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## Real-Time RT-PCR Assay for the Quantitation of Polyomavirus BK VP1 mRNA Levels in Urine

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UNIVERSITÀ DEGLI STUDI DI TORINO

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**1Real-time RT-PCR assay for the quantitation of polyomavirus BK VP1 mRNA levels**  
**2in urine.**

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8

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10

11**Running title:** Real-time RT-PCR for urine BKV VP1 mRNA.

12

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20

## 1Abstract

2In renal transplant recipients, polyomavirus BK can reactivate resulting in graft  
3nephropathy. Screening for BK virus replication may allow for earlier intervention with  
4reduced allograft loss. The measurement of urinary cell BKV VP1 mRNA for identify viral  
5replication levels at risk of developing nephropathy has been proposed. In this paper the  
6development, optimization, and standardization of a Taqman Real-time RT-PCR assay for  
7the quantitation of BKV VP1 mRNA levels in urine is described. Subsequently, the method  
8has been validated on urine specimens obtained from renal transplant recipients. The use  
9of VP1 mRNA measurement as a marker for viral replication and a tool for non invasive  
10diagnosis of nephropathy should be regarded with great caution, given the potentially  
11limited positive predictive value and the drawbacks associated with the complexity of the  
12real-time RT-PCR assay requiring an expert well trained operator and the relatively poor  
13cost-efficiency ratio.

14

15

16

## 17Key words

18Polyomavirus BK; Real-time RT-PCR; VP1 mRNA; BK virus-associated nephropathy;  
19renal transplantation.

20

## 21Abbreviations

22**BKV**, BK virus

23**RT**, reverse transcription

## 11. Introduction

Human polyomavirus BK (BKV) is a highly prevalent virus characterized by latency in uroepithelial cells and other sites. In renal transplantation, in the context of intense immunosuppression, BKV can reactivate from latency resulting in BKV-associated nephropathy with interstitial nephritis and/or ureteral stenosis in 1-10% of the patients, leading to graft failure and return in hemodialysis in 30 up to 80% of the cases (1-3). Screening for polyomavirus replication, by urine and serum viral load quantitation (4,5), may allow for earlier intervention with reduced allograft loss (2). Ding et al. (6) proposed the measurement of urinary cell BKV VP1 mRNA for identify viral replication levels at risk of developing nephropathy. Although there is no definitive cut-off for BKV VP1 mRNA levels diagnostic of nephropathy, Dadhania et al. (7) found that the median BKV VP1 copy number in renal transplant patients with BKV replication without nephropathy was significantly lower in comparison to that reported in renal allograft recipients with biopsy proven nephropathy (267 BKV VP1 copies/pg of total RNA vs. 34,000,  $p = 0.004$ ). In this paper the development, optimization, and standardization of a Taqman Real-Time reverse transcription (RT)-PCR assay for the quantitation of BKV VP1 mRNA levels in urine is described. Subsequently, the method has been validated on urine specimens obtained from renal transplant recipients.

## 12. Materials and methods

### 22.1 Clinical samples

3Two-hundred-seventeen urine and 217 serum specimens from 114 renal transplant  
4recipients (61 males, 53 females; men age  $\pm$  standard deviation,  $56.9 \pm 9.7$  years; range,  
529-77) were studied. The occurrence of BKV-associated nephropathy was diagnosed by  
6histopathological evaluation on kidney graft biopsy, that was collected in the presence of  
7renal function abnormalities and/or on the basis of clinical features suggestive of  
8nephropathy and/or rejection.

9

### 102.2 Nucleic acid extraction

11Automated extraction of total nucleic acids from urine samples was performed with the  
12NucliSens easyMAG platform (bioMérieux, Marcy l'Etoile, France). As regards total RNA,  
13extracted specimens were subjected to RQ1 RNase-Free DNase digestion (Promega,  
14Milan, Italy), following the manufacturer's instruction. A second automated extraction was  
15performed in order to remove the DNase buffer that could interfere with the amplification.  
16The absence of contaminating DNA was ascertained by PCR amplification (see section  
172.3), following the second extraction, that tested negative.

18

### 192.3 Real Time PCR for BKV-DNA quantitation in serum and urine samples

20BKV DNA quantitation was performed by Real Time TaqMan PCR using a commercial kit  
21(BKV Q-PCR Alert Kit; Nanogen Advanced Diagnostic, Milano, Italy) for the detection of  
22the target viral gene encoding for the large T-antigen of BKV with the 7300 Real Time PCR  
23System (Applied Biosystems, Foster City, CA), according to the manufacturer's  
24instructions and as described previously (4). Briefly, PCR amplifications were set up in a  
25reaction volume of 25  $\mu$ l that contained 5  $\mu$ l of extracted sample or negative control (sterile

1 double-distilled H<sub>2</sub>O) or plasmid. The assay is linear in the range 10<sup>1</sup>-10<sup>6</sup> copies per  
2 reaction, corresponding to an absolute quantitation of 10<sup>3</sup> copies/ml.

3

#### 42.4 Plasmids

5 The plasmid pBKV (ATCC 33-1 [ATCC, Manassas, VA]), containing the complete genome  
6 sequence of BKV (linearized using restriction enzyme BamHI) and cloned into the pBR322  
7 (Invitrogen, Carlsbad CA) vector, was used to determine the sensitivity and linear range of  
8 the PCR assay.

9 The plasmid pGAPDH containing a fragment of 512 bp within the region between exon 6  
10 and 8 of the housekeeping gene Glyceraldehyde-3-phosphate-dehydrogenase (GenBank  
11 accession no. **J04038**; primer F 5'-GCCAAAAGGGTCATCATCTC-3'; primer R 5'-  
12 GGGGCCATCCACAGTCTTCT-3'; probe VIC-5'-TGGTATCGTGGAAGGA-3'-MGB  
13 designed using the Primer Express 3.0 software (Applied Biosystems) was cloned into  
14 pCR<sup>®</sup>2.1- TOPO-TA<sup>®</sup> cloning (Invitrogen) and propagated in TOP10 high-efficiency  
15 chemically-competent cells (Invitrogen), according to the manufacturer's instructions.

16

#### 172.5 cRNA standard production

18 A large amount of cRNA of the pBKV was obtained by using the T7 RiboMAX Express  
19 Large Scale RNA Production System (Promega), according to the manufacturer's  
20 instructions. After the transcription, the DNA template was removed by adding RQ1  
21 RNase-free DNase to a concentration of 1 U/μg DNA. After incubation at 37°C for 15 min.,  
22 the RNA was purified by performing two automated extractions with the NucliSens  
23 easyMAG instrument (bioMérieux). The concentration of BKV-cRNA was estimated by  
24 spectrophotometric reading at OD<sub>260</sub>. To determine the sensitivity of the RT-PCR assay,  
25 stock preparations of BKV-cRNA were diluted to 10<sup>-1</sup> copies/μl by serial 10-fold dilutions.

1To simulate the natural specimen, BKV-cRNA was extracted in parallel with the nucleic  
2acid of the sample.

3

#### 42.6 Reverse transcription

5The RT reaction was done by a random cDNA synthesis from 10 µl of extracted sample  
6RNA in two step: first a reaction mixture containing random primers (50 ng/ml)(Invitrogen)  
7and 10 mM dNTPs (Invitrogen, Carlsbad, CA) was added and incubated at 65°C for 5 min.  
8using the 9800 Fast Thermal Cycler (Applied Biosystems, Monza, Italy); subsequently, a  
9second reaction mixture containing 0.1 M DTT, 10 x Buffer (200 nM Tris-HCl [pH 8.4], 500  
10nM KCl), RNase OUT™ (40 unit/µl)(Invitrogen) and reverse transcriptase (50 unit/µl)  
11(SuperScript™ II RT, Invitrogen), giving a total volume 20 µl, was used. After incubation for  
1210 min. at 25°C, 50 min. at 42°C and 15 min. at 70°C, the cDNA mixture was used as  
13target cDNA.

14

#### 152.7 Optimization and standardization of the Real-time RT-PCR assay for BKV VP1 mRNA

16Primers and probe for BKV VP1 mRNA were obtained from the literature (6) and were as  
17follows: primer F 5'-TGCTGATATTTGTGGCCTGTTTACTA-3'; primer R 5'-  
18CTCAGGCGGATCTTAAATATCTTG-3'; probe 5'-FAM-  
19AGCTCTGGAACACAACAGTGGAGAGGCC-TAMRA-3'. For the optimization of the assay,  
20various conditions that affect amplification were tested, such as concentrations of primers  
21(0.12 mM; 0.5 mM; 0.9 mM) and probe (0.1 mM; 0.18 mM; 0.25 mM), and MgCl<sub>2</sub> (3 mM; 4  
22mM; 5 mM). Two µl of cDNA, retrotranscribed BKV-cRNA standard or negative control  
23(sterile H<sub>2</sub>O) were added to 23 µl of a reaction mix containing 1X master mix (Invitrogen),  
24primers and probe, giving a final volume of 25 µl. Amplification profile was optimized for  
25the 7300 Real Time PCR System (Applied Biosystems), as described above.



1The sensitivity of Real Time PCR assays is defined as the lowest concentration of target  
2quantified at a frequency of 100%. The dynamic range (or linearity rate) was evaluated  
3using 10-fold dilutions (from  $10^{10}$  to  $10^0$  copies/reaction) of BKV-cRNA standard. Precision  
4or intra- and inter-assay variability (CV) was evaluated using different concentrations of  
5standard BKV-cRNA (ranging from  $10^5$  to  $10^2$  copies/reaction) within a single run ( $n = 10$ )  
6or different run experiments ( $n = 10$ ) with single replicates of each concentration  
7considered as an unknown specimen. The primers were tested for potential cross-  
8reactivity with related viral sequences of polyomaviruses JC, SV40, KI and WU based on  
9the data available at the BLAST alignment software. Similarly, BLAST was also performed  
10on short fragments (primers lacking at the 3'end down to 15 nucleotides).

11In order to ascertain the identity of the resulting products a cycle sequencing was  
12performed. PCR amplification products were used as templates for DNA sequencing using  
13the BigDye Terminator chemistry, following the manufacturer's instructions (BigDye  
14Terminator v3.1 Cycle Sequencing Kit [Applied Biosystems, Warrington, UK]). Cycle  
15sequencing was performed with the 9800 Fast Thermal cycler (Applied Biosystems) under  
16the following conditions: an initial denaturation step of 1 min. at  $96^\circ\text{C}$ ;  $96^\circ\text{C}$  for 10 sec.,  
17 $50^\circ\text{C}$  for 5 sec,  $60^\circ\text{C}$  for 4 min for twenty-five cycles. In order to purify the sequencing  
18product from not-incorporated BigDye Terminators, the mixture was loaded direct onto  
19Centrisep columns (Applied Biosystems). Subsequently, sequencing products were  
20subjected to capillar gel-electrophoresis using the 3130x1 Genetic Analyzer (Applied  
21Biosystems).

22A number of precautions were undertaken to prevent the occurrence of false-positive  
23results. Each RT-PCR run included control reactions lacking template (no-template  
24controls) to test for the presence of contamination or the generation of nonspecific  
25amplification products under the assay conditions used. The optimization criteria for the  
26Real Time PCR assay for the housekeeping gene GAPDH were the same of VP1 mRNA.

## 22.8 Quantitation of the urine BKV VP1 mRNA level

3For Real-time RT-PCR quantitation, a standard curve was created in a 4-log range by 10-  
4fold serial dilutions of the BKV-cRNA and pGAPDH. Urine VP1 mRNA levels were  
5measured by normalizing the number of mRNA copies on the basis of the number of  
6urinary cells. This was achieved by amplifying separately the housekeeping gene GAPDH  
7(considering the linearity range of the pGAPDH ranging from  $10^2$  to  $10^5$ ) and the viral  
8target. In this study, a patient was classified as BKV replication positive if the urine VP1  
9mRNA copy number was  $>10^3/10^3$  cells.

10

## 112.9 Statistical analysis

12The coefficient of variation for samples quantified in repeated measurements was  
13calculated. Descriptive statistics were expressed as absolute value and percentage. For  
14statistical analysis the t test was used. Operating characteristics, including sensitivity,  
15specificity, negative predictive value (NPV), and positive predictive value (PPV) were  
16evaluated by using a commercially available software (MedCalc; version 9.2.1.0).

## 13. Results

### 23.1 Optimization, standardization, and validation of Real Time PCR assays

The following primer/probe and  $\text{MgCl}_2$  concentrations were chosen: 0.25 mM/0.18 mM and 43 mM  $\text{MgCl}_2$ , and 0.5 mM/0.25 mM and 3 mM  $\text{MgCl}_2$  for VP1 and GAPDH, respectively. The dynamic range of the home-made Real Time PCR for VP1 was evaluated by carrying out serial dilutions of BKV-cRNA (from  $10^{10}$  to  $10^0$  copies/reaction), and ranged from  $10^8$  to  $10^2$ . The sensitivity of the assays was 10 copies/reaction. The precision, defined as the level of concordance of the individual test results within a single round (intra-assay precision) and from one run to another (inter-assay precision), was evaluated using different concentrations of BKV-cRNA, ranging from  $10^5$  to  $10^2$  copies/reaction, and is reported in Table 1.

12

### 3.2 Clinical samples

Results are summarized in Table 2. Based on urine VP1 mRNA measurement, BKV replication was found in 31/217 (14.3%) specimens from 20/114 (17.6%) patients; in particular, 15 specimens (6.9%) of 9 patients (7.9%) with levels  $>10^4$  copies/ $10^3$  cells and 5 (2.3%) of 3 (2.6%)  $>10^5$ . As regards viremia and viruria, BKV-DNA resulted positive (above  $10^3$  copies/ml) in 18 (8.3%) and 23 (10.6%) serum and urine specimens, respectively from 9 (7.9%) and 15 (13.2%) patients. A histologically confirmed diagnosis of BKVAN was made in two patients, both with VP1 mRNA levels  $>10^5$  (66.7% of patients above this cutoff level) and with serum BKV-DNA  $>1 \times 10^6$  copies/ml (66.7% of patients above this cutoff level). Urine VP1 mRNA was detected in all the cases, except five, in association to viremia and in all the cases to viruria. Considering the highest level in each patient, median urine VP1 mRNA level was 31236 copies/ $10^3$  cells; no difference of VP1 mRNA levels was found between the two patients who developed BKVAN and the highest values

1in patients who did not developed BKVAN. Operating characteristics of VP1 mRNA  
2measurement for levels  $>10^5$  copies/ $10^3$  cells were as follows: sensitivity 100%; specificity  
357.1%; positive predictive value 25%; negative predictive value 100%.

## 14. Discussion

In this paper the optimization and standardization of a Real-time RT-PCR assay for the quantitation of urine VP1 mRNA levels is described. This method was first proposed by Ding et al. (6) as a noninvasive and accurate tool for diagnosing BKVAN and has been used more recently to quantify BKV replication in monitoring renal transplant recipients, thus possibly representing an alternative approach to serum and urine viral load measurement. However, as pointed out by Nickeleit et al. (8) and Hirsch (9), some factors could affect the relevance of VP1 mRNA quantitation, in particular the high susceptibility of the extraction of mRNA from urinary cells to erroneous results and the degree of viral genomic VP1 DNA contaminating the VP1 cDNA preparation, that could be overcome by the quantitation of VP1 signals after omitting reverse transcription or after prior DNase digestion. In the present study, the potential drawbacks associated to mRNA extraction have been overcome by performing an automated extraction of total nucleic acids, a DNase digestion of extracted specimens, and a second automated extraction to remove the DNase buffer that could interfere with the amplification. This procedure, by removing viral genomic VP1 DNA contaminating potentially the VP1 cDNA preparation, should allow for a more correct quantitation of BKV VP1 mRNA.

The Real time RT- PCR assay for BKV VP1 mRNA designed by Ding et al. (6) target VP1, a major capsid protein responsible for the characteristics icosahedron feature and enabling BKV to entry into cells. This target was chosen because the transcription and translation of VP1 are contingent upon viral DNA replication, so that VP1 mRNA are detected only in infected productively cells. The design of primers and probe was made to target a highly conserved region of the VP1 region and incorporated multiple sequence mismatches with the polyomavirus JCV VP1 region. The assay was optimized by examining different primer and probe concentrations and was found to be very sensitive with a minimum cut-off for

1detection of 10 copies/reaction. The dynamic range was evaluated by carrying out serial  
2dilutions of BKV-cRNA and ranged from  $10^8$  to  $10^2$ . When increased sensitivity and broad  
3dynamic range are combined, it is possible to quantify template from samples containing a  
4wide range of concentrations, as in clinical samples. This avoids the need for dilution of  
5the amplicon prior to conventional detection or repetition of the assay using a diluted  
6sample because the first result falls outside the detection limits. The assay reproducibility  
7was high with a median intra-assay and inter-assay variability of 0.445% (range 0.31% to  
80.49%) and 1.98% (range 1.90% to 2.33%), respectively.

9To confirm the specificity of the resulting product and that the assay did not cross-react  
10with related viral sequences of polyomaviruses JC, SV40, KI and WU, the primer set was  
11evaluated by the BLAST alignment software and a cycle sequencing of the PCR  
12amplification product was performed.

13The assay has been validated subsequently on clinical specimens from a group of renal  
14transplant recipients in which two case of confirmed nephropathy was diagnosed. The  
15availability of a reliable quantitative test permitted to evaluate different cut-off levels in  
16relation to the presence of viral replication and the development of BKV-associated  
17nephropathy. The identification of viral replication represents a tool to evaluate the risk of  
18development of nephropathy, although high levels of viral replication may occur even in  
19the absence of it (4). Polyomavirus BK replication occurs in the urothelial cells and results  
20in shedding of urothelially derived decoy cells in high number and only replicating BKV  
21leads to these cytopathic changes and the shedding of decoy cells in urine. In this study,  
22the measurement of VP1 mRNA copies has been normalized on the basis of the number  
23of urinary cells, rather than on the amount of total RNA. Because decoy cells mostly  
24contain BK virus antigens (9), it is likely that extracted mRNA originated from these cells.  
25Therefore, the availability of a RT-PCR that normalized the measurement of BKV VP1  
26mRNA on the basis of the number of cells could be useful for investigating the relationship

1between these findings and other markers of viral replication, such as urine cytology (i.e.  
2decoy cells), viremia and viruria, in kidney transplant recipients at risk of BKV-associated  
3nephropathy. Nevertheless, it has to be taken into account that the use of the  
4housekeeping gene GAPDH as quantification control might be affected by different factors  
5such as treatments and biological processes, thus potentially influencing its role in  
6normalisation when considering severely altered or dead cells as shed renal tubular cells  
7**(10)**. Polyomaviruses replication may occur without clinical effects along the urothelium of  
8healthy individuals and kidney allograft recipients. There are established protocols to  
9screen for BKV-associated nephropathy, based on BKV-DNA detection by real time PCR  
10in blood and urine **(4,11)**; these screening methods already provide a reliable basis for  
11further treatment recommendation. Therefore, the use of VP1 mRNA measurement as a  
12tool for non invasive diagnosis of nephropathy should be regarded with great caution,  
13given the low prevalence of confirmed nephropathy in our study and the potentially limited  
14positive predictive value, even considering high VP1 mRNA values, and the drawbacks  
15associated with the complexity of the real-time RT-PCR assay requiring an expert well  
16trained operator and the relatively poor cost-efficiency ratio.

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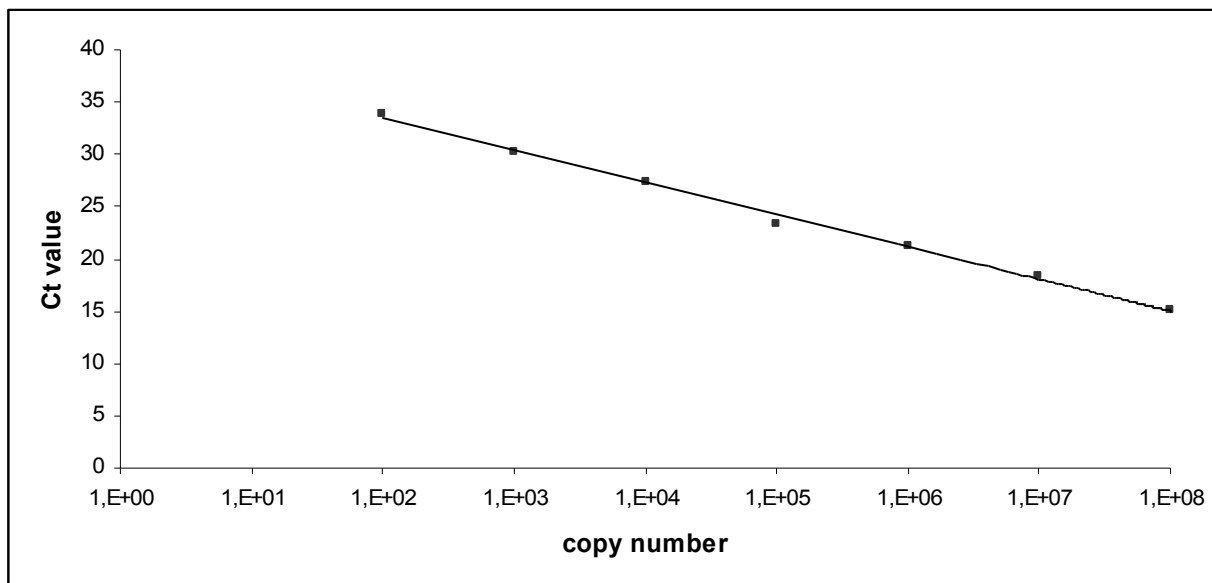
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**Figure 1.** Dynamic range of BKV VP1 mRNA quantitation with the Real-time RT-PCR assay. Number of cycle threshold (Ct) is plotted versus copy number (from  $10^2$  to  $10^8$ ).

3



4

**Table 1. Intra- and inter-assay variability of standard BKV-cRNA by Real Time PCR.**

<b>BKV-cRNA (copies/reaction)</b>	<b>Intra-assay variability (%)</b>	<b>Inter-assay variability (%)</b>
10 <sup>2</sup>	0.49	2.33
10 <sup>3</sup>	0.48	1.97
10 <sup>4</sup>	0.41	1.90
10 <sup>5</sup>	0.31	1.99

**Table 2. Results of BKV VP1 mRNA measurements in renal transplant recipients.**

\*Two (66.7%) with confirmed diagnosis of BKVAN.

<b>Urine VP1 mRNA (copies/10<sup>3</sup> cells)</b>	<b>Specimens N (%)</b>	<b>Patients N(%)</b>
	<b>Total = 217</b>	<b>Total = 114</b>
<b>&gt;10<sup>3</sup></b>	<b>31 (14.3%)</b>	<b>20 (17.6%)</b>
<b>&gt;10<sup>4</sup></b>	<b>15 (6.9%)</b>	<b>9 (7.9%)</b>
<b>&gt;10<sup>5</sup></b>	<b>5 (2.3%)</b>	<b>*3 (2.6%)</b>

3

4