

HEK293 Residual DNA Sizing: Transitioning from a Microfluidic Chip–Based Electrophoresis Method to Droplet Digital™ PCR

Joshua Lehrer, Lovedeep Grewal, Nathan Sepulveda, Stephen Bolaris, Daniela Beltran-Hernandez, Prasanthi Bhagavatula
Bio-Rad Laboratories, Inc., Digital Biology Center, 5731 W Las Positas Blvd, Pleasanton, CA 94588

Abstract

This application note introduces the VeriCheck ddPCR™ HEK293 Res DNA Size Kit for Droplet Digital PCR as a test for residual host cell HEK293 genomic DNA (gDNA) contamination and compares it to a microfluidic chip–based electrophoresis method to determine the median size, percentage greater than 200 bp, and mass of partially degraded HEK293 DNA samples. The VeriCheck ddPCR HEK293 Res DNA Size Kit is a powerful alternative to using a microfluidic chip–based electrophoresis assay and has many advantages, such as greater throughput, sample matrix compatibility, and dynamic range.

Introduction

HEK293 cells are the dominant producer cell type for cell and gene therapy viral vector products like recombinant adeno-associated virus (rAAV) and lentivirus. These products initially contain residual DNA from the host cell and vectors or plasmids from which they are produced. Cell lysate may be treated with endonucleases prior to downstream purification. Residual host cell DNA may also be a contaminant in other sample types, such as chimeric antigen receptor (CAR) T cells, if lentivirus is used for gene transfer. Residual DNA contaminant regulatory guidelines require less than 10 ng per parenteral dose and that the fragment size be less than 200 bp for patient safety, due to the risk of oncogenic DNA (U.S. Department of Health and Human Services Guidance for Industry 2010). The VeriCheck ddPCR HEK293 Res DNA Size Kit effectively provides quality control for estimating the size of contaminating DNA.

ddPCR technology uses a water-oil emulsion technology. A sample is partitioned into 20,000 droplets, and PCR amplification of the template molecules occurs in 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 simpler, highly sensitive, and precise. The VeriCheck ddPCR HEK293 Res DNA Size Kit generates estimates of median size, and total mass and mass percentage of fragments greater than 200 bp in each sample.

One method for quantitatively monitoring the size of residual host cell DNA is microfluidic chip–based electrophoresis. This generates an electrophoretogram on which a smear analysis is performed to determine the concentration of each size region. A side-by-side comparison of this method and Droplet Digital PCR was conducted in this study. This ddPCR Assay is designed to replace this step of the quality control workflow, and provides higher sensitivity, dynamic range, and throughput than the alternative method. A key difference between each method is that the chip-based electrophoresis method is not HEK293 specific. This means that when determining the amount of oncogenic HEK293 DNA in a sample (for example, CAR T cells), any contaminating plasmid or viral or human gDNA will obfuscate the result. In contrast, this kit is HEK293 specific and detects only the host cell DNA. Full-length DNA may clog microfluidic channels. To avoid clogging, this sizing kit is compatible with restriction enzymes and in-well digestion allows the user to process samples containing full-length DNA without compromising the results. The ddPCR platform demonstrates tolerance to reagents in rAAV and lentiviral production and formulation, whereas the electrophoretic products used for this purpose are limited to water or Tris-EDTA buffer. This may require the user to extract the sample or attempt buffer exchange. The dynamic range of the VeriCheck ddPCR HEK293 Res DNA Size Kit Assay is 25 pg–300 ng, and 30 samples can be tested per plate using ddPCR instruments.

Materials and Methods

Three samples (S1, S2, and S3) containing 1 µg of gDNA from HEK293 cells (American Type Culture Collection [ATCC], catalog #CRL-1573) were fragmented by 0.1 U Benzonase (Millipore Sigma, #706643), 0.2 mM Mg²⁺, and 0.1 mg/ml of bovine serum albumin in 50 mM Tris for 4 (S3), 12 (S2), and 25 (S1) minutes at 37°C. The reaction was quenched with ethylenediaminetetraacetic acid (EDTA) and purified with the Monarch PCR & DNA Cleanup Kit (New England Biolabs, Inc., #T1030S) according to the manufacturer's protocol. The purified DNA concentrations were measured with a Qubit 4 Fluorometer (Thermo Fisher Scientific Inc. #Q33238) using the Qubit dsDNA BR Assay (#Q32850) and diluted serially. Each sample was tested (n = 3) using the chip-based electrophoresis kit, following the manufacturer's protocol.

A smear analysis was performed for each replicate and a region between 40 bp and 9 kb was selected to find the mass of the sample from the given concentration. A second region was chosen that divided the area of the curve into equal parts, which indicated the median size of the sample. A third region from 200 bp to the upper marker was selected to obtain the percentage greater than 200 bp from the percentage of total column.

Three tests (three wells per test) were performed using the VeriCheck ddPCR HEK293 Res DNA Size Kit (Bio-Rad™ Laboratories, Inc., #12016183) on a single plate. The input volume and mass per test were kept constant on each platform and the same sample dilution was used to control for variance in pipetting and to remove the risk of error in dilution. The data exported from QX Manager Software, Regulatory Edition, version 1.2 (Bio-Rad, #12012172), was analyzed with the Sizing Worksheet, which generates five outputs. The Sizing Worksheet determines whether the sample is less than 200 bp, estimates median size and percentage greater than 200 bp, and calculates mass in pg/µl of each target. This value is multiplied by the total volume of the three wells to determine the input total mass. The study workflow is shown in Figure 1.

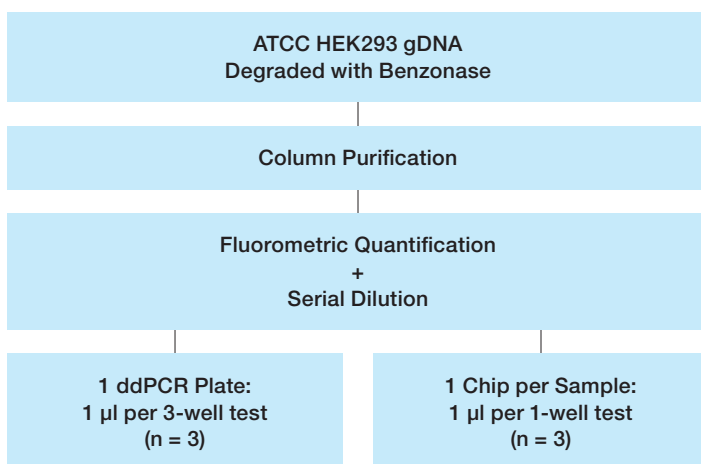


Fig. 1. Study workflow.

Results and Discussion

The electrophoretograms for each sample are shown in Figure 2. The fragment sizes increase as incubation time in Benzonase decreases, where the peak shifts toward the upper marker at 10 kb. The 2-D plots from each sample tested using the VeriCheck ddPCR HEK293 Res DNA Size Kit Assay are shown in Figure 3. As the fragment population increases in size, the HEX-positive green and orange clusters increase in density.

The Sizing Worksheet provides three outputs that will be compared to the microfluidic chip–based electrophoresis method. The percentage greater than 200 bp is defined as the mass percentage from DNA fragments greater than 200 bp. In Droplet Digital PCR, the mass is determined from concentration (copies/µl) with a correction factor based on the copy number of the target and the mass of the entire genome. In samples containing other sources of DNA, these values would greatly vary between platforms.

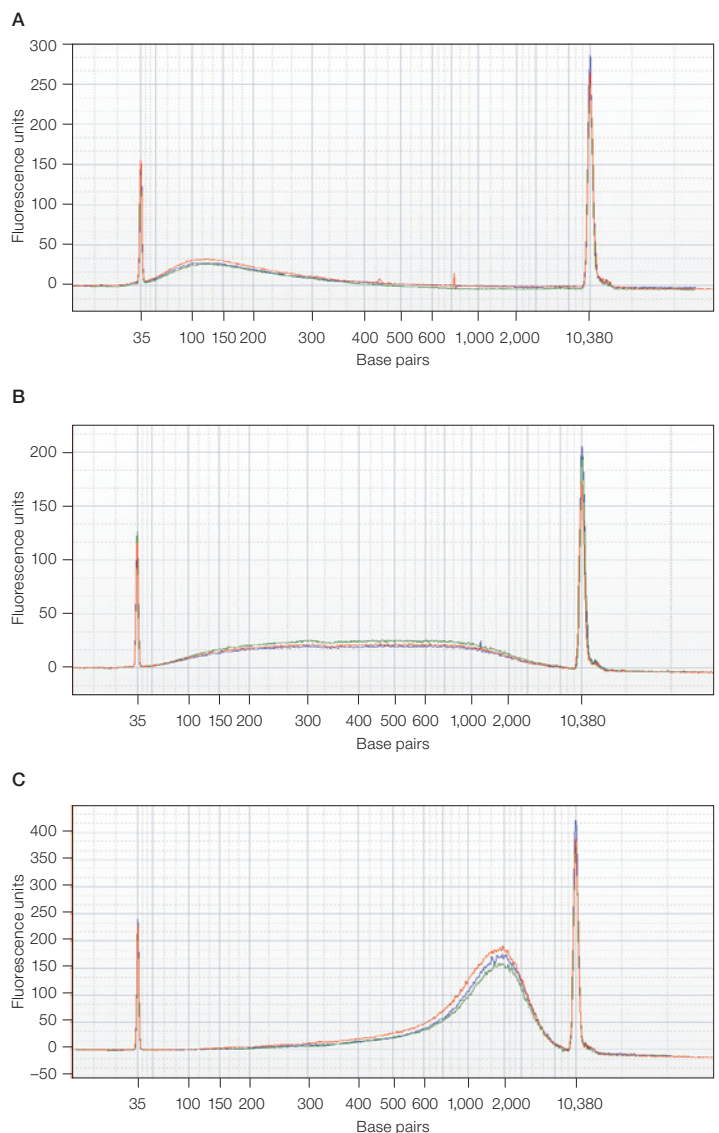


Fig. 2. Electrophoretograms (n = 3, overlaid) from each sample. A, S1; B, S2; C, S3. Replicate 1 (—); replicate 2 (—); replicate 3 (—).

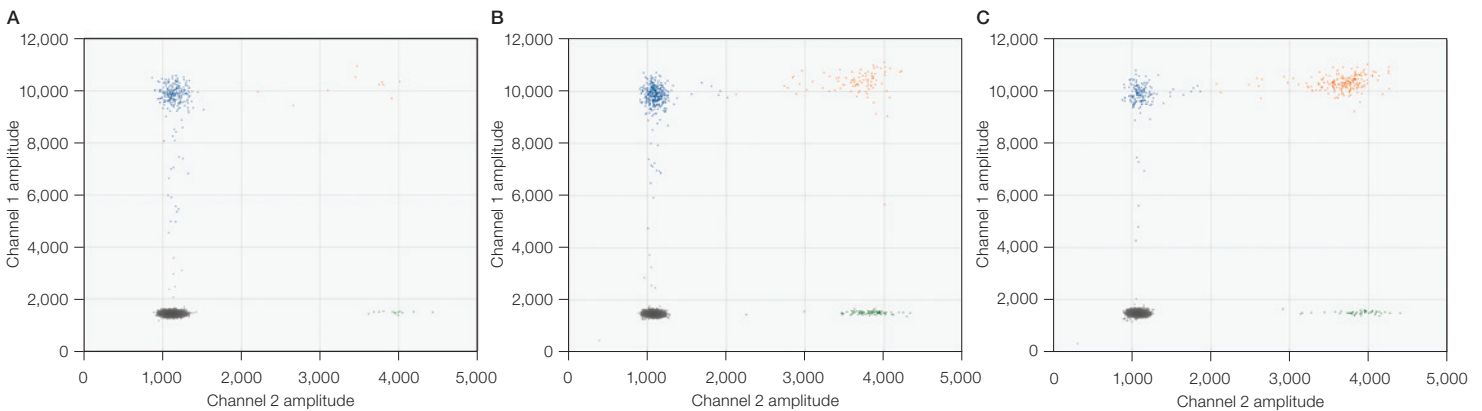


Fig. 3. 2-D plots of the ddPCR Assay readout from each sample. Droplet clusters are small fragments (FAM, blue cluster), medium fragments (HEX, green cluster), double-positive large fragments (FAM + HEX, orange cluster), and negative fragments (gray cluster). As the fragments increase in size the green and orange clusters increase in density. **A**, S1; **B**, S2; **C**, S3.

The results of each platform for each sample were compared and reported in Tables 1–3 and shown in Figures 4A–C. Samples were unknowns, and no conclusions should be made as to accuracy. Percentage difference between experimental averaged values are reported in Tables 1 and 3, where the electrophoresis method was treated as the reference.

$$\text{Percentage Difference} = \left(\frac{\text{Droplet Digital PCR} - \text{Electrophoresis}}{\text{Electrophoresis}} \right) \times 100$$

The ddPCR measurements corresponded well with the electrophoresis method, with less than 20% difference between median sizes predicted, which is below the inherent error expected for the chip-based kit. In highly degraded samples, greater sensitivity is demanded to detect and quantify the remaining signal from fragments greater than 200 bp and accuracy may be reduced. This may contribute to the larger discrepancy between the percentage greater than 200 bp in sample S1, which is more fragmented (Table 2). The mass of each sample was within the expected range and variation between samples tracked across platforms. Droplet Digital PCR detected less mass in each sample. More replicates are needed to determine the significance of any of the observed differences in each instance.

Table 1. Comparison of values determined for median size. Averaged values (n = 3) for each platform and the percentage difference between each average.

| Sample | Droplet Digital PCR | | Electrophoresis | | Difference, % |
|--------|-------------------------|--------------------|-------------------------|--------------------|---------------|
| | Average Median Size, bp | Standard Deviation | Average Median Size, bp | Standard Deviation | |
| S1 | 138 | 8.50 | 156 | 2.65 | 11.32 |
| S2 | 309 | 16.44 | 353 | 3.06 | 12.48 |
| S3 | 1,350 | 375.31 | 1,217 | 90.89 | 10.90 |

Table 2. Comparison of values determined for percentage greater than 200 bp. Averaged values (n = 3) for each platform and the absolute difference between each average.

| Sample | Droplet Digital PCR | | Electrophoresis | | Absolute Difference |
|--------|---------------------|--------------------|-------------------|--------------------|---------------------|
| | Average % >200 bp | Standard Deviation | Average % >200 bp | Standard Deviation | |
| S1 | 16.8 | 7.08 | 34.7 | 1.15 | 17.84 |
| S2 | 68.1 | 1.74 | 76.7 | 0.58 | 8.57 |
| S3 | 94.4 | 2.54 | 95.3 | 0.58 | 0.89 |

Table 3. Comparison of total mass detected. Averaged values (n = 3) for each platform and the percentage difference between each average.

| Sample | Droplet Digital PCR | | Electrophoresis | | Difference, % |
|--------|---------------------|--------------------|------------------|--------------------|---------------|
| | Average Mass, pg | Standard Deviation | Average Mass, pg | Standard Deviation | |
| S1 | 569 | 7.83 | 685 | 84.36 | 16.94 |
| S2 | 996 | 26.15 | 1,121 | 178.09 | 11.15 |
| S3 | 740 | 43.41 | 842 | 95.63 | 12.09 |

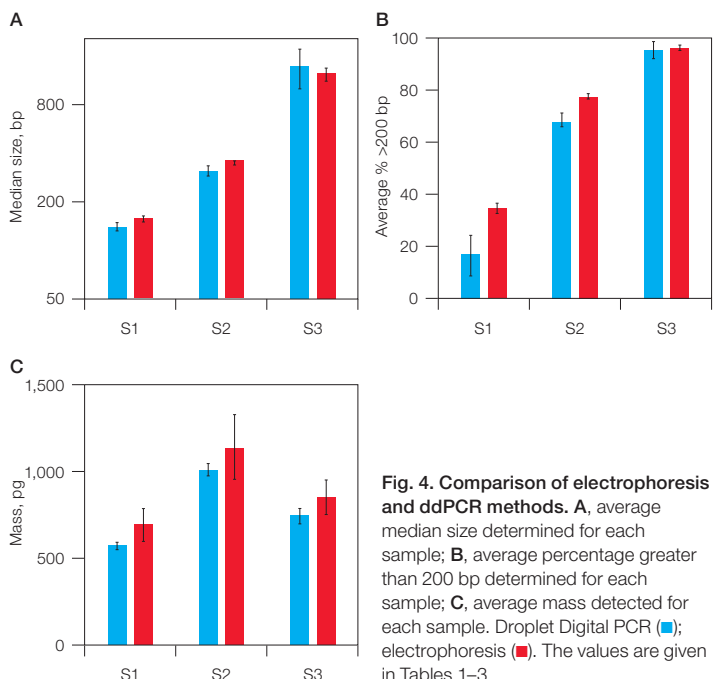


Fig. 4. Comparison of electrophoresis and ddPCR methods. **A**, average median size determined for each sample; **B**, average percentage greater than 200 bp determined for each sample; **C**, average mass detected for each sample. Droplet Digital PCR (■); electrophoresis (■). The values are given in Tables 1–3.

Conclusions

This application note provides a side-by-side comparison and introduction to transitioning from a microfluidic chip-based electrophoresis workflow to Droplet Digital PCR for HEK293 DNA sizing analysis. The primary advantages of Droplet Digital PCR are its sensitivity, dynamic range, throughput, and specificity to HEK293 DNA. With the VeriCheck ddPCR HEK293 Res DNA Size Kit, one can quantify mass and determine median size and percentage DNA greater than 200 bp, addressing the U.S. Food and Drug Administration guidance for residual HEK293 DNA fragment size.

Reference

U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research (2010). Guidance for Industry: Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications. <https://www.fda.gov/media/78428/download>, accessed June 8, 2022.

Visit [bio-rad.com/ddPCR-VeriCheck-HEK-Size](https://www.bio-rad.com/ddPCR-VeriCheck-HEK-Size) for more information.

BIO-RAD, DDPCR, and DROPLET DIGITAL are trademarks of Bio-Rad Laboratories, Inc. in certain jurisdictions. All trademarks used herein are the property of their respective owner. © 2022 Bio-Rad Laboratories, Inc.

Purchase of Digital PCR and/or Single-Cell NGS Sample Preparation products (the "Products") from Bio-Rad Laboratories is subject to Bio-Rad Laboratories, Inc. Standard Terms and Conditions of Sale, which can be accessed at <https://www.bio-rad.com/en-us/terms-conditions>. Unless we expressly state otherwise in additional Terms and Conditions, no rights are granted for you to distribute or resell the Products. Unless we expressly state otherwise in additional Terms and Conditions, no rights are granted for the development or commercialization of diagnostic assays for use with the Products without a license from Bio-Rad. The Products and/or their use are covered by U.S. and foreign patents and/or pending patent applications owned by or under license to Bio-Rad Laboratories, Inc. See <https://www.bio-rad.com/en-us/trademarks>.



**Bio-Rad
Laboratories, Inc.**

Life Science
Group

Website [bio-rad.com](https://www.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

