HEK293 Host Cell DNA Residual Testing: Transitioning from Quantitative PCR to Droplet Digital PCR™

Mackenzie Scherr, Lovedeep Grewal, Parveen Alam, and Joshua Lehrer
Bio-Rad Laboratories, Inc., Digital Biology Center, 5731 W Las Positas Blvd, Pleasanton, CA 94588

Abstract

This application note discusses the transition from quantitative PCR (qPCR) to Droplet Digital PCR (ddPCR™) for HEK293 residual DNA quantification. A bridging study between the qPCR and ddPCR formats demonstrates the differences and similarities to consider between these two methods. Compared to qPCR, Droplet Digital PCR provides sensitive HEK293 quantification with high specificity and reproducibility without the need for extraction.

Introduction

Immortalized cell lines, most commonly HEK293, are used to manufacture biotherapeutic products such as recombinant adeno-associated virus (AAV) and lentivirus. Residual host cell DNA (HCD), often carried over during the viral vector production process, can harbor oncogenic sequences that could be transmitted through the biotherapeutic to the human recipients.

While various workflows and methods exist to remove residual HCD, samples are typically treated with endonucleases prior to downstream purification. Because of the risk of oncogenic DNA, the U.S. Food and Drug Administration guidelines require residual DNA to be less than 10 ng/dose. Accurate quantification of residual HEK293 DNA is essential to produce a safe and appropriate therapeutic dose.

Quantitative PCR, one of the traditional methods for monitoring residual HCD, has drawbacks, including amplification bias, nonspecific signal, and lack of reproducibility. Furthermore, qPCR requires sample extraction and calibration standards for quantification of samples. In contrast, ddPCR technology provides a solution for accurate HEK293 quantification that is HEK specific, extraction free, and does not require standard curves. Specificity is particularly important for chimeric antigen receptor (CAR) T-cell workflows because CAR T-cell DNA needs to be distinguished from the oncogenic HEK293T residual DNA. In this case, a generic human quantification kit could inaccurately indicate an overload of contaminated DNA.

In qPCR, a bulk measurement is taken for each sample and compared to a reference control to determine the concentration of DNA in the sample of interest. In contrast, ddPCR methodology uses water-oil emulsion technology to partition the reaction into approximately 20,000 droplets, and PCR amplification of the template molecules occurs within each individual droplet. ddPCR technology provides an absolute count of target DNA copies per input sample without the need for running standard curves, making this technique ideal for measurements of target DNA. Furthermore, Droplet Digital PCR is run to endpoint prior to data acquisition, making it more inhibitor tolerant and allowing for the use of a broad range of unextracted samples.

Figure 1 provides example data readouts for a HEK293 qPCR assay and the Vericheck ddPCR HEK293 Res DNA Quant Kit Assay. For the qPCR assay, the quantification cycle (Cq) is calculated as the cycle number at which the amplification curve crosses a predetermined threshold. A standard curve is run alongside samples and used to calculate the HEK293 concentration in each bulk sample reaction. In the ddPCR Assay, the HEK293 concentration is calculated directly using the number of FAM-positive droplets.

Materials and Methods

Study Design

Three sample sets (Table 1) were tested in parallel using a commercially available HEK293 qPCR kit and the Vericheck ddPCR HEK293 Res DNA Quant Kit (Bio-Rad Laboratories, Inc., catalog #12016814).

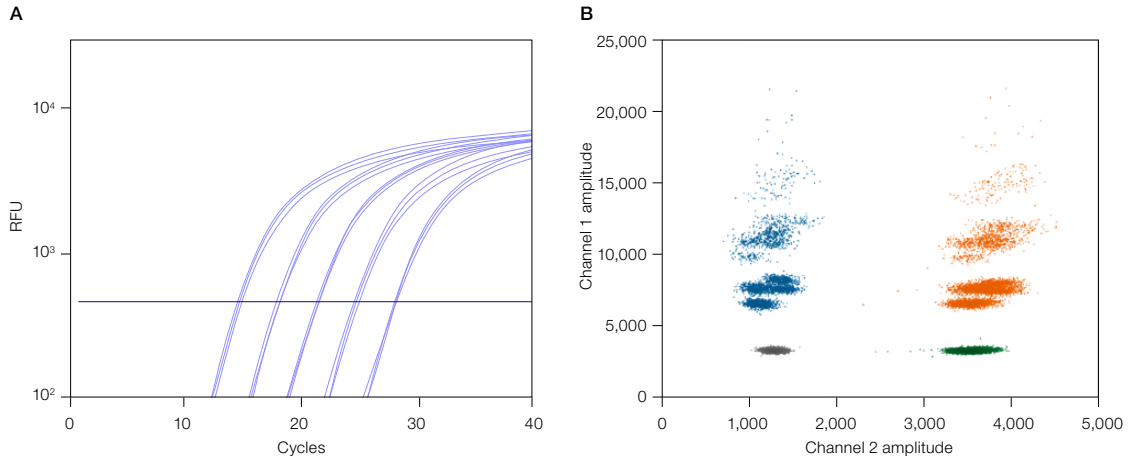

Fig. 1. Readouts for qPCR and ddPCR HEK293 Assays. A, the qPCR amplification plot for the standard curve generated with HEK293 genomic DNA (gDNA) from 0.3 pg to 3 ng/reaction; **B**, 2-D plot of the ddPCR Assay positive control, 5 ng/reaction. The HEK293 signal is in channel 1 (FAM) and the internal control signal is in channel 2 (HEX). The gray cluster is double-negative for HEK293 and internal control, the blue clusters are positive for HEK293, the green cluster is single-positive for internal control, and the orange clusters are positive for HEK293 and internal control. The assay included in the kit detects five targets in the FAM channel. The higher-amplitude FAM-positive clusters represent higher-occupancy droplets that contain multiple HEK293 target molecules. RFU, relative fluorescence units.

Table 1. Three sample sets tested with qPCR and Droplet Digital PCR.

Sample Set	Category	Sample Identification	N
1	Sensitivity, linearity, and reproducibility	HEK293 gDNA control: 0.3 pg–30 ng/reaction	3 each
2	Specificity	Negative-specificity samples — human, Vero, or CHO gDNA: 5 ng/reaction	1 each
		Positive-specificity samples — HEK293 gDNA: 1 ng/reaction and human, Vero, or CHO gDNA: 5 ng/reaction	1 each
3	Extraction-free workflow	HEK293 gDNA with PBS and HSA spike-in: 2 ng/reaction	2 extracted 2 unextracted

CHO, Chinese hamster ovary; gDNA, genomic DNA; HSA, human serum albumin; PBS, phosphate buffered saline.

Sample Preparation

A HEK293 gDNA control from a commercially available qPCR kit was used to prepare tenfold serial dilutions from 30 ng to 0.3 pg/reaction. This dilution series was used to evaluate the sensitivity, linearity, and reproducibility of the qPCR and ddPCR Assays. Human gDNA (Raji, Bio-Rad), Vero gDNA (American Type Culture Collection [ATCC]), and CHO gDNA (United States Pharmacopeial Convention) were prepared at 5 ng/reaction as negative-specificity samples. Three positive-specificity samples were prepared with 1 ng/reaction HEK293 gDNA (ATCC) digested with Csp6I (CviQI) (Thermo Fisher Scientific Inc.) and 5 ng/reaction human, Vero, or CHO gDNA. A sample containing Csp6I-digested HEK293 gDNA at 0.4 ng/µl, PBS and HSA, and water was prepared and split into two aliquots. The first aliquot was tested directly with qPCR and Droplet Digital PCR as an unextracted sample. The second aliquot was extracted using a commercially available magnetic bead–based extraction kit prior to testing with qPCR and Droplet Digital PCR. For sample set 3, the final concentration of HEK293 DNA was 2 ng/reaction and the final concentration of PBS and HSA was 10% PBS and 0.25% HSA per qPCR reaction and 15% PBS and 0.375% HSA per ddPCR reaction.

qPCR and ddPCR Protocol

qPCR and ddPCR reactions were set up according to the respective kit user guides. Five microliters of sample were added to each reaction for both qPCR and Droplet Digital PCR. CviQI restriction enzyme (New England Biolabs, Inc., # R0639S) at a concentration of 5 units/reaction was added to all sample wells in the ddPCR Assay. The CFX96 Touch Real-Time PCR System (Bio-Rad, #1855195) was used for qPCR, and the QX200 AutoDG Droplet Digital PCR System (Bio-Rad, #1864100) was used for Droplet Digital PCR. The qPCR data were analyzed using CFX Maestro Software (Bio-Rad, #12013758), and the threshold was set with the software's automatic thresholding function. The wells containing 0.3–3,000 pg/reaction of HEK293 gDNA control were used to generate a standard curve. The ddPCR data were analyzed with QX Manager Software, Regulatory Edition, version 1.2 (Bio-Rad, #12012172), and thresholds were set using positive control–based automatic thresholding. Picograms per 20 µl reaction were calculated by multiplying the molecular weight provided by software (in pg/µl) by 20.

Additional Extraction Samples

To provide guidance on the expected results when transitioning from extracted to unextracted samples, four additional samples were prepared and tested unextracted and extracted with only Droplet Digital PCR. Two Benzonase treated HEK293 samples, one Csp6I-digested HEK293 DNA sample, and one AAV sample were tested. Each sample was prepared in bulk and aliquoted according to guidelines in Table 2. One aliquot was reserved and tested directly with Droplet Digital PCR. The remaining aliquots were extracted in parallel using a commercially available magnetic bead–based extraction kit, then tested with Droplet Digital PCR. Droplet Digital PCR was run according to the instrument user guide.

Table 2. Additional samples tested with only Droplet Digital PCR. Each sample was diluted in water to reach the final concentration per reaction. Median sizes were determined using an Agilent 2100 Bioanalyzer Instrument (#G2939BA).

Sample Number	N (aliquots)	Sample Name	Approximate [HEK293 DNA] per reaction, ng
1	1 unextracted 3 extracted	GeneCopoeia, Inc. AAV and associated residual HEK293 DNA	6.5
2	1 unextracted 3 extracted	Benzonase treated ATCC HEK293 DNA (783 bp median size)	4
3	1 unextracted 3 extracted	Benzonase treated ATCC HEK293 DNA (1,898 bp median size)	4
4	1 unextracted 2 extracted	HEK293 DNA digested with Csp6I	4

Results

Sensitivity, Linearity, and Reproducibility

Sensitivity, linearity, and reproducibility were assessed for each assay using a HEK293 gDNA control ranging from 0.3 pg to 30 ng/reaction (Figure 2). The qPCR and ddPCR Assays detected DNA down to 0.3 pg/reaction (Table 3). The dynamic range of the qPCR assay is 0.3 pg–3 ng. The dynamic range of the ddPCR Assay is 0.8 pg–50 ng. The qPCR assay had lower coefficient of variation percentage (CV%) values at lower concentrations, in line with its dynamic range. In contrast, the ddPCR Assay had lower CV% values at higher concentrations, as these concentrations fall well within the dynamic range of the assay (Table 3). Both assays were linear with $R^2 > 0.99$ and a slope between 0.95 and 1.05 (Figures 3 and 4).

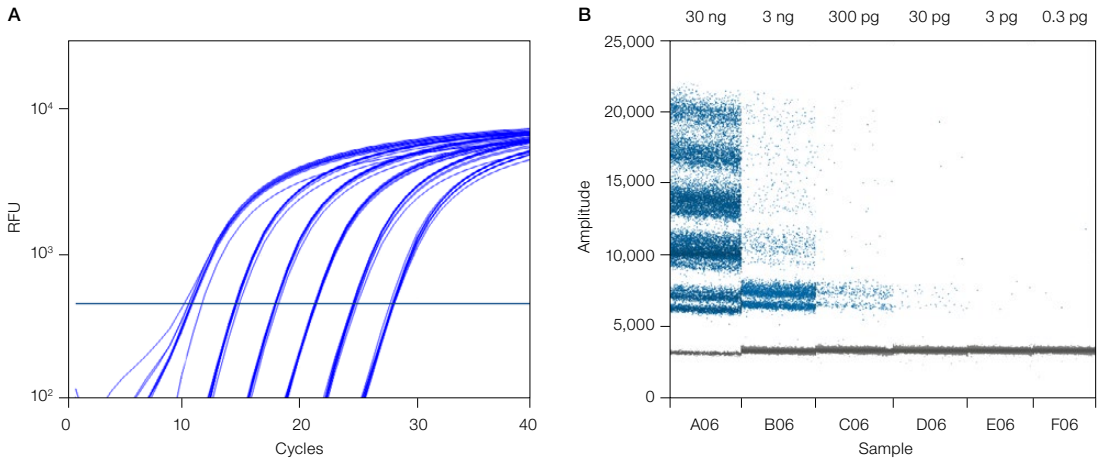

Fig. 2. qPCR and ddPCR readouts from 0.3 pg to 30 ng/reaction of HEK293 gDNA control. A, qPCR amplification curves; **B**, ddPCR 1-D plots. RFU, relative fluorescence units.

Table 3. Comparison of qPCR and ddPCR sensitivity and reproducibility.

		qPCR Results				ddPCR Results				
Target Concentration, pg/reaction	n	Average, Cq	Average Measured, pg/reaction	SD	CV, %	Average, copies/µl	Number of Positive Tests	Average Measured, pg/reaction	SD	CV, %
30,000	3	10.80	42,131.76	3,524.87	8.37	3,708.91	3/3	26,973.92	200.34	0.74
3,000	3	14.59	3,081.15	207.99	6.75	357.00	3/3	2,596.35	21.97	0.85
300	3	17.99	292.99	10.86	3.71	36.69	3/3	266.84	6.10	2.29
30	3	21.31	29.51	0.32	1.10	3.14	3/3	22.83	1.20	5.25
3	3	24.61	3.02	0.15	5.06	0.24	3/3	1.74	0.57	32.44
0.3	3	27.94	0.30	0.02	5.10	0.07	3/3	0.52	0.20	38.23

Cq, quantification cycle; CV, coefficient of variation; SD, standard deviation.

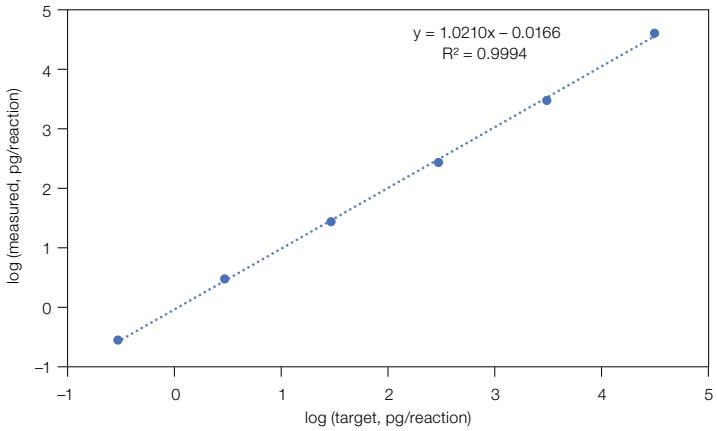

Fig. 3. Measured vs. target HEK293 concentration detected by qPCR. $R^2 > 0.99$ and slope of 1.02.

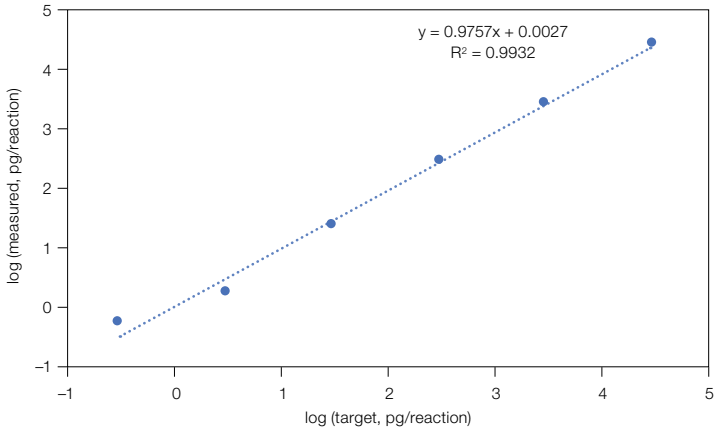

Fig. 4. Measured vs. target HEK293 concentration detected by Droplet Digital PCR. $R^2 > 0.99$ and slope of 0.98.

Specificity

The qPCR assay cross-reacts with human and Vero gDNA and reports higher than expected concentrations for those samples (Table 4). In contrast, the ddPCR Assay is HEK293 specific and accurately quantifies HEK293 DNA in a background of human, Vero, or CHO gDNA (Figure 5).

Table 4. Specificity results for the qPCR and ddPCR Assays. The qPCR assay detects human and Vero DNA. The ddPCR Assay shows no cross-reactivity with human, Vero, and CHO DNA.

		qPCR Results			ddPCR Results		
Sample ID	n	Measured, Cq	Expected, pg/reaction	Measured, pg/reaction	Measured, copies/µl	Expected, pg/reaction	Measured, pg/reaction
Human –	1	13.94	0.00	4,822.32	0.00	0.00	0.00
Human +	1	13.32	1,000.00	7,379.40	131.61	1,000.00	957.20
Vero –	1	14.63	0.00	2,988.26	0.00	0.00	0.00
Vero +	1	13.98	1,000.00	4,688.57	123.76	1,000.00	900.06
CHO –	1	33.67	0.00	0.01	0.00	0.00	0.00
CHO +	1	15.23	1,000.00	1,969.00	130.08	1,000.00	946.05

CHO, Chinese hamster ovary; Cq, quantification cycle.

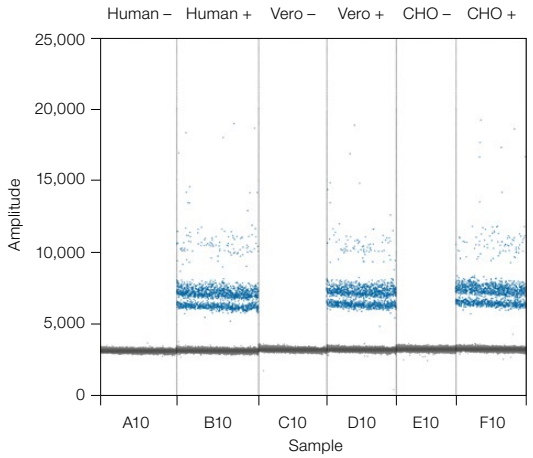

Fig. 5. 1-D plot for wells containing human, Vero, or CHO gDNA. Wells marked with – contain no HEK293 DNA. Wells marked with + contain 1 ng/reaction HEK293 gDNA in addition to the human, Vero, or CHO gDNA. CHO, Chinese hamster ovary.

Extraction-Free Workflow

Both assays detected HEK293 DNA in the unextracted and extracted samples (Table 5). For the qPCR assay, the unextracted sample concentration was 1.4x higher and the extracted sample concentration was 1.8x higher than expected. The ddPCR Assay detected the unextracted sample at the expected concentration. The extracted sample concentration was 1.4x higher than expected.

Table 5. Results for unextracted and extracted samples tested with qPCR and Droplet Digital PCR.

		qPCR Results					ddPCR Results				
Sample Identification	n	Average, Cq	Expected, pg/reaction	Average Measured, pg/reaction	SD	CV, %	Average, copies/µl	Expected, pg/reaction	Average Measured, pg/reaction	SD	CV, %
Unextracted	2	14.75	2,000.00	2,754.16	261.92	9.51	266.57	2,000.00	1,938.67	27.23	1.40
Extracted	2	14.36	2,000.00	3,592.73	327.37	9.11	394.08	2,000.00	2,866.06	7.70	0.27

Cq, quantification cycle; CV, coefficient of variation; SD, standard deviation.

To determine the expected differences when running unextracted and extracted samples with Droplet Digital PCR, additional samples were run only with the ddPCR Assay (Figure 6). For the 783 bp Benzonase treated, 1,898 bp Benzonase treated, and restriction enzyme–digested samples, the extracted sample concentration was 1.3x, 1.2x, and 1.4x greater than the unextracted sample concentration, respectively. For the AAV sample, the concentration of the extracted sample was within 2 standard deviations of the concentration of the unextracted sample.

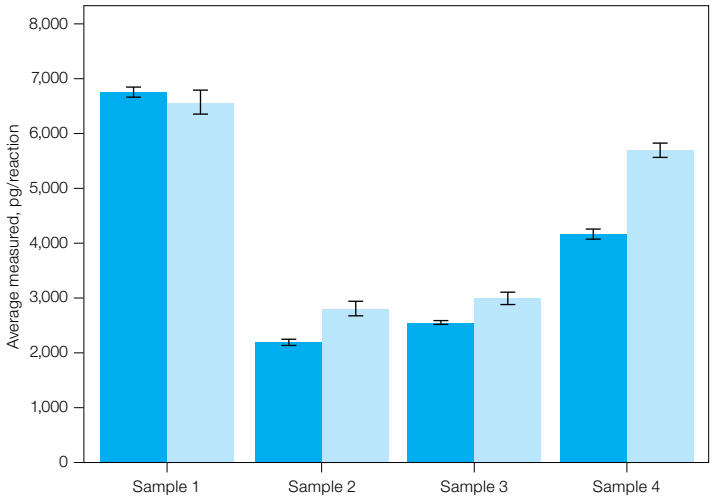

Fig. 6. Concentration comparison for unextracted and extracted samples. Sample 1, AAV and associated residual HEK293 DNA; sample 2, Benzonase treated HEK293 sample, 783 bp median size; sample 3, Benzonase treated HEK293 sample, 1,898 bp median size; sample 4, HEK293 DNA digested with Csp6I. For the AAV sample, the unextracted and extracted sample concentrations were within 2 standard deviations. For all other samples tested, the extracted sample concentration was higher than the unextracted sample concentration. Error bars are ±2 standard deviations. Unextracted (■); extracted (■).

Discussion

The purpose of this study was to compare the output for qPCR and Droplet Digital PCR when run with the same samples to aid in the transition between the platforms. The qPCR assay was run on the CFX96 Touch System and automatic thresholding was done using CFX Maestro Software. The standard curve and sample concentrations for the qPCR data were derived manually instead of using the commercially available qPCR kit software.

Conclusion

The qPCR and ddPCR Assays both detected HEK293 DNA down to 0.3 pg/reaction. When directly compared with Droplet Digital PCR, qPCR results showed concentrations up to approximately 2-fold higher than ddPCR concentrations (average of 1.5-fold difference). The qPCR assay detected normal human and Vero gDNA, resulting in inaccurate results for samples containing normal human or Vero contamination. The ddPCR Assay was specific to HEK293 and detected no human, Vero, or CHO DNA. The ddPCR Assay is linear and highly reproducible within the dynamic range. In conclusion, the Vericheck ddPCR HEK293 Res DNA Quant Kit provides a highly specific, extraction-free method for residual DNA quantification without need of a standard curve.

Visit bio-rad.com/ddPCR-Vericheck-HEK-Quant for more information.

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Website *bio-rad.com* ***USA*** *1 800 424 6723* ***Australia*** *61 2 9914 2800* ***Austria*** *00 800 00 24 67 23* ***Belgium*** *00 800 00 24 67 23* ***Brazil*** *4003 0399*
Canada *1 905 364 3435* ***China*** *86 21 6169 8500* ***Czech Republic*** *00 800 00 24 67 23* ***Denmark*** *00 800 00 24 67 23* ***Finland*** *00 800 00 24 67 23*
France *00 800 00 24 67 23* ***Germany*** *00 800 00 24 67 23* ***Hong Kong*** *852 2789 3300* ***Hungary*** *00 800 00 24 67 23* ***India*** *91 124 4029300* ***Israel*** *0 3 9636050*
Italy *00 800 00 24 67 23* ***Japan*** *81 3 6361 7000* ***Korea*** *82 2 3473 4460* ***Luxembourg*** *00 800 00 24 67 23* ***Mexico*** *52 555 488 7670*
The Netherlands *00 800 00 24 67 23* ***New Zealand*** *64 9 415 2280* ***Norway*** *00 800 00 24 67 23* ***Poland*** *00 800 00 24 67 23* ***Portugal*** *00 800 00 24 67 23*
Russian Federation *00 800 00 24 67 23* ***Singapore*** *65 6415 3188* ***South Africa*** *00 800 00 24 67 23* ***Spain*** *00 800 00 24 67 23* ***Sweden*** *00 800 00 24 67 23*
Switzerland *00 800 00 24 67 23* ***Taiwan*** *886 2 2578 7189* ***Thailand*** *66 2 651 8311* ***United Arab Emirates*** *36 1 459 6150* ***United Kingdom*** *00 800 00 24 67 23*

Bulletin 3314 Ver A US/EG *22-0261 0422 Sig 0122*