and BKPyV template DNA using the primers described in Table 1 (0.3 μM each) without the use of the fluorescent probes. PCR was performed using High Resolution Melt mastermix (Roche Diagnostics) on a LightCycler 480 apparatus (Roche) with 2.5 mM MgCl₂ under the following conditions: 1 cycle of 95°C, 5 minutes followed by 50 cycles of 95°C, 10 seconds; 60°C, 10 seconds; 72°C, 10 seconds (data collection step). After the PCR, amplicons were melted at 95°C for 1 minute and cooled to 40°C for 1 minute. Melt profiles were generated in the presence of HRM dye by continuously monitoring the fluorescence as the amplicons were heated from 70°C to 95°C, with 25 data acquisitions/second.

**Assay sensitivity and specificity using clinical samples.** Results generated using the triplex qPCR method were compared to a triplex nested conventional PCR that detects BKPyV, JCPyV, and SV40 and differentiates the amplicons by size (15). Conventional nested PCR used an ABI GeneAmp 9700 thermocycler (ABI) and second-round PCR products were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide. For the JCPyV qPCR assay, sensitivity was measured using 105 urine, plasma, or CSF samples that were positive only for JCPyV by conventional PCR along with 41 samples that were positive for both JCPyV and BKPyV. The sensitivity of the BKPyV qPCR assay was assessed by analyzing 89 BKPyV positive samples and 41 double positive samples. The specificity of both assays was measured using 91 samples that were negative by conventional PCR.

**Detection limit and assay reproducibility.** Dilutions of plasmid DNA containing the JCPyV or BKPyV genome were used to assess the detection limits of the qPCR method.
For the detection limit, assays were performed at 100, 50, and 10 copies/qPCR assay and the detection limit was specified as the number of genome copies that was detected in >95% of assays (11). Interassay reproducibility was measured by calculating the coefficient of variation (CV) of the Cp values in six replicate qPCR assays, while intraassay reproducibility was assessed using the Cp values of replicates within a single qPCR assay.

Results

Template specificity of each PyV assay. To determine whether each of the three qPCR assays to be combined into triplex format was specific for the template of interest, we determined the amount of fluorescence generated from each nonspecific template with each PCR primer/TaqMan probe set in 1-plex format on the ABI 7500 Fast instrument (Figure 1). Each of the assays was specific for the template of interest, with no fluorescence generated from any of the unintended templates. We also measured the signal generated by each TaqMan probe in the presence of each nonspecific amplicon (e.g., JCPyV template and primers with the BKPyV probe). None of the three TaqMan probes generated any fluorescent signal with nonspecific amplicon (Figure 1). In addition, none of the assays generated any signal using human DNA, KIPyV (2) genomic DNA or WUPyV (16) genomic DNA as template; however the JCPyV assay generated a weak signal (Cp >30) with >10⁹ copies of SV40 genomic DNA (data not shown).

The JCPyV primer/probe set has been used extensively on a wide range of clinical samples and shown to have very high specificity (27, 34). In addition, BLAST analysis of the JCPyV primers indicated that they share 100% nucleotide identity to over 250 isolates of various origins deposited in GenBank (not shown). The BKPyV assay described herein
was targeted to the highly conserved large T antigen, and BLAST analysis indicated that
the primers and probe share 100% nucleotide identity with approximately 150 isolates of
various origins in GenBank (data not shown). Therefore, both of the PyV assays
described here are expected to detect the target viral genomes in a very wide range of
clinical samples.

High resolution melt analysis of amplicons generated in triplex format. Various
mixtures of the PyV genomes were amplified and subjected to HRM analysis. The
JCPyV amplicon melted at a temperature of 78.2°C, while the BKPyV amplicon had a
Tm of 75.0°C (Figure 2A). Both amplicons were detected in unequal mixtures of the two
templates ($10^7+10^2$ PyV genome copies) (Figure 2B).

Triplex qPCR performance using single and mixed templates. The triplex qPCR
assay on the LightCycler 480 used a FAM-labeled probe for BKPyV, a Cy5-labeled
probe for JCPyV, and a VIC-labeled probe for MuPyV. To differentiate the signal
generated from the VIC fluorophore (MuPyV) from that generated from the FAM
fluorophore (BKPyV), color compensation was applied on the LightCycler 480 according
to the manufacturer’s recommendations. Using either individual PyV plasmids or equal
mixtures of both PyV plasmids, linear standard curves were generated, which enabled the
quantification of PyV genomes over at least seven orders of magnitude (Figure 3A). The
Cp values for the mixtures were an average of 2.18 (BKPyV assay) or 2.61 (JCPyV
assay) lower than those obtained with individual plasmids (Figure 3A). The triplex assay
was used to quantify defined mixtures of JCPyV and BKPyV plasmid DNA. When
JCPyV and BKPyV were mixed in equivalent amounts, the quantification was accurate
over seven orders of magnitude (Figure 4A). Moreover, the signal from the internal
control was detectable in all copy numbers of target PyV genomes tested (Figure 3A, Figure 4A, B). In addition, this assay enabled the detection of both JCPyV and BKPyV genomes in highly unequal mixtures (million-fold excess of one PyV genome over the other), although amounts below 100 copies of both JCPyV and BKPyV were underestimated when the other viral genome was in vast (>10^5-fold) excess (Figure 4B). However, the underestimation of the minority PyV did not affect the sensitivity of the assay, since the low copy number samples were positive.

For the ABI 7500 Fast configuration, both JCPyV and BKPyV generated linear standard curves over six or seven orders of magnitude of copy numbers using both single templates and mixtures of equivalent copy numbers of both plasmids (Figure 3B). However, the fluorescent signal from the internal control (NED) was inhibited in the presence of more than 10^4 copies of each PyV genome (Figure 3B).

**Assay detection limit and reproducibility.** The LightCycler 480 was used to assess the detection limit and reproducibility of the triplex qPCR assay. The JCPyV assay had a slightly lower limit of detection than the BKPyV assay, with as few as 10 genome copies detected in >95% of assays compared with 50 genome copies for the BKPyV assay (Table 2). In these low copy number samples, the coefficient of variation ranged from 0.92-3.54%, with the lowest copy number samples exhibiting the highest variation. The interassay variability of both assays was generally lower in the higher copy number samples, and was comparable to the results obtained within a single qPCR run (Table 3). The variability of the MuPyV at 100 copies/qPCR assay was very low when the template was added directly to the qPCR mastermix (0.64% CV inter- and intraassay) (Table 3).
The variability of this assay increased somewhat when the MuPyV plasmid was added prior to DNA extraction for use as an internal amplification control (Table 3).

**Assay sensitivity and specificity using clinical samples.** We compared the performance of the triplex qPCR assay in both formats to that of a triplex nested conventional PCR assay (15) in both single and double positive samples. Using the LightCycler 480 configuration, the sensitivity for JCPyV was 97.2% in single infections but 100% when both polyomaviruses were present (Table 4). The BKPyV assay was 100% sensitive in single infections and 80.5% sensitive in double infections. Overall, the sensitivities of the two assays were 98% (JCPyV) and 93.8% (BKPyV). Neither assay was 100% specific; one sample was negative for JCPyV using conventional nested PCR but positive by triplex qPCR and 7 samples were BK negative by conventional PCR and positive by qPCR (Table 4).

Using the ABI 7500 Fast configuration with clinical samples, the assay sensitivity was 96% for JCPyV (n=113) and 94% for BKPyV (n=90) in single infections. For samples with both JCPyV and BKPyV genomes, the sensitivity was 100% for JCPyV and 48% for BKPyV. Considering both single and double infections, the assay sensitivities using this dye configuration were 97% (JCPyV, n=136)) and 85% (BKPyV, n=113). The specificities of this dye configuration were 97% (JCPyV) and 99% (BKPyV) (n=88).

**Discussion**

The importance of determining quantitatively the viral load of JCPyV and/or BKPyV in clinical samples is being increasingly recognized. A number of qPCR assays for human PyV have been reported. However, these assays are typically designed to amplify and quantify either BKPyV (23) or JCPyV (9, 10) alone, or to detect both viruses, but in
separate assays (17, 28, 29, 34). Other assays have combined the detection of JCPyV and BKPyV into a single reaction. For example, Elfaitouri et al. describe an assay that quantifies BKPyV, JCPyV, and SV40 in a single reaction, but this assay targets a conserved region such that positive results must be typed by sequencing or methods specific for each of the viruses (14). Biel et al. (5) describe a set of assays that can differentiate and quantify the two human polyomaviruses, but the quantitative aspect of the assay does not distinguish the two viruses and a separate, seminested PCR is required for this purpose. Another multiplex PCR is capable of distinguishing the three viruses, but it is a nested, nonquantitative conventional PCR that differentiates the viruses on the basis on amplicon size (15).

In this study, we have described an internally controlled, triplex quantitative PCR assay for JCPyV and BKPyV in clinical samples. The use of an internal amplification control (IAC) to monitor extraction efficiency and PCR inhibition in clinical samples is considered essential to avoid the possibility of false negative results (18). We have chosen to use a plasmid containing the genome of MuPyV as internal control. This plasmid is added prior to DNA extraction and therefore monitors both extraction efficiency and PCR inhibition. The IAC is added at a low level to maximize its responsiveness to a loss of DNA during extraction and to minimize potential interference with the detection of the target DNAs (18); we found that the addition of IAC to a level such that 100 copies are present in the qPCR assay was consistently detected with a low Cp variability and a low rate of IAC negative samples (Table 3).

We designed this assay for the LightCycler 480 system, which is a platform that performs very well for viral diagnostics, particularly for multiplex applications (31). The
The dye configuration for this platform featured detection of BKPyV on the FAM channel, the IAC on the VIC/HEX channel, and JCPyV on the Cy5 channel. To correct for signal crosstalk between FAM and VIC on the LightCycler 480, we used color compensation as suggested by the manufacturer. This dye configuration performed well with single templates as well as with equal or unequal artificial mixtures, and the IAC was detected in all mixtures tested (Figure 2A, 4A). The assay using this dye configuration was therefore characterized more thoroughly.

The detection limits, as well as the intraassay and interassay variabilities, were consistent with those reported for other nonmultiplexed PyV-targeted qPCR assays (4, 9, 14, 28, 30). Using clinical samples, we found that the triplex qPCR assay compared very favorably to the conventional nested triplex assay described by Fedele et al. (15), with specificities >93% (Table 4). The BKPyV qPCR assay showed a low sensitivity compared with the conventional PyV PCR assay in double infections (80.5%), in spite of the fact that the assay detected a very wide range of mixtures of the two PyV genomes. However, the samples that were BKPyV negative by qPCR and double positive by conventional PCR had very high levels of JCPyV genomes (mean of $5 \times 10^8$ genomes/assay); high levels of JCPyV genomes can yield an artifactual band the same size as BKPyV in the presence of high JCPyV copy numbers (T. Dumonceaux, unpublished observation), suggesting that the apparent lower sensitivity of the qPCR assay for BKPyV is due to false positive detection of BKPyV in samples with high copy numbers of JCPyV by the conventional assay. Neither the JCPyV nor the BKPyV assays were perfectly concordant with the conventional assay using PyV negative samples; this
is likely due to the presence of false negatives in the conventional assay, as has been reported elsewhere (4).

We also designed an assay for the ABI7500 Fast system, which is another commonly used platform for viral diagnostics (32, 36). This assay used the following dye configuration: detection of BKPyV on the FAM channel, JCPyV on the VIC channel, and MyPyV on the NED channel. Using this configuration, we were able to detect and quantify accurately JCPyV and BKPyV alone or in artificial mixtures when the two target genomes were present in similar amounts (Figure 3B). However, we found that the detection of the IAC was inhibited when either of the target PyV genomes was present in excess in the template mixture, and it was rendered undetectable in the presence of larger amounts (more than 1000-fold excess) of PyV genomes (Figure 3B). This observation in itself does not affect the assay results, since the detection of the target PyV genome(s) means that amplifiable DNA is present and the result can be accepted as positive (18). However, the detection of both the IAC (NED) and BKPyV (6-FAM) were adversely affected when JCPyV (VIC) was present in excess (not shown). As a result, using this dye configuration, any sample that was positive for JCPyV with $>10^4$ genomes in the qPCR could be falsely interpreted as negative for BKPyV. This was reflected in the assay sensitivity with this dye configuration for BKPyV using clinical samples, which was 94% for single infections but only 48% for double infections. We found clear evidence that the BKPyV amplicon was generated in the presence of excess JCPyV (Figure 2), suggesting that the detection of the 6-FAM-labeled TaqMan probe was inhibited in the presence of a strong signal from the VIC-labeled JCPyV probe.
In summary, the triplex qPCR assay described in this study performed very well in detecting either or both PyV genomes in a single reaction. Multiplex detection using the LightCycler 480 provided a robust assay with no apparent effect on assay detection limit, as has been reported for other viral targets (31). This assay not only makes the detection of JCPyV and BKPyV in clinical samples more convenient and cost effective, it can also yield results that might be of clinical benefit when unexpected PyV reactivations are detected. This assay can therefore offer benefits over other single target qPCR assays for PyV.

Acknowledgements

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gastrointestinal tract after exposure to virions or viral DNA. The Journal of Virology 75:10290-10299.


Figure Legends

Figure 1. Template specificity of each of the assays included in the PyV triplex reaction mixture. A. JCPyV assay. The PCR primers and probes described in Table 1 were used to generate product using $10^7$ genome copies of JCPyV ($\Diamond$), BKPyV ($\Box$), or MuPyV ($\triangle$). In addition, the JCPyV probe was used in combination with the BKPyV amplicon ($\blacksquare$) and with the MuPyV amplicon ($\sigma$). Fluorescence was monitored on the VIC channel with an ABI7500 Fast instrument. B. BKPyV assay. PCR products were generated with the BKPyV primers and detected on the FAM channel with the BKPyV TaqMan probe described in Table 1 with JCPyV ($\Diamond$), BKPyV ($\Box$), or MuPyV ($\triangle$) template DNA. The BKPyV-specific TaqMan probe was also used to detect amplicon generated using JCPyV ($\blacksquare$) or MuPyV ($\sigma$) PCR primers and template. C. MuPyV assay. PCR products were generated with the MuPyV primers and detected on the NED channel with the MuPyV TaqMan probe with JCPyV ($\Diamond$), BKPyV ($\Box$), or MuPyV ($\triangle$) template DNA. The MuPyV TaqMan probe was also used to detect amplicon generated using JCPyV ($\blacksquare$) or BKPyV ($\sigma$) PCR primers and template.

Figure 2. Detection of JCPyV and BKPyV amplicons in mixed template format using high resolution melt analysis. A. Detection of JCPyV and BKPyV single templates. The JCPyV amplicon had a melting temperature of 78.2°C and the BKPyV amplicon melted at 74.6°C. B. High resolution melt analysis of mixed templates. Mixtures of $10^7$ JCPyV+10^2BKPyV and 10^2JCPyV+10^7BKPyV were used as template with JCPyV and BKPyV PCR primers.
Figure 3. Typical standard curves generated using mixtures of PyV plasmid DNA or individual PyV plasmid DNA using the LightCycler 480 dye configuration (A) or the ABI 7500 Fast dye configuration (B). Mixtures containing identical copy numbers of JCPyV (♦) and BKPyV (■) plasmids, or JCPyV (◇) or BKPyV (□) alone, were assayed and the Cp (A) or Ct (B) for each copy number was determined. In the case of the mixed standards, the Cp/Ct for MuPyV (*) was also determined in each sample.

Figure 4. Detection of defined mixtures of PyV template DNA using the LightCycler 480 system. A. Equivalent mixtures of JCPyV and BKPyV templates (10⁷ – 10¹ genome copies each) with 100 copies of MuPyV in each reaction. B. Unequal mixtures of JCPyV and BKPyV templates (10⁷ JCPyV + 10¹ BKPyV – 10¹ JCPyV + 10⁷ BKPyV) with 100 copies of MuPyV in each reaction. For both A and B, the number of PyV genomes in each mixture was calculated based on a standard curve of the corresponding template. The crossing point of the IAC was monitored in each mixture.
### Table 1. PCR primers/TaqMan probes for the amplification and detection of JCPyV, BKPyV, and MuPyV

<table>
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<tr>
<th>Viral Target</th>
<th>primer/probe sequence (5’-3’)</th>
<th>probe fluorophore&lt;sup&gt;a&lt;/sup&gt;</th>
<th>probe quencher&lt;sup&gt;b&lt;/sup&gt;</th>
<th>amplicon location&lt;sup&gt;c&lt;/sup&gt; and size</th>
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<td>JCPyV primer</td>
<td>AGAGTGTTGGGATCTGTGTTTT</td>
<td></td>
<td></td>
<td>4352-4375 (78 bp)</td>
<td>(27, 34)</td>
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<td>JCPyV primer</td>
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<td></td>
<td></td>
<td></td>
<td>this study</td>
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<tr>
<td>probe&lt;sup&gt;d&lt;/sup&gt;</td>
<td>TCATCAGGCAACAT</td>
<td>VIC®</td>
<td>MGB™</td>
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<td>probe&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>NED™ or VIC®</td>
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<sup>a</sup>fluorophores: VIC, 6-carboxyrhodamine; FAM, 6-carboxyfluorescein; Cy5, cyanine-5

<sup>b</sup>quenchers: MGB, minor groove binder; BHQ-2, black hole quencher-2

<sup>c</sup>location of primer hybridization in JCPyV (GenBank accession no. NC_001699), BKPyV (GenBank accession no. NC_001538), or MuPyV (strain A2, GenBank accession no. NC_001515)

<sup>d</sup>JCPyV or MuPyV probe for ABI 7500 Fast platform

<sup>e</sup>JCPyV or MuPyV probe for LightCycler 480 platform

<sup>f</sup>JCPyV or MuPyV probe for LightCycler 480 platform (internal standard) on the LightCycler 480 platform and the ABI 7500 Fast platform.

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Triplex quantitative PCR assay for JC and BK polyomaviruses
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<thead>
<tr>
<th>Polyomavirus</th>
<th>#genome copies/24qPCR assay</th>
<th>#positive/24</th>
<th>%positive</th>
<th>mean Cp</th>
<th>standard deviation</th>
<th>%CV</th>
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Table 2. Detection limit for triplex polyomavirus assay.

Triplex quantitative PCR assay for JC and BK polyomaviruses
Table 3. Interassay and intraassay reproducibility for triplex polyomavirus assay.

<table>
<thead>
<tr>
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<th>log # genomes</th>
<th>interassay variability</th>
<th>intraassay variability</th>
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<tr>
<td></td>
<td>average Cp</td>
<td>Standard deviation Cp</td>
<td>%CV</td>
</tr>
<tr>
<td>JCPyV</td>
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<td>35.09</td>
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<td>2^d</td>
<td>36.73</td>
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</table>

^a number of replicate qPCR runs analyzed
^b number of individual replicates within one qPCR run analyzed
^c MuPyV plasmid DNA added to qPCR master mix prior to amplification
^d MuPyV plasmid DNA added to clinical samples prior to DNA extraction

Triplex quantitative PCR assay for JC and BK polyomaviruses
<table>
<thead>
<tr>
<th></th>
<th>JC/BK</th>
<th>n</th>
<th>JC only</th>
<th>n</th>
<th>combined</th>
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<td>Conventional nested multiplex PCR result:</td>
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<tr>
<td>triplex specificity (negative) or sensitivity (positive):</td>
<td>1.000</td>
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<td>(0.956-0.988)</td>
<td>105</td>
<td>(0.968-0.991)</td>
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<td>0.980</td>
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<td>BK assay (FAM)</td>
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<tr>
<td>Conventional nested multiplex PCR result:</td>
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<td>triplex specificity (negative) or sensitivity (positive):</td>
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<td>41</td>
<td>(0.743-0.867)</td>
<td>89</td>
<td>(0.917-0.960)</td>
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Table 4. Sensitivity and specificity of triplex polyomavirus assay on the Light Cycler 480 platform compared with nested conventional multiplex PCR (15). Ranges of sensitivity are given based on the 95% confidence interval for the number of samples analyzed (3).