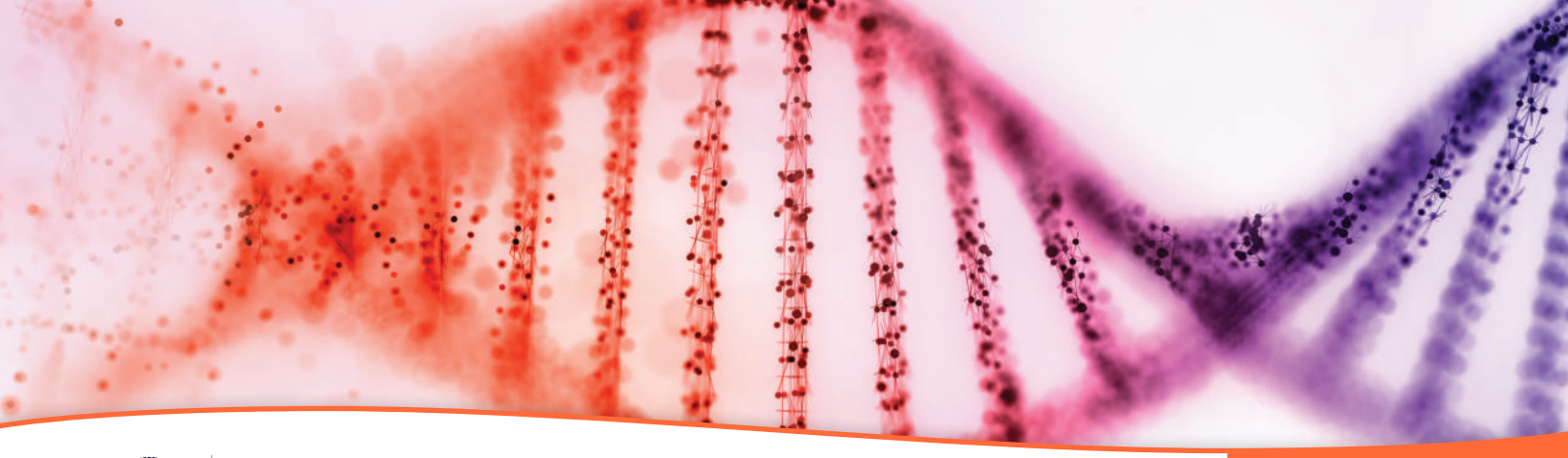

Application Note

Application of an MRC-5 gDNA control in the detection of residual host cell DNA in biopharmaceuticals

Abstract

Residual host cell genomic DNA (gDNA) poses a significant safety risk in biopharmaceutical manufacturing. The precise detection and quantification of this impurity is, therefore, essential. In this study, we demonstrate the application of our high-quality quantitative gDNA control material in validating quantitative PCR (qPCR) assays designed to detect residual host gDNA impurities in biologics.

Introduction

When manufacturing biopharmaceuticals such as vaccines and other biologics, cell substrates are often used to produce the desired product. One of the primary concerns when using these complex expression systems is the potential presence of residual host cell gDNA in the final product, even after downstream purification processes. The effective removal of this impurity is essential as host gDNA may harbor oncogenic or viral sequences that can put patient safety at risk. In response to this concern, regulatory agencies such as the U.S. Food & Drug Administration (FDA),[1] European Medicines Agency (EMA),[2] and World Health Organization (WHO),[3] have set criteria about the maximum amount of residual host gDNA allowed in such products (< 10 ng/dose, ≤ 200 bp in length).

A widely accepted methodology for the precise quantification of residual host cell gDNA is quantitative PCR (qPCR). This approach is a highly sensitive and specific high-throughput technology that can detect femtogram amounts of residual host gDNA in samples.[4-7] While qPCR offers many advantages, biases associated with different protocols or reagents can affect the resulting data. Therefore, it is essential to thoroughly validate assay performance using high-quality, authenticated reference materials to ensure reliable, reproducible, and comparable results.

To support the need for highly qualified reference materials, USP and ATCC have collaborated to develop gDNA control materials derived from host cell lines commonly used to manufacture vaccines and other biologics. These commercially available controls are manufactured, tested, and quantitated using robust processes, providing high-quality controls for evaluating qPCR detection methods.

In this report, we demonstrate the utility and performance of our MRC-5 gDNA control material against three highly sensitive PCR assays developed to detect residual human gDNA.[4-7] These assays target highly repetitive sequences in the human genome (18S rRNA genes or *Alu* transposable elements) and were chosen to evaluate our control material as they were proven to be highly sensitive and specific methods for quantitating small amounts of human gDNA.

Materials and Methods

In this study, we used gDNA (catalog #1592112) extracted from the MRC-5 cell line (ATCC® CCL-171™). This gDNA product was evaluated for critical attributes such as concentration, total amount, purity, and integrity as well as, most importantly, its utility as a reliable qPCR control material.

Development and quality control of the reference material

Product integrity and purity were evaluated via classical gel electrophoresis and the Agilent TapeStation system (Agilent Technologies, Inc). Restriction digestion using *Bam*HI (New England Biolabs, catalog number R0136S) was conducted according to the manufacturer's recommendations. Undigested gDNA was incubated under the same conditions as the treated sample (i.e., temperature, duration, buffer) but without the restriction enzyme.

qPCR assays

Table 1 summarizes the assays we used in this study. These assays have been shown to have a wide dynamic range and high sensitivity for detecting traces of human gDNA.

Table 1: Published assays developed to detect residual gDNA from human cells

Authorship	Target Seq.	Detection range (pg/reaction) in the publication	Linear range (pg/reaction) in the publication	Testing range (pg/reaction) in this study
Zhang *et al. et al,* 2014[4]	*Alu*	0.125–1250	0.125–1250	0.025-125
Funakoshi *et al.*, 2017[6]	*Alu*	0.00001–1000	0.00001–1000	0.005-1000
Andre *et al.*, 2016[5]	18S rRNA	5–50000	5–50000	1-5000

qPCR experiments were performed in a 20 µL reaction containing 10 µL of iTaq™ Universal Probes Supermix (Bio-Rad, catalog number 1725131). The primer and probe sequences used were those from published assays (**Table 2**),[4-6] and the Taqman probes were tagged with a FAM dye and quenched by a combined ZEN/Iowa Black fluorescent quencher from Integrated DNA Technologies (IDT). The cycling parameters were performed as summarized in **Table 3**; in some instances, the master mix formula was adapted to use reduced concentrations of primers to help increase the Cq values of the no template controls (NTCs) while still allowing efficient amplification of the DNA samples (**Table 4**). All samples were tested in triplicate.

Table 2: Primers and probes used in the study

Assay Publication	Oligo names	Oligonucleotide sequences	Amplicon Size (bp)
Zhang *et al.*, 2014[4]	HS_Alu_F1	GAGGCGGGCGGATCA	94
	HS_Alu_R2	CCCGGCTAATTTTTGTATTTTTAGTAG	
	HS_Alu_P1	CAGCCTGGCCAACATGGTGAAACC	
Andre *et al.*, 2016[5]	HS_RIB_F_123	GCAATTATTCCCCATGAACG	123
	HS_RIB_R	GGCCTCACTAAACCATCCAA	
	HS_RIB_P	AAGTCCCTGCCCTTTGTACACACCG	
	HS_RIB_F_254	AACAGGTCTGTGATGCCCTT	254
	HS_RIB_R	GGCCTCACTAAACCATCCAA	
	HS_RIB_P	AAGTCCCTGCCCTTTGTACACACCG	
Funakoshi *et al.*, 2017[6]	Funakoshi_Alu_F1	GGTGAAACCCCGTCTCTACT	105
	Funakoshi_Alu_R1	GGTTCAAGCGATTCTCCTGC	
	Funakoshi_Alu_P1	CGCCCGGCTAATTTTTGTAT	

Table 3: Cycling parameters

Publication	Initial Denature [Cycles x Target °C/Hold Time (min:sec) / Ramp Rate in °C/sec]	PCR Cycling [Cycles x Target °C/Hold Time (min:sec) / Ramp Rate in °C/sec]	Instrument
Zhang *et al.*, 2014[4]	1 × 95°C/15:00/3.3	40 × (95°C/00:30/3.3 – 60°C/01:00/3.3)	CFX96™ (Bio-Rad)
	1 × 95°C/15:00/1.6	40 × (95°C/00:30/1.6 – 60°C/01:00/1.6)	QuantStudio™ 3 (Thermo Fisher Scientific)
Andre *et al.*, 2016[5]	1 × 95°C/10:00/3.3	40 × (95°C/00:10/3.3 – 62°C/00:30/3.3 – 72°C/00:10/3.3) + 1 × (40°C/00:30/3.3)	CFX96™ (Bio-Rad)
	1 × 95°C/10:00/1.6	40 × (95°C/00:10/1.6 – 62°C/00:30/1.6 – 72°C/00:10/1.6) + 1 × (40°C/00:30/1.6)	QuantStudio™ 3 (Thermo Fisher Scientific)
Funakoshi *et al.*, 2017[6]	1 × 95°C/10:00/3.3	50 × (95°C/00:15/3.3 – 56°C/00:30/3.3 – 72°C/00:30/3.3)	CFX96™ (Bio-Rad)
	1 × 95°C/10:00/1.6	50 × (95°C/00:15/1.6 – 56°C/00:30/1.6 – 72°C/00:30/1.6)	QuantStudio™ 3 (Thermo Fisher Scientific)

Table 4: Master mix formulation

	Assay		
Component	Zhang *et al.*, 2014[4] Final Concentration	Andre *et al.*, 2016[5] Final Concentration	Funakoshi *et al.*, 2017[6] Final Concentration
qPCR Reaction Mix	1×	1×	1×
10 µM Forward Primer	700 nM	500 nM	200 nM
10 µM Reverse Primer	700 nM	500 nM	200 nM
10 µM Probe	350 nM	200 nM	250 nM
PCR-Grade water	variable	variable	variable
Template	variable	variable	variable

During this study, we observed that PCR-grade water products were not well-suited as negative controls for human assays. We evaluated several PCR-grade water products from various companies; despite their claims to be free of human gDNA, these products showed positive PCR results. Those with the least human gDNA burden were from Amerigo Scientific and QIAGEN; therefore, these products were used interchangeably in this study.

Another aspect of data quality and reproducibility pertains to serial dilution, especially the diluent. Despite using low-bind or siliconized tubes, some amounts of gDNA still bind to the inner wall of these vessels. This phenomenon confounds PCR results, especially at dilutions containing very low gDNA concentrations. We were able to solve this problem by using Poly(A) buffer as a diluent; Poly(A) aids DNA recovery by coating the inner walls of the storage tubes and it protects nucleic acids from degradation by serving as a substrate for contaminating nucleases. In our study, we used 0.25 mg/mL Poly(A) (Millipore Sigma/Roche, catalog number 10108626001) solution as a diluent for serial dilutions. Serial dilutions were made in low-bind tubes (Thomas Scientific, catalog number 1149X75), and PCR-grade water (QIAGEN, catalog number 17000-10, or Amerigo Scientific, catalog number PER1136265AMP) was used throughout all experiments.

PCR inhibition assessment

We used FBS (Sigma-Aldrich, catalog number F0926) and molecular-grade BSA (Thermo Fisher Scientific, catalog number B14) for spike-in PCR experiments.

Results

We conducted a series of tests to assess the quality and applicability of the MRC-5 gDNA product.

Assessing the quality of the gDNA control

We assessed gDNA integrity via the Agilent TapeStation system (**Figure 1**). As expected, the uncut gDNA was a single band >48 kb. In contrast, the product appeared as a <48 kb smear following treatment with the *Bam*HI restriction enzyme. These findings indicate that the product has no contaminating material residues from extraction. We found similar results when analyzing the gDNA via classical gel electrophoresis (data not shown). No traces of residual RNA were observed. We also assessed product purity via spectroscopy. The A_{260}/A_{280} values were within 1.7-2.0, which are industry-acceptable purity parameters and within specifications outlined in the product Certificate of Analysis (data not shown).

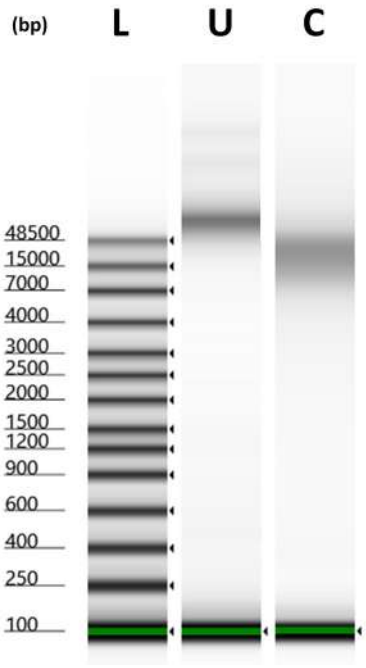

Figure 1: Evaluation of the integrity and purity of the MRC-5 gDNA. Analysis of cut (**C**) and uncut (**U**) gDNA via the Agilent TapeStation system. The sizes of resultant products were compared against the reference ladder (**L**).

Assessment of the qPCR assay sensitivity and linearity with the gDNA control material

We assessed the utility of the product as a qPCR control in three independent and highly sensitive assays.[4-6] Using the MRC-5 gDNA, we successfully confirmed the linearity, repeatability, intermediate precision, and published lower limit of quantitation (LLOQ). These qPCR experiments were executed repeatedly by two users and using two separate instruments on separate days. **Figure 2** summarizes the results from the Zhang *et al.* assay[4]. We paid particular attention to the lower end of the dynamic range to better assess the quality and utility of the gDNA as a reference material. The assay maintained linearity at concentrations up to 5× below the published LLOQ, and these results are consistent within each instrument. Minor variations observed between devices are due to inherent thermocycling conditions, hence inherent instrument design.[8-9] Variations in thermocycling patterns among PCR instruments could affect assay performance; therefore, users might be able to avoid potentially unexpected outcomes of sensitive PCR-based assays by designing their PCR protocols and establishing reliable workflows with the understanding that thermocycling conditions could vary among instruments. The data obtained using the André *et al.*[5] and Funakoshi *et al.*[6] assays were similar to those generated using the Zhang *et al.* assay and are shown in the supplementary information.

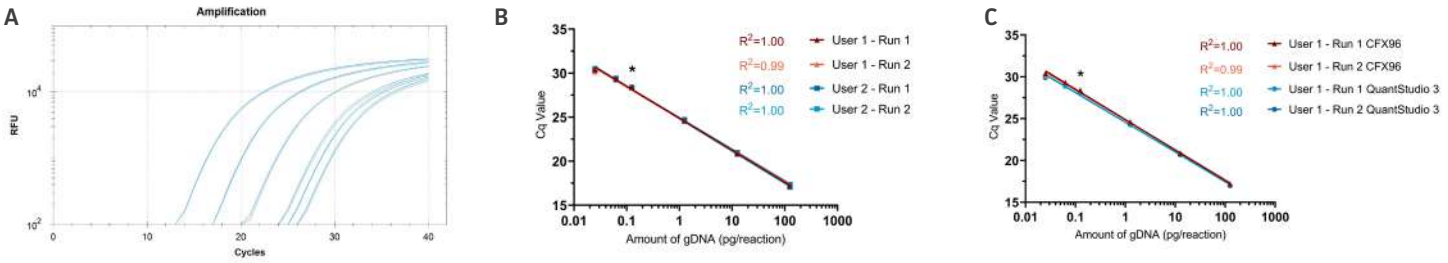

Figure 2: Comparative qPCR results summary regarding linearity and sensitivity of the Zhang *et al.*[4] assay using MRC-5 gDNA. (**A**) Amplification plots were generated by running the assay with serial dilutions ranging from 125 pg gDNA/reaction to 25 fg gDNA/reaction. (**B**) qPCR data between two users using a CFX96™ thermocycler (Bio-Rad). (**C**) qPCR data executed by one user using CFX96™ (Bio-Rad) or QuantStudio™ 3 (Thermo Fisher Scientific) thermocycler instruments. All samples were tested in triplicate. * = Lower Limit of Quantitation claimed by original assay developers in their publication.[4]

Evaluating the gDNA product as a control for assessing PCR inhibition during the bioproduction process

To evaluate the utility of the gDNA control in assessing the extent of qPCR inhibition during the bioproduction process, we diluted the MRC-5 gDNA in materials that mimic in-process samples or the final product. Then we compared the overall performance of the Zhang *et al.* assay under each condition (**Figure 3**). As a cell lysate surrogate (i.e., in-process sample), we used fetal bovine serum (FBS) due to its rich protein content and high amounts of salts, lipids, carbohydrates, and other constituents; FBS also has a pH that is not a critical PCR inhibition factor. As a highly purified protein material (i.e., final product), we used molecular-grade bovine serum albumin (BSA). Poly(A) was used as a control.

While the PCR containing FBS remained linear even with gDNA below the assay's LLOQ, the efficiency appeared significantly reduced: ΔCq ~5, suggesting a significant decrease in efficiency. The fluorescence of the reaction was also reduced by half as compared to that of the reactions containing the Poly(A) control and BSA diluents. As a result, the estimated concentration of the gDNA diluted in FBS was 4.8 pg/reaction versus 125 pg/reaction when diluted in the Poly(A) control, representing a 26× underestimate. These results were similar to those reported in the literature, showing cell lysates inhibit PCR.[7] In contrast, the PCR containing BSA, as pure protein and end product surrogate, had a minimal but not negligible impact on assay performance. At higher gDNA concentrations, the estimated gDNA in the BSA mixtures is, on average, up to 15% off the pure gDNA, which is within the acceptable range for the qPCR predictions. At lower gDNA concentrations, the assay overestimated the gDNA amount in the BSA mixtures between 1.6-2.5× (relatively lower Cq/Ct qPCR values). Overall, these results demonstrate the MRC-5 gDNA product can be used as a reliable control material for assessing the extent of PCR inhibition during the development and optimization of bioproduction processes.

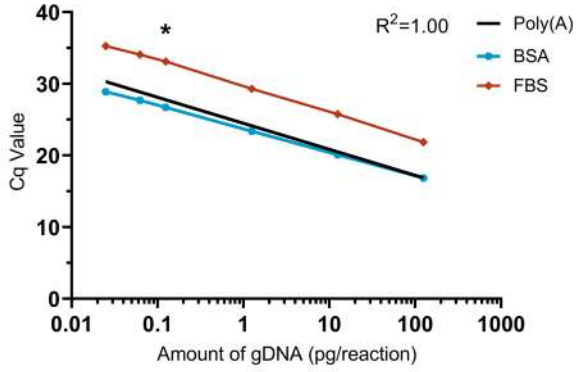

Figure 3: Using MRC-5 gDNA to assess PCR inhibition. Comparative performance of the Zhang *et al.*[4] PCR assay using the MRC-5 gDNA diluted in Poly(A) (control), FBS as a cell lysate surrogate, and BSA as a highly purified protein product. All samples were tested in triplicate. * = Lower Limit of Quantitation claimed by original assay developers in their publication.[4]

Conclusion

In this study, we demonstrate the quality of the MRC-5 gDNA product and its applicability as a reliable control material for qPCR assays designed to detect residual host gDNA in biologics. The gDNA is free of impurities, has high integrity, and is quantifiable and amplifiable. Furthermore, it is compatible with various highly sensitive qPCR assays developed for host residual gDNA detection. We evaluated three qPCR assays using the MRC-5 gDNA and successfully confirmed their linearity, sensitivity, repeatability, intermediate precision, range, and LLOQ. The PCR assays yielded consistently positive results with very low gDNA concentrations, even below their originally claimed LLOQ, indicating that the MRC-5 gDNA has good quality and is a reliable PCR control material.

Product Utility Recommendations

- Test the drug substance and process intermediates to assess clearance of residual gDNA using fully validated PCR assays with an analytical reference material derived from an authenticated cell line.
- According to ICH[10] and USP[11] guidelines, it is essential to develop and thoroughly validate assay performance using high-quality, authenticated reference materials to ensure reliable, reproducible, robust, and comparable results.
- Users might be able to avoid potentially unexpected outcomes of sensitive PCR-based assays by designing their PCR protocols and establishing reliable workflows with the understanding that thermocycling conditions could vary among instruments.

References

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SUPPLEMENTARY INFORMATION

MRC-5 gDNA performance in the André *et al.*[5] 123 bp amplicon assay.

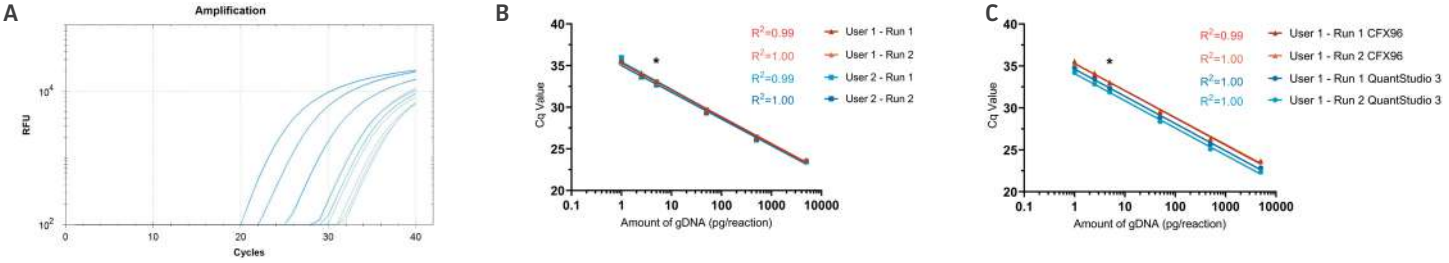

Figure S1: Comparative qPCR results summary regarding dynamic range and sensitivity of the André *et al.*[5] 123 bp amplicon assay using MRC-5 gDNA. (**A**) Amplification plots were generated by running the assay with serial dilutions ranging from 5000 pg - 1 pg gDNA/reaction. (**B**) qPCR data between two users using a CFX96™ thermocycler (Bio-Rad). (**C**) qPCR data executed by one user using CFX96™ (Bio-Rad) or QuantStudio™ 3 (Thermo Fisher Scientific) thermocycler instruments. All samples were tested in triplicate. * = assay developers' LLOQ .[5]

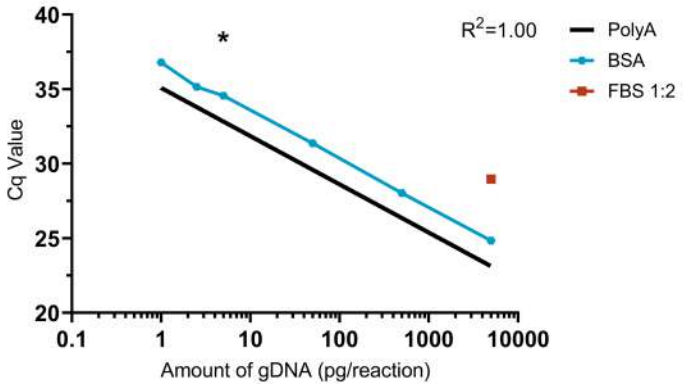

Figure S2: Using MRC-5 gDNA to assess PCR inhibition. Comparative performance of the André *et al.*[5] 123 bp PCR assay using the MRC-5 gDNA diluted in Poly(A) (control), FBS as a cell lysate surrogate, and BSA as a highly purified protein product. All samples were tested in triplicate. * = assay developers' LLOQ.[5]

MRC-5 gDNA performance in the André *et al.*[5] 254 bp amplicon assay.

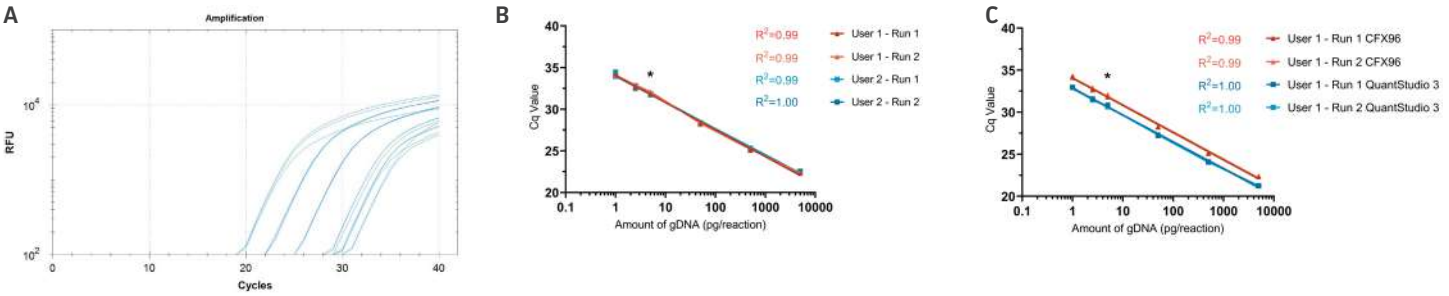

Figure S3: Comparative qPCR results summary regarding dynamic range and sensitivity of the André *et al.*[5] 254 bp amplicon assay using MRC-5 gDNA. (**A**) Amplification plots were generated by running the assay with serial dilutions ranging from 5000 pg - 1 pg gDNA/reaction. (**B**) qPCR data between two users using a CFX96™ thermocycler (Bio-Rad). (**C**) qPCR data executed by one user using CFX96™ (Bio-Rad) or QuantStudio™ 3 (Thermo Fisher Scientific) thermocycler instruments. All samples were tested in triplicate. * = Lower Limit of Quantitation claimed by original assay developers in their publication.[5]

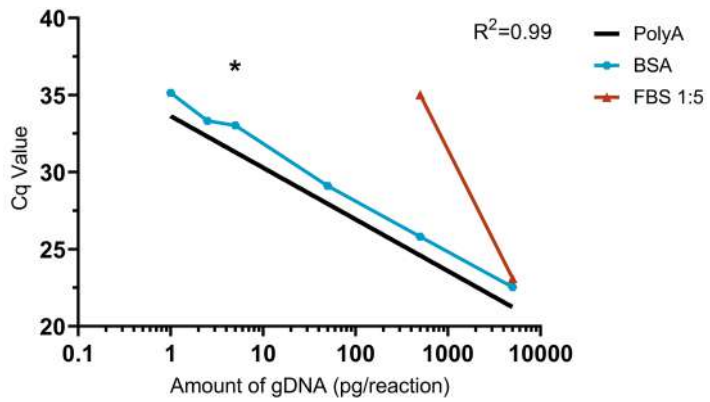

Figure S4: Using MRC-5 gDNA to assess PCR inhibition. Comparative performance of the André *et al.*[5] 254 bp PCR assay using the MRC-5 gDNA diluted in Poly(A) (control), FBS as a cell lysate surrogate, and BSA as a highly purified protein product. All samples were tested in triplicate. * = assay developers' LLOQ.[5]

MRC-5 gDNA performance in the Funakoshi *et al.*[6] assay.

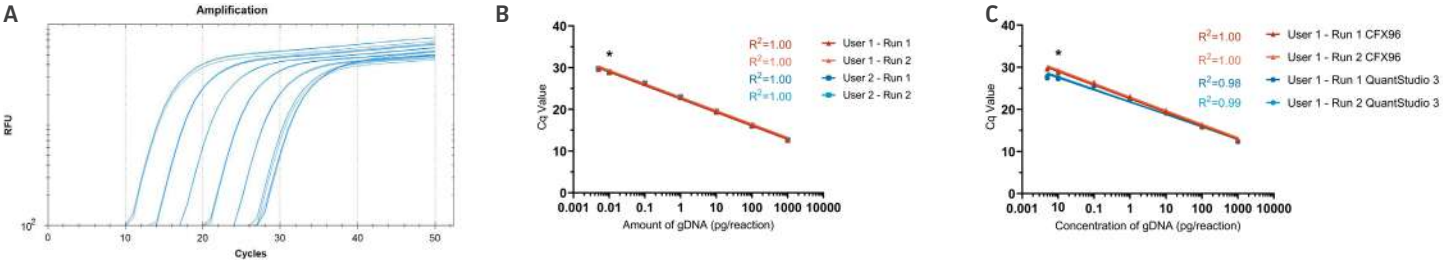

Figure S5: Comparative qPCR results summary regarding linearity and sensitivity of the Funakoshi *et al.*[6] assay using MRC-5 gDNA. (**A**) Amplification plots were generated by running the assay with serial dilutions ranging from 1000 pg - 5 fg gDNA/reaction. (**B**) qPCR data between two users using a CFX96™ thermocycler (Bio-Rad). (**C**) qPCR data executed by one user using CFX96™ (Bio-Rad) or QuantStudio™ 3 (Thermo Fisher Scientific) thermocycler instruments. All samples were tested in triplicate. * = assay developers' LLOQ.[6]

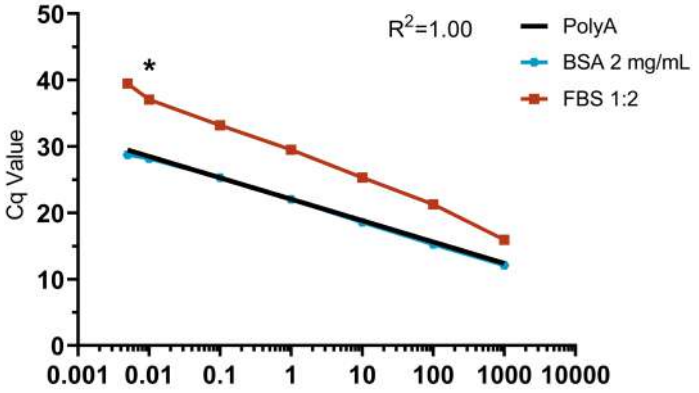

Figure S6: Using MRC-5 gDNA to assess PCR inhibition. Comparative performance of the Funakoshi *et al.*[6] PCR assay using the MRC-5 gDNA diluted in Poly(A) (control), FBS as a cell lysate surrogate, and BSA as a highly purified protein product. All samples were tested in triplicate. * = assay developers' LLOQ.[6]

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