

An Update on a Candidate BK Virus DNA Standard Reference Material

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Abstract

The polyomavirus, BKV, is widespread in the population due to primary infection during childhood and largely remains latent in the majority of individuals. However, illnesses as a result of BKV occur in immunocompromised patients, organ transplant, HIV/AIDS-infected, or diabetic patients. Two common illnesses resulting from BKV, hemorrhagic cystitis and nephropathy, occur in transplant patients, as prescribed anti-rejection medications can weaken the immune system and this often ultimately leads to renal allograft failure. To treat BKV, accurate quantitation of viral load is necessary for proper adjustment of anti-rejection medications. Currently, no international "higher order" standard for BKV exists, making chronic monitoring of the virus difficult. Therefore, we propose a candidate standard reference material (SRM) to aid in precise measurements of viral load across laboratories and time. We have amplified and sequenced six full-length BKV genomes. Various primer and probe sets were designed and optimized for qPCR and digital PCR (dPCR). The availability of a panel of BKV DNA genotypes as potential reference materials will enable traceability for manufacturers of calibrant materials. This will ultimately improve upon the consistency and accuracy of viral load quantitation measurements across laboratories, leading to improved dosing regimens in infected patients.

Development Strategies

Utility of multiple strains

Feedback from the clinical community triggered the design of a candidate reference material containing multiple genotypes.

Viral DNA was extracted from six available clinical isolates with distinct genotypes (Ia, Ic, III, IV, V, VI) of the BK virus (Hoffman, et. al, 2008). Sample digestion by BamHI or EcoRI, followed by long-range PCR with Takara LA Taq DNA polymerase (Clontech) and genotype-specific primers surrounding BamHI or EcoRI, allowed amplification of some, but not all six genotypes (Figure 1). This was likely due to strain polymorphisms within primer binding sites and/or the existence of multiple, instead of single BamHI or EcoRI sites predicted from known sequence information.

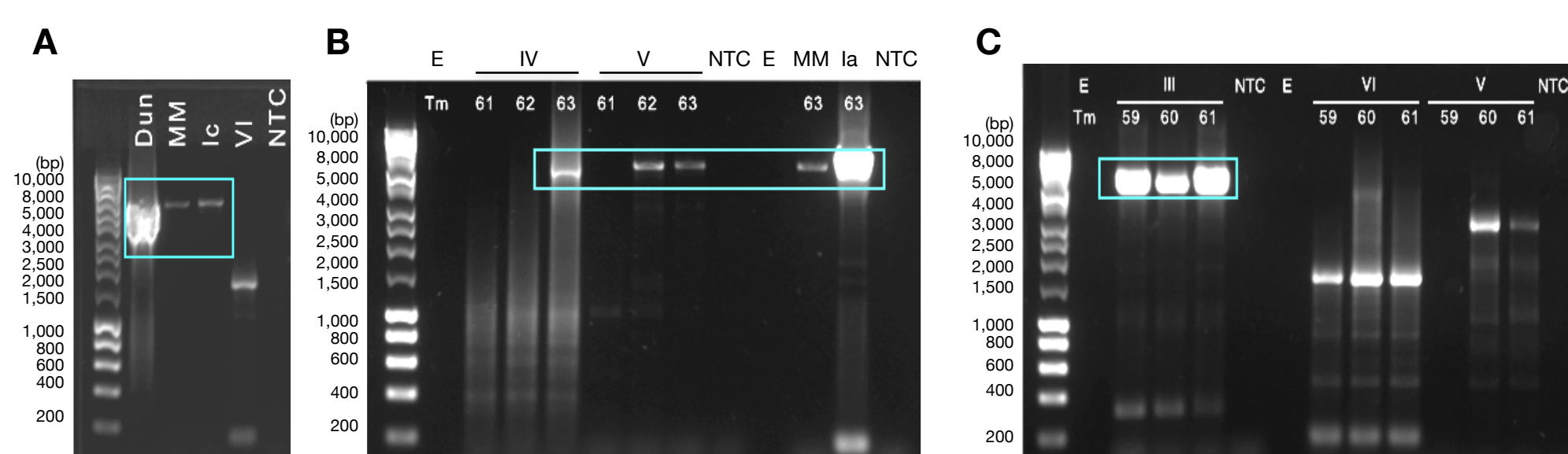


Figure 1. Long-range PCR of six BKV clinical samples. 1.8% Agarose gel electrophoresis of long-range PCR products. Complete genomes of genotypes Ia, Ic, IV, and V (boxed in blue), but not genotypes III and VI, were successfully amplified. The Dunlop (Dun) and MM strains were used as Ia genotype positive controls. NTC = no template control, E = empty well.

Whole genome amplification using φ29 DNA polymerase (Epicentre) and random RNA hexamers, successfully amplified BKV genomes from each clinical isolate, Figure 2. Next-generation sequencing was performed using the Nextera XT library prep and MiSeq v3 600-cycle chemistry.

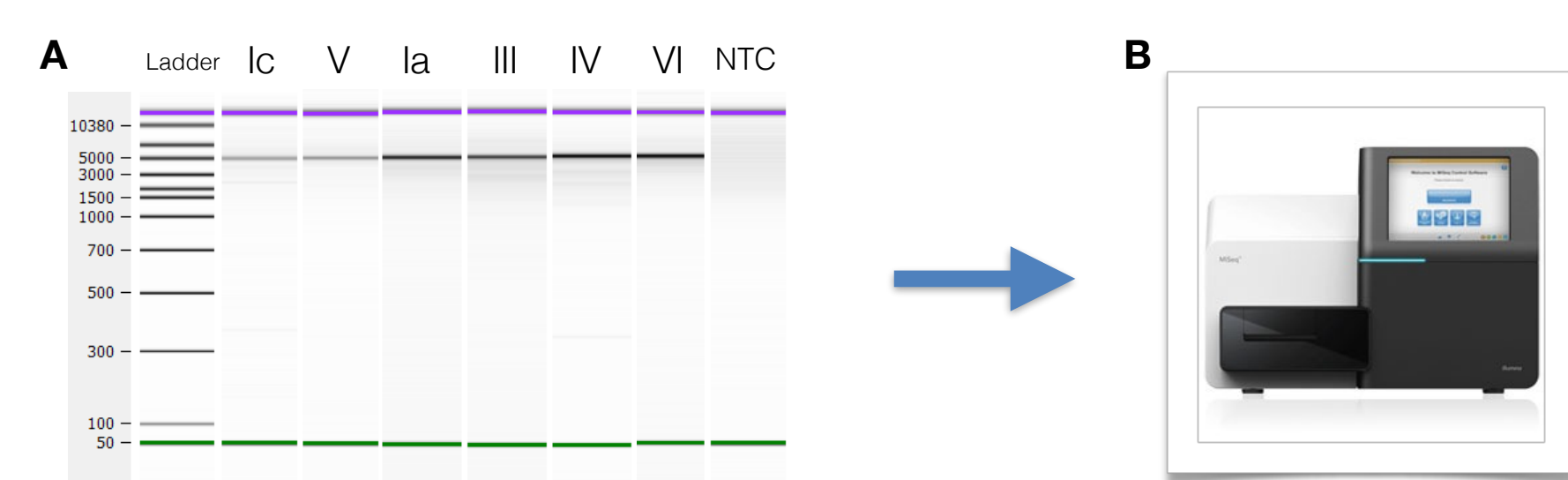


Figure 2. Whole genome amplification of BKV genomes prior to NGS. Rolling circle amplification created BKV genome concatamers, which were either left in tact or digested with a single cut restriction enzyme (HhaI, EcoRI, or BamHI). Quality was assessed via the Agilent Bionalyzer, A, prior to NGS, B (representative of each cut genotype shown).

BK Verification of Isolate Genotypes prepared by WGA

Following quality base trimming (Q<30) and alignment to the BK Dunlop strain, V01108.1, variant calling at a 5% frequency was performed with CLC Genomics Workbench, Figure 3. To determine concordance, the resulting variants were compared to two publications documenting genotype SNPs, Figure 4 and Table 1. Each sample had a minimum coverage of 1000x.



Figure 3. NGS polymorphism distribution across the BKV genome for six genotypes. For each clinical isolate, 1-2 digested replicates and the non-digested replicate from WGA were used for NGS. Shown are polymorphisms in the non-digested samples, compared to the Dunlop strain, V01108.1. As expected, genotypes III and IV have the highest number of polymorphisms, as they are the most divergent of all of the genotypes.

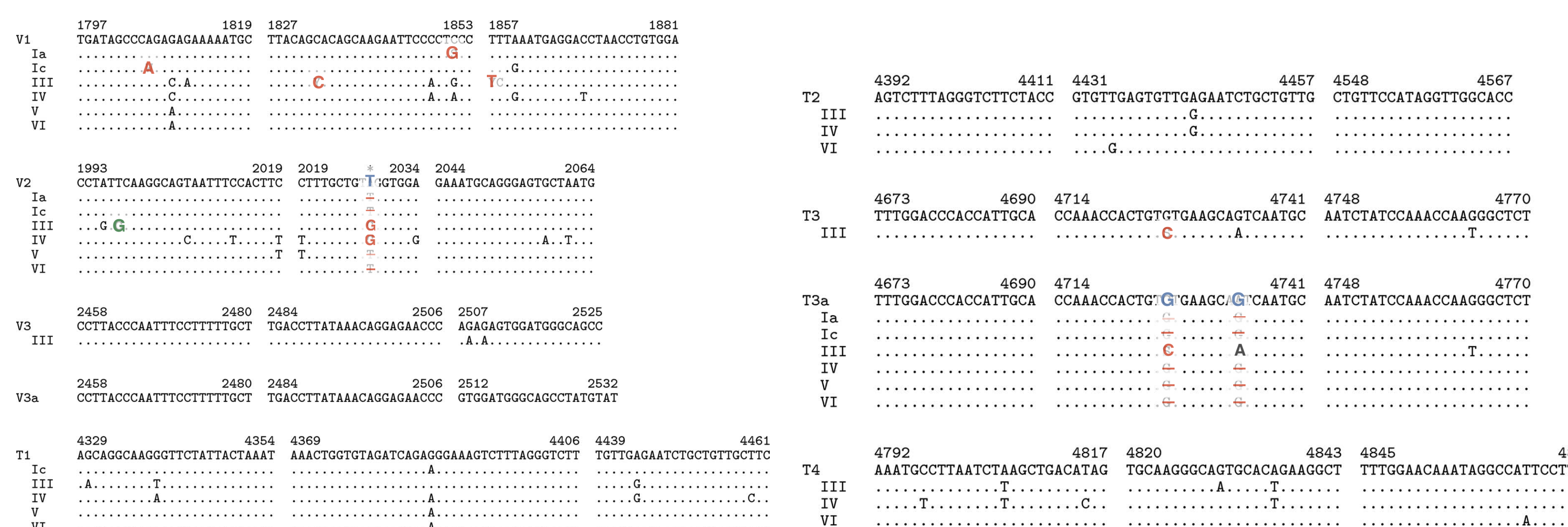


Figure 4. Concordance of BKV WGA-prepped clinical isolates with previously published Sanger sequence data. NGS identified previously ambiguous bases (red). Three nucleotides were discordant from previous Dunlop strain sequence data (blue). Genotype SNPs previously reported were confirmed, with the exception of genotype III (green), n=2-3 separate WGA preparations per clinical sample. Figure adapted from Hoffman, et. al, 2008.

Table 1. Concordance of WGA-prepped BKV clinical isolates with previously published Sanger sequence data. Of the 235 SNPs documented in Sharma, et. al, 2006, the Ia clinical isolate sequenced was the only concordant genotype.

	Ia	Ic	III	IV	V	VI
Number of SNPs discordant	0	1	>10	>10	4	3
Number of new SNPs found	0	0	-	-	1	1

Synthetic plasmid construct and preparation

The wild-type BKV genomic sequence (Accession No. JQ713822.1) was synthesized, ligated into the pUC57 vector, and transformed into *E. coli* (Genewiz) in preparation for SRM production, Figure 5. The plasmid will be propagated in *E. coli*, giga-prepped, purified, and linearized with restriction enzymes unique to the pUC57 backbone, leaving the entire BKV sequence in tact. Linearization of the plasmid is necessary for accurate digital PCR quantitation, Figure 6.

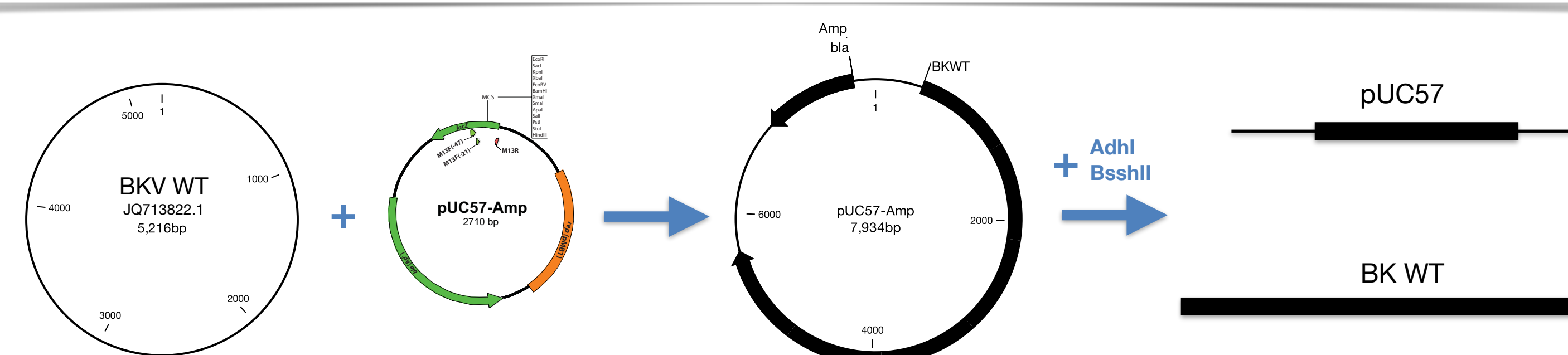


Figure 5. Candidate BKV SRM construct. Plasmid maps for the BKV WT sequence, JQ713822.1, pUC57, and candidate BKV SRM to be certified for concentration.

Characterization Techniques

Certification for concentration

Digital PCR, which allows for the calculation of absolute copy number per volume, will be used to certify the BKV material for concentration. Further, multiple assays will be used across droplet digital and chamber digital platforms (ddPCR using BioRad QX100 and cdPCR using BioMark Fluidigm).

Sequence Data

Next-generation sequencing data will be provided in the SRM certificate as informational value.

Assay optimization

Seven BK-specific assays were designed and tested at 2-3 T_ms (58, 60, 62, 64°C) for qPCR, ddPCR, and cdPCR, Figure 7.

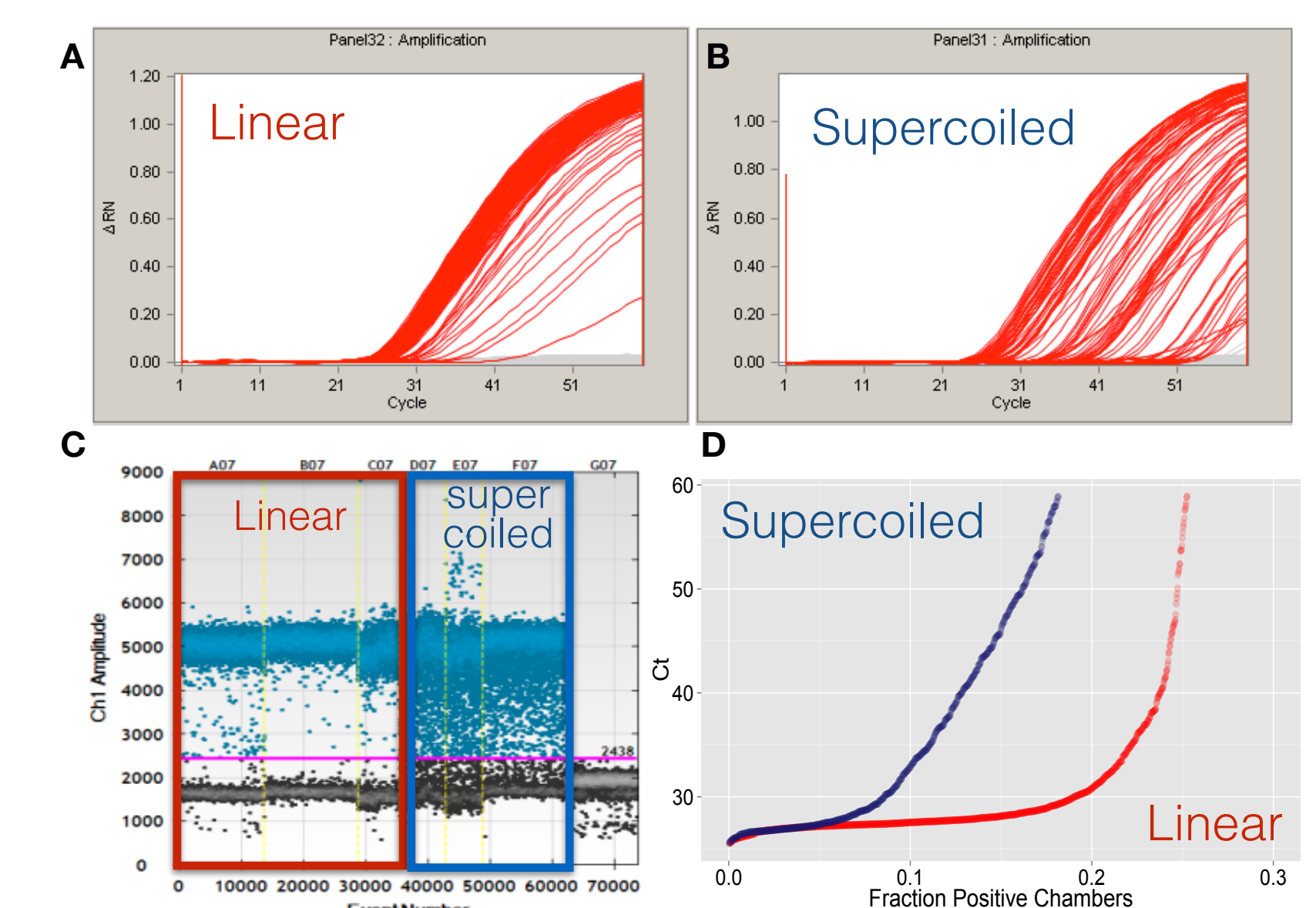


Figure 6. The target plasmid conformation matters for digital PCR. As observed in both ddPCR (C) and cdPCR (A, B, D), there are more targets amplifying at later cycles in the supercoiled plasmid compared to the linear plasmid. Further, at 60 cycles, the supercoiled plasmid is still being amplified (D), but the linear plasmid has fully amplified.

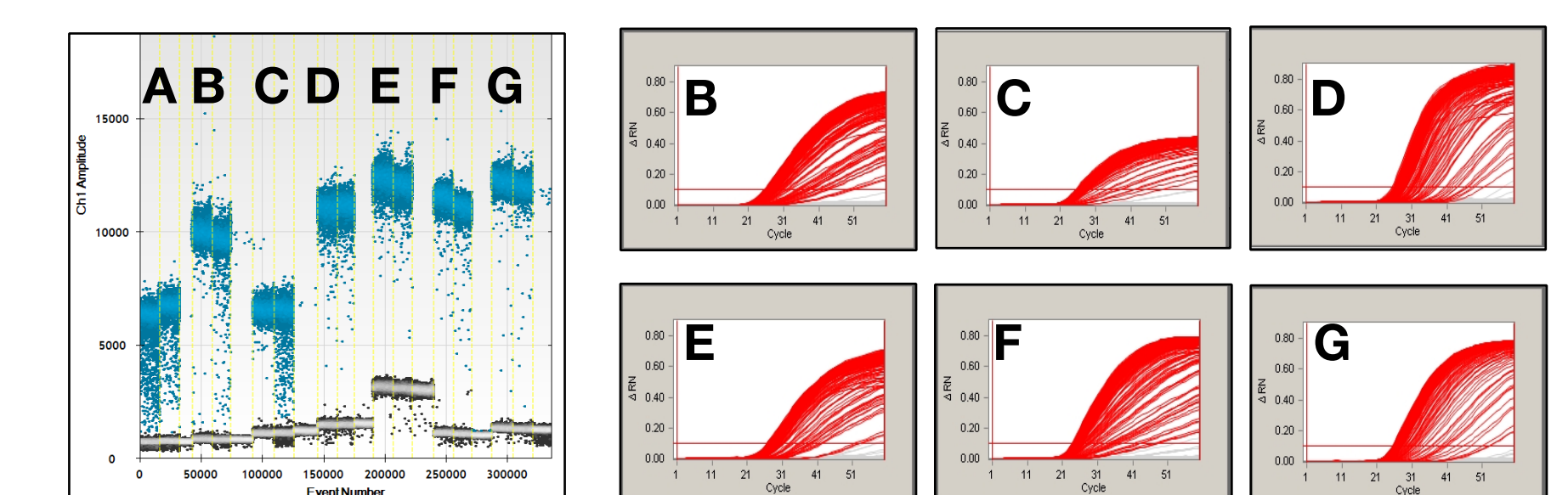


Figure 7. BKV assays optimized for dPCR. Five (B, D, E, F, G) of seven assays designed within coding regions of the BKV genome, showed optimal positive/negative droplet separation and curve morphologies for dPCR (left panel) and cdPCR (right panel). Note: Assay A was not used for cdPCR.

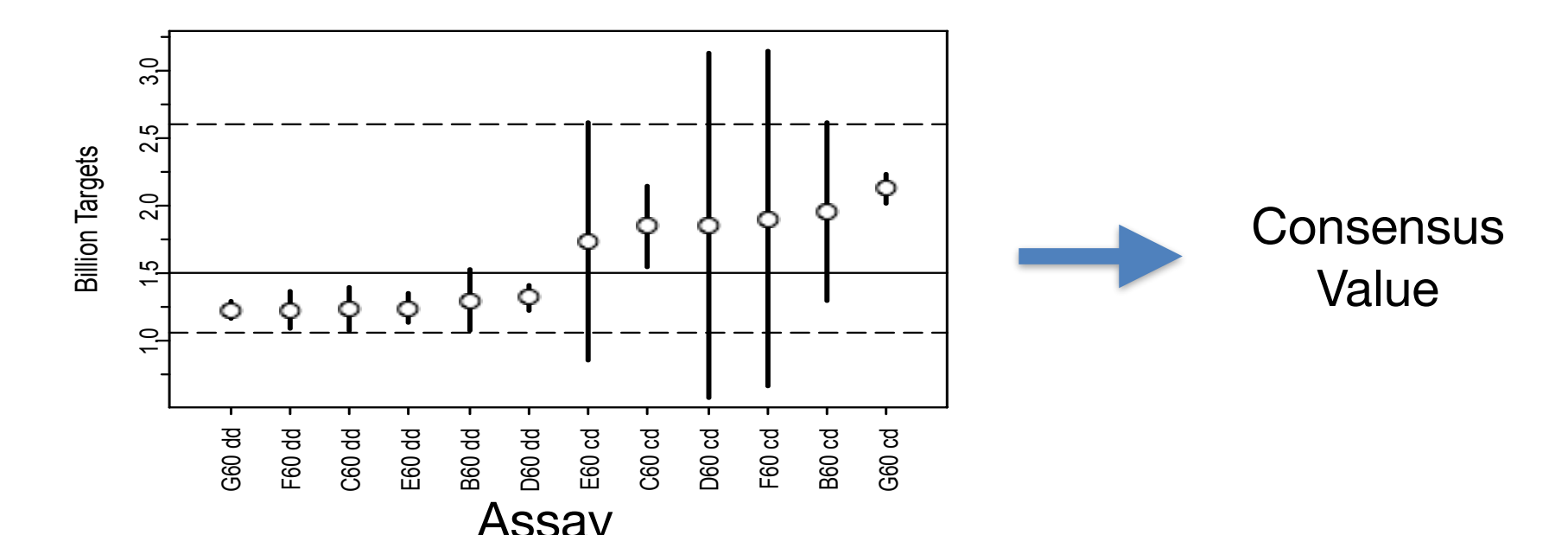


Figure 8. Certification of SRMs utilizing multiple assays. By treating the results of multiple assays across platforms as an interlaboratory study, we can use the DerSimonian-Laird approach to calculate a consensus value.

Conclusions and Future Directions

Amplification and cloning challenges have prompted NIST to redirect its efforts to characterization of synthetic constructs, as they are easier to prepare, can be stored indefinitely in *E. coli*, and will allow for quicker production of reference materials. Additionally, we will be providing a JC virus construct along with the BKV construct. By integrating data from multiple assays and platforms, we can expand the measurement uncertainty, which may arise due to different master mixes and/or inconsistencies in droplet/chamber volumes.

Scan the QR code to tell us your needs for DNA- or RNA-based reference materials!

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