A QUANTITATIVE REAL TIME PCR ASSAY FOR DETECTING BK VIRUS IN SERUM, PLASMA AND URINE

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ABSTRACT

BK is a non-enveloped virus in the Polyomavirus family, closely related to SV40 and JC virus. Primary infection with BK generally occurs during childhood without specific symptoms, and is widespread in the population, with approximately 80% of adults infected globally. The virus remains latent in the urogenital tract, but can become reactivated. Asymptomatic reactivation and sporadic shedding of BK virus in urine can happen spontaneously in immunocompetant patients.

BK virus has been associated with hemorrhagic cystitis in bone marrow transplant patients, as well as with ureteral stenosis and transplant-associated nephropathy in patients receiving kidney transplants. BK virus allograft nephropathy (BKVAN) is distinguished by persistent graft dysfunction, often resulting in graft loss. BKVAN is seen in up to 8% of kidney transplant recipients, frequently targeting renal epithelial tubules. New intensive immunosuppressive regimens are considered a risk factor for BKVAN. Detection of BK has traditionally been done using culture, which is slow, or by electron microscopy, which can be rapid but displays low sensitivity. The use of quantitative real time PCR for BK can provide a valuable tool for the clinician in diagnosing BKVAN as well as monitoring the patient's response to treatment.

We validated a quantitative real time PCR assay on the HT7900 Sequence Detection System (Applied Biosystems) to detect BK virus in serum, plasma and urine. This assay is in an ASR format, and targets the viral VP1 gene using a primer and Eclipse hybridization probe set from Nanogen/Epoch Biosciences. With each new lot of Taq master mix or BK ASR reagent, a new standard curve is generated using a lyophilized plasmid clone, and this curve is stored for quantitation of subsequent assays. Prior to extraction, an exogenous internal control is added to the sample, providing the ability to detect PCR inhibition and extraction failures. To limit the risk of contamination, the assay is run in a 96-well, closed tube format, using UNG and dUTP in the master mix. Accuracy was evaluated by comparing the results of this assay with those obtained using a TaqMan assay performed at another reference lab. The reportable range of the assay is 390 to 390,000,000 copies/mL. Data for precision, linearity and specificity will also be presented.

INTRODUCTION

Two common approaches to performing quantitative real time PCR assays are: 1) running a standard curve on each run, or 2) generating a single standard curve and applying it to multiple runs. Besides increased costs, a drawback to running a standard curve on each run is the difficulty in reproducibly amplifying the lowest standard. Since patient results less than this standard cannot be quantitated, it is desirable for this standard to be as low as possible. However, as the quantity of this standard approaches the assay's limit of detection, a test becomes vulnerable to having this standard fail to amplify. This results in a loss of the lowest part of the standard curve, and consequently, an inability to report patient results in this range. For this reason, we chose to use a stored standard curve. Four replicates of each standard are run, and the average of each crossing threshold is used in generating a stored standard curve (the lowest standard is required to amplify in 3 of 4 replicates). The slope and Y-intercept of this curve are stored in and used for quantitation of patient samples tested on subsequent runs.

To minimize potential variability in assay performance, we generate a new standard curve with each new lot of reagents (BK ASR reagents and Roche LightCycler Hybridization Probes master mix), and then sequester these reagents for subsequent BK runs. Whenever either of these reagents is replaced, a new standard curve is generated. Advantages of this approach include lower reagent costs and an enhanced ability to quantitate samples with low copy numbers. We use a plasmid containing the cloned amplicon to generate the standard curve. Dilutions of the plasmid (stock concentration = 10^7 copies/mL) from 10^{-1} down to 10^{-7} provide a quantifiable range for the assay from 390 to 390,000,000 copies per mL. The stock standard material is lyophilized to limit potential deterioration of its performance.

RESULTS

The amplification plot for a typical standard curve is shown in **Figure 1a** (7 standards, 4 replicates each). Plotting the log concentration versus crossing threshold for each standard generates the graph shown in **Figure 1b**. The standards are linear over the entire quantifiable range of the assay and the PCR efficiency is approximately 100%.

Table 1a shows the reproducibility amplification for a typical standard curve. **Table 1b** shows the mean, standard deviation and coefficients of variation for multiple standard curves generated over the period of 2 months, verifying the stability of our lyophilized stock standard.

As part of our validation, we tested a minimum of 20 positive and 10 negative urine, plasma and serum samples that had been previously tested at a major reference laboratory. To evaluate the accuracy of our assay, we compared our results with those performed at the reference lab. A Deming regression plot of these data is shown in **Figure 2**. (For reference, the dotted line represents unity.) The correlation was satisfactory, with an R^2 value of 0.83 and a slope of 0.948.

Linearity plots for BK virus in serum, plasma and urine are shown in **Figure 3**. The limits of detection are approximately 400 copies/mL for serum, 250 copies/mL for plasma and 3200 copies/mL for urine. No cross reactivity was detected for any of the following organisms: JC virus, HSV, CMV, EBV, VZV, HHV-6 (types A and B), Borrelia (Lyme), Toxoplasma and Parvovirus.

Since polymorphisms within the amplicon can affect amplification and detection of target in a real time assay, we prepared synthetic oligonucleotides corresponding to three different BK strains found in the NCBI database. The amplicon sequences for these 3 strains (Dunlop, HC-u9, HI-u8) are illustrated in **Figure 4**, as well as the sequences of the assay primers and probe (bottom). Polymorphisms within the primer or probe sequences are indicated in red. Dunlop is a perfect sequence match with the primers and probe. HI-u8 has a single polymorphism beneath the probe, which would be predicted to lower the T_m for this species. Finally, the HC-u9 strain carries a SNP at the 3' end of the upstream primer (which overlaps the probe-binding site) as well as 2 polymorphisms beneath the probe. These SNPs would be predicted to lower the T_m and decrease the efficiency of the PCR reaction. **Figure 5** shows a melting curve analysis following amplification of each of these variants. As predicted, the Dunlop strain displays the highest T_m (no SNPs beneath the probe), followed by HI-u8 (a single SNP) and HC-u9 (2 SNPs). A 3 point standard curve (log dilutions) for each of the sequence variants is presented in **Figure 6**. As anticipated, while the Dunlop and HI-u8 strains display essentially 100% efficiency, the PCR efficiency for the HC-u9 target is reduced to approximately 60%.

Figure 1a

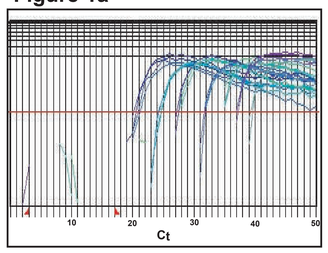


Figure 1b

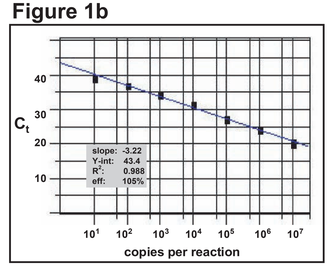


Table 1a

	10^-1	10^-2	10^-3	10^-4	10^-5	10^-6	10^-7
Replicate #1	20.3	25.0	28.1	30.8	34.1	36.5	38.5
Replicate #2	20.4	24.2	27.9	31.1	33.9	37.2	40.5
Replicate #3	20.5	24.5	27.3	31.0	33.1	37.6	38.9
Replicate #4	20.7	24.3	26.9	31.2	33.6	36.7	40.5
Mean	20.47	24.49	27.56	31.03	33.69	36.98	39.61
StDeviation	0.20	0.36	0.56	0.18	0.44	0.51	1.03

Table 1b

	10 e-1	10 e-2	10 e-3	10 e-4	10 e-5	10 e-6	10 e-7
mean	20.73	24.59	28.02	31.35	34.50	37.65	40.74
S.D.	0.57	0.50	0.64	0.52	0.81	1.15	2.20
%C.V.	2.70	2.00	2.20	1.70	2.30	3.10	5.40

Figure 2

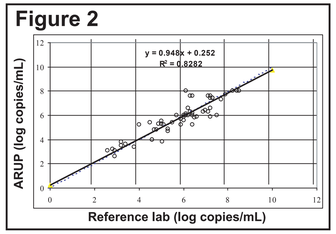


Figure 3

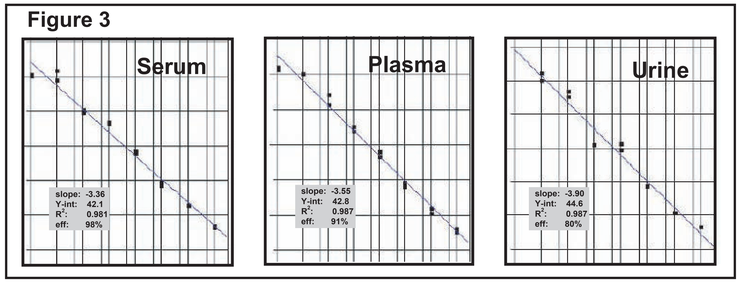


Figure 4

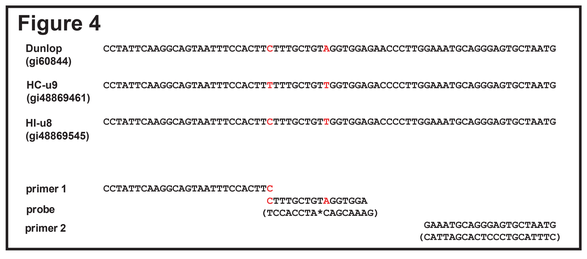


Figure 5

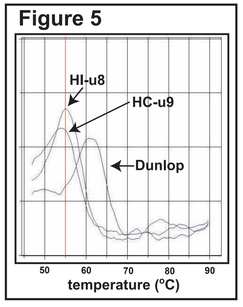
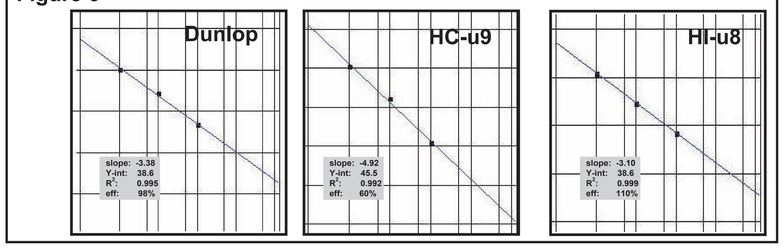


Figure 6



MATERIALS and METHODS

Extractions:

Extractions were performed on the Qiagen 9604 Robot using the recommended protocol. Internal control plasmid is added to the lysis buffer to give a final concentration in the extracted samples of 100 copies per μL (=1000 copies per 10 μL extracted sample).

Amplification and detection:

The BK primer and probe reagents are purchased from Nanogen/Epoch Biosciences as an ASR that also includes primers and probe for an internal control plasmid. The BK probe is labeled at the 5' end with a non-fluorescent quencher (NFQ) and a minor groove binding molecule (MGB). The 3' end of the BK probe is labeled with FAM. The internal control probe is also labeled at the 5' end with a non-fluorescent quencher (NFQ) and a minor groove binding molecule (MGB). The 3' end of the IC probe is labeled with TET. The asterisk indicates an adenosine that has been modified to base pair using three hydrogen bonds instead of the typical two. Purple indicates primer sequences, green indicates probe sequences, and nucleotides in red indicate regions where a primer and probe sequence overlap.

BK:

- upstream primer: 5' - CCTATTCAAGGCAGTAATTTCCACTTC - 3'
- downstream primer: 5' - CATTAGCACTCCCTGCATTTC - 3'
- probe: 5' - NFQ - MGB - TCCACCTA*CAGCAAAG - FAM - 3'

CCTATTCAAGGCAGTAATTTCCACTTCTTTGCTGTAGGTGGAGAACCCTTGGAAATGCAGGGAGTGCTAATG

IC:

- upstream primer: 5' - GCAATCGTATTACCTCTTATCGCAG - 3'
- downstream primer: 5' - CAACCATCGTCATCGTCAGGAAAC - 3'
- probe: 5' - NFQ - MGB - GGCAAAGTCCCATCGTT- TET - 3'

GCAATCGTATTACCTCTTATCGCAGCTGGTTCCTATTTGGCAAAGTCCCATCGTTTCCTGACGATGACGATGGTTG

We perform the assay using 10μl of extracted DNA in a 50μl total reaction volume. In addition to the target DNA, we include the following reagents:

	final conc.
BK/IC primer mix	1x
BK/IC probe mix	1x
$MgCl_2$	4 mM
UNG (Perkin Elmer)	0.5 U
LC FastStart Hyb Probe Mix (Roche)	1x
PCR Enhancer (Epicentre)	1x

The final reaction concentration of the primers is 1 μM each for the BK and internal control reactions. Both probes are present in the reaction at a concentration of 200 nM.

The cycling conditions for amplification and detection are as follows:

1) 50°C 2 minutes
2) 95°C 8 minutes

3) 95°C 15 seconds
4) 58°C 30 seconds
5) 76°C 30 seconds

(3–5: 50 cycles)

6) 95°C 15 seconds
7) 45°C 15 seconds
8) 95°C 15 seconds

Synthesis of artificial templates:

Overlapping pairs of oligonucleotides were ordered for each of the synthetic targets to be generated (Dunlop, HC-u9, HI-u8). Each pair was PCR amplified using the following protocol:

5 minutes at 95°C; 5 cycles of 1 minute at 94°C, 1 minute at 63°C, 1 minute at 72°C; 30 cycles of 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C; 10 minutes at 72°C.

Following amplification, the PCR products were sequenced to confirm the presence of the predicted polymorphisms.

CONCLUSIONS

We developed a real time assay for the detection and quantitation of BK virus in plasma, serum and urine. (We recently validated this assay for whole blood.) This assay is multiplexed with a heterologous plasmid internal control that is co-extracted and co-amplified with the BK target, monitoring the extraction as well as the PCR reaction. Adding the internal control reaction has no detectable effect on the sensitivity of the BK assay. Using a stored standard curve has proven to be a satisfactory option for quantitation, and provides accurate results when compared to another reference lab. Lyophilizing the stock standard appears to be an excellent solution for stabilizing this material, and is also being used to stabilize the BK positive controls. As with any real time assay, this test may be vulnerable to polymorphisms that occur within the amplicon. At ARUP, when a sample fails to generate an amplification plot, examination of the melting curve has proven helpful in detecting sequence variants.