# *Design and Optimization of Primer and Probe Sets for BK Virus Candidate SRM 2365*

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## Abstract

BK virus is a polyomavirus that infects approximately 80% of the population [1]. The virus remains latent in most people, but may cause illnesses in immunocompromised patients, including organ transplant, HIV/AIDS-infected, and diabetic patients. BK virus may cause nephropathy or hemorrhagic cystitis in renal transplant patients because they receive anti-rejection medication which weakens the immune system [2]. These diseases frequently cause renal allograft failure. In order to treat BK virus, accurate quantitation of viral load is necessary to optimize immunosuppressant dosing regimens. In preparation for PCR, the BK Dunlop plasmid was linearized using the restriction enzymes BamHI and EcoRI (ThermoScientific). Multiple primer sets were successfully designed and optimized for droplet digital PCR (ddPCR). In the future, primer sets will be tested using chamber digital PCR to show concordance across platforms. Five of the seven assays designed within coding regions of the WT BK Virus genome showed optimal positive/negative droplet separation in ddPCR and curve morphologies for quantitative PCR (qPCR).

## Materials and Methods

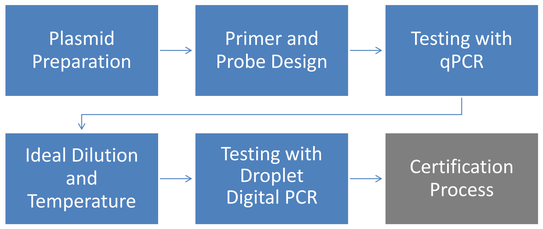


**Figure 1. Experimental Design** Depicted above is the general process followed prior to starting the certification process of a DNA-based SRM: plasmid preparation, primer and probe design, testing with qPCR, determining ideal dilution and temperature for digital PCR, and testing with droplet digital PCR.

Primers and probes were designed using Biosearch software. The coding regions were targeted because of increased conservation across BK virus genotypes. These primers and probes were checked for secondary structures, such as primer dimers and hairpins, using Beacon Designer software. Primer and probe sets were ordered from Operon (Figure 5).

For qPCR, primer and probe sets were tested at three melting temperatures: 60.0˚ C, 62.0˚ C, and 64.0˚ C. At each temperature, 5 ten-fold dilutions of ~15 ng/µl stock ($10^{-3}$ to $10^{-7}$) were tested. TaqMan PCR Master Mix (Applied Biosystems) was used according to manufacturer guidelines. Samples were amplified and analyzed using the Applied Biosystems 7500 (Figure 2).

**Figure 2. Applied Biosystems 7500**

For ddPCR, primer and probe sets were tested initially at seven dilutions in order to determine the dilution at which there were ~40-50% positives (Figure 7). The primers and probes were then tested at three melting temperatures, 58.0, 60.0, and 62.0˚ C with a 1:1 dilution of $10^{-5}$ from a ~15 ng/µl stock solution. ddPCR Super Mix (Bio-Rad) was used according to manufacturer guidelines. Droplets were created using the Qx100 Droplet Generator, amplified for 60 cycles using the Veriti (Applied Biosystems), and analyzed using the Qx100 Droplet reader and software, Quantasoft (Figure 3).

**Figure 3. Bio-Rad Qx100**

## Results

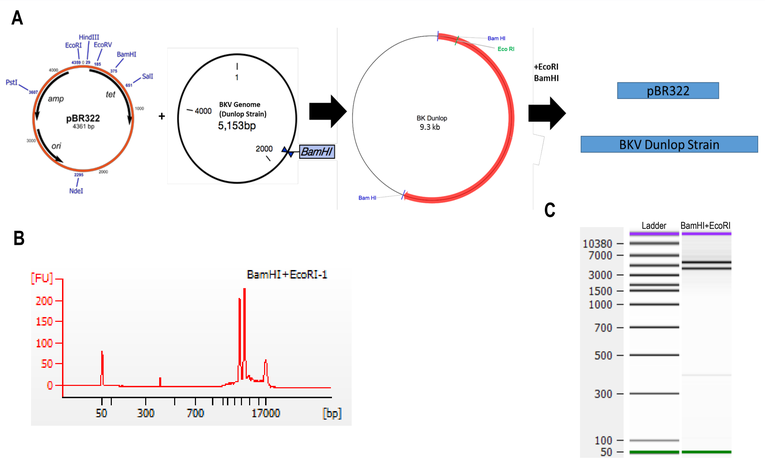


**Figure 4. Preparation of BK (Dunlop) Plasmid** The Dunlop plasmid used in this experiment was received from ATCC ligated into a pBR322 backbone. In order to prepare it for PCR, it was linearized using the restriction enzymes BamHI and EcoRI **(A).** Following plasmid preparation, the sample was run on the Agilent Bioanalyzer to ensure complete digestion. The electropherogram and gel indicate the expected fragment lengths of ~380, 4300, and 5000 bp **(B,C).**

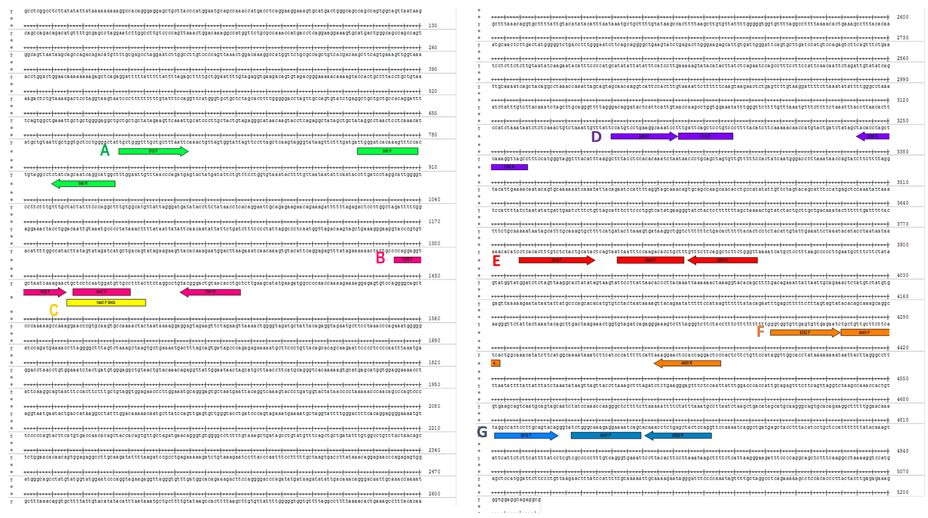


**Figure 5. Position of Primer and Probe Sets** Depicted above are the 7 sets of primers and probes designed for the BK virus genome. Primer sets B and C share forward and reverse primers, but have different probes. Primer set C has a Black Hole Quencher (BHQ) probe while primer set B has a BHQplus probe. All other sets have BHQplus probes.

**Table 1. Efficiency of qPCR reactions** Depicted below are the efficiencies of the qPCR reactions for each primer and probe set. This efficiency is calculated using the formula $E = -1+10^{(-1/slope)}$ where "slope" is the slope of the standard curve.

| Primer Set | Probe Type | Amplicon Length | Slope 60.0° C | Efficiency 60.0° C | Slope 62.0° C | Efficiency 62.0° C | Slope 64.0° C | Efficiency 64.0° C |
|---|---|---|---|---|---|---|---|---|
| A | BHQ Plus | 129 | -3.53 | 92.04% | -3.68 | 86.81% | -3.81 | 83.13% |
| B | BHQ Plus | 80 | -3.51 | 92.81% | -3.57 | 90.71% | -3.58 | 90.43% |
| C | BHQ | 80 | -3.42 | 96.21% | -3.52 | 92.49% | -3.52 | 92.40% |
| D | BHQ Plus | 103 | -3.50 | 93.04% | -3.51 | 92.77% | -4.46 | 67.65% |
| E | BHQ Plus | 78 | -3.49 | 93.60% | -3.52 | 92.21% | -3.63 | 88.52% |
| F | BHQ Plus | 114 | -3.496 | 93.22% | -3.58 | 90.30% | -3.40 | 96.84% |
| G | BHQ Plus | 71 | -3.43 | 95.67% | -3.51 | 92.81% | -3.41 | 96.37% |

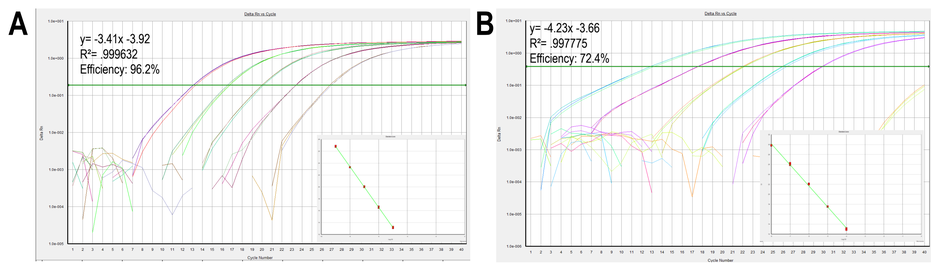


**Figure 6. High Efficiency Reaction vs. Low Efficiency Reaction** Depicted above are the amplification plots of two qPCR primer and probe sets, along with their respective standard curves (inset). Graph **A** is an example of a high efficiency reaction, with a sharp curve and an efficiency of 96.2%, while Graph **B** is an example of a low efficiency reaction with a dull curve and an efficiency of 72.4%.

## Results

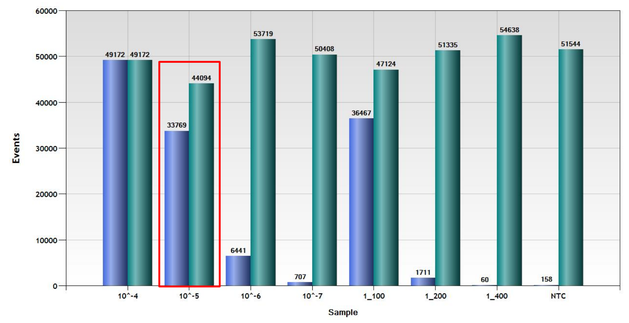


**Figure 7. ddPCR Dilution Series** Depicted above are the positives (blue) compared to the total counts (green). At $10^{-5}$ there are ~80% positives, so a 1:1 dilution of $10^{-5}$ would yield within the optimal range of 40- 50% positives. Dilutions were tested using primer set B.

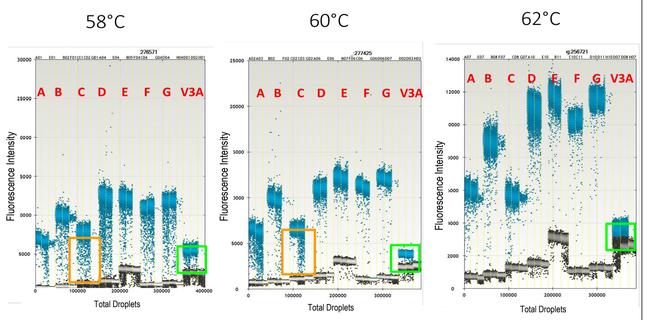


**Figure 8. Droplet Digital PCR Plots** Droplet digital PCR plots are displayed on the left (n=3 replicates, 2 shown). Positive (blue) and negative (black) droplets should have distinct separation in a good assay. Sets A and C have far less separation than the others (Set C boxed in orange). V3A, a published assay [3] was included for comparison, and shows little to no separation (boxed in green) at these temperatures.

**Table 2. Droplet Digital Concentrations** The table below lists the mean copies/µl (concentration) for each primer set and its respective standard deviation. These tables show that all of the primer sets yield similar results.

| | 58°C | | 60°C | | 62°C | |
|---|---|---|---|---|---|---|
| Primer Set | Mean | Standard Deviation | Mean | Standard Deviation | Mean | Standard Deviation |
| A | 649 | 18.0 | 597 | 52.9 | 655.3 | 45.6 |
| B | 649 | 26.8 | 623 | 54.1 | 731 | 33.6 |
| C | 762 | 52.6 | 593 | 39.2 | 719.3 | 97.2 |
| D | 770 | 35.0 | 578 | 68.8 | 799.3 | 10.3 |
| E | 700 | 67.3 | 633 | 22.9 | 695.3 | 46.9 |
| F | 727 | 58.2 | 594 | 26.1 | 723 | 18.4 |
| G | 742 | 73.5 | 588 | 33.6 | 721.7 | 41.3 |
| V3A | 732 | 29.5 | 586 | 15.0 | 687 | 52.3 |

## Conclusions

5 of the 7 primer and probe sets designed show optimal positive/negative separation on ddPCR and curve morphologies on qPCR. These 5 primer sets, B, D, E, F, and G will be used to complete the certification process.

## Future Studies

In the future, the BK Virus primers and probes must be optimized from chamber digital PCR to show concordance across platforms. Primer and probes for the JC Virus must also be optimized for qPCR, dPCR, and cdPCR. Once optimized, BK Virus and JC Virus SRMs will be prepared for certification.

## References

[1] Egli A, et al, 2009. "Prevalence of Polyomavirus BK and JC Infection and Replication in 400 Healthy Blood Donors". *Journal of Infectious Disease.* 199: 837-46.
[2] Gupta, G, et al, 2006. "Low Incidence of BK Virus nephropathy after simultaneous kidney pancreas transplantation". *Transplantation.* 82 (3): 382-8.
[3] Hoffman, N. G., Cook, L., Atienza, E. E., Limaye, A. P., & Jerome, K. R. (2008). Marked Variability of BK Virus Load Measurement Using Quantitative Real-Time PCR among Commonly Used Assays. *Journal of Clinical Microbiology*, *46*(8), 2671–2680.